CHROMATOGRAPHY, THE STATE OF THE ART
Volume I

Symposia Biologica Hungarica 27

CHROMATOGRAPHY THE STATE OF THE ART Volume I

27

Akadémiai Kiadó, Budapest

CHROMATOGRAPHY
THE STATE OF THE ART

Edited by

H. KALÁSZ and L. S. ETTRE

(Symposia Biologica Hungarica 27)

The volume contains the papers presented at the different sessions of the Budapest Chromatography Conference which was held in Budapest, Hungary, June 1–3, 1983.

The book includes altogether 9 chapters dealing with "Theoretical aspects", "Stationary phases for chromatography", "Chromatography of amines and amino acids", "Separation of peptides and proteins," "Separation of drugs and metabolites", "Thin-layer chromatography", "Gas chromatography", "Calculation and optimization methods" as well as "Various topics".

The papers of this book present the most recent theoretical considerations, practical approaches and results as well as the trends of the latest development.

Since the authors of the articles are either specialists of chromatography or the users of the separation methods in their everyday work, the papers published in the book are up-to-date, the figures and tables help the understanding of the written text.

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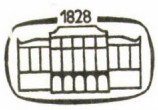

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PREFACE

This volume contains the proceedings of the *Budapest Chromatography Conference*, held in Budapest, Hungary, June 1-3, 1983.

The Budapest Chromatography Conference was part of the series of American-Eastern European Symposia on Liquid Chromatography initiated in 1981, and held annually ever since. The number of participants grew from about 30 to 60, 200 and 350 scientists in 1981, 1982, 1983 and 1984, respectively. The material of the First Symposium was published in the *Journal of Liquid Chromatography*. Later, the increased number of manuscripts made it possible to publish them each year in book form. After the Fourth Symposium, the question arises as to the reason behind the increasing interest in these Symposia. Several factors, such as the expertise and activity of the organizers, ensured the high scholarly standard and comfort. The skill of the local organizers, headed by Dr. Gabriella Lázár (Szeged, 1981, 1982 and 1984) and Mrs. Ágnes Rubányi (Budapest, 1983) contributed greatly to the success of these meetings. The participation of Soviet and American scientists was organized and sponsored by Dr. Ljudmilla Kolomiets and Mr. Chuck Rudd, and the Hungarian chromatographers were organized by Professors L. Leisztner and L. Szepesy. Dr. Tibor Dévényi, who was the chairman and the *spiritus rector* of the Symposia, avoided the limelight but provided the necessary support to every organizer, participant and technical staff member. That is the reason why this volume is dedicated to him.

This book consists of 62 papers presented during the Budapest Chromatography Conference. Professor Knoll's introductory

lecture is published in the same form as it was delivered at the Conference. The other lectures and posters have been revised by the authors according to the formal requirements of the publisher.

We are indebted to Dr. L.S. Ettre who was responsible for an essential part of the editing, including the grammatical revision.

Special thanks are due to Mrs. Edith Róth for espousing the proceedings of the symposia series and Dr. Judit Kerpel-Fronius and Mrs. Katalin Csóka for the technical editorial work.

Thanks are due to the coworkers of the Publisher and the Printing House for their conscientious support of this book, and to the authors of the papers who - hopefully - wrote interesting and informative pages.

Huba Kalász

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VARIOUS TOPICS

Chromatography, the State of the Art
H. Kalász and L.S. Ettre (Eds)

INTRODUCTORY REMARKS

J. KNOLL

Department of Pharmacology, Semmelweis University of Medicine, Budapest, Hungary

Ladies and Gentlemen,

I am one of those medical doctors who were attracted during their student years by the greatest marvel of nature, the work of the brain. I began to analyse 35 years ago the problems of drives; the question, what happens in the brain objectively when we have the subjective feeling of the need of something. The deeper I penetrated into the relationships between brain physiology and behavior, the more and more I realized that appropriately developed selective pharmacological agents are the revolutionary new tools in our times for the analysis of the physiological and behavioral problems I encountered, so I became a pharmacologist.

Analysing the nature of drives by combining the physiological methods with the use of pharmacological tools I was pressed by the facts to hypothesize the existence of unknown regulatory substances and became confronted with the problems to prove the presence of these endogenous substances in the body. I desperately needed the help of the science of chromatography to solve my problems and was lucky enough to invite more than a decade ago Drs Kalász and Nagy in my laboratory who helped me to make benefit of the chromatographic techniques.

I hope you will allow me now to show you just a few examples giving small examples of the usefulness of chromatographic techniques in my research.

It was in 1953 during my early meetings with the revolutionary new ideas in psychopharmacology, paving the way

for biological psychiatry. that the physiological problems of drives arrested my attention. I looked in vain for a feasible proposal in literature regarding the rate limiting objective, chemical change in the brain responsible for the subjective feeling of hunger and regarding the essential chemical alteration in the brain which transforms our unpleasant sensation of hunger to the pleasant feeling of being satieted. The careful experimental analysis of drive motivated behavior in rats compelled me to develop a new physiological theory for the interpretation of drives /Knoll, 1969/.

Based on this theory, the working hypothesis visualized in Fig. 1 seemed to me in the early 70s very promising for searching the essential chemical machinery in the brain inseparable from the subjective sensations of hunger and its relief.

According to this hypothesis satiety is regulated, hunger is the lack of satiety. Fig. 1 is an utmost simplified scheme to visualize the putative chemical basis for the hunger-drive and its elimination following feeding. A blood-borne substance, named satietin, is supposed to play the role of the rate limiting satiety signal in the negative feed-back of food intake. Feeding increases the blood concentration of active satietin, consequently the population of satietin-sensitive neurons, the 'satiety center', becomes activated and the subject has the feeling of being satieted. Digestion of food eliminates the fullness of the gastrointestinal tract leading to a decrease in the concentration of satietin in blood, the 'satiety center' loses satietin, the unpleasant sensation of hunger is proportionally increasing, the drive for searching food is operating again.

The satietin hypothesis of the regulation of food intake seemed to be in excellent agreement with old and new physiological experiences. The lack of appropriate separation techniques, however, hindered for years the approval of the validity of this hypothesis.The existence of satietin was finally successfully demonstrated in human serum by the aid of gel chromatography /Knoll, 1979/ and using affinity chromatography we were also able to isolate the substance /Nagy, Kalász and Knoll, 1983/.

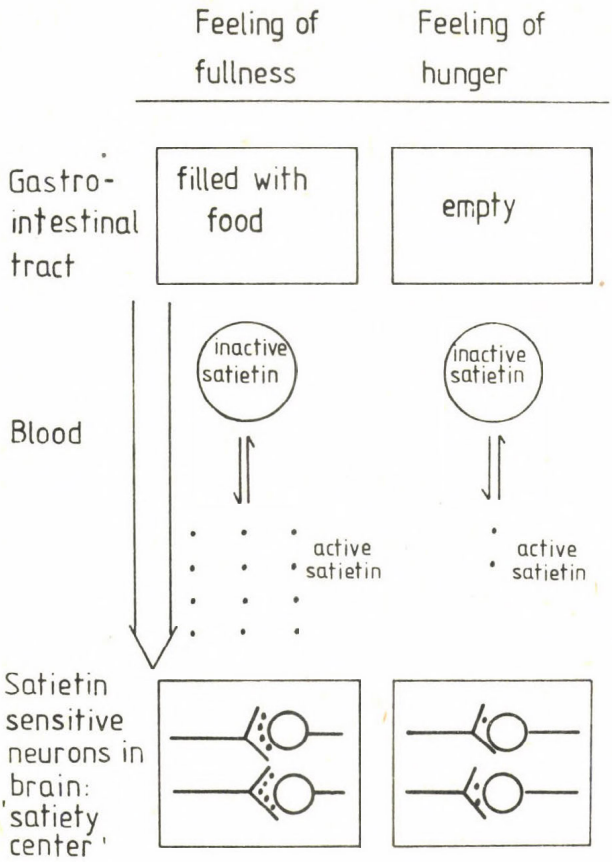


Fig. 1. Scheme visualizing the hypothesis that satietin, a blood-borne substance, plays the role of a rate limiting satiety signal in the negative feed-back of food intake.

Fig. 2 shows the flow-sheet of the preparation of satietin, a 50.000 dalton glycoprotein, which proved to be an extremely potent anoretic substance with unique selectivity. The chances now to develop a safe new pharmacological method for the control of body weight are good. If satietin research, as we hope,

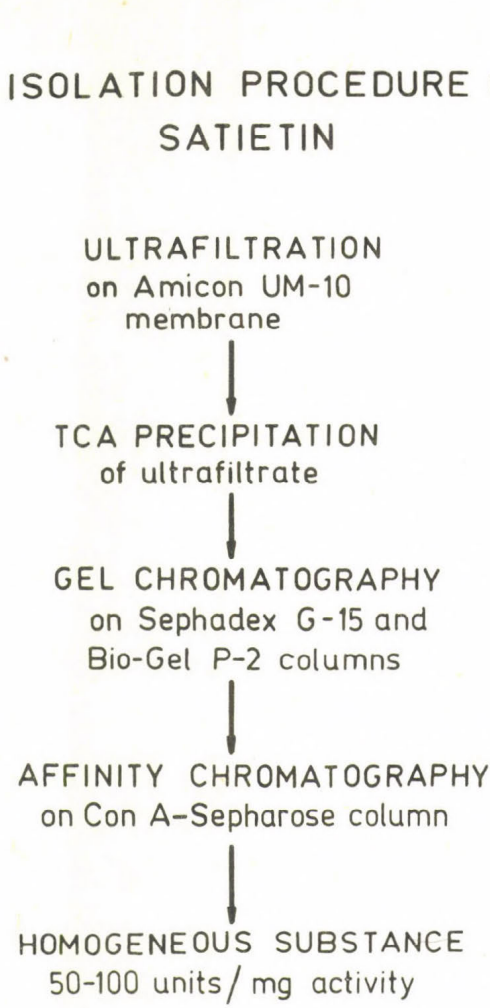


Fig. 2. Flow-sheet of the isolation of human serum satietin.

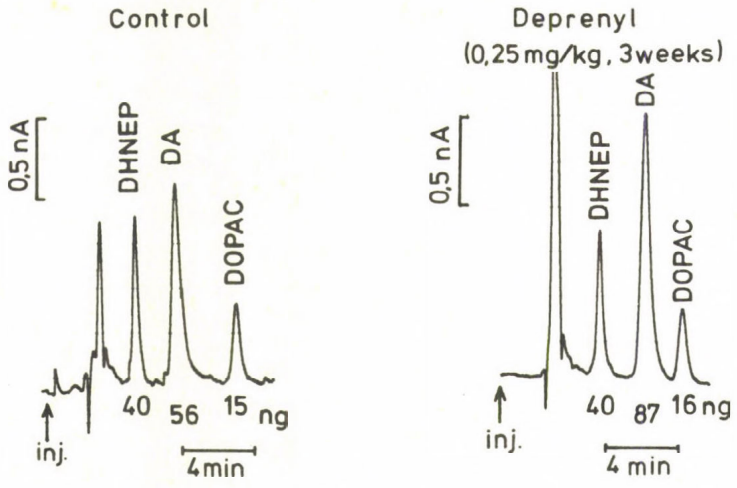
will help to solve the serious medical problem of overweight and obesity, we could hardly overestimate the contribution of chromatography in this progress.

And may I mention briefly a second example how chromatography helped me in solving another special biological problem. We developed a new drug, /-/deprenyl, the first and still the

only selective inhibitor of the so-called B-type monoamine oxidase /MAO/, which is now widely used as an adjuvant in the levodopa treatment of Parkinson's disease. It is the most interesting feature of this substance that it activates with a unique mechanism the dopaminergic system in the brain and can be administrated for years with reasonably low incidence of side effects.

The possibility to increase dopaminergic tone in the brain by a safe drug which can be administered for years is of great practical importance as the dopamine content of the brain decreases in an age-related manner. It is firmly established by now that over age 45 the decline of brain dopamine is about 13% per decade. The age-related loss of dopamine seems to be an unavoidable biochemical lesion of aging and might play a serious role in the well-known high incidence of depression and parkinsonian symptoms in the aged, as well as in the decline of the sexual vigor in the aging males. Thus, the proposal /Knoll, 1982/ to improve the quality of life in senescence by preventing the age-related decline of the dopaminergic tone in the brain, or at least counteracting its consequences, deserves careful clinical scrutiny. Detailed analyses with /-/deprenyl spoke in favour of the assumption that /-/deprenyl enhances the dopaminergic tone on the brain /Knoll, 1978/ but we needed direct evidence. The part of the brain richest in dopamine is the striatum. We needed to compare how much dopamine and dioxyphenylacetic acid /DOPAC/, the metabolite of dopamine, is emitted from the dopaminergic nerve terminals in the striatum during the stimulation of the dopaminergic neurons in untreated and /-/deprenyl treated rats. The only technique which allowed us to measure these changes was HPLC. Using this method we were able to demonstrate that in response to the stimulation of the dopaminergic nerves of a striatum taken from a rat pretreated daily with /-/deprenyl for 21 days releases significantly higher amounts of dopamine and lower amounts of DOPAC than a striatum taken from a rat treated with a daily injection of saline for 21 days. Fig. 3 shows an experiment. Direct evidence for such an effect of /-/deprenyl seems to support substantially our view regarding the potential usefulness of

The effect of Deprenyl pretreatment on DA and DOPAC release.



Superfused isolated striatal slices of the rat.
Stimulation: 20 mmol/l KCl

Fig. 3. The demonstration of the enhancement of dopamine release from the striatum of a rat pretreated with /-/deprenyl, by the aid of HPLC.

the long term administration of our drug to improve the quality of life in senescence.

I do not want to go into more details with regard to our biological problems, I just wanted to show you two examples of my personal successful encounters with the science of chromatography.

Ladies and Gentlemen,

The Hungarian Pharmacological Society followed with great attention the two conferences on chromatography in Szeged, initiated and organized by Dr. Dévényi and gladly joined to this meeting as its sponsor. We are aware of the invaluable importance of your science. I myself am pleased to see here in Budapest so many comparative chromatographers and express my admiration for your scientific achievements from which I derived so much benefit.

I wish you a successful meeting, pleasant days in Budapest and further progress in the development of more and more sophisticated gelchromatographic methods.

REFERENCES

Knoll, J.: The Theory of Active Reflexes. Akadémiai Kiadó, Budapest, and Hafner, New York, 1969. p. 131.

Knoll, J.: Satietin: a highly potent anorexogenic substance in human serum. Physiology and Behavior, 23, 497-502, 1979.

Nagy, J., Kalász, H., Knoll, J.: Isolation and characterization of a highly selective anorexogenic substance by chromatography and electrophoresis. In: Chromatography and Mass Spectrometry in Biomedical Sciences 2, /Ed.: A. Frigerio/, Elsevier, Amsterdam, 1983, pp. 421-42.

Knoll, J.: The possible mechanism of action of /-/deprenyl in Parkinson's disease. J. Neural Transmission, 43, 177-198, 1978.

Knoll, J.: Selective inhibition of B type monoamine oxidase in the brain: a drug strategy to improve the quality of life in senescence. In: Strategy of Drug Research. Ed.: J.A. Keverling Buisman, Elsevier, Amsterdam, 1982, pp. 107-135.

THEORETICAL ASPECTS

Chromatography, the State of the Art
H. Kalász and L.S. Ettre (Eds)

QUO VADIS HPLC

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The separation method invented and named chromatography by the Italorussian botanist M.S. Tswett at the beginning of this century has become the most widely used analytical technique and the most powerful tool for separating biochemical substances. The last stage in the evolution of chromatography began about a score of years ago when high performance liquid chromatography has emerged. Now the acronym HPLC is a word on its own is almost every language, synonymous with advances in analytical chemistry, separation science and life sciences. In the last ten years we have witnessed a particularly rapid growth of this chromatographic technique and its wide ranging impact on science and technology.

The roots of HPLC go back to the introduction of partition chromatography by A.J.P. Martin and R.L.M. Synge in 1941 who have firmly established linear elution development as the preeminent mode of chromatography for the decades to come and made possible the quantitative treatment of the chromatogram by using the plate theory. In those days, lack of suitable adsorbents and sufficient column efficiency thwarted the efforts by others to use the by then popular adsorption chromatography in the linear elution mode and to make displacement chromatography efficacious.

A new feature of chromatography has commenced upon the introduction of paper chromatography by Martin and co-workers in 1944; it has become a microanalytical technique of great versatility with particular significance in the life sciences.

The next milestone was associated with the invention of gas chromatography by Martin and James in 1952 which procreated the chromatograph. With the instrument, the chromatographic process could be controlled conveniently and with high precision. Instrumentation made it possible for chromatography to benefit from many technological advances such as those in electronics and data handling. In turn a sizable industry devoted to the manufacture of chromatographic instruments and supplies has evolved over the years. Furthermore, the theory of linear elution chromatography has made vast progress in gas chromatography, which made it possible to obtain precision data with great facility. Thus, the meteoric growth enjoyed by gas chromatography was caused by cross-fertilization of theoretical and practical developments besides the wide ranging applications of the technique.

In the early sixties the groundwork had been laid to integrate all the above features of chromatography. The amino acid analyzer which evolved from the work by Stein and Moore in 1958, was the first "liquid chromatograph" ,but as an instrument dedicated to the analysis of only one kind of sample it did not have the versatility that characterized the gas chromatograph, and it was comparatively slow. For a general approach to design a liquid chromatograph in the early sixties, theoretical considerations suggested that when the mobile phase is a liquid, to attain the speed and efficiency typical for gas chromatography the column inlet pressure had to be much higher than it was customary in gas chromatography and in traditional liquid chromatography. Therefore, columns and instruments were constructed from steel and high pressure pumps were used to generate eluent flow.

Theory also predicted that the column material should consist of particles having a 5-10 micron diameter. However, no reasonably homodisperse sorbents of this particular size range were available in the early sixties. The problem was bypassed by the development of pellicular sorbents which were mechanically stable and size graded by screening because they yielded significantly higher efficiency than totally porous sorbent particles of the same size. At the end of the sixties, particle

technology advanced sufficiently to obtain 10 micron silica gel particles by air classification and the relatively large pellicular sorbent particles were largely phased out in the early seventies.

Silica gel had already been the prime stationary phase in traditional solid-liquid chromatography and in microparticular form together with ion-exchangers served at the beginning of HPLC as the most frequently used, high efficiency stationary phases. A major step forward came about by the introduction of silica-bound hydrocarbonaceous stationary phases. The concept goes back to the work by Howard and Martin in 1948 who treated siliceous support with trimethyl chlorosilane and formed a molecular fur of hydrocarbonaceous silane functions covalently bound to the surface by siloxane bridges. The hydrophobic support thus obtained was used in reversed phase partition chromatography introduced by these authors.It has been recognized in the late sixties and early seventies that silica gel with a long chain hydrocarbonaceous moiety bound to the surface can serve as a broadly useful stationary phase per se in reversed phase chromatography with hydro-organic eluents. The use of bonded phases not only eliminated the numerous practical problems arising from energetically heterogeneous surface of native silica gel, but also made possible the use of aqueous mobile phases, thus the chromatography of biochemical substances. The wide dynamic range of eluent polarity and the operational simplicity and convenience of reversed-phase chromatography has been responsible for the rapid acceptance of HPLC as the prime method of chemical analysis and thereby greatly expanded the scope of instrumental liquid chromatography.

Although life sciences are a major beneficiary of HPLC today, the technique was not widely considered as a useful tool of biochemical analysis in its early years. Life scientists, who employ chromatographic methods more extensively than the members of any other profession, appeared to experience three phobias: barophobia, the fear of high pressure; siderophobia, the fear of working with steel columns and equipment; and, lithophobia, the fear of contacting delicate biological substances with siliceous stationary phases. The facility, ef-

ficiency and convenience of reversed-phase chromatography were important factors to overcome these phobias and to accept HPLC in life sciences. Perhaps this is the reason that HPLC is quite frequently identified with reversed-phase chromatography.

Since the dominant features of modern liquid chromatography have been recognized, the acronym HPLC is attributed to mean high performance liquid chromatography a technique which results in rapid separations with high efficiency and uses precision instruments under tightly controlled conditions. Sophisticated pumps and sample injections facilitate reproducible and accurate results whereas sensitive detectors make it possible to quantify sample components at the picogram level. With the high efficiency columns available today, HPLC is used in almost every branch of science and technology.

Although it has been said by many experts a few years ago that we have reached the peak because everything had been accomplished as far as the development of HPLC is concerned. I don't think so! In my opinion, it has been just a beginning and we shall witness HPLC burgeoning in the future.

As the ultimate tool of chemical analysis, at some point in time the liquid chromatograph will find its place on every bench. The trend toward miniaturization stems from the recognition that a liquid chromatograph of the size of a bread box or typewriter could handle the analysis of a few micrograms of material and there is no need for the large instruments which we have been accustomed to. Consequently, reduction of column dimensions is inevitable, and after the formidable engineering problems are solved, HPLC will follow suit in the way as computers have done.

Chemistry features prominently in liquid chromatography. Upon accumulating more and more precision data, our understanding of the physico-chemical process underlying chromatographic separation will increase to the point that we shall have sufficient insight into the separation process either by rigorous theory or through well examined case histories. The understanding of chromatography will not be the privilege of a select few, but de rigueur for the many who will use the technique. Besides analyzing minute quantities, HPLC will also

contribute to the physico-chemical characterization of the sample components because chromatographic data entail a great deal of information on their molecular behaviour.

Recently, strides have been made in HPLC of macromolecules particularly with proteins and nucleic acids. Although it is not clear yet which branch of chromatography, i.e. size exclusion, affinity or hydrophobic chromatography, will be most widely accepted, the advantages of the technique have been clearly recognized, and it is only a matter of time that HPLC becomes the method par excellence for the rapid analysis of biopolymers.

New types of stationary phases are needed to exploit the potential of HPLC in the different areas of applications. Not long ago the success of reversed phase chromatography with octyl- and octadecyl-silica might have implied that only a small number of column types are required in liquid chromatography and the variation of the eluent composition suffices to handle any kind of separation on problems. As the scope of HPLC is being expanded we are recognizing the constraints imposed on the mobile phase composition by the properties of the sample components, and concomitantly by their interactions with certain mobil phase constituents which give rise to conformational changes, secondary equilibria, denaturation and to untoward kinetic phenoma resulting in poor separation efficiency.

In columns packed with 2-3 micron particles diffusional resistances to the chromatographic phase - exchange process are reduced to a level where the separation efficiency for complex eluite molecules largely depends on the relatively slow kinetics of certain secondary processes or desorption under the usual operating conditions. In practice optimization of the separation involves finding an appropriate combination of the mobile and stationary phases which yields sufficient retention and selectivity without encountering significant band spreading due to processes which are superimposed on the actual chromatographic process and have a characteristic time on the time scale of the separation. When manipulation of the mobile phase composition, which is often limited, does not solve the problem, it is expedient to employ another type of column. The

same considerations apply to chromatography of biopolymers when denaturation has to be avoided. In large scale chromatography it is even conceivable that the stationary phases have to be taylored to the separation problem proper in order to maximize throughput and to facilitate product recovery from the effluent.

Silica gel has been the more useful support material for bonded phases. It is relatively easy to manufacture with desirable pore morphology and dimensions and its surface can be readily modified to impart the functionality required. In fact, siliceous stationary phases are used in HPLC almost exclusively. It appears that the potentiality of silica gel have not been exhausted yet. Our knowledge of the surface topography and its role in the chromatographic retention process is on the increase and we can expect that upon refinement of the bonding chemistry the arrangement of the functional groups at the silica surface can be designed to obtain various stationary phases with optimal properties for different types of separations. It is hoped that these advances are accompanied by progress in specifying the properties of the stationary phases so that they can be characterized in a systematic fashion.

As the processes involved in analytical separations are increasingly understood, more attention is paid to preparative liquid chromatography which is benefitting from this development. The emerging biotechnology needs not only efficient techniques for process control and product analysis, but also for industrial scale separation processes and liquid chromatography promises to become a strong candidate. We should keep in mind, however, that there is a great deal of difference between separating submicrogram quantities for analytical purposes and kilograms to obtain a pure product. Therefore, optimum design of each system requires different approaches. The equipment, the stationary and mobile phases are poorly utilized in linear elution chromatography because linearity is maintained by keeping the concentrations of elutes low. In preparative chromatography, one attempts to operate at the highest possible concentration of the feed components to be separated and as a result the conditions of linear elution chromatography do not apply. It may be expedient, therefore,

to employ the displacement mode of chromatography for large scale operations. This nonlinear chromatographic process had been overshadowed by linear elution chromatography which is used exclusively in analytical work. Furthermore, high column efficiency and the availability of suitable sorbents are prerequisites to the successful use of displacement chromatography and they have only recently been met in liquid chromatography. In this technique the non-linearity of adsorbtion isotherms at sufficiently high concentrations is exploited and when the separation is accomplished upon the effect of the displacer all components move with the same velocity as adjacent bands. Whereas practical experience in the selection of the column and displacer is still at an inchoate stage and the perception of, and the transition to non-linear chromatography is not without difficulties, the advantages of displacement chromatography will inevitably engender a percipient following.

The cradle of HPLC was the liquid chromatograph and instrument design has remained a fertile ground for further advancement of the technique. New ideas abound to make instrumentation more convenient to use, to engage novel principles for detection and to expand the scope and versatility of the liquid chromatograph.

HPLC has been the protagonist in the recent revolution in analytical chemistry and likely to remain so at least in the next few years. Nurtured by an infusion of ideas, it will gain wider and wider application and remain an exciting area for research for both scientists and engineers.

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ELECTROCHEMICAL DETECTION IN CHROMATOGRAPHY

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INTRODUCTION

Although spectrophotometry has retained its leading position as flow-through detection principle, among the complementary detectors electroanalytical sensors have an eminent role. They are ideally suited for automation because of their simplicity and because they give directly electrical signals.

They have been used recently in areas such as segmented and unsegmented flow analysis /especially for serial analysis of individual samples/, monitoring of different,e.g. physiological processes and liquid chromatography.

The selectivity, sensitivity and working range of electrochemical detectors can widely vary. Their selectivity is different from that of spectrophotometric and other detectors due to the different operation principle.

Amperometric and potentiometric detection in chromatography

Amperometric and potentiometric detection can be used in GC and LC but most applications are in the latter. Since 1972 about six hundred publications have dealt with HPLC-EC.

Amperometric detectors include dropping mercury electrodes and electrodes with stationary metal or carbon surface. The basic process at the electrode is usually the oxidation or reduction of the detectable compounds, but adsorption phenomena can also be used to advantage /1/. The range of detectable

species consists therefore mainly of compounds which can be reduced or oxidized at the above electrodes in the chromatographic eluent or after a make-up fluid has been mixed to the eluent. In any case a certain electrical conductivity of the solution is necessary. Among the substances that can be easily determined by amperometry we find many biologically active substances. These are often determined by RP-LC in aqueous or partly aqueous eluents which is favourable for EC detection. For these reasons amperometric detectors have found yet widest chromatographic application in the analysis of compounds of biological importance, foods, etc.

Amperometric detection has a very low detection limit which is somewhat surprising even for electrochemists. Concentration detection limits below 10^{-10}M have been achieved. This should be contrasted to earlier literature on other uses of amperometric detection with detection limits around 10^{-7}- 10^{-8}M. Amperometric detectors had been used in flow analysis already before they became generally known as HPLC detectors. Experience gathered in such applications may be a useful aid in HPLC-EC. The branch of electrochemical /amperometric/ measurements under mixed convective and diffusive transport has been termed hydrodynamic voltammetry /2/. A number of detailed studies are available /3/ which describe the relation between the current passing through the working electrode and the physical parameters of the transport process, e.g. bulk concentration of the analyte, flow rate, viscosity and electrode geometry /Table 1./ These results may prove very useful in the design and operation of amperometric detectors for HPLC.

Amperometric detectors are usually operated at constant potential vs. a suitable reference electrode. In this way lower detection limits can be achieved then by potential sweep methods, which are preferred in batch analysis. At constant potential the background current is often fairly stable, there is no capacitive charging current and no current connected to surface reactions evoked by potential change. The constant background current can be efficiently compensated by electronic means. To achieve very low detection limits suitable new

Table 1. Limiting current equations for voltammetric electrodes

Electrode shape	Equation
Rotating disc	$i_L = knFAc_o D^{2/3} \nu^{-1/6} \omega^{1/2}$
Planar /parallel/	$i_L = knFb.h^{1/2} c_o D^{2/3} \nu^{-1/6} V^{1/2}$
Tubular	$i_L = knFR^{2/3} x^{2/3} c_o D^{2/3} V^{1/3}$
Disc /opposite/	$i_L = knFAL^{1/2} c_o D^{2/3} \nu^{-1/6} V^{1/2}$

i_L is the limiting current
k is constant
n is the number of electrons taking part in the electrode reaction
F is the Faraday constant
A is the geometric surface area of the working electrode
c_o is the concentration of the electroactive species in the moving solution
D is the diffusion coefficient of the electroactive component
ν is the kinematic viscosity of the solution
V is the flow rate
ω is the rotation speed
b, h, R, x and L are the geometric characteristics of the electrodes

electronic devices are needed, because earlier designs were not meant for measuring currents in the pA range.

Detector performance depends very much on the electrode material and geometry. Residual current, which is an important factor in determining the lower detection limit, may be increased disadvantageously by surface reactions of the electrode material itself. The quality of the working electrode's surface also influences adsorption processes which may lead to electrode desactivation.

The lower detection limits of amperometric detectors for various substances can be very different. Three factors are very important in this respect: the oxidation /or reduction/ potential of the substance at the electrode, the number of electrons exchanged /involved/ in the electrode process at the electrode surface per molecule and the ease of electron transfer. Increased detection limits can be expected if the working potential of the electrode is too low for the given reaction, if only one or two electrons are exchanged per molecule and if the electron transfer needs a high activation energy /overpotential/ /"irreversible processes"/ Working at high potentials may increase sensitivity /i.e. current/concentration ratio/ but it also increases the background current and the noise. For any given substance there is usually an optimum working potential /range/. When many substances have to be determined the choice of the working potential must be usually a compromise.

A frequently encountered problem with electrochemical detectors has been surface desactivation by film formation. A non conducting film can be deposited if e.g. in the first step of the oxidation process free radicals are produced which polimerize in a consecutive chemical reaction. Filming typically occurs with certain phenolic compounds and aromatic amines. With present day amperometric detectors for liquid chromatography filming is not always a serious problem. This can be attributed to several factors:

- at the very low concentration range filming is less probable
- high linear flow rates help to keep the electrode surface clean

- in the analytical problems solved with these detectors film forming compounds either are not present or are eliminated by sample preparation.

Amperometric detectors are usually working in the simple DC mode. It is well known, however, that there are other methods of electroanalysis, e.g. pulse methods, which have lower detection limits in stationary solutions than the DC method. Expectations that the same might apply to amperometric detectors do not hold /4/. Problems with electrode surface reactions may be responsible for this.

There are other non-classical electrochemical techniques which can be applied more successfully in chromatographic detection. The ring-disc electrode,for example, has its counterpart in LC-EC, the so-called series dual-electrode detection /5/.

Determinations based on electrochemical reduction are at least as important as oxidation reactions. The classical electrode for reduction, the dropping mercury electrode is, however, not so easily adapted to the small cell volumes allowed by LC band broadening requirements.

Much work has been done to design chromatographic systems with dropping mercury, mercury pool and mercury coated solid /metal and graphite/ electrodes as working electrodes. Actually,there are only few detectors containing dropping mercury electrode on the market /PAR Co, USA; Tacussel, France/ and there is much less experience with this type of detectors than with the solid electrode containing ones. In certain cases, the use of solid electrodes enables the determination of reducible components, too. The interference caused by the reduction of dissolved oxygen can be overcome with the help of the dual electrode detector mentioned above.

Potentiometric detectors, i.e. metal electrodes and ISE-s have a much more limited applicability in chromatography. Although they are usually very selective to a single compound, they have a number of drawbacks. Most electrodes are sensitive to inorganic species. Selective detection of these species is seldom requested, although application in ion chromatography

has been reported /6/. Here, detection by ISE-s may make the suppressor column superfluous. Organic species can be detected by potentiometric sensors in two ways. There exist composite electrodes /called sensitized electrodes/ which can measure very selectively organic compounds by using an enzyme reaction /the enzyme is immobilized on the electrode surface/. Early prototypes of enzyme electrodes showed, however, too slow response. Recent reports confirm that fast signals can also be measured by these electrodes. A further possibility to detect organic species by ISE-s is to use postcolumn reactor where the separated organic substances react with an inorganic species sensed by the electrode. There have been reports on reacting amino acids with metal ions /e.g. Cu^{2+} /7// and measuring the change in metal ion concentration.
Ion selective electrodes usually have wide dynamic range. Their sensitivity is sufficient under the stable conditions of a chromatographic flow-through cell. Detection limits are above 10^{-7}M, in some cases only about 10^{-5}M.

The working of amperometric and potentiometric sensors is connected with heterogeneous reactions at solid surfaces /except for mercury electrodes/. Heterogeneous reactions are rather sensitive to surface quality. For this reason, these detectors may require more care than e.g. spectrophotometric detectors.

Electrochemical detectors have been investigated in our laboratory mainly in conjunction with flow analysis. This means that we use a system which is equivalent to an LC system without separation column. Such and similar systems have been used for speeding up serial analyses /8-10/. The detector signal is peak shaped and the band broadening is determined by what is called extracolumn effects in chromatography. In the actual system we have used in this work /LKB 2115 Multiperpex pump, MTS Slide Valve 24144 two-loop plastic injector, home made wall jet detector cell, PAR 174A polarograph/ and at flow rates of about 1 ml/min we observed peaks with several seconds width at half peak height. Sample sizes were quite large /40 and 120 µl, respectively/. The implications of such a system for chromatographic detection are clear. One can study the effects of possible eluent compositions and different

analytes on the response of the electrochemical detector in much shorter time than with a chromatographic system. Costs are also saved: a cheap peristaltic pump can be used and no column packing material is needed. Approximate chromatographic detec - tion limits can be calculated by considering the differences in peak broadening between the two systems. Not all results can be transferred directly to chromatography, however.The signal to noise ratio may be influenced e.g. by differences in the hydraulic system. For our investigations we have used the well known thin layer cell design /11/ and a modified wall jet design. The latter one can accomodate almost any type of electrode, also ISE-s Calibration lines /peak height vs. concentration of injected sample/ are shown in Figs.1,2. for dopamine and serotonine, resp. Linear calibration line was observed down to 10^{-9} and $2x10^{-10}$M, respectively. The latter figure is roughly equivalent to detecting 10^{-13} moles or about 20 pg serotonine in a chro- matographic peak of 1 ml baseline width. Since we were not using a column, the actually detected quantity was about 2 pg. With dopamine, similar calculations result in an expected chromatog- raphic detection limit near 100 pg. An actual chromatogram with 100 pg dopamine is shown in Fig. 3.

It should be noted that in the chromatographic literature detection limits are usually given as quantity detectable at signal/noise=2. We have found that results given in this form characterize the performance limit obtained less reliably than showing the smallest reproducibly detected chromatographic peak together with a sufficiently long portion of the baseline, and stating the molar concentration of the detected species at the maximum of this peak. While the recorder trace of the peak would show the really observed detection limit which is an index of system performance, the concentration at this peak's maximum would characterize the performance of the detector. There have been reports in the literature /e.g. 12,13/ on the chromatographic detection of a few pg-s of biogenic amines with amperometric detection. This is roughly equivalent to measuring these substances at a concentration level of 10^{-11}M at the peak maximum.

Our experiments and the quoted results show that amperometric

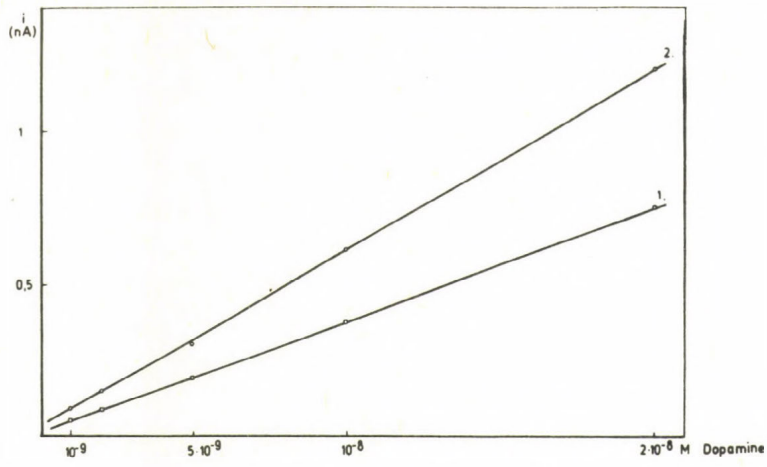

Fig. 1 Amperometric calibration lines obtained with flow injection technique for dopamine
1. Injected volume: 40 µl
2. Injected volume: 120 µl

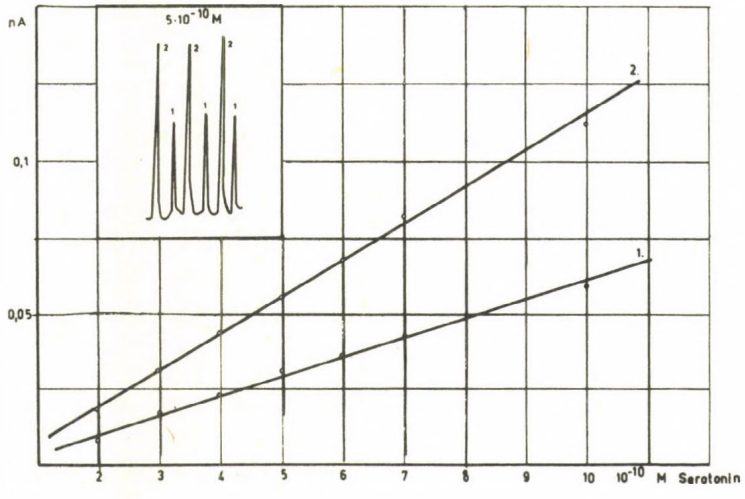

Fig. 2 Amperometric calibration lines obtained with flow injection technique for serotonin
1. Injected volume: 40 µl
2. Injected volume: 120 µl

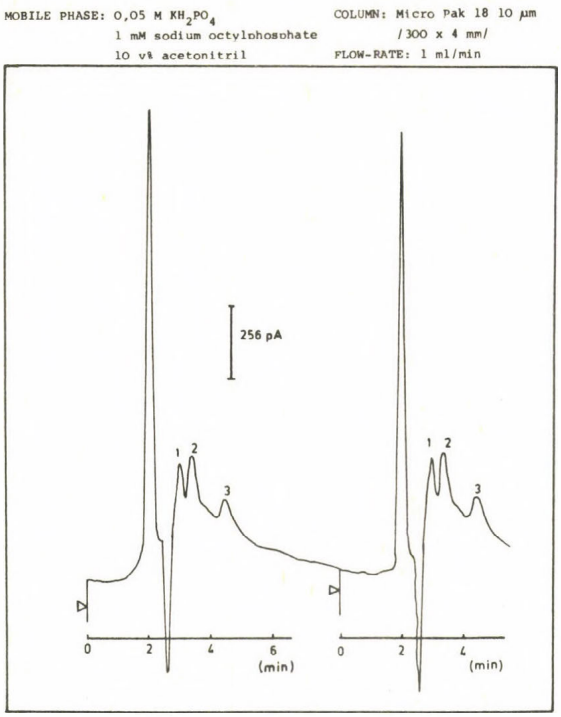

Fig. 3 Chromatogram of catecholamines recorded with amperometric detector

1. nor-epinephrine 132 pg
2. epinephrine 169 pg
3. DOPA 98 pg

detection is one of the most sensitive concentration detection techniques and it appears to have the best performance/cost ratio in this respect. Although chromatographic applications are in the foreground at present, we hope to be able to utilize this remarkable sensitivity in other areas of chemical analysis as well.

A further interesting topic is the use of electroanalytical detectors based on conductance measurements. However, the electrical conductance is never a selective parameter of a given species, rather a signal characteristic to the sum of all ionic species being present in the solution. Despite this, one can use it under definitive conditions for analytical determination of the concentration of a component.

The measurements can be carried out in two ways: either with measuring cells incorporating electrodes being in galvanic contact with the solution, or with cells in which the electrodes are isolated from the solution tested. The former is called the conductometric method, the latter one is the so called oscillometric technique. Permittivity is to be measured also with cells having galvanic contact between the electrodes and solution.

Conductometric and permittivity detectors were used in the recent time for flow analysis, especially for chromatographic investigations. Pecsok and Saunders /14/ Tesarik and Kalab /15/ Stankoviansky, Cicmanec and Kamiansky /16/, Jackson /17/, Kambara and Tachikawa /18/, Duhne and Sancher /19/, Svoboda and Marsal /20/ and in recent literature Jupille, Togami and Burger /21/, furtheron Evans and Stolz /22/ have shown important applications of the conductometric technique for solving first of all chromatographic problems.

The permittivity measuring methods with micro cells were applied without exceptions to chromatography. Haderka /23/ Vespalec and Hana /24/ Poppe and Kuysten /25/ Krejci and Pospisilova /26/ Krejci, Vespalec and Sirec /27/ Alder and Thoer /28/ have published papers dealing with permittivity measurements in chromatography.

Both of these techniques show the disadvantage that the direct contact of solution and electrode gives rise to a lot of unwanted reactions. Therefore it seemed very useful to

elaborate oscillometric technique for flow analysis and chromatography /29/. We used a condenser type microcell, the equivalent circuit of which is represented in Fig. 4. In our microcell the stray capacity is small in comparison to the measuring capacity which makes possible a direct measurement of the signal depending on permittivity of the cell having a high electric resistance.

The theoretical description of the cell leads to the following expression

$$K = \frac{\omega^2 R C_s^2}{1+\omega^2 R^2 (\varepsilon C_m + C_s)^2}$$

where
- K a.c. conductance
- ω angular frequency
- R d.c.resistance
- C_s stray capacity
- C_m measuring capacity
- ε the permittivity

When C_s is negligible compared to C_m and in the denominator 1 is small compared to the second term, then

$$K = \frac{C_s^2}{\varepsilon^2 R\, C_m^2}$$

while $C_m = \varepsilon\, C_{m,o}$

where ε = permittivity

$C_{m,o}$ = vacuum capacity of the cell, then

$$K = \frac{C_s}{\varepsilon^2\, R.C_{m,o}^2}$$

One may measure the ε, if R is high and constant.

The oscillometric capacitive cell developed by us has a dead volume of 10-20 µL. Recently we have constructed cells in µL dimensions. A model of such a cell is given in Fig. 5.

Using the micro oscillometric cell for a.c. conductance measurement we established that the calibration line is linear between 10^{-6}M and 10^{-3}M. The limit of detection is in µg ionic amount.

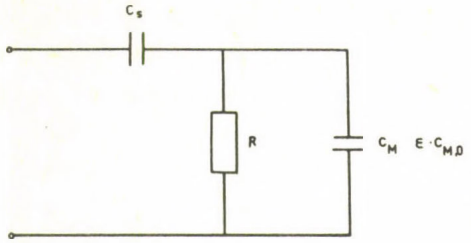

Fig. 4 Equivalent circuit of a condenser type oscillometric cell

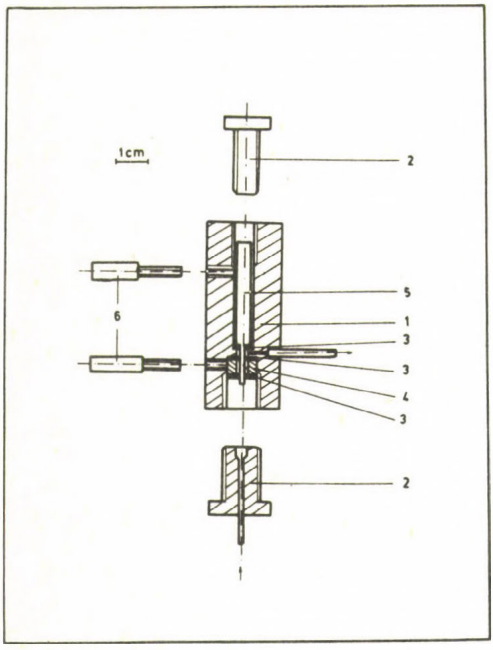

Fig. 5 Oscillometric microcell

1. Cell body
2. Fixing screw
3. Teflon sealing
4. Grounding electrode
5. Measuring electrode
6. Cell connection

In aqueous solutions /high permittivity/ conductance measurements were carried out. Fig. 6 shows the results of experiments with injected KCl solution. The amounts injected were 1.10^{-10} mole, 2.10^{-10} mole and 10^{-9} mole of potassium chloride. The relative standard deviation of the measurements was less than 0,5% above 10^{-9} mole and below it its value was about 1,5%.

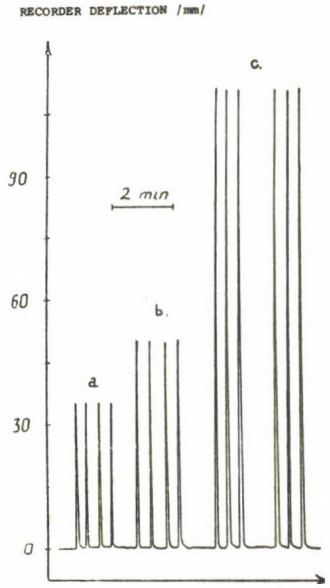

Fig. 6 Recorder trace obtained with an oscillometric micro-cell as potassium chloride is injected
a. 10^{-10} mole
b. 10^{-10} mole
c. 10^{-10} mole

The methods mentioned above were developed for application in flow analysis. However, the cells were constructed in a way that their dead volume is very low, and they are applicable to liquid chromatography in general. Our investigations in this direction are in progress. The preliminary results show the validity of the idea.

For the improvement of the capability of electrochemical detectors in chromatography attempts are made in different aspects. One of the permanent requirements lies in the improve-

ment of the detection limit, for which e.g. detector cells of extremely small volume have been designed /30,31/. The improvement of the parameters of the electronic circuitry of the electrochemical detector helps in this respect, too.

To achieve a better selectivity - especially in the case of amperometric detectors - various efforts have been made, e.g. fast scan a.c. voltammetry has been used for this purpose /32/. The use multiple electrode detectors can serve the same purpose.

With the aim of broadening the range of application of electrochemical detectors different post-column reactions have been employed.

Moreover, the potentiality of electrochemical detection allows to draw more information about the system by the use of known electrochemical techniques, e.g. cyclic voltammetry, ring-disc voltammetry.

Besides this, new type of detectors e.g. oscillometric one opens up new areas, especially with ion chromatography.

SUMMARY

The paper gives a short survey on electrochemical detection in chromatography. The amperometric, potentiometric as well as oscillometric detectors are discussed first of all with electroanalytical aspects. A few examples are shown for the potential use of the sensors and a brief outlook of the subject was shown.

Acknowledgement

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References

1 Kutner,W., Behr,B., Kemula,W., Z.Anal.Chem. 312 /1982/ 121

2 Jordan,J., Javick,R.A. and Ranz,W.E., J.Am.Chem.Soc. 80 /1958/ 3846

3 Levich,V.G., Physicochemical Hydrodynamics, Prentice Hall, Englewood Clifts, N.J. 1962.

4 Dieker,J.W., Van der Linden,W.E., Poppe,H., Talanta 26 /1979/ 511

5 Roston,D.A., Shoup,R.E., Kissinger,P.T., Anal.Chem. 54 /1982/ 1417A

6 Slanina,J., Bakker,F.P., Jongejan,P.A.C., Van Lamoen,L., Möls,J.J., Anal.Chim.Acta, 130 /1981/ 1

7 Loscombe,C.R., Cox,G.B., J.Chromatogr. 166 /1978/ 403

8 Nagy,G., Fehér,Zs., Pungor,E., Anal.Chim.Acta 52 /1970/ 47

9 Fehér,Zs., Nagy,G., Tóth,K., Pungor,E., Analyst, 99 /1974/ 699

10 Pungor,E., Fehér,Zs., Nagy,G., Tóth,K., Horvai,G., Gratzl,M., Anal.Chim.Acta 109 /1979/ 1

11 Kissinger,P.T., Refshauge,C.J., Dreiling,R., Blank,L., Freeman,R., Adams,R.N., Anal.Lett., 6 /1973/ 465

12 Michand,R.L. Bannon,M.J. Roth,R.H., J.Chromatogr. 225 /1981/ 335

13 Grossman,P., Chimia 37 /1983/ 91

14 Pecsok,R.L., Saunders,D.L., Anal.Chem. 40, /1968/ 1765

15 Tesarik,K., Kaláb,P., J.Chromatogr. 78, /1973/ 357

16 Stankoviansky,S., Cicmanec,P., Kaniansky,D., J.Chromatogr. 106, /1975/ 131

17 Jackson,A., J.Chem.Educ. 42, /1965/ 447

18 Kambara,T., Tachikawa,T., J.Chromatogr. 32, /1968/ 728

19 Duhne,C., Sancher,O., Anal.Chem. 34, /1962/ 1074

20 Svoboda,V., Marsal,J., J.Chromatogr. 148 /1978/ 111

21 Jupille,R., Togami,D., Burger,D., Abstract 242 Pittsburgh Conference 1982

22 Evans,B.,Stolz,J., Abstracts 247 Pittsburgh Conference 1982

23 Haderka,S., J.Chromatogr. 57, /1971/ 181
24 Vespalec,R., Hana,K., J.Chromatogr. 65, /1972/ 53
25 Poppe,H., Kuysten,J., J.Chromatogr. 132, /1977/ 369
26 Krejci,M., Pospisilova,N., J.Chromatogr. 73, /1972/ 105
27 Krejci,M., Vespalec,R., Sirec,M., J.Chromatogr. 65, /1972/ 333
28 Alder,J.F., Thoer,A., J.Chromatogr. 178, /1979/ 15
29 Pungor,E., Oscillometry and Conductometry, Pergamon Press, Oxford,London,Edinburgh,New York,Páris 1965
30 Slais,K., Kourilova,D., J.Chromatogr. 258 /1983/ 57
31 Slais,K., Krejci,M., J.Chromatogr. 235, /1982/ 21
32 Trojanek,A., De Jong,H.G., Anal.Chim.Acta, 141, /1982/ 115

Chromatography, the State of the Art
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DEVELOPMENT OF TRANSPORT DETECTORS FOR LIQUID CHROMATOGRAPHY

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For detection of low-volatile and non-volatile substances separated on an LC column, transport or transport-destructive detectors have been proposed /1-3/ /a typical transport detector is the moving-wire detector/. Despite their relatively complicated design and low reliability in operation, these detectors are still of interest, particularly when used in connection with other detectors /4/. Transport detectors offer the following advantages: a. high sensitivity, b. independence of the eluent composition, c. versatility.

The use of such detectors in quantitative analysis requires extremely careful calibration and maintenance of constant analysis conditions, particularly flow rates /5/. In order to maintain the experimental conditions constant and to ensure that the entire experiment is conducted in a strictly controlled atmosphere, we have developed a special arrangement in which the transport detector is completely isolated from the environment, and the transport detector can be used with different carrier gases. We know of no other system in which a similarly designed transport detector is used.

The following design features were planned: /a/ using a tape instead of the wire to enhance the capacity of the eluate-carrying element; /b/ enclosing the system in a chamber to intensify the processes of evaporation and pyrolysis, simplify the design, increase its reliability, improve the operator's working conditions, etc.; /c/ using a forced flow of gas to evaporate the eluent; and /d/ using gas locks.

INSTRUMENTATION AND EXPERIMENTAL PROCEDURE

For detection in liquid chromatography we have developed a device shown in Fig. 1. Mounted on base 1 are two airtight compartments 2 accomodating drive pulley 3 and pulleys 4 and 5 guiding the tape. Arranged between the compartments are the evaporation chamber 6, the pyrolysis chamber 7 and the purification chamber 8, all three integrated into quartz tube 9. Unions 10, 11, 12 and 13 are arranged between the chambers and the compartments, provided with fine-adjustment valves. Electric heater coils heat the above-mentioned chambers. The quartz tube is hermetically coupled to the compartments. The guide pulleys carry the endless metal tape 15. The capillary feeder 16 dispenses the eluate from the LC column onto the tape.

The device operates as follows. The eluate from the LC column is applied onto the tape via the capillary feeder. The tape with the eluate is continuously fed through the evaporation chamber where the volatile eluent is evaporated from the eluate at a certain temperature, then through the pyrolysis chamber in which the substance being detected is vaporized at an elevated temperature or pyrolyzed, and finally through the purification chamber where the tape is heated to a temperature higher than in the pyrolysis chamber. Finally, the tape is reversed through this chamber with the aid of the pulley system. The vaporized substance or pyrolysis products are delivered from the pyrolysis chamber into the GC detector by the carrier gas. Compartments 2 are purged through special valves. The eluate is brought in and applied on the tape in a microchamber.

This device includes two airtight compartments with the drive mechanism and guide pulleys for the tape, between which a quartz tube with an internal diameter of 3 mm is arranged and provided with gas flow inlets and outlets. The tube sections corresponding to the evaporation, pyrolysis and purification chambers are heated by electric coils wound directly on the tube. The temperature in the chambers is maintained by stabilized voltage regulators and controlled by means of thermocouples. The eluate-carrying tape is made of nichrome, 0.1 mm thick and 2 mm wide. It is fed at a rate of 1.2 m/min. The

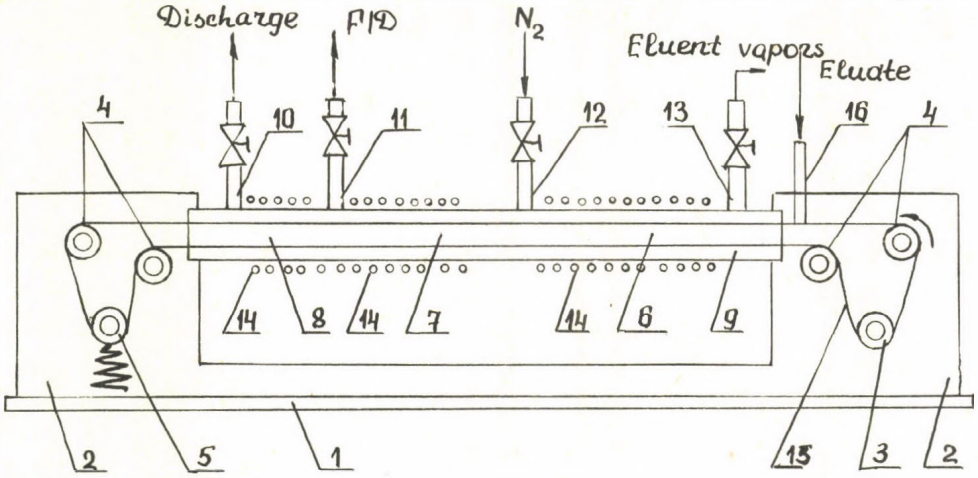

Fig. 1: Transport detector for liquid chromatography

1 - base
2 - airtight compartments
3 - drive pulley
4, 5 - guide pulleys
6 - evaporation chamber
7 - pyrolysis chamber
8 - purification chamber
9 - quartz tube
10, 11, 12, 13 - unions with fine-adjustment valves
14 - electric heater coils
15 - metal tape
16 - capillary feeder.

carrier gas is ultra-pure nitrogen. Its pressure in the detecting device is 1 atm. The carrier gas flow rates are 300 to 500 ml/min through the evaporation chamber, 30 to 50 ml/min through the pyrolysis chamber, and 3 to 5 ml/min through the purification chamber.

The relationship between the chromatographic peak area and pyrolysis chamber temperature has been established. The optimum

pyrolysis temperature is in the range of 575 to 685°C. The carrier gas is directed from the pyrolysis chamber into the FID via a flow restrictor. The eluent applied on the tape through the capillary feeder is tetrahydrofuran, the flow rate being 0.5 ml/min.

In order to determine the reproducibility of the detecting device, 10- μl amounts of calibrating solutions of polystyrene in tetrahydrofuran were injected into the eluent flow for 5 seconds with the aid of a microvolume syringe. The following temperatures were maintained: 100°C in the evaporation chamber, 600°C in the pyrolysis chamber, and 700°C in the purification chamber.

RESULTS AND DISCUSSION

Chromatograms of the calibration solutions consisting of polystyrene in tetrahydrofuran at concentration of 0.001 %, 0.01 % and 1 % were obtained. The results are summarized in Table I. The noise level in the detecting device was 2 % on a 10^{-10}A scale, i.e. $2x10^{-12}$A. The relationship between peak area and sample size has been determined. It indicates that the linear range of the detecting device reaches 1: 1000. The minimum detectable amount of analyzate is $2x10^{-8}$ g.

The developed detecting device has a sensitivity /slope/ of $1.98x10^{-8}$A (mg/s) and a sensitivity threshold of $0.93x10^{-9}$g/s, which is lower by one order of magnitude than the specifications of the commercial detector of Pye-Unicam /7/, in which conversion to methane is additionally used.

An advantage of our detector is that pyrolysis is conducted in a controlled atmosphere at constant gas flow rates, which provides for a lower noise level and high stability of the detector's operation. Another important advantage is the possibility of using the detector at subatmospheric pressures in the system, which, in our opinion, is of interest as far as the incorporation of our device in a combined LS-MS system /8/.

Table I. Results of Determining the Detector Sensitivity[x]

Sample concentration %	Amount of analyzate g	FID scale A	Peak height mm	Peak area mm^2	Peak area red. to 10^{-10}A mm^2	Relative mean standard deviation of the peak area %
0.001	8.10^{-8}	10^{-10}	23	67	67	1.76
0.01	8.10^{-7}	10^{-10}	139.7	659	659	1.53
0.1	8.10^{-6}	10^{-9}	177.5	778	7780	1.18
1.0	8.10^{-5}	10^{-8}	138.5	762	76200	0.96

[x] Sample size - 10 µl

In the case of chromatographic separation of substances exhibiting electron affinity, an electron capture detector should preferably be used. In these case the minimum detectable amount will decrease by 2 to 3 orders of magnitude.

CONCLUSION

Ways to improve transport detectors for liquid chromatography have been investigated and a new design is proposed. One of the first models of the newly designed transport detector has been tested and described.

REFERENCES

1. R. Scott, British Patent No. 998,107; Cl. B I X, 1965.
2. H.W. Johnson, E.E. Seibert and F.H. Stross, Anal. Chem., 40, 403 /1968/.
3. V.G. Berezkin, J.M. Savinov and Ya. I. Yashin, J. Chromatogr., 150, 200 /1978/.
4. E. Kenndler and E.R. Schmid, in J.E.K. Huber /Editor/, Instrumentation for High-Performance Liquid Chromatography. p. 163. Elsevier Amsterdam, 1978.
5. Z. Deyl, K. Macek, J. Janak, eds., LIQUID COLUMN CHROMATOGRAPHY, Chpt. 8. Elsevier, Amsterdam, 1975.
6. V.G. Berezkin, L.N. Kolomiets, A.A. Korolev, U.S.S.R. Inventor's Certificate, No 711,466 /1979/; Byull. Izobret., No. 3 /1980/.
7. R.P.W. Scott and J.C. Lawrence, J. Chromatogr. Sci., 8, 65 /1970/.
8. D.E. Games, P. Hirter, W. Kuhnz, E. Lewis, N.C.A. Weerasinghe, S.A. Westwood. J. Chromatogr., 203 131. /1981/.

Chromatography, the State of the Art
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HP PRECIPITATION CHROMATOGRAPHY - A NEW TECHNIQUE FOR THE ELUCIDATION OF THE MOLECULAR STRUCTURE OF COPOLYMERS

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ABSTRACT

The chemical composition distribution of copolymers has been measured by means of HPLC gradient elution. The gradient has been built up by adding a solvent to a nonsolvent which mainly fractionates by composition. Investigating the chemical composition of poly/styrene-co-acrylonitrile/ and poly/ α-methylstyrene-co-acrylonitrile/ we have used tetrahydrofuran as a solvent and n-hexane as a precipitant. With an appropriate gradient it was possible to separate copolymers which differed in acrylonitrile content by no more than 4 wt %.

INTRODUCTION

Synthetic polymers consist of macromolecules which differ in degree of polymerization /the latter indicates the number of repeating monomeric units per macromolecule/. In most cases this molar mass distribution /MMD/, which is a consequence of the reactions yielding macromolecules, strongly influences the properties of a polymeric material. Hence, the evaluation of MMD is not only a matter of scientific interest but also has practical importance, for instance in plant control or product development.

According to the present level of technology, MMDs are mostly measured by means of size-exclusion chromatography /SEC/. This method is much more effective than classical fractionation. In SEC the cumbersome isolation of fractions and their consecutive drying, weighing and measuring are abolished. MMDs can be derived from the records of appropriate detectors with the help of calibration functions.

A copolymer also has an MMD. In addition, it usually has a distribution with respect to chemical composition (CCD). If the latter distribution is broad it often deteriorates the properties of the copolymer. For this reason, copolymers are sometimes investigated by means of SEC using two detectors, one of which is sensitive to composition. Unfortunately, this technique fails in many cases. For instance, it cannot reveal even a broad CCD if it is of equal shape in all the SEC fractions.

In order to obtain the desired information about MMD and CCD, copolymers must be investigated by means of cross fractionation. This technique means subdividing the complex molecular distribution into slices along a certain direction and subsequently fractionating these slices in another direction which should diverge as far as possible from that of the first fractionation. With the classical technique, cross fractionation requires a lot of work: about 50-100 fractions must be isolated and characterized. The classical cross fractionation is based upon solubility differences as expressed by the equation /Ref. 1/:

$$\varphi''_{x,P} / \varphi'_{x,P} = \exp [P(\sigma + K.x)] \qquad /1/$$

Here, $\varphi''_{x,P}$ and $\varphi'_{x,P}$ are the volume fractions of the polymeric material in the gel phase and the sol phase, respectively. The quantity P indicates the degree of polymerization, and x is the volume fraction of one of the constituent monomers forming the copolymer. K and σ are interaction parameters determining the direction of fractionation. The principle of cross fractionation can be described in such a way that K must be small in comparison with σ during the first fractionation, but large during the perpendicular second fractionation.

It is challenging to use chromatographic methods for measuring CCD and MMD of copolymers. This requires the combination of two procedures, one of which mainly separating by molar mass, the other by composition. Combinations of this kind have been attained by thin-layer chromatography /TLC/ + SEC /Ref. 2/ and by column adsorption chromatography + SEC /Ref. 3/. Mention has

also been made of the combination SEC + TLC /Ref. 4/.

The use of SEC for the primary fractionation has advantages and disadvantages. The fact that SEC separates by hydrodynamic volume and not strictly by molar mass is one of the drawbacks. Among the advantages are the following characteristics:

/i/ All fractions are obtained in an eluent of constant quality.

/ii/ SEC chromatograms are "short", since the distribution constants are within the limits 0 and 1. Therefore, the fractions are only slightly diluted.

/iii/ SEC can cope with rather large samples. About 1 mg of polymer solute can be injected even into analytical columns /e.g. d_c = 7.8 mm/. Hence, the fractions contain at least 20 to 50 µg of polymer.

The experimental technique for the subsequent fractionation in a perpendicular direction must be capable of revealing the CCD of such small fractions. The amount of polymer in the eluate of the second chromatographic separation should be continuously measured by a suitable detector. The solvents used must not disturb this detection.

One of the solutions of this problem is the use of a UV detector in the case of polymers which absorb in an appropriate region, and of transparent solvents. The magnitude of the detector signal indicates the amount of solute in any portion of the eluate, and the retention time should indicate the composition.

Column adsorption chromatography can be applied for the separation by composition. This method has recently been used for the investigation of some copolymers /Ref. 5-8/. Applying this technique one must carefully avoid the difficulties involved in the extremely nonlinear adsorption isotherms of polymers. As far as we know, combination of column adsorption chromatography and SEC has not yet been reported.

Another possibility of separating copolymers by composition is based upon their solubility behaviour. Precipitation chromatography of polymers was suggested by Baker and Williams as early as 1956 /Ref.9/. The method of these authors was aimed at estimating the MMDs of homopolymers. Numerous papers by sci-

entists from laboratories all over the world proved the utility of the "Baker-Williams fractionation" in MMD analysis /Ref. 10/. Attempts have also been made to use the method for CCD estimation /Ref. 11/.

The Baker-Williams technique makes use of the dependence of solubility on solvent quality and temperature. An antiparallel temperature gradient is applied to the column, i.e. the temperature at the top is about 20 or 30 degrees higher than at the outlet /See Fig. 1/. The temperature gradient is combined with a likewise antiparallel elution gradient. The elution starts with a poor solvent whose dissolving power is gradually increased by the addition of a good solvent in the course of the run.

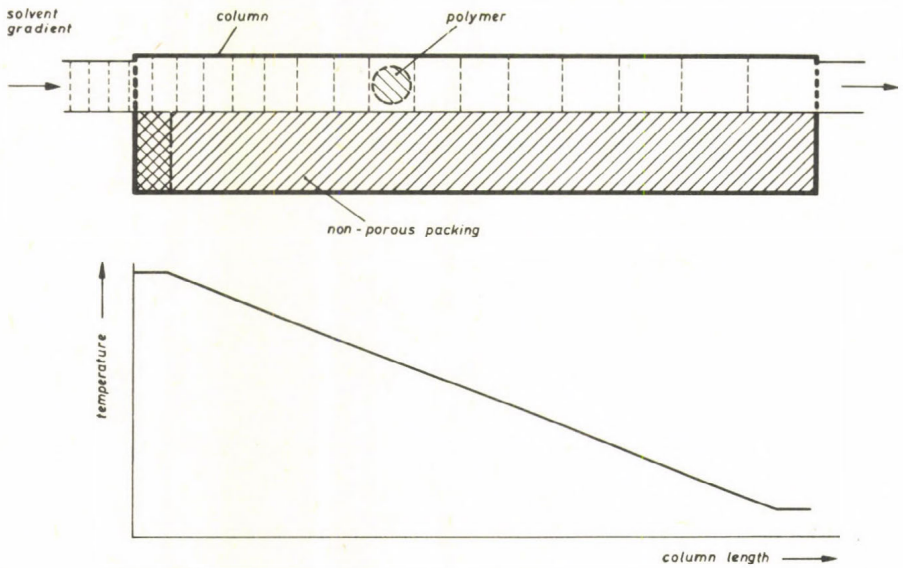

Fig. 1. Baker-Williams fractionation: schematic representation of column arrangement, antiparallel elution gradient, and indication of temperature gradient.

The column is packed with glass beads. The sample is applied as a thin film on the beads of the first parts of the column. In the classical Baker-Williams fractionation the coating procedure is performed outside the column by pouring a solution containing about 300 mg of sample into an appropriate amount of

dry beads. The mixture is stirred during the evaporation of the solvent.

The sample bed is installed at the top of the column where the temperature is higher than in the regions below. Here, even a comparatively poor solvent may dissolve certain constituents of the sample. They will be carried by the eluent into the cooler zones below where they precipitate and remain stationary until a solvent of higher dissolving power will take them up again.

The combination of temperature gradient and elution gradient causes repeated precipitation and redissolution of each constituent of the polymer. Due to this multistage mechanism the Baker-Williams fractionation is also called precipitation chromatography.

For MMD analysis it has been superseded by SEC. Among the reasons for this replacement are the laborious application of the samples which demands dismantling of the column, as well as the need for isolation of fractions and their characterization. In some cases, the temperature control caused difficulties. We fully share the opinion that SEC is the up-to-date method for the estimation of MMD, but the situation is different in the case of CCD. Here, a solubility-based chromatography offers more separating power than SEC.

We successfully applied an improved technique of precipitation chromatography for revealing the CCD of copolymers /Ref. 12, 13/. The following improvements were realized:

/i/ The use of HPLC equipment and the UV detection of the polymer.

/ii/ The injection of the sample solution into the streaming eluent in conformity with the common injection technique in HPLC.

/iii/ Reduction of sample mass to about 10 µg.

/iv/ Replacement of the temperature gradient by the possibilities offered by porous packing materials.

If the pore size is such that polymer molecules are excluded, the volume available to the eluent is about twice the volume available to the polymer. In the case of total exclusion, the latter is restricted to the interstitial volume. The excluded

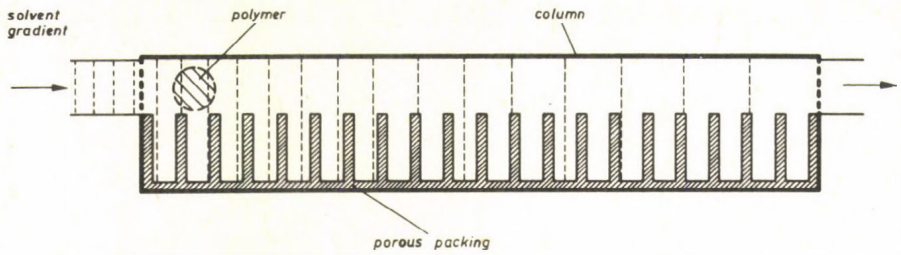

Fig. 2. HP precipitation LC; schematic representation of column arrangement and antiparallel elution gradient. The molecules of the solvent enter the pores of the packing whereas the polymer molecules are excluded.

polymer is transported along the column with a higher velocity than any change in eluent composition. Hence, the dissolved polymer rushes into the solvent running in front, which is too poor to keep the polymer in solution. It precipitates and remains at that spot until it will be reached by a mixture of higher dissolving power /Fig. 2/.

MATERIALS AND METHODS

Samples. Random copolymers of styrene and acrylonitrile (SAN) with an acrylonitrile content (AN) ranging from 16.1 to 30 wt % and molar mass values M between 41,000 and 480,000 g/mole. Random copolymers of α-methylstyrene and acrylonitrile, 15.4--45.9 wt % AN, M: 50,000-445,000 g/mole.

Solvents. Tetrahydrofuran /THF/, peroxide-free, kept under N_2, with addition of 10 vol.-% of methanol. n-Hexane or /alternatively/ isooctane.

Columns. /i/ L = 25 cm, d_c = 4.6 mm, packed with LiChrosorb® RP-18, /ii/ L = 15 cm, d_c = 4.6 mm, packed with LiChrosorb RP-18, or /iii/ L = 15 cm, d_c = 4.5 mm, packed with LiChrospher® SI 1000 bonded layer RP-8.

Apparatus. Part of the work was performed by using a HEWLETT-PACKARD 1084 B apparatus and UV detection at 254 nm. The experiments with THF/isooctane mixtures were accomplished by using a VARIAN Model 5000 with a SCHOEFFEL Instruments Co. GM 770 monochromator /set at 259 nm/ and Spectroflow SF 770 monitor.

All HP precipitation chromatography runs were carried out by means of gradient elution. The initial eluent consisted of 90 % of an alkane hydrocarbon and of 10 % of the THF/methanol mixture /"solvent B"/. The large portion of hydrocarbon nonsolvent was necessary to gain precipitation of the polymer from a solution in pure THF, the injecting solvent. /The SEC was carried out by using this solvent./ The copolymers left the column together with an eluent containing 60-80 % of "B".

RESULTS

The higher the acrylonitrile content, the longer was the retention of a sample. The influence of composition was much more striking than the increase of retention with molar mass. The molar mass dependence /given in % nonsolvent/ was a linear function of $M^{-0.5}$. This finding agreed well with earlier results /Ref. 14/.

For a copolymer of a certain composition the detector signal varied directly with sample load. At the wavelengths mentioned, the intensity of the UV absorption increased with the increasing amount of aromatic structural units in the copolymer /styrene or α-methylstyrene/. By means of calibration the detector signal can be converted into the solute amount.

The features of the chromatograms and the retention times of the sample components were strongly influenced by the shape of the gradient. This offered the possibility of adjusting the chromatographic resolution to the width of the CCD of the sample under investigation.

Crude copolymers often yielded rather broad HPPLC traces, but fractions from SEC, which are homogeneous in hydrodynamic

volume, produced distinct peaks. SEC fractions of a mixture of two copolymers differing in AN content by 4 % yielded HPPLC chromatograms which clearly showed both components. The copolymer with the higher AN content also had a higher molar mass, hence both influences on retention worked in the same direction.

For a more rigorous test we mixed a copolymer sample of high molar mass, which was poor in AN, with a copolymer of low molar mass but high AN content. In this case, the molar influence counteracted the influence of the composition. From SEC fractionation we collected slices, each 0.5 ml in volume and containing 20-50 µg of copolymer. /The total amount of sample investigated in the SEC run was 0.87 mg./ From the eluate slices 100 µl each were injected into HPPLC without any additional treatment. The chromatograms showed the predominance of the sample having a high AN content in the fractions of low molecular mass, and the increasing portion of the component poorer in AN with increasing molar mass of the SEC fractions /Ref. 15/.

DISCUSSION

For polymer scientists the method may be of interest as a tool for effective investigation into the molecular structure of copolymers, especially since the successful injection of SEC eluate has proven that a direct coupling of the two chromatographic methods, and hence automation is within reach.

Here we shall discuss the mechanism of separation. The arguments in favour of the suggested precipitation and redissolution mechanism are:

/i/ The eluent composition at peak position /as derived from chromatograms obtained by gradient elution at 40°C/ plotted vs. AN content of the samples yields a curve similar to that one which can be derived from turbidimetric titration of SAN copolymers dissolved in THF. In the latter experiments, n-hexane was used as a precipitant.

/ii/ The molar mass dependence of retention yields exactly the same relation between solvent composition and $M^{-0.5}$ as found by means of turbidimetric titration.

/iii/ At 50°C all components of a mixed sample showed shorter retention than at 25°C. This observation corresponds to the improved solubility at 50°C. If the separation had been controlled by adsorption, the retention would have been longer at 50°C, because the adsorption of polymers generally increases with temperature.

Another question concerns the risk of blocking the column. Right from the beginning of these experiments we carefully watched the column pressure during the run, but never observed an alarming increase. There was no pressure difference between a blank run and a run with injection of, e.g., 20 µg of polymer into a column 4.6 mm in diameter. The reason is that the polymer is injected as a solution in pure THF. To ensure precipitation the THF content must be reduced to about 40 %. This is done by the starting eluent with a high content of nonsolvent, which fills the pores of the column packing. For the necessary reduction in THF concentration, the injected volume must migrate a certain distance. Hence, the precipitated polymer is spread over such a large surface area that the thickness of the film is much smaller than the width of the interstitial channels of the column.

REFERENCES

1. Topčiev, A.V.; Litmanovic, A.D., Stern, V.Ja. Dokl. Akad. Nauk SSSR 147 1389-1391 /1962/
2. Taga, T.; Inagaki, H. Angew. Makromol. Chem. 33, 129-142 /1973/
3. Inagaki, H.; Tanaka, T. Pure and Appl. Chem. 54 309-322 /1982/
4. Belenkij, B.G.; Gankina, E.S. J. Chromatogr. 141 13-90 /1977/
5. Taranachi, S.; Hasegawa, A.; Shima, Y.; Akatsuka, M.; Nakajima, M. Macromolecules 12 992-996 /1979/
6. Belenkij, B.G. Pure and Appl. Chem. 51 1519-1535 /1979/
7. Danielewicz, M.; Kubin, M. J. Appl. Polymer Sci. 26 951-956 /1981/

8. Danielewicz, M.; Kubin, M.; Vozka, S. J. Appl. Polymer Sci. 27 3629-3631 /1982/
9. Baker, C.A.; Williams, R.J.R. J. Chem. Soc. /London/2352--2362 1956
10. Cooper, A-R. "Column Fractionation of Macromolecules by Gradient Elution" in: "Chromatography of Synthetic and Biological Polymers", vol. 1, p. 240-265 /R. Epton ed./, Ellis Horwood Ltd. Publishers; Chichester U.K. 1978
11. Müller, K.-D., Thesis, Dresden 1971
12. Glöckner, G.; Kroshwitz, H.; Meissner, Ch. Acta polymerica 33 614-616 /1982/
13. Glöckner, G. Pure and Appl. Chem., in press /24 th PMM, July 11-14, 1983/
14. Glöckner, G. Z. physik. Chem. 229 98-109 /1965/
15. Glöckner, G.; Koningsveld, R. Makromolekulare Chemie, Rapid Communications 4 529 /1983/.

Chromatography, the State of the Art
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EVALUATION OF SHORT AND EFFICIENT REVERSED-PHASE COLUMNS FOR THE FAST ANALYSIS OF THEOPHYLLINE BY HPLC

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ABSTRACT

Separation of theophylline from theobromine, 1,7-dimethylxanthine, β-hydroxyethyltheophylline /internal standard/ and caffeine was accomplished within three minutes using a 5cm SUPELCOSIL® LC-18 column and a ternary mobile phase consisting of 1 % acetonitrile, 1 % tetrahydrofuran and 98 % 1.75 mM H_3PO_4. Moderate flow rate and low column back-pressure contributed to long column lifetime, reduced solvent consumption and decreased cost per analysis. A silica column extraction technique was used for cleanup of serum samples. Fifty-four drugs were investigated with respect to chromatographic retention and/or extractability. Chlorothiazide and metronidazole interfered with the internal standard and theophylline, respectively. Levels of 1,7-dimethylxanthine were generally below 1 µg/ml, and independent of the caffeine concentration.

INTRODUCTION

The development and commercial availability of second-generation HPLC columns have resulted in the widespread application of high-performance liquid chromatography /HPLC/ in therapeutic drug monitoring /TDM/. Efficiences of 18,000 theoretical plates for a 25 cm column, and 10,000 for a 15 cm column packed with spherical 5 µm /bonded phase/ silica particles can be obtained under the manufacturer's test conditions. Although these efficiencies are generally not realized in "real" samples, it is apparent that, in many TDM analyses, only 1,500 to 3,000 theoretical plates are required for separation. Under optimum conditions, 5 cm columns can generate about 3,500 theoretical

plates. Consequently, instead of 25 or 15 cm columns, a 5 cm column is sufficient for most TDM analyses. Examples of drugs that are readily analyzed with these short columns include procainamide, quinidine, lidocaine, disopyramide, theophylline, acetominophen, propranolol and chloramphenicol. For the analysis of these drugs, 5 cm columns can provide faster analysis time and thus reduced cost per analysis. Additional advantages include a reduction in solvent consumption and operation of the column at lower back pressure. Finally, short columns are less expensive than conventional columns and are generally more stable.

In this paper, we will discuss our experience with the use of short columns for routine therapeutic drug monitoring of theophylline and related xanthines in a clinical setting. Evaluation of 5 cm SUPELCOSIL LC-18 columns for therapeutic monitoring of theophylline was carried out at the Toxicology Department of the South Bend Medical Foundation over the past two years.

MATERIALS AND METHODS

Chromatography

HPLC analyses were performed with a Model SP-8000 liquid chromatograph /Spectra Physics, San Jose, CA 95134/ equipped with a Model 7010 injector /Valco, Houston, TX 77055/, a Model SF-740 variable wavelength UV detector /Kratos, Ramsey, NJ 07446/, and with a system consisting of a Constametric III pump /LDC, Riviera Beach, FL 33404/, a Model 7125 injector /Rheodyne, Cotati, CA 94928/, a Model UV-10 variable wavelength UV detector /Varian, Palo Alto, CA 94303/, and an Omniscribe B-5000 recorder /Houston Instruments, Austin, TX 78753/. The mobile phase was made up from HPLC-grade solvents by adding 10 ml acetonitrile or methanol and 10 ml tetrahydrofuran to 980 ml 1.75 mM H_3PO_4 /pH = 2.8/. All columns /50 x 4.6 mm and 150 x 4.6 mm ID/ contained SUPELCOSIL® LC-18 /Supelco, Inc., Bellefonte, PA 16823/, spherical 5 µm silica bonded with octa-

decyl-dimethylchlorosilane and "end capped" with trimethylchlorosilane.

Extraction

Serum /0.1 ml/ was extracted with the aid of Clin-Elut 1000 M columns /Analytichem, Harbor City, CA 90710/ according to the method described by Frethold /1/. The evaporated sample extract was reconstituted in 150 µl mobile phase of which 40 µl was injected. Quantitation of serum theophylline levels was done by peak height using the internal standard ratio method. The absolute recovery of theophylline was 78 % over the entire therapeutic range.

RESULTS AND DISCUSSION

One of the main advantages of HPLC in TDM is its ability to separate the drug of interest from its metabolites and related chemicals. Since theophylline belongs to the same chemical class as caffeine and theobromine /common in many beverages/, the analysis must allow for adequate separation of caffeine, theophylline, theobromine, and an appropriate internal standard. Metabolism of caffeine can also lead to other potential interference such as those found with the caffeine metabolite 1,7-dimethylxanthine /paraxanthine/. Finally, potential interferences from medications often prescribed concurrently with theophylline, such as antibiotics, diuretics, analgesics, and phenobarbital, must be eliminated.

As other authors /2, 3/ have reported, we found it necessary to mix a small percentage of tetrahydrofuran with acetonitrile or methanol to affect separation of paraxanthine from theophilline. A quaternary mobile phase consisting of acetonitrile, methanol, tetrahydrofuran and buffer did not lead to further improvements. A standard mixture of theobromine,acetaminophen, paraxanthine, theophylline, diphylline, β -hydroxyethyltheophylline and caffeine was injected on a 5 cm and 15 cm SUPELCOSIL LC-18 column /see Fig. 1/. At a flow rate of

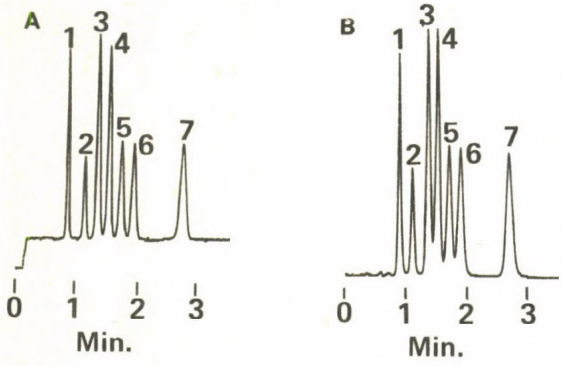

Figure 1. Theophylline analysis on 5 and 15 cm LC-18 columns.

Mobile Phase: 1 % tetrahydrofuran, 1 % acetonitrile, 98 % 1.75 mM H_3PO_4; Temperature: 30°C; Detection: UV at 274 nm; Injection: 10 µl of each compound at 10 µg/ml in mobile phase; Sample: theobromine /1/, acetominophen /2/, paraxanthine /3/, theophylline /4/, diphylline /5/, β-OH-ethyltheophylline /6/, caffeine /7/.

A. SUPELCOSIL LC-18, 50 x 4.6 mm; Flow Rate: 2 ml/minute; Sensitivity: 0.2 AUFS; Pressure: 740 psi.

B. SUPELCOSIL LC-18, 150 x 4.6 mm; Flow Rate: 6 ml/minute; Sensitivity: 0.1 AUFS, Pressure: 4800 psi.

2 ml minute, caffeine elutes from the 5 cm column within three minutes. Although the same mixture was better resolved on a 15 cm column, excessive pressure at a flow rate of 6 ml/minute was necessary to obtain the same analysis time as on the 5 cm column. Thus, for fast analysis of a relatively small number of compounds, one benefits from the use of short columns in terms

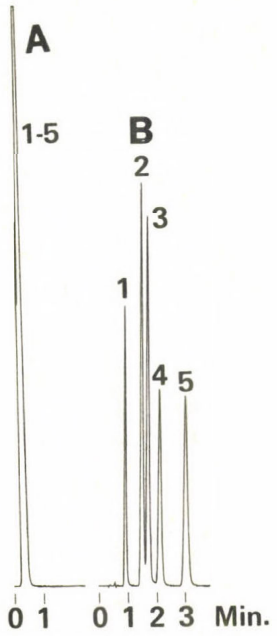

Figure 2. Retention behaviour before and after wetting.

SUPELCOSIL LC-18, 50 x 4.6 mm; Mobile Phase: 1 % tetrahydrofuran, 1 % methanol, 98 % 1.76 mM H_3PO_4; Flow Rate: 2 ml/minute; Pressure: 815 psi; Temperature: 30°C; Detection: UV at 254 nm; Sensitivity: 0.4 AUFS; Injection: 10 µl of each compound at 0.2 mg/ml in mobile phase; Sample: theobromine /1/, paraxanthine /2/, theophylline /3/, β-OH-ethyltheophylline /4/, caffeine /5/.

A. Dry column.
B. After wetting with 100 µl methanol /see text/.

of lower pressure drop, solvent consumption, and price. The additional advantage of increased sensitivity for a fixed sample volume is of minor importance for this particular analysis, although Figure 1 illustrates a two-fold gain for the 5 cm column when using a constant injection volume.

Some minor precautions must be taken to assure satisfactory results on a 5 cm column. Since the 5 cm column is 3 to 5 times

shorter than the 15 and 25 cm columns, it dries out quicker during shipping and storage. Therefore, the column should be washed with 1 ml methanol before equilibrating with the theophylline mobile phase /1 % acetonitrile, 1 % tetrahydrofuran, 98 % 1.75 mM H_3PO_4/. This procedure assures that the surface of the C18 reversed-phase silica is properly wetted. No retention of the analytes was observed when a dry column was equilibrated directly with the ternary mobile phase, as shown in Figure 2A.A 100 µl injection of methanol can be used as an alternative to the 1 ml methanol wash. Once properly wetted, a reversed phase column can be operated at any desired percentage of organic modifier. Note that the described wetting procedure is similar to that used with reversed phase type sample cleanup columns and hydrophobic membrane filters.

With solid phase extraction for sample preparation, at least 1,000 serum extracts could be injected without the use of a guard column before reduction of column efficiency warrants a column change. Since the column packing does not show a depression during its useful lifetime, we believe that contaminations from the sample and the sampling valve are the cause of gradual degradation of resolution. Although not restricted to the use of short columns, we noticed as occasional loss of efficiency due to partial blockage of the column inlet frit. This blockage does not necessarily give rise to increasing column pressure. In such cases, we were able to restore the original separation after reversing the flow through the column.

During this operation, the column is disconnected from the detector to prevent particulates from entering the detector cell. It is sufficient to operate the column in this reversed flow direction for only a short time at a high flow rate. The analysis can then be continued with the flow going in either direction. Protection of the analytical 5 cm column by a 2 cm disposable guard column packed with either pellicular /40 µm/ or porous /5 µm/ C18 bonded silica resulted in less instrument downtime and extended column life. Using a 2 cm disposable pellicular guard column /a total of three were actually used/ more than 3,000 serum extracts were injected without serious loss of resolution.

TABLE I. Extractable compounds with HPLC retention times less than 5 minutes

Compound	Retention Time in Minutes	Serum Concentration[1] in µg/ml
Acetaminophen	1.2	100.0
β-OH-ethyltheophylline	2.0	100.0
Caffeine	2.8	100.0
Chlorothiazide /Diuril/[2]	2.0	100.0
Diphylline	1.8	100.0
Hydrochlorothiazide	2.4	100.0
Metronidazole[3]	1.2	100.0
Paraxanthine	1.5	100.0
Theobromine	1.0	100.0
Theophylline	1.7	100.0

[1] Using a sample volume of 100 µl of serum in the extraction procedure, 10 µg/ml of compound in mobile phase would be equivalent to 100 µg/ml of compound in the extracted serum, assuming 100 % recovery.

[2] Coelutes with β-OH-ethyltheophylline.

[3] Tailing edge coelutes with theophylline.

Clinically useful and cost effective monitoring of theophylline requires rapid sample preparation, adequate separation of analytes and interferences, and short chromatographic analysis time. Theophylline sample preparation for HPLC ranges from direct injection of serum after precipitation of protein by acetonitrile to complex extraction procedures. A multitude of published methods have appeared over the past three years /4-25/. Precipitation and extraction methods each have inherent advantages. The authors feel that the elimination of nonextracted interferences justifies the additional time required to prepare samples using a solid phase or solvent extraction procedure. Therefore, in the study presented here, a solid phase adsorption extraction system was used to prepare all samples /1/.

A variety of compounds were tested for potential interferences and are listed in Tables I and II. The retention times

TABLE II. Compounds with retention times greater than 10 minutes[1]

Compound	Amount Injected / µg/ in Mobile Phase	Compound	Amount Injected / µg/ in Mobile Phase
α-Phenylglutarimide	12.5	Mesantoin	12.5
Amobarbital	12.5	Methaqualone	12.5
Barbital	12.5	Metharbital	12.5
Butabarbital	12.5	Methsuximide	12.5
Butalbital	12.5	4-Methylprimidone	12.5
Carbamazepine	12.5	Methylprylon	12.5
Cefamandole	20.0	Motrin	20.0
Cephalothin	20.0	Nirvanol	25.0
Cephapirin[2]	20.0	Nordiazepam	12.5
Chloramphenicol	20.0	Oxazepam	6.25
Chlordiazepoxide	12.5	Papaverine	20.0
Cimetidin	20.0	Pentobarbital	12.5
N-Desmethsuximide	25.0	Phenacetin	12.5
Diallybarbiturid Acid	12.5	Phenobarbital	20.0
Diazepam	12.5	Phensuximide	12.5
Dilantin	17.5	Phenylbutazone	12.5
Diphenylhydantoin	20.0	PEMA	15.0
Ethinamate	12.5	Primidone	12.5
Ethosuximide	50.0	Secobarbital	12.5
Ethotoin	12.5	Sulfamethoxazole	20.0
Furosimide	20.0	Sulfisoxazole	20.0
Glutethimide	12.5	Talbutal	12.5
Lidocaine	12.5	Tobramycin	20.0
Mephobarbital	12.5	Trimethoprim	20.0
Meprobamate	18.75		

[1] Since these drugs did not interfere with the chromatography, we did not test them in our extraction procedure.

[2] Cephapirin has a retention time of 3.5 minutes but did not extract at a serum concentration of 100 µg/ml in the procedure outlined.

of those drugs that are extracted and have chromatographic retention times of less than 5 minutes /under the conditions of Figure 1/ are given in Table I. Other drugs that showed retention times longer than 10 minutes are listed in Table II together with the concentration /in mobile phase/ that was in-

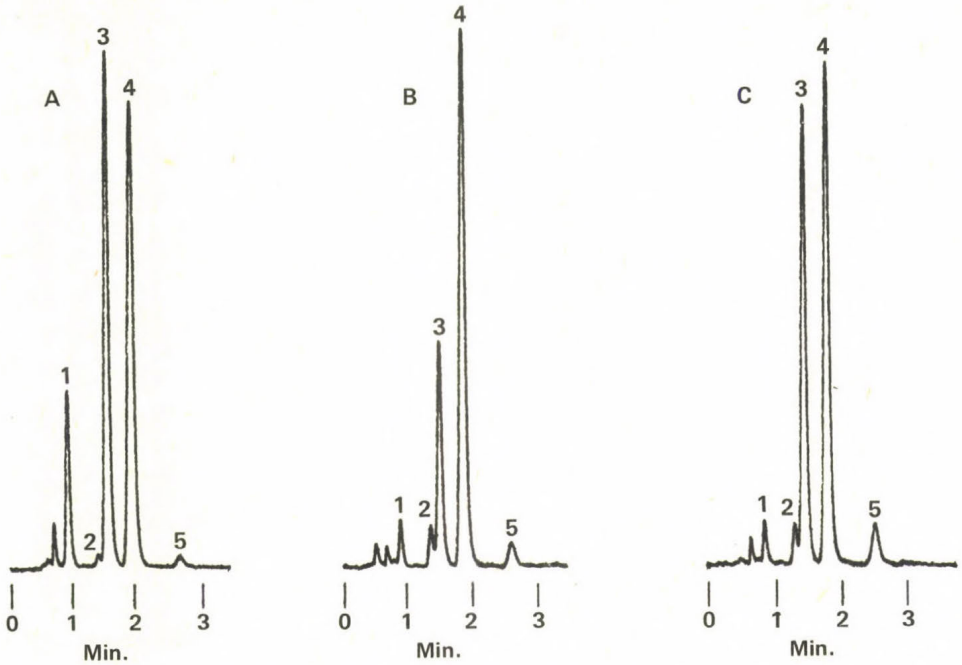

Figure 3. Theophylline patient serum samples.

Column and conditions as in Figure 1A except for 40 µl sample volume. Peaks are labeled as in Figure 1. Theophylline concentrations: A = 11.0 µg/ml, B = 4.3 µg/ml, C = 8.3 µg/ml.

jected. These drugs were not taken through the extraction procedure. Cephapirin eluted in 3.5 minutes but did not extract. The chromatograms from three patient serum samples containing varying amount of theophylline, theobromine and paraxanthine are shown in Figure 3. In our experience, the concentration of paraxanthine rarely exceeds 1 µg/ml regardless of the concentration of caffeine.

The analysis of theophylline by HPLC using short 5 cm columns, combined with a solid phase serum extraction procedure, has proven to be reliable and cost effective with a minimum of interferences.

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REFERENCES

1. D. Frethold, Theophylline Applications Bulletin, Analytichem International, 1979
2. L.A. Broussard, Clin. Chem. 27 1931. /1981/
3. J.R. Miksic, B. Hodes, Clin. Chem. 25 1866. /1979/
4. D.A. Jowett, Clin. Chem. 27, 1785. /1981/
5. S. van der Wal, L.R. Snyder, Clin. Chem. 27 1233. /1981/
6. A. Marion, L.J. Lesko, C. Oliver, Ther. Drug. Monit. 3, 107. /1981/
7. P. van Aerde, E. Moerman, R. van Severn, P. Braeckman, J. Chromatogr. 222 467. /1981/
8. K.H. Valia, C.A. Hartman, N. Kucharczyk, R.D. Sofia, J. Chromatogr. 222 170. /1980/
9. A.J. Quattrone, R.S. Putman, Clin. Chem. 27 129. /1981/
10. D.B. Bowman, M.K. Aravind, R.E. Kauffman, J.N. Miceli, Clin. Chem., 26 1622. /1980/
11. T. Foenander, D.J. Birkett, J.O. Miners, L.M. Wins, Clin. Biochem. 13 132. /1980/
12. J.W. Dolan, S. van der Wal, S.J. Bannister, L.R. Snyder, Clin. Chem. 26 871. /1980/
13. P.J. Naish, M. Cooke, R.E. Chambers, J. Chromatogr. 163 363. /1979/
14. D.A. Asdeppa, S.D. Lipton, Clin. Chem. 26 788. /1980/
15. H.H. Farrish, W.A. Wargin, Clin. Chem. 26 524. /1980/
16. N. Weidner, D.N. Dietzler, J.H. Ladenson, G. Kessler, L. Larson, C.H. Smith, T. Janes, J.M. McDonald, Am. J. Clin. Pathol. 73 79. /1980/
17. P.J. Naish, R.E. Chambers, M. Cooke. Ann. Clin. Biochem. 16 254. /1979/

18. J.R. Miksic, B. Hodes, J. Pharm. Sci. 68 1200. /1979/
19. G.P. Butrimovitz, V.A. Raisys, Clin. Chem. 25 1461 /1979/
20. L.T. Mann, Jr., Clin. Chem. 25 1336./1979/
21. L.W. Bond, D.L. Thornton, Clin. Chem. 25 1186. /1979/
22. C.A. Robinson, Jr., J. Dobbs, Clin. Chem. 24 2208. /1978/
23. K. Nakatsu, J.A. Owen, K.Scully, Clin. Biochem. 11 148 /1978/
24. C.A. Robinson, Jr., B. Mitchell, J. Vasiliades, S.L. Siesel, Clin. Chem. 24 1847. /1978/
25. F.L.S. Tse, D.W. Szeto , J. Chromatogr. 226 231. /1981/

Chromatography, the State of the Art
H. Kalász and L.S. Ettre (Eds)

HPLC OF NUCLEOTIDES. II. GENERAL METHODS AND THEIR DEVELOPMENT FOR ANALYSIS AND PREPARATIVE SEPARATION. THE APPROACH TO SELECTIVITY CONTROL*

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ABSTRACT

In order to develop the efficient separation methods of nucleotides according to their size and heterocyclic composition the application of ion-exchange, reverse-phase and normal-phase adsorption HPLC has been studied. The comparative investigation of retention power and selectivity of various packings (nonpolar bonded phase and amino-silicas) in relation to the nucleotides' length and composition results in some data for their reasonable selection and formulates the way to prepare the new packing for the particular separation problem. Thus the new anion exchanger with high selectivity and dynamic mass transfer has been prepared for fractionation of large oligonucleotides. The effect of the eluent pH and composition (organic modifier, salt) on retention, selectivity and resolution in ion-exchange and reversed-phase HPLC has been studied. The optimum separation conditions comprising elution with oppositely directed gradients of the salt and the modifier have been established. Also, a method for the selection of the most suitable packing for a pre-column providing the best protection for the main column without any loss of its efficiency and a method for the determination of the optimum gradient program

*This review has been presented at the Conference as the report and two posters. Part of it was included into papers /1/ and /2/. Abbreviations: r - denotes ribonucleotides; d - (deoxy) is omitted; p - denotes terminal phosphate groups; internucleotide phosphate groups are also omitted.

for the desired retention of the component of interest have been developed. The inter-relation between loading and sample concentration has been studied. The system for gradient elution was improved. It was shown that two-dimensional separation proves to be the most reliable and informative method for the preparation of homogeneous oligonucleotides. The model of hydrophobic-pair ion-exchange mechanism was proposed for the so-called ion-pair chromatography. High separation selectivity of protected and partially deblocked oligonucleotides, chemically synthesized for genetic engineering studies, has been shown for adsorption (normal-phase) HPLC which was efficient for gradient elution with isohydric eluents. The analysis of monomeric composition of nano- (pico-) molar amount of oligonucleotides has been developed; it involves their microcolumn digestion with immobilized enzyme followed by microcolumn separation of the nucleoside-mononucleotide mixture. A new slurry method for packing stable non-shrinked HPLC columns with tightly consolidated bed of particles was developed.

INTRODUCTION

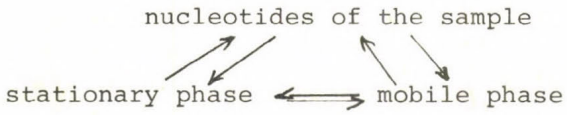
Aut viam inveniam aut faciam

The rapidly developed biotechnology including genetic engineering, molecular biology and bioorganic chemistry; general and clinical biochemistry with the newest methods of diagnostics; pharmaceutical chemistry and related fundamental and applied sciences necessitated the development of rapid, selective, sensitive and productive chromatographic methods for the separation of mono- and oligonucleotides. Ten years ago traditional chromatography on DEAE-cellulose lasting from several hours to a few days was considered as sufficient in the case of the investigation of chemical-enzymatic synthesis of functionally active DNA-structures /3/ Today the computer-controlled oligonucleotide synthesizer may prepare during one day several oligonucleotides containing some tens of monomeric residues. Ten years ago such synthesis would have to be continued from

several months up to a year. However, the development of perfect chemical methodology providing high yields in intermediate synthetic steps /4/ required the employment of high performance and informative chromatography for the isolation of the product in question especially those synthesized with solid-phase method /5/ without isolation of the intermediates. Such chromatography will be able to provide isolation of the directly identified product of interest from the complex mixture of unreacted intermediate products, as well as from the products with shortened and elongated chains and the side products with modified heterocycles. The isolated oligonucleotide block is suitable for the subsequent faultless, enzyme-catalyzed incorporation into the reconstructured structure of a functionally active DNA segment.

The chromatography of natural nucleotides - that is nucleotide pool of living cell or biological fluids of the organism, the products of DNA or RNA digestion - should solve even more complex problems.

The development of such chromatography is our goal. We studied the possibility and expediency of applying known methods by testing the commercial adsorbents and columns taking into account the role of the mobile phase components and the relative contribution of molecular and ionic interactions in a dynamic three component system:



For the HPLC of larger oligonucleotides a new packing material with easily regulated selectivity and fast mass-transfer is required in order to solve the problems of genetic engineering.

We have also carried out some research concerning the general instrumental aspects of modern liquid chromatography such as improvement of the column protection against undesirable admixtures in the eluent, and concerning the sample, optimization of the gradient elution system, development of slurry-

packing method for long-lasting HPLC-columns with stable sorbent bed which does not collapse even at high viscosity during the repeated change or pulsation of the mobile phase.

Natural nucleotides and their synthetic analogues are heterocyclic N-glycosides of ribose or deoxyribose linked in RNA or DNA polymeric chains via phosphoric acid residues. They are distinguished by the chain length, composition and the sequence of alternating heterocycles. The size of the chain is directly correlated with the number of phosphate groups and thus with their total charge. Therefore ion-exchange (IE) and reverse-phase (RP) HPLC based on the difference in the net charge of the molecules and their hydrophobicity i.e. on ionic and molecular dispersive interactions with the stationary phase are widely used. Size-exclusion chromatography of nucleotides is used rather rarely since the available packings are either not completely inert to nucleotides and their adsorption interactions distort the molecular weight distribution /6/, or have a very low efficiency.

EXPERIMENTAL

Incipit

Chemicals

"Chemically pure" MeOH and CH_3CN were additionally purified as described in /7/; $CHCl_3$ was dried over P_2O_5, distilled over K_2CO_3 and stored with 0.5% EtOH in a dark vessel away from air. NaOAc solutions (pH 4.3) were prepared from CH_3COOH distilled over $KMnO_4$ and NaOH. NH_4OAc solutions were prepared from "high purity" grade salt with the addition of aqueous ammonia or CH_3COOH for the required pH. 1 M NaH_2PO_4-H_3BO_3 solution was prepared from 1 M "high purity" grade Na_2H_4PO and H_3BO_3 solutions and the necessary pH was adjusted with Na_3PO_4 or H_3PO_4. Sodium dodecyl sulfate (SDS) was recrystallized from water up to optical purity at 254 nm. All solutions were filtered through 2.7 μm GF/D and 0.7 μm GF/F (Whatman) glass-fiber filters and degassed for 10 min in vacuo. Deoxymononucleotides

(Sigma) were used at 10 AU_{254}/ml concentrations. Deoxy-oligonucleotides were chemically synthesized by our colleagues from Prof. M.N.Kolosov's laboratory by the "phosphotriester" method. Ribooligoadenylates $rA(pA)_n$ were prepared by alkaline hydrolysis of polyadenylic acid followed by terminal dephosphorylation by E.coli alkaline phosphatase.

Apparatus

The following liquid chromatographs were used: 1) Model 830 (DuPont) with pneumatic amplifier pump, Model 838 gradient former, Rheodyne-7120 injector (loops from 10 µl to 10 ml) and built-in UV_{254}-photometer (cuvette volume 8 µl with an optical path length of 10 mm and cuvette volume 20 µl with an optical path length of 1 mm); 2) Model 8520 (Varian) with Rheodyne-7125 injector and UV-spectrophotometer (DuPont); 3) Model 8820 (DuPont) with microprocessor-controlled three-head pump, column oven compartment with Rheodyne-7125 injector, UV_{254}-photometer and UV-spectrophotometer and flotation degasser; 4) microcolumn Model LC-1305 (Special Design Office of Analytical Instruments Production, USSR Academy of Sciences) with a single syringe pump and preformed gradient, spectrophotometric detector (cuvette 0.8 µl and an optical path length of 1.5 mm; the output signal 10 mV full scale was recorded through an additional capacity with the sensitivity of about 1 mV full scale by a Model A-25 (Varian) recorder, which was equal to 0.025 A.U.F.S., and a time constant of about 10 sec). Chromatographic data were calculated with a Varian Model SDS-111 Data System. The HPLC-columns were packed using the DST-150A (Haskel) pneumatic amplifier pump.

Methods

For the RPC-5 system preparation and fractionation of the microparticles and column packing see Wulfson et al. /1,2/.

The following commercial pre-packed HPLC-columns were used: Micropak-CH (Varian), 0.2x25 cm; Zorbax-Sil, -TMS,-C8, -ODS, $-NH_2$, -SAX (DuPont), 0.46x25 cm; Ultrasphere-C8, -ODS,

Ultrasil-NH_2 (Altex/Beckman 0.46x25 cm; Nucleosil-C8, -ODS, -NH_2, -SB (Macherey-Nagel), 0.4x25 cm. The home-made packed columns containing Separon-NH_2 and Silasorb-NH_2 (spherical and irregular particles respectively, d_p = 10 μm, Chemapol, ČSSR) were 0.46x25 cm. Our new anion-exchanger (5 μm particles) was packed into 0.46x15 cm columns. All columns with bonded-phase packings were flushed with MeOH before operation at less than 30 bars, then with water - MeOH mixtures (1:9, 3:7, 1:1 and 9:1). The operating flow rates were up to 1 ml/min for the 0.46 cm ID columns, 15 ml/min for the 2.12 cm ID column with bonded-phase packings and up to 50 ml/min for the columns with silica. Both RP and IE HPLC were carried out at 35°C.

Bonded-phase adsorbents (d_p = 5 and 10 μm) were packed upwards into stainless steel columns with polished inside under 600 bar. Two successive reservoirs (60 ml) connected with the pump were used. The pump reservoir was filled with 150 ml of 50% aqueus MeOH with 0.1 M NH_4OAc, the next reservoir (a stainless-steel tube) was filled with MeOH, while the packing reservoir was filled with the slurry of the packing material in MeOH or acetone; the amount of the adsorbent was sufficient to fill the column and the adaptor (0.46x4 cm) between the column and the reservoir up to 2-3 cm. After running the first solution the column was flushed with 200 ml of 50% MeOH containing 2.5 M NH_4OAc and then with 200 ml of MeOH.

For the guard-columns (0.46x5 cm) LiChroprep-RP2 (Merck; fractionated by sedimentation, d_p = 25-35 μm), Vydac-RP (Separation Group), Co-Pel-ODS (Whatman) and Permaphase-ODS (DuPont) were used. These columns were dry packed, flushed with MeOH at up to 15 ml/min, checked for the completeness of packing; if necessary, additional adsorbent was added.

HPLC on silica was performed in $CHCl_3$ with MeOH containing 5.2% of water /9/ using 0-10%/30 min or 0-30%/60 min linear gradient of MeOH, for the analytical columns, while the preparative columns were run isocratically.

The procedure for the analysis of the monomeric composition of oligonucleotides was described by Wulfson et al. /1,2/.

For the determination of K' (retention factor; $K' = (t_R - t_o)/t_o$ where t_R is the retention time and t_o is the elution time of

a non-retained compound) t_o was estimated according to elution of the weak component of the mobile phase: $CHCl_3$ in $CHCl_3$/MeOH in the case of NP-HPLC; water in H_2O/MeOH and H_2O/NH_4OAc in RP and IE-HPLC.

RESULTS AND DISCUSSION

De rebus omnibus et quibusdam aliis

In spite of many known methods for the HPLC separation of nucleotides there is no unambiguous procedure which is based on only one type of interaction and would therefore possess predictability. Such a method should be informative and should allow the identification of nucleotides in relation to their retention and eluent concentration, as it had been possible by the widely applied chromatographic method on DEAE-cellulose developed by Tomlinson and Tener /10/. We shall consider below how ionic and molecular interactions are revealed and how they can be used and controlled.

One of the most efficient methods of nucleotide separation is partition (liquid-liquid) IE RP chromatography, the so-called RPC-5 system /11/. The support consists of porous fluorocarbon beads (Plascon CTFE) coated with water-insoluble trialkyl quaternary amine (Adogen 464). We succeeded to obtain the narrow 6-8 µm fraction of the microparticles and to use them for packing high efficient columns (see Fig. 1). Unfortunately, these columns exhibit a low permeability. When the flow rate was increased the enhanced backpressure, even at 5-10 bar, compressed the support and this process was over only at about 300 bar. Therefore only those columns were stable which were packed at 400-500 bar, but they had to be operated at a high backpressure. Their efficiency was low, apparently due to the compression of the support's pores and the decrease of its specific surface area. At the same time, RPC-5 is unsuitable because of a lack of positive structural information due to the mixed ionic and hydrophobic interactions of the nucleotides with the charged ammonia groups and hydrophobic aliphatic

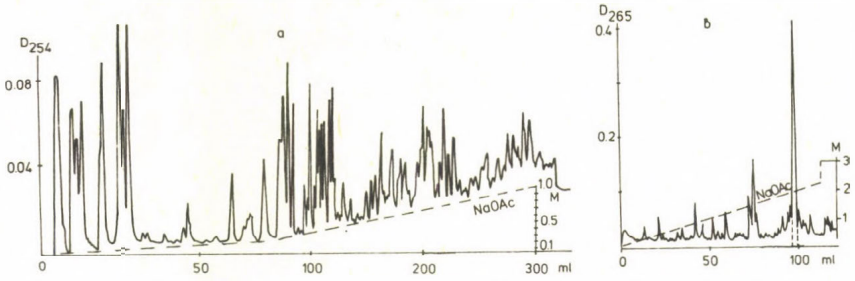


Fig. 1. RPC-5 High performance liquid chromatography of mono- and oligonucleotides: a) RNase digest of yeast RNA; b) synthetic mixture of undecanucleotide CCTTATAAATC preparation (column 0.8x11 cm; conditions the same as for (a), see /1,2/).

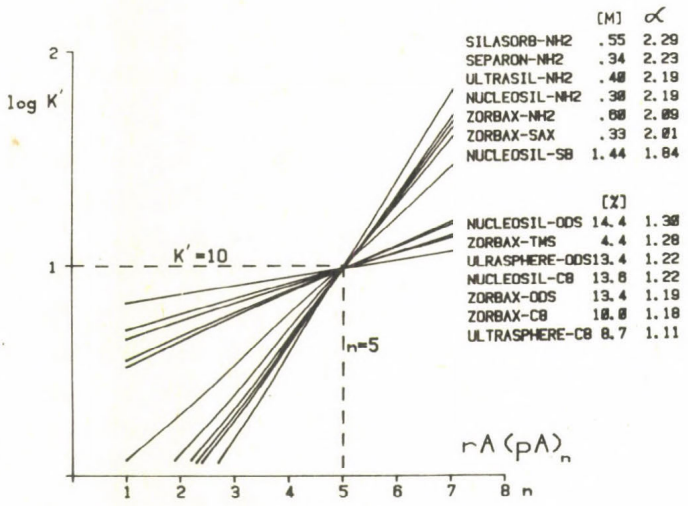


Fig. 2. Analysis of the selectivity of bonded-phase anion-exchangers and non-polar modified silicas to oligonucleotide length (number of mononucleotide units, n). Sample: mixture of oligo-riboadenilates $rA(pA)_n$. Elution conditions: [M] - NH_4OAc (molar) concentration (pH 7); % - MeOH concentration 0.2 M NH_4OAc (pH 7.2) is added to MeOH . α - selectivity coefficient (K'_n/K'_{n-1}) of the adjacent homologues

chains of the stationary phase. Evidently this type of chromatographic separation may only be used for fingerprinting.

Chromatography on bonded-phase anion-exchangers

Exceptis ecipiendis

Fractionation of oligonucleotides according to their length may involve not only IE, but also RP mechanism of HPLC /12/. We have compared widely used bonded-phase packings for RP and IE HPLC from the point of their selectivity to the length of the oligonucleotides in order to determine the preference of one of these methods. Such comparison can be graphically presented as linear dependences of logK'on the number of mononucleotide units (n) in oligoriboadenylates $-rA(pA)_n$ used as the sample (Fig. 2). Selectivity of the packing materials is determined by the relative retention α ($\alpha = K'_n/K'_{n-1}$), in these coordinates $\alpha = \log K'_n - \log K'_{n-1}$. It can be seen from Fig. 2 that on the anion exchangers tested, α= 1.8-2.3, while on the RP packings, α= 1.1-1.3. In other words, the latter are 1.6-1.8 fold less selective to the length of the oligonucleotides. It can also be deduced from Figs 2 and 3 that the adsorbents of each group are markedly different in their retention power (or in eluent concentration, see these data for IE under [M]; RP-adsorbents will be discussed below). As it is known, these packings are similar since they are prepared from silicas with a mean pore size of 40-80 Å, and a specific surface area of 300-500 m^2/g, and they have the same γ-aminopropyl stationary phase (Zorbax-SAX and Nucleosil-SB contain only quaternary amino groups while all the others are primary amines). The only possible explanation of such a difference is based on the variations in the technology of their production. The two packings Nucleosil-NH_2 and Nucleosil-SB exhibit a predictable increase in the retention which correlates with the increase in basicity during the transfer from primary to quaternary amine (see /13/, /14/ for more details about bonded-phase packings).

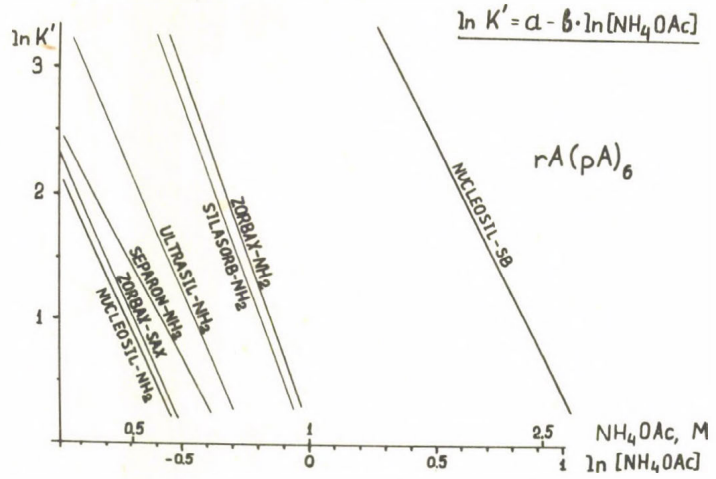


Fig. 3. Analysis of the capacity of γ-aminopropyl-silicas. Dependence of the retention factors (K') on the salt concentration in the mobile phase (pH 7). The sample is $rA(pA)_6$.

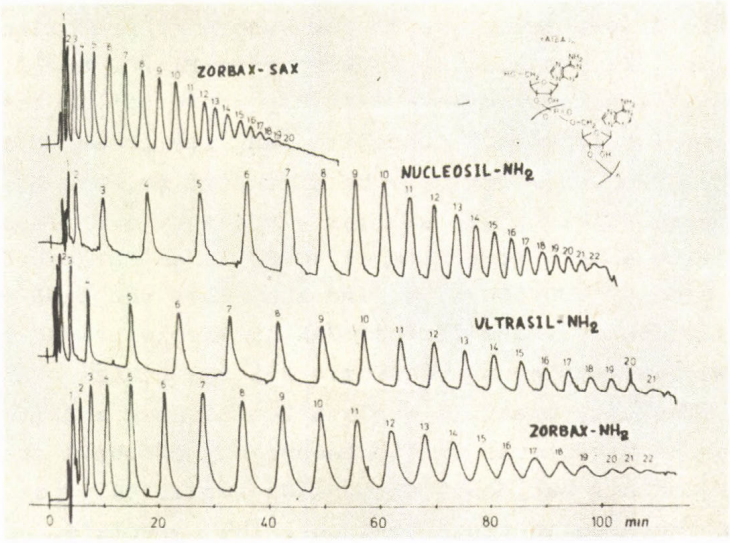


Fig. 4. Anion-exchange HPLC of oligoriboadenylates $rA(pA)_n$ ($n \approx 30$) on commercial columns. Elution conditions: linear gradient of NH_4OAc (pH 7) 0.4 - 2 M/100 min in 20% MeOH, 1 ml/min. Temperature, 25°C.

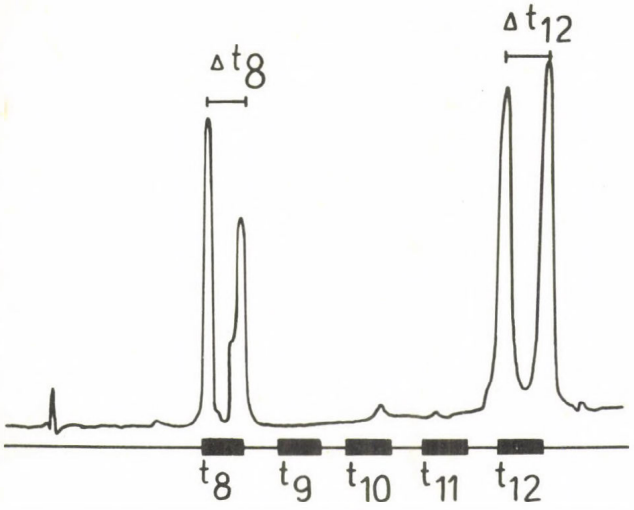


Fig. 5. Ion-exchange chromatography of the mixture of octanucleotides TATGATAT and AGCTTCCT and dodecanucleotides GAGAGATTTTAC and TAACCATGTCCA on an Ultrasphere-NH_2 column. For the conditions see Fig. 4 except that the gradient is 0.6 - 2 M/80 min.

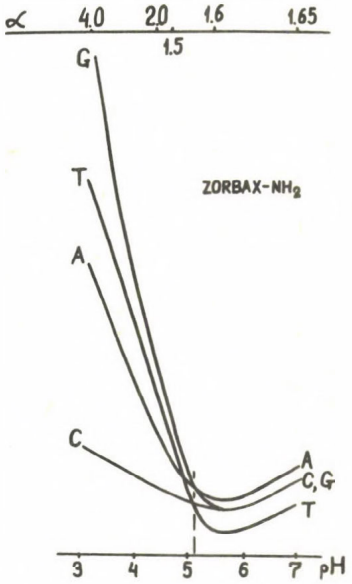


Fig. 6. Dependence of the retention and selectivity of separation of mononucleotides on the pH of the eluent. Column: amino-silica; eluent: 0.4 M NH_4OAc; temperature, 25°C.

Such comparison of the adsorbents for IE-HPLC permits to select the most suitable material depending on the size of the nucleotides (the number of phosphate groups involved) to be separated, the selectivity and the retention which is related to the salt concentration needed for elution. One should remember that salts markedly increase the mobile phase viscosity thus decreasing the efficiency of mass transfer.

The commercial anion-exchangers tested permits to separate the full set of homologous oligonucleotides, e.g., riboadenylates with the length of up to 20 monomer units (Fig. 4). Separation of the larger oligomers is limited by both the stronger retention by the stationary phase and, probably, by the pore size of the silica. In contrast to this packing material the silica having 100-120 Å pores with adsorbed polyethyleneimine prepared recently by Regnier and Pearson /15/ exhibits a higher resolution for the fractionation of larger oligomeres, but this support has a slow mass transfer and thus, the separation of, e.g., 30 oligoadenylates in a 5 cm column lasts about 4-5 hours.

IE-HPLC may be more informative if the dependence of the retention from nuclectide length is known. Fig. 5 demonstrates graphically the problem of linear calibration "retention versus oligonucleotide length". This problem involves the search for such separation conditions where the adjacent time windows (Δt_{n-1}, Δt_n and Δt_{n+1}) for the isoplytes with different composition will not be overlapped (the analysis of commercial packings for this purpose has been presented by Wulfson et al. /1,2/). Since the molecular interactions which make the separation of isoplytes according to their heterocyclic composition possible occur together with ionic interactions, we studied the effect of the mobile phase on the nucleotides and the stationary phase in order to minimize the molecular interactions and thus to make the time windows of isoplytes narrow.

The change in pH has a strong effect on the charge of the nucleotide /16/ and on the basic properties of the stationary phase /17/. According to Fig. 6, the pH value when the molecular interactions would be minimal, is pH=5.2.

The resolution of isoplytes of different composition is markedly influenced by the addition of an organic modifier. It results in an increase in the hydrophobic selectivity (Fig. 7). This unexpected effect may be accounted for by the change in the steric structure of the bonded-phase radicals. The increase in the concentration of the organic modifier leads to a destruction of the hydrophobic associates of the alkyl chains of the stationary phase. This effect gives rise to a change of its capacity but also to an undesirable selectivity to heterocycles. Therefore, it is possible to propose that for oligomeric branched stationary phases, the molecular interactions should be gradually enhanced in relation to the "untwisting" of the chains as the concentration of the organic modifier is increased. On the other hand, for a monolayer stationary phase the maximum of the selectivity corresponding to the "untwisting" of the chains should be observed followed by a decrease in the contribution of molecular interactions to total retention. (A more detailed information about adsorbents will be published elsewhere.)

The study of commercial anion-exchangers makes it possible to develop a new anion exchanger. This exhibits a more efficient mass transfer and, therefore, higher resolution and it permits to fractionate larger oligonucleotides (see Fig. 8). The selectivity of this adsorbent to heterocycles can be easily regulated by the addition of an organic solvent since, in contrast to commercial anion exchangers, the increase in the methanol concentration up to 50% practically eliminates the separation of isoplytes according to their composition (see Table I and Fig. 9). Under these conditions the identification of large oligonucleotides according to their size (according to total charge) is possible.

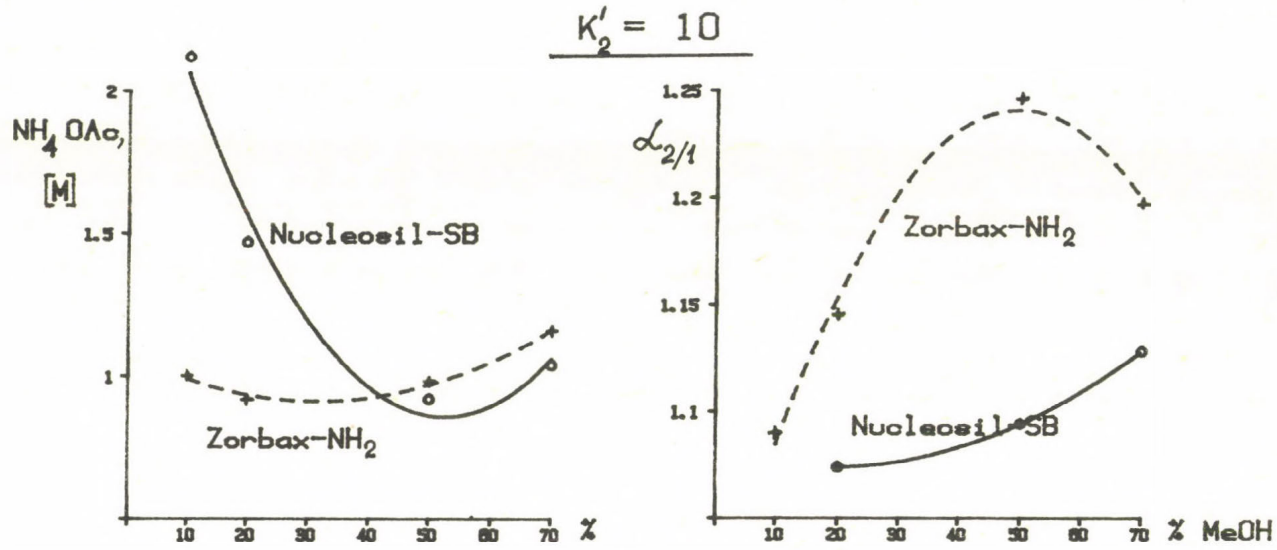


Fig. 7. Dependence of the salt concentration and of the selectivity ($\alpha_{2/1}$) of isoplyte separation on the concentration of organic solvent (MeOH) in the eluent. Sample: dodecanucleotide TAACCAGTTCCA (1) and TAACC ACTACA (2). Column: amino-silicas (NH_2-primary and SB quaternary amino group). Data were obtained at constant retention, K'_2=10, pH=7.

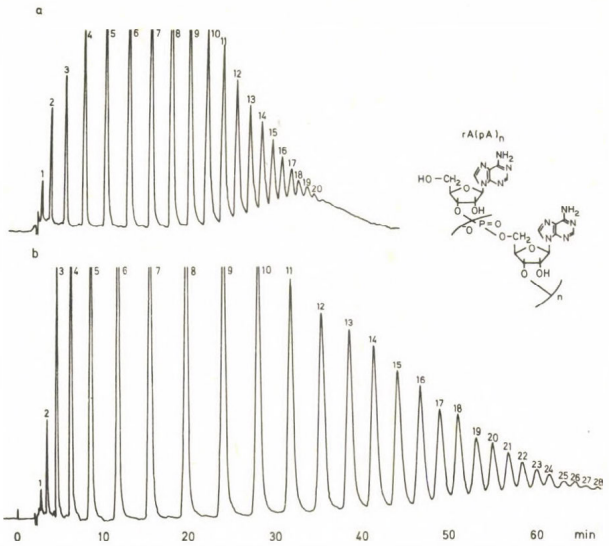


Fig. 8. Ion-exchange chromatography of oligoribo-adenylates on the new anionite. Column (0.46x15) cm is packed with 5 μm particles. (The efficiency is 6650 theoretical plates for the pentanucleotide $rA(pA)_4$ at K' = 3.5). Elution conditions: linear gradient of NH_4OAc (pH 7) in 50% MeOH, a- 0.13-1 M/174 min; b- 0.1 - 1 M/300 min; flow rate, 0.84 ml/min. Temperature, 40°C. Pressure, 70 bar.

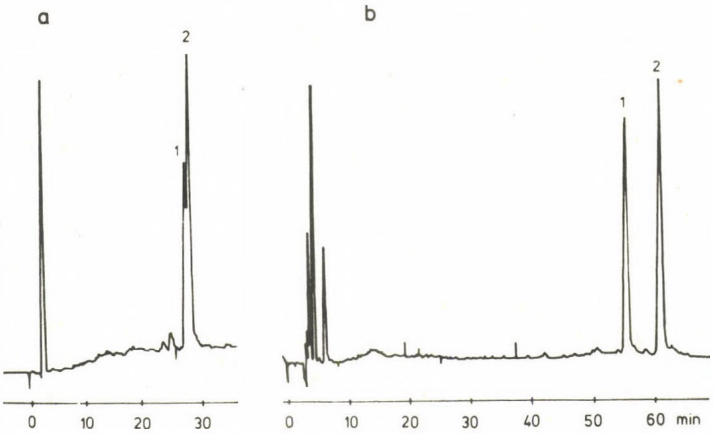


Fig. 9. Chromatography of the hexanucleotides GGTACC (1) and GAATTC (2) on the new anion-exchanger. The effect of MeOH concentration on the selectivity to the isoplyte composition is shown. The conditions are the same as in Fig. 8 except the eluent composition: a- in 50% MeOH, b- in 20% MeOH.

Table I. Dependence of the resolution (R_s) and the reduced resolution (R_s/t_{R2}) of isoplytes on the new anion-exchanger on the methanol concentration of the eluent. Column: 0.46x15 cm, $\bar{d}_p$:5 µm. Sample: hexanucleotides GGTACC and GAATTC. Flow rate: 0.7 ml/min. Temperature: ambient. (See Figs 9 and 13.)

Methanol [%]	Ammonium acetate [M]	R_s	t_{R2}	R_s/t_{R2}
50	0.1 - 1 <200 min>	0.28	26.5	0.011
30	0.1 - 1 <80 min>	1.82	33.0	0.055
20	0.1 - 1 <40 min>	3.52	62.5	0.056
20 - 100 <200 min>	1	3.16	23.0	0.137

Reversed-phase HPLC. Optimization of the separation of nucleotides according to their composition

Argumentum ad rem

RP-HPLC becomes an indispensable instrument for the separation of nucleotides due to the high selectivity of non-polar packings (usually ODS silicas) to the hydrophobic heterocycles of nucleotides. RP-HPLC (with µBondapak-C_{18}) has been used the first time for the separation of nucleotides by Fritz et al. (18). Today, besides µBondapak-C_{18}, other RP packings are also applied for this type of separation. Since the properties of individual RP packings are different, it would be interesting to find the proper objective criteria for the selection of the suitable support, for a certain separation problem.

The difference in retention of these packings has already been demonstrated in Fig. 3 (compare the methanol concentration (see under %) used for the elution of the same nucleotide $rA(pA)_5$ with equal retention from various columns). The dependence of the retention factor on the eluent (methanol) concentration is more clearly expressed in Fig. 10: as seen the

lnK' vs. ln[MeOH] relationship is linear. Among the data given in Fig. 10 one should pay particular attention to the similar relationships for three sorbents of the same type, apparently, produced by using the same technology. These are: Zorbax-TMS (trimethylsilyl, C_1-phase), Zorbax-C8 (C_8 phase) and Zorbax-ODS (C_{18}-phase). Plotting these data in the coordinates ln[MeOH]-ln C_i (C_i is the length of the stationary phase radical), we obtained a linear relationship between the length of the alkyl radical of the stationary phase and the concentration of the eluent needed for elution with equal retention of the same sample (Fig. 11). We assume that such strong dependence should take place for a monomolecular stationary phase, prepared by the modification of the silanol groups with monochlorosilanes. On the contrary, silicas modified with polyfunctional reagents having polycondensed structures on the surface exhibit a weak dependence, like the one given in the upper part of Fig. 11. The proposed method for the evaluation of the packings can help to solve the problems resolved by HPLC. We do not consider here the advantages of a monomolecular stationary phase discussed in detail in the books of Unger (13) and Snyder and Kirkland (14).

Selectivity of the packing material to the compounds to be separated remains one of the main questions. Such a comparison of the selectivity of various packings has been carried out by the separation of the mixture of four deoxymononucleotides. The significant differences between the high selectivity of RP-adsorbents and the relatively low selectivity of IE-adsorbents can be seen in Fig. 12. (It is noteworthy that the comparison was performed at a neutral pH when the amino groups of the heterocyclic bases are not protonated and thus, the separation of mononucleotides on both types of packings proceeds due to molecular rather than ionic interactions.) It can be also seen from the presented data that the composition of the eluent has a strong effect on the molecular interaction of the nucleotides with the stationary phase. The substitution of 1% of acetonitrile for 1% of methanol exhibits a different influence on the adsorbent selectivity. This effect can be attributed to the specific aspects of their production.

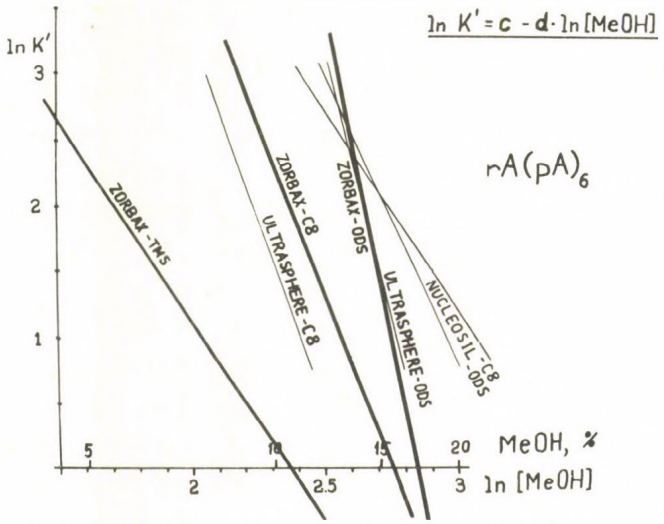


Fig. 10. Dependence of the retention factor of an oligonucleotide on the MeOH concentration in 0.2 M NH_4OAc (pH 7.2), using bonded-phase non-polar silicas. Sample: $rA(pA)_6$.

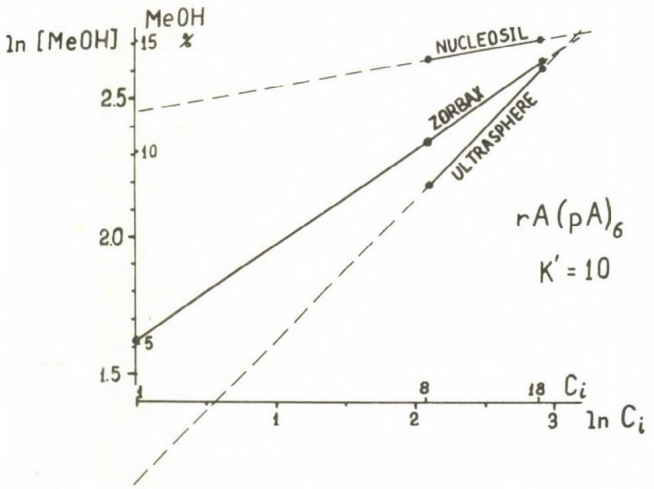


Fig. 11. Dependence of the eluent concentration (MeOH, %) necessary for the elution of an oligonucleotide with K' = 10 on the length of the stationary phase radical (C_i). Sample: $rA(pA)_6$. 0.2 M NH_4OAc (pH 7.2) is always added to the eluent.

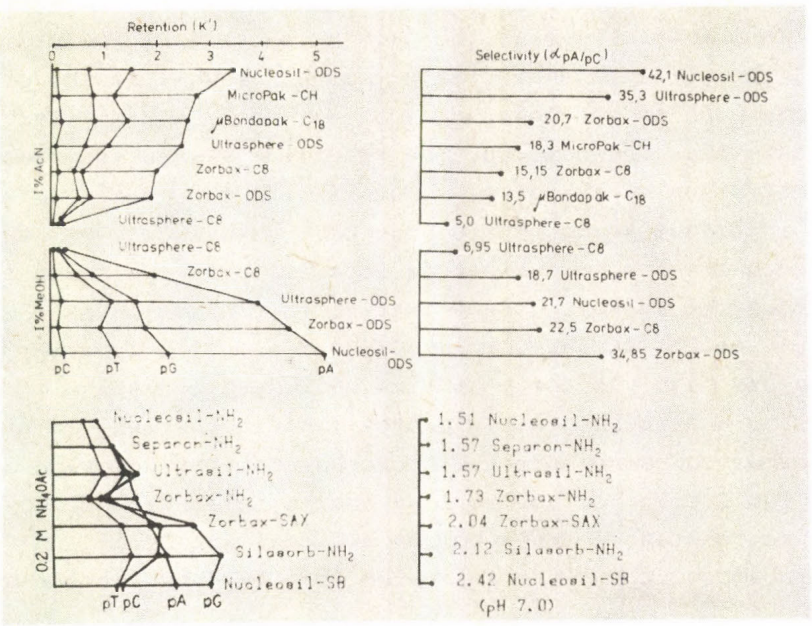


Fig. 12. Comparison of commercial columns containing bonded-phase nonpolar and amino-silicas in respect of the retention of and their selectivity to mononucleotides (from /1,2/).

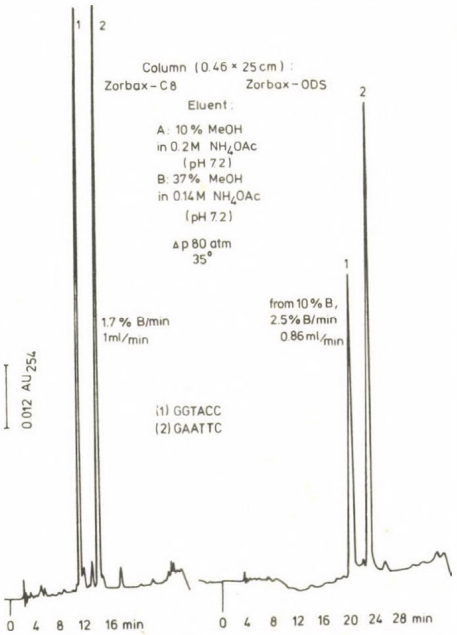


Fig. 13. RP-HPLC of hexanucleotides on Zorbax-C8 and Zorbax-ODS columns. The conditions are given on the figure.

In the selection of the packing material we consider these data, the obtained selectivity values, as well as the economic aspects such as the lower cost of methanol in comparison with acetonitrile. In addition, the retention values and thus the concentration of the organic modifier in the mobile phase are also important to accomplish the best resolution. For example, in the case of oligonucleotide separation it was found that the required methanol concentration for Zorbax-C8 is 15-20% while that for Zorbax-ODS is 30-40% (Fig. 13). However, an anomalous high viscosity at such MeOH concentrations in water leads to a decrease in efficiency (and resolution) in the case of Zorbax-ODS as compared with Zorbax-C8. The sorption-desorption equilibrium of nucleotides having molecular weights below 5000 is constant on this column and does not depend on the sample amount up to the following peak heights (at 254 nm):

0.46 cm diameter column: 10 AU (about 0.4 mg)
0.94 cm diameter column: 40 AU
2.12 cm diameter column: 200 AU.

Half of these loads results in a 40-50% decrease in column efficiency while the full load results in a twofold decrease in the resolution. It is known that the sample capacity of the columns depends on the concentration of the sample /19-21/ and this has been confirmed in our studies. We have also found that the optimal concentration of the injected sample corresponds to each load (Fig. 14). Taking into account that in genetic engineering oligonucleotides are employed from a fraction of a mg up to several milligrams, the loadability of even analytical columns is sufficient in most cases.

Further optimization involves the search for the optimum pH to achieve the best selectivity. We could not establish this value, as it is situated in the alkaline region (Fig. 15). It is known that the silica-based packings are rapidly destroyed in alkaline medium due to the hydrolysis of the silyl ether groups and the solubility of silica. Nevertheless a rather high selectivity was observed at pH 7-7.2 which is non-hazardous for the packings and for the nucleotides.

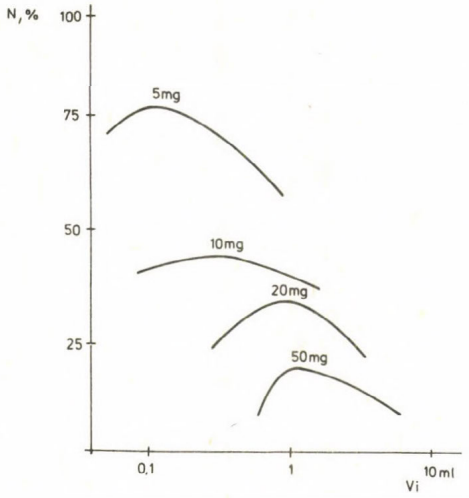


Fig. 14. Dependence of the efficiency (N: the number of theoretical plates) on the load and the sample concentration (V_i) for a Zorbax-C8 preparative column (2.3x25 cm). Sample: benzyl alcohol, K' = 1.5. Eluent: MeOH/H_2O 45:55, flow rate: 10 ml/min.

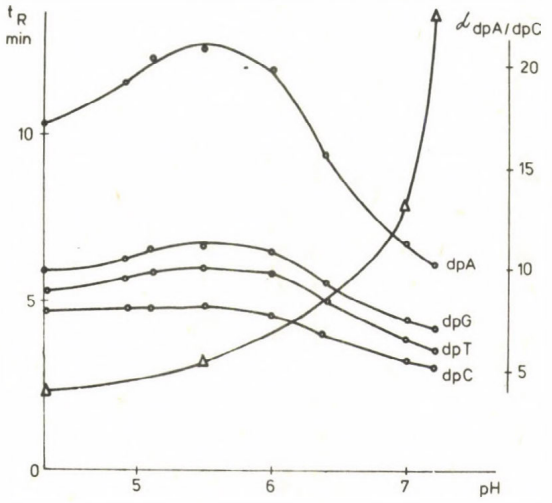


Fig. 15. The effect of pH on the retention and selectivity ($\alpha_{dpA/dpC}$) of mononucleotides on Zorbax-C8. Eluent: 1% MeOH in 0.1 M NH_4OAc.

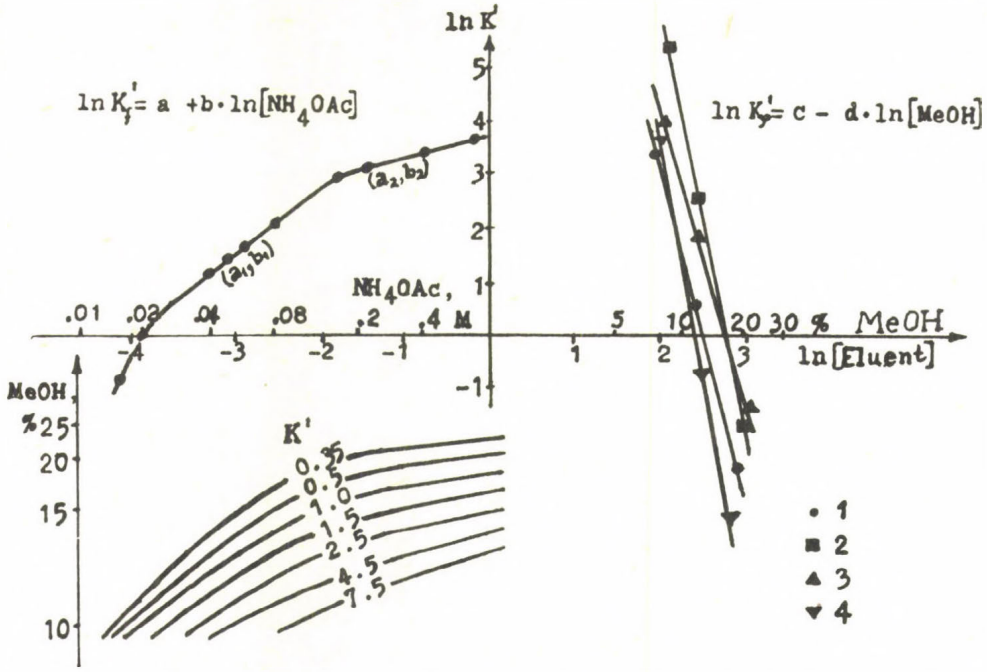


Fig. 16. Dependence of the retention factor of nucleotides in RP-HPLC on the concentration of NH_4OAc (pH 7.2) and MeOH in the mobile phase. Zorbax-C8 column. 1- TAAGCAGATGCCA, 2- ATCTTACTGCAT, 3- AATTCGGTT, 4- CGAATTCG. K'_f: at 10% MeOH, K'_ψ: at 0.2 M NH_4OAc. The lower graph is for sample 1.

We have also studied the effect of the eluent's composition and the concentration of its components on the retention. The addition of the dissociated salt proves to be very important. (We used ammonium acetate as it is volatile and can be removed from the nucleotide fractions.) It can be seen in the left part of Fig. 16 that at low salt concentrations (<0.02 M) the value of the retention factor is less than 1. This can be explained by the electroexclusion of the negatively charged nucleotides from the pores having a negative surface charge. When the concentration of the salt is increased above 0.02 M the retention factor is enhanced up to charge neutralization and further increase (above 0.14 M) reveals a slight salting-out effect.

The dependences of the retention on the concentration of the salt and the organic solvent are similar (Fig. 16): they are linear in the logarithmic coordinates lnK' - ln E (where E is the eluent, salt or the solvent) but have the opposite sign.

Optimization of the mobile phase composition. Prediction of the optimum gradient program for the desired retention of the component of interest.

Viribus unitis ... et mutatis mutandis

It can be seen in the lower part of Fig. 16 where the relationships between the concentrations of methanol and the salt are given as a function of K' that each K' value may be achieved by various combinations of methanol and salt concentrations. It is necessary to find the best concentration regions. Since the effects of methanol and salt on the retention are opposite we tested three combinations (No 1, 2, 3) from nine possible ones. In this diagram gradient with increased concentration is denoted as "↗", gradient with decreased concentration is denoted as "↘" and unchanged concentration as "-":

[MeOH]	↗	↗	↗	-	-	-	↘	↘	↘
[NH_4OAc]	↗	-	↘	↗	-	↘	↗	-	↘
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)

The combinations 4, 5, 7 and 8 have no practical use while combinations 6 and 9 require high salt concentration accompanied with high viscosity and decrease in efficiency. The best results were obtained (see Table II) in the case of combination 3, with increasing methanol and simultaneously decreasing salt concentration (detailed information were given by Wulfson et al. /1,2/).

Gradient elution is necessary for the separation of complex multicomponent mixtures of nucleotides, either chemically synthesized or of natural origin. For the detection of all the components in the mixture gradient elution chromatography

Table II. Dependence of the resolution (R_s) and the reduced resolution (R_s/t_{R2}) on the conditions of gradient elution. RP chromatography of hexanucleotides GGTACC and GAATTC on Zorbax-C8 (0.46x25 cm). (For details see /1,2/)

Combination No	Gradient program (100 min duration) [MeOH], %	[NH_4OAC], M	R_s	t_{R2} min	R_s/t_{R2}
1	10-37	0.2-03	6.0	14.2	0.42
2	10-37	0.2	6.4	14.5	0.44
3	10-37	0.2-0.15	6.7	14.8	0.45

requires much less time. At the same time a well defined gradient program provides the same sensitivity for both early and late eluting components.

Working with a certain type of mixture, and especially for analytical purposes, it is convenient to carry out separation under fixed conditions, i.e. with a suitable standard gradient. However, when one component of the mixture should be isolated, steep ("survey") gradient is often irrelevant because the resolution of the adjacent zones upon gradient elution is poorer than in the case of isocratic analyis. The experimental determination of the optimum program for each new mixture is time consuming, while for its calculation on the basis of known equations, a series of coefficients must be estimated, and this may also be difficult. The theory and practical information about gradient elution is given by Snyder /22/).

We have developed a simple method for the determination of the optimum gradient program for the desired retention of the component of interest. It consists of the determination of the eluent concentration (which happens to be equal to 10% MeOH in the example given in Fig. 17) at which the component of interest is eluted isocratically for a desired time (10 min in this particular case). The gradient program must involve this

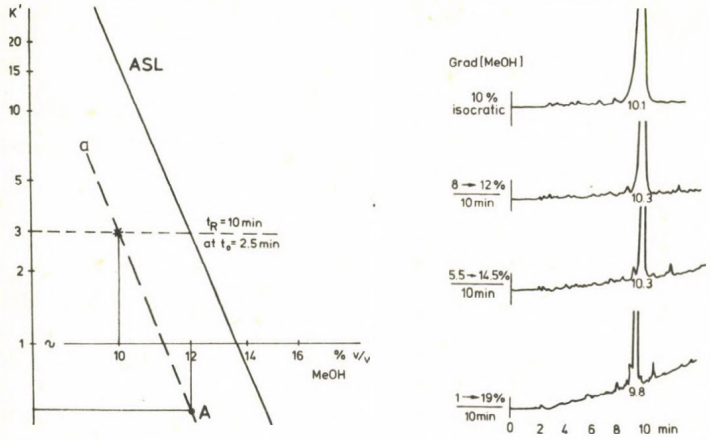


Fig. 17. Schematic presentation of the method for the prediction of the gradient program with a given elution time of the component in question. Left: estimation of the MeOH concentration ("*") corresponding to a given (10 min) retention time; ASL - average straight line for the retention of oligonucletides (according to the data given in Fig. 16). Right: the chromatograms of the same sample at various gradient slope symmetrical in relation to the found (*, 10%) MeOH concentration. Sample injection in the gradient cycles with a 2.5 min delay after the gradient start (see text).

concentration as a middle point (1-19%, 2-18%, 9-11% etc) for the desired 10 min. Then, the elution time of the sample remains constant (10 min) regardless of the steepness of the gradient. The resolution between the substance of interest and the adjacent substances will be the best in case of a slow gradient. The concentration of the eluent set for the middle of the program is found either on the basis of one isocratic chromatogram at any eluent concentration (point A at Fig. 17) followed by drawing from this point a direct line parallel to the average straight line (ASL) found earlier for a series of substances (it was plotted by averaging the data given in Fig. 16) or on the basis of two isocratic chromatograms (point A and the second point at another, e.g., lower MeOH concentration).

Selection and installation of the pre-column. Widening of its functions

Abusus non tollit usum

Guard-columns are used for the protection of analytical columns from failure due to adsorption of strongly retained and chemisorbed substances originated from the eluent or the samples. There are two approaches for the preparation of guard-columns: 1) to use the same packing material as in the main column; 2) to use larger particles of pellicular packings /23/. Since the sample band passing through the guard column undergoes broadening, the guard-column in the first case is considered as an extension of the main column from the top that is the mixture starts to be separated in it and the loss the efficiency of the whole system would be minimal in the case of a minimum volume of the connections. In the second case, the band broadening leads to a higher decrease of the system's efficiency. Thus, the volume of the guard-column and the volume of the connections have in both cases a negative effect on efficiency and resolution, especially in the case of early eluting peaks. The volume of the guard-column can be decreased but this will diminish its effectiveness due to the decrease of its capacity. It is possible to decrease the volume of the connections but it is difficult /24/ . All there remarks refer to isocratic opertation.

We found that the limitations due to the volumes of the guard column and its connections with the analytical column are practically unimportant in gradient elution if the adsorbent for the guard column is properly chosen. For its selection we used a simple procedure. A typical mixture containing oligonucleotides was chromatographed on a guard-column (0.46x5 cm), dry packed with 30-50 μm particles of the adsorbent. The chromatographic conditions for the quard-column were the same as for the main column (Fig. 18). After testing a few packings (see Table III.) we selected those which had a low retention of the components of interest, but retained the undesired components within the working range of the eluent concentration.

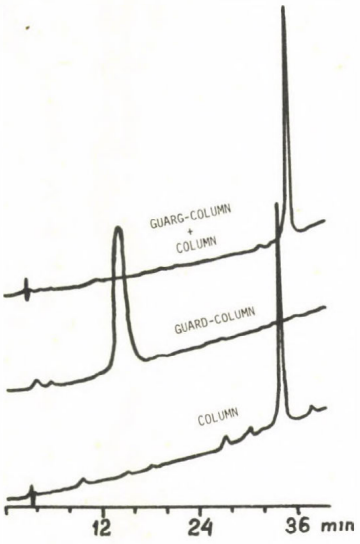


Fig. 18.

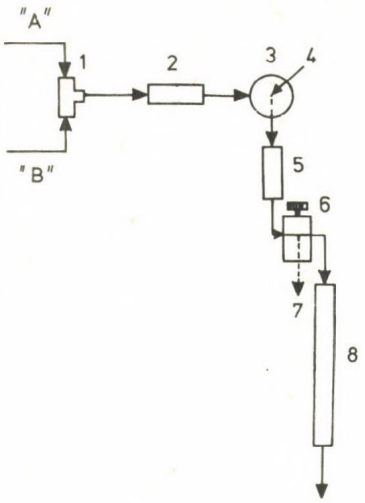


Fig. 19.

Fig. 18. Illustration of the test method for the selection of a guard-column. The conditions for gradient elution are the same in all cycles; MeOH gradient 0 - 37%/50 min. Sample: dodecanucleotide. Column: Zorbax-C8 with a guard-column (0.46x5 cm) packed with Permaphase-ODS.

Fig. 19. Schematic presentation of the gradient elution system with two pre-columns. A and B - time-programmable eluent flows, 1- T-shaped union, 2- precolumn (0.46x5 cm) for the mobile phase and static mixer, 3- injector for the sample (4), 5- guard-column for the sample (0.46x5 cm), 6- 3-way valve, 7- drain for guard-column washing, 8 column (see text).

Since the components of the mixture are eluted earlier from such guard-column than they start their movement along the main column, the volumes in which they are introduced into the guard-column and in which they enter the main column are not critical. Thus the efficiency of the system does not decrease and the limitations on the volume on the connections between the guard column and the main column are eliminated. Moreover it is possible to connect the guard column and the main column

Table III. Studies of guard-column packing materials for RP-HPLC. Sample: hexanucleotide GAATTC containing a not identified impurity.

	Methanol concentration		Peak broadening at peak width of half height, %
	hexanucleotide	impurity	
Zorbax-C8	19.7	50	0
Vydac-RP	14.7	45	
Co-Pell-ODS	11.9	25	
Lichroprep-RP2	~0	50	
Permaphase-ODS	3.5	43	
Vydac-RP + Zorbax-C8	22.4		49.0
Co-Pell-ODS + Zorbax-C8	20.5	50	4.5
Lichroprep-RP2 + Zorbax-C8	21.1	60	4.5
Permaphase-ODS + Zorbax-C8	20.0	60	3.0

Column-0.46x25 cm
Guard-column-0.46x5 cm

Methanol concentration:
linear gradient 0→ 37%/50 min
column clean-up 37→ 70%/20 min

with a 3-way valve in order to be able to wash the guard-column only with a strong eluent for gegeneration.

In addition to the guard-column we recommend to use a precolumn (2 in Fig. 19) mounted between the pump and the sample injector, in order to preserve the column from undesired substances present in the eluent, to filter it from particulates and to mix the flows of eluents A and B. The substitution of a dynamic (electro-magnetic) mixer and a porous metal filter to the precolumn with a free volume of only 0.5 ml leads to a

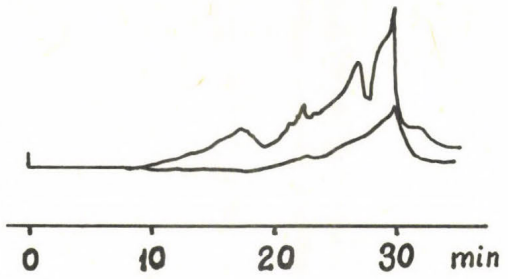


Fig. 20. Demonstration of the degree of purification of the mobile phase with pre-columns. Gradient: conventional chemical grade MeOH, 0 - 50%. Detection: UV_{254}, 0.02 AUFS. The upper line - Ultrapack-C8 analytical column. The lower line - the same analytical column with a guard-column containing Permaphase-ODS and a pre-column containing LiChroprep-RP2.

decrease of the volume before the column, thus diminishing the gradient delay time and improving the reproducibility of the gradient program. This also permits us to use common cost chemically pure solvents instead of the very expensive HPLC-grade solvents (Fig. 20). The packing material used for the precolumn should not retain the components of the eluent (e.g., methanol from water in RP, methanol from chloroform in NP and methanol and salt in IE-HPLC) and should have a particle size above 30 μm for efficient mixing (in most cases Lichroprep-RP2 fulfills these requirements for RP- and IE-HPLC and silicas with a specific surface up to 300 m^2/g are suitable for NP-HPLC).

The application of all the optimum conditions found for RP-HPLC of oligonucleotides is demonstrated in Fig. 21 in the case of isolation of the dodecanucleotide GTTGTTGACATT, one of the fragments of promotor of RNA polymerase E.coli.

However, the apparent isolation of an individual oligonucleotide by RP-HPLC (as in Fig. 21, right) does not always prove its homogeneity, since in some cases nucleotides different in size and having heterocyclic composition have the same retention (see Fig. 22a). Therefore, it is worthwhile to perform a preliminary isolation of the oligonucleotide of interest by ion-exchange HPLC which is more selective to the

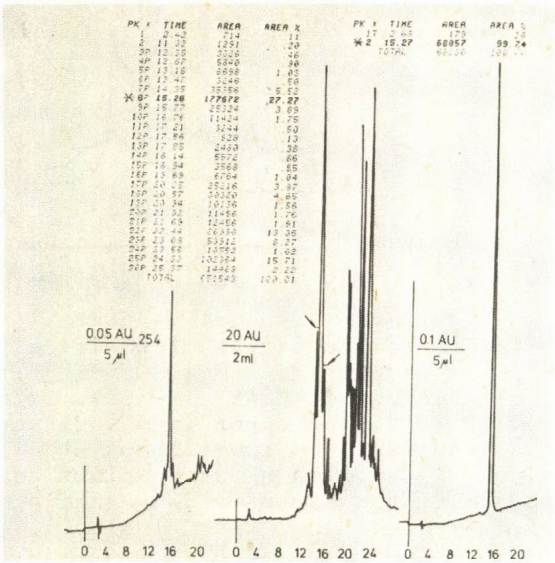


Fig. 21. Isolation of the synthetic dodecanucleotide GTTGTTGACATT by RP-HPLC with programming of the mobile phase composition at a given elution time (15 min) Column: Zorbax-C8 (0.46x25 cm), guard-column: Permaphase-ODS (0.46x5 cm). Eluent: A- 10% MeOH, 0.2 M NH_4OAc (pH 7.2); B- 37% MeOH, 0.145 M NH_4OAc, linear gradient. Flow rate: 1 ml/min. Temperature: 35°C. Detection: UV_{254}, 0.06, 3.88, and 0.06 AUFS (from left to right). The detector output signal with a maximum response of 2.56 AU is attenuated X 1.5

Gradient programs:

analysis of the mixture (left): 10-18% MeOH/15 min; preparative cycle (center) and analysis of sample homogenity (right): 12-16% MeOH/15 min (symmetrical to 14% MeOH); after 15 min, rapid increase of the gradient in order to elute the late peaks.

Sample injection delayed 3.5 min after the start of the gradient program. the data for the dodecanucleotide peak are marked in the report by an asterisk (*), in the case of purification and homogeneity analysis.

nucleotides and permits their identification according to their size. The final purification should be carried out by RP-HPLC, which is more selective to heterocyclic composition (see Fig. 22b). All synthetic oligonucleotides (more than 350) - DNA fragments - were isolated by a similar way, and we can

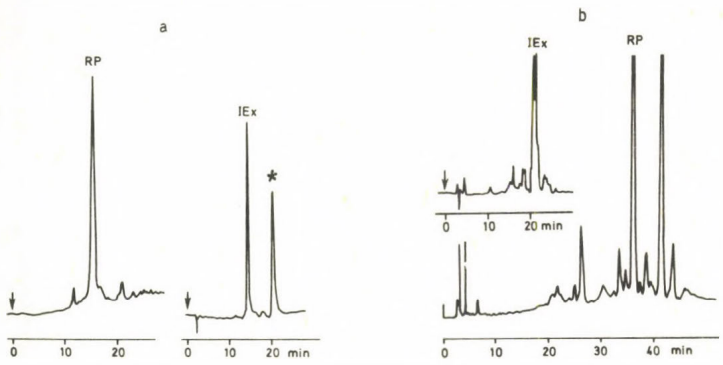


Fig. 22. Two-dimensional HPLC of oligonucleotides. a- Analysis of a synthetic sample (GAACTGGTTACCG, marked "*"); b- isolation of isoplytes of dodecanucleotides by IE-HPLC and RP-HPLC. The conditions are similar to those given in Figs 21 and 8.

conclude that such two-dimensional approach guarantees the high purity of oligonucleotides, which are successfully used for the construction of functionally active DNA fragments and for other studies in molecular biology.

Concerning the mechanism of the so-called "ion-pair" chromatography

Cessante causa, cessat effectus

We are interested in this method as an alternative approach to the separation of nucleotides according to their heterocyclic composition. If alkylsulfonates, e.g. dodecylsulfate (SDS), are added to the mobile phase, mono- and oligonucleotides (and nucleosides) are fractionated with high selectivity according to their composition as in RP-chromatography (see Fig. 23). However they are actually fractionated as in ion-exchange chromatography. This is confirmed by the fact that retention depends on the pH and the elution sequence of the nucleotides in acidic media corresponds to a positive charge arising on the amino groups of the heterocycles upon their protonation /16/ (Fig. 24a). The decrease in the reten-

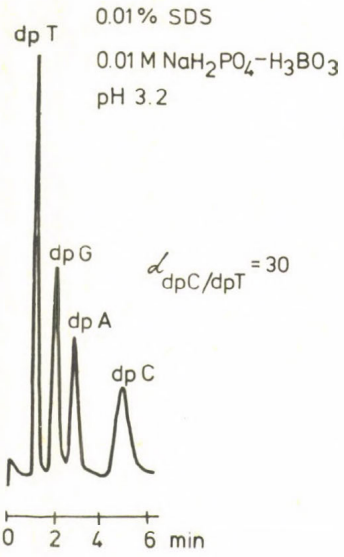


Fig. 23. Separation of mononucleotides on non-polar packing (Micropack-CH column, packed with LiChrosorb-RP18) in the presence of SDS. Flow rate: 0.3 ml/min. Temperature: 25 °C.

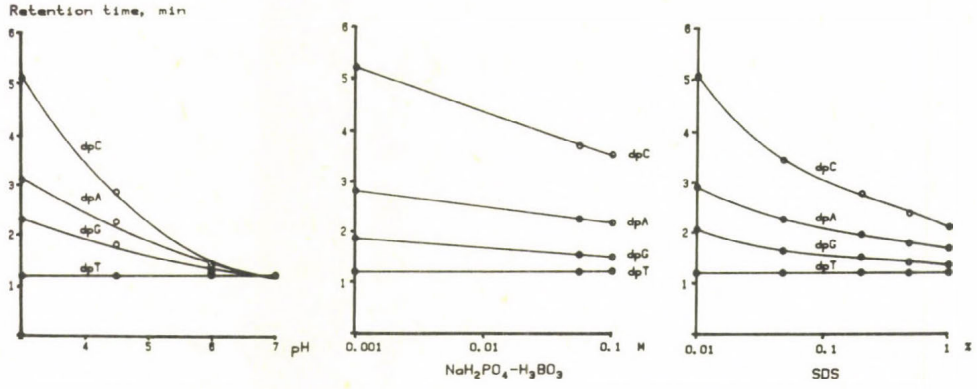


Fig. 24. Dependence of the retention of mononucleotides on the pH (0.01% SDS in 0.01 M NaH_2PO_4–H_3BO_3), the buffer concentration (in 0.01% SDS, pH 3.2) and the SDS concentration (in 0.01 M buffer, pH 3.2) in "ion-pair" chromatography. Other conditions are as in Fig. 23.

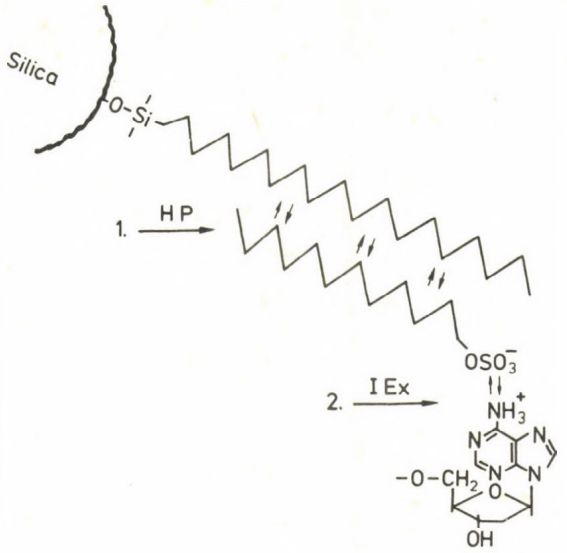


Fig. 25. Schematic presentation of hydrophobic-pair (HP) ion-exchange (IEX) mechanism.

tion when the concentration of the salt and the ionic modifier is increased also supports an ion-exchange mechanism. How can this happen in the case of chromatography on the non-polar C_{18}-silica?

Apparently the separation mechanism of water-soluble polyelectrolytes in such conditions involves two main processes (such mechanism has been first proposed by Kissinger /25/):1) due to hydrophobic affinity the modifier from the water-salt mobile phase intercalates into the non-polar stationary phase layer (C_{18} or C_8) orienting with the ionic groups toward the mobile phase; 2) the stationary phase modified in this way with SDS sulfo-groups participates in the ionic interactions with the positively charged protonated amino groups of the heterocycles. Such a model (Fig. 25) clarifies why a pure ion-exchange separation like on a typical cation-exchanger /26/ is observed in the case of "ion-pair" chromatography. Providing that the first stage - formation of a hydrophobic pair [stationary phase — modifier] - did not take place, i.e., the dodecylsulfate ions in the solution (in mobile phase) would form ion pairs with the nucleotides and therefore, separation

would proceed on the unmodified stationary phase, then the retention of nucleotides would be increased with the enhancement of the SDS concentration in the eluent; however in fact, we have observed the reverse of this (Fig. 24c). Thus, the first step is a determining step in the process. Therefore, we suggest to call the separation of water-soluble electrolytes on a non-polar support in the presence of ionic hydrophobic modifier as hydrophobic-pair ion-exchange chromatography. Another independent confirmation of such mechanism is the opposite dependence of the retention on the pH and the concentration of the ionic but hydrophilic modifier -ammonium acetate - observed in RP chromatography (see Figs. 15 and 16), which may be considered as actual ion-pair chromatography.

Normal-phase adsorption HPLC of protected oligonucleotides

Te, aqua, laudamus

NP adsorption chromatography of nucleotides was applied only recently after the introduction of the so-called phosphotriester method of oligonucleotide synthesis (see for example /4,5/). During such synthesis the internucleotide condensation products are derivatized (protected) over all polar (phosphate, amino and hydroxy) groups. The application of NP-HPLC for the purification of the intermediates during oligonucleotide synthesis, as shown in Fig. 26, provides high purity and increased productivity of the synthesis and it markedly reduces the consumption of the starting compounds and reagents /1,2/. Diastereomers, which are formed due to the chirality of the triester phosphate groups, should not be separated because at the end of the synthesis the protecting groups are removed and the isomery disappears.

The most efficient and productive method is gradient NP-HPLC which has been recently used rather rarely. However, after the development of isohydric solvents (see Thomas et al. /27/) it became as simple as the other LC methods. The fast analyses of dinucleotides different in the heterocycle of the second unit and the separation of protected and partially deprotected

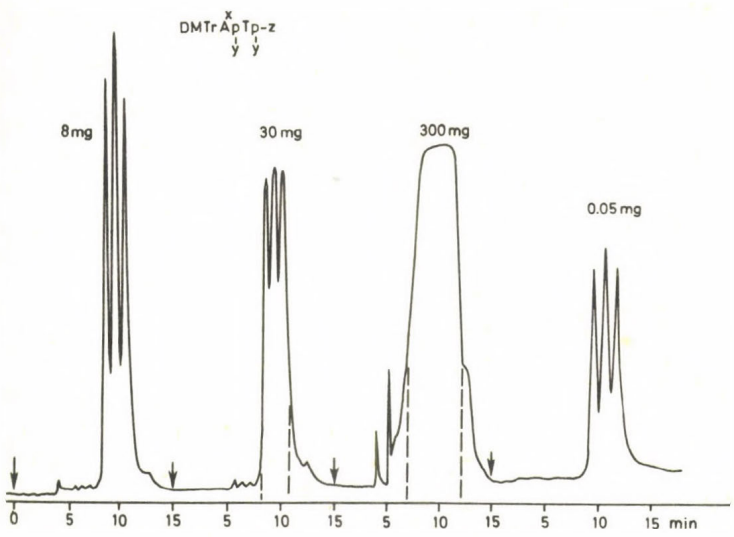


Fig. 26. Normal-phase adsorption HPLC of a protected dinucleotide (DMTr: dimethoxytrityl, x: benzoyl, y: p-(C -phenyl, z: CNEt). Column: LiChrosorb Si-60 (2.3x25 cm), 10 µm particles. Eluent: $CHCl_3$/MeOH 96:4 (MeOH contains 5.2% water). Detection: UV_{254}, 1 mm cuvette, 25.6 AUFS maximum response.

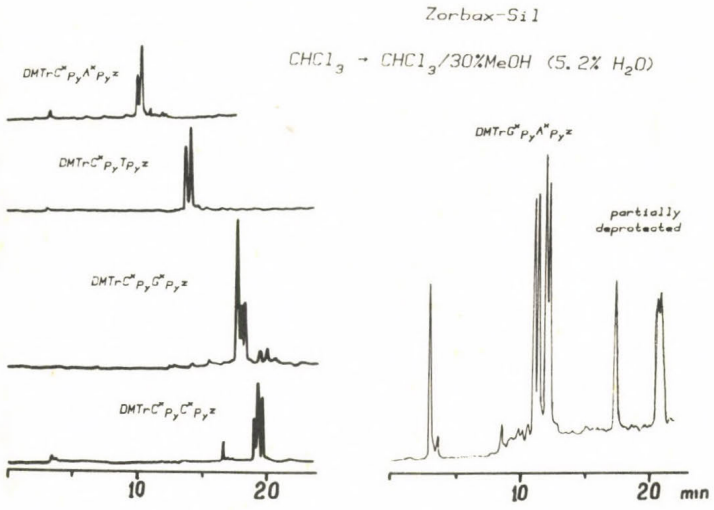


Fig. 27. Analysis of the homogeneity of protected dinucleotides by NP-HPLC with gradient elution in isohydric conditions. Column: Zorbax-Sil (0.46x25 cm). Gradient time: 60 min. For the symbols of the protective groups, see Fig. 26.

Fig. 28. NP-HPLC of a totally protected and a 5'-detritylated hexanucleotide. Conditions as in Fig. 26 except that $CHCl_3$/MeOH 9:1.

dinucleotides are demonstrated in Fig. 27. Large oligonucleotides can also be separated with high selectivity; see e.g. the separation of protected hexanucleotide and the same nucleotide without dimethoxytrityl group, i.e. with free 5'-hydroxy group (Fig. 28). Note that the equilibration (conditioning) of the silica column with the starting solvent after the gradient cycle in isohydric conditions takes less than 10 minutes.

Automatic microcolumn system for the determination of the composition of oligonucleotides

A minori ad majus

The determination of the monomeric composition of oligonucleotides was replaced recently by fingerprinting the partial hydrolysates of terminally labelled oligonucleotides /28/ designed for the elucidation of their primary structure. However, we assume that this substitution is not fully adequate,

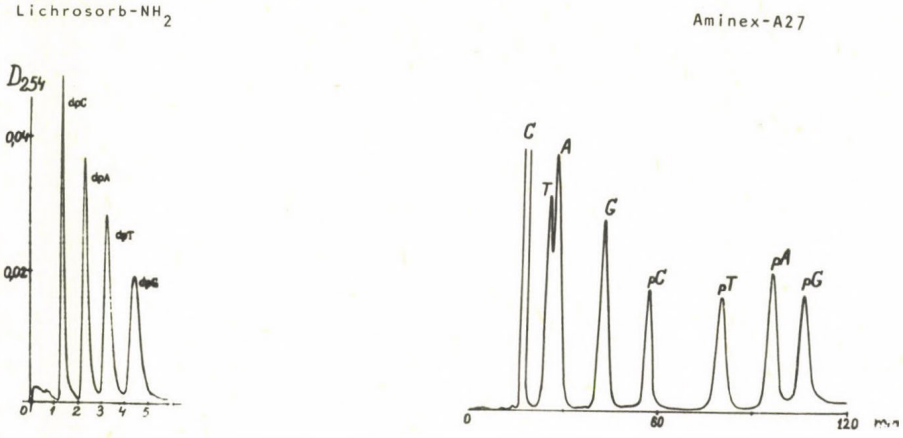


Fig. 29. Ion-exchange chromatography of the monomeric constituents of deoxyoligo- and polynucleotides.

Left: Analysis of a mononucleotide mixture (total amount: 0.005 AU_{254}). Column: 0.2x25 cm; particle diameter: 10 µm. Eluent: 25 mM phosphateborate buffer (pH=3.3); flow rate: 50 ml/hr. Temperature: 50°C.

Right: Microcolumn ion-exchange chromatography of an equimolar mixture of nucleosides and mononucleotides (total amount: 0.0- AU_{254}). Column: 0.5x100 mm packed with Aminex A27. Eluent: NaOAc (pH=4.3) gradient 0-IM/2ml starting at 50 min: flow rate: 1 ml/hr. Temperature: 20°C. The sample was dissolved in 4 µl of 0.05M phosphate buffer (pH=8.2), standard deviation of the peak area of mononucleotides from $\pm$0.5% (pC) to $\pm$2.5% (pG).

since the terminal enzymatic phosphorylation performed before partial enzymatic hydrolysis may proceed incompletely and selectively (in the case of a nonhomogeneous sample). In this case, the fingerprint together with the sequence shows the composition of not the whole sample but only of its labelled component. Such occasions were registered in our practice and the inhomogeneity of the oligonucleotide was determined after the analysis of its total degradation products.

Aminopropyl-silicas can be employed for the separation of mononucleotides. However, Aminex-A27 or A28 was found to be suitable for the separation of mononucleotides and nucleosides (from terminal nonphosphorylated units) (see Fig. 29). Both steps - enzymatic cleavage and chromatography of the hydrolysate - are performed in microcolumns. The first column (volume: 10 µl) is filled with venom phosphodiesterase immobilized on cellulose, while the second column (volume: 40 µl) is filled with Aminex. One syringe micropump delivers 0.5-2 µl of the oligonucleotide solution and the phosphate buffer for hydrolysis into the first column, then the hydrolyzate is directly displaced into the second column, where it is fractionated in a sodium acetate gradient. The oligonucleotide consumption is 0.01 AU_{254} (10^{-9} mole), while column efficiency is only 300-500 theoretical plates and the detector sensitivity is 0.025 AUFS. Thus there is a 50-fold reserve as microcolumns with an efficiency of 5000 theoretical plates can be prepared and the detector sensitivity can be increased up to 0.005 AUFS.

Method to pack stable HPLC-columns by means of viscous consolidation

Qui nimis propere, minus prospere

Modern HPLC columns produced by many companies exhibit high performances including selectivity, efficiency, peak symmetry and quite often permeability. Such columns can be used to one year performing several thousands of separations. However, this takes place under conditions close to test conditions: without pulsation of the mobile phase and without often changing the composition and viscosity of the mobile phase. Under such conditions the columns would lose their high performance rather soon. When, for example, we took out the inlet head of a 25-cm column which exhibited reduced permeability, bad peak symmetry and low efficency, we observed a cavity of up to 1 cm depth. This demonstrates that in many cases the adsorbent particles are packed rather uniformly (this is the main requirement for high efficiency) but not sufficiently tight that is they are not consolidated.

The effect of the mobile phase leading to such alterations in the column is viscous friction (drag force) that entrains the microparticles resulting in the repacking of the support bed observed as a shrinkage in a non-tightly packed column.

A few methods exist to overcome these problems. Examples are the "Radial Pack system" (Waters Associates), the radial compression of the packed column i.e. the tightening of the most friable boundary zone of the adsorbent bed; the "intra-column injection system" /29/, i.e., the insertion of the inlet capillary below the frit, deeper than the possible shrinking of the bed. However, these methods are employed for non-stable columns. We decided to eliminate the main reason of column failure - the shrinking of the column bed. This can be achieved only by the consolidation of the adsorbent microparticles along the whole column bed. During the packing of HPLC-columns we used the same approach as in the case of packing thoroughly fractionated cellulose /30/, the so-called viscous "finish-packing" procedure. It consists of rapid slurry packing from a nonviscous solvent followed by washing the column with a viscous mobile phase at the same pressure which was applied for packing. "Finish-packing" or viscous consolidation may be performed with a solvent which poorly wets the adsorbent particles (see Experimental). In such a solvent the column cannot be packed immediately, as the microparticles form friable agglomerates. However, in a prepacked column under the action of such a solvent, the loosening of the most tight zones occurs mainly in the axis part of the column followed by transferring the particles in the direction from the axis to the loose peripheria under the action of viscous drag forces. This process is favoured by an irregular flow of the solvent along the column cross section, as the highest flow velocity is in the most friable peripheria part of the prepacked bed. This method is illustrated by the diagram and the schematic model in Figs 30 and 31.

After "finish-packing" and before removal the column has to be washed at the same pressure with a solvent wetting well the particles in order to eliminate the electrostatic repulsion of the particles.

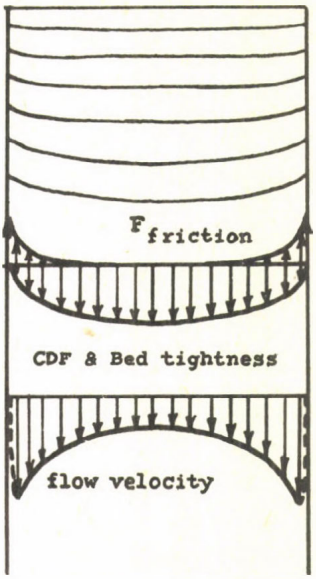


Fig. 30.

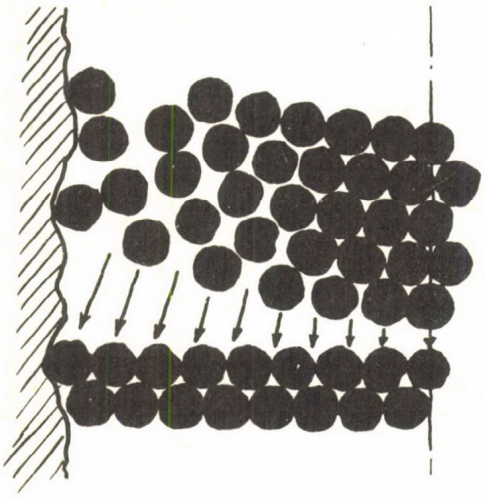


Fig. 31.

Fig. 30. Schematic diagram of the distribution of cumulate drag force (CDF), bed tightness and wall friction along the column diameter. The lower diagramm depicts the distributiion of flow velocity.

Fig. 31. Schematic presentation of the transfer of particles in "finish-packing" consolidation, from the tightly packed axial zone toward the friable perifery, under the action of viscous drag forces upon the flow of viscous solvent. See Experimental.

All columns packed with our method exhibit good permeability, which corresponds to the size of the particles used, and good efficiency (HETP/$\bar{d}_p$ less than 2), when packed with a fraction of the particles of which more than 80% have the diameter of 5 $\pm$ 1 µm or 10 $\pm$ 2 µm according to microscopic determination. (A detailed description of this method, together with the theoretical consideration of the packing process is under preparation.)

CONCLUSIONS

Vita brevis, HPLC longa

We have mainly focused our attention on studies in selectivity which is the basis of the chemical nature of chromatography and close to our mind, as chemists. The selectivity of the adsorption phenomena is the basis of the whole chromatographic system, which governs the relative contribution of the chemical and physical effects resulting in an efficient separation. The higher the chemical selectivity the lower can be the requirements to physical perfection of the separating system; the simpler the system, the easier its operation and control.

After the chemical aspects, but not at the second place in importance, are the physico-chemical questions, primarily those which define the dynamics of mass transfer. The combined effects of chemical selectivity and of the physico-chemical dynamics of the adsorption-desorption process provide the basis for the perfection of the separation.

And, finally, in combination with high level physics of the mass transfer, chromatography will become fully perfect, really modern with its high efficiency and productivity corresponding to the spirit and the requirements of our time.

We hope that our concept of chromatography and the results obtained upon following it will represent a small contribution to the discussion whether chromatography is a science, a technique or a method.

AKNOWLEDGEMENTS

Motu proprio
et more majorum

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REFERENCES

1. Wulfson A.N., Yakimov S.A. Bioorgan. Khim. 9, 365-390 (1983).
2. Wulfson A.N., Yakimov S.A. J.Liquid Chrom., 1983 (in press).
3. Khorana H.G. et al. J.Mol.Biol., 72, 209-426 (1972).
4. Dobrynin V.N., Filippov S.A., Bystrov N.S., Severtsova I.V., Kolosov M. Bioorgan. Khim. 9, 706-710 (1983).
5. Ohtsuka E., Ikehara M., Söll D. Nucl.Acids Res., 10, 6553-6570 (1982).
6. Molko D., Derbyshire R., Gyu A., Roget A., Teoule T., Boucherle A., J.Chromatogr., 206, 493-500 (1981).
7. Gordon A.J., Ford R.A., The Chemist's Companion, Wiley, New York, 1972.
8. Pearson R.L., Weiss J.F., Kelmers A.D. Biochim. Biophys. Acta, 228, 770-774 (1971).
9. Berlin Yu.A., Diakov V.L., Kolsov M.N. Biokhimiya 39, 747-750 (1974).
10. Tomlinson R.V., Tener G.M. J.Amer.Chem.Soc., 84, 2640-2645 (1962).
11. Kelmers A.D., Weeren H.O., Weiss J.F., Pearson R.P., Stulberg M.P., Novelli G.D. Methods Enzymol., 1971, Part C, pp. 9-34.
12. McFarland G.D., Borer P.N., Nucl. Acids Res., 7, 1067-1080 (1979).

13. Unger K.K., Porous Silica: Its Properties and Use as a Support in Column Liquid Chromatography. Elsevier, Amsterdam 1979.

14. Snyder L.R., Kirkland J.J. Introduction to Modern Liquid Chromatography: Wiley, New York, 2nd ed., 1979.

15. Pearson J.D., Regnier F.E. J.Chromatogr., 256, 137-149 (1983).

16. Smith J.D. In: The Nucleic Acids, Chargaff E., Davidson N. Editors, Academic Press New York, 1955, vol. 1, p. 267.

17. Ref. 13, p. 264-268.

18. Fritz H.-J., Belagaje R., Brown E.L., Fritz R.H., Jones R.A., Lees R. ., Khorana H.G. Biochemistry, 17, 1257-1267 (1978).

19. Klinkenberg A. In: Gas Chromatography 1960, Scott R.P.W., editor, Butterworths, London, 1960, p. 182.

20. Scott R.P.W., Kucera P. J.Chromatogr. 119, 467-482 (1976).

21. Barford R.A., McGraw R., Rothbart H.L. J.Chromatogr., 166, 365-372 (1978).

22. Snyder L.R. Gradient Elution. In: HPLC: Advances and Perspectives. Horváth, Cs., Editor, Academic Press. New York, 1980, vol. 1, pp. 208-312.

23. Kirkland J.J., Yau W.W., Stoklosa H.J., Dilks C.H. J.Chromatogr.Sci., 15, 303 (1977).

24. The use of Guard Columns in HPLC, Their Selection and Installation. Bulletin No. 781, Bellefonte, PA (USA), 1979.

25. Kissinger P.T. Anal.Chem., 49, 883-885 (1977).

26. Gere R. In: Modern practice of Liquid Chromatography Kirkland J.J. Editor, Wiley, New York, 1971.

27. Thomas J.-P., Brun A., Bounine J.-P. J.Chromatogr., 139, 21-44 (1977).

28. Sanger F. In: Virus Research Fox C.F., Robinson W.S., Editors. Academic Press, New York, 1973, p. 573.

29. Katz E., Scott R.P.W. J.Chromatogr., 246, 191-197 (1982).

30. Berlin Yu.A., Wulfson A.N., Kolosov M.N., Korobko V.G., Yakimov S.A., Bioorgan. Khim. 3, 22-3 (1977).

Chromatography, the State of the Art
H. Kalász and L.S. Ettre (Eds)

ANALYSIS OF PRODUCTS OF ANTHRACENE SULFONATION BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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INTRODUCTION

Sulfonation of anthracene represents an important step in the synthesis of intermediates in the organic dyestuff industry.

The research whose aim is to optimize and to control the sulfonation reaction requires fast and accurate information about the composition of the reaction mixture. The analytical methods applied so far to these compounds have been based on paper chromatography /1/, thin-layer chromatography /2, 3/, gel chromatography /2/ and on nuclear magnetic resonance spectroscopy /3, 4/. Paper and thin-layer chromatography, still widely used and helpful in detecting organic impurities are only of qualitative and semiquantitative importance. Quantitation obtained by using the elution technique in paper and thin-layer chromatography or by using low-pressure columns, is usually very complicated and time-consuming. Both methods based on nuclear magnetic resonance spectroscopy lead to large deviations and errors. However, high-performance liquid chromatography has resulted in being the most important technique for reducing the time of analysis and in achieving quantitative data in the analysis of mixtures of aromatic sulfonic acids /5-10/.

This contribution deals with the separation and quantitative determination of all sulfonic acids present in the reaction mixture after sulfonation of anthracene /anthracene-1-sulfonic acid, anthracene-2-sulfonic acid, anthracene-9-sulfonic acid, anthracene-1,5-disulfonic acid, anthracene-1,6-

-disulfonic acid, anthracene-1,7-disulfonic acid, anthracene--1,8-disulfonic acid, anthracene-2,6-disulfonic acid and anthracene-2,7-disulfonic acid/ on a reversed-phase column packing. The elaborated analytical method consists of two isocratic operations: the first for the separation of monosulfonated products and the second for the separation of anthracene disulfonic acids.

EXPERIMENTAL

Reagents

Water - methanol /70 : 30, v/v/, 0.00025 M aqueous solution of sodium sulfate. Distilled water was used, methanol and sodium sulfate were of analytical grade and were purchased from LACHEMA /Brno, Czechoslovakia/.

Standard samples of anthracene sulfonic acids were obtained as sodium and potassium salts by repeated crystallization of the technical products from water. Anthracene sulfonic acids were prepared by Ing. J. Haase and Ing. G. Adler, from the Laboratory of Organic Technology, in our research institute.

Apparatus

The chromatographic equipment consisted of a modular liquid chromatograph /Laboratorni přistroje, Praha, Czechoslovakia/ equipped with the high-pressure pump HPP 4001, a UV - detector /254 nm/ and sample injector. Stainless steel columns /25 x 0,4 cm I.D./ were prepacked with Separon SI-C_{18} /silica gel modified with octadecyl trichlorsilane/.

Procedure

The samples of the reaction mixture after sulfonation of anthracene were diluted with distilled water to a concentration of about 10 mg of the total amount of anthracene-sulfonates to 100 ml and a few drops of concentrated sodium hydroxide solution were added /neutral reaction to phenolphthaleine papers/.

All sample solutions were filtered through 0,45 μm organic filters so that the residual amounts of the starting anthracene could be removed.

An appropriate amount /1-2 microliters/ of the sample solution was injected into the chromatographic system.

Chromatographic procedure of the total mixture of anthracene sulfonic acids consists of two isocratic operations. Operation A: Monosulfonated derivatives of anthracene and the sum of anthracene disulfonic acids were separated using water - methanol /70 : 30/ as the mobile phase. Operation B: 0.00025 M aqueous solution of sodium sulfate was applied for the separation of anthracene disulfonic acids. Another separating column, or the column after operation A, washed with 30 ml filtered distilled water and 30 ml of sodium sulfate solution, was used in this case.

The conditions of both procedures are given in Fig. 1 and Fig. 2.

The analysis of laboratory-prepared samples of known composition was performed in the same way.

The quantitative evaluation of the chromatograms was carried out by measuring the peak areas of the isomers in the chromatograms of technical samples and of those in the chromatograms of standard mixtures.

RESULTS AND DISCUSSION

The separation of anthracene monosulfonic acids and the sum of anthracene disulfonic acids by using water - methanol /70 : 30/ mixture as the mobile phase is shown in Fig. 1. All three compounds and the sum of the disulfonic acids were eluted close to each other with highly symmetrical peaks in the following sequence: the sum of anthracene disulfonic acids, anthracene-9-sulfonic acid, anthracene-1-sulfonic acid, anthracene-2-sulfonic acid. On the other hand, the complete separation of all anthracene disulfonic acids was obtained when 0,00025 M aqueous solution of sodium sulfate was applied as the mobile phase. Anthracene disulfonic acids were eluted with sharp

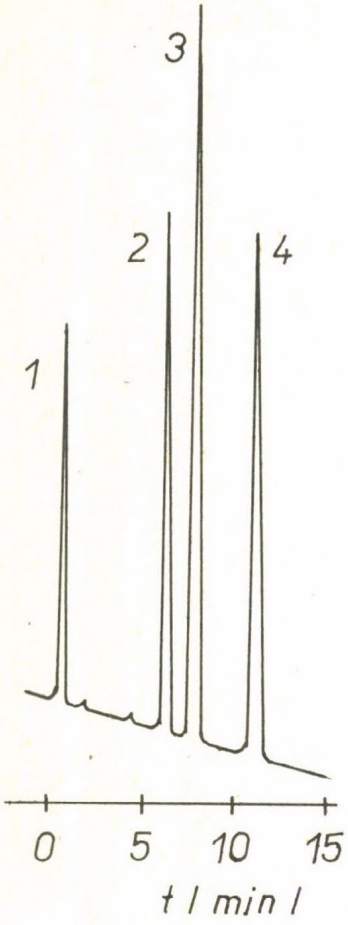

Fig. 1. Reversed-phase HPLC separation of anthracene monosulfonic acids. Isocratic elution with methanol - water /30 : 70/. Column: 25 x 0,4 cm I. D. Packing: Separon SI-C_{18}, 10 µm. Flow-rate: 0,6 ml/min. Detection: UV, 254 nm. Injection volume: 1 µl. Sample concentration: 0.5 mg of sodium salts/ 1 ml. Compounds: 1 = sum of anthracene disulfonic acids: 2 = anthracene-9-sulfonic acid; 3 = anthracene-1--sulfonic acid; 4 = anthracene-2-sulfonic acid

symmetrical peaks in the following sequence: anthracene-1,5--disulfonic acid, anthracene-2,6-disulfonic acid, anthracene--2,7-disulfonic acid, anthracene-1,7-disulfonic acid, antracene--1,8-disulfonic acid /Fig. 2/. Under these conditions, anthracene monosulfonic acids have the long elution time and thus it

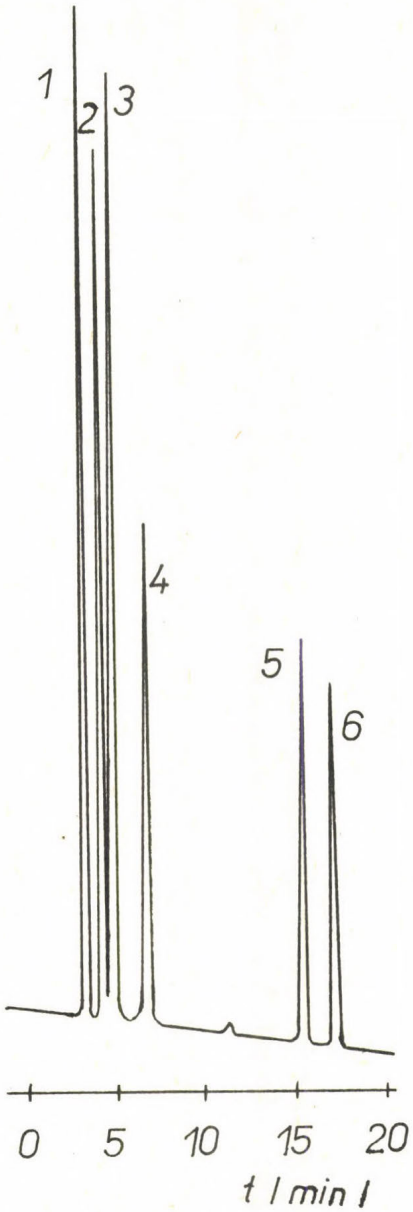

Fig. 2. Reversed-phase HPLC separation of anthracene disulfonic acids. Isocratic elution with 0,00025 M aqueous sodium sulfate solution. Column: 25 x 0,4 cm I.D. Packing: Separon SI-C_{18}, 10 µm. Flow-rate: 1 ml/min. Detection: UV, 254 nm. Injection volume: 1 µl. Sample concentration: 1 mg of sodium salts/ 1 ml. Compounds: 1 = anthracene-1,5-disulfonic acid; 2 = anthracene-2,6-disulfonic acid; 3 = anthracene-1,6-disulfonic acid; 4 = anthracene-2,7-disulfonic acid; 5 = anthracene-1,7-disulfonic acid; 6 = anthracene-1,8-disulfonic acid

is necessary to wash the column with water and water - methanol mixture.

This method for the chromatographic analysis permits the separation and quantitative determination of all anthracene mono- and disulfonic acids from their mixture. The described method has been used in the analyses of laboratory anthracene sulfonation products.

REFERENCES

1. K. Obruba, A. Koloničný, A. Novák, K. Růžička, Chem. průmysl, 27, 119 /1977/.
2. K. Obruba, A. Koloničný, K. Růžička, Chem. průmsyl, 26, 584 /1976/.
3. J. O. Morley, J. Chem. Soc. Perkin II, 1554 /1976/.
4. A. Koeberg-Telder, H. Cerfontain, Rec. Trav. Chim., 91, 22 /1972/.
5. H. U. Ehmcke, H. Kelker, K.-H. König, H. Ullner, Z. Anal. Chem., 294, 251 /1979/.
6. L. Rossineli, H. Thies, W. Richarz, Chimia, 33, 451 /1979/.
7. P. Jandera, J. Churáček, J. Chromatogr., 197, 181 /1980/.
8. C. Prandi, T. Venturini, J. Chrom. Sci, 19, 308 /1981/.
9. T. D. Rotsch, W. R. Cahill, D. J. Pietrzyk, F. D. Cantwell, Can. J. Chem., 59, 2179 /1981/.
10. A Kraslawski, J. M. Kulawinek, J. Chromatogr., 254, 322 /1983/.

Chromatography, the State of the Art
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SEPARATION OF STEROID KETOALCOHOLS AND DIOLS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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We earlier reported[1] the asymmetric hydrogenation of steorid ketones via hydrosilylation catalyzed by enantiomeric chiral rhodium-DIOP complexes. The hydrogenation produced a mixture of steroid ketoalcohols and diols. We describe here the separation of stereoisomeric steroid ketoalcohols and diols by high-performance liquid chromatography (HPLC) on a Partisil 10/25 column (Whatman) with detection by UV absorption at suitable wavelengths and by refractive index.

The steroid models for reduction were oestrone-3-methyl ether (1), pregna-3,5-dien-20-one (4) and 5β-pregnane-3,20-dione (7).

The 17α and 17β-alcohols (2 and 3) were separated with chloroform-hexane (2:1) as the solvent. The 17α-isomer (3) had the shorter retention time (Fig. 1).

Reduction of the 20-keto group was studied on pregna-3,5-dien-20-one (4)[2]. The stereoisomers of the 20-alcohols were separated using the same solvent as before. The 20β-alcohol (5) had the shorter retention time (Fig. 2).

Separation of the mixture of partially and totally reduced products of pregna-3,20-dione (7) was rather complicated. A solvent system suitable for the separation of seven substances was hexane-tetrahydrofuran (1:1). Besides the starting material (7) six substances (8-13) (Fig. 3) could be identified via their retention times by the application of standard steroid diols and ketoalcohols in the HPLC. Moreover the substances corresponding to each HPLC peak were collected and their structures were elucidated by NMR spectroscopy.

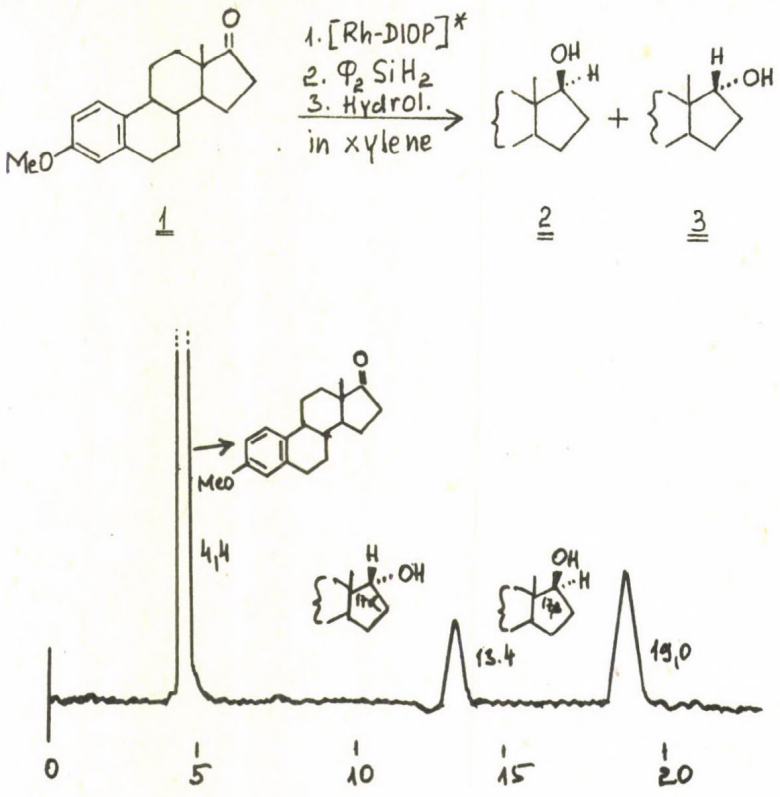
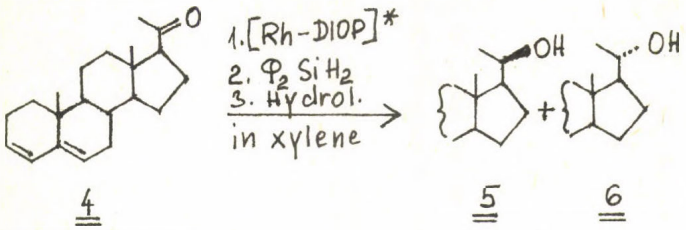


Fig. 1.

As Fig. 4 shows, the retention time of the 3β,20β-diol (10) was shorter than that of the 3α,20α-diol (13), and the retention time of the 3β,20α-diol (9) was shorter than that of the 3α,20β-diol (12). In the series of ketoalcohols the retention time of the 3-one, 20 α-ol (8) was shorter than that of the 3α-ol,20-one (11).

CONCLUSIONS

The separation data indicated that the retention times of the β isomers were shorter than those of the α isomer of steroid alcohols and diols in the pregnane series. With the estrone series the situation is the opposite. This holds for a


O
1.[Rh-DIOP]*
2. Φ2 SiH2
3. Hydrol.
in xylene
OH
OH
4
5
6

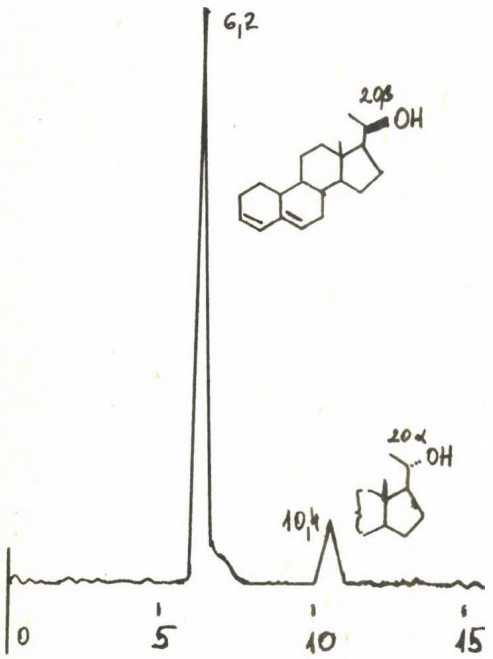

6,12
20β
OH
20α
OH
10,11
0
5
10
15

Fig. 2

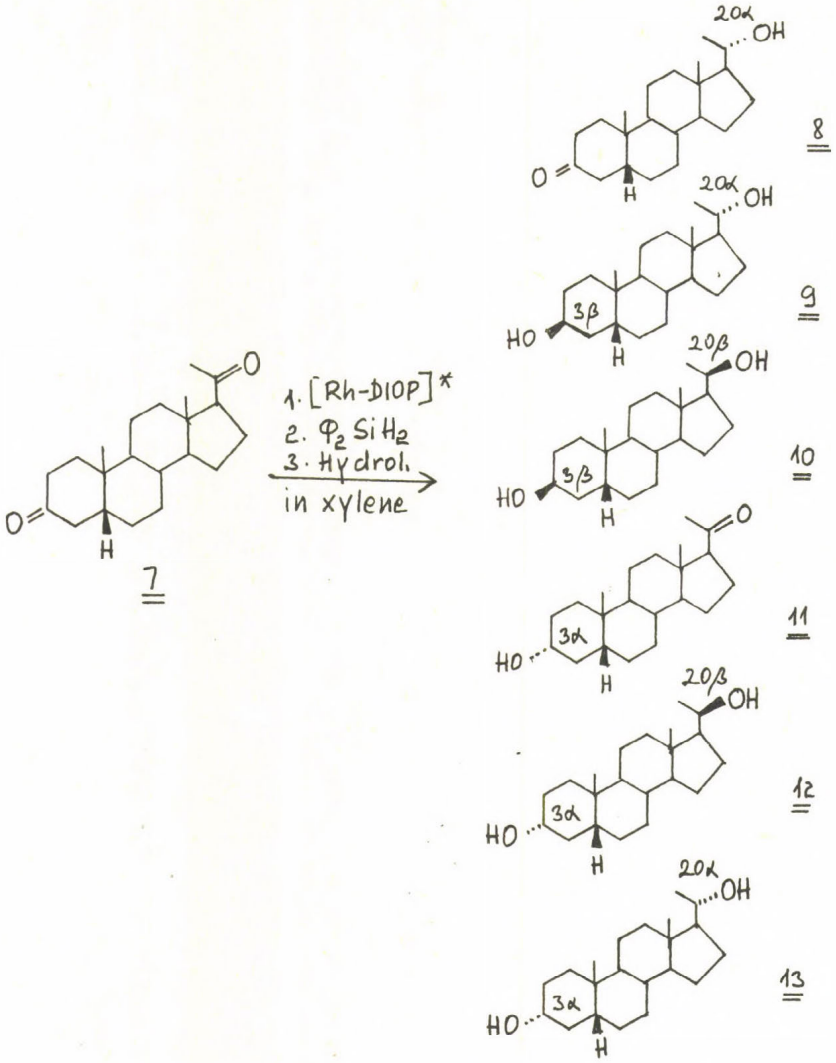


Fig. 3

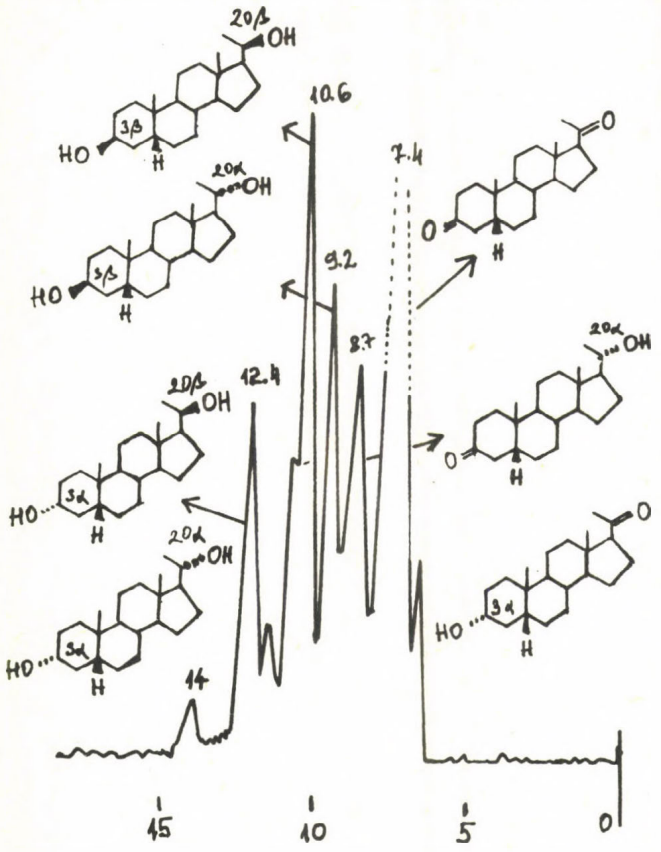


Fig. 4.

Partisil column with the specified solvent systems at room temperature.

REFERENCES

(1) Göndös, Gy., and Orr. J. C., J. Chem. Soc. Chem. Commun., 1982, 1238.

(2) Romeo, J., Romeo, M., Djerassi, C., and Rosenkranz, G., J. Am. Chem. Soc., 73, 1528 (1951).

Chromatography, the State of the Art
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DETERMINATION OF CAROTENOIDS AND RETINOL IN HUMAN SERUM

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SUMMARY

Fractionation and quantitative determination of serum carotenoids /carotenes, β-cryptoxanthin, lutein, zeaxanthin/ and retinol was achieved by normal-phase high-performance liquid chromatography using Chromsil-NH_2 packing and an isocratic mobile phase of 1.2% /v/v/ methanol in benzene.

INTRODUCTION

It has been reported by several authors that human cancer risk is inversely correlated with the blood retinol and carotenoid level /1/. Certain carotenoids, in addition to their vitamin A activity, may also be involved in other biochemical processes in humans: e.g., some of them have the capability of acting as cytoprotective agents while some are used in the treatment of diseases of photosensitivity /2, 3/. For this reason, the usual determination of the "total carotene level" by spectrophotometric method is unsuitable as part of a diagnostic test in clinical laboratories. Instead of this, a simple and fast assay for serum retinol and carotenoids has been developed using HPLC.

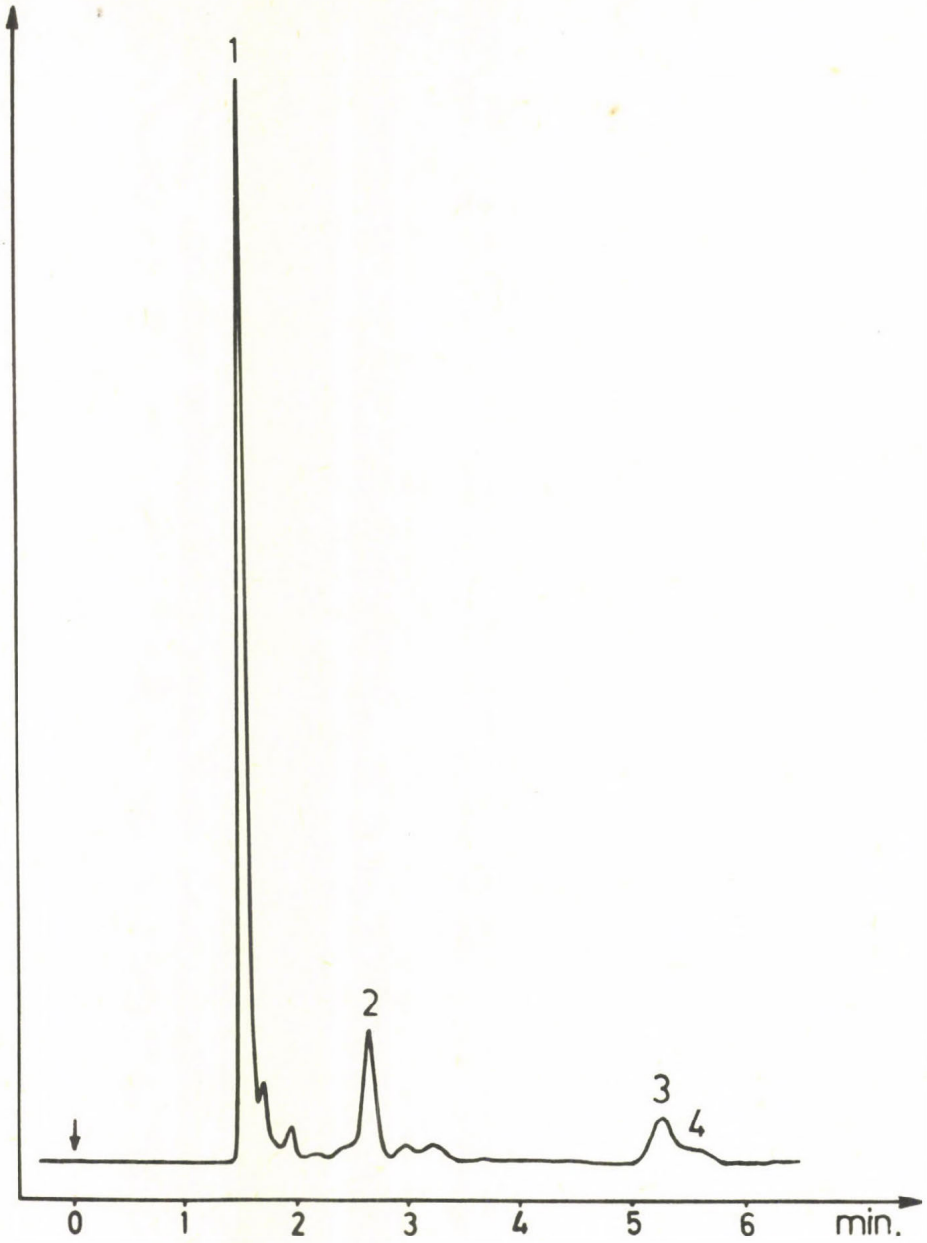

Fig. 1. Separation of serum carotenoids without saponification. 1 Carotenes, 2 β-cryptoxanthin, 3 lutein, 4 zeaxanthin. Detection at 450 nm.

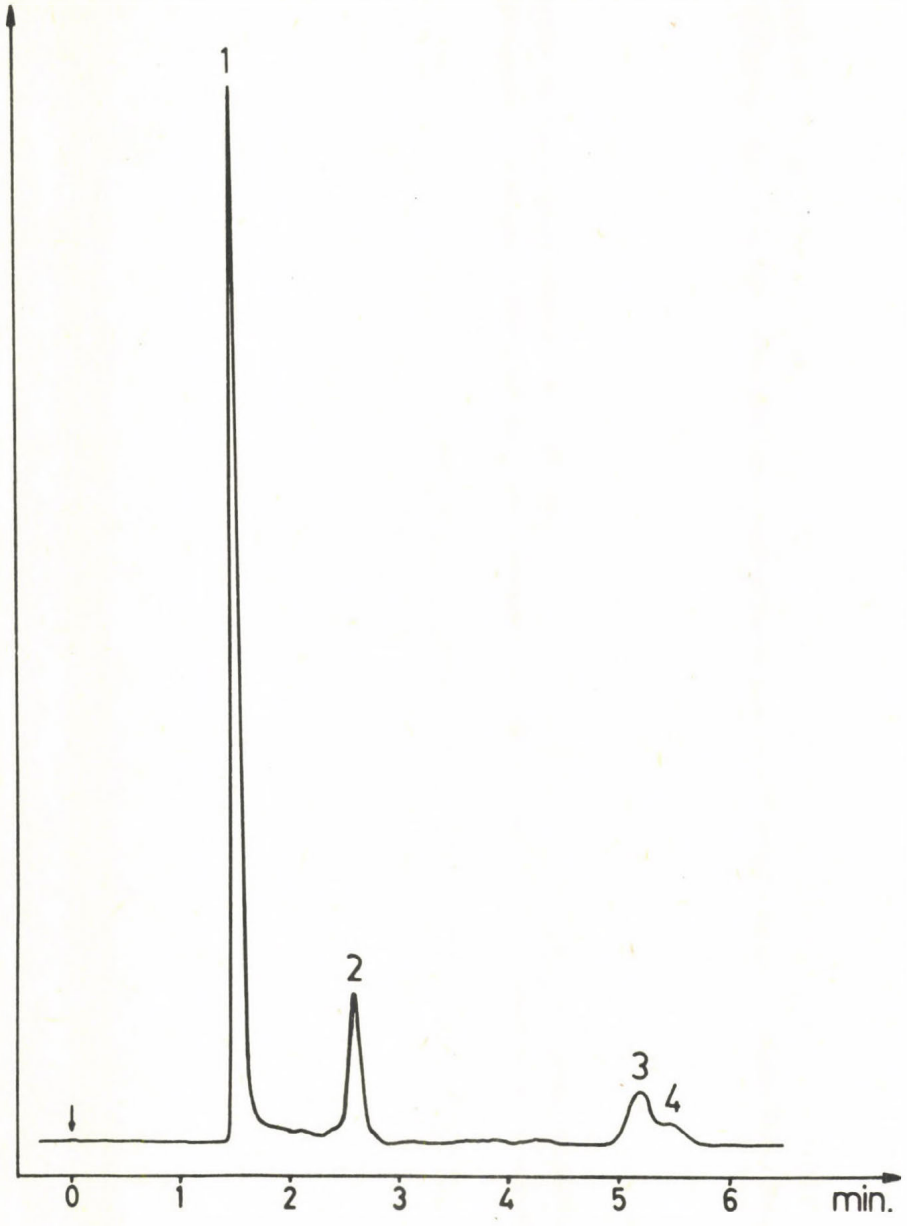

Fig. 2. Separation of serum carotenoids after saponification.
1 Carotenes, 2 β-cryptoxanthin, 3 lutein, 4 zeaxanthin.
Detection at 450 nm.

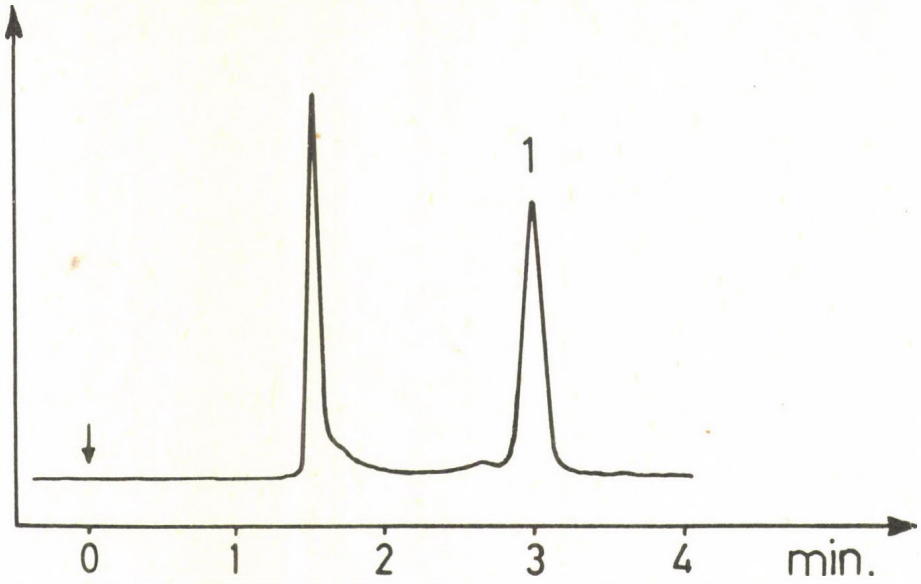

Fig. 3. Retinol profile of a serum extract without saponification. 1 Retinol. Detection at 328 nm.

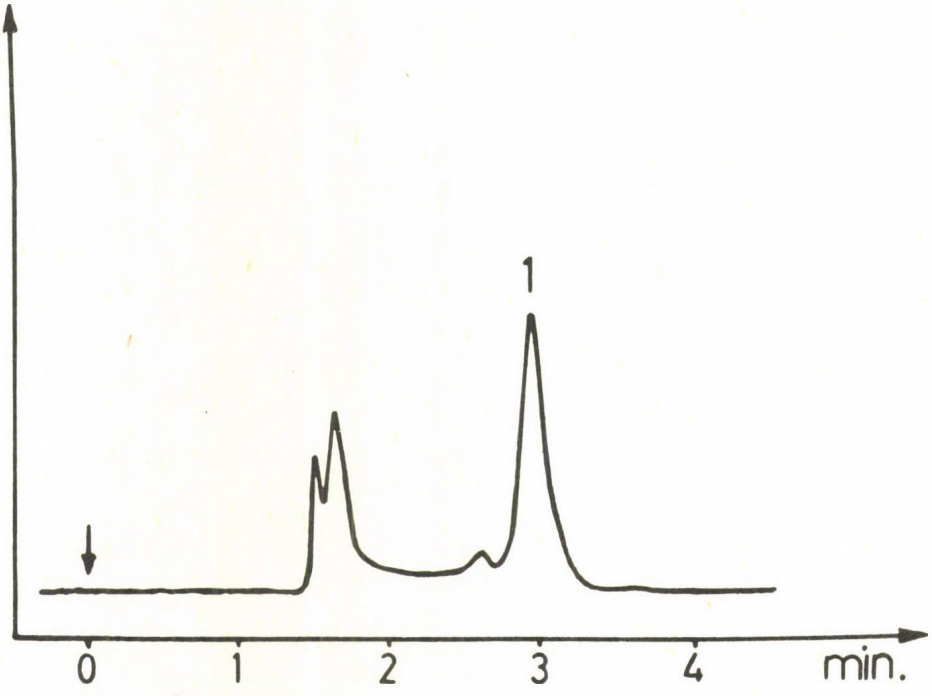

Fig. 4. Retinol profile of a serum extract after saponification. 1 Retinol. Detection at 328 nm.

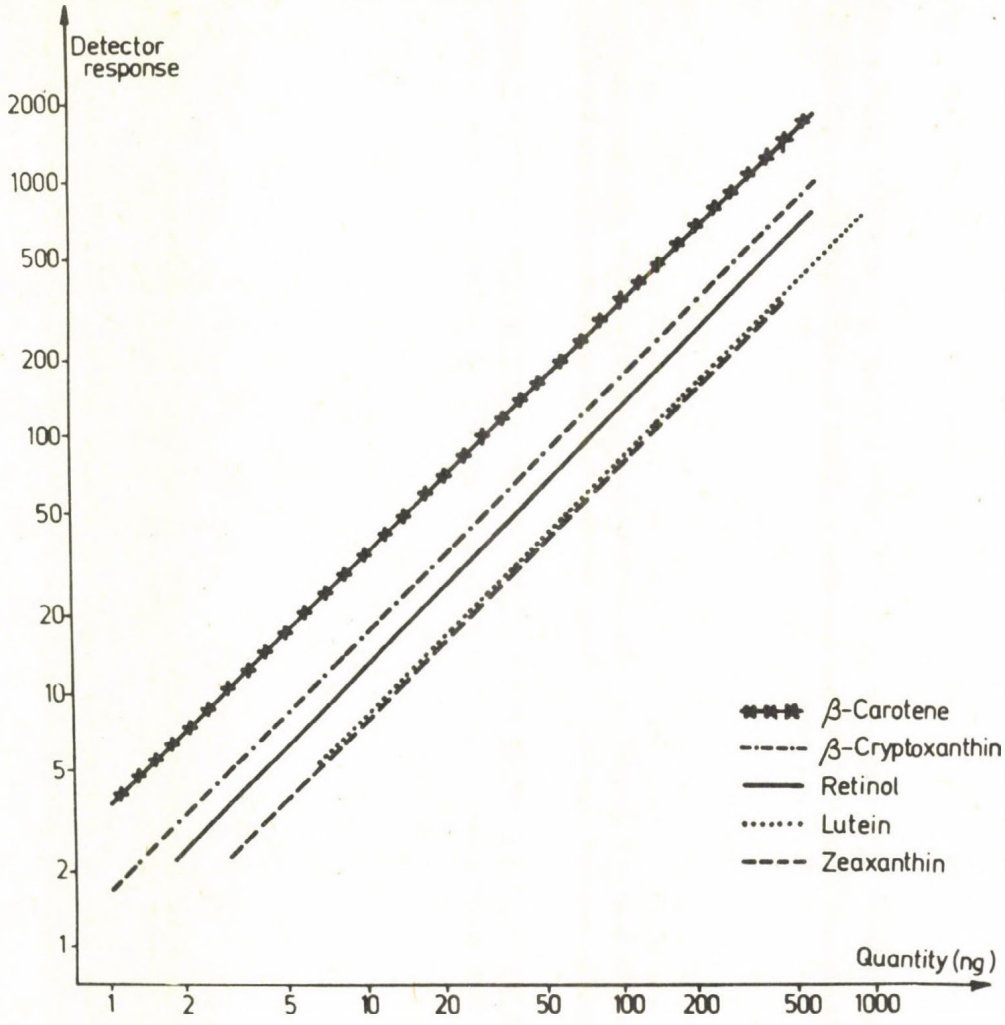

Fig. 5. Calibration curves for carotenoids and retinol.

EXPERIMENTAL

Preparation of samples: 0.5 ml of a serum sample was extracted with 3 ml of n-hexane by shaking for 10 min. The organic layer was removed after centrifugation, evaporated to dryness under reduced pressure and reconstituted with 200 µl of the mobile phase.

Chromatographic procedure: The Liquochrom OE 307 liquid chromatograph /Labor MIM, Budapest, Hungary/ was equipped with a Model SV 7 sample injection valve /Glenco, USA/ with a 50 µl loop. The column /250 x 4.6 mm Type 323 Labor MIM/ was packed with Chromsil-NH_2 10 µm /Labor MIM/ /4/. The spectroscopic detector was set at 450 nm for the carotenoids and 328 nm for retinol, the isocratic mobile phase consisted of 1.2% /v/v/ methanol in benzene.

RESULTS

The above procedure provides a simple and very rapid assay for serum carotenoids and serum retinol. The total analysis time is only 10 min. Figs 1 and 2 show the carotenoid profile of a human serum extract before and after saponification. The retinol profile of the serum is shown in Figs 3 and 4. The main carotenoids in human serum are: carotenes /mainly β-carotene/, β-cryptoxanthin, lutein, and zeaxanthin. Vitamin A esters are not found in detectable quantities in human serum /in case of an empty stomach/. Serum carotenoids and retinol can be determinded by using this system without saponification, and it is suitable for clinical purposes. The calibration graphs are shown in Fig. 5. As seen, they are linear over the explored concentration range.

The development of a method for the separation of β-carotene from other carotenes is currently in progress.

REFERENCES

/1/ Peto, R., Doll, R., Buckley, J.D., Sporn, M.B., Nature 290 201 /1981/

/2/ Jávor, T., Tóth, Gy., Szabolcs, J., Bata, M., Morón, F., Mózsik, Gy., Int. J. Tissue Reactions /in press/

/3/ Mathews-Roth, M.M., JNCI 69 279 /1982/

/4/ Ohmacht, R., Matus, Z., in: New Approaches in Chromatography. p. 71. Ed.: Kalász, H., Akadémiai Kiadó - Elsevier, Budapest, 1984.

STATIONARY PHASES FOR CHROMATOGRAPHY

Chromatography, the State of the Art
H. Kalász and L.S. Ettre (Eds)

NEW "CHROMSIL" PACKINGS

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SUMMARY

The Chromsil-CN and Chromsil-C_8 packings were examined. It was found that pore structure and column efficiency are good. Packing homogeneity was characterized by using the column from the packing direction, and after this reversing it and using from the other side. Several applications on these packings are presented.

INTRODUCTION

In our previous paper (1) dealing with Chromsil HPLC packings we demonstrated the good quality of this type of silica gel and the two bonded phases /octadecyl- and amino-phases/ made from Chromsil by chemical reaction. In the present study we report on investigations on the structure and chromatographic performance of two new chemically bonded liquid-chromatographic stationary phases: Chromsil-C_8 and Chromsil-CN. These packing materials have been developed by the University Medical School, Pécs in collaboration with Labor Instrument Works, Budapest.

Both packing materials /octyl- and cyano-phases/ have been prepared by the chemical reaction of Chromsil silica with the appropriate alkoxy- or chlorosilane. With a special capping reaction the residual silanol content has been decreased in

order to minimize the undesirable influence of unreacted accessible silanol groups on solute retention.

The pore structure /pore size distribution, pore volume, specific surface area/ has been measured by size-exclusion chromatography. The specific surface area measured by this method is usually 30% higher than measured by nitrogen adsorption (2).

The efficiency of the columns packed with Chromsil-C_8 and Chromsil-CN have been characterized by the van Deemter equation:

$$H = A + \frac{B}{u} + C \cdot u$$

where H is the height equivalent to a theoretical plate, u is the linear flow velocity, A, B and C are characteristics of the column, the packing material, the solvent, and the solute. We used the complete form of this equation for the characterization of columns packed with particles with about 5 μm diameter. For the characterization of 10 μm particles we investigated a simplified form of the van Deemter equation:

$$H = A^{*} + C^{*} \cdot u$$

Experimental

We used Liquochrom Model 2010 liquid chromatograph /Labor MIM, Budapest, Hungary/ consisting of a pulseless solvent delivery pump, an injector valve with a 20-μl loop, a variable-wavelength UV detector and a strip-chart recorder /Type OH-814/1, Radelkis, Budapest, Hungary/.

250x4.6 mm stainless steel columns /Type OE-323, Labor MIM, Budapest, Hungary/ were packed with the cyano- and octyl-phases. The packing procedure based on a modified "balanced-viscosity" method has been specially developed by Labor MIM. The packed columns were equilibrated with the eluent for an hour before use.

The components of all mobile phases were freshly distilled. The test mixtures were prepared by mixing analytical-grade standard substances.

Results and Discussion

1. Chromsil-CN packing material

This packing material has medium polarity. Using non-polar eluents we have a normal-phase chromatographic system; on the other hand, using a polar eluent we have a reversed-phase separation system.

The average pore diameter was found as 82 Å, the specific pore volume was 0.76 cm^3/g, while the specific surface area was 370 m^2/g.

The efficiency of this phase was measured with n-heptane eluent /saturated with water/ using perchloroethylene /k'=0/, benzene /k'=0.1/, o-nitrotoluene /k'=0.8/ and 2.4-dinitro-toluene /k'=3.2/ as the solutes. The van Deemter curves /Figs 1, 2, 3/ show that the good HETP is a result of the small A term, and particularly of the low C term. Fig. 4 shows the separation of the above-mentioned solutes at close to optimum flow-velocity.

The linear-peak asymmetry - measured at 10% peak height - was always better than 1.5.

Two simple separations in Figs 5 and 6 demonstrate further application fields of Chromsil-CN packing.

2. Chromsil-C_8

The separation mechanism of octyl phases is similar to that of octadecyl phases except that at identical solvent composition retention is shorter than the retention measured on octadecyl phases. We have found that the average pore diameter is 90 Å, the specific pore volume is 0.76 cm^3/g, and the specific surface area is about 340 m^2/g.

From this packing material we have only investigated the 10 µm particle fraction. The quality of the surface coverage was controlled by methyl-red adsorption and by measuring the retention of basic solutes (3). It was found that the surface coverage and capping of residual silanol groups are optimum.

The efficiency of the columns was tested using water : methanol mixture as the eluent. Benzene /k'=0.4/, pyrene

/k'=1.3/ and benzo/a/pyrene /k'=2.1/ was injected /Figs 7 and 8/. The measurement was made in both flow directions.

Figs 9 and 10 present two applications of Chromsil-C_8. Fig. 9 demonstrates a rapid separation of six different carboxylic acids. Fig. 10 shows the separation of an anodyne drug.

References

1. Ohmacht, R., Matus, Z.: in: New Approaches in Chromatography. /Ed: Kalász, H./ p. 71. Akadémiai Kiadó - Elsevier, Budapest, Amsterdam, 1984.
2. Ohmacht, R., Halász, I.: Chromatographia 14, 155 /1981/
3. Ohmacht, R.: Magyar Kémiai Folyóirat /in press/

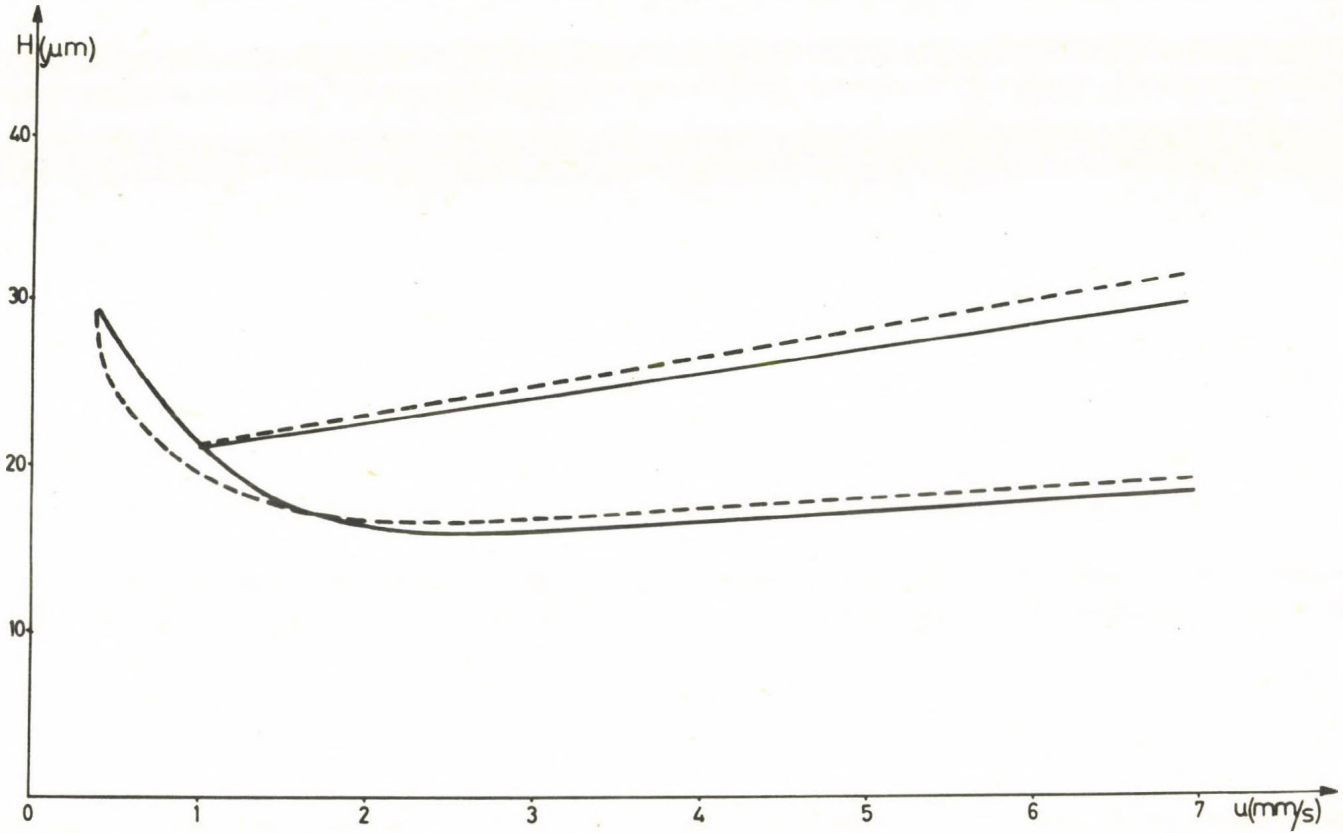

Fig. 1. H-u plots on Chromsil-CN for benzene (k' = 0.1), Eluent: n-heptane (water saturated), Detector: 254 nm; 0.1 A, Upper lines: column with 10 µm packing material, Lower lines: column with 6 µm packing material, Solid lines: measured by having the flow direction corresponding to the packing direction, Dashed lines: measured by inverted flow direction

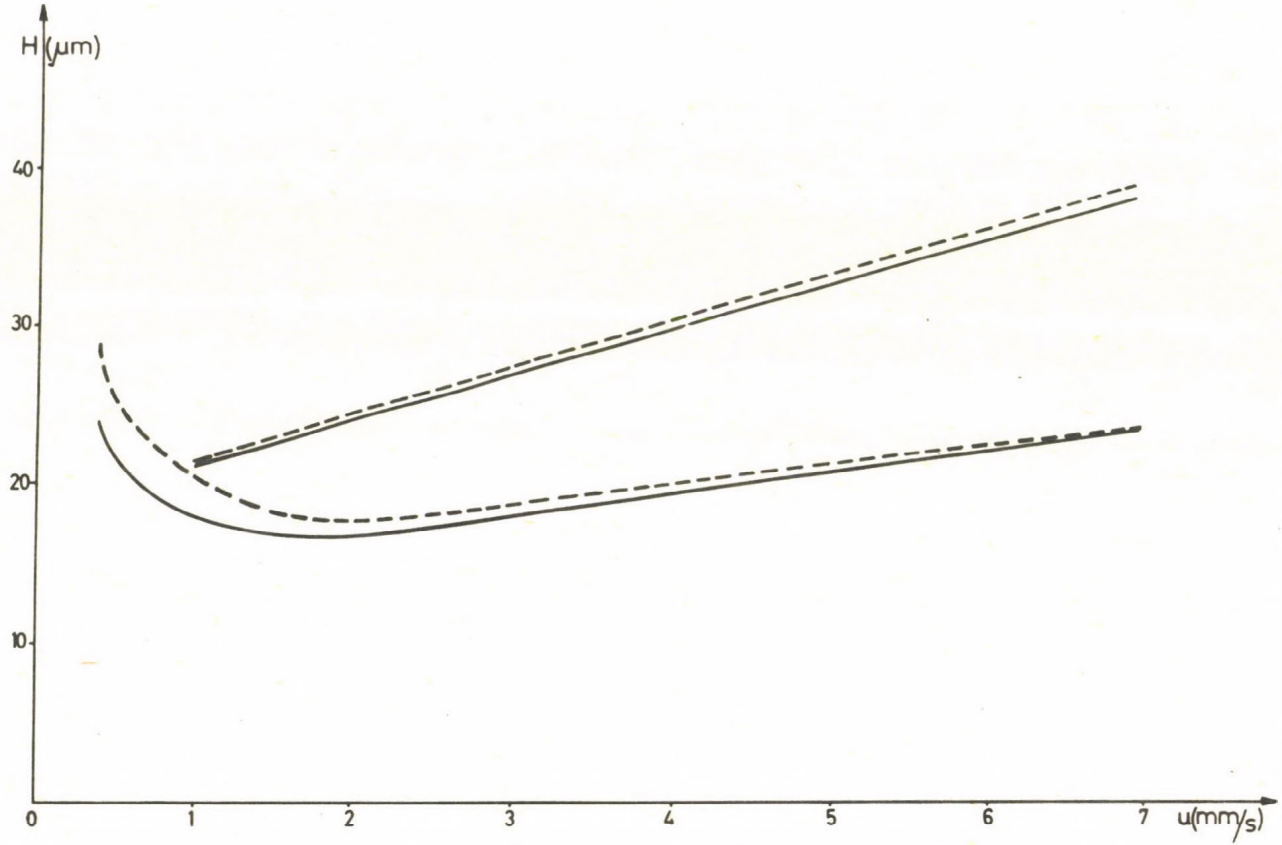

Fig. 2. H-u plots on Chromsil-CN for o-nitrotoluene (k' = 3.2), Explanation as in Fig. 1.

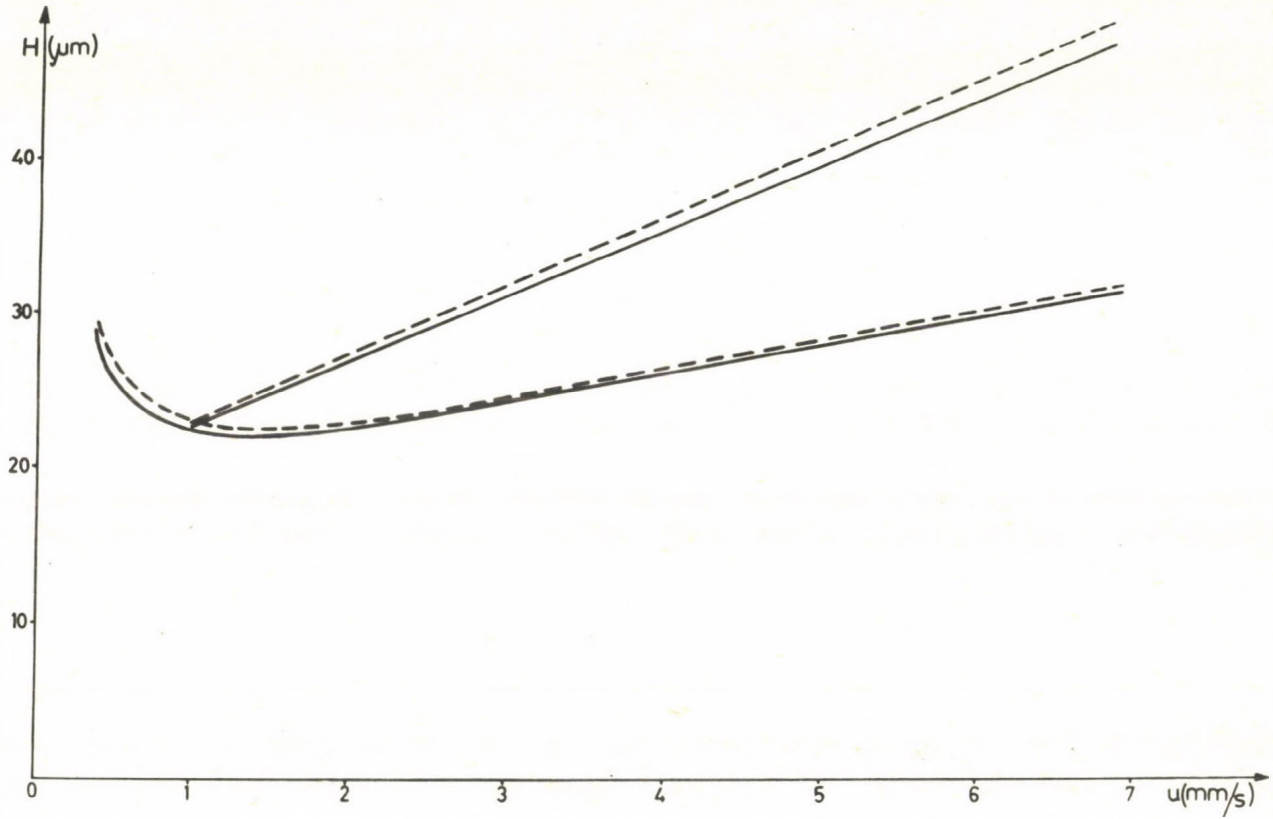

Fig. 3. H-u plots on Chromsil-CN for 2.4-dinitrotoluene (k' = 3.2), Explanation as in Fig. 1.

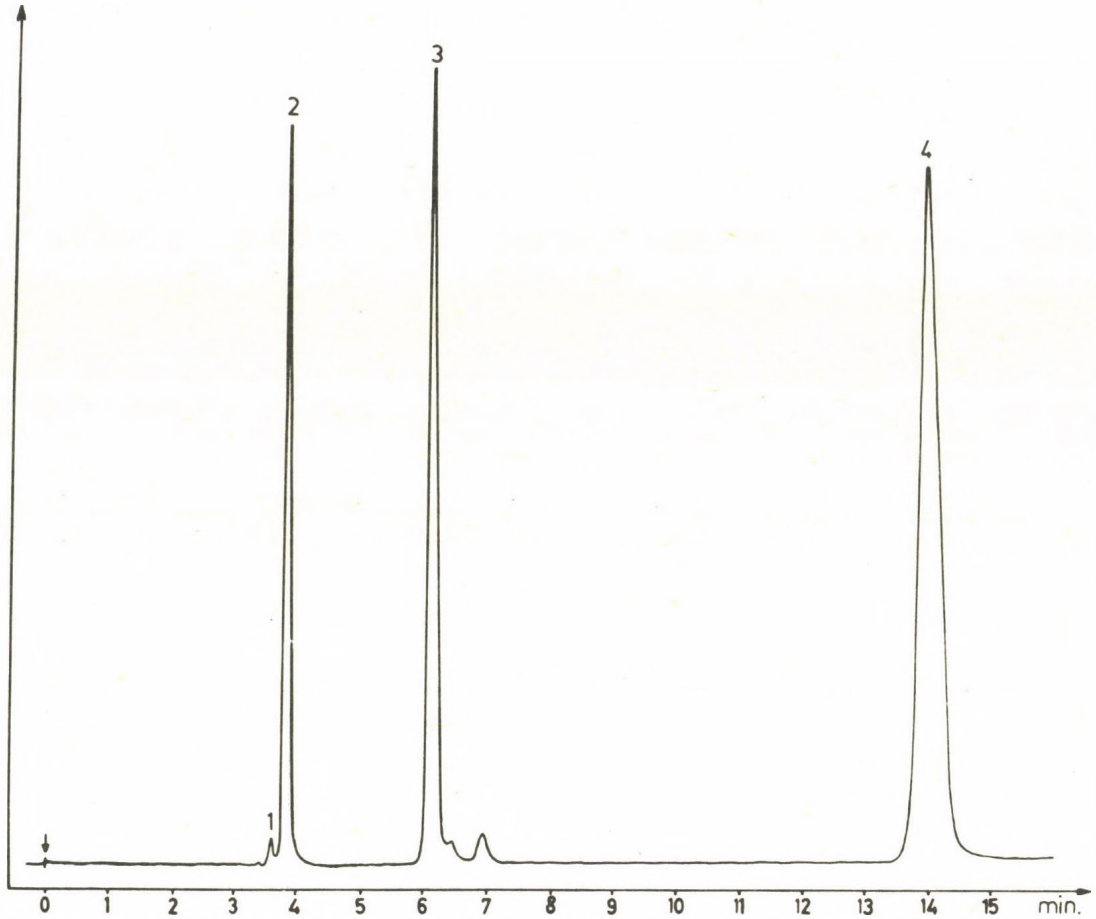

Fig. 4. Separation of a test mixture, Column: 250 x 4.6 mm, Packing material: Chromsil-CN 10 µm Eluent: n-heptane (water saturated), u = 1.16 mm/sec, Detector: 254 nm; 0.1 A, Peaks: 1. Perchlorocthylene, 2. Benzene, 3. o-Nitrotoluene , 4. 2.4-Dinitrotoluene

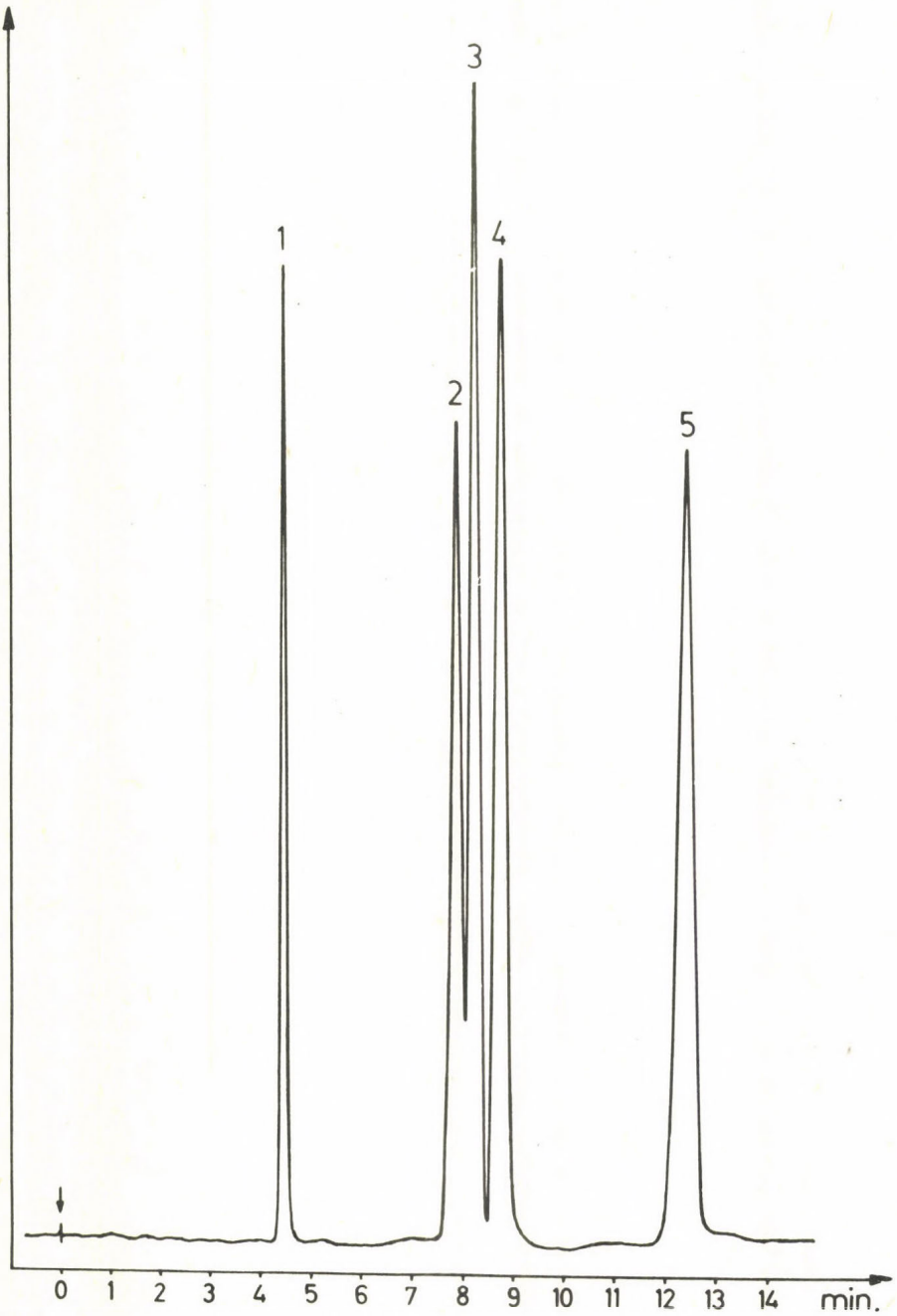

Fig. 5. Separation of xylenols, Column: 250 x 4.6 mm, Packing material: Chromsil-CN 10 m, Eluent: 0.15% Acetic acid, 2% acetonitrile in n-hexane, u = 2.6 mm/sec, Detector: 275 nm; 0.1 A, Peaks: 1. 2.6-xylenol, 2. 2.5-xylenol, 3. 2.4-xylenol, 4. 2.3-xylenol, 5. 3.4-xylenol

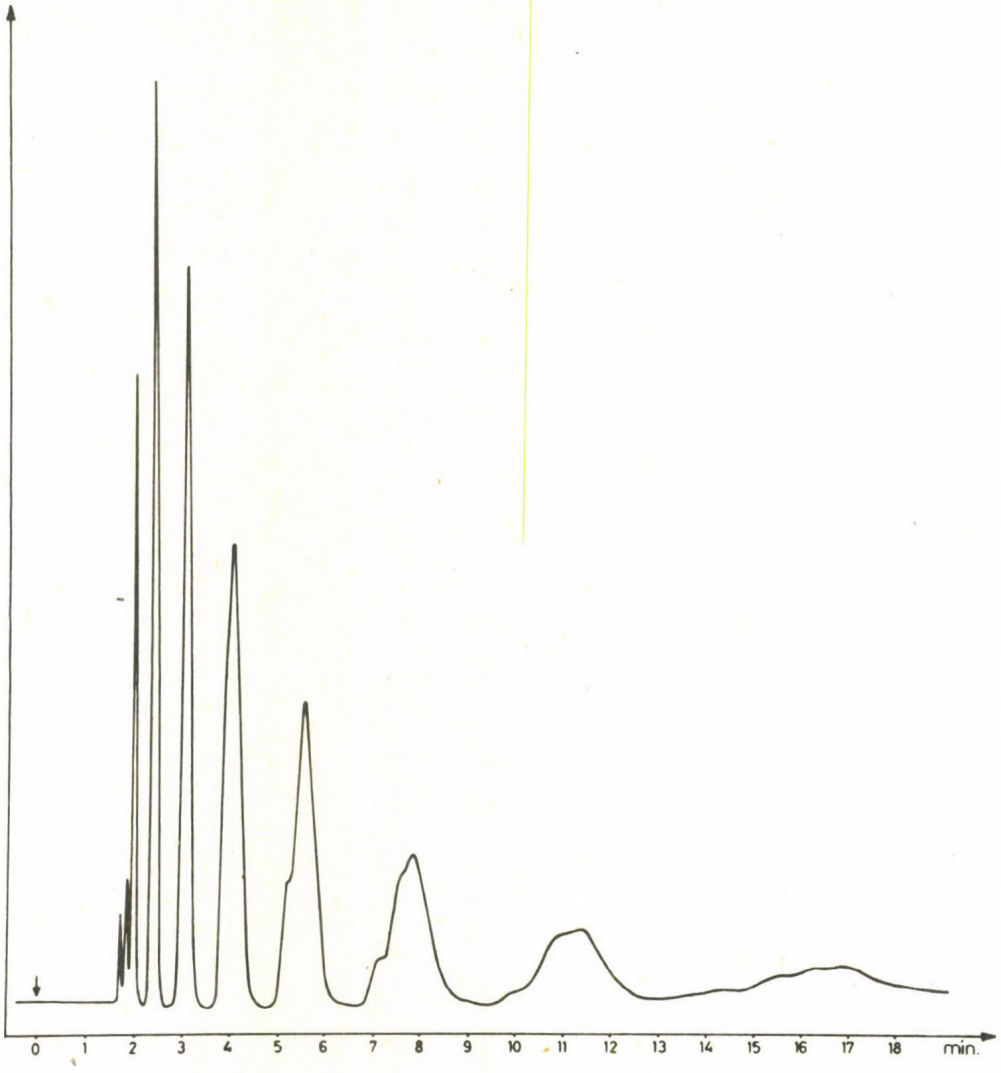

Fig. 6. Separation of polystyrene oligomers, Column: 250 x 4.6 mm, Packing material: Chromsil-CN 10 µm. Eluent: Isooctane dichloromethane (15:1), u = 2.5 mm/sec, Detector: 254 nm; 0.1 A, Peaks: Components of a 600 MW polystyrene standard

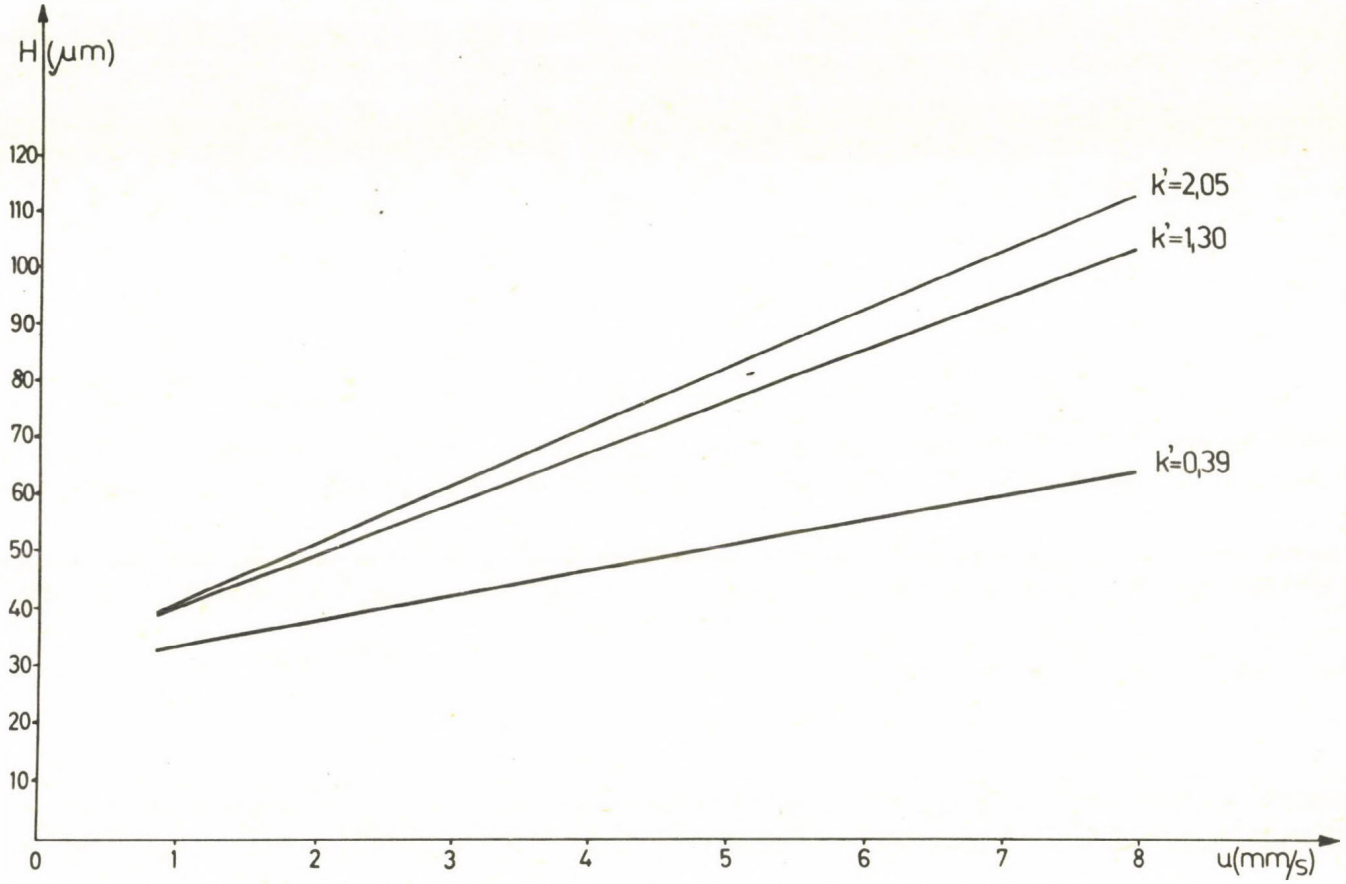

Fig. 7. H-u plots on Chromsil-C_8 (10 µm), Eluent: 17% Water in methanol, Detector: 254 nm; 0.1 A

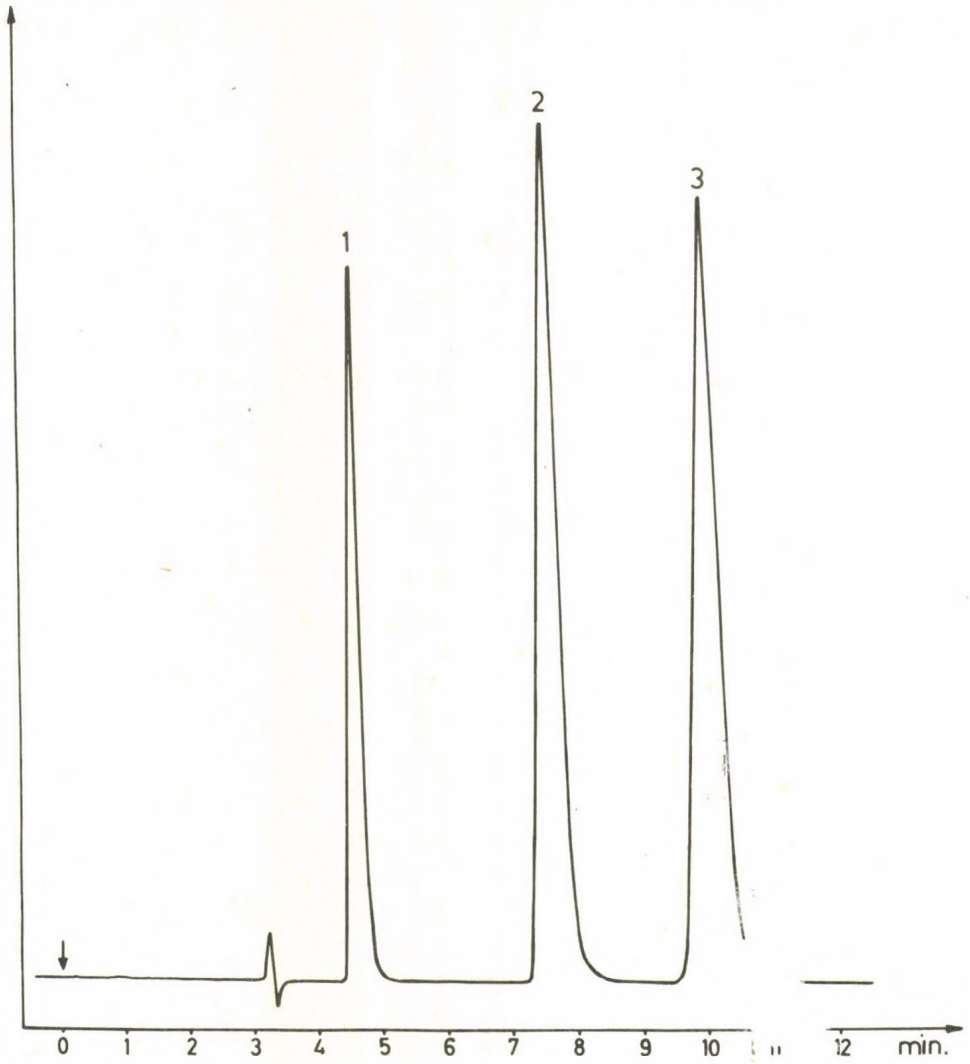

Fig. 8. Separation of a test mixture on Chromsil-C_8 (10 µm), Column: 250 x 4.6 mm, Eluent: 17% Water in methanol, u = 1.3 mm/sec, Detector: 254; 0.1 A, Peaks: 1. Benzene, 2. Pyrene, 3. Benzo(a)pyrene

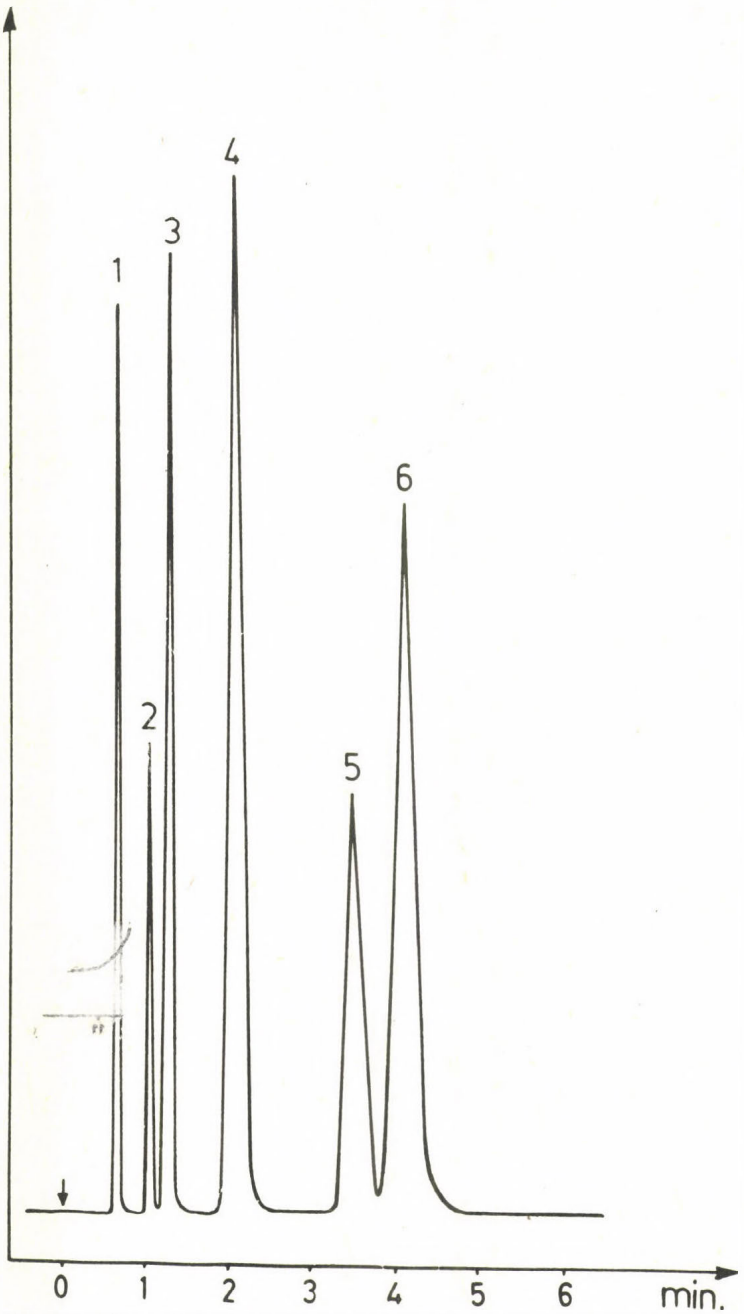

Fig. 9. Separation of carboxylic acids, Column: 250 x 4.6 mm, Packing material: Chromsil-C_8 10 µm, Eluent: Water: methanol (60:40) +0.1% acetic acid, u = 2.8 mm/sec, Detector: 240 nm; 0.1 A, Peaks: 1. Maleinic acid, 2. Fumaric acid, 3. o-Nitrobenzoic acid, 4. 3.5-dinitrobenzoic acid, 5. p-Nitrobenzoic acid, 6. Benzoic acid

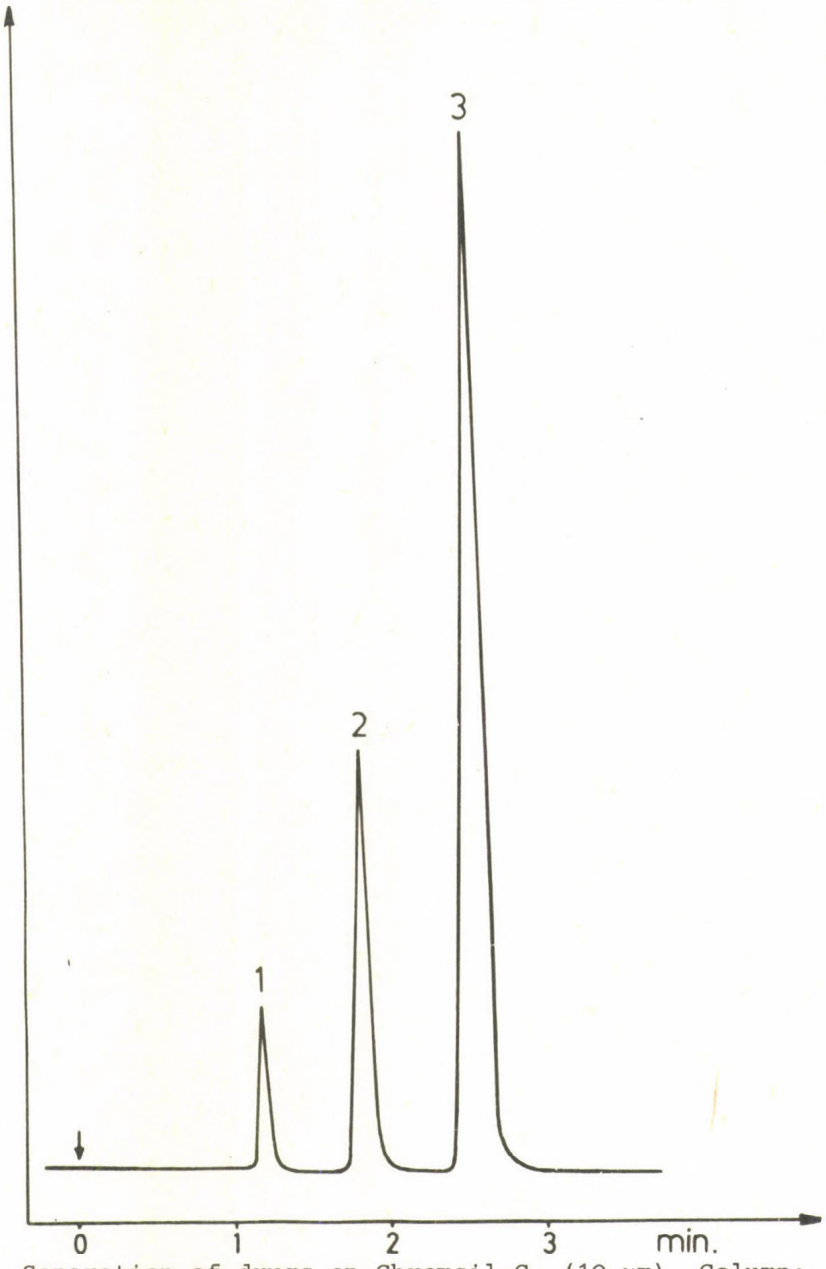

Fig. 10. Separation of drugs on Chromsil-C_8 (10 µm), Column: 250 x 4.6 mm, Eluent: Water: methanol (40:60) +0.1% NH NH_4OH, u = 3.5 mm/sec, Detector: 223 nm; 0.1 A, Peaks: 1. Phenobarbital, 2. Caffeine, 3. Phenacetin

Chromatography, the State of the Art
H. Kalász and L.S. Ettre (Eds)

SYNTHESIS, CHARACTERIZATION AND ANALYTICAL APPLICATION OF CHEMICALLY MODIFIED SILICA IN HPLC

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INTRODUCTION

Reversed-phase packings are the most often used stationary phases in HPLC and a great number of separations can be achieved with these materials by varying the mobile phase composition (org. modifier, ternary mixtures, ion-pair formation, etc.). Nevertheless there are some separations as well as some on-line clean-up steps which require a specially tailored stationary phase[1,2,3]. One of these examples is the separation of enantiomers. Although a separation can be performed by a reversed-phase column with a suitable mobile phase[3], all can be done in a simple way with a special modified phase[4,5]. In this paper we want to show how the synthesis of a chiral stationary phase can be developed and how its structure can be controlled by spectroscopic means.

MATERIALS AND METHODS

Preparation of a chiral proline-phase according to Foucault et al. (Pro-phase)[6]

A 7.5-g (0.065 mole) amount of L-proline was added to 17 ml (0.075 mole) of 3-(trimethoxysilyl)-propylamine in 175 ml of pyridine. The mixture was stirred for 15 h at room temperature, refluxed under anhydrous conditions for 5 h, then kept at $0^{o}C$ for 15 h. The unreacted L-proline (about 2.3 g) was separated

by filtration under argon atmosphere and the solution was evaporated. Subsequently the solution was vacuum dried at 44°C ($5 \cdot 10^{-2}$ Torr) and 5.6 g of Pro-amide were obtained. Elemental analysis gave C=50.1%, H=8.8%, N=8.9%. The optical rotation of the chiral silane was $\alpha_{579}^{20°C} = -25°$ (for C=1.5%, pyridine). A 5-g amount of dried Nucleosil 100-5 was suspended in 70 ml of dry toluene, and adsorbed water removed by azeotropic distillation with toluene. Then 10 ml of Pro-amide were added. The mixture was then refluxed under anhydrous conditions for 24 h.

The bonded silica was washed with dry toluene, methylene chloride, methanol, water. Bonded silica was vacuum dried at 40°C ($1 \cdot 10^{-3}$ Torr) for 24 h.

Elemental analysis gave C=9.5%, H=2.1%, N=2.8%. After filtration Pro-amide was recovered by evaporation; no racemisation was observed.

Schematic reaction: see Fig. 1

Preparation of a chiral diacetyltartaric acid-phase.[7] (Dws-phase)

Under anhydrous conditions (argon atmosphere) and ice water 10.8 g (0.050 mole) of L(+)-diacetyltartaric acid anhydride dissolved in 50 ml of absolute tetrahydrofuran was dropped into 10 ml (0.056 mole) of 3-(trimethoxysilyl)-propylamine (AMMO). The mixture was stirred at 0°C for 3 h. The solution was evaporated (tetrahydrofuran: 20°C, 100 Torr). Subsequently the yellow oil was vacuum dried to remove remaining AMMO (AMMO: 44°C, $5 \cdot 10^{-2}$ Torr) and 18,9 g of Dws-amide were obtained. Yield: 100% of theory.

The reaction occurs without racemisation and the optical rotation of the chiral yellow viscose silane was found to be $\alpha_{360}^{20°C} = +15.7°$ to $+17.4°$ (for C=1%, tetrahydrofuran).

Elemental analysis gave C=40.1%, H=6.0%, N=3.4%.

A 5 g amount of silica gel (Nucleosil 100-5) was dried for 24 h at 150°C to remove surface water. Under anhydrous conditions the warm silica gel was suspended in 30 ml of absolute tetrahydrofuran and 5 g of Dws-amide dissolved in 40 ml of absolute tetrahydrofuran was added. The mixture was then re-

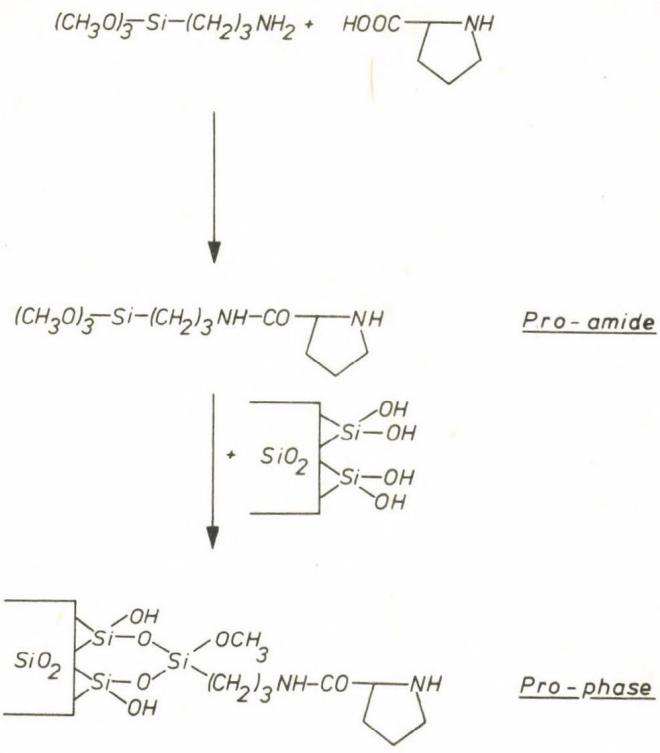

Fig. 1. Reaction scheme for preparing the L-proline phase

fluxed under anhydrous conditions for 30 h. The bonded support was filtered and washed with dry tetrahydrofuran, methanol, water and methanol (70 ml portions). The Dws-phase was vacuum dried at 40°C ($1 \cdot 10^{-3}$ Torr) for 24 h.

Elemental analysis gave C=7.6%, H=1.4%, N=1.1%.

After filtration, Dws-amide was recovered by evaporation; no racemisation was observed.

Schematic reaction: see Fig. 2.

Elemental Analysis

Elemental analyses were obtained by "Mikroanalytisches Laboratorium Beller", Göttingen, FRG.

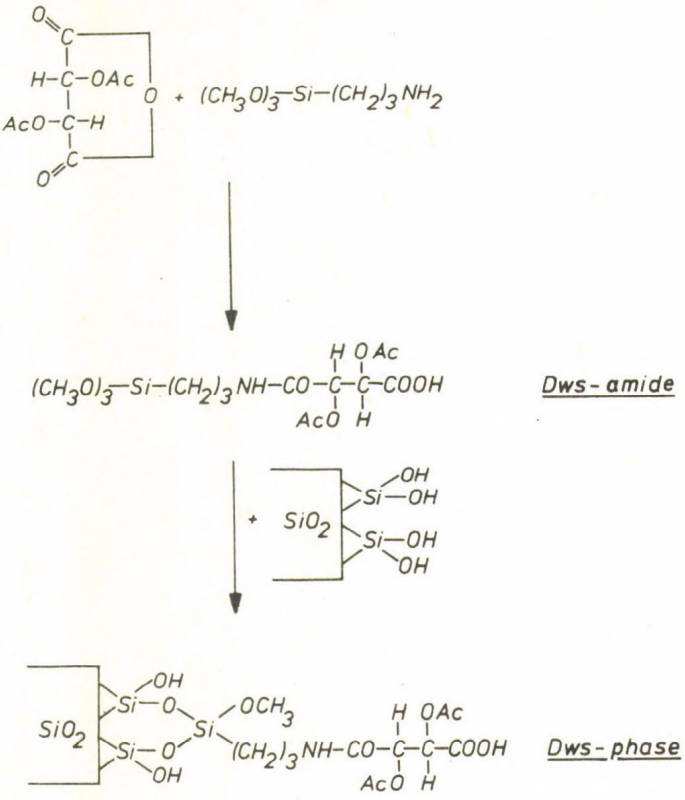

Fig. 2. Reaction scheme for preparing the L(+)-diacetyltartaric acid phase

Infrared Spectra

Infrared spectra were obtained with a Nicolet NIC-7199 Fourier transform infrared spectrometer. Samples were prepared in KBr pellets (silica gel or modified silica gel : KBr=1:20).

NMR-Spectra

The ^{1}H-NMR and ^{13}C-NMR were obtained on a Brucker WP 250 (250 MHz) Fourier transform spectrometer. All samples were dissolved in $CDCl_3$, only the immobilized silica was suspended in D_2O.

Optical Rotation

Optical rotation was obtained with a Perkin-Elmer Model 241 polarimeter.

Chromatographic Measurements

Columns (125 mm x 4 mm ϕ) were filled by pumping a slurry of the appropriate silica-gel in 2-propanol with methanol into the columns. All chromatographic measurements were carried out with a Model 6000 A pump, a Model 440 UV-detector (Waters Associates, Königstein, FRG) and a Model 7125 Rheodyne valve. Mobile phases consisted of different organic-aqueous buffer mixtures and are given in the figures.

RESULTS AND DISCUSSION

A chiral stationary phase can be synthesized in two ways:

(I) in a two-step reaction: first reacting the silica with an appropriate silane (e.g. aminopropyltrimethoxysilane) and in a second step reacting this "amino-silica" with a chiral compound

(II) by synthesizing a chiral silane which is then bonded to silica.

Way (II) has the advantage that during the preparation of the silane its structure can be easily controlled as well as the maintenance of the optical activity. Especially this point is very important and nearly impossible to confirm by way (I).

Spectroscopic confirmation of the chiral phases

A spectroscopic confirmation can be performed by ^{13}C-spectra and FT-IR-spectra. The ^{13}C-NMR spectra of the two chiral phases are given in Fig. 3. The coincidence with the free diacetyltartaric acid amide silane, e.g., shows that the immobilization occurs without changing of the silane. This is also confirmed by the FT-IR-spectra of the appropriate silica gels (Fig. 4).

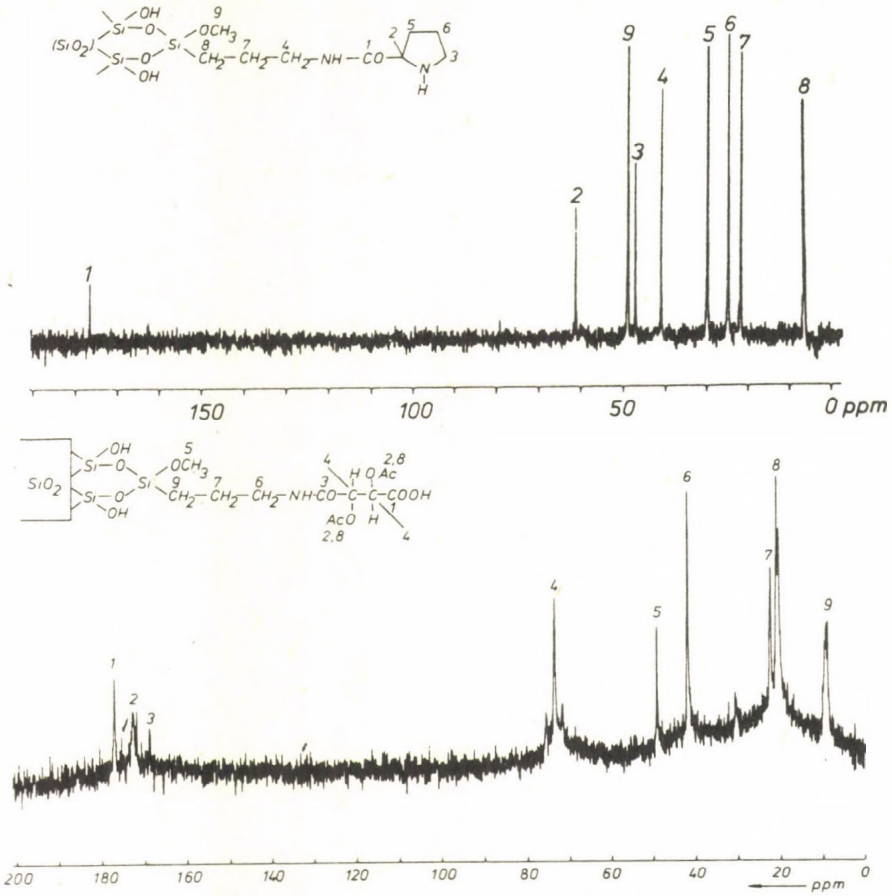

Fig. 3. ^{13}C-NMR spectra of Nucleosil 100-5 immobilized by Dws-amide and Pro-amide, suspended in D_2O

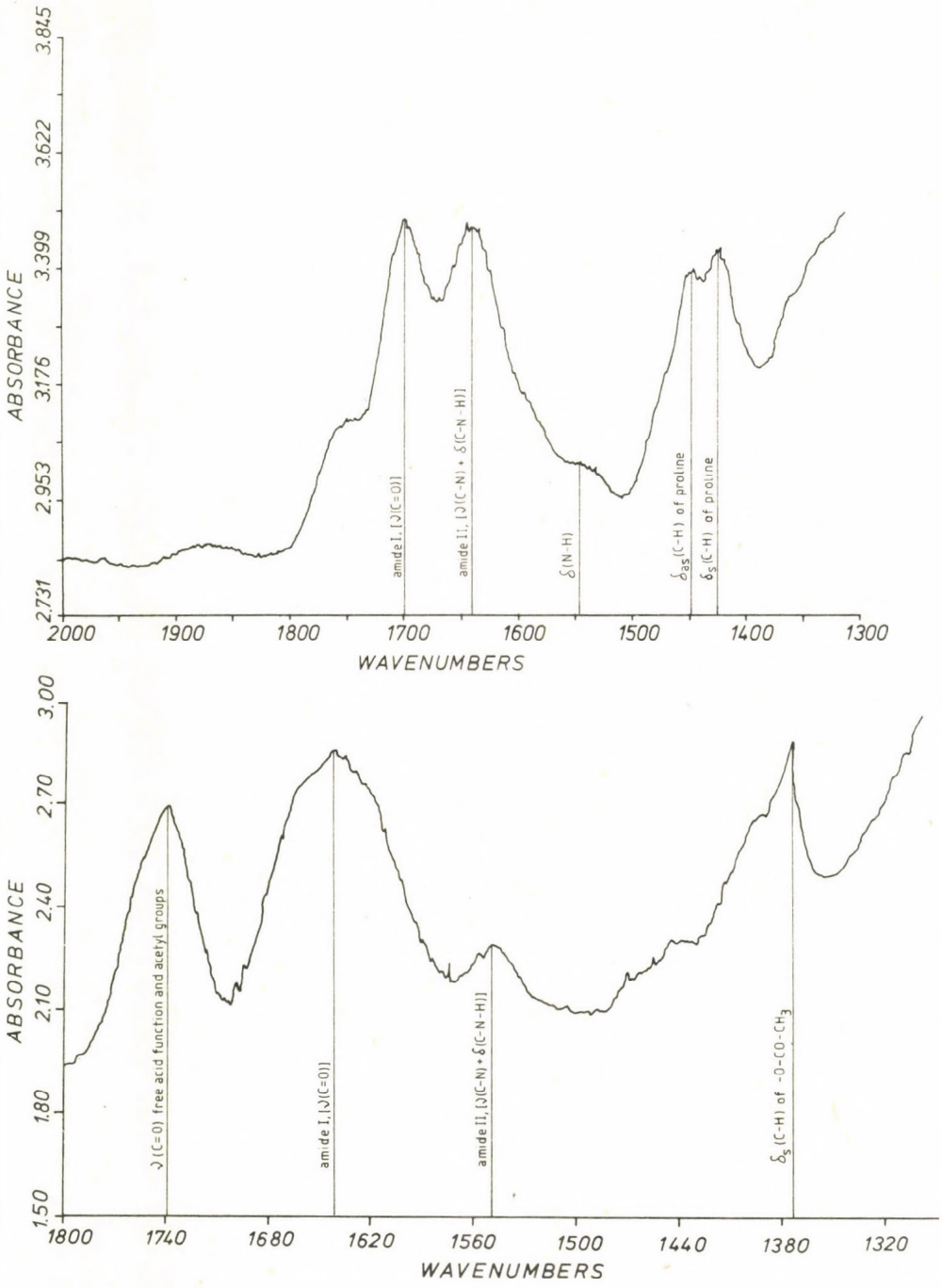

Fig. 4. FT-IR differential spectra of Nucleosil 100-5 immobilized by Dws-amide and Pro-amide in the region of 1800-1320 cm^{-1} and 2000-1300 cm^{-1} respectively.

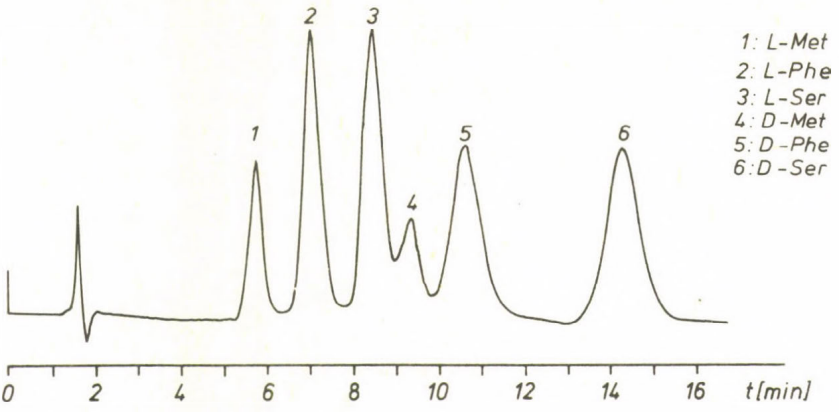

Fig. 5. Separation of D,L-dansyl-amino acids on the copper-loaded Pro-amide phase.
Eluent: $CH_3CN/2 \cdot 10^{-4}$M $CuSO_4$ in 0.1M NH_4Ac, pH = 8.7 (50:50)
Temperature: 55°C, flow rate: 1 ml/min

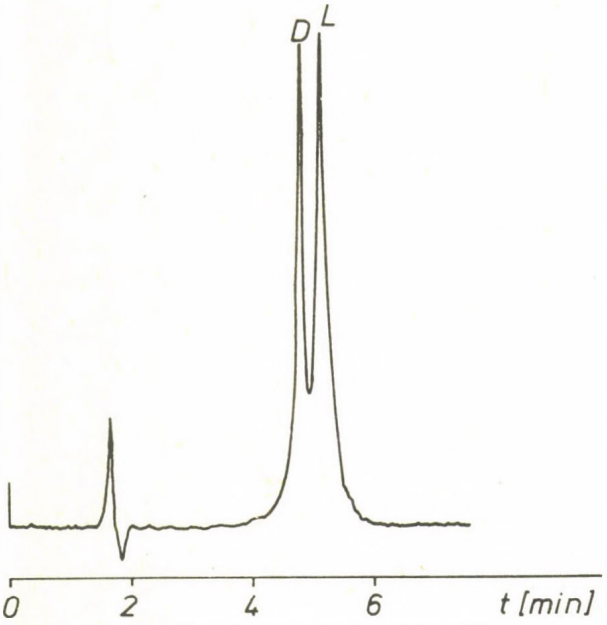

Fig. 6. Separation of D,L-epinephedrin on the copper-loaded tartaric acid phase
Eluent: $2 \cdot 10^{-4}$M $CuSO_4$ in 0.1 M KH_2PO_4, pH = 4.5
Temperature: ambient, flow rate: 1 ml/min

Application of the chiral phases in HPLC

From the elemental analysis the surface concentration was calculated to 4.2 μmol/m^2 (Pro-phase) and 3.8 μmol/m^2 (Dws-phase). These values are high enough to assure a reasonable interaction in chromatography between the stationary phase and the substrates to be separated provided that no racemisation has occurred during the immobilisation. Fig. 5 shows the separation of three dansyl-amino acids on the proline-phase, and Fig. 6 shows the separation of epinephedrin on the tartaric acid-phase (obtained after cleavage of the protective groups). In this way, it is confirmed that both silanes are bonded to silica in that state as they are synthesized and characterized by spectroscopic data plus optical rotation measurements.

Further investigations with other chiral stationary phases are in progress.

ACKNOWLEDGMEMENTS

The authors wish to thank Prof. Dr. H. Marsmann (Universität-GH Paderborn) for the FT-NMR-spectra, Mr.H.Henning (Universität-GH Essen) and Dr. H.H.Belz (Nicolet Instruments, Offenbach) for the FT-IR-spectra, and Dynamit Nobel AG for donating the silylating agent.

REFERENCES

1. W. Holstein, Chromatographia 14, 469 (1981)
2. Ch. Werkhoven-Gowie, P. Kwakman, R.W. Frei, U.A.Th. Brinkman, W. Maasfeld, T. Seshadri, A. Kettrup, J. Chromatogr. submitted for publication
3. V.A. Davankov, A.S. Bochkov, A.A. Kurganov, P. Roumeliotis, K.K. Unger, Chromatographia 13, 677 (1980)
4. G. Gübitz, W. Jellenz, W. Santi, J. Chromatogr. 203, 377 (1981)

5. W. Linder, Naturwissenschaften 67, 354 (1980)

6. A. Foucault, M. Caude, L. Oliveros, J. Chromatogr. 185, 345 (1979)

7. H.G. Kicinski, A. Kettrup, Z. Anal. Chem. 316, 39 (1983)

Chromatography, the State of the Art
H. Kalász and L.S. Ettre (Eds)

NATURAL CHROMATOGRAPHIC SYSTEMS IN BIOLOGICAL OBJECTS

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SUMMARY

There is hypothesis on the existence of a phenomenon based on the chromatographic separation of biologically active substances in a mixture, which occurs in natural biological chromatographic systems.

A great number of such systems may be discovered at various morphological and structural levels (reticulum, blood capillary, nephron, pulmonary system, etc.).

As a result, standard functioning of these chromatographic systems under certain conditions can control the transportation of biological molecules, whereas their malfunction could lead to a disease.

INTRODUCTION

It has been believed until now that chromatography is only a method of separation of substance mixtures (based on certain practical and theoretical developments [1,2] concerning the interactions of phases and the mixtures of substances to be separated) for a further qualitative and quantitative analysis of the components.

Since the moment of discovery of chromatography by Tswett in 1903, the thoughts and efforts of researchers working in the field have been directed at the improvement of known versions of chromatographic systems [3], the elaboration of new types of

chromatography [4], the analysis of separation mechanisms [5], the development of equipment and instruments, etc. The idea about the existence of indigenous chromatographic systems in nature has never been generated and formulated since the attention of theorists, researchers, and users of the method has been given to the necessity and efficiency of a most rapid application of chromatography for scientific and commercial purposes. It seems that for this particular reason objects similar to chromatographic systems have not yet been found in nature.

DISCUSSION

Consider the morpho-physiological characteristics of a blood capillary of an animal (Figs 1,2). The wall of the capillary consists of endothelium cells and the lumen is filled with flowing blood. Blood consists of cells - erythrocytes, leucocytes, platelets - and liquid plasma. In large vessels the velocity of plasma flow and that of the motion of blood cells are identical while in a capillary they are different, i.e. in a capillary a flow of plasma with respect to the wall and blood cells is observed. Thus, a capillary is a natural chromatographic column where the endothelium and blood cells act as the sorbent and blood plasma acts as the eluent. In other words, it is a natural chromatographic system. For the separation effect to occur in this system under natural conditions, a mixture of substances should enter the capillary column in a pulsed manner; otherwise, equilibrium will set up. Such situations are observed in the living organism and are due to the exogenous supply of a mixture of substances and to the endogenous synthesis of biomolecules. After intravenous administration of a mixture of medicinal preparations, separate components will reach target cells where their pharmacological action will be displayed at different times and in a certain sequence because of the effect of chromatographic separation in the blood capillary. A similar situation will occur in the activation of the biosynthesis of metabolites by tissues, e.g. when steroid

hormones are ejected into the blood as a result of the action of stress.

A great many systems of this kind may be found in the tissues of a living object on different morphological and structural levels. Endoplasmic reticulum (Figs 3,4) performing metabolism in the cell is in fact an ultracapillary column. It can be suggested that the control of the cellular transport of substances is dependent on the parameters of this ultrachromatographic system.

Tubules and collecting tubes of the kidney (Fig. 5) may be considered as an analogue of a microcapillary chromatographic system where the wall of the tubule acts as the stationary phase, secondary and primary urine are the mobile phase, and the metabolites filtered in the glomerulus are the separated phase.

The lungs of the animals and humans (Fig. 6) seem to be an interesting chromatographic system. It is evident that the trachea, bronchi, bronchioles, and alveoli are a system of consecutive chromatographic columns with different diameters and different properties of the internal surfaces acting as sorbent. The air is the separated mixture where biologically neutral nitrogen can be interpreted as the mobile phase with a reciprocal motion. During inhalation and exhalation, the content of oxygen, carbon dioxide, and nitrogen at the entrance of the system (trachea) changes:

Composition, %	Inhaled (atmospheric) air	Exhaled air	Alveolar air
O_2	20.94	16.3	14.2-14.6
CO_2	0.03	4.0	5.5-5.7
N_2	79.03	79.7	80.0

It can be assumed that during inhalation some oxygen is sorbed on the endothelium cells of the conductive respiratory tracts and for this reason, the percentage of oxygen in the alveolar air at the beginning of the gas exchange process is

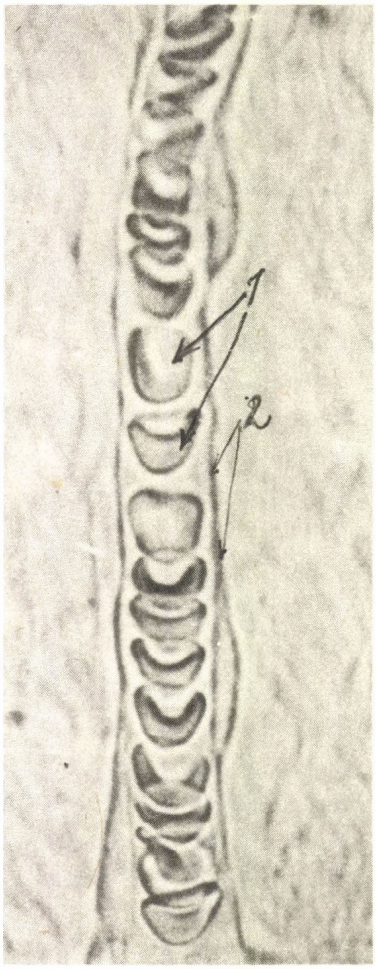

Fig. 1

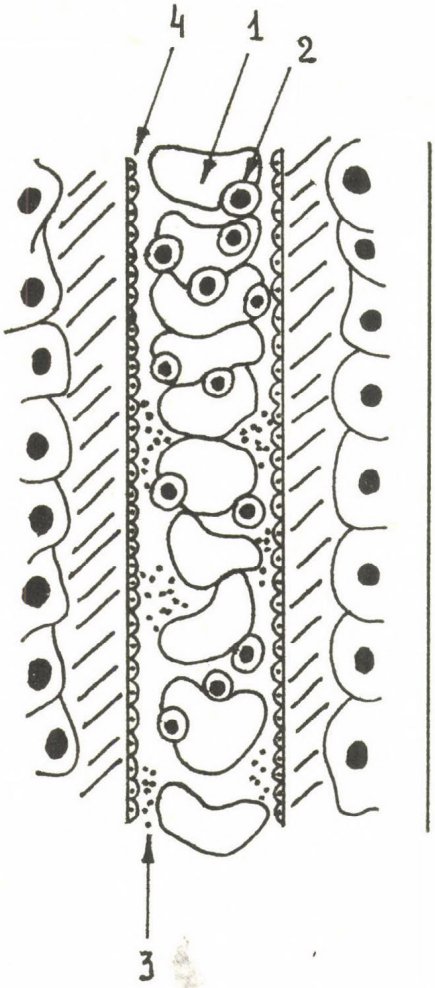

Fig. 2

Fig. 1. Blood capillary of an animal (600x), 1, erythrocytes; 2, endothelium

Fig. 2. Scheme of a blood capillary, 1, erythrocytes; 2, leucocytes; 3, platelets; 4, endothelium

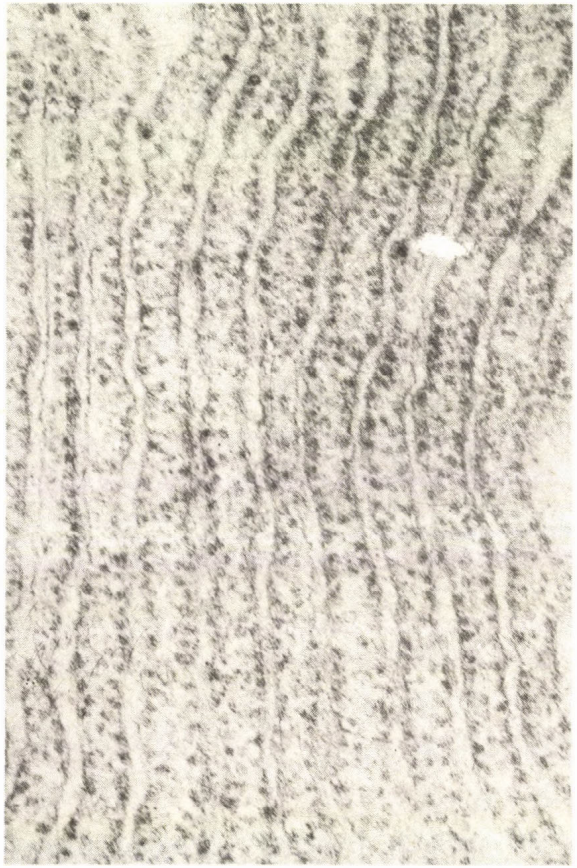

Fig. 3. Electron micrograph of endoplasmic reticulum (75000x)

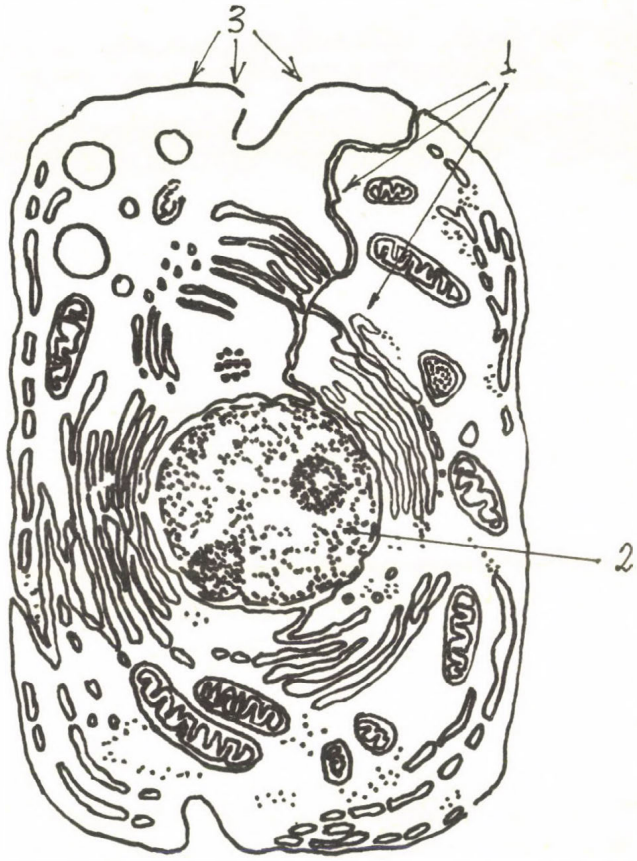

Fig. 4. Drawing of a typical cell, 1, endoplasmic reticulum; 2, nucleus; 3, plasma membrane

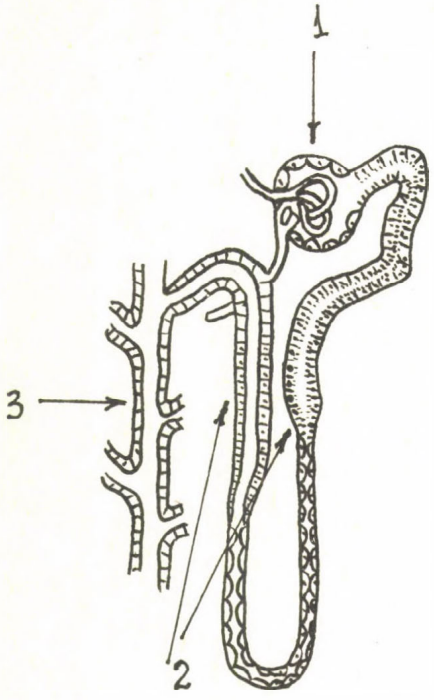

Fig. 5. Scheme of the lungs, 1, renal corpuscle; 2, tubules; 3, collecting tubes

slightly lower than in the atmosphere. It is to be added that the higher the sorption activity of the internal surface of the bronchi, the larger the amount of sorbed oxygen and the lower the quantity of oxygen in the alveoli. During exhalation, the whole system is regenerated. In the cases when the mucous membrane of the respiratory tract is modified by surface-active agents (coal, cement dust, etc.), oxygen sorption will be stronger and the content of oxygen in the alveolar air lower than the normal value. As a result, in such diseases as anthracoses and silicoses (or other pneumoconioses) this phenomenon may become a cause of hypoxia.

It is likely that in total a normal functioning of the macro-, micro-, and ultrachromatographic biological systems may ensure the control of the transport of biomolecules at

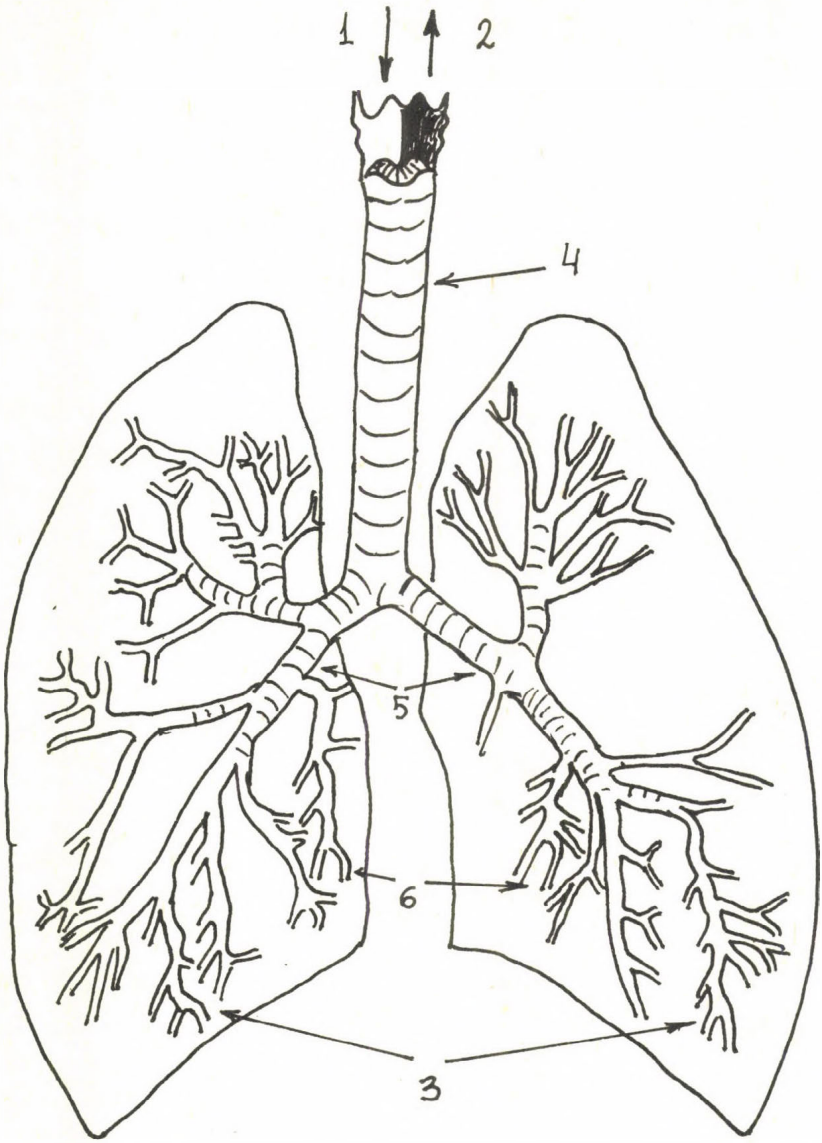

Fig. 6. Scheme of the lungs, 1, inhalation air; 2, exhalation air; 3, alveolar air; 4, trachea; 5, bronchi; 6, bronchioles

certain stages; on the other hand, a disturbance in the functioning of these systems will lead to pathological changes.

It is obvious that indigenous chromatographic systems and also indigenous chromatographic processes exist in the organic world as an independent phenomenon and can be easily found in any living object; plants and viruses, microbes and protozoa, and finally in animal tissues and organs.

Thus, chromatography as a phenomenon occurs in any biological object; hence, it is rightful to speak of chromatographic biology.

It is quite probable that in the inorganic world natural chromatographic systems have existed and operated for millions of years, being one of the mechanisms ensuring the circulation of matter. This problem should be solved by experts in the corresponding fields, e.g. geologists or geochemists.

REFERENCES

1. Bristow P.A., LC in Practice, HETP Publ., Handforth, Wilmslow, Cheshire, U.K., 1976.
2. Snyder L.R. and Kirkland J.J., Introduction to Modern Liquid Chromatography (Second Edition), John Wiley & Sons, Inc., New York, 1979.
3. Parris N.A., Instrumental Liquid Chromatography: A Practical Manual, Elsevier, New York, 1976.
4. Terweij-Groen C.P. and Kraak J.C., J. Chromatogr., 138 (1977) 245.
5. Engelhardt H., High Performance Liquid Chromatography, Springer, Berlin, 1979.

CHROMATOGRAPHY OF AMINES AND AMINO ACIDS

Chromatography, the State of the Art
H. Kalász and L.S. Ettre (Eds)

POLYAMINES AND THEIR DERIVATIVES AS MARKERS OF NEOPLASTIC GROWTH

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SUMMARY

In previous years the natural polyamines putrescine, spermidine, and spermine, and some of their derivatives have been explored as potential markers of neoplastic growth. However, the influence of alterations of polyamine catabolism on observable polyamine patterns in urine have not been taken into consideration. From our work, it appeared that aminoguanidine is not only capable of inhibiting the catabolism of putrescine and cadaverine, but also the transformation of spermine and spermidine into the corresponding amino acids, which are excretory products. Aminoguanidine is therefore a tool for the study of polyamine balance in vivo.

Using rats with Morris hepatoma in a model study, it appeared that the polyamines excreted in the urine represent only about 36 percent of the total amount destined to be catabolised or excreted. Inhibition of ornithine decarboxylase by administration of α-difluoromethylornithine (DFMO) (2 percent in the drinking fluid) reduced total polyamine excretion by 75 percent. The corresponding value for animals not treated with aminoguanidine was 57 percent. This example shows that alterations in the synthetic activity may not be adequately reflected by the observable changes of urinary polyamines, unless the terminal catabolism of the polyamines is inhibited.

Administration of DFMO did not reduce cadaverine excretion. Since decarboxylation of lysine in tissues is catalysed by a DFMO-inhibitable enzyme, it is evident that the major portion of urinary cadaverine in contrast with putrescine, is of bacterial origin.

The total amount of polyamines excreted by hepatoma rats was at no time during tumoral growth significantly larger than that of control animals. On the contrary: during a period of loss of body weight, polyamine excretion was significantly reduced. It increased again with the increase of tumor mass.

Disregarding a slight but significant increase in the excretion of N^1-monoacetylspermidine in rats with a large tumor, the polyamine patterns of normal and hepatoma bearing rats were the same. These results demonstrate that even a rapidly growing tumor, or a large tumor burden, may not cause significant alterations of the urinary polyamine pattern. Several factors contribute to these observations. Among them, the following are assumed to be most important: (a) reduction of polyamine producing tissues (loss of body weight); (b) utilisation of putrescine and spermidine, which were formed in certain organs, such as liver, for the formation of spermidine and spermine, respectively in the rapidly growing tumor.

INTRODUCTION

In the course of the last years evidence has accumulated that putrescine, spermidine, and spermine are essential for normal cell growth and proliferation [1-3]. Fig. 1 shows the structures of these simple aliphatic amines, and those of their derivatives which occur either in tissues or in body fluids of vertebrates, including man.

Based on observations of polyamine patterns in neoplastic cells in culture and in rapidly growing tissues [4] the following generalizations have been suggested [5]:

(a) increased accumulation of intracellular putrescine in tissues indicates initiation of growth process;

(b) accumulation of spermidine concomitant with ribosomal RNA prescribes increased mass or hypertrophy, which may or may not be followed by DNA synthesis and cell division;

(c) increased spermine appears to be a marker of differentiation, whereas decreased spermine parallels the de-differentiation processes.

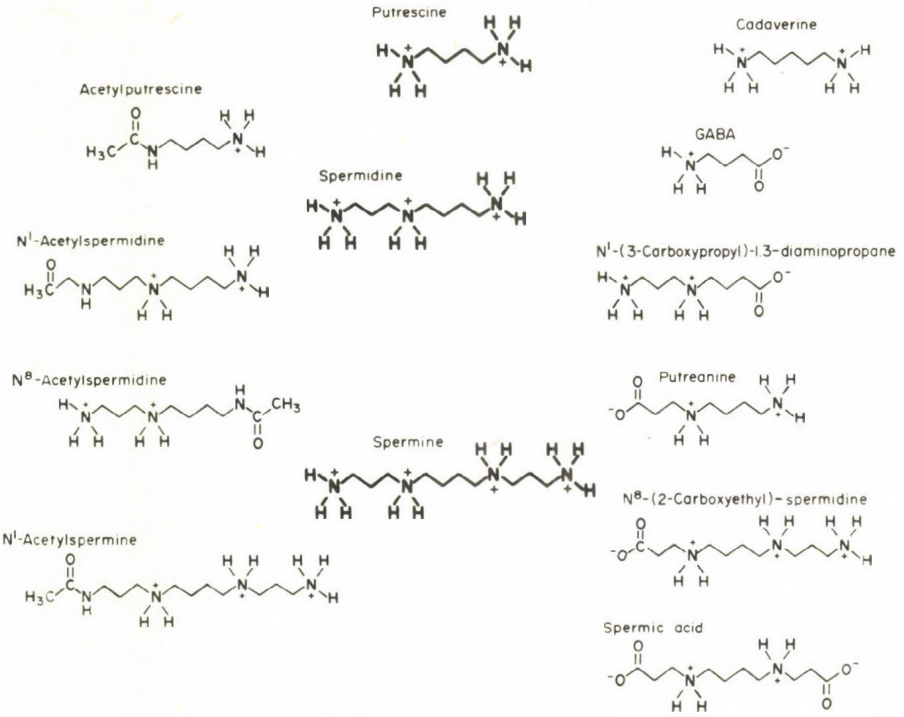

Fig. 1. Structural formula of polyamines, polyamine conjugates and metabolites.

It was expected that intracellular alterations of the polyamine patterns are followed by altered extracellular levels in various pathological states. Following the first work of D. H. Russell [6], polyamines were studied in urine, serum, plasma, blood, blood cells and CSF as potential in vivo markers of altered cellular polyamine metabolism, with special emphasis on neoplasms. The polyamines became thus the most recent class of naturally occuring amines for which sophisticated analytical methods had to be applied in clinical studies.

In recent reviews [5, 7-10] the results of the very numerous efforts to clarify the usefulness of polyamines as tumor markers were summarized. The following areas are presently considered as especially promising:

(a) evaluation of tumor status and burden;

(b) long-term monitoring of extracellular polyamines, in order to recognize remission or relapse of tumor patients;

(c) determination of urine or plasma polyamines for the evaluation of cell kinetics (correlation with autoradiographic data of bone marrow and tumor cells);

(d) measurement of spermidine in plasma as a means to determine cell kill (correlation with clinical response to therapy).

These applications of polyamine analyses are based on the following assumptions [5]:

(a) In a growing tumor, intracellular and extracellular concentrations of putrescine are proportional to the number of cells in the cell cycle, i.e. the growth fraction of the tumor.

(b) Intracellular spermidine concentration is related to the amount of ribosomal RNA in the tumor. Extracellular spermidine levels are proportional to the spontaneous cell loss factor of the tumor.

(c) During and after therapy by cytotoxic agents or radiation, extracellular spermidine is considered to be proportional to tumor cell kill, and an index of the effectiveness of the therapy.

(d) Extracellular putrescine concentration is believed to be proportional to the growth fraction of the tumor, suggesting that putrescine may be excreted from cells traversing cell cycle, and to the tumor cell kill, i.e. release of the intracellular pool of putrescine.

In these considerations, potential alterations of polyamine biosynthesis were regarded exclusively. However, it was shown in the course of the last years that polyamines undergo several catabolic reactions in vivo [11]. Some reactions lead to terminal products, which cannot be transformed again into putrescine, spermidine, or spermine. In addition conjugates are formed which serve as excretory products, and as intermediates of catabolic reactions.

Catabolic reactions may be enhanced or reduced in their activities in pathological states and by drugs. It can be expected that alterations of catabolism are reflected in changes of polyamine concentrations of certain tissues and body fluids.

In studies with human tumor patients, it is difficult to control the numerous parameters which may influence polyamine excretion. Therefore we used in our work an animal tumor model, namely Morris hepatoma. This solid tumor is rapidly growing and highly reproducible.

Since the tumor mass was increased during the experimental period from a palpable size (< 500 mg) to 10-20 g, it was possible to study the influence of tumor burden on polyamine excretion. Our major emphasis was on the comparison of the effects of inhibition of polyamine biosynthesis and oxidative deamination on polyamine concentration in urine.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS AND TUMOR MODEL

Buffalo rats (bred in our Center) were individually housed in stainless steel metabolic cages. They were kept under their usual 12 h light, 12 h dark cycle and had access to standard diet and water ad libitum. Each treatment group consisted of four animals. A suspension of cultured Morris 7288C hepatoma (HTC) cells (2×10^6 cells in 0.2 ml 0.9% NaCl solution) was injected into the hind leg muscle. Urine was collected from control and hepatoma bearing rats in regular intervals until day 26 after tumor cell inoculation, and body weight and tumor size were controlled. The cross section of the tumor was calculated and taken as a measure of tumor size. Some groups of tumor free and tumor bearing rats received a 2% solution of α-difluoromethylornithine (DFMO) in tap water as drinking fluid.

URINE COLLECTION AND POLYAMINE ANALYSIS

Urine samples were collected in regular intervals as follows: after collection of a first 24 h urine sample, the animals received 50 mg/kg of aminoguanidine sulfate (AG) intraperitoneally, and another 24 h urine sample was collected in 50 ml flasks containing 25 ml of ethanol. The volume was adjusted with ethanol to 50 ml and the polyamines were determined in 25 µl aliquots, which had been diluted with 225 µl of 0.2 N $HClO_4$ (containing 50 pmoles of 1,7-diaminoheptane as internal standard). The chromatographic procedure was described previously [12].

Creatinine was determined in 100 µl aliquots of the urine samples using Jaffe's reaction [12].

For polyamine determinations in urine hydrolysates 1 ml aliquots were acidified with 0.5 N HCl and evaporated in vacuo. The residues were dissolved in 0.5 ml 6N HCl and after sealing heated for 16 h at 120°C. 50 µl Portions of the hydrolysates were brought to 1 ml with 0.2 N $HClO_4$ (containing the internal standard). 200 µl Samples of these dilutions were directly applied to the reversed phase column.

METHODS OF POLYAMINE ANALYSIS

The development of sensitive methods for the assay of polyamines in tissues and body fluids is charateristic for analytical developments in general and resembles for obvious reasons especially the history of the development of the methods for amino acids: at one time or the other all newly created potentially useful separation methods, starting with paper chromatography and electrophoresis, and ending with GLC-MS techniques, have been applied to polyamines. [For review, see 14, 15]. Table I gives a brief account of the various methods in use presently.

The physical characteristics of the polyamines are not suitable for their sensitive and specific determination. Either one produces derivatives, such as benzoyl, or dansyl derivatives, and separates the polyamine derivatives, or one separates the free amines and produces derivatives suitable for sensitive detection after completed separation. Both approaches can give adequate results. Initially the interest was restricted to the determination of putrescine, spermidine, and spermine. Several procedures are suitable for this purpose. However, with the detection of the acetylated polyamines it was necessary to improve separations, for the determination of these compounds. Two dansylation procedures, three ion-exchange column chromatographic procedures and separations of ion-pairs of the polyamines on reversed phase columns are presently in use for this purpose. Fig. 2A shows the efficacy of the latter method. It allows to establish a complete pattern of the polyamines within less than 30 min in tissues. In urine a somewhat longer program is necessary, and monoacetylputrescine is usually interfering with other urinary constituents.

With the detection of the amino acids deriving from spermidine and spermine (see Fig. 1) a new analytical problem arose, that has not yet been adequately solved. Fig. 2B shows a chromatogram of the ion pairs

TABLE I

SELECTED METHODS OF POLYAMINE ASSAY IN TISSUES AND BODY FLUIDS

Separation method		Mode of detection	Approx. sensitiv. pmoles	Suitable for acetyl-polyamine assay	Ref.
TLC	Dansyl derivatives; silica gel layer	Fluorescence	10-20	yes	16
HPLC	Dansyl derivatives; silica gel column	Fluorescence	5-10	yes	17
HPLC	Dansyl derivatives; reversed phase column	Fluorescence	5-10	no	18,19
HPLC	Benzoyl derivatives; reversed phase column	UV abs.	20-50	no	20
HPLC	Ion pairs of the non-derivatized amines, reversed phase column; o-phthalaldehyde	Fluorescence	5	yes	21,22
HPLC	Cation exchange column chromatography of non-derivatized amines; o-phthalaldehyde	Fluorescence	10-50	Yes no	23,24 25
GLC	Trifluoracetyl derivatives	Flame ioniz.	200-600	no	26
GLC	Ethyloxycarbonyl derivatives	Flame ioniz.	5000	yes	27
GLC	Pentafluorbenzoyl derivatives	Electron capt.	1	no	28
GLC-MS	Trifluoracetyl derivatives		1	no	29
GLC-MS	Heptafluorobutyryl derivatives		1	no	30
Radioimmuno assay	Purified antibodies		0.05-0.5	no	31,32
Enzymatic assay	Bacterial oxidases;	Det. of H_2O_2	10000-20000	no	33,34
	Placental diamine oxidase	Det. of H_2O_2	5000	no	35

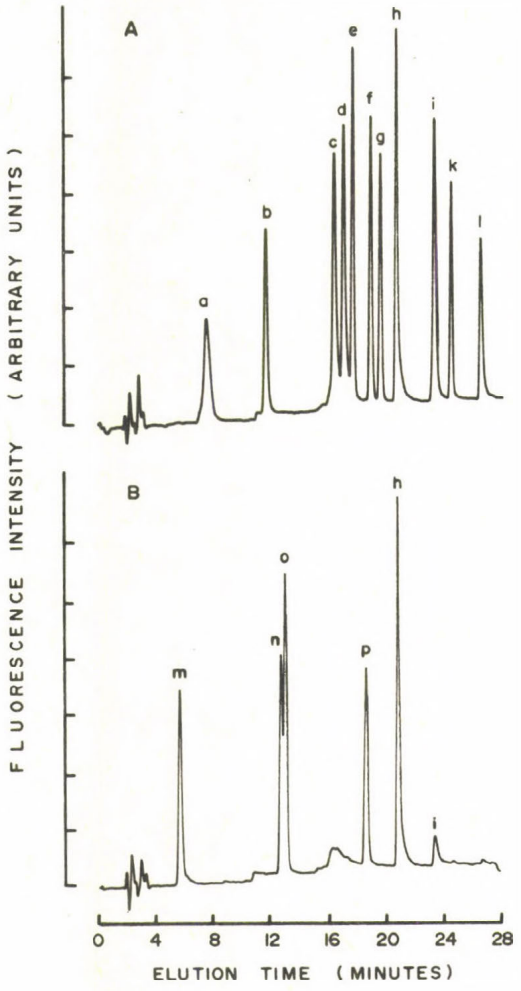

Fig. 2. Separation of polyamines, acetylpolyamines and related compounds as ion pairs with octane-sulfonic acid usinga μ Bondapak C_{18} column.

Elution by a linear gradient formed by mixing 0.1 M sodium acetate (pH 4.5) and a 10:3 (v/v) mixture of 0.2 M sodium acetate + acetonitrile. Both buffers contained 10 mM octanesulfonate. Stepness of the gradient: 4 percent increment.

(Continued next page)

of polyamine derived amino acids on a reversed phase column. Putreanine, isoputreanine, and the lactam of isoputreanine, the actual excretory form of isoputreanine [36], are clearly separated. However, only isoputreanine and its lactam can be determined in urine with this method. A GLC-MS method, separating isoputreanine from other urinary constituents has been reported, but the derivatization procedure forms isoputreanine lactam as artifact [30]. Nevertheless it allowed to demonstrate the presence of excessive amounts of isoputreanine in hydrolyzed samples of the urine of a patient with a Burkitt-type lymphoma. No method is available presently for the determination of spermic acid.

A BRIEF ACCOUNT OF POLYAMINE METABOLISM

In order to understand the influence of metabolic parameters on the polyamine pattern in urine, it is necessary to understand the key steps of polyamine metabolism. Fig. 3 summarizes the reactions involved in the formation, degradation and elimination of the polyamines. Putrescine is formed from ornithine by decarboxylation and serves as substrate of an enzyme, which forms spermidine by coupling an aminopropyl moiety obtained from S-adenosylmethionine with putrescine. An analogous reaction forms spermine from spermidine.

As far as polyamine catabolism is concerned, we can distinguish two types of catabolic reactions: (a) the intracellular degradation of spermine to spermidine and of spermidine to putrescine; (b) the terminal conversion of putrescine, spermidine, and spermine into products which cannot be transformed again into polyamines. The products of pathway (a) can be reutilized in the synthetic branch of

←

A: (a) monoacetylputrescine, (b) monoacetylcadaverine, (c) putrescine, (d) cadaverine, (e) histamine, (f) N^1-acetylspermidine, (g) N^8-acetylspermidine, (h) 1,7-diaminoheptane (internal standard), (i) spermidine, (k) N^1-monoacetylspermine, (l) spermine.

B: (m) putreanine, (n) N-(3-aminopropyl)pyrrolidin-2-one (isoputreanine lactam), (o) isoputreanine, (p) N^8-(2-carboxyethyl)spermidine.

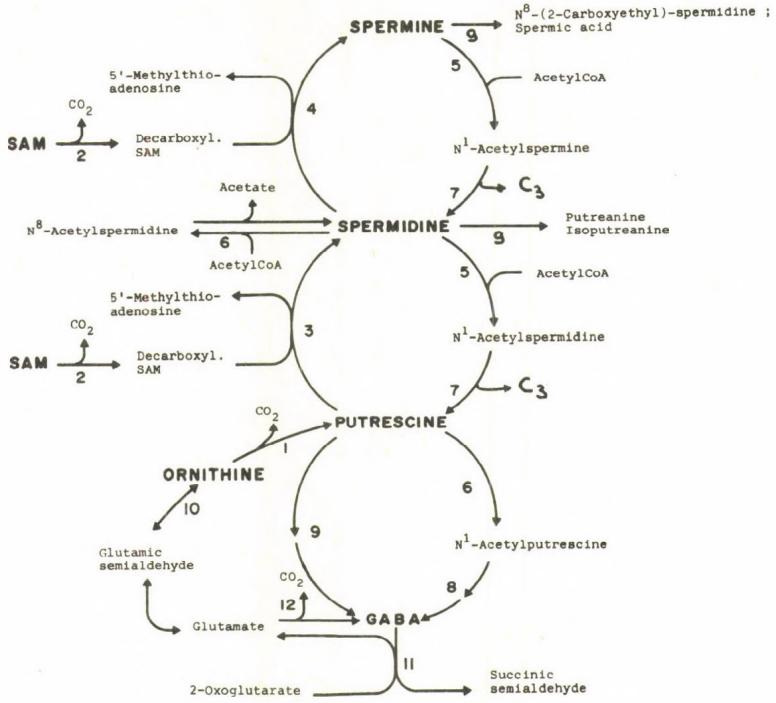

Fig. 3. Reactions involved in polyamine biosynthesis and catabolism: 1: ornithine decarboxylase; 2: S-adenosylmethionine decarboxylase; 3: spermidine synthase; 4: spermine synthase; 5: acetylCoA: spermidine N^1-acetyltransferase (cytoplasmic); 6: acetylCoA: spermidine N^8-acetyltransferase (nuclear); 7: polyamine oxidase; 8: monoamine oxidase; 9: diamine oxidase (aminoguanidine sensitive); 10: ornithine: 2-oxoacid aminotransferase; 11: 4-aminobutyrate: 2-oxoacid aminotransferase; 12: glutamate decarboxylase.

polyamine metabolism. This pathway is called the interconversion pathway [11]. Both the transformation of spermine into spermidine and the formation of putrescine is initiated by an acetylation step which forms the corresponding monoacetyl derivatives. The acetyl derivatives can either be degraded by polyamine oxidase, or they can be excreted with the urine. In the case of spermidine, a second nuclear acetylase

forms N^8-acetylspermidine, which is also a normal urinary excretion product. The same acetylase can form monoacetylputrescine from putrescine.

The interconversion pathway does not lead to losses of the putrescine moiety, if we disregard the fact that N^1-acetyl-spermidine is a normal urinary constituent. The set of oxidative deaminations form N^8-(2-carboxyethyl)-spermidine and spermic acid from spermine, isoputreanine lactam and putreanine from spermidine [37, 38], and 4-aminobutyric acid from putrescine. The latter can be further degraded [39]. The enzyme responsible for these transformations is either the classical diamine oxidase (DAO; E.C. 1.4.3.6) or a closely related Cu-containing amine oxidase [40]. The reaction products of DAO and related enzymes constitute together with the polyamines and acetylpolyamines, which are excreted, the irreversible losses of the polyamine metabolic cycle. In order to compensate for these losses, putrescine has to be formed from ornithine. Of course, any net increase of cellular polyamine concentration is also covered by putrescine formation.

POLYAMINES IN RAT HEPATOMA TISSUE DURING TUMORAL GROWTH

Five to six days after inoculation of HTC cells into the hind limb muscle of buffalo rats the tumor reached a palpable size. Growth was very rapid during the following three weeks, as can be seen from the increasing cross section of the tumor (Fig. 4). At day 26 the tumor mass was between 10 and 20 g.

Although tumoral growth was rapid during the entire experimental period, significantly increased levels of tissue polyamines were mostly observed during the first half (Fig. 4).

Treatment of the animals with α-difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase (ODC; E.C. 4.1.1.17) [41] restricts putrescine formation. This causes depletion of tissue putrescine and spermidine concentrations. The increase of spermine concentration is most probably due to the fact that S-adenosyl-methionine decarboxylase is induced in DFMO-treated animals [42]. Since putrescine is not available as substrate, spermidine is used. In other words, the decrease of spermidine concentration is partially due to a

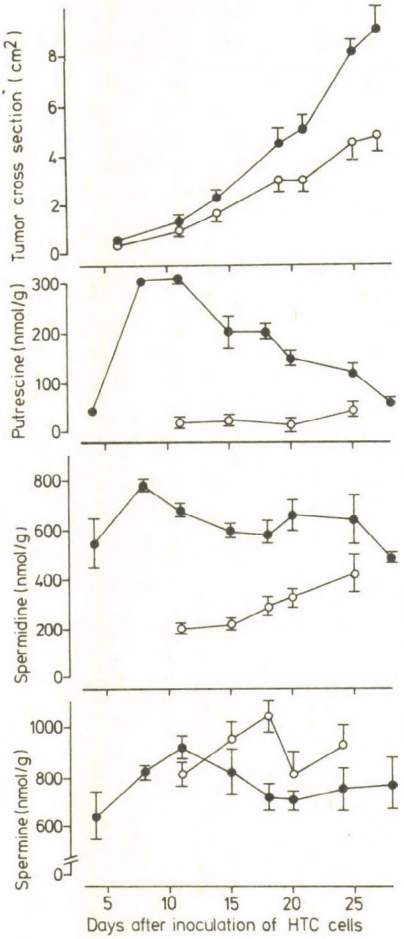

Fig. 4. Tumor cross section and polyamine concentrations in the tissue of Morris hepatoma during tumoral growth.
Closed circles: untreated animals;
Open circles : animals receiving 2 percent DFMO in the drinking fluid starting on day 4 after tumor cell inoculation.

decreased rate of formation, and partially due to its transformation to spermine. A consequence of polyamine limitation is a decreased rate of tumoral growth [See also 43, 44].

THE URINARY POLYAMINE PATTERN OF NORMAL AND HTC TUMOR RATS

In human urine the polyamines are nearly entirely excreted in the form of their monoacetyl derivatives [45, 46]. In contrast, rat urine contains, if any, only traces of conjugated putrescine, and the amount of non-conjugated spermidine is comparable to that of N^1-acetyl-spermidine. Spermine content is negligibly low [46]. The above mentioned separation of ion pairs of the polyamines allows therefore to establish a complete polyamine pattern in rat urine in one run. That practically all of the putrescine is excreted as free amine was shown by comparison of the polyamine contents of hydrolyzed and non-hydrolyzed urine samples of normal and HTC tumor bearing rats. The results were, within the error of the method, identical.

Despite a large tumor burden between day 19 and day 26 after tumor cell inoculation, the polyamine pattern of HTC tumor rats was not significantly different from that found in the urine of healthy controls, if we disregard from a small, but significant increase of N^1-acetylspermidine excretion (Table II). The result was essentially the same if the data were expressed in nmoles per mg creatinine (Fig. 5-7) or in total excretion per 24 h.

Treatment of the animals with DFMO affected mainly the excretion of putrescine, but N^1-acetylspermidine and spermidine excretion was also significantly reduced. But this treatment did not reveal any differences between animals with a large tumor burden and normal controls (Fig. 5.).

Cadaverine excretion was not affected by treatment with DFMO. There is evidence for the decarboxylation of lysine by mammalian ODC [47]. Our results suggest, therefore, that the cadaverine found in urine is of bacterial origin, whereas the major portion of urinary putrescine is formed within the various tissues.

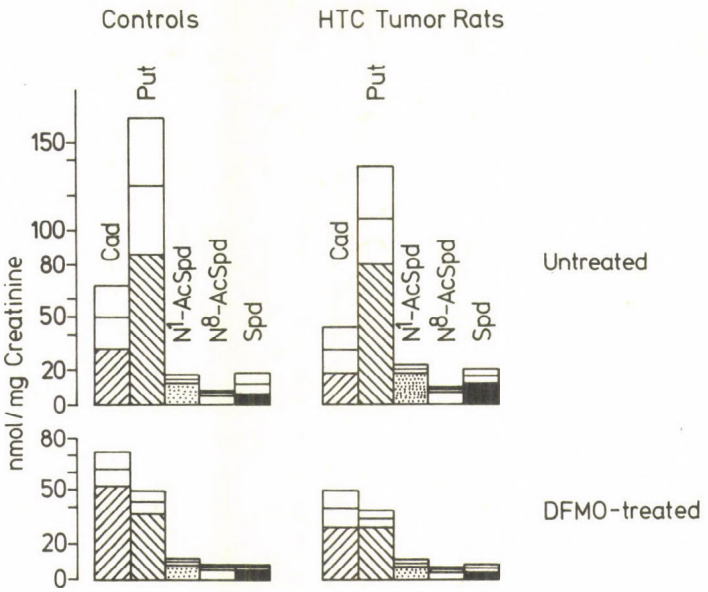

Fig. 5. Urinary polyamine patterns of buffalo rats and rats with a large Morris hepatoma (19-26 days after tumor cell inoculation). The effect of treatment with DFMO.
Cad: cadaverine; Put: putrescine; N^1-AcSpd: N^1-acetylspermidine; N^8-AcSpd: N^8-acetylspermidine; Spd: spermidine. The non-hatched parts of the columns represent 2 x S.D.

THE INFLUENCE OF CATABOLIC REACTIONS ON URINARY POLYAMINE EXCRETION

In a homeostatic system synthetic rates are in balance with catabolic reactions and excretion. As we have seen above, inhibition of putrescine formation by DFMO reduces according to the expectations the amount of excreted polyamines. However, it is unclear to what proportion polyamine catabolism is involved in the physiologic elimination of the polyamines. In other words, one has to ask the question which proportion of the expendable polyamines is eliminated by excretion, and which proportion is catabolised to compounds which cannot be re-converted into polyamines? These reactions comprise , as was mentioned, formation of 4-aminobutyrate (GABA) from putrescine, and

oxidative deamination of spermidine and spermine to the corresponding amino acids (Fig. 3). It has been known a long time that the major degradative pathway of putrescine is via DAO [39] but it has only recently been shown that spermidine, N^1-acetylspermidine, and spermine are also substrates of either DAO or an enzyme very closely related to DAO [38, 40].

Treatment of rats with 50 mg/kg of aminoguanidine sulfate (AG), a well known specific inhibitor of DAO turned out to be sufficient for complete inhibition of the above mentioned terminal catabolic reactions of the polyamines. This was demonstrated by showing that amino acid formation from injected spermine, spermidine, and N^1-acetylspermidine was blocked by this amount of AG, and that urinary excretion of isoputreanine (formed from endogenous precursor) was reduced to unmeasurable levels [36, 40]. Thus AG proved to be a suitable tool for the determination of the proportion of polyamines that is normally degraded, and does therefore not appear in the urine.

Fig. 6 demonstrates the effect of a single dose of 50 mg/kg of AG on the urinary polyamine concentrations of normal buffalo rats. From a quantitative point of view, only putrescine and cadaverine levels are dramatically elevated. The effects on spermidine and N^1-acetylspermidine are small but significant. No change of N^8-acetylspermidine excretion was demonstrable, although this compound is also a substrate of the DAO-like enzyme.

It cannot be shown here in detail, but unpublished results (N. Seiler, B. Knödgen and F.N. Bolkenius) have demonstrated that only a part of the putrescine appearing in urine is formed directly from ornithine by decarboxylation. A considerable portion is formed from spermidine, respectively from N^1-acetylspermidine along the interconversion pathway (Fig. 3). This finding suggests that in considerations of the metabolic balance of the polyamines - these include all in vivo studies of polyamine excretion - it is advantageous to compare total polyamine excretion (or more precisely excretion of putrescine equivalents), since changes in the activity of enzymes of the interconversion pathway may considerably alter the relative amounts of excreted putrescine and N^1-acetylspermidine, without influencing greatly total excretion of putrescine equivalents.

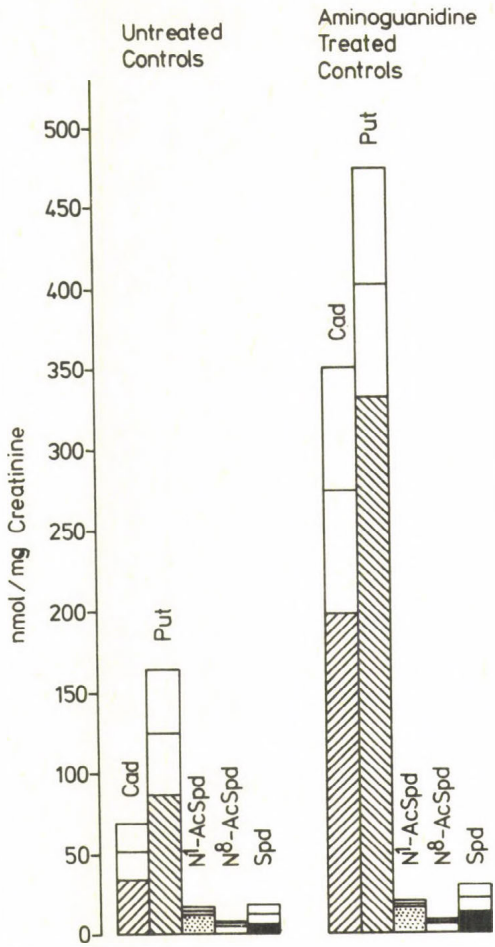

Fig. 6. The effect of treatment with 50 mg/kg of aminoguanidine sulfate on the urinary pattern of buffalo rats.
Cad: cadaverine; Put: putrescine; N^1-AcSpd: N^1-acetylspermidine; N^8-AcSpd: N^8-acetylspermidine; Spd: spermidine. The non-hatched parts of the columns represent 2 x S.D.

Owing to its bacterial origin cadaverine has to be excluded from balance studies.

TOTAL POLYAMINE EXCRETION IN NORMAL AND HTC TUMOR RATS

In Fig. 7, total polyamine excretion of normal buffalo rats is compared with polyamine excretion of rats with growing HTC tumors. Moreover, the polyamine excretion after administration of AG is compared with that of untreated animals, and finally the effect of treatment of DFMO is shown in both groups. The complete data are summarized in Table II. Control animals excreted polyamines at constant amounts over the entire experimental period, although they increased their average body weight from 334 to 345 g. The comparison of polyamine excretion after inhibition of the catabolic reactions with that of untreated animals demonstrates that more than 60 percent of the polyamines destined for disposal are degraded by AG-sensitive amine oxidases. (The proportion of polyamines accumulating in certain organs, such as liver, kidney and small intestine of AG-treated rats is negligible in comparison with the excreted amount [40]).

Treatment with DFMO in the drinking fluid reduced the expendable amount of polyamines by 75 percent in the AG-treated rats. The corresponding figure for animals not treated with AG was 57 percent. The DFMO inhibitable portion of the polyamines is of tissue origin. The residual polyamines excreted by DFMO-treated rats originate most probably to a considerable part from intestinal bacteria. This assumption is based on the fact that DFMO is not capable of inhibiting ODC of *E. coli* [48] and on the assumed analogy to cadaverine formation from lysine (see above). Since it is not possible to inhibit tissue ODC in an intact animal completely, a precise figure of the proportion of urinary polyamines originating from tissues, cannot be given. Our data suggest, however, that it is above 80 percent.

In contrast to the expectations (see Introduction), the HTC tumor bearing rats did not excrete more polyamines than normal controls at any time during tumoral growth (Table II and Fig. 7). On the contrary: a very significant decrease of urinary polyamine concentrations was observed in the urines collected on day 13 (after treatment with AG). This minimal polyamine excretion occured at a time when the tumor mass was relatively modest, and polyamines in the tumor tissue were elevated (Fig. 4). It also correlated with the minimum body weight (Fig. 7). The subsequent increase in total body weight and urinary polyamines has to be ascribed to the increase of the tumor mass.

TABLE II

POLYAMINE EXCRETION BY BUFFALO RATS. THE EFFECT OF TREATMENT WITH AMINOGUANIDINE SULFATE (AG) AND α-DIFLUOROMETHYLORNITHINE (DFMO) ON THE URINARY POLYAMINE PATTERN OF NORMAL AND HTC TUMOR BEARING RATS.

The data in this table are expressed in nmoles per mg creatinine. They are mean values ±S.D. from several urine collections of four animals per treatment grpup. In the case of untreated or AG - treated (50 mg/kg, i.p.) normal rats all 24 h urines collected between day 5 and 26 were used for the determination of the mean values (N = 16). In the case of DFMO administration (2 percent in the drinking fluid) the data were obtained from urines collected between day 12 and 26, i.e. from the treatment period, during which the effect of DFMO was maximal (N = 12). Urinary polyamine patterns of HTC rats with a large tumor burden were obtained by evaluating the data of the urines collected between day 19 and 26 (N = 6). (For details of the experimental protocol see the Methods section). (+ Cadaverine not included).

A. Normal rats. Body weight: untreated 325-360g; DFMO - treated 320-330g

Treatment AG	Treatment DFMO	Cadaverine	Putrescine	N^1-Acetyl-spermidine	N^8-Acetyl-spermidine	Spermidine	Total polyamines+
-	-	51 ± 18	125 ± 39	15 ± 2	7 ± 1	12 ± 6	160 ± 47
+	-	274 ± 76	402 ± 71	18 ± 2	7.1±0.3	21 ± 8	441 ± 74
-	+	62 ± 10	43 ± 6	10 ± 2	6.6±0.9	7.1±0.9	69 ± 9
+	+	172 ± 20	87 ± 7	14 ± 4	6.6±0.7	8 ± 2	118 ± 10

B. Rats with a large HTC tumor (19 - 26 days after inoculation of HTC cells)

Body weight: untreated 290-335g; DFMO - treated 245-305g

Treatment AG	Treatment DFMO	Cadaverine	Putrescine	N^1-Acetyl-spermidine	N^8-Acetyl-spermidine	Spermidine	Total polyamines+
-	-	31 ± 13	107 ± 29	20 ± 2	7.5±0.8	16 ± 4	154 ± 34
+	-	157 ± 43	311 ± 82	27 ± 8	9 ± 2	19 ± 10	370 ± 94
-	+	39 ± 11	33 ± 5	9 ± 2	5 ± 1	6 ± 2	53 ± 5
+	+	97 ± 17	66 ± 14	11 ± 4	5 ± 1	6 ± 1	88 ± 16

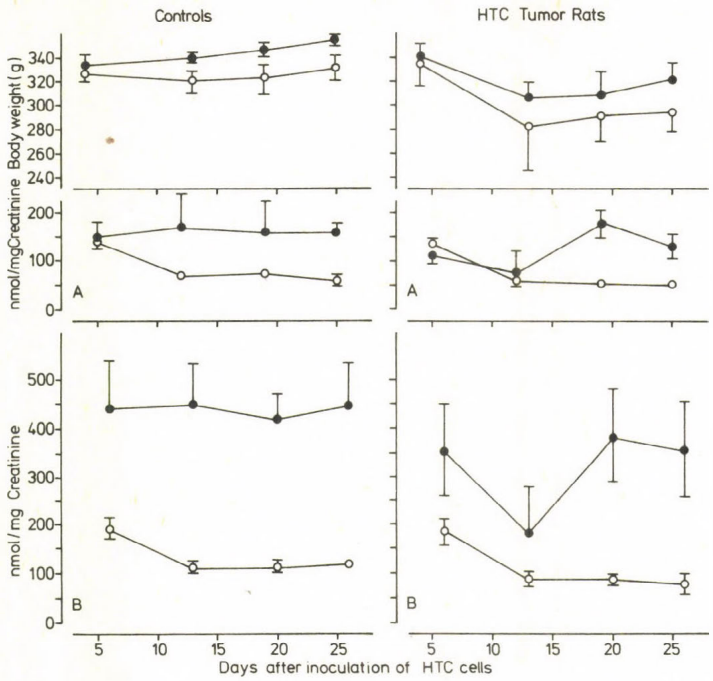

Fig. 7. Total urinary polyamines of control and Morris hepatoma bearing buffalo rats. The effect of treatment with DFMO on body weight and polyamine excretion.

A: Polyamine excretion before treatment with aminoguanidine sulfate.

B: Polyamine excretion after administration of 50 mg/kg of aminoguanidine sulfate.

Closed circles: animals receiving tap water as drinking fluid.

Open circles : animals receiving a 2 percent solution of DFMO as drinking fluid.

The bars indicate S.D.

Treatment with DFMO abolishes the difference in polyamine excretion between normal and HTC tumor rats, demonstrating that the amounts of expendable polyamines is reduced to the same level.

DISCUSSION

Our brief review of analytical methods revealed that at present several adequate methods are available for the sensitive determination of polyamines and most of their derivatives, in tissues and body fluids. The actual problem in using polyamines as tumor markers lies in our still inadequate understanding of the basis of the method.

Enhancement of polyamine excretion after treatment of pregnant rats with AG has been previously demonstrated [49]. But only the finding that a complete blockade of terminal catabolic reactions can be achieved with this compound [36-40] established AG as a tool for polyamine balance studies.

The proportion of polyamines which is catabolised by rats to terminal products is with 64 percent high (See Fig. 7., Table II). Human data are not yet existing. Since there is no evidence for the saturation of this catabolic system, an increased production of polyamines is presumed to be inadequately reflected in the urine of rats, unless the excessively produced polyamines are for some reason not exposed to the catabolic enzymes. The major site of oxidative deamination of the polyamines by AG-sensitive oxidases are the intestines [40] but other organs might contribute as well.

It is known that several types of tumors have enhanced DAO activity. Increased serum DAO activities have also been shown in pregnancy, and in a variety of diseases [50]. Since a larger portion of the expendable polyamines is expected to be catabolised under these conditions than in normal controls, even a significant overproduction of polyamines may not be detected. In a clinical test for detection of a tumor one would obtain a false negative.

In contrast, treatment of patients with certain drugs that impair polyamine catabolizing oxidases should give false positives. In both cases could pretreatment with AG and determination of total polyamines in urine correct the picture.

Our experiments with DFMO demonstrate that the major portion of the urinary polyamines is of tissue, and not of bacterial origin. This seems, however, not to be the case for cadaverine. Analytical methods,

which are not distinguishing between putrescine and cadaverine are therefore of restricted value for the diagnostic use of the polyamines.

It is self evident that our findings with HTC tumor rats cannot be generalized. However, they demonstrate clearly that one cannot expect a priori that a rapidly growing tumor produces excessive amounts of polyamines which can be used in one way or the other, for diagnostic purposes. The rate of polyamine formation may in fact limit in some tumors the rate of growth. The previous demonstrations of restricted tumoral growth after specific inhibition of putrescine formation [43, 44] are suggestive in this regard. Morris hepatoma growing in the leg muscle of Buffalo rats belong perhaps to the type of rapidly growing solid tumors with a greater demand for polyamines than can be covered by its synthetic capacity. Polyamine excretion by this tumor was at least too small, as to be detected in urine, even after blockade of polyamine catabolism.

The increase of N^1-acetylspermidine concentration in urine of HTC tumor rats, though significant, is too small, as to be useful for diagnostic purposes. Our previous explanation for this finding [51] that polyamine interconversion is impaired in the large tumor, due to a restricted oxygen supply, still seems valid.

REFERENCES

1 C.W. Tabor and H. Tabor, Ann. Rev. Biochem., 45 (1976) 285.

2 H.G. Williams-Ashman and Z.N. Canellakis, Perspect. Biol. Med., 22 (1979) 421.

3 A.E. Pegg and P. McCann, Am. J. Physiol., 243 (1982) C212.

4 J. Jänne, H. Pösö and A. Raina, Biochim. Biophys. Acta, 473 (1978) 241.

5 D.H. Russell in:T. Ming Chu (Editor), Biochemical Markers for Cancer, Marcel Dekker, 1982, p. 241.

6 D.H. Russell, Nature (London), 233 (1971) 144.

7 D.H. Russell and B.G.M. Durie, Polyamines as Biochemical Markers of Normal and Malignant Growth, Raven Press, 1978.

8 G. Scalabrino and M.E. Ferioli, in Advances in Cancer Research, Vol. 35, Part I, Academic Press, 1981, p. 151; Vol. 36, Part II, 1982, p. 1.

9 D.H. Russell, in CRC Critical Reviews in Clinical Laboratory Sciences, Vol. 18, Issue 3, CRC Press, 1983, p. 261.

10 W. Kersten, Dtsch. Med. Wschr., 108 (1983) 243.

11 N. Seiler, F.N. Bolkenius and O.M. Rennert, Med. Biol., 59 (1981) 334.

12 N. Seiler and B. Knödgen, J. Chromatog. Biomed. Appl., 221 (1980) 227.

13 F. Loken, Scand. J. Clin. Lab. Invest., 6 (1954) 325.

14 N. Seiler, Clin. Chem., 23 (1977) 1519.

15 N. Seiler, in J.M. Gaugas (Editor), Polyamines in Biomedical Research, John Wiley, 1980, p. 435.

16 N. Seiler and B. Knödgen, J. Chromatog. Biomed. Appl., 164 (1979) 155.

17 M.M. Abdel-Monem and J.L. Merdinck, J. Chromatog., 222 (1981) 363.

18 N. Seiler, B. Knödgen and F. Eisenbeiss, J. Chromatog. Biomed. Appl., 145 (1978) 29.

19 Y. Saeki, N. Uehara and S. Shirakawa, J. Chromatog. Biomed. Appl., 145 (1978) 221.

20 R. Porta, R.L. Doyle, S.B. Tatter, T.E. Wilens, R.A. Schatz and O.Z. Sellinger, J. Neurochem., 37 (1981) 723.

21 N. Seiler and B. Knödgen, J. Chromatog. Biomed. Appl., 221 (1980) 227.

22 J. Wagner, C. Danzin and P. Mamont, J. Chromatog. Biomed. Appl., 227 (1982) 349.

23 M.H. Mack, H. Kersten and W. Kersten, J. Chromatog., 223 (1981) 51.

24 D.H. Russell, J.D. Ellingson and T.P. Davis, J. Chromatog. Biomed. Appl., 273 (1983) 263.

25 L.J. Marton and P.L.Y. Lee, Clin. Chem., 21 (1975) 1721.

26 C.W. Gehrke, K.C. Kuo, R.W. Zumwald and T.P. Waalkes, in D.H. Russell (Editor), Polyamines in Normal and Neoplastic Growth, Raven Press, 1973, p. 343.

27 S.Y. Yamamoto, M. Yokogawa, K. Wakamatsu, H. Kataoka and M. Makita, J. Chromatog., 233 (1982) 29.

28 M. Makita, S. Yamamoto and M. Kono, Clin. Chim. Acta, 61 (1975) 403.

29 R.G. Smith, G. D. Daves, Jr. and D.P. Grettie, in R.A. Campbell, D.R. Morris, D. Bartos, G.D. Daves and F. Bartos (Editors), Advances in Polyamine Research, Vol. 2, Raven Press, 1978, p. 23.

30 F.A.J. Muskiet, C.M. Stratingh, D.C. Fremouw-Ottevangers and M.R. Halie, J. Chromatog. Biomed. Appl., 230 (1982) 142.

31 D. Bartos, R.A. Campbell, F. Bartos and D.P. Grettie, Cancer Res., 35 (1975) 2056.

32 P. Chaisiri, M.E. Harper, R.W. Blamey, W.B. Peeling and K. Griffiths, Clin. Chim. Acta, 104 (1980) 367.

33 U. Bachrach and B. Reches, Anal. Biochem., 17 (1966) 38.

34 S. Kubota, M. Okada, K. Imahory and N. Ohsawa, Cancer Res., 43 (1983) 2363.

35 T. Matsumoto, O. Suzuki, Y. Katsumata, M. Oya, Y. Nimura, M. Akita and T. Hattori, Clin. Chim. Acta, 112 (1981) 141.

36 N. Seiler and B. Knödgen, Int. J. Biochem., 1983, in press.

37 N. Seiler, B. Knödgen, M. W. Gittos, W.Y. Chan, G. Griesmann and O.M. Rennert, Biochem. J., 200 (1981) 123.

38 N. Seiler, B. Knödgen and K. Haegele, Biochem. J., 208 (1982) 189.

39 N. Seiler, Physiol. Chem. and Physics, 12 (1980) 411.

40 N. Seiler, B. Knödgen, G. Bink, S. Sarhan and F.N. Bolkenius, in U. Bachrach, A. Kaye and R. Chayen (Editors), Advances in Polyamine Research, Vol. 4, Raven Press, 1983, p. 135.

41 B.W. Metcalf, P. Bey, C. Danzin, M.J. Jung, P. Casara and J.P. Vevert, J. Am. Chem. Soc., 100 (1978) 2551.

42 P.S. Mamont, C. Danzin, J. Wagner, M. Siat, A.-M. Joder-Ohlenbusch and N. Claverie, Eur. J. Biochem., 123 (1982) 499.

43 N. Seiler, C. Danzin, N.J. Prakash and J. Koch-Weser, in N. Seiler, M.J. Jung and J. Koch-Weser (Editors), Enzyme-Activated Irreversible Inhibitors, Elsevier, 1978, p. 55.

44 J. Bartholeyns and J. Koch-Weser, Cancer Res., 41 (1981) 5158.

45 M.M. Abdel-Monem and K. Ohno, J. Pharm. Sci., 66 (1977) 1195.

46 N. Seiler, J. Koch-Weser, B. Knödgen, M. Richards, C. Tardif, F.N. Bolkenius, P.J. Schechter, G. Tell, P.S. Mamont, J.R. Fozard, U. Bachrach and E. Grosshans, in C.M. Caldarera, V. Zappia and U. Bachrach (Editors), Advances in Polyamine Research, Vol. 3, Raven Press, 1981, p. 197.

47 A.E. Pegg and S. McGill, Biochim. Biophys. Acta, 568 (1979) 416.

48 A. Kallio and P.P. McCann, Biochem. J., 200 (1981) 69.

49 N. Rojansky, E. Neufeld and R. Chayen, Biochim. Biophys. Acta, 586 (1979) 1.

50 S.B. Baylin and G. Luk, in D.R. Morris and L.J. Marton (Editors), Polyamines in Biology and Medicine, Marcel Dekker, 1981, p. 377.

51 N. Seiler, A. Graham and J. Bartholeyns, Cancer Res., 41 (1981) 1572.

Chromatography, the State of the Art
H. Kalász and L.S. Ettre (Eds)

DETERMINATION OF POLYAMINES BY OVERPRESSURED THIN-LAYER CHROMATOGRAPHY (OPTLC)

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INTRODUCTION

The natural polyamines, putrescine, spermidine and spermine are widely believed to have a regulatory role in cellular metabolism /for review see 1/. They are essential growth factors /2, 3/, stabilize the structure of cells /4/, membranes /5/, ribosomes /6/ and nucleic acids /7, 8/. These facts emphasize the importance of their analysis. High-performance liquid chromatographic /9, 10/, thin layer chromatographic /11/, gas-liquid chromatographic /12/ and thin-layer ion-exchange chromatographic /13/ etc. methods have been developed to determine the basic amino acid and polyamine content of biological samples.

In mammals, the synthesis of polyamines /Fig. 1/ starts from ornithine. Carbon dioxide is split off the molecule and putrescine is formed. In the subsequent steps putrescine is coupled with propylamine group and is converted to spermidine, then spermine is formed by the uptake of a second propylamine group, the polyamines are polycations, therefore one can easily separate them by ion-exchange chromatography.

MATERIALS

Chromatography was carried out on FIXION 50x8 thin-layer cation-exchange chromatosheets /Reanal, Budapest, Hungary/. All the amino acids and polyamines were purchased from Sigma /Hei-

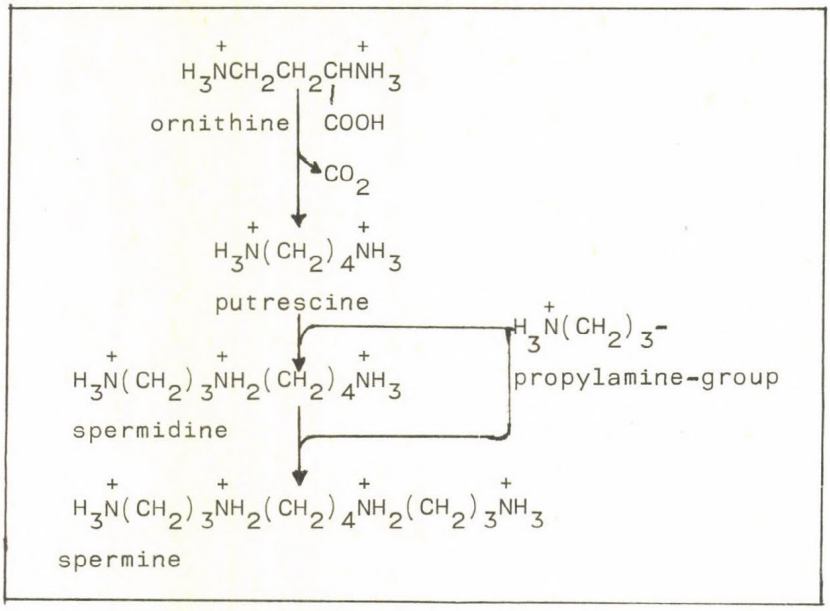

Fig. 1 Biosynthesis of polyamines

delberg, FRG/. All other materials were Reanal /Budapest, Hungary/ products of analytical grade.

METHODS

Tissue samples of mice and rats were homogenized in 3 volume /w/v/ of 10% trichloroacetic acid, then centrifuged /10 min, 5000 x g/. The clear supernatants were applied to FIXION 50x8 ion-exchange thin-layer chromatosheet in Na^+ cycle.

RESULTS

For the determination of basic amino acids and the polyamine content of biological samples, 20-50 µl of the superna-

tants and 5-5 nmol ornithine, arginine, putrescine, spermidine and spermine were applied to the chromatosheets as references. The sheets were run in phosphate buffer. The changes in R_f as a function of pH are shown in Fig. 2.

At least pH 7.5 is necessary for the separation. Relatively high amount of sodium, i.e. 2-3 mol/l gives the best resolution /Fig. 3/.

Therefore the sheets were run for about 4 hours at room temperature in 300 mmol/l potassium phosphate buffer, pH 7.5, containing 2 mol/l NaCl.

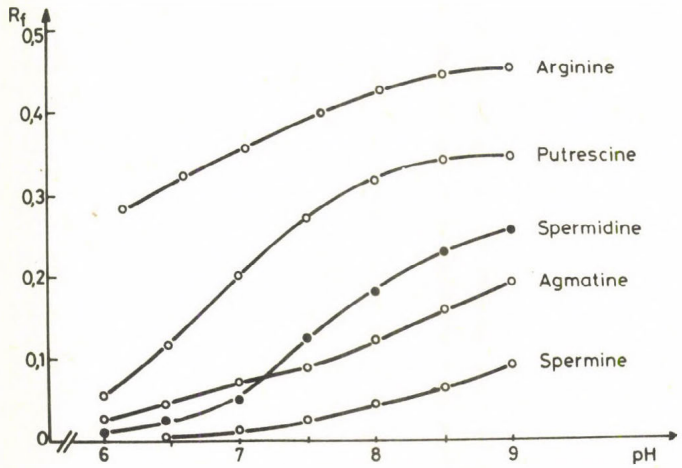

Fig. 2 The changes in R_f as a function of pH

Trichloroacetic acid present in the extract does not interfere with the determinations, because it runs with the front.

After chromatography the sheets were dried and developed with ninhydrin cadmium acetate reagent, as suggested by Dévényi /16/.

The basic amino acids and polyamines are readily separated on cation-exchange chromatosheet. The R_f values of basic amino acids vary between 0.72 and 0.41, while those of polyamines between 0.28 and 0.07 /Table I/.

The resolution could be improved by exhaustive chromatography, over-running the eluent. A filter-paper strip was placed

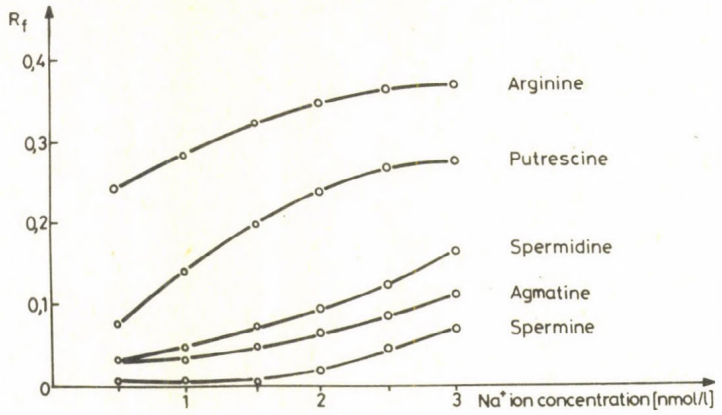

Fig. 3 The changes in R_f as a function of the sodium concentration

Table I R_f Values of Amino Acids

Amines	R_f
Methylamine	0.81
Ornithine	0.72
Arginine	0.41
Cadaverine	0.37
Putrescine	0.28
Spermidine	0.14
Agmatine	0.10
Spermine	0.07

horizontally on the top of the sheet, in its whole length. Care was taken to ensure the contact between the paper and the sheet. The running time was about 10 hours. In this case the basic amino acids ran off the sheet, and escaped detection.

The running time can be reduced by performing the chromatography in a "Chrompres 10" overpressured thin-layer chromatograph /Labor MIM, Hungary/.

In this equipment, the eluent feeder micropump delivers the solvent into the sorbent-layer by an adjustable overpressured flow. The chromatosheet is covered in the chamber by a water-cushion system, which is maintained by pressure, so the vapour space above the layer is eliminated. Three edges of the sheets have to be isolated with a special filling solution, "IMPRES" /Labor MIM, Hungary/, which forms a solid layer and forces the eluent to run only in one direction.

The migration of the solvent front in linear OPTLC is described by the equation.

$$k^{OPTLC} = \frac{x}{t},$$

where k is the velocity of the front, x is the distance of the solvent front from the start, and t is the separation time. The input pressure of the eluent increases in linear proportion to the distance, and makes the run-velocity of the solvent front constant.

In our experiments 10.5 bars water-cushion pressure was used, and the input pressure of the eluent was 3.5 bars. In this case, the 8-10 hour running time of the separation of polyamines by over-run chromatography was reduced to about 30 minutes /Fig.4/.

For quantitative determination, the density of ninhydrin spots of polyamines and basic amino acids were measured with a TELECHROM-S OE 976 reflective video-densitometer /Chromatronix, Palo Alto, California,USA/ by the method of Dévényi /17/.

The change in density of ninhydrin spots of polyamines as a function of their amount is linear up to 100 nmols /Fig.5/.

DISCUSSION

Under the described condition, the overpressured thin-layer chromatography combined by video-densitometry is suitable for the separation and measurement of polyamines in the range of

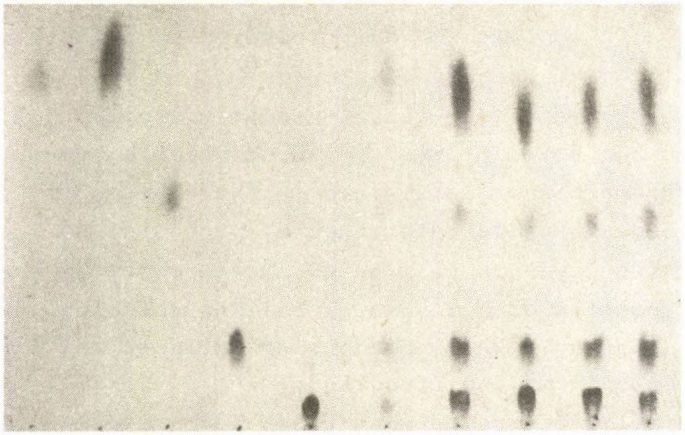

Fig. 4 OPTLC of polyamines. The spots are ornithine /1/, arginine /2/, putrescine /3/, spermidine /4/, spermine /5/, references /6/, and tissue extracts of mouse liver /7/, lungs /8/, thymus /9/ and kidney /10/.

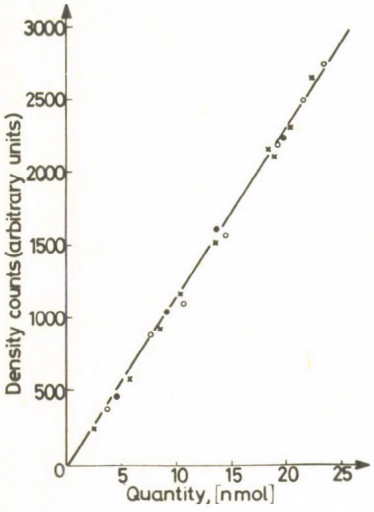

Fig. 5 Density of ninhydrin spots as a function of their amount

2-60 nmols, with ± 3% error. The sensitivity can be improved up to 0.1 nmol by adjusting zoom optics to the densitometer, but this increases the error to ± 5%. Further advantages of OPTLC are the small volume of eluent necessary for separation, the large number of samples that can be applied to the same sheet, and the short running time.

According to our experiments, the polyamine content of tissue extracts and other biological samples can be investigated without any previous purification.

ACKNOWLEDGEMENT

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REFERENCES

1 Polyamines in Normal and Neoplastic Growth. /Ed: Russel, D.H./ Raven Press, New York, 1973

2 Herbst, E.J., Snell, E.E., J. Biol. Chem. 181, 47-54 /1949/.

3 Goyns, H.H., J. Theor. Biol. 97, 577-589 /1982/.

4 Bachrach, U., Function of Naturally Occurring Polyamines, Academic Press, New York and London, 1973; pp. 1-21.

5 Tabor, H., Tabor, C.W., Adv. in Enzymol. 36, 203-268 /1972/

6 Stevens, L., Pascoe, G., Biochem. J. 128, 279-289 /1972/.

7 Tabor, C.W., Tabor, H., Ann. Rev. Biochem. 45, 285-306 /1976/.

8 Raina, A., Jänne, J., Siimes, M., Biochim. Biophys. Acta 123, 197-201 /1966/.

9 Simpson, R.C., Mohammed, H.Y., Veening, H., J. Liquid Cnromatogr. 5, 245-264 /1982/.

10 Prussak, C.E., Russell, D.H., J. Chromatogr. 229, 47-56 /1981/

11 Seiler, N., Knödgen, B., J. Chromatogr. 164, 155-158 /1979/.

12 Yamamoto, S., Itano, H., Kataoka, H., Makita, M., J. Agric. Food. Chem. 30, 435-439 /1982/.

13 Bardócz, S., Karsai, T., J. Chromatogr. 223, 198-201 /1981/.
14 Mincsovics, E., Tyihák, E., Kalász, H., J. Chromatogr. 191, 293-300 /1980/.
15 Kalász, H., Tyihák, E., Mincsovics, E.: in Recent Developments in Chromatography and Electrophoresis. 10, /ed.: Frigerio, A., McCamish, M./ Elsevier, Amsterdam, 1980; pp. 289-299.
16 Dévényi, T., Acta Biochim. Biophys. Acad. Sci. Hung. 11, 1-10 /1976/.
17 Dévényi, T., Acta Biochim. Biophys. Acad. Sci. Hung. 5, 435-440 /1970/.

Chromatography, the State of the Art
H. Kalász and L.S. Ettre (Eds)

MEASUREMENT OF DA AND DOPAC RELEASE FROM RAT STRIATAL PREPARATIONS "IN VITRO" USING HPLC WITH ELECTRO-CHEMICAL DETECTION

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SUMMARY

The release of dopamine (DA) and 3,4-dihydroxyphenylacetic acid (DOPAC) was measured from "in vitro" preparations of the striata of the rat using reverse phase HPLC with electrochemical detection. The effect of (-)deprenyl, a potent and specific B type MAO inhibitor was studied on the DA and DOPAC outflow applying thin slice and bulky tissue preparations. 20 mmol/l KCl was administered to stimulate the transmitter outflow. Under "in vitro" circumstances (-)deprenyl did not exert any remarkable effect on DA release and decreased the outflow of DOPAC. After three weeks of chronic pretreatment, both (-)deprenyl and clorgyline enhanced the output of DA and decreased the release of DOPAC. These findings substantially support the view that the beneficial effect of (-)deprenyl in Parkinson's disease is due to the enhancement of DA release in the striatum.

INTRODUCTION

(-)deprenyl, a potent MAO inhibitor with peculiar spectrum of pharmacological activity was developed by Knoll et al. in 1965. It was demonstrated that (-)deprenyl inhibits the uptake of tyramine (Knoll et al., 1968) and is a selective inhibitor of B-type MAO (Knoll and Magyar, 1972). As the only MAO inhibitor which does not potentiate the effects of tyramine, levodopa, etc., it was introduced into therapy as a valuable adjuvant of levodopa treatment in Parkinson's disease (Birkmayer, 1977). Knoll demonstrated that the most important central effect of (-)deprenyl is the potent, long-lasting enhancement of the dopaminergic tone in the brain (for review see Knoll, 1976) and proposed that this mechanism plays a role in the efficiency of (-)deprenyl in Parkinson's disease (Knoll, 1978). This view was supported by the analysis of the effect of (-)deprenyl on the release of ACh in isolated striatum of the rat (Hársing et al., 1979), but no direct dopamine release measurement has been done so far in the striatum of (-)deprenyl treated animals. The aim of our work presented in this paper was to analyze the effect of (-)deprenyl on striatal dopaminergic transmission by direct measurements of dopamine and 3,4-hydroxyphenylacetic

acid released to 20 mM potassium stimulation of the tissue. Recent developments in reverse phase high-performance liquid chromatography with electrochemical detection provided us a specific and sensitive analytical method to measure few hundred pmoles of catecholamines in samples of biological origin (reviewed by Kissinger et al., 1981). This paper serves evidence that dopamine and 3,4-hydroxyphenylacetic acid release can be measured from isolated striatal preparations of the rat and (-)deprenyl pretreatment increases dopamine outflow.

METHODS AND MATERIALS

Apparatus

A Liquopump 312 solvent delivery system with injection valve fitted with 20 µl loop (Labor MIM Hungary) was used. The column used was a 250x4.6 mm I.D. Chromsil C_{18}, 10 µm particle size (Labor MIM Hungary). The wall-jet type electrochemical detector cell was developed by the Institute for General and Analytical Chemistry, Technical University Budapest, Hungary. The working electrode was packed with paraffin oil based carbon paste, and an electronic controller was developed by the Biomedical Engineering Workshop of the Department of Pharmacology. The signal from the amplifier was visualized on an OH 814/1 Potentiometric Recorder (Radelkis, Hungary).

Reagents

3,4-dihydroxy-norephedrine HCl (Cal-Biochem, Los Angeles, USA) Dopamine HCl (Serva Heidelberg, BRD) Noradrenaline bitartarate (Gedeon Richter Hungary), 3,4-dihydroxyphenylacetic acid (Fluka AG, Buchs, Switzerland), (-)deprenyl (Chinoin, Hungary), alumina (Brockmann Grade II. neutral, Merck) octane sulphonate sodium (Kodak USA). All the other chemicals were of the commercially available highest quality.

ISOLATION OF STRIATA AND SAMPLE COLLECTION

Albino Wistar rats weighing 120-150 g of both sexes were decapitated. Striata were quickly removed according to the method described by Glowinski and Iversen (1966). The striata were either halved into two parts or cut into 0.3-0.5 mm thin slices. The slices were superfused and the halved preparations were soaked in carbogenated (95% O_2 5% CO_2) Krebs solution (composition in mmol/l: NaCl 111, KCl 4.7, $CaCl_2$, $MgSO_4$ 1.64, $NaHCO_3$ 25, KH_2PO_4 1,2, glucose 11. 50 mg/l ascorbic acid and 20 mg/l Na_2EDTA added). Four striata were pooled in one organ bath. In the case of soaked preparation, after one hour of equilibration the bath was replaced for a fresh one, and then it was changed in every 10 minutes. The third organ bath contained 20 mmol/l KCl and less NaCl by a proper quantity to make an isoosmotic solution. The elevated level of KCl stimulated the DA release. In the next 10 minutes we returned to the original Krebs solution. At the superfused preparation the striatal slices were put in a 0.5 ml volume plexi glass superfusion chamber. Krebs solution was delivered through the chamber by a flow rate of 6 ml/hour using a syringe pump. The

superfusate collected in the first hour was discarded, and then 20 min samples were collected. In the third collection period we switched to a solution with a higher KCl concentration (see above). After 20 minutes we returned to the original Krebs solution. Temperature was kept at 37°C. Drugs studied were present in appropriate concentrations during the total experiment.

In the studies of chronic pretreatments, animals received daily a single s.c. injection of the drug for three weeks. 24 hours after the last injection the rats were killed, and the release was measured. There was no drug present in the nutrient solutions.

SAMPLE PREPARATION

Immediately after obtaining the superfusate 40 ng dihydroxy norephedrine HCl (DHNEP) was added as an internal standard. Half the sample volume of pH 8,6 3 mol/l Tris-HCl buffer was added and the mixture was transferred onto a 90 mg alumina containing micro-column. Alumina was prepared as described by Anton and Syre (1962). Then the columns were washed twice with distilled water and once with 20% acetonitrile (v/v) in distilled water solution. This working step greatly reduced the front size in the chromatography. Catechols were eluted from the columns with 0.1 mol/l trichloroacetic acid containing 0.5 µg/ml ascorbic acid. The eluent was then injected onto the HPLC system.

CHROMATOGRAPHY

The mobile phase was 0.14 mol/l monochloroacetic acid solution, pH set to 3 with 4 mol/l NaOH. This buffer contained 50 mg/l octane sulphonate sodium as an ion pairing agent and 0.05 mmol/l Na_2 EDTA to reduce the detector's spike activity. 6% acetonitrile and 1% tetrahydrofurane (v/v) were added to make the final mobile phase. Flow rate was 1 ml/min.

CALCULATIONS

The amount of DA and DOPAC relased was calculated on the basis of their peak height ratio to that of the internal standard DHNEP, data are given in $pmol g^{-1} min^{-1}$, means and S.E.M. For statistical analysis Student's t test for two means was used. Level of signifiance was $p < 0.05$.

RESULTS

Figure 1 demonstrates the relative sensitivity of the system to noradrenaline, dopamine, 3,4-dihydroxyphenylacetic acid versus the internal standard dihydroxynorephedrine. The ratio is linear to the amount of catecholamines as small as 100 pg per injection. In Fig. 2 we demonstrate two chromatograms of the purified biological sample. Measurable amount of DA and DOPAC is released even under resting circumstances.

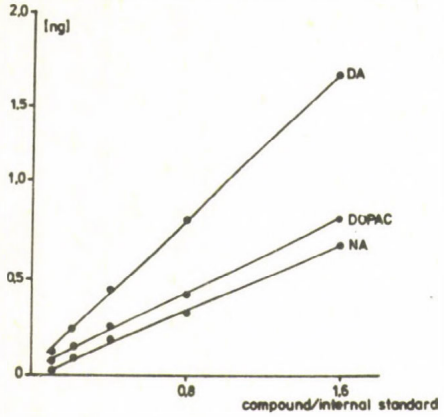

Fig. 1 Relative sensitivity of the system to dopamine, DOPAC and noradrenaline versus internal standard (DHNEP).

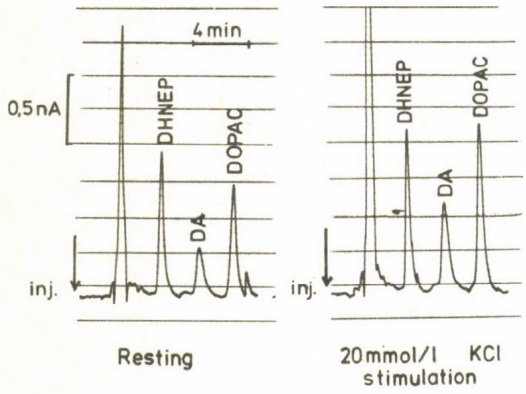

Fig. 2 Chromatograms of the purified superfusate. pH=3 monochloroacetic acid buffer 6% acetonitrile 1% THF, 20 µl sample. Internal standard DHNEP (1.6 ng added before alumina adsorption).

THIN SLICE PREPARATIONS

This preparation released 44.5±7.6 DA and 56.3±13.1 $pmol\,g^{-1}\,min^{-1}$ DOPAC as resting values. These changed to 115.0±9.1 DA and 55.4 $pmol\,g^{-1}\,min^{-1}$ DOPAC following 20 mmol/l KCl stimulation. It is worth remarking that there is no increase of DOPAC levels after stimulation which is possibly due to the thin preparation, DA can easily escape from the tissue. (-)Deprenyl in increasing concentrations did not change the DA outflow, and slightly decreased the DOPAC output (Fig. 3). After chronic

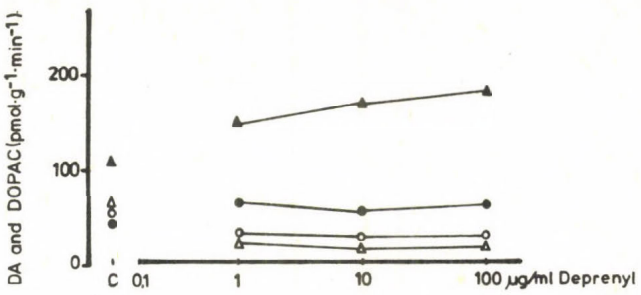

Fig. 3 The effect of (-)deprenyl "in vitro" on the DA and DOPAC release from thin slice striatal preaparation. ● DA resting, o DOPAC resting, ▲ DA stimulated, Δ DOPAC stimulated. C = control, without drug.

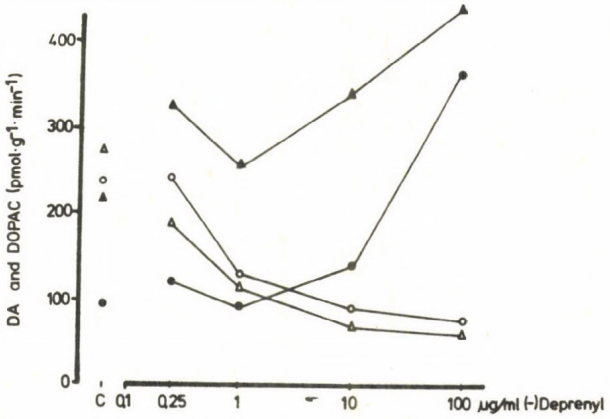

Fig. 4 The effect of (-)deprenyl "in vitro" on the DA and DOPAC release from Glowinski-Iversen preparation. ● DA resting, o DOPAC resting, ▲ DA stimulated, Δ DOPAC stimulated, C = control, without drug.

treatment (0.25 mg/kg for three weeks) DA release was strongly augmented either using (-)deprenyl or clorgyline. There was no significant change in DOPAC release (Table 1).

GLOWINSKI - IVERSEN PREPARATION

This preparation released 91.1±7.2 DA and 258,3±18.5 $pmol g^{-1} min^{-1}$ DOPAC under resting conditions. 20 mmol/l KCl stimulation elevated the DA and DOPAC release, 200±25,8 and 291.5±29,5 $pmol g^{-1} min^{-1}$ respectively. "In vitro" (-)deprenyl treatment resulted in a concentration dependent decrease in DOPAC release (Figure 4), but there was no significant change

TABLE I

The effect of 0.25 mg/kg deprenyl and clorgyline (daily injections for 3 weeks) on DA and DOPAC release from rat striata using 0.3-0.5 mm thin slices preparation. Mean and S.E.M. are shown in $pmol g^{-1} min^{-1}$. *marks significance ($p<0.05$) compared to the control value

	Resting		20 mmol/l (K+)		
	DA	DOPAC	DA	DOPAC	n
Control	44.5±7.6	56.3±13.1	115.0±9.1	55.4±36.3	10
0.25 mg/kg deprenyl	106.9±9.1*	58.7±17.9	263.0±57.5*	33.3±6.2	6
0.25 mg/kg clorgyline	231.7±15.3*	26.4±7.0	439.0±108.0*	29.3±10.3	4

TABLE II

The effect of 0.25 mg/kg deprenyl and clorgyline (daily injections for 3 weeks) on DA and DOPAC release from rat striata using Glowinski-Iversen preparation. Mean and S.E.M. are shown in $pmol g^{-1} min^{-1}$. *marks significance ($p<0.05$) compared to the control value

	Resting		20 mmol/l (K+)		
	DA	DOPAC	DA	DOPAC	n
Control	91.1±7.2	258.3±18.5	200.0±25.8	291.5±29.5	10
0.25 mg/kg deprenyl	503.1±20.6*	71.8±10.1*	1451.2±183.1*	120.8±36.3*	6
0.25 mg/kg clorgyline	406.0±66.5*	87.7±22.0*	711.1±118.2*	84.7±10.9*	4

in DA outflow except at very high (-)deprenyl concentration (100 µg/ml) which is far beyond any therapeutic range. Three weeks' pretreatment with (-)deprenyl and clorgyline respectively, caused a marked and significant increase in the output of DA and a decrease in the outflow of DOPAC (Table 2). There was no significant difference between the effects of clorgyline and (-)deprenyl.

DISCUSSION

The HPLC-ECD method with the three component solvent system - acetonitrile - tetrahydrofurane - water - at pH 3 provided a useful media for quick, efficient separation and sensitive detection of the catecholamines of brain tissue origin. The method enabled us to measure the unlabeled DA and DOPAC release from different isolated rat striata preparations (Figure 1). Using the internal standard dihydroxynorephedrine (DHNEP) made possible to obtain correct results in the measurement regardless on the changes in efficiency of the purification step (average recovery between 60-70%). The washing step with acetonitrile included substantially reduced the chromatographic front.

Under "in vitro" circumstances, (-)deprenyl had practically no effect on slice preparation and exerted only a decreasing effect on DOPAC release on the other preparation (Figs. 3-4). These findings do not elucidate the mechanism of action of this drug. After three weeks of chronic pretreatment, both the specific MAO-A and MAO-B inhibitor compound showed a strong effect on the DA and DOPAC release - specially on a more bulky Glowinski-Iversen preparation. These findings support substantially the view (Knoll, 1978) that the beneficial effect of (-)deprenyl in Parkinson's disease is due to the enhancement of DA release in the striatum.

REFERENCES

Anton, H.A. and Sayre, D.F.: A study of the factors affecting the aluminium oxide-trihydroxyindole procedure for the analysis of catecholamines. J. Pharm. exp. Ther. 138, 360-372 (1962).

Birkmayer, W., Riederer, P., Ambrozi, L. and Youdim M.B.H.: Implications of combined treatment with "Madopar" and l-deprenyl in Parkinson's disease. The Lancet 1, 439-443 (1977).

Glowinski, J. and Iversen, L.L.: Regional studies of catecholamines in the rat brain I. The disposition of ^{3}H-norepinephrine, ^{3}H-dopamine and ^{3}H-DOPA in various regions of the brain. J. Neurochem. 13, 655-669 (1966).

Hársing, L. G. Jr., Magyar, K., Tekes, K., Vizi, E.S. and Knoll,J.: Inhibition by deprenyl of dopamine uptake in rat striatum: A possible correlation between dopamine uptake and acetylcholine release inhibition. Pol. J. Pharmacol. Pharm. 31, 297-308 (1979).

Kissinger, P.T., Craig, S.B. and Shoup, R.E.: Neurochemical applications of liquid chromatography with electrochemical detection. Life Sciences 28, 455-465 (1981).

Knoll, J., Ecseri, Z., Kelemen, K., Nievel, J.G. and Knoll, B: Phenylisopropylmethylpropinylamine (E-250): a new spectrum psychic energizer. Arch. Int. Pharmacodyn. Ther., 155, 154-164 (1965).

Knoll, J.: Selective inhibition of B type monoamine oxidase in the brain: A drug strategy to improve the quality of life in senescence. Strategy in Drug Research ed.: J.A. Keverling Buisman,Elsevier Scientific Publishing company, Amsterdam pp. 107-135 (1982).

Knoll, J.: Analysis of the pharmacological effects of selective monoamine oxidase inhibitors. In: Monoamine oxidase and its inhibition. CIBA Foundation Symposium 39. Elsevier, Excerpta Medica, North Holland, Amsterdam 1976 (Eds.: G.E.S. Wolstenholme, J. Knigh) pp. 135-161, (1976).

Knoll, J. and Magyar, K.: Some puzzling pharmacological effects of monoamine oxidase inhibitors. Advances in biochemical Psychopharmacology Vol. 5. 393-408 (1972).

Knoll, J., Vizi, E.S. and Somogyi, G.: E-250, an MAO inhibitor antagonizing the effects of tyramine. Arzneim. Forsch., 18, 109-112 (1968).

Knoll, J.: The possible mechanism of action of (-)deprenyl in Parkinson's disease. J. Neural Transmis. 43, 177-198 (1978).

Chromatography, the State of the Art
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IN VITRO EFFECT OF L-DEPRENYL ON DOPAMINERGIC AND CHOLINERGIC NEURAL TRANSMISSIONS IN CAUDATE NUCLEUS OF THE RAT

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SUMMARY

The in vitro effect of l-deprenyl, a selective inhibitor of type B monoamine oxidase /MAO/ was measured on the resting and KCl /22 mmol/1/ induced release of dopamine /DA/, 3,4-dihidroxyphenylacetic acid /DOPAC/ and acetylcholine /ACh/ in incubated striatal slices of the rat. DA and DOPAC were determined in the incubation fluid by using high-performance liquid chromatography coupled with electrochemical detection /HPLC-ED/ and ACh was measured by bioessay on guinea-pig ileum. Inhibition by l-deprenyl of ^{3}H-DA uptake was also determined in striatal homogenates. l-Deprenyl /10^{-6}-10^{-4} mol/1/ enhanced the release of endogenous DA, whereas the efflux of DOPAC was changed in the opposite direction. ^{3}H-DA uptake was inhibited by identical concentration of l-deprenyl /10^{-4} mol/1/ that increased the release of DA. As l-deprenyl was added to the striatum in concentration of 10^{-6}-10^{-4} mol/l, the release of ACh was reduced. Correlation was found between stimulation of DA release and inhibition of ACh release elicited by l-deprenyl.

It is concluded that l-deprenyl stimulates the nigro-striatal dopaminerg neurotransmission in the rat and this effect might be associated with inhibition of type A MAO. Besides MAO inhibitory property of l-deprenyl, its DA uptake inhibitory effect can also take part in facilitation of DA release. l-deprenyl decreases the activity of striatal cholinergic interneurons indirectly by activation of dopaminergic neural transmission. The enhanced dopaminergic and reduced cholinergic neural activities elicited by l-deprenyl might have importance in its antiakinetic action.

INTRODUCTION

Anden et al. /1/ demonstrated for the first time that neural fibers arising from the substantia nigra pars compacta and projecting to the caudate nucleus operate with secretion of dopamine /DA/. Since then several lines of investigations indicate that the nigrostriatal dopaminergic impulse transmission

has an important role in controlling the activity of neural circuits within the basal ganglia. Although the termination of this pathway is not completely known, it is believed that a population of striatal interneutrons that utilizes acetylcholine /ACh/ possesses inhibitory dopaminergic inputs /2-7/.

The pathological importance of the nigrostriatal tract in malfunction of the basal ganglia became evident in the light of the observation of Ehringer and Hornykiewicz /8/. These authors demonstrated that the DA content in the striatum of Parkinsonian patients is markedly reduced. DA deficiency in the basal ganglia characteristic for Parkinson's disease led to the introduction of l-dopa in the clinical treatment of Parkinsonian syndrome /9/. In 1975 Birkmayer et al. /10/ described the potentiation of the antiakinetic effect of l-dopa treatment by l-deprenyl, a selective inhibitor of type B monoamine oxidase /MAO/ /11/ in patients suffering from Parkinson's disease.

Some previous observations suggest, however, that l-deprenyl might influence the nigrostriatal dopaminergic function not only with its MAO inhibitory property but with DA uptake inhibition as well. This became evident from our investigation which showed that l-deprenyl prevents the elevation of striatal ACh release elicited by intracerebroventricular injection of 6-hydroxydopamine /12/. In the present study striatal slices prepared from rat brain were used as a tool for investigation of l-deprenyl's action on dopaminergic and cholinergic neurotransmissions. We found that l-deprenyl shifts dopaminergic/cholinergic balance in the striatum by increasing the release of DA from the nerve endings of the nigrostriatal neurons which leads to reduction of the release of ACh from cholinergic interneurons.

METHODS

1. Measurement of dopamine and 3,4-dihydroxyphenylacetic acid /DOPAC/ release from striatal slices of the rat

Rats were decapitated and striatum was dissected as described by Glowinski and Iversen /13/. Three rats' striata were suspended in 1 ml of Krebs bicarbonate buffer containing Na_2EDTA /27 µmol/l/ and ascorbic acid /0.28 mmol/l/ at 37°C

and bubbled with 5% CO_2 in oxygen. After a 60 min preincubation period serial samples of 20 min were collected. The release of DA and DOPAC was determined in resting condition and in the presence of various release stimulatory agents such as KCl, ouabain or veratridine.

The collected incubation fluid was lyophilized then reconstituted in 200 µl of 0.2 mol/l perchloric acid-0.11 mmol/l ascorbic acid, centrifuged and 20 µl of the supernatant was injected onto the column.

A Biotronik high-performance liquid chromatography-electrochemical detection /HPLC-ED/ system /Biotronik Wissenschaftliche Geräte GmbH, Frankfurt am Main, GFR/ was used for the determination of DA and DOPAC in the incubation fluid. The analytical column was 150 x 4.6 mm, prepacked with Nucleosil C_{18} reversed-phase resin, particle size 5 µm and it was protected by a guard column, 30 x 4.6 mm. The injection valve /Rheodyne 7125, Berkeley, CA/ was equipped with a 20 µl sample loop. For electrochemical detection an ESA system /Environmental Sciences Associates Inc., Bedford, MA/ was used with a guard cell /ESA Model 502o/ with voltage setting of +0.6 V, a dual electrode analytical cell /ESA Model 5010/ with voltage setting of -0.5 and +0.3 V and a control modul /ESA Model 5100 A/. The signal of the oxidation electrode was recorded on a two-channel recorder.

The mobile phase was 0.1 mol/l sodium acetate/citric acid buffer, pH 4, containing 8% MeOH and 0.4 mmol/l octyl sodium sulfate and delivered with a flow rate of 0.7 ml/min. Concentrated stock solutions for DA and DOPAC were made and kept at -20°C. Dilutions of the standards were prepared from the stocks before each experiment.

2. Measurement of ^{3}H-dopamine uptake in striatal homogenates

Measurement of ^{3}H-DA uptake in striatal homogenates was made as described by Snyder and Coyle /14/. Rats were killed by decapitation, the brain was removed from the skull and the striatum was dissected according to Glowinski and Iversen /13/.

The tissue was homogenized in 8 volumes of 0.32 mol/l of saccharose and the homogenates were centrifuged at 1000 x g for 10 min. All procedures were carried out at $0^{\circ}C$. The incubation mixture /0.2 ml supernatant, 2.6 ml Krebs-Henseleit bicarbonate buffer containing ascorbic acid 0.2 mg/ml, Na_2EDTA 0.05 mg/ml, pargyline 1.25×10^{-4} mol/l and 0.1 ml water or l-deprenyl/ was preincubated for 5 min at $37^{\circ}C$ under 95% O_2 - 5% CO_2 then ^3H-DA 10^{-7} mol/l was added. Following a 5 min incubation the reaction was stopped by cooling to $0^{\circ}C$. The incubation mixture was centrifuged at 48.000 x g for 30 min at $4^{\circ}C$, the radioactivity in the supernatant and pellet was determined and the tissue/medium ratio was calculated. Protein was measured by the method of Lowry et al. /15/.

3. Measurement of acetylcholine release from striatal slices of the rat

Rat striatum slices were prepared as described above. The striatum was suspended in 2 ml of Krebs bicarbonate buffer containing 3 µmol/l eserine sulfate at $37^{\circ}C$ and bubbled with O_2 and CO_2 gas mixture /95% : 5%/. After a 60 min preincubation period serial samples of 10 min were collected and the amount of ACh released was determined in resting condition and in the presence of 22 nmol/l of KCl.

ACh concentration in the incubation fluid was determined by bioassay using guinea-pig ileum preparation /16/. In some cases spasmogenic substance in the perfusate was identified as ACh by HPLC /17/.

4. Calculation of data

The release of DA, DOPAC and ACh was calculated as follows:

$$\text{Release} = \frac{\text{concentration in sample pmol/ml} \times \text{incubation volume}}{\text{incubation time /min/} \times \text{tissue weight /g/}}$$

and was expressed as pmol g^{-1} min^{-1} for DA and DOPAC and as nmol g^{-1} min^{-1} for ACh.

For statistical comparison of the data Paired t Statistic and t Statistic for Two Means were used.

5. Drugs used in this study

The following drugs and chemicals were used: DA, DOPAC, uric acid, homovanillic acid /HVA/, 5-hydroxyindole-3-acetic acid /5-HIAA/, veratridine, Sigma; l-norepinephrine /NE/, Koch-Light Lab.; d,l-normethanephrine HCl /NMN/, 3-methoxy--4-hydroxyphenethylene-glycol /MOPEG/, d,l-3,4-dihydroxymandelic acid /DOMA/, d,l-3,4-dihydroxyphenylglycol /DOPEG/, Aldrich; octyl sodium sulfate, Merck; l-deprenyl, Chinoin; acetylcholine iodine, BDH; eserine sulfate, ouabain, Calbiochem; pargyline HCl, Abbot; tetradotoxin, Sankyo; 7,8-^{3}H--dopamine, Amersham, spec act. 1.43 TBq/g; Na_2EDTA, ascorbic acid, Reanal.

RESULTS

1. HPLC elution of dopamine and DOPAC

Fig. 1 shows the detector response to external standards containing different amounts of DA and DOPAC. The detector response was found to be linear in the range of 0.5-30 ng of authentic DA and DOPAC used for estimation the concentrations of these substances in biological samples. The best-fit line of linear relationship between the peak heights and ngs of DA and DOPAC injected onto the column was determined by linear regression /Fig. 2/. DA and DOPAC present in striatal perfusate were identified by retention times of standards and quantified by the peak heights. In our HPLC condition the retention times for DOPAC and DA were found to be 5.3 and 8.1 min, respectively. The chromatographic properties of some compounds expressed as capacity factor are listed in Table I. None of the compounds tested showed interference with either DA or DOPAC.

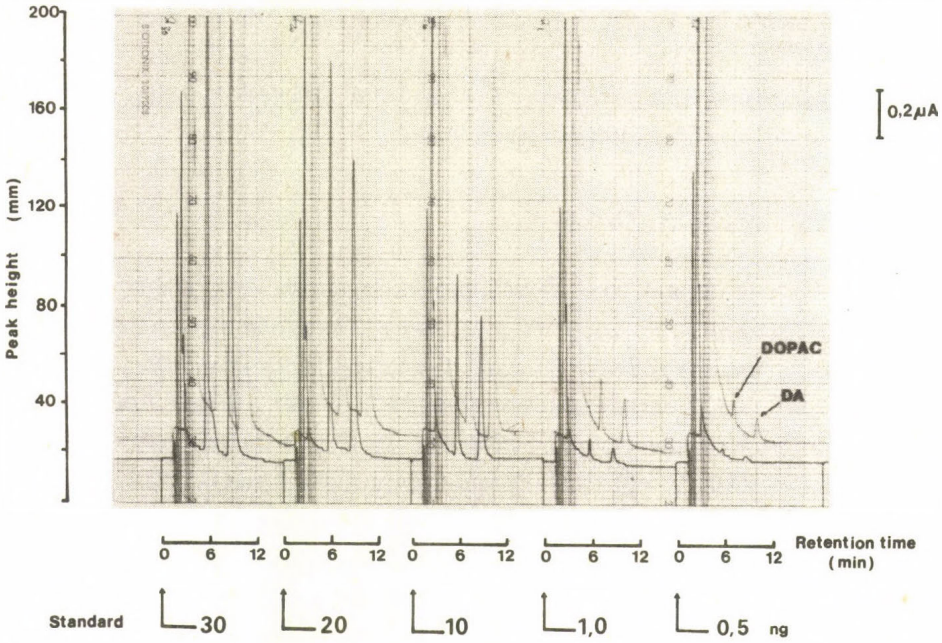

Fig. 1 HPLC chromatogram for external standards containing DA and DOPAC /0.5-30 ng/. Concentrated stock solution of each compound was diluted in 1 N HCl containing 0.11 mmol/l ascorbic acid and was kept at -20°C. From the stock solutions standards were diluted in 0.2 mol/l perchloric acid that also contained ascorbic acid. Injection volume: 20 µl.

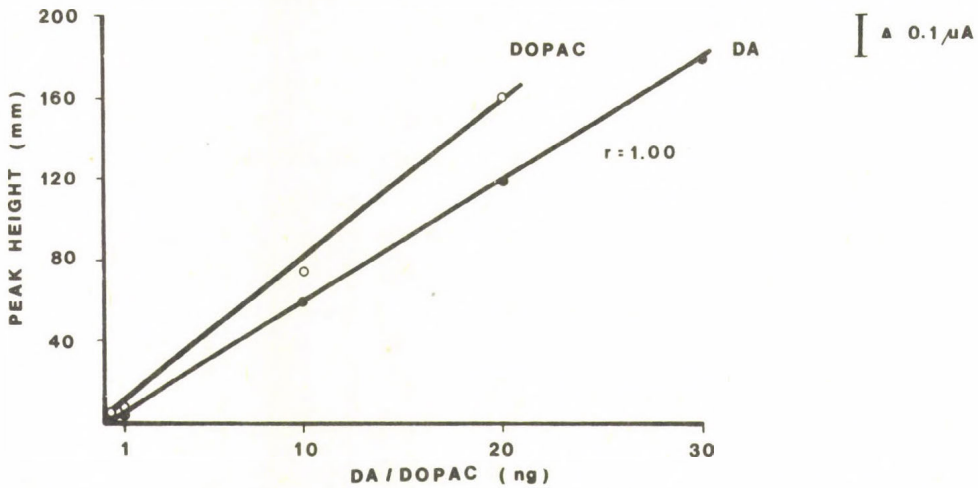

Fig. 2 Standard curves for DA and DOPAC. The best-fit line of linear relationship between the peak heights and ngs of DA or DOPAC injected onto the column was determined by linear regression.

2. The release of dopamine and DOPAC from striatal slices of the rat

Peaks with retention times corresponding to authentic DA and DOPAC were found in HPLC chromatogram of striatal perfusate /Fig. 3/. The rate of DA and DOPAC efflux measured from striatal tissue gradually decreased by extension of incubation time and

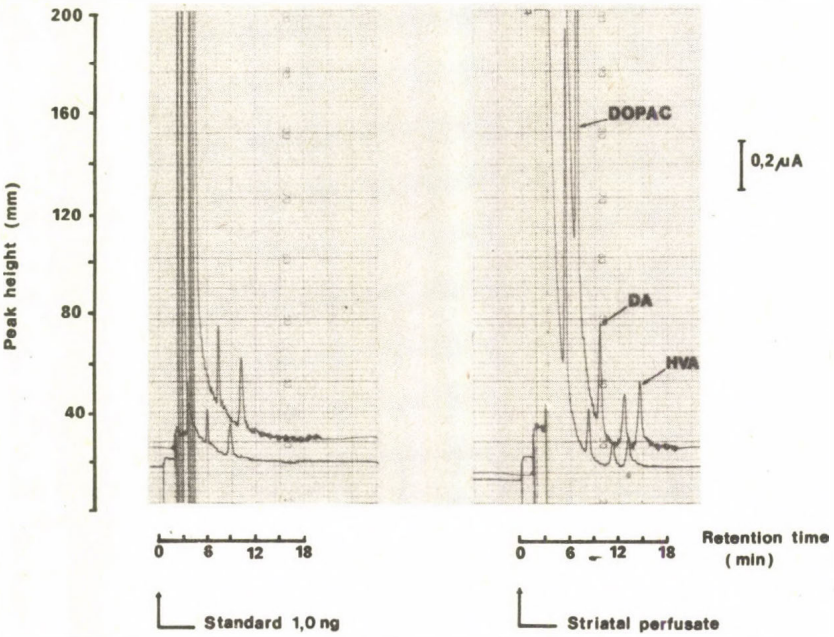

Fig. 3 HPLC elution of endogenous DA and DOPAC released from incubated striatal slices of the rat /left panel/. An unknown compound was eluted following DA peak with a retention time of 10.8 min. The last peak was identified as HVA.

Right panel: HPLC elution of external standard that contained 1 ng of DA and DOPAC.

reached an equilibrium by the end of 60 min preincubation. The release of DA measured in resting condition was 39.5 $\pm$ 5.6 pmol g^{-1} min^{-1} and that was increased to 85.7 $\pm$ 8.5 pmol g^{-1} min^{-1} by addition of 22 mmol/l of KCl, n = 18, $p < 0.05$. These values represent 0.33 and 0.70% of total DA content released min^{-1} in rest and in response to high potassium medium. The KCl induced release of DA was Ca^{2+} dependent /Table II/. Ouabain and ve-

Table I Chromatographic properties of some compounds tested expressed as capacity factor

Compound	Capacity factor
Uric acid	0.39
Norepinephrine	0.73
MOPEG	1.17
DOPAC	1.54
DOPEG	1.89
NMN	2.18
DA	2.88
DOMA	3.57
5-HIAA	4.07
HVA	5.03

Capacity factor = (peak retention time/void volume time) /26/
Void volume time: 2.09 min.
10 ng of each compound was injected onto the column.
For abbreviation of compounds see Method 5.
For HPLC-ED condition see Method 1.

ratridine also stimulated the DA efflux from striatum preparation and the later proved to be tetradotoxin sensitive /Table II/.

KCl which induced a two fold increase of DA efflux from incubated striatal slices of the rat, failed to affect the rate of DOPAC release. The resting DOPAC outflow was 131.8 $\pm$ 19.6 pmol g^{-1} min^{-1} and it was 117.4 $\pm$ 25.3 pmol g^{-1} min^{-1} in the presence of 22 mmol/l of KCl, n = 17, $p > 0.30$. As it is shown in Fig. 4 lack of correlation occurredbetween striatal DA and DOPAC release evoked by either KCl or ouabain.

TABLE II Modification of dopamine release by different chemical agents in striatal slices of the rat

Condition of slices	Concentration	Release of dopamine resting	Release of dopamine stimulated /pmol g^{-1} min^{-1}/
KCl	22 nmol/l	39.5±5.6	85.7±8.5/18/*
KCl Ca^{2+} omitted	22 nmol/l	46.1±9.0	51.4±8.4/4/
Ouabain	$2x10^{-5}$ mol/l	45.2±11.9	143.3±14.7/10/*
Veratridine	$5x10^{-5}$ mol/l	43.9±12.3	250.4±10.8/3/*
Veratridine + tetradotoxin	$5x10^{-5}$ mol/l 10^{-6} mol/l	30.0±3.3	26.1±2.9/3/

Striatal slices were incubated for 120 min, samples of 20 min were collected. Resting release of DA was determined during the 80th to 100th min of experiment then slices were exposed to KCl, ouabain or veratridine during the 100th to 120th min of incubation.
Mean± S.E.M., number of experiments in brackets, *$p < 0.05$, Paired t Statistic.

3. Effect of l-deprenyl on dopamine and DOPAC release from striatal slices of the rat

For determination of the effect of l-deprenyl on the release of DA and DOPAC, striatal slices were incubated for 120 min in the presence of various concentrations /10^{-6} - - 10^{-4} mol/l/ of this drug. The effect of l-deprenyl on the net DA release i.e., 22 mmol/l KCl induced release- resting

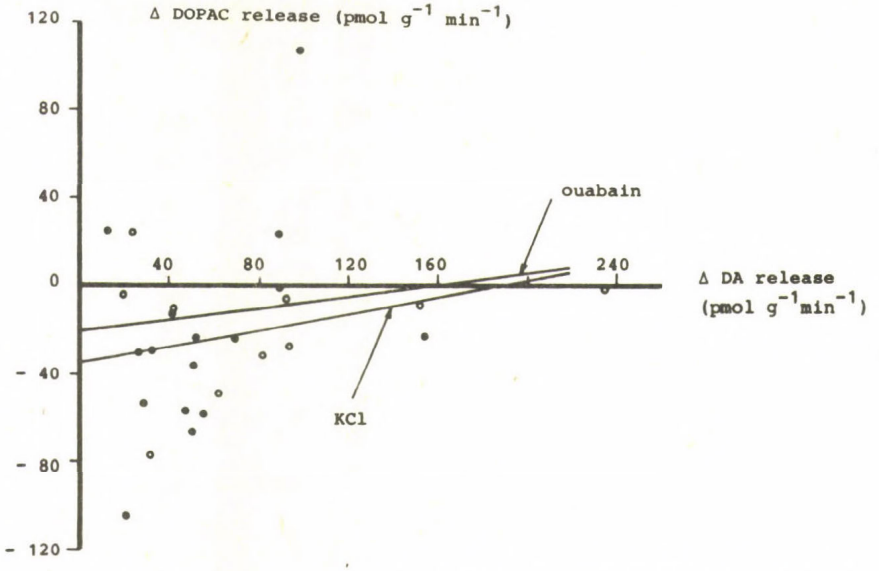

Fig. 4 Lack of correlation between DA and DOPAC release evoked by KCl /22 mmol/l, full circle/ or ouabain 2×10^{-5} mol/l, open circle measured from incubated striatal slices of the rat. Δ release: stimulated release- resting release. For KCl stimulation r = = 0.1732, $p > 0.10$, n = 15. For ouabain stimulation: r = 0.1870, $p > 0.10$, n = 10.

release is shown in Fig. 5. A. A significant enhancement of DA release was observed in the presence of 10^{-4} mol/l of l-deprenyl. By contrast, the release of DOPAC measured in either resting condition or in the presence of 22 mmol/l of KCl was reduced by l-deprenyl in a concentration dependent manner /Fig. 5 B/.

4. Effect of l-deprenyl on ^{3}H-dopamine uptake in striatal homogenates

Table III shows the effect of l-deprenyl on ^{3}H-DA uptake measured in striatal tissue homogenates. l-Deprenyl induced a concentration dependent inhibition of ^{3}H-DA uptake and the estimated IC_{50} value was found to be 10^{-4} mol/l.

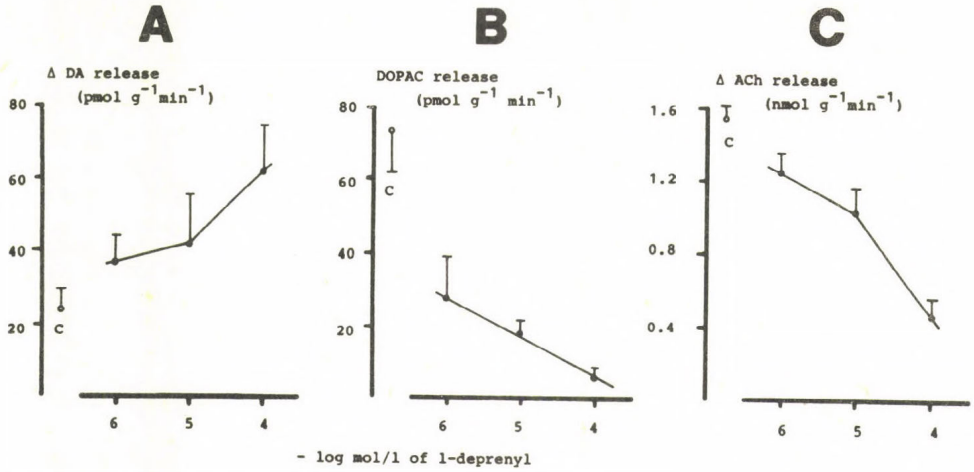

Fig. 5.A Effect of l-deprenyl on the release of endogenous DA /22 mmol/l KCl induced release- resting release/ from striatal slices of the rat. C indicates control release, 24.6 ± 5.5/6/ pmol g^{-1} min^{-1}.

B Effect of l-deprenyl on the release of DOPAC measured from striatal slices of the rat in resting condition. C indicates control release, 74.5 ± 2.9/6/ pmol g^{-1} min^{-1}.

C Effect of l-deprenyl on the release of ACh /22 mmol/l KCl induced release- resting release/ from striatal slices of the rat. C indicates control release, 1.54 ± 0.09/4/ nmol g^{-1} min^{-1}.

For these experiments striatal slices were incubated in the presence of various concentrations of l-deprenyl for 120 min. Mean ± S.E.M., number of experiments 3-6.

5. Effect of l-deprenyl on acetylcholine release from striatal slices of the rat

Due to the well-characterized transsynaptic modulation of striatal cholinergic interneurons by nigrostriatal dopaminergic pathway, we also examined the effect of l-deprenyl on the release of ACh. In control condition the resting release of ACh was found to be 0.13 ± 0.02 nmol g^{-1} min^{-1} and it was stimulated by 22 mmol/l of KCl to 1.68 ± 0.12 nmol g^{-1} min^{-1}, n = 4, $p < 0.05$. The calculated net ACh release /i.e., KCl

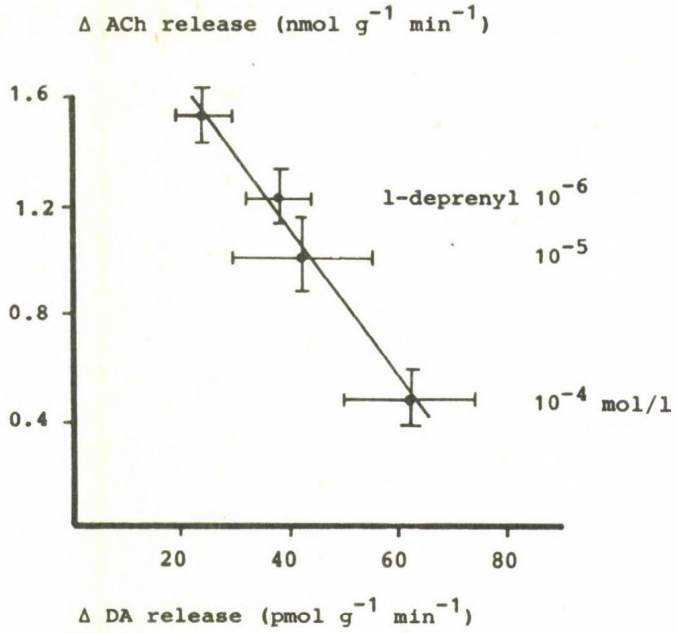

Fig. 6 Correlation between DA and ACh release measured from striatal slices of the rat. Δ release was calculated as KCl/22 mmol/l/ induced release-resting release. The slices were incubated in the presence of l-deprenyl /10^{-6} - 10^{-4} mol/l/ for 120 min. r = 0.9950, p<0.001, n = 3-6. Mean ± S.E.M.

Table III Effect of l-deprenyl on ^{3}H-dopamine uptake in striatum homogenates

Concentration of l-deprenyl mol/l	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Inhibition of ^{3}H-DA uptake /%/	17.8± 0.7	21.2± 4.4	37.9± 1.3	55.5± 1.2	73.1± 3.2

^{3}H-DA uptake in control condition was 6.34 ± 0.01 pmol mg protein^{-1} min^{-1}. Mean ± S.E.M., n = 5.

stimulated release- resting release/ was 1.54 $\pm$ 0.09 nmol g^{-1} min^{-1}. Fig. 5 C shows that the net ACh release was inhibited by l-deprenyl in a concentration dependent manner. As the net ACh release was plotted against net DA release measured in the presence of different concentration of l-deprenyl, a very strong correlation was found between the releases of the two neutrotransmitters /Fig. 6/.

DISCUSSION

HPLC combined with electrochemical cell provided selective detection for DA and DOPAC in striatal perfusate. Electrochemical detector with dual electrode operating in redox mode led to obtain clear HPLC chromatograms excluding interferences of unknown compounds with either DOPAC or DA.

The resting release of endogenous DA measured from incubated striatal slices of the rat was similar to the values reported by others /18, 19/. Application of various chemical release stimulants such as high potassium, ouabain or veratridine, all produced elevation of striatal DA release. The effect of KCl was Ca^{2+} dependent, that of veratridine was tetradotoxin sensitive. These facts indicate that the release of endogenous DA that we measured, follows the general rules of transmitter release processes.

In contrast to DA, the release of DOPAC proved to be fairly insensitive to either KCl induced depolarization or ouabain evoked inhibition of Na^{+}-K^{+}-activated adenosinetriphosphatase. This finding is contradictory with those of Kapoor /18/ and Arbuthnott /19/ who reported increase of DOPAC efflux from striatal tissue elicited by either KCl or electrical stimulation. The fact that DA release could be stimulated by chemical agents whereas the DOPAC efflux not, indicates different mechanisms in the release processes of DA and DOPAC. DA that reaches cytoplasmic sites by leakage out from the vesicules, is rapidly metabolized to DOPAC by MAO and the formed DOPAC is not retained by neural structures.

L-Deprenyl enhanced the release of DA from striatal slice preparation. This effect of l-deprenyl seems to be associated with inhibition of MAO since as the release of DA was increased the rate of DOPAC efflux was reduced. The concentrations of l--deprenyl required for development of these effects, however, were too high for selective inhibition of type B MAO. Using m--iodo-benzylamine or phenylethylamine, both are MAO-B substrates, the IC_{50} value of l-deprenyl for 50% inhibition of their deamination was reported to be 10^{-8}-10^{-7} mol/l /11, 20/. It was suggested that DA is substrate for type A MAO in the rat striatum /12, 21, 22/; moreover, the nigrostriatal dopaminergic pathway contains MAO-A /22/. The IC_{50} value of l-deprenyl for inhibition of DA deamination was found to be $5x10^{-6}$ mol/l in mitochondrial homogenates of rat whole brain /22/. Our preliminary experiments /23/ also indicate that about $5x10^{-6}$ mol/l of l--deprenyl exerts 50% inhibition on DA deamination in incubated striatal slices, preparation we used for investigation the effect of l-deprenyl on DA release. Thus, it is highly probable that in our experimental condition the applied concentration of l-deprenyl inhibited both types A and B MAO in incubated striatum.

The increase of DA release elicited by l-deprenyl might be a consequence of non-selective MAO inhibition. Protection of DA from metabolic degradation by MAO inhibition leads to enhancement of DA content in presynaptic vesicles and cytoplasmic stores as well. Accumulation of DA within the presynaptic vesicules after MAO inhibition results in an increased efflux rate of DA in response to high potassium medium. Besides MAO inhibition, other mechanisms can be also taken into account for explanation of the effect of l-deprenyl. We found that l-deprenyl inhibits ^{3}H-DA uptake in rat striatal homogenates with an IC_{50} value of 10^{-4} mol/l. This value is corresponding to the observation of Lai et al. /24/ and Azzaro and Demarest /25/. Although l-deprenyl proved to be a weak inhibitor of DA uptake in the striatum, its IC_{50} value on DA uptake inhibition was identical with the concentration that enhanced DA release. Thus, one can speculate that inhibition of DA uptake by l-deprenyl might be responsible for DA releasing action of this compounds.

Concentrations of l-deprenyl that enhanced the release of DA inhibited the ACh release from incubated striatal slices. Previously we analysed the action of l-deprenyl on striatal cholinergic neural transmission in details /12/. The correlation observed between DA and ACh releases might indicate that l-deprenyl reduces the amount of ACh released by enhancement of DA efflux from the nerve endings of the nigrostriatal fibers. Therefore, we suggest that l-deprenyl exerts indirect inhibitory effect on striatal cholinergic neurotransmission by facilitating the dopaminergic tone.

We conclude from these data that l-deprenyl increases the release of DA from rat striatum by non-selective concentrations that already inhibit type A MAO. The DA releasing property of l-deprenyl might be a consequence of inhibition of DA deamination and/or DA uptake inhibition. Since the dopaminergic/cholinergic synaptic connection in the striatum is inhibitory in nature, l-deprenyl as it stimulates dopaminergic transmission, concomitantly inhibits striatal cholinergic neural activity. By this action l-deprenyl can modify the reduced dopaminergic and enhanced cholinergic dysbalance characteristic for Parkinson's disease.

REFERENCES

1. N.E. Anden, A. Carlsson, A. Dahlström, K. Fuxe, N.A. Hillarp and K. Larsson, Life Sci. 3, 523-530 /1964/
2. H. Stadler, K.G. Lloyd, M. Gaden-Ciria and G. Bartholini, Brain Res. 55, 426-480 /1973/
3. Y. Agid, P. Guyenet, L. Glowinski, J.C. Beaujouan and F. Jaroy, Brain Res. 86, 488-492 /1975/
4. P.G. Guyenet, Y. Agid, F. Jaroy, J.C. Beaujouan, J. Rossier and L. Glowinski, Brain Res. 84, 227-244 /1975/
5. E.S. Vizi, L.G. Hársing and L. Knoll, Neuroscience, 2, 953-961 /1977/
6. S. Console, H. Ladinsky, R. Samain, S. Bianchi and D. Ghezzi, Brain Res. 155, 45-54 /1978/

7. C. Eurard, L. Premont, C. Oberlander, L.R. Boissier and J. Bockaert, Arch. Pharmacol. 309, 241-245 /1979/
8. H. Ehringer and O. Hornykiewicz, Wien, Klin. Wschn. 38, 1236-1239 /1960/
9. W. Birkmayer and O. Hornykiewicz, Wien, Klin. Wschr. 73, 787-789 /1961/
10. W. Birkmayer, P. Riederer, M.B.H. Youdim and W. Linauer, J. Neural Transm. 36, 303-326 /1975/
11. J. Knoll and K. Magyar, in Monoamine Oxidase - New Vistas. Adv. Biochem. Psychopharmacol., eds. E. Costa and M.Sandler, Raven Press, New York, 1972; Vol 5, pp. 393-408.
12. L.G. Hársing, Jr., K. Magyar, K. Tekes, E.S. Vizi and J. Knoll, Pol. J. Pharmacol. Pharmac. 31, 297-307 /1979/
13. J. Glowinski and L.L. Iversen, J. Neurochem. 13, 655-669 /1966/
14. S.H. Snyder and J.T. Coyle, J.Pharmacol. Exp. Ther. 165, 78-86 /1968/
15. O.H. Lowry, H.T. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193, 265-275 /1951/
16. U.D.M. Paton and E.S. Vizi, Br. J. Pharmacol. 35, 10-28 /1969/.
17. L.G. Hársing, Jr. and E.S. Vizi, in preparation
18. V. Kapoor, J. Physiol. 322, 51P /1982/
19. V. Kapoor and G.W. Arbuthnott, Br. J. Pharmacol. 76, 235P /1982/
20. H.Y.-T. Yang and N.H. Neff, J. Pharmacol. Exp. Ther. 187, 365-371 /1973/
21. P.C. Waldmeier, A. Delini-Sthula and L. Maitre, Arch.Pharmacol. 292, 9-14 /1976/
22. K.T. Demarest, D.J. Smith and A.J. Azzaro, J. Pharmacol. Exp. Ther. 215, 461-468 /1980/.
23. L.G. Hársing Jr. and G. Zsilla, unpublished data.

Chromatography, the State of the Art
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MEASUREMENT OF PLASMA L-DOPA LEVEL BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED WITH ELECTROCHEMICAL DETECTION IN PARKINSONIAN PATIENTS TREATED WITH MADOPAR®

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INTRODUCTION

From the original discovery of Ehringer and Hornykiewicz /1/ the primary importance of impaired nigro-striatal dopaminergic neurotransmission has been focused in the development of Parkinson's disease. L-dopa treatment in this disease aims at restoration of dopaminergic neural function in condition when several dopaminergic neurons are damaged /2/. Combined administration of l-dopa with peripheral decarboxylase inhibitors /carbidopa, benserazide/ assures penetration of higher percentage of orally given l-dopa into the brain by reduction of its breakdown before crossing the blood-brain barrier /3/. While introduction of l-dopa treatment in the early stage led to the conclusion that correlation exists between l-dopa level in the blood and the motor performance of Parkinsonian patients, later on the generality of this correlation was queried by some authors /4/.

Our main goal was to determine whether l-dopa concentration in plasma changes parallel with clinical symptoms of Parkinson's disease in patients treated with Madopar® /ratio of L-dopa to benserazide = 4.1/, Roche. For measurement of plasma l-dopa level we used high-performance liquid chromatography combined with electrochemical detection /HPLC-ED/ and the condition of the determination is described in this study.

METHODS

Instrumentation

The Biotronik chromatographic system /Biotronik Wissenschaftliche Geräte GmbH, Frankfurt am Main , /FRG/ consisted of a Model BT 3020 pump, a Model 7125 injector valve /Rheodyne Berkeley, CA/ and a stainless steel column prepacked with Nucleosil C_{18} reversed-phase resin /5 µm average particle size, 4.6 x 150 mm, Bischoff Analysentechnik/. Additional pulse dampening was obtained by placing stainless steel tubing between the pump and the sample injector. The sample loop connecting to the injector system had a volume of 20 µl. The electrochemical detection system was a dual electrode analytical cell, ESA Model 5010 /Environemtal Sciences Associates, Inc., Bedford, MA/ coupled with a guard cell /ESA Model 5020/ with control module /ESA Model 5100 A/. The porous graphite working electrodes were set at potentials of +0.6 V /guard cell/ and -0.5 and 0.3 V /analytical cell/ versus paladium reference electrodes. The signals of the oxidation electrode were recorded on a two-channel recorder operating with different sensitivities.

The analytical mobile phase, 0.1 mol/l sodium acetate citric acid and 0.4 mmol/l sodium octyl sulfate buffered to pH 4, was delivered at a flow rate of 0.7 ml/min. The eluent was filtered and degassed before use.

Sample preparation

Blood from human was collected from cubital vein to glass tubes containing sodium citrate /3.8% w/v 0.2 ml to 1 ml of blood/ and centrifuged at 1000 r.p.m. for 10 min at 4°C. The plasma was removed and kept at -20°C until analysis. The extraction of l-dopa from plasma was carried out according to Hallman et al. /5/ and Watson /6/. 0.2 ml plasma was added to a 5 ml vial and 6.5 nmol of protecatechuic acid was added as internal standard. Approximately 20 mg alumina was added to the samples and the pH was adjusted to 8.6 by the addition of 1.8 ml of 0.5 mol tris/hydroxymethyl/ aminomethane hydrochloride /Tris-HCl/ buffer. The vials were shaken manually for 15 min,

the plasma was discarded and the alumina was washed with water. L-dopa was desorbed by addition of 100 µl of 0.2 mol/l perchloric acid containing 0.11 mmol/l ascorbic acid. The samples were centrifuged and 20 µl of the supernatant was injected onto the column.

Drugs and chemicals

L-dopa /Fluka/ and protecatechuic acid /Sigma/ were dissolved in 1 N HCl-0.11 mmol/l ascorbic acid and the stock solutions were stored at -20°C. Diluted standards were prepared and passed through alumina as it was described in the preparation of samples. Alumina was purchased from Woelm Eschwege, FRG. All the chemicals used were of analytical reagent grade.

RESULTS AND DISCUSSION

Figure 1/A shows a typical HPLC chromatogram of external standard containing 0.15 nmol of l-dopa and 6.5 nmol protecatechuic acid after alumina absorption. The retention times were 4.0 min for l-dopa and 10.8 min for protecatechuic acid while the solvent front appeared 1.5 min after the injection. A linear relationship between the peak height ratio of l-dopa/protecatechuic acid and the amount of l-dopa injected onto the column is demonstrated in Figure 2, where the best-fit line as determined by linear regression was plotted. A peak with retention time corresponding to authentic l-dopa was obtained in the plasma extract. A typical HPLC elution profile for plasma extract is shown in Figure 1/B. The identity of the l-dopa peak was confirmed by the addition of a small amount of l-dopa to the sample which resulted in an increase of peak height without affecting the shape of the peak. An unknown peak was also observed in the chromatogram of the plasma extract eluted after l-dopa but it appeared at a different retention time /5.1 min/ than l-dopa. Although we did not make any attempt for the identification of this unknown compound it did not prove to be identical with norepinephrine which appeared in the chromatogram at a retention time of 6.9 min.

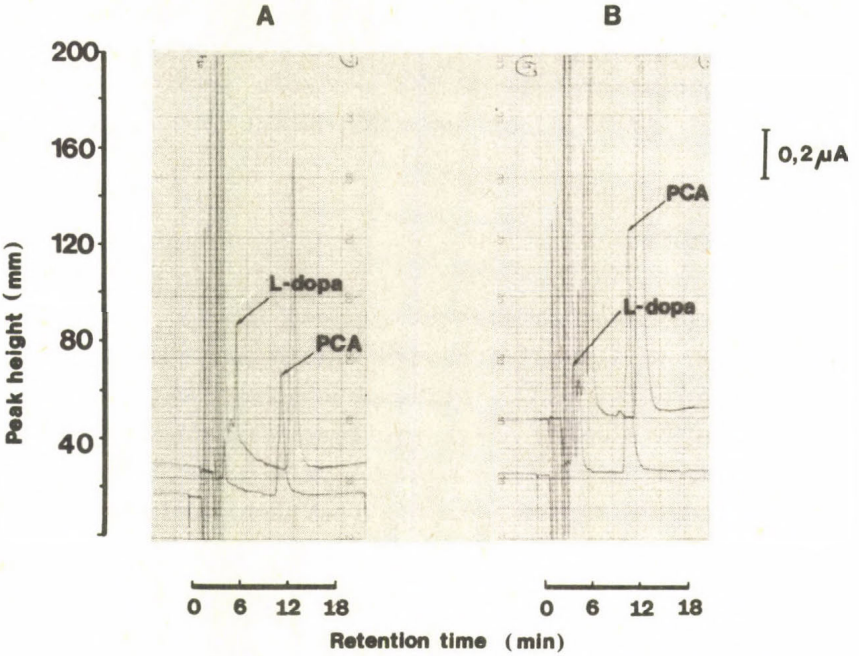

Figure 1/A Typical HPLC elution profile of an external standard containing l-dopa and protecatechuic acid /PCA/ as the internal standard. The standard was submitted to alumina adsorption as desribed in Methods.

Figure 1/B Typical HPLC chromatogram of a plasma sample after purification with alumina adsorption. Unknown peak can be seen after l-dopa elution. For sample preparation and HPLC conditions see Methods.

L-Dopa concentration in the plasma obtained from patients suffering from Parkinson's disease and treated with Madopar® varied in the range of 0.005-16.5 μg/ml. Figure 3 shows the changes in the l-dopa concentration in plasma following a 12 hours period. A clearcut correlation was observed between the plasma l-dopa level and the oral intake of Madopar® capsules /200 mg l-dopa + 50 mg benserazide/.

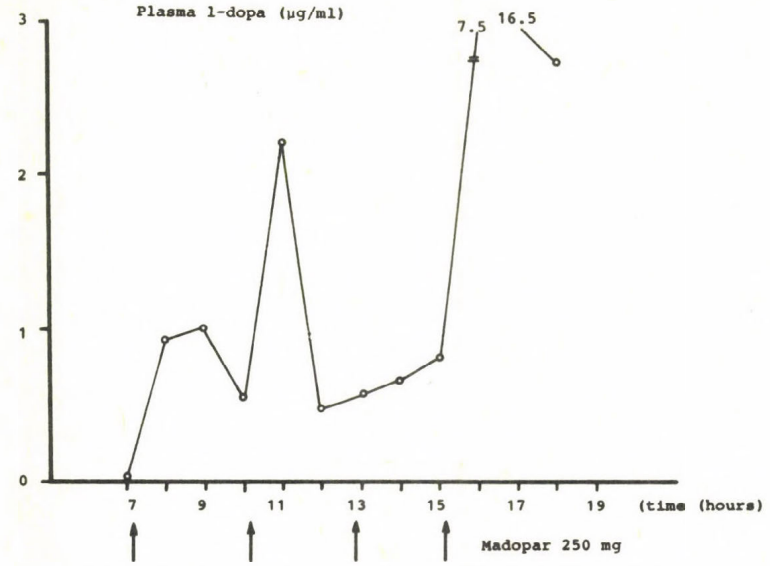

Figure 2 Standard curve for 1-dopa measured with HPLC-ED. The standards contained increasing amounts of 1-dopa and a constant amount of protecatechuic acid /PCA/ used as the internal standard. Each point represents the mean of four determinations.

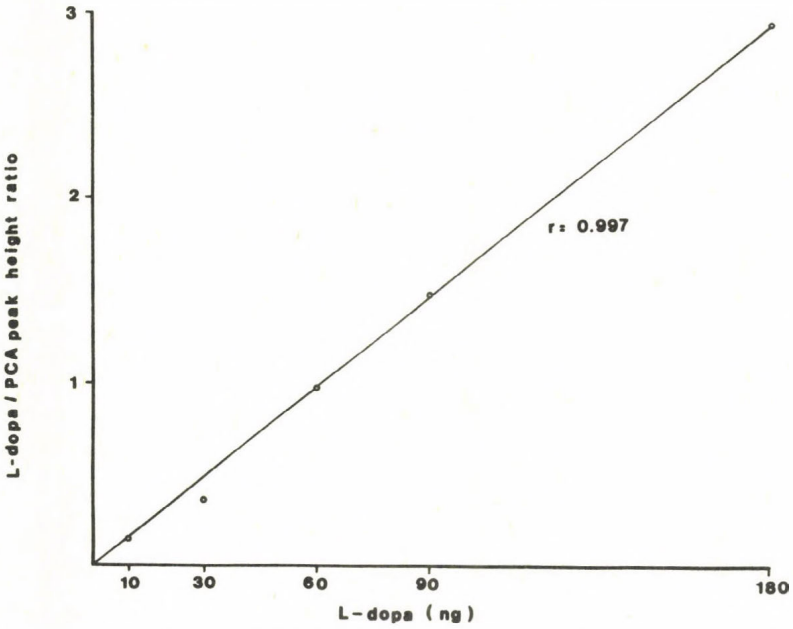

Figure 3 Change of plasma 1-dopa concentration /patient G.T., female, 46 years/ in response to Madopar® /250 mg/ treatment during a 12 hours observation period.

SUMMARY

We used HPLC in combination with electrochemical detection for the measurement of plasma l-dopa level in Parkinsonian patients treated with Madopar ®. Application of an analytical cell with dual electrode operating in redox mode resulted in a clear chromatogram for plasma l-dopa after purification by alumina adsorption. While an unidentified peak appeared in the HPLC elution profile of the plasma extract, it did not interfere with either l-dopa or protecatechuic acid used as internal standard in the determination. Plasma l-dopa concentration was found to be varied in the range of 0.005-16.5 μg/ml in patients treated with Madopar ®. We are currently testing in our laboratories whether any correlation exists between the l-dopa concentration of plasma and the clinical symptoms of Parkinson's disease in patients treated with Madopar ® capsule.

REFERENCES

1. H. Ehringer and O. Hornykievicz, Klin. Wsch. 38, 1236-1239 /1960/
2. W. Birkmayer and O. Hornykiewicz, Wien. Klin. Wschr. 73, 787-789 /1961/.
3. G. Bartholini, W.P. Burkhard, A. Pletscher, and V.M. Bates, Nature /London/, 215, 852-858 /1967/.
4. I. Shoulson, G.A. Glaubiger, and T.N. Chase, Neurology /Minneapolis/ 25, 1144-1148 /1925/.
5. H. Hallman, L.O. Farnebo, B. Hamberger and G. Jonsson, Life Sci. 23, 1049-1052 /1978/.
6. E. Watson, Life Sci. 28, 493-497 /1981/.

SEPARATION OF PEPTIDES AND PROTEINS

Chromatography, the State of the Art
H. Kalász and L.S. Ettre (Eds)

IDENTIFICATION OF POSITIONAL ANALOGUES OF SPDP-β-MSH DERIVATIVES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF DANSYL AMINO ACIDS

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SUMMARY

3-/2-Pyridyldithio/ propionyl derivatives of β-melanotropin /β-MSH/ were synthesized for the purpose of preparing biologically active thiolated β-MSH. The free amino groups of the peptide /Asp #1, Lys #6, Lys #17/ were substituted with N-succinimidyl 3-/2-pyridyldithio/ propionate /SPDP/. The mono-, bis- and tris-substituted peptide derivatives were separated by reversed-phase HPLC. The positions of SPDP-substitutions were delineated by subtractive dansylation: unsubstituted amino groups were dansylated; the dansylated peptide derivatives were hydrolyzed with 6N HCl at 110° C and the dansyl amino acids were quantitated by RPLC. Dansyl norleucine was included as internal standard. Our results show that positional analogues of SPDP-substituted peptides can be resolved by RP-HPLC.

INTRODUCTION

We have shown that a conjugate of β-MSH and daunomycin had receptor-mediated cell-specific cytotoxicity against murine melanoma cells /1/. Although the β-MSH-daunomycin conjugate had an increased selective cytotoxicity in vitro, the experiments with tumor-bearing animals were less successful because of unfavorable pharmacokinetics /2/ and unexpected toxic side effects /3/. We have realized that part of our difficulties could have originated from the choice of toxin we have used for con-

jugation. Daunomycin is a relatively stable anthracyclin antibiotic and therefore, it may destroy a large number of "bystander" cells following its liberation from the conjugate. It would be advantageous to use a more potent cytotoxic agents such as ricin-A /4/ which is susceptible to intracellular inactivation or drugs such as p-quinon derivatives which have enhanced toxicity against melanoma cells /5/. Trenimon /2,3,5-tris-ethylenimino-p-benzoquinone/, an anti-cancer alkylating agent /6/, could be used for this purpose. However, conjugation of β-MSH to either ricin-A or to trenimon would require a free thiol group in the peptide. The amino acid sequence of porcine β-MSH is: Asp-Glu-Gly-Pro-Try-Lys-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp. Thiol groups can be introduced by substituting the free amino groups /N-terminal Asp, Lys #6 or Lys #17/ with SPDP, and reducing the disulphide group with DTT /7/. When the octadecapeptide is substituted, the formation of a mixture is anticipated, consisting of three mono-, three bis- and one tris-SPDP-β-MSH conjugates. Since some of the amino-acid residues of β-MSH are needed for biological activity in an unaltered form while others can be modified /8,9/, the SPDP-β-MSH derivatives have to be characterized and separated from each other in order to avoid diluting the active molecular species by inactive derivatives. As we show in this report, separation of SPDP substituted β-MSH derivatives can be achieved in one step by using Reversed-Phase High Performance Liquid Chromatography /RP-HPLC/ and delineation of positional analogues of SPDP-β-MSH can be done by RP-HPLC analysis of dansyl amino acids.

EXPERIMENTAL

Materials

The dansyl amino acids were obtained from the British Drug House Ltd. /England/ and from Sigma /St. Louis, MO, USA/; SPDP was from Pharmacia Fine Chemicals /Piscataway, NJ, USA/. The following chemicals were supplied by J.T. Baker Chemical Co.

/Phillipsburg, NJ, USA/: NaH_2PO_4, Na_2HPO_4, $NaHCO_3$, Acetonitrile /HPLC grade/, HCl, Acetone. ß-MSH was a gift from Dr. Lande of our department.

Instrument

The Liquid Chromatograph consisted of a Model 420 Beckman solvent programmer /Berkeley, CA, USA/, two Model 110A Beckman pumps, a Beckman Model 210 sampling valve with a 100-µl sample loop, a Model 153 Beckman U.V. detector and a Perkin-Elmer /Norwalk, CT, USA/ Model 650 10 LC fluorescence spectrophotometer. Chromatograms were obtained with a Kipp & Zonen BD 41 /Delft, Holland/, dual channel recorder. The column effluent was monitored at 280 nm for the U.V. detector; fluorescence detection was carried out at 345 nm and 470 nm for excitation and emission respectively. We used Waters /Milford, MA, USA/ µBondapak C_{18}/3.9 mm I.D. x 300 mm/ column.

Procedures

1 mg HPLC-purified ß-MSH in 0.2 ml PBS was reacted with 0.31 mg SPDP for 5 minutes at room temperature then 0.1 ml of ethanolamine /0.1 M/ was added. The resulting mixture was kept at room temperature for 60 minutes and purified by RP-HPLC with an acetonitrile gradient. Seven fractions were collected, freeze-dried, and used for dansylation.

Dansylation: 1 mg of each fraction was dissolved in 100 µl of 0.2 M $NaHCO_3$. A 150 µl portion of dansyl chloride solution /2.5 mg/ml in acetone/ was added and the reaction was allowed to proceed for 1 hour at 37°C. The dansyl peptide derivatives were precipitated with 1 ml of acetone, centrifuged, washed four times with acetone and dried. The dried residues were hydrolyzed by 100 µl HCl /6N/ at 110°C for 16-17 hours. The hydrolyzate was dried by purging with helium. The dansyl- amino acids were dissolved in 100 µl of water/acetonitrile /1:1/ solution. Aliquots /50 µl/ were analyzed by RPLC. The determination of ε-dansyl-L-lysine and dansyl-L-aspartic acid in the dansyl peptide hydrolyzate was performed by RP-HPLC analysis,

using the internal standard plot method. We prepared a series of standard solutions for this purpose containing the same amount of the internal standard /dansyl-DL-norleucine/. Aliquots /80 µl/ of the standard solution were injected and the peak heights of the internal standard and the compounds of interest were calculated from the resulting chromatogram. The ratios of the peak heights were plotted versus the molarity of ε-dansyl-L-lysine and dansyl-L-aspartic acid, respectively. An aliquot of the dansyl peptide hydrolyzate containing internal standard, was injected into the HPLC system and the molarity of ε-dansyl-L-lysine and dansyl-L-apartic acid was determined on the basis of the ratio of peak heights by using the calibration curves.

RESULTS

Figure 1 shows the separation of SPDP-β-MSH derivatives by reversed-phase high-performance liquid chromatography. The method was /RP-HPLC/ a modification of our earlier technique /10/. Open peaks represent fractions obtained without adding β-MSH to the reaction mixture. The line-shaded peak shows the position of unsubstituted β-MSH. The peaks of SPDP-β-MSH derivatives are shaded black. Figure 2A shows the separation of dansyl amino acids obtained by hydrolyzing dansylated β-MSH. A similar analysis, carried out on Fraction D, is shown in Figure 2B. The detection was carried out by using simultaneous U.V. and fluorescence detection. The separation was achieved by linear gradient elution from 0 to 100% acetonitrile in 20 mM phosphate buffer. Peak #1 corresponds to dansyl-L-aspartic acid, peak #2 to ε-dansyl-L-lysine and peak #3 to dansyl-DL--norleucine. In chromatogram A the intensity of peak 2 is higher than that of peak #1 as was expected. In chromatogram B dansyl-L-aspartic acid is absent. In this figure, we can see that the ratio of dansyl-L-aspartic /ε-dansyl-L-lysine is similar in both U.V. and fluorescence detections, but the sensitivity is higher when fluorescence detection is used. Figures 3 and 4 show the calibration curves obtained with U.V.

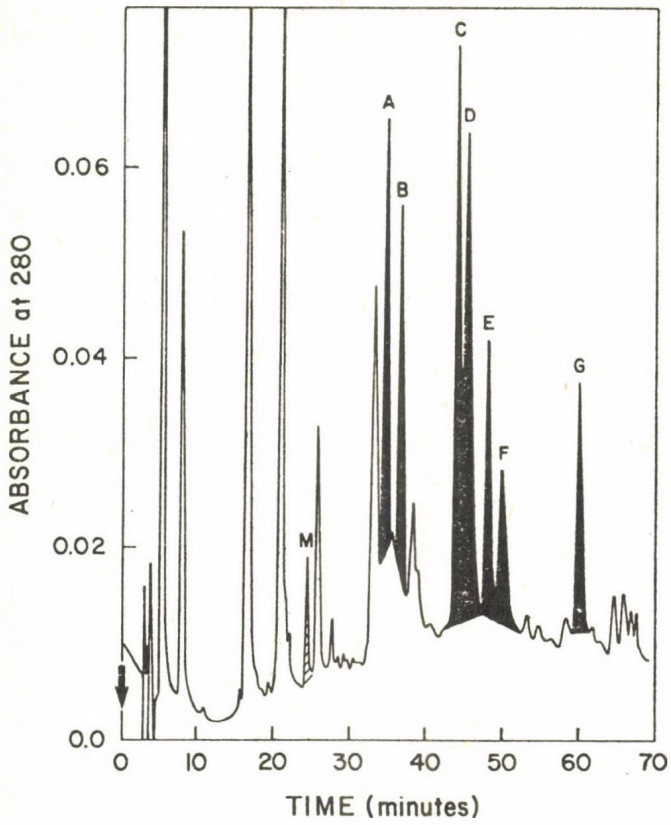

Figure 1 RP-HPLC of SPDP-βMSH derivatives. Gradient elution: Solvent A, Aqueous solution of 0.05% TFA; Solvent B, 0.05% TFA in Acetonitrile. The gradient conditions were from 10% B to 20% B in A in 5 min, from 20% to 30% B in A in 15 min, from 30% to 40% B in A in 35 min, from 40% to 60% B in A in 5 min. Column: 10 µm µ Bondapak C_{18}, 300 x 3.9 mm; flow rate; 1 ml/min: Open peaks show fractions obtained without adding β-MSH to the reaction mixture. The shaded peak shows the position of unsubstituted β-MSH. Black peaks show the positions of SPDP-β-MSH derivatives

and fluorescence detection respectively for dansyl-L-aspartic acid and ε-dansyl-L-lysine. In both detections, the analysis shows that the yield is higher for ε-dansyl-L-lysine than for dansyl-L-aspartic acid. The curves are linear within the limits of concentrations explored. The results of three separate experiments are summarized in Table I.

Table I Delineation of the Position of Substitution by SPDP

Fraction	Recovery of dansyl amino acids (n mole)*		Position of Substitution by SPDP
	L-Asp	L-Lys (ε-amino)	
A	2.6	11.0	N-terminal Asp
B	5.3	17.8	Lys #6 or #17
C	3.2	12.5	Lys #6 or #17
D	1.5	5.1	N-terminal Asp + Lys #6 or #17
E	1.3	21.3	?
F	1.4	4.4	N-terminal Asp + Lys #6 or #17
G	1.3	1.7	Tris-substituted

*average of three measurements

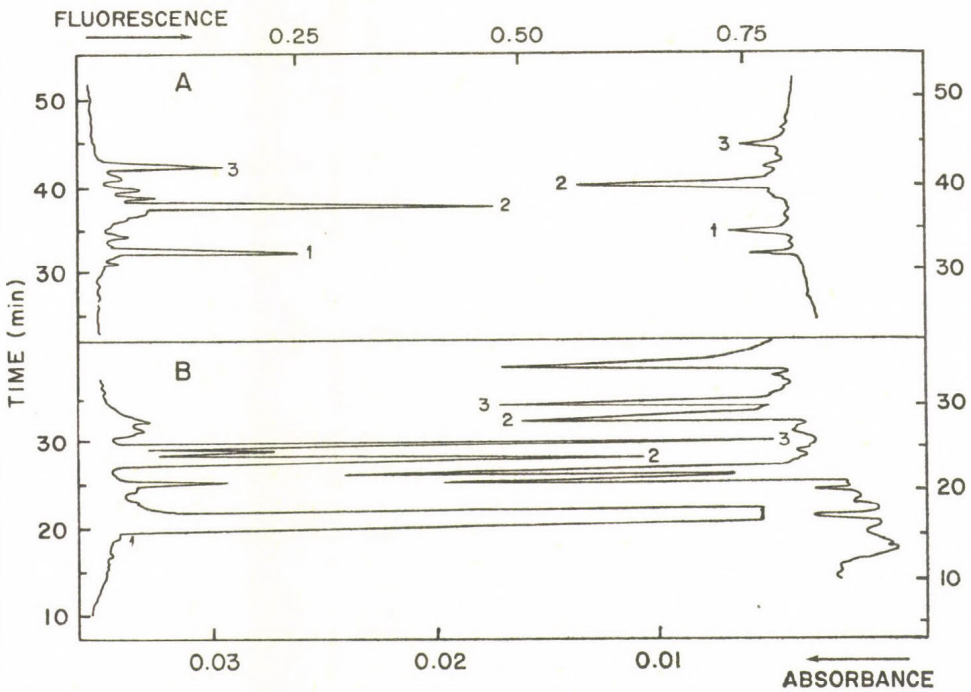

Figure 2. Separation of dansyl amino acids obtained hydrolysis of dansylated β-MSH (chromatogram A) and dansylated Fraction D (chromatogram B); Column: Waters μ Bondapak C_{18}, 300 x 3.9 mm; flow rate: 1 ml/min: Volume: 80 μl; mobile phase: linear gradient from 0% to 100% acetonitrile in 20 mM phosphate buffer pH 3.2 in A and pH 6 in B. U.V. detection: 280 nm; Fluorescence: 470 nm emission; and 345 nm exitation.

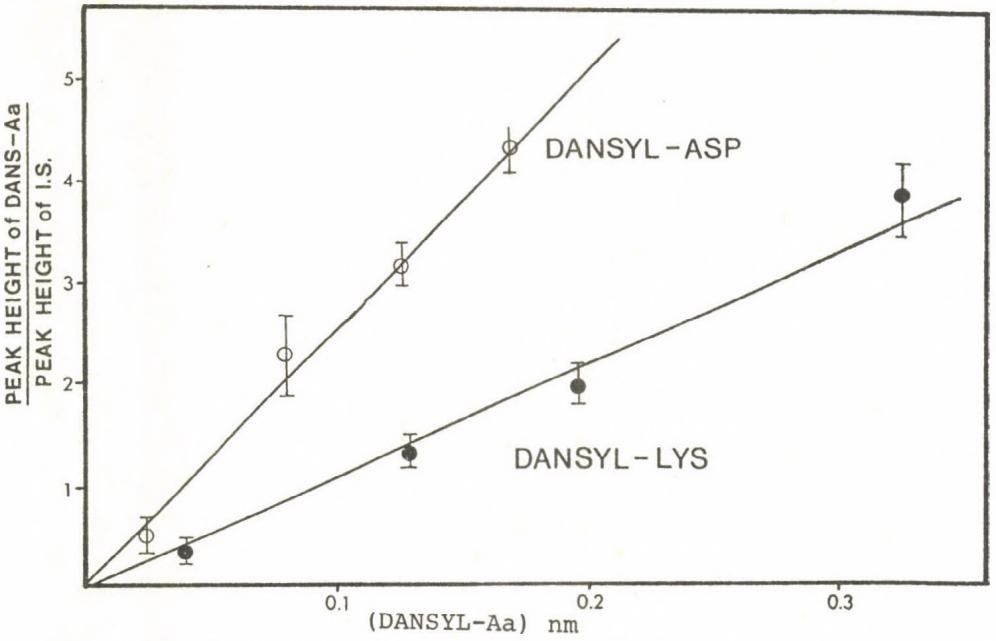

Figure 3. Standard curve for dansylated amino acids under the conditions mentioned in the text; U.V. detection was used

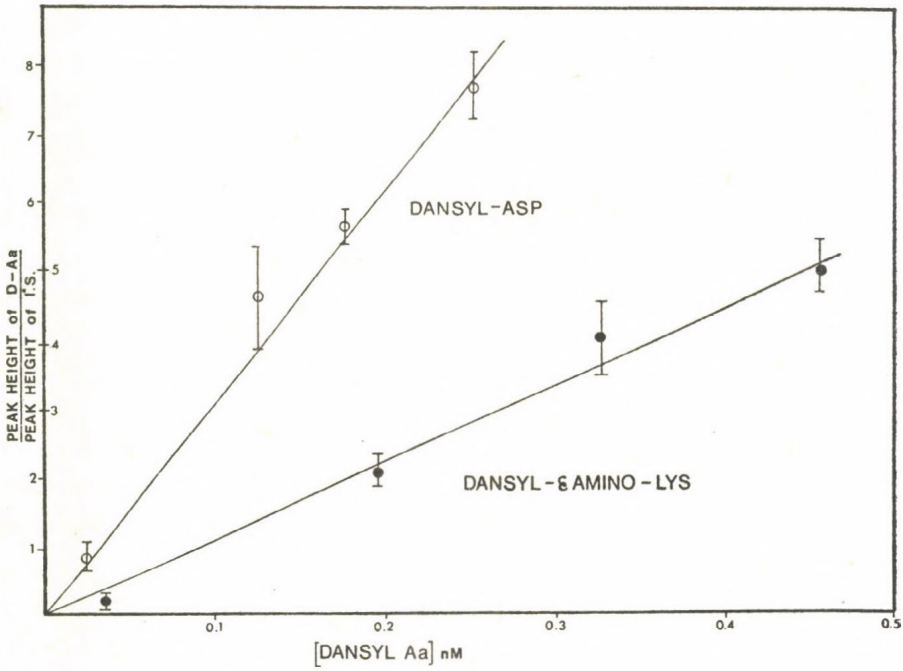

Figure 4. Standard curve for dansylated amino acids under the condition mentioned in the text; fluorescence detection was used

DISCUSSION

We have published a method for the preparation and partial separation of SPDP-β-MSH derivatives in a previous volume of this series /10/. We have now optimized the method of β-MSH derivatization to favor the production of mono-substituted conjugates. In addition, we have increased the resolution of our RP-HPLC technique and delineated the positions of SPDP substitutions by subtractive dansylation and quantitation of dansyl amino acids by RP-HPLC.

RP-HPLC has been used by others for the separation of dansyl amino acids /11/ and for the quantitation of N-terminal amino acids /12/. However, this is the first report on the application of RP-HPLC to delineate positional analogues of peptide derivatives. Currently, RP-HPLC is the fastest and most sensitive method for the quantitation of dansyl amino acids /11, 12/. The major limitation of the method is the low degree of reproducibility as shown by the large standard deviations when data from several experiments are averaged /Figures 3, 4/. This problem, however, derives not from the method of separation /RP-HPLC/, but from the chemistry of dansylation and hydrolysis of dansylated peptides /11, 12/. It is well known that when subtractive dansylation is used for N-group analysis, an unknown portion of dansyl amino acids is destroyed during hydrolysis /13/. We have attempted to circumvent this last problem by using dansyl-DL-norleucine as an internal standard, assuming that during hydrolysis, this compound decomposes with the same rate as the dansyl amino acids /derived from Asp and Lys/ we want to quantitate. We chose norleucine because the peptide does not contain this amino acid and also, because the dansyl derivative is well separated from the substances of interest. The amounts of dansyl amino acids recovered from peaks A to G of Figure 1 and the tentative positions of SPDP substitutions are listed in Table I. As we can see, we were able to identify most of the anticipated mono-, bis- and tris-substituted peptides. The position of substitution in peak E is uncertain. In bis-substituted peptides, analysis of dansyl amino acids, obtained after proteolytic digestion and/or

cleavage with CN-Br would be required to delineate which of the two lysine residues /#6 or #17/ are substituted. However, since the mono-substituted peptides /peaks A, B, C/ have the highest biological activity /unpublished observations/, further structural analysis on the multi-substituted β-MSH derivatives is beyond the scope of our objectives.

ACKNOWLEDGEMENTS

We thank Dr. Saul Lande for the generous supply of β-MSH. This investigation was supported by PHS Grant No. CA 26081 awarded by the National Cancer Institute, DHHS and by the National Foundation for Cancer Research.

REFERENCES

1. Varga, J.M., Asato, N., Lande, S. and Lerner, A.B., Nature 267: 56 /1977/.
2. Varga, J.M., Asato, N., Wiesehahn, G. and Hearst, J.E., IN "Pigment Cell 1981" /M.Seiji, Ed./ Univ. Tokyo Press, Tokyo, 1981, pp 399-405.
3. Varga, J.M. and Asato, N., IN "Targeted Drugs" /E.Goldberg, Ed./ John Wiley and Sons, New York, 1982, pp 73-88.
4. Olsens, S. and Phil, A., Biochemistry 12: 3121 /1973/.
5. Chavin, W., Jelonek, E.J., Jr., Reed, A.H. and Binder, L.R., Science 208: 408 /1980/.
6. Linford, J.H., Chem. Biol. Interactions 6:149 /1973/.
7. Carlsson, J., Dievin, H. and Axen, R., Biochem. J. 173: 723 /1983/.
8. Schwyzer, R. and Eberle, A., Front. Horm. Res. 4: 18 /1977 /1977/.
9. Lambert, D.T., Stachelek, C., Varga, J.M. and Lerner, A.B., J. Biol. Chem. 257: 8211 /1982/.

10. Varga, J.M., Airoldi, L. and Davila-Huerta, G.

11. Wilkinson, J.M., J. Chrom. Sci. 16: 547 /1978/.

12. Kaneda, N., Sato, M. and Yagi, K., Anal. Biochem. 127: 49 /1982/.

13. Varga, J.M. and Richards, F.F., Anal. Biochem. 53; 397 /1973/.

Chromatography, the State of the Art
H. Kalász and L.S. Ettre (Eds)

PRINCIPLES IN ISOLATION OF PEPTIDE FACTORS FROM URINES WITH RETAINED BIOLOGICAL ACTIVITY, EXAMPLE FROM SCHIZOPHRENIA

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INTRODUCTION

Urinary peptide factors that are metabolically and behaviourally active have been found in both normal and diseased states. A sleep-inducing muramyl-peptide has been found in urine /Krueger et al. 1982/. Also in neoplastic states have peptides been found such as a factor that stimulates emigration of endothelial cells /Chodar et al. 1981/ and various growth inhibitory peptides /Burzynski et al. 1976/. Releasing factors may also be found in urines such as growth hormone release inhibiting factor /Kronheim et al. 1977/ LHRH like activity /Root et al. 1977/ and TRH like activity. Considerable evidence has also been forwarded that the acid-soluble peptides excreted in women and rats are mainly determined by the endogeneous formation of peptides rather than dietary peptide inputs /Noguchi et al. 1982/. We have found in several psychiatric disorders that the level of benzoic acid precipitable peptide - protein complexed in the urine is considerably increased /Trygstad et al. 1980 ; Reichelt et al. 1981; Saelid et al. 1983/. Some of these peptides have extensive behavioural effects /Hole et al. 1979; Reichelt et al. 1981/. A peptide that blocks the cephalic phase of digestion /Coy et al. 1981; Schally et al. 1982/ has been isolated /Reichelt et al. 1969/ although not inducing anorexia as originally thought.

Many of the available techniques are based on relatively pure starting material or often also known synthetic peptides. This circumvents the real life problems of working from a bio-

logical starting point where the contamination material is far more dominant than the interesting peptide. Working up unknown samples also requires a bioassay and we have tried to follow a parallel in vitro and in vivo assay to concentrate on substances of interest. A relatively specific peptide assay is also used. When working with biologically obtained material it is also vital that each chromatographic peak always be checked for amino acid composition by acid hydrolysis and by amino-acid analyses. This is especially true whenever changes in the state of the biological staring material are present. To obtain large enough samples of the active material, conventional columns are used followed by HPLC for the final preparation.

Overall scheme of purification in principle

The aim is to separate the raw material into as many subgroups as possible from a chemical viewpoint, guided by bioassays in vitro and in vivo in parallel.

Step A: Precipitation of glycoprotein-peptide benzoic acid and glycoprotein-peptide-purine derivative complexes by precipitation with benzoic acid /Chalmers et al. 1958; Reichelt et al. 1981/. Hereby low MW compounds such as bilirubin and not complexed peptides and amino acids are removed.

Step B: Gel filtration to separate according to size or according to affinity to gels. In the Sephadex G25 step pH 8.5 proteins are separated from retarded peptide-amino acid, benzoic acid complexes and peptide glycoprotein-purine complexes.

On BioGel P2 gels glycoprotein is separated from peptides and peptides from benzoic acid or purines. P2 gel has a cut off at about 2000 daltons.

Step C: Anion exhange: To separate anionic compounds from neutral and basic compounds, cysteic acid and taurine is poorly retained on cation exchangers and it is of advantage to have these removed from the N-substituted peptides.

Step D: Cation exhanger: At pH 2 all the amino-free peptides and amino acids are retained. The N-substituted compounds pass through without retention. Alpha-amino-free compounds are eluted with 2M pyridine.

Step E: Sephadex LH 20 to separate in organic solutions olephinic from hydrophilic compounds.

Hydrophilic → straight-phase silica

Step F: HPLC

Hydrophobic → reversed-phase ODS

NB! At low pH many peptides have a somewhat more hydrophobic nature and reversed-phase may also be used.

METHODS:

a/ Selection of patients: This was performed by established psychiatrists and in close accord with the Research Diagnostic criteria of Feighner /Feighner et al. 1972/. Thus the hard-core schizophrenics who show clearcut symptoms have been studied by us, and all the patients were without medication for at least 3 months /Reichelt et al. 1981/.

b/ Preparation of urinary material: Precipitation of urines with benzoic acid was first performed by Chalmers /Chalmers et al. 1958/. To work with larger quantities of urine it is better to remove the bilirubin derivatives present as they interfere in the purification. 24 hour complete urines were taken as the basis to avoid changes in the circadian rhythms. In diseased states change in diurnal rhythm may also make hormone assessment of specific times hazardous. Urines from different hospitals and different countries have been studied to avoid dietery bias and regional differences in treatment, etc.

The urines were acidified to pH 4.3 with HCl and 10% by volume of ethanol saturated with benzoic acid was added with

stirring. The pH was again checked and adjusted to between 4 and 4.3 with NaOH. The suspension was allowed to precipitate overnight in the cold room at $4^{o}C$.

After careful decanting the precipitate was centrifuged at 3000 g, 20 min at $4^{o}C$ and resuspended with stirring in ethanol. Centrifugation and resuspension in WTH stirring in ethanol were performed until the final OD 280 nm was close to 0.300 OD units with a 1 cm lightpath in the Zeiss PMQ II. This step in important as glycoprotein - peptide complexes are extremely soluble and therefore the benzoic acid cannot be completely removed. Also the retention of these complexes on Sephadex G25 will not occur if the protein-complexed benzoic acid is removed.

Columns used, with comments

1. Sephadex G25 superfine 2.6 cm x 150 cm in 0.1M NH_4HCO_3 buffer. The final precipitate from the above washings was dissolved in NH_4HCO_3 buffer at pH 8.5 and centrifuged at 100 g 20 min at $4^{o}C$. Each 24 hour urine was dissolved in 40 ml of buffer and the G25 column was in the cold room. Rate of flow was 30 ml/h and fractions of 15 ml were collected. Continuous UV monitoring of the eluent at 280 nm was used.

Comment: The glycoproteins seem to have their surface partly covered with benzoid acid or uric acid derivatives. These substituents retard these compounds on the Sephadex G25 gel. Relatively typical patterns are found in different disorders /Trygstad et al. 1980; Reichelt et al. 1981/. If every trace of UV 280 nm material is washed out in the washing procedure no peak equivalent to the benzoic acid elution place is obtained. The complexes that are filtrated are made up of peptide glycoprotein and aromatic compounds /Reichelt et al. 1981/.

2. The individual peaks from the Sephadex G25 run were lyophilised on rotavapor and dissolved in acetic acid to a final concentration close to 0.5M. The Bio Gel

P2 gels were of dimensions 1.6 x 90 cm and run in 0.5M acetic acid at 3 ml/10 min. Application volume was 5-10 ml and fractions of 3 ml were collected. Aliquots of about 10 vol% were analyzed by alkaline hydrolysis and ninhydrin colouring /Reichelt and Kvamme, 1967/.

Fractogel MG 2000 in 0.2M/l formic acid of dimensions 1.6 x 70 cm. Application volume was 6 ml of freeze-dried samples from the P2 run dissolved in 0.2M formic acid. Flow rate was 3 ml/10 min and fractions of 3 ml were collected.

Comment: This column has high affinity for acid groups such as aspartic acid, serin-phosphate etc. and gives good peptide resolution.

Dowex 1: anion exchange in the formate form. Dimensions 1 x 20 cm, discontinuous elution 23 ml H_2O, 32 ml 2M formic acid.

Comment: The neutral and basic amino acids are not retained.

Dowex 50: 1 x 20 cm in a buffer of pH 2 was eluted with 4 ml fractions of 0.35M NaCl with 0.25M formic acid pH 2 buffer to a total of 34 ml. This was followed by 40 ml 2M pyridin.

Comment: The N-substituted peptides were not retained at this low pH. The high salt concentration assures that free amino acids and polyamines that tend to ride "piggy back" on the peptides of interest are retained.

Sephadex G10 1.6 x 90 cm: Application volume 10 ml in 0.15 ml acetic acid. Rate of elution was 3 ml/10 min and 3 ml fractions were collected.

Comment: Desalting step as salts are problematic in most in vitro and intracranial bioassays.

Fractogel MG 2000 in 1M/l acetic acid and 40 mM/l HCl. Dimensions of the column 1.6 x 90 cm. Application volume was

6 ml and the rate of elution was 3 ml/10 min; 3 ml fractions were collected.

Comment: The higher ionic strength ensures more separation especially of aggregated peptides /piggy back phenomena/. A high resolution column.

HPLC: straight phase: Hypersil of 10 x 25 cm dimensional was used as reversed-phase column and gave very little retention of our compounds. Buffer was 70 parts of 60% n propanol/ water and 30 parts of 100 mM trifluoracetic acid pH 2. Elution rate was 1 ml/min; the eluent was monitored at 205 nm using an LDC UV detector Application volume was 1 ml.

HPLC: reversed phase: prp-10

Hamilton C-18 ODS. Resistant to pH down to 1 was run in a n propanol 50 nm trifluoroacetic buffer, pH 1.5. Column dimension was 5 x 200 mm. The eluent was monitored at 205 nm and by offline hydrolysis and ninhydrin development.

Peptide detection: On high purification OD 205 nm could be used on-line. Usually, however, ninhydrin after alkaline hydrolysis was used /Reichelt and Kvamme, 1973/. Aliquots of 5-10% of each fraction were dried overnight and hydrolyzed in 0.2 ml 2M KOH for 2 hours at 100°C in a boiling water bath. This was neutralized and the ninhydrin colour developed in an acetate/ cyanide buffer.

Comment: This procedure has the advantage of

a/ splitting of the N terminal blocking groups and opening pyroglu rings,

b/ it multiplies the ninhydrin colourable groups with the number of peptide bonds split, thus increasing the sensitivity,

c/ by comparing hydrolyzed samples with unhydrolyzed samples, a measure of the peptide-bound amino acids is obtained. With our method the specific absorption is almost the same for the different amino acids making calculations easier.

Criteria of purity:

a/ a constant integral number of the amino acids after hydrolysis measured on the amino acid analyzer /Chromaspec/ in spite of further purification;

b/ same composition of a peak from the column at its start, its middle and its end;

c/ only 1 C terminal or 1 N terminal /after opening the N terminal pyroglu in HCl/methanol/ using dansylation /Gray, 1967/ or the Edman procedure /Gray, 1967b/.

Comment: Up to the introduction of high salt cation separation at pH 2 of the free amino acids riding piggy back on the peptides were a problem. These were largely removed by the Dowex 50 and the Fractogel 1M acetic acid 40 mM HCl step. However, salt removal has not been easy, although necessary before the in vivo bioassays. Desalting was best performed by running 1.6 x 90 cm Sephadex G-15 columns.

Protein identification:

The glycoproteins present in the individual peaks were studied by crossed immunoelectrophoresis and the rocket technique. The proteins identified by the antibodies do not account for all the unknowns.

BIOASSAYS

a/ Dopamine uptake inhibition by a factor /Hole et al. 1979/ was shown in synaptosomes from hypothalamus and striatum but not hippocampus. The factor was active in a concentration of $<10^{-6}$M estimated as free amino acids. Dopamine was tested in concentrations.

b/ In vivo dopaminergic hyperfunction: /Hole et al. 1979/ was shown for the same factor in unilaterally, nigro-striatally lesioned animals, using stereotactically placed 6OH-dopamin, induced turning as if amphetamine had been given and opposite

to that found for apomorphine. This indicates that in vivo dopaminergic hyperactivity may be induced /Hole et al. 1979/ by a factor containing 2.5 nanomoles of free amino acid as peptide injected intracranioventricularly. The effect of the factor was blocked by haloperidol /Hole et al. 1979/.

c/ GABA release: Hamberger and collaborators precharged slices of hippocampus with labelled GABA, and studied the release to the perfusion fluid from the slice in a small perfusion cell. One peptide gave a powerful increase in GABA release over the background /Hamberger et al. in preparation/. To check the intactness of the synaptosomes, a high K^+ pulse was given, after the effect of the applied factor had worn off. As another transient increased release was obtained, this effect seems to be physiological. So far the factor has been found active down to 10^{-8}M amino acids, with increasing specific activity through 7 purification steps. The release of GABA could be separated from that of glutamate pointing to some specificity, and induced longlasting immobility in test animals /Jørgensen et al., to be published/.

d/ Muscarinic receptor binding factor was found by Ledaal and Øye at the Department of Pharmacology. They used QNB binding to synaptosome membrane preparation in a Hepes/TRIS buffer. The factor replaced QNB in high dilution 10^{-10}M /measured as amino acids/ and was also active on the guinea pig ileum preparation /Ledaal et al., to be published/.

e/ Catatonia-like state inducing factor was found to inhibit the uptake of GABA by Hole in synaptosome preparation. This factor is probably a substituted tripeptide.

f/ Analgesia: This factor seems to be different from the enkephalin or endorphin series as the amino acids relased by hydrolysis are different. The intracranioventricularly applied peptide-containing factor /2.5 nanomoles of amino acid/ was tested by the tailflick test under an infrared heat source. The analgesia induced was reversible by haloperidol and only partly reversible by naloxone /Hole et al. 1979/.

RESULTS

Fig. 1 shows the pattern obtained on Sephadex G-25 gels for normals giving the range of 27 persons of ages 15-60. In the same figure the two main types of schizophrenia patterns obtained are plotted with three individual cases randomly picked of each kind. Type 1 has a big late peak and type 2 little or no late peak. As we have standardized the washing procedure of the benzoic acid precipitate, the area under the UV 280 nm trace can be taken as representing the total amount of complex in each peak. The relationships clearly show an increase in the diseased state compared to normals with $p<0.01$ using Wilcoxon nonparametric statistics /Table I/.

Table I. Quantitative aspects of schizophrenia>
Area under 280nm peak area in $cm^2 \pm$ SD

Category	N	Peak 600-900 ml	Peak 1100-1800 ml
Type 1	8	17.3 ± 3.6	30.3 ± 9.3
Type 2	12	23.4 ± 6.6	8.7 ± 6.8
Normal	17	7.9 ± 3.7	3.5 ± 2.8
Low mw ninhydrin groups after hydrolysis /nanomoles/24 h/			
Type 1	8	44719 ± 11479	51072 ± 2350
Type 2	12	33813 ± 13929	10500 ± 3300
Normal	17	7222 ± 1306	6700 ± 1260

Normal population age range 15-60 years

Lyophilizing on rotavapor the peak from 600-900 ml and dissolving the material in 0.5M acetic acid and applying as described gave Bio Gel P2 gel filtration patterns as shown in Fig. 2. The hydrolyzed ninhydrin-colourable material is shown measured at 570 nm. Expressed in nanomoles per 24 hour diuresis it is similarly clear from Table I that there is a statisti-

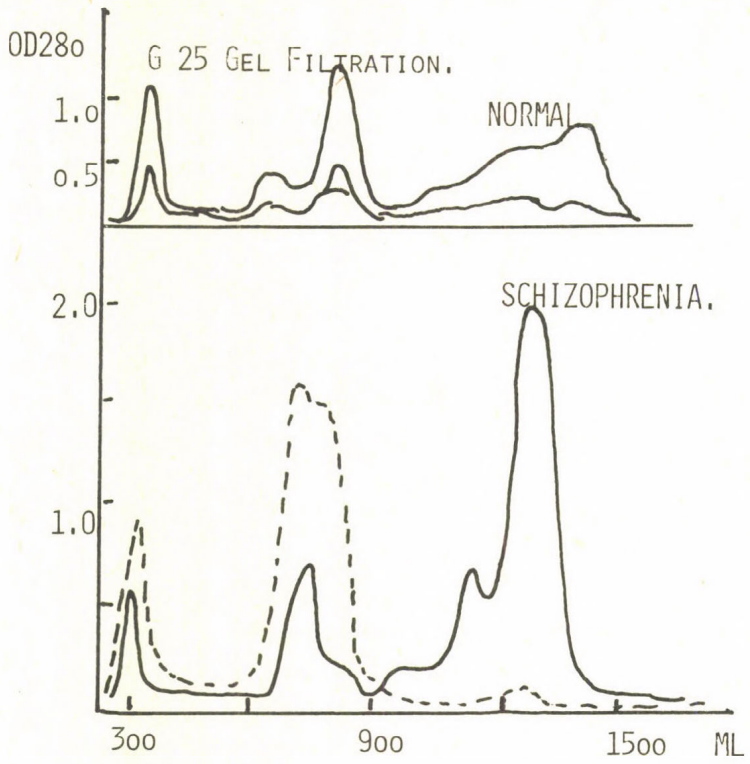

Fig. 1. The Sephadex G-25 gel filtration pattern for 17 normals is shown in the upper part of the figure with max. and min. values obtained. The two types of schizophrenic patterns obtained are shown in the lower part. The peak from 600-900 ml contains glycoproteins, peptides and benzoic acid, the late peak glycoproteins, peptides and purine or uric acid derivatives /Reichelt et al., 1981/. Type 1 has large late peak, type 2 little or no late peak

cally significant /$p<0.01$/ increase in hydrolyzable material colourable with the ninhydrin reagent in schizophrenic urines. This agrees with similar finding in serum by Drysdall et al. /1982/. Type 1 correlates with slow onset schizophrenia and type 2 more with acute onset and more paranoid schizophrenia but the division is not absolute and we do not yet understand the dichotomy found.

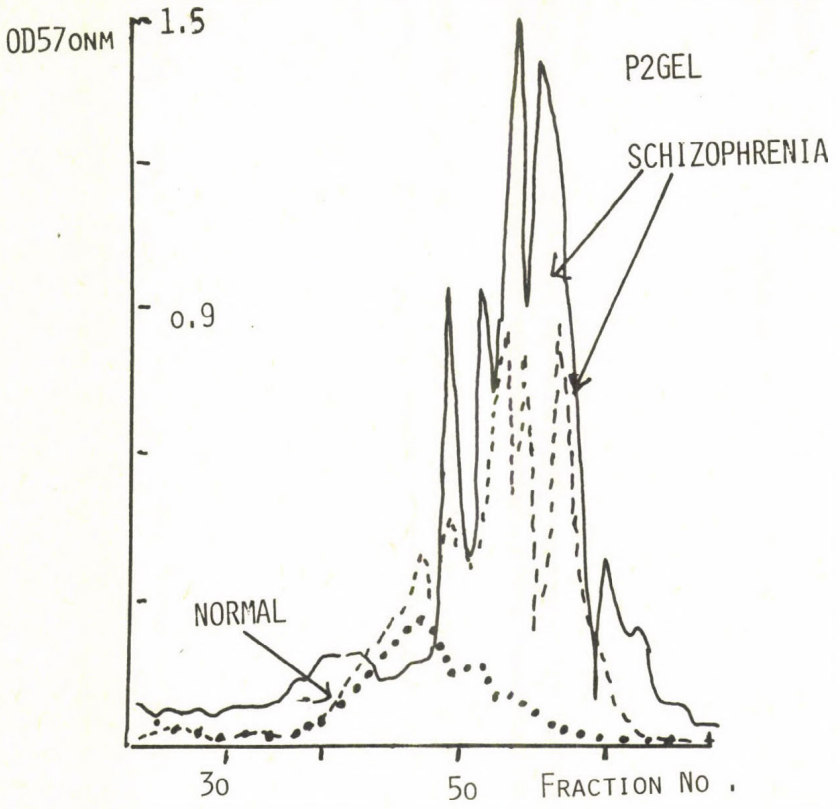

Fig. 2. The computer drawn ninhydrin coloured pattern obtained on gel filtration of the 600-900 ml peak from G25 columns /Fig. 1/. The ninhydrin colour after alkaline hydrolysis is shown. P2 gels of dimension 1.6 x 90 cm. The material from the G25 columns were lyophilized and extracted with 0.5M acetic acid. Without hydrolysis the ninhydrin colour was less than 10% of that of Fig. 2. This step separates peptides and proteins from benzoic acid

Fig. 3 shows the overall purification scheme and the more important K_{av} values for the dopamine uptake inhibitor /circled/ and the GABA releasing factor /dotted circle/. For an overview of all the activities found see Table II Fig. 4 shows some of the hydrolyzed patterns obtained from different columns, with the two activites singled out for study in this paper.

These factors are peptidic because they yield ninhydrin-colourable material only on hydrolysis or increase their nin-

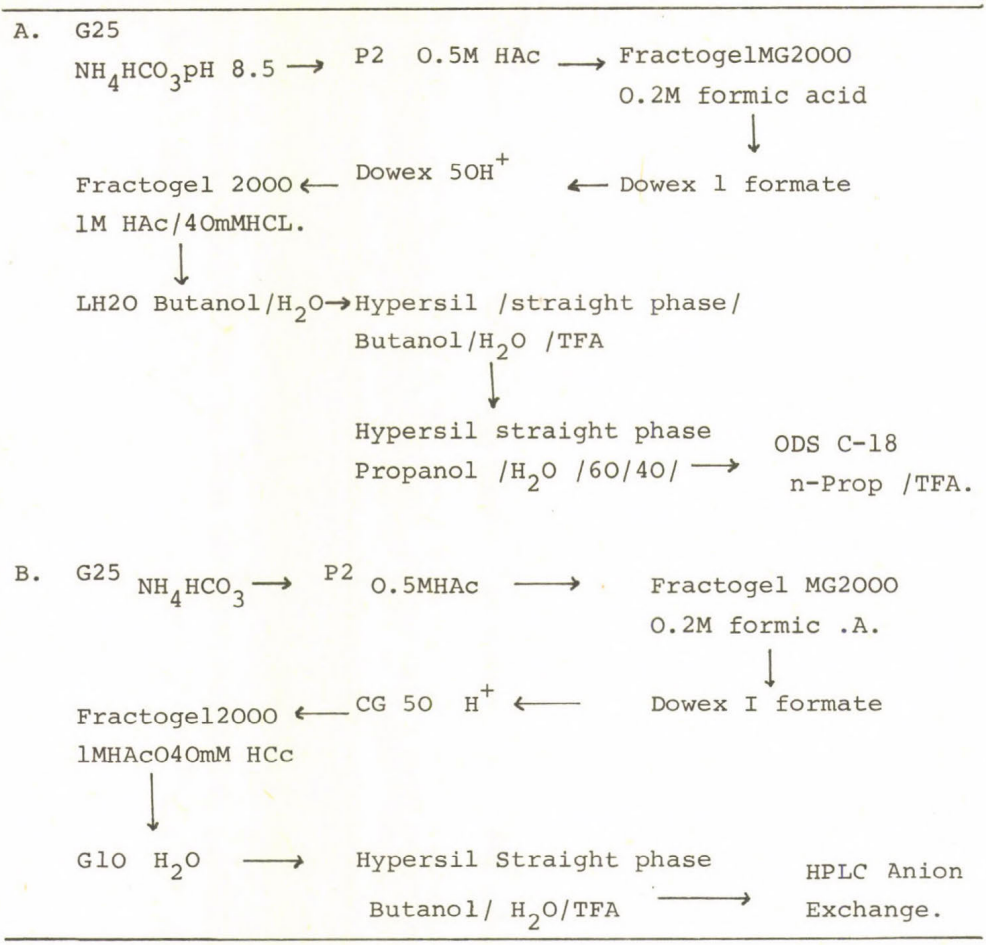

Fig. 3. The purification scheme of the dopamine uptake blocking factor is shown at the top and that of the GABA releasing factor at the bottom. For details see text

hydrin colourable groups on hydrolysis. After hydrolysis increase in amino acids is found, measured on the Chromaspeck amino acid analyzer, and on high purification the factors have a clearcut absorption at 205 nm. Many of the activities tested have N-terminally substituted groups such as pyroglu as they are not colourable by ninhydrin. This explains their passage through a Dowex 50 column in NaCl/formate buffer, pH 2. Hydrolyzed fractions were inactive.

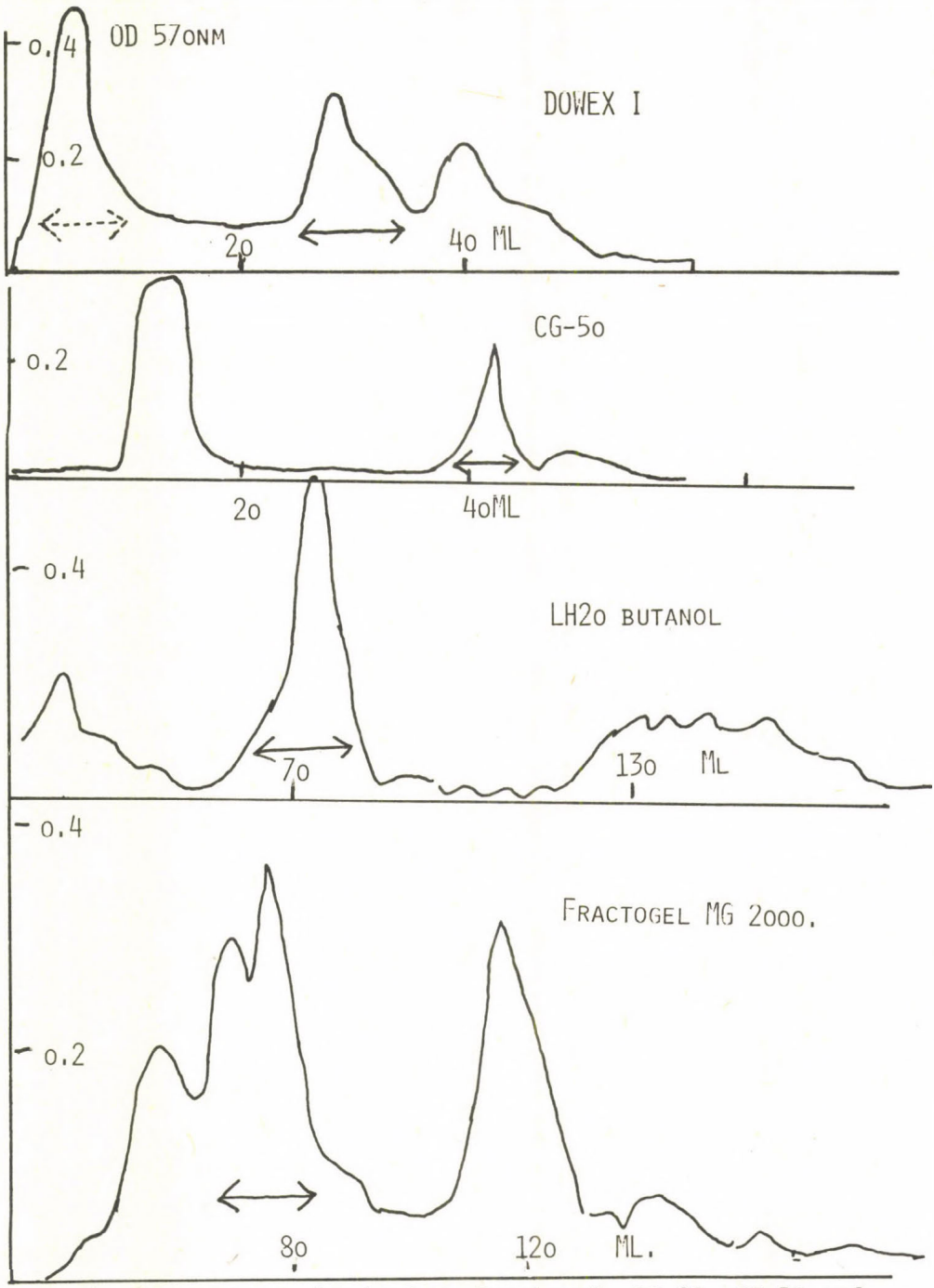

Fig. 4. The pattern obtained on Fractogel, LH 20, CG 50 and Dowex 1 is shown. In all cases the ninhydrin colour obtained after hydrolysis is shown. The solid arrow shows the dopamine uptake inhibitory factor, the dotted line the GABA releasing factor

Table II. The various peptidic activities found

Disorder	Peak		Activity	Tested by
Schizophrenia /9/	3.	a/	Analgesia reversed by haloperidol	K. Hole, Bergen
		b/	Striatal inhibition of dopamine uptake in synaptosomes	K. Hole, Bergen
		c/	Strong release stim. of GABA in hippocampal synaptosomes	Hamberger, Gøteborg*
	5.	d/	Rigidity Inhib. of GABA uptake in synaptosomes	K. Hole, Bergen*
	3.	e/	Increase dopaminergic activity in vivo	K. Hole, Bergen
	3.	f/	Muscarinic receptor binding to synaptosomal membranes	Ledaal, Oslo*

*In preparation

Properties of the two peptides:

a/ Dopamine uptake inhibitor behaves like an anion because it is retarded on anion exchanger. It is not retained on reversed-phase HPLC at pH 2 and on Sephadex LH 20 column and hardly contains any hydrophobic groupings.

It passes through the Dowex 50 column even at pH 2 and must therefore probably be N-substituted or cyclic. It is not retained on G 10 filtration in water and therefore probably does not contain any aromatic groups.

The GABA releasing factor /Hamberger/ passes through both Dowex 50 at pH 2 and is probably N-substituted. Indeed pyroglu has been found to be split off on by pyroglutaminase and anion chromatography. It is not retained on the anion exchanger in the formate form and therefore it contains either a neutral or C-terminally amidated amino acid. It is not retained on reversed-phase ODS columns even at pH 2 nor on LH 20 columns and therefore hardly contains any lipophilic sequences. No retention on Sephadex G 10 in water also points to the lack of aromatic amino acids.

We are at the present trying to synthesize these compounds for detailed testing.

Discussion

The increase in peptide found in schizophrenia has been confirmed by Drysdall et al. /1982/. Some of their factors show effects similar to ours /Hole et al. 1979/. What is clear is that there is an increase in peptidic material associated with glycoproteins in this disorder. Furthermore, it is not a one peptide disorder, but a whole family of peptides are increased.

There is considerable evidence that the breakdown of several releasing factors is regulated by feedback from endocrine target tissues /Bauer, 1976; Griffiths et al. 1975; Kuhl et al. 1978/. We have elsewhere argued extensively that the genetic basis of schizophrenia may be a peptidase insufficiency /Reichelt et al. 1981/. The genetic basis of schizophrenia is well documented /Kallman, 1946; Kringlen, 1964; Rosenthal, 1971/. Could an increased release by any number of inputs to the peptidergic neurones outstrip the breakdown? As peptides generally act as initiators and controllers of complex and sequential processes /Reichelt and Edminson, 1978/ it would not be surprising that where, if they are not broken•down, they could cause prolonged states of altered behaviour quite re-

sistant to the patient's wishes or to the therapist's verbal efforts.

Such a model is given increased credence by the fact that peptidase like aminopeptidase that breaks down enkephalin is inhibited by beta - endorphin /Hui et al. 1982/.

A very intriguing hypothesis has been forwarded by Dohan on peptides from the food causing schizophrenia /Dohan, 1982/. It is of course entirely possible that our peptides are alimentary. However, the fact that these peptides do have effects on the CNS makes it possible that there must be endogeneous peptides of similar structure that cause similar actions on neurones and behaviour. As yet we must leave this question for future studies. However, hypothalamically lesioned animals have very large increases in peptide secretion /Holm, unpublished data/ and the dipeptide homocarnosin injected intracranioventricularly is to a large measure found in the urine /Ziesler et al., in press/.

RELEVANCE TO SCHIZOPHRENIA

The antipsychotic drugs used act mostly by blocking dopamine activity. This is why parkinsonian-like symptoms may be induced. A dopaminergic theory as the cause of schizophrenia has therefore been advanced /Carlsson, 1978/ and especially with regard to the mesolimbic nuclei such as n. accumbens /Stevens, 1979/. It should be noted that EEG anomalies have been registered from these areas in schizophrenics /Stevens et al., 1979/. A decreased blood flow in the medial frontal cortex has also been reported /Ingvar, 1982/.

The increase in peptidic material in the urine of severely ill schizophrenics from different institutions is hardly accidental. It makes sense that we do find a family of peptides which cause profound disturbance as seen in rats /Hole et al. 1979/, when injected intracranioventricularly and also causes increased dopaminergic activity in vivo /Hole et al. 1979/. The lack of direct feedback from the n. accumbens to the A-10 area of the tegmental dopaminergic neurones could cause even more

activation of the dopaminergic neurones caused by GABA release /inhibiting an inhibitor gives increase in activation/. It is also interesting that we do find muscarinic receptor binding, as this receptor has also been indicated as relevant to schizophrenia /Singh and Ray, 1979/. The innervation of n. accumbens is clearly involving the relevant transmitter types, /Fig. 5/. GABA and dopamine inhibition of the 5HT /serotonin/ nuclei could both be involved in the preponderance of auditory hallucinations, which are common to compounds that inhibit serotonergic neurones.

The fact that a whole set of peptides are increased /not measurable in normals with our techniques/ could indicate that indeed peptidases may be the primary insufficiency. This is not known. However, our model could easily incorporate more dynamic psychological mechanisms, by increasing one or several inputs to the peptidergic neurones.

REFERENCES

Burzynski, S.R., Loo, T.L., Ho, D.N., Rao, P.N., Georgiades, G. and Kratzenstein, H. Biologically active peptides in human urine III. Physiol. Chem. Phys. 8, 13-22 /1976/

Carlsson, A. Does dopamine have a role in schizophrenia? Biological Psychiatry, 13, 3-21 /1978/

Chalmers, T.M., Kerwick, A. and Pawan, G.L.S. On the fatmobilising activity of human urine. The Lancet, 26.April, 866-869 /1958/

Chodar, G.W., Schreiner, C.J. and Zeitler, B.R. Urine from patients with transitional cell carcinoma stimulates emigration of capillary endothelial cells. New England J. Med. 305, 869-874 /1981/

Coy, D.H., Jaworek, J., Kontorek, S.J., Kwiecien, N., Radecki, T., Schally, A.V. and Tasler, J. Effects of anorexogenic peptide on gastric and pancreatic secretion. J. Physiol. /Lond./ 314, 225-235 /1981/

Dohan, F.C. Schizophrenia: possible relationship to cereal grains and celiac disease. Schizophrenia /Ed.: Sandkar, D.V.S./. P.J.D. Publications, 539-551 /1969/

Drysdall, A., Deacon, R., Lewis, P., Olley, J., Electicwala, A.A. and Sherwood, R. A peptide containing fraction of plasma from schizophrenic patients which binds to opiate receptors and induces hyperreactivity in rats. Neuroscience 7, 1567-1574 /1982/

Feighner, P.J., Robbins, S.E., Guze, S.B., Woodruff, R.A., Winokur, G. and Munoz, R. Diagnostic criteria for use in psychiatric research. Archiv General Psychiat. 26, 57-63 /1972/

Gray, W.R. Dansylchloride procedure. In Methods in Enzymology /Ed.: Hirs, C.H.W./ A.P. New York XI, 139-151 /1967/

Gray, W.R. Sequential degradation plus dansylation. in Methods in Enzymology /Ed : Hirs, C.H.W./. A.P. New York, XI 469-478 /1967/

Griffiths, E.C., Hooper, K.C., Jeffcoate, S.L. and Holland, D.T. The effect of gonadectomy and gonadal steroids on the activity of hypothalamic peptidase inactivating luteinizing hormone - releasing hormone /LHRH/. Brain Research 88, 384-388 /1975/

Hole, K., Bergslien, J.A., Jørgensen, H., Berge, C.G., Reichelt K.L. and Trygstad, O.E. A peptide containing fraction in urine of schizophrenic patients which stimulates opiate receptors and inhibits dopamine uptake. Neuroscience 4, 1883-1893 /1979/

Hui, K.S., Graf, L. and Lajtha, A. β-endorphin inhibits met-encephalin breakdown by a brain aminopeptidase. Biochem. Biophys.Res.Comm. 105, 1482-1487 /1982/

Ingvar, D.H. /1982/. Mental illness and regional brain metabolism. Trends in Neuroscience 5, 199-203 /1982/

Kallman, H. The genetic theory of schizophrenia. An analysis of 691 index families. Amer. J. Psychiat. 103, 309-322 /1946/

Kastin, A.J., Coy, D.H., Schally, A.V. and Miller, L.H., /1978/ Peripheral administration of hypothalamic peptides results in CNS changes. Pharmacol.Res.Comm. 10, 293-312 /1978/

Kringlen, E. Schizophrenia in male monozygotic twins. Acta Psychiat.Scand. Suppl. 178, 1-76 /1964/

Kronheim, S., Berelowitz, M. and Pimstone, B.L. The characterization of growth hormone released inhibiting hormone like immunoreactivity in urine. Clin.Endocrinol. 7, 343-347 /1977/

Krueger, J.K., Pappenheimer, J.R. and Karnovsky, M.L. The composition of sleep-promoting factor isolated from human urine. J.Biol.Chem. 257, 1664-1669 /1982/

Kuhl, H., Rosniatowski, C. and Taubert, H.D. /1978/. The activity of an LHRH - degrading enzyme in the anterior pituitary during the rat oestrus cycle and its alteration by injection of sex hormones. Acta Endocrinol. 87, 476-484 /1978/

Noguchi, T., Okiyama, A., Natto, H., Koneko, K. and Koike, G. Some nutritional and physiological factors affecting the urinary excretion of acid soluble peptides in rats and women. Agricult.Biol.Chem. 46, 2821-2828/1982/

Reichelt, K.L. and Kvamme, E. Histamine dependent formation of N-acetyl-aspartyl-peptides in mouse brain. J.Neurochem. 21, 849-859 /1973/

Reichelt, K.L., Foss, I., Trygstad, O.E., Edminson, P.D., Johansen, J.H. and Bøler, J.B. /1978/. Human control of appetite II. Neuroscience 3, 1207-1211 /1978/

Reichelt, K.L., Hole, K., Hamberger, A., Saelid, G., Edminson, P.D., Braestrup, C.B., Lingjaerde, O., Ledaal, P. and Ørbeck, H. Biologically active peptides containing fractions in schizophrenia and childhood autism. Adv. in Biochem.Psychopharmacol. 28, 627-643 /1981/

Rosenthal, D., Wender, P. and Kety, S. The adopted away offspring of schizophrenics. Amer. J. Psychiat. 128, 307-311 /1971/

Saelid, G., Haug, J.O., Heiberg, T. and Reichelt, K.L. Peptide containing fractions in depression. Biol. Psychiat: In press.

Schally, A.V., Medina-Santillan, R., Colacula, J., Carter, W.H., Redding, T.-W., Jones, J., Altamaro-Bustamente, P. and Coy, D.A. Inhibition of sham feeding - induced gastric secretion and serum hormonal responses by analogs of /pyro/ Glu-His-GlyOH. Proc.Soc.Exp.Biol. and Med. 170, 264-271 /1982/

Singh, M.M. and Ray, S.R. Therapeutic antagonism between anticholinergic antiparkinsonian agents and neuroleptics in schizophrenia. Implications for a neuropharmacological model. Neuropsychobiology 51, 74-86 /1974/

Stevens, J.R., /1979/. Schizophrenia and dopamine regulation the mesolimbic system. T.I.N.S. 2, 102-104 /1979/

Stevens, J.R., Bigelow, L., Denney, D., Lipkin, J., Livermore, A.H., Rauscher, F. and Wyatt, R.J. Telemetered EEG-EOG during psychotic behaviours of schizophrenia. Arch.Gen.Psychiat. 36, 251-262 /1979/

Trygstad, O.E., Reichelt, K.L., Foss, I., Edminson, P.D., Saelid G., Bremer, J., Hole, K., Ørbeck, H., Johansen, J.H., Bøler, J.B.,Titlestad, K. and Ofstad, P.K. /1980/. Patterns of peptides an protein - associated - peptide complexes in psychiatric disorders. Brit.Journ.Psych. 136, 59-72 /1980/

Chromatography, the State of the Art
H. Kalász and L.S. Ettre (Eds)

RP-HPLC IN THE ANALYSIS, SYNTHESIS AND REACTIONS OF NEUROPEPTIDES AND THEIR DERIVATIVES

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INTRODUCTION

The development of HPLC with permanently bonded non-polar, so-called reversed-phased packings has been very useful in the liquid chromatographic analysis of different types of peptides, such as peptide hormones, antibiotics, their metabolites and derivatives. Extensive work by Horváth, Molnár, Hearn, Hancock and Rivier /1-4/ demonstrated the superior analytical performance which can be achieved by using reversed-phase, chemically bonded packings in the field of peptide-HPLC /5/.

In this paper the potential of RP-HPLC is demonstrated for the analysis of some neuropeptides, such as α-MSH /α-melanocyte stimulating hormone/, CCK /cholecystokinin/ and ACTH /adrenocorticotropin/ peptides, MIF /melanotropin inhibiting factor/, enkephalins and their analogs, fragments and derivatives. Methods have been developed for the monitoring of stages in their synthesis, for the analysis of protected, half protected and free fragments, metabolites and for the purity control of the full peptides. Peptide couplings, hydrolysis of peptide esters and side reactions have been studied by HPLC.

MATERIALS AND METHODS

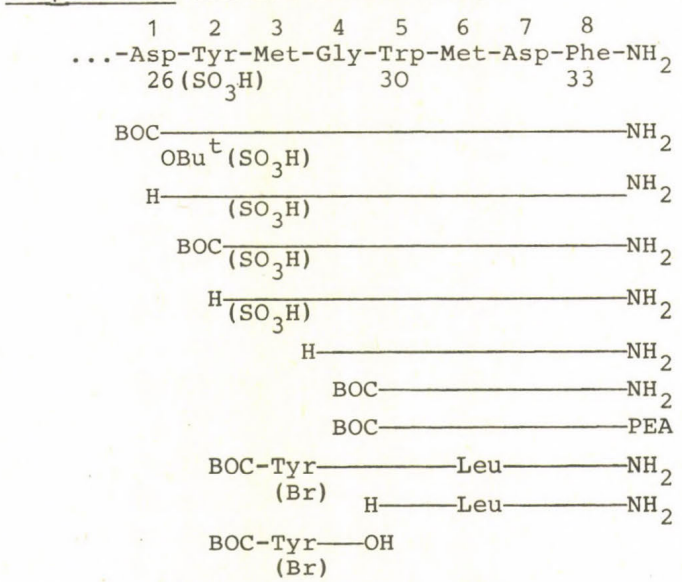
Column packing materials used mainly consisted of ODS-Hypersils /marketed by Shandon Southern Products, Runcorn, Great Britain/, the characteristics of which have been de-

scribed by Knox and Pryde /6/. The chromatograph used was assembled in our laboratory; its principal points were an Orlita DMP 1515 pump/Giessen, F.R.G./ and a variable-wavelength photometer fitted to an 8-µl flow cell /CECIL Model 212, Cambridge, Great Britain/. In some cases a Waters M 420 fluorimeter was coupled to the instrument. Columns were 125 and 250 mm long with 4 mm i.d. Samples were injected by a Model 7011, 10-µl loop-injector /Rheodyne, Inc. Berkeley, USA/. The peptides to be investigated were synthesized by peptide research groups in Hungary /in the Polypeptide Research Group of the Hungarian Academy of Sciences, Budapest, and in the University Medical School of Szeged/.

The substances investigated are listed in Tables 1 and 2.

Table 1. The investigated substances, I.

CCK, CCK-OP and its derivatives



Z: $C_6H_5CH_2O-\overset{O}{\overset{\|}{C}}-$

BOC: $(CH_3)_3C-O-\overset{O}{\overset{\|}{C}}-$

TOS: $CH_3-C_6H_4-SO_2-$

PEA: $C_6H_5CH_2CH_2NH-$

OMe: $-OCH_3$

MeCN: CH_3CN

TFA: CF_3COOH

OBut: $-OC(CH_3)_3$

Ac: CH_3CO-

β-LPH fragments, derivatives

... - Tyr-Gly-Gly-Phe-Met-...

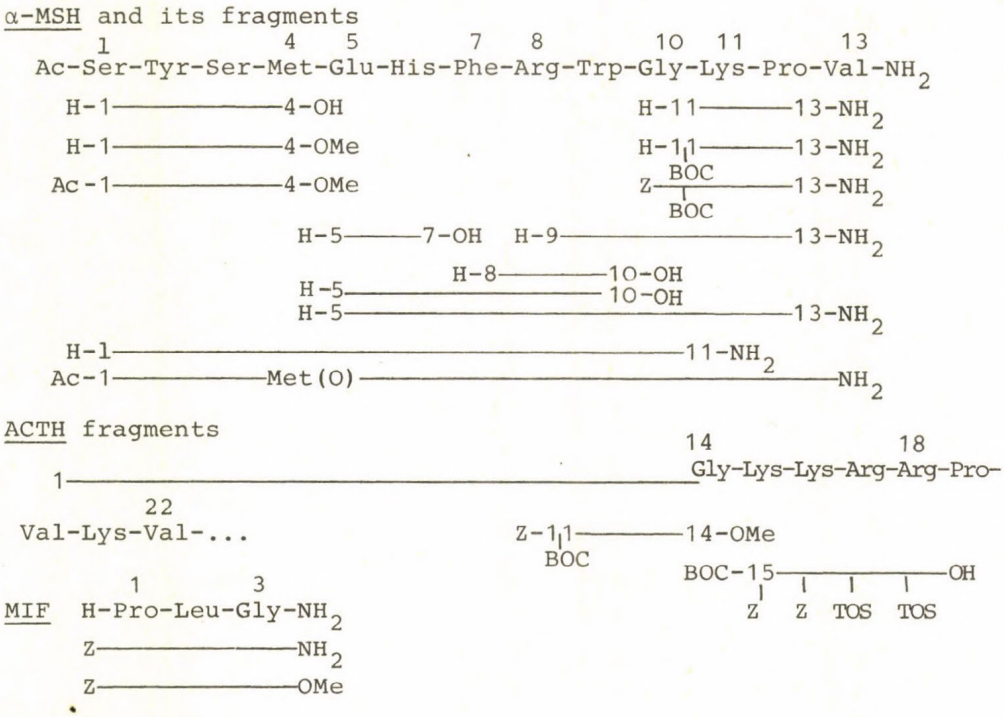
Z——Leu-OH

Z——Leu-OMe

H——Arg-PheOH

Z——Lys-Lys-Gly-Glu-OBut and free fragment
(BOC) (BOC)

Table 2. The investigated Substances, II.



The peptides were chromatographically characterized by peak-numbers, retention times and capacity factors.

The derivatization reagent was prepared by dissolving 27 mg of o-phthalaldehyde /OPA/ in 0.5 ml of ethanol. Boric acid solution /0.4 M/ was adjusted to pH 9.5 with 1 M NaOH. 20 microliters of 2-mercapto-ethanol and the OPA solution was added to 5 ml of the borate buffer. The reagent mixture was allowed to stand for 24 hrs prior to use. 100 microliters of the reagent was used for labeling 20-30 µg of peptide fragments.

RESULTS AND DISCUSSION

In peptide analysis the control of synthesis and purity requires adequate resolution in order to give a complete

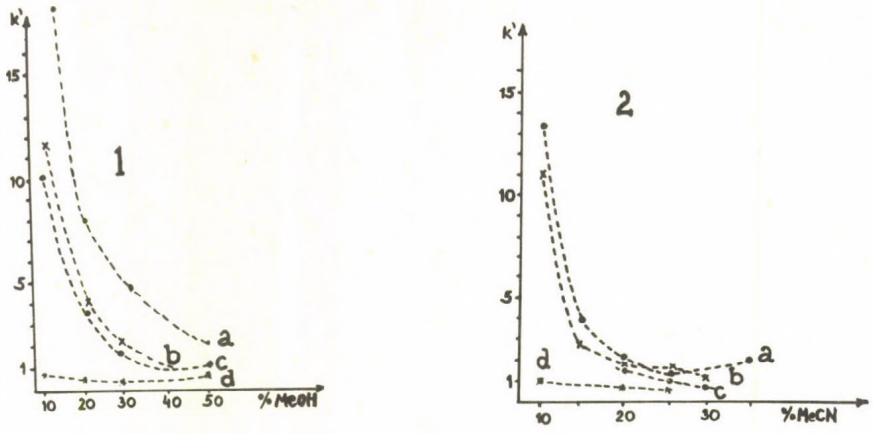


Fig. 1. Optimization with α-MSK fragments, I.
Column: ODS-Hypersil. Mobile phase: 0.01M phosphate buffer /pH2/.
Plots: o H $\frac{1\quad 4}{}$ OH
X H $\frac{5\quad 7}{}$ OH
o H $\frac{8\quad 10}{}$ OH
X H $\frac{11\ 13}{}$ NH_2

Fig. 2. Optimization with α-MSH fragments, II.
Column: ODS-Hypersil. Mobile phase contains 0.1% trifluoroacetic acid.
For the plots see Fig. 1

separation of the mixtures of protected and free peptides, starting materials, and by-products of the coupling reactions. Furthermore, for any determination, isolation, and investigation of the metabolism or the enzymatic degradation the applied method must also provide baseline separations of the different fragments. Therefore the systems used were always optimalized /see Figs 1-2 in the case of α-MSH fragments/.

In general 2-20 μg of the peptides were chromatographed. Chromatography required about 20 min. The mobile phases consisted of water and acetonitrile or methanol as organic modifiers. In the case of peptides having free amino, carboxyl or other ionizable groups, the eluents were buffered /because of tailing/ to pH 2, 4, 4.5, or 7.5 with phosphate and acetate

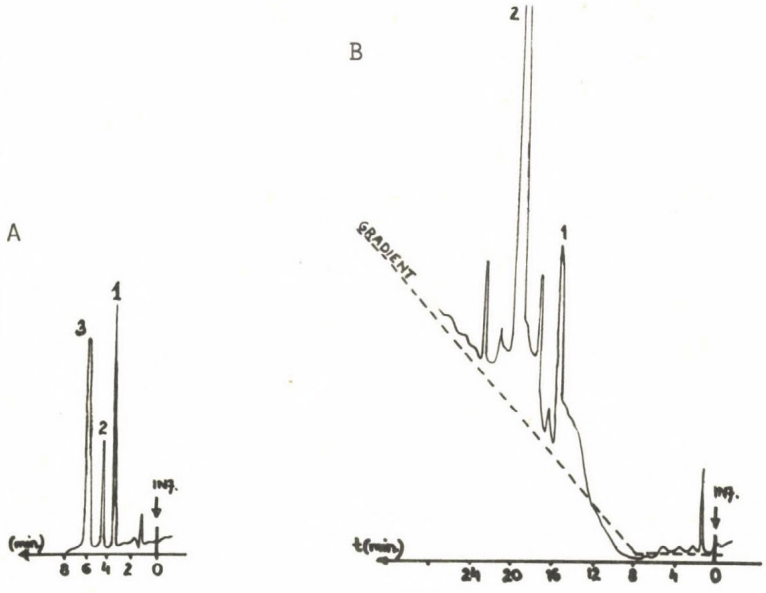


Fig. 3/a. Chromatogram of α-MSH fragments.

Column: 125 x 4 mm, ODS-Hypersil, 5 µm. Mobile phase: 30:60 methanol-0.1M phosphate buffer /pH 2/. Flow rate: 1.2 cm^3/min /65 bar/ Detection: UV at 220 nm, A=0.05.

Peaks: 1 H $\frac{1 \quad 4}{\quad\quad}$ OH /3.2 µg, k'= 2.5/

2 H $\frac{1 \quad 4}{\quad\quad}$ OMe /2.1 µg, k'= 3.5/

3 AC $\frac{1 \quad 4}{\quad\quad}$ OMe /4.0 µg, k'= 4.8/

Fig. 3/b. Purity control of 10 µg synthetic α-MSH.

Column: 125 x 4 mm, ODS Hypersil, 5 µm. Solvents: /A/ 20:80:0.1 acetonitrile-water-trifluoroacetic acid; /B/ 60:40:0.1 acetonitrile-water-trifluoro-acetic acid. Gradient: 1% B per minute. Flow rate: 1.4 cm^3/min /70 bar/.
Detection: UV at 220 nm, A= 0.02.

Peaks: 1 Met /SO/ α-MSH /k'= 11.0/,

2 α-MSH /k'= 15.0/

solutions. Trifluoroacetic acid used as an ionpairing reagent changed the retention times of bulky peptides /7/.

The pressure varied between 30 and 120 bars; flow rates were between 1 and 2 cm^3/min. The column effluents were

Table 3. Retention data of CCK peptides on C_{18} columns*

Peptides	t_R /min/	k'	Eluents
a/			
BOC-CCK8-SE	16.7	15.6	A : B_1
BOC-CCK7-SE	2.8	1.8	15 : 85
CCK8-SE	12.1	5.66	A : B_2
CCK7-SE	16.2	8.0	25 : 75
CCK6	14.2	6.88	
b/			
CCK8-SE	13.2	5.0	
Met/O/-CCK8-SE	11.8	4.3	
CCK8-SE	6.85	3.03	A : B_3
CCK8	8.7	3.53	25 : 75
Met/O/-CCK8-SE	6.1	2.60	
c/			
BOC-CCK4	6.6	5.60	A : B_4
BOC-CCK4-PEA	11.1	10.10	30 : 70
BOC-Tyr/Br/^{1}Leu^4-CCK^6	18.8	17.8	M : B_5 : A
BOC-Tyr/Br/Gly-OH	2.8	1.8	30 : 60 : 40
Leu^2-CCK^4	1.8	0.8	

* SE - sulfat ester

Columns: a/ ODS Hypersil 250 x 4 mm
b/ µBondapak C_{18} 300 x 3.9 mm
c/ ODS Hypersil 125 x 4 mm

Flow rates a, c/ 1.1 - 1.3 cm^3/min
b/ 2.0 cm^3/min

Solvents: A - CH_3CN
M - MeOH
B_n - 0.01 M buffer B_1 NaOAc /pH 4/
B_2 TEA phosphate /pH 6.5/
B_3 NH_4OAc /pH 4.2/
B_4 phosphate /pH 7.5/
B_5 phosphate /pH 2.1/

Table 4. Retention data of some neuropeptides and their derivatives on a ODS-Hypersil column*

Peptides	t_R /min/	k'	Eluents
MIF			
Pro-Leu-Gly-NH_2	1.4	0.75	M : H_2O : A
Z-Pro-Leu-Gly-NH_2	3.0	2.7	30 : 30 : 40
Z-Pro-Leu-Gly-OMe	5.3	5.6	
Enkephalins			
Z-Tyr-Gly-Gly-Phe-Leu-OMe	3.2	2.2	A : M : H_2O
Z-Tyr-Gly-Gly-Phe-Leu-OH	2.35	1.35	20 : 50 : 30
Met^5-enkephalin-Arg^6Phe^7 a/	7.2	3.5	M : B_4 55 : 45
Z-enkephalin-Lys^6(BOC)-Lys^7(BOC)-Gly^8-Glu^9-OBu^t	3.9	2.85	M : B_5 : A 30 : 30 : 40
ACTH'			
BOC-11(/Z/)———14-OMe	3.30	2.30	
BOC-11(/Z/)———14-OH	1.90	0.90	M : H_2O : A 30 : 30 : 40
BOC-15(Z Z Tos Tos)———19-OH	2.80	1.80	

*Columns: 125 x 4 mm, except a/ where it is 250 x 4 mm
Flow rates: 1.1, 1.4 and 1.5 cm^3/min
Solvents: for explanation see the footnote of Table 3

monitored at 215, 220, 254 or 280 nm depending on the protecting groups and amino acid residues.

From the point of view of a peptide chemist the analysis of protected peptide fragments is also very important. Generally, the protecting groups increase the retention times on RP columns because of their higher hydrophobicity, /e.g.: Z. BOC, Tos as amino-, alkyl and benzylesters as carboxyl-protecting

Table 5. Retention data of α-MSH derivatives on the ODS Hypersil column*

Peptides	t_R /min/	k'	Eluents
α-MSH			
α-MSH	19.8	15.0	A : H_2O : TFA gradient
α-MSH-Met/O/[4]	15.6	11.0	
Z-11(BOC)-13-NH_2	3.1	2.2	30 : 30 : 40 M : H_2O : A
H-1-4-OH	3.4	2.5	M : B_5
H-1-4-OMe	4.3	3.5	30 : 60
Ac-1-4-OMe	5.9	4.8	
H-11-13-NH_2	1.8	0.1	A : B_5
H-5-7-OH	2.7	1.3	30 : 90
H-8-10-OH	3.9	1.6	
H-1-4-OH	5.0	2.0	
OPA-11-13-NH_2	21.5	9.2	A : H_2O : TFA gradient
OPA-8-10-OH	18.8	8.5	

*Column: 125 x 4 cm
Flow rates: 1.4 cm^3/min /gradient/ and 1.2 cm^3/min /isocratic/
Solvents: for explanation see the footnote of Table 3

groups, respectively; see Tables 3, 4/. At the same time these groups also give favourable chromophors for detection.

Table 3 shows /see data of CCK analogs/ that, after coupling, the fully protected endproduct has greater retention than the starting N- and C-terminal fragments.

Deprotection always results in faster elution in the same chromatographic system.

The HPLC monitoring of the hydrolysis of α-MSH, ACTH, enkephalin and MIF derivatives show /see Table 4/ that in all cases the acid /i.e. the peptide with free C terminal/ being more polar than the methylesters or amides, elutes before the

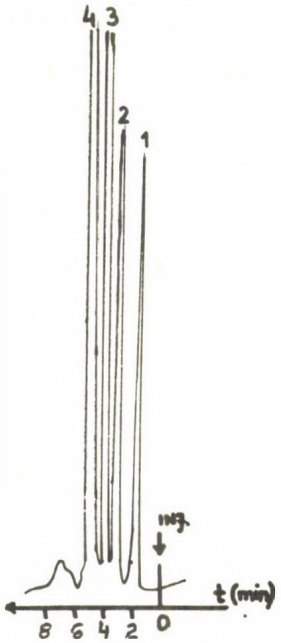


Fig. 4. Chromatogram of α-MSH fragments.
Column: 125 x 4 mm, ODS-Hypersil, 5 µm. Mobile phase: 10:90 acetonitrile - 0.1M phosphate buffer /pH 2/.
Flow rate: 1.1 cm^3/min /60 bar/.
Detection: UV at 220 nm; A=0.1.

Peaks: 1 H $\overset{11 \quad 13}{\underline{\quad\quad}}$ NH_2 /5 µg; k'= 0.1/

2 H $\overset{5 \quad 7}{\underline{\quad\quad}}$ OH /5 µg; k'= 1.3/

3 H $\overset{8 \quad 10}{\underline{\quad\quad}}$ OH /2 µg; k'= 1.6/

4 H $\overset{1 \quad 4}{\underline{\quad\quad}}$ OH /10 µg;k'= 2.0/

corresponding esters. In the case of acetylated, esterified, fully and half-protected fragments α-MSH 1-4, baseline separation was achieved /see Fig. 3 / isocratically. This shows the possibility to use HPLC in the guality control of the various steps in peptide synthesis /8/.

For the separation of different fragments /1-4, 5-7, 8-10, 11-13; see Table 5 and Fig.4 / of α-MSH, the chromatographic system was optimized in respect of the concentration of both the methanol-phosphate buffer and the acetonitrile-TFA mixture /see Figs 1-2/.

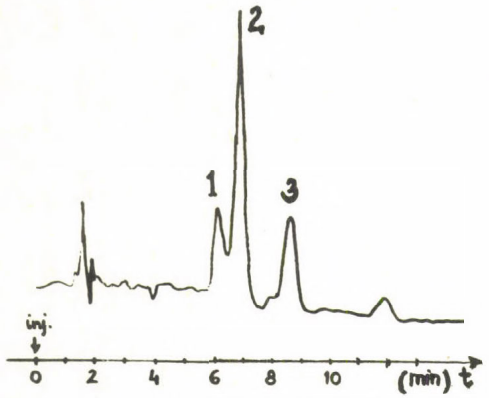


Fig. 5. Chromatogram of synthetic CCK-OP.

Column: 300 x 3.9 mm μBondapak C_{18}. Mobile phase: 25-75 acetonitrile - 0.01M ammonium acetate buffer /pH 4.2/. Flow rate: 2 cm^3/min.
Detection: UV, 220 nm, A=0.2.

The peak at 6.85 min corresponds to CCK-OP /k'= 3.03/. The peaks at 6.1 and 8.7 min corresponds to the respective impurities of Met/o/ and des /SO_3H/-CCK-OP.

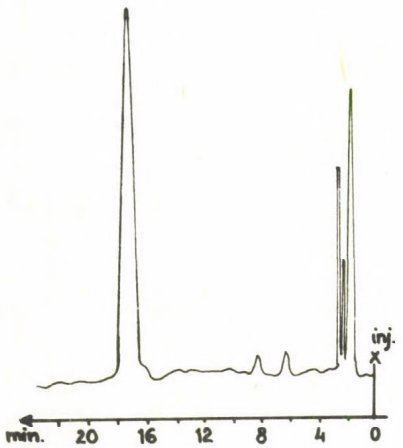
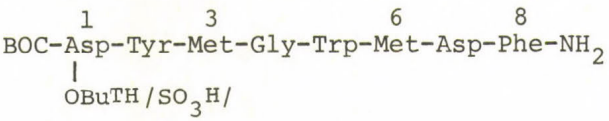


Fig. 6. Chromatogram of protected CCK-OP.

Column: 250 x 4 mm, ODS-Hypersil, 6 mm. Mobile phase: 15:85 acetonitrile - 0.01M sodium acetate buffer /pH 4/. Flow rate: 1.3 ml/min. Detection: UV, 214 nm, A=0.05.

The peak at 16.7 min corresponds to



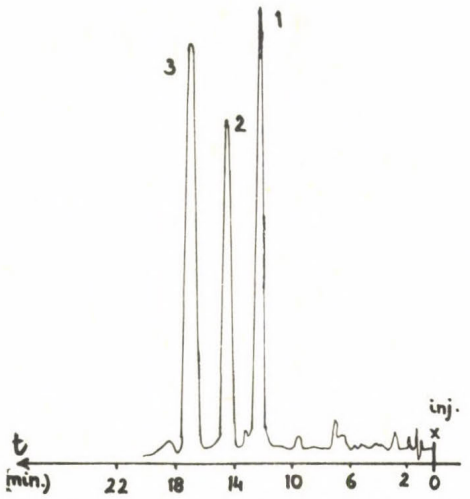
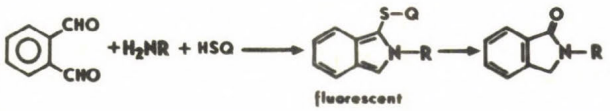


Fig. 7. Chromatogram of CCK-fragments.

Column: 250 x 4 mm, ODS-Hypersil, 6 µm. Mobile phase: 25:75 acetonitrile - TEA-phosphate buffer /pH 6.5/. Flow rate: 1.1 cm^3/min. Detection: UV at 220 nm, A=0.05.

Peaks correspond to 2-10 µg fragments of 1 CCK-8 sulfate ester, 2 CCK-6, 3 CCK-7 sulfate ester



	R	Q	STRUCTURE identified
SIMMONS-COMPOUND	C_3H_7–	–C(CH₃)₃ –CH₂CH₂OH	(mp.58°) by IR, NMR, MS (9)
AMINO ACIDS	–CH(R")–COOH	–CH₂CH₂OH	"only" analogy
GLYCINE ESTER	–CH₂COOEt	–C(CH₃)₃	by IR, NMR
LYSINE AMIDE	Z–HNCH(CH₂)₄–CONH₂	–C(CH₃)₃	by IR, NMR
PEPTIDES	–NH–CHCONHCHCO– (R, R)	–CH₂CH₂OH	analogy

peptide hormones
fragments of α-MSH, ACTH

Fig. 8. Precolumn derivatization with o-phthalaldehyde

The resolution obtained gives a possibility to study the degradations of α-MSH /for example, its enzymatic degradation or metabolism in blood serum/.

α-MSH could be separated well from its sulfoxide impurity at the ^{4}Met-residue /see the chromatogram of crude synthetic α-MSH in Fig. 3/. The efficiency of the separation was increase by gradient elution. The sulfoxide derivative has a lower retention than the pure hormone because of its somewhat higher polarity.

The HPLC analysis of CCK peptides requires very high efficiency because of the presence of sensitive residues: sulfated Tyr and Met. By a recently elaborated isocratic system both fragments and side products were separated.

Table 3 lists the retention times of some free and protected CCK peptides on the ODS-Hypersil column while Figs 5, 6, 7 show the analytical results.

The CCK-octapeptides are eluted in the following order: oxidized, sulfated and desulfated compounds /Fig. 5/, in agreement with the expectation for reversed-phase behaviour on the basis of the Rekker-constants or Meek-data /9-11/. The BOC and OBu^{t} protected derivative has an increased retention time /Fig. 6/. The system can differentiate between the fragments of CCK /Fig. 7/: hydrophilic residues as aspartyl and sulfat ester cause a faster elution. The substitution of the C terminal amide group by a phenyl-ethylamide unit results in longer retention /Table 3./.

Sensitivity enhancement is also very important in the analysis and determination of peptides, their metabolites and fragments existing in very small quantities in biological and clinical samples. The sensitivity of detection can be increased by the application of simple pre-column derivatization /12/: we used o-phthalaldehyde /OPA/ as the reagent for peptides /12, 13/. OPA reacts with the free NH_2 groups of peptides in alkaline media in the presence of a reducing agent such as 2-mercapto-ethanol to form a substituted isoindole /14/. This highly fluorescent derivative may be excited at 340 nm resulting in an emission at 455 nm. OPA-amino acids have been used in amino acid analyzers since they can be separated easily /15,

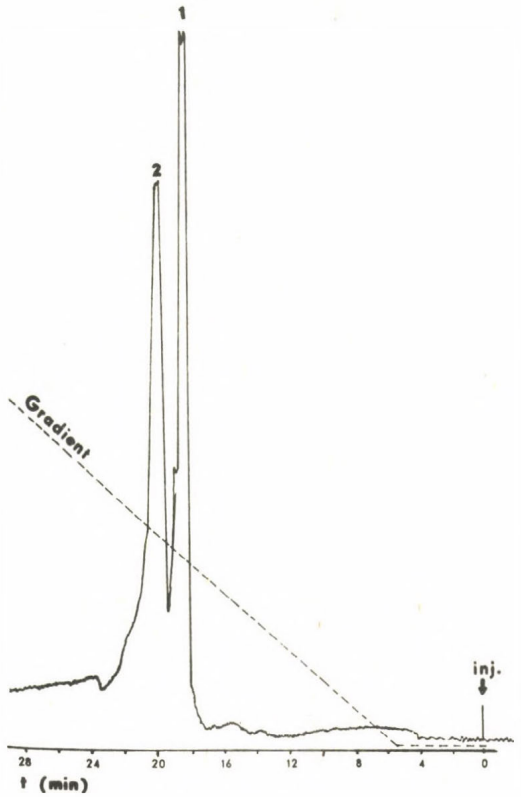


Fig. 9. Chromatogram of o-phthaldehyde derivatives of α-MSH fragments.

Column: 125 x 4 mm ODS Hypersil 5 µm. Solvents: /A/ 15:85:0.1 acetonitrile-water-trifluoro-acetic acid; /B/ 90:10:0.1 acetonitrile-water-trifluoro-acetic acid. Gradient: 2.2% of B per minute. Flow rate: 1.4 cm^3/min /70 bar/. Detection: waters M20 fluorimeter.

Peaks: 1 H-8-10-OH /0.3 µg, k'= 8.5/, 2 H-11-13-NH_2 /0.2 µg, k'= 9.2/.

16/. Their application being based on analogy only, we also investigated the structures of the OPA-derivatives of amino acids and peptides /see Fig. 8/. Their isoindole ring was identified by NMR and IR spectroscopy. The effectiveness of pre-column derivatization by OPA has been demonstrated in our laboratory for monitoring fragments of α-MSH /Fig. 9 /. 20-30 µg of the peptide fragments were labeled and 0.1-0.4 µg of the peptides were detected. The efficiency of the separation on the

ODS-Hypersil column was increased by gradient elution using acetonitrile-water eluent containing 0.1 % of trifluoro acetic acid. This method is well applicable to the study of the metabolism and enzymatic degradation of neuropeptides if the samples are pre-purified on SEP-PAK cartridges /Waters/, with 40-80% recovery for the α-MSH fragments /16/.

SUMMARY

Liquid chromatographic systems with ODS-Hypersil reversed-phase packing material were elaborated for the separation of CCK-OP and α-MSH fragments and for the analysis of CCK-OP, α-MSH, MIF, enkephalin, ACTH peptides and their derivatives. Methanol-acetonitrile-water mixtures containing trifluoro acetic acid, acetate or phosphate buffer used as the mobile phases. After optimization baseline separation could also be achieved. The different steps of peptide synthesis were checked and studied by HPLC. The sensitivity of detection was increased by the application of precolumn derivatization. o-Phthalaldehyde was used as the reagent to form fluorescent peptide-derivatives.

Our methods are well applicable not only for controlling the purity of neuropeptides, but also for the investigation of their enzymatic degradation and metabolic pathways.

REFERENCES

1. I. Molnár, Cs. Horváth: J. Chromatogr. 142 /1977/ 623
2. W.S. Hancock, C.A. Bishop, M.T.W. Hearn: J. Chromatogr. 153 /1978/ 391
3. J.E. Rivier: J. Liquid Chrom. 1 /1978/ 343
4. E.C. Nice, M.J. O'Hare: J. Chromatogr. 162 /1979/ 401
5. J.H. Knox, Gy. Szókán: J. Chromatogr. 171 /1979/ 439
6. J.H. Knox, A. Pryde: J. Chromatogr. 112 /1975/ 171

7. C.A. Browne, H.P.J. Bennett, S. Solomon: Biochem. 20 /1981/ 4538

8. M.A. Chlenov, E.V. Titova, L.J. Kudryashov: Bioorg.khim. 8 /1982/ 914

9. L. Pradayrol, N. Vaysse, J. Cassigneul, A. Ribet: Hormone Receptor in Digestion and Nutrition (edited by G. Rosselin, P. Fromageot, S. Bonfilis), Elsevier/North Holland, Amsterdam, the Netherlands, 1979, p. 95.

10. M. Deschodt-Lanckman, N.D. Bui, M. Noyer, J. Christophe: Regulat. Peptides 2 /1981/ 15

11. M.C. Beinfeld, R.T. Jensen, M.J. Brownstein: J. Liquid Chrom. 3 /1980/ 1367

12. Gy. Szókán: J. Liquid Chrom. 5 /1982/ 1493

13. Gy. Pintér: Dissertation, Budapest /1980/

14. S.S. Simmons, D.F. Johnson: J. Chem. Soc. Chem. Commun. 11 /1977/ 374

15. J.C. Hodgin: J. Liquid Chrom. 2 /1979/ 1047

16. P. Lindroth, K. Mopper: Anal. Chem. 51 /1979/ 1667

Chromatography, the State of the Art
H. Kalász and L.S. Ettre (Eds)

STRUCTURE ANALYSIS OF BRANCHED CHAIN POLY- AND ISOPEPTIDES BASED ON HPLC OF THEIR DANSYL DERIVATIVES

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ABSTRACT

Liquid chromatographic systems with ODS-Hypersil and Partisil-IO PAC packing materials were developed for the separation of α, ε and bis-Dns-lysine, other Dns-amino acids and Dns-OH.

These methods were applied to the determination of the primary structure of synthetic lysine oligo-, poly- and isopeptides and used for the analysis of the surface topography of polylysine-based branched-chain polypeptides.

The results obtained by HPLC were compared with the data of the amino acid analysis of branched polypeptides.

The dansylation procedure of synthetic oligo- and polypeptide is also described.

INTRODUCTION

Synthetic branched polypeptides have been extensively used in biological research as synthetic antigens for a molecular understanding of basic immunology (1) and as macromolecular carriers of various biologically active materials such as antitumor drugs (2), synthetic fragments of influenza virus (3) and diphtheria toxin (4).

Extending this utilization range to investigate their immunomodulatory potential, we have developed a new group of branched polypeptides with short poly-DL-alanine side chains

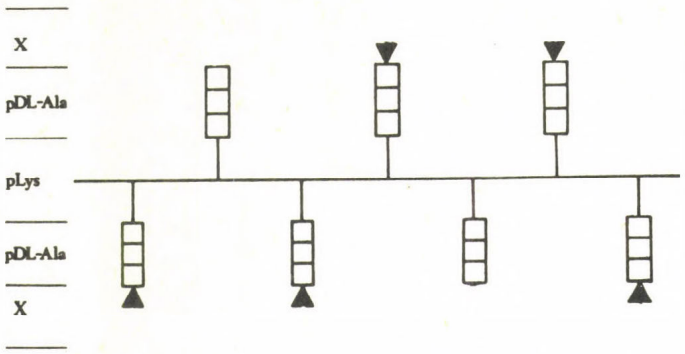

Fig. 1. General scheme of the new group of branched polypeptides. (X = Leu, Ile, Nle, Val, Tyr, Pro, Phe, Glu)

grafted to the ε-amino groups of a poly-lysine backbone and with the ends of the poly-DL-Ala side chains partly covered by one particular amino acid (5).

Much emphasis was placed on the manysided physico-chemical examination of these polypeptides, among others, by sedimentation analysis, thin-layer and column gel chromatography and conformational studies (6).

In the present paper we report on our studies of the primary structure of the macromolecules including sequence determination and surface topography based on their amino acid analysis, parallel with HPLC analysis of the hydrolysates of dansylated polypeptides.

Dansyl chloride (1-dimethylaminonaphthalene-5-sulfonyl chloride, Dns-Cl) has been used for both the detection of amino acids and the determination of the amino terminus in peptide chemistry. In the past Dns-amino acids were separated by thin-layer chromatography (7) and, more recently, by high-performance liquid chromatography (HPLC) using silica gel (8) or chemically-bonded (9-12) packings.

In our study the application of HPLC for rapid separation was also investigated in the quantitative determination of the composition of the Dns-amino acid mixture (also containing 1-dimethylaminonaphthalene-5-sulfonyl acid, Dns-OH). Our methods were then adapted for the qualitative and quantitative

analysis of the hydrolysates of several synthetic lysine oligo-, poly- and isopeptides (group A) and extended to the analysis of poly-lysine-based branched polypeptides (group B), not investigated previously in a similar way.

Using these methods we determined the N-terminal amino acids of branched polypeptides also drawing some conclusions concerning their surface topography. The results were compared with data obtained by amino acid analysis.

MATERIALS AND METHODS

For standard measurements Dns-amino acids and Dns-chloride were acquired in a kit from Pierce Chemical Co (Rockford, Ill. USA), ε-Dns-Lys was obtained from Sigma Chemical Co (St. Louis, Mo., USA). The oligo- and polypeptides were synthesized in our laboratory as described elsewhere (5, 13).

Dansylated synthetic lysine oligo-, poly- and isopeptides (group A) and dansylated branched polypeptides (group B) were obtained by the adaptation of Hartley's method (7) in the following way: 1 mg (1-5 μM) oligo- or polypeptides were dissolved in 2 cm^3 0.2 M $NaHCO_3$ solution. 0.25 cm^3 (0.125 - 0.625 μM) of the polypeptide solution was lyophilized and treated with a 8-10 fold (group A) or a 2-3 fold (group B) molar excess of Dns-Cl in acetone (2.5 mg/cm^3). The same volume (80 - 600 μl) of distilled water was added. The solution was incubated at 25°C for 3 h, 12 h or 18 h and lyophilized without purification prior to hydrolysis which was carried out in 6 N HCl (300 - 1000 μl) at 105°C for 15 h in sealed tubes. The HCl was removed in vacuo, and the hydrolysate redissolved in 2-3 cm^3 of methanol for injection into the column.

Acetonitrile and methanol used as the solvents were of UV grade, "distilled in glass"; glacial acetic acid was of p.a. quality. The buffer was prepared from analytical-grade chemicals and glass-distilled water. The following solvent systems were used:

System 1: methanol - 0.03 M phosphate buffer, pH 7.3
(55 : 45)

System 2: acetonitrile - water - acetic acid
(35 : 65 : 1)
System 3: acetonitrile - water - methanol - acetic acid
(25 : 65 : 10 : 1)
System 4: acetonitrile - water - methanol - acetic acid
(35 : 65 : 10 : 1)

Amino acid analysis was carried out on a Model OE 975 analyser (Chinoin, Budapest, Hungary) after hydrolysis in 6N HCl at 105°C for 8 h.

HPLC separations were performed on a laboratory-assembled instrument whose principal components were: a reciprocating piston pump (Type 1515; Orlita, Giessen, F.R.G.), a sample injector (Model 7011 loop injector; Rheodyne, Berkeley, Cal. USA) and a variable-wavelength ultraviolet monitor with a flowcell having a pathlength of 10 mm and a volume of 10 µl (Model 212; Cecil, Cambridge, Great Britain). The column effluents were monitored at 254 nm. The columns (4.6 mm x 250 mm) were of internally polished stainless steel. Peaks were recorded on a chart recorder (Type OH-814/1; Radelkis, Budapest, Hungary). ODS-Hypersil (Shandon Southern Products, Runcorn, Great Britain) and Partisil-10 PAC (10-µm particle size, pre-packed, Whatman, Clifton, N.J., USA) were used as the packing materials. All chromatograms were run at room temperature. Flow rates were between 0.6 and 1.1 cm^3/min. The areas under the peaks were calculated using Simpson's rule.

Solutions of standard dansyl amino acids and of hydrolysates were prepared in methanol prior to liquid chromatography.

RESULTS AND DISCUSSION

We have found that the preparations described above yield hydrolysates containing small (group A) or large (group B) amounts of dansyl acid. Therefore, in addition to the good resolution of dansyl amino acids, the dansyl acid contents also

had to be taken into consideration when choosing the suitable chromatographic conditions.

Two different columns and four different solvent systems were tested for the separation of Dns-amino acids with respect to the α, ε, bis-Dns-lysines, and Dns-OH (for group A) and with respect to the α, ε, bis-Dns-lysines, Dns-Ala, Dns-X (X=Leu, Ile, Nle, Val, Tyr, Pro, Phe, Glu) and Dns-OH (for group B), respectively. The dansyl derivatives were characterised by retention time (t_R) and capacity factor (k').

Good resolutions were achieved isocratically on ODS-Hypersil column with methanol-phosphate buffer eluent (13) (see Fig 2). Retention times and capacity factors concerning different Dns-amino acids and Dns-OH are summarized in Table I.

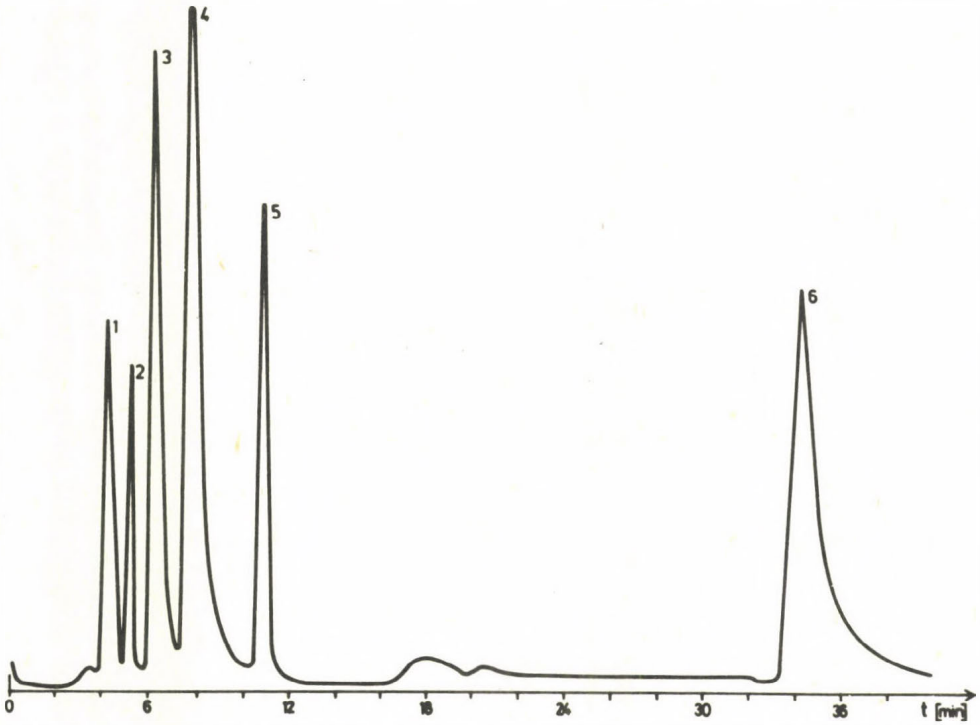

Fig. 2. Chromatogram of dansylated lysines, alanine, phenylalanine and Dns-OH. Column: ODS - HYPERSIL (4.6 mm x 250 mm); solvent (system 1): 55 : 45 methanol - 0.03 M phosphate buffer (pH 7.3); flow rate: 0.6 cm^3/min. Detector range: 0.05 A.U.F.S. at 254 nm. 1 = Dns-OH; 2 = Dns-Ala; 3 = Dns-Lys; 4 = Lys(Dns); 5 = Dns-Phe; 6 = Dns-Lys(Dns).

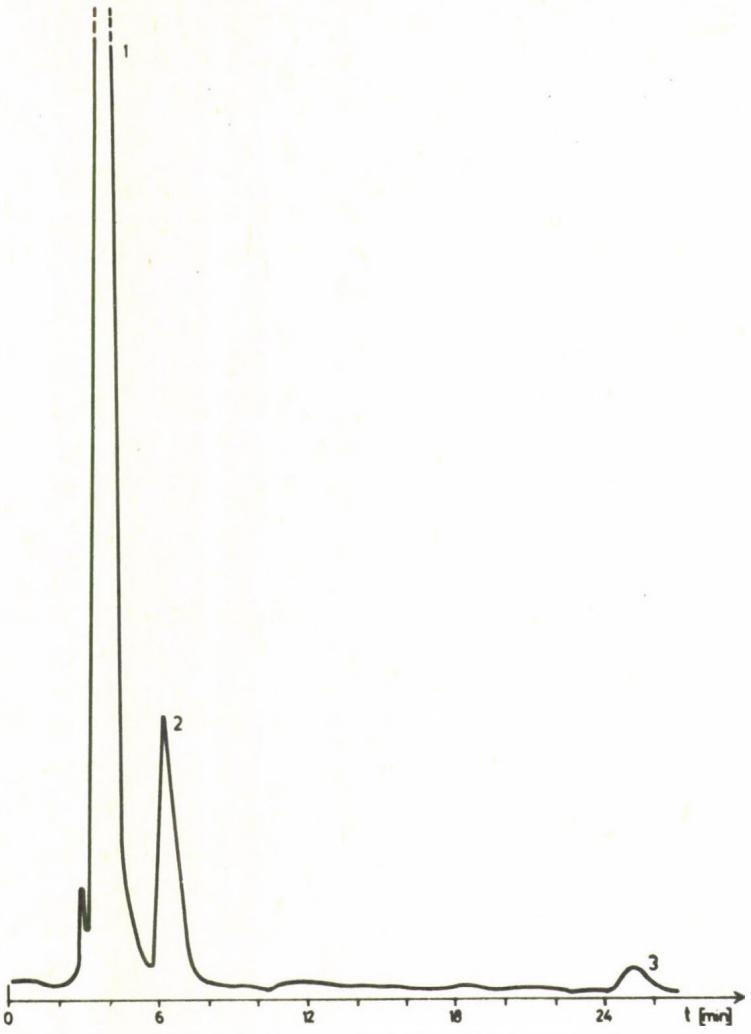

Fig. 3. Chromatogram of Dns-amino acids obtained from poly--lysine. Conditions as described in Fig. 2. Flow rate: 0.8 cm^3/min. 1 = Dns-OH; 2 = Lys(Dns); 3 = Dns-Lys(Dns).

System 1 was applied for the chromatography of the hydrolysates of dansylated poly-lysine (Fig. 3), poly-ε-lysine and two lysine isodipeptides (Table II). The α-peptides gave sharp peaks for ε- and bis Dns-Lys, while the ε-peptides showed sharp peaks for α- and bis-Dns-Lys. Poly-lysine gave a sharp

Table I. Retention times and capacity factors of dansyl amino acid derivatives on ODS-HYPERSIL under the conditions given in Figure 2.

Derivative	Retention time (min)	Capacity factor
Dns-OH	4.2	0
Dns-Ala	5.0	0.19
Dns-Tyr	5.4	0.28
Dns-Lys	6.2	0.48
Dns-Pro	6.8	0.62
Lys(Dns)	8.0	0.90
Dns-Leu	9.6	1.28
Dns-Phe	11.0	1.62
Dns-Lys(Dns)	34.4	7.19

Table II. Applicability of solvent system 1 for the determination of α- and ε-peptide bonds in lysine peptides

Peptides	Peaks			Type of peptide bond
	Dns-Lys	Lys(Dns)	Dns-Lys(Dns)	
Lys - Lys	-	+	+	α
Lys(Lys)	+	-	+	ε
Lys(Phe)	+	-	-	ε
Lys(Gly)	+	-	-	ε
poly(α-Lys)	-	+	(+)	α
poly(ε-Lys)	+	-	(+)	ε

peak for ε-Dns-Lys with a small peak for bis-Dns-Lys while poly-ε-lysine gave a sharp peak for α-Dns-Lys and a small peak for bis-Dns-Lys.

On this basis it may be concluded that this column and solvent system can be used in the structure analysis of lysine

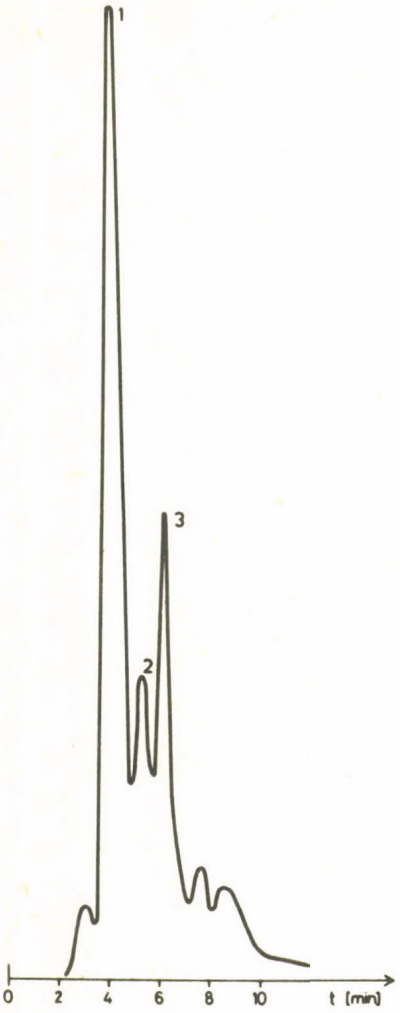

Fig. 4. Chromatogram of Dns-amino acids obtained from poly--Lys-poly-DL-Ala. Conditions as described in Fig. 2. Flow rate: 0.8 cm^3/min. 1 = Dns-OH; 2 = Dns-Ala; 3 = Lys(Dns)

oligo-, poly- and isopeptides (group A), for the determination of the α and ε peptide bonds as shown in Table II. In the case of the chromatography of branched-chain poly-Lys--poly-DL-Ala (AK) polypeptide three different peaks were obtained (Fig.4), but their resolution was not significant enough, presumably because of the presence of a large amount of Dns-OH.

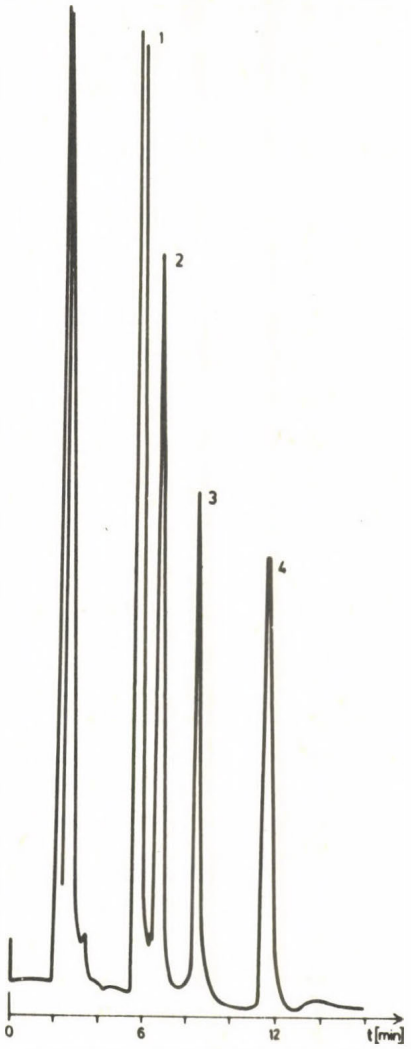

Fig. 5. Chromatogram of dansylated lysines and alanine on Partisil PAC (4.6 mm x 250 mm); solvent system 2: acetonitrile - water - acetic acid (35 : 65 : 1); flow rate: 1 cm^3/min. Detector range: 0.05 A.U.F.S. at 254 nm. 1 = Lys(Dns); 2 = Dns-Lys(Dns); 3 = Dns-Lys; 4 = Dns-Ala.

Good resolution of standard Dns-amino acids and Dns-OH was achieved on Partisil-10 PAC column (10, 14, 15) using acetonitrile - water containing 1% acetic acid (solvent system 2,

Table III. Retention times and capacity factors of dansyl amino acid derivatives and dansyl sulfonic acid (Dns-OH) on Partisil PAC under the conditions given in Figure 6.

Derivative	Retention time (min)	Capacity factor
Lys(Dns)	2.4	0
Dns-Lys(Dns)	4.4	0.83
Dns-Ile	4.8	1.00
Dns-Nle	5.0	1.08
Dns-Leu		
Dns-Val		
Dns-Ala	5.5	1.29
Dns-Pro	6.0	1.50
Dns-Glu	6.5	1.71
Dns-Tyr	6.8	1.83
Dns-OH	13.0	4.42

see Fig. 5) or acetonitrile - water - methanol - acetic acid (solvent system 3, see Table III) as the eluent.

System 3 was used for the qualitative and quantitative analysis of branched-chain poly-Lys--poly-DL-Ala and also for the determination of the N-terminal amino acids of polypeptides belonging to group B and containing the following amino acids at the end of the poly-DL-Ala side chains: Leu, Ile, Nle, Val, Tyr, Pro, Glu. It was found that the addition of acetonitrile to solvent system 3 results in a better resolution of Dns-Ala and Dns-Phe. Therefore system 4 was applied to the HPLC of the hydrolysates belonging to the Phe-containing dansylated branched polypeptides.

From the chromatogram of the hydrolysate of AK-s the proportion of the amounts of Dns-Ala and Lys(Dns) can also be determined. The results show that 90-95 per cent of the total number of Lys residues in poly-lysine were substituted by Ala.

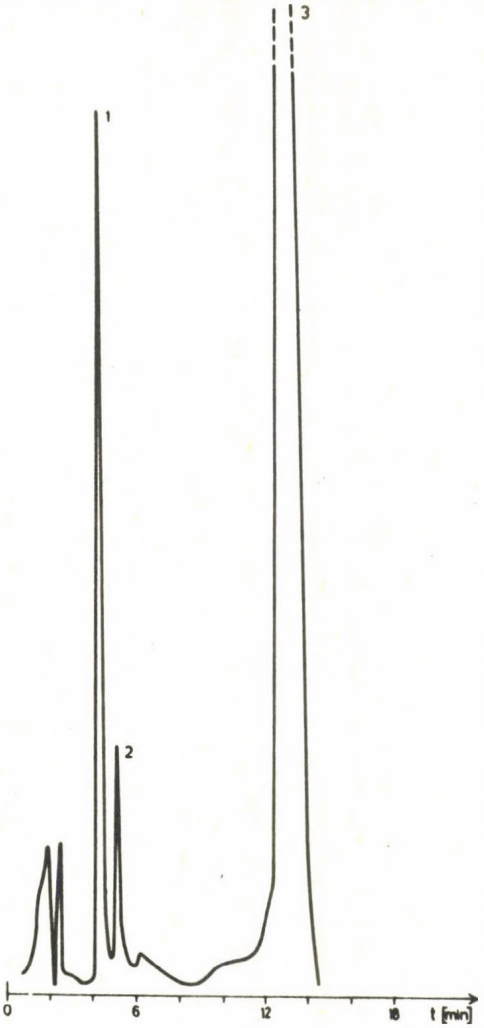

Fig. 6. Chromatogram of Dns-amino acids obtained from poly--Lys--poly-DL-Ala-Leu (LAK) on Partisil PAC (4.6 mm x x 250 mm); solvent system 3: acetonitrile - water - methanol - acetic acid (25:65:10:1); flow rate: 0.8 cm^3/min. Detector range: 0.05 A.U.F.S. at 254 nm. 1 = Dns-Leu; 2 = Dns-Ala; 3 = Dns-OH.

A typical chromatogram of the hydrolysate of dansylated branched polypeptides can be seen in Fig. 6. The Dns-OH peak was separated from the Dns-derivatives and shifted to the end

Table IV. Applicability of solvent system 3 for the determination of amino acid ratios of branched-chain polypeptides in comparison with the data obtained by amino acid analysis

Polypeptide*	HPLC**				Amino acid analysis***			
	Dns-Ala/Dns-X	Lys	: Ala	: X	Lys	: Ala	: X	
LAK 174	0.33	1	3	0.75	1	3	0.7	
IAK 276	0.04	1	3	0.96	1	3	0.95	
NLAK 277	0.09	1	3.1	0.92	1	3.1	0.95	
VAK 278	0.08	1	3	0.93	1	3	0.75	
YAK 152	0.33	1	3.5	0.75	1	3.5	0.8	
PAK 143	1.38	1	3.1	0.42	1	3.1	0.5	
FAK 279	0.11	1	3.1	0.9	1	3.1	0.9	
D-FAK 280	0.04	1	3.1	0.96	1	3.1	0.92	
EAK 268	0.16	1	2.9	0.86	1	2.9	0.81	
D-EAK 274	0.18	1	2.9	0.85	1	2.9	0.87	

* For the identification of the polypeptide see Table V.

** Amino acid ratio obtained by the hydrolysis of the dansylated branched polypeptides followed by the HPLC determination of Dns-Ala/Dns-X.

*** Amino acid ratio obtained by the hydrolysis of branched polypeptides

of the chromatogram, so the large excess od Dns-acid did not interfere with the quantitative evaluation.

Relative quantities of the Dns-amino acids obtained from the N-terminal side chains of branched polypeptides were calculated by measuring the peak areas (Dns-Ala/Dns-X, see Table IV). These data were converted into the ratio of amino acids forming the macromolecules. The amino acid ratios obtained by the HPLC of the dansylated branched polypeptide and

by amino acid analysis are compared in Table IV . As can be seen, results obtained by different methods are in quite good agreement.

ABBREVIATIONS

Letter code	Polypeptide
LAK	poly-Lys-poly-DL-Ala-Leu
IAK	poly-Lys-poly-DL-Ala-Ile
NLAK	poly-Lys-poly-DL-Ala-Nle
VAK	poly-Lys-poly-DL-Ala-Val
YAK	poly-Lys-poly-DL-Ala-Tyr
PAK	poly-Lys-poly-DL-Ala-Pro
FAK	poly-Lys-poly-DL-Ala-Phe
D-FAK	poly-Lys-poly-DL-Ala-D-Phe
EAK	poly-Lys-poly-DL-Ala-Glu
D-EAK	poly-Lys-poly-DL-Ala-D-Glu

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REFERENCES

(1) Sela, M., Mozes, E.: Springer Semin. Immunopathol. 2 119 (1979)

(2) Szekerke, M., Driscoll, J.S.: Europ. J. Cancer 13 529 (1977)

(3) Müller, G.M., Shapiro, I.M., Arnon, R.: Proc. Natl. Acad. Sci. USA 79 569 (1982)

(4) Audibert, F., Jolivet, M., Chedid, L., Arnon, R., Sela, M.: Proc. Natl. Acad. Sci. USA 79 5042 (1982)

(5) Hudecz, F., Szekerke, M.: Coll. Czech. Chem. Commun. 45 933 (1980)

(6) Votavova, H., Hudecz, F., Šponar, J., Blaha, K., Szekerke, M.: Coll. Czech. Chem. Commun. 47 3437 (1982)

(7) Hartley, B.S.: Biochem. J. 119 805 (1970)

(8) Bayer, E., Grom, E., Kaltenegger, B., Uhmann, R.: Anal. Chem. 48 1106 (1976)

(9) Wilkinson, J.M.: J. Chromatogr. Sci. 16 547 (1978)

(10) Hsu, K.T., Currie, B.L.: J. Chromatogr. 166 555 (1978)

(11) Kaneda, N., Sato, M., Yagi, K.: Anal. Biochem. 127 49 (1982)

(12) Grego, B., Hearn, M.T.W.: J. Chromatogr. 255 67 (1983)

(13) Szókán, Gy., Gyenes, M., Tyihák, E., Szende, B.: Proc. of 17th Eur. Peptide Symp. (1982) Prague (in press)

(14) Szókán, Gy.: J. Liquid Chromatogr. 5 1493 (1982)

(15) Szókán, Gy., Pintér, Gy.: Proc. of 17th Eur. Peptide Symp. (1982) Prague (in press)

Chromatography, the State of the Art
H. Kalász and L.S. Ettre (Eds)

REVERSE PHASE HPLC OF HYDROPHOBIC AND HYDROPHILIC MODIFIED PEPTIDE HORMONES

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ABSTRACT

We have synthetized 23 vasopressin, cholecystokinin, gastrin and gonadotropic releasing hormone analogoues. A very mild and rapid method was used for preparative purification of labile peptides loading the reaction mixture directly onto a preparative HPLC column. We found a good correlation between the hydrophobicity and the retention time of synthetic peptide hormone analogues using the same chromatographic conditions.

INTRODUCTION

High-performance liquid chromatography /HPLC/ is a revolutionary method for the isolation and purification of peptides /1/. This method is very versatile, and it is suitable for the analysis and purification of both hydrophobic /protected, iodinatid/ and hydrophilic /free or sulfonated/ peptide using various adsorbents and solvent systems /2 , 3/. Rapid and perfect separation, mild chromatographic conditions, high sensitivity and good reproducibility characterize this method. It is the method of choice for the isolation and purification of labile peptides where the other classical methods are not successful /4/. Our aims were to work out the separation conditions necessary for analytical purity control of various peptide hormones we synthesized, and to purify these synthetic peptide hormone analogues in preparative scale /to gram quantities/.

PEPTIDE SYNTHESIS

All peptide hormones and analogues were synthesized in our laboratory. Arginine-vasopressin /AVP/ was synthesized by the original Merrifield method /5/. Cholecystokinin octapeptide

sulphate ester or the non-sulphated form and cholecystokinin analogues with compound number 15 and 16 were synthesized by the conventional solution method /7, 8/. Hexagastrin, GnRH, their analogues and the other CCK analogues were prepared on solid phase by the Rivier method /9/. The structures of synthesized peptides are shown in Table I.

APPARATUS AND METHODS

The instruments used consisted of a Varian LC-5000 liquid chromatograph coupled with a Waters WISP automatic injector, LKB 2151 Variable Wavelength Monitor and Varian Vista CDS 401 data system, or a Beckman 100A pump coupled with a Waters U6K injector, Waters 440 absorbance detector and Infotronics 110 integrator. For preparative scale purification the instrument used was a Waters Model LPC 500A liquid chromatograph. The columns used were the following:

a./ Lichrosorb RP18 10 µm 4.6 x 250 mm, Knauer
b./ Lichrosorb C 18 10 µm 4.6 x 250 mm, Vydac
c./ Nucleosil 5 C 18 5 µm 4.6 x 250 mm, Chrompack
d./ Lichroprep C 18 30 µm 9.0 x 250 mm, Vydac
/for semipreparative use/
e./ Plastic cartridge Lichroprep C 18 10 µm
60 x 300 mm, Vydac
/preparative use only/

The elutions were effected in the following solvent system:

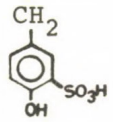
	SOLVENT A	SOLVENT B
a./	NH_4OAc 0.1 M pH 6.5	60 % Acetonitrile in solvent a.
b./	Trifluoracetic Acid /TFA/ 0.1 %	Acetonitrile
c./	Triethylammonium-phosphate /TEAP/ 0.25 M pH 6.3	Acetonitrile
d./	NH_4OAc 0.01 M pH 4	Methanol
e./	TEAP 0.25 M pH 3	Methanol
f./	NH_4OAc 0.01 M pH 4	Acetonitrile,
g./	TFA 0.09 %	90 % Acetonitrile, 9.91 % H_2O, 0.09 % TFA

Table I. The Structure of Peptides

1. H-CYS-TYR-PHE-GLN-ASN-CYS-PRO-ARG-GLY-NH_2 /AVP/
2. 2-IODO-TYR-AVP
3. 2-DIIODO-TYR-AVP
4. 2-/3-SULFO-TYR/-AVP
5. H-ASP-TYR/SO_3H/-MET-GLY-TRP-MET-ASP-PHE-NH_2 /CCK 8 SE/
6. H-ASP-TYR-MET-GLY-TRP-MET-ASP-PHE-NH_2 /CCK 8 NSE/
7. H-ASP-TYR/I_2/-MET-GLY-TRP-MET-ASP-PHE-NH_2
8. AC-TYR/SO_3H/-MET-GLY-TRP-MET-SER/SO_3H/-PHE-NH_2
9. AC-TYR-MET-GLY-TRP-MET-SER/SO_3H/-PHE-NH_2
10. AC-TYR/SO_3H/-MET-GLY-TRP-MET-THR/SO_3H/-PHE-NH_2
11. AC-TYR-MET-GLY-TRP-MET-THR/SO_3H/-PHE-NH_2
12. AC-TYR/SO_3H/-MET-GLY-TRP-MET-HYP/SO_3H/-PHE-NH_2
13. AC-TYR-MET-GLY-TRP-MET-HYP/SO_3H/-PHE-NH_2
14. AC-TYR/SO_3H/-MET-D-ALA-TRP-MET-ASP-PHE-NH_2
15. BOC-PHE/SO_3H/-MET-GLY-TRP-MET-ASP-PHE-NH_2
16. H-D-TYR-ASP-TYR/SO_3H/-MET-GLY-TRP-MET-ASP-PHE-NH_2
17. GLP-GLN-ASP-TYR-THR/AC/-GLY-TEP-MET-ASP-PHE-NH_2
18. GLP-GLN-ASP-TYR/SO_3H/-THR-GLY-TRP-MET-ASP-PHE-NH_2 /CAERULEIN/
19. H-TYR-GLY-TRP-MET-ASP-PHE-NH_2 /HEXAGASTRIN/
20. H-TYR/SO_3H/-GLY-TRP-MET-ASP-PHE-NH_2
21. GLP-HIS-TRP-SER-TYR-GLY-LEU-ARG-PRO-GLY-NH_2 /GnRH/
22. GLP-HIS-TRP-SER/SO_3H/-TYR-GLY-LEU-ARG-PRO-GLY-NH_2
23. GLP-HIS-TRP-SER-TYR/I_2/-GLY-LEU-AFG-PRO-GLY-NH_2

H_2N - CH - COOH

3-SULFO-TYR =



Methanol and acetonitrile were used without any purification as supplied by Merck. Water was glass-distilled and deionized. The prepared buffers were filtered through a 0.45 μm Millipore HA filter. The samples were also filtered before injection. Loading capacity was 1 μg to 1 mg in analytical columns 1 mg to 50 mg in the semipreparative column and from 50 mg to several grams in the preparative column.

RESULTS AND DISCUSSION

1. Vasopressin and its analogues

We chromatographed these peptides in 0.01 M ammonium acetate buffer isocratically, with methanol or acetonitrile. The pH value was 4. The same amount of methanol means about 50 % longer retention time /t_R/ than acetonitrile. The iodine incorporation resulted in a marked increase in the retention times owing to the hydrophobicity of the iodo group/s/. The sulfonation of AVP decreased the retention time by about 50 percent, due to the smaller hydrophobicity of the tyrosine residue. The retention times are listed in Table II.

Table II Chromatography of Vasopressin and its Analogues[x]

Compound	Retention time, min	Solvent system
vasopressin /AVP/	6.6	f /50%/
vasopressin /AVP/	7.3	d /60%/
vasopressin /AVP/	10.0	d /50%/
vasopressin /AVP/	12.9	f /25%/
2- monoiodo-Tyr AVP	15.6	f /25%/
2- diiodo-Tyr AVP	21.5	f /25%/
2- sulfo-Tyr AVP	4.6	d /50%/
2- sulfo-Tyr AVP	6.4	d /40%/

[x] Column: Lichrosorb RP 18 10 μm, 4.6 x 250 mm.
Flow rate: 1.5 ml/min

2. Acetyl CCK heptapeptide analogues

These peptides were chromatographed with 0.1 M ammonium acetate buffer, pH 6.3 /A component/. The B component was 60 % acetonitrile in 0.1 M ammonium acetate pH 6.3. We chromatographed with gradient elution from 5 % to 95 % B component in 20 minutes. The retention time values are listed in Table III.

Table III Chromatography of Acetyl CCK Heptapeptide Analogues[x]

Compound	Retention time, min	Solvent system
Ac-[Tyr^2,Ser/SO_3H/^{7}] CCK/2-8/	12.8	a
Ac-[Tyr/SO_3H/^{2},Ser/SO_3H/^{7}] CCK/2-8/	14.2	a
Ac-[Tyr/SO_3H/^{2}, Thr/SO_3H/^{7}] CCK/2-8/	13.0	a
Ac-[Tyr^2,Thr/SO_3H/^{7}]CCK/2-8/	14.3	a
Ac-[Tyr/SO_3H/^{2}, Hyp/SO_3H/^{7}] CCK/2-8/	12.5	a
Ac-[Tyr^2, Hyp/SO_3H/^{7}] CCK/2-8/	13.5	a
Ac-[Tyr/SO_3H/^{2}, D-Ala^4]CCK/2-8/	14.3	a

[x] Column: Lichrosorb C17 10 µm, 4.6 x 250 mm.
Flow rate: 2.0 ml/min.

3. Cholecystokinin octapeptides and analogues

We characterised the natural octapeptide sulphate ester in five different solvent systems. The R_f values strongly depend on the organic solvent, its concentration and the buffer ion concentration. The non-sulphated peptide has a higher retention time value because this peptide is more hydrophobic. The iodine incorporation into the non-sulphated form of cholecystokinin made reverse-phase HPLC impossible: it is practically insoluble in water or water containing organic solvents. The p-sulfo Phe containing analogue runs near the octapeptide under the same conditions. The retention time of the nonapeptide analogue with D-tyrosine in the N terminal position dif-

fers in three slightly modified solvent systems. It strongly depends on the concentration of the organic solvent. The chromatography was carried out isocratically with 22, 25, 30 % B solvent. The retention time values are listed in Table IV.

4. Caerulein

The two analogues of caerulein were chromatographed in 0.1 M ammonium acetate, pH 6.3 with acetonitrile. The B component was 60 % acetonitrile in ammonium acetate buffer. The gradient was 5 % B to 95 % B in 20 minutes, flow rate 2.0 ml/min. The retention time values are listed in Table V.

Table IV Chromatography of Cholecystokinin Octapeptides and Analogues[x]

Compound	Retention time, min	Solvent system
cholecystokinin, sulphated	4.8	f /25%/
cholecystokinin, sulphated	4.1	d /50%/
cholecystokinin, sulphated	28.5	e xx
cholecystokinin, sulphated	10.3	c /25%/
cholecystokinin, nonsulphated	12.6	c /25%/
1-[BOC-p-sulfo-Phe] CCK/2-8/	9.4	c /25%/
H-D-Tyr-CCK/1-8/, sulphated	30.9	c /22%/
H-D-Tyr-CCK/1-8/, sulphated	12.4	c /25%/
H-D-Tyr-CCK/1-8/, sulphated	6.7	c /30%/

x Column: Lichrosorb RP 18 10 µm, 4.6 x 250 mm.
Flow rate: 1.5 ml/min.

xx The /e/ system was a gradient, 30% → 80% in 25 minutes.

Table V Chromatography of Caerulein[x]

Compound	Retention time, min	Solvent system
5-[Thr/Ac/] caerulein, non-sulphated	8.5	a
Caerulein	8.0	a

[x] Column: Lichrosorb C18, 10 µm, 4.6 x 250 mm

5. Hexagastrin and its sulphate ester

These peptides were run in 0.25 M triethylammonium phosphate buffer /pH 6.3/ with 25 % acetonitrile. The elution was carried out isocratically, the flow rate was 0.1 ml/min. The retention time values are listed in Table VI.

In this case the sulphate ester incorporation resulted a slight difference in the retention time. The less hydrophobicity of hexagastrin sulphate ester led to a 2.3 minutes decrease in the retention time.

Table VI Chromatography of Hexagastrin[x]

Compound	Retention time, min	Solvent system
Hexagastrin	15.8	c/25%/
1-[Tyr/SO_3H/] hexagastrin	13.5	c/25%/

[x] Column: Nucleosil 5C18 5 um, 4.6 x 250 mm

6. Gonadotrop releasing hormone /GnRH/ and its analogues

The GnRH analogues were chromatographed in ammonium acetate buffer /pH 6.3; 0.1 M/. The B component was 60 % acetonitrile in the same buffer. Gradient elution was made from 5 % to 95 % B in 30 minutes. The sulfatisation of the serine residue made a very little change in the retention time, but the iodine incorporation caused a marked increasing in the retention time /Table VII/.

Table VII Chromatography of Gonadotrop-releasing Hormone[x]

Compound	Retention time, min	Solvent system
GnRH	15.8	a
5-[diiodo-Tyr] GnRH	29.2	a
4-[Ser/SO_3H/] GnRH	16.3	a

[x] Column: Lichorosorb C18, 10 um, 4.6 x 250 mm.
Flow rate: 2.0 ml/min.

Preparative purification of synthetic peptides

Most of our peptides were very labile towards oxidation, so in order to hinder peptide decomposition and side reactions caused by light and elevated temperatures the following special method was used. The crude reaction mixture /in a water-miscible organic solvent/ was diluted with water to 10-100 volumes. The pH was adjusted to the appropriate value with NaOH, NH_4OH or acetic acid. The solution was filtered and loaded directly onto the preparative column. Gradient elution was applied in solvent system a b or c. Fractions were rapidly controlled on an analytical column in an isocratic solvent system. Pure fractions were pooled and lyophilised. Buffers with ammoniumacetate content were lyophilised twice, triethylammonium phosphate was changed to solvent system c /trifluoroacetic acid/ on another preparative reverse-phase HPLC column before lyophilisation.

Most peptide syntheses give impure products. Generally purification is impossible using classical methods. For example analytical HPLC chromatograms of two synthetic paptide hormones before preparative HPLC purification are shown in Fig. 1. There are about 10-15 % of impurities present with the main product. After preparative purification the analytical control of the appropriate fractions is shown in Fig. 2. The HPLC of the resulting pure peptides is shown in Fig. 3.

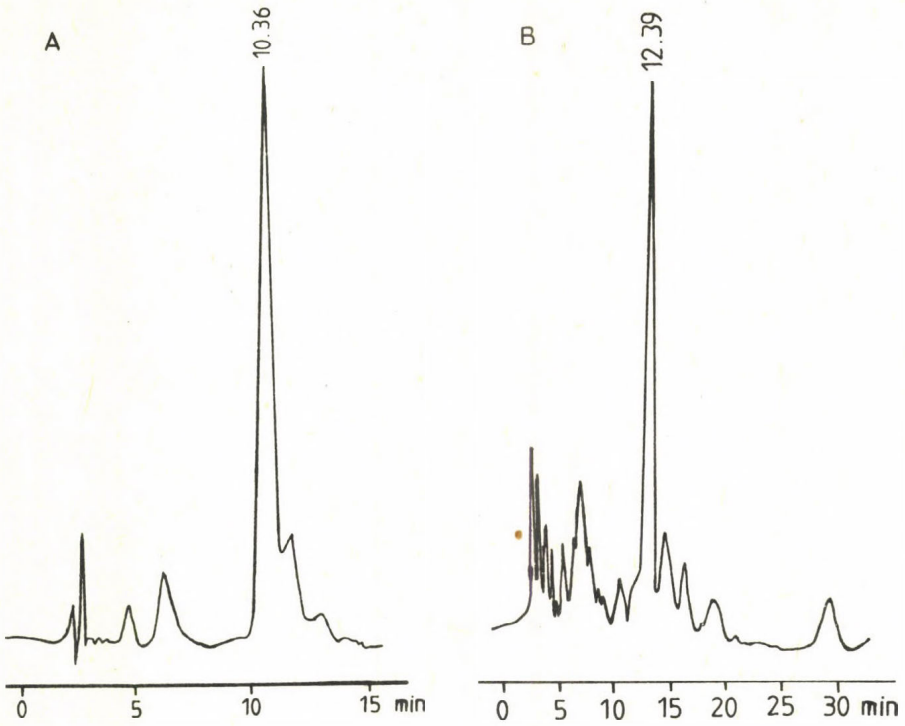


Fig. 1. Analytical HPLC chromatograms from the crude product of cholecystokinin octapeptide /A/ and compound 16 /B/. Lichrosorb RP-18 column /10µm, 4.6 x 250 mm/, solvent sytem: 75% TEAP /0.25M,pH 6.3/ - 25% acetonitrile, flow rate 1.5 ml/min

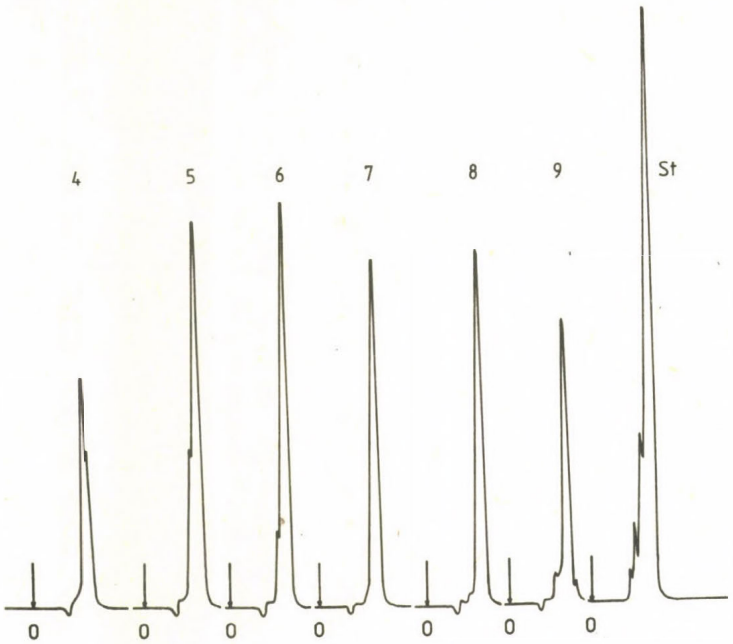


Fig. 2. Analytical control of the fractions /4-9/ of the preparative HPLC peak of caerulein. Pure peptide eluted in fractions 7-8. St standard caerulein obtained from Serono Company. Lichrosorb C-18 column /10 μ, 4.6 x 250 mm/, solvent system: 80% NH_4OAc /0.1 M, pH 6.3/ - 20% acetonitrile, flow rate 1.5 ml/min

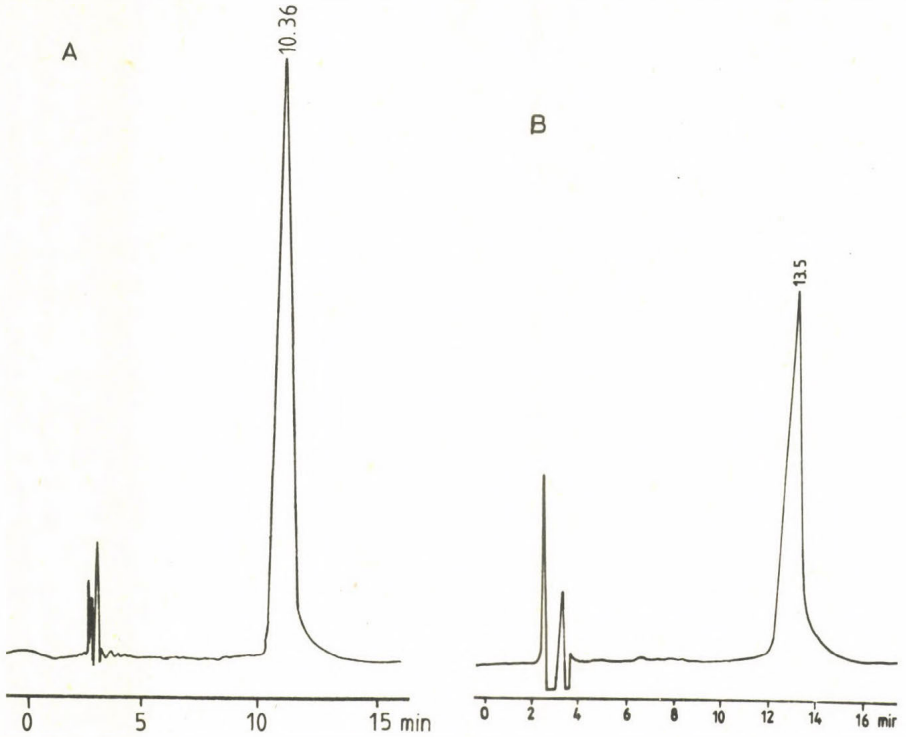


Fig. 3. Checking of the purity of HPLC purified cholecystokinin octapeptide /A/ /chromatografic system as Fig. 1./ and hexagastrin sulphate ester /B/ /Nucleosil 5C18 column /5µm, 4.6 x 250 mm/, solvent system: 75% TEAP /0.25 M, pH 6.3/ - 25% acetonitrile, flow rate 1.0 ml/min/

REFERENCES

1. Burgus, R., Rivier, J., Peptides 1976: Proc. of 14th Eur. Peptide Symp, ed. Loffet, A., l'Université de Bruxelles, Bruxelles, 1976, p. 85.
2. Gabriel, T.F., Jimenez, M.H., Felix, A.M., Michalewsky, J., Meienhofer, J., Int. J. Pept. Prot. Res. 9, 129, /1977/.
3. Rivier, J.R., J. Liq. Chrom. 1, 343, /1978/.
4. Scarso, A., Brison, J., Durieux, J.P., Loffet, A., Peptides 1980: Proc. of 16th Eur. Peptide Symp, ed. Brunfeldt, K., Scriptor, Copenhague, 1981, p. 321.

5. Merrifield, R.B., J. Amer. Chem. Soc. 85, 2149 /1963/.
6. Janáky, T., Roy, J., Morgat, J.L., László, F.A., J. Label. Comp. submitted for publication.
7. Penke, B., Tóth, G., Zarándy, M., Nagy, A., Kovács, K., Peptides 1980: Proc. of 16th Eur. Peptide Symp, ed. Brunfeldt, K., Scriptor, Copenhague, 1981, p. 253.
8. Penke, B., Zarándy, M., Tóth, G., Kovács, K., Fekete, M., Telegdy, G., Pham, P., Peptides 1982: Proc. of 17th Eur. Peptide Symp., eds. Bláha, K., Malon, P., W. de Gruyter, Berlin-New York, 1983, p. 569.
9. Rivier, J., Kaiser, R., Galyean, R., Biopolymers, 17, 1927, /1978/.

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APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE RAPID ESTIMATION OF THE MOLECULAR WEIGHTS OF PROTEINS

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Since the introduction of gel filtration, or gel exclusion chromatography, by Porath and Flodin (1) in 1959, this technique has been rapidly and widely accepted in the field of biochemistry as a powerful and versatile method for the separation of proteins and other biopolymers according to their size. However, an ambiguous word "size" does not generally mean or parallel with the protein molecular weight, and actually corresponds to the effective hydrodynamic volume of proteins in this case. Therefore, approaches to make use of gel filtration as a means of estimating the molecular weight of proteins were unsuccessful until Fish, Mann and Tanford (2), as well as others, measured the elution volume of reduced and alkylated proteins in 6 M guanidine hydrochloride. However, in spite of the theoretical uniqueness, this method has not become popular, since conventional gel filtration using soft gel matrices requires too long a time, namely one or two days, for the completion of a single run and lacks sufficient resolving power.

Recent development of rigid silica gel-based matrices suitable for HPLC seems to have overcome these difficulties in conventional

gel filtration. By using them, we have shown that a single analysis can be completed within 50 min or even less. The resolving power as well as the reproducibility and sensitivity has revealed that the present procedure is suitable for the rapid estimation of the molecular weights of protein polypeptide chains (3, 4).

THEORETICAL CONSIDERATIONS

Gel filtration is a process of partition between the mobile solvent outside the gel and the solvent inside the pores. The distribution coefficient, K_d, can be expressed as follows:

$$K_d = (V_e - V_o)/(V_t - V_o) \qquad [1]$$

where V_e is the elution volume of proteins, which can be measured from the distance between the point of sample application and the location of each protein peak on the recorder chart. V_o and V_t are the void volume and the total available volume of the column, respectively.

At first, let us consider a simple protein constituting of amino acid residues alone. In concentrated solutions of guanidine hydrochloride, all the proteins, if their disulfide bonds have been disrupted, are dissociated into constituent polypeptide chains. These polypeptide chains behave hydrodynamically as randomly coiled linear homopolymers, whose radius of gyration, R_G, is a simple function of the number of amino acid residues in the chain, and thus of the molecular weight, M. This relationship is expressed by the simple empirical equation derived by Tanford and his associates [cf. (2)],

$$R_G = a\, M^{0.555} \qquad [2]$$

where a is a constant.

The radius of gyration is known to be directly proportional to the Stokes radius, R_e, which is the radius of a sphere of equivalent hydrodynamic properties. Equations relating K_d and R_e have been proposed by some investigators on the basis of theoretical treatment of gel filtration. If we take the simple equation of Porath (5), namely

$$K_d^{1/3} = A - B\, R_e \qquad [3]$$

where A and B are constants, the relationship between R_e and M is given by the following equation

$$K_d^{1/3} = A - B'\, M^{0.555} \qquad [4]$$

where B' is a constant. Therefore, if we plot $K_d^{1/3}$ against $M^{0.555}$, a linear curve should be obtained, as will be shown later in our experiments.

In the case of glycoproteins, proteins which are widely distributed in nature and known to have various important biological functions, the situation may not be as simple as that of simple proteins not containing carbohydrate. Reduction of disulfide bonds in glycoproteins gives branched heteropolymers, instead of linear homopolymers, in concentrated guanidine hydrochloride solutions. Since no theory except for branched homopolymers (6, 7) has been available, it would be important at the present stage to know the extent of deviation of the chromatographic behavior of glycoproteins from that of simple proteins.

MATERIALS AND METHODS

Proteins, except for those lacking disulfide bonds, were dissolved in 6 M guanidine hydrochloride containing 1 M Tris-HCl buffer, pH 8.0, and 2 mM EDTA and reduced by dithiothreitol added in at least 5 molar excess of the amount required theoretically. The mixture was allowed to stand at 22°C for 2 h and the resultant SH groups were carboxymethylated by the addition of 2 molar equivalents of iodoacetamide (pH 8.0). After incubation for 20 min, the mixture was dialyzed against 6 M guanidine hydrochloride containing 10 mM phosphate buffer, pH 6.5, and 1 mM EDTA.

For high-speed gel filtration experiments, a column (usually 60 X 0.75 cm) of TSK-GEL G 3000 SW or G 4000 SW, packed with spherical silica gel chemically bonded with hydrophilic compounds, was obtained from Toyo Soda Mfg. Company, Tokyo. Before use, the column was thoroughly washed and equilibrated with the medium of 6 M guanidine hydrochloride containing 10 mM phosphate buffer, pH 6.5, and 1 mM EDTA, which was used for the preparation of samples. Gel filtration was carried out in a Hitachi 635 high-performance liquid chromatograph equipped with a Hitachi 034 double-beam effluent monitor. The flow rate was fixed at 0.5 ml/min and the absorbance at 280 nm of the effluent was recorded. The sample size for each protein was usually 50 μg or less.

RESULTS AND DISCUSSION

Simple proteins --- When high-speed gel filtration was carried out in 6 M guanidine hydrochloride using a column of silica gel-based support, G 3000 SW or G 4000 SW, the results obtained were satisfactory. The flow rate could be increased up to 1.5 ml/min, but with some sacrifice of resolution. Therefore, in most experiments, a flow rate was fixed at 0.5 ml/min. The total time required for a single run using a column of 60 cm long is only 50 min. At present, we are testing short columns (30 cm) packed with smaller spherical particles. In this case, the time required is a half and the resolution even higher than that of 60 cm-columns has been obtained.

A typical elution profile obtained with an artificial mixture of protein polypeptide chains using a G 3000 SW column (60 cm) is shown in Fig. 1. It is seen that α-chymotrypsinogen A (M, 25,700), soybean trypsin inhibitor (M, 20,100), ribonuclease A (M, 13,700) and histone H4 (M, 11,300) are almost completely separated from each other, indicating that the resolving power is much higher than that found in conventional gel filtration using soft gel matrices.

To test the validity of Eq.[4], a number of proteins with known molecular weights were reduced and alkylated, except for those not containing a disulfide bond, and then subjected to high-speed gel filtration. Reproducibility was confirmed by the finding that the standard deviation of elution volumes was approximately 0.5 % of the mean values.

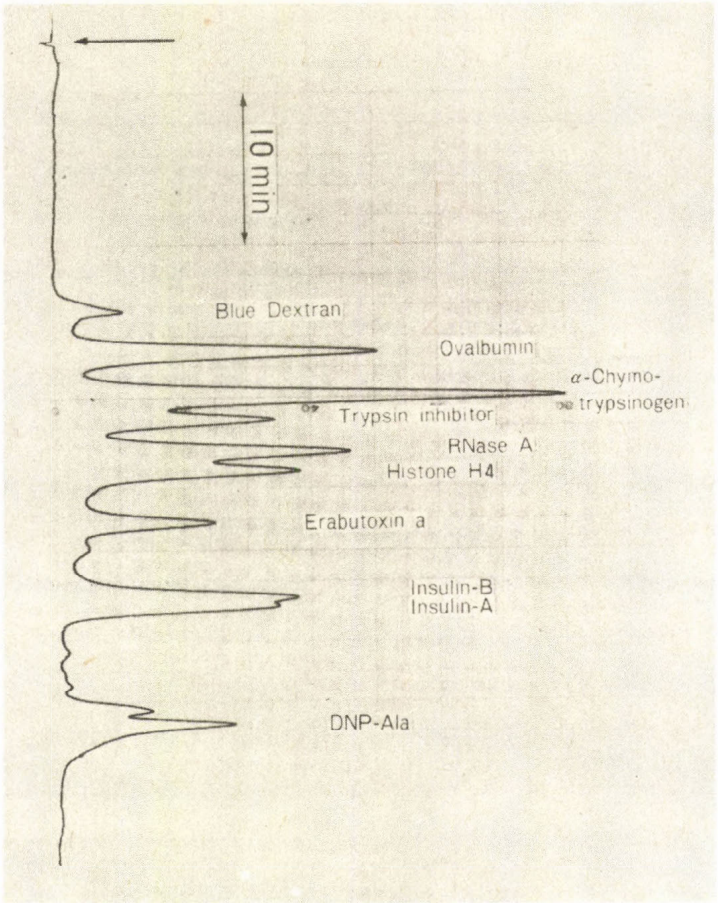

Fig. 1. High-speed gel filtration profile of protein polypeptide chains in the presence of 6 M guanidine hydrochloride obtained with a column of G 3000 SW (60 X 0.75 cm). The arrow at the left represents the position of time of sample application. The flow rate was 0.5 ml/min. Absorbance at 280 nm was recorded at an absorbance full scale of 0.08.

Figure 2 shows a curve obtained when $M^{0.555}$ was plotted as a function of $K_d^{1/3}$ calculated from the data for a column of G 3000 SW. The linear relationship is apparent. A similar result was obtained also with a G 4000 SW column capable of measuring proteins with higher molecular weights.

Using a standard curve as shown in Fig. 2, the molecular weight of protein polypeptide chains in question can be estimated easily and accurately. It is also to be noted that, owing to its rapidity and high resolution, the present method is suitable for the study of subunit structure of proteins. This has been shown by our work on adenosine triphosphatase prepared from thermophilic bacteria (8).

Glycoproteins --- As discussed previously, glycoproteins give branched heteropolymers, instead of linear homopolymers, on reduction of disulfide bonds. Therefore, behavior of reduced glycoproteins in high-speed gel filtration in 6 M guanidine hydrochloride would not be the same as that of simple proteins.

To study the extent of deviation, the elution volume of 11 reduced-alkylated glycoproteins with known molecular weights were measured in a G 3000 SW column. Using a standard curve prepared with proteins not containing carbohydrate, an apparent molecular weight of each glycoprotein was calculated and compared with the actual value.

As shown in Fig. 3, the gel filtration behavior of the most of

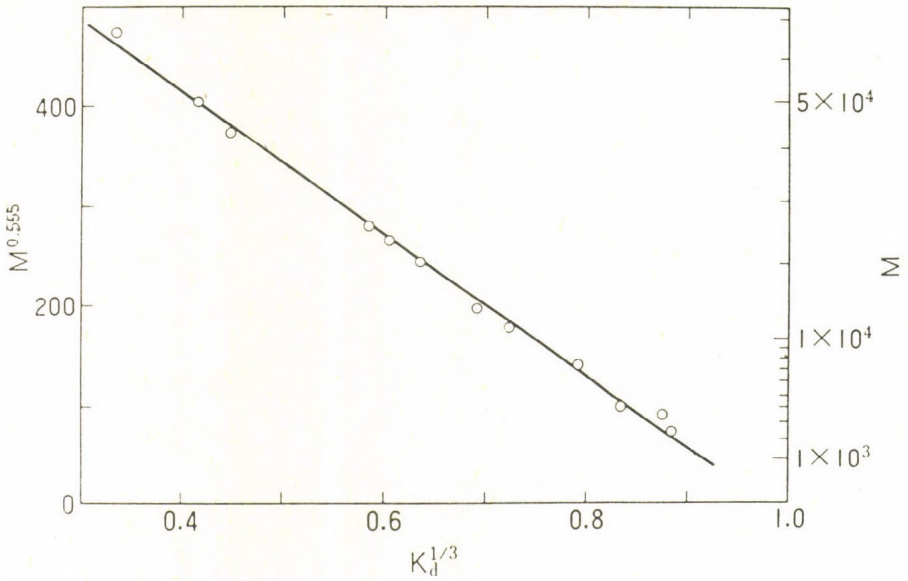

Fig. 2. Calibration curve for high-speed gel filtration in 6 M guanidine hydrochloride of protein polypeptide chains using a G 3000 SW column. Proteins used are bovine serum albumin (M = 66,300), bovine γ-globulin, H chain (51,500), ovalbumin (43,000), α-chymotrypsinogen A (25,700), bovine γ-globulin L chain (23,500), soybean trypsin inhibitor (20,100), bovine ribonuclease A (13,700), histone H4 (11,300), erabutoxin a (7,430), clupein (4,110), insulin B chain (3,420) and insulin A chain (2,380), in the order of decreasing molecular weight. See text for details.

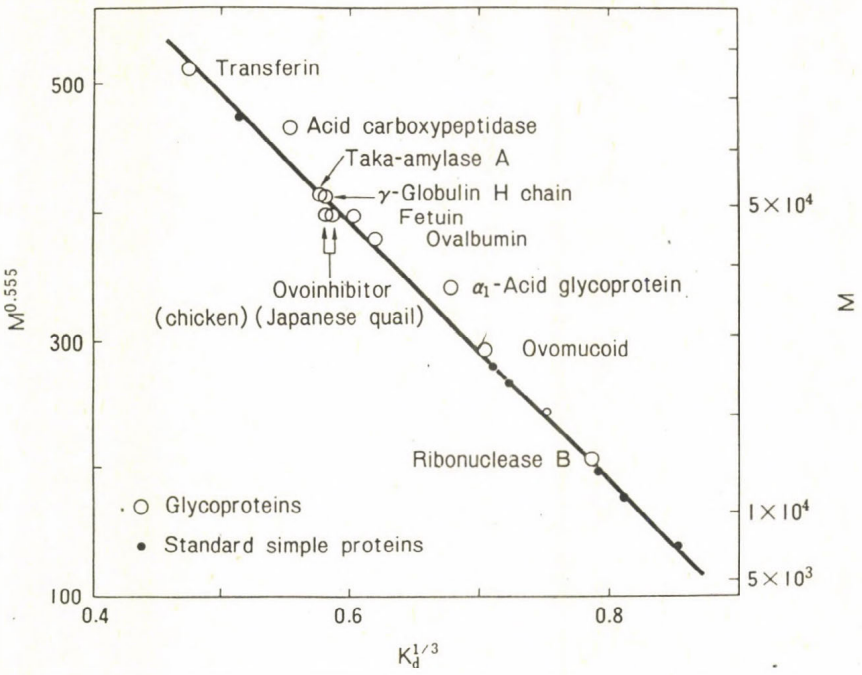

Fig. 3. Calibration plot for high-speed gel filtration in 6 M guanidine hydrochloride of glycopolypeptides (open circles). The straight line was drawn using the data for simple protein polypeptides (closed circles) so that the extent of deviation with glycopeptides can be easily observed.

branched-chain glycopolypeptides studied did not deviate appreciably from that of the linear polypeptides obtained from simple proteins. Particularly, in the case of glycoproteins with a carbohydrate content of less than 10 %, the apparent and actual molecular weights coincided within the limits of experimental error and the uncertainty in the estimated "actual" value.

However, two carbohydrate-rich glycopolypeptides studied, α_1-acid glycoprotein and acid carboxypeptidase, with 41.3 % and 21.6 % carbohydrate contents, respectively, gave appreciably underestimated apparent molecular weights (-13.7 % and -10.0 %, respectively). It was also noticed that, although the carbohydrate content of fetuin and ovomucoid is similar to that of acid carboxypeptidase, the deviation was not as high as that of the latter protein having a higher molecular weight. Hence, further study is needed to see the effect of branched carbohydrate chains on the gel filtration behavior of glycoproteins.

Recently, we studied the gel filtration behavior of standard preparations of linear polysaccharide, pullulan, which are poly(maltotriose) of bacterial origin, with different molecular weights. When compared with the linear calibration curve obtained with several simple proteins, data for pullulan did not fit the curve well. The deviation of the apparent molecular weight was especially evident at the high molecular weight range (unpublished observation).

HPLC combined with low angle light scattering --- For proteins for which reliable molecular weight is hard to be obtained by HPLC alone, such as highly carbohydrate-rich proteins, what can we do? Recent studies have shown that low angle laser light scattering in combination with HPLC seems to be promising (9).

Thyroglobulin is a protein which plays an important role in the biosynthesis of thyroid hormones. This protein has a molecular weight as high as 670,000 and contains approximately 10 % of carbohydrates. If thyroglobulin is dissolved in 6 M guanidine hydrochloride, it partially dissociates into half-sized subunits, and the rest is resistant to dissociation. On reductive cleavage of disulfide bonds in thyroglobulin, no simple pattern was obtained in either HPLC or SDS-polyacrylamide gel electrophoresis. This has brought about long-lasting debates concerning the size of the elementary polypeptide chain(s) constituting thyroglobulin. In cooperation with Dr. Takagi of the Institute for Protein Research, Osaka University, we adopted low-angle laser light scattering technique in combination with high-speed gel filtration and have been able to confirm that the molecular weight of the major constituent polypeptide chains is 330,000 (10).

CONCLUSION

In conclusion, the present method of high-speed gel filtration has proved useful for the rapid estimation of the molecular weights not only of simple proteins but also of glycoproteins. It seems that

this method is a superior substitute for SDS-polyacrylamide gel electrophoresis, a method now quite popular for the same purpose, although further improvement in resolving power of high-speed gel filtration remains to be explored.

REFERENCES

1. Porath, J. & Flodin, P. (1959) Nature 183, 1657-1659.
2. Fish, W.W., Mann, K.G., & Tanford, C. (1969) J. Biol. Chem. 244, 4989-4994.
3. Ui, N. (1979) Anal. Biochem. 97, 65-71.
4. Ui, N. (1981) J. Chromatogr. 215, 289-294.
5. Porath, J. (1963) Pure Appl. Chem. 6, 233-244.
6. Casassa, E.F. & Berry, G.C. (1966) J. Polym. Sci. A-2, 881-897.
7. Berry, G.C. (1968) J. Polym. Sci. (1968) A-2, 1551-1554.
8. Yoshida, M., Sone, N., Hirata, H., Kagawa, Y. & Ui, N. (1979), J. Biol. Chem. 254, 9525-9533.
9. Takagi, T. (1981) J. Biochem. 89, 363-368.
10. Ui, N. (1980) In "Thyroid Research VIII" (Stockigt, J.R. & Nagataki, S., eds.), p. 182-185, Australian Acad. Sci.

Chromatography, the State of the Art
H. Kalász and L.S. Ettre (Eds)

CHROMATOGRAPHIC AND FUNCTIONAL ANALYSIS OF COMPLEMENT PROTEINS IN α-INTERFERON PREPARATIONS

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SUMMARY

The α-interferon preparations (salt precipitation and gel filtration) with $3 \cdot 10^5$ IU/mg activity contained approximately 0.6 μg hemolytically active factor $\bar{D}$, one of the basic enzymes of complementary alternative pathway. C3, C5 and B complementary components were not found in these preparations. An original method was developed for the purification of factor $\bar{D}$. Factor $\bar{D}$ isolated by this method with a yield of 40% was homogeneous in SDS-PAG electrophoresis and was 125,000 times purer. Antiviral activity of the interferon was retained after diisopropylfluorophosphate treatment. Individual factor $\bar{D}$ did not possess antiviral activity. The possible role of factor $\bar{D}$ in antitumor activity of interferon preparations is discussed.

INTRODUCTION

Interferon preparations are known to have a wide mode of action both in vivo (antiviral /1/ and antiproliferative /2/ effects) and in vitro (macrophage activation /3/, influence on antibody synthesis /4/, etc.). Interferon content in treatment preparations usually count approximately 0.1-5.0% (10^6 - $5 \cdot 10^7$ IU/mg of protein /5,6/). These drugs also contain such contaminats as lymphokines, cytokines, monokines /7,8/, human serum proteins /9/, several enzymes produced by macrophages /10/

together with low-molecular weight compounds and salts introduced at different stages of the drug production.

At present time it seems, therefore, impossible to determine biological properties of interferon as an individual compound and the possible explanations of its mechanism of action have to take into account the probable influence of contaminants /11/.

The problem of individual interferon action must apparently be solved with the development of its production by means of recombinant DNA methods and purification using monoclonal antibodies to some epitopes of recombinant molecule.

At present the majority of researchers consider antiviral action of interferon preparations to be determined strictly by the interferon molecules /12/, but at the same time it is not clear yet if antitumor interferon effect is caused by pure interferon or if it is related to the contaminants /13,14/.

Production method analysis of α-interferon to specific activity ~ 10^5 IU/mg of protein which we have developed, revealed the possibility of the presence of $\bar{D}$-factor, the key component of complement alternative pathway activation, which can play some role in antitumor activity of the drug. Accordingly the goal of our research was to analyse the contents of separate complement components (notably $\bar{D}$-factor) in currently produced drugs. As far as we are informed the question about the presence and physiologic role of complement system factors in human α-interferon preparations is proposed for the first time.

MATERIALS AND METHODS

Normal human serum was obtained from fresh donor blood by centrifugation of the clot and cells.

$\bar{D}$-factor depleted serum was used as a reagent for the $\bar{D}$-factor (RD). The serum (250 ml) was chromatographed in VBS (isotonic veronal buffer pH 7.4) in (5 x 5 cm) CM-Sepharose CL-6B column. To the obtained reagent (unabsorbed fraction)

EGTA was added to the final concentration of 10 mM. It was stored at -70°C.

We used CM-Sepharose CL-6B, a set of calibrated proteins for electrophoresis-LMW (Pharmacia), DEAE-TSK Toyopearl (Toyo Soda MFG, Co. Ltd, I-7-7 Akasaka Tokyo), polyethylene glycol 6000, ammonium sulphate, DFP (Serva), EGTA (Sigma), Ultrogel ACA-54 (LKB), acrylex P-60 (Reanal, Hungary), Igla MEM medium (Difco), cattle serum (Minmedprom, USSR), liquid chromatograph SP 8000 (Spectra-Physics), 0.7 x 60 cm column TSK-SW 3000 (Altex), detector SF 770 (Kratos).

Production of interferon preparations

Interferon biosynthesis was done in short-term culture of donor blood leucocytes isolated by double hemolysis with ammonium chloride of leucocyte containing erythrocytal mass, then centrifuged at 1500 g. Isolated leucocytes were resuspended in Igle MEM medium, containing 2% (V/V) of normal human serum. Interferon induction was according Cantell/16/ with slight modification using "H" strain of Newcastle disease virus as an interferonogene. Interferon activity was controlled by the microplate titration of inhibiting cytotoxic activity of vesicular stomatitis virus Indiana strain /17/. Each titration included α-interferon laboratory standard the activity of which was counted in MPC B69/19 units.

Crude interferon activity at this stage of production was $5\text{-}10 \cdot 10^3$ IU/mg with a protein content of 2-2.5 mg/ml.

Interferon concentration was carried out by total protein sedimentation with 70% $(NH_4)_2SO_4$. The precipitate was collected by centrifugation at 30,000 g for 10 min and redissolved in 0.1 M phosphate buffer (pH=7.0), containing 8M urea. The obtained concentrated interferon was purified on Ultragel ACA-54 (LKB) or acrylex P60 (Reanal, Hungary) columns.

Determination of the functional activity of complementary components in leucocyte interferon preparations

Hemolytic test was used for the determination of complementary components (C3, C5, B, $\bar{D}$). The reagents R3, R5, RB for protein titration in interferon preparations were prepared according to Kozlov et al. /18,19/. Sensibilized sheep erythrocytes (definition C3 and C5) /20/ and rabbit erythrocytes (definition B and $\bar{D}$) were used as target cells for lysis in VBS-Mg-EGTA system /21/.

For the determination of the $\bar{D}$-factor in fractions of the isolation methods the hemolytic microtitration method was used which rapidly revealed $\bar{D}$-factor in the fractions.

$\bar{D}$-factor inhibition by DFP

$\bar{D}$-factor in interferon preparation was inactivated by DFP. To 1 ml of sample, containing $14 \cdot 10^3$ IU interferon and 30 ng $\bar{D}$-factor $5 \cdot 10^{-3}$ to 0.1 M DFP was added, and incubated for 30 min at 37°C. The inhibitor surplus was removed by dialysis against VBS (2000 ml, 18h, 4°C), then by means of hemolytic titration the $\bar{D}$-activity was measured. An analogous method was used to block the activity of the $\bar{D}$-factor in serum and in the drug containing individual $\bar{D}$-factor.

Isolation of the $\bar{D}$-factor

Modified method described by Johnson et al. /22/ was used for the isolation of the $\bar{D}$-factor. An original method was developed for the purification of $\bar{D}$-factor; it includes two chromatographic procedures.

In the first step serum proteins were fractionally sedimented from 3000 ml of blood with PEG 6000 at concentrations of 5% and 20%. The fraction with $\bar{D}$-hemolytic activity was applied to the 5 x 30 cm CM-Sepharose CL-6B column, equilibrated with 0.02 M Tris-HCl buffer containing 0.1 M NaCl and 0.01 M EACA at pH=7.4. The column was washed with the same buffer up to the initial absorption level. The $\bar{D}$-factor was eluted by linear

NaCl gradient (0.1-0.5 M). The total buffer volume for the gradient elution was 6000 ml.

The fractions containing factor $\bar{D}$ hemolytic activity were pooled and $(NH_4)_2SO_4$ was added to 50% saturation. The suspension was stirred for 2h at 4°C, then centrifuged at 10,000 g for 2h. $(NH_4)_2SO_4$ was added to the supernatant to give 70% saturation and the suspension was stirred for 2h at room temperature. The precipitate was dissolved in Tris-HCl buffer 0.05 M-0.01 M EACA, pH=7.4, and after dialysis against the same buffer (1000 ml, 18h, 4°C), applied to a column (0.9 x 60 cm) under moderate pressure (Δp=5 atm) with DEAE-TSK Toyopearl. The $\bar{D}$-factor was eluted by linear NaCl gradient (0-0.5 M) at a flow rate of 1 ml/min. The total buffer content was 500 ml.

The preparation containing the $\bar{D}$-factor was dialysed against Tris-HCl 0.02 M containing 0.1 M NaCl and 0.01 M EACA, pH=7.4, 0.02% NaN_3 was added and then stored at 4°C.

Chromatography of leucocyte interferon preparation

Leucocyte interferon preparation (2 ml) dialysed against 0.005 M Tris-HCl buffer, pH=7.4 (1000 ml, 18h, 4°C), interferon activity $2 \cdot 10^5$IU, 0.5 mkg of factor $\bar{D}$, was applied to a column (0.9 x 60 cm) DEAE-TSK Toyopearl, equilibrated in the same buffer. The interferon preparation components were eluted by linear NaCl gradient (0-0.3 M). The total buffer content was 500 ml. The chromatographed fractions were examined on factor $\bar{D}$ and interferon activity by the mentioned methods.

Molecular weights of α-interferon proteins in the preparations were measured by highly effective exclusion chromatography using liquid chromatograph SP 8000 and the column (0.7 x 60 cm) TSK SW 3000 equilibrated with 0.3 M NaCl. The column was run at a flow rate of 1 ml/min, Δp=32 atm. Detection wave-length was 280 nm. The column was previously calibrated by definite protein mixtures. $5 \cdot 10^4$ IU of interferon was dissolved in 100 µl of water, then the total volume was analysed. Fractions (1 ml each) were analysed on their antiviral activity.

SDS/polyacrylamide gel electrophoresis

Electrophoresis was carried out as described by Laemmli /23/ using 12.5% polyacrylamide gel. Proteins were stained using Comassie brilliant blue.

Protein samples (20-200 μg) in 20 mM-iodoacetamide 0.05 M, Tris-HCl, 8 M urea, 2% (U/V), SDS, pH=6.8, were incubated for 20 min at 70°C, then 2% (V/V) β-mercaptoethanol was added and reincubated for 30 min at 70°C. Molecular weights of factor $\bar{D}$ and interferon were calculated using the following marker proteins: phosphorylase B (94000 d), albumin (67000 d), ovalbumin (43000 d), carboanhydrase (30000 d), trypsin inhibitor (20100 d), α-lactalbumin (14400 d).

RESULTS

The α-interferon production and purification scheme given in the present work provides rather reproducibly partially pure interferon preparations with specific antiviral activity up to $3 \cdot 10^5$ IU/mg (Fig. 1). The preparation contains 10^4IU of interferon lyophilized from 0.1 M phosphate buffer with 0.001 M EDTA and 1% lactose. The analysis with high-performance size exclusion chromatography (HPSEC) (Fig. 2) revealed some contaminants with molecular mass $1.5 \cdot 10^5$D and $0.68 \cdot 10^5$D which obviously correlate with immunoglobulin G and serum albumin molecular masses. These data are in a good agreement with the results of SDS-PAG electrophoresis (Fig. 5) and immunoelectrophoresis (Fig. 6).

The hemolytical test of the interferon preparation did not reveal C3, C5 and B complementary components, but the hemolytically active factor $\bar{D}$ content was rather high.

In order to calculate the specific activity of the individual factor $\bar{D}$ and to analyse its possible influence upon the antiviral interferon activity the active factor $\bar{D}$ was isolated from human serum by a few chromatographic steps. For this purpose we used both the conventional method of Johnson et al./22/

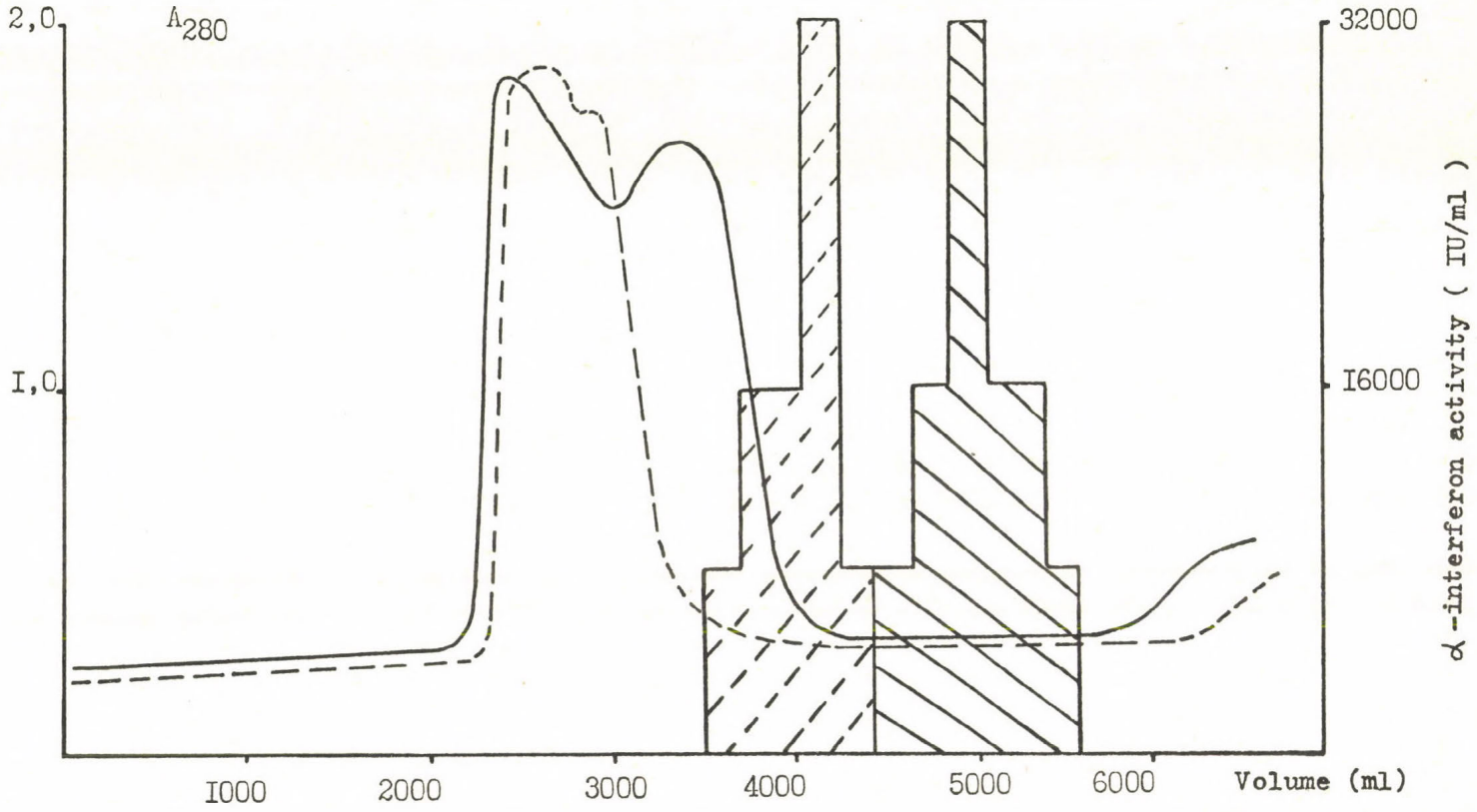

Fig. 1. Large scale purification of human α-interferon. Chromatography on the column (10x100 cm) packed with Ultrogel ACA-54 (——), and with Acrylex P-60 (----); 0.1 M phosphate buffer, pH=7.6; flow rate 0.4 l/hr. 250 ml protein concentrate was obtained from 10 l of crude interferon and applied to the column

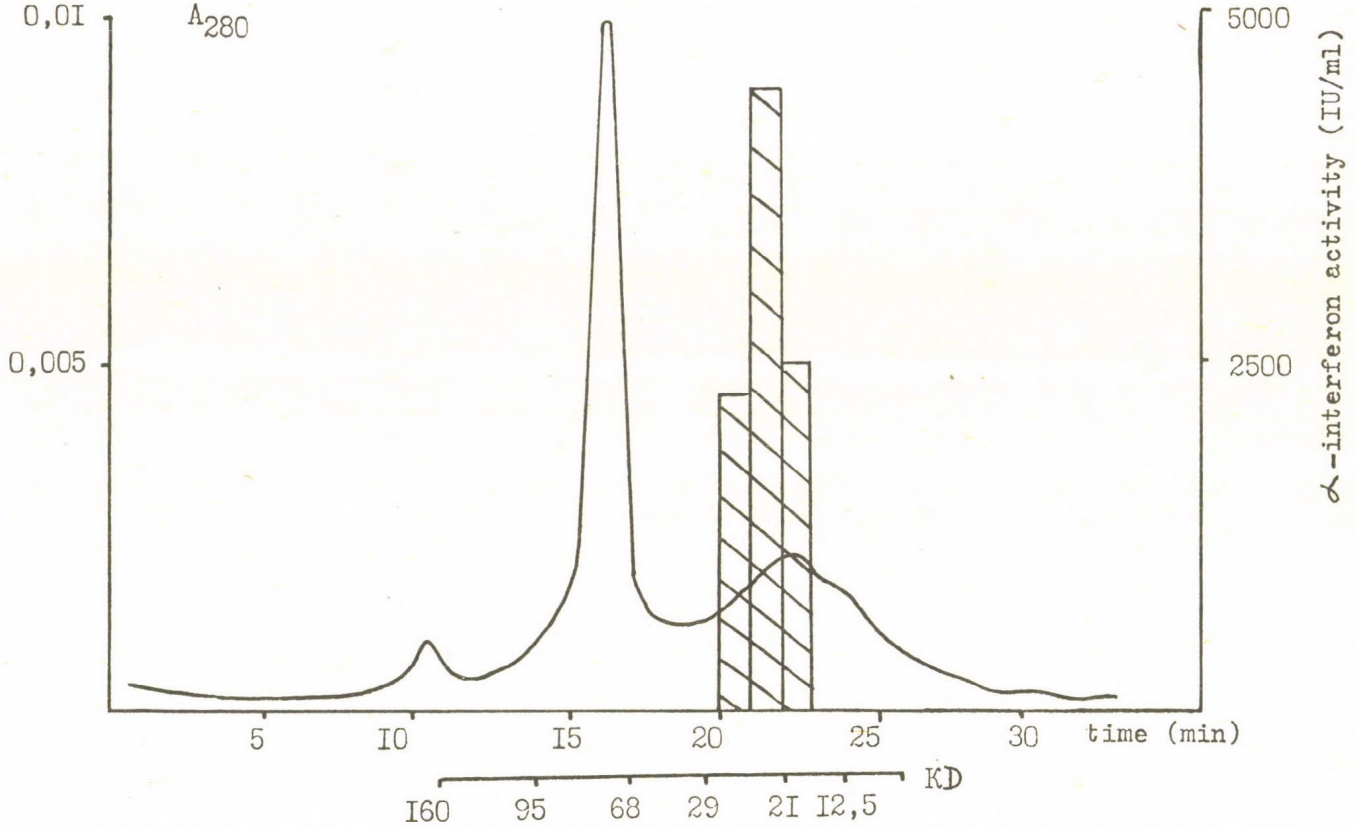

Fig. 2. Analytical exclusion chromatography of human α-interferon after the first purification stage. One ampule of product (10^4 IU, $5 \cdot 10^4$IU/mg) was dissolved in 100 µl of water. Column, 0.75x60 cm, TSK SW 3000; mobile phase, 0.3 M NaCl; flow rate,1 ml/min; sample volume, 100 µl; detector, SF 770, λ=280 nm; Spectra-Physics SP-8000 liquid chromatograph. One milliliter fractions were collected and tested on interferon activity

Table I. Isolation of factor $\bar{D}$ from human serum

Fraction	Volume (ml)		Total protein A280 (mg)	
	1	2	3	4
Normal human serum	1950	3000	120,000	172,000
PEG 6000 precipitation	200	600	78,000	126,000
CM-Sepharose CL-6B	51	1350	51.3	81
50-70% $(NH_4)_2SO_4$ - precipitation (pellet)	-	11	-	42
DEAE-Sepharose CL-6B	26	-	12	-
CoN-A Sepharose	12	-	5.2	-
TSK SW-50 gel filtration	4.5	-	0.97	-
DEAE - TSK TOYOPEARL	-	4.6	-	1.8*

*The final recovery of factor $\bar{D}$ hemolytic activity was $1.98 \cdot 10^{13}$ $\frac{\text{effective molecules}}{\text{mg}}$ with 125,000-fold purification

Table II. Comparison of factor $\bar{D}$ purification methods

Methods	Steps of purification	% Recovery (hemolytic activity)	Fold purification (hemolytic activity)
Johnson, Gagnon Reid (22)	6	22	60,000
Pasechnik et al. (present report)	4	40	125,000

(Table I, columns 1,3) slightly modified by us and our original method (Table I, columns 2,4).

The second method is obviously less laborious and results in higher purification of factor $\bar{D}$ (125,000 times higher) evaluated by hemolytic activity. The additional advantage of

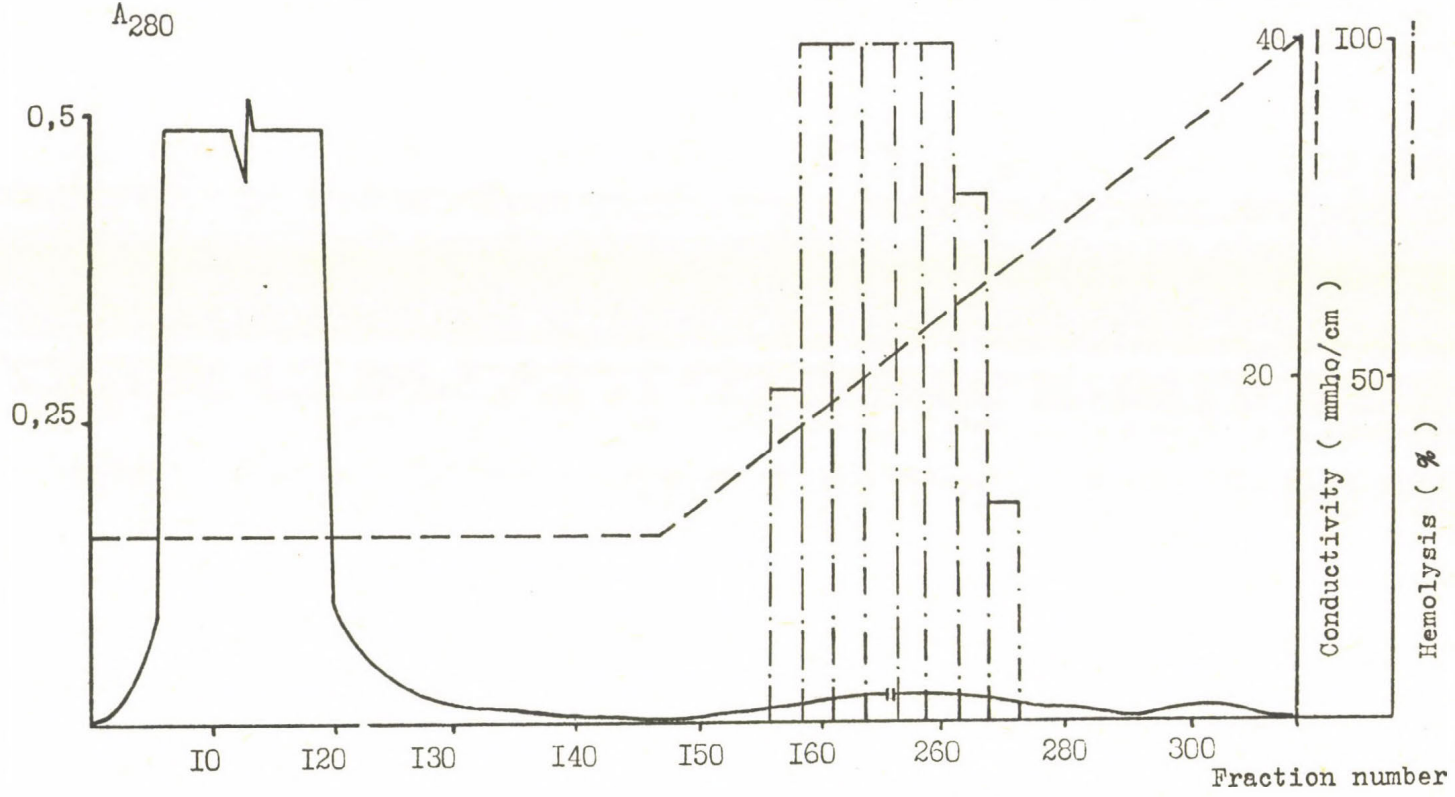

Fig. 3. Ion-exchange chromatography of human serum proteins after PEG-6000 (5-20%) precipitation on CM-Sepharose CL-6B. Column, 5x30 cm; eluent, 0.02 M tris-HCl buffer containing 0.1 M NaCl, pH=7.4; flow rate, 2 ml/min; linear NaCl (0.1-0.5 M) gradient

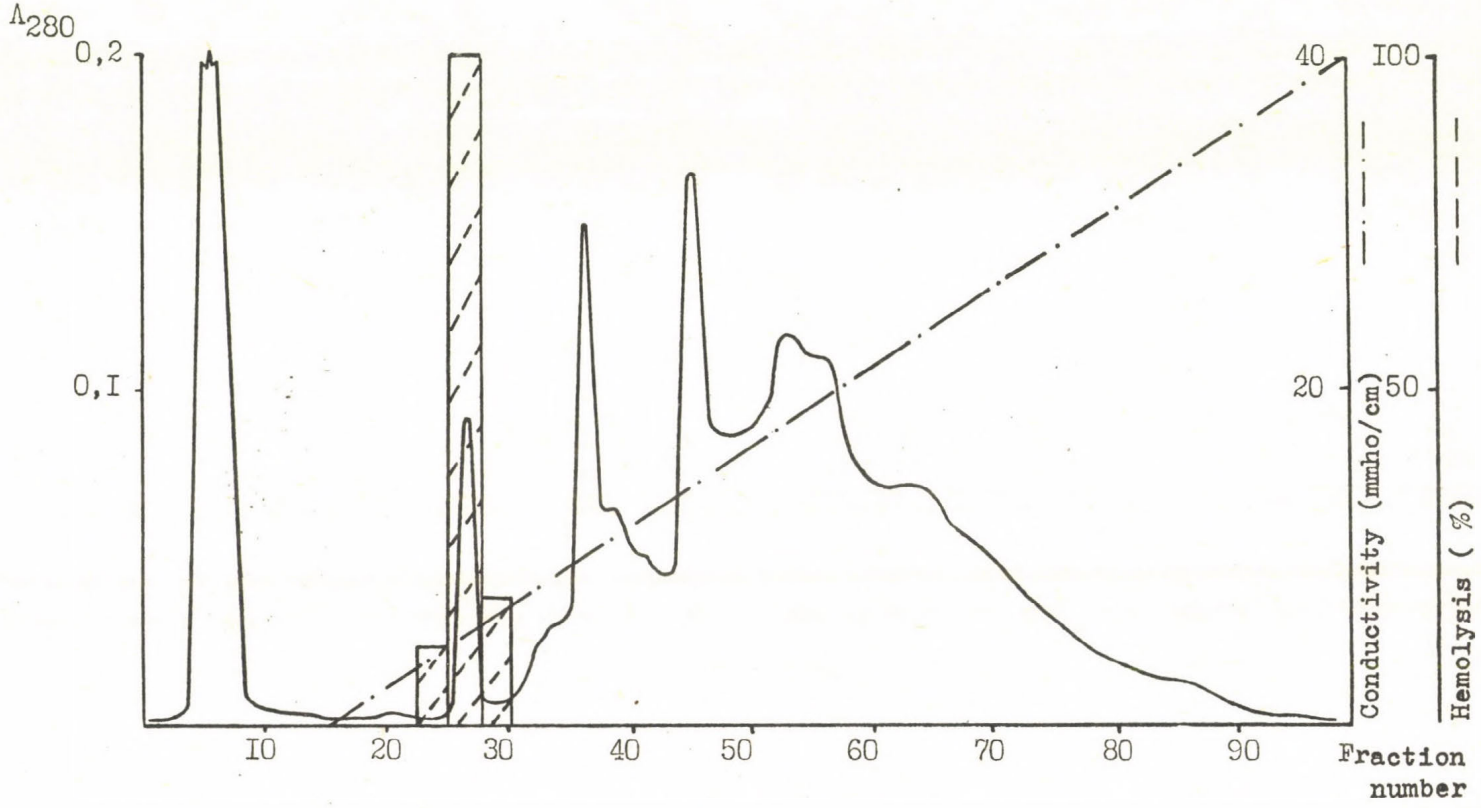

Fig. 4. Ion-exchange chromatography of the fraction after CM-Sepharose CL-6B and ammonium sulphate (50-70%) precipitation on TSK-DEAE-Toyopearl. Column, 0.9x60 cm; eluent, 0.02 M tris-HCl buffer, pH=7.4; flow rate, 1 ml/min; linear NaCl gradient (0-0.5 M)

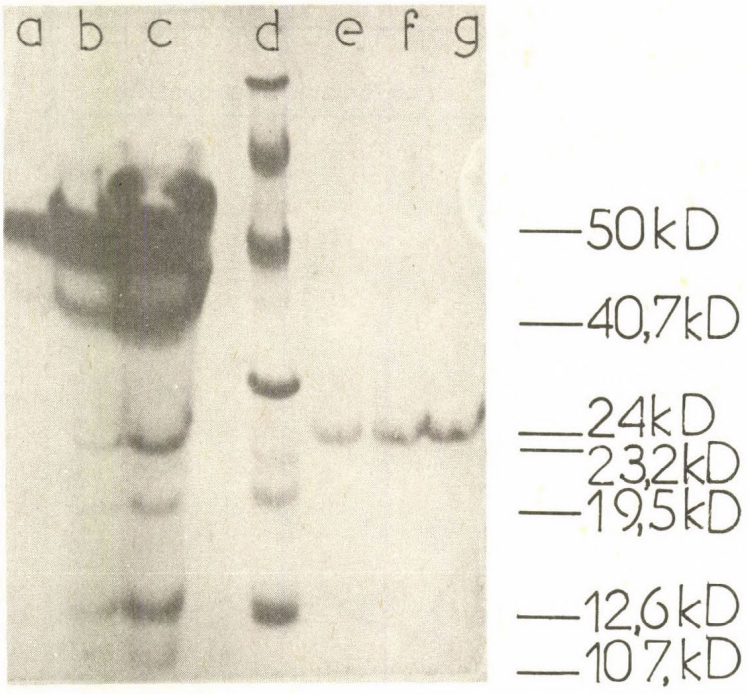

Fig. 5. SDS-polyacrylamide gel electrophoresis. a, b, c - α-interferon samples containing 20, 100 and 200 μg total protein respectively; d - markers LMW; e, f, g - highly purified factor D̄ (20, 50 and 100 μg respectively)

this method is that the yield is twice as high (Table II). Chromatograms shown in Figs 3 and 4 demonstrate the isolation of factor D̄ by the original method.

The purified factor D̄ appears to be homogeneous on SDS-PAG electrophoresis under reducing conditions; its apparent molecular mass is 24 KD (Fig. 5).

By means of the isolation methods described here the yield of D̄ was 0.97 mg and 1.8 mg at the first and second isolations respectively. Hemolytic titration revealed no loss of pure factor D̄ activity during a period of up to 3 weeks.

It should be noted that our method for factor D̄ isolation proved to be advantageous: during 1 cycle (7h) using a column (5x5 cm) CM-Sepharose CL-6B we obtained such an amount of the reagent which is sufficient for 2500 measurements.

Micro-hemolytic titration on hemolytic plates was used for the qualitative demonstration of factor D̄ in fractions during

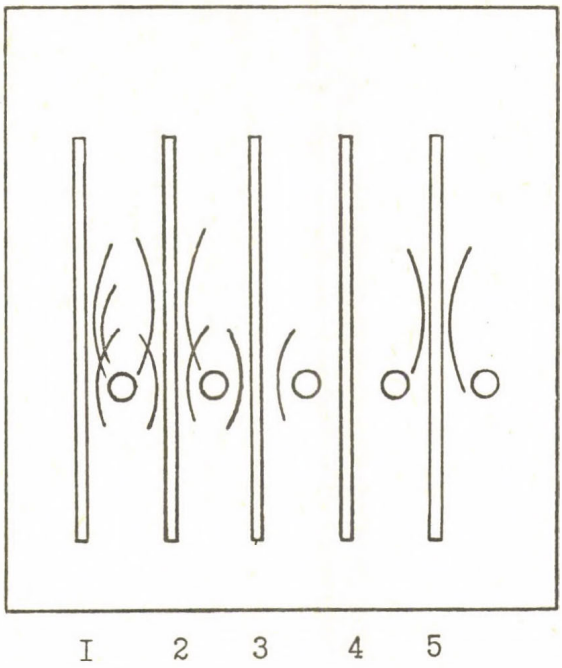

Fig. 6. Immunoelectrophoresis of α-interferon. Immunoelectrophoresis agarose contained 20 mM veronal buffer pH=8.6, 1.3% agarose (Sigma). Constant current: 10 mA, V=40 Ov/cm, 1 hr. 20 µl α-interferon with activity 10^6IU was placed in the well. 200 µl anti-NHS (1), anticultural medium without interferon inductor (2), anti-human IgG (3), anti-human IgA (4), and anti-human albumin (5) were placed in each trough

the isolations steps. Reagent outlay was diminished by a factor of three, and the time of the experiment was shortened to one-fifth in comparison to the quantitative method.

The last method revealed the presence of factor $\bar{D}$ in α-interferon preparations in the amount of 10-25 ng with 10^4IU of activity. Calibration from hemolytic units to weights was carried out according to pure factor $\bar{D}$. Ion exchange chromatography was used to localize factor $\bar{D}$ activity which was nearly the same as one of the active interferon form (Fig. 7).

The same column, DEAE-TSK Toyopearl, was used for the isolation of factor $\bar{D}$ from partly purified protein mixtures in CM-Sepharose CL-6B serum and in interferon preparation. When adsorption took place at equal value of pH (7.4), then factor

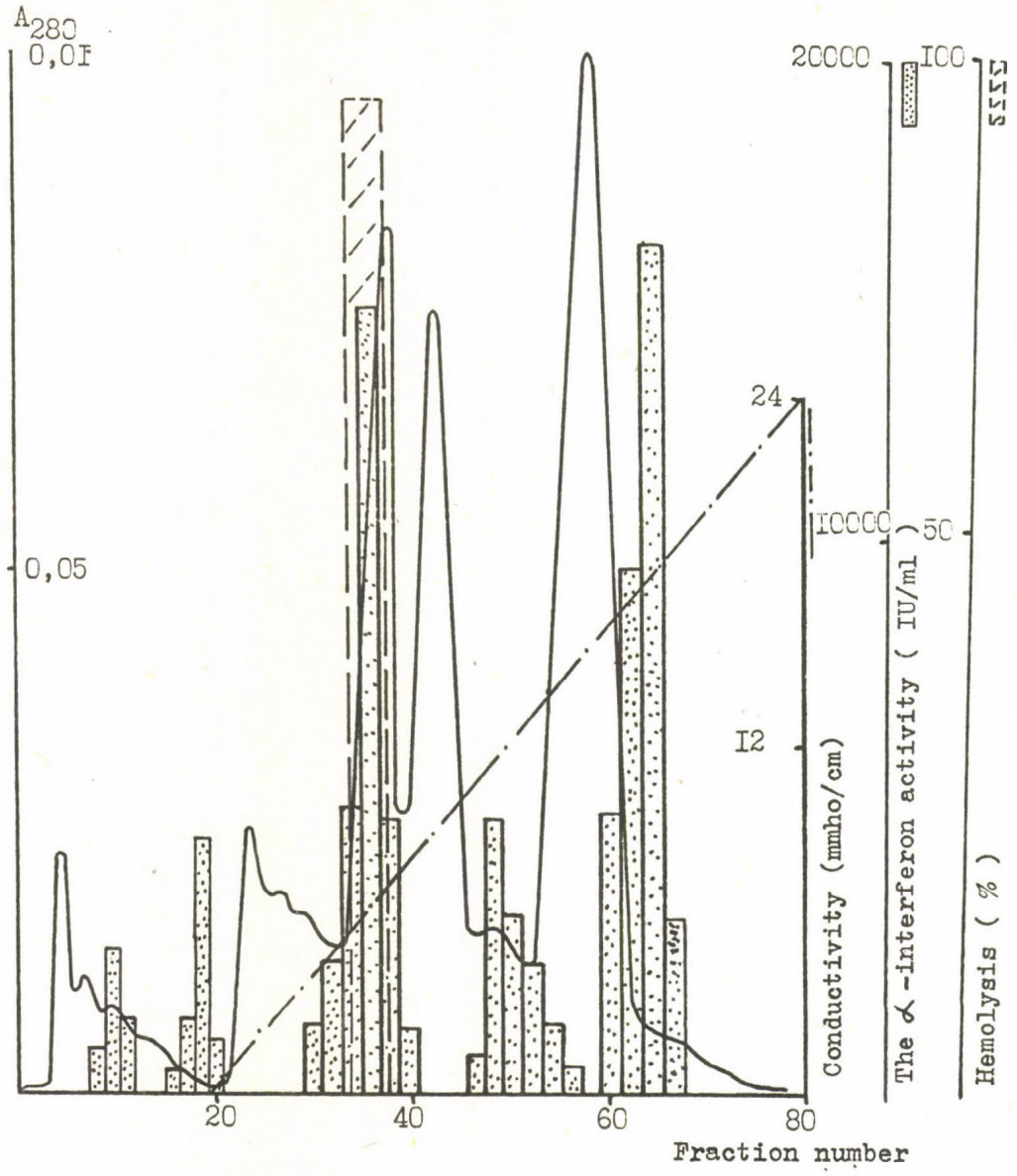

Fig. 7. Ion-exchange chromatography of α-interferon on the TSK-DEAE Toyopearl column. The sample containing $2 \cdot 10^5$ IU of α-interferon and 0.5 μg of factor D was applied to the column (0.9x60 cm). Eluent, 0.005 M tris-HCl buffer, pH=7.4; flow rate, 1 ml/min; linear NaCl (0-0.3 M) gradient

$\bar{D}$ was eluted in both cases at the same ion force (~ 0.08 M NaCl).

In order to reveal the antiviral activity of the interferon free of factor $\bar{D}$ functional activity these preparations were treated with DFP, which is an irreversible serine protease inhibitor, inactivating factor $\bar{D}$.

The hemolytic activity of the individual factor $\bar{D}$ was also blockaded by DFP. (DFP blockades 50% of purified factor $\bar{D}$, and in the interferon, $\bar{D}$ is blockaded in the ratio $3.5 \cdot 10^{-3}$ M/I ng of factor $\bar{D}$). Highly purified factor $\bar{D}$ had no antiviral activity.

DISCUSSION

Analysis of interferon preparations on the content of complementary system proteins (C3, C5, B, $\bar{D}$) using highly sensitive functional tests revealed a hemolytic active factor $\bar{D}$ in the amount of 20-25 ng on 10^4IU. It seems that the serum (its $\bar{D}$ content was 100 ng/MI) added to the medium during leucocyte cultivation was the main source of complementary system factor $\bar{D}$.

Chromatographic separation of α-interferon on DEAE-TSK Toyopearl (Fig. 7) revealed several zones of interferon activity, in one of which factor $\bar{D}$ activity was localized. The peaks in the interferon chromatogram are possibly connected with different molecular weights of protein, as shown by other researchers who used different methods /29/.

Thus we can produce $\bar{D}$-containing and $\bar{D}$-free preparations regarding the method of interferon purification.

Factor $\bar{D}$ is the key ferment in alternative pathway of complementary activation which is responsible for positive control mechanism (Fig. 8). According to this we can assume that factor $\bar{D}$ introduced exogenously or activated from precursor can produce fragments of complementary components ($\overline{C3}b$, C3a, $\bar{B}b$, Ba, etc.) with immune regulating functions /25-27/. In certain conditions it can possibly explain the immune regulation ability of interferon.

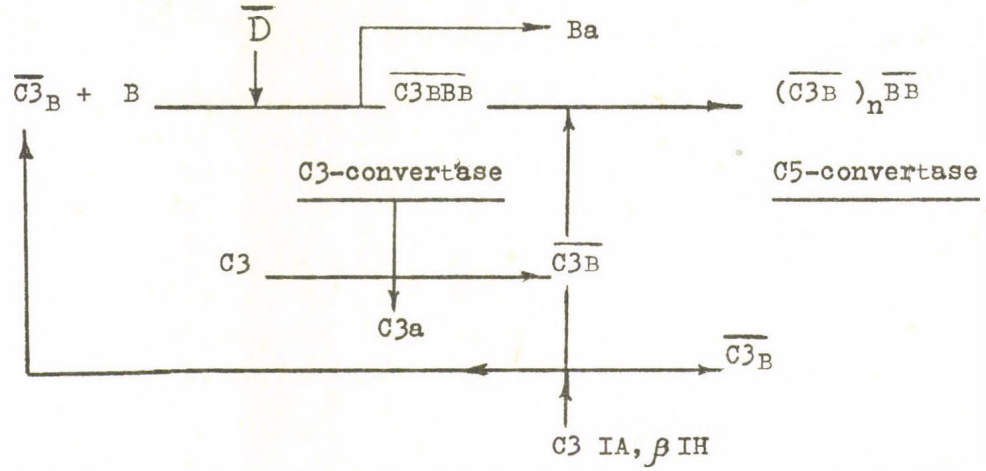

Fig. 8. Basic representation of the molecular interaction during the alternative pathway

One of the forms of the activity of interferon preparations mediated by factor $\bar{D}$ may be an antitumor activity. It is connected with C3 and C5 convertases which are the main and the last ferments in the formation of the lytic complex of cells and tumor cells are also opsonized by $\overline{C3}b$ /28/.

In order to test this thesis it is necessary to examine the antitumor and immune modulating characteristics of isolated factor $\bar{D}$ in combination with interferon in model experiments in vitro and in vivo.

It is also important to analyse the presence of factor $\bar{D}$ in interferon preparations manufactured by different firms. It is also necessary to compare these data to their biological activity.

The contribution of factor $\bar{D}$ to the antiviral action of interferon was tested in special experiments. The different sensitivity of factor $\bar{D}$ and interferon to DFP were used for these experiments. It was shown that the antiviral activity of interferon is independent of the presence of factor $\bar{D}$. Individual factor $\bar{D}$, without antiviral activity, isolated separately was used in vitro to prove this conclusion.

CONCLUSIONS

1. Preparations of human α-interferon purified by chromatography on AcA-54 up to $3 \cdot 10^5$ IU/mg specific activity contained 0.6 μg hemolytically active factor $\bar{D}$, one of the basic enzymes of complementary alternative pathway. Active complementary components C3, C5 and B were not found in these preparations.
2. An original method was developed for the purification of factor $\bar{D}$ resulting in a higher purity (125,000 fold) with a 40% yield.
3. Antiviral activity of the interferon was retained after treating by DFP. The individual factor $\bar{D}$ did not possess antiviral activity.
4. It is important to analyse the interferon preparations manufactured by different firms for the presence of factor $\bar{D}$ and to investigate the role of the exogeneous factor $\bar{D}$ in the antitumor activity of interferon preparations.

ABBREVIATIONS

EGTA-ethylene glycol bis(2 amino-ethyl ether)-N,N-tetraacetic acid
VBS-Mg-EGTA-VBS - containing 5 mM $MgCl_2$ and 0.01 M EGTA
DFP -diisopropylfluorophosphate
EACA-e -aminocapronic acid
SDS - sodium dodecylsulphate

ACKNOWLEDGEMENTS

We thank Ms.L.A.Diodorova for performing the antiviral activity analyses and Ms,L.A.Seleznojva and Ms.I.Yu.Sokolova from the Department of Science for their assistance in bibliographic search.

REFERENCES

1. J. Lindenmann, in Interferon N 3, Academic Press, London, 1981, pp. 1-13.
2. N.O. Kaplen and S. Slimmer, in L.W. Mozes (Editor), Miami Winter Symposia, Vol. 18, Academic Press, New York, 1981, pp. 443-454.
3. R.T. Dean and J.L. Virelizier, Clin. and Exper. Immunol., 51 (1983) 501.
4. E. Jirrillo and S. Antonaci, Cell. Immunol., 71 (1982) 208.
5. G.M. Scott and D.A.J. Tyrrell, Brit. Med. J., N 6231 (1980) 1558.
6. S.L. Warren, Ann. Allergy, 45 (1980) 37.
7. A. Billiau, Arch. Virol., 67 (1981) 121.
8. J. Wietzerbin, E.P. Kolb and A. Senik, Immunobiol., 163 (1982) 298.
9. A.M. Attallah, J.C. Petricciani and G.J. Galasso, J. Infec. Dis., 142 (1980) 300.
10. C. Bentley, B. Zimmer and U. Haddiny, in E. Pick (Editor), Lymphokines, Vol. 4, Academic Press, New York, 1981, pp. 197-231.
11. Ch. Chany, in E. Pick (Editor), Lymphokines, Vol. 4, Academic Press, NY, 1981, pp. 409-427.
12. W.R. Struebring and P.J. Chapple, in Human interferon. Production and clinical use. Plenum Press, New York, 1978.
13. R.M. Shultz, M.A. Chirigos and U.J. Heine, Cell. Immunol., 35 (1978) 84.
14. H. Strander and K. Cantell, Ann. Med. Exp. Biol. Fenn., 44, (1966) 265.
15. R.G. DiScipio, Immunology, 45 (1982) 587.
16. K. Cantell and S. Hirvonen, J. Gen. Virol., 39 (1978) 541.
17. S. Rubinstein, P.C. Familleti and S. Pestka, J. Virol., 37 (1981) 755.

18. L.V. Kozlov, Yu.I. Krylova and V.P. Chikh, Bioorganich. Khimiya, 8 (1982) 652.

19. L.V. Kozlov and L.S. Solyakov, Bioorganich. Khimiya, 8 (1982) 342.

20. E. Kabot and M. Mayer, in "Experimental immunochemistry", World, Moscow, 1968, pp. 140-159.

21. H.V. Dijk, P.M. Rademaker and J.M.N. Willers, J. Immunol. Meth., 36 (1980) 29.

22. D.M.A. Johnson, J. Gagnon and K.B.M. Reid, Biochem. J., 187 (1980) 863.

23. V.K. Laemmli, Nature, 227 (1970) 680.

24. P.H. Lesavre and H.J. Müller-Eberhard, J. Exp.Med., 148 (1978) 1498.

25. M.E. Medof and K. Iida, J. Exp. Med., 156 (1982) 1739.

26. M.P. Dierich, O. Scheiner and H.H. Mussel, Mol. Immunol., 19 (1982) 1255.

27. P. Carlson, S. Ruddy and D.H. Conrad, Immunology, 46 (1982) 163.

28. M. Daveau, M. Fontaine and D. Gibbert, Ann. D'Immunol., 132C (1981) 339.

29. S. Rubinstein, P.C. Familletti and R.S. Muller, Proc. Nat. Acad. Sci., USA, 76 (1979) 640.

Chromatography, the State of the Art
H. Kalász and L.S. Ettre (Eds)

NOVEL APPLICATION OF SUPEROXIDE DISMUTASE

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ABSTRACT

Two specific enzyme sensors (superoxide dismutase and catalase) and a Clark electrode system were used for the quantitative determination of superoxide anion radical (O_2^-) and H_2O_2, respectively.

The preparation of these enzyme electrodes and the range of their sensitivity are described.

INTRODUCTION

Recently, several papers discussed the importance, occurrence, isolation and properties of the enzymes superoxide dismutase (SOD) and catalase (C-ase) (1-6) which are generally considered to be essential for the aerobic life. An interesting practical application of these enzymes seems to be the construction of an enzyme electrode for the highly specific, quantitative determination of their substrates.

At the same time, the rapid quantitative analysis of the anion radical O_2^- and of H_2O_2 is a long-standing biological problem. If a good sensor could be found for these compounds, the experimental problems could readily be solved. For this purpose, an O_2 (Clark) electrode was tested. We have checked the properties of this system and the possible use of it for the measurements.

MATERIALS AND METHODS

The compounds used were the products of Reanal (Budapest, Hungary), Sigma (St. Louis, USA), Bohringer (Mannheim, FRG), Serva (Heidelberg, FRG), and Calbiochem (Luzern, Switzerland).

The O_2 (Clark) electrode was purchased from Rank Brothers (Bottism, UK).

Enzyme membrane preparation: 1 mg enzyme (Cu. Zn-SOD or bovine liver catalase) freeze-dried or preserved with ammonium sulphate (Serva) and 1 mg bovine serum albumine were dissolved in 50 µl of 50 mM/liter phosphate buffer, pH 7.0, and 2.5 µl of 25% glutaraldehyde was added. The resulting enzyme solution was added drop by drop to dry porcine intestine and dried at room temperature onto the surface of the intestinal layer. If SOD and C-ase were applied together, first the C-ase solution was transferred onto the intestine, and the SOD solution was subsequently layered onto the active surface of the C-ase.

The enzyme layer was deposited above the teflon membrane covering the Pt-electrode below the flexible neoprene O-ring. Thus, the measuring enzyme layer was located between the O_2 sensor and the solution to be measured. The scheme of the apparatus is presented in Fig. 1, while Fig. 2 shows the electrode arrangement.

RESULTS

Fig. 3 illustrates the quantitative determination of H_2O_2 by means of catalase. It can be seen that the measured quantity of O_2 linearly increases, as a function of the H_2O_2 concentration.

Fig. 4 shows an example for the quantitative measurement of O_2^- with the electrode consisting of C-ase and SOD. Use of this combined electrode reveals two surprising phenomena:

- an O_2 consumption can be observed in the system;
- the O_2 consumption linearly increased with the substrate concentration.

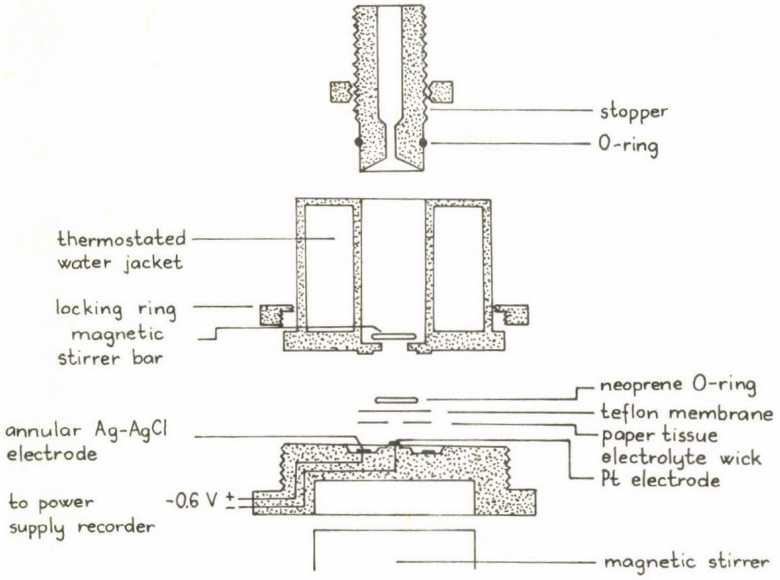

Fig. 1. Schematic arrangement of the apparatus

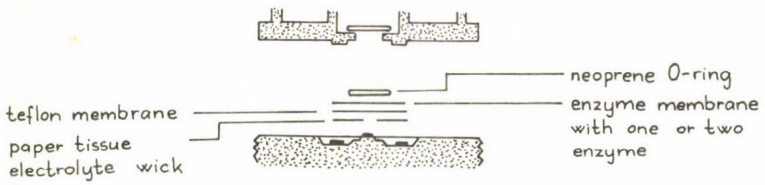

Fig. 2. Electrode arrangement

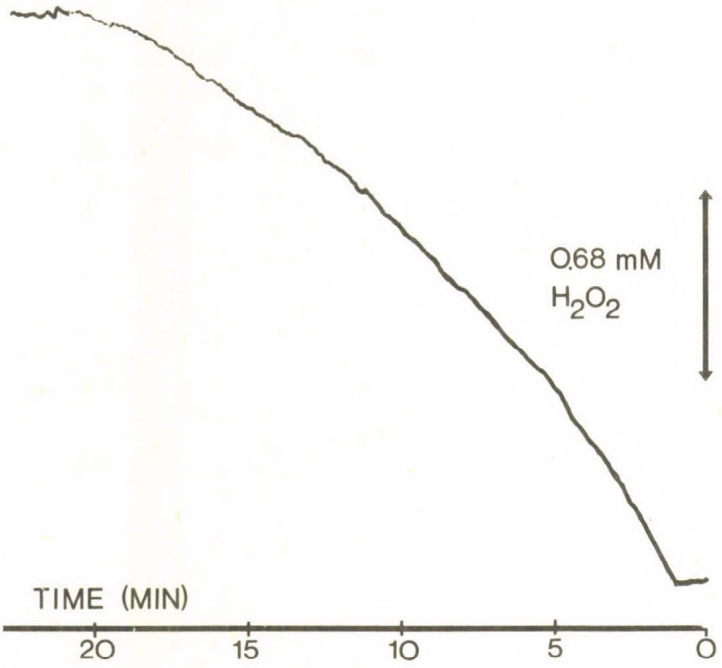

Fig. 3. Quantitative determination of H_2O_2 with C-ase electrode

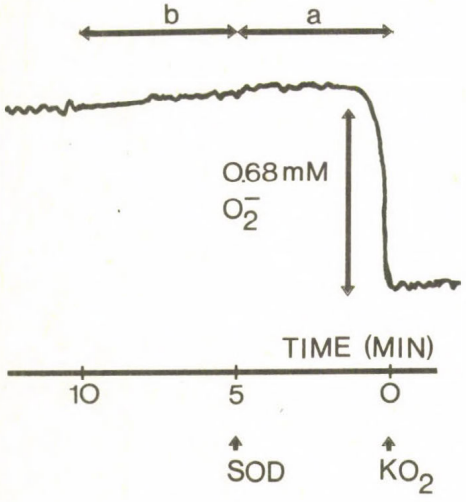

Fig. 4. Quantitative measurement of O_2^- with combined C-ase and SOD electrode

The spontaneous dismutation of the radicals released from potassium superoxide (KO_2) is recorded in section (a). Section (b) demonstrates that the addition of SOD to the KO_2 enhances the spontaneous dismutation.

Fig. 5 shows the change in the O_2 concentration measured with a traditional Clark electrode. The SOD - adrenaline - adrenochrome system consumes O_2 and the rate of this consumption is decreased in the presence of SOD.

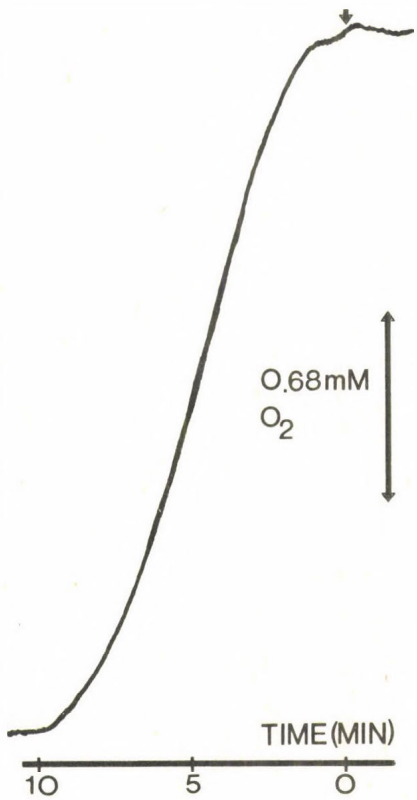

Fig. 5. Direct determination of changes in the concentration of O_2 on the effect of SOD in the presence of epinephrine at pH 10.2.

DISCUSSION

The essential points of this investigation have been the application of specific catalysts to the reactions and finding a proper sensor for O_2 produced in the reactions

$$O_2^- + O_2^- + 2\,H^+ \longrightarrow O_2 + H_2O_2 \qquad \text{(reaction 1)}$$

$$H_2O_2 + H_2O_2 \longrightarrow O_2 + 2\,H_2O \qquad \text{(reaction 2)}$$

The electrodes were formed from SOD and C-ase for reactions 1 and 2, respectively, as well as from the combination of these two enzymes. The system was set up according to Fig. 2.

The properties of the system have been evaluated. It was found that quantitative measurements are possible with them. The adaptation of the system for a chromatographic (non-segmented flow-through) sensor is in progress.

REFERENCES

(1) K. Asada, M. Urano and M. Takahashi, Eur. J. Biochem. 36, 257 (1973).

(2) I. Fridovich, Ann. Rev. Biochem., 44, 147 (1975).

(3) P.H. Hien, K. Kovács and B. Matkovics, Enzyme, 18, 341 (1974).

(4) B. Matkovics and L. Szabó, in: "New Approaches in Chromatography", Ed.: H. Kalász, Elsevier, Amsterdam, 1984, p. 245.

(5) G.A. Rechni, Chem. Eng. News, 1975, 29.

(6) H.M. Hassan and I. Fridovich, in: "Enzymatic Basis of Detoxification". Ed.: W.B. Jakoby, Academic Press, New York, 1980, p. 311.

Chromatography, the State of the Art
H. Kalász and L.S. Ettre (Eds)

STUDIES ON THE PURIFICATION AND PROPERTIES OF SATIETIN, AN ANOREXIGENIC GLYCOPROTEIN OF BIOLOGICAL ORIGIN

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ABSTRACT

Satietin, an anorexigenic glycoprotein, was separated from human serum by a series of purification techniques. Prior to the fine separations, the plasma was fractionated by means of ultrafiltration, trichloroacetic acid precipitation and centrifugation. Essential purification steps were performed by using gel chromatography and resulted in partially purified substances which contained specific anorexigenic activity. Successful utilization of affinity chromatography on Con A-Sepharose and proteolytic digestion followed by gel chromatography on Bio-Gel P-2 column produced homogeneous substances. The properties of these materials were studied by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and analytical isoelectric focusing. All of these were classified as glycoproteins. These substances have relatively low percentage of peptide in an average of 14-22% and extremely high carbohydrate content (60-75%) with a molecular weight range of 48,000-50,000 daltons. Homogeneous substances isolated from the plasma of humans using a different step in the course of separation exist in isoelectric forms of 3.0-3.1 and 7.0-7.1, respectively. Thermal stability and water content of these substances were determined by thermal gravimetric analysis. The molar extinction coefficient of the homogeneous human product was found to be $\varepsilon_{282\ nm}=30,000\ M^{-1}\ cm^{-1}$.

INTRODUCTION

The existence of a new selective anorectic substance named satietin was discovered by Knoll (1) in human serum. Satietin, which was proposed to be a glycoprotein, appeared to inhibit the food intake in normally fed as well as food-deprived rats without exerting any other central and peripheral effects. This is in contrast to endogenous peptides with anorectic effects like cholecystokinin, calcitonin and to the drug pair, amphetamine and fenfluramine which represent the two main types of antiobesity agents (2, 3, 4).

Later on, satietin-activity was also detected in the sera of different mammals (rat, guinea pig, rabbit, cat, dog, horse, cattle) and in poultry (goose) and was proposed by Knoll to play the role of a rate limiting, blood-borne satiety signal in the negative feed-back of food intake (2, 3).

An anorexigenic endogenous protein with a molecular weight of 50,000 daltons which regulates feeding has recently been isolated from urine of rats by Kinoshita et al.(5) and was suggested to be satietin. Also, a partially purified anorexigenic substance was found in the feces of rat and mouse by Tsuda et al.(6). They experienced that this large molecular weight substance contained protein but it might not be a protein as the biological activity was unchanged by pronase and pepsin digestion. A certain amount of carbohydrate (neutral hexoses) was also found in these preparations. However, we have previously shown that those substances which have been isolated in the last few years in our laboratory containing specific anorectic effect were found to be glycoproteins. More recent discoveries (7, 8) show that these glycoproteins obtained from different sources contained the same sugars, a high amount of lysine and behaved as stable compounds, although the biological effect could be diminished or lost by proteolytic digestion under special conditions.

The intent of this paper is to focus on the possible purification studies of the satietin-like substances obtained from the plasma of humans and to characterize and analyse the substance by the methods here described. Moreover, the application

of these methods allows us to prepare substantial amounts of homogeneous or highly purified anorectic glycoproteins which are required for a series of biological and pharmacological experiments as well as enabling chemical structural investigations.

EXPERIMENTAL PROCEDURES

Materials

Human plasma was purchased from the National Institute of Haematology and Blood Transfusion, Budapest. Ampholines were from LKB Produkter, Sweden.

Sephadex G-15, Con A-Sepharose gels were obtained from Pharmacia Fine Chemicals, Sweden. Bio-Gel P-2 (100-200 mesh) gel, Aminex A-5 ion-exchange resin, Low Molecular Weight Standard Protein Mixture and the reagents for SDS-gel electrophoresis were purchased from Bio-Rad Laboratories, Richmond, California. 4 N methanesulfonic acid was purchased from Pierce Chemical Company. Amino acid standard mixture, trypsin and α-chymotrypsin were from Serva, Feinbiochemica, Heidelberg and precoated silica gel layers from E. Merck, Darmstadt, Germany. Fixion 50x8 ion-exchange thin-layer plates were obtained from Chinoin Pharmaceutical Co., Budapest. All other solvents and reagents were of analytical grade from Reanal, Budapest.

Methods

Ultrafiltration was performed on Amicon YM-10 membrane as previously described (5) and in large scale on an Amicon system equipped with hollow fiber having a cut-off of 50,000 daltons. Gel chromatographic separations were performed on Pharmacia columns and LKB ReCyChrom System as reported earlier (7, 8). Uvicord II monitors were used to follow the separations at 280 nm.

Affinity chromatography was permitted on Con A-Sepharose columns equilibrated and washed with 15 column volumes starting buffer (0.02 M Tris-HCl, 0.5 M NaCl, 0.001 M $MnCl_2$ and 0.001 M $CaCl_2$, pH 7.0). Column dimensions were 1.7x37 cm. Eluent was

0.02 M Tris-HCl, pH 7.0 buffer with a linear gradient of 0-0.5 M α-D-methylmannoside. The fraction volume was 2.5 ml.

Tryptic-chymotryptic digestion was carried out in the following way: the highly purified salt-free product (20 mg) was suspended in 0.1 M Tris to a concentration of 5 mg/ml, the pH adjusted to 8.2, and 0.1 mg each of trypsin and chymotrypsin per mg of human satietin were added. After incubation of the mixture for 5 h at 38°C, one-half of the original amounts of trypsin and chymotrypsin were added to the mixture and the digestion was prolonged for 19 h. After the 24 h digestion, it was cooled to 0°C and 55% (w/v) trichloroacetic acid was added to a final concentration of 5% (w/v). The turbid solution was left to stand for 1 h at 0°C and clarified by centrifugation at 10,000 xg for 20 min. The optically clear supernatant was then subjected to gel chromatographic separation on Bio-Gel P-2 column.

Desalting process and final purification of serum satietin samples were achieved on a column (2.6x90 cm) of Bio-Gel P-2 (100-200 mesh) in deionized water and 10 ml of fractions were collected.

Microbiuret method was carried out by a modification of the method described by Honn and Chavin (9).

SDS gel electrophoresis was performed on polyacrylamide rods in a 12 tube gel electrophoresis cell (Reanal, Budapest) according to the method described by Laemmli (SDS-Tris PAGE) (10) and Weber and Osborn (SDS-phosphate PAGE) (11). 25-50 µg loads of the purified materials were prepared for electrophoresis by treatment with 2% of SDS (w/v) in the presence of 1% mercaptoethanol, 10% glycerol, 0.001% bromophenol blue in 0.0625 M Tris, pH 6.8 buffer. Samples were then incubated at 80°C for 10 min. Gels were stained for proteins with 0.5% Coomassie brilliant blue R-250 (CBB) in acetic acid - methanol - water (1:5:5, v/v/v) for 20 to 60 min (depending on how freshly prepared the dye mixture was). The gels were destained by dif-

fusion in 10% (v/v) acetic acid. The periodic acid-Schiff staining (PAS) for carbohydrates was adapted from that described by Köiv (12) and Glossmann and Neville (13).

Isoelectric focusing was performed in polyacrylamide slab gels containing Ampholines with pH range of 3 to 10 according to Radola (14). Protein bands were stained with CBB R-250.

Amino acid analysis. Free amino acids were liberated from the glycoproteins by acid hydrolysis in 6 N HCl at 105°C. Cysteine was determined as cysteic acid after performic acid oxidation followed by acid hydrolysis as described by Walker et al. (15). Inspection of the tryptophan content was carried out after 4 N methanesulfonic acid hydrolysis. The qualitative examination of the liberated amino acids were performed on precoated silica gel thin-layer chromatoplates and Fixion 50x8 ion-exchange thin-layer plates in standard Desaga glass tanks. Developing solvent systems were:

System I Phenol - water (80:20, v/v)

System II Na^+ citrate buffer 0.2 N Na^+, pH 2.2 to 3.7

System III Pyridine (1%, v/v)-acetic acid buffer, pH 2.5-7.5.

Ninhydrin spray reagent was prepared by dissolving 2.0 g ninhydrin and 0.25 g copper sulfate in 800 ml ethanol and 200 ml acetic acid. Amino acid content was determined on a Bio-Cal, Model BC-200 automatic amino acid analyzer using a single column (0.9x50 cm) of Aminex A-5, and an adaptation of two buffers described by Dévényi (16).

Thermal gravimetric analysis (TG) was carried out on a Du Pont 990 System. The flow rate of air was 10 liter/hour. The heating rate was 10°C/min.

Thermal evolution analysis (TEA) was performed in N_2 atmosphere. The flow rate was 1.8 liter/hour. The heating rate was 8°C/min.

The molar extinction coefficient of the satietin-active human glycoprotein was determined by taking spectra in the

250-300 nm ultraviolet absorption range on a Specord UV/VIS Zeiss spectrophotometer, Jena, GDR, in the following solvents:

Solvent I :0.1 N NaOH - HCl, pH 2.0
Solvent II :0.1 N NaOH - HCl, pH 7.0
Solvent III:0.1 N NaOH - HCl, pH 10.0

The concentration of the sample was 1 mg/ml in every case.

RESULTS AND DISCUSSION

The preparation of partially purified samples from human serum and the final isolation procedure were detailed in previous papers (7, 8). Even the final step which delivered homogeneous glycoprotein from human plasma with neutral isoelectric point (pI=7.0-7.1) was first obtained by means of an affinity column of Con A-Sepharose. We systematically studied the possible ways of the separation of satietin-active serum glycoproteins by affinity chromatography on Con A-Sepharose columns starting from the assumption that these substances contained α-D-mannopyranosyl and/or α-D-glucopyranosyl residues derived from their glycoprotein nature. Moreover, the possibility of proteolytic digestion in order to remove the undesirable serum protein impurities which were accompanied by the satietin in the highly purified preparations was also investigated. We supposed that these protein and peptide contaminants might be faster digested than the satietin-active glycoprotein molecule which earlier proved to be more stable. In addition, it was found that the application of preparative electrophoresis was not necessary prior to the affinity step.

The complete isolation procedure which is being applied currently is outlined in Fig. 1. The last several years' experiences proved that after the trichloroacetic acid treatment and the two gel chromatographic steps produced highly purified preparations with significantly high biological activity which were easy to handle and suitable for the most frequent chemical analyses. As described earlier (8), affinity chromatography proved to be one of the most promising ways for the isolation of human satietin. A small affinity column of Con A-Sepharose

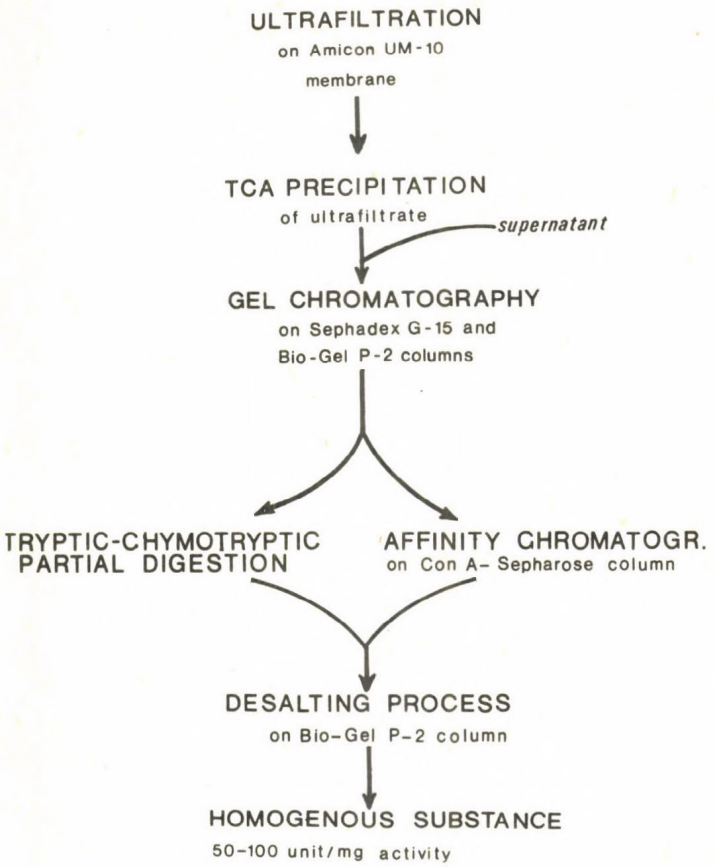

Fig. 1. Sequence of the purification steps for the isolation of satietin and satietin-D from human plasma

was enough to gain homogenous material which was bound on the column (component B) and eluted by the effect of a linear gradient of α-D-methylmannoside at neutral pH. The non-carbohydrate like materials and the insoluble were excluded from the bioaffinity column (i.e. not bound and appeared as component A as it is shown in Fig. 2). Approximately 22% of the total amount of substance (20 mg) passed through the column without being adsorbed. The optimal concentration of NaCl in the buffer was found to be 0.5 M and was used in the starting buffer as well as in the eluting buffer. Regeneration of the column occurred without repacking the gel according to the following procedure:

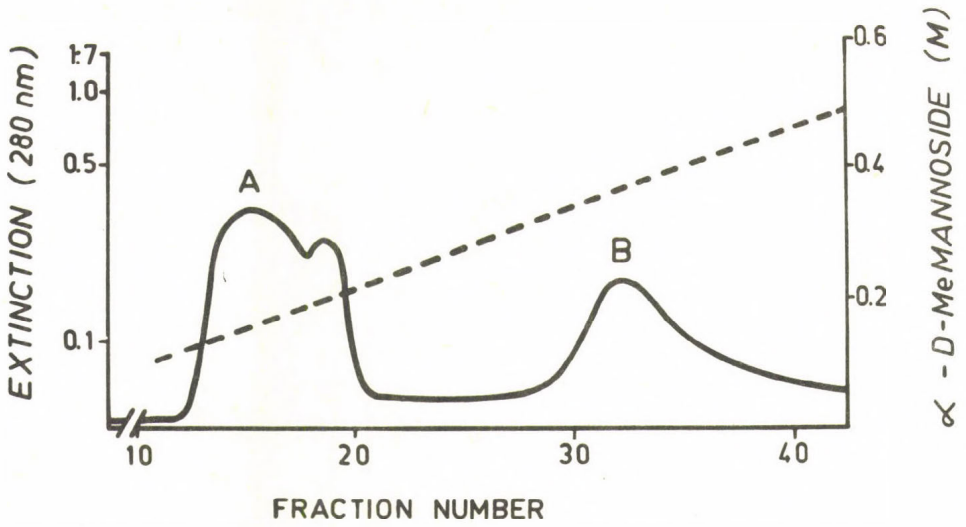

Fig. 2. Isolation of human satietin by affinity chromatography on Con A-Sepharose column. Column dimensions: 1.7x37 cm (bed volume, 84 ml). Eluent: 0.02 M Tris-HCl, pH 7.0 buffer with a linear gradient of 0.0-0.5 M α-D-methyl-mannoside. Fraction volume: 2.5 ml. Component A, the mixtures of proteins and glycoproteins unbound to Con A-Sepharose, component B specially bound on the bio-affinity column and eluted by the effect of linear gradient of α-D-methylmannoside. Separation was followed by measuring the UV extinction with LKB Uvicord II instrument.

1. Wash with 10 column volumes of 0.1 M Tris - HCl buffer containing 0.5 M NaCl, pH 8.5.
2. Wash with 10 column volumes of 0.1 M sodium acetate buffer containing 0.5 M NaCl, pH 4.5.
3. Re-equilibration with starting buffer.

The final desalting procedure of component B on Bio-Gel P-2 column offered further separation (Fig. 3) and the large glycoprotein molecule was excluded from the gel column appearing as a very sharp peak (marked S), while some others, probably smaller glycopeptides retarded on the column and this step finally resulted in pure salt-free substance after lyophilization.

The peak marked S (Fig. 3) has been classified as a glycoprotein and judged to be homogeneous by SDS-polyacrylamide gel

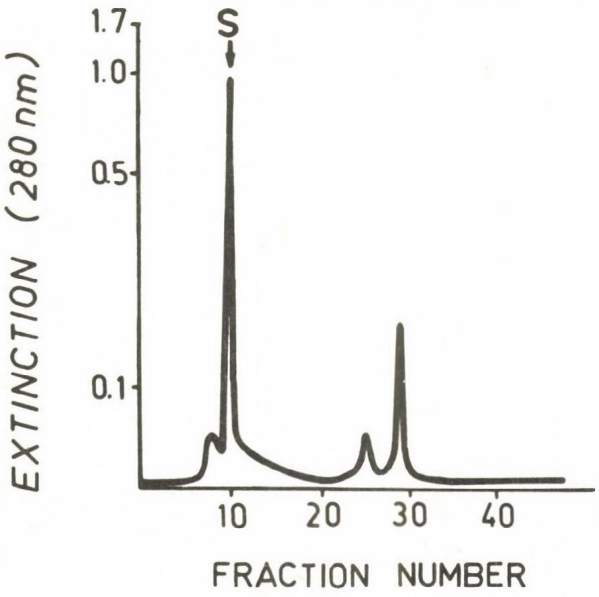

Fig. 3. Desalting process and separation of the pure plasma satietin (component B) on Bio-Gel P-2 column (2.5x90 cm) in deionized water. S shows satietin-active fractions excluded from the column. The UV extinction was recorded on an LKB Uvicord II instrument

electrophoresis and analytical isoelectric focusing. Thus, the isoelectric point of this pure glycoprotein was found to be pI=7.0-7.1 in agreement with our previous findings (8). Proteolytic digestion as another possible way for the isolation of human satietin was applied. Kinetic studies of the incubation were done to claim the optimal conditions. Fig. 4 demonstrates the gel electrophoretic patterns of samples digested for different periods of times indicating that digestion of 24 h completely eliminates the plasma protein impurities.

The final purification step of the enzymatically treated substance was accomplished by gel chromatography on a column of Bio-Gel P-2. Fig. 5 shows a very efficient separation of human plasma satietin digested with trypsin and chymotrypsin. Those fractions excluding from the gel (S) were pooled and lyophilized. This way, a white powder was obtained showing 50-100 units/mg satietin activity. This substance was named satietin-D.

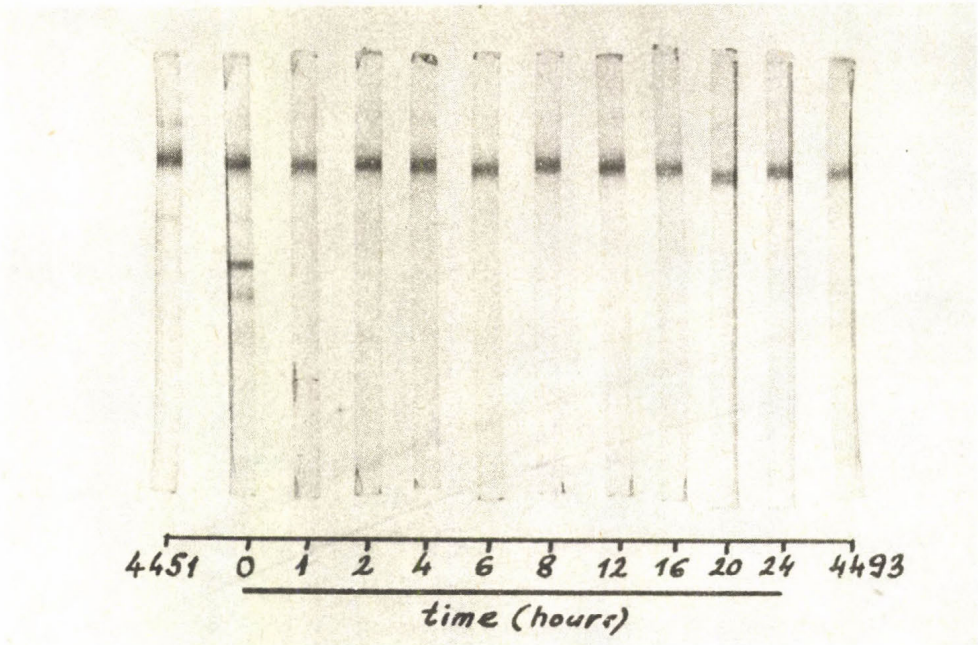

Fig. 4. Kinetic studies of proteolytic digestion of plasma satietin by SDS-Tris PAGE. Symbols: 4451, highly purified satietin; 4493, satietin-D after proteolytic digestion. The acrylamide concentration was 13%. During the digestion 50 µl aliquots were withdrawn at the indicated times mixed with 75 µl sample buffer followed by heating at 80°C for 10 min and subjected to electrophoresis according to Laemmli (10). The satietin concentration in digestion mixture was 0.5% (w/v). The gels were stained with Coomassie brilliant blue.

The overall yield was 60% calculated for the digestion and the desalting procedures. Fig. 6 shows the gel electrophoretic patterns of human satietin-D before and after the final isolation step. The pure substance could be stained with CBB as well as PAS, which proves the two bands to be identical (this is another evidence for the glycoprotein nature of the compound) and the mobility has not been changed during this separation step. As shown in this figure, in polyacrylamide gel rods loaded with identical amounts of satietin samples, most of the intense staining was found in bands of molecular weights around of 50,000 daltons. The molecular weight determination was carried out by using SDS-Tris PAGE (10) containing 13% acrylamide.

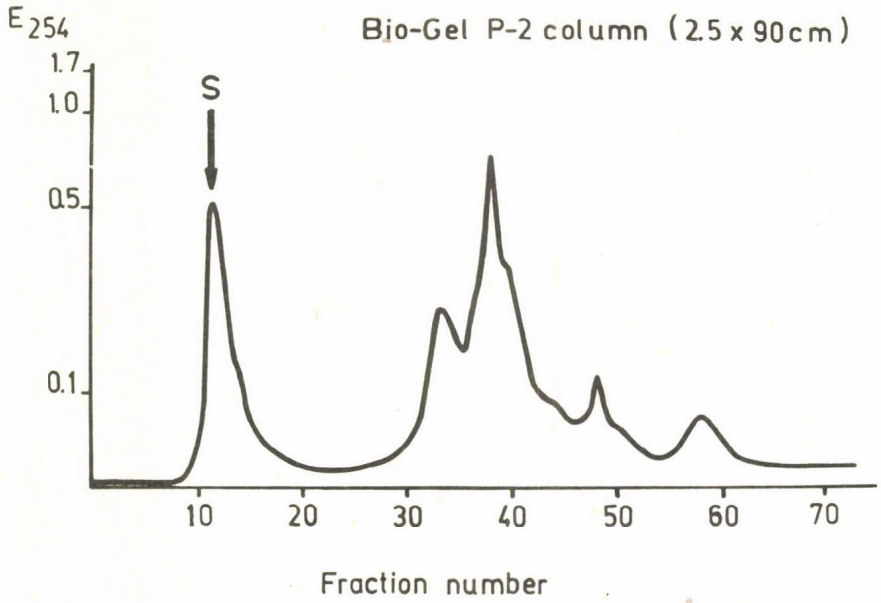

Fig. 5. Gel chromatographic separation of the highly purified satietin preparation treated with trypsin and chymotrypsin on Bio-Gel P-2 column. Separation was followed by recording the UV extinction on an LKB Uvicord II instrument. S represents the satietin-active fractions.

The electrophoretic mobilities calculated by the method of Weber and Osborn (11) for marker proteins were plotted against the log of their molecular weights (Fig. 7). On the basis of electrophoretic mobility of pure human satietin, a molecular weight of 49,000 daltons was found. We also determined the exact correlation coefficient for the five marker proteins by using a TI-59 programmable calculator that proved to be 0.990. Similar results were obtained when SDS-phosphate PAGE was applied for the same purpose.

Glossmann and Neville (13) found that the molecular weights of glycoproteins can be estimated with ca. 10% deviation in an apparent range between 30,000-40,000 daltons when the relative mobilities of the glycoproteins in SDS-PAGE were used to calculate the apparent molecular weights from a calibration curve obtained with the standard marker proteins listed there.

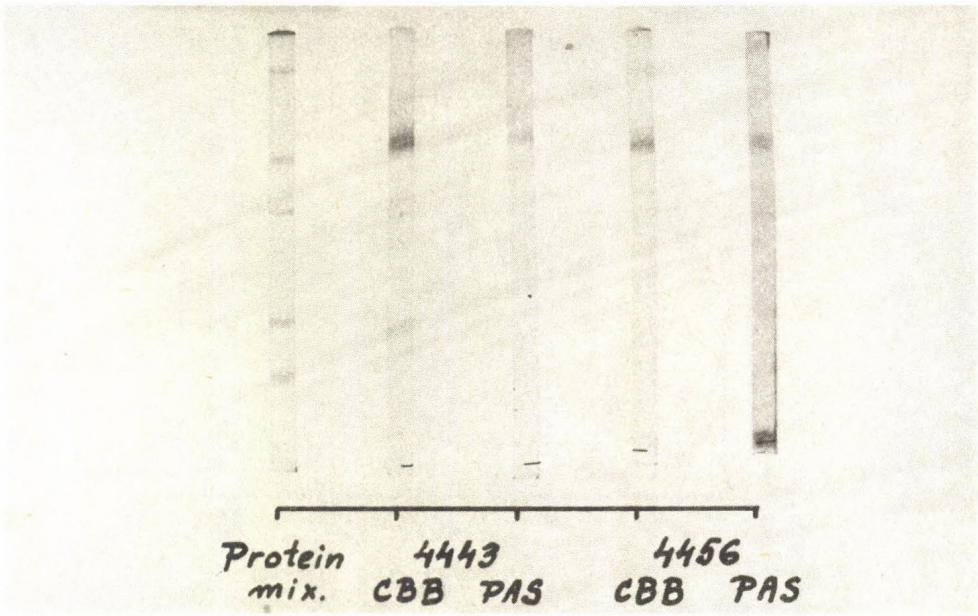

Fig. 6. The SDS-Tris PAGE of satietin-D before and after proteolytic digestion. Symbols: Protein mix, from top to bottom: phosphorylase B (92,500); ovalbumin (45,000); carbonic anhydrase (31,000); soybean trypsin inhibitor (21,500); lysozyme (14,400); 4443, highly purified satietin; 4456, satietin-D after proteolytic digestion. The total acrylamide concentration was 13%. The gels were stained CBB and PAS, respectively.

When examining the pure satietin-D sample by isoelectric focusing we found that it existed as a single band in the pI range of 3.0-3.1 providing further support to that the isolated glycoprotein was homogeneous (Fig.8).

The carbohydrate and protein content of the pure material was determined after acid hydrolysis. The analysis of sugars was kindly performed by Dr. M. Mészáros (Organic Chemistry Department, Eötvös Loránd University, Budapest) as published earlier (8).

As a first step in analysing the amino acid content of these highly purified preparations, thin-layer chromatography has been used. The purified samples were hydrolysed in 6 N HCl for 18 hours, at 105^{o}C and the samples were dried in vacuo over NaOH pellets and redissolved in 0.01 N HCl. Aliquots were then

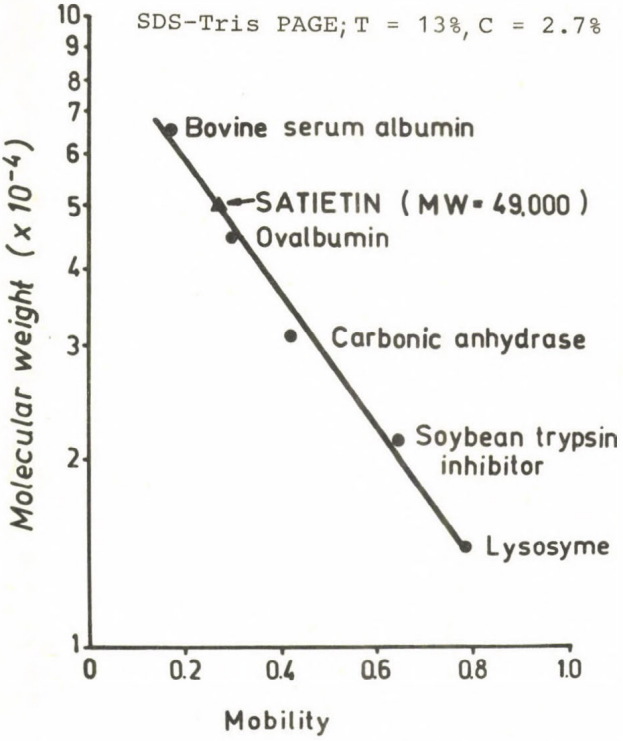

Fig. 7. Determination of the molecular weight of human plasma satietin-D by means of SDS-Tris polyacrylamide gel electrophoresis. The five marker proteins used were: bovine serum albumin (67,000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,400). All proteins were run on duplicate gels.

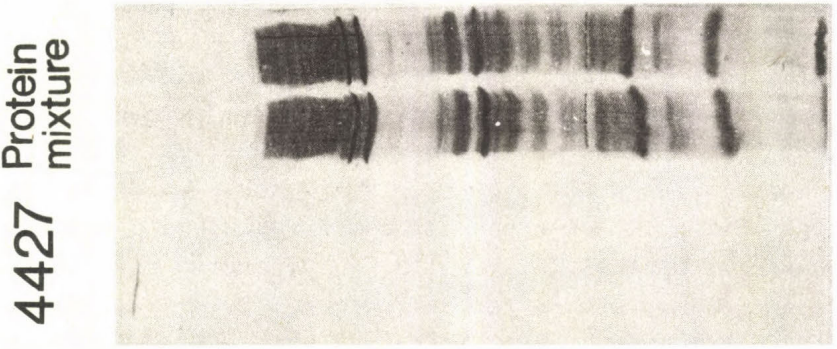

Fig. 8. Isoelectric patterns of pure human satietin-D (4427) and standard protein mixture in a pH range of 3.0-10.0. LKB ampholine and a Pharmacia Horizontal Apparatus were used for the isoelectric focusing studies according to Radola (14).

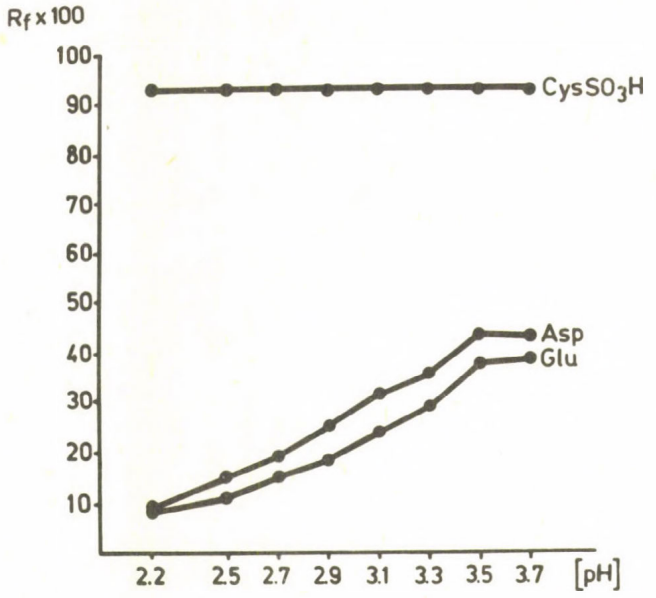

Fig. 9. pH dependence of the separation of acidic amino acids on Fixion 50x8 ion-exchange resin coated thin-layer developed in system II in a pH range of 2.2 to 3.7.

applied onto a 20x20 cm silica gel layer and chromatographed by using system I. Overrun mode of development was carried out which increased the resolution of the individual amino acids even in the case of one-dimensional thin-layer technique. This system provided the separation of almost all the essential amino acids. Since the substantial amounts of lysine and aminosugars, namely glucosamine and galactosamine, caused a traffic jam in the lower R_f region, ion-exchange thin-layer chromatography was used and that ensured the selectivity of the separation for those amino acids which showed up very poorly on silica gel. The application of ion-exchange coated thin-layers was first introduced by Dévényi et al. (17). We attempted to optimize this ion-exchange method for our purposes regarding the separation of acidic amino acids, lysine and aminosugars which components occurred in higher quantity in our preparations of biological origin. The results obtained by us in the separation of acidic amino acids are illustrated in Fig. 9, when system II was the solvent and the optimum pH range was found to be

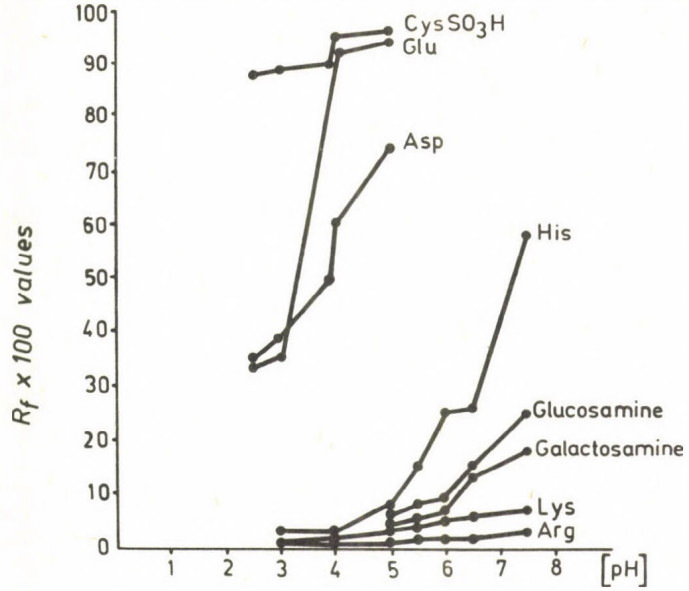

Fig. 10. pH dependence of the separation of acidic and basic amino acids and aminosugars on Fixion 50x8 ion-exchange chromatoplate developed in System III in a pH range of 2.5 to 7.5.

around 3.0-3.1. The separation of basic amino acids and aminosugars could be solved by doing chromatography in system III at pH 7.5 (Fig. 10).

The qualitative informations for the amino acid content in glycoprotein hydrolysates were confirmed by quantitative evaluation carried out on automatic amino acid analyser using standard ion-exchange procedure. The optimum time of hydrolysis of these glycoproteins were checked after 4, 8, 12, 18, 24 hours treatment in 6 N HCl at 105°C. The results obtained are shown in Fig. 11. In each case the hydrolysate was dried, dissolved in the application buffer and subjected to the column of amino acid analyser. The exact content of the individual amino acids was plotted against the time of hydrolysis. Fig. 12 represents one of the typical chromatograms of the hydrolysed pure human preparation (satietin-D), while its amino acid composition is listed in Table I. Detectable amounts of tryptophan was not found beside the essential amino acids when 4 N methanesulfonic acid was used for hydrolysis. The huge lysine peak (the lysine

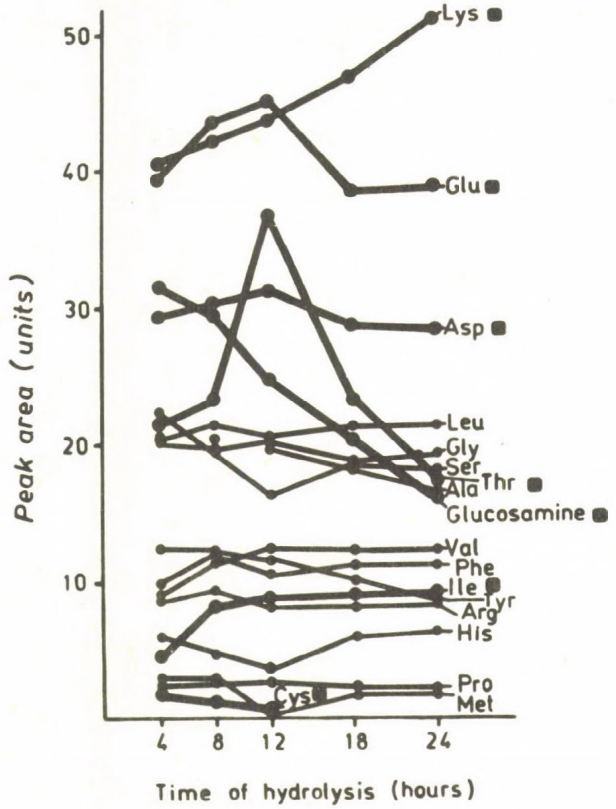

Fig. 11. Change of the free amino acid content of a satietin hydrolysate vs. time of hydrolysis in 6 N HCl at 105°C.

peak might be covered by its N-methylated derivatives or other rare amino acids behaving chromatographically very similarly and being eluted at the same place in our system) was found to be significant for the human material and for other satietin active substances obtained from different sources. Table I compares the amino acid composition and the total peptide content of the neutral (pI=7.0) human preparation with the acidic (pI=3.0)substance named satietin-D after acid hydrolysis.

The extremely high content of lysine and the acidic amino acids as aspartic and glutamic acid in both preparations is in good correlation with other hitherto known glycoproteins. From this consideration it is imaginable that the ratio between the acidic amino acids and lysine possessing free carboxyl and

Table I Amino acid composition of pure satietin preparations from human plasma hydrolysed with 6 N HCl

Amino acid	After Con A-Sepharose purification (Satietin)		After tryptic - chymotryptic treatment (Satietin-D)	
	Found %	Nearest integer	Found %	Nearest integer
Asp	1.2	5	2.5	10
Thr	0.7	3	1.4	6
Ser	0.7	4	1.1	7
Glu	1.9	7	3.1	12
Pro	0.5	2	1.3	5
Gly	0.4	3	0.7	5
Ala	1.6	11	0.9	5
Val	0.6	2	0.8	3
Cys*	0.4	2	0.4	2
Met	0.1	1	0.4	2
Ile	0.3	1	0.7	3
Leu	0.9	3	1.2	4
Tyr	0.3	1	1.1	3
Phe	0.4	1	0.8	2
Lys	3.8	15	3.4	13
His	0.2	1	0.2	1
Arg	0.5	2	0.7	2
Total	14.5%	64 MW = 6,938	20.7%	82 MW = 9,486

*Cys calculation based on the measurement of $CysSO_3$

ε-amine groups may result in a wide range of substances with different pI values, which probably have similar chemical structure. This implies that these substances having various isoelectric points do not show major differences in molecular weight and sugar composition but are substantially different in the ratio of the acidic and basic amino acids.

The results of thermal analysis of the freeze-dried human satietin-D sample are seen in Fig. 13. As it turned out, the adsorptive bound water (0-5%) was eliminated from the sample between 25-75°C. Chemically bound water was not found. Applying a linear temperature gradient up to 600°C, it was found that the decomposition of the substance analysed began at 150°C (see the TG and DTG curves). This also means that this compound was found to be heat stable until 150°C. The thermal evolution

Table II Characteristics of human satietin samples isolated by different ways

	Con A-Sepharose column	Enzymatic treatment
Molecular weight (daltons)	48,000-50,000	48,000-50,000
Isoelectric point (pI)	7.0-7.1	3.0-3.1
Molar extinction coefficient ($\varepsilon_{282} M^{-1} cm^{-1}$)	-	30,000
Protein content (w %)		
Amino acid analysis	14-15	20-22
Microbiuret method	14-16	21-24
Carbohydrate content (w %)	17-75	55-60
Glucosamine content (w %)	3-4	4-5
Water content (adsorptive, w %)	-	5-6
Biological activity		
units/mg	50-100	50-100
*ID_{50} in rats, µg	10-20	10-20

*ID_{50} = the dose which suppressess food intake in rats during the first day of feeding after a 96-hour food deprivation to half of the amount eaten by untreated controls.

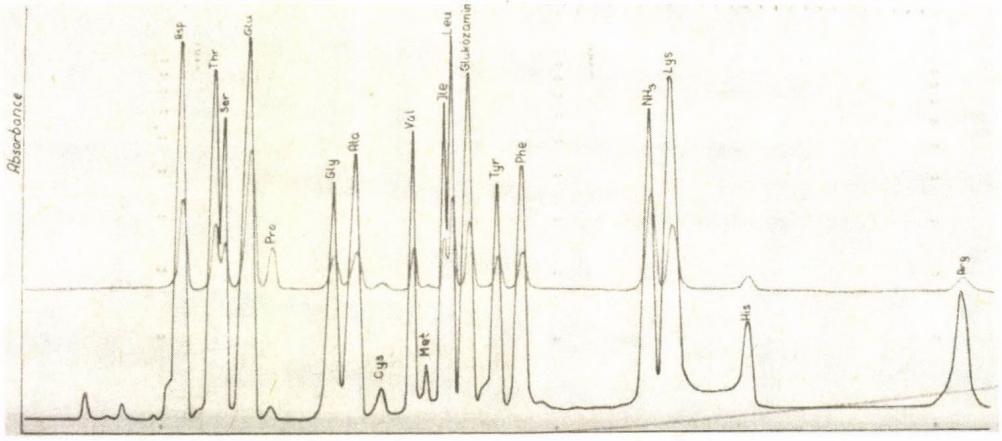

Fig. 12. Analysis of human satietin hydrolysate on automatic amino acid analyzer (Bio-Cal BC-200) using one column and two buffer systems. Column: Aminex A-5 (0.9x50 cm) at 52°C. Buffers: A, 0.2 N Na^+ citrate, pH 3.25; B, 0.8 N Na^+ citrate, pH 4.25. Buffer B was switched over at Pro.

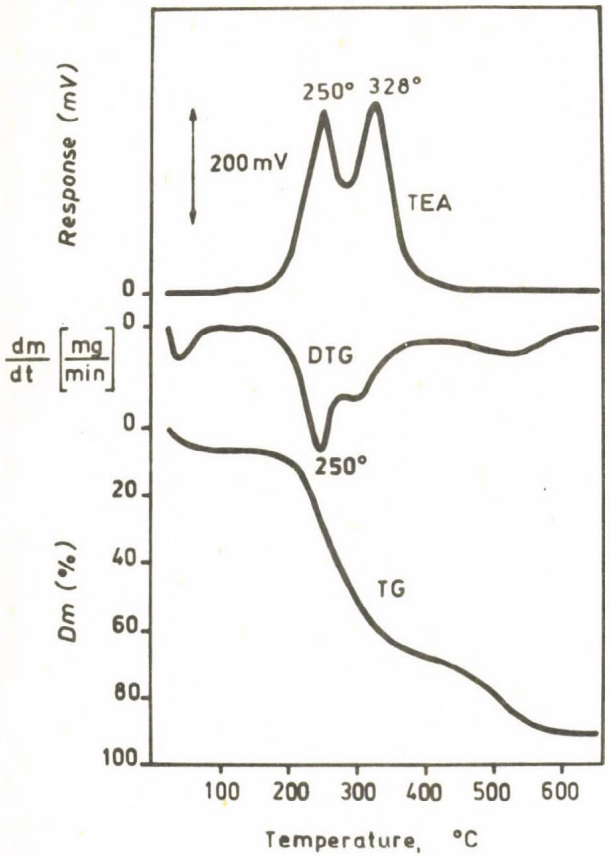

Fig. 13. Thermal gravimetric and thermal evolution analyses of a human satietin sample. Conditions are described under "Experimental Procedures".

analysis (TEA) curve (Fig. 13, on the top) shows the evolution of gaseous organic degradation products.

The ultraviolet absorption spectra of human satietin-D at different pH values can be seen in Fig. 14. The absorption maximum was found at 282 nm and no essential spectral changes were detected between pH 2 and pH 10. The molar extinction coefficient was calculated from the molecular weight (50,000 daltons) based on SDS-Tris PAGE determination. The value found was $\varepsilon_{282\,nm} = 30{,}000\ M^{-1}\ cm^{-1}$.

The protein content of both preparations was also estimated by microbiuret method and compared to those values calculated from amino acid analysis. The average difference determined

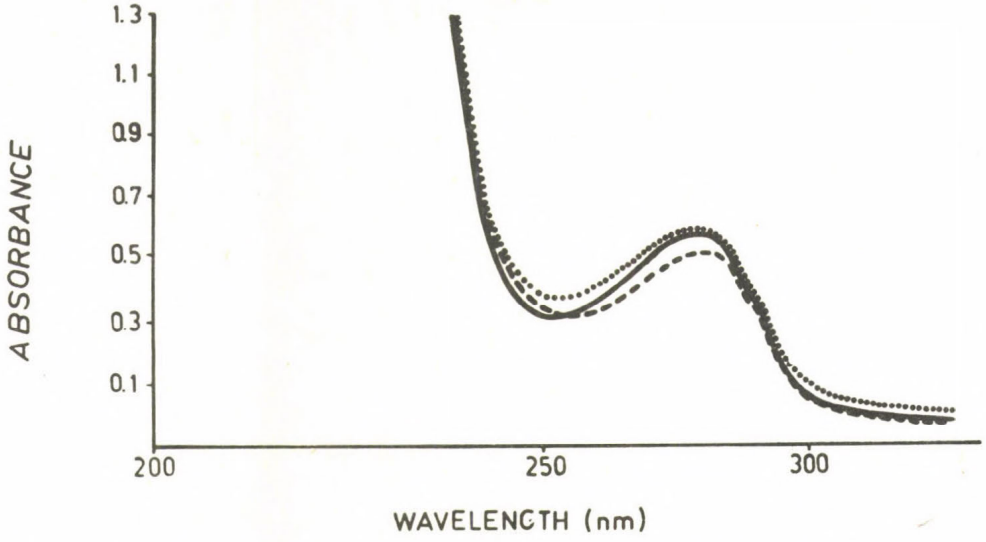

Fig. 14. Ultraviolet absorption spectra of human plasma satietin in variation with pH, in Solvent I, pH 2 ....; Solvent II, pH 7——; Solvent III, pH 10 ----.

by these two methods was found as low as 2.0%. The properties of these plasma proceeded glycoproteins documented in Table II strongly suggest that the similarity may be valid not only in the biological effect but for the basic chemical structure as well.

ACKNOWLEDGEMENT

The valuable advice and help of Dr. Sándor Harmath (Veterinary Biologicals Co.) for the electrofocusing studies are gratefully acknowledged. We also thank Dr. György Pokol for TGA and TEA analyses. Thanks are due to Ms. Maria Dancs for her excellent technical assistance.

REFERENCES

1. Knoll, J.: Physiol. Behav. 23, 497-502 (1979).
2. Knoll, J.: in: Advances in Pharmacology and Therapeutics II. Vol. 1 CNS Pharmacology - Neuropeptides (Eds.: Yoshida, H., Hagihara, Y., Ebashi, S.) Pergamon Press, Oxford - New York, pp. 147-162 (1982).
3. Knoll, J.: Pol. J. Pharmacol. Pharm., 34, 3-16 (1982).
4. Knoll, J.: in: Regulatory Peptides: From Molecular Biology to Function (Eds.: Costa, E. and Trabucchi, M.) Raven Press, New York, pp. 501-509 (1982).
5. Kinoshita,C.M., Lichton, I.J. and Ako, H.: Biochim. Biophys. Acta 719, 232-237 (1982).
6. Tsuda, T.T., Kishino, Y. and Katsunuma, T.: Physiol. Behav. 29, 519-525 (1982).
7. Nagy, J., Kalász, H. and Knoll, J.: Pol. J. Pharmacol. Pharm., 34, 47-52 (1982).
8. Nagy, J., Kalász, H. and Knoll, J.: in: A. Frigerio (Ed.), Chromatography and Mass Spectrometry in Biomedical Sciences, 2.Elsevier Scientific Publishing Company, Amsterdam, pp. 421-432 (1983).
9. Honn, K.V. and Chavin, W.: Anal. Biochem. 68, 230-235 (1975).
10. Laemmli, U.K.: Nature 227, 680-685 (1970).
11. Weber, K. and Osborn, M.: J. Biol. Chem. 244, 4406-4412 (1969).
12. Köiv, E. and Gronwall, A.: Scand. J. Clin. Invest. 4, 244-246 (1952).
13. Glossmann, H. and Neville, D.M.: J. Biol. Chem. 246, 6339-6346 (1971).
14. Radola, B.J.: Electrophoresis, 1, 43-56 (1980).
15. Walker, W.H., Kearney, E.B., Seng, R.L., Singer, Th. P.: Eur. J. Biochem. 24, 328-331 (1971).

16. Dévényi, T.: Acta Biochim. Biophys. Acad. Sci. Hung. 3, 429-432 (1968).

17. Dévényi, T., Hazai, I., Ferenczi, S. and Báti, J.: Acta Biochim. Biophys. Acad. Sci. Hung. 6, 385-388 (1971).

Chromatography, the State of the Art
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ISOLATION OF THE RETINOL BINDING PROTEIN (RBP) FROM THE SERUM OF FARM ANIMALS

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INTRODUCTION

Two types of retinol binding proteins, in the metabolism of Vitamin A are involved. One of the carrier (RBP) (5, 7, 13) and the other one is a cellular receptor (cRBP) (3) of Vitamin A. The first investigations regarding RBP were carried out in the late sixties (7) and early seventies (13). Isolation and partial characterisation were carried out by different chromatographic (ion exchange, gel filtration), electrophoretic (agar-, PAG, IEF) and ultracentrifugation steps (5, 6, 7, 8, 13, 14). These methods require extensive laboratory equipments, materials and, last but not least, time. The affinity chromatographic separation of proteins is more specific and usually needs shorter time. This possibility was used by coupling Vitamin A derivative (retinoic acid, Rac) to activated Sepharose (4). In the present work affinity chromatographic separation was performed on a cellulose support for the isolation of RBP from the serum of some farm animals (cow, swine, goose, chicken).

MATERIALS

Affinity chromatographic materials:
Aminohexylcellulose(AHC) with 100 µeq/ml functional group content and N-cyclohexyl-N-/beta(N-methylmorpholino)ethyl/-carbo-

diimide-p-toluene-sulphonate (CMC) were received as a gift from E. Merck (Darmstadt, FRG).

Gel filtration materials:
Sephadex G-25 (Pharmacia, Uppsala, Sweden), Ultrogel AcA 44 (LKB, Bromma Sweden) Acrylex P-6 (Reanal, Budapest, Hungary) were used.

Buffer materials for phosphate buffer solution (PBS); lipid extraction materials; guanidine HCl (Fluka, Buchs, Switzerland); retinoic acid (Rac) (Fluka, Switzerland) were research grade.

Blood samples were taken from animals of the University's experimental station. After blood-clotting, the serums were separated and freshly used.

EQUIPMENT

All the chromatographic processes were carried out in a refrigerator (at 4°C) on a LKB system (Bromma, Sweden) consisting of a Microperpex pump, an Ultrograd gradient former, a Uvicord S photometer, and a RediRac fraction collector. The chromatograms were recorded by a potentiometric recorder (Radelkisz, Budapest, Hungary).

METHODS

Elimination of Vitamin A to yield apo-RBP:
Fifty ml serum was mixed overnight by rotary movement with a delipidation mixture (petrolether: ether, 96:4, v/v). The aqueous phase was separated by centrifugation (2500 g, 4°C, 30 min) and collected.

Affinity chromatography:
20 mg retinoic acid was dissolved in 1 ml chloroform, then filled to 50 ml with dioxane. Moist AHC (5 g) was suspended in 50 ml PBS (pH 6). The two parts were collected and 200 mg CMC was added as the coupling agent. The whole mixture protected from light was gently shaked for two days, then

filtrated. The moist AHC-Rac affinity matrix was mixed with the collected aqueous phase (overnight at $4^{o}C$). The column (0.8 cm x 10 cm) was filled with the suspension and the non-bound proteins washed out by pumping (7 $cm.h^{-1}$) PBS (pH 7.5, containing 0.15 mole NaCl) for 8 h. The bound protein (RBP) was eluted by the same buffer containing a deforming substance (6 mole/l guanidine-HCl). For the concentration of the protein content of these fractions Acrylex P-6 (exclusion limit 6 x 10^3 M_m, swelling 7.5-9.5 ml/g), was added (1 g per 10 ml).

Molecular mass (M_m) determination:
Two columns (1.6 cm x 60 cm) were filled with the gel filtration media, the first with Sephadex G-25 fine, and the second with Ultrogel AcA 44. They were connected to each other. Buffer (PBS, pH 7.4 containing downward 0.15 mole NaCl) was pumped through the columns, in a downward direction through the first column and in an upward direction through the second column. The column system was calibrated by the solution of (in the order of decreasing molecular weight) Blue Dextran (BD), Yellow Dextran (YD), Cytochrome C (C-c), and Vitamin B_{12} (B_{12}). The protein fractions which remained bound to the AHC-Rac affinity support after the concentrating process were chromatographed and their elution profile compared to the calibration results. The methods are summarized in Fig. 1.

RESULTS

The results are presented in Figs 2 and 3. From the AHC-Rac column a large amount of proteins could be washed out in the first step (Fig. 2). These proteins are not connected to the matrix i.e. they are the non-bound proteins. The elution was performed until the OD was decreased to the start value. At the 8th hour the elution buffer was changed to another containing 6 mole guanidine HCl as the deforming agent. After this treatment a new peak appeared in the proteinogram. This corresponded to the bound protein wich was associated with the immobilized retinoic acid. The elution volume (V_e) on the gel

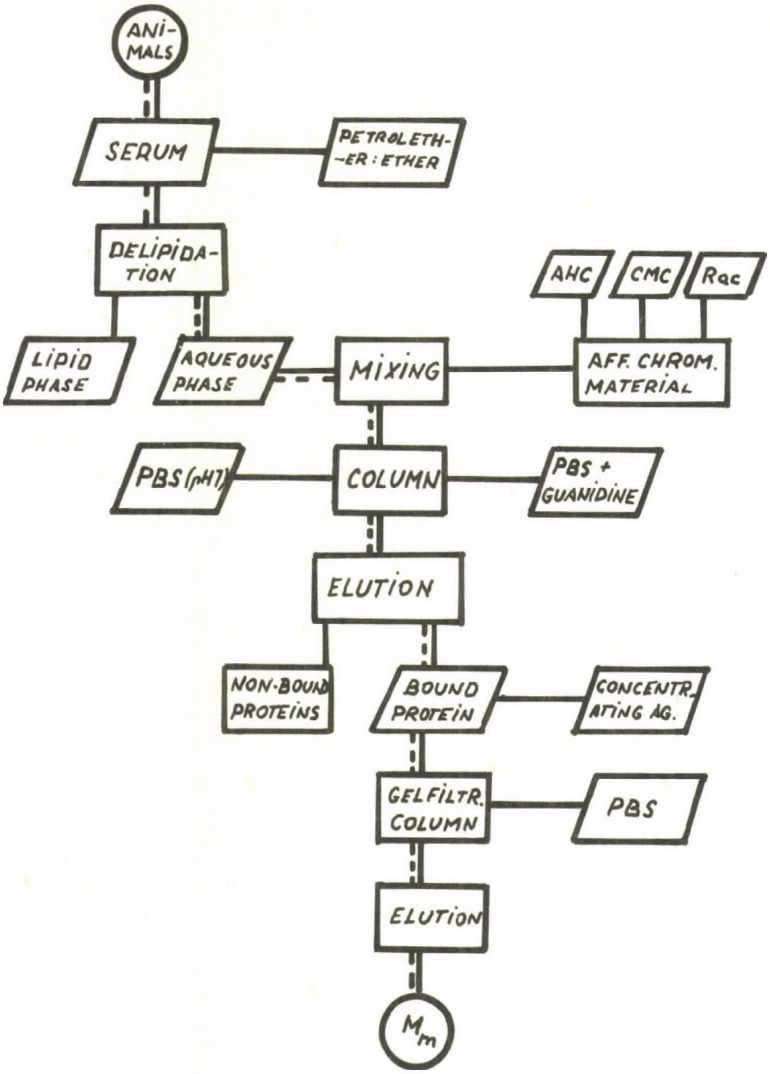

Fig. 1. The separation scheme. The route of RBP (═══)

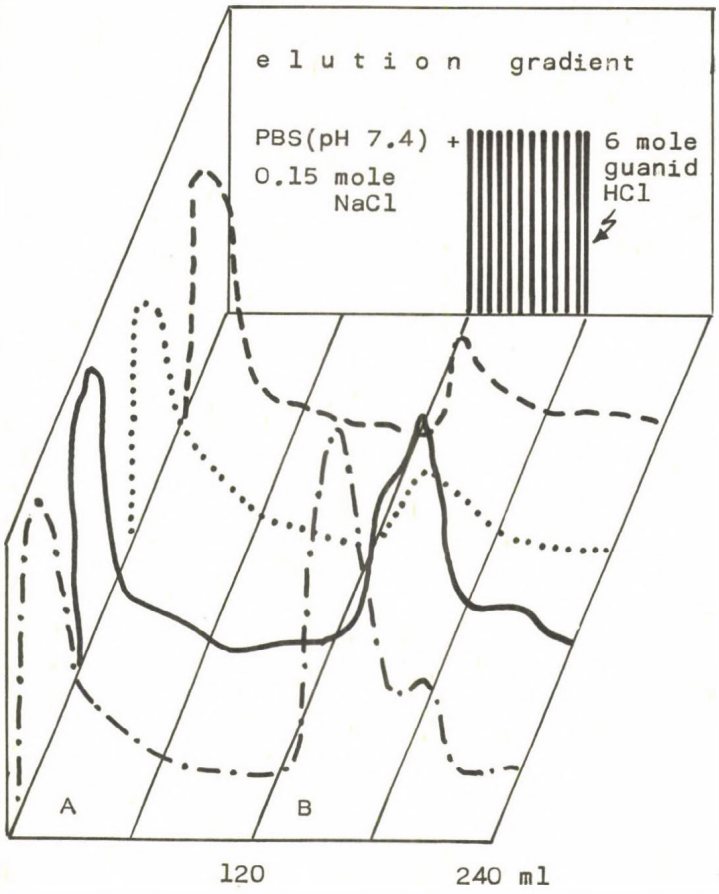

Fig. 2. Affinity chromatography of cow (- -), pig (....), goose (——) and chicken (-·-) serum on the AHC-Rac column. A = non-bound proteins, B = bound proteins (RBP)

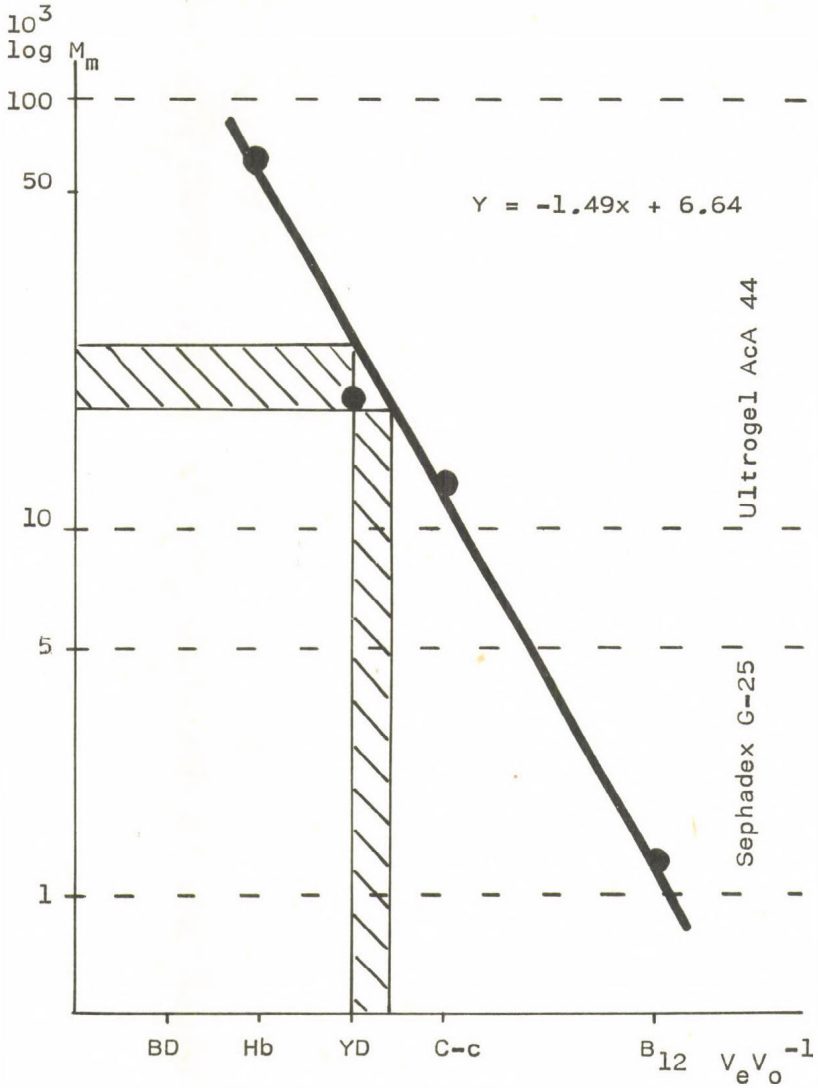

Fig. 3. Molecular mass (M_m) determination of the bound-protein fractions (RBP/////)

filtration columns were to identical the V_o of Yellow Dextran (M_m = 2 x 10^3) (Fig. 3).

DISCUSSION

There are different statements in the literature concerning the association and/or dissociation properties of the retinol - RBP - prealbumin complex. The RBP free of retinol (apo-RBP) is unable (12) or able (8.9) to couple with prealbumin. Retinol was washed out with diethyl ether by Kopelman et al. (8). In our experiment the same process was carried out with petrolether : diethyl ether (96:4) which delipidation mixture is regularly used for the colorimetric determination (1) of the retinol/retinyl ester ratio (16). The proteins obtained by delipidation had an ability for coupling to the immobilized retinol derivative (Rac). This binding was carried out at the physiological ionic strength (0.15 mole NaCl) and was stable, when the elution was performed under the same condition. By this treatment only the non-bound proteins could be washed out from the affinity matrix. When guanidine HCl was added to the buffer, a characteristic peak appeared in all the cases (Fig. 2). The shapes and heights (OD at 206 nm) of the peaks were different, depending on the investigated animal species. The highest peaks were obtained for birds (chicken and goose). These animals were in period thereby the mobilization of the Vitamin A (as retinol) from the liver to the ovarium was intensive. The smallest peak occurred in the chromatogram obtained from the serum of the swine. Swine exhibits a usually small Vitamin A level (10). For this reason Muto et al. (11) included a concentration step in the preparation of swine RBP. As can be seen, we could detect a valuable peak without any concentration before the affinity chromatography of swine serum (Fig. 2). Guanidine HCl initiates the proteins into coil-shaped forms which are related only to the molecular mass (M_m) which, naturally, depends on the amino acid chain length (13). On the calibrated column system the elution volumes (V_e) of the bound proteins are similar for the investigated farm animals. These volumes correspond to Yellow Dextran ($M_m = 2 \times 10^4$). (Fig. 3). The simple purification of apo-RBP by affinity chromatography facilitates the preparation of this protein for futher studies e.g. for immunization. The cellulose base material (AHC) served

as an excellent affinity support for the isolation of cow, swine, goose and chicken retinol-transporting protein.

ACKNOWLEDGEMENTS:

We thank E. Merck (Darmstadt, FRG) for the gift of AHC and CMC, and Miss K. Karchesz for her assistance.

REFERENCES

1. Bárdos L. Bull.Univ.Agr.Sci. Gödöllő pp. 11-20 (1975)
2. Cogan, U.,Kopelman, M., Mokady, S., Shinitzky, M. Eur. J. Biochem 65 71 (1976)
3. Chytil, F., Page, D.L., Ong, D.E. Int.J.Vit.Nutr.Res. 45 293 (1975)
4. Fex, G., Hansson, B. Biochim. Biophys. Acta 537 358 (1978)
5. Glover, J. Vitam. Horm. 31 (1973)
6. Heller, J. J. Biol. Chem. 250 6549 (1975)
7. Kanai, M., Raz, A., Goodman, DeWitt S. J.Clin.Invest. 47 2025 (1968)
8. Kopelman, M., Mokady, S., Cogan, U. Biochim. Biophys. Acta 439 442 (1976)
9. Kopelman, M., Cogan, U., Mokady, S., Shinitzky, M. Biochim. Biophys. Acta 439 449 (1976)
10. Moore, Th. The Vitamin A Elsevier Amsterdam,(1957)
11. Muto, Y., Smith, R.F., Goodman, DeWitt S. (1973) J.Lipid Res. 14 525 (1973)
12. Natelson, S., Natelson, E.A. Principles of Applied Clinical Chemistry Vol.3. Plenum Press, (1980)
13. Peterson, P.A. J.Biol.Chem. 246 44 (1971)
14. Rask, L. Eur.J.Biochem. 44 1 (1974)
15. Vahlquist, A. Int.J.Vit.Nutr.Res. 44 375 (1974)

Chromatography, the State of the Art
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INHIBITION OF MYOMETRIAL CONTRACTIONS IN VITRO BY A SUBSTANCE IN EXTRACT OF SOW OVARIES. THE RELATION OF THE SUBSTANCE TO STANDARD RELAXIN

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SUMMARY

Although the mouse symphysis assay is widely held to be the specific test system for monitoring purification of relaxin from its tissue source, the ability of relaxin to inhibit in vitro myometrial contractions is also accepted by many as a less specific, but inherent property of the molecule. Endogenous substances, distinct from relaxin, with unidentified biological activity, although have been less extensively studied until recently, are certainly present in various ovarial extracts. If they affect myometrium contractions, but are without any effect on the pubic symphysis, they may easily escape detection if their purification is monitored solely with the aid of the symphysis assay. This was the main reason, why we have decided to monitor the attempted purification of the non-relaxin components assumedly present in the ovarial extract used in these experiments, by isolated myometrium preparations, instead of the pubic symphysis assay.

In the experiments presented here, the effect of a standard relaxin preparation /NIH-R-P-1/ on myometrium contractions was also studied, and subsequently compared to that of one of the SG-25 fractions obtained from sow ovaries which, among all fractions, disclosed the most marked activity in a biodetection system /BDS/ based on the use of an isolated myometrium strip, as described elsewhere / 3, 5 /. The relaxin content of the SG-25 fractions was determined with help of a specific RIA method.

Concerning their chemical composition, a standard relaxin preparation proved to be heterogenous. Its lowest myometrium contractions affecting concentration was found to be 5 $\mu g\ ml^{-1}$. In sharp contrast to this, although the already mentioned SG-25 fraction contained immunoreactive relaxin in a concentration as low as $4.1 \times 10^{-3}\ \mu g\ ml^{-1}$, it produced a more marked inhibition of the myometrium contractions than the heterogenous standard relaxin preparation in a much higher concentration. Expressing quantitatively, the SG-25 fraction was at least 3000 times more active on the myometrium strips than standard relaxin.

Based on the data presented in this paper we conclude that ovarial extracts contain a hitherto unidentified endogenous substance with a more potent inhibitory potential on myometrial contractions than standard relaxin. This substance appears to be closely related to relaxin, as indicated by its moderate cross-reactivity with specific relaxin antisera. Nevertheless, it seems to be distinct from relaxin, as judged on the basis of its other properties.

INTRODUCTION

It has previously been demonstrated that ovaries of different species /rat, sow, human/ are rich sources of a large number of peptides with various biological activity detectable under in vitro or in vivo conditions /1/. One of these peptides was identified as relaxin, a 6300 molecular weight peptide which, among other effects, also inhibits myometrium contractions both in vivo and in vitro /2/.

It has also been demonstrated that the water-soluble fraction of acidic-acetone extract prepared from the ovaries of pregnant sows also includes a strikingly large number of biologically active endogenous substances, some of which also has the capability of affecting contractions of pregnant myometrium strips in vitro /3/, in one way or the other.

It may easily occur that endogenous substances closely related to relaxin, nevertheless distinct from it, which may or may not have an effect on pubic symphysis, escape detection

if their purification is monitored solely with the aid of the symphysis assay /4/. This was the main reason, why we have decided to monitor the purification of the agents present in ovarial extracts, by applying the myometrium contraction assay, instead of the pubic symphysis assay.

METHODS

Ovaries obtained from pregnant sows were extracted by acidic-acetone as described by Doczi and Griess /2/. The water-soluble fraction of the extract was fractioned by Sephadex G-25 /fine//SG-25/ chromatography /3/. The UV light-absorbing capacity of the eluent was continuously monitored at 254 nm, and the eluted solution collected in 2.6-ml fractions. The biological activity of the SG-25 fractions and standard relaxin was tested on spontaneously contracting uterine strips prepared from rats in the 20th day of their pregnancy /5/. The isometric contractions of myometrium strips were continuously registered. The biologically active fractions were characterized by their K_{av} values calculated at the maximum of their biological activity.

Relaxin immunoreactivity was determined by a specific porcine radioimmunoassay, as described by Sárosi et al. /6/. Standard relaxin /NIH-R-P-1/ was rechromatographed on a SG-25 column, and its UV absorption was continuously monitored at 254 nm.

The dose-response curve and ID_{50} value of standard relaxin were determined by the use of the biodetection system mentioned earlier. Finally, the effect of one of the SG-25 fractions eluted in the immediate vicinity of the void volume was compared to the effect of standard relaxin, by using the isolated myometrium strip assay.

RESULTS

The acidic-acetone extract of pregnant sow ovaries could be resolved by SG-25 chromatography into several fractions with a UV light-absorbing capability at 254 nm /Fig. 1./. The fractions with K_{av} values ranging between 0 and 0.35 differently affected the myometrium contractions /Fig. 2./. The K_{av} value of the SG-25 fraction which eluted in the immediate vicinity of the void volume and disclosed the most marked inhibitory action on isolated myometrium, was estimated to be 0.17. Immunoreactive relaxin concentration of this fraction was found to be 40 pg ml^{-1}, whereas that of the fraction eluted in the void volume was 4100 pg ml^{-1} /Fig. 3./.

By SG-25 chromatography, the standard relaxin could also be resolved into three distinct fractions with a UV light absorbing capability at 254 nm /Fig. 4./. The K_{av} values of these relaxin-related fractions were found to be 0.07; 0.51; and 1.02, respectively.

The biological activity of the heterogeneous standard relaxin preparation was also tested on isolated myometrium. Its dose-response curve determined by the biodetection system could be described by the following equation:

$$y = 24.4 \lg x + 28.5$$

where y stands for effect /in per cent of the control/, and x for dose /in µg/. ID_{50} value of standard relaxin was calculated to be 7.6 µg.

The myometrium contraction inhibiting capability of 5 µg standard relaxin and that of 20 pg of the SG-25 fraction with a K_{av} value of 0.17 were compared on the same myometrium preparation. It was found that the SG-25 fraction disclosed a more marked inhibitory effect on the myometrial contractions than standard relaxin, although its immunoreactive relaxin concentration was nearly 3000 times less than that of standard relaxin.

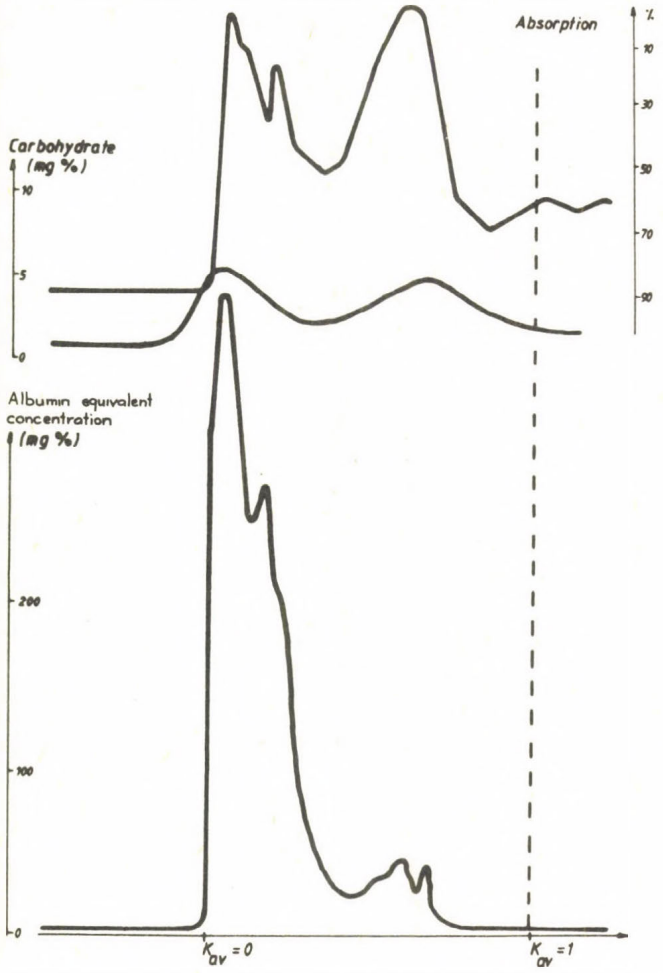

Fig. 1. Upper curve: UV absorption at 254 nm; middle curve: carbohydrate content measured by the orcin-sulfuric acid method; lower curve: peptide content expressed in albumin equivalent as measured by the Lowry method. Base line: K_{av} values. Measurements were made on fractions of sow ovarian origin, separated on a Sephadex G-25 column

DISCUSSION

It has been indicated in the literature that pregnant ovaries of widely different species are rich sources of various biologically active peptides /1/. One of them is relaxin, a

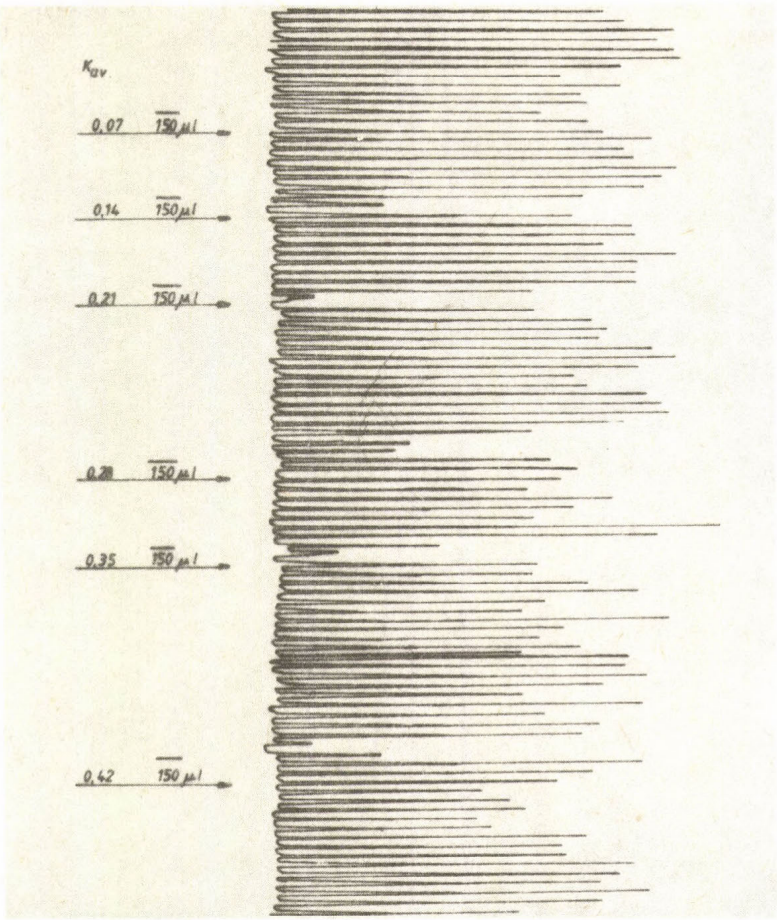

Fig. 2. Biological activity of Sephadex G-25 fractions of sow ovarium origin eluted at various K_{av} values as measured on a spontaneously contracting myometrium strip prepared from a rat at the 20th day of its pregnancy

peptide with a molecular weight of 6300 /2/, and also with a myometrium contraction inhibiting capability. In the experiments presented in this paper, the extract of pregnant sow ovaries was partially purified on a SG-25 column. Biological activity of the partially purified fractions was detected in a myometrium contraction assay system which, although seems to be a rather sensitive assay, cannot be regarded as a strictly specific test system if used for monitoring the purification of endogenous substances with myometrium contraction inhibitory

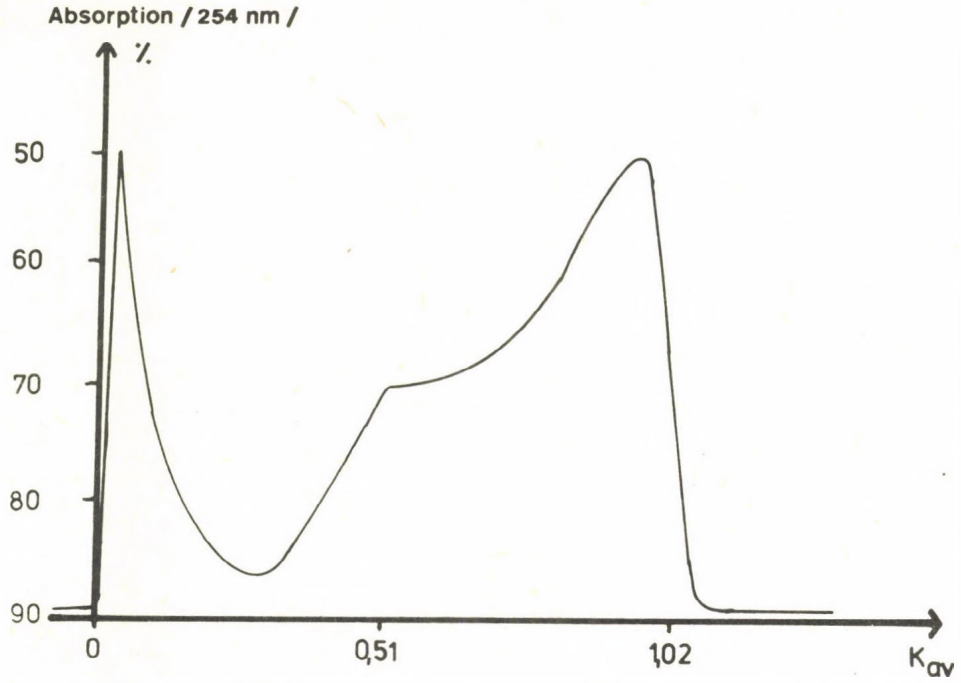

Fig. 3. Immunoreactive relaxin content of Sephadex G-25 fractions of sow ovarium origin eluted at various K_{av} values

activity /7/. Results presented in this paper indicated that the SG-25 fraction eluted in the void volume displays a marked relaxin immunoreactivity. This is far from being a surprise for the following reason. Due to the inherent separation properties of SG-25 gels, substances with a molecular weight of 5000 or higher are eluted, as a rule, in the void volume of a SG-25 column. Consequently, relaxin, having a molecular weight of 6300, should also be eluted in the void volume of such a column. Relaxin is known to be present in an ovarian extract as that used in these experiments /1/. Thus, the high immunoreactive relaxin content of the SG-25 fraction eluted in the void volume is to be attributed to relaxin itself present in this fraction. On the other hand, the immunoreactive relaxin content of the SG-25 fraction eluted at a K_{av} value of 0.17 and thus, in the immediate vicinity of the relaxin-containing fraction eluted in the void volume, was found to be negligible. However, this

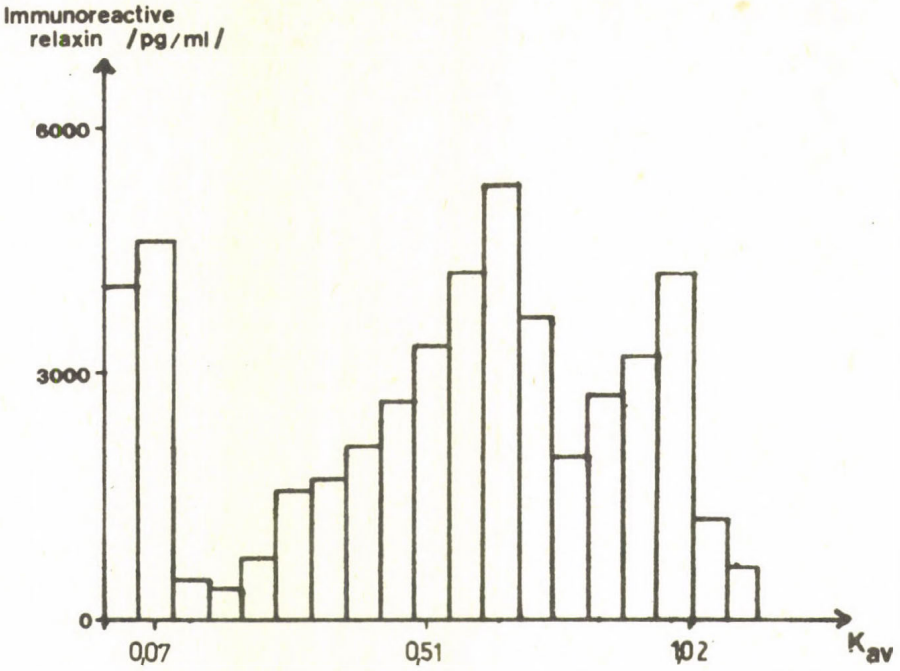

Fig. 4. Sephadex G-25 profile of a standard relaxin preparation /NIH-R-P-1/. Abscissa: absorption at 254 nm; ordinate: K_{av} values

fraction, poor in relaxin, proved to be an inhibitor of myometrium contractions in vitro, about 3000 fold more potent than standard relaxin. This finding gave a firm support to our assumption, that in the acidic-acetone extract of pregnant sow ovaries, an agent with a striking myometrium contraction inhibitory capability is present which, although seems to be physico-chemically closely related to relaxin, nevertheless it is distinct from it.

To our great surprise, SG-25 chromatography revealed the striking heterogeneity of the standard NIH relaxin preparation. It was also shown that this heterogeneous standard relaxin preparation disclosed only a moderate inhibitory action on isolated myometrium. This raises the possibility that, in the standard relaxin preparation, the myometrium contraction inhibiting effect is not due to relaxin per se, but to one of the contaminating substances present in the standard relaxin pre-

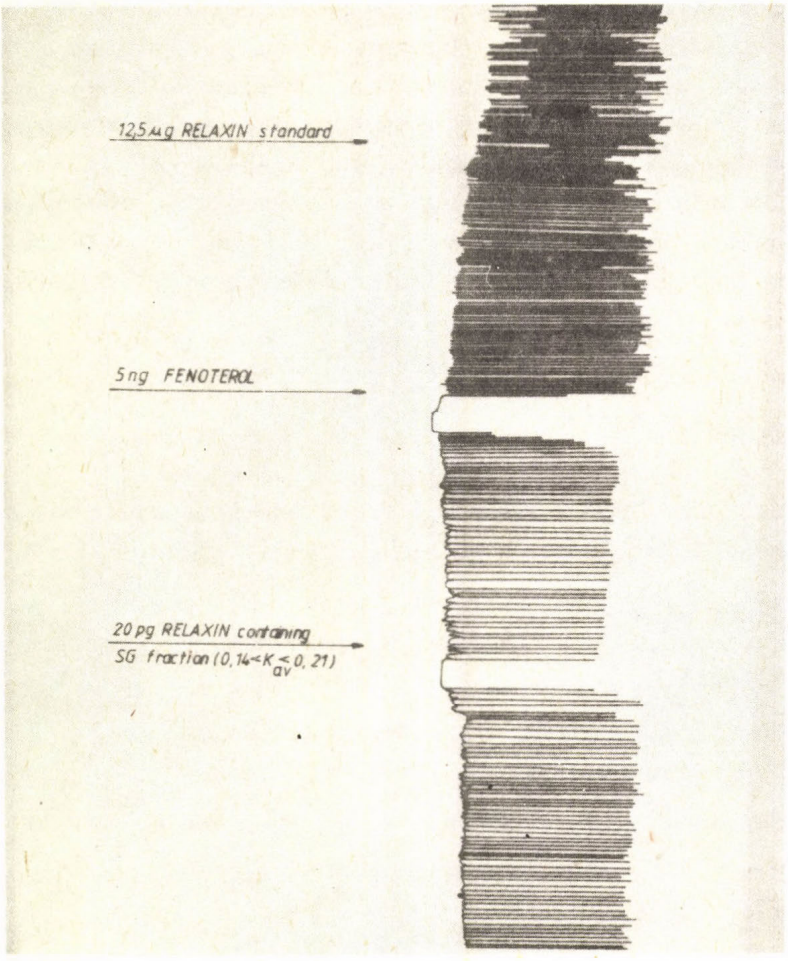

Fig. 5. Effect of standard relaxin, Fenoterol /Partusisten, Boehringer/ and the Sephadex G-25 fraction of sow ovarium origin eluted at a K_{av} value of 0.17 /range: 0.14 - 0.21/ on an isolated myometrium preparation prepared from a pregnant rat

paration. For the same reason it might also be assumed that the moderate myometrium contraction inhibiting capability of the relaxin-containing fraction eluted in the void volume is, in reality,not due to relaxin per se, but to its contamination with the agent/s/ present in the SG-25 fraction eluted in the immediate vicinity of the void volume, which has a powerful myometrium contraction inhibitory potential. Accordingly, the

specific action of relaxin would be directed selectively on the pubic symphysis, whereas the myometrium contraction inhibitory capability would be attributed to other component/s/ equally present in the standard relaxin preparation and the ovarian extract used in our experiments and also by others.

In order to substantiate this assumption, the myometrium contraction inhibiting SG-25 fraction, poor in relaxin, has been subjected to further purification as well as characterization in our laboratory.

REFERENCES

1. Schwartz, N.B.: Research Frontiers in Ovarian Fertility Regulation. Vol. 2. N°.2. pp: 1-11, 1982.
2. Schwabe, C., Steinetz, B., Weiss, G., Segaloff, A., McDonald, J.K., O'Byrne, E., Hochman, J., Carriere, B., Goldsmith, L.: Rec. Prog. Horm. Res. 34, 123-211 /1978/.
3. Guoth, J., Horváth, K., Gróf, J., Menyhárt, J.: Kisérletes Orvostudomány, 34, 343-347 /1982/.
4. Sherwood, O.D.: Endocrinology, 104, 886-891 /1979/.
5. Guoth, J., Idei, M., Menyhárt, J.: Kisérletes Orvostudomány. In press.
6. Sárosi, P., Schmidt, C.F., Essig, E., Steinetz, B.G., Weiss, G.: Am. J. Obstet. Gynecol. 145, 402-405 /1983/.
7. Steinetz, B.G.: 1st International Congress on Human Relaxin. Firenze, 1982.

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APPLICATION OF ISOLATED ORGAN PREPARATIONS AS SENSITIVE AND SELECTIVE DETECTORS IN THE PRACTICE OF LIQUID CHROMATOGRAPHY

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INTRODUCTION

In the chromatographic practice, selection of the appropriate detector is a step of paramount importance, and often it is a rather difficult task. Many times, the substance to be separated is present in a fairly large matrix of compounds whose physico-chemical and chemical properties are closely related to those of the substance to be detected. Although, today, a considerable number of highly selective detectors is available due primarily to their highly specific character, none is suitable as a general purpose instrument (1, 2). Thus, e.g., one usually encounters substantial difficulties when a substance with a given biological activity would have to be separated from, and detected in, one of the body fluids that ordinarily represent a complex matrix of compounds with closely related molecular characteristics.

In this paper we intend to show that appropriately selected isolated organ preparations, such as those extensively used in several fields of biomedical research, can excellently be used as chromatographic detectors (CDs), under appropriate conditions. An up-to-date, sensitive CD is expected to meet the following criteria (1, 2):

(a) it must be sensitive enough (e.g., sensitivity of a modern UV detector is around 10-20 ng/ml (1));

(b) it must be highly selective;

(c) it must respond quickly to changes in the composition of the effluent;

(d) it must generate a signal which is proportional to the quantity of the sample to be detected;

(e) it must be stable against noise and drift.

As will be illustrated below, isolated organs share many of these criteria with up-to-date and sensitive CDs. In addition, by using isolated organs as CDs, one may obtain valuable additional informations concerning the chemical structure of the detected substance.

For example, vasa deferentia are richly supplied with highly sensitive opiate receptors in mice (3, 4). This is indicated by the capability of the isolated mouse vas deferens preparation (MVD) to detect Met-enkephalin, a widely known endogenous opiate compound, in a concentration as low as 35 nmol/l, or 20 ng/ml. On the other hand, research conducted on endogenous opiate compounds soon revealed that manifestation of the opiate activity of a molecule requires the simultaneous presence of: (a) a group positively charged at physiological pH values; (b) an aromatic ring coplanar with the positively charged group; and (c) a peptide chain of appropriate length between the aromatic ring and the positively charged group (5, 6, 7, 8, 9). As a consequence, if a compound is found to display opiate activity, we may be certain that it possesses the structural characteristics mentioned above. As various isolated organs respond differently to chemical substances with distinct structural characteristics, further information on the molecular structure of a certain compound can easily be collected by comparing its biological activity on different isolated organ systems. For example, by the comparative use of MVD and guinea-pig ileum assay systems, one may obtain information on the flexibility or rigidity of the detected compound. The effect of an opiate-active compound on MVD and the lack of the effect of an opiate-inactive substance is demonstrated in Fig. 1, where it is also demonstrated that the opiate effect can be prevented by naloxone, an opiate antagonist. Apart from MVD, isolated rat uterus strip preparations (RUS) were the second type of isolated organs we used as a CD. This preparation also has the capability of responding with remarkable sensitivity to compounds with appropriate chemical structure

Table I. Sensitivity of the isolated rat uterus strip

compound	ID_{50} (mol/l)	effect
oxytocin	2×10^{-10}	contraction
histamine	10^{-7}	relaxation
epinephrine	10^{-7}	relaxation
norepinephrine	10^{-7}	relaxation
acetylcholine	10^{-7}	contraction

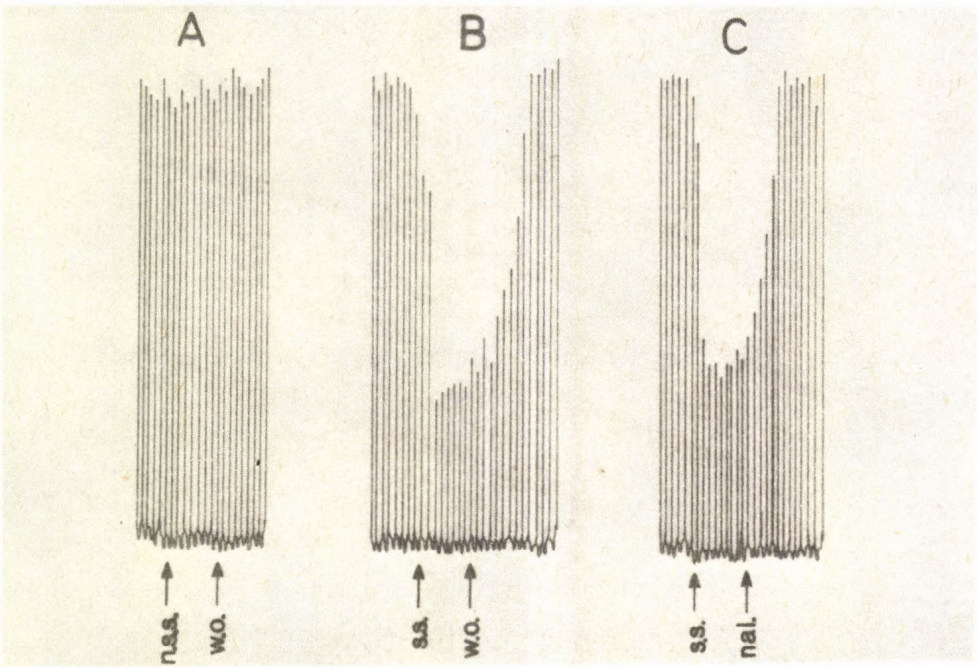

Fig. 1. Effect of non-schizophrenic (A), and schizophrenic serum (B) on an isolated mouse vas deferens preparation, and the antagonizing effect of naloxone (C). n.s.s. = non-schizophrenic sample; s.s. = schizophrenic sample; w.o. = wash out; nal. = naloxone

(Table I). The effects that can be elicited on a rat uterus strip preparation by chemical stimuli are demonstrated in Fig. 2. As seen, different biologically active agents may differently affect the organ preparations: they may increase or

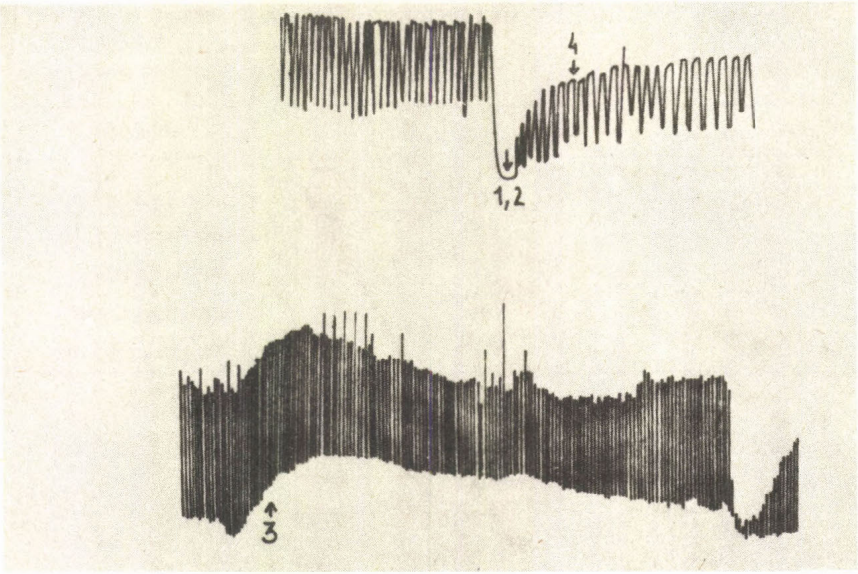

Fig. 2. Effects of Sephadex G-25 fractions obtained from an acidic-acetone extract prepared from pregnant sow ovaries on an isolated rat uterus strip preparation. 1: decrease in tone; 2: decrease in amplitude; 3: increase in tone; 4: decrease in frequency.

decrease the tone, amplitude or the frequency of the contractions, etc.

METHODS

Deproteinized sera from healthy volunteers (controls), and from schizophrenic patients were separated on a Sephadex G-25 column. The biological activity and UV light-absorbing capability (at 254 nm) of the separated fractions were simultaneously detected on a MVD system and a Uvicord S. detector (LKB), respectively (11, 12, 13). In a second experiment, acidic-acetone extract of pregnant sow ovaries were fractioned on a Sephadex G-25 column. Detection of the separated fractions was similarly performed as in the former experiment with the exception that, instead of MVD, RUS was used as a CD (14).

Preparation of MVD

White mice (CFLP), 20 g in weight, were killed by decapitation. Their vasa deferentia were prepared, and hanged in a perfusion vessel of appropriate size filled up with a Krebs-Ringer solution saturated with carbogen gas at 30° (3). A schematic drawing of the system used for the detection of biologically active compounds is shown in Fig. 3. The MVD was electrically stimulated with the following parameters: train rate: 1.2/10 sec; train duration: 2.2/100 ms; stimulation rate: 1.1/10 pps; delay: 0.5/0.1 ms; duration 1/1 ms; voltage: 75/100 V; stimulation mode: train.

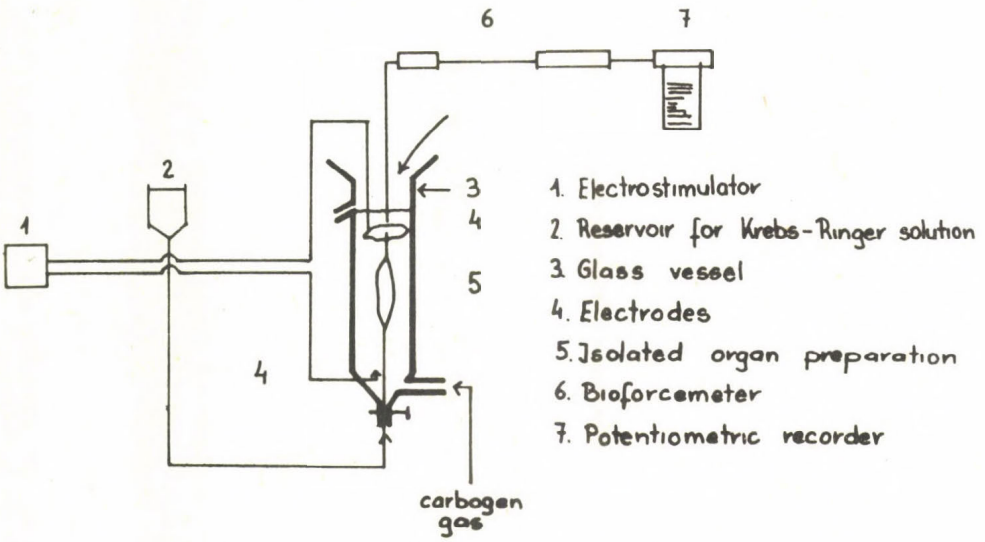

Fig. 3. Schematic drawing of the system used for the detection of compounds affecting isolated organ preparations

Preparation of RUS

RUSs were prepared from CFY rats at the twentieth day of their pregnancy, and treated like MVDs. In this case, the parameters of electric stimulation were selected as follows: train rate: 8/10 sec; train duration: 10/1000 ms; stimulation

rate: 5/10 pps; delay: 0 ms, duration: 1/1 ms; voltage: 80/100 V; stimulation mode: train.

RESULTS

Analysis of schizophrenic samples

Both normal and schizophrenic samples could be resolved by SG-25 chromatography into three distinct peaks with practically identical K_{av} values (Fig. 4). On the schizophrenic profile, however, an additional peak with a K_{av} value of 0.51

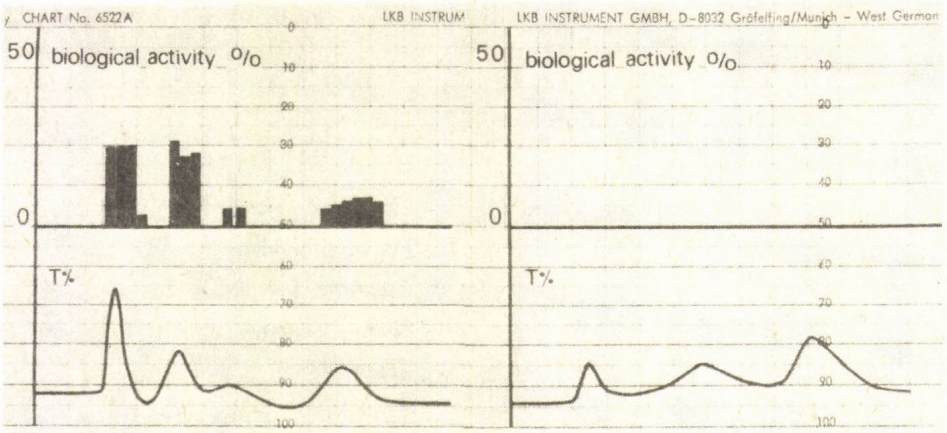

Fig. 4. Sephadex G-25 profile (lower part of the figure) and biological activity (upper part of the figure) of schizophrenic and non-schizophrenic sera. Chromatographic parameters: V_t = 67.7 ml; elution fluid: 0.9% NaCl, flow rate: 0.6 ml min^{-1}; volume of collected fractions: 2.4 ml; paper speed: 40 mm h^{-1}; sample volume: 3 ml; chromatographic detector: Uvicord S (LKB) (254 nm); biological detector: isolated mouse vas deferens preparation.

could also be observed, which was regularly absent from the normal samples. The ratio of the corresponding peaks from the two samples also differed to some extent. On the whole, however, no substantial difference between the two profiles could be observed by conventional detection at 254 nm alone. In sharp contrast to this, the corresponding fractions from the two samples disclosed striking differences when their actions on MVD have been compared. The control fractions were completely inactive on MVD, whereas the schizophrenic fractions markedly inhibited contractions of MVD. This experiment clearly demonstrated the occasional superiority of a biological detector over a conventional detector. The sole use of the conventional detector would not permit to recognize the significant differences in the composition of the two samples. As the components carrying MVD activity in schizophrenic sera were present in a large matrix of compounds whose chemical and physicochemical properties were closely related or even identical, the very small amount of the MVD-active component(s) in schizophrenic sera would have remained undetected by using a conventional detector only. From the results presented above, the highly specific character of the biological detector used can also be recognized. At this stage of purification, none of the schizophrenic fractions were homogeneous in composition. However, the use of a biological detector allowed the detection of the biologically active agents, even in these highly heterogeneous fractions.

Analysis of acidic-acetone extract of sow ovaries

By applying conventional detection alone, the extract could be only unsatisfactorily resolved by SG-25 chromatography into six fractions with K_{av} values of 0.0, 0.12, 0.22, 0.66, 1.08 and 1.25, respectively. Biological detection, however, secured a significantly higher resolution power and disclosed the presence of at least 13 components with different biological activities. Superiority of the biological detection was especially obvious in the case of the fraction with a K_{av} value of 0.17. It happened namely that we had to decide whether this

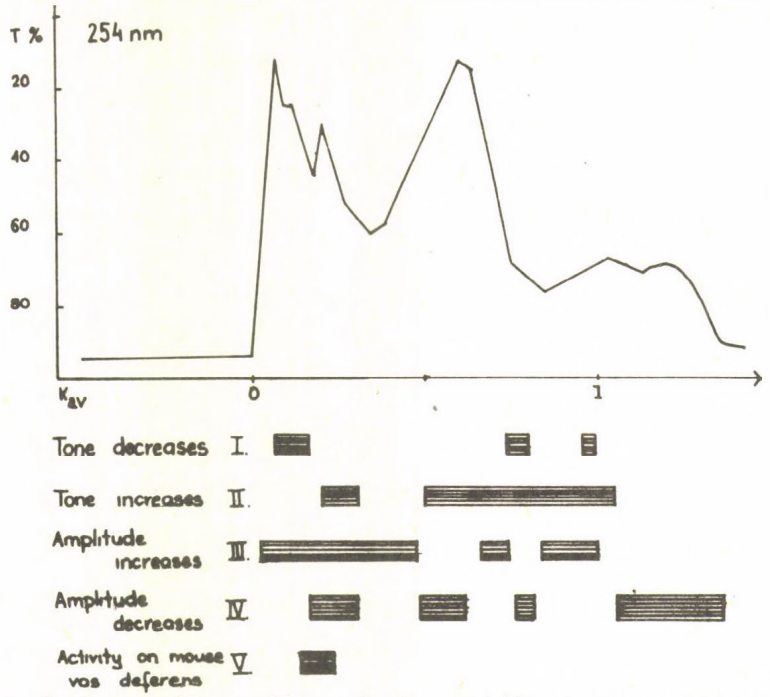

Fig. 5. Sephadex G-25 profile of the acidic-acetone extract from pregnant sow ovaries and biological actions of the separated fractions. Chromatographic parameters: V_t = 84.4 ml; elution fluid: 0.9% NaCl; volume of collected fractions: 5 ml; paper speed: 60 mm h^{-1}; sample volume: 5 ml; chromatographic detector: Uvicord S (LKB) (254 nm); biological detector: isolated rat uterus strip

fraction contained or not relaxin, a compound of physiological importance, which was predictably present in the extracts used, and whether other component(s) with relaxin-like biological activity was or was not included in the fraction in question. In our system the K_{av} value of standard relaxin was estimated to be 0.13. As the K_{av} value of the fraction in question was about 0.17, the presence or the absence of relaxin in this fraction could not be determined with certainity on the basis of the K_{av} values only. On the other hand, the problem could be easily resolved by performing two, relatively simple, biological assays: one with RUS and another with MVD. Relaxin disclosed an activity on RUS, but was ineffective on MVD. On the other hand, our SG-25 fraction displayed a marked effect on both systems. Thus, what we could not do with the aid of a con-

ventional detector, we could do relatively simply by using bio-detection which clearly established the presence of another component, distinct from relaxin in this fraction. The finding that this non-relaxin component inhibited contractions of both RUS and isolated human uterus strip indicates the potential physiological importance of the non-relaxin component.

DISCUSSION

We believe that the results presented in this paper convincingly demonstrated that isolated organ preparations may occasionally be applied as CDs with excellent success. This is possible because isolated organs share essential properties with up-to-date conventional CDs: (a) they are highly sensitive; e.g., Met-enkephalin can be detected by MVD in a conc. of 35 nmol/l, or 20 ng/ml, as contrasted to 10-20 ng/ml, the lowest limit of the detection capacity of a modern UV detector. Taking into account the volume of the perfusion vessel applied in these experiments, the quantity of Met-enkephalin we could detect with the aid of MVD was as low as 0.15 nmol. This sensitivity, e.g., exceeds that of an UV detector (0.5 nmol) applied in a HPLC system used for Met-enkephalin separation as described in a recent publication (15); (b) they respond almost as quickly as conventional detectors to changes in the effluent composition; (c) their response is proportional to the log. concentration of the substance detected; (d) they are highly specific, by being provided with the capability to detect a biologically active agent in an occasionally highly complex matrix of compounds with similar physico-chemical and chemical properties, but without similar biological activity.

Application of biological detectors seems to be especially fruitful in the following research fields: (a) in extensive areas of physiological, pharmacological, and biological research; (b) as it was also demonstrated in this paper, biological detectors may occasionally be of great help in revealing the molecular etiology of various pathological conditions; (c) in all instances, when a compound with optional biological

activity has to be detected in a complex matrix of compounds we are not interested in; (d) in cases, where quickly obtainable information of a compound is required without its prior isolation. Naturally, like other detectors, biological detectors also have their shortcomings and limits of application. For example, isolated organs are rather susceptible to external influences and their application needs the rather strict safeguarding of physiological conditions. The presence of toxic agents or organic solvents in their environment, even in trace amounts, may easily kill the isolated organ preparations. Thus, such contaminants should be eliminated, or at least neutralized prior to the onset of biodetection. This may not always be easy, and may require excess work. However, the several advantages what biological detection offers, usually deserve extra efforts to be invested.

SUMMARY

In the present study, the use and applicability of appropriately selected isolated organ preparations as chromatographic detectors was demonstrated. Deproteinized sera from healthy volunteers and from schizophrenic patients, as well as acidic acetone extracts from sow ovaries were separated on a Sephadex SG-25 column. The fractions eluted were subsequently analyzed in a system which allowed the continuous and simultaneous monitoring of their UV absorbance (254 nm), and their effect on isolated mouse vas deferens and rat uterine strip preparations. Profiles obtained by registering UV absorbance and biological activity were then compared.

Results presented in this paper gave a firm support to the idea that isolated organ preparations selected according to the needs of particular experiments meet essential criteria of conventional chromatographic detectors in terms of sensitivity, specificity, and rapidity of response. The use of isolated organs allows the detection of biologically active substances in a matrix composed of several physico-chemically closely related, but biologically distinctly different fluid com-

ponents. Apart of from being nearly as sensitive and specific as conventional chromatographic detectors, biological detectors may also provide useful, additional information concerning the chemical structure of unidentified biologically active agents.

REFERENCES

1. L.R. Synder, J.J. Kirkland, Introduction to Modern Liquid Chromatography. J. Wiley and Sons, New York, 1974
2. L. Szepesy, Gas Chromatography. Akadémiai Kiadó, Budapest, 1971, pp. 118-120
3. J. Hughes, T.W. Smith, H.W. Kosterlitz, Linda A. Fothergill, B.A. Morgan, H.R. Morris, Nature: 258 577-579 (1975)
4. A. Rónai, A Biológia Aktuális Problémái. (Actual Problems of Biology) (In Hungarian) Vol. 22. Medicina, Budapest, 1982, pp. 122-123
5. L. Gráf, ibid. pp 26-32
6. L. Gráf, G. Cseh, E. Barát, A. Rónai, J.I. Székely, A. Kenessey, S. Bajusz, Ann. N.Y. Acad. Sci. 297 63 (1977)
7. A. Rónai, A Biológia Aktuális Problémái (Actual Problems of Biology) (In Hungarian). Vol. 22. Medicina, Budapest, 1982, pp. 78-83
8. A. Barclett, A.F. Casy, J. Pharm. Pharmacol. 6 986 (1954)
9. A.S. Horn, J.R. Rodgers, Nature (London) 260 795 (1976)
10. A. Rónai, J.I. Székely, I. Berzétei, E. Miglécz, S. Bajusz, Biochem. Biophys. Res. Comm. 91 1239-1249 (1979)
11. M. Idei, J. Gróf, J. Menyhárt, A. Pajor, XLVI. Annual Meeting of the Hungarian Physiological Society, 1981, Budapest, Abstracts, p. 83
12. M. Idei, Zs. Ribai, I. Felmérai, Proc. of the 21st Hungarian Annual Meeting for Biochemistry, 1981, Veszprém, p. 195

13. J. Szilárd, Gy. Gál, J. Gróf, J. Menyhárt, M. Idei, in: Detoxikationsverfahren bei den neuropsychiatrischen Erkrankungen. Edited by K. Ernst and K. Seidel. VEB Verlag Volk and Gesundheit, Berlin, 1983, pp 43-49.

14. J. Guoth, K. Horváth, J. Gróf, J. Menyhárt, Kisérletes Orvostudomány 34 343-347 /1982/

15. S. Mause, D. Mullett, D. Couri, Life Sci. 29 61-68 (1981)

SEPARATION OF DRUGS AND METABOLITES

Chromatography, the State of the Art
H. Kalász and L.S. Ettre (Eds)

STUDY OF DEPRENYL METABOLISM

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Deprenyl /phenyl-isopropyl-methyl-propinylamine.HCl; JUMEX/, a B-type selective monoamine oxidase /MAO/ inhibitor, has been discovered in 1964 /1/ but relatively little was known of its fate in the body and mainly of its metabolism. The beneficial pharmacological properties of the inhibitor, including its strongest MAO-inhibitory potency are due to its /-/-enantiomer /2/. Therefore /-/-deprenyl was used in all of our studies.

Post-mortem experiments in human brain tissue showed by gas-chromatographic analysis that after deprenyl treatment of parkinsonian patients amphetamine was present in a concentration of up to 56 ng/g /3/.

The authors suggested that the production of amphetamine and methamphetine metabolites from deprenyl and the inhibition of 2-phenyl-ethylamine oxidation are two ways that, in addition to the blockade of dopamine metabolism, may also be important in the pharmacological effects of the drug. This finding fastened the experimental efforts to know more about the fate of deprenyl, mainly of its metabolism. The aim of this paper is to summarize the essence of our results accumulated in this field during the past decades.

In 1968 we published /4/ that ^{14}C-deprenyl is well absorbed after oral and subcutaneous administration to mice. The highest blood level was reached half an hour after subcutaneous and one hour after oral administration /Fig. 1/.

Whole-body autoradiography in mice showed that ^{14}C-deprenyl rapidly enters the central nervous system following intravenous

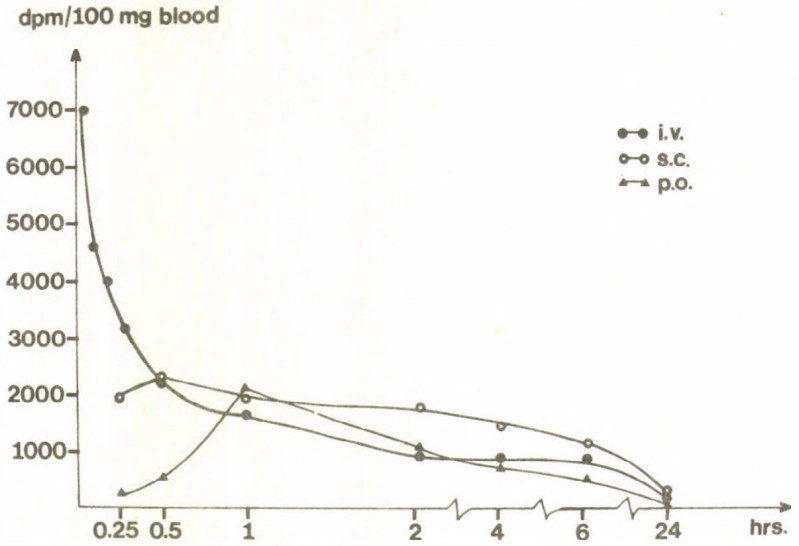

Fig. 1. Concentration changes of blood radioactivity in mice at different times after oral, subcutaneous and intravenous treatments with ^{14}C-deprenyl. Male mice weighing 20±1 g were used. 5 µCi of ^{14}C-Deprenyl were given to each mouse. Specific activity of ^{14}C-deprenyl. HCL: 10.85 µCi/mg; labeled at C_1 position.

administration. Within 30 sec after the injection, the brain and spinal cord contain fairly high concentrations of the isotope. Body lipids, as the brown-fat tissue contain high level of radioactivity as well. The substance is excreted by the gastric mucosa at this time which event is characteristic of the basic compounds /Fig. 2/.

The 5 min autoradiogram shows, in contrast to the 30 sec one, that the cortex contains almost only the background of radioactivity while the lipids and other organs show fairly high level /Fig. 3/.

The rapid rise in ^{14}C-deprenyl concentration is followed immediately by a sudden decrease in the radioactivity of the brain /Fig. 4/. The changes of ^{14}C-deprenyl concentrations in the blood are also represented in this figure.

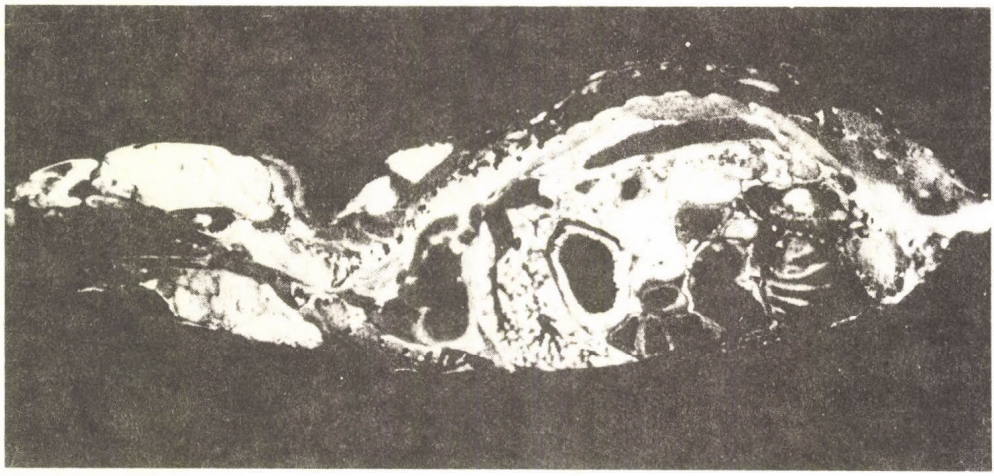

Fig. 2. Whole body autoradiography of mice after 30 sec of intravenous treatment with ^{14}C-deprenyl. Specification of the mice and the dose of ^{14}C-deprenyl shown in Fig. 1.

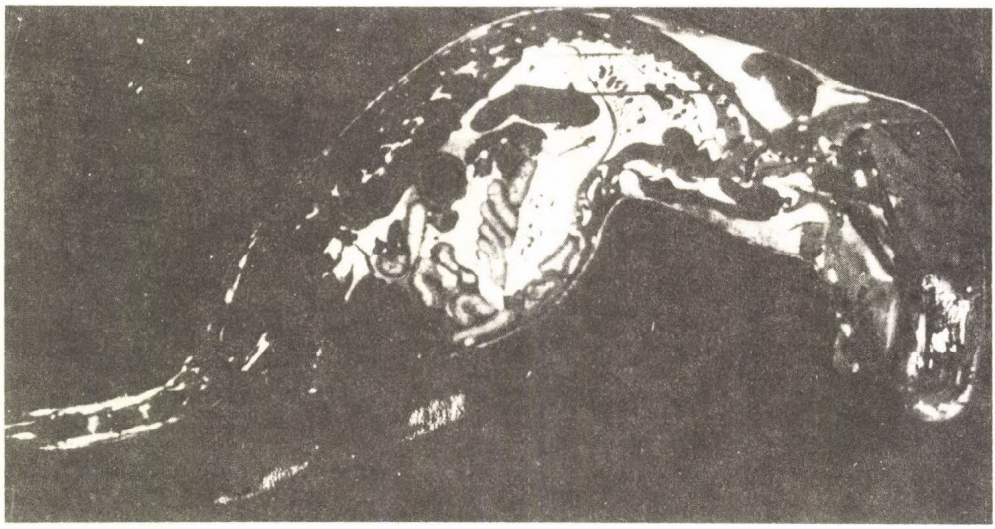

Fig. 3. Whole body autoradiography of mice after 5 min of intravenous treatment with ^{14}C-deprenyl. Experimental conditions as in Fig. 1.

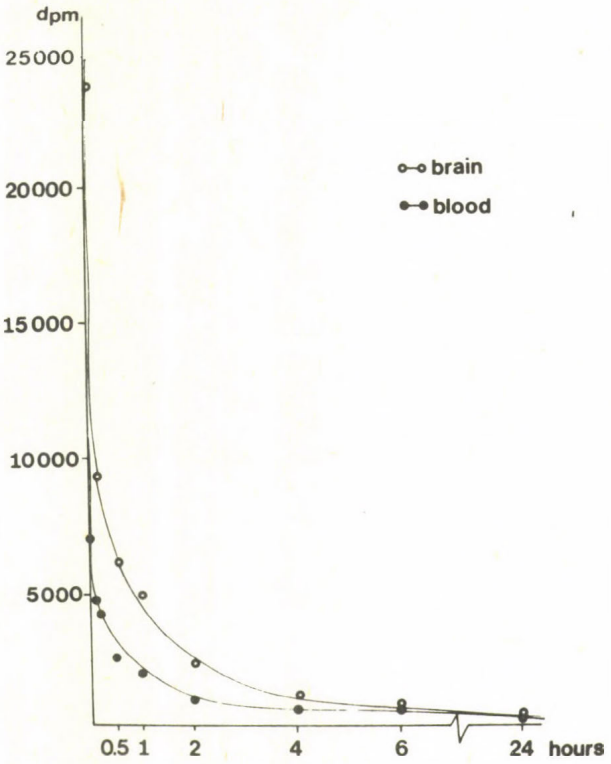

Fig. 4. Concentration changes of radioactivity in the blood and brain of mice at different times after intravenous administration of ^{14}C-deprenyl. Experimental conditions as in Fig. 1.

The very rapid penetration of deprenyl through the blood-brain barrier must be due to the lower polarity and basicity of the compound comparing with amphetamine derivatives having primary or secondary amino group /Table I /.

Tissue concentrations of the brain and the blood as well as that of other organs were quantitatively determined by liquid scintillation technique. The results are summarized in Table II.

Kinetic analysis of the numerical data of Table II showed that three exponential equations can be adjusted to the organ levels.

The half life times /T 1/2/ in the blood and in the brain calculated form the equations were 2-25-643 min and 1-48-767

Table I. The distribution of ^{3}H-Noradrenaline, ^{3}H-Amphetamine and ^{14}C-Deprenyl in water-hexane phases

Compounds HCl	In hexane %	In water %	Recovery %
^{3}H-Noradrenaline	0.00	93.38	93.38
^{3}H-Amphetamine	0.32	94.02	94.34
^{14}C-Deprenyl	82.50	18.14	100.60

$$\frac{\text{c Deprenyl}}{\text{c Amphetamine}} = 25.8 \text{ /in hexane/}$$

Table II. Radioactive concentrations in tissues of mice after i.v. injection of ^{14}C-Deprenyl

Time, min	blood	brain	liver	kidney	lung	heart	brown-fat
0.5	6 996	24 583	8 786	51 248	53 834	16 625	101 943
5	4 733	9 371	25 278	33 005	24 819	15 549	51 714
10	4 065	9 537	25 284	44 315	20 852	11 626	29 095
30	2 340	6 723	29 020	29 705	11 495	10 620	15 427
60	1 868	5 163	18 218	26 609	14 158	5 373	6 165
120	864	2 685	17 636	14 675	16 299	3 232	5 412
240	714	1 561	7 539	6 674	4 093	1 887	2 226
360	781	942	5 072	3 636	3 648	1 441	1 103
1 440	125	309	2 125	952	1 907	587	933
2 800	133	214	1 298	751	737	334	903

Values: dpm/100 mg tissues

The injected dose was: 23 mg/kg; 5 μCi/20 g mice.

The specific activity of ^{14}C-deprenyl: 10.85 μCi/mg

min, respectively. Further kinetic analysis of these data - correlation coefficients of the linear regression between the concentration curves of the organs - showed that the blood and the other organs examined, except liver, followed similar distribution kinetics, e.g. fast equilibration was developing between the blood level and that of the organs /Table III/. The radioactivity concentration of the liver, in this respect, seems to be more independent of the blood level.

In the light of our distribution studies and kinetic analysis the sudden decrease of deprenyl concentration in the central nervous system must be considered, regarding the pharmacological effect of the inhibitor. The relatively low level of deprenyl or of its metabolites, left in the brain after some seconds following a single i.v. injection of the compound can explain the lack of psychostimulant /amphetamine-like/ activity. This, otherwise, could be expected if doses of amphetamine or methamphetamine equivalent to deprenyl had been administered. The distribution kinetics of deprenyl may be responsible for this phenomenon, even if the inhibitor were completely converted to methylamphetamine and amphetamine, as it was registered by other authors /3/.

For HPLC analysis of the deprenyl metabolites, rats /200±5 g/ were treated intraperitoneally with 5 mg/kg of ^{14}C-deprenyl and urine was collected during 24 hrs. The urinary metabolites were separated on a Sephadex G-15 column. Results are shown in Fig. 5, where radioactivity was detected by liquid scintillation method. Three fractions were separated, concentrated by evaporation and further analysed by HPLC. Metabolites were followed by radioactivity monitoring. As Fig. 6. shows fraction I of the gel-chromatographic separation proved to be homogeneous, but fraction III gave three well separable components /Fig. 7/. These metabolites seem to have basic character because radioactivity can be completely extracted into organic solvent /ether/ at pH 10. These compounds could be deprenyl, amphetamine and methamphetamine.

Further analysis of fraction III on two-dimensional thin-layer chromatography proved this hypothesis /Fig. 8/.

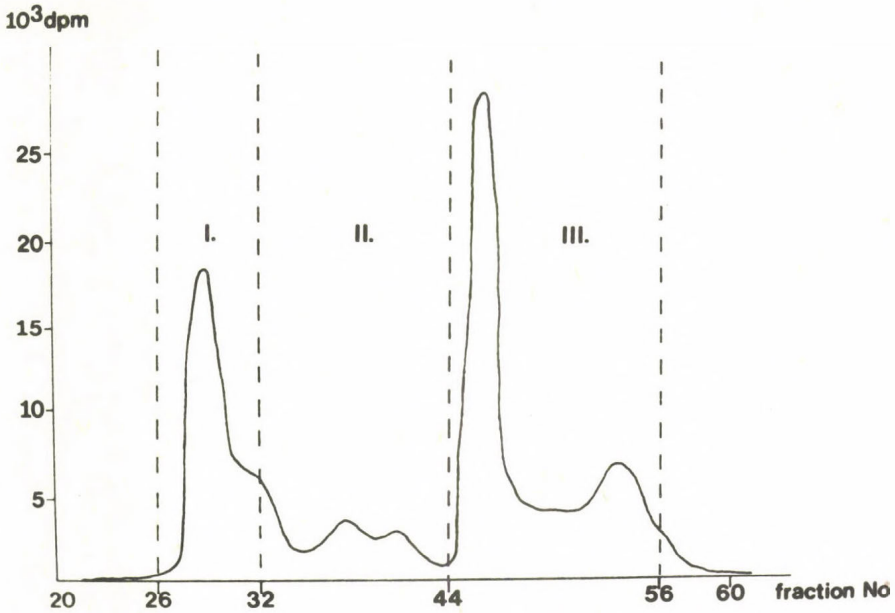

Fig. 5. Gel-chromatographic separation of ^{14}C-deprenyl metabolites in rat urine.

Treatment: 5 mg/kg deprenyl, i.p.;
Specific activity of ^{14}C-deprenyl: 6.89 μCi/mg, labelled at C_2 positions.
Column: Sephadex G-15 25x900 mm
Eluent: 0.01 N HCl
Detection: Liquid scintillation method.

After acidic hydrolysis /6N HCl; 100°C; 24 hrs/ the first peak of the gel-chromatographic separation disappeared, but a new metabolite appeared on the gel chromatogram /Fig. 9/. HPLC analysis of fraction III/a showed that the new metabolite was homogeneous /Fig. 10/. The short retention time of this metabolite suggests that it is highly polar in its chemical character. In addition to its higher polarity, this compound should also contain a basic amino group, because at pH 10 it can be extracted into an organic solvent. We assume that it could be p-OH-methylamphetamine or p-OH-amphetamine but we were not successful in its identification because of the lack of reference

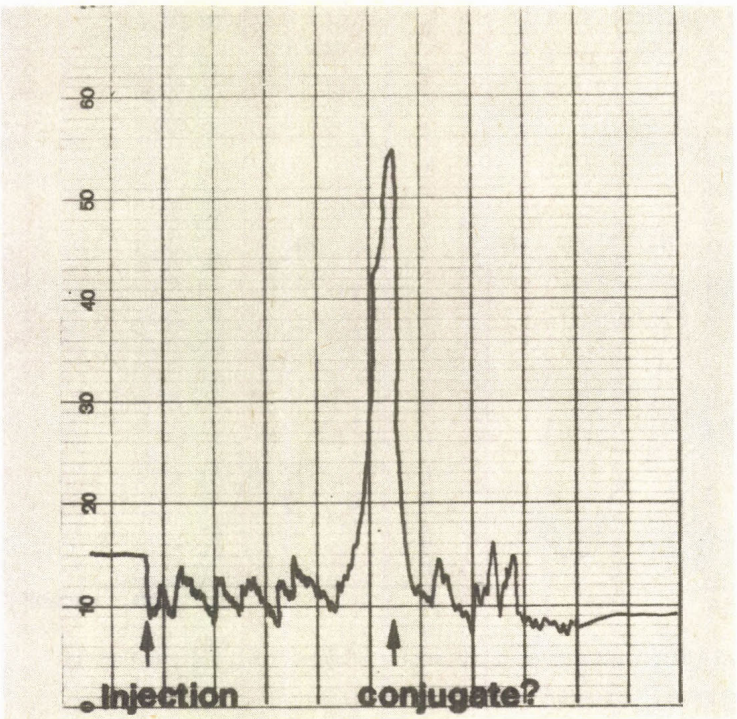

Fig. 6. HPLC analysis of fraction I of the gel chromatogram /see Fig. 5/
Column: Chromspher-Sil 6 μm
Fluent: Triethanolamine-PO_4 buffer /pH 3/
Flow rate: 1 ml/min.
Equipment: Liquochrom 2010/Labor MIM/
Detection: Radioactivity monitor LB 503 /Berthold/

compound. These series of experiments suggest that fraction I of the gel-chromatographic separation of the urine contains a conjugate of p-OH-methylamphetamine or p-OH-amphetamine.

Our experimental results can be summarized as follows: Deprenyl was metabolized in rat to N-propinyl-amphetamine, methamphetamine and amphetamine. At least two further metabolites were also formed which were not yet identified, but most probably were having aromatic hydroxyl group.

In the course of the further metabolic studies with deprenyl the following should be taken into consideration:

Table III Correlation coefficients of multiple regressions between organs

	blood	brain	liver	kidney	lung	heart	brown-fat
blood	1	0.95	0.44	0.93	0.94	0.96	0.96
brain	0.96	1	0.28	0.89	0.96	0.87	0.97
liver	0.44	0.28	1	0.65	0.29	0.64	0.20
kidney	0.93	0.89	0.65	1	0.87	0.94	0.81
lung	0.94	0.98	0.29	0.87	1	0.85	0.96
heart	0.96	0.87	0.64	0.94	0.85	1	0.87
brown-fat	0.95	0.97	0.20	0.81	0.96	0.87	1

The data of Table II were analysed.

1. N-oxidation of tertiary amines is known as an important metabolic route /5/, thus one of the main human metabolites of N,N-dimethylamphetamine is the N-oxide. Deprenyl has also a tertiary amino-group and because of this the N-oxide formation should also be considered as a metabolic route, however in vivo reduction can render its determination rather difficult. Competitive pathways may also interfere with N-oxide formation.

2. Sterical factors also play some role in the metabolism of amphetamine derivatives. While the extent of metabolism was in linear correlation with the chain length of alkyl substituents of N-alkyl-/+/-amphetamine derivatives, there was no correlation in the case of /-/-amphetamines /5/ which corresponds to the deprenyl metabolites.

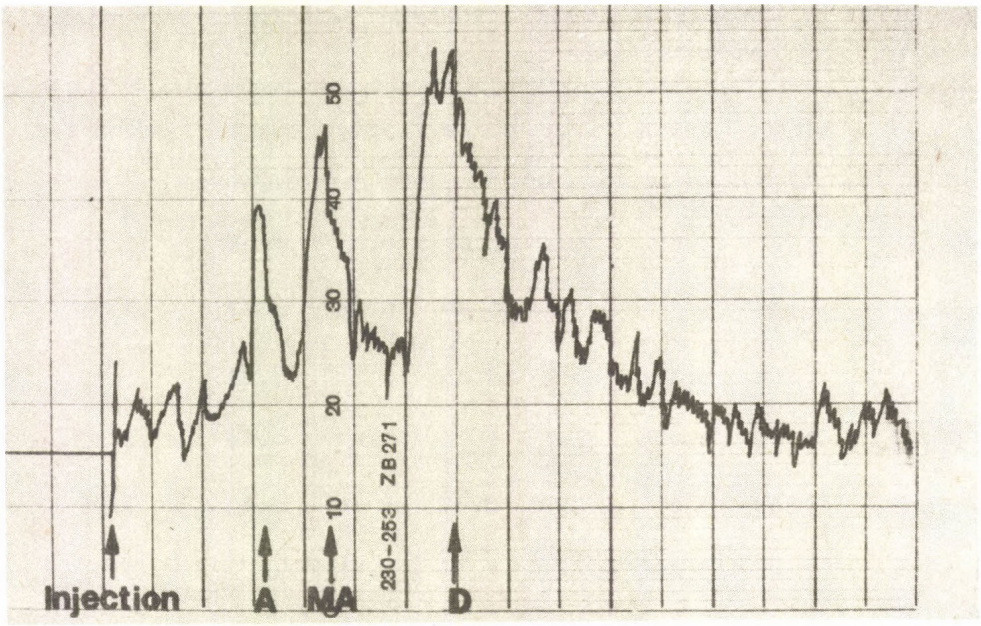

Fig. 7. HPLC analysis of fraction III of the gel chromatogram /see Fig. 5/.

3. Replacement of the ethyl with 2-cyanoethyl group to give N-cyanoethyl amphetamine, increases N-dealkylation, which is probably due to the strong electron-withdrawing effect of the -CN group /5/. We assume that the C≡C bond in the propinyl--group has a similar influence and this may promote the depropinylation of deprenyl.

4. It is very important to stress that species variations in the case of amphetamine derivatives are well known /5/. In the rat, the main metabolic route is aromatic hydroxylation while in man, rabbit and guinea pig the deamination process is of higher significance.

Due to the fact that competitive metabolic routes are less substrate-dependent and influenced mainly by species differences this phenomenon must also be very much considered in the deprenyl metabolism.

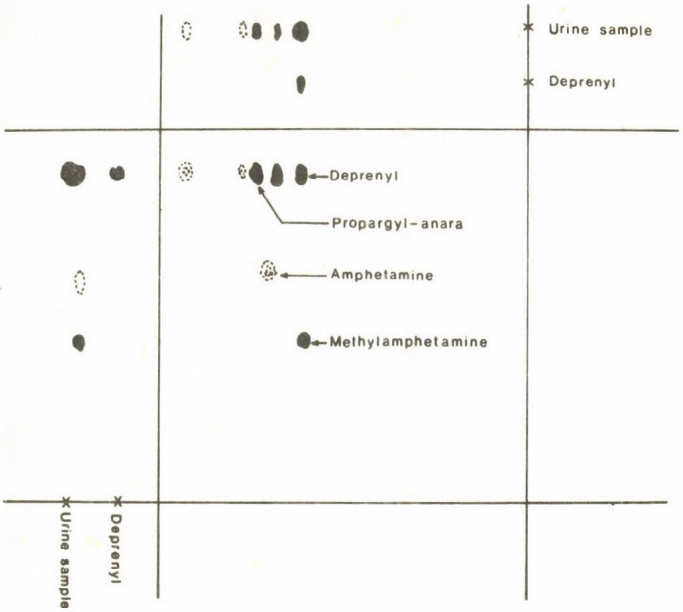

Fig. 8. Two-dimensional thin-layer chromatogram of fraction III of the gel chromatogram /Fig. 5/
First solvent: butan-1-ol-ammonia-water-methanol 20:1:4:2 v/v/
Second solvent: Butan-1-ol-acetic acid-water 3:1:1 /v/v/

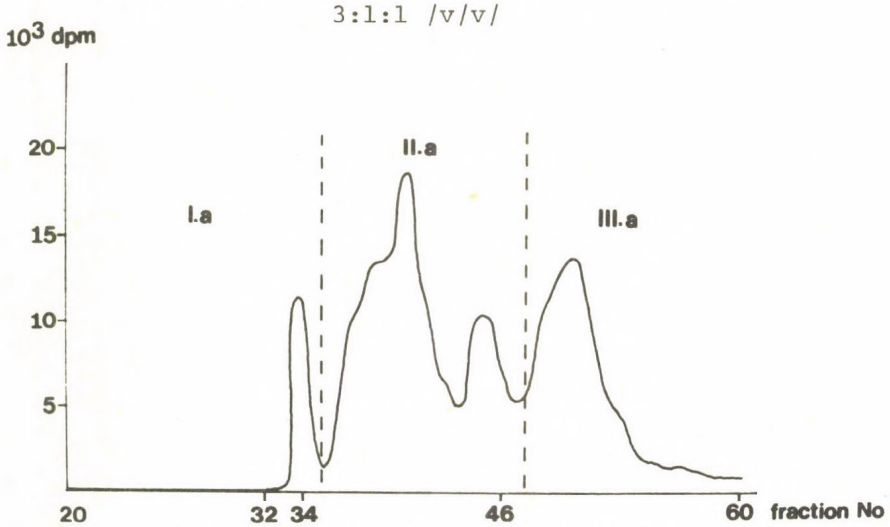

Fig. 9. Gel-chromatographic separation of ^{14}C-deprenyl metabolites in urine after acidic hydrolysis.
Hydrolysis: 6N HCl; 100°C; 24 hrs.
Conditions: as in Fig. 5.

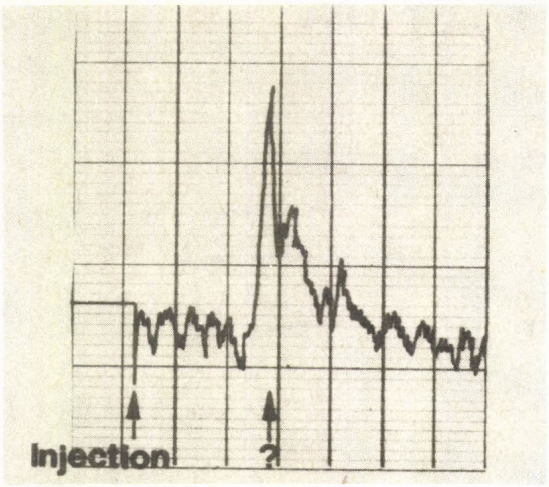

Fig. 10. HPLC analysis of fraction III/a of the gel chromatogram /see Fig. 9/.
Conditions: as in Fig. 6.

Our further aim is to study the metabolic conversions of deprenyl in parkinsonian patients during chronic deprenyl treatment.

REFERENCES

1. J. Knoll, Z. Ecseri, K. Kelemen, J. Nievel and B. Knoll, Archs. int. Pharmacodyn. 155, 154 /1965/.
2. K. Magyar, E.S. Vizi, Z. Ecseri and J. Knoll, Acta Physiol. Hung. 32, 377 /1967/.
3. G.P. Reynolds, U.D. Elsworth, K. Blau, M.Sandler, A.J. Lees and G.M. Stern, Br. J. Clin. Pharmac. 6, 542 /1978/.
4. K. Magyar, L. Skolnik and J. Knoll, in V. Conferencia Hungaria pro Therapia et Investigatione in Pharmacologia. /Ed. G.P. Leszkovszky/, Akadémiai Kiadó, Budapest, 1968, p. 103.
5. B. Testa and P. Jenner, Drug Metabolism: Chemical and Biochemical Aspects. N. Dekker, Inc., New York, 1976.

Chromatography, the State of the Art
H. Kalász and L.S. Ettre (Eds)

SOME ASPECTS OF STRUCTURE-CHROMATOGRAPHIC BEHAVIOR RELATIONSHIPS IN TLC OF BARBITURATES

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INTRODUCTION

Therapeutic applications of barbituric acid[2,4,6(1H,3H,5H) pyrimidinetrione] derivatives create needs for appropriate analytical procedures for these compounds. Various chromatographic techniques have been widely used for the separation and identification of barbiturates.

We tried to establish the relationship between the molecular structure of barbiturates and their chromatographic properties. In our previous papers we have investigated the TLC of barbiturates on the polar adsorbent silica gel[1] and on non-polar adsorbents[2] using binary solvent mixtures as the mobile phases.

The correlation analysis of different structural parameters of barbiturates and their chromatographic behavior yielded significant results discriminating important factors of both structural and chromatographic nature[2,3-8]. It was found that the first order valence molecular connectivity indices[9] give good correlations with the R_F values found by TLC[2,6], but the validity of these findings for 5,5-dialkylbarbiturates was limited by small amount of therapeutically useful compounds of this class which were the subject of analysis. Therefore we decided to expand this group by some non-medicinal barbiturates and to check their chromatographic behavior in RP-TLC in relation to some structural features in the C_5 substituents.

Another problem was to evaluate the possibility of influence of electronic effects of C_5 substituent on the R_F values. A

specially designed group of 1,3-dimethyl-5-arylidenebarbiturates was investigated in this respect and the results of TLC on silica gel were correlated with the Hammett σ constants.

MATERIALS AND METHODS

The compounds used in the study /Table I and IV/ were commercial products /nos. 1-4/, investigational samples /nos. 5--10/ or compounds /nos. 11-22/ synthesized according to known methods[10-12].

Stationary phases - precoated TLC plates: silica gel 60 F_{254} /Merck-5715/, silica gel 60 F_{254} silanised /Merck-5747/, HPTLC RP-8 F_{254} /Merck-13725/, HPTLC RP-18 F_{254} /Merck 13724/, Nano-SIL C_{18}-50, 75 and 100 UB_{254} /Macherey-Nagel 81 1062-4/, KC_{18}-F /Whatman 4803-600/.

Mobile phases for RP-TLC:organic solvents and 2% aqueous NaCl solution in different ratios; for TLC on silica gel: acetone : chloroform : acetic acid /36 : 40 : 24/.

All reagents were of analytical reagent grade.

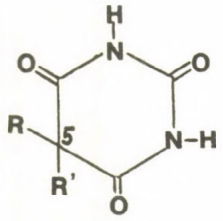
Sample application - 2 µl of 1% solutions of the investigated compounds in ethanol or dry 1,4-dioxane.

Visualisation of spots: UV_{254} light.

Correlations were evaluated by the least squares method using Student's test for their significance. Intercepts /a/ and slopes /b/ of the straight lines and statistical parameters: correlation coefficients /r/ significance levels / α / and standard errors /s/ were calculated on Cyber 72 computer and Hewlett Packard 32E calculator.

RESULTS AND DISCUSSION

5,5-Dialkyl derivatives of barbituric acid of the general formula with the C_5 substituents presented graphically in Table I



were separated by TLC in different chromatographic systems with non-polar stationary phases. Highly significant linear correlations were found between the R_F values and the first-order valence molecular connectivity indices /Table I/.

For the chain isomers branching does not change essentially the R_F values /cf. compounds no. 3 and 5 or 6, 4 and 10; see Table I/ and the position of branching /cf. compounds 5 and 6; see Table I/ have no effect on the R_F values although the results of stability[13-16] and biological activity[17,18] studies evidence some special features of branching at the C_1 atom of the substituent due to the steric effect of shielding of the ring.

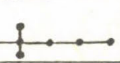
The addition of 5-spirobarbiturates slightly lowers the correlation coefficient /Table I/ and significant differences are observed in the R_F values for the three carbon spiroring /comp. no. 11/ and its open-ring analog /comp. no. 1/. This might be due to the highly strained three-membered spirocyclopropane system of special electronic state[19] because the five-membered spirocyclopentane ring /comp. no. 12/ yields R_F values similar to those of barbital /comp. no. 2/.

For eight 5,5-dialkylbarbiturates /comp. nos. 3-10/ with the R_F values in the 0.2 - 0.7 range different chromatographic systems were checked to prove the influence of chromatographic conditions on the significance of correlations.

The results presented in Table II show that the difference in the stationary and mobile phases did not significantly change the correlation coefficients and all correlations have significance levels better than 0.05. On the other hand, the slopes of the regression line /parameter b/ are different and can be taken as a measure of selectivity of particular chromatographic system. We did not observe substantial changes in the correlation coefficients depending on the percentage of silylation of the gel on the Macherey-Nagel plates.

Table III presents the results of variations of statistical parameters with the change of a percentage of organic solvent in the mobile phase. The optimal chromatographic conditions were those for the methanol : water /65 : 35/ mobile phase.

Table I R_F values, chromatographic systems, molecular connectivity indices and results of correlation analysis for 5,5-dialkyl-barbiturates

C_5 substituents /.CH_2 or CH_3 groups/	$^1\chi^v$	R_F						
		A	B	C	D	E	F	G
1.	3.1123	0.77	0.88	0.78	0.84	0.59	0.78	0.76
2.	4.2336	0.69	0.72	0.64	0.69	0.57	0.71	0.71
3.	5.7336	0.50	0.41	0.35	0.55	0.38	0.49	0.60
4.	.7.2336	0.31	0.12	0.10	0.43	0.29	0.28	0.54
for comp. 1-4 r =		0.995	0.998	0.998	0.994	0.974	0.991	0.993
5. 	5.5894	0.52	0.42	0.38	0.56	0.39	0.49	0.62
6. 	5.6544	0.52	0.42	0.38	0.55	0.39	0.49	0.61
7. 	6.1543	0.44	0.26	0.19	0.46	0.30	0.36	0.54
8. 	6.5814	0.40	0.25	0.18	0.45	0.29	0.34	0.54
9. 	6.6544	0.39	0.24	0.17	0.45	0.28	0.34	0.54
10. 	7.2336	0.37	0.18	0.15	0.45	0.27	0.30	0.54
for comp. 1-10 r =		0.990	0.995	0.989	0.985	0.968	0.987	0.975
11.	3.3194	0.98	0.93	0.87	0.91	0.96	0.92	0.91
12.	4.3194	0.69	0.72	0.64	0.69	0.57	0.72	0.71
for comp.1-12 r =		0.962	0.993	0.986	0.975	0.889	0.980	0.931
a =		1.283	1.549	1.430	1.203	1.145	1.336	1.058
b =		-0.135	-0.199	-0.188	-0.113	-0.129	-0.149	-0.077
s =		0.02	0.01	0.02	0.02	0.06	0.03	0.05
α =		0.001	0.001	0.001	0.001	0.001	0.001	0.001

Chromatographic systems: KC_{18} Whatman Art. 4803-600, A-acetanitrile-water /5:5/, B-methanol-water /6:4/, TLC plates silica gel 60 F_{254} Art. 5715 /Merck/ silanised, C-methanol-water /5:5/, D--isopropanol-water /5:5/, E-acetonitrile-water /5:5/, F-ethanol--water /5:5/, G-n-propanol-water /5:5/.

Table II Results of correlation analysis for different RP-TLC systems for 5,5-dialkylbarbiturates /compounds no. 3-10/

Chromatographic system[x]	Correlation equation	Statistical parameters		
		r	s	α
TLC plates silanised /Merck/				
acetonitrile 50%	$R_F=0.795-0.089\,{}^1\chi^v$	0.969	0.01	0.001
methanol 55%	$R_F=1.308-0.159\,{}^1\chi^v$	0.970	0.02	0.001
ethanol 50%	$R_F=1.231-0.132\,{}^1\chi^v$	0.964	0.02	0.001
n-propanol 60%	$R_F=0.800-0.026\,{}^1\chi^v$	0.921	0.07	0.01
isopropanol 40%	$R_F=0.841-0.087\,{}^1\chi^v$	0.918	0.02	0.01
RP-8 /Merck/				
acetonitrile 60%	$R_F=1.472-0.129\,{}^1\chi^v$	0.997	0.01	0.001
methanol 70%	$R_F=1.560-0.185\,{}^1\chi^v$	0.977	0.02	0.001
RP-18 /Merck/				
acetonitrile 50%	$R_F=1.577-0.182\,{}^1\chi^v$	0.981	0.02	0.001
methanol 60%	$R_F=1.341-0.177\,{}^1\chi^v$	0.975	0.01	0.001
SIL C_{18}-50 /Macherey-Nagel/				
acetonitrile 55%	$R_F=1.112-0.114\,{}^1\chi^v$	0.964	0.02	0.001
methanol 70%	$R_F=1.453-0.147\,{}^1\chi^v$	0.987	0.02	0.001
SIL C_{18}-75 /Macherey-Nagel/				
acetonitrile 50%	$R_F=1.094-0.117\,{}^1\chi^v$	0.974	0.02	0.001
methanol 65%	$R_F=1.331-0.154\,{}^1\chi^v$	0.971	0.02	0.001
SIL C_{18}-100 /Macherey-Nagel/				
acetonitrile 50%	$R_F=0.960-0.119\,{}^1\chi^v$	0.965	0.02	0.001
methanol 70%	$R_F=1.509-0.162\,{}^1\chi^v$	0.971	0.02	0.001
KC_{18} /Whatman/				
acetonitrile 50%	$R_F=1.117-0.106\,{}^1\chi^v$	0.957	0.04	0.01
methanol 60%	$R_F=1.445-0.179\,{}^1\chi^v$	0.963	0.03	0.001

[x] stationary phase and percent of organic solvent in water

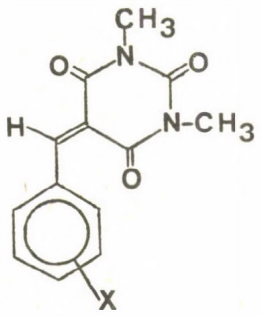
Since our previous results[2,6] suggested that electronic effects of C_5 substituents may contribute to some extent to the chromatographic behavior of barbiturates we synthesized the 1,3-dimethyl-5-arylidenebarbiturates where the N-methylation ruled out the possibility of hydrogen bonding by the imide groups and the whole C_5 substituent had varied electronic nature due to different substituents and their position at the phenyl

Table III Dependence of parameters of correlation analysis on the composition of the mobile phase for compounds nos. 3-10

Chromatographic system	Correlation equation	Statistical parameters		
		r	s	α
RP-18 /Merck/				
methanol : water				
50 : 50	$R_F=0.467-0.059\,{}^1\chi^v$	0.918	0.02	0.01
55 : 45	$R_F=0.956-0.127\,{}^1\chi^v$	0.942	0.02	0.01
60 : 40	$R_F=1.341-0.177\,{}^1\chi^v$	0.975	0.01	0.001
65 : 35	$R_F=1.451-0.186\,{}^1\chi^v$	0.977	0.01	0.001
70 : 30	$R_F=1.437-0.174\,{}^1\chi^v$	0.962	0.02	0.001

moiety. The results of adsorption chromatography on silica gel of these compounds are given in Table IV along with the pertinent Hammett σ constants for the substituents[20]. The spread of R_F values is relatively small which is in agreement with our previous finding that the C_2 carbonyl group plays an important role in the adsorption of barbiturate molecule on silica gel

Table IV R_F values and Hammett substituent constants for 1,3-dimethyl-5-arylidenebarbiturates



Compound No.	Substituent X	R_F value[x]	σ	$lg(\frac{R_{FX}}{R_{FH}})$
13.	p-NO_2	0.61	0.78	-0.0576
14.	m-NO_2	0.63	0.71	-0.0437
15.	m-Cl	0.66	0.37	-0.0256
16.	p-Cl	0.68	0.23	-0.0126
17.	p-Br	0.68	0.23	-0.0125
18.	H	0.70	0.0	0.0
19.	m-CH_3	0.72	-0.07	0.008
20.	p-CH_3	0.74	-0.17	0.0276
21.	p-OCH_3	0.84	-0.27	0.0823
22.	p-$N(CH_3)_2$	0.83	-0.83	0.0719

x in the system: TLC plates silica gel 60 F_{254} Art. 5715 /Merck/
acetone - chloroform - acetic acid /36:40:24/

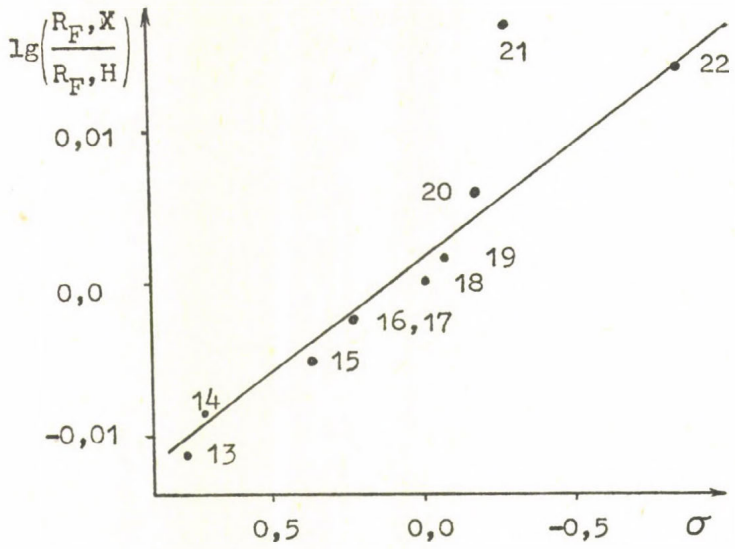


Fig. 1 Hammett type correlation for 1,3-dimethyl--5-arylidenebarbiturates /cf. Table IV/.

Correlation analysis:

$r = 0.9930$ /comp. no. 21 excluded/

$s = 0.02$

$\alpha = 0.001$

$$\lg \left(\frac{R_F,X}{R_F,H} \right) = 0.006 - 0.0787$$

but the highly significant Hammett-type correlation was observed for the investigated compounds as presented in Fig. 1. The only substituent which deviates from the straight line is the OCH_3 group in the para position at the phenyl moiety. This deviation may be due to the strong resonance effect of this group or its direct involvement in the adsorption process.

These results indicate that electronic effects of substituents should be taken into account in the chromatographic behaviour of barbituric acid derivatives.

ACKNOWLEDGEMENT

The authors express their thanks to Dr. A.O. Geiszler /Abbott Laboratories, North Chicago, Ill, U.S.A./ for the investigational samples of barbiturates and to Mr. J. Taylor /Whatman Ltd., Springfield Mill, Maidstone, England/ and Mr. J. Reiff /Macherey-Nagel GmBH, Düren, F.R.G./ for the TLC plates used in this study.

REFERENCES

1. L.Ekiert, Z. Grodzinska-Zachwieja, J. Bojarski, Chem. Anal. /Warsaw/ 24, 1045 /1979/.
2. L.Ekiert, Z. Grodzinska-Zachwieja, J. Bojarski, Chromatographia 13, 472 /1980/.
3. M.C.Bonjean, C. Luu Duc, Eur. J. Med. Chem. 13, 73 /1978/.
4. J.S.Millership, A.D. Wolfson, J. Pharm. Pharmacol. 32, 610 /1980/.
5. M.J.M.Wells, C.R. Clark, R.M. Patterson, J. Chromatogr. Sci. 19, 573 /1982/.
6. J.Bojarski, L.Ekiert, Chromatographia 15, 172 /1982/.
7. A.H.Stead, R.Gill, A.T. Evans, A.C.Moffat, J. Chromatogr. 234, 277 /1982/.
8. J.Bojarski, L.Ekiert, J. Liquid Chromatogr. 6, 73 /1983/.
9. L.B.Kier, L.H. Hall, J. Pharm. Sci. 70, 583 /1981/.
10. Y.M. Slobodin, I.N. Shokhor, Sbor. Stat. Obsh. Khim. 2, 850 /1953/ through C.A. 49, 6960c /1955/.

11. G.S.Skinner, G.Limperos, R.H.Pettebone, J. Am. Chem. Soc. 72, 1648 /1950/.
12. V.M.Vvedenskii, Khim. Geterosikl. Soed. 1092 /1969/.
13. S.O.Friksson, A.Holmgren, Acta Pharm. Suecica 2, 293 /1965/.
14. S.O.Eriksson, Acta Pharm. Suecica 2, 305 /1965/.
15. E.R.Garrett, J.T.Bojarski, G.J.Yakatan, J. Pharm. Sci. 60, 1145 /1971/.
16. J.M.A.Sitsen, J.A.Fresen, Pharm. Weekblad 109, 61 /1974/.
17. G.D.Daves, R.B.Belshee,W.R.Anderson, H.Downes, Mol. Pharmacol. 11, 470 /1975/.
18. P.R.Andrews, G.P. Jones, D.Lodge, Eur. J. Pharmacol. 55, 115 /1979/.
19. R.W. Kierstead, R.P. Linstead, B.C.Weedon, J. Chem. Soc. 1952, 3610.
20. J.Shorter, Correlation Analysis in Organic Chemistry, Clarendon Press, Oxford 1973, p. 9.

Chromatography, the State of the Art
H. Kalász and L.S. Ettre (Eds)

REVERSED-PHASE HPTLC AND HPLC STUDIES OF SOME PYRIDAZINE DERIVATIVES

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SUMMARY

Some pyridazine derivatives of pharmaceutical interest were investigated by reversed-phase HPTLC and HPLC. Stationary phases were RP 8 HPTLC plates and an RP 8 HPLC column were used as the stationary phase. The mobile phases consisted of mixtures of methanol and aqueous buffer solutions. The values of log k and R_M were studied as the function of mobile phase composition. In the case of two compounds HPTLC and HPLC data were compared. Variation of the relative retention values as a function of mobile phase composition was in good agreement when using the two methods.

INTRODUCTION

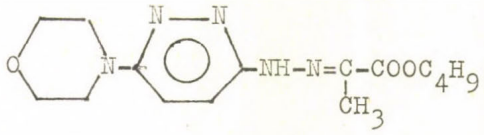
Reversed-phase /RP/ adsorbents are widely used in the chromatographic separation of different organic compounds. One of the advantages of this method is that the decomposition of substances having a strong mutual interaction with silica gel can be avoided during the chromatographic process. In addition, it was observed /1, 2/ that in RP chromatography retention data yielded by TLC can be more easily converted into column chromatography retention data than in the case of silica gel chromatography.

In a previous communication /3/ the RP chromatography of some pyridazine derivatives of pharmaceutical interest was re-

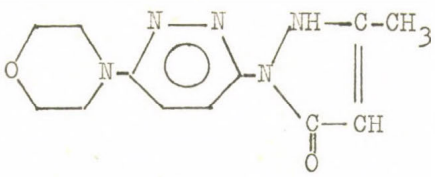
ported. The stationary phase was silica gel impregnated by paraffin oil in order to avoid decomposition of these compounds. In the present work chemically bonded /RP 8/ reversed-phase material was used as the stationary phase in HPTLC and HPLC. The values of R_M and log k were studied as the function of mobile phase composition in both methods. In the case of two compounds retention data yielded by HPTLC and HPLC were compared.

MATERIALS AND METHODS

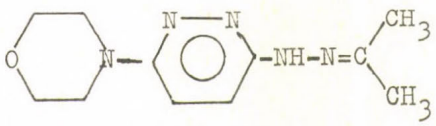
The following compounds were chromatographed:



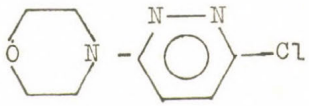
I



II



III



IV

Compounds II and III are the possible degradation products of I.

HPTLC studies were carried out on 10x10 cm RP 8 HPTLC plates /Merck/. The plates were developed in sandwich chamber /CAMAG/. The spots were made visible in 254 nm UV light. HPLC was performed on a Tracor instrument on a 4x200 mm RP 8 /5 μm/ column. Flow rate was 2 ml/minute, and detection was done at 280

nm. The mobile phases consisted of mixtures of methanol and 0.1 M /pH = 8/ acetate buffer.

RESULTS AND DISCUSSION

The relation between retention data and mobile phase composition was studied in both methods. In Fig. 1 the R_M values obtained in HPTLC of the four compounds were plotted against the methanol content of mobile phase. In the case of I, II and IV linearity was found, showing an increase in the retention with increasing water content of the mobile phase. Similar linearity was found and reported in the literature for several substances /2, 4/. The behaviour of compound III, however, differed from that of I, II and IV. The R_M value of III - considering the experimental error - was independent of the composition of the mobile phase. The strong retention shown by this compound in pure methanol did not increase with an increasing water content of the mobile phase /Fig. 1/.

Fig. 2 demonstrates the relationship between the retention of I and IV and the mobile phase composition in HPLC. For the measurement of the t_o values needed to calculate log k an easily detectable compound was chosen which had an R_f=1 value in all the mobile phases investigated. This compound was sulphosalicylic acid. The time of appearance of sulphosalicylic acid in the chromatogram was equal to t_o. A linear relationship was obtained, similar to that found in HPTLC.

Subsequently the retention data of I and IV obtained by HPTLC and HPLC were compared. The values of log k /HPLC/ were plotted against R_m /HPTLC/ measured in the same mobile phase. A linear relation was observed, similar to the results reported in the literature for other substances /2/. Consequently, the retention data characterizing both methods can be converted into each other.

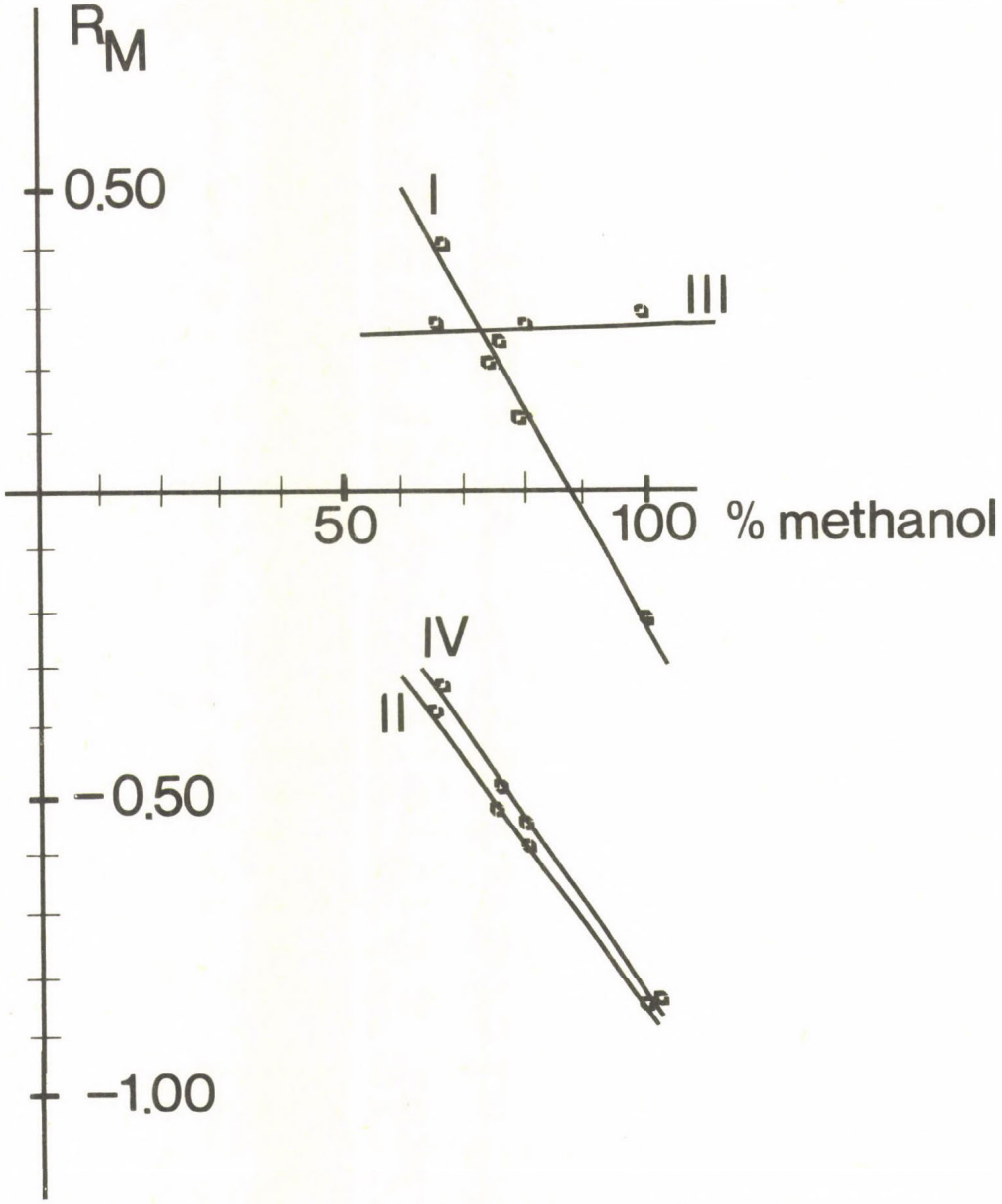


Fig. 1. R_M values of I, II, III and IV plotted against the methanol concentration of the mobile phase. Plate: RP 8 HPTLC /Merck/.
Development: sandwich chamber /CAMAG/.
Mobile phase: mixtures of methanol and 0.1 M /pH = 8/ acetate buffer.

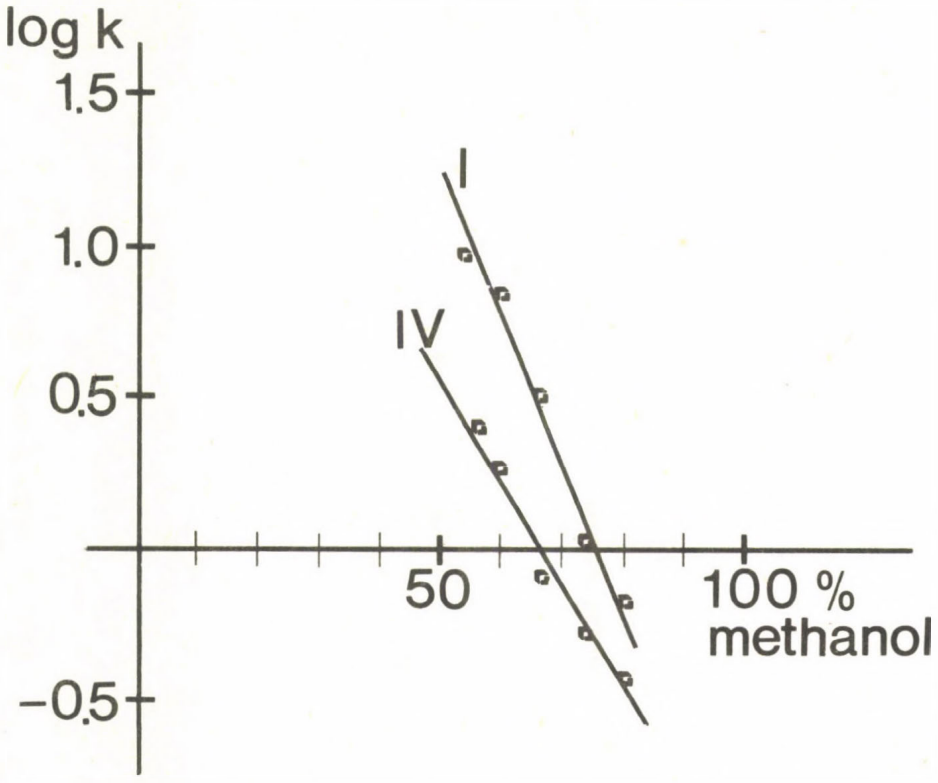


Fig. 2. log k values of I and IV in HPLC plotted against the methanol concentration of the mobile phase. Column: 4x200 mm RP 8 /5 μm/. Flow rate: 2 ml/min. Mobile phase: mixtures of methanol and 0.1 M /pH = 8/ acetate buffer.

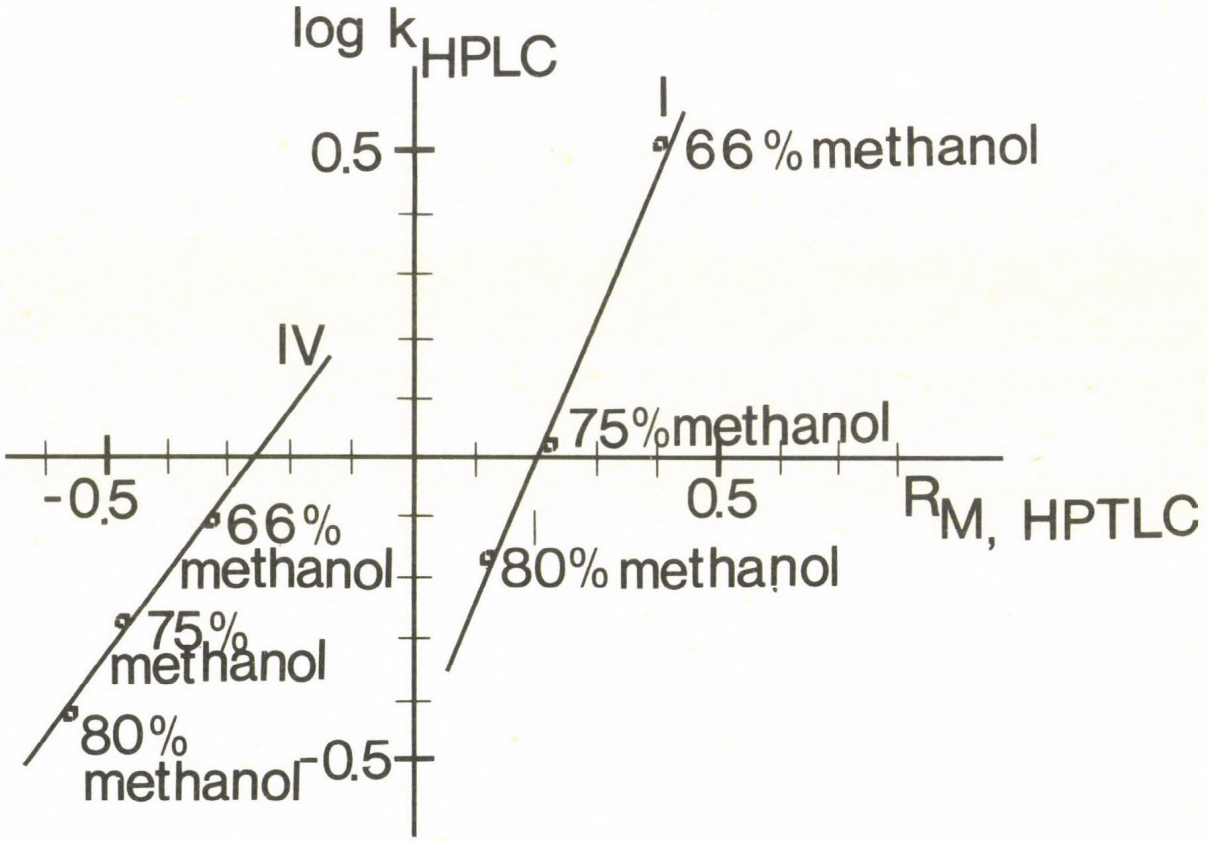


Fig. 3. log k/HPLC/ plotted against R_M /HPTLC/ of compounds I and IV.

Stationary phase: RP 8.
Mobile phase: mixtures of methanol and 0.1 M /pH = 8/ acetate buffer.

CONCLUSIONS

Reversed-phase HPLC and HPTLC proved to be good methods for the qualitative and quantitative chromatographic investigation of pyridazine derivatives. Quantitative evaluation of the HPTLC plates by densitometry is also possible. With proper selection of the mobile phase in HPTLC R_f differences suitable for separation can be obtained and decomposition observed on silica gel can be avoided. Comparison of HPTLC and HPLC data shows that HPTLC can be used for modelling the HPLC conditions.

LITERATURE

1. U.A. Th. Brinkman, G. de Vries, J. Chromatogr. 192. 331. /1980/.
2. M. Bieganowska, E. Soczewinski, J. Chromatogr. 205. 451. /1981/.
3. M. Bidló-Iglóy, Progress in Chromatography /2nd Danube /Symp./, Carlsbad, 1979
4. J. Draffehn, K. Ponsold, B. Schönecker, J. Chromatogr. 216. 69. /1981/.

Chromatography, the State of the Art
H. Kalász and L.S. Ettre (Eds)

HPLC SEPARATION OF BROMFENVINPHOS AND ITS METABOLITES IN PHYSIOLOGICAL SAMPLES

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SUMMARY

Bromfenvinphos (2-brom-1(2,4-dichlorphenyl)-vinyldiethylphosphate) is a new Polish phosphororganic insecticide exhibiting a high insecticidal against the various bioindicators, especially the potato beetle. It is a bromic analog of chlorfenvinphos produced by Shell Co. under the trade name of Birlane. Bromfenvinphos is a very strong poison and the control of this substance and the level of its biotransformation products in living organisms is necessary in this connection.

This paper presents possibilities of the application of various chromatographic techniques to the analysis of Bromfenvinphos and its three main metabolites. Special attention is devoted to the optimization of separation of these substances by ion-pair liquid chromatography using columns containing chemically bonded C_{18} phase. Examples of these techniques for the determination of Bromfenvinphos metabolites in biological samples (rat urine extracts) are presented.

The possible improvement in the detection of these substances in physiological samples is studied.

INTRODUCTION

A new phosphororganic insecticide named Bromfenvinphos (code name IPO-62) synthesized in the Warsaw Organic Industry Institute (1) was the subject of the investigations described

in this paper. The active component of this product is 2-brom-1 (2,4-dichlorphenyl)-vinyl-diethylphosphate (chemical name according to the IUPAC nomenclature) which exhibits a high insecticidal activity against the various bioindicators, especially the potato beetle. The technical product Bromfenvinphos is a yellow-brown oilferous heavy volatile liquid (boiling point 131-135°C at 0.003 mm Hg). It is insoluble in water but soluble in methanol, chloroform, trichlorethylene and kerosene. The active compound content in the technical product varies from 86.4 to 91%. The chemical structure of the compound can be seen in Fig. 1 (I).

The toxicity of Bromfenvinphos against rats is less than half than the toxicity of chlorfenvinphos.

The LD_{50} value of technical Bromfenvinphos for male rats varies in the range of 36.9-63.5 mg/kg(2,3). The DL_{50} per os value of chlorfenvinphos for rats is 10-15 mg/kg (5), and the LD_{50} value of Bromfenvinphos for dogs is 2730 mg/kg per os and 112.75 mg) kg i.p. (5). Metabolism of phosphororganic triesters leading to the formation of less toxic diesters can take place by anhydric bond destruction caused by phosphatization or microsomic oxydation and also by the destruction of the P-O-alkyl group (O-dealkylation process (6,7). Results of the in vitro and in vivo investigations of Bromfenvinphos transformation in dogs (64, 61%) showed that ethylbromfenvinphos (II-Fig. 1), the product of O-dealkylation (6,7,8), is the main product of its metabolism. This metabolite can be considered as a weakly toxic substance because its LD_{50} per os value is 716.9 mg/kg. Paper and thin-layer radiochromatography of urine extracts from rats and dogs fed by ^{14}C-vinyl Bromfenvinphos in amounts of 20 and 50% exhibited 9-10 spots from which six were identified.

The main metabolites in a rat are 2.4-dichlormandelic acid-(29.26%), (III-Fig. 1), monodesethylbromfenvinphos(10.96%) (II-Fig. 1) and 2.4-dichlorbenzoic acid(9%) (IV-Fig. 1).

Very important type of intoxicational reactions of xenobiotics are synthesis (conjugation) reactions. These reactions have not yet been studied in details for phosphororganic insectides. In our studies we first investigated that part of the

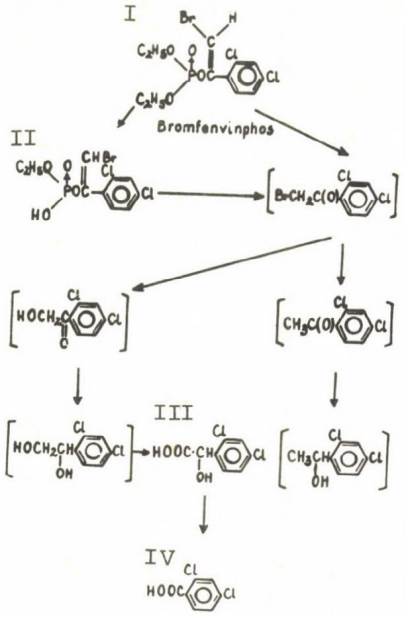

Fig. 1. The metabolism of ^{14}C-Bromfenvinphos in rats and dogs. Compounds in brackets were not isolated

molecule which contains the phosphorus atom. Investigations of the Bromfenvinphos intoxication processes showed that for a rat, the conjugated substances represent 46.47% of the total amount of metabolitic products while for a dog, they only represent 9.53%.

On the basis of experimental data one can establish a hypothetical degradation sequence of Bromfenvinphos. This is illustrated in Fig. 1. This sequence corresponds to the general sequence known for the biotransformation process of chlorfenvinphos (10).

Furthermore, Bromfenvinphos is partially degraded to carbon dioxide because in the expired air of rats small quantities of this substance (about 1% of the total amount) were detected.

The presence of undecomposed substances has not been detected in either rat of dog urine. From this fact it can be concluded that Bromfenvinphos is intensively metabolised (9) and should not stay in biotope.

In order to investigate the metabolism of Bromfenvinphos a rapid method must be developed for its determination.

This paper presents possibilities of the application of various chromatographic techniques to the analysis of Bromfenvinphos and its three main metabolites. A special attention was given to the optimization of the separation of these substances by ion-pair liquid chromatography using columns containing chemically bonded C_{18} phase.

APPARATUS

Chromatographic measurements were carried out using a Pye Unicam Model LC 20 liquid chromatograph combined with a UV-detector (254 and 280 nm) equipped with a 8-µl measuring cell.

Absorption spectra measurements in the ultraviolet range showed that the typical detector wavelength, 254 nm, will be the most suitable (Fig. 2).

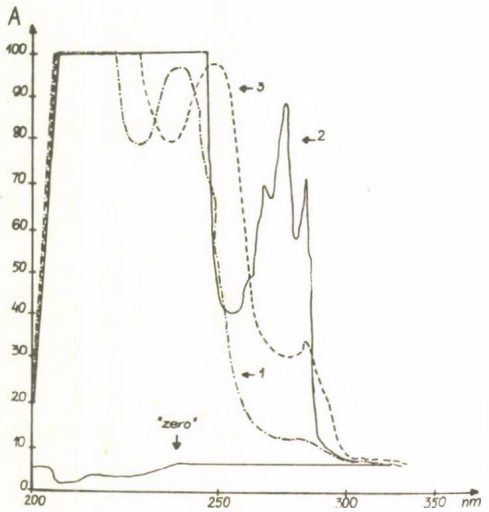

Fig. 2. UV absorption spectra of investigated substances
1. 2.4-dichloromandelic acid
2. 2.4-dichlorobenzoic acid
3. monodesethyl-Bromfenvinphos

MATERIALS AND METHODS

The examined samples were injected into the column using Rheodyne Model 7220 pressure valve; the volume of the injecting loop was 10 µl. The flow rate of the mobile phases applied in our experiments was 1 ml/min.

Solvents and reagents: redistilled water; methanol, acetic acid, phosphoric acid, and disodium phosphate, all p.a. purity (P.O.Ch., Gliwice, Poland); diethyl ether p.a. purity (E. Merck, Darmstadt, FRG); tetrabutylammonium iodide, pure (Chemapol, Prague, Czechoslovakia); and tetrabutyl ammonium chloride, pure (Fluka, Buchs Switzerland).

In order to determine Bromfenvinphos and its derivatives standard columns of 100 mm length and 4 mm internal diameter were used. Depending on the applied technique the following stationary phases were used:

1. Adsorption chromatography: LiChrosorb Si 60, d_p = 5 µm
2. Reversed-phase chromatography:
   a/ LiChrosorb RP-8, d_p = 10 µm
   b/ LiChrosorb RP-18, d_p = 12 µm

The columns were filled by the upward slurry low pressure viscosity method. The reduced Knox parameter and the flow resistance factor (17) characterizing the stationary phases and columns used in our investigations are listed in Table I.

Table I. Column Parameters

Column	Sample: diphenyl			
	k'	h_{min}	ν_{min}	φ
LiChrosorb Si-60	0.59	2.50	4.90	720
LiChrosorb RP-8	6.33	3.90	7.50	680
LiChrosorb RP-18	6.21	3.90	7.50	530

RESULTS AND DISCUSSION

Numerous methods are described in the literature for the determination of enolphosphate substances by chromatographic methods, especially by paper and thin-layer radiochromatography (8,14,15).

Although these methods give satisfactory results, they are, complicated, time consuming and expensive because of the need for labelled standards and complex radioisotopic apparatus.

For these reasons great efforts were undertaken for the development of a rapid and effective method adapting the advantages of HPLC and ion-pair chromatography.

Using thin-layer chromatography (9) Bromfenvinphos derivatives have been separated on silica gel; for this reason, the conditions of TLC separation were transformed onto high-performance column liquid chromatography (Table II).

By liquid adsorption chromatography three derivatives were separated, but the determination of Bromfenvinphos is impossible probably because of its irreversible adsorption on the stationary phase (Fig. 3). For this reason further investigations were carried out on systems containing chemically bonded stationary phases having various length of the alkyl chain. LiChrosorb RP-8 and RP-18 represent such materials.

Comparing the obtained results (Fig. 4 a,b) significant changes can be observed in the resolution of the separated substances. However, Bromfinvenphos is still eluted together with its monodes ethyl derivative. The results of the separation on the C_8-LiChrosorb column are not satisfactory because of the poor separation of the two first substances; also, the column filled by RP-8 packing became contaminated after limited use.

Further optimization of the separation conditions was based on the change of the dissociation constant by the addition of acetic acid to the binary methanol-water mobile phase (Fig. 5). Change in the pH causes an increase in the peak capacity between 2.4-dichlormandelic acid, 2.4-dichlorbenzoic acid and the monodesethyl derivative.

Another possibility for the modification of the mobile phase is ion-pair HPLC (19,20). Tetrabutylammonium iodide was

Table II. Methods and the composition of the mobile phases

Chromatography method	Column	Mobile phase composition	
adsorption	Si-60	n-hexane	100
		n-hexane - diethyl ether - - methanol - acetic acid	60:30:10:1
reversed-phase	RP-8	methanol - water	60:40
		methanol - water	40:60
	RP-18	methanol - water	70:30
		methanol - water	40:60
		methanol - water - acetic acid	90:10:1
ion pair	RP-18	methanol - water	60:40
		phosphate buffer	pH=4
		tetrabutyl ammonium iodide	0,005M
		methanol - water	60:40
		phosphate buffer	pH=4
		tetrabutyl ammonium chloride	0,005M

used as the modifier in the first series experiments. This substance forms ion pairs with the substances examined, causing an increase in the hydrophobicity of the separated acidic substances. Fig. 6a presents the chromatogram of this separation.

As the separation of 2,4-dichlormandelic acid and 2,4-dichlorbenzoic acid is still not satisfactory under these conditions, the applications of tetrabutyl ammonium chloride (TBA) as a modifier of the mobile phase the use of tetrabutyl ammonium chloride (TBA) was tried as a modifier. TBA shows a greater degree of dissociation and thus is a more effective substance to form ion-pairs (Fig. 6b). This is undoubtedly related to a greater ionic strength and thus to the formation of more stable complexes (larger pH range). The application of TBA improves significantly the separation of Bromfinvenphos (peak 4 in Fig. 6a and b/ and its metabolites (peaks 1 and 3,

1. 2,4-dichloromandelic acid
2. 2,4-dichlorobenzoic acid
3. monodesethyl - - Bromfenvinphos

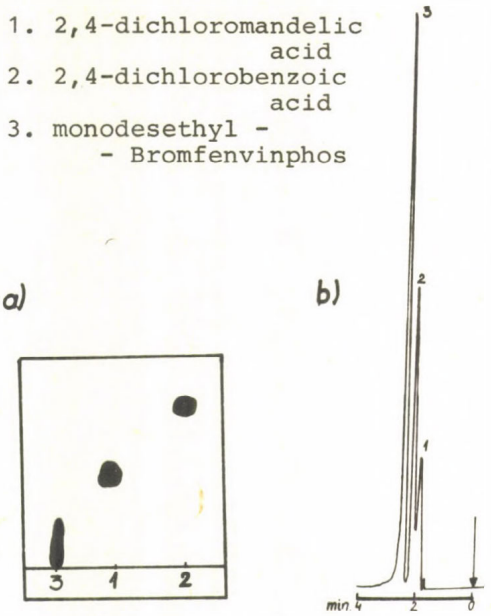

Fig. 3. Comparison of TLC and HPLC separation of Bromfenvinphos metabolites under similar analytical conditions

1. 2,4-dichloromandelic acid
2. 2,4-dichlorobenzoic acid
3. monodesethyl-Bromfenvinphos

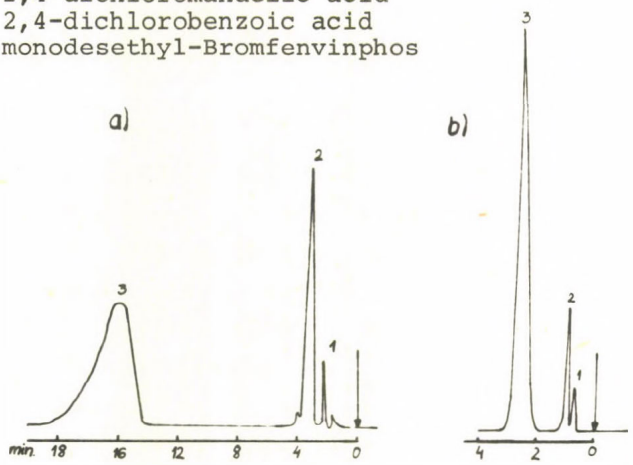

Fig. 4. The separation of three Bromfenveinphos metabolites a/ column RP-8* b/ column RP-18**

*mobile phase: methanol - water (40:60 v/v)
**mobile phase: methanol - water (70:30 v/v)

1. 2,4-dichloromandelic acid
2. 2,4-dichlorobenzoic acid
3. monodesethyl-Bromfenvinphos

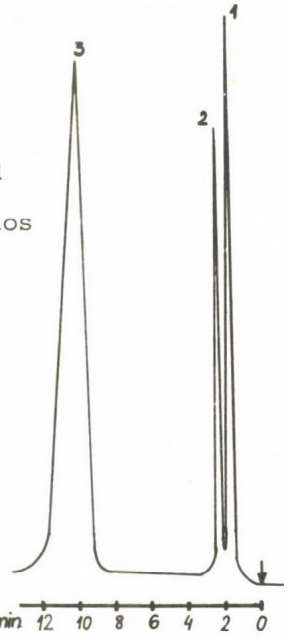

Fig. 5. Example of urine analysis. Column: RP 18, mobile phase: methanol - water - acetic acid (90:10:1 v/v)

1. 2,4-dichloromandelic acid
2. 2,4-dichlorobenzoic acid
3. monodesethyl-Bromfenvinphos
4. Bromfenvinphos
5. naphthalene

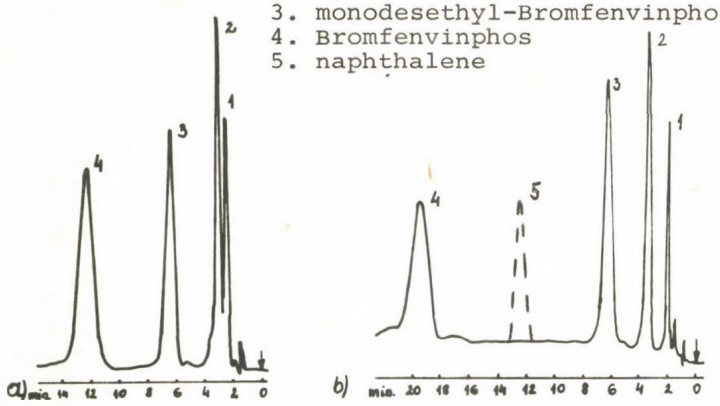

Fig. 6. Example of urine analysis. Complexing reagents 0.005M: a/ tetrabutylammonium iodide, b/ tetrabutylammonium chloride;
mobile phase: methanol - water (60:40 v/v) pH = 4,
column: RP-18 (see Table II)

Fig. 6a and b) A good separation of peaks 3 and 4, and also of peaks 1 and 2 can be observed. It appears that the use of

TBA as the mobile phase modifier gives the best results in the analysis of Bromfenvinphos and its metabolites by ion-pair chromatography.

More exact optimization of the chromatographic separation using TBA requires a more precise selection of the pH and the mobile phase composition. The effect of changes in the pH value was studied in the pH range of 4-7.5. At pH = 4 good separation of the two acidic metabolites was obtained. Furthermore, the influence of this pH value on the chemically bonded C_{18} phase is rather small, while at lower pH values destruction may take place. Reduction of the a methanol concentration in the mobile phase somewhat improves the separation of the two acidic components),but the analysis time of the two undissociated compounds is increased also, an increase in peak asymmetry is observed. The optimum composition of the methanol-water mixture is 60:40% v/v.

The amount of the complexing agent which must be added to mobile phase was also evaluated. The optimum value, found, 0.005 M TBA, is in good agreement with the literature data (19,20).

We have also determined the detection limits of the individual substances under various analytical conditions. The values obtained are listed in Table III. The data presented in this table show that the best results are obtained for the optimum conditions i.e. when the mobile phase containing TBA is used.

Increase in the resolution between substances 3 and 4 permits the use of naphthalene as the internal standard. Prior to quantitative analysis, the linearity of the detector response was checked. For this purpose pure substances were then added to animal urine extracts. Solutions prepared in this way were purified by extraction and then concentrated by evaporation. The chromatographic measurements showed a linear relationship between the detector signal and sample concentration for all substances in the concentration range of 2×10^{-4} - 7.6 mg/ml. As shown in Table IV good reproducibility of the obtained quantitative results was observed. The maximum loss of the determined substances was less than 6%.

Table III. Detection of the various substances in the analysis of rat urine samples

Mobile phase \ Solute	Detection limit g/ml			
	1	2	3	4
CH_3OH - H_2O - CH_3COOH 90 : 10 : 1	$4 \cdot 10^{-3}$	$1 \cdot 10^{-4}$	$2 \cdot 10^{-5}$	$2 \cdot 10^{-5}$
CH_3OH - H_2O 60 : 40 phosphate buffer pH=4 tetrabutyl-ammonium iodide 0.005M	$8 \cdot 10^{-5}$	$3 \cdot 10^{-6}$	$4 \cdot 10^{-6}$	$4 \cdot 10^{-6}$
CH_3OH - H_2O 60 : 40 phosphate buffer pH=4 tetrabutyl-ammonium chloride 0.005M	$4 \cdot 10^{-6}$	$2 \cdot 7 \cdot 10^{-7}$	$4 \cdot 10^{-7}$	$2 \cdot 10^{-7}$

1. 2,4-dichloromandelic acid
2. 2,4-diclorobenzoic acid
3. monodesethyl-Bromfenvinphos
4. Bromfenvinphos

The concentration of a substance in the sample was calculated according to the following equation:

$$c = H \cdot \frac{c_s}{H_s} \cdot \frac{h_x}{c_x}$$

C - concentration of a substance in the sample

H - peak height of the substance in the sample

H_s - peak height of the standard (naphthalene) in the sample

c_s - concentration of the standard in the sample

$h_x = \frac{h_s}{h_m}$ - ratio of the peak heights of naphthalene and the substance in the standard mixture

$c_x = \frac{c_s}{c_m}$ - ratio of the concentration of naphthalene and the substance in the standard mixture

Table IV. Reproducibility of the analysis of standard urine samples

Samples No.	Solute	Concentration of sample g/ml	Measured concentration of sample g/ml	Loss %
I	1*	$1.5 \cdot 10^{-4}$	$1.44 \cdot 10^{-4}$	3.8
	2	$1.5 \cdot 10^{-3}$	$1.41 \cdot 10^{-3}$	5.7
	3	$1.5 \cdot 10^{-4}$	$1.45 \cdot 10^{-4}$	3.0
	4	$1.5 \cdot 10^{-4}$	$1.42 \cdot 10^{-4}$	5.3
II	1	$1 \cdot 10^{-3}$	$9.68 \cdot 10^{-4}$	3.2
	2	$1 \cdot 10^{-2}$	$9.49 \cdot 10^{-3}$	5.1
	3	$1 \cdot 10^{-3}$	$9.71 \cdot 10^{-4}$	2.9
	4	$1 \cdot 10^{-3}$	$9.50 \cdot 10^{-4}$	5.0
III	1	$1.5 \cdot 10^{-3}$	$1.45 \cdot 10^{-3}$	3.4
	2	$1.5 \cdot 10^{-2}$	$1.41 \cdot 10^{-2}$	6.0
	3	$1.5 \cdot 10^{-3}$	$1.45 \cdot 10^{-3}$	3.4
	4	$1.5 \cdot 10^{-3}$	$1.42 \cdot 10^{-3}$	5.4

* - 1. 2,4-dichlorobenzoic acid, 2. 2,4-dichloromandelic acid, 3. monodesethyl-Bromfenvinphos, 4. Bromfenvinphos

CONCLUSIONS

The results of our investigations indicate that the concentration of a given substance can be determined precisely and reproducibly. The method described in this paper may be used for the determination of Bromfinvenphos and its metabolites.

REFERENCES

1. Śledziński B. Biol. Urz. Pat. PRL, 23, 34 (1973) (I)
2. Wysocka-Paruszewska B., Osicka A., Brzeziński J., Gradowska J., Bromat. Chem. Toksykol., 7, 195, (1974)
3. Majda A., Chrusielska B., Toxicological Symposium, Poznan 1974

4. Hutson D.H., Hotwey D.E., Biochem. Pharmac. 15, 946 (1967)

5. Bronisz H., Ochyński J., Pestycydy 1, 21 (1977)

6. Menzer R.E., Danterman U.C. J. Agr. Food Chem., 18, 1031 (1970)

7. Danterman W.C., Bull. Wld. Hlth. Org., 44, 193 (1971)

8. Hutson D.H., Akintonwa A., Hathway D.E., Biochem. J. 102, 133 (1967)

9. Ochyński J., Toxicological Symposium, Lublin 1976

10. Ochyński J., I Conf. of Polish Toxicological Society, Warszawa 1981

11. Suprynowicz Z., Buszewski B., Lodkowski R., Trocewicz J., Chem. Anal., 25, 749 (1980)

12. Suprynowicz Z., Buszewski B., Lodkowski R., Trocewicz J., Polish Patent No 110372 (1979)

13. Suprynowicz Z., Buszewski B., Lodkowski R., Trocewicz J., Polish Patent No 120844 (1979)

14. Baynon K.J., Wright A.N., J. Sci. Fol. Agric., 18, 143 (1967)

15. Baynon K.J., Wright A.N., J. Sci. Fol. Agric., 19, 146 (1968)

16. Knox J.H., Parcher J., Anal. Chem., 41, 1599 (1969)

17. Bristow P.A., Knox S.H., Chromatographia 10, 279 (1977)

18. Suprynowicz Z., Buszewski B., Lodkowski R., Ochyński J., "Study on the HPLC separation of Bromfenvinphos metabolites". Symposium on Chromatography, Siófok, 1981 (Hungary)

19. Bidlingmeyer B.A., J. Chromatogr., Sci., 18, 525 (1980)

20. Tomlinson E., Jefferies T.M., Riley C.M., J. Chromatogr., 159, 315 (1978)

Chromatography, the State of the Art
H. Kalász and L.S. Ettre (Eds)

BIOEQUIVALENCE STUDY OF SENSIT® AND SENSIT-β-CYCLODEXTRIN COMPLEX BY HPLC AND RADIOISOTOPIC METHODS

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INTRODUCTION

SENSIT, fendiline hydrochloride, N-/2-benzhydril-ethyl/-N-/1-phenyl-ethyl/-amine hydrochloride, /Fig. 1./ is a coronary vasodilator and calcium antagonist of CHINOIN Pharmaceutical and Chemical Works, /Budapest/ /1, 2/.

Pharmacokinetic studies of the compound were performed by Thiemann GMBH /Lünen, FRG/in both humans and animals /3/. Using the labelled derivative they followed up the changes of plasma concentration after oral and intravenous administration of the compound. Rats of 250 g average body-weight were treated with

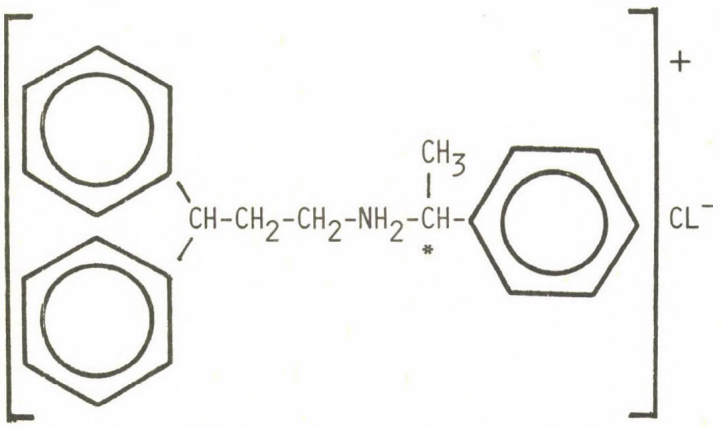

Fig. 1. Chemical structure of Fendiline · HCl /Sensit®/ labelled with ^{14}C

5 mg/kg of the ^{14}C-labelled compound diluted in 1:4 ratio with inactive fendiline hydrochloride. Peak plasma concentrations were measured 100 minutes after treatment. Examinations were also performed with unlabelled fendilin hydrochloride administered in oral doses of 25 mg/kg body-weight. Serum concentration were determined by gas-chromatography using a nitrogen-specific thermionic detector. Peak concentrations were found 1 hour following administration, but the values showed great individual variation /0.03 to 0.34 µg/ml/.

It is known that complexation with β-cyclodextrin increases the solubility of poorly soluble substances, thus leading to higher blood levels and increased bioavailability of the drugs. /4/.

Fendilin hydrochloride dissolves poorly in water /1%/, therefore to improve water-solubility its β-cyclodextrin /BCD/ complex was prepared at the Biochemical Laboratory of CHINOIN /5/. The active ingredient content of the complex was 10% calculated for fendilin hydrochloride. In view of the better water-solubility of the BCD complex it appeared justified to assume that the biologic utilization of the preparation would also be improved. Better utilization of a drug is characterized by its more rapid appearance in the blood, elevated serum concentrations, and an increase in the area under the concentration curve.

One molecule of fendiline base is included in two β-cyclodextrin molecules, as proved by thermoanalytical examinations. In vitro experiments have revealed that fendiline. HCl is even better dissolved in artificial gastric fluid. In the knowledge of these results our aim was to prove also in <u>in vivo</u> experiments that the improved absorption qualities of the complex are also reflected in the blood concentration changes and bioavailability of the drug.

EXPERIMENTAL

Materials and apparatus

HPLC examinations were performed with bidistilled water and chemicals of analytical-grade purity. The preparations used were SENSIT /CH-81-07/3/, and Sensit BCD /CHBK 985/SÁ/.

Packing of the column: Zorbax ODS /5 µm/ Apparatus: Biotronik BT 3020 /pump/, BT 3030 UV detector, Sp 4100 integrator, K 23 Janetzky centrifuge, Vortex /Kutesz/.

Extraction of sera was carried out as described in the literature /3/. Blood was allowed to stand for one hour at room temperature, then centrifuged at 3000 rpm. Two-ml serum was alkalified with 2N NaOH, homogenized in a Vortex apparatus, extracted with 10 ml ether, washed into a test tube, and frozen on dry ice. The supernatant was evaporated to dryness and kept in a refrigerator until used.

Considering that no data were available in the literature on the HPLC determination of fendiline. HCl, we first had to elaborate a suitable method for it. The UV spectrum of the compound showed two maxima: at 220 and 259 nm. The latter value is more favourable from the technical aspect, but the expectable serum concentrations were not detectable in this range, therefore the 220 nm maximum had to be taken into consideration. As it can be seen from Fig. 2. the serum proteins did not disturb the measurement after extraction.

Fendiline base obtained by extraction had an absorption maximum at 221 nm, and the detectability limit was 1 µg/ml sensitivity: 0.04 AUFS/.

Based on the structural formula of fendiline base, the development of an ion-pair chromatographic method appeared to be most suited for separation and determination. Separation was performed on a 15 x 4.6 mm I.D. Zorbax ODS /5 µm/ column. Elution was made by a 30:70 mixture of water and methanol containing 0.2% decylamine. pH was adjusted to 8 with perchloric acid. Under such conditions the calibration curve was linear in the concentration range of 5 to 20 µg/ml; recovery was 50%.

CFY male rats of 200-250 g body-weight were used for the experiments. The amimals were administered fendiline hydro-

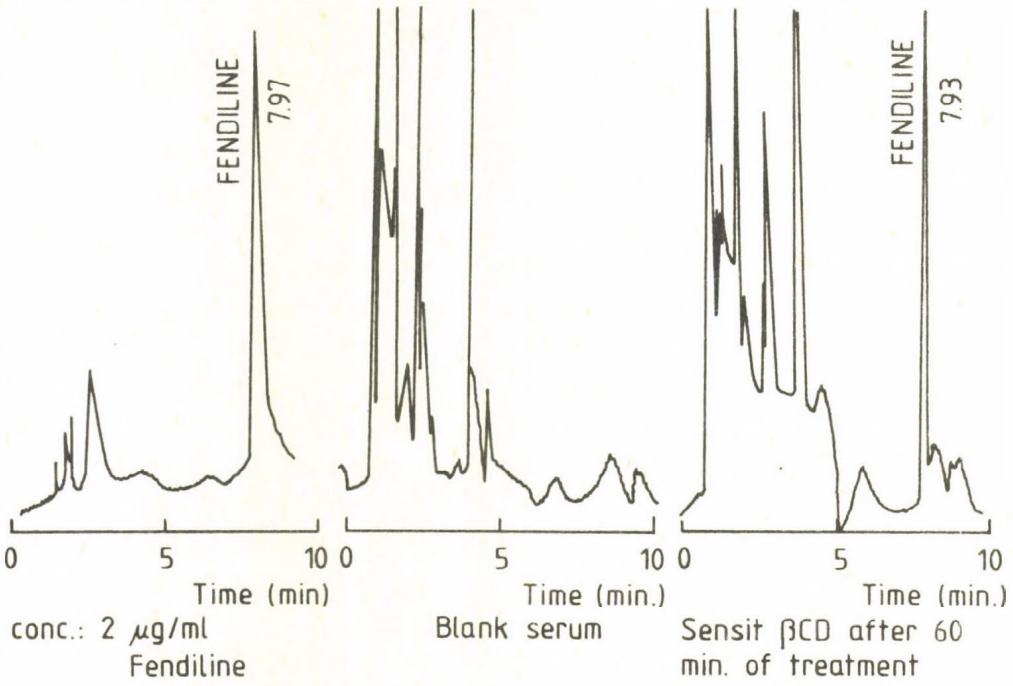

Fig. 2. HPLC chromatograms

chloride and its BCD complex in a dose of 250 mg/kg body-weight in 1% methylcellulose suspension, through a gastric tube. Blood samples were withdrawn at 15, 30, 60, 120, and 240 minutes following treatment. Fendiline content of the blood samples was determined by the method elaborated in our laboratory.

MATERIALS AND APPARATUS

Investigations performed with fendiline hydrochloride-^{14}C

The preparations used were: fendilin hydrochloride-^{14}C /GYOKI 449/1982/ and fendilin hydrochloride-BCD-^{14}C /GYOKI 450/1982/. Bidistilled water, chemicals of analytical-grade purity and Insta-gel /Packard 601300// were used. Unlabelled fendiline. HCl and its BCD complex were the same as used for HPLC measurements. The liquid scintillation spectrometer used was a Packard TriCarb 300C type apparatus; calculator type: HP 97.

METHOD

The rats were administered 5 mg/kg fendilin hydrochloride-^{14}C and 20 mg/kg fendilin hydrochloride, and the same amounts of the labelled and unlabelled BCD complexes, respectively, in 1% methylcellulose suspension orally. The body-weights ranged from 250 to 300 g. Blood samples were taken 5 and 30 minutes, 1, 2, 4, 8, 12, 24, 36 and 48 hours following administration. Blood samples were taken from the orbital sinus in superficial anaesthesia. The activities of whole blood and serum were determined for each sample.

Processing of blood samples: 100 µg whole blood was pipetted into the 1:1 mixture of 600 µg isopropanol and water, bleeched with 300 µl 30% H_2O_2 solution, homogenized and washed with instagel into 10 ml cuvettes. The remaining part of the blood was stored at $+5^{o}$ for 25 hours, and the sera were processed as described above. Activity of the samples was expressed in dpm after 10 minutes' measurement. A quench series extending from No. 1 to 10 was used.

RESULTS AND CONCLUSIONS

Determination of fendilin hydrochloride and of its BCD complex by HPLC in case of the applied doses is not sensitive enough. Therefore we have used 10 times higher doses than described in the literature. In the knowledge of the recovery rate /%/ the serum concentration of fendilin hydrochloride can be calculated. Fig. 3. shows the changes of the serum concentrations in the function of the time after treatment measured by HPLC.

It can be seen from the figure that fendilin hydrochloride reaches peak serum concentrations in both cases 30 minutes after treatment.

However, the serum concentrations of the rats treated by the BCD complex are by 40 per cent higher than those of the animals treated by the uncomplexed drug. The area under the curve in case of the animals treated by the BCD complex is also

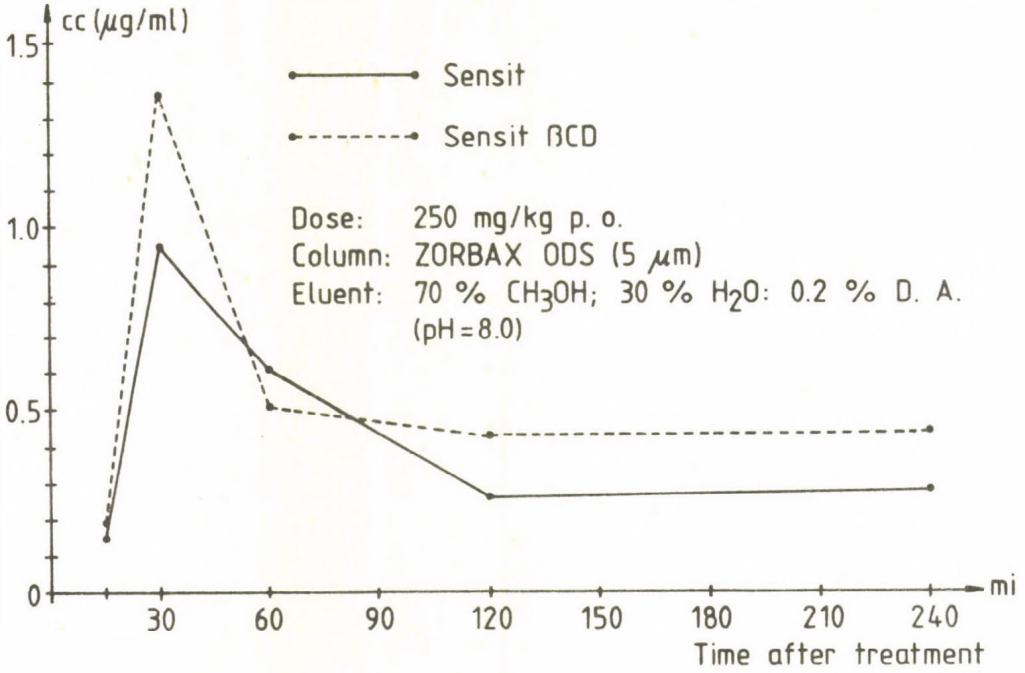

Fig. 3. Serum level of Sensit and Sensit BCD by HPLC determination

by 32 per cent larger. These results are in good correlation with the in vitro experiments, and unanimously indicate the better biological utilization of fendilin hydrochloride BCD complex. In order to confirm the results of the HPLC method and the conclusions drawn from them, we performed a pharmacokinetic study with the ^{14}C-labelled compound. In that case the doses described in the literature could be applied. The use of the labelled compound made possible an increased number of blood samplings, and extraction had not to be performed. The previous experiment was thus repeated with 5 mg/kg doses of fendilin hydrochloride-^{14}C and with its BCD complex containing the same amount of the active ingredient. In this experimental series not only the serum concentrations, but the ^{14}C-content of whole blood was determined.

The obtained results proved the validity of our presumption. It can be clearly seen from the figures that the absolute values of the blood- and serum concentrations in the animals

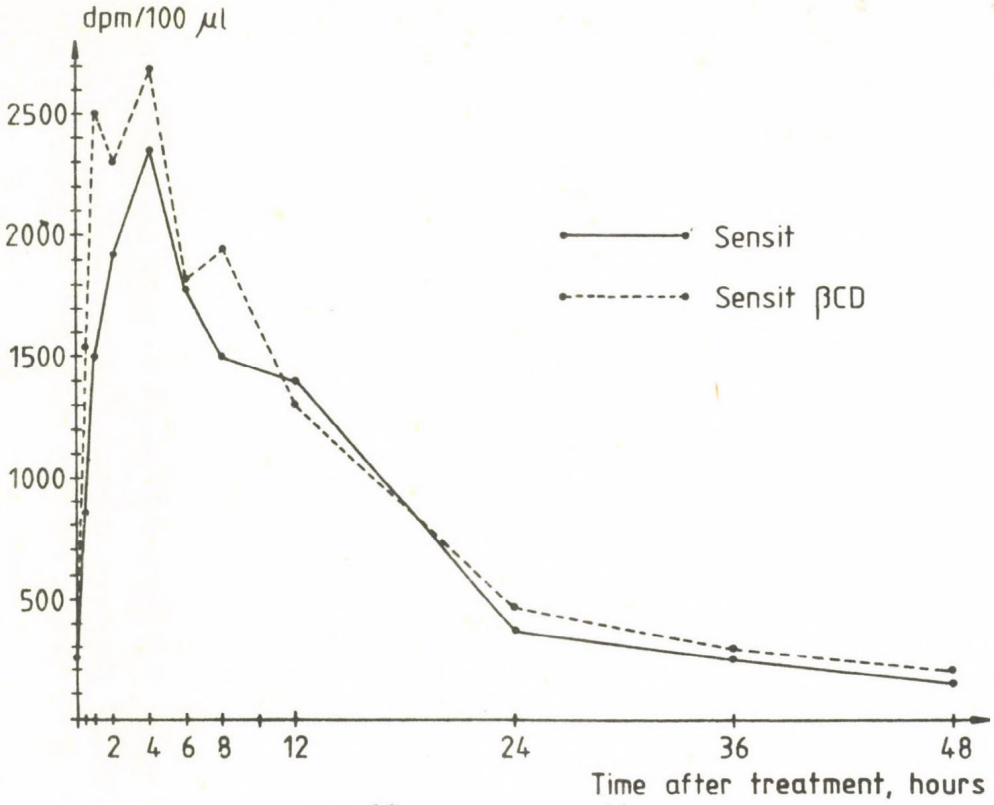

Fig. 4. Serum level of ^{14}C Sensit and ^{14}C Sensit BCD

treated orally with fendilin hydrochloride-BCD-^{14}C are higher and reach a peak earlier than in animals treated under identical conditions with the uncomplexed drug. Peak concentrations are achieved 30 minutes following administration and persist for 4 hours. In the rats treated with fendilin hydrochlorid-^{14}C peak concentrations were measured in whole blood and serum only 4 hours following treatment. With both preparations the blood and serum levels decreased more rapidly until the 24th hour, whereafter their decrease was slower up to the 48th hour. The areas under the curve were by 15% larger for the animals treated with fendilin hydrochloride-BCD-^{14}C than for those treated with the uncomplexed preparation.

Both our HPLC measurement and the results of our pharmacodynamic studies performed with the isotope technique are in good agreement with the results of earlier in vitro examina-

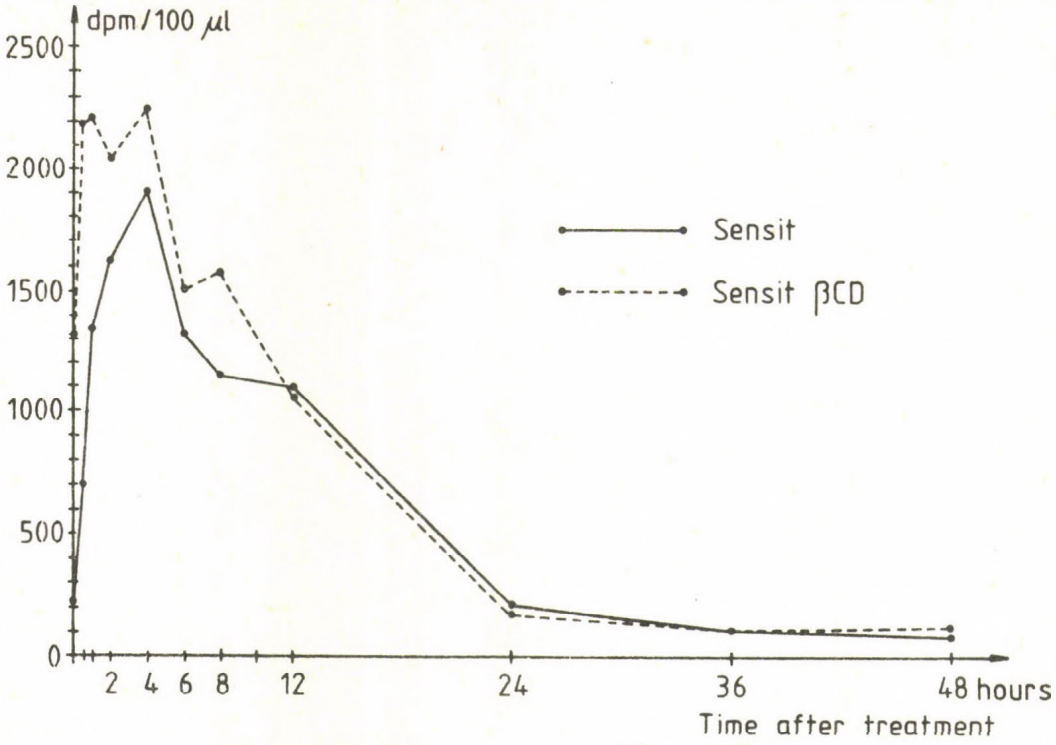

Fig. 5. Blood level of ^{14}C Sensit and ^{14}C Sensit BCD

tions. The conclusion can therefore be drawn that complexation with BCD increases the solubility and absorption of fendilin hydrochloride and thereby improves the bioavailability of the drug.

REFERENCES

1. Hung. Pat. 1505 34 /1961/
2. Knoll József: Gyógyszertan. Medicina Budapest, 1983; Vol. 1. p. 427.
3. W.R. Kukovetz, F. Brunner, E. Beubler, R. Weyhenmeyer, R. Lohaus, M. Grob and D. Mayer: Single dose pharmacokinetics of fendiline in humans. Europ. J. Drug Met. Pharmacokin. 7, 105 /1982/.

4. J. Szejtli and L. Szente: Interaction beetween Indometacin and β-cyclodextrin. Die Pharmazie 36, 694 /1981/.

5. A. Stadler-Szőke, M. Vikman and J. Szejtli: Inclusion complexes of fendilinhydrochloride. Personal communication.

Chromatography, the State of the Art
H. Kalász and L.S. Ettre (Eds)

CHARACTERIZATION OF NOVICARDIN WITH CHROMATOGRAPHIC METHODS

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INTRODUCTION

It has been known from traditional therapy that the teas of cherry peduncle have beneficial diuretic effect.

Novicardin, its wax-free substance prepared by acetone extraction /1/ has a particular effect on the heart. According to pharmacological trials it has positive inotropic effect which is not adrenaline and digitalis like /2/. During clinical applications Novicardin proved to be an orally active cardiotonic that in itself is able to cease slight and average decompensation without any appearance of toxic symptoms and keep on compensation for a long period.

The dragee containing Novicardin as active ingredient is planned to be put into circulation by Alkaloida Chemical Works in the near future. For this purpose it was necessary to develop an analytical method for the determination of the ratio of components. Since even the structures of the main components have been unknown so far, it was our task to identify them as well.

MATERIALS AND METHODS

TMS-derivatization

10 mg of Novicardin is dissolved in 100 µl of the 1:1 mixture of N,O-Bis-trimethylsilyl-trifluoracetamide/BSTFA, Carlo Erba/ and pyridine /freshly distilled and absolutized/ at room temperature, injected after 15 minutes.

Gas Chromatography

Fractovap 2300 AC gas chromatograph of Carlo Erba equipped with flame ionization detector was used in our work. The deriva-

tives / 1µl/ were chromatographed on a glass column /2mx3mm/ packed with OV-1 /3%/ on Gas Chrom Q /80-100 mesh/. The nitrogen flow rate was 25 ml/min, the oven temperature was programmed from 80 to 300°C at 10°C/min and kept at 300°C for 18 min. Both injector and detector temperatures were maintained at 275°C. For GC/MS investigations a Hewlett-Packard 5710A gas chromatograph with OV-101 fused-silica capillary column /25 m/ was used. The temperature of the column was programmed from 70 to 280°C at 6°C/min.

High Performance Liquid Chromatography

A Hewlett-Packard 1084B liquid chromatograph was used equipped with a HP-1030A variable wavelength UV detector and a Knauer prepacked analytical /120x4.6mm/ and guard /40x4.6mm/ column containing LiChrosorb RP-8 /5µm/.

Solvent A contained 0.01 mol/l phosphoric acid and potassium dihydrogen-phosphate in methanol-water /20:80 v/v/, while solvent B was methanol. The elution profile was: 0-10 min, 5-25%B /gradient/, 10-20 min, 25-65%B /gradient/, 20-21 min, 80%B /isocratic/, 21-22 min, 65-5%B /reverse gradient/ and 22-24.5 min, 5%B /isocratic for conditioning/. The temperature of the oven thermostat was set at 35°C. The flow rate was 1.50 ml/min and the column inlet pressure was 210-230 bar. The detector was set at 280 nm.

Novicardin samples of 1% solution in methanol were applied to the column by means of the autoinjector set at 20 µl. For quantitative determination imino-dibenzyl /product of Alkaloida, recrystallized twice/ was used as the internal standard /ISTD/ in concentration of 0.1 mg/ml. Calibration was carried out with a standard solution containing 0.12 mg/ml Naringin /5,4'-dihydroxy-7-/2-O-rhamnosido/glycosyloxy-flavanone, isolated at the Kossuth Lajos University, Debrecen/ and the ISTD. From the available authentic flavonoid standards methanolic solutions of 0.1-0.2 mg/ml were prepared.

For the isolation of the Novicardin components a Knauer preparative column /250x16mm/ was used. In order to remove the eluents more easily the phosphate buffer was substituted with

0.5% acetic acid and the flow rate and the sample volume were increased to 8 ml/min and 100 µl, respectively.

For the determination of the isocratic relative retention times /RRT/ of the isolated Novicardin components we used eluents with 10, 20, 40%B and 3,4,5-trimethoxybenzoic acid, cinnamic acid and 6-hydroxy flavone, respectively, as the internal standard.

To compare the relative retention times on normal phase a Hewlett-Packard prepacked analytical column /200x4.6mm/ of LiChrosorb Si-100 /5µm/ was used. Eluent A was n-hexane, while eluent B was n-hexane:methanol:chloroform:diethylamine 600:300:100:1. The flow rate was 1.5 ml/min and the concentration of eluent B was 100, 80 /ISTD=cinnamic acid/, 50 and 30% /ISTD=6-hydroxyflavone/.

Spectra of the Novicardin components and authentic flavonoid standards were scanned from 200 to 400 nm by a HP-79875A variable wavelength detector.

Mass Spectrometry

Electron impact mass spectra were obtained by a VG-7035 GC/MS/ Data System /VG Micromass/ at an ionization voltage of 70 eV and an ion source temperature 150°C.

RESULTS

According to literature data /3-5/ we expected the presence of flavonoids and their glycosides in Novicardin. Since some authors reported gas chromatographic investigations of tea flavanol /6,7/, catechins, flavones, flavanones and other phenolic compounds /8/ after their trimethylsilylation, our first choice was this method for the determination of the ratio of Novicardin components.

Figure 1 illustrates that by this way we could achieve the separation of more than 30 components.

Their ratio was nearly the same even if BSTFA + 1% TMCS and stronger conditions for the derivatization /15 hours at 80°C/ were applied. In spite of this we were afraid of hydro-

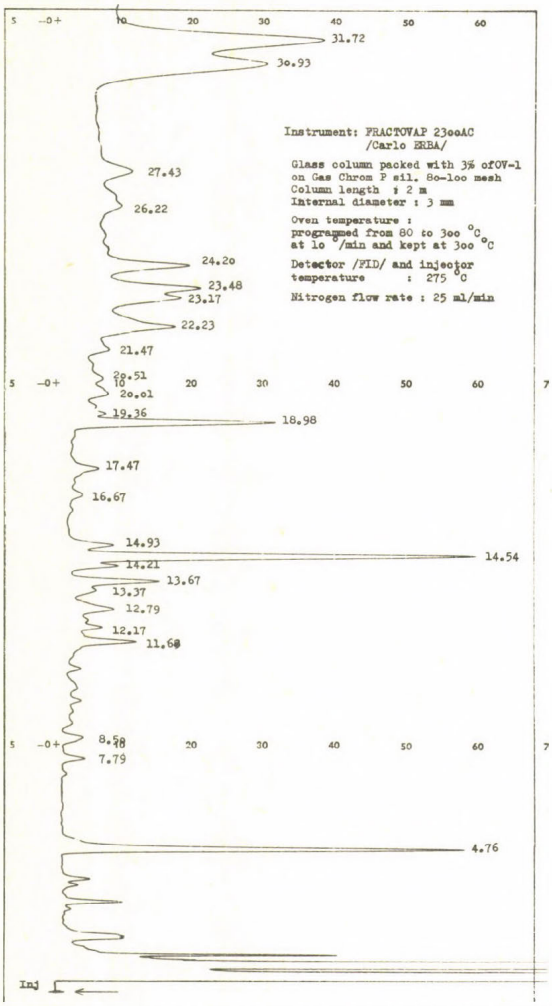


Figure 1. Gas chromatogram of trimethylsilylated Novicardin on glass column /2mx3mm/ packed with OV-1 /3%/ on Gas Chrom Q /80-100 mesh/. Linear temperature program from 80 to 300°C.

lysis of the glycosides and that additional transformation, e.g. enolization or ring-opening /9/ may occur. The identification of the peaks was proven by GC/MS investigations: the peaks of scan numbers 1103, 1136, 1405 on the total ion chromatogram of trimethylsilylated Novicardin seen in Figure 2 were identified according to their spectra as TMS derivatives of flavonoids transformed in such a way. Although we succeeded in identifying some aromatic-acidic components of Novicardin, we gave up the

application of gas chromatography for analytical purposes and for the identification of flavonoid constituents.

In 1974 Ward and Pelter /10/ published the first application of High-Performance Liquid Chromatography to flavonoid analysis and at present HPLC became the method of choice for their separation and quantification. There is no need for derivatization and when using the reverse-phase technique, there is no danger that some highly polar substance/s/ may be irreversibly retained /11/.

We have tried RP-8 and RP-18 columns, eluents with different pH values /0.01 M/l sodium hydrocarbonate, 0.15% acetic acid and phosphate buffers/ and methanol and acetonitrile as the organic modifier. The best results were obtained with acidic eluents. When suppressing the ionization of the acidic components, the retention and separability increased and the peak shapes proved to be correct.

Figure 3 illustrates that after the first unvaluable and unrecorded ballast peaks about 20 components can be separated from which the 15 most characteristic peaks were denoted with letters /Novi-A,.Novi-B, ... Novi-O/. For quantification we used iminodibenzyl as the internal standard and calibration was carried out with Naringin, which and its aglycone were isolated from different Prunus species /3,4,5/, and were available to us.

Table I contains the averaged relative retention times /RRT, calculated relative to the ISTD/, and the maximum and minimum of the analysed values /AMT/ of the 15 main components of the manufactured Novicardin substances. The same table lists the averaged values of the standard deviations of the analysed values calculated for parallel injections, weighings and repeated analyses at different dates /$\bar{s}1$, $\bar{s}2$, $\bar{s}3$, n=3/. The standard deviations of RRT, averaged for the 15 components, were 0.15, 0.15 and 0.58%, respectively.

For the identification of the components we isolated 12 on the preparative HPLC column in 0.1-1 mg quantities with a purity better than 95%. First we compared their relative retention time with those of the 100 authentic flavonoid standards and related compounds according to our analytical method. In order to determine the RRT values more exactly we have carried

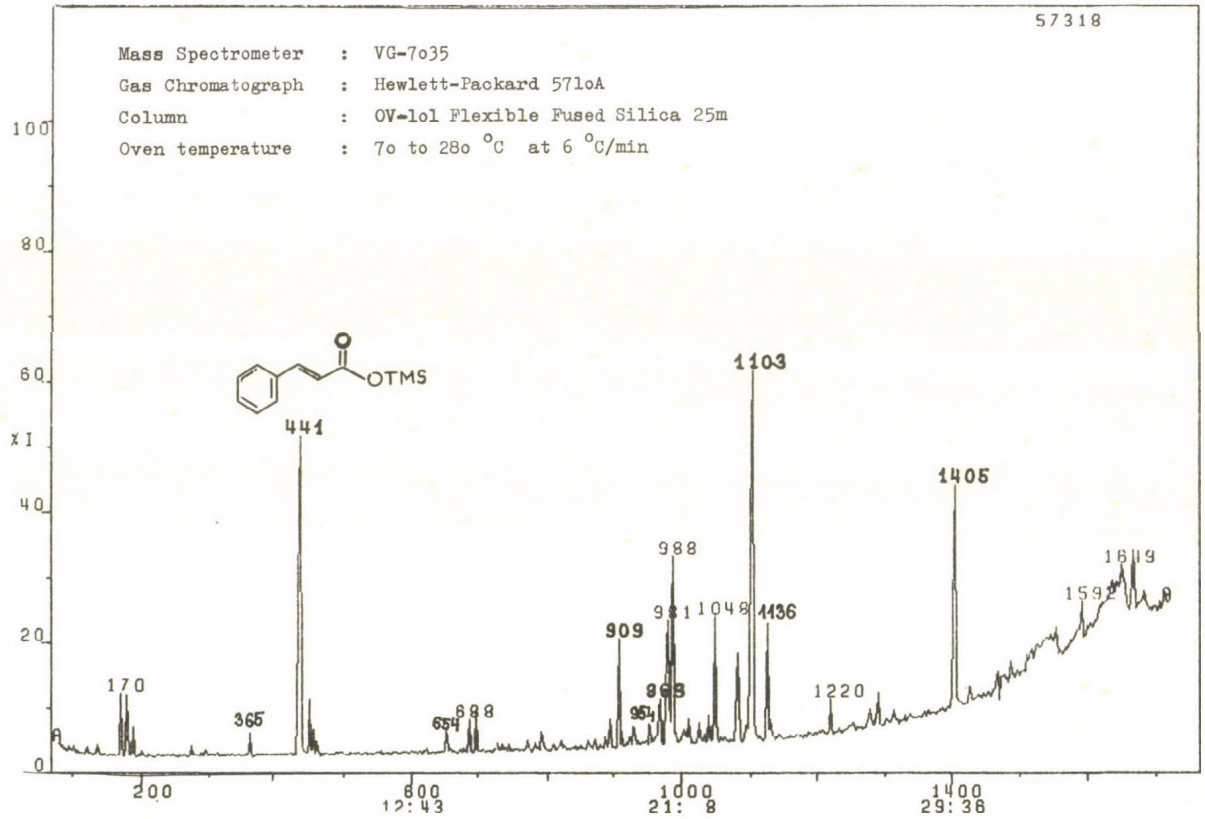
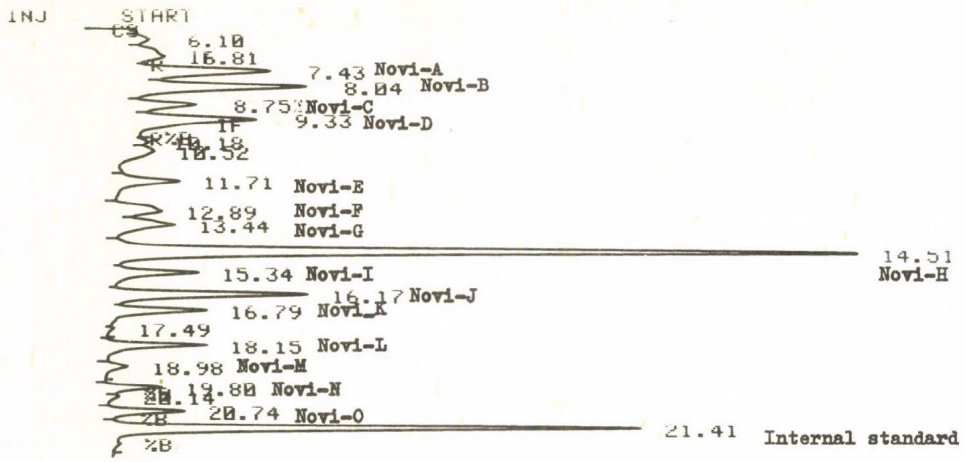


Figure 2. Total ion chromatogram of trimethylsilylated Novicardin on OV-101 fused silica capillary column. Peaks with scan numbers 365, 441, 654, 909, 954, 969 were identified according to their spectra as the TMS-derivative of benzoic acid, cinnamic acid, benzylalcohol, 4-hydroxycinnamic acid, ferrulic and caffeic acid, respectively.



BTL: 25 RUN # 1 Novicardin o8o583

ISTD

RT	EXP RT	AREA	CAL #		AMT %
6.81		55530			0.167
7.43		213500		A :	0.641
8.04		257200		B :	0.773
8.75		80220		C :	0.241
9.33		190400		D :	0.572
10.52		50010			0.150
11.71		99220		E :	0.298
12.89		95700		F :	0.287
13.44		126200		G :	0.379
14.51		975000		H :	2.929
15.34	15.37	123300		I :	0.370
16.17		245600		J :	0.738
16.79		129900		K :	0.390
18.15		114600		L :	0.344
18.98		28350		M :	0.085
19.80		58310		N :	0.175
20.74		80180		O :	0.241
21.41	21.42	566900	R	1	

DF: 9.6690 E+ 0
ISTD AMT: 1.0020 E- 1

Figure 3. HPLC analysis of Novicardin on a Knauer prepacked column /250x4.6 mm/ of LiChrosorb RP-18 /5µm/ with a guard column /40x4.6 mm/ of the same packing material. For the eluting system see Materials and Methods. Quantification of all peaks was carried out with the internal standard method /ISTD=imino-dibenzyl/ using the same response factor as determined for Naringin.

out the measurements under isocratic circumstances. In the last case we applied three different eluent compositions /%B=10,20 and 40/ and internal standards because of the different polarities of the individual components. We have found one or more

Table I. Quantitative HPLC analysis of Novicardin

Component	RRT /to ISTD/	AMT /w%/ Max/min	$\bar{s}1$ %	$\bar{s}2$ %	$\bar{s}3$ %
Novi-A	0.340	0.873/0.623	1.36	1.50	1.81
Novi-B	0.368	0.854/0.767	0.98	1.37	2.13
Novi-C	0.401	0.306/0.240	1.59	1.64	1.33
Novi-D	0.429	0.607/0.524	1.22	1.33	1.50
Novi-E	0.543	0.313/0.279	1.09	1.37	2.05
Novi-F	0.601	0.468/0.396	1.24	1.37	1.03
Novi-G	0.630	0.370/0.252	1.68	2.01	2.49
Novi-H	0.678	3.022/2.442	0.82	1.06	2.26
Novi-I	0.715	0.512/0.358	1.32	1.43	2.44
Novi-J	0.753	0.978/0.755	0.81	1.01	2.02
Novi-K	0.783	0.393/0.352	1.26	1.56	2.76
Novi-L	0.847	0.577/0.303	1.01	1.25	2.53
Novi-M	0.886	0.195/0.075	2.60	0.88	3.54
Novi-N	0.923	0.243/0.178	1.50	0.88	2.65
Novi-O	0.965	0.268/0.228	1.37	1.41	2.43
ΣNovi A-O	-	9.119/8.643	1.03	0.81	1.53
Averaged for A to O	-	-	1.32	1.34	2.20

standards behaving similarly in the case of seven Novicardin components.

After repeating their examination on normal phase there were only three components showing the same retention as that of the standards. For the same peaks we also investigated the UV spectra scanned by the HP-79875 variable wavelength detector: only Novi-I was behaving similarly to Naringin, and Naringenin and Novi-M to Sakuranetin /Table II/. To corroborate these results and to identify the other components we turned to mass spectrometry.

The direct mass spectrometry of the available flavonoid glycoside standards showed that since their volatility and stability were not satisfactory, we can only obtain information on the aglycones. From the electron impact mass spectra of the

12 isolated Novicardin components we could solve the fragmentation and thus identify the structure of Novi-A,B,H,I,J,M and O. The results are given in Table III. In this way the HPLC/UV identification of Novi-I and Novi-M was supported.

The identified aglycones are known flavonoids. The mass spectra of the main component indicated as Novi-H and the solved fragmentation pattern can be seen in Figures 4 and 5.

DISCUSSION AND CONCLUSION

We have developed an HPLC method to separate the components of Novicardin and to determine their relative quantities. This method permits the comparison of the products extracted from cherry peduncle of different origin and the establishment of the quality requirements, which is one of the main conditions for introducing the Novicardin dragee into circulation. The comparative examinations will be carried out in the second half of this year.

We have isolated by a preparative HPLC column twelve Novicardin components but found only two which had the same retention on both reversed and normal phase and the same UV characteristics as one of the authentic flavonoid standards available for us.

Their HPLC/UV identification was confirmed with direct mass spectrometric method. We can identify the aglycone by the same way in the case of five other Novicardin components. These proved to be flavanones /Dihydrowogonin, Pinobanksin, Dyhidro-öroxylin, Naringenin, Sakuranetin/, a flavone /Prunetin/ and an isoflavone /Chrysin/ which are known in literature.

We are planning to identify their glycosides with mass spectrometric examination following permethylation and per-deuteromethylation.

Table II. Retention and UV spectral data of isolated Novicardin components together with those of authentic standard flavonoids and related compounds characterized by the similar retention on a reverse phase column

Substance	Relative Retention Time[+] Reverse Phase Gradient	Reverse Phase Isocratic	Normal Phase Isocratic	Spectral Data λMax/Min
Novi-A	0.340	0.511/10/	> 2 /100/	290/260
7-Hydroxycumarine	0.336	0.515/10/	0.840/100/	
Novi-B	0.368	0.592/10/		284/254
Novi-D	0.429	0.794/10/		288/254
Novi-E	0.543	0.521/20/	1.144/80/	
4',7-Dimethoxy isoflavone	0.536	0.517/20/	1.607/80/	
3,4,5-Trimethoxy-benzoic acid		0.502/20/	1.052/80/	
Novi-G	0.630	0.752/20/	1.217/80/	
3,4,5-Trimethoxy-cinnamic acid	0.627	0.778/20/	1.316/80/	
Fisetin/1/	0.634	0.769/20/	3.5 /80/	
Novi-H	0.678	0.352/40/		288/254
Novi-I	0.715	0.415/40/	2.650/50/	292/252
Naringin /2/	0.716	0.415/40/	2.664/50/	292/252
Naringenin /3/	0.733	0.418/40/	2.645/50/	292/252
Floretin /4/	0.735	0.418/40/	3.210/50	
Novi-J	0.753	0.452/40/	2.882/50/	262/236
Cinnamic acid		0.457/40/	4.4/50/	
Novi-L	0.847	0.753/40/		292/256
Novi-M	0.886	0.948/40/	1.533/30/	292/252
Sakuranetin /5/		0.947/40/	1.554/30/	292/252
Novi-N	0.923	1.199/40/		264/240
Novi-O	0.965	1.606/40/	0.567/30/	270/240
7-Methoxy flavanone	0.967	1.641/40/	0.560/30/	278/250
Chrysin /6/	0.963	1.578/40/	1.661/30/	
7-Methoxy-3',4-methylenedioxy flavanone	0.966	1.606/40/	0.676/30/	
4',7-Dimethoxy-flavanone	0.967	1.647/40/	0.639/30/	

[+]See Chromatographic parameters in Materials and Methods. For isocratic elution %B is in parentheses. The internal standard was imino-dibenzyl for gradient elution /RT= =21.41±0.05/, 3,4,5-trimethoxy benzoic acid for reversed phase %B=10 /RT=13.35±0.04/, cinnamic acid for reversed

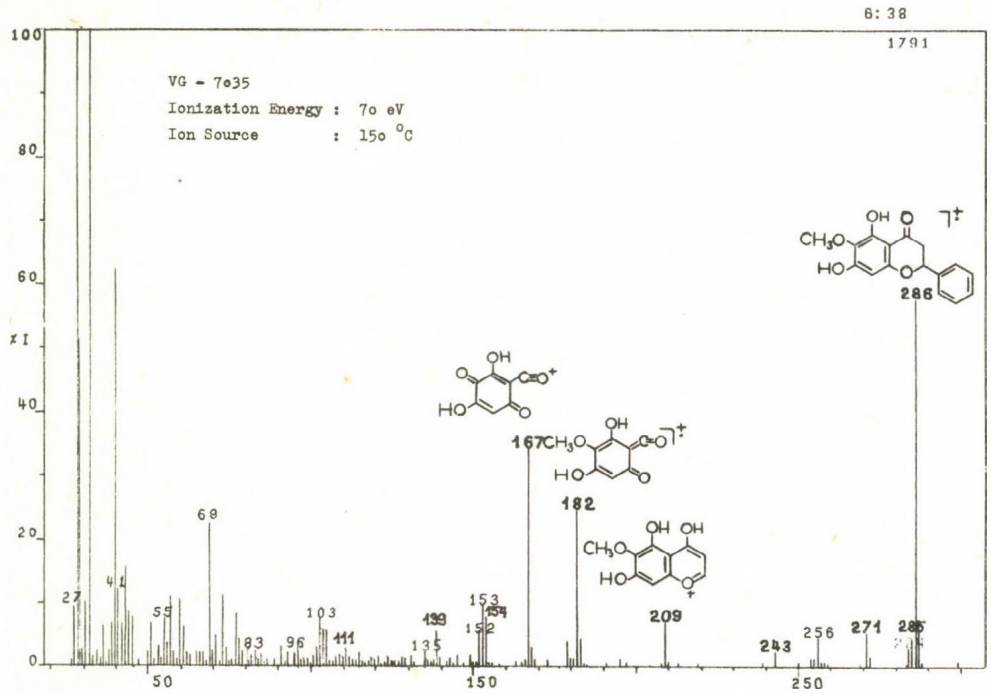


Figure 4. Electron impact mass spectra of Novi-H.
Ionization voltage 70 eV, ion source temperature 150°C.

phase %B=20 /RT=13.24±0.07/ and for normal phase %B=100 and 80% /RT=3.00 and 4.32/, 6-hydroxy flavone for reversed phase %B=40 /RT=9.00±0.07/ and for normal phase %B=50 and 30% /RT=3.42 and 5.48/.

/1/ 3,7,3',4'-Tetrahydroxy flavone, /2/ 5,4'-Dihydroxy-7-/2-O-rhamnosido/glycosyloxy flavanone, /3/ 5,7,4'-Trihydroxy flavanone, /4/ 4,2',4',6'-Tetrahydroxydihydrochalcone, /5/ 5,4'-Dihydroxy-7-methoxy flavone, /6/ 5,7-Dihydroxy flavone

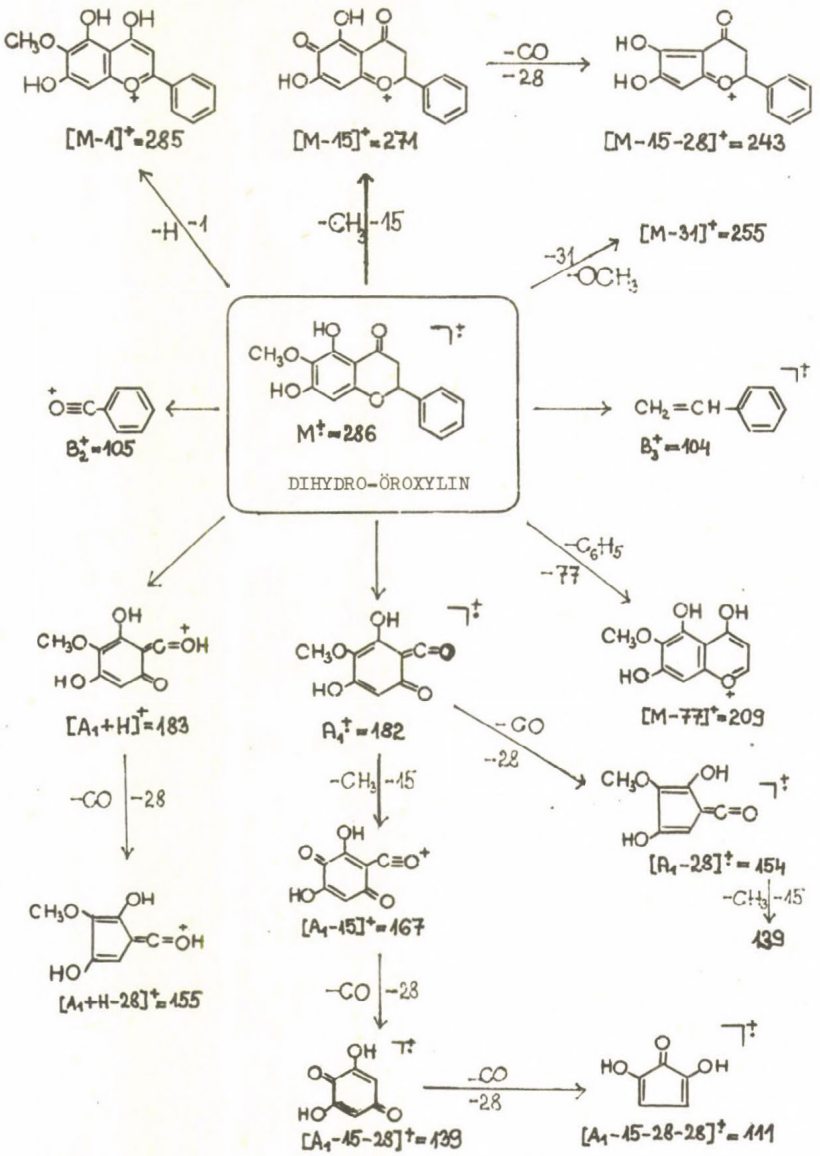
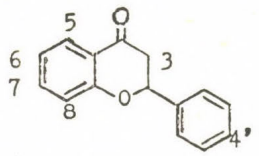
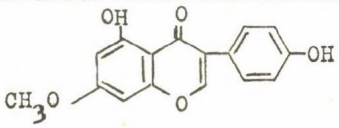
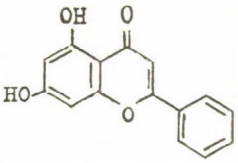


Figure 5. Fragmentation of Dihydroöroxylin, aglycone of Novi-H

Table III. Mass Spectrometric Identification of Novicardin Components

Novicardin component	Structure of aglycone determined by Mass Spectrometry
	Flavanones 
Novi-A	5,7-Dihydroxy-8-methoxyflavanone Dihydrowogonin
Novi-B	3,5,7-Trihydroxyflavanone Pinobanksin
Novi-H	5,7-Dihydroxy-6-methoxyflavanone Dihydroöroxylin
Novi-I	4′,5,7-Trihydroxyflavanone Naringenin
Novi-M	4′,5-Dihydroxy-7-methoxyflavanone Sakuranetin
Novi-J	 4′,5-Dihydroxy-7-methoxyisoflavone Prunetin
Novi-O	 5,7-Dihydroxyflavone Chrysin

REFERENCES

/1/ L. Králik, Hung. Pat. 150.217 /1960/

/2/ L. Frank, Report on the pharmacological trials of Novicardin /Alkaloida Chemical Works, 1983/

/3/ M. Hasegawa, T. Shirato, J. Am. Chem. Soc. 74, 6114 /1952/

/4/ M. Hasegawa, J. Am. Chem. Soc. 76, 5560 /1954/

/5/ M. Hasegawa, J. Am. Chem. Soc. 79, 1738 /1957/

/6/ A. R. Pierce, H. N. Graham, S. Glassner, H. Madlin, J. G. Gonzalez, Anal. Chem. 41, 298 /1969/

/7/ P. D. Collier, R. Mallows, J. Chromatogr. 57, 29 /1971/

/8/ F. Dravert, G. Leupold, Chromatographia 9, 605 /1976/

/9/ M. D. Woodward, Phytochemistry, 21, 1403 /1982/

/10/ R.S. Ward and A. Pelter, J. Chromatogr. Sci. 12, 571 /1974/

/11/ K. V. Casteele, C. F. van Sumere, J. Chromatogr., 240, 81 /1982/

Chromatography, the State of the Art
H. Kalász and L.S. Ettre (Eds)

APPLICATION OF MICRO-HPLC FOR THE DETERMINATION OF THERAPEUTIC LEVELS OF DRUGS

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SUMMARY

A micro high-performance liquid chromatographic precolumn method has been developed, which determines an antiatherosclerotic drug(CH123) and its metabolite (MZ672) at therapeutic levels from directly injected human serum without any sample pretreatment. Internal standard was used to eliminate sample size fluctuation.

INTRODUCTION

Considerable interest has recently been focused in high-performance liquid chromatography on column miniaturization (1-7). The advantages of micro-HPLC are: the running cost is low because of small solvent and packing material consumption; the low flow rate should facilitate direct interfacing to other special detectors (e.g. mass-spectrometer) (2,8); the detection limit can be reduced (9); and the speed of separation can be increased (3,10).

The first micro instrument was the FAMILIC-100, of JASCO which has been available for a few years. The column of this instrument is made of a teflon tube with 0.5 mm inner diameter. As peak-broadening in the column is small, micro-LC is specially suitable for trace analysis (9,11-13).

A micro HPLC method has been developed, which determines therapeutic levels of an antiatherosclerotic drug (CH123) and

its metabolite (MZ672) from human serum. In the development of methods using liquid chromatography for the analysis of biological samples, one of the most time-consuming steps which introduces considerable sources of error is sample pretreatment prior to injection. We have adopted a micro precolumn sample enrichment technique (11) for the direct injection of body fluids. S353 was used as the internal standard in order to eliminate sample-size fluctuation.

MATERIALS AND METHODS

Instrument and columns

The FAMILIC-10ON micro HPLC instrument (JASCO, Japan) was used in our work. The instrument utilizes a syringe-type pump and the flow rates can be selected in the range of 1 to 29 µl/min. In addition to a regular UV detector, the UVIDEC-II detector of JASCO can also be utilized. Here, the quartz capillary cell with a volume of 0.3 µl is fixed on the micro-cell casette. Sample injection is carried out with a 0.3 µl loop.

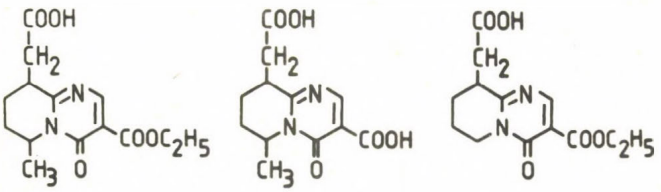
The analytical micro column consisting of a teflon tube, 14 cm x 0.5 mm i.d. was packed by the slurry technique as described earlier (1) with Zorbax C8 (DuPont). The micro precolumn for enrichment was prepared by packing LiChrosorb C8 (10 µm, Merck) in a teflon tube, 2 cm x 0.5 mm i.d. The micro precolumn was connected to the analytical column with 10 mm x 0.35 mm i.d. stainless-steel tubing.

Reagents

Analytical reagent-grade chemicals were used without any further purification. All solutions were prepared from distilled and deionized water.

The components used in the test mixture for the investigation of column performance were in the order of their elution: benzene, naphthalene, and anthracene. Acetonitrile/water (70/30 v/v) was used as the mobile phase.

For standard samples, CH123, MZ672 and the internal standard S353 (from CHINOIN) were dissolved in a 0.05M acetate buffer (pH=3).



CH 123 MZ 672 S 353

Formulae

The mobile phase used for the analysis was 0.05M acetate buffer (pH=3) containing 17% methanol as the organic modifier. The serum was adjusted to pH=3 with 2M HCl solution.

Sample injection into the precolumn

The micro precolumn was washed with 25 µl buffer solution prior to sample injection. A 25 µl aliquot of the human serum was injected into the precolumn for about 5 min with a syringe by hand and then the precolumn was washed with 25 µl of the buffer solution and was connected to the analytical column.

RESULT AND DISCUSSION

Column performance

Fig. 1 shows a typical test chromatogram on the micro column packed with Zorbax C8. The van Deemter curves (H vs. u) are shown in Fig. 2; the plate height is smaller at lower flow rates. However, a low flow rate would greatly increase the analysis time; therefore, the selected flow rate is typically about 10 µl/min.

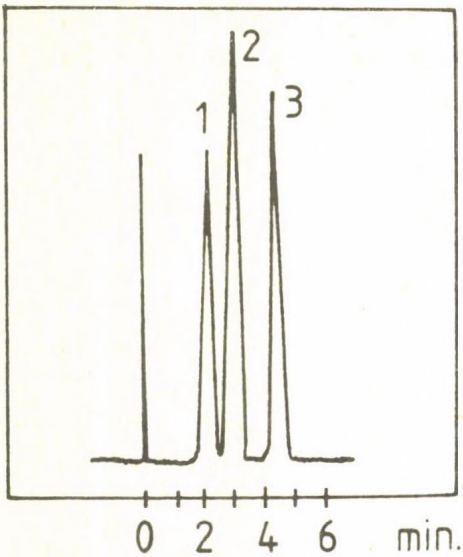


Fig. 1. Test chromatogram on a 14 cm x 0.5 mm i.d. micro column packed with Zorbax C8. Test mixture: 1 benzene (k'_1=1.33); 2 naphthalene (k'_2=2.15); 3 anthracene (k'_3=3.78). Mobile phase: acetonitrile/water (70/30). Detection: UV,254 nm. Flow rate: 20 µl/min.

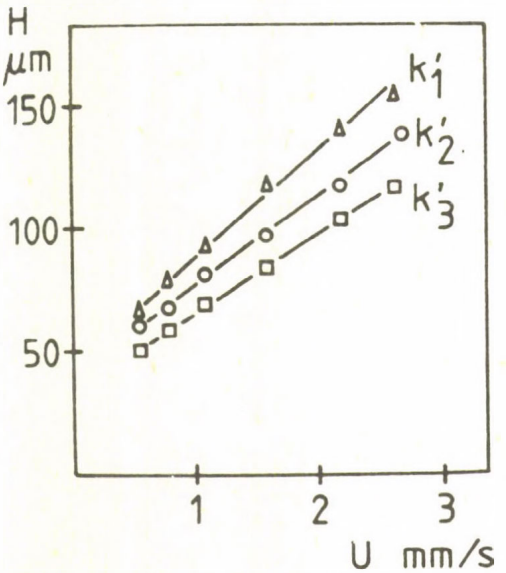


Fig. 2. Plots of HETP(H) vs. linear velocity (u) for the three test substances. For conditions (except flow rate) see Fig. 1.

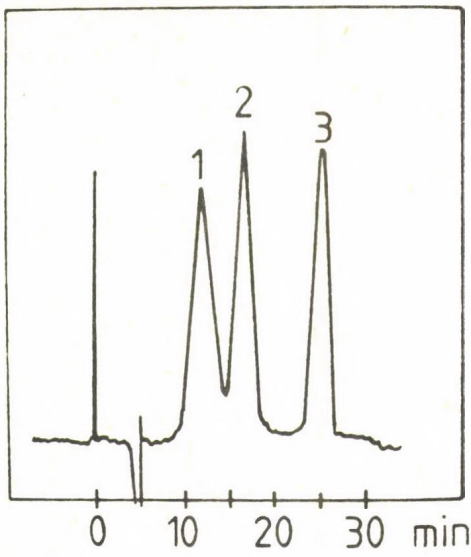


Fig. 3. Chromatogram of a standard solution. Mobile phase: 0.05 M acetate buffer (pH=3) containing 17% methanol. Detection: UV, at 302 nm. Mobile phase flow rate: 7 µl/min. Sample components present: 1 MZ672 (40 ng), 2 S353 (30 ng), 3 CH123 (40 ng). Sample volume: 0.03 µl.

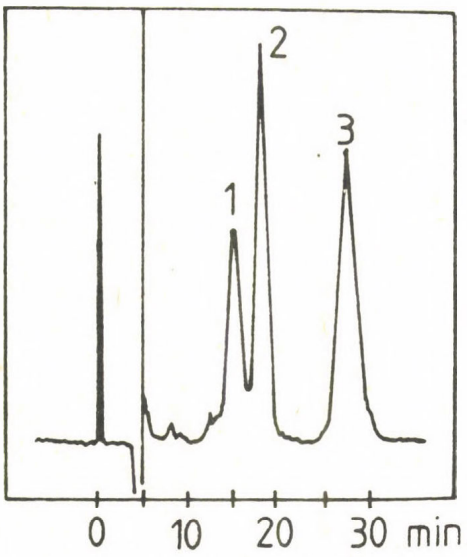


Fig. 4. Chromatogram of 25 µl of human serum injected into the micro precolumn. To the 25 µl of human serum 50 ng of each of 1 MZ672, 2 S353, and 3 CH123 were added. Conditions the same as in Fig. 3.

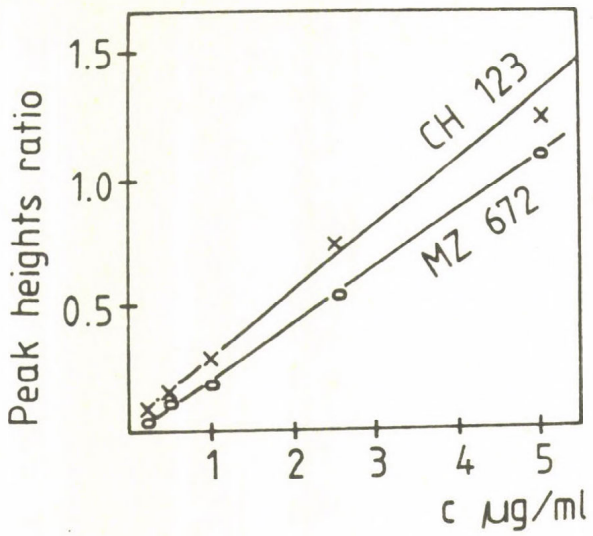


Fig. 5. Calibration curves for CH123 and MZ672. 2.5 µg/ml S353 was used as the internal standard.

Determination of CH123 and MZ672 with the internal standard method

Separation of the standard solution containing CH123, MZ672 and the internal standard S353 is shown in Fig. 3. The standard solution was injected with the 0.3 µl loop injector.

A typical chromatogram of directly injected human serum, to which CH123, MZ672 and S353 were added, is shown in Fig. 4. In the procedure of sample enrichment with the micro precolumn 2.5 µg/ml S353 was used as the internal standard in order to eliminate sample-size fluctuation.

The calibration curves were found to be linear up to the ca. 100 ng injected amounts (Fig. 5). The detection limit is 0.2 µg/ml of CH123 and 0.3 µg/ml of MZ672. This could be improved by the injection of 100 µl serum.

CONCLUSIONS

Micro HPLC with a micro precolumn is a very suitable technique for the determination of therapeutic levels of drugs in human serum, without any sample pretreatment.

REFERENCES

1. D. Ishii, K. Asai, K. Hibi, T. Jonokuchi and M. Nagaya, J. Chromatogr. 144 (1977) 157.
2. T. Takeuchi, K. Hirata and Y. Okumura, Anal. Chem. 50 (1978) 659.
3. R. P. W. Scott and P. Kucera, J. Chromatogr. 169 51 (1979).
4. R. P. W. Scott and C. F. Simpson, J. Chromatogr. Sci. 20 62 (1982).
5. T. Tsuda and M. Nocorny, Anal. Chem. 50 (1978) 1807.
6. T. Takeuchi and D. Ishii, J. Chromatogr. 190 150 (1980).
7. F.J. Yang, J. Chromatogr. Sci. 20 241 (1982).
8. C. Eckers and C.S. Skrabala, Clin. Chem. 28 1882 (1982).
9. R. P. W. Scott and P. Kucera, J. Chromatogr. 185 27 (1979).
10. F.J. Yang, J. High Resolut. Chromatogr. Chromatogr. Commun. 4 83 (1981).
11. D. Ishii, K. Hibi, K. Asai and M. Nagaya, J. Chromatogr. 156 173 (1978).
12. M. Goto, T. Nakamura and D. Ishii, J. Chromatogr. 226 33 (1981).
13. T. Takeuchi, S. Saito and D. Ishii, J. Chromatogr. 258 125 (1983).

Chromatography, the State of the Art
H. Kalász and L.S. Ettre (Eds)

OPTIMIZATION OF CHROMATOGRAPHIC SEPARATIONS IN THE PHARMACEUTICAL ANALYSIS

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INTRODUCTION

Chromatography became one of the most important methods in pharmaceutical analysis. The many different types of analytical tasks and objects, however, make the use of uniform chromatographic systems impossible: the applicability of a chromatographic system is defined by the character of the analytical problem. For this reason it is very important that the advantages and disadvantages of the method used should be known furthermore, that the capability of a particular method, the analytical objects and claims should be synchronized.

In our paper we shall introduce the screening and control system developed out on the basis of our recent experience. The selection of the most suitable chromatographic procedure is based on the data obtained through a series of different screening and control steps considering the physico-chemical properties /such as the molecular weight, volatility, solubility, presence of ionizable functional groups, detectability etc./ of the compounds investigated.

The screening steps contain the recommended phase systems. In the case of GC-methods stationary phases having different polarities are used, while in the cases of TLC and HPLC methods the separations are performed only on a limited number of stationary phases. The composition of the mobile phase has been varied to increase the selectivity,

efficiency and sensitivity of the separations. In the control steps the results and chromatographic data obtained in the screening steps are evaluated by system suitability data and estimation.

The estimation serves to decide:

- which chromatographic technique should be selected for the next screening step comparing the results obtained by different techniques;
- possible degradation during chromatography or in solution should be identified in the chromatograms;
- whether the elution order obtained by any technique or method is favourable or not, considering the different analytical objects;
- which elution mode should be selected for solving the given analytical tasks, etc.

On the whole, those considerations which cannot be calculated are given by estimation.

The system used for the selection of the most suitable chromatographic method can be divided into the following steps;

- characterization of the compounds investigated on the basis of pre-investigation data;
- first screening step using recommended phase systems;
- first control step to evaluate the data of the first screening step, and to select the further directions of research, as well as the most suitable chromatographic methods;
- second screening step using new recommended phase systems and optimizing the recent ones;
- second control step to evaluate the data of the second screening step, to select the most suitable systems and to decide the possible necessity of using special chromatographic techniques;
- third screening step to finish the optimization of the recent systems including sample preparation, derivatization procedure etc.; furthermore to study the chromatographic

behaviour of the compounds investigated by using special chromatographic techniques such as separation on glass capillary columns in GC, ionpair and molecular complexation chromatography in HPLC and overpressurized thin-layer chromatography in TLC.

- third control step to evaluate the data of the third screening step, to select the most suitable systems and compare them with the recent ones;
- final step to select the method to be used for solving the given analytical problem and for controlling the results obtained.

It has to be noted that in our paper only the general considerations and conclusions are discussed. The results obtained by using this screening and control system have been recently published /see references 1-6 for ergot alkaloids and references 6-11 for eburnane alkaloids/; for this reason, details on the methods are not given here.

DISCUSSION

Characterization of the compounds investigated on the basis of pre-investigation data

For the selection of the starting chromatographic conditions /first screening/the following non-chromatographic data are required which can be obtained from the literature:

- structure of the compounds investigated including possible isomerism and molecular weights;
- volatility of the compounds, data on thermal stability /possibly given by TGA analysis/;
- solubility or polarity data;
- presence or absence of ionizable functional groups;
- detectability data given by UV spectrophotometric, fluorimetric or electrochemical measurements;
- chromatographic data found in the literature;
- stability data;

- presence or absence of functional groups suitable for derivatization.

Table I collects the data on the characterization of the compounds from such points of view.

Table II presents the summary of pre-investigation data.

On the basis of the data collected in Table II, ergot and eburnane alkaloids can be characterized as follows:

ergot alkaloids: GC-B/C-I_1-S_2-D_1-TLC-BD/BA-b_{2x}-U_{11}/F_1-I_{1-3}-$S_{2,3}$-D_2-LC-BD/BA-b_{2x}-U_{11}/F_1-$I_{1,2}$-$S_{2,3}$-D_4

eburnane alkaloids: GC-C/D-I_1-$S_{1,2}$-D_1-TLC-DB/BD-b_{2x}-U_{11}-$I_{1,2}$-S_1-D_3-LC-DB/BD-b_{2x}-U_{11}-I_1-S_1-D_4

First screening step.

The recommended GC and TLC systems are collected in Table III and the recommended HPLC systems in Table IV

In the case of GC and TLC systems the most generally applicable stationary phases and mobile phase compositions have been selected.

In the case of HPLC systems the first screening contains two elution modes differing in selectivity /separation on silica and octadecyl-silica/; in reversed-phase chromatography "ion-supression" has been used to exclude the ionization of the compounds during chromatography. As it can be seen in Table IV the solubility and polarity of the compounds have a great importance when the eluent strength is chosen using the polarity data of Snyder et al. /12-14/ for the calculation.

The strongest recommended eluent has been used first on both silica and octadecyl silica in order to avoid the long retention of any compound in the model mixture.

Table I.

Characterization of the compounds on the basis of pre-investigation data

I. Molecular weight - volatility

GC-A	GC investigation of the compounds is impractical or impossible
GC-B	GC investigation of the compounds is questionable
GC-C	The compounds can possibly be investigated after derivatization
GC-D	The compounds can possibly be investigated without derivatization

II. Solubility - polarity

/Solubility requirement: the solvent should dissolve the compounds in a concentration of 0.1 % or more/

AA	The compounds can be dissolved	only in water
AB-BA	"	in water and methanol
BB	"	only in methanol
AC-CA	"	in water and acetonitrile
BC-CB	"	in methanol and acetonitrile
CC	"	only in acetonitrile
BD-DB	"	in methanol and chloroform
CD-DC	"	in acetonitrile and chloroform
DD	"	only in chloroform
DE-ED	"	in chloroform and hexane
EE	"	only in hexane
FF	the compounds cannot be dissolved in these solvents	

Notes: the first letter refers to the better solubility, e.g. BD means that the compounds can be dissolved better in methanol than in chloroform but 0.1 % concentration solution can be prepared with both solvents. If the second solvent is marked with an asterisk it means that 0.1 % solution cannot be prepared with this solvent, but the compounds are well soluble in a 1:1 mixture of the first and second solvent. All solubility data would be measured for the non-ionized forms /free base or acid form/ and "S"-mark is added if the acid, "B" when the base form can be used for the dissolution.

III. Presence of ionizable functional group.

	pK-values
s_1	$<10^{-2}$
s_2	10^{-2} - 10^{-5}
s_3	$>10^{-5}$
b_1	$<10^{-2}$
b_2	10^{-2} - 10^{-5}
b_3	$>10^{-5}$

Notes: if the strength of the acid or base is unknown the functional group is marked with the subscript "x" /s_x or b_x/; if the molecule contains more than one acidic or basic functional group the strength of the first /strongest/ is marked and the other is marked with an "x", independently of the number of the groups /b_{2x} or s_{2x}/; if the molecule contains both acidic and basic functional groups the indication is $s_x b_x$ or $b_x s_x$ depending on the relative strengths of the acidic or basic functional groups.

IV. Detactability.

U_{11} the compounds can be well detected by a UV detector /$\varepsilon > 10000$/; the selectivity can be increased by changing the detection wavelength

U_{12} the compounds can be well detected by a UV detector; the selectivity cannot be increased by changing the detection wavelength

U_{21} detectability of the compounds by a UV detector is moderate /$\varepsilon \simeq 1000$ - 10000/; the selectivity can be increased by changing the detection wavelength

U_{22} detectability of the compounds by a UV detector is moderate; the selectivity cannot be increased by changing the detection wavelength

U_3 detectability of the compounds by a UV detector is bad /$\varepsilon < 1000$/

F_1 the compounds can be detected by a fluorimetric detector; the selectivity can be increased as compared to the UV detector

F_2 the compounds can be detected by a fluorimetric detector; the selectivity cannot be increased as compared to a UV detector

E_1 the compounds can be detected by an electrochemical detector; the selectivity can be increased as compared to a UV detector

E_2 the compounds can be detected by an electrochemical detector; the selectivity cannot be increased as compared to UV detector

T the compounds cannot be detected with these detectors

V. Literature data

I_1 no data are found in the literature

I_2 literature data are available, but these are considered to be unapplicable

I_3 the method found in the literature can be used directly or after modification

VI. Stability of the compounds

S_1 the compounds are stable during the investigation

S_2 the stability of the compounds is questionable; possible degradation can occur during chromatography

S_3 the stability of the compounds is questionable; possible degradation can occur in solution

S_4 the instability of the compounds is well known

VII. Derivatization

D_1 by derivatization the chromatographic properties of the compounds can be improved

D_2 by derivatization the selectivity of the separation can be increased

D_3 by derivatization the detectability of the separated compounds can be improved

D_4 no functional group exists which is suitable for derivatization

Note: if derivatization can improve more than one chromatographic parameter it is indicated by a combination of numbers.

Table II

Summary of the used pre-investigation data

Method	Pre-investigation I	II	III	IV	V	VI	VII
GC	A B C D	-	-	-	I_1 I_2 I_3	S_1 S_2 S_3 S_4	D_1 D_2 D_3 D_4
TLC	-	AA AB-BA BB AC-CA BC-CB CC BD-DB CD-DC DD DE-ED EE FF	s_1 s_2 s_3 b_1 b_2 b_3	U_{11} U_{12} U_{21} U_{22} U_3 F_1 F_2 T	I_1 I_2 I_3	S_1 S_2 S_3 S_4	D_1 D_2 D_3 D_4
HPLC	-	AA AB-BA BB AC-CA BC-CB CC BD-DB CD-DC DD DE-ED EE FF	s_1 s_2 s_3 b_1 b_2 b_3	U_{11} U_{12} U_{21} U_{22} U_3 F_1 F_2 E_1 E_2 T	I_1 I_2 I_2	S_1 S_2 S_3 S_4	D_1 D_2 D_3 D_4

Table III

GC and TLC systems recommended in the first screening

I GC

System	Stationary phase	McReynolds constant
A	Carbowax 20M	2308
B	AN-600	1793
C	Dexsil 410	952
D	Dexsil 300	474
E	OV-101	229
L	found in the literature	

II TLC

Chromatoplate: Kieselgel 60 F_{254} /Merck, Darmstadt, FRG/

System	Eluent composition
A	n-butanol - acetic acid - water
B	isooctane - ethyl acetate - acetic acid
C	chloroform - acetone - diethylamine
D	chloroform - methanol - cc. ammonia
E	benzene - chloroform - methanol
F	chloroform - methanol /ammonia vapour if needed/
L	search in the literature

Table IV

HPLC systems recommended in the first screening

	Solubility	Presence of ionizable functional group	Estimated polarity of the eluent on C-18	Estimated polarity of the eluent on silica
1	AA	-	0 - 0.6	aqueous eluent
		s	pH 2-3	with small amount
		b	pH 7-8.5	of organic modifier
2	AB-BA	-	0.4 - 0.8	>4.0
		s	pH 2-3	+ acetic acid
		b	pH 7-8.5	-
3	AC-CA	-	0.7-1.1	3.3-4.0
		s	pH 2-3	+ acetic acid
		b	pH 7-8.5	-
4	BB, CC,	-	0.9-1.3	3.0-3.5
		s	pH 2-3	+ acetic acid
		b	pH 7-8.5	-
5	BD, DB	-	1.2-1.8	2.0-3.1
		s	pH 2-3	+ acetic acid
		b	pH 7-8.5	-
6	DD	-	1.6-2.1	1.5-2.2
		s	pH 2-3	+ acetic acid
		b	pH 7-8.5	-
7	DE-ED	-	1.9-2.4	1.0-1.7
		s	pH 2-2	+ acetic acid
		b	pH 7-8.5	-
8	EE	-	>2.4	0.5-1.2

First control step.

In this step the results obtained in the first screening are evaluated. Based on these data, one has to decide whether all chromatographic techniques should be optimized or a limited number of techniques can be selected for the next steps. The results of the first screening are tabulated containing all important chromatographic data /capacity factors and their dependences on the temperature - program rate and solvent strengths; selectivity and its dependence on the eluent composition, resolution, peak asymmetry, order of elution etc./ The capability of the systems is estimated, involving the identification of possible degradation, comparison of different phase systems and elution modes, clarification of a possible derivatization procedure and its advantage for obtaining better peak shape, selectivity and sensitivity as well as to decide which way should the elution order or selectivity be changed.

On the basis of the tabulated data /"T-I"/ the most suitable methods are selected /OGC-I, OTLC-I and OLC-I/, and the most important improvements decided.

Second screening step.

The second screening step has a double object: the possible improvement of chromatographic systems used previously in order to increase the selectivity and efficiency of the separation, and if needed, the selection of new stationary phases and new phase systems.

Table V presents the recommended GC, TLC and HPLC experiments.

When octyl-silica stationary phase is used in the HPLC investigations, the values of the recommended eluent polarity have to be modified as indicated in Table VI.

In the case of TLC and HPLC experiments the solvents

Table V

Investigations during the second screening step.

I GC

System	Stationary phase	McReynolds constant
F	Silar 10C	3682
G	FFAP	2546
H	OV-225	1949
I	Dexsil 400	587
J	OV-1	222

II TLC

Chromatoplate: Polygram Sil GF_{254} /Macherey Nagel, G.F.R./

System	Eluent composition
G	Acetonitrile - acidic buffer solution
H	Methanol - acidic buffer solution
I	Acetone - ethanol - 0.1 M aqueous ammonium carbonate

Chromatoplate: HPTLC - Kieselgel

Eluents: A - F

III HPLC

Silica	C-18	Nitrile	C-8
/A/ to clarify the dependence of the selectivity on the types of organic modifiers, based on Snyder's triangle-theory		the same experiments are carried out as in the first screening performed on silica and C-18,	
/B/ Investigation of pH and salt concentration used in the eluent on C-18 phase			

used as the components of the eluent are divided into four groups; nonpolar, polar and moderator solvents /see reference 7/ and highly polar solvents /pyridine, acetic acid and water/.

On silica the moderator solvent was first varied, then the optimum solvent composition established and the polar and nonpolar organic modifiers were changed if necessary to select the best one.

On octadecyl-silica, in accordance with literature data /14-17/, the eluent composition resulting in optimum k'-values was selected and the change in selectivity studied using different types of solvents according to Snyder's triangle theory. In every case have clarified the dependence of the capacity factors on the concentration of each individual organic modifier in order to establish the possible change in the elution order which is coused by the significant difference in the dependence of the capacity ratios of the individual compounds on the eluent polarity /which is independent of the type of organic modifier/ and is due to the different selectivities of the solvent used /depending on the type of organic modifier/.

At this stage of the investigations, the effect of the pH of the eluent on the selectivity and efficiency of the separation is also studied.

Second control step.

In this step the results obtained in the second screening step are evaluated. The most important decisions made in this step are:

- establish whether we have all the methods needed for solving the given analytical tasks;
- which non-chromatographic analytical experiments can be optimized /clean-up, sample preparation, derivatization reaction etc./;

Table VI

Estimated polarity of the eluent on C-8 phase

	Solubility	Estimated polarity of the eluent
1	AA	0 - 0.4
2	AB-BA	0.3 - 0.6
3	AC-CA	0.5 - 0.9
4	BB, CC, BC, CB	0.7 - 1.1
5	BD, CD	1.0 - 1.5
6	DB, DC	1.3 - 1.7
7	DD	1.5 - 1.9
8	DE-ED	1.8 - 2.4
9	EE	>2.4

Table VII

GC, TLC and HPLC investigations recommended in the third screening

I GC.

/A/ Optimization of the previously investigated chromatographic systems:

- determination of the optimum liquid stationary phase loading
- optimum temperature or temperature program
- flow rate
- if derivatization is required it is studied in detail
- detection limit and sample capacity are determined
- optimization of the sample preparation procedure

/B/ The separations are performed on a glass capillary column similar in polarity to the best packed column.

II TLC.

/A/ Optimization of the previously investigated chromatographic systems:

- comparison of the separation in saturated and unsaturated chambers,
- temperature-dependence of the separation is studied,
- by comparison of the chromatograms obtained with spot and line-applications, the sample application is decided,
- optimization of densitometric evaluation, wavelength selection for detection
- if development is required, comparison of different developing reagents in respect of selectivity and sensitivity as well as colour stability,
- detection limit and sample capacity are determined
- optimization of the sample preparation procedure

/B/ The separations are performed by overpressurized thin-layer chromatography using microparticulate silica and previously optimized eluent system

III HPLC.

/A/ Optimization of the previously investigated chromatographic systems:

- determination of the optimum flow rate, temperature, column length and particle size
- detector selection and determination of its operating conditions
- detection limit and sample capacity are determined
- optimization of the sample preparation procedure

/B/ The separation of the compounds are investigated by using

- reversed-phase ion-pair chromatography
- normal-phase molecular complexation chromatography.

- the need for special chromatographic methods to improve the selectivity, efficiency or sensitivity of the separation.

The results obtained are tabulated containing all the important chromatographic data mentioned above including also the detection limit of the method the sample capacity, the pH-dependence of the capacity factors and the selectivity as well as data indicating the "salting out effect" using inorganic salts in the eluent.

The estimations are similar to the first control step for the new phase systems, but they are also extended to a comparison of the different phase systems and eluent compositions, as well as to the suitability of the system for preparative isolation of unknown compounds or the applicability of the GC-methods in a combined GC-MS investigation.

On the basis of the tabulated data /"T-II"/ the most suitable methods are selected /OGC-II. OTLC-II, OLC-II/ and the further directions of the experiments are decided.

Third screening step.

The recommended experiments carried out in this step are summarized in Table VII.

As it can be seen in Table VII the model investigations are completed in this step and all the experimental conditions are determined.

Concerning the use of special chromatographic techniques, in the case of glass capillary GC and overpressurized TLC the optimum conditions are similar to those determined earlier /for glass capillary GC, the stationary phase selection is based on a similar polarity as on a packed column while in OPTLC, microparticulate silica and the previously optimized mobile phase is first used/.

The recommended ways for the optimization of reversed-

phase ion-pair and normal-phase molecular complexation chromatographic systems are summarized in Table VIII.

Third control step.

In this step the results obtained in the third screening step are evaluated. The chromatographic data are tabulated and we have all the information needed for the solution of the given analytical problems. The line diagram of the optimization procedure of the phase systems is shown in Fig.1.

On the basis of the tabulated data /"T-III"/, the most suitable methods are selected /OGC-III, OTLC-III, OLC-III/.

Final step.

The main object of this step is the selection of the final analytical method suitable for solving the analytical problem and for controlling the results obtained.

The different analytical tasks, objects and required chromatographic parameters are summarized in Table IX.

Considering the given analytical problem, our procedure is summarized as follows:

- the most suitable chromatographic method is selected to solve the analytical problem;
- the control method is selected;
- the sample preparation procedure and sample loading are decided;
- the experimental conditions /eluent composition, flow rate, detection wavelength or temperature program/ are determined to obtain the optimum k'-values, resolution, detectability and analysis time;
- if derivatization is required, the reaction conditions are selected;
- if TLC is used, the optimum analytical conditions are selected;
- the mode of quantitative evaluation is decided; if

Table VIII

Optimization of reversed-phase ion-pair and normal-phase molecular complexation chromatographic methods

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I Reversed-phase ion-pair chromatography:

- the separations are performed on octadecyl silica,
- the eluent composition is selected so that in the absence of an ion-pair reagent in the eluent, the capacity factors of the compounds would be as small as possible but all the components have retention /$k' \simeq 0.1 - 1.0$/;
- the pH of the eluent should be selected so that the compounds exist in perfectly ionized form in the eluent;
- that ion-pair reagent is selected first for the investigations where the weakest reaction should be expected;
- the dependence of the capacity factors on the concentration of ion-pair reagent is clarified;
- the dependence of the capacity factors on the pH of the eluent using optimum concentration of the pairing agent is clarified;
- the dependence of the capacity ratios on the concentration of the organic modifier using optimum pH and reagent concentration is studied;
- dependence of the capacity factors on the types of organic modifiers using optimum pH, reagent concentration and polarity value of the eluent is studied;
- dependence of the capacity factors on the inorganic salt concentration using optimum eluent composition /pH, solvent type and polarity, reagent concentration/ is studied;
- we start the whole investigation again with the second ion-pair reagent after perfect regeneration of the column.

II Normal-phase molecular complexation chromatography:

- the separation experiments are started on a "nitrile" column;
- the polarity of the eluent used is selected so that in the absence of a pairing reagent the capacity factors of the compounds would be as small as possible but all the components have retention /$k \simeq 0.1 - 1.0$/;
- the dependence of the capacity factors on the reagent

concentration is studied increasing the reagent concentration from 5×10^{-4} mole/dm^3 to 10^{-1} mole/dm^3. If the change in the capacity ratios in the presence of the reagent suggests ion-pair formation, the polarity of the eluent is changed so that the capacity factors of the compounds investigated would be high /$k' > 10$/ in the absence of a pairing reagent.

- the dependence of selectivity and efficiency on the diethylamine concentration is studied, changing the ratio of reagent and diethylamine /the concentration of one of them is kept constant/. /see Ref. 6./
- using the reagent and diethylamine in optimum concentration, the types of organic modifiers are changed, first the type of the moderator solvent, then the polar solvent, and finally that of the non-polar solvent;
- if enantiomer separation is required, the D-; L- and racemicforms of the reagent are used, and the change in the selectivity of the separation is studied. /See Ref. 7/

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internal standards are used, the suitable standard is selected;

- the analytical methods /both the selected and control methods/ are validated, their reproducibility and precision are determined;
- the analytical investigations are carried out with both the selected and control methods and the results are statistically evaluated;
- the analytical file is prepared.

Set of Table X indicates the recommended methods and conditions for pharmaceutical analysis.

On the basis of the recommended methods and experiences obtained during the analytical investigations system suitability data are tabulated /see Table X / indicating the analytical task, the most important compounds, selected and control methods, as well as the values of the capacity factors, the minimum required resolution and HETP; the maxi-

Table IX

Types of analytical tasks in pharmaceutical analysis

I Analysis of natural products

/A/ Types	/B/ Objects
- Analysis of plant extracts - Analysis of extracts of animal organs - Analysis of fermentation mixtures	Separation of some well-defined main components in the presence of other unknown, so-called "background" compounds.

/C/ The most important requirements of the method are:

- the chromatographic method should have the necessary efficiency and selectivity; the peaks of the main components should not overlap the peaks of any detectable component;
- the capacity factors of the main components should be within the range of 2-5; the resolution of the main component/s/ and the next eluting compound should be higher than 1.25;
- the detection wavelength should be selected so that the uninteresting background compounds cannot be detected or only a small part of them should appear on the chromatogram, and the desired detectability can be achieved. The detectability can be increased by pre-concentration of the sample or increased sample loading,
- the relative standard deviation of the method /including sample preparation, application, chromatography and evaluation/does not exceed $\pm$ 5.0 %.

II Analysis of intermediates

/A/ Types	/B/ Objects
- Analysis of intermediates, raw products	Separation of the most important impurities from the main components
- Analysis of reaction mixtures	Separation of the initial and formed compounds and by-products
- Analysis of mother liquors	Separation and determination of the main important impurities, identification and isolation of the by-products and unknown impurities

C. The most important requirements of the method:

- high selectivity: the impurities should be separated from each other and from the main components. In HPLC the impurities should be eluted before the main components;
- suitable efficiency: the resolution between the main component an impurity eluted next to it should be higher than 1.25. When preparative isolation is carried out in the analytical system the resolution for this component and another eluting closest to it should be more than 1.50.
- the capacity factors of the impurities should be within the range of 1-7 and for the main components within the range of 7-10,
- more than 0.1 % impurity present in the sample should be detectable;
- the relative standard deviation of the method used for the determination of impurities present in less than 1 % concentration should not be more than $\pm$ 5 %.

III Analysis of pharmaceutical substances

/A/ Types	/B/ Objects
- purity tests	Separation and quantitative determination of impurities present in the sample
- determination of related substances present in similar concentration	Separation and quantitative determination of closely related compounds such as isomers, homologues etc.
- determination of the active ingredient content	Separation of the main component/s/ from the impurities and quantitative determination
- stability trial	Separation of impurities originating from the manufacture, and decomposition products of the main components

/C/ The most important requirements of the method are

- as in II /C/ extended with the following:
- high precision. For the determination of the content of active ingredient the relative standard deviation depends on the acceptable limit:for purity test it is less than 10 % if the amount of impurities is less than 1 % and not more than 5 % if the impurity level is between 1 and 10 %,
- detectability of the method permits the identification of more than 0.01 % impurity,

- if the active ingredient content is determined the capacity ratio of the main component should be between 2 and 5.

IV Analysis of formulated products

/A/ Types	/B/ Objects
- determination of active ingredient content	Separation and determination of the active ingredient/s/ from their impurities and other compounds present;
- Content uniformity tests	determination of the active ingredients occurring in low concentration from single solid dosage forms, in the presence of other substances;
- purity tests	Separation of impurities formed during the manufacturing process;
- stability trial	Separation of impurities and decomposition products of the active ingredients and other compounds formed during storage.

/C/ The most important requirements of the method are similar to III /C/ extended with the following:

- the efficiency and selectivity of the method is much more critical than in III /C/
- the suitable sample preparation procedure is very important

V Determination of pharmaceutical substances and their metabolites in biological medium

The objects and requirements are the same as in I

VI Other /special/ investigations

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mum permitted peak asymmetry, detection limit and precision data. The representative chromatogram should help in comparing the separation obtained at different times. Table X refers to the separation and quantitative determination of ergocristine in "ergocristine-rich"plant extracts. The representative chromatograms of selected and control methods can be found in our recently published papers for the selected method, see Ref. 5, while for the control method, see Ref. 3.

CONCLUSIONS

The series of the experiments used in the screening and control system can be seen in Fig.1. This figure collects the recommended phase systems used in the different steps. The most important conclusions on the control steps should be tabulated /"T-I "-"T-III"/. The optimized methods should be applicable in the pharmaceutical analysis considering also the system suitability data /see Table X/.

The advantages of this system can be summarized as follows;

- its use results in a set of different chromatographic procedures and in the case of a new analytical task the suitable method can be practically immediately selected;
- the optimization procedure gives a guarantee that, by using the selected method, reliable results and information will be obtained;
- the set of the different methods provides the possibility for flexible selection of the best method;
- the collection of different chromatographic methods provides a possibility to control the results; when unexpected results are obtained, their reason can easily be found and correction can be made.

Fig.1.

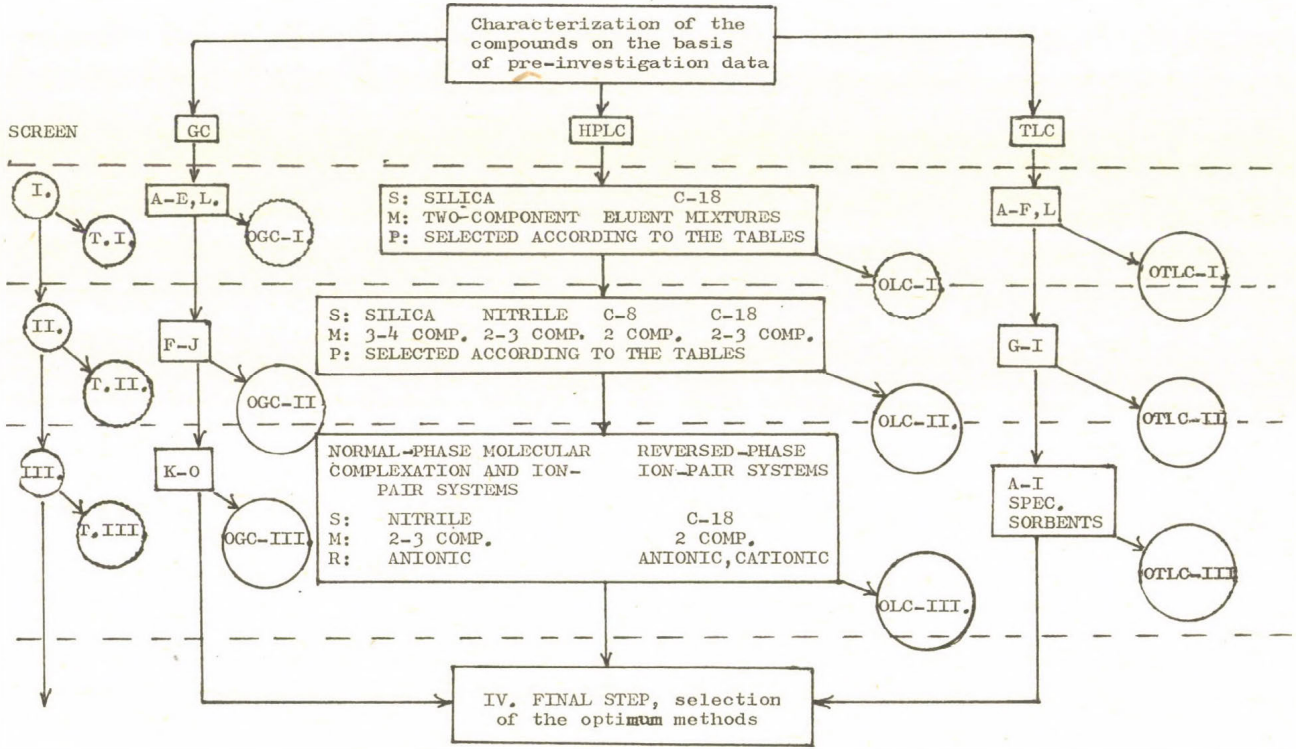
Characterization of the compounds on the basis of pre-investigation data
SCREEN
GC
HPLC
TLC
I.
T.I.
A-E,L.
OGC-I.
S: SILICA C-18
M: TWO-COMPONENT ELUENT MIXTURES
P: SELECTED ACCORDING TO THE TABLES
OLC-I.
A-F,L
OTLC-I.
II.
T.II.
F-J
OGC-II.
S: SILICA NITRILE C-8 C-18
M: 3-4 COMP. 2-3 COMP. 2 COMP. 2-3 COMP.
P: SELECTED ACCORDING TO THE TABLES
OLC-II.
G-I
OTLC-II.
III.
T.III.
K-O
OGC-III.
NORMAL-PHASE MOLECULAR COMPLEXATION AND ION-PAIR SYSTEMS
REVERSED-PHASE ION-PAIR SYSTEMS
S: NITRILE C-18
M: 2-3 COMP. 2 COMP.
R: ANIONIC ANIONIC, CATIONIC
OLC-III.
A-I SPEC. SORBENTS
OTLC-III.
IV. FINAL STEP, selection of the optimum methods

Table X.

RECOMMENDED METHODS FOR PHARMACEUTICAL ANALYSIS

SYSTEM	STATIONARY PHASE /COLUMN/	ELUENT	TEMPERATURE	FLOW RATE	DETECTION	COMPOUND TO BE INVESTIGATED
GC-A	2 % Dexsil 410 on Anachrom ABS (90/100 mesh) 1 m x 2 mm I.D. glass, spiral shape	N_2	170°C up to 280°C with 10°C/min program rate	30 cm^3/min	FID	ergocornine ergocryptine
TLC-A	Kieselgel 60 F_{254} /Merck/ 20x20 cm	Chloroform--acetonitrile--cc.ammonia (8:24:2)	ambient unsaturated chamber	-	by densitometry reflection mode at 280 and 310 nm	agroclavine elimoclavine
LC-B	LiChrosorb SI-60 5 µm (250 x 4.6 mm. I.D.)	Hexane--chloroform--acetonitrile (56:22:22)	ambient	100cm^3/min	by UV at 320 nm	ergocornine-ergocryptine

SYSTEM SUITABILITY DATA FOR METHODS USED

ANALYTICAL TASK		MOST IMPORTANT COMPOUNDS	SELECTED METHOD	SYSTEM SUITABILITY DATA						CONTROL METHOD	Number of representative chromatograms
TYPE	DESCRIPTION			k'	R_S (min)	H.mm (min)	A_{sf} (max)	Detection limit	Relative standard deviation		
I.	Determination of ergocristine in "ergocristine-rich" plant extract	ergocristine ergocristinine	LC-B	4.40 1.10	4.0	0.07	1.8	$\geq$ 40 ng ergocristine	$\pm$2.5 %	LC-A	E-17

SUMMARY

A screening and control system used for the optimization of the chromatographic procedure in pharmaceutical analysis is introduced. This system starts with the characterization of the compounds investigated, on the basis of non-chromatographic pre-investigation data followed by three screening steps containing recommended phase systems, and three control steps to evaluate the results of the different screening steps and to determine the necessary improvements in the next screening step. Finally, in the last step, from a set of different chromatographic methods the most suitable method is selected for solving the given analytical task.

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REFERENCES

1., G. Szepesi, M. Gazdag, J. Chromatogr. 122 479 /1976/

2., L. Szepesy, I. Fehér, G. Szepesi, M. Gazdag, J.Chromatogr. 149 271 /1978/

3., P. Horváth, G. Szepesi, A. Kassai, Planta Med. 33 407 /1978/

4., G. Szepesi, J. Molnár, Sz. Nyiredy, Z. Anal. Chem. 294 47 /1979/

5., G. Szepesi, M. Gazdag, L. Terdy. J. Chromatogr. 191 101 /1980/

6., G. Szepesi, M. Gazdag, R. Ivánesics, J. Chromatogr. 241 153 /1982/

7., G. Szepesi, M. Gazdag, R. Ivánesics, J. Chromatogr. 244 33 /1982/

8., G. Szepesi, M. Gazdag, J. Chromatogr. 204 341 /1981/

9., G. Szepesi, M. Gazdag, J. Chromatogr. 205 57 /1981/

10., M. Gazdag, G. Szepesi, K. Csomor, J. Chromatogr. 243 315 /1982/

11., M. Gazdag, K. Mihályfi, G. Szepesi, Z. Anal. Chem. 309 105 /1981/

12., L.R. Snyder, J. L. Glajch J. Chromatogr. 214 1 /1981/

13., J. L. Glajch, R. L. Snyder, J. Chromatogr. 214 21 /1981/

14., J. L. Glajch, J. H. Kirkland, Analyt. Chem. 55 319 /1983

15., P. J. Schoenmakers, H. A. H. Billiet, L. de Galan, Chromatographia 15 205 /1982/

16., L. B. Kier, J. Pharm. Sci. 70 930 /1981/

17., J. J. Issaq, J. R. Klose, K. L. McNitt, J. E. Haky, G. M. Muschik, J. Liquid Chromatogr. 4 2091 /1981/