CHROMATOGRAPHY '87

37

Symposia Biologica Hungarica

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Akadémiai Kiadó, Budapest

CHROMATOGRAPHY '87

Ed. by H. KALÁSZ and L.S. ETTRE

Symposia Biologica Hungarica Vol. 37

Chromatography is a useful tool for the preparative and analytical separations of substances present either in multicomponent mixtures or in more or less pure solutions. In recent years two congresses have been organized in Hungary discussing this subject, with international participation. The present volume includes the papers read at the meetings comprising a wide range of topics: thin-layer chromatography, column chromatography and various separation techniques.
The book may be of interest to chemists, biochemists and pharmacists dealing with chromatographic problems at different levels.

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CHROMATOGRAPHY '87

Proceedings of the Advances of Liquid Chromatography (Balatonszéplak, 1986) and the Budapest Chromatography Conference (Budapest, 1987)

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PREFACE

Chromatography '87 contains the papers presented at two symposia: the Sixth and Seventh Annual American-Eastern-European Symposium on Liquid Chromatography, held in 1986 and 1987 , in Balatonszéplak and Budapest, respectively.

The papers - lectures and posters - presented at these two symposia cover a wide variety of topics, from theoretical discussions to practical applications. Some of the papers draw theoretical conclusions from well-established, practical research while others describe the practical use of theoretical considerations. Although the title of the symposia specifies liquid chromatography, a few papers dealing with other branches of chromatography are also included. This broad range of the presented reports characterizes our symposium series.

Another characteristic of our symposium series is the wide geographical distribution of the participants: they came from the United States of America, Asia, Western and Eastern Europe. This wide geographical distribution provides an excellent opportunity for the mutual exchange of ideas, particularly to those who in general do not have the opportunity to attend the principal, large international chromatography meetings outside their own area. We, the organizers of these symposia are always very careful to provide the opportunity to the scientists from the west and the east for personal meetings. Although not every participant submitted the manuscript of his presentation for this volume, all of them actively participated in the informal exchange of ideas. We are very grateful for this.

Obviously, the contents of this volume reflect the fields of interest of the participants. Thus, not every topic which is generally considered as a major research area in chromatography is handled in the papers. Reviews of our past Proceedings have sometimes mentioned this as a shortcoming. We do not question this, however, the editors cannot invent subjects for the Proceedings: by its nature, such a volume always represents the cross-section of the research interests of the participants.

Another question mentioned by reviewers of past volumes in this series is the slow publication of the Proceedings. Again, we do not quarrel with them, however, one has to understand that this is often beyond the control of the editors. Coordination of authors from many countries most of whom having only a limited knowledge in English, and dealing with a limited capability for printing in that language is a very difficult task and obviously requires compromises in a number of questions. The situation is not different in this volume either which combines the papers presented at two symposia: hence, the publication of the 1986 Symposium papers is inevitably delayed while the papers presented at the 1987 Symposium are published relatively quickly.

As in the past volumes, Dr. Leslie S. Ettre has again served as the coeditor of the Proceedings. His help had been invaluable in reviewing the papers, suggesting modifications to the authors, in correcting their manuscripts, and - I should add - also in participating at the Symposium, always willing to discuss with the participants their problems, offer them suggestions for their future work, and in general, acting as an elder statesman with an over-thirty-years experience in the various chromatographic techniques.

On September 16, 1987, Dr. Ettre celebrated his sixty-fifth birthday. While others retire at this age, he has no intention to do this: he is as vigorous as ever, and more active than many men much younger than he. I would like to dedicate this volume to his honor, wishing him good health and continued enjoyment in both his professional and private life.

Budapest, May 1988

Huba Kalász

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HPLC ANALYSIS OF ECDYSTEROIDS IN PLANTS AND ANIMALS

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Ecdysteroids represent a large family of steroids (more than 100 compounds) widespread among Invertebrates (Zooecdysteroids) and Plants (Phytoecdysteroids)[1]. In Invertebrates (at least in Arthropods and probably also Nematodes) they are important hormones which control both development and reproduction, and it is also presently conjectured that they do so in several other Invertebrate groups. Their presence in Plants, on the other hand, is considered as a protective means against non-adapted phytophagous Insects [2] , but this assumption has in fact received up to now only limited experimental support.

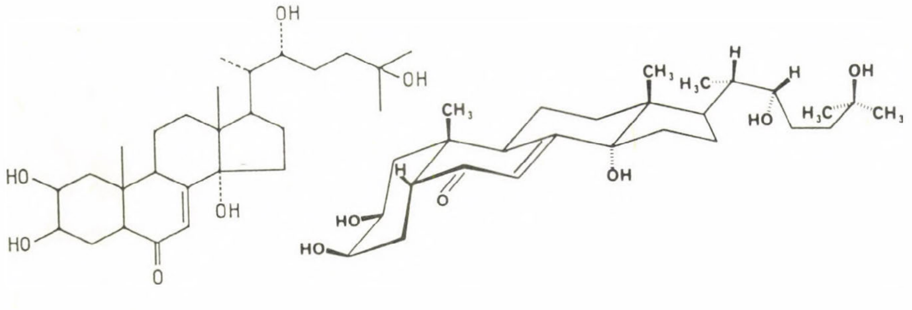

Figure 1. Structure of the ecdysone molecule

From a chemical point of view, ecdysteroids are characterized by several common features, i.e. (1) they have retained the entire skeleton of cholesterol (C-27 ecdysteroids) or of homologues bearing a methyl (or ethyl) group at the C-24 position (2) they have a 5β hydrogen (non-planar steroid nucleus), (3) almost all bear a 7-en-6-one chromophore (maximum absorbance at 242-3 nm in methanol), and (4) they contain several hydroxyl groups that render them rather water-soluble, in any case much more than Vertebrate steroid hormones.

Besides these common features, ecdysteroids may present several variations which can strongly modify their polarity and consequently their chromatographic properties. We can therefore recognise :

- very polar ecdysteroids
    + non-ionic : GLUCOSIDES
    + ionic : PHOSPHATE/SULFATE ESTERS, ECDYSONOIC ACIDS

- medium-polarity compounds
    + ECDYSONE and 20-HYDROXYECDYSONE
    + metabolites bearing one additional -OH group
    + various 28-C and 29-C compounds (essentially in plants)
    + immediate precursors of ecdysone
    + ACETATES, GLYCOLATES
    + DOUBLE CONJUGATES (e.g. BENZOATE+GLUCOSIDE)
- apolar compounds
    + low-polarity precursors (e.g. KETODIOL=2,22,25 trideoxyecdysone)
    + FATTY ACID ESTERS

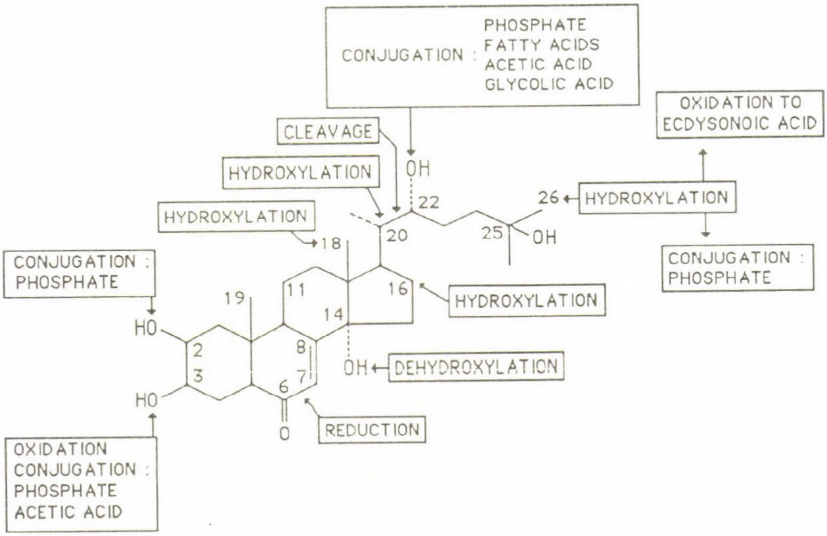

Figure 2 . Major metabolites of the ecdysone molecule in animals

Of course these different types of compounds have to be analyzed using specific chromatographic procedures. We will focuse here on the various HPLC systems currently used.

THE CHROMATOGRAPHIC SYSTEMS

We have previously reviewed the first systems used (1968-1980) [3] and only systems presently used will be described here (see also [4]).

Normal phase systems

The systems generally use silica columns. Sometimes Diol- or Aminopropyl- bonded phases are used for specific purposes. Bonded phases allow in theory to use gradient elution and to separate in a single run compounds of very different polarity, but they give significantly lower efficiencies and finally this possibility is not really used.

Solvent systems consist of either binary mixtures (directly derived from the solvent systems used for TLC), or ternary and even quaternary mixtures. In practice binary systems give generally poor efficiencies with peak tailing and do not allow to separate complex mixtures. By contrast, more evolved solvents give much better results, regarding both peak symmetry and selectivity. Among them it seems that mixtures of methylene chloride, isopropanol and water are the most widely used. The original mixture (125:25:2) was used to separate compounds having one OH less or more than ecdysone [5]. For less polar compounds the percentage of isopropanol has to be decreased (e.g. 125:20:1.5 ; 125:15:1 or 250:15:1) and of course for more polar compounds (e.g. 26-hydroxyecdysone and 20,26-dihydroxyecdysone) more eluting mixtures (e.g. 125:40:3) can be used with a good efficiency (figure 3).

The water content in these solvents is just below saturation, and is essential for peak symmetry. It allows to perform partition rather than absorption chromatography, and recovery of the injected compounds is very good. In addition, it is possible to rinse the columns with a few ml of methanol and they re-equilibrate very quickly (after about 5 column volumes).

In our hands, columns from different manufacturers gave very different results, and in several cases (probably due to silica deactivation) their performances decreased sharply after only a few days of use. Best results (regarding efficiency and reproducibility upon prolonged use) were obtained with Zorbax®-SIL columns.

Aminopropyl-bonded columns (eluted with dichloroethane/methanol/isopropanol) appeared to provide interesting selectivities for the separation of 3β-, 3α- and 3-oxo-ecdysteroid mixtures, although the authors report that retention times were not reproducible upon continuous use [6].

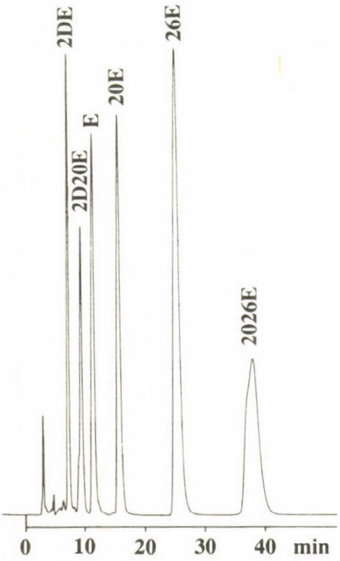

Figure 3. Separation of a mixture of reference ecdysteroids on a normal-phase column. Operating conditions : column Zorbax®-SIL 250 mm long, 4.6 mm i.d., solvent methylene chloride/ isopropanol/water (125:40:3), flow-rate 1 ml/min. 2DE = 2-deoxyecdysone; 2D20E = 2-deoxy-20-hydroxyecdysone; E = ecdysone; 20E = 20-hydroxyecdysone; 26E = 26-hydroxyecdysone; 2026E = 20,26-dihydroxyecdysone

Reverse-phase systems

They are the most commonly used, essentially with ODS-bonded columns. Other columns (C8, C6 or Phenyl-bonded phases) have also been used and they give rather identical results.

The most common use of ODS column is its application to the separation of ecdysone and 20-hydroxyecdysone (sometimes also 2-deoxyecdysone) from biological samples prior to radioimmunoassay, and this may be achieved with methanol/water mixtures. When more complex samples are to be analyzed, especially during metabolic studies, it appears more advisable to use a buffer instead of water (e.g. Tris, citrate or phosphate) [7,8]: this results in a better separation of

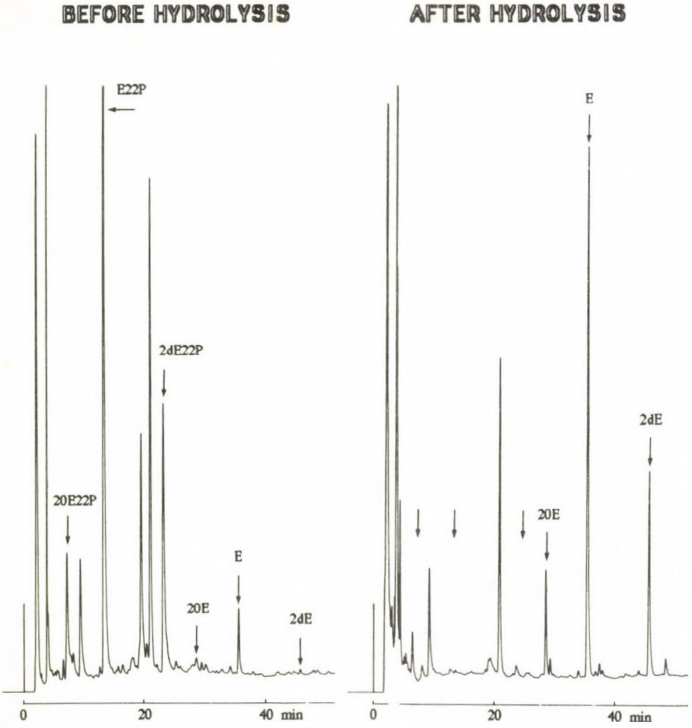

Figure 4. Analysis of ecdysteroids in *Locusta migratoria* eggs using a Spherisorb-ODS 2 column (250 mm long, 4.6 mm i.d.) eluted with a linear gradient (60 min) from 8% to 40% acetonitrile in 20 mM Tris/$HClO_4$, pH 7.5. Flow-rate 1 ml/min.

[E] = ecdysone; [20E] = 20-hydroxyecdysone; [2DE] = 2-deoxyecdysone; [22P] = 22-phosphate conjugate

ionic components (ecdysonoic acids and/or conjugates) that elute as sharp peaks. Acetonitrile is generally preferred to methanol : it gives higher efficiencies and allows working at much lower pressures, which results in a longer lifetime of the columns. An application of such a system to Locust eggs is given in figure 4.

The selectivity of reverse-phase systems varies with their origin : the order of elution of a set of reference compounds may differ when using columns from different manufacturers [9]. In addition, it is possible to improve the separation of a given pair of compounds by using different organic solvents. Thus when a pair of compounds seem difficult to separate with methanol or acetonitrile, it may be of interest to check tetrahydrofuran, or a mixture of two organic solvents. This problem has been investigated in details by Wilson et al. [9] (figure 5).

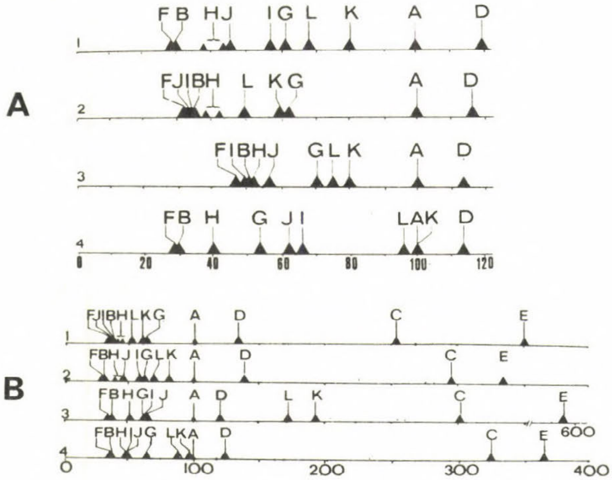

Figure 5. Variations in the selectivity of reverse-phase chromatographic systems due to the columns (A) or the solvent systems used (B) (from reference [9], reproduced with permission of Elsevier).

A : Selectivity differences between Spherisorb-ODS eluted with (1) acetonitrile/water (15:85) or (2) methanol/water (35:65) and Nucleosil-ODS eluted with (3) methanol/water (50:50) or (4) acetonitrile/water (20:80).

B : Comparison of different solvents with a Spherisorb-ODS column : (1) methanol/water (35:65), (2) acetonitrile/water (15:85), (3) tetrahydrofuran/water (10:90) and (4) dioxan/water (20:80).

Retention is given relative to ecdysone [A] which is given a value of 100.

[A] = ecdysone; [B] = 20-hydroxyecdysone; [C] = 2-deoxyecdysone; [D] = 2-deoxy-20-hydroxyecdysone; [E] = ponasterone A; [F] = polypodine B; [G] = makisterone A; [H] = inokosterone; [I] = cyasterone; [J] = poststerone; [K] = ajugasterone C; [L] = muristerone A

The use of acetonitrile/water mixtures may in some cases lead to excessive peak tailing even with non-ionic compounds, and this problem is overcome by using 0.1% trifluoroacetic acid in water (acetic acid also works but needs higher concentrations).Such acidic conditions appear especially adapted to the analysis of mixtures containing 3-oxo ecdysteroids [10]. In counterpart, they give poor results with conjugates or ecdysonoic acids.

Ion-pair and ion-exchange chromatography

Most polar ecdysteroids are anionic compounds, and thus some specific chromatographic systems may be used for their analysis. They have until now been used in a few cases only.

Ion-pair chromatography uses reversed-phase columns and a mobile phase which contains a cationic detergent (cetyltrimethylammonium bromide or tetrabutylammonium hydrogen sulfate), the presence of which leads to an increased retention of anionic ecdysteroids whereas non-ionic compounds elute at the same place as in the absence of detergent. This method has received little applications [7,11,12].

Anion-exchange columns have been more often used, essentially for the purification of phosphate conjugates, for instance from the eggs of *Schistocerca gregaria* [13]. They use in that case a mobile phase based on acetatebuffer [8].

DETECTION PROCEDURES

Many methods may of course be used for the detection of ecdysteroids in column effluents, with of course some limitations linked with the nature of the mobile phase.We will describe here only two methods which are rapidly developing.

UV Detection

Most ecdysteroids show a strong UV absorbance ($\varepsilon \approx 12{,}000$ at 242 nm). This allows the easy detection of these compounds in the nanogram range, either when using a spectrophotometer set at this maximum of absorbance or a (cheaper) fixed-UV (254 nm) detector.

In many cases, when analyzing biological samples, it would be of interest to obtain UV spectra of the different peaks. Thus in addition to the coelution with a reference ecdysteroid, any UV-absorbing peak would be further characterized by its absorbance spectrum, which represents an additional evidence for its identity and purity. Of course this can be achieved through off-line procedures, i.e. by collecting the different peaks evaporating the solvent and dissolving again in methanol. It appears that (1) it is rather time-consuming and (2) it does not work with low amounts of compounds, due to the simultaneous presence of various impurities in the effluent. In this respect, diode-array detectors eliminate both of these drawbacks by providing on line data with a good sensitivity. Thus it was possible to obtain a good spectrum with less than 100 ng of

20-hydroxyecdysone contained within an haemolymph sample from *Pieris brassicae* pupae [14] (figure 6 shows the 3-D map obtained) and it seems that even lower amounts could be processed to give useful data. Of course when ecdysteroid concentrations are large, as is the case in plant extracts, diode array detectors allow immediately to decide which peak may belong to the ecdysteroid family.

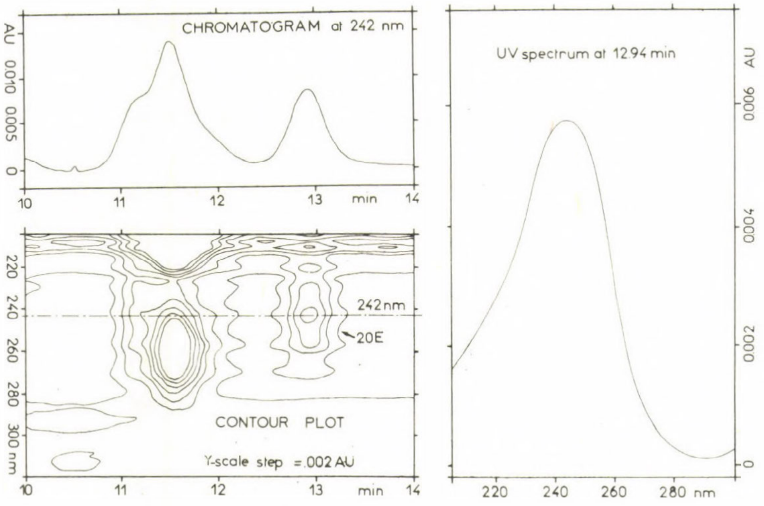

Figure 6. Analysis of a haemolymph extract from *Pieris brassicae* pupae by normal phase chromatography (Zorbax-SIL column, 250 mm long, 4.6 mm i.d., eluted at a flow-rate of 2 ml/min with isooctane/isopropanol/water 100:40:3), using a diode-array detector (Waters 990).The contour plot presented here represents the part of the chromatogram where 20-hydroxyecdysone (20E) is eluted (at 12.94 min), and gives evidence that the compound which elutes at that time shows a maximum of absorbance at 242 nm. Of interest is to notice the high sensitivity reached, as the 20E peak gave a maximal absorbance of 0.0085 AU only, which was enough to obtain a characteristic UV spectrum after correcting for background and smoothing (data from ref. [14], courtesy of Drs Beydon, Fabre and Goichon)

The possibility to use such detectors is linked with the use of solvents which do not absorb UV. This is not at all the case with the normal-phase solvents, which contain either chloroform or methylene chloride (cut-off respectively at 245 and 233 nm). This led us to design solvents based on hydrocarbons (hexane or isooctane) mixed with isopropanol/water as previously mentioned.

The efficiency of such systems is slightly lower, and their selectivity is significantly different from that of the methylene chloride-based solvents, but they work quite well with biological samples [14]. As an alternative, it would be possible to use of hexane/ethanol/methanol/acetonitrile (85:15:3:3) [15].

Radioactivity monitoring

Several radioactivity monitors have become available in the last years. They allow both sensitive and convenient analyses of samples from metabolic studies. The advantages of such apparatus over conventional fraction collection are numerous :

- with a 0.5 ml detection cell and respective flow-rates of 1 ml/min for the column effluent and 3 ml/min for the scintillation cocktail, the resolution is equivalent with the collection of 0.15 or 0.20 ml fractions, which for a 60 min run would correspond to 3-400 fractions !
- the result is directly available, even when performing double-labeling experiments, with peak integration and complete report;
- there is a direct correspondence of radioactivity with the data from the UV monitor, which are processed simultaneously, and this may be very important when two compounds are eluting very close together.

The method works well with reversed-phase systems (figure 7), and its use for normal-phase implies that non-quenching solvents are used. This problem is in fact similar to that mentioned above with diode-array detectors, and it may be solved by using isooctane-based solvents.

PREPARATION OF BIOLOGICAL SAMPLES

Sample preparation is classically performed by using methanol (or ethanol) extraction, followed by solvent partition(s) and silica column chromatography [7,16-18].

Recently some new procedures for sample processing were designed, which introduce the adsorption of ecdysteroids dissolved in water or aqueous methanol on C-18 bonded silica mini-columns (e.d. Sep-Pak® cartridges) followed by elution with a step-gradient of methanol in water, which is able to separate the three classes of compounds mentioned above [8,12,19-21]. The use of Florisil® cartridges (normal-phase) has also been reported as a substitute to silica column chromatography step : elution is performed by a step gradient of ethanol in chloroform [22].

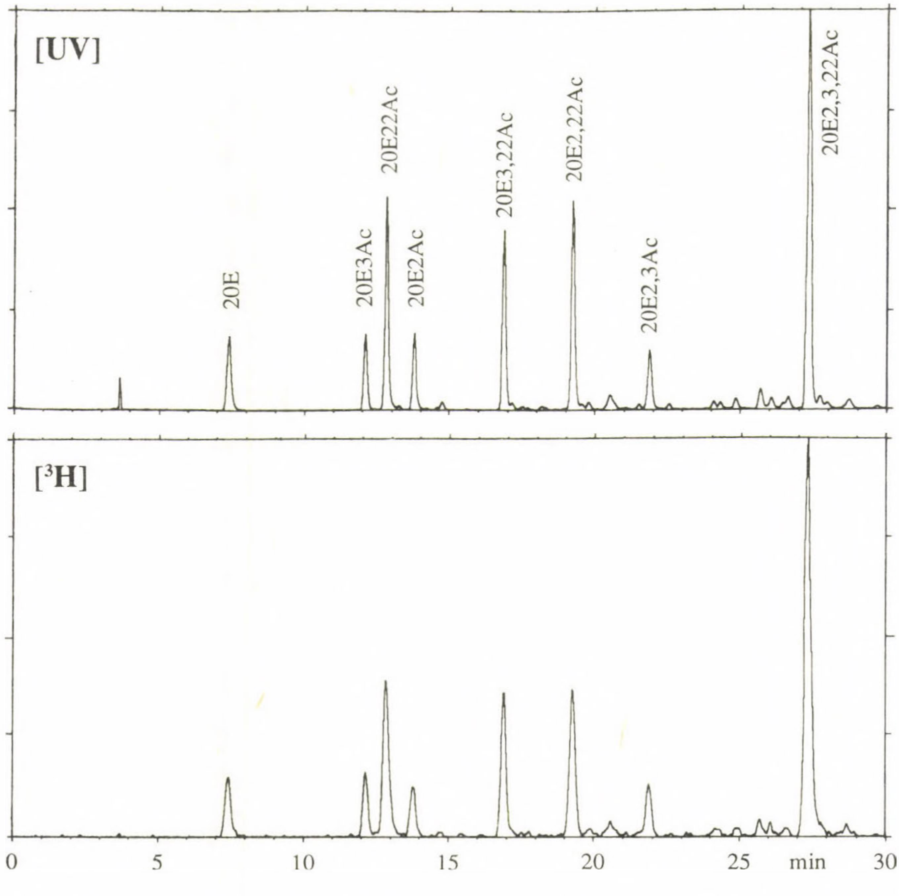

Figure 7. Separation of 20-hydroxyecdysone and its acetates on a Spherisorb-ODS 2 column (250 mm long, 4.6 mm i.d.) using a linear gradient (in 45 min) from 20% to 70% acetonitrile in 0.1% TFA, flow-rate 1 ml/min. Radioactivity was continuously monitored with a Flo-One model IC detector (Radiomatics, Tampa, Florida) : detector cell volume 0.5 ml, scintillation cocktail Lumaflow III, flow-rate 3 ml/min

Of course many other methods may be used for a more or less selective preparation of ecdysteroid fractions, depending on the sample size. Small samples can be processed using disposable cartridges, but larger ones need either conventional procedures, flash-chromatography or droplet-counter-current chromatography [23]. This last method allows to obtain quite pure fractions.

SOME EXAMPLES OF APPLICATION

HPLC techniques are widely used in the ecdysteroid field for many applications which include (1) the separation of all the major ecdysteroids contained in animal (insect) extracts using RIA for their detection (e.g. [24]+ figure 8) or [^{3}H] monitoring in metabolic studies (e.g. [25] + figure 9), (2) the isolation in pure form of specific compounds which are then characterized by physico-chemical methods (mass spectrometry, NMR,...).Generally two successive HPLC steps are enough to get pure compounds. Table I gives some HPLC systems classically used for the three different classes (polar, medium polar and apolar) of ecdysteroids.

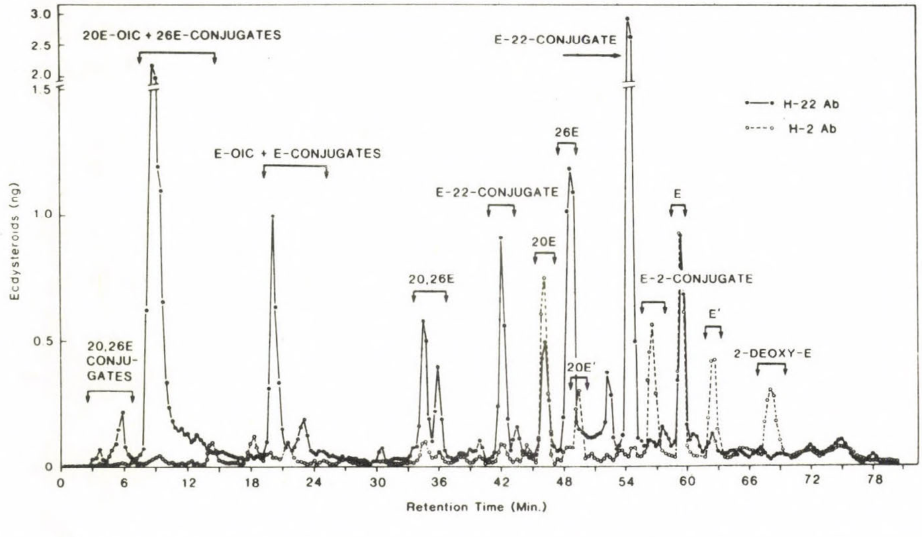

Figure 8. HPLC-RIA analysis of ecdysteroids from the gut of day 14 female developing adults of *Manduca sexta*. Operating conditions : column μBondapak (Waters) 15 cm long, 3.9 mm i.d., flow-rate 1 ml/min. Gradient elution from 100% solvent A (5% acetonitrile: 95% 20 mM tris/perchlorate buffer, pH 7.5) to a mixture of 85% solvent A 15% solvent B (95% acetonitrile: 5% tris/perchlorate buffer) in 60 min, followed by 5 min isocratic 15%B/85%A and then a 10 min cleansing shift to 100%B. Collected fractions were assayed by RIA using two different antisera (H-2Ab and H-22Ab). Abbreviations : [E] = ecdysone; [20E] = 20-hydroxyecdysone; [20E-oic] = 20-hydroxyecdysonoic acid; [E-oic] = ecdysonoic acid; [20,26E] = 20,26-dihydroxyecdysone; [26E] = 26-hydroxyecdysone; [20E'] = 3-epi-20-hydroxyecdysone; [E'] = 3-epi-ecdysone; [2-deoxy-E] = 2-deoxyecdysone. (reproduced from reference [24] by courtesiy of Marcel Dekker, Inc.)

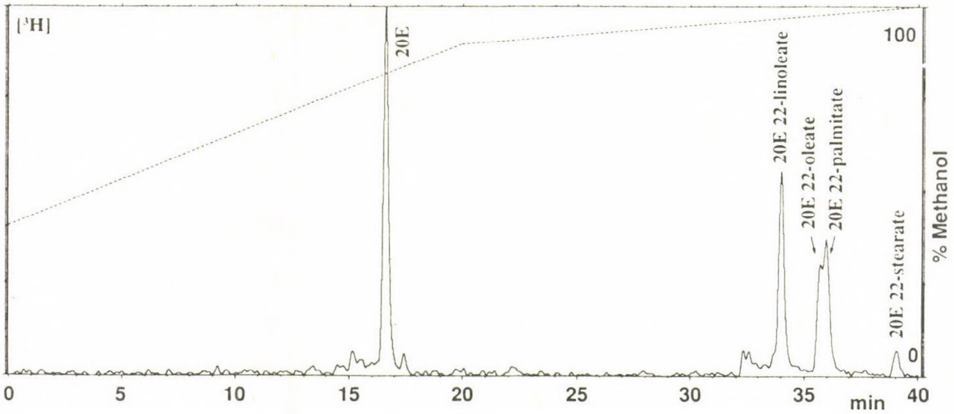

Figure 9. RP-HPLC analysis of [^{3}H]20-hydroxyecdysone metabolites from the frass of *Heliothis virescens* larvae fed with this compound. Column : Spherisorb-ODS 2 (250 mm long) eluded with methanol/0.1% TFA at 1 ml/min. Gradient conditions : from 40% to 90% methanol in 20 min, then 90% to 100% methanol in 20 min, then 100% methanol. Radioactivity monitoring was performed as described in figure 7

CONCLUSION

HPLC represents a very efficient means for the analysis of ecdysteroids. Some of its future developments might include fluorescent derivative formation for a detection in the picogram range [35,36], and on-line HPLC-MS coupling.

But HPLC is by no means the sole technique available and, according with specific needs, other methods may be preferred.

HPTLC [37-40] may be regarded as an alternative means of analysis particularly suitable for routine metabolic studies.

DCCC [23] on the other hand is particularly interesting for preparative-scale experiments (up to 1 g). It is essentially used for plant extracts [41] (which contain large amounts of ecdysteroids) but it works as well with insect extracts (it was for instance applied to *Bombyx* pupae [23]).

GLC was used since the early 70 s [16], but it is now of limited use, although GLC/ECD represents a highly sensitive method [42]. Only GLC/MS (SIM mode) seems to be used presently, in order to identify ecdysteroids present at very low concentrations, e.g. in Helminths [43,44].

Thus indeed many methods are currently available for ecdysteroid analyses, among which HPLC is presently the most versatile and widely used one.

Table I. Selected examples of chromatographic systems commonly used for the analysis of ecdysteroids [the use of gradient elution allows a single system to separate the three classes of ecdysteroids in a single run]

Class of ecdysteroids	Type of chromatography	Reference (e.g.)
POLAR	REVERSE-PHASE CHROMATOGRAPHY	
	Methanol/water	[18]
	Methanol/sodium acetate	[11]
	Acetonitrile/Tris-$HClO_4$	[7,26]
	Acetonitrile/Tris-HCl	[18]
	Acetonitrile/sodium citrate	[26]
	ION-PAIR CHROMATOGRAPHY	
	Acetonitrile/Cetrimide-Phosphate	[7]
	Methanol/Tetrabutylammonium	[11,12]
	ION-EXCHANGE CHROMATOGRAPHY	
	Ammonium acetate (Partisil SAX)	[8]
MEDIUM	REVERSE-PHASE CHROMATOGRAPHY	
	Ethanol/water	[27]
	Methanol/water	[28]
	Acetonitrile/water	[29,30]
	Acetonitrile/0.1% trifluoroacetic acid	[31]
	Acetonitrile/buffer	[5]
	Tetrahydrofuran/water	[9]
	Dioxan/water	[9]
	NORMAL-PHASE CHROMATOGRAPHY	
	SILICA : dichloromethane/isopropanol/water	[3,5,7]
	isooctane/isopropanol/water	[14]
	hexane/ethanol/methanol/acetonitrile	[15]
	DIOL : dichloromethane/isopropanol/water	[3]
	APS : dichloromethane/isopropanol/methanol	[6]
APOLAR	REVERSE-PHASE CHROMATOGRAPHY	
	Methanol/Tris-$HClO_4$	[32]
	Methanol	[33]
	Acetonitrile/isopropanol	[34]
	NORMAL-PHASE CHROMATOGRAPHY	
	SILICA : dichloromethane/isopropanol/water	[32]

Abbreviations : SAX = strong anion exchanger ; APS : aminopropyl silane

ACKNOWLEDGEMENTS

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EFFECTIVENESS OF CHROMATOGRAPHIC COLUMNS FILLED WITH POLYMER SURFACE-LAYER SORBENTS

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About 15 years ago a new type of sorbents, i.e., surface-layer sorbents were successfully introduced in the practice of gas chromatography (1, 2).

In gas-adsorption chromatography the time of the molecular adsorption itself is usually very short (3). Therefore the adsorption kinetics are mainly defined by the diffusion in the gas to the adsorbent surface: to the outer surface of the particles of non-porous adsorbents and additionally, to the inner surface in the pores of the particles of porous adsorbents.

Sorption processes in surface-layer sorbents occur not in the whole volume of the particle but in the surface layer of small depth, equal to the thickness of the active sorbent layer. Resistance to the mass transfer for such type of sorbents, as compared with ordinary (volumetric) sorbents, is decreasing, since the length of the diffusion routes into the depth of the particle decreases. Consequently the diffusion time which will be determined not only by the particle diameter (4), but also by the thickness of the active layer, is also decreasing.

Separation processes on porous polymer sorbents according to Hollis (5) differ from those in gas-liquid or gas-adsorption chromatography. Chromatography on porous polymer sorbents is realized in the whole volume of the particles. At present there is no single opinion on the separation mechanism with the porous polymer.

We have studied the properties of the surface-layer variant of the polymer sorbent Polysorb-1 (60:40 co-polymer of styrene and divinylbenzene) and also in intself, as column packing.

The properties of the sorbents were compared using similar column lengths (1.5 m) and the same column diameter (3 mm). C_1-C_4 hydrocarbon gases, C_5-C_{10} hydrocarbons, C_1-C_8 alcohols and some acids were used as model mixtures.

A surface-layer sorbent was prepared by mechanical shaking of the solid support Chromosorb W (particle size: 0.16-0.20 mm) with fine dispersive Polysorb-1 powder, in the mass ratio of 1:4. Polysorb-1 (particle size: 0.16-0.2 mm) was also used as a column packing. The column temperature was 140°C. Thermal conductivity detector was used. Helium was the carrier gas at a flow rate of 30 ml/min for C_1-C_4 hydrocarbon gases, and 50 ml/min in the other cases.

Table I lists the values of the adjusted retention volumes and the asymmetry coefficients for the investigated sample components on the studied sorbents. As shown unsaturated hydrocarbons are eluting earlier from the column than saturated hydrocarbons with the same number of carbon atoms, and the other components are eluting according to their boiling temperature, on both sorbents.

The retention volumes on the surface-layer sorbent are less than on the volumetric sorbent; consequently the analysis time decreases. With surface-layer sorbents the peaks obtained are more symmetrical, since in this case, the sorption and desorption processes defining the spread of the chromatographic zones occur only in the thin layer of the active sorbent. The symmetry of the peaks indicates the establishment of adsorptive equilibrium.

One of the main characteristics of the sorbents used in gas chromatography is the separation factor (6). When comparing columns with surface and volumetric sorbents, we have used the specific characteristic K/t_R, i.e., the ratio of the separation factor to the mean time of the component output for two neighbouring peaks (7). This parameter characterizes the separation on the column relative to the analysis time.

As shown in Table II, the separability on the column with surface-layer sorbent relative to the analysis time is higher than with the volumetric sorbent.

Table I. The reduced retention volumes (V'_R) and asymmetry coefficients (K_a) of individual sorbates on the studied sorbents

Sorbate	V'_R		K_a	
	Polysorb-1	Polysorb-1 + chromosorb W	Polysorb-1	Polysorb-1 + chromosorb W
CH_4	0.73	0.53	1.0	1.0
C_2H_4	1.41	1.04	1.02	1.0
C_2H_6	2.79	1.51	1.25	1.13
C_3H_6	3.29	2.11	1.37	1.25
C_3H_8	6.78	4.84	1.13	1.10
C_4H_{10}	7.73	6.69	1.58	1.22
pentane	2.64	1.90	1.20	1.01
hexene-1	3.68	2.95	1.60	1.40
hexane	4.61	3.49	1.33	1.27
heptane	5.16	4.69	1.37	1.22
octene-1	6.27	4.91	1.25	1.13
octane	6.69	5.55	1.54	1.13
nonane	7.36	6.21	1.32	1.15
decane	8.21	6.78	1.58	1.22
methanol	2.11	1.13	1.22	1.05
ethanol	2.61	1.39	1.54	1.12
iso-propanol	3.21	1.77	1.20	1.01
n-propanol	3.79	2.62	1.60	1.40
n-butanol	4.95	4.10	1.33	1.20
n-pentanol	6.54	4.70	1.25	1.12
n-hexanol	7.85	5.84	1.30	1.13
n-neptanol	8.68	6.70	1.37	1.25
n-octanol	9.09	7.24	1.67	1.30
formic acid	4.10	2.82	1.33	1.07
acetic acid	8.53	5.80	1.25	1.07
propionic acid	25.77	20.81	1.30	1.13

For model mixtures given in Table I we calculated the uniformity criteria being the analog to the selectivity coefficient for the separation of multicomponent mixtures, and fast-action coefficients, the values of which are summarized in Table III.

Fast-action coefficients, being the criterion of the generalized type, are defined by the sorbent selectivity relative to the worst separable pair, the column sorptive

Table II. Coefficients of separation of certain component pairs with account of analysis time

Sorbent components	Polysorb-1	Polysorb-1 + chromosorb W
C_2H_4-C_2H_6	0.01	0.14
C_2H_6-C_3H_8	0.09	0.12
C_3H_6-C_3H_8	0.02	0.06
C_3H_8-C_4H_{10}	0.06	0.14
pentane-hexene-1	0.12	0.18
hexene-1-hexane	0.13	0.20
hexane-heptane	0.73	1.10
heptane-octene-1	0.84	1.06
octene-1-octane	0.75	1.03
methanol-ethanol	1.01	1.50
ethanol-propanol	1.51	2.20
propanol-butanol	1.60	1.86
butanol-pentanol	1.62	1.80
pentanol-hexanol	1.73	1.93
hexanol-heptanol	1.70	1.91

Table III. Comparison of uniformity criteria and fast action coefficients for multicomponent mixtures of the studied sorbents

Sorbent model mixture	Polysorb-1		Polysorb-1 + Chromosorb W	
CH_4-$C_2H_6C_2H_6$-C_3H_6-C_3H_8-C_4H_{10}	0.121	$0.398 \cdot 10^{3}$	0.493	$0.627 \cdot 10^{-3}$
pentane-hexene-1-hexane-octane-1 octane-nonane-decane	0.193	$0.449 \cdot 10^{-3}$	0.533	$0.521 \cdot 10^{-3}$
methanol-ethanol-propanol(iso-)-n, propanol-butanol-pentanol-hexanol-heptanol-octanol	0.231	$0.357 \cdot 10^{-3}$	0.454	$0.388 \cdot 10^{-3}$
formic acid-acetic acid propionic acid	0.122	$0.296 \cdot 10^{-3}$	0.306	$0.326 \cdot 10^{-3}$

Table IV. Values of N/t_R for individual components on surface-layer and volumetric sorbents

Sorbent	N/t_R	
Sorbate	Polysorb-1 volumetric sorbent	29 % Polysorb-1 +Chromosorb W
pentane	881	1,372
hexane	622	1,733
neptane	612	1,580
octane	889	2,155
nonane	913	1,892
decane	836	1,706
methanol	284	648
ethanol	322	756
propanol	225	615
butanol	248	654

capacity, its effectiveness and the dimension of the peak distribution on the chromatogram.

This table shows, that under similar conditions of the experiment the separation of the model mixtures occurs with better parameters on the surface-layer sorbent than on volumetric one.

To compare the work of the columns with different types of sorbents, we have calculated the effective number of theoretical plates per unit of the time (N/t_R) (7) (Table IV).

Thus, the use of the surface-layer sorbents gives certain advantages; under similar conditions of the experiment the effectiveness of the columns with surface-layer sorbent is higher than with volumetric one, the time of analysis decreases, the chromatographic zone spread is removed.

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A HIGH-SENSITIVE PHOTOIONIZATION DETECTOR FOR GAS CHROMATOGRAPHY

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SUMMARY

A new high-sensitivity photoionization detector for use with both packed and capillary columns is presented. The design of the detector and of the UV source used are described. Advantages and disadvantages of the applied UV lamp are discussed.

Performance data and relative response values are given for selected hydrocarbons.

INTRODUCTION

The analytical performance of a gas chromatograph essentially depends on the detector used. In most cases only some of the established detectors are available. Among these are the thermal-conductivity detector (TCD) the flame-ionization detector (FID), the electron-capture detector (ECD), the thermionic detector (TID) and the flame-photometric detector (FPD). It is to be expected that the photoionization detector (PID) characterized by excellent analytical properties will also be added to the generally used detectors. The PID is a nondestructive, concentration-dependent detector which can be successfully operated with packed columns. A concentration-sensitive detector has a natural advantage over a mass-flow detector such aş the FID for capillary column analyses, particularly where sensitivity is important, because its response increases with decreasing flow rate.

For many compounds, especially for olefins and aromatics, the sensitivity of the PID is greater than that of the FID (1-3). It is possible to realize relatively simple designs of the photoionization detector which are easy to use. A proper design enables a low background current and a very low noise level to be obtained which is the prerequisite for reaching a low minimum detectable level. The PID is applicable with several carrier gases such as nitrogen, hydrogen, carbon dioxide, all noble gases, particularly helium (He) and even with pure air. Various investigations and improvements show that interest in this type of detector has steadily increased. The present paper describes a new PID with a high-intensity 10.20 eV-UV source and presents the performance data of this detector.

APPARATUS AND EXPERIMENTAL

Figure 1 shows the scheme of the PID. Its most essential parts are the UV source and the ionization chamber with two electrodes and the gas lines. The heart of the PID is the UV source. In this case a low pressure hydrogen discharge lamp with a hollow cathode is applied as the UV source. The hollow cathode discharge tube is a radiation source using the radiation in the range of the negative glow of a gas discharge.

Figure 2 shows such a hollow cathode discharge tube. The precise balance between the cathode size, the cathode material and the filling gas pressure is important for the proper functioning of the lamp. Due to the excitation conditions the radiation of the lamp is characterized by sharp spectral lines of neutral and ionized compounds of the cathode material and of the filling gas. Since for the PID the resonance radiation of the filling gas is of importance rather than the atomic line of the atomized cathode material a cathode material has to be selected whose atomizability is as small as possible. Such a material is e.g., tungsten or molybdenum. The ultraviolet transmitting faceplate consists of vacuum-grown magnesium fluoride crystal (MgF_2). By means of a disk it is connected vacuum tight with the glass tube of the UV source. The described UV source is filled with ultrapure hydrogen. In the glow

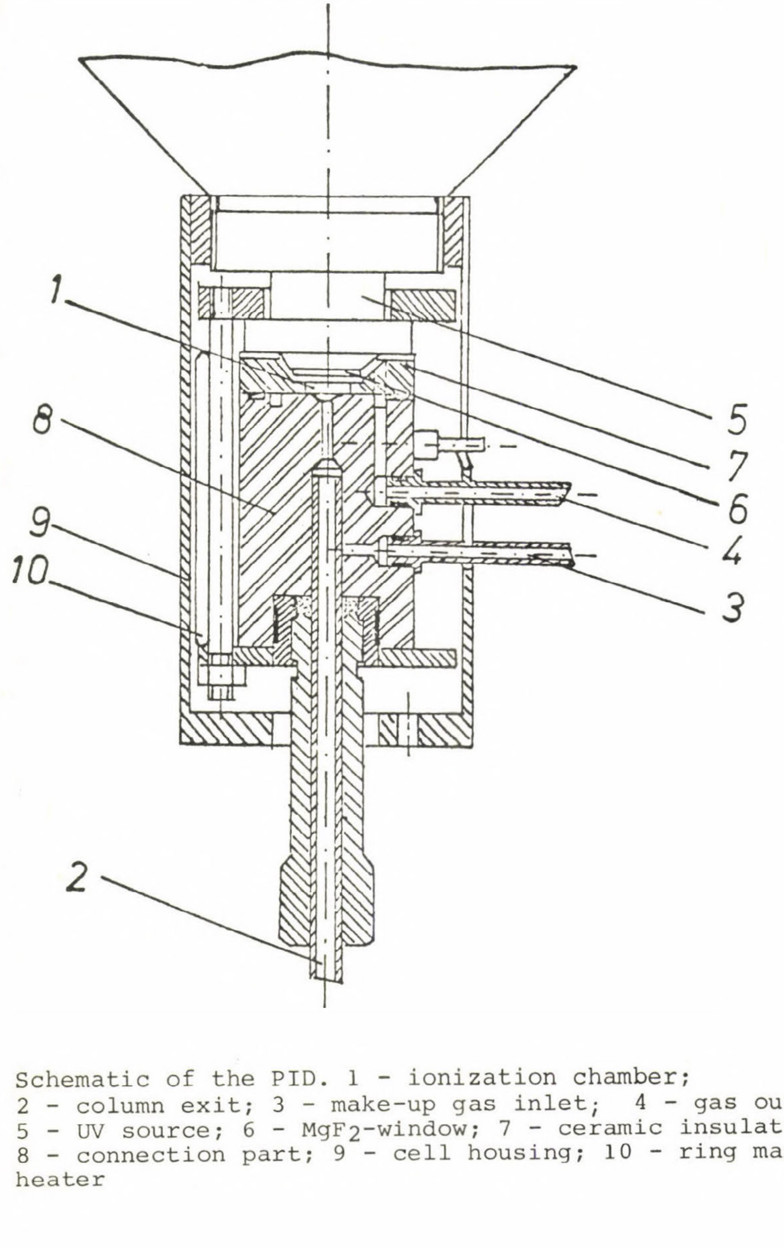

Fig. 1. Schematic of the PID. 1 - ionization chamber; 2 - column exit; 3 - make-up gas inlet; 4 - gas outlet; 5 - UV source; 6 - MgF_2-window; 7 - ceramic insulator; 8 - connection part; 9 - cell housing; 10 - ring mantel heater

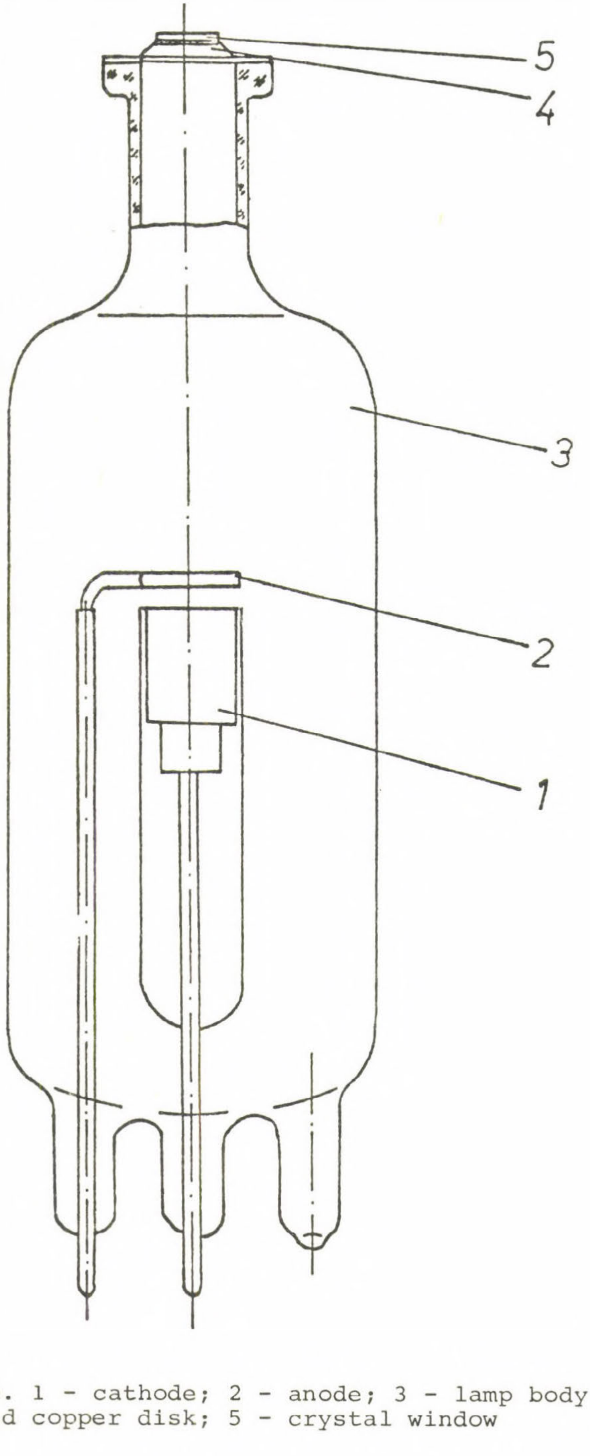

Fig. 2. UV source. 1 - cathode; 2 - anode; 3 - lamp body; 4 - gilded copper disk; 5 - crystal window

discharge mainly the resonance line of atomic hydrogen is excited with a wavelength of 121.568 nm (Lyman alpha line).

The maximum radiation intensity of the lamp amounts to $1 \cdot 10^{10}$ photons per second. In continuous work the lamp can be operated at a current of 5 mA over a long time. At this current the lifetime of the lamp would be at least two years. Over a relatively short period discharge currents from 30 to 50 mA are possible. However at high discharge currents an increasing cathode atomization takes place, which in turn causes increasing gas sorption.

The ionization chamber of the PID is formed by the source window and two insulators made of KER 710 ceramic. The electrodes are positioned so that the photons from the UV source cannot fall on them. A parallel arrangement of the electrodes is possible. However, a cylindrical shape of the chamber can also be chosen where the electrodes are placed as circular segments on the inner side of the upper insulator. This insulator part has four gas outlet bores and is shaped so that the crystal window is precisely placed between the electrodes and that the inner surface of the part forms a ring slit with the gilded copper disk supporting the window. Through this ring slit the gas leaves the ionization chamber. This arrangement results in a short time constant. The carrier gas flows into the ionization chamber through an axial bore in the second insulator, the so-called connection part, sealing the chamber at the bottom.

Capillary columns as well as other chromatographic columns can be fitted into this connection part. In addition, it contains the make-up gas inlet, the gas outlet and the receptacles for the collecting and polarizing electrodes. On its upper side the connection part has a ring-shaped groove for withdrawing the gas from the ionization chamber. The two-piece detector cell is placed in a ring mantel heater with a maximum power consumption of 160 W.

The detector was used on a modified GCH 18.3 gas chromatograph (VEB Chromatron, Berlin). The separation columns were packed with Chromosorb W coated with 10 % SE-30 and methylsilicone were connected directly to the detector cell. The carrier gas was pure helium or nitrogen with a column flow of 15-50 ml/min. The PID was investigated with two various cylindrical ionization chambers, which had different volume.

RESULTS AND DISCUSSION

For the two ionization chambers (100 and 200 µl effective volume) the detection limit and the sensitivity of the PID were determined at various lamp currents.

Table I. Performance data of the PID*

Lamp current	Cell volume, 200 µl		Cell volume, 100 µl	
(mA)	NG (pg/s)	E (A.s.)/g	NG (pg/s)	E (A.s.)/g
3	0.42	0.08	0.62	0.06
5	0.24	0.14	0.36	0.10
8	0.17	0.21	0.23	0.16
11	0.12	0.30	0.16	0.22

*Test substance, benzene; carrier gas, nitrogen; flow rate, 16.7 ml/min. Detection limits and sensitivities are given in terms of pg/s and (A.s.)/g to permit comparison with the performance of the mass-flow dependent FID.

The results are given in Table I. From the data it can be seen that better results were obtained by using the chamber with 200 µl cell volume. However, the differences in the detection limit and sensitivity between the two chambers are not considerable.

When helium (purity: 99.99 %) is used as the carrier gas it is possible to reach a detection limit of $5 \cdot 10^{-14}$ g/sec benzene. The linear dynamic range of the detector is 10^5. In all the measurements the polarization voltage was 80 V. Even at relatively high lamp currents the noise level of the PID was low: it did not exceed $1.6 \cdot 10^{-14}$ A. The background current of the PID was 1-5 pA.

The sensitivity of the PID decreases with increasing operating temperature. The reason for this phenomenon is the change in the radiation intensity of the UV lamp. At high temperatures, not only the transmission limit of the crystal window shifts toward longer wavelengths but also an increased absorption of UV rays takes place. In addition the increase in the temperature causes an increase in the pressure in the lamp. In turn, the pressure increase disturbs the optimum process of the gas discharge between the lamp electrodes.

Table II. Performance data of the present detector

Performance data	
sensitivity [(A.s.)/g]	0.3 (carrier gas: nitrogen) 0.6 (carrier gas: helium)
detection limit for benzene (pg/s)	0.1 (carrier gas: nitrogen) 0.05 (carrier gas: helium)
background current (pA)	5
noise level (a)	$1.5 \cdot 10^{-14}$
linear dynamic range	10^5
operating temperature ($^{\circ}$C)	up to 250
carrier gas	pure nitrogen or helium

Table III. Relative response factors (r) related to benzene (r=100)

Substance	r
benzene	100
toluene	175
m-xylene	300
2-hexyne	120
3-hexyne	45
2-heptyne	90
hexane	2.3
heptane	3.2
octane	3.0
nohane	2.7

Table II presents the performance data of the present PID. It can be seen that our PID is particularly characterized by a very low detection limit, small background current and low noise level.

Relative response values for some compounds related to benzene are given in Table III. It can be seen that the sensitivity to aromatic hydrocarbons is higher than to aliphatic hydrocarbons and that the sensitivity for alkynes is higher than for alkanes.

CONCLUSIONS

A new photoionization detector is presented. It is characterized by a UV lamp with a very large photon flux (10^{10} photons/sec) and a long lifetime (about 2 years) and permits the detection of a great number of substances with high sensitivity.

The extent of the linear dynamic range amounts to five orders of magnitude. A disadvantage in comparison to known PID's is the relatively large dimensions of the UV lamp.

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DIFFERENT SEPARATION TECHNIQUES FOR THE EVALUATION OF GLYCOHEMOGLOBIN LEVELS IN COWS

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INTRODUCTION

According to the elution behaviour on a cation-exchange resin the adult human hemoglobin (HbA) can be divided into subfractions. A few minor fast-hemoglobin fractions (HbAla, -b, -c) are appearing before the main component (HbA0). These findings have been first reported in the late fifties (1). According to later results, an electrophoretically less positive hemoglobin fraction can be detected in elevated quantity in diabetic patients (10). From the main component of the chromatographically fast fractions, HbAlc was identical with this diabetic fraction. After structural analysis the chemical nature of this hemoglobin component was elucidated: it is a glycoprotein (3). It was found that hemoglobin glycolisation takes place by non-enzymatic processes in the mature red blood cells. The percentages of this post-translational glycolisation depends on the blood sugar level (7). The developing process and the structure of the HbAlc are summarized in Fig. 1.

Because the existence of the glycohemoglobin is equal with the life span of the red blood cells, its determination is a powerful tool for the evaluation of the retrospective blood sugar status. For this reason glycohemoglobin measurement is in the focus of interest of diabetologists.

The possibilities of glycohemoglobin determination are originated from its chemical nature. According to this, cation-exchange chromatographic (by macro- or micro-column, and bath-

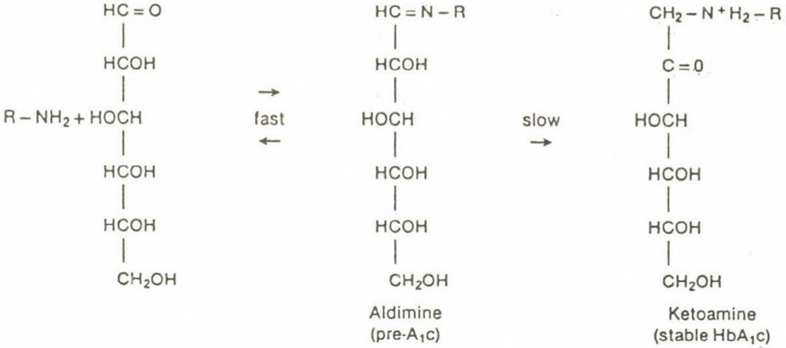

Fig. 1. The formation of glycohemoglobin. R = the beta chain of hemoglobin A

wise technique), affinity chromatographic, electrophoretic, isoelectric focusing, immuno, spectrophotometric, fluorimetric and colorimetric assays exist. These were summarised and well-documented in the review of Miedema and Casparie (9).

Because the glycohemoglobin level is a background mirror of the glucose status of a diabetic patient, literary data are dealing mostly with humans. Only a few reports are available on non-human investigations (4, 5, 7, 13, 14).

According to our opinion, the investigation of the glycohemoglobin level helps us with the evaluation of the chronic energy-deficit (critically low blood-sugar) of cows.

MATERIALS AND METHODS

Venous blood samples were taken from cows into heparinised tubes. The urine of the cows after calving was tested with sodium nitroprusside ($Na_2Fe[CN]_5NO$) for ketone bodies. The centrifugated packed cells were three times washed with physiological saline solution and subsequently they were hemolysed with a detergent solution (0.3 mM sodium dodecys sulfate). The investigations were carried out with these hemolysates.

Glycohemoglobin can be determined in the following ways:

Colorimetry: By the modified tiobarbituric acid (TBA) method (12) with a test Kit (Reanal, Budapest, Hungary).

Affinity chromatography: On Glyco-Gel B (m-aminophenyl boronic acid, covalently coupled to cross-linked 6 % agarose) with a test Kit (Pierce Chemical Company, Rockford, Ill., USA).

Cation-exchange chromatography: On CM-Sephadex C-50 (Pharmacia, Uppsala, Sweden) matrix in column (I.D. 1.6 cm; bed height 10 cm). Buffers: A.: 10 mmol sodium acetate/acetic acid pH 5 (11); B.: 0.1 mol NaOH containing 0.5 mol NaCl. Gradient: 0 to 100 % B.

Batch chromatography: A 100 µl hemolysate aliquot was mixed with 3 ml CM-Sephadex A-50 slurry (approx. 1:1 sodium acetate buffer:gel, v/v). After a short centrifugation the optical density of the supernatant was measured.

Isoelectric focusing: With LKB Multiphor instrument on thin-layer polyacrilamide gel containing beta-alanine (6). The quantitative evaluation of the isoelectrophoretogram was carried out by a soft laser densitometer of LKB.

Photometric measurements were taken by Specord UV-VIS (C.Zeiss Jena, GDR) at 443 nm in the case of the TBA reaction and at 415 nm in the other chromatographic determinations.

RESULTS AND DISCUSSION

The isoelectrofocusing and one typical densitogram of bovine hemolysate are shown in Fig. 2. The Hb component, placed at a higher distance to the anode than the main component (HB0), has lower electric charge due to glycolisation. This Hb fraction is the GHb, i.e., the HbAlc. The mobility of the bovine Hb in the electric field is similar to human Hb (6), in identical circumstances. As the isoelectrofocusing shows, the distance between the major and minor component is very narrow, i.e., the isoelectric points distinguish only a little these proteins (11).

We compared the results of affinity chromatographic separation and colorimetric determination of bovine GHb. The values, given in percentages, are summarised in Table I. The slightly higher mean of the affinity chromatographic determination is

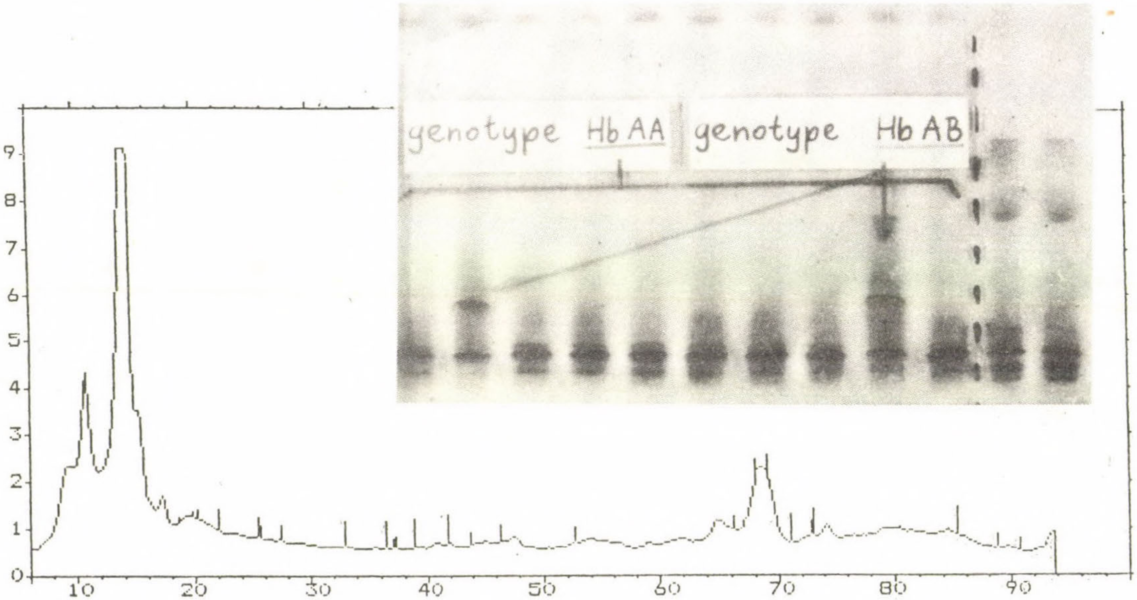

Fig. 2. Isoelectrofocusing of bovine and human hemolysates and a typical densitogram. Thin-layer polyacrilamide gel containing beta alanine, pH gradient 6-9 by Ampholyne

Table I. Determination of GHb in ten cattles by affinity chromatography and colorimetry. Correlation of the values between the two methods: r = 0.07

	Glycohemoglobin %	
No.	Affinity chromatography	Colorimetry TBA reaction
1.	2.60	2.50
2.	2.90	2.72
3.	2.31	2.25
4.	1.82	1.94
5.	2.62	2.55
6.	2.70	2.60
7.	2.80	2.35
8.	2.65	2.65
9.	3.45	2.85
10.	2.50	2.17
mean	2.63	2.45
S.D.	0.41	0.27

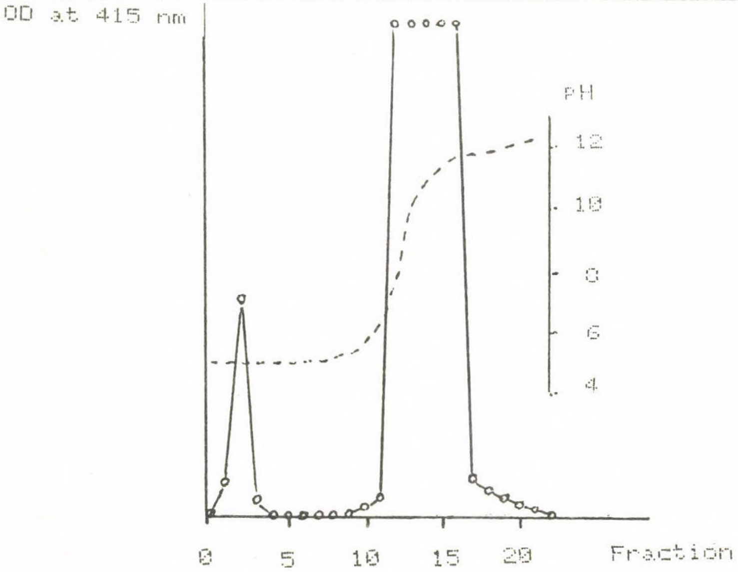

Fig. 3. Elution profile of a bovine hemolysate on CM-Sephadex C-50. Column 1.6 cm i.d., bed height 10 cm, gradient elution with buffer A (10 mM sodium acetate/acetic acid, pH 5) to buffer B (100 mM NaOH containing 500 mM NaCl; 0 to 100 %). Fraction volume 5 ml

perhaps due to the binding affinity of the Schiff base form (Pre HbA1c) to the immobilised boronic acid matrix.

The elution profile of the hemolysate on the CM-Sephadex C-50 column can be seen in Fig. 3. The first peak appeares immediately at the start of the elution. Subsequently, by the increasing of both the pH and pI of the buffer a large colored fraction (HbA0) was eluted. The proteins of the first peak cannot bind to the cation-exchange gel because its electric charge is decreased because of glycolisation. In this separation we could not distinguish among the GHb-s (HbA1a, HbA1b, HbA1c), but because HbA1c is the major component among the minor fractions, this would be, according to our opinion, not so important. The column chromatographic techniques are relatively time consuming in the case of the mini-column methods also. Therefore we have developed a batch chromatographic separation of GHb, as described in details under the methods. This proved to be a quick and reproducible technique.

The results of bovine GHb measurement are presented in a distribution diagram (Fig. 4). The normal level of the bovine GHb is near to 2.5 % of the total hemoglobin. This is half of the human value (4.5-6.5 %)(9, 12). The life span of human and cattle red blood cells is similarly 120 days. The explanation of the lower bovine GHb concentration is the lower blood sugar level of the ruminants.

In cattles the physiological blood sugar level is one-third (1.6-3.3 mmol/l) of that observed in other mammals. This is a phylogenetical property, originating from the carbohydrate fermentation in their rumen. For this reason, when the pathways of the blood sugar producing glyconeogenetic steps are hindered, the quantity of ketone bodies increases easily. This develops a keto-acidotic status, the so-called ketosis (2). The ketotic animal loses the appetite, and its milk production decreases.

Ketonuria is an indicator of the intensive ketone body production. This condition is formed if the easily oxidable blood sugar is not enough for the energy production needed for the metabolism. Ketonuric cows have significantly (<5 %) lower GHb level than non ketonuric cows (Fig. 4). This result indicates that their blood sugar has been in the lower range in the

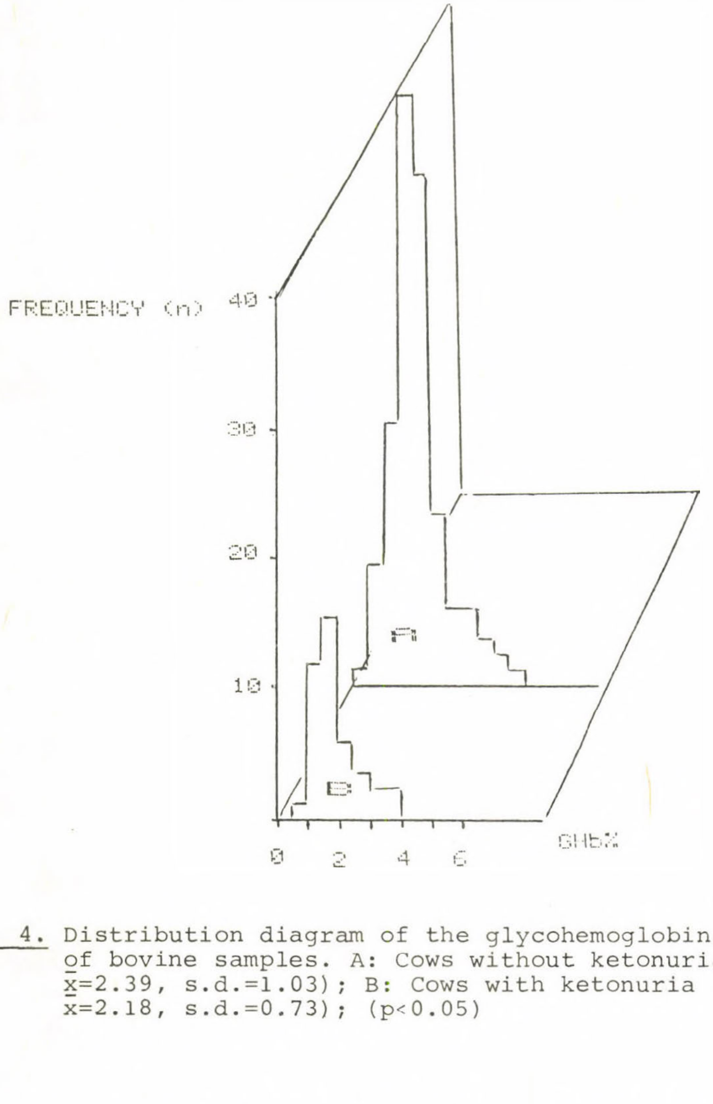

Fig. 4. Distribution diagram of the glycohemoglobin content of bovine samples. A: Cows without ketonuria (n=125, $\underline{x}$=2.39, s.d.=1.03); B: Cows with ketonuria (n=36, $\bar{x}$=2.18, s.d.=0.73); ($p<0.05$)

last two months. The lower GHb level of cows with subclinical ketosis (ketonuria) proves the energy-defficiency of weeks before.

ACKNOWLEDGEMENTS

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CHROMATOGRAPHIC INVESTIGATION OF TOMATO FRUIT METABOLITES DURING RIPENING

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INTRODUCTION

The advent of high-performance liquid chromatography (HPLC) opened new possibilities for the study of food constituents. The use of this technique can result in faster, simple and perfect analysis of complicated samples like carotenoids, organic acids and carbohydrates.

Separation and identification of tomato fruit pigments were achieved by thin-layer and column chromatography with different adsorbents and (1-5) eluents. These chromatographic systems were not so accurate and convenient to be used as analytical methods for the determination of both the micro and macro constituents. Moreover, oxidation and isomerization take place during the separation process since colour substances are very sensitive to light, oxygen and some organic solvents. Zakaria et al. (6) developed a new HPLC method to isolate carotenoid pigments from tomato fruits using several stationary phases with chloroform in acetonitrile at different ratios, as the mobile phase. Attention was paid only to α- and β-carotenes and lycopene in tomato fruit samples saponificated before analysis. In previous work tomato fruit pigments were separated on Chromsil C_{18} column with acetone water as the mobile phase (7). The use of acetone with Chromsil C_{18} was very harmful and oxidation of the pigments could not be avoided.

Determination of organic acids in fruits and vegetables was carried out by several chromatographic techniques including paper chromatography (8-9), gas chromatography (10) and liquid chromatography (11, 12). These methods may lack specificity, may be time-consuming or may require derivatization of the acid before analysis. Recently, HPLC methods have been used for the rapid specific and direct determination of organic acids in food products. However, these techniques were either specific for the determination of one organic acid such as e.g., citric (13) and ascorbic acid (14) or require derivatization before analysis (15).

For the past several years gas chromatography and, more recently, HPLC have been used for the determination of sugars in foods. HPLC separation and detection of soluble carbohydrates have been developed with many advantages (16, 17). The application of HPLC systems for the determination of sugars in some food products is satisfactory and gives accurate results (18, 19). These methods are used in both basic research and industrial operations.

The objective of this study was to develop and apply HPLC methods for the separation of coloured substances, organic acids and carbohydrates and to follow their metabolic pathways during the ripening stages of tomato fruits.

EXPERIMENTS

Tomato fruits (Lycopersicum esculentum var ventura) at different stages of ripening were obtained from experimental fields of the Chemistry Department, University of Horticulture, Budapest. The fruits were directly stored at refrigeration temperature after harvesting.

Tetrabutyl ammonium hydroxide was purchased from Sigma. Sugar standards were from Supelco; organic solvents, citric acid, tartaric acid and malic acid were from Reanal. Other organic acids were from Sigma.

Sample preparation methods

Pigment solutions. Four tomato fruits were cut into small pieces and 10 g samples, in duplicates, were disintegrated in a mortar with quartz sand and homogenized in 30 ml methanol. The initial homogenate was filtered through a funnel containing a piece of cotton. The residues were recovered for extraction by mechanically shaking with 60 ml of 4:2 carbon tetrachloride - methanol for 30 minutes at room temperature. The pigments soluble in the first methanol fraction were recovered by shaking the methanol fraction with 30 ml of carbon tetrachloride and let to stand for 5 minutes; then the lower carbon tetrachloride fraction was separated with a separatory funnel and remixed with the carbon tetrachloride-methanol extracting mixture. After mechanical shaking the organic lower phase was separated by a separatory funnel and dried on anhydrous sodium sulfate. The solvent was removed under vacuum in a rotary evaporator at ambient temperature. The solid material was redissolved in a suitable volume of the HPLC eluent with a minimal volume of chloroform.

Samples containing organic acids and soluble carbohydrates. Twenty grams of tomato fruits (random sample) were disintegrated in a mortar with quartz sand and homogenized with 40 ml of 2 % metaphosphoric acid. The initial homogenate was mechanically shaken at ambient temperature for 30 minutes and filtrated through a filter paper (Rundfilter MN640 d). The clear filtrate was stored in a refrigerator (4^oC) for the determination of the organic acid and in a freezer (-20^oC) for carbohydrate analysis.

HPLC methods

Pigment separation. A Beckman series liquid chromatograph equipped with a Model 114 M pump, a Model 340 organizer fitted with a 20 µl sample loop, a Model 420 controller and a Model 165 variable wavelength detector was used. The detector signal was electronically integrated by a Shimadzu C-R3A integrator.

The pigments were separated with a 250 x 4.6 mm i.d. column packed with Chromsil C_{18} 10 μm particles. A 39:57:4 mixture of acetonitrile-isopropyl alcohol-water and a 9:1 mixture of acetone-water were used as the mobile phase, at a flow rate of 1 ml/min. The separated pigments were detected at 438 nm.

Organic acid separation. The same liquid chromatograph as described above was used. The standards and the organic acids in the tomato fruit were separated on a 250 x 4.6 mm i.d. column packed with LiChrosorb C_{18} (10 μm) using 0.75 nM tetrabutyl ammonium hydroxide in 97.5:2.5 0.01 M KH_2PO_4-methanol as the mobile phase, at 1 ml/min. Detection was done at 225 nm.

Separation of carbohydrates. A Liquochrom 2010 series liquid chromatograph equipped with a Model 312 Liquopump (Labor MIM) a refractive index detector (KNAUER) and a Model OH-814/1 potentiometric recorder was used. The sugars were separated on a 250 x 4.0 mm i.d. column containing LiChrosorb NH_2 (5 μm), using 8:2 acetonitrile-water as the mobile phase, at 1 ml/min and 50°C.

Peak identification

Identification of tomato fruit pigments was based on the retention times and was compared with available standards such as β-, ζ-carotene and lycopene. Since retention times alone are not sufficient for accurate identification, stopped-flow visible spectra of the chromatographic peaks were also obtained using the scanning system of the Beckman equipment. The maxima between 300-700 nm of each compound were compared with literature data (20-23).

The peaks of organic acids and sugars were identified according to the retention times in comparison with the standards as well as by co-chromatography with the standards. Direct scanning of the organic acid peaks was also achieved between 200-300 nm for both standard and sample organic acids.

RESULTS AND DISCUSSION

Separation of pigments

Acetone has been used as the mobile phase for the separation of carotenoids of plant origin in reversed-phase C_{18} (24-25). However, the use of acetone with C_{18} adsorbent was found to have many disadvantages: (a) It loosens the C_{18} phase causing a decrease in the separation ability; (b) in the presence of acetone many coloured substances are strongly bond on the functional group of the adsorbent; and (c) it promotes oxidation of some pigments during the separation process.

The changes in the retention times of many pigments from tomato fruit after several injections into a Chromsil C_{18} column eluted with 9:1 acetone-water can be seen in Table I. It was found that the retention time of some pigments such as lutein increased while the retention times of lycopene violet xanthophyll and ß-carotene decreased when acetone-water was used as the mobile phase. Unstability of the capacitiy factor with acetone elution necessitated the use of a new mobile phase for the HPLC separation of pigments with a minimal harmful effect on the C_{18} column. Therefore, many eluents having similar polarity as acetone-water have been applied for the separation of tomato fruit pigments. Among several eluent mixtures 39:57:4 acetonitrile-isopropanol-water was found as the best for this purpose. Stability of retention times, a capacity factor range of 1.07-10.24 and short elution time are some of the advantages of this mobile phase (Tables I and II).

The different pigments were identified at different stages of ripening of the tomato fruit by direct scanning of the absorption maxima of each pigment separated on C_{18} column with the new mobile phase and comparison with values published in literature. This method is rapid and solves the problem when two components have the same retention time but appear at different stages of ripening such as pyropheophytin B and violet xanthophyll which differ in their absorption spectra.

Application of the newly elaborated HPLC system to the simultaneous fractionation of tomato fruit pigments resulted in an additional information about the nature of the pigments

Table I. Change in retention time of some pigments during several injections

No injection	Acetone-isopropanol-water 39:57:4				Acetone-water 9:1			
	Lutein	Violet xanthoph	Lycopene	β - Carotene	Lutein	Violet xanthoph	Lycopene	β-Carotene
	Retention time (minutes)				Retention time (minutes)			
5	4.47	5.60	7.57	10.240	4.1	7.6	12.56	17.2
10	4.45	5.61	7.59	10.244	4.22	7.45	12.16	17.0
15	4.45	5.59	7.63	10.23	4.36	6.88	12.08	16.75
20	4.45	5.61	7.58	10.25	4.42	6.75	11.9	16.55
25	4.48	5.62	7.62	10.27	4.49	6.5	11.75	16.44
Δ*	+0.03	+0.03	+0.06	+0.03	+0.39	-0.43	-0.81	-0.8

Δ*The difference between maximum and minimum retention time after 25 injections

Table II. Retention time (t_R) and capacity factor (k')* of tomato fruit pigments separated by HPLC

Pigments**	Acetone-isopropanol-water		Acetone-water	
	t_R	k'	t_R	k'
Zeaxanthine	3.72	1.07	3.06	0.63
Lutein	4.47	1.50	4.1	1.21
Chlorophyll B	5.08	1.83	5.78	2.05
Violet xanthophyll	5.60	2.11	7.02	2.74
β-Pyropheophytin	5.9	2.30	7.6	3.0
Lycopene	7.57	3.22	12.56	5.6
cis Neurosporene	8.15	3.55	13.05	5.9
Neurosporene	8.76	3.85	13.66	6.2
β-Carotene	10.24	4.7	17.2	8.05

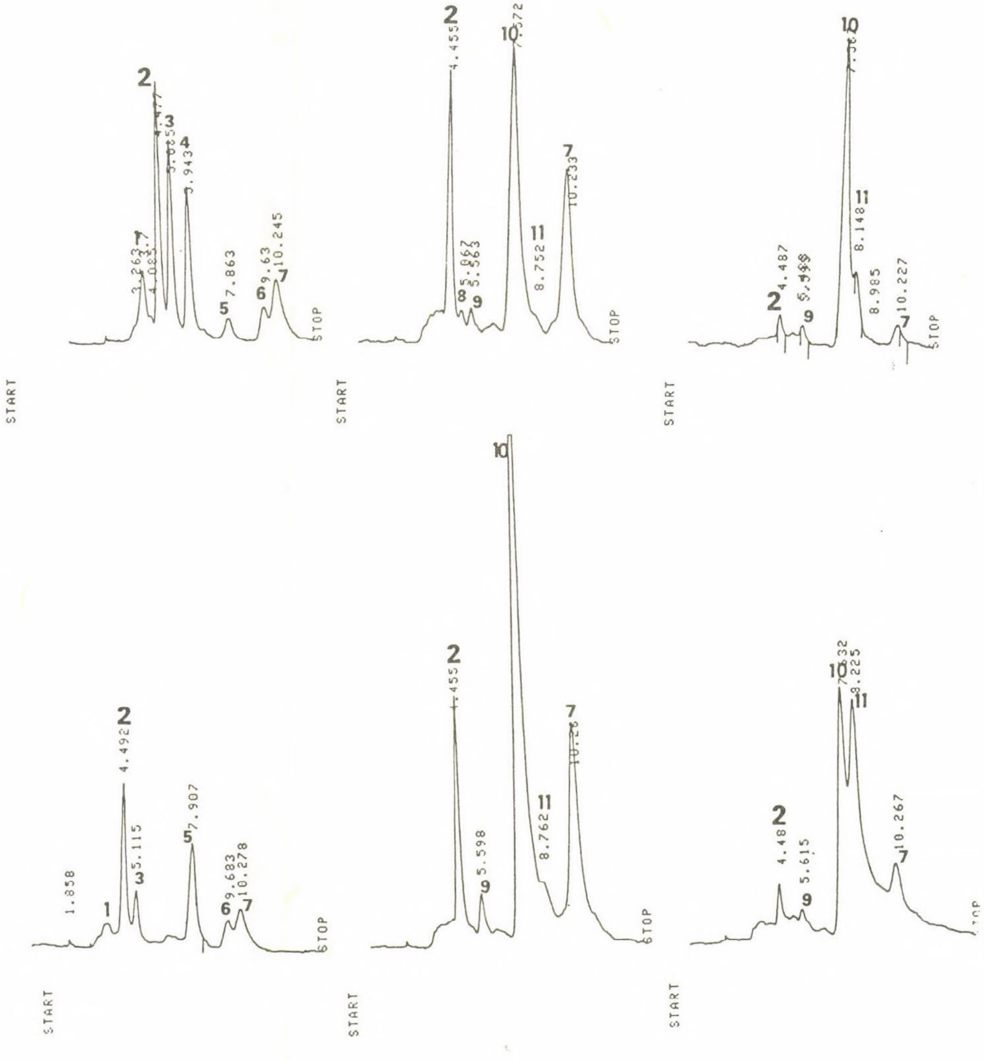
* $k' = \frac{t_R - t_o}{t_o}$ where t_o = retention time of the solvent.

Zeaxanthin, lutein, chlorophyll β, pyropheophytin and cis neurosporene were found in green fruits, whereas, violet xanthophylls, lycopene, neurosporene and β-carotene were found in orange and red fruit.

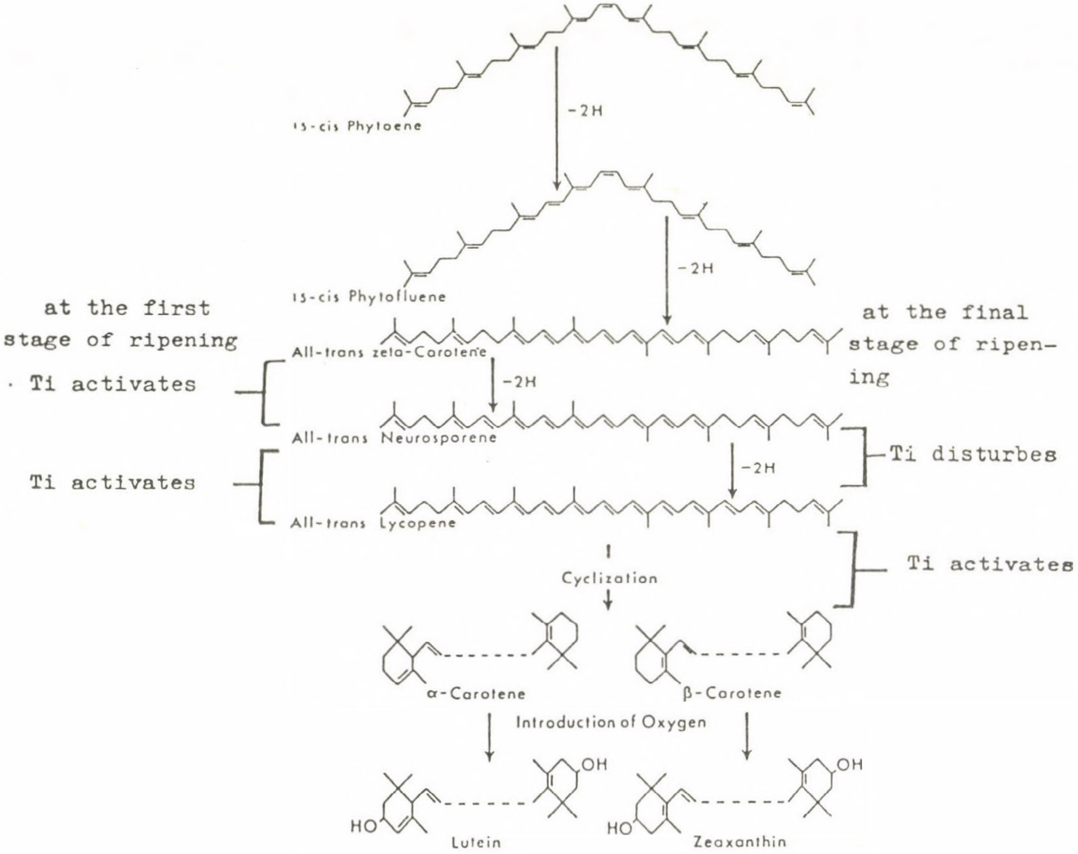
and their metabolic changes during the stages of ripening. Among several pigments zeaxanthin, lutein, chlorophyll B pyropheophytion B, neoxantin, lycopene, neurosporene, and β-carotene were separated and identified (Fig. 1). The peak at a retention time of 9.6 min was found to have two areas of visible spectra, the first at 405 nm and 652 nm while the second was at 470 nm, 503 nm and 534 nm. These maximum values are due to a chlorophyll derivative and carotene isomers, so, this peak may be due to chlorophyll-carotenoid complex which disappears when the fruit becomes yellow.

Biosynthesis of carotenoids in tomato fruits was found to start with the conversion of chlorophyll to pyropheophytin followed by accumulation of cis-neurosporene. Disappearance of cis-neurosporene was accompanied by the accumulation of neurosporene and lycopene in yellow fruit indicating that isomerization reaction of cis to trans neurosporene was necessary for the subsequent development of red pigments. The proposed reactions of the first steps of chlorophyll discolourisation includes conversion of chlorophylls to pyropheophytins followed by rapid deamination reactions leading to accumulation of phytoene and phytofluene. The next steps are the same with the general scheme of carotenoid biosynthesis shown in Fig. 2 with the exception that cis-neurosporene accumulated at the beginning of chlorophyll decolourisation phase followed by the appearance of trans neurosporene indicating that isomerization of cis to trans form is required for the development of yellow and red pigments.

Effect of titavit treatment (spraying the plant twice with Titanium ascorbate) on the biosynthesis of carotenoids is also shown in Fig. 3. The treatment caused an increase in the rate of biosynthesis of the yellow and red pigments at the first stages of ripening but, at the final stage the lycopene content on the treated fruits was lower than of the control samples. On the other hand the β-carotene content of treated samples was three times higher than of the control fruits. These observations led to the suggestion that titanium affected the biochemical pathway of carotenoid synthesis at several points as shown in Fig. 2. The effects of titanium on pigment biosynthesis can be summarized as:



<u>Fig. 1</u>. Typical HPLC chromatograms of tomato fruit pigments extracted at different stages of ripening. 1 = Zeaxanthin, 2 = Lutein, 3 = Chlorophyll B, 4 = Pyropheophytin B, 5 = Cis-neurosporene, 6 = Unidentified chlorophyll-carotenoid complex, 7 = β-carotene, 8 = Neoxanthin, 9 = Lycoxanthin, 10 = Lycopene, 11 = Neurosporene



at the first stage of ripening

Ti activates

Ti activates

at the final stage of ripening

Ti disturbes

Ti activates

Fig. 2. Symplified pathway of carotenoid biosynthesis with the proposed attack of Titavit treatment

(a) The treatment promotes biosynthesis of pyropheophytion in green fruit.
(b) Conversion of cis-neurosporene to neurosporene and then to lycopene was faster in treated fruit at the first stage of ripening.
(c) At the final stage of ripening lycopene biosynthesis was disturbed to some extent in treated fruit with high increase in neurosporene and β-carotene concentration.

These results indicated that titanium treatment disturbed the dehydrogenation of neurosporene to lycopene and/or promoted the cyclization of lycopene to β-carotene at the final stage of ripening.

Determination of organic acids

In a previous work Biacs and co-workers (26) elaborated a new ion-pair chromatography system for organic acid separation. In this system tetrabutyl ammonium served as the coupling ion and phosphate as the counter ion. The separation was improved by increasing the concentration of the coupling ion and adding methanol to the eluent. Application of this system for organic acid determination in tomato fruit and following their metabolic pathways resulted in rapid, accurate and also specific determination without any derivatization. Capacity factor and retention time of several organic acid standards are shown in Table III.

Figure 4 illustrated a typical chromatogram of organic acids in mature, green and red tomato fruits. The first two peaks are a mixture of unidentified water soluble compounds which were unretained on the column. These are followed by glucorunic γ-lacton, an organic metabolite of ascorbic acid biosynthesis, ascorbic acid, iso-ascorbic, malic, tartaric, isocitric, citric, oxalic, fumaric, gallic and an unidentified aromatic acid. Aromatic and ascorbic acids strongly absorb at 225 nm and even although their peaks in the chromatogram are large, the actual amount present is smaller than that of other acids such as citric, tartaric and malic which appear as small

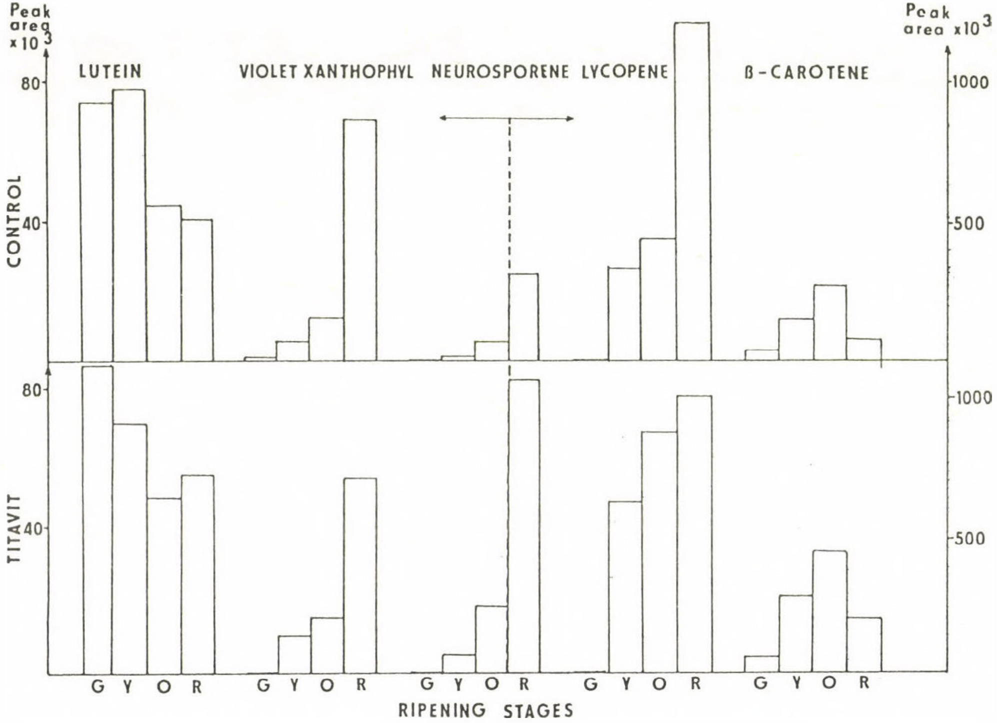
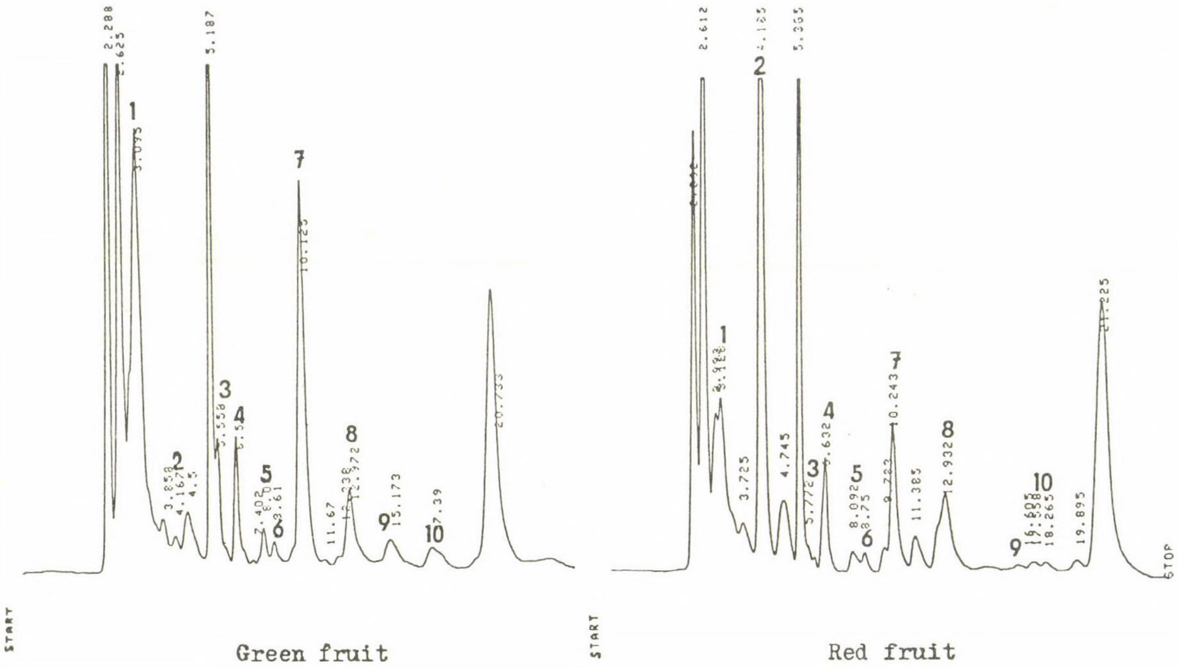


Fig. 3. Changes in the concentration of the major pigments in tomato fruit during ripening. G = green, Y = yellow, O = orange, R = red



Green fruit

Red fruit

Fig. 4. HPLC chromatograms of organic acids extracted from green and red ripe tomato. 1 = Glucorunic-γ-lacton, 2 = Ascorbic acid, 3 = Malic acid, 4 = Tartaric acid, 5 = Isocitric acid, 6 = Succinic acid, 7 = Citric acid, 8 = Oxalic acid, 9 = Fumaric acid, 10 = Gallic acid

Table III. The retention times (t_R) and capacity factors (k') of organic acids

Name	t_R	k'
Glucoronic acid γ-lactone	3.34	0.7
Galacturonic acid	3.66	0.87
Glycolic acid	4.17	1.12
Chinic acid	4.21	1.14
Shikimic acid	4.27	1.18
Glucoronic acid	4.44	1.26
Ascorbic acid	4.67	1.38
Formic acid	4.81	1.45
Iso-ascorbic acid	4.96	1.53
Acetic acid	5.03	1.57
Lactic acid	5.32	1.71
Malic acid	5.86	1.98
Tartaric acid	6.66	2.39
Iso citric acid	7.34	2.74
Succinic acid	8.15	3.15
Propionic acid	9.54	3.87
Citric acid	11.92	5.08
Oxalic acid	12.89	5.88
Malonic acid	13.80	6.04
Fumaric acid	14.64	6.48
Oxalacetic acid	16.72	7.53
Gallic acid	18.64	8.51
Isovaleric acid	19.79	9.1

peaks. Green tomato fruit contains large amount of glucuranic γ-lacton and small amount of ascorbic acid. The amount of glucuronic γ-lacton then decreased with a high increase in the ascorbic acid content indicating that glucuronic γ-lacton is an intermediate of ascorbic acid biosynthesis in tomato fruit.

These results show that tomato fruit contain mainly citric, tartaric, ascorbic, succinic, iso-citric and oxalic acid (Table IV).

Table IV. Organic acid content of the tomato fruit at different stages of ripening

Organic acid	Concentration of organic acid in the tomato mg/100 g			
	Green	Yellow	Orange	Red
Ascorbic	1.27	42.35	28.8	19.3
Malic	236	150.5	20.63	23.14
Tartaric	41.9	60.4	102.7	183.6
Succinic	9.4	93.2	75.7	68.8
Isocitric	17	12.6	29.4	32.6
Citric	1555	824	693	604.4
Fumaric	0.35	0.14	none	none
Gallic	1.12	0.345	none	0.22

At the final stages of ripening the ascorbic acid content decreased as a result of oxidation, therefore, the maximum amount of vitamin C could be found in yellow fruit (Fig. 5). The technologically most important acid of tomato fruit is citric. The concentration of citric acid was high in green fruit then decreased with the development of ripening. Other organic acids of tricarboxylic cycle exhibited the same behaviour except tartaric and some unidentified acids which slightly increased. Unlike in sweet potato (27) the inverse correlation between citric and malic acid has not been observed. Being stable at low level malic acid was believed to be the precursor of sugar synthesis in tomato fruit.

HPLC investigation of the organic acid content of tomato fruit resulted in a new information about the effects of Titavit treatment on the biochemical pathways of some organic acids. These effects could be summarized as:

(a) In general Titavit treatment increased the concentration of most organic acids in tomato fruit.

(b) Unlike in control samples the concentration of fumaric and oxalo-acetic acid increased in Titavit-treated fruits revealing that titanium activated the succinic acid dehydrogenase enzyme (Fig. 6).

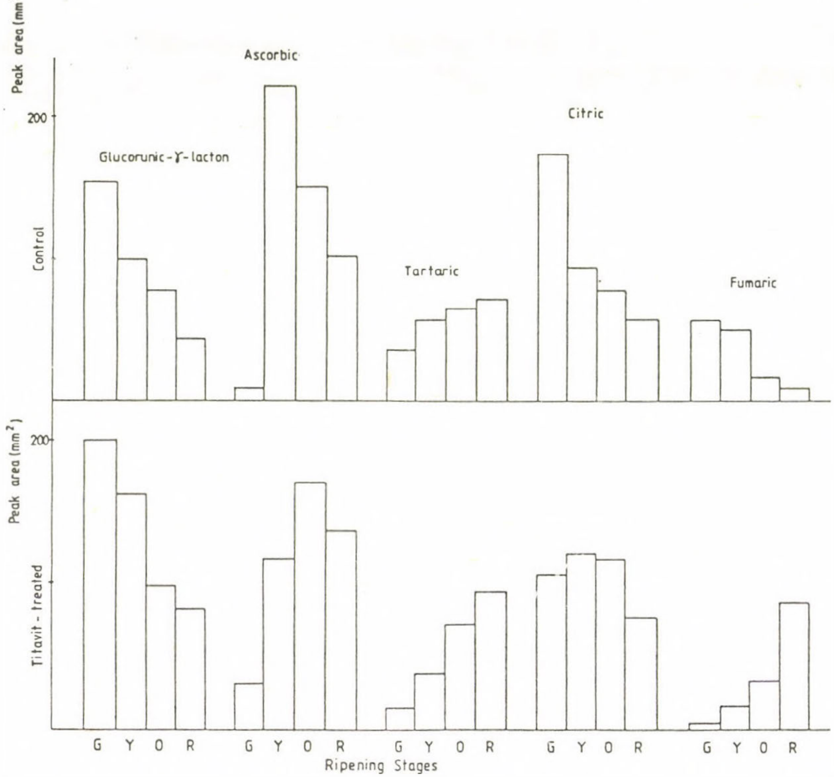


Fig. 5. Changes in the concentration of organic acids content in tomato fruit during ripening. G = green, Y = yellow, O = orange, R = red

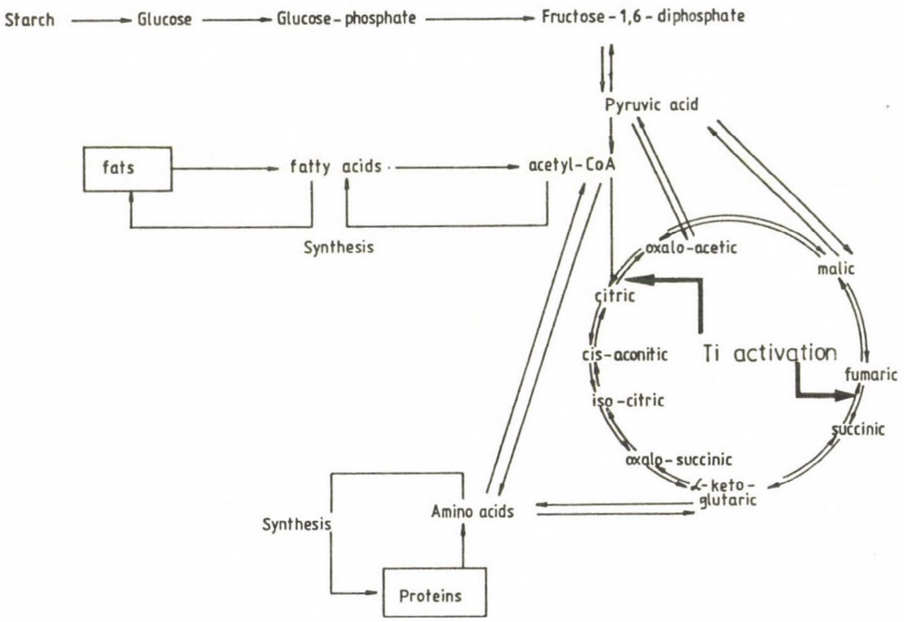


Fig. 6. Biosynthesis of carbohydrates, lipids, and proteins in relation to the tricarboxylic acid cycle, with the proposed attack of titanium treatment

(c) At the final stage of ripening ascorbic acid oxidation in titavit-treated fruits was lower than in the control samples.

(d) Titavit treatment promoted the biosynthesis of some aromatic acids.

These results are of nutritional and technological importance since, as a result of titativ treatment, a significant increase was estimated in the ascorbic and citric acid content of tomato fruit.

Determination of soluble carbohydrates

Detector response to all sugars tested was linear in the concentration range of 0.1-10 %. The variability in the fructose, glucose and sucrose concentration was found less than 2.52, 1.94 and 0.8 % respectively among several injections of the same sample. The detection limit of the system was as low as 0.1 % of each sugar.

The extraction method used for organic acids was also appropriate for sugar extraction and no specific procedure was necessary since organic acids and other impurities are not detectable when refractometric detector is used.

Figure 7 illustrates a typical chromatogram of sugars separated from ripe tomato fruits. The major sugars, fructose and glucose, eluted within 6-8 minutes followed by sucrose which was detectable only at the first stages of ripening.

The presence of fructose, glucose and also sucrose indicated that the biosynthesis of monosaccharides started through sucrose inversion and other metabolic pathways during the biological ripening of the tomato fruit. As the amount of inverted sucrose is not parallel with the amount of glucose and fructose formed during the ripening process, sugar biosynthesis may involve the inverse correlation with some organic acid like malic, as mentioned before.

Glucose concentration decreased when the fruit became yellow, whereas the fructose concentration increased (Fig. 8). This indicates that the conversion of chlorophylls to yellow

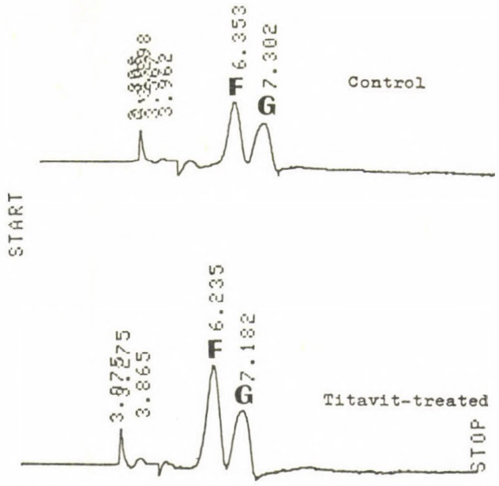


Fig. 7. Typical HPLC chromatograms of tomato fruit sugars.
F = Fructose, G = Glucose

and red pigments required glucose exhaustion as energy source.

Inversion of sucrose to fructose and glucose was faster in Titativ-treated fruits leading to a higher accumulation of invert sugars in comparison with the control samples. During the ripening process of Titavit-treated fruits the subsequent development in carotenoid biosynthesis (in orange fruit) exhausted much fructose. This abnormality in energy requirement supported the suggestion that the normal biosynthesis of red carotenoid has been affected and disturbed by the Titavit-treatment.

During freeze-storage for 6-months sugar exhaustion for energy supply was also much more in the control fruit than in Titavit-treated fruit. This observation indicated that the Titavit treatment improved the storage stability of tomato fruits.

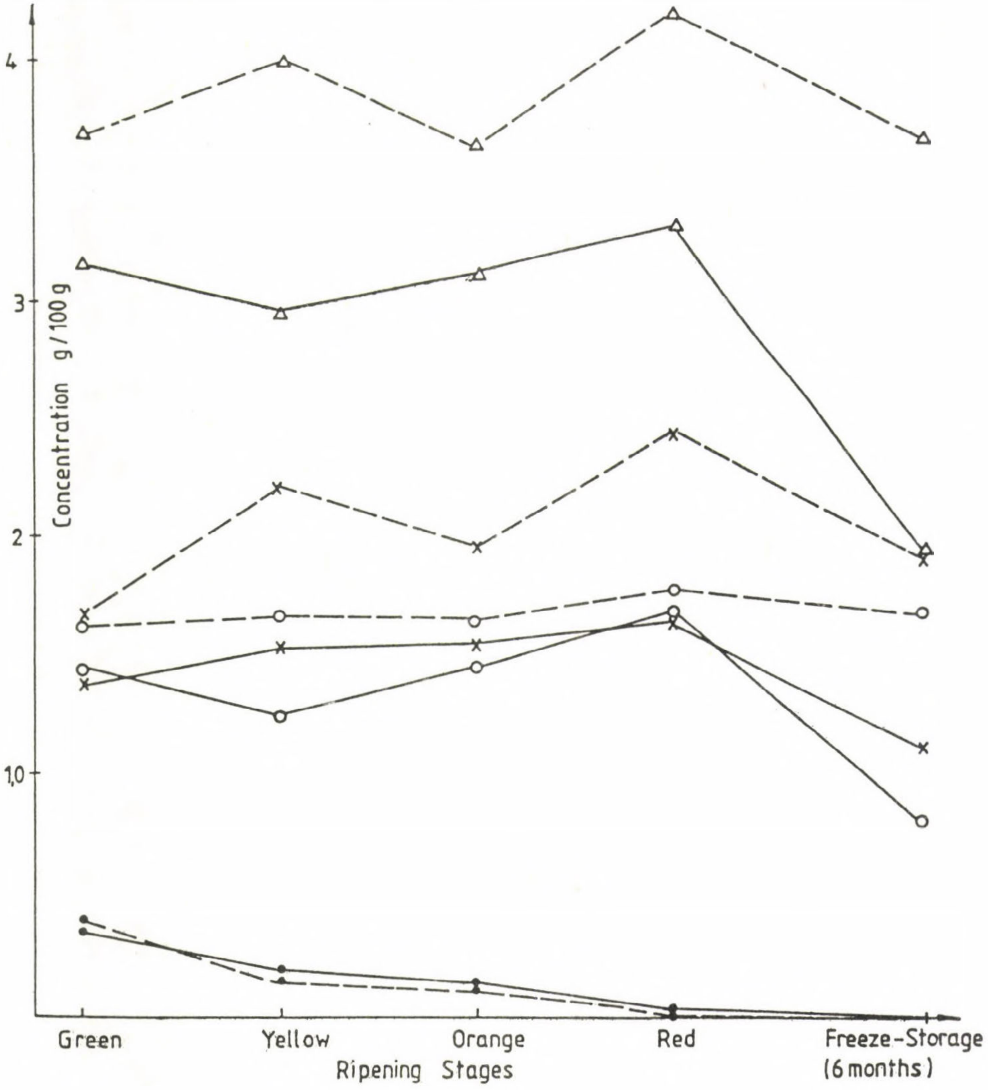


Fig. 8. Changes in the sugar concentration of tomato fruit at different stages of ripening and during freeze storage.
● Sucrose, o Glucose, x Fructose, Δ Total sugars,
---- Titavit-treated, —— Control

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H. Kalász and L.S. Ettre (Eds)

NEW ADVANCES IN CHIRAL STATIONARY PHASES - A REVIEW

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Chromatographic separation of enantiomers is a relatively young but quite active and broad research field.

During the 1985 Budapest Symposium I have discussed this subject with a special emphasis on its pharmaceutical applications (1). Now I would like to be more specific and concentrate on the advances in the field of chiral stationary phases which are used for the direct resolution of optical isomers.

There are many excellent reviews on the chromatographic separations of racemates, discussing among others the direct methods of chiral separation by gas chromatography (2-6) and liquid chromatography (2, 4, 5, 7-12), using different types of stationary phases. The present paper will be limited to the results published in the last two years, with special attention to phases used in high-performance liquid chromatography.

In my opinion, the greatest breakthrough during this time was the commercial introduction of different chiral stationary phases and their availability for the chromatographic resolution of enantiomers of different types of compounds.

Historically the first, introduced even earlier, were columns filled with stationary phases developed by William Pirkle of the University of Illinois. Their chiral selectors were the 3,5-dinitrobenzoyl derivatives of D- and L-phenylglycine and L-leucine bonded to the silica support by the aminopropyl spacer with covalent (Fig. 1.) or ionic linkage. Manufacturers from the United States (Regis, J.T. Baker), Europe (Serva), and Japan (Sumimoto) distribute various types of these phases for analytical and preparative purposes.

Until recently these phases were the most popular and their use is widely documented in the literature. In addition to the pharmaceutical applications mentioned previously (1) additional examples include the resolution of enantiomers of amphetamine (13), N-acylated heterocyclic amines (14), hydroxy derivatives of polycyclic aromatic hydrocarbons (15, 16), phosphine oxides (17), 2-carboxyindoles and N-aryl-α-amino esters (18), ephedrine and related compounds (19), β-naphthylcarbamate derivatives of pharmaceutically important amino compounds (20), primaquine and its metabolites (21), a new antidepressive compound with cyclopropane moiety (22) and methamphetamine (23).

Recently new stationary phases developed by Pirkle (Fig. 2.) were introduced by Regis Chemical Co. Their chiral cores are D- and/or L-naphthylalanine and naphthylvaline (24). Columns containing these phases separate efficiently the dinitrobenzoyl derivatives of amide, carbamate and urea types obtained from amines, amino acids, alcohols and thiols by an appropriate derivatization with 3,5-dinitrobenzoyl chloride or 3,5-dinitrophenyl isocyanate (24).

Daicel Chemical Industries Ltd. offers different chiral columns based on poly(triphenylmethyl methacrylate)(Chiralpak OT and OP), cellulose derivatives (Chiralcel OA, OB, OC, OE and OK) and ligand-exchange stationary phases with amino acid-copper complexes (Chiralpak WH and WM). The chemical structures of Chiralpak (+)OT and (+)OP are shown in Fig. 3.

These stationary phases owe their chirality and recognition properties to the helicity of the polymer. Recently published papers of Okamoto et al. (25, 26) give a deeper insight into their properties, application range and also provide references to earlier studies. Applications of (+)poly (triphenylmethyl methacrylate) to optical resolution of some stilbestrol analogues (27) and 10,10'-dihydroxymethyl-9,9'-biphenanthrene (28) have also been reported.

Chiralcel stationary phases (Fig. 4.) are differently modified ether or ester type cellulose derivatives bonded to macroporous silica. Their synthesis and performance were

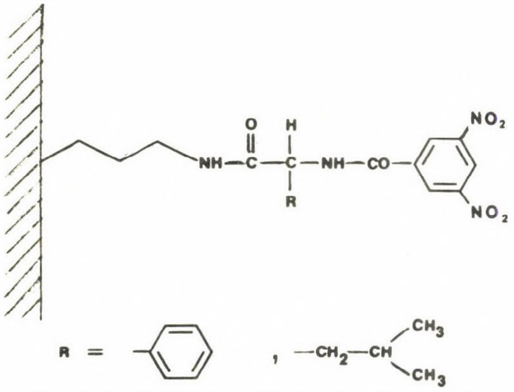

Fig. 1. Covalently bonded stationary phase of Pirkle

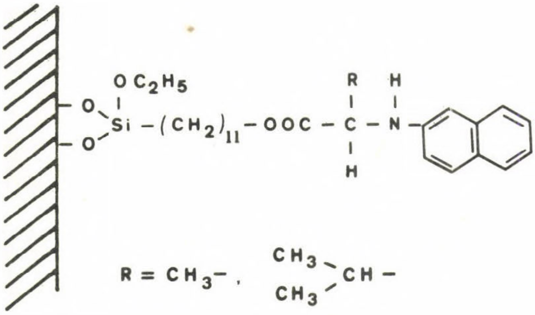

Fig. 2. New stationary phases of Pirkle

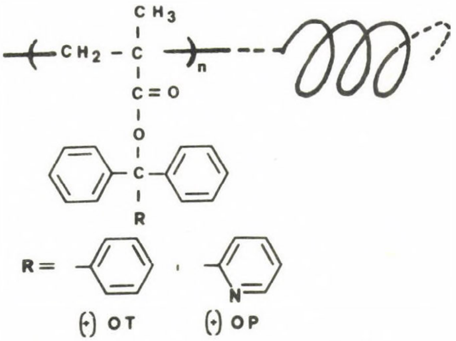

Fig. 3. The chemical structure of Chiralpak (+)OT and (+)OP stationary phases

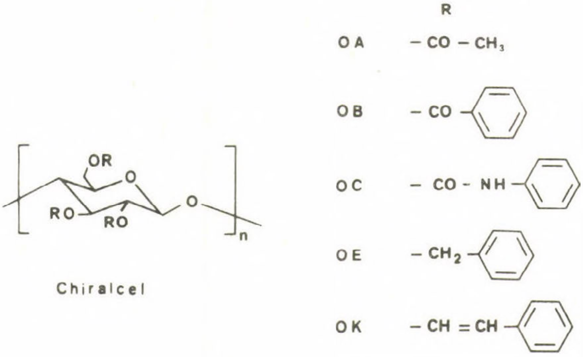

Fig. 4. Chiralcel stationary phases

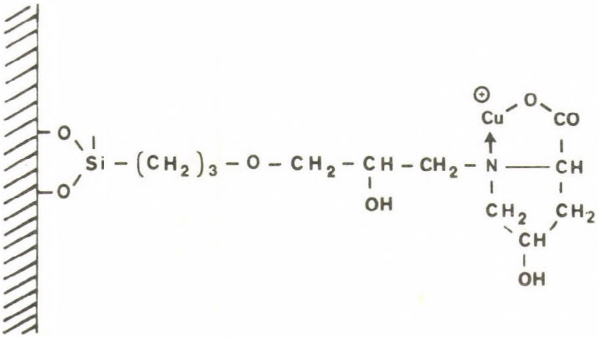

Fig. 5. Nucleosil Chiral-1 stationary phase

described in 1984 (29) and recently Wainer et al. reported successful resolutions of enantiomers of amides (30) and sulfur compounds (31) on these phases. Manufacturer's brochures provide further examples of separations of enantiomers on Chiralcel and Chiralpak packings (32).

Among other commercially available chiral stationary phases we can list Cyclobond I, which has β-cyclodextrin covalently bonded to silica gel, distributed by Advanced Separation Technologies Inc. (USA). A similar phase is also distributed by Serva. This phase was synthesized by Armstrong (33, 34) and several studies have been published on its use for the separation of enantiomers of dansyl derivatives of amino acids (35), drugs (35, 36), metallocenes (37) and binaphthylidyl crown ethers (38). A significant improvement was recently announced in the columns containing this phase (39) but closer experimental details have not been disclosed.

Enantiopac and Resolvosil columns, manufactured by LKB and Macherey and Nagel, respectively, represent chiral stationary phases with the support coated with proteins. These phases are classified as "affinity phases" (40) because their separation mechanism is based on the principle of bioaffinity, and as such, strongly depends on several factors which influence the protein structure and function, including the temperature, pH, ionic strength and concentration of the organic modifier in the mobile phase.

In the Enantiopac column the protein is α_1-acid glycoprotein (orosomucoid), broadly investigated by Hermansson (41) and others (42) for the analytical and pharmaceutical applications in the chiral separation of different drugs.

Bovine serum albumin is a chiral selector in the Resolvosil column, designed on the basis of studies of Allenmark et al. (43). Many compounds, mainly of biochemical and pharmacological interest, were successfully resolved on this chiral phase (44-46).

Macherey and Nagel also offer Nucleosil Chiral-1 stationary phase (Fig. 5.) which separates enantiomers according to the principle of chiral ligand exchange chromatography (CLEC).

The immobilized complex of hydroxyproline and copper can resolve racemic mixtures of several amino acids.

The stereoselectivity of the chiral stationary phase consisting of (2S, 4R, 2'RS)-4-hydroxy-1-(2'-hydroxydodecyl) proline-copper complex is also based on CLEC (47). This phase covers the reversed-phase type silica gel and forms a thin layer on the chiral plate, used for the thin-layer chromatographic separation of enantiomers. The resolutions of enantiomers of amino acids (48), L-DOPA (49), penicillamine derivative with thiazolidine structure (50), methyldopa (51) and stereoisomeric dipeptides (52) are examples for such separations. The same manufacturer also distributes cross-linked acetylated

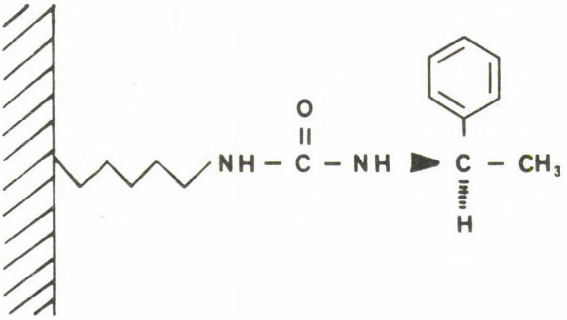

Fig. 6. Supelco LC-(R)urea stationary phase

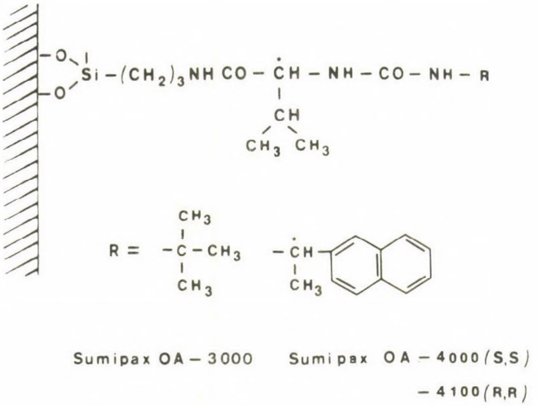

Fig. 7. Examples of Sumipax stationary phases

cellulose for chiral separations.

Supelco, Inc. introduced a chiral stationary phase under the name Supelcosil LC-(R)urea. Its structure is given in Fig. 6.

The manufacturer claims good resolution for racemic mixtures of phenylhydantoin-type derivatives of amino acids (53). Phases synthesized by Ôi et al. (54-57) are among the chiral phases distributed by Sumimoto Chemical, Osaka, Japan, under the name of Sumipax with different designations. Typical examples of the chemical structures of these phases are shown in Fig. 7.

Similarly to the phases developed by Pirkle the chiral recognition and separations on these phases are also due to hydrogen bonding, π-π, dipole-dipole and steric interactions.

For those who are seeking for a practical guide among different commercially available chiral stationary phases, the recent review by Däppen et al. (40) may be of excellent assistance. "It gives some information about the recommended eluents and also about the compatibility with achiral phases for column switching. Special hints and possibilities for preparative separations are mentioned" as well (40).

Although the choice among different, commercially available chiral stationary phases seems to be rich, there is a constant effort to design new chiral phases with better properties for the optical resolution of different groups of compounds. The factors contributing to this evolution are easier and more efficient synthesis, lower cost and broader access to the starting materials. A brief review of these studies will be given below, using the classification of phases based on their separation principles, as proposed by Däppen et al. (40).

LIGAND EXCHANGE PHASES

The ligand exchange phase used for the preparation of Chiral-plate was also employed in HPLC for the determination of the enantiomeric purity of D-penicillamine, an important pharmaceutical product (58).

Audebert and coworkers investigated a new, optically active phase (Fig. 8a) which is a copolymer of acrylamide and vinylpyridine monomer substituted by L-proline moiety. This phase was coated on silica and complexed with cupric ions. The packing has been tested for the separation of amino acid enantiomers and compared with another packing of this type with the chiral phase shown in Fig. 8b (59, 60). In general, the separation factors were higher for the former chiral phase.

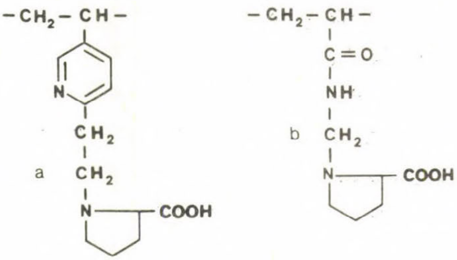

Fig. 8. Stationary phases of Audebert et al. (59)

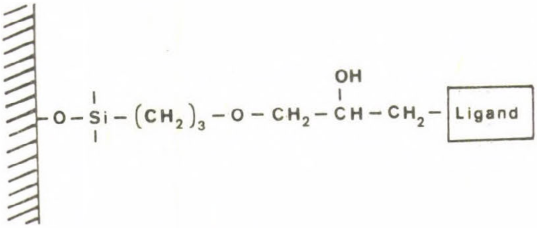

Fig. 9. Phases of Gübitz

Another chiral stationary phase with (+)L-tartaric acid bonded to the silica support via 3-trimethoxysilylpropylamine was used for the determination of enantiomeric catecholamines by CLEC (61).

Gübitz (62) studied the application of chiral stationary phases of the general type shown in Fig. 9. where the appropriate chiral ligand, able to complex metal ions, is bonded to the silica via 3-glycidoxypropyltrimethoxysilane. Those ligands represent mainly amino acids, such as L-proline, L-hydroxyproline, L-valine, L-histidine, L-phenylalanine, but also diamines (L-propylenediamine), amino alcohols (L-ephedrine) and hydroxyacids (L-tartaric acid). These phases were used for the resolution of enantiomers of amino acids and their derivatives, glycyl dipeptides and hydroxy acids (62).

One of the phases of Gübitz, that with L-proline as the chiral ligand, was prepared directly by on-column synthesis and utilized for the resolution of some aromatic α-amino acid racemates. Grierson and Adam claim a reasonable stability of this column, good peak symmetry and selectivity with a reasonable degree of efficiency (63). In all these studies the temperature, pH and mobile phase composition were varied for optimization purposes.

The so-called "clay chromatography", studied by Yamagishi, where the chiral stationary phase is Λ-Ru-tris(1,10-phenantrethroline)$^{2+}$ on montmorillonite, seems to separate also enantiomers of some aromatic compounds according to the CLEC principle (64-65).

HELICAL POLYMER PHASES

Polymers which posses the property of helicity such as e.g., poly(triphenylmethyl methacrylate) or cellulose, may resolve enantiomers due to their steric fit between the layers of the helix.

Cellulose derivatives and other polysaccharides are extensively studied as potential or already used chiral stationary phases. An excellent survey of these studies appeared recently in the Journal of Liquid Chromatography (66).

Triacetylcellulose seems to be the most frequently used material of this type (67). The preparation of the silica support coated with this phase was reported by Okamoto et al. (68), while the separation of enantiomers of a trial drug oxindazac (69) and other drugs (7) or polychlorinated biphenyls (71) exemplify its usefulness.

Francotte and coworkers (72) studied the influence of the supramolecular structure of cellulose triacetate and found that heterogeneously acetylated cellulose is the only form of cellulose triacetate which is able to resolve a broad variety of racemic compounds. The crystal structure of this polymer has an important impact on the chiral discrimination ability.

Mannschreck et al. (73) described preparative separation on triacetylcellulose with an optimization of the particle size and column loading and a use of axial compression of the column to shorten the time of separation. Applications of tribenzoylcellulose (68, 74) and different phenylcarbamate derivatives of polysaccharides (75, 76) as chiral stationary phases were also reported.

Native cellulose was used for the resolution of some chiral indoles (77) and racemic 2,4-dinitrophenyl derivatives of amino acids (78).

Although much work was devoted to the elucidation of the mechanism of chiral discrimination of polysaccharides, their behavior was regarded "as confusing as the case of the five blind men and the elephant" (66). However, due to the relatively cheap and naturally occurring raw material, we can anticipate further research in this field and an expanding range of applications of such phases for the chromatographic separation of enantiomers on both analytical and preparative scale.

CAVITY PHASES

The principle of separation of enantiomers on the so-called "cavity phases" is similar to that for the helical polymer phases, but different steric fit is required for the inclusion of optical antipodes into more or less well-defined cavity structures formed within the polymer matrix or inside the larger molecules attached to the solid support. One of the examples may comprise chiral polyacrylamides and polymethacrylamides broadly investigated by Blaschke and coworkers (9) and used primarily for the preparative resolution of various chiral drugs (70, 79).

Similar phases (Fig. 10.) were synthesized by Japanese investigators and represent a polyamide which has an anti head-to-head coumarin dimer component with different substituents on and between the nitrogen atoms (80) and the polyamides synthesized from (-)1,2-diphenylethylenediamine and dichlorides of appropriate dicarboxylic acids (81).

Different racemic mixtures were resolved on these phases, among others, those of benzoin, mandelamide and 2,2'-dihydroxy-1,1'-binaphthyl.

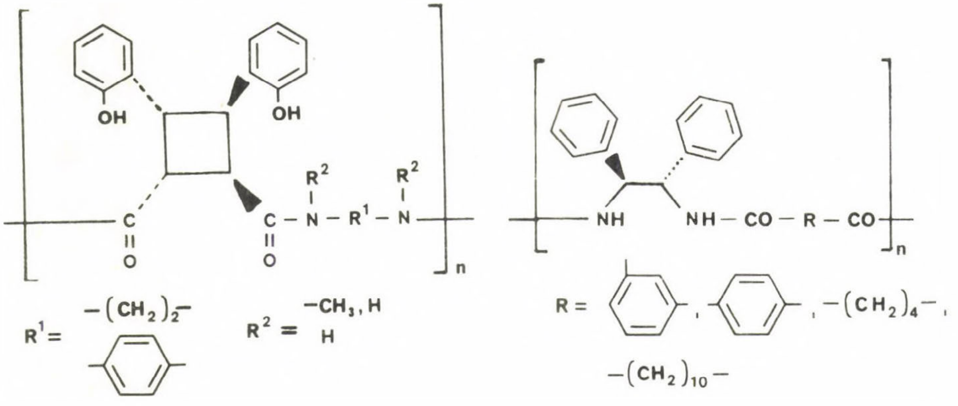

Fig. 10. Chiral polyamide stationary phases

Cyclodextrin molecules are cyclic oligosaccharides formed from glucose units bonded through α-1,4-linkages and designated by α, β, and γ for species with 6, 7 and 8 glucose molecules in the toroid, respectively. The OH-groups of the glucose units may be modified by etherification, esterification or substitution by other substituents, expanding the structural diversity of these phases. Columns commercially available with the β-cyclodextrin stationary phase were already mentioned.

Feitsma et al. (82) investigated the resolution of enantiomers of some aromatic carboxylic acids, structurally related to oxyphenonium, a potent anticholinergic drug, by HPLC on the β-cyclodextrin phase prepared by themselves. They reported a success in the case of cyclohexylphenylglycolic acid and cyclohexylphenylacetic acid and also evaluated the effect of the mobile phase composition, column temperature and sample concentration on the retention time and selectivity; however, the application of these results for the analytical control in pharmacokinetic studies seems doubtful.

A similar phase was used for the optical resolution of mandelic acid esters (83) and effects of structural changes in the chiral structure on the separation factors were discussed.

Modified cyclodextrins were also used as chiral phases. 6-Deoxyamino-β-cyclodextrin has been immobilized through its epoxy glyceride group on hydrophobic gel beads (84), while the so-called "cyclodextrin polymers", i.e. cyclodextrins coupled and cross-linked by appropriate di- or polyfunctional reagents, were used as gel beads for the chromatographic separation of enantiomers of different alkaloids (85-87).

The details on the discrimination of enantiomers on the cyclodextrin phases are still obscure and remain to be elucidated. Although the inclusion complexation is by all means a decisive factor, it is not the only one. For examples, it was reported that the enantiomers of warfarin, an anticoagulant drug, cannot be resolved on the β-cyclodextrin column (36), but their partial resolution was accomplished by complexation with β-cyclodextrin and subsequent chromatography on Sephadex gel (88).

There are also phases with chiral cavities which are produced by the process of imprinting them inside the polymer matrix with the appropriate chiral template molecules. After a removal of these molecules, the chiral cavities thus formed may discriminate between enantiomers of the same or complementary structure. This mechanism is similar to the highly stereoselective enzyme action in biological systems. Recently published papers give excellent examples for the preparation of these phases (89) and the resolution of racemic mixtures of some amino acid derivatives (90).

"BRUSH TYPE" PHASES

Finally, some "brush type" chiral stationary phases will be discussed. The name comes from the similarity of the structures with different chiral molecules attached to the surface of the silica support to this well known object and was originally given to the reversed phases, where hydrocarbon chains of different length were attached to the silica. The previously discussed stationary phases of Pirkle belong to this class and his research group is leading in their synthesis and the evaluation of the chiral recognition mechanism for these chromatographic media.

Figure 11 represents a phase with the chiral selector having 5-α-naphthylhydantoin structure. The structural variatons

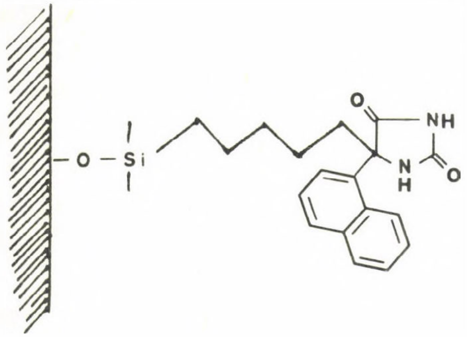

Fig. 11. 5-α-naphthylhydantoin stationary phase

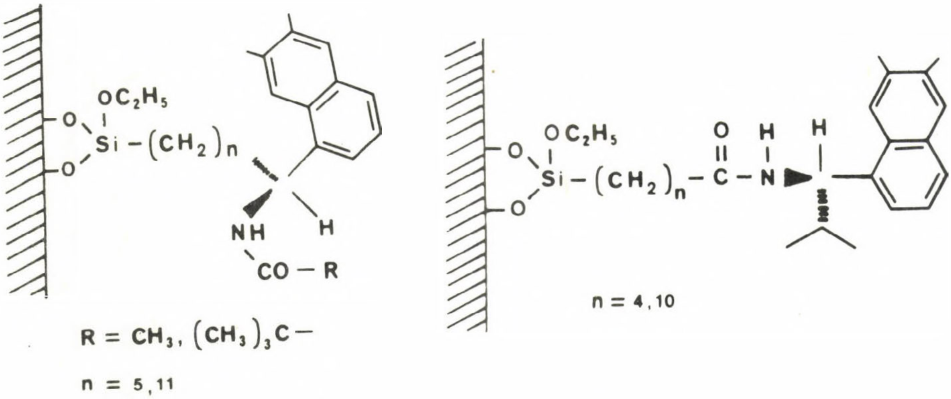

Fig. 12. Phases with α-naphthylalkylamine moiety

include the presence of a methyl group at position 1 of the hydantoin ring or the presence of two methyl groups in positions 6 and 7 of the naphthalene moiety.

Figure 12 shows phases derived from α-naphthylalkylamines (92, 93) where their moieties are bonded to the silica support by varied length hydrocarbon spacer.

On these phases many enantiomers of amino acids and other compounds with amino and hydroxy groups were separated after derivatization with 3,5-dinitrobenzoyl chloride or 3,5-dinitrophenyl isocyanate (93-96). Analogous phases were also synthesized with the urea moiety between the chiral center and the hydrocarbon spacer instead of the amide linkage (97).

It was found that two different chiral recognition processes may be involved in the overall separation mechanism on these phases. These processes are of a competitive character and are based, on one hand, on the dipole-dipole stacking interactions, which lead to intercalation of the hydrophobic part of the enantiomer molecule into "the brush" of the chiral stationary phase, and, on the other hand, on hydrogen bonding interactions of non-intercalative process. The enantioselectivity of these processes leads in opposite directions, what may explain some phenomena of changing the order of elution of enantiomers of even closely related racemic compounds, depending on their structure, which may favor the first or the second process.

The density of the chiral "brush" on the surface of silica gel may also influence the discrimination of enantiomers, depending on the predominant chiral recognition mechanism described above (94).

Besides the interactions just mentioned, other types such as e.g., π-acceptor - π-donor (charge transfer) bonding forces may add to the enantioselectivity effects and therefore, one can often encounter various aromatic moieties in the chiral phases. Many "brush type" chiral stationary phases have been synthesized with this principle in mind.

Figure 13 shows a phase analogous to one of the phases by Pirkle; the only difference is in the substitution of pentafluorobenzoyl moiety for the 3,5-dinitrobenzoyl moiety (98). The strong electron-withdrawing effect of fluorine atoms makes the aromatic ring very electron-deficient and this results in smaller capacity factors in comparison with those obtained on a similarly loaded column prepared with Pirkle's phase and in the higher separation factors for some racemic solutes on the pentafluorobenzoyl phase.

Four new chiral stationary phases, with the general structure and combination of substituents as shown in Fig. 14., were synthesized by Berndt and Krüger (99) and tested for the separation of enantiomers of isopropyl esters of N-(3,5-dinitro-

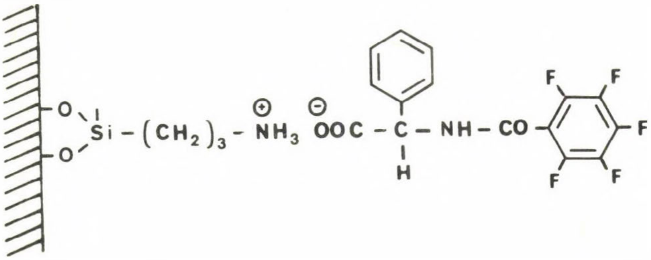

Fig. 13. Stationary phase with the pentafluorobenzoyl moiety

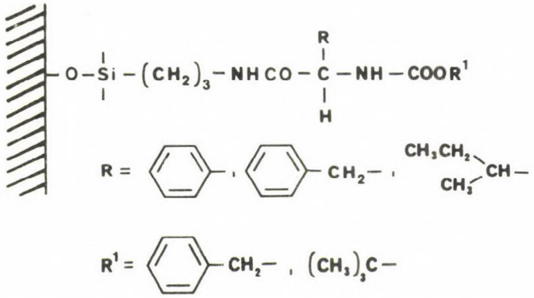

Fig. 14. Phases synthesized by Berndt and Krüger

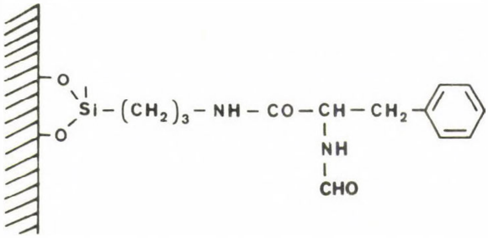

Fig. 15. N-formyl-β-phenyl-α-alanine phase

benzoyl)-α-amino acids. Only the combinations with R being phenyl and R' being benzy or tert-butyl proved to be useful for this purpose.

Benzodiazepine-type drugs, camazepam and temazepam were successfully resolved on the chiral phase shown in Fig. 15., constructed from N-formyl-β-phenyl-α-alanine covalently bonded to the silica gel (70).

Some brush-type phases have not only one but more chiral centers. For the phase shown in Fig. 16., when R = phenyl, there are two chiral centers. The phenylgylcyl moiety has the R configuration, while naphthylethylamine component may be in the R or S configuration. When isopropanol-hexane was used as the mobile phase there were small differences between these phases in the chiral discrimination of enantiomers of 3,5-dinitrobenzoyl amino acid esters, but these differences become significant when dichloromethane was substituted for isopropanol (100). The same chiral selector, 1-(α-naphthyl)ethylamine, was in the chiral phases synthesized recently by Däppen et al.(101).

Two chiral centers are also in the chiral phase designed by Feibush et al. (102)(Fig. 17.).

This structure was carefully planned, taking into account the possibility of hydrogen bond formation between its appropriate parts, such as the nitrogen atoms of the amide or pyridine ring and the complementary substructures of drugs such as barbiturates, glutarimides and hydantoins. Some racemic representatives of these classes were successfully resolved, thus proving the correctness of the applied approach.

Very interesting phases were reported by Rosini and coworkers (103). A molecule of quinine, a Cinchona alkaloid, was used as the chiral selector and they coupled it via its vinyl group with the silica gel support through 3-mercaptopropylsilane spacer. The acetyl derivative of this phase was also prepared (Fig. 18.). These phases show enantioselectivity toward racemic mixtures of binaphthol derivatives. The mechanism of their resolution has also been proposed, taking into account possible interactions between the chiral selector and the selectand molecules.

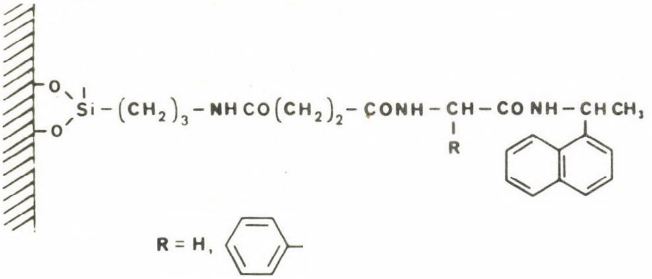

Fig. 16. Chiral stationary phases of Lloyd (100)

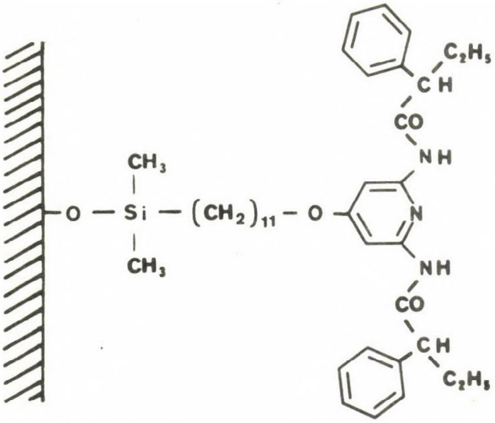

Fig. 17. Chiral phase of Feibush et al. (102)

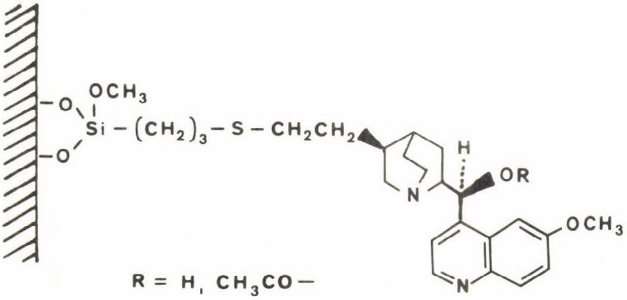

Fig. 18. Chiral phase of Rosini et al. (103)

Another naturally occurring material, D-glucose, served for the preparation of stationary phases shown in Fig. 19.

They were obtained by binding appropriately substituted 1-isothiocyanato-D-glucopyranosides to aminopropyl silica gel (104). Depending on the substituents in the sugar moiety, different enantioselectivities were observed for the derivatives of amino acids and β-adrenoreceptor blocking drugs. It was found that the introduction of an amide function at the C-2 atom facilitates chiral discrimination. Even better results were obtained after derivatization, which made possible additional charge transfer interactions, due to the presence of the dinitrophenyl group.

Figure 20 shows some chiral stationary phases where the chiral selectors were substituted tartramide (105) or N-acyl-L-valine (106) moieties. The chiral resolution on these phases was discussed in terms of the formation of weak hydrogen bonds with appropriate solutes.

Similar stationary phases may be generated "in situ" by dynamic coating of silica gel by chiral reagents with L-valine tert-butylamide moiety (106, 107) added to the mobile phase.

Chiral stationary phases built from L-valine, L-alanine and L-proline tripeptide bonded to the silica gel were also reported, as capable of discrimination of chiral dipeptides (108).

Di-n-butyl-L-tartrate coated on a solid support formed the chiral stationary phase used for the resolution of enantiomers of ephedrine and its analogs by ion-pair chromatography with hexafluorophosphate as the counter ion (109).

CONCLUDING REMARKS

Let us finish this short review of recent research on the chiral stationary phases with some general remarks and an anticipation of the future trends.

There is no doubt that the future will bring us other chiral stationary phases with better properties than those available now. However, we are just at the beginning of more detailed studies on the mechanism of chiral recognition. At

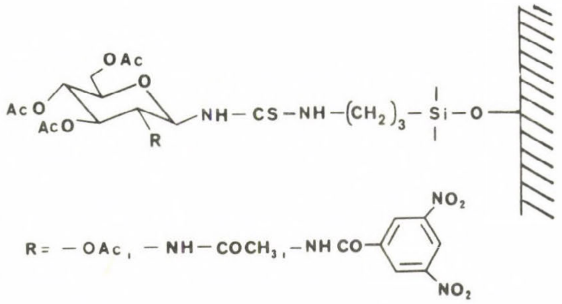

Fig. 19. Derivatives of D-glucose as chiral phases

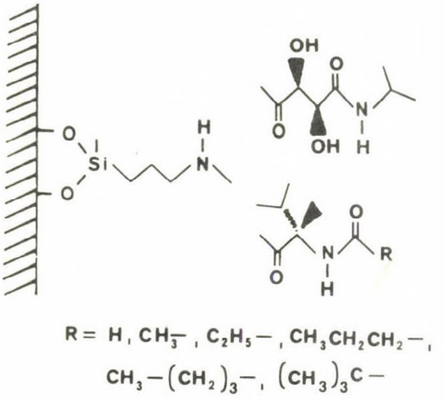

Fig. 20. Chiral phases of Hara et al. (105, 106)

present we are able to understand and use it for qualitative predictions only in rather simple cases. There is certainly a need for broader investigations on the relationship between the chemical structure of the chiral selectors and selectands and the magnitude of chiral discrimination.

A quantitative approach to the problem of chromatographic separation of enantiomers on chiral stationary phases is still a melody of the future. However, probably the first tones of this melody were just sounded when the recently published papers reported the beginning of a theoretical analysis on the different conformations of the stationary phases of Pirkle and on the energy changes during their interconversions (110, 111). Quoting Lipkowitz, Landwer and Darden we can also believe that "with the aid of molecular graphics, molecular mechanics, molecular orbital theory and some common sense we can pinpoint the important interactions in chiral recognition and via computer simulation help design improved versions of the chiral surface used for enantiomer separation" (111).

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DIGITAL HPLC OF NATURAL CIS-DIOL COMPOUNDS

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OBJECTIVES

One of the major goals in modern bioanalytical HPLC is the quantitative detection of single compounds present in highly complex body fluids such as blood, serum, plasma, urine, milk, lymphatic fluid and liquor. Despite its high resolution power, sensitivity, precision and practicability, HPLC analysis of biological matrices is restricted by the pretreatment and processing of an appropriate sample. Often sample preparation represents the weakest chain segment in an analytical HPLC-procedure as it still involves many elaborated, manually performed and thus error-prone and time consuming work-up steps. Thus, the goal in sample preparation should be a rapid and if possible an automated, HPLC-system integrated procedure with a high selectivity and specificity for the analyte. This also implies that the recovery of the analyte should be quantitative and that the purity of the extract should already be as high as possible.

In order to fulfill this criteria the classical liquid-liquid extraction methods are more and more substituted by solid-phase extraction procedures, which use silica- or polymer-bonded phase materials filled in disposable cartridges or small, conventional columns. This strategy allows a sample preparation in a manually or semi-automatically performed off-line mode under reduced-pressure conditions or in an on-line mode under HPLC-system internal conditions. Prerequisite for an on-line sample clean-up is a column switching technique between the sample processing pre-column and a suitable analytical column.

The key to obtaining high recoveries of a purified compound is to identify the bonded phase that is most selective for that compound. The selectivity of a given phase material is determined by the different affinities of the sorbent with respect to the compound of interest and the residual matrix constituents. This is expressed by the different distribution coefficients or capacity factor (k') of the analyte versus a chemically-bonded phase and a mobile phase. Dependent from the relative interaction energies, which occur in the corresponding phase, the analyte will be retained at the stationary phase of the pre-column or it moves with the mobile phase. Taking into account the relative energy amount of the different interactions (ionic, hydrogen bond, dipole-dipole, van der Waals), the ideal interaction with the bonded phase would be a covalent one. Prerequisite for such an ideal sample processing, however, would be only a temporary covalent fixation. This means that a total retention ($k'>1000$) of the analyte is followed by a total elution ($k'<10^{-3}$). This "all-or-nothing" or "on-off" principal for extractive sample processing represents a special kind of high-performance affinity chromatography (HPAC) and can be described as digital HPLC.

Figure 1 shows the investigated biomolecules (unmodified, modified and hypermodified ribonucleosides as well as the parent catecholamines), the quantitation of which in body fluids is of importance in pathobiochemistry and clinical chemistry as these compounds serve as diagnostic marker molecules for a variety of distinct metabolic disorders.

For affinity chromatography, i.e. digital chromatography, the cis-diol system of these compounds was chosen as a selectivity criterion, as this structural moiety reversibly forms under alkaline conditions a cyclic diester with tetrahedral configured boronic acid (1). This biospecific affinity ligand was immobilized via its m-aminophenyl derivative to various gel supports, e.g. agarose, cellulose, polyacrylamide, and used for the manual or partially automated (2) clean-up of ribonucleosides (3-12) and catecholamines (13) under low

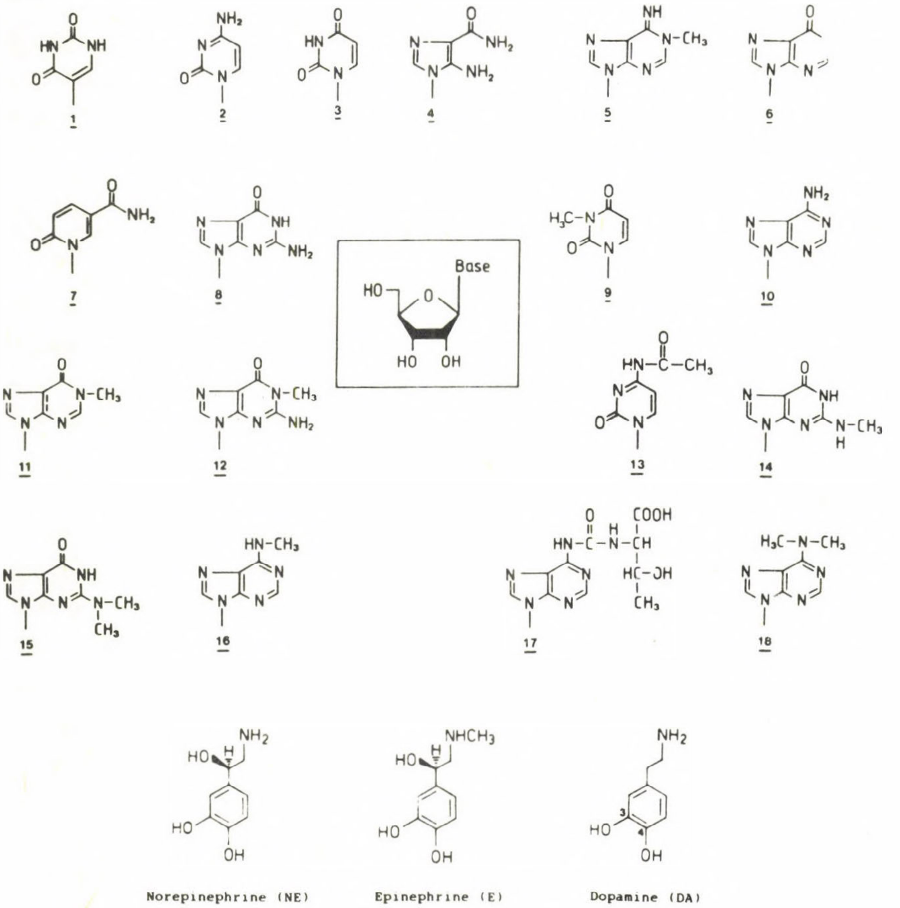

Fig. 1. Structural formula. 1 = pseudouridine (Ψ); 2 = cytidine (Cy^{d}); 3 = uridine (Urd); 4 = 5-amino-imidazole-4-carboxamido-N-ribofuranoside (AICAR); 5 = N^1-methyladenosine (m^1Ado); 6 = inosine (Ino); 7 = guanosine (Guo); 8 = 2-pyridone-5-carboxamido-N-ribofuranoside (PCNR) = 1,6-dihydro-6-oxo-1-(β-D-ribofuranosyl)-3-pyridinecarboxylic amide; 9 = N^3-methyluridine (m^3Urd); 10 = adenosine (Ado); 11 = N^1-methylinosine (m^1Ino); 12 = N^1methylguanosine (m^1Guo); 13 = N^4-acetylcytidine (ac^{4}Cyd); 14 = N^2-methylguanosine (m^2Guo); 15 = N^2-dimethylguanosine (m22Guo); 16 = N^6-methyladenosine (m^6Ado); 17 = N-carbamoylthreonyladenosine (t^6Ado); 18 = N^6-dimethyladenosine (m62Ado)

pressure, i.e. off-line conditions.

After these sample-pretreatment steps, HPLC-analysis of ribonucleosides and catecholamines is usually performed on reversed-phase materials either under isocratic conditions or in a gradient elution mode.

A methodological improvement was achieved when we succeeded with the preparation of a m-aminophenylboronic acid-substituted chromatography (HPAC)(14). On the basis of this affinity material and a column-switching technique, we set up an instrumentally-connected two column liquid chromatographic on-line system that can be used for a system-integrated direct clean-up and analysis of ribonucleosides (15-19) and catecholamines (20, 21) in protein-free fluids.

More recently, we prepared a new bonded-phase material (patent pending) which, for the first time, allows the direct application and subsequent on-line analysis of proteinaceous fluids, such as serum or milk (22). The pre-column material is a chemically modified, semi-rigid gel and allows a simultaneous performance of two different modes of high-performance liquid chromatography. First, by virtue of its gel-permeation properties, macromolecules (e.g. proteins) can be quantitatively separated from the solute (SEC: size-exclusion chromatography). Secondly, by support, high-performance affinity chromatography (HPAC) or digital HPLC of cis-diol containing compounds can be carried out.

In cooperation with E. Merck (Darmstadt, FRG) we finally built a fully automated and commercially available HPLC-analyzer for ribonucleosides and catecholamines. This unique device is distinguished by its practicability with respect to routinely quantifying and profiling these compounds in the biological fluids of individuals with different diseases and thus should encourage more extensive research in the biochemical as well as in the clinical field.

ANALYTICAL PROCEDURE

Instrumentation

The HPLC-analyzer is built up with modular units from E. Merck (Darmstadt, FRG). As shown in Fig. 2 the apparatus is composed of a L 5000 LC-controller Model (LC-C), two Model 655A-12 pumps (P1, P2), a Model 655A-40 autosampler (AS), a Model ELV 7000 automatic valve switching system (ASV), a Model F-1100 fluorescence detector (FD) for catecholamine analysis, a Model 655A-22 UV-detector (UV) for ribonucleoside analysis, a Model D-2000 integrator (I), a precolumn (PC) and an analytical column (AC). For the optional use of post-column derivatization the Model 655A-13 reaction-system (RS) was integrated.

In order to perform a dual-column switching technique the pre-column and the analytical column were connected via an automatic six-port valve, the configuration of which is shown in Fig. 3.

This instrumental set-up allows the independent use of the basic HPLC-system besides the dual mode. Thus, reliability of the overall system performance can easily be controlled by comparing the off-line (RPLC) analysis of a standard mixture with the on-line (HPAC-SEC/RPLC) analysis of an adequate sample.

For post-column fluorogenic derivatization of the catecholamines norepinephrine and epinephrine to the corresponding trihydroxyindole derivative (cf. Fig. 5) a reaction system, which is illustrated in Fig. 4, is placed between the analytical column outlet and the fluorescence detector.

The aromatic cathecholamine ring system is stepwise oxidized in the first knitted reaction coil to the corresponding adrenoquinone (1) and adrenochrome (2) respectively (cf. Fig. 5) by the action of a buffered ferricyanide solution (E), which is delivered by the synchronized pump 1 (SP-1).

In order to stabilize the fluorophore generated at the first step as well as to destroy excess ferricyanide a reducing agent (ascorbic acid) is added by pump 2 (SP-2) before the alkalization process (cf. Fig. 5).

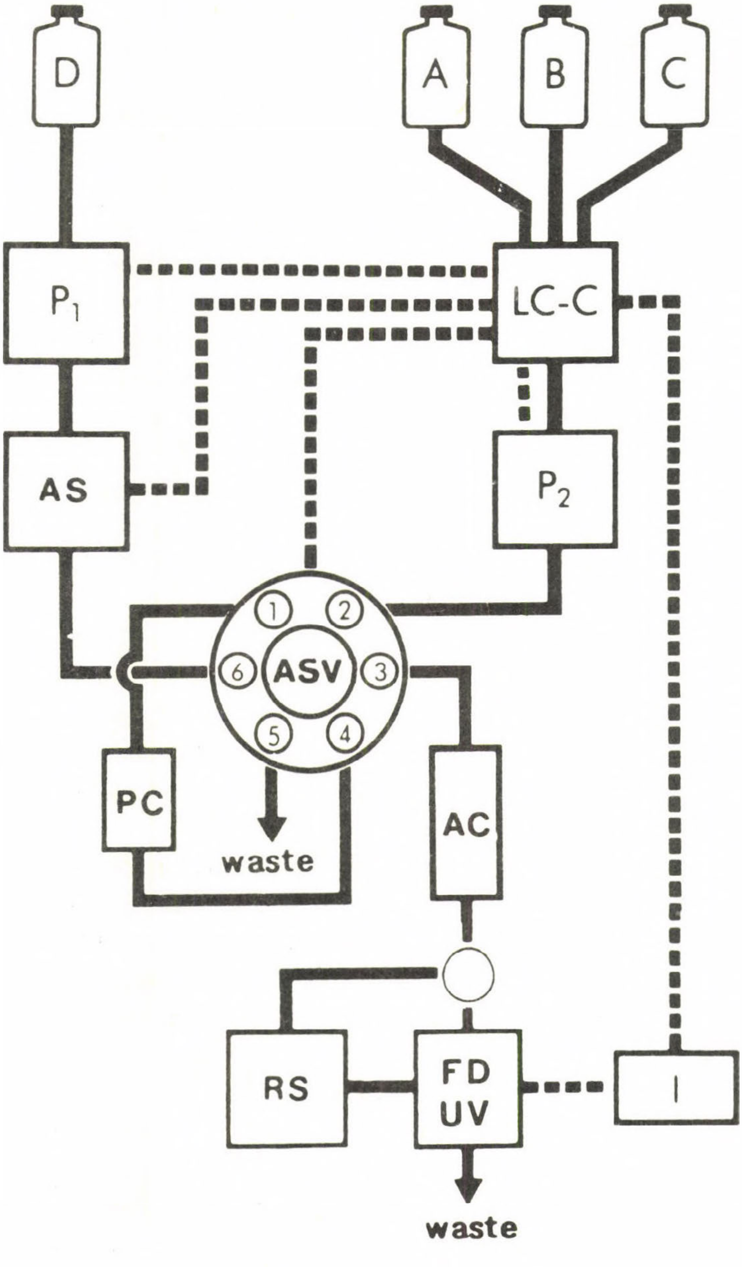

Fig. 2. Apparatus flow-diagram

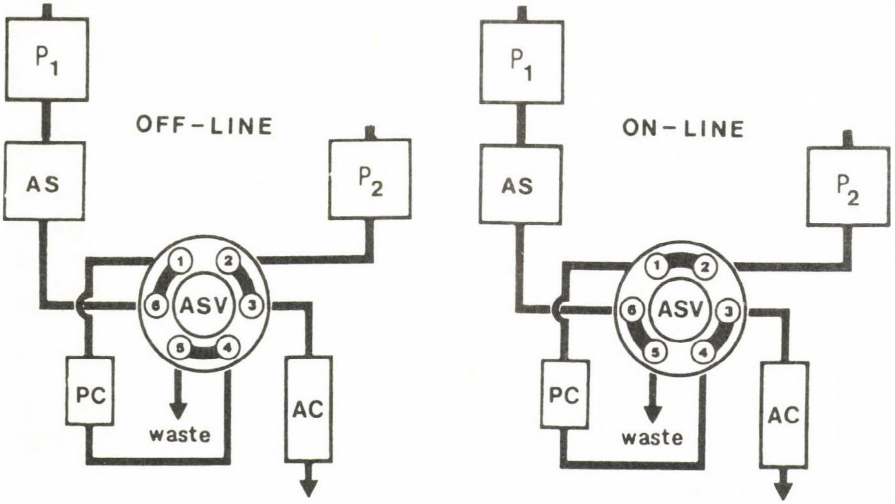

Fig. 3. Valve switching positions. Left: off-line mode; pre-column (PC) and analytical column (AC) can be run separately. Right: on-line mode; pre-column and analytical column are series-connected through valve positions 2-1-4-3. P1, pump 1; P2, pump 2; AS, autosampler; ASV, automatic switching valve

The intramolecular rearrangement of the adrenochrome is induced by NaOH in the third reaction coil. This process generates stabile trihydroxyindole derivatives (3) with high fluorescence quantum yield at an excitation wavelength of 410 nm and an emission wavelength of 520 nm.

Digital Chromatography

Figure 6 schematically shows the chromatographic properties of the newly developed pre-column material for the on-line sample processing, i.e. digital chromatography of cis-diols. The stationary phase is a chemically modified, semi-rigid gel which tolerates pH-values from 1 to 12 and a back pressure up to 2000 psi.

The column material allows the simultaneous performance of two different modes of high performance liquid chromatography.

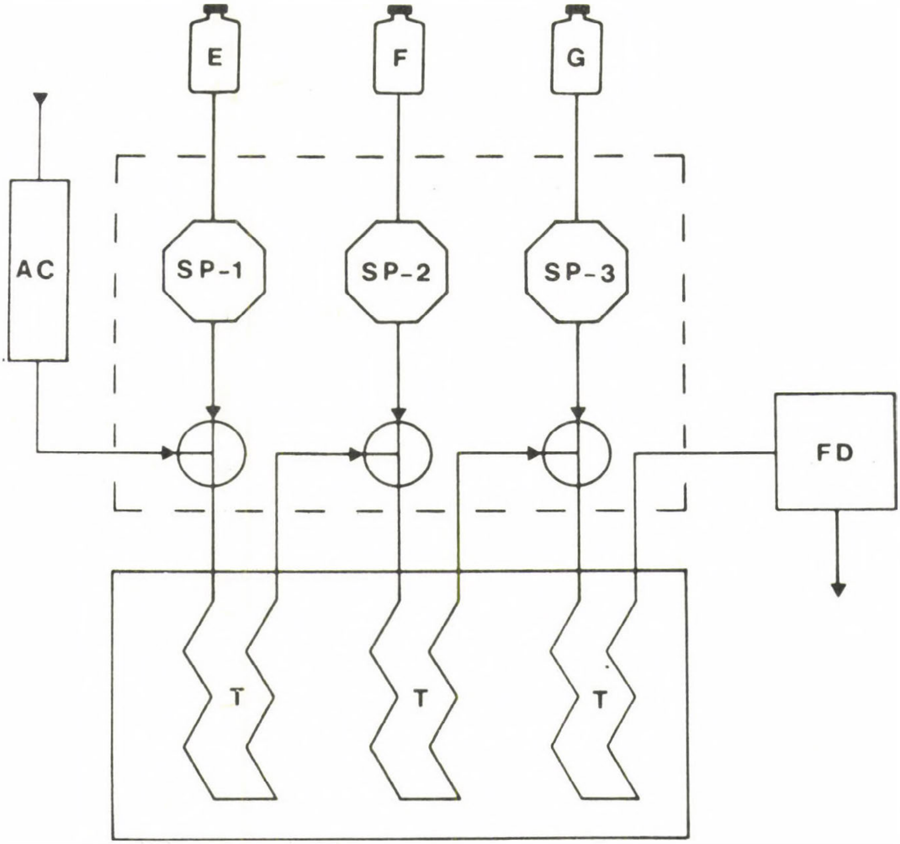

Fig. 4. Chemical reaction-system for the post-column derivatization.
AC, analytical column;
FD, fluorescence detector;
SP, synchronized pumps;
T, thermostatic reaction coils;
E, potassium hexacyano-ferrate III;
F, ascorbic acid;
G, sodium hydroxide

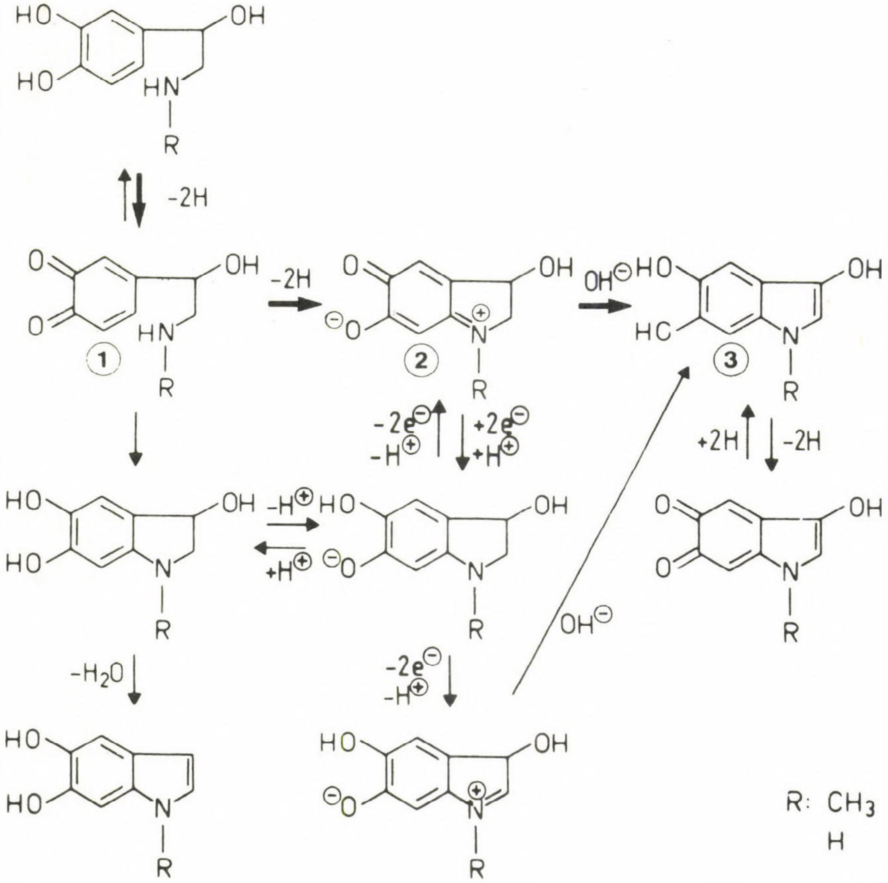

Fig. 5. Reaction diagram for the generation of trihydroxy-indole derivatives.
(1), adrenoquinone;
(2), adrenochrome;
(3), trihydroxyindole

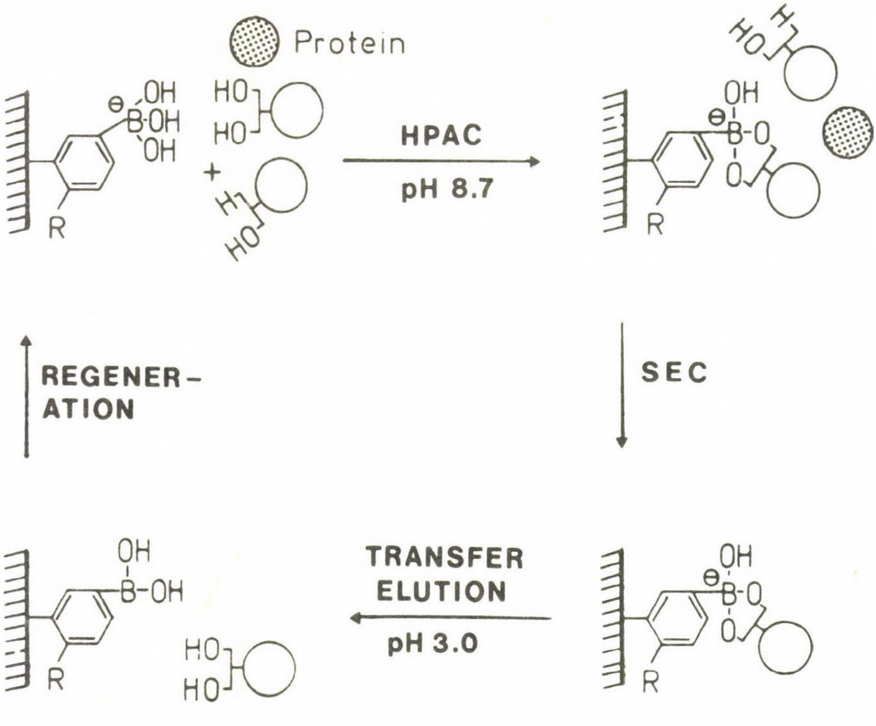

Fig. 6. Chromatographic properties of the pre-column. HPAC, high-performance affinity chromatography; SEC, size-exclusion chromatography

First, by virtue of its size-exclusion (SEC) properties, macromolecules (e.g. proteins) can be quantitatively separated from the solute.

Secondly, by immobilizing a specifically modified phenylboronic acid to the gel support, high-performance affinity chromatography (HPAC) can be performed. The affinity ligand, i.e. tetrahedral configured boronic acid, is capable of a pH-controlled, i.e. reversible, covalent and thus digital interaction with the diol group of catecholamines and ribonucleosides by the chemospecific formation of a cyclic boronate ester (cf. Fig. 7).

Although the binding characteristic of boronic acid with vicinal diols is highly selective, secondary interactions of the bonded phase with other functional groups can cause an

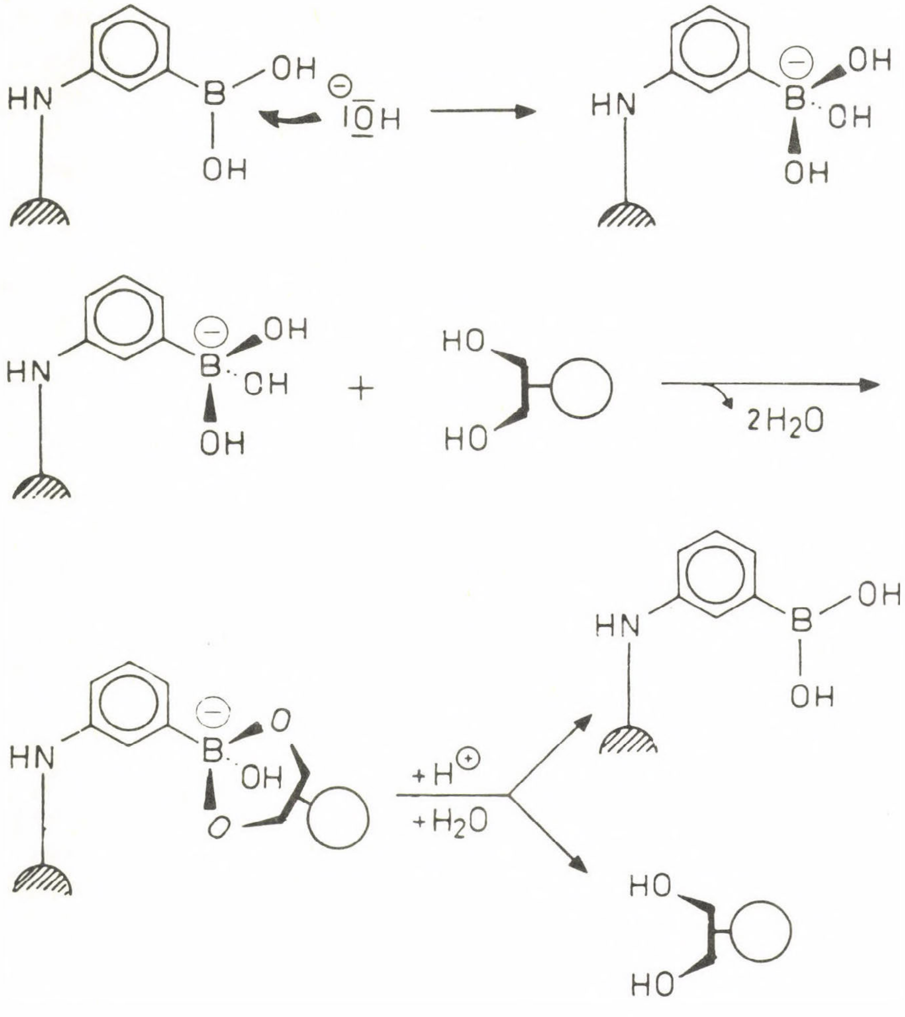

Fig. 7. Principle of boronic acid-based digital chromatography

unspecific retention of sample contaminants. These interactions (hydrogen bonding, charge transfer complexes, ion exchange phenomena, hydrophobic interactions) have been diminished by an optimized buffer composition, molarity and pH value. In order to keep the elution volume of the analytes from the pre-column at a minimum, i.e. one column bed volume, the acidic elution buffer was optimized with regard to pH value, ionic strength and capacity. In addition, this buffer had to serve as mobile phase for the subsequent analytical separation of the investigated diols.

Analysis Cycle

In principle, the desired group-selective prefractionation and on-line clean-up of ribonucleosides and catecholamines is carried out by a simple pH-step elution, followed by the analytical resolution under reversed-phase conditions.

The overall on-line analysis cycle is characterized by five discrete steps:

(1) Sample application (10-500 µl) via the autosampler in valve-position "off-line" (cf. Fig. 3) followed by chemo-selective binding (cf. Figs 6, 7) as well as enrichment of the cis-diol compounds on the affinity ligand on the pre-column (cf. Fig. 2) under slightly alkaline, i.e. buffer D conditions ("HPAC-STEP", cf. Figs 6, 7).

(2) Simultaneous, quantitative elution of the residual matrix constituents from the pre-column into the waste ("SEC-STEP", cf. Fig. 6).

(3) Microprocessor controlled valve-switching into position "on-line" (cf. Fig. 3). Quantitative, group-selective elution of the cis-diol compounds from the pre-column (cf. Fig. 6, 7) by acidification (buffer A) of the immobilized cyclic boronate ester and simultaneous on-line transfer in a single, narrow elution-band through position 2-1-4-3 of the valve to the top of the series-connected analytical column ("TRANSFER-STEP").

(4) Automated valve-switching into position "off-line" (cf. Fig. 3) and separation of the cis-diol compounds on the analytical column under reversed phase chromatography conditions (SEPARATION-STEP).

(5) Reconditioning of the tetrahedral trihydroxyboronyl functionality (cf. Figs 6, 7) for a new extraction cycle during the analytical run with the initial eluent D (REGENERATION-STEP).

RESULTS

The detailed experimental conditions are described for ribonucleoside analysis in ref. (23) and for catecholamine analysis in ref. (24).

Figure 8 represents typical chromatograms, which demonstrate that the investigated compounds can be easily identified and quantitated using the described analytical approach.

CONCLUSIONS

Our approach to use a coupled dual column system for the on-line processing and trace analysis of catecholamines and ribonucleosides succeeded in the development of the first fully automated HPLC-analyzer for these compounds in highly complex and even protein-containing biological matrices.

The dual column technique applied is based on a newly developed and unique column material for digital chromatography and combines the selectivity of bioaffinity- and size-exclusion-chromatography with the high resolution and speed of analysis of reversed-phase chromatography.

The high analytical precision and excellent long-term stability of the HPLC-analyzer is documented by the very low intra- and interassay values of the coefficient of variation for retention times and quantitation. The broad linear measuring range covers by far the concentrations found in normal and pathological samples.

The high accuracy, which primarily is based on the quantitative and matrix-independent recovery of the investi-

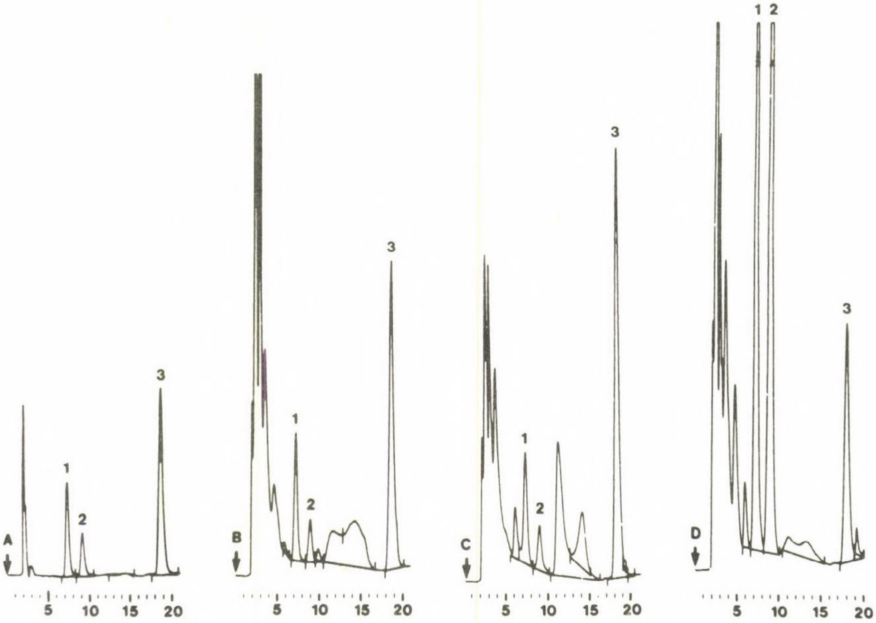

Fig. 8. Automated on-line analysis of free urinary catecholamines with native fluorescence detection.
Sample volumes: 100 µl
Peaks: 1 = norepinephrine; 2 = epinephrine;
3 = dopamine.
(A) Standard. Concentrations: 1 = 0.0047 nmol;
2 = 0.016 nmol; 3 = 0.130 nmol.
(B) Native urine. Concentrations: 1 = 0.063 nmol;
2 = 0.016 nmol; 3 = 0.250 nmol.
(C) 24-hr urine. Concentrations: 1 = 0.056 nmol;
2 = 0.015 nmol; 3 = 0.343 nmol.
(D) Pathological urine (phaeochromocytoma).
Concentrations: 1 = 0.259 nmol; 2 = 0.293 nmol;
3 = 0.161 nmol

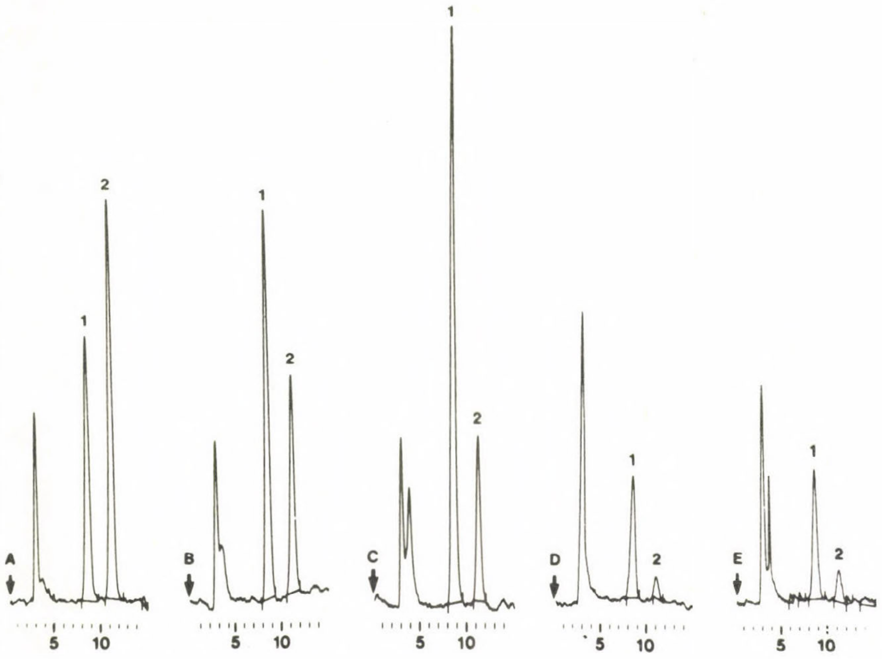

Fig. 9. Automated on-line analysis of free urinary, plasma and serum catecholamines with trihydroxyindole fluorescence detection. Peaks: 1 = norepinephrine; 2 = epinephrine.

(A) Standard: sample-volume 100 µl. Concentrations:
1 = 3.78 pmol; 2 = 2.72 pmol.
(B) Native urine; sample volume 20 µl. Concentrations:
1 = 7.25 pmol; 2 = 1.69 pmol.
(C) 24-hr urine; sample volume 20 µl. Concentrations:
1 = 10.81 pmol; 2 = 1.24 pmol.
(D) Plasma; sample volume 500 µl. Concentrations:
1 = 1.97 pmol; 2 = 0.23 pmol.
(E) Serum; sample volume 500 µl. Concentrations:
1 = 1.85 pmol; 2 = 0.25 pmol

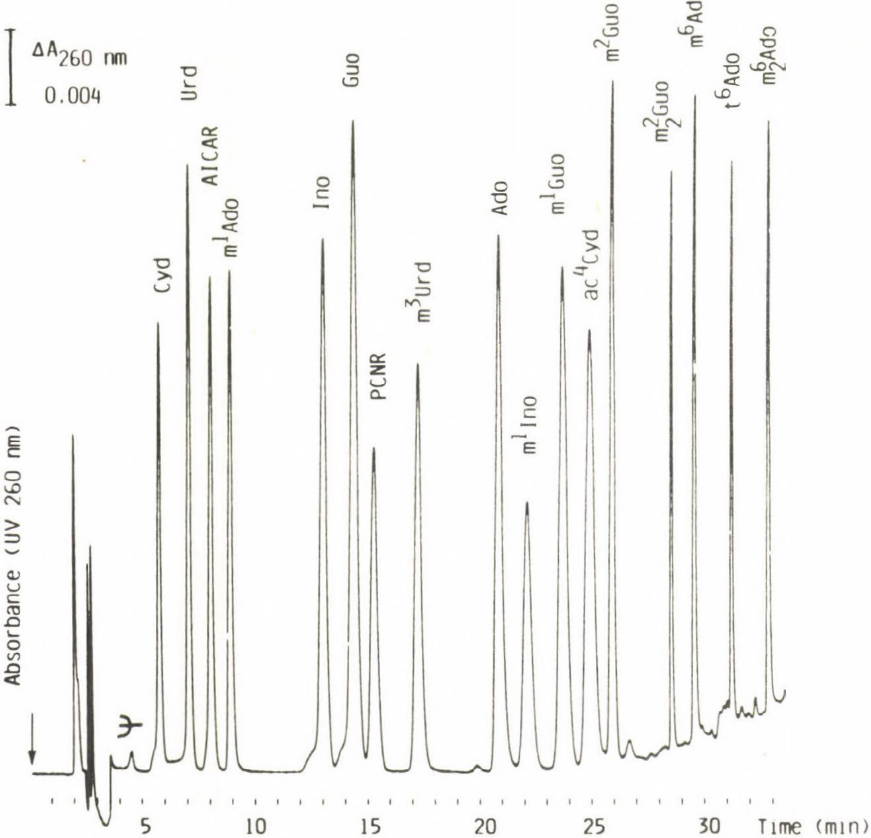

Fig. 10. On-line run of a synthetic mixture of ribonucleosides (cf. Fig. 1)

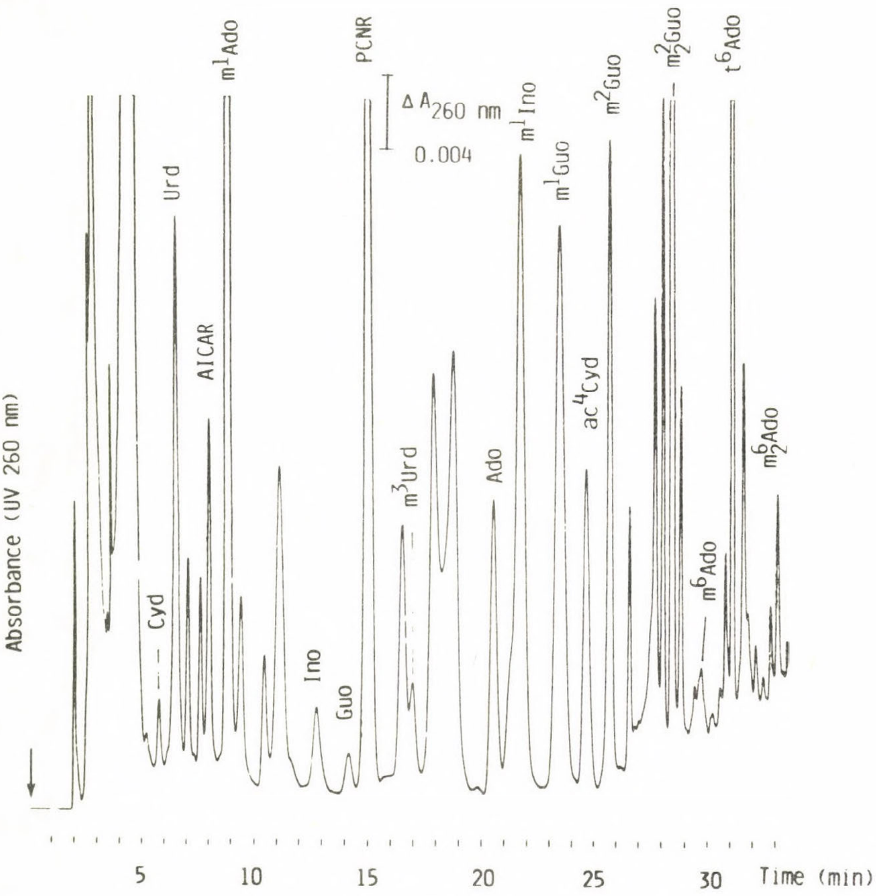

Fig. 11. On-line analysis of ribonucleosides in normal human urine

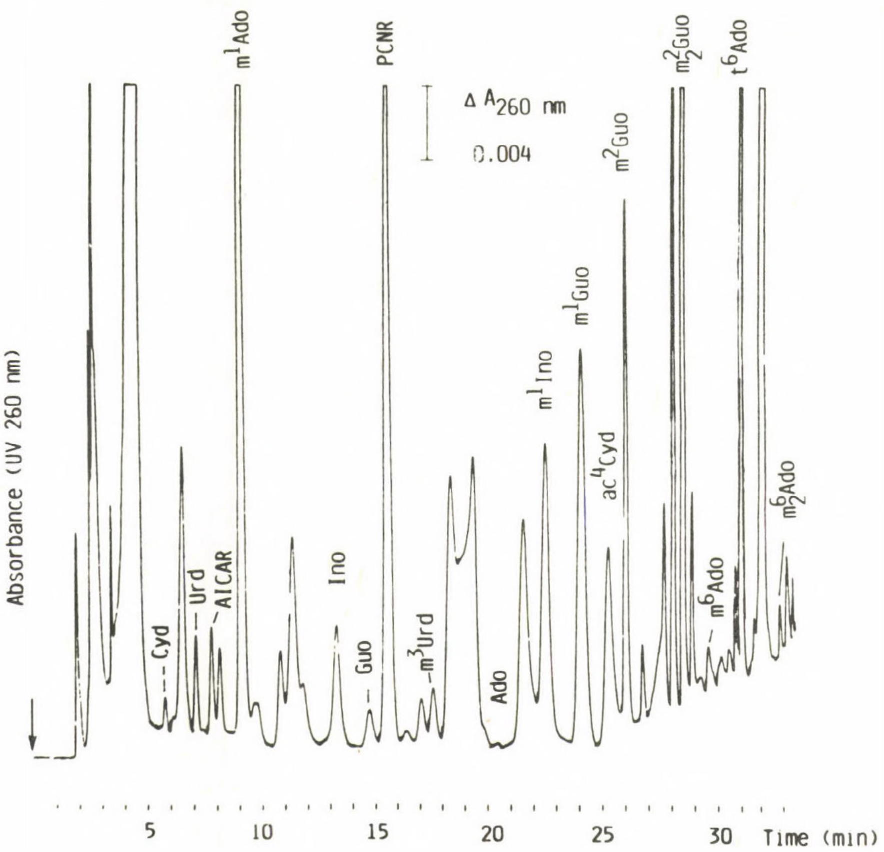

Fig. 12. On-line analysis of urinary ribonucleosides of a patient suffering from breast cancer

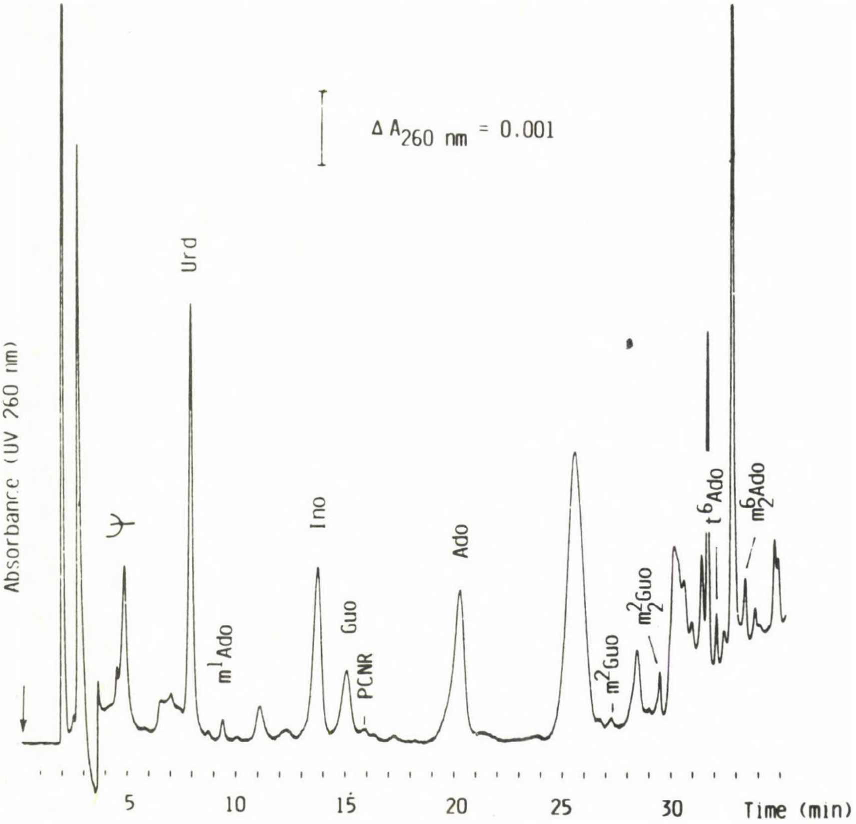

Fig. 13. On-line analysis of ribonucleosides in normal human serum

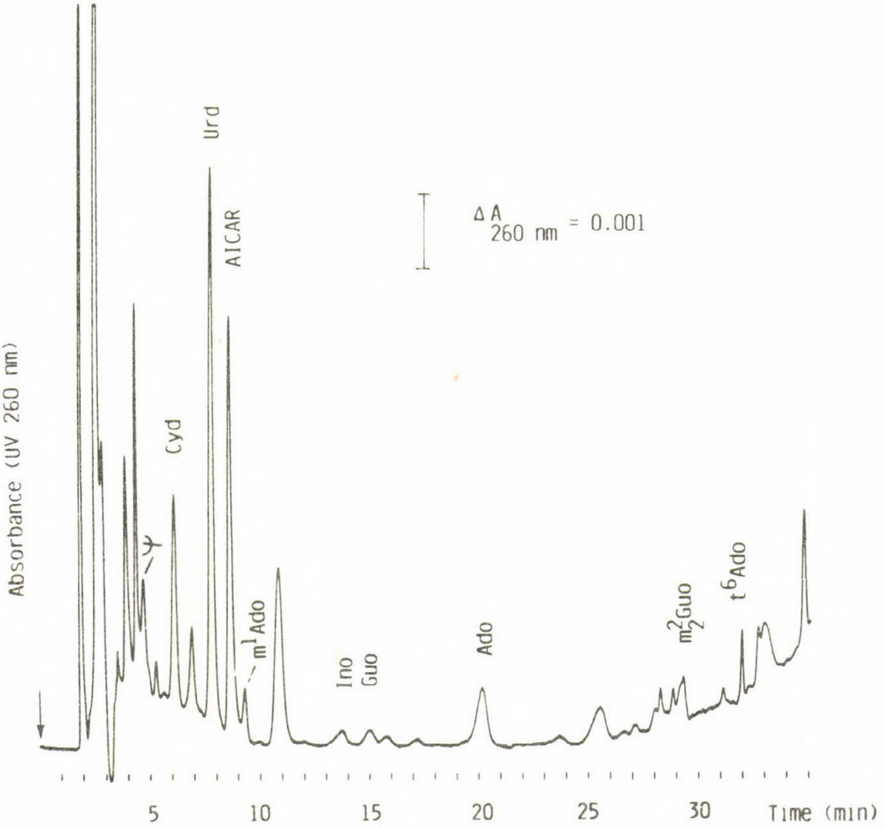

Fig. 14. On-line analysis of ribonucleosides in breast milk

gated analytes, the practicability and the commercial availability of the HPLC-analyser offer the system as a powerful analytical method for investigations in the biochemical as well as in the clinical reasearch field.

Further applications of this method will be: (i) trace enrichment for the structural characterization of diol-containing compounds in biological fluids; (ii) small-scale preparation of natural diol-containing compounds; (iii) investigation of disorders in catecholamine, ribonucleoside, ribonucleotide and/or RNA metabolism; (iv) a non-invasive screening test (urinary modified ribonucleosides) for cancer diseases in humans; (v) investigation of renal reutilization processes; (vi) therapeutic drug monitoring during nucleoside or catecholamine chemotherapy.

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HPLC OF SOME PEPTIDE HORMONES

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The preparation of highly purified peptide hormones is an important problem due to their wide application in medicine. Reversed-phase high-performance liquid chromatography (RP-HPLC) is presently the most popular method for the analysis and purification of these hormones (1, 2). Mixture of acetonitrile (MeCN) or isopropanol with acidic aqueous buffers or acid solutions are the most widely used mobile phases in these applications. Gradient elution using a mixture of MeCN with aqueous 0.1% trifluoroacetic acid (TFA) is very often applied. However, systematic studies dealing with the principles of sorbent selection are almost absent in the literature. Various silica-based packing materials with different porosity and modified with hydrocarbon groups with different chain lengths are used for RP-HPLC of peptide hormones. These sorbents are varied in the degree of surface modification of the silica particles and in using of end-capping processes which block the residual silanol groups (3). Thus there are a wide variety of packings which can be used for the analysis of peptide hormones. Therefore it was of interest to find the most suitable packings for the analysis of some peptide hormones differing in properties (pI, hydrophobicity, etc.) and molecular weight (MW). We compared some commercial packings differing in the aforementioned properties as well as a silica-based packing with bonded butyldimethyl groups followed by end-capping prepared in the USSR (4).

The chromatographic separations have been performed with a Millipore-Waters liquid chromatograph consisting of two M-501 pumps, an M-680 gradient controller, a Rheodyne injector 7125, an M-441 photometer ($\lambda = 280$ nm) and an M-740 computing integrator. The injected volume of the peptide solution (1 mg/ml in component A) was 20-30 μl. Gradient elution in RP-HPLC was carried out from 20% to 70% B for 30 min (A: 10% MeCN in 0.1% TFA; B: 90% MeCN in 0.1% TFA), whereas in the case of chromatography on silica columns from 100% to 20% B for 30 min. The flow rate was 1 ml/min in both cases. The chromatographic behaviour of the following peptide hormones was studied: porcine calcitonin (CT) comprising 32 amino acids (AA)(pI~8.5); porcine adrenocorticotropic hormone (ACTH) having 39 AA (pI~7.5); human somatotropic (or growth) hormone (STH) comprising 191 AA (pI~5.0); and a preparation of porcine follicle stimulating hormone (FSH) the sum of glycoproteins with a MW of about 30 KD (pI~4.5) also containing albumin (MW~60 KD). All hormones were of USSR Pharmacopoeia grade.

The following silica-based RP-packing materials were investigated:

(1) modified by octadecyl (C-18) groups:

- (I) Zorbax ODS (particle size 6 μm, pore size 80 Å; duPont, USA)
- (II) Nova Pack C-18 (4 μm, 80 Å, Waters, USA)
- (III) Vydac 218 TP (5 μm, 300 Å, Separations Group, USA)

(2) modified by octyl (C-8) groups:

- (IV) Zorbax PEP RP1 (5 μm, 120 Å, duPont, USA)
- (V) Silasorb C-8 (12 μm, 80 Å, Lachema, Czechoslovakia)

(3) modified by butyl (C-4) groups:

- (VI) Vydac 214TP (5 μm, 300 Å, Separations Group, USA)
- (VII) material prepared by us (4) from Lichrosphere Si500 (Merck, FRG) by chemical modification (10 μm, 500 Å)

The following silica packings have also been studied:

- (VIII) Silasorb 600 (10 μm, 60 Å, Lachema, Czechoslovakia)
- (IX) Silasorb 300 (10 μm, 80 Å, Lachema, Czechoslovakia)
- (X) Lichrosphere Si500 (10 μm, 500 Å, Merck, FRG)

All sorbents were investigated in columns having the same dimensions (250 x 4.6 mm I.D.), except packings II and IV where the column dimensions were 150 x 3.9 mm I.D., and 80 x 6.2 mm I.D., respectively. Packings I-IV and VI were available in commercial columns while sorbents V, VII and VIII-X were slurry-packed in our laboratory at 400 bar, with isopropanol or acetone.

On the basis of literary data one can assume that all these packings are suitable for the analysis of proteins and peptides at the selected elution conditions (1, 2). However, our experiments demonstrated that these sorbents exhibited quite different selectivity for the analysis of the studied peptide hormones. The chromatographic behaviour of three hormones CT, ACTH and STH used as the model mixture (1 mg/ml of each hormone) on various octadecyl packings is compared in Fig. 1. The results obtained showed that sorbent I was not suitable for the separation of large hormones under the selected elution conditions since STH eluted as a broad peak. The situation was similar in the case of packing II, while sorbent III exhibited good separation of all the studied hormones: the peaks were narrow and well separated. Similar results were obtained on the analysis with these packings of a FSH preparation, which, as can be seen in Fig. 2, is a mixture of substances with close structure plus albumin. Again the best separation of this mixture was achieved on the column packed with sorbent III. As demonstrated by the analysis of a model mixture sorbent I failed to separate the complex mixture of close substances, while sorbent II exhibited only partial separation of the FSH sample. Thus the nature of the silica matrix, i.e., the property of its surface including the degree of coverage with the hydrocarbon groups, the percentage of residual silanol groups and, probably to the greatest extent, the pore size of the particles have a substantial effect on the separation of relatively large hormones. In the case of C-18 bonded packings those which are end-capped and have a pore size of about 300 Å and moderate degree of coverage seem to be optimal for the separation of various peptide hormones.

It was shown earlier (5) that packings consisting of silica modified by shorter (octyl and especially butyl) groups

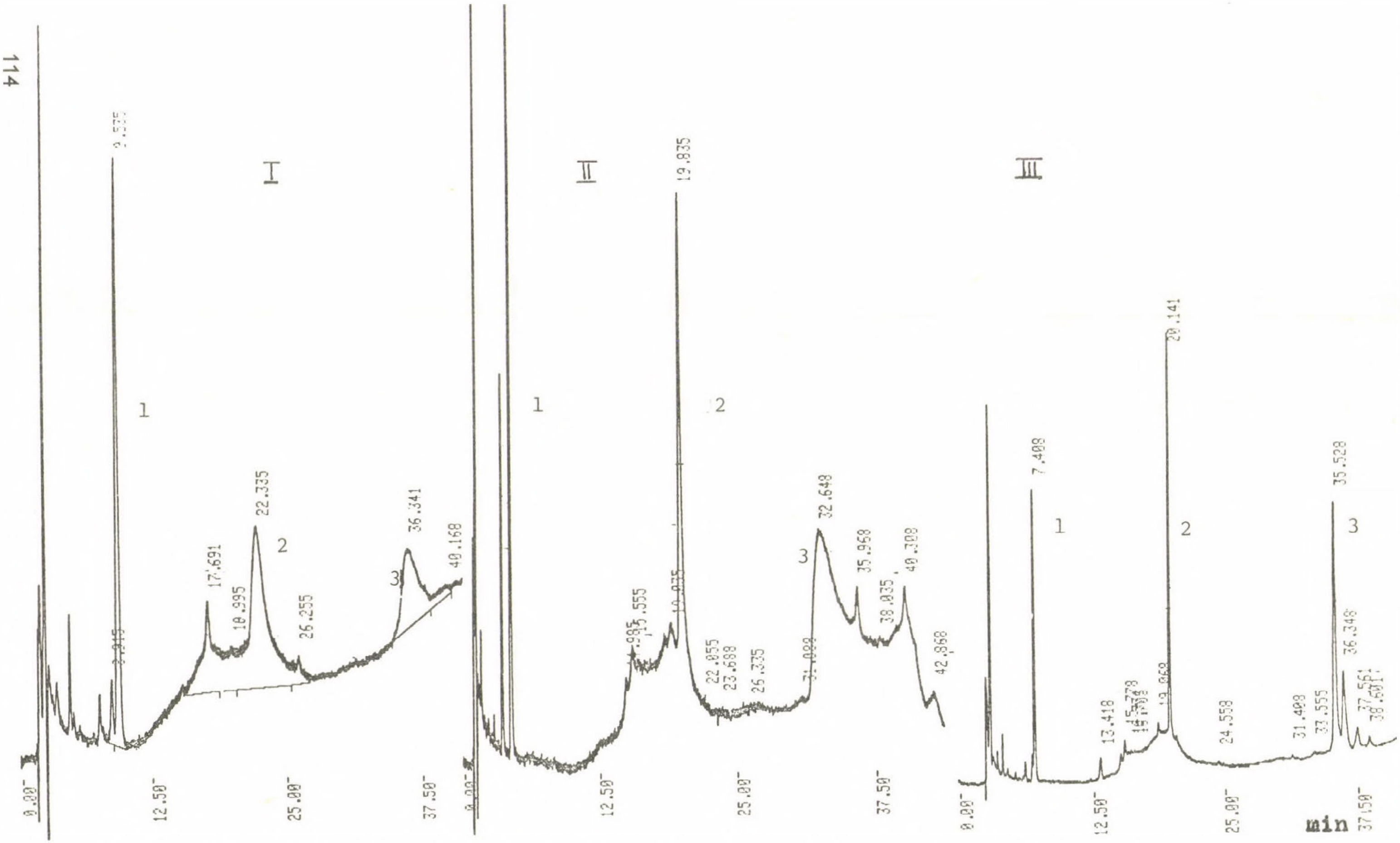
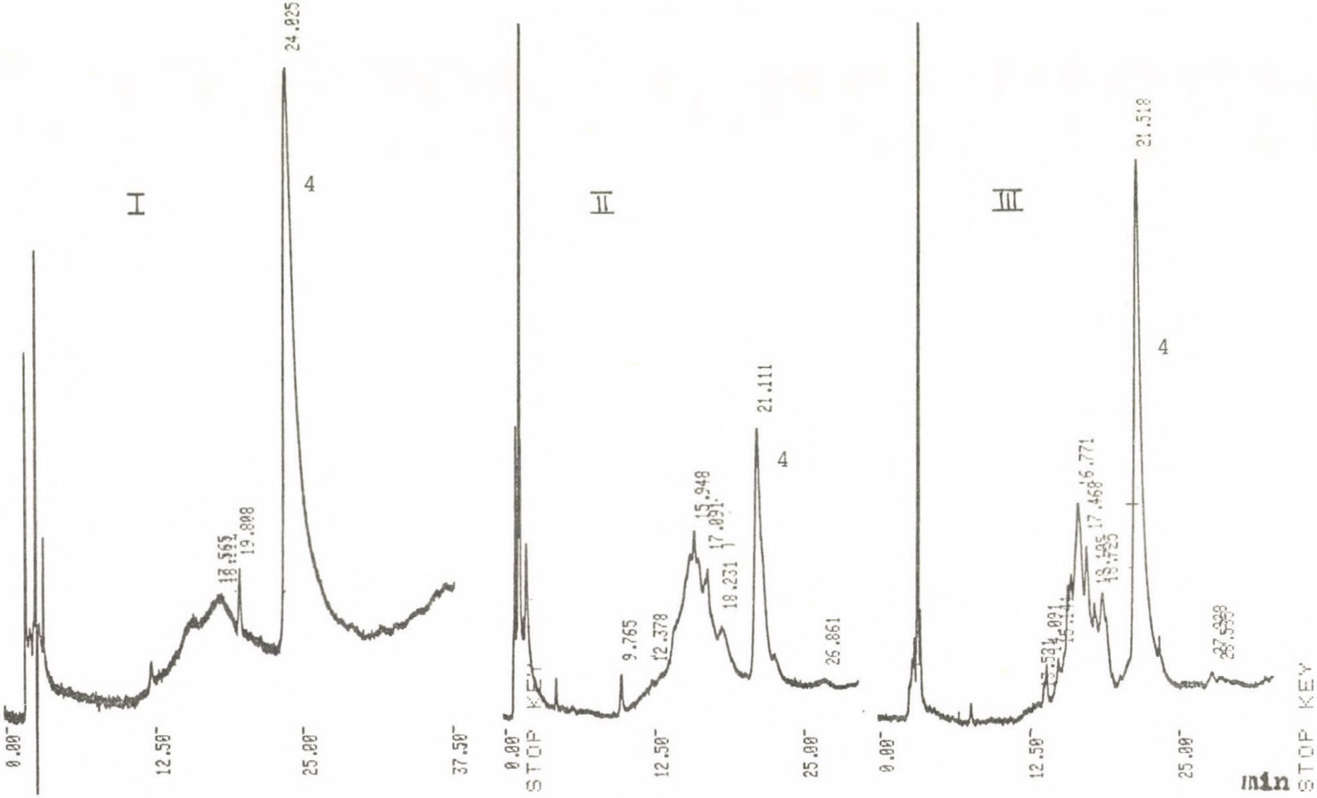


Fig. 1. Chromatograms of the model mixture consisting of ACTH (1), CT (2) and STH (3), obtained on the columns packed with sorbents I, II, III (see text). Solvent system: A: 10% MeCN in 0.1% TFA; B: 90% MeCn in 0.1% TFA. Gradient was 20%-70% B in 30 min, flow rate 1 ml/min



<u>Fig. 2</u>. Chromatograms of FSH preparation, containing also albumin (4) obtained on the same columns as in Fig. 1. Elution conditions are the same as in Fig. 1

possessed some advantages in the RP-HPLC analysis of rather high molecular weight peptides and proteins (MW>25 KD). However, comparison of two C-8 packings demonstrated that sorbent IV specially developed for the separation of proteins and peptides was not suitable under the selected conditions for the analysis of large hormones, since STH and FSH failed to be eluted from the column. At the same time sorbent V was not inferior to Zorbax ODS, although the column efficiency was lower due to the 12 µm particle size of the packing (Fig. 3).

The sorbents modified by butyl groups (VI and VII) appeared to be quite suitable for the analysis of all the studied hormones: this is evident from the high selectivity and efficiency of the obtained separation (Fig. 3). It should be emphasized that these sorbents have wide pores (300 and 500 Å), which favours a better permeability of the sorbate molecules into pores and their interaction with the stationary phase. The good characteristics of these sorbents for the separation of relatively large hormones shown in the analysis of the model mixture, are confirmed by the separation of FSH components (Fig. 4) where high resolution could be observed. The FSH components were also successfully separated also using packing V, although the separation efficiency was a little lower.

The results obtained show that the retention of peptide hormones depends on the nature of the RP sorbent. Thus similar sorbents III and VI differing in the length of the bonded hydrocarbon chain demonstrated varied retention of the same hormones. As expected, sorbent VI (C-4) exhibited a lower retention than sorbent III (C-18), which can be seen from the comparison of the retention times of the same hormones under identical separation conditions (Table I). Among two C-4 packings sorbent VII had stronger retention of the peptide hormones as compared with VI which can be the result of the higher degree of surface modification of the silica in the case of sorbent VII as compared to VI. It should also be noted that the selectivity obtained with the home-made packing VII was not inferior to that of the Vydac type material (VI), although the peptide peaks are markedly wider, which is connected to the lower column efficiency, since the particle size was 10 µm.

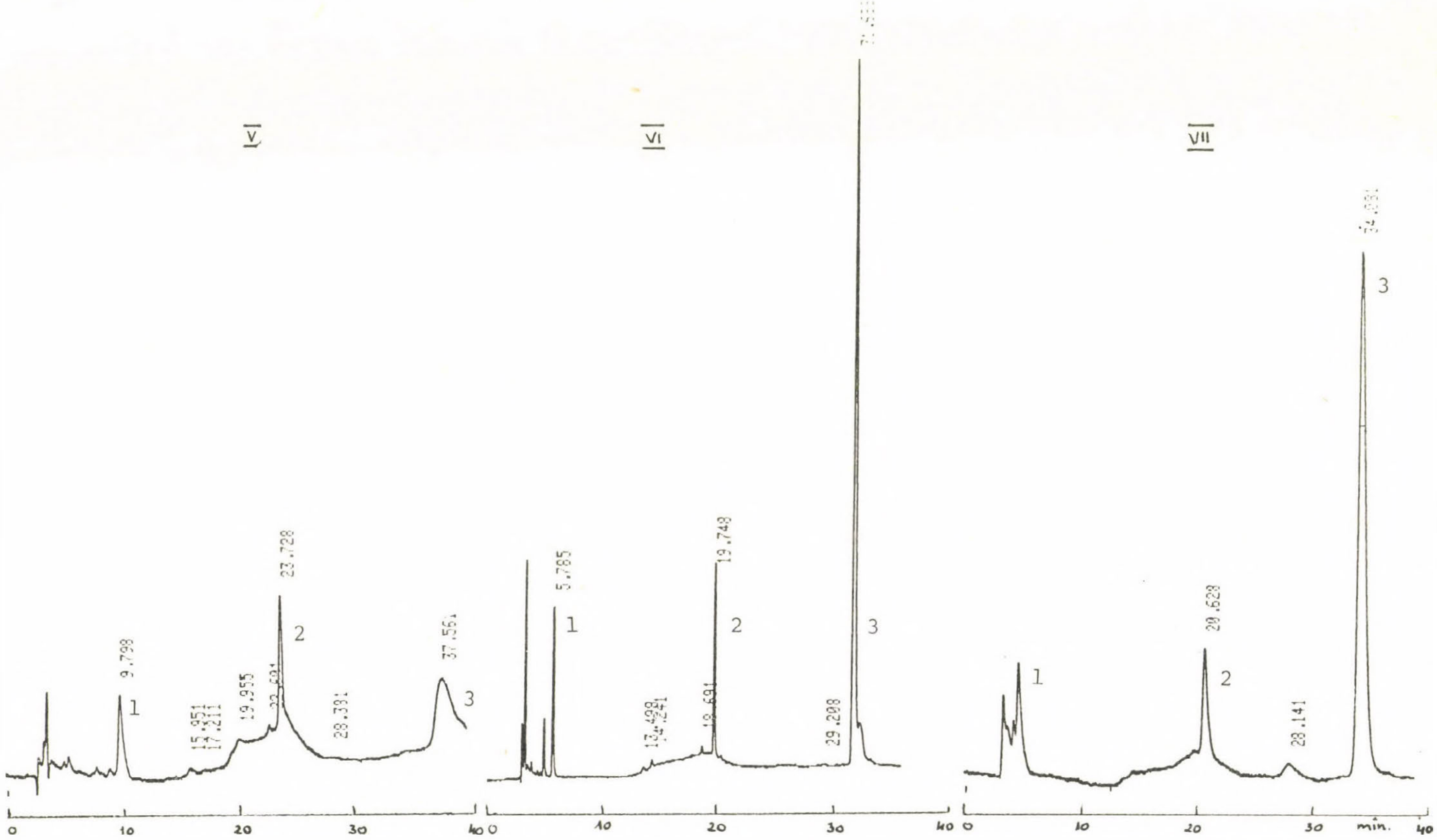


Fig. 3. Chromatograms of the model mixture obtained on the columns packed with sorbents V, VI and VII (see text). Abbreviations and elution conditions are the same as in Fig. 1

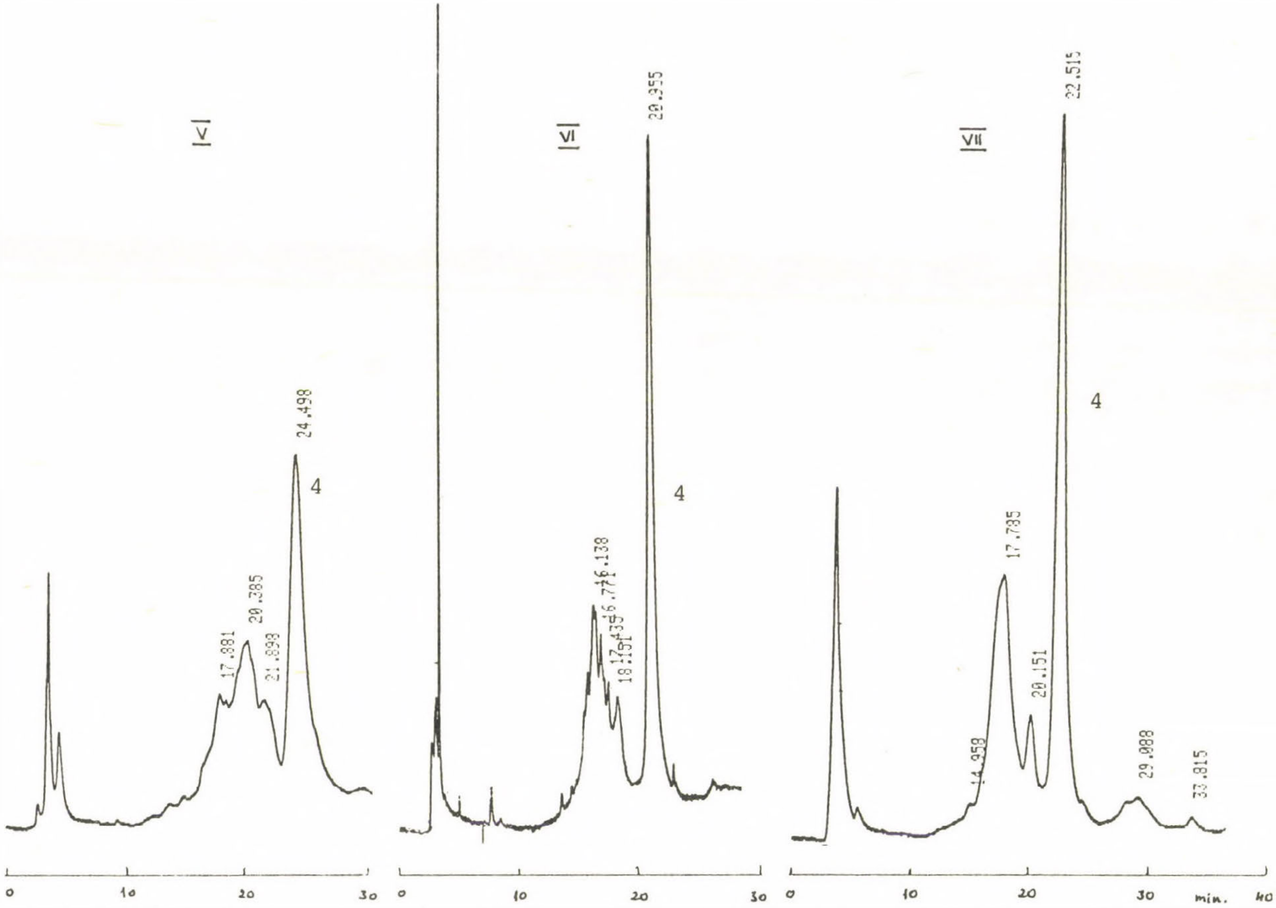


Fig. 4. Chromatograms of FSH preparations obtained on the same columns as in Fig. 3. Abbreviations and elution conditions are the same as in Fig. 2

Table I. Comparison of the retention times (in minutes) of peptide hormones on different RP columns

	I	II*	III	V	VI	VII
ACTH	9.5	3.8	7.4	9.8	5.8	4.5
CT	22.3	19.8	20.1	23.7	19.7	20.6
STH	36.3	32.6	35.5	37.5	31.7	34.1
Albumin in FSH	24.0	21.1	21.5	24.5	20.9	22.5
FSH	17.5	15.9	16.8	20.4	16.1	17.8

*column dimensions: 150 x 3.9 mm

There are also some other differences in the retention of the peptide hormones on the studied sorbents.

It can be concluded that the most suitable packings for RP-HPLC of peptide hormones are wide-porous (pore size 300,500 Å) silica-based materials with bonded C-18 or C-4 groups.

As it was mentioned earlier the most popular packing materials for the HPLC of peptide hormones consist of bonded-phase silica with alkyl chains of various lengths. The possibility of the application of unmodified silica for the separation of these substances is considered at far less extent in the literature. It was for example shown that peptide hormones (e.g., human and salmon calcitonin, insulin and glucagon) can be separated on Porasil-type sorbents under isocratic conditions (eluent: MeCN-ammonium acetate, pH 4). However, systematic studies dealing with the effect of the pore size and other silica properties on the separation of peptide hormones are almost absent.

We have studied the chromatographic behaviour of our model mixture of three peptide hormones and FSH preparation on three different silica packings (VIII). It was found that the chromatographic separation of the hormone mixture can be carried out under a gradient profile opposite to that of RP-sorbents, that is with increased concentration of the polar component (0.1% TFA). It was found that all studied hormones could be eluted with this gradient (apart from FSH on sorbent VIII) from the column packed with unmodified silicas. It should be noted that the retention of hormones is highly dependent on the nature of silica (Table 2), and it is the stronger the smaller is the pore diameter of the packing. Thus the retention time of STH changed from 19.3 min to 41.9 min when changing from sorbent X (pore size 500 Å) to sorbent VIII (pore size 60 Å) while in the latter case STH eluted as a broad peak (see Fig. 5). It should be emphasized that the separation selectivity for the hormone mixture and the FSH preparation in particular was much lower than in the case of RP-packings (Figs 5, 6). For example the FSH preparations could not be separated on sorbents VIII and IX and only very poorly on sorbent X.

Table 2. Comparison of the retention times (in minutes) of peptide hormones on different silica columns

	VIII	IX	X
ACTH	3.0	3.5	4.1
CT	28.8	20.1	19.3
STH	41.9	24.3	19.3
Albumin in FSH	-	28.5	23.1
FSH	-	30.2	22.1

Taking into account the gradient profile, the order of elution of hormones and the dependence of their retention time on pore size and, therefore, on the specific surface area of the silica, one can assume in this case the presence of partition chromatography on the hydrophilic absorbed layer formed on the silica surface. It should be mentioned that silica happened to be suitable for the separation of relatively low molecular weight hormones, e.g., calcitonin. Thus sorbent VIII could be applied to the analysis of various samples of porcine CT.

Finally, the comparison of different packings consisting of unmodified silica and silica with bonded alkyl groups and used under gradient elution conditions demonstrated that the packings with bonded C-18 and C-4 groups and a pore size of

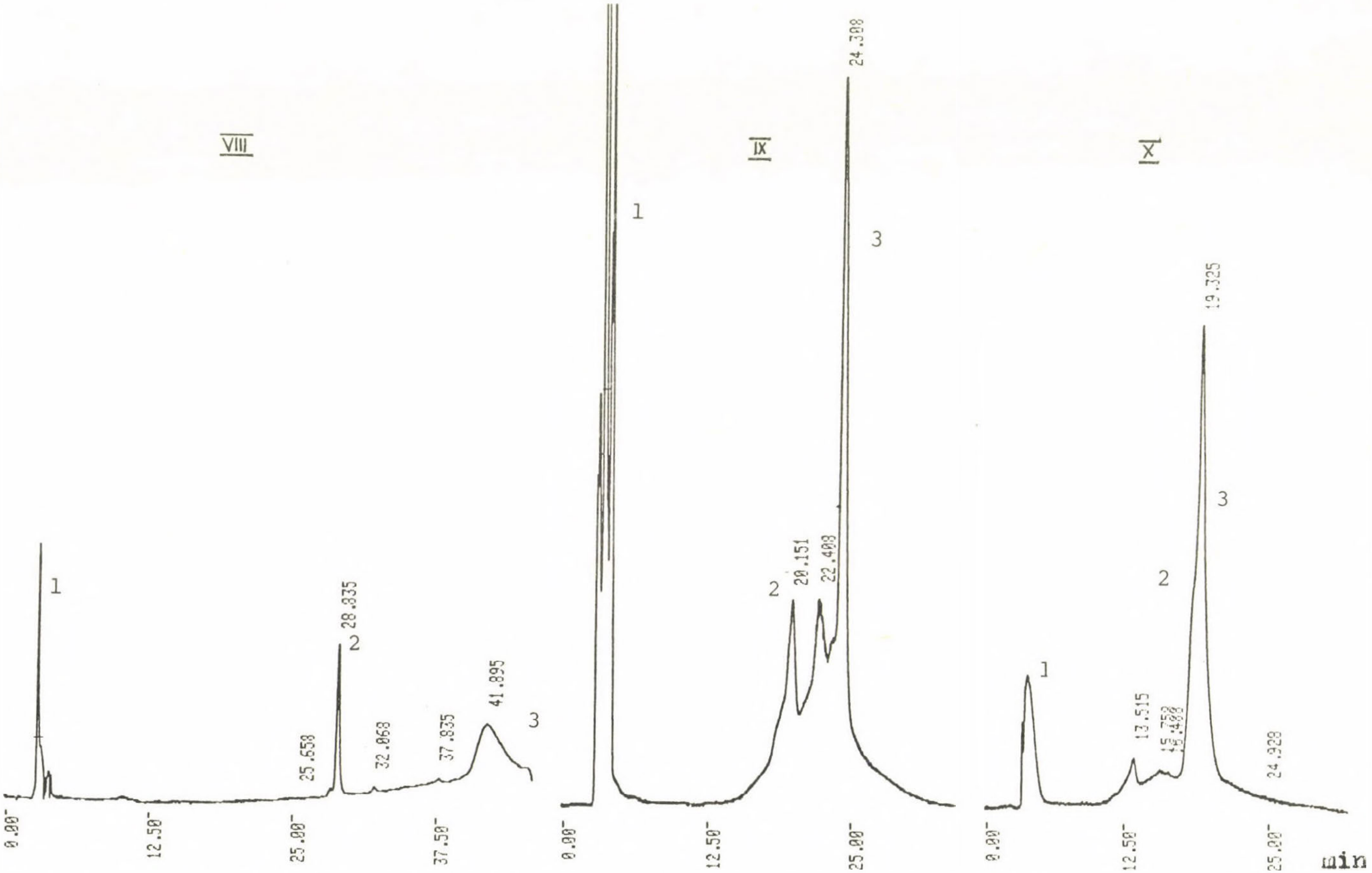


Fig. 5. Chromatograms of the model mixture obtained on silica columns VIII, IX and X (see text). Solvent system: A: 10% MeCN in 0.1% TFA; B: 90% MeCN in 0.1% TFA. Gradient: 100%-20% B in 30 min, flow rate 1 ml/min

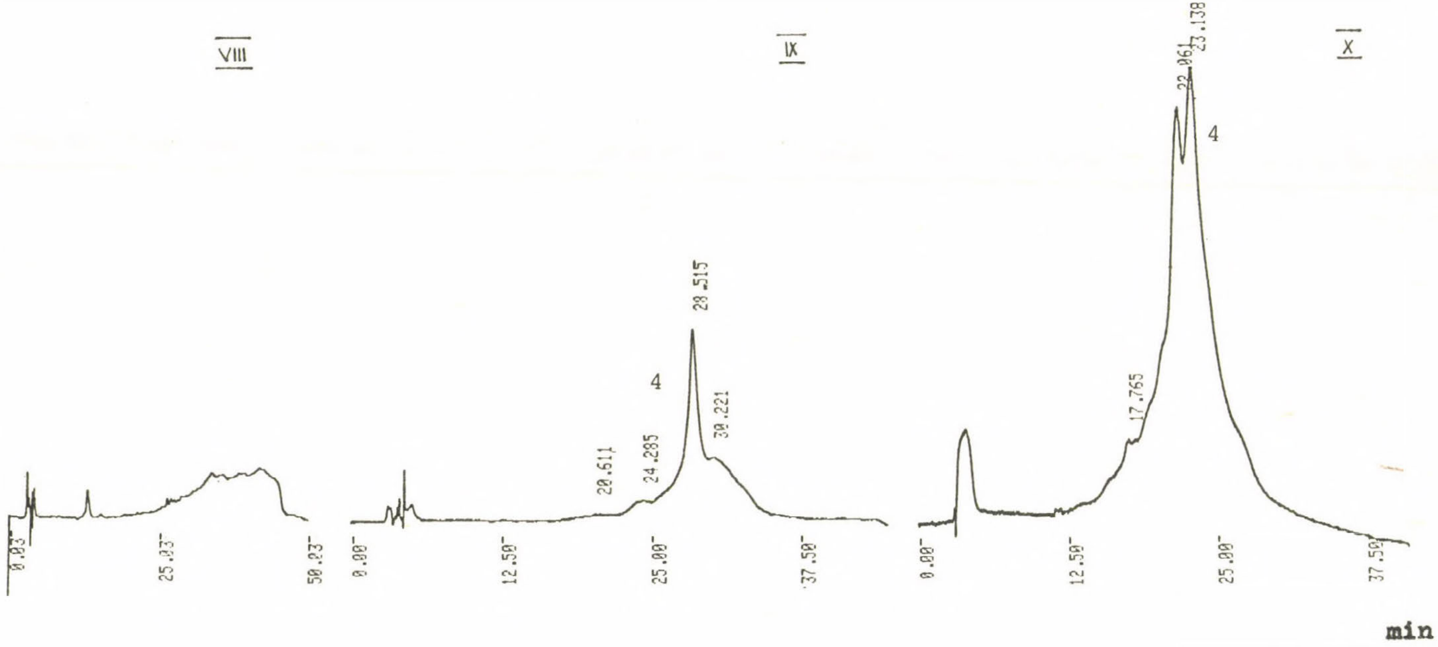


Fig. 6. Chromatograms of FSH preparation obtained on the same columns as Fig. 5. Abbreviations and elution conditions are the same as in Fig. 5

300 Å are the most suitable for the analysis of relatively large hormones (MW>20 KD) while bare silica can be used for the analysis of relatively small hormones.

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STUDIES ON THE SEPARATIVE PROPERTIES OF ZEOLITE-CONTAINING TUFFS OF VARIOUS DEPOSITS IN HUNGARY

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At present, synthetic zeolites are widely used in the practice of the gas chromatography as adsorbent for the separation of different gaseous mixtures and, in certain cases, also for liquid systems. The use of zeolites of natural origin is more restricted, although separation of some mixtures, e.g., oxygen-nitrogen, on these zeolites can be performed with higher resolution than on synthetic zeolites (1). It had been shown (1-3) that with mordenite-containing tuff of the Ratevani deposits (Georgian SSR), with 50% zeolite content in the rock, there is a possibility of full separation of oxygen and nitrogen; enrichment of the zeolite by calcium cation improves this separation while modification by strontium or barium favourably affects the argon-oxygen separation.

The present paper reports on the chromatographic properties of zeolite-containing tuffs of Hungarian deposits with various (30-81%) mordenite content in the rock and of the same materials modified by calcium, potassium and ammonium cations. The separation of gas mixtures consisting of helium, neon, argon, oxygen, nitrogen, methane and carbon oxide will be investigated. Table I gives the composition of natural mordenite and of the substances enriched in cations. Composition is expressed in terms of oxides.

As shown by Table I, the mordenite content in rocks covers the range of 30 to 81%. For samples A and B with low and medium mordenite content the potassium cations are predominant; only in samples A-4 and B-4 predominate the calcium cations

Table I. Chemical composition of (A) natural mordenite-containing rock; of samples enriched in (B) calcium and (C) potassium cations, and (D) of decationized samples obtained by the calcination of ammonium mordenite, at 1,050°C

Samples		Mordenite content in rocks %	Chemical composition expressed in terms of oxides
A	1	30	$0.58K_2O$; 0.17CaO; $0.17Na_2O$; 0.01MgO; Al_2O_3; $0.03Fe_2O_3$; $10.0SiO_2$; $2.7H_2O$.
	2	46	$0.42K_2O$; 0.25CaO; $0.17Na_2O$; 0.01MgO; Al_2O_3; $0.03Fe_2O_3$; $9.9SiO_2$; $3.8H_2O$.
	3	60	$0.50K_2O$; 0.25CaO: $0.25Na_2O$; 0.01MgO; Al_2O_3; $0.03Fe_2O_3$; $9.8SiO_2$; $3.6H_2O$.
	4	81	$0.18K_2O$; 0.45CaO; $0.27Na_2O$; 0.01MgO; Al_2O_3; $0.04Fe_2O_3$; $10.3SiO_2$; $6.6H_2O$.
B	1	30	$0.58K_2O$; 0.25CaO; $0.08Na_2O$; 0.01MgO; Al_2O_3; $0.03Fe_2O_3$; $10.0SiO_2$; $2.9H_2O$.
	2	46	$0.42K_2O$; 0.42CaO; $0.08Na_2O$; 0.01MgO; Al_2O_3; $0.03Fe_2O_3$; $9.8SiO_2$; $3.9H_2O$.
	3	60	$0.45K_2O$; 0.45CaO; $0.09Na_2O$; 0.01MgO; Al_2O_3; $0.03Fe_2O_3$; $10.8SiO_2$; $3.9H_2O$.
	4	81	$0.09K_2O$; 0.64CaO; $0.18Na_2O$; 0.01MgO; Al_2O_3; $0.04Fe_2O_3$; $10.3SiO_2$; $6.7H_2O$.

	Samples	Mordenite content in rocks %	Chemical composition expressed in terms of oxides
C	1	30	$0.86K_2O$; $0.06Na_2O$; $0.004CaO$; $0.002MgO$; Al_2O_3; $0.024Fe_2O_3$; $10.0SiO_2$; $2.1H_2O$.
	2	46	$0.84K_2O$; $0.06Na_2O$; $0.008CaO$; $0.003MgO$; Al_2O_3; $0.03Fe_2O_3$; $10.2SiO_2$; $3.4H_2O$.
	3	60	$0.86K_2O$; $0.09Na_2O$; $0.004CaO$; $0.001MgO$; Al_2O_3; $0.02Fe_2O_3$; $10.3SiO_2$; $3.0H_2O$.
	4	81	$0.82K_2O$; $0.02Na_2O$; $0.04CaO$; $0.001MgO$; Al_2O_3; $0.03Fe_2O_3$; $10.3SiO_2$; $5.5H_2O$.
D	1	30	$0.55K_2O$; $0.08Na_2O$; $0.004CaO$; $0.003MgO$; Al_2O_3; $0.03Fe_2O_3$; $10.2SiO_2$; $3.1H_2O$.
	2	46	$0.36K_2O$; $0.04Na_2O$; $0.004CaO$; $0.003MgO$; Al_2O_3; $0.02Fe_2O_3$; $10.1SiO_2$; $4.5H_2O$.
	3	60	$0.41K_2O$; $0.07Na_2O$; $0.004CaO$; $0.001MgO$; Al_2O_3; $0.03Fe_2O_3$; $10.3SiO_2$; $4.1H_2O$.
	4	81	$0.05K_2O$; $0.03Na_2O$; $0.04CaO$; $0.002MgO$; Al_2O_3; $0.03Fe_2O_3$; $10.8SiO_2$; $7.6H_2O$.

over the sodium and potassium ions. For all samples C, independent of the zeolite content of the rock, the potassium cations predominate over sodium, calcium and magnesium ions. In the case of decationized mordenites (samples D) it is noteworthy that the total sum of the oxide coefficients (except for Al_2O_3 and SiO_2) is much lower than unity. This phenomenon is obviously caused by the fact that during the calcination of these samples at 1050°C a deammoniation takes place at the initial stage (up to 100°), resulting in hydrogen-containing and decationized mordenites.

Experiments were carried out under the following conditions:

column length: 50-100 cm
column diameter: 5 mm
particle size of the samples: 0.5-1.0 mm
activation temperature: 300 and 450°C
column temperature: generally, room

Helium was mainly used as the carrier gas but in some cases, neon, argon, nitrogen and carbon dioxide were also used. The carrier gas flow rate was between 10 and 150 ml/min.

The effect of the mordenite content in the rock, as well as its modification with potassium, calcium and $NH_4^+(H^+)$ cations, and of the thermal activation temperature on the specific retention volumes of the sample components is given in Tables II and III. Tables IV and V indicate the influence of the carrier gas on the chromatographic results.

It can be seen in Tables II and III that mordenites containing calcium cations, as all other zeolites, show a special selectivity which is characteristic for molecules which have quadrupole moments, such as e.g., for nitrogen. Both for the natural samples (A) and for samples enriched in calcium cation (B), the retention volumes of argon, oxygen, and especially of nitrogen increase up to a limit with an increase in the zeolite content of the rock. There is however, a decrease of the retention volumes of all gases in the case of samples A-4 and B-4. It is difficult to explain this, however, obviously, it is connected to the cation composition and the location of these cations in the elementary cell of the zeolite.

Table II. The specific retention volume (ml/g substance in the column) of inert gases, as a function of the mordenite content of the rocks and of the thermal activation temperature. Carrier gas: helium, flow rate: 100 ml/min

	Samples							
	A*-1		A*-2		A*-3		A*-4	
	Activation temperature							
	300°C	450°C	300°C	450°C	300°C	450°C	300°C	450°C
	Specific retention volume of gases (ml/g)							
Argon	1.1	1.9	2.2	3.5	2.3	3.7	1.5	3.2
Oxygen	1.3	2.1	2.3	4.2	2.5	4.4	1.7	4.0
Nitrogen	3.7	20.3	7.8	45.0	10.3	49.0	4.2	41.0

Table III. The specific retention volumes (ml/g substance in the column) of inert gases, as a function of the modification of mordenites with calcium, potassium and NH_4^+ (H^+) cations. Activation temperature: 450°C; column temperature: room; carrier gas: helium; flow rate: 100 ml/min.

Modified mordenite sample		Argon	Oxygen	Nitrogen
		Specific retention volume (ml/g)		
B	1	3.7	4.5	63.0
	2	5.2	7.7	122.5
	3	5.4	7.9	161.5
	4	2.3	5.0	60.0
C	1	2.0	1.9	3.4.
	2	2.1	2.5	4.1
	3	1.9	2.0	3.4
	4	1.2	2.3	2.7
D	1	1.9	1.9	3.8
	2	2.4	2.7	3.2
	3	2.2	2.2	3.1
	4	2.3	3.0	3.3

As shown in Table III the specific retention volumes of the studied gases are not high for samples C and D and change little in spite of the increase in the mordenite content of the rock from 30 to 81% and of a thorough activation (450^{o}C). Hence, practically identical results have been obtained. These samples are characterized by considerably worse separation properties as compared with samples A and B. This can obviously be explained for samples C by the blocking of the entrance windows to the zeolite cavity, by the large potassium cations while in the case of samples D a widely porous H^+ mordenite is obtained due to deammoniation.

The sequence of the elution of the gases on samples A and B is: He-H_2-Ar-O_2-N_2. Oxygen and nitrogen are separated for certain samples (e.g., B-3) all components are fully separated over the temperature range of 20 to 160^{o}C. With an increase in the zeolite and calcium cation content of the rock selectivity gradually increases relative to nitrogen, consequently, the separation of oxygen and nitrogen improves up to a limit. In the case of samples A-4 and B-4 the separation of this mixture becomes poorer. In general, however, separation is better on adsorbents (B) enriched by Ca^{2+} cations than on the natural mordenites (A) (see Figs 1 and 2). It is also noteworthy, that the highly activated sample B-3 is the most selective for nitrogen, the retention time of which is very sensitive to the increase of the column temperature.

The nature of the carrier gas considerably affects the retention times of the gases present in the mixture. The use of argon instead of helium reduces the retention times to one-half or one-third and the peaks become more symmetrical. Carbon dioxide has an even stronger influence: the retention times of oxygen and especially nitrogen significantly decrease, essentially resulting in a worsening of the separation of oxygen and nitrogen (see Table IV).

In spite of certain differences in the retention volumes of argon and oxygen, these could not be separated on those mordenite samples where the peaks of the chromatographed substances significantly broadened. However, using mordenite sample B-3, after saturation of the adsorbent with carbon

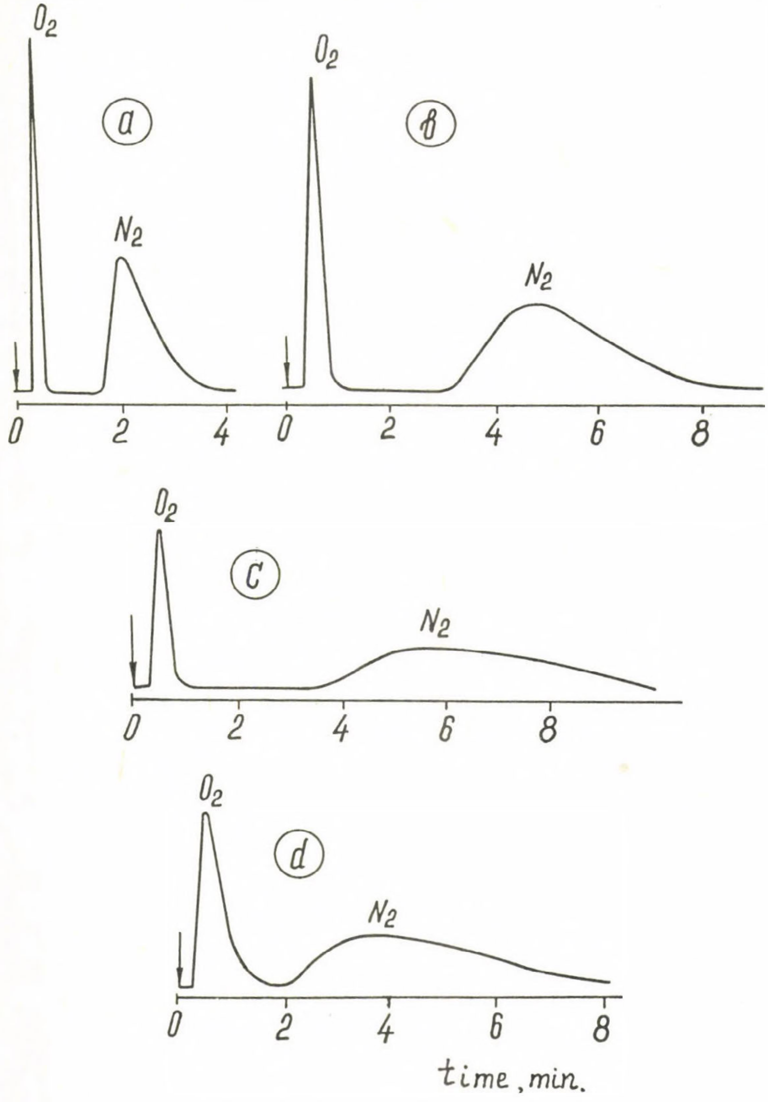

Fig. 1. Separation of oxygen and nitrogen on the initial natural mordenite-containing rocks. Activation temperature of the samples: 450°C; column temperature: room; carrier gas (helium) flow rate: 100 ml/min. (a) sample A-1; (b) sample A-2; (c) sample A-3; (d) sample A-4

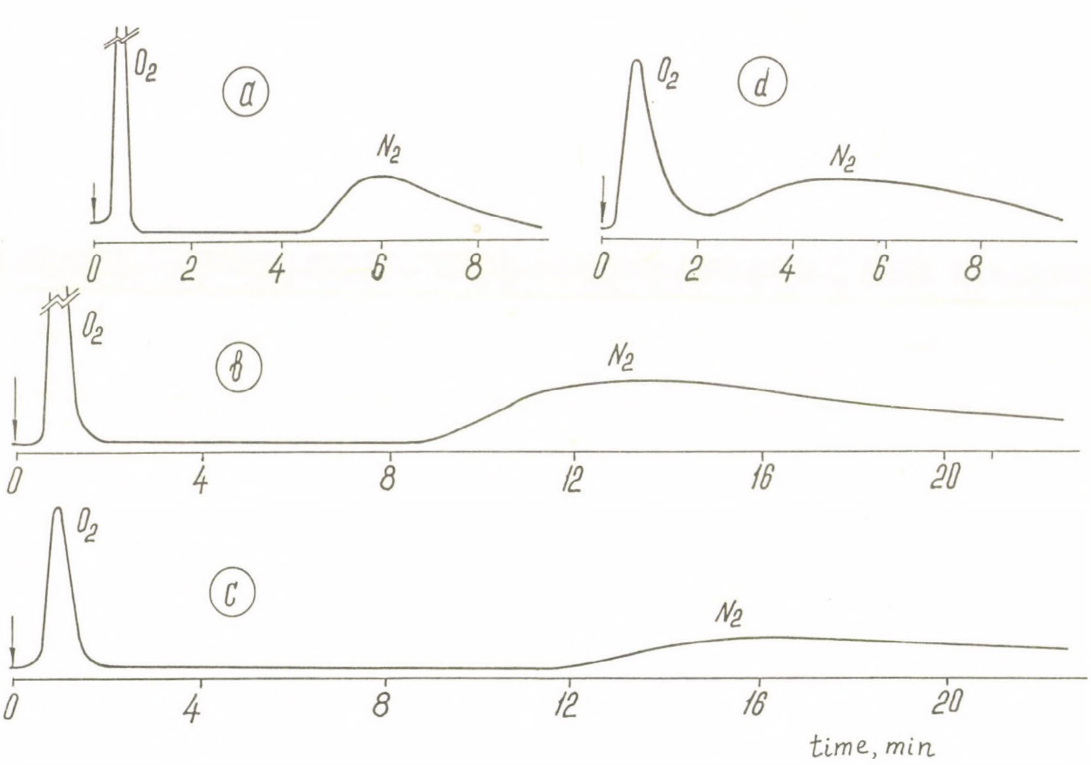

Fig. 2. Separation of oxygen and nitrogen on mordenite-containing rocks, enriched by calcium cations. Activation temperature of the samples: 450°C; column temperature: room; carrier gas (helium) flow rate: 100 ml/min. (a) B-1; (b) B-2; (c) B-3; (d) B-4

Table IV. The specific retention volumes (ml/g substance in the column) of oxygen and nitrogen as a function on the carrier gas. Sample: B-3; activation temperature: 450°C; column temperature: room; carrier gas flow rate: 100 ml/min

	Carrier gas				
	Helium	Neon	Argon	Nitrogen	Carbon dioxide
	Specific retention volume (ml/g)				
Oxygen	8.0	7.3	4.3	3.0	0.3
Nitrogen	161.5	146.5	47.5	-	1.0

Table V. Degree of separation (%) of a helium-hydrogen mixture at room temperature, as a function of the carrier gas, its flow rate and the column length

Carrier gas flow rate ml/min	Carrier gas			
	Argon		Neon	
	Column length			
	50 cm	100 cm	50 cm	100 cm
100	42 %	91 %	58 %	96 %
50	56 %	96 %	69 %	98 %
20	57 %	98 %	77 %	100 %

dioxide, and repeated activation at 450° B-3 sample using helium as the carrier gas, incomplete separation of the Ar-O_2 pair is possible at room temperature.

It is also difficult to separate the helium-hydrogen binary mixture; this requires the use of rather long columns filled with synthetic zeolites CaA and NaX and the proper selection of the carrier gas (4). We have studied the effect of the following factors on the separation of the helium-hydrogen mixture; the nature of the carrier gas and its flow rate, the mordenite content in the original sample, its cation modification, the activation temperature, and the column length. It was found that the nature of the carrier gas significantly affects the separation. When using nitrogen or carbon dioxide there is no separation. The use of argon or neon results in a

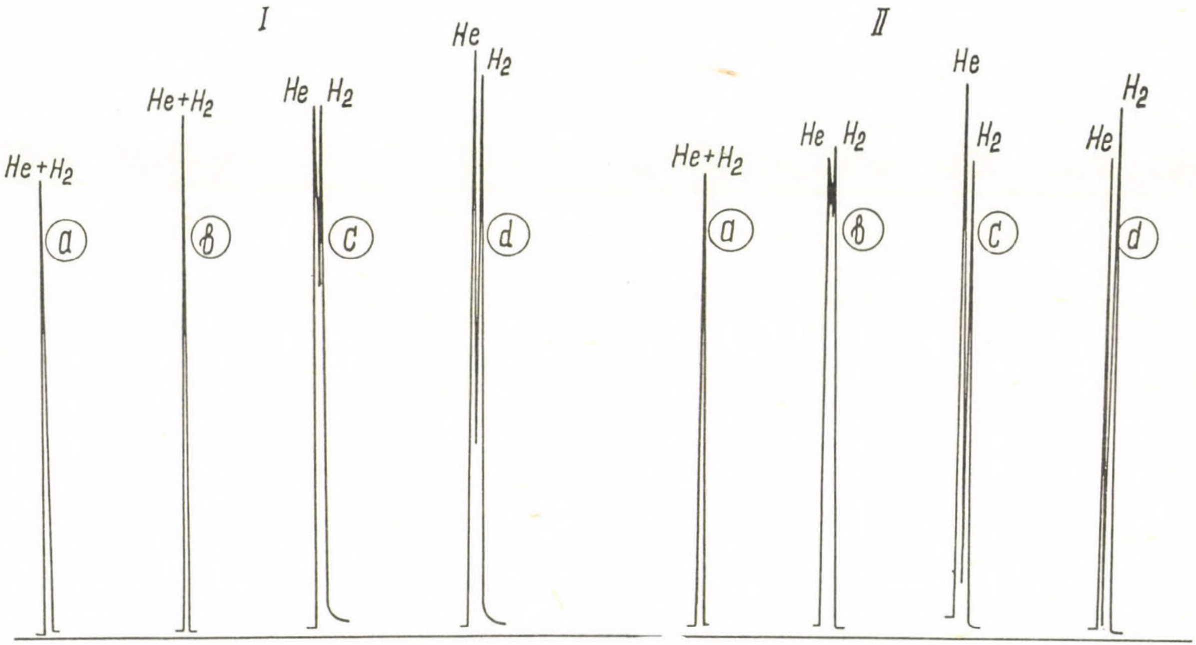

Fig. 3. Effect of the selection of the carrier gas and the length of the column on the separation of helium and hydrogen. Adsorbent: B-4 activated at 450°C; column temperature: room; carrier gas flow rate: 100 ml/min. I: column length: 50 cm; II: column length: 100 cm; carrier gas: (a) CO_2; (b) N_2; (c) Ar; (d) Ne

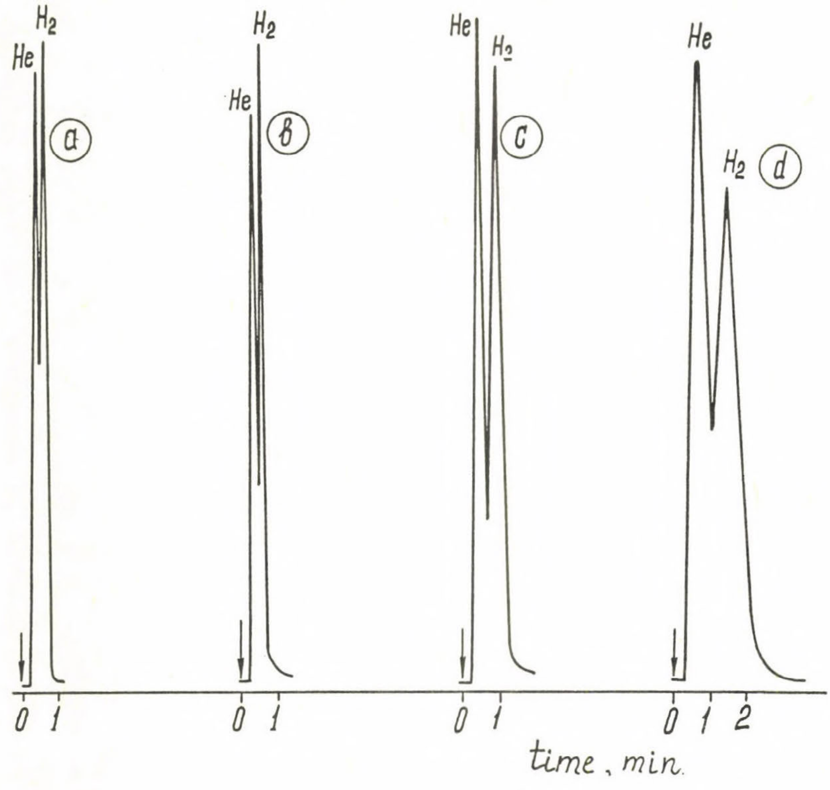

Fig. 4. Effect of the carrier gas flow rate on the separation of helium and hydrogen. Sample B-4 activated at 450°C; column temperature: room; column length: 50 cm; carrier gas: neon; carrier gas flow rate: (a) 100; (b) 50; (c) 20; (d) 10 ml/min

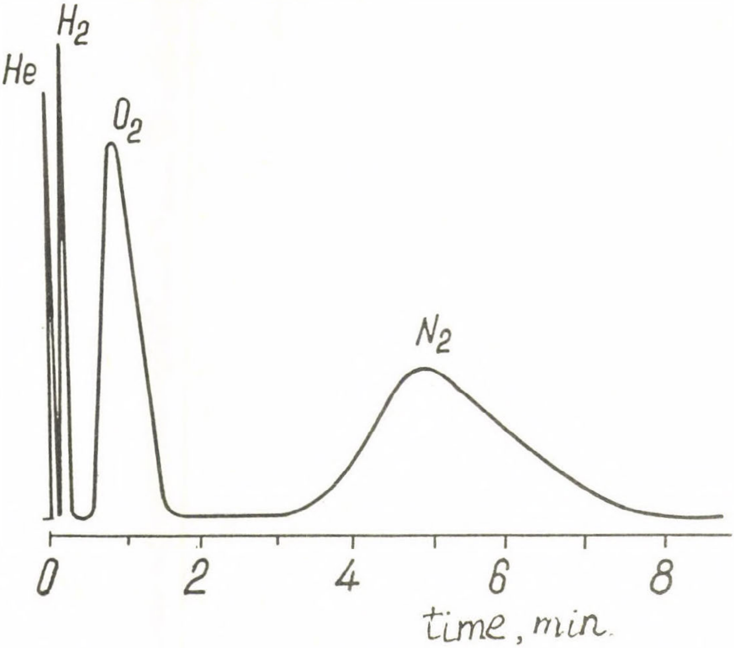

Fig. 5. Separation of the mixture $He-H_2-O_2-N_2$. Sample: A-4; column length: 100 cm; carrier gas (Ne) flow rate: 100 ml/min, column temperature: room

partial separation of this pair with this elution sequence; the separation with neon is better (Table V, Fig. 3). A decrease of the carrier gas flow rate to a definite limit also improves the separability of helium and hydrogen (Fig. 4). An increase in the mordenite content and enrichment by calcium cation results in a better separation of this pair. Increasing the column length from 50 cm to one meter results in complete separation (Table V), while with synthetic zeolites longer columns are needed (4).

As a conclusion, mordenite-containing tuffs with high mordenite content in the rock, both untreated and enriched with calcium cations, can be suggested for the separation of $He-H_2$ and O_2-N_2 binary systems as well as of the $He-H_2-O_2-N_2$ mixture (Fig. 5).

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CONFORMATIONAL EFFECTS IN HIGH-PERFORMANCE HYDROPHOBIC-INTERACTION CHROMATOGRAPHY OF CONALBUMIN

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SUMMARY

Secondary equilibrium effects resulting from on-column denaturation of conalbumin in high-performance hydrophobic-interaction chromatography (HIC) under isocratic and gradient conditions, were investigated. Mobile phase composition, flow-rate, and contact time of the protein with the hydrophobic stationary phase were the parameters evaluated in order to study the propensity of conalbumin to undergo conformational variation within the chromatographic time scale. Absorption ratio measurements with a photodiode array detector are consistent with the interpretation that anomalous chromatographic behavior of conalbumin are due to slow interconversions between native and partially or fully unfolded forms.

INTRODUCTION

Some proteins are eluted from chromatographic columns in unusually broad peaks, or even in discrete multiple peaks. This has been ascribed to several causes: (i) heterogeneity in the interactive sites of the stationary phase (1); (ii) aggregation (2); (iii) gradient artifacts (3); (iv) denaturation. Proteins are stabilized by a combination of hydrogen bonding, electrostatic interactions, and hydrophobic interactions. The same forces are also involved in the chromatographic processes, so that conformational changes induced by the mobile or stationary phase may occur, leading to a loss of bioactivity and denaturation. The native and denaturated forms can be resolved if conformational equilibrium is slow relative to the time scale of migration through the column; otherwise, a single broad peak will be obtained which is the weighted average of the forms in equilibrium. Strop and co-workers (4) ascribe the broad or multiple HIC peaks of human serum albumin and α-fetoprotein to normal conformational variations in the tertiary structure. The implication is that a protein can assume different conformations which can be adsorbed on the surface of the packing material with varying binding forces and, therefore, may be desorbed at varying times in gradient elution. Several authors have examined the effects of temperature on conformational behavior of proteins in reversed-phase chromatography (RPC)(5-8), as well as in hydrophobic-interaction chromatography (HIC)(9-12) and electrostatic-interaction chromatography (EIC)(13) with HPLC column and instrumentation. In spite of the fact that HIC is performed under non-denaturating conditions by using stationary phases having mild interactive properties ("soft" surfaces) and aqueous eluents, conformational effects can be exhibited, depending on the mobile phase, the column temperature, the retentivity of the stationary phase, and, especially, the lability of the protein. In the present study the propensity of conalbumin to undergo conformational variation within the chromatographic time scale has been assessed in terms of band broadening and peak multizoning phenomena associated with both isocratic and gradient elution.

EXPERIMENTAL PART

Proteins and reagents

The chromatographed proteins were α-chymotrypsinogen A, (CHY), β-lactoglobulin A (LAC-A), conalbumin (CON), cytochrome C (CYT), lysozyme (LYS), ovalbumin (OVA) and ribonuclease A (RNase). These proteins were selected because their retention behavior had already been studied in detail (14-16). All were purchased from Sigma (St.Louis, MO, USA). Reagent-grade monosodium and disodium hydrogen phosphate, sodium sulphate, phosphoric acid, sodium hydroxide, as well as HPLC-grade water were obtained from Carlo Erba (Milan, Italy).

Equipments

The experiments were performed with a Beckman (Berkeley, CA, USA) Model 342 liquid chromatograph, consisting of two Model 114 M solvent delivery pumps, a Model 420 system controller, a Model 210 sample injection valve with a 20-μl sample loop, a Model 340 dynamically-stirred high-pressure mixer, and a Model 163 variable UV detector. A Varian (Walnut Creek, CA, USA) Model Polychrom 9060 photodiode array detector with a Hewlett-Packard (Palo Alto, CA, USA) printer was used for the on-line spectroscopic analysis of conalbumin. Chromatograms were recorded with a Hewlett-Packard Model 3390 A integrator or with a Model D 5117 Omniscribe Recorder (Houston Instrument, Gistel, Belgium).

Chromatography

Data were obtained using a Toyo Soda (Tokyo, Japan) TSK Phenyl 5 PW column (75 x 7.5 mm). According to the manufacturer (17), this new support was developed by introducing phenyl groups (ca. 0.1 mmol/ml) with ether linkage into TSK gel G 5000 PW, which is a hydrophilic-polimer based material (polyacrylamide) of large pore size (1000 Å) for size-exclusion chromatography (particle diameter 10 μm). Mobile phases were prepared by adding the correct weight of salt and buffer to a volumetric flask containing previously degassed HPLC water. The pH was adjusted to the appropriate value with either phosphoric acid or sodium hydroxide, and a small amount of degassed HPLC water was added to the mark. The 50 mM phosphate buffer was prepared by mixing 25 mmol/l monobasic sodium phosphate with 25 mmol/l dibasic sodium phosphate. Samples were eluted isocratically, starting at 100 % eluent B and repeating at decreasing concentration of B until the protein was completely retained on the column. This generated a series of retention times for each protein. Eluent A was 1.0 M sodium sulphate, 50 mM phosphate buffer, 0.02 % sodium azide (pH 6.9). Eluent B was 50 mM phosphate buffer 0.02 % sodium azide (pH 6.9). Chromatograms were obtained under isocratic conditions at a nominal flow rate of 1.0 ml/min. After each change of mobile phase composition a period of 20 min ($\pm$ 0.5 min) was allowed for equilibration. Protein solutions (7-14 mg/ml) were freshly made up in HPLC water. When not in use, the samples were stored at -5°C. A 20-μl sample size was injected. The observed solvent pertubation peak was taken to indicate the retention time of an unretained component. Retention times were converted into retention factor (k') values by the equation: $k' = t_R - t_0/t_0$; where t_R is the retention time of the protein of interest, and t_0 is the retention time of an unretained component. A standard 20-min linear gradient from 0 to 100 % B was used in most experiments with a flow rate of 1.0 ml/min. Proteins were detected by monitoring the column effluent at 280 nm. Exception to these conditions are noted in the paper.

RESULTS AND DISCUSSION

Table I lists the elution order, and the retention volumes of a series of proteins chromatographed on the TSK Phenyl 5 PW column by a linear decreasing salt gradient. Except cytochrome C, all the proteins listed in the table were strongly retained and not eluted when chromatographed isocratically with the starting mobile phase. The effect of salt concentration on protein retention was investigated under isocratic conditions with mobile phases having different salt concentration. Isocratic elution was achieved by using 50 mM phosphate buffer (pH 6.9), containing various percentage of a solution of 1.0 M Na_2SO_4 in 50 mM phophate buffer (pH 6.9). A representative plot of the retention factor of conalbumin and lysozyme against the salt concentration in the eluents is shown in Fig. 1. The band dispersion of conalbumin and lysozyme is plotted in Fig. 2 against the retention factor. The band dispersion was estimated as the peak width (w_h) measured at the half of the total height of the chromatographic peak. The w_h values were obtained with a recording data processor for chromatography. Compared with

Table I. Retention volumes (V_e) of a series of proteins chromatographed under gradient elution conditions (see Experimental)

Protein	V_e (ml)
CYT	2.53
RNase	5.62
LAC-A	6.45
CON	7.74
OVA	9.06
LYS	12.02
CHY	18.44

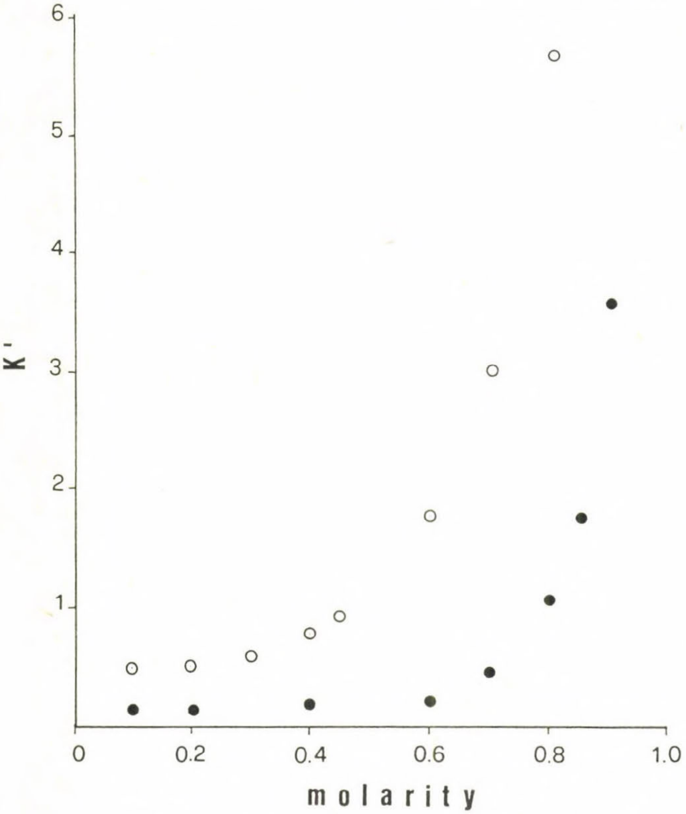

Fig. 1. Plot of the logarithmic retention factor of conalbumin (●) and lysozyme (o) against the Na_2SO_4 concentration in the eluent. Background electrolyte: 50-mM phosphate buffer, 0.02 % sodium azide (pH 6.9); flow rate: 1.0 ml/min. Samples: 10 mg/ml conalbumin, 12 mg/ml lysozyme, 20 µl injected. Detection at 280 nm; column temperature: 25°C

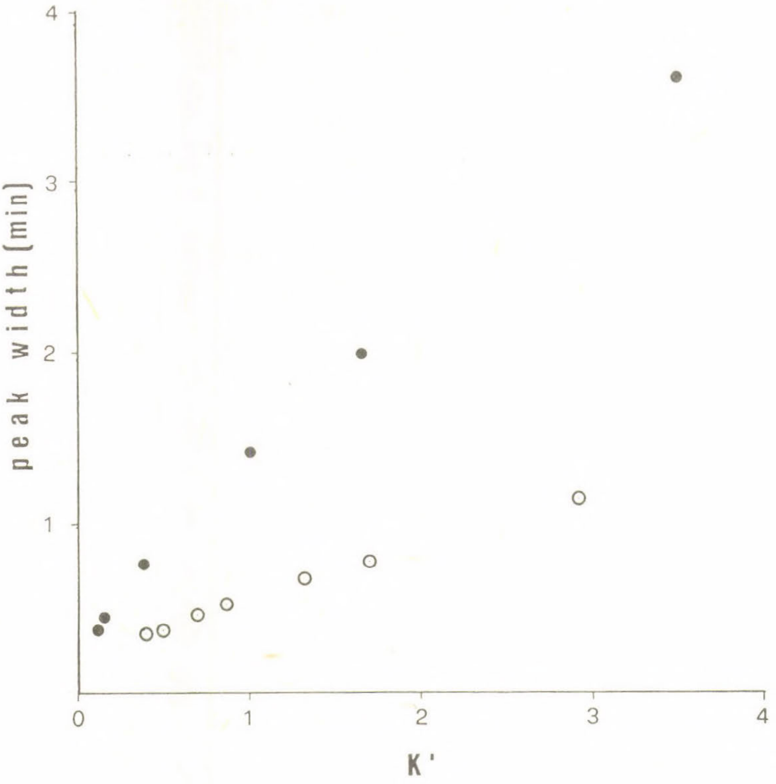

Fig. 2. Plot of the peak width (measured at half height) against the retention factor (k') of conalbumin (●) and lysozyme (o) chromatographed at various Na_2SO_4 concentrations in the eluent. Conditions as reported in Fig. 1

the data of lysozyme, the increase in band dispersion observed for conalbumin was much more dramatic as the relative retention was increased.

Lysozyme is known to be a very stable protein for which the thermal denaturation temperature in water at pH 7.0 is around 70°C (18). Karger and co-workers (11) showed that lysozyme is also stabile under HIC conditions and undergoes no conformational changes. Furthermore, Fausnaugh and Regnier (19) reported that the enzymatic activity of lysozyme is retained when this protein is chromatographed isocratically on the TSK Phenyl 5 PW column at various salt concentrations. The role of secondary retention effects including protein conformational changes on band dispersion in reversed-phase chromatography was extensively focused by Hearn and Grego (20). Therefore, one explanation which would account for the large peak width value differences at equivalent k' values, noted in the present investigation between the stable lysozyme and the more highly structured conalbumin, is the occurrence of slow interconversions between native and partially or fully unfolded forms of conalbumin during the chromatographic run.

Figure 3 presents the chromatographic gradient elution of conalbumin as a function of the contact time of the protein with the stationary phase. In this experiment an aqueous solution of the protein was injected into the column equilibrated with the starting mobile phase containing 1.0 M sodium sulfate, and an isocratic hold was maintained for a period of time before starting the linear decreasing salt gradient. The time from injection until the start of the gradient is defined as the incubation time. When a zero incubation time was used, it can be seen from Fig. 3a that conalbumin elutes as a relatively sharp peak. When a 10-min incubation time preceded the linear gradient a shoulder on the downslope of the chromatographic band was observed (Fig. 3b). This peak-splitting was proportionally more marked with the increase of the incubation time. We collected the fraction eluted over the first peak of the chromatogram obtained with 40-min incubation time. This fraction was rechromatographed under the same condtions of Fig. 3e and a similar chromatogram was observed. This result leads to the conclusion that the phenomena arise from conalbumin itself and not from impurities. Most likely the peak-splitting is due to either multiple conformers of the protein yielding similar but not identical retention or/and kinetic processes of conformational interchange.

Figure 4 illustrates the chromatographic gradient behavior of conalbumin as a function of mobile phase flow rate. In this experiment, the gradient rate (t_G; percentage of solution B per min) was changed in the same proportion as the flow rate (F) in order to maintain a constant gradient volume (i.e., $V_G = t_G \times F$). According to Snyder and co-workers (21), holding

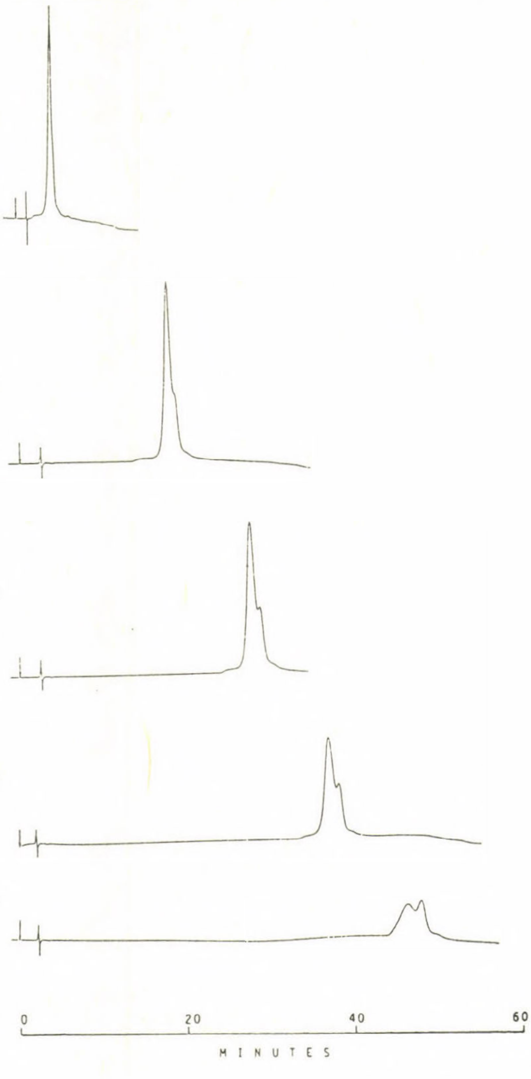

Fig. 3. Chromatographic behavior of conalbumin as a function of the on-column incubation time. Mobile phase A: 1.0 M Na_2SO_4, 50 mM phosphate buffer, 0.02 % sodium azide (pH 6.9); mobile phase B: 50 mM phosphate buffer, 0.02 % sodium azide (pH 6.9); gradient rate: 5 % mobile phase B/min, 20-min linear gradient; flow rate: 1.0 ml/min; sample: 10 mg/ml conalbumin, 20 μl injected; detection at 280 nm; column temperature 25°C; incubation times: a = 0 min, b = 10 min, c = 20 min, d = 30 min; e = 40 min

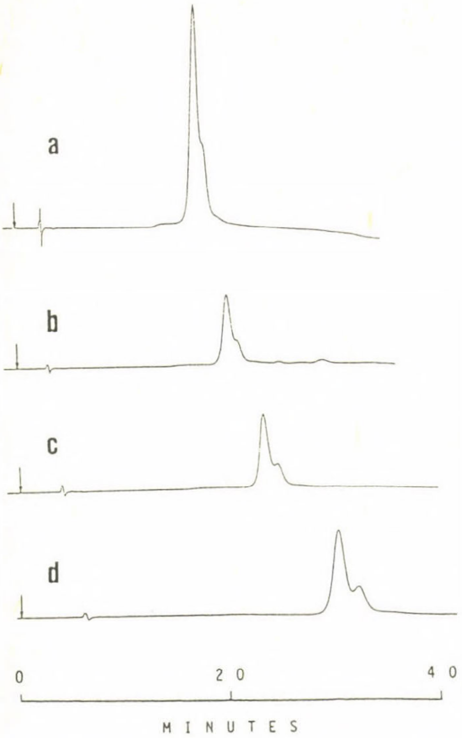

Fig. 4. Chromatographic behavior of conalbumin as a function of flow rate (F) and gradient time (t_G) at constant gradient volume (V_G); incubation time: 10 min; other conditions as reported in Fig. 3

V_G constant, the retention factor ($\bar{K}'$) as the protein passes the center of the column, is also maintained constant. It can be observed in Fig. 4 that the lower the flow rate the more pronounced is the band broadening and simultaneously, the greater is the peak-splitting. In this experiment, the time the protein spends travelling through the column varies inversely with the flow rate. For example, for F = 0.4 ml/min, the protein will spend twice as much time in the moblie phase as at a flow rate of 0.8 ml/min. Thus, apparently similar phenomena are occurring in either the stationary phase or/and the mobile phase.

Proteins exhibit UV-absorption spectra characteristic of their component amino acids, particularly the aromatic residues phenyl-alanine, tyrosine and tryptophan. Generally these residues will be found in the interior of a globular, water-soluble protein, since they are relatively hydrophobic. Conformational changes may bring one or more of the aromatic residues to the surface of the protein. These newly exposed residues can lead to small variations in the UV spectrum of the protein. We used a photodiode array detector to display variations in the UV spectra of the eluted protein at the wavelength characteristic for UV-absorption of the aromatic amino acid residues. Absoprtion at 292 nm is mainly due to tryphtophan, whereas at 274 nm both tryptophan and tyrosine may contribute. We selected the 273 nm/292 nm ratio to detect changes in tyrosine exposure. Table II presents data on the on-line spectroscopic analysis across the peak profile. Absorbance ratios are listed for a position before the peak apex (1), at the peak apex (2),and after the peak apex (3). Increases in the absorbance ratios proportional to the contact time of the protein with the hydrophobic stationary phase suggest that tyrosine amino acid residues have moved from the interior to the surface of conalbumin. Spectroscopic analysis was also conducted at four flow rates (1.0, 0.8, 0.6, and 0.4 ml/min), while the gradient volume was maintained constant. All the gradients were preceded by 10-min incubation time. Absorption ratios were calculated for four positions over the chromatogram, one before (1), and two after (3, 4) the peak apex (2). As shown in Table III, at a flow rate of 1.0 ml/min the spectral characteristics are similar across the peak and close to those obtained without any incubation time. Differences in the absorption ratios across the peak are observed at the lower flow rate, but the general trend is followed all over the peak, that the lower the flow rate the higher the absorption ratio. Thus, changes in tyrosine exposure seem to be related to the time that the protein spends in either the stationary or the mobile phase.

Table II. Absorption ratios of the UV-spectra of conalbumin eluted under the conditions represented in Fig. 3

F (ml/min)	t_G (min)	I_t (min)		273 nm/292 nm
1.0	20	0	1	1.45
			2	1.45
			3	1.44
1.0	20	10	1	1.46
			2	1.46
			3	1.45
1.0	20	20	1	1.47
			2	1.47
			3	1.47
1.0	20	30	1	1.51
			2	1.50
			3	1.51

F = flow rate (ml/min); t_G = gradient rate (%/min);
I_t = incubation time

Table III. Absorption ratios of the UV-spectra of conalbumin eluted under the conditions represented in Fig. 4

F (ml/min)	t_G (min)	I_t (min)		273 nm/292 nm
1.0	20	0	1	1.45
			2	1.45
			3	1.44
1.0	20	10	1	1.46
			2	1.46
			3	1.45
0.8	25	10	1	1.45
			2	1.46
			3	1.46
			4	1.45
0.6	33.3	10	1	1.46
			2	1.47
			3	1.46
			4	1.46
0.4	50	10	1	1.65
			2	1.63
			3	1.63
			4	1.62

F = flow rate (ml/min); t_G = gradient rate (%/min);
I_t = incubation time

CONCLUSIONS

We have used several artifacts to promote protein denaturation during the chromatographic process. The data confirm that when a protein is conformationally labile, the HIC peak is broad. This property is independent of the overall affinity for the stationary phase surface, since at a high salt concentration under isocratic conditions, conalbumin was eluted in extremely broad peak but had appreciably shorter retention time than lysozyme. Furthermore, the results show that conformational changes are related to the contact time of the protein with the hydrophobic stationary phase. The broad and splitted peak may represent the combined retention of the folded and unfolded protein, with the differences in binding strength due to the newly exposed hydrophobic groups being so small that there is a band overlap. At the same time, some broadening could arise from slow interconversion in the mobile phase. The simplest expression of this process can be depicted as a reversible two-state model, similar to those proposed by Karger et al. for RPC (6), and by Parente and Wentlanfer for EIC (13):

$$\begin{tikzcd}
\text{FOLDED}_{\text{mp}} \arrow[r, shift left] \arrow[d, shift left] & \text{UNFOLDED}_{\text{mp}} \arrow[l, shift left] \arrow[d, shift left] \\
\text{FOLDED}_{\text{sp}} \arrow[r, shift left] \arrow[u, shift left] & \text{UNFOLDED}_{\text{sp}} \arrow[l, shift left] \arrow[u, shift left]
\end{tikzcd}$$

Subscripts mp and sp represent the mobile and the stationary phase. The intermediates between the folded and the unfolded forms can be omitted if their lifetime is irrelevant on the chromatographic time scale. For a fast interconversion between folded and unfolded forms, one peak corresponding to the average retention time would be expected. If the kinetic process of conformational change is slow relative to the chromatographic time scale, two peaks would be predicted. For an intermediate equilibration rate, broad and asymmetrical bands may result. Thus by changing the chromatographic time scale, secondary equilibrium effects resulting from on-column denaturation may be detected.

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THE TLC-HPLC STUDY OF IPSAPIRONE (TVX Q 7821) METABOLISM IN RATS

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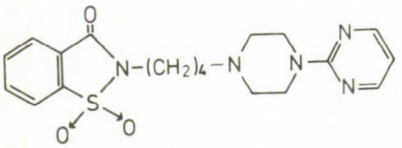
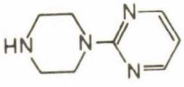
INTRODUCTION

Ipsapirone (TVX Q 7821) is a new anxiolytic drug which interacts with the central serotonergic system (1). It contains in the molecule pyrimidinyl-piperazine moiety a system which is active biochemically and pharmacologically (2). As buspirone another anxiolytic agent with the chemical structure similar to that of TVX Q 7821 was metabolized in rats to 1-(2-pyrimidinyl)-piperazine (1-PP) and the latter metabolite - acc. to Caccia et al. (3) - may contribute to the pharmacological effect of the parent drug, it is possible that also TVX Q 7821 - when given to rats - is biotransformed to 1-PP, the latter compound being capable of playing some role in the pharmacological effects of TVX Q 7821 (Fig. 1). Therefore we have performed a chromatographic (TLC, GC-MS and HPLC) investigation of the urine and the brain of rats receiving single dose of TVX Q 7821 to establish whether TVX Q 7821 is metabolized in rats to 1-PP (Fig. 1) and if so, whether the metabolite is accumulated in the central nervous system (CNS) of these animals.



TVX Q 7821



1-PP

Fig. 1. Chemical structure of TVX Q 7821 and 1-PP

MATERIALS AND METHODS

The experiments were carried out on male Wistar rats (200-250 g) kept on a natural day-night cycle at room temperature during autumn and spring. The animals were fed with the standard granulated diet (Bacutil) and had free access to tap water.

1. Isolation and identification of 1-PP from the urine of rats by TLC and GC-MS

Rats received a single dose (30 mg/kg ip) of TVX Q 7821 (ipsapirone hydrochloride, Troponverke) and were placed in a metabolic cage. Their urine was collected for 24 h and then it was extracted with the 20 % solution of methanol in chloroform (1:1). The organic extract was concentrated under the diminished pressure to 1 ml and put on a preparative chromatoplate (PSC Kieselgel 60 F-254, Merck) against a standard of 1-PP (1-(2-pyrimidinyl)-piperazine hydrochloride, Mead Johnson) and TVX Q 7821. The chromatoplate was developed in the chloroform-methanol system (9:1), and the spots and bands were localized in the UV light (254 nm). The band that corresponded with the standard of 1-PP post (Rf = 0.37) was removed from the plate and extracted with 5 ml of chloroform by mechanical shaking for 15 min; the chloroform extract was concentrated under the diminished pressure to the volume approx. 0.1 ml and rechromatographed on the preparative chromatoplate in methanol--conc. ammonium hydroxide system (9.5:0.5). The procedure was repeated twice. The final chloroform extract was evaporated to dryness and the residue was analysed by GC-MS (LKB-9000S).

2. Quantitative analysis of 1-PP in the rat brain by HPLC

a. Calibration curve

Brains of intact animals were homogenized with 0.1 N HCl (1:4) the homogenate was centrifuged (2000 g for 15 min), and to 1 ml of the supernatant there was added a standard solution of 1-PP and TVX Q 7821 to give a final concentration 2; 4; 6; 8 and 10 μg of 1-PP and TVX Q 7821/sample (each sample was prepared in triplicate). Then the samples were alkalized with 1.0 N NaOH to pH = 10.4 and extracted twice with 3 ml of chloroform. Combined chloroform extracts were evaporated under a diminished pressure to dryness, the residue was dissolved in 2 ml of methanol, and 20 μl of methanol solution was injected into the HPLC system (Kipp und Zonen LC 771) equipped with the Waters stainless column (8 mm x 10 cm) packed with Nova Pak C_{18}, UV detector (254 nm) and Orlita pump. Mobile phase: methanol-acetonitrile (2:1). Sensitivity approx. 2 μg of 1-PP per 1 ml of the supernatant. The linear relationship between 1-PP concentration and the height of the peaks on the chromatogram was found within the concentration range of 2 μg-10 μg of 1-PP per 1 ml of the supernatant (r = 0.89)(Fig. 2). Under described conditions only trace amount of TVX Q 7821 was extracted into organic phase.

b. Assay of 1-PP in the rat brain

Rats received TVX Q 7821 in a single dose of 30 mg/kg ip and 30 min later they were killed and their brains were dissected.

Simultaneously an internal standard and tissue blanks were prepared. In this aim the intact animals were killed,

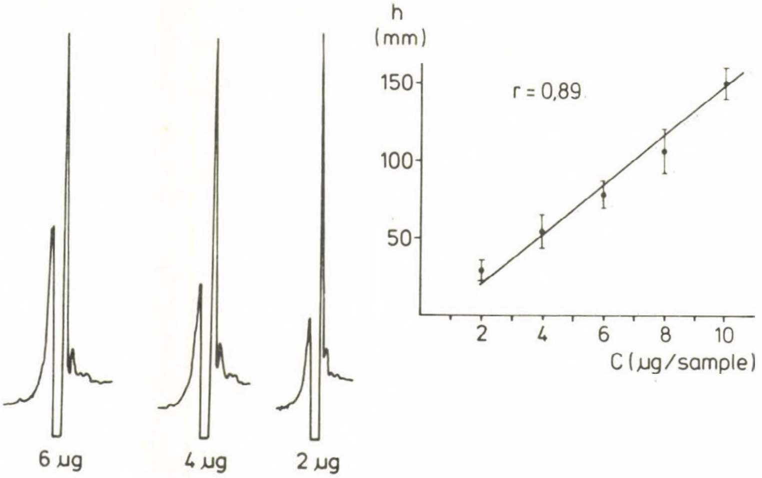


Fig. 2. Calibration curve of 1-PP. Abscisse: 1-PP concentration in the sample; ordinate: peak height; n = 3. On the left side an example of the chromatograms of samples containing different amounts of 1-PP

their brains were isolated, homogenized and 1 ml samples of supernatant were used for internal standards (10 µg of 1-PP/1 ml of supernatant) and tissue blanks perparation. 1-PP was assayed in the samples by HPLC as described before.

RESULTS

As indicated the results of TLC analysis of the urine of rats TVX Q 7821 - when given to rats in a single dose of 30 mg/kg ip - was metabolized among others to 1-PP, the metabolite was excreted with the urine (Fig. 3).

The chemical structure of 1-PP ,isolated from the urine of rats treated with a single dose of TVX Q 7821, was confirmed by GC-MS (Fig. 4).

The results of quantitative HPLC analysis of the rat brains showed that 30 min after administration of TVX Q 7821 to rats in a single dose of 30 mg/kg ip, 1-PP was accumulated in the brain of these animals in amount approx. 1 µg/g of the brain tissue (Fig. 5).

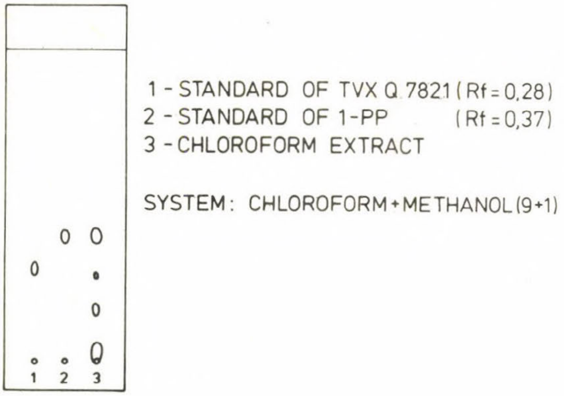


Fig. 3. Chromatoplate of chloroform-methanol extract of urine of rats receiving TVX Q 7821 in a single dose of 30 mg/kg ip

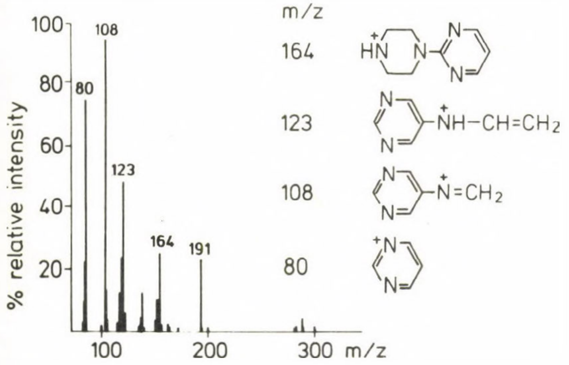


Fig. 4. Mass-spectrum of 1-PP isolated from the urine of rats receiving TVX Q 7821 in a single dose of 30 mg/kg ip

CONCLUSION

As indicated presented results of TLC, GC-MS and HPLC analysis of the urine and brains of the rats treated with TVX Q 7821, this compound, similarly to buspirone, is metabolized

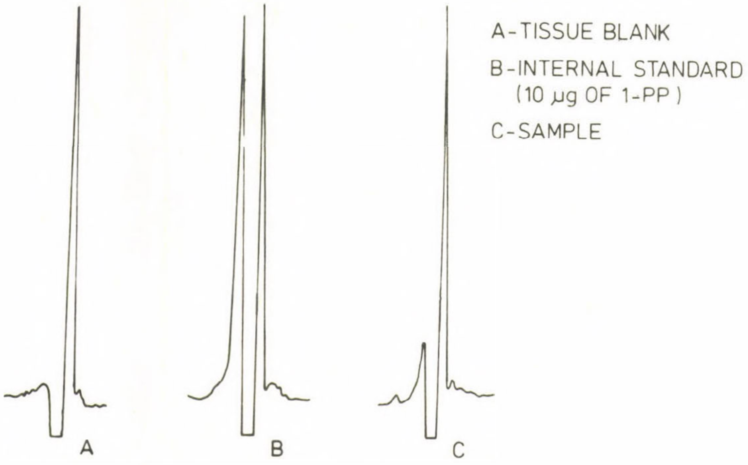


Fig. 5. Chromatograms of the rat brains. Animals received TVX Q 7821 in a single dose of 30 mg/kg ip. A - tissue blank; B - internal standard of 1-PP (10 µg); C - sample from the brain

in rats to 1-PP and the metabolite is accumulated in the rat brain. Therefore it can be assumed that 1-PP may contribute to the pharmacological effect of the parent drug. The parent compound and the metabolite differ in their in vitro interaction with brain receptors; TVX Q 7821 appears to be active on 5-HT_1 receptor (1), 1-PP was highly effective in the interaction with cortical α_2 receptor (3). It is of interest to assess the role of 1-PP in the anxiolitic effect of the parent compound to determine which of these in vitro receptor activities is present when TVX Q 7821 is administered to rats.

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NEW MOBILE PHASE FOR THE HPLC SEPARATION OF PIGMENTS IN SOME VEGETABLES

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SUMMARY

Chromatographic separation of individual colour substances of foodstuffs always faces the problem that oxidation and isomerization may take place during the separation process. The chromatographic investigation of food pigments was carried out using mobile phases containing either acetone or chloroform alone or as a gradient system. These organic solvents were found to be harmful to the chromatographic material and to the separated pigments as well.

In our work a new mobile phase was developed and used for the separation of chlorophyllys, xanthophyllys and carotenoids of vegetable origin with low harmful effect. The new mobile phase was composed of acetone-isopropanol-water (39:57:4) and it was applied for the investigation of the pigment composition of tomato fruit, paprika powder and carrot with improved separation characteristics on Chromsil C_{18} 10 μm stationary phase.

Compound identification was achieved by direct scanning of the maximum absorption spectra of each component during HPLC separation as well as by TLC separation on Kieselgel 60 using two mobile phases.

INTRODUCTION

The use of HPLC for the analysis of carotenoids and other natural pigments is rapidly replacing classical gravity-flow column chromatographic methods. The reason for the growing use of HPLC in pigment analysis lies in the rapidity, nondestructiveness and analytical nature of these methods. Both normal and reversed-phase columns are used for carotenoid and retinoid analysis. Normal-phase solvent systems routinely contain a primary nonpolar solvent, such as n-hexane or petroleum ether, with low or increasing concentrations of a more polar solvent such as diethyl ether, acetone or an alcohol. On the other hand, reversed-phase solvent systems normally utilize mixtures of water with alcohols or acetonitrile (1).

Mixtures of water and acetone were used for the chromatographic separation of naturally occurring pigments of some vegetables (2-4) on LiChrosorb or Chromsil C_{18} by reversed--phase chromatography. However, acetone is harmful to both the stationary phase and the separated pigments.

The objective of this work was to develop a new mobile phase for the separation of different pigments of vegetable origin with minimal harmful effect and to establish the corresponding chromatographic characteristics using a C_{18} column.

MATERIALS AND METHODS

Materials

Tomato fruit (Lycopersicum esculentum cv. Venture and Bulka)were obtained from the University of Horticulture in Budapest while carrot (Daucus Carote cv. Nanti) was obtained from the local market and paprika powder from Paprika Processing plant in Szeged. All organic solvents used were from Reanal.

Sample extraction and preparation

The method of Urlich & Mackinney (5) was applied with some modification for the extraction of different pigments from tomato fruit and carrot. The method and our modifications are outlined in Fig. 1. Pigments and paprika powder were extracted by shaking 0-5 g of powder with 100 ml of acetone

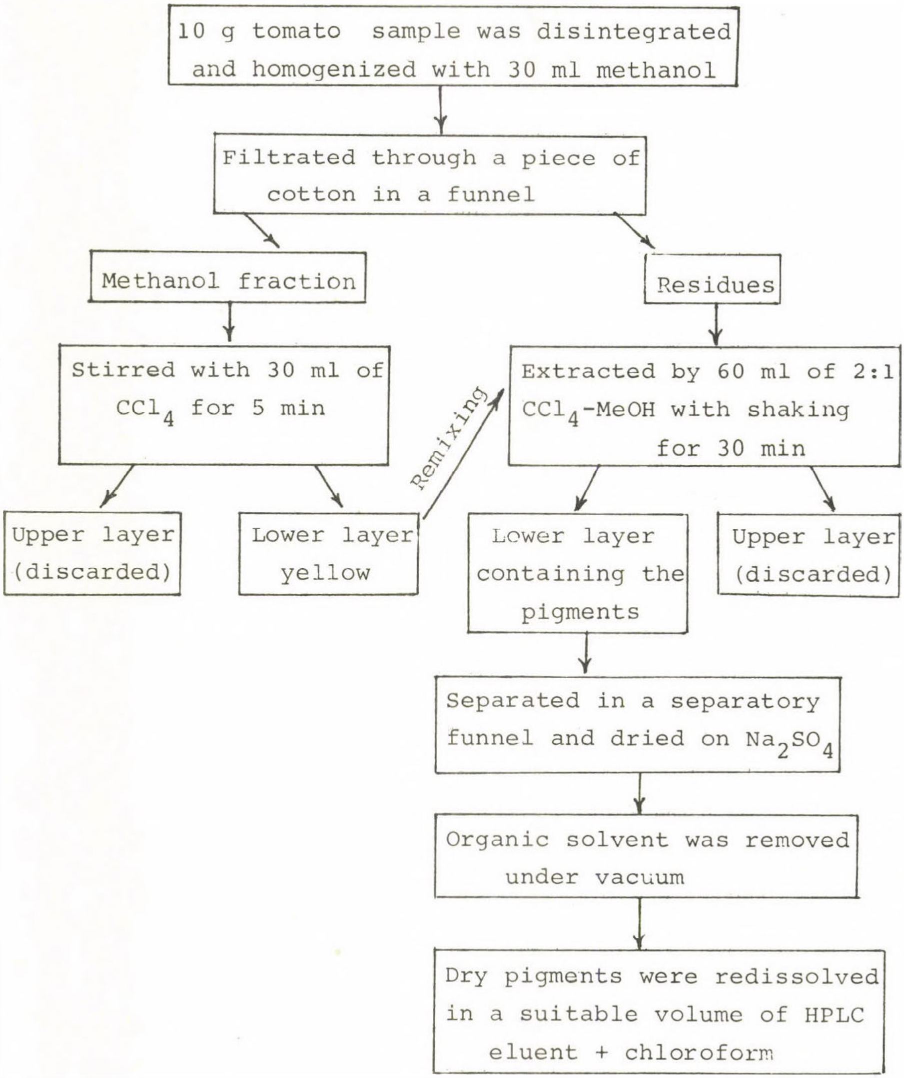

Fig. 1. Scheme illustrating the extraction and preparation steps of tomato fruit pigments for HPLC investigation

for 30 minutes. A 20 ml aliquot of the clear extract was evaporated to dryness under vacuum with a rotary evaporator at room temperature. The dry material was then redissolved in the HPLC mobile phase containing 25-50% chloroform.

TLC separation

Kieselgel 60 preparative TLC plates (Merck) were used to separate the different pigments with two developing solvents:

80:10:5:5	Hexane-benzene-acetone-acetic acid; and
85:10:5	Hexane-benzene-acetone

300 µl of the redissolved pigment was spotted at the 6 cm line on the TLC plate and developed with the eluent for 1 hour.

HPLC analysis

Two HPLC systems were used:

a) Beckman liquid chromatograph equipped with a Model 114 M pump, a Model 420 controller and a Model 165 variable wavelength UV detector. The detector signal was electronically integreted by a Shimadzu Model C-R3A integrator.

b) Liquochrom 2010 (Labor MIM Hungary) series liquid chromatograph.

The pigments were separated on a 250 x 4.6 mm i.d. column packed with Chromsil C_{18} 10 µm phase and eluted with the following mobile phases:

90:10	acetone-water;
85:10:5	acetone-water-hexane; and
39:57:4	acetonitrile-siopropanol-water.

Flow rate was 1 ml/min and detection was carried out at 438 nm.

Peak identification

The peaks from tomato fruit and carrot pigments were identified according to their retention time compared with some available standards such as β-carotene, lycopene and zeaxanthin. The peaks were also spectrally scanned between 300-700 nm maxima using the Beckman HPLC system. The absorption maxima of the peaks were also compared with the values reported in the literature (6-9).

The Red pepper pigment peaks were identified according to Biacs et al. (3). The method included TLC separation, alkaline hydrolysis of each band, measurement of the visible spectra and HPLC detection of the individual pigments.

RESULTS AND DISCUSSION

The carbon tetrachloride-methanol mixture used for the extraction of pigments from fresh vegetable tissues was very efficient and unharmful to the extracted pigments. Some yellow pigments of ripe vegetables could not be extracted from the first methanol fraction because of their higher solubility in polar solvents such as water and methanol. These pigments belong to the flavonone group of plant pigments since their absorption maxima was at 277 nm and 315 nm and no absorption was noticed in the visible region of the spectra.

Separation and identification of pigments

Chromatograms of some vegetable extracts are shown in Fig. 2. In reversed-phase chromatography, the more polar compounds elute first such as zeaxanthin, lutein, chlorophylls and their derivatives in green tomato and capsanthin, capsorubin, and some esters in red pepper powder (10).

It was found that β-carotene overlapped with lycopene on the tomato extract chromatograms and its area seemed to be larger than actual when dissolved in the mobile phase. This problem could be solved by dissolving the pigments in the mobile phase to which 20% chloroform or hexane was added (Fig. 3.).

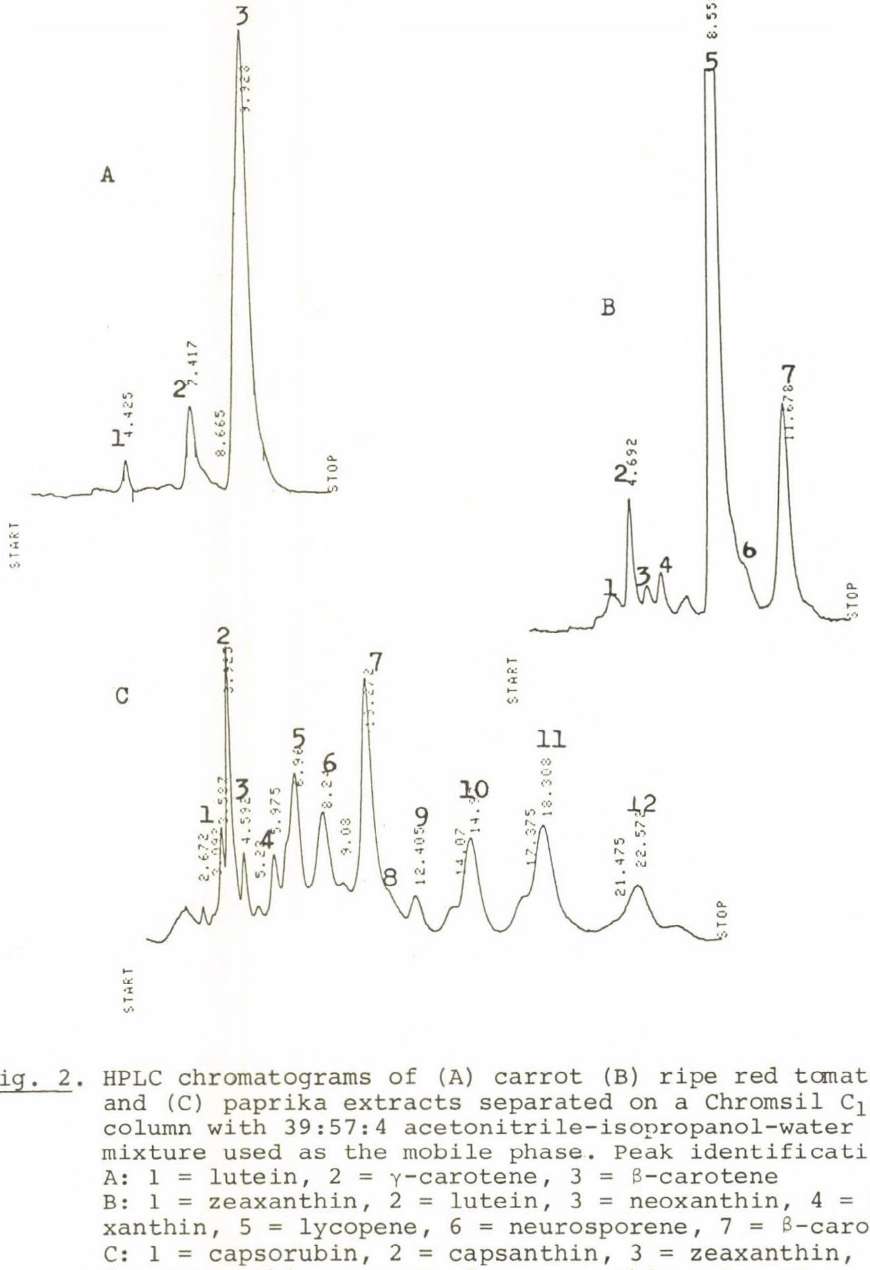

Fig. 2. HPLC chromatograms of (A) carrot (B) ripe red tomato, and (C) paprika extracts separated on a Chromsil C_{18} column with 39:57:4 acetonitrile-isopropanol-water mixture used as the mobile phase. Peak identification:
A: 1 = lutein, 2 = γ-carotene, 3 = β-carotene
B: 1 = zeaxanthin, 2 = lutein, 3 = neoxanthin, 4 = lycoxanthin, 5 = lycopene, 6 = neurosporene, 7 = β-carotene
C: 1 = capsorubin, 2 = capsanthin, 3 = zeaxanthin, 4 = capsanthin ester I, 5 = capsanthin ester II, 6 = zeaxanthin ester, 7 = β-carotene, 8 = capsorubin ester I, 9 = capsorubin ester II, 10 = capsanthin ester III, 11 = capsanthin ester IV, 12 = oxidation product

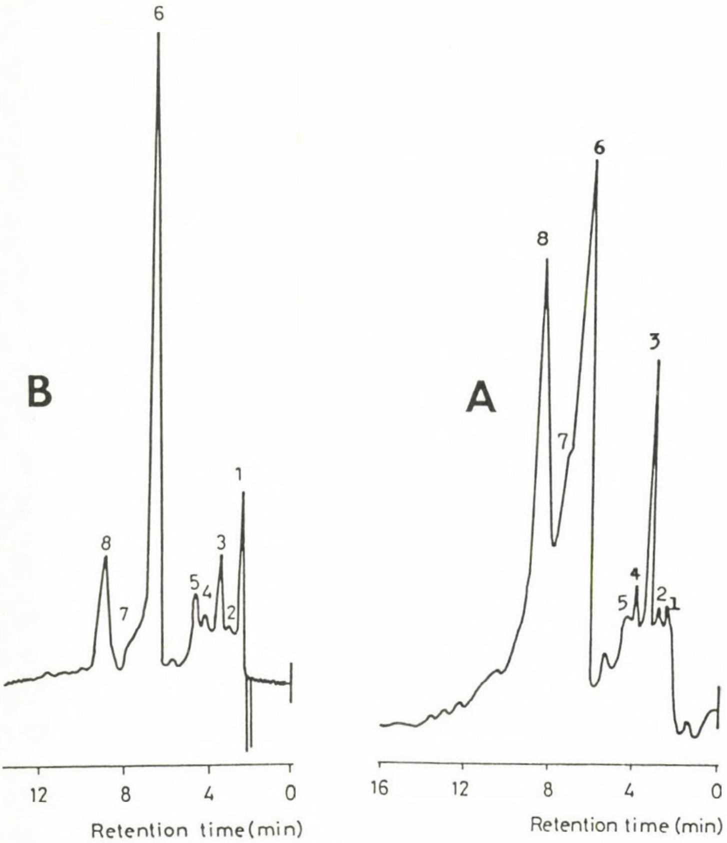

Fig. 3. Effect of pre-injection solvation of tomato fruit pigments on the RP-HPLC separation on a Chromsil C_{18} column using 39:57:4 acetonitrile-isopropanol-water the mobile phase. Peaks: 1 = solvent, 2 = zeaxanthin, 3 = lutein, 4 = neoxanthin, 5 = lycoxanthin, 6 = lycopene, 7 = neurosporene, 8 = β-carotene
A = the pigments were dissolved in the mobile phase;
B = the pigments were dissolved in the mobile phase containing 25% chloroform

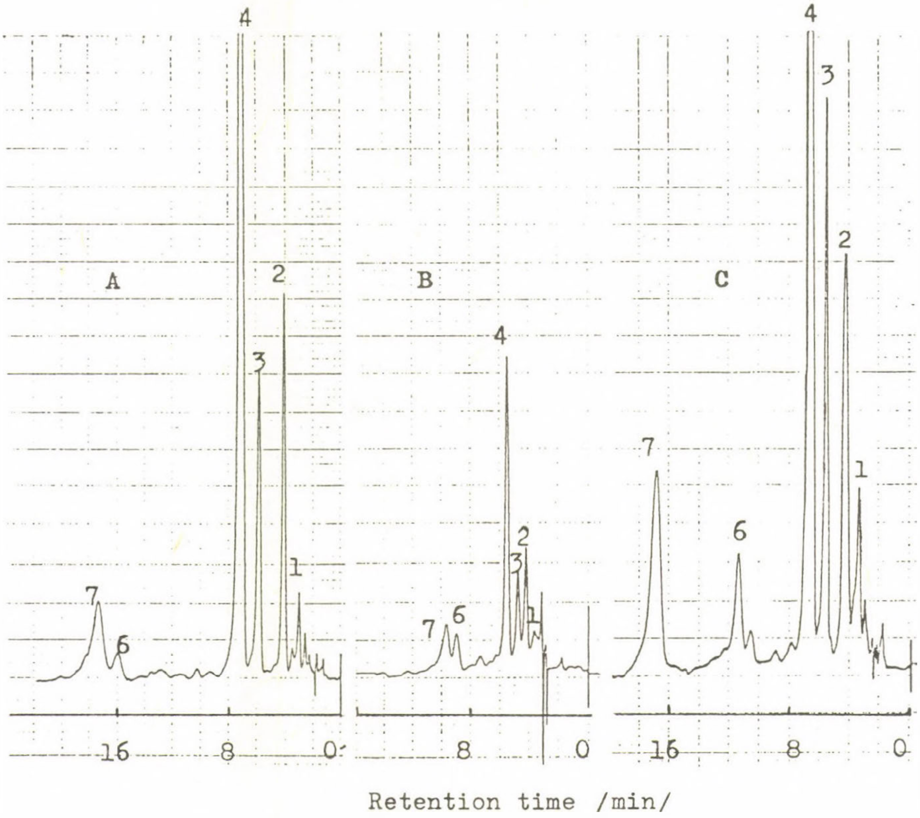

Fig. 4. HPLC separation of green tomato fruit pigments on a Chromsil C_{18} column using different mobile phases. A: 90:10 acetone-water; B: 39:57:4 acetonitrile-isopropanol-water; C: 85:10:5 acetone-water-hexane. Peaks: 1 = zeaxanthin, 2 = lutein, 3 = chlorophyll B, 4 = pypropheophytin B, 5, 6 = unidentified, 7 = β-carotene

Separation of pigments on the C_{18} column using different mobile phases is shown in Fig. 4. The best separation with the lowest detrimental effect on the C_{18} material was achieved with the acetonitrile-isopropanol-water mobile phase. The addition of hexane at 2.5-5% concentration to the acetone-water mobile phase resulted in a better separation on the C_{18} column. It is believed that hexane, as a non-polar solvent, neutralized the high polarity caused by the impurities present in acetone, leading to a remarkable improvement in the characteristics of the column.

However, the application of such a mobile phase, is not advisable since acetone and hexane cannot be used for an extended period as the mobile phase in a C_{18} column. The utilization of a non-polar solvent as the mobile phase constituent with a C_{18} column had been reported by Zakaria and Simpson (11) who used a 8:92 chloroform acetonitrile mixture to separate provitamin A carotenes from tomato fruits using Bondapack C_{18} and Partisil-PXS columns.

One of the advantages of the acetonitrile-isopropanol--water mobile phase is the short time needed for the complete elution of the pigments in comparison with other mobile phase systems. The fast elution reduces exposure of the pigment to oxidation during the separation process. Table I lists the retention time and capacity factor values of different pigments present in red pepper powder, eluted with two mobile phases. The capacity factor and retention time values of most pigments were also stable when acetonitrile-isopopropanol-water was used. This indicated the properness of this mobile phase for the separation of different pigments with a less influence on the C_{18} groups of the packing material.

Spectral scanning of the pigments

Figure 5 shows the visible absorption spectra of tomato fruit pigments scraped off the TLC plate and automatically measured using Specord Model 40 scanner (Carl-Zeiss). The spectrum of some pigments such as β-carotene and the violet xanthophylls separated on one-dimensional TLC plate showed more maxima than those of the pure components indicating the existence of more than one component in the same band. Some pigments such as neurosporene could be separated from the β-carotene band by redevelopment with a less polar solvent system such as 95:5 hexane-benzene, but other pigments such as xanthopylls could not be recovered since these pigments are very sensitive to oxygen and light and are rapidly damaged. Isomerization of colour substances on TLC with silica gel

Table I. Retention times (t_R) and capacity factor values (k') of individual pigments of red pepper powder

	Acetonitril-isopropanol-water		Acetone-water	
	t_R	k'	t_R	k'
Capsorubin	3.68	1.25	2.5	1.53
Capsanthin	3.95	1.35	2.75	1.75
Zeaxanthin	4.6	1.74	3.26	2.3
Lutein	5.27	2.14	4.4	3.45
Capsanthin ester I	6.07	2.6	5.9	4.94
Capsanthin ester II	7.05	3.2	6.8	5.86
Zeaxanthin ester I	8.32	3.9	8.36	7.4
β-Carotene	10.24	5.09	9.2	8.28
Capsorubin ester I	11.20	5.66	9.6	8.62
Capsorubin ester II	12.53	6.4	16.4	15.46
Capsanthin ester III	15.09	8.0	20.6	19.63
Capsanthin ester IV	18.3	9.8	25.8	24.85
Oxidation product	22.4	12.3	33.15	32.18

plates could also be observed by the appearance of two bands having the same or close absorption spectra as violet xanthophylls (Fig. 6.).

Direct scanning of the absorption spectra between 300 nm and 700 nm combined with the comparison of the TLC data with those reported in the literature represents the best way for the rapid identification of a wide variety of colour substances. Table II presents the absorption maxima of different colour substances when scanned during their separation on the C_{18} column and eluted with acetonitrile-isopropanol-water mobile phase.

Scanning techniques permitted us to distinguish between two components which have close retention times but appear at different stages of ripening, such as e.g., propheophytin in green tomato and lycoxanthin in the red fruit. Spectral scanning of different pigments can be seen in Figs 7 and 8. The other advantage of this technique is that complex metabolites can also be detected; e.g., the peak with a retention time of 0.6 min was found to be a chlorophyll-carotenoid complex, since its spectral scanning showed two absorption areas, the first at 409 and 654 nm and the second at 470,

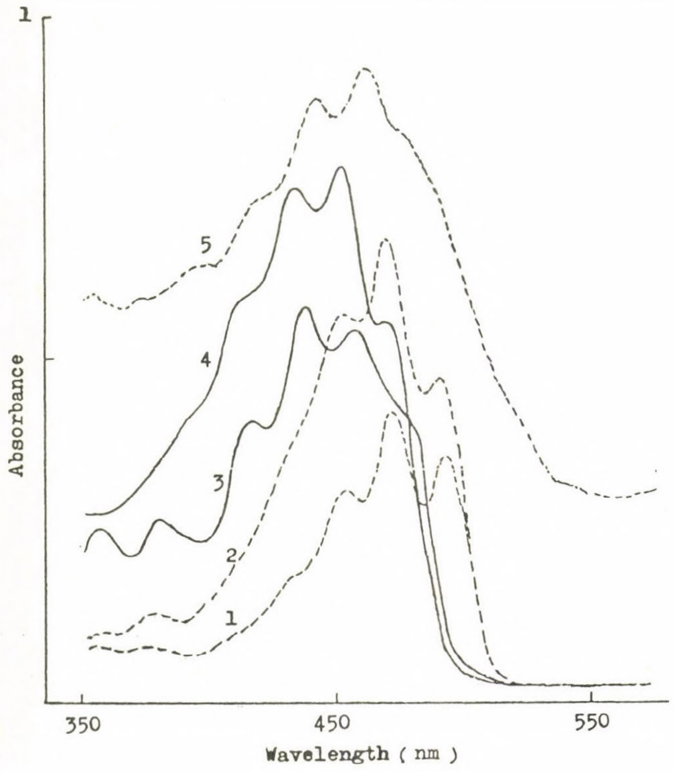

Fig. 5. Visible spectra of tomato fruit pigments scraped off the TLC plate and redissolved in the appropriate solvent. 1 = prolycopene, 2 = lycopene, 3 = ζ-carotene + β-carotene, 4 = β-carotene, 5 = neurosporene

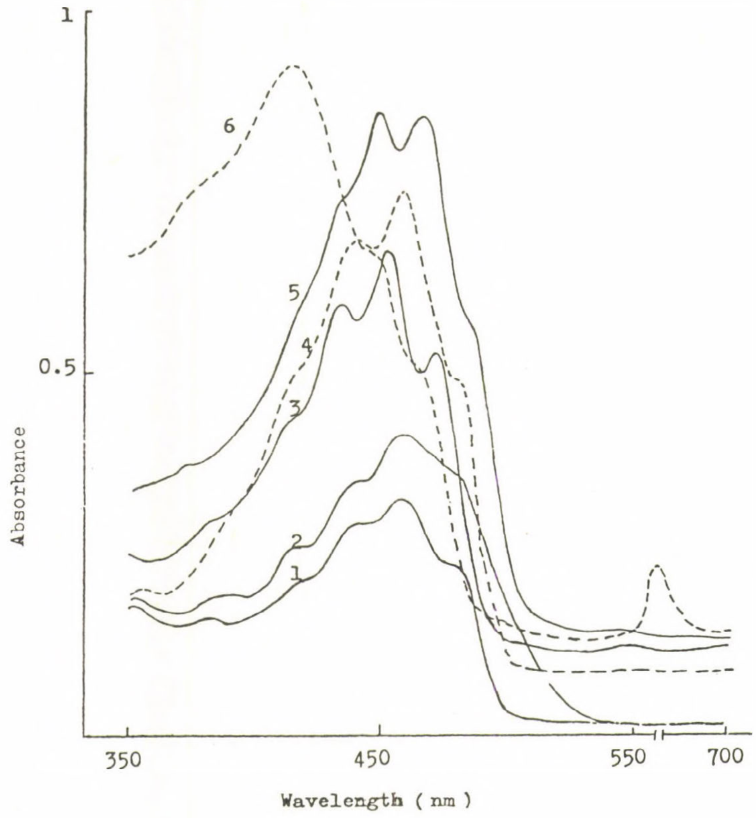

Fig. 6. Visible spectra of some xanthophylls and chlorophylls of tomato fruit. 1 = violaxanthin 2 = unidentified violet pigment, 3 = lutein, 4 = cis mutatoxanthin, 5 = neoxanthin, 6 = pheophytin

517 and 534 nm. The majority of the absorption corresponded to the chlorophyll moiety.

Table II. Absorption maxima of different pigments directly scanned during the HPLC separation using a Chromsil C_{18} column with 39:57:4 acetonitrile-isopropanol-water mixture as the mobile phase

Components	Absorption Maxima (nm)	Source
Chlorophyll B	462, 646	Green tomato
Pyropheophytin B	440, 666	
Carotenoid-chlorophyll (unidentified)	409, 470, 503, 534, 656	
Lutein	415, 430, 457	Ripe red tomato
Zeaxanthin	422, 447, 471	
β-Carotene	456, 476	
β-Carotene	446, 477, 510	
Neoxanthin	427, 445, 480	
Lycoxanthin	451, 479, 506	
Lycopene	456, 479, 509	
Capsanthin	462, 475, 517	Red pepper
Capsanthin ester I	452, 475, 486	
Capsanthin ester II	458, 481	
Capsanthin ester III	466, 492, 498	
Capsanthin ester IV	465, 490	
Capsorubin	452, 476, 517	
Capsorubin ester I	488, 507	
Capsorubin ester II	475, 492	

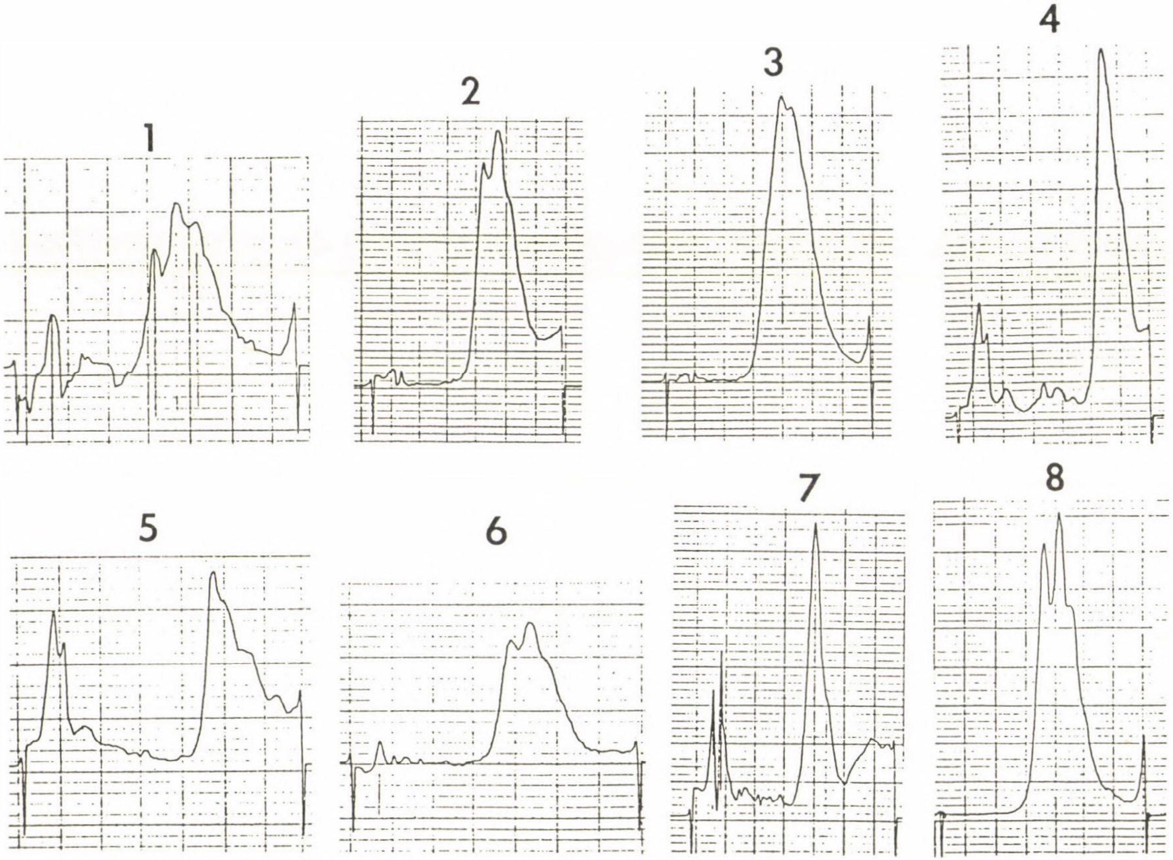

Fig. 7. Spectral scanning of the major pigments of tomato fruit as separated by HPLC. 1 = lycoxanthin, 2 = lutein, 3 = β-carotene, 4 = unidentified chlorophyll-carotenoid complex, 5 = pheophytin B, 6 = neoxanthin, 7 = chlorphyll B, 8 = lycopene

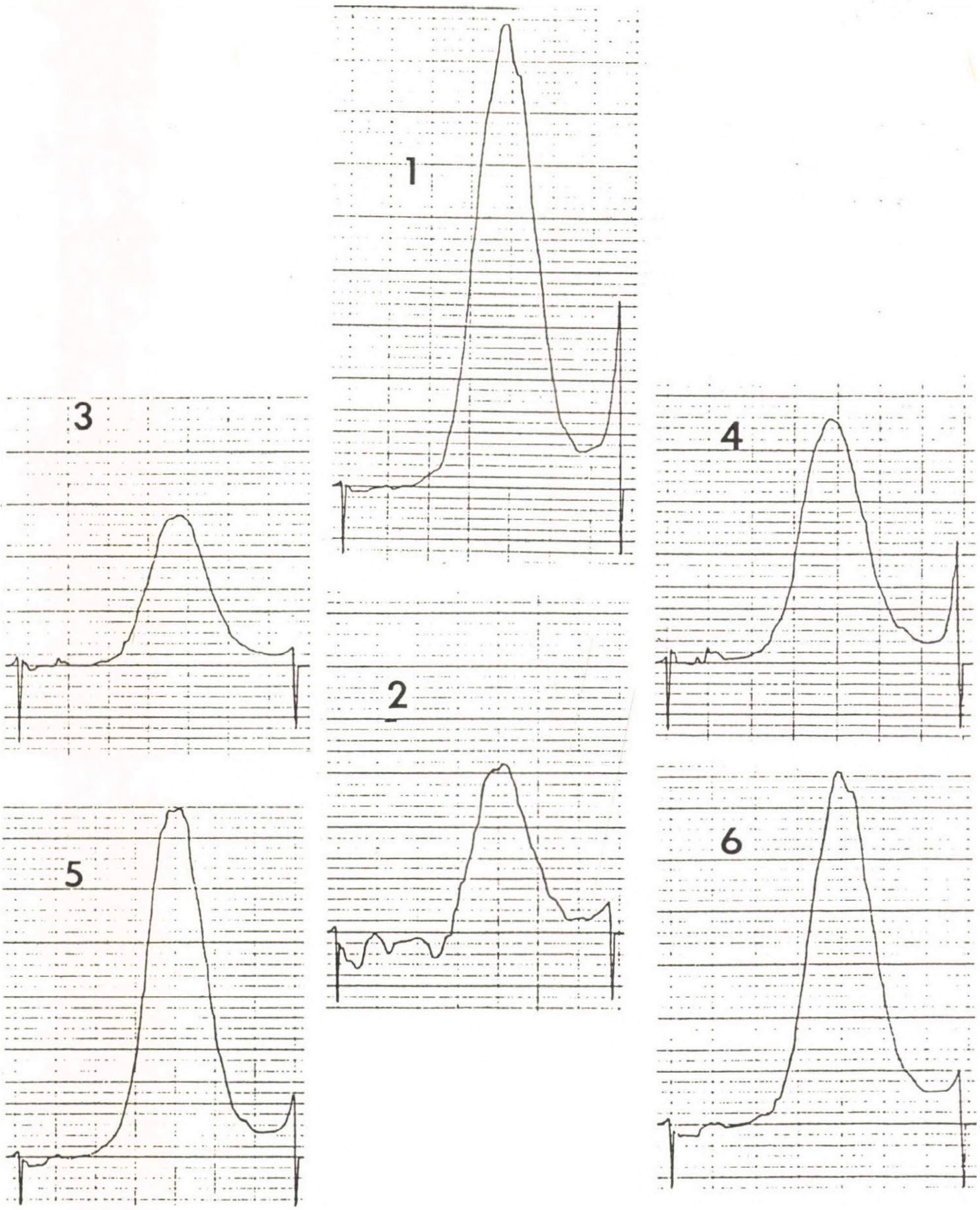

Fig. 8. Spectral scanning of paprika pigments separated by HPLC
1 = capsorubin, 2 = capsanthin, 3 = capsorubin ester I,
4 = capsorubin ester II, 5 = capsanthin ester I,
6 = capsanthin ester II

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MULTI-ELEMENT ANALYSIS BY SOLVENT EXTRACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF SOIL SAMPLES

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SUMMARY

High-performance liquid chromatography (HPLC) was used after preliminary solvent extraction for the complete separation of trace amounts of metal chelates prior to their non-selective determination. A UV detector set to the most intense absorbance peak of the reagent (used for the solvent extraction) was used as a universal detector for all the metal chelates at approximately the same sensitivity.

Normal-mode HPLC using diethyl dithiocarbamates was applied to the analysis of a series of soil samples. These were obtained as soil extracts in molar (1M) hydrochloric acid, prior to their extraction with a solution of diethyldithiocarbamate. The results were compared with those of the analysis of the same samples by means of atomic absorption spectroscopy (AAS).

INTRODUCTION

As applied to soil investigation, the principal function of total trace element analysis is connected with mineralogy and assists in determining the soil genesis and classification (1, 2). It is also used in the study of long term changes in a soil, however, the determination of the total amount of an element will reveal its inadequacy.

In order to establish a fairly simple and routine multi-element method for monitoring trace elements in soil samples, HPLC, after preliminary solvent extraction, was investigated for the complete separation of trace amounts of metal chelates prior to their non-selective determination. In this way the overall selectivity of the organic reagents can be increased up to the point at which they will be useful for the rapid routine fingerprinting of groups of trace metals, and comparable to the use of derivative gas-liquid chromatography for organic compounds. If the separation is complete, an ultraviolet detector set to the most intense absorbance peak of the reagent itself can be used as a universal detector for all the metals at approximately the same sensitivity. A sulphur-containing reagent, diethyldithiocarbamate (DDTC)(3), was chosen because of its high molar absorptivity and because it forms strong complexes with heavy trace metals, such as lead and mercury.

In this study HPLC was used in the absorption mode because metal chelates are typically very soluble in non-polar solvents such as chloroform. This high solubility has proved to be an advantage because, with the high distribution ratios of the complexes in such solvents, it was possible to use relatively small volumes for the extraction. This practice avoids the need for a slow evaporation step prior to direct injection of the aliquot onto the HPLC column.

The soil samples were initially dried (or ignited) and digested with a hydrofluoric-perchloric-nitric acid mixture and finally extracted with hydrochloric acid, before being extracted with the DDTC solution.

EXPERIMENTAL

Reagents

All reagents were of Analar grade unless stated otherwise.

Diethylammonium diethyldithiocarbamate (DDTC) solution. Dissolve 0.50 ± 0.01 g of DDTC in 100 ± 1 ml of dry chloroform (prepare freshly every week and store in a refrigerator after use).

Buffer solution. Dissolve 45.2 g ammonium chloride and 9.6 ml of concentrated ammonia solution (sp.gr.0.880) in 1000 ml of water in a graduated flask. This solution should have a pH of 8.5. Dilute 10-fold before use.

Chloroform. Dry over a molecular sieve.

HPLC eluent. Dry spectro-grade benzene (Fisons Scientific Instruments, Loughborough, Great Britain) with a molecular sieve and degas before use.

Apparatus

The HPLC system used was the model PU4000 modular instrument of Pye Unicam, Cambridge, England. The atomic absorption spectrophotometer used was the model GBC-901 of GBC Scientific Equipments Pty Ltd., Australia.

Sample tubes (20 x 50 mm) were used for the collection of the organic phases after solvent extraction of the samples. These tubes should be fitted with Snap-on polyethylene lids. Other apparatus consisted of 400 and 600 ml graduated borosilicate (pyrex) glass beakers and 500 and 1000 ml separating funnels fitted with ground-glass stoppers and taps (if possible Interflow polyethylene taps); 1^{P}s silicone-treated phase separating paper (Whatman labsales, Springfield Mill, Maidstone, Great Britain) was used.

Procedure (including calibration)

Add an appropriate aliquot of the standard (see Note a below) or sample (Note b) solutions to a clean 600-ml graduated pyrex glass beaker and adjust the pH of the solution to about 8 with either 2M hydrochloric acid or 2M sodium hydroxide solution. Add an appropriate amount of buffer to maintain the pH of the metal solution at 8.5 ± 0.1 (Note c). Dilute the solution to 300 ± 1 ml in a beaker or in a large measuring cylinder.

Transfer the solution into a clean 500-ml separating funnel, add 5.0 ± 0.05 ml of DDTC (Note d) solution in chloroform with a clean pipette as required, stopper the funnel, shake it vigorously for 3 min ± 15 sec, and allow it to stand for 5 min ± 30 sec.

Separate the organic from the aqueous phase by filtration through the phase-separating paper into a sample tube and fit the lid (Note e). The HPLC stage must be completed during the same working day.

Inject 2-10 μl of this chloroform solution of the separated metal chelates onto an already equilibrated chromatographic column by the stop-flow syringe mode of injection. Mark the injection point on the recorder trace. Measure the peak heights and compare with calibration standards.

Blank determination. A blank must be run with each batch of determinations (e.g., up to 10 samples) using the same batch of reagents as for the samples and also the sample eluent for the HPLC run. Carry out the above steps again, but replace the standard or sample solution with distilled water and make up to mark as for samples.

Calibration. Stock standard solutions (Note a) are used for calibrations. Duplicate calibration standard should be run with each batch of determination (e.g.,up to 10 samples). Using an appropriate aliquot of the stock standard solution, carry out the steps. The linearity of the calibration graph should be checked from time to time, by plotting peak height against concentration of sample solution in the aqueous phase (the volume ratio, V_{aq}/V_o, used is 100, i.e., 500 ml of aqueous phase to 5 ml of organic phase).

Notes. (a) Stock standard solutions should be prepared by dissolving the appropriate metal or stoichiometric salt (Analar or 99.9% purity) at a concentration of 1000 ppm.
(b) Sample pre-treatment. The soil samples were sampled according to recommended Procedures (4, 5). Accurately weight about 0.1 g of oven-dry, 0.15 mm soil into a platinum crucible and moisten with a drop or two of water. Add 5 cm^3 of hydrofluoric acid and 0.5 cm^3 of perchloric acid, nearly cover the crucible with its lid and heat on a sand-tray at 200°C until the liquid has evaporated. Any unoxidized organic matter deposited on the upper part of the crucible or its lid can be

oxidized with the flame of a Meker Burner (6).

After cooling, add 5 cm^3 of 6M hydrochloric acid and half fill the crucible with water. Place the crucible in an electrothermal Bunsen and gently boil the contents for 5 minutes. If this treatment does not completely dissolve the solids, evaporate to dryness and repeat the acid digestion. When the residue has been quite dissolved, transfer to a 100-cm^3 volumetric flask and dilute to volume. (c) Do not use more than 100 ml of buffer solution, in order to avoid the introduction of errors. (d) It was observed that some of the commercial diethyldithiocarbamic acids produce oxidation products that are liable to interfere in the determination of nickel and manganese dithiocarbamate, especially at lower levels. In order to remove these two interferents from the reagent, it is recommended that the reagent should be back-washed to remove oxidation products before being used for the preparation of metal complexes. (e) All samples and standards should be processed to this stage before proceeding to the HPLC stage.

RESULTS AND DISCUSSION

The HPLC behaviour of the metal chelates of DDTC used in this work were studied on silica gel columns with spectrograde benzene as the mobile phase. Typical HPLC conditions giving the best compromise between efficiency, speed and sensitivity of separation were: column, stainless steel (150 mm x 4.6 mm i.d.); packing, Hypersil (5 μm particles); eluting agent, spectro-grade benzene; flow rate, 1.25-1.50 $ml \cdot min^{-1}$ at ambient temperature; detector, ultraviolet at a wavelength of 280 nm and with 0.05 a.u.f.s.d. With these chromatographic conditions, the limits of detection of the metals and the reproducibility are those given in this paper.

Figure 1 shows an example of the chromatogram obtained by using a standard mixture of transition metals, after separation as the DDTC complexes. The resolution between successive peaks is nearly complete in most instances and the complete profile can be recorded in about 8 minutes. Each peak of the chromatogram corresponds to about 100 ng of the metal,

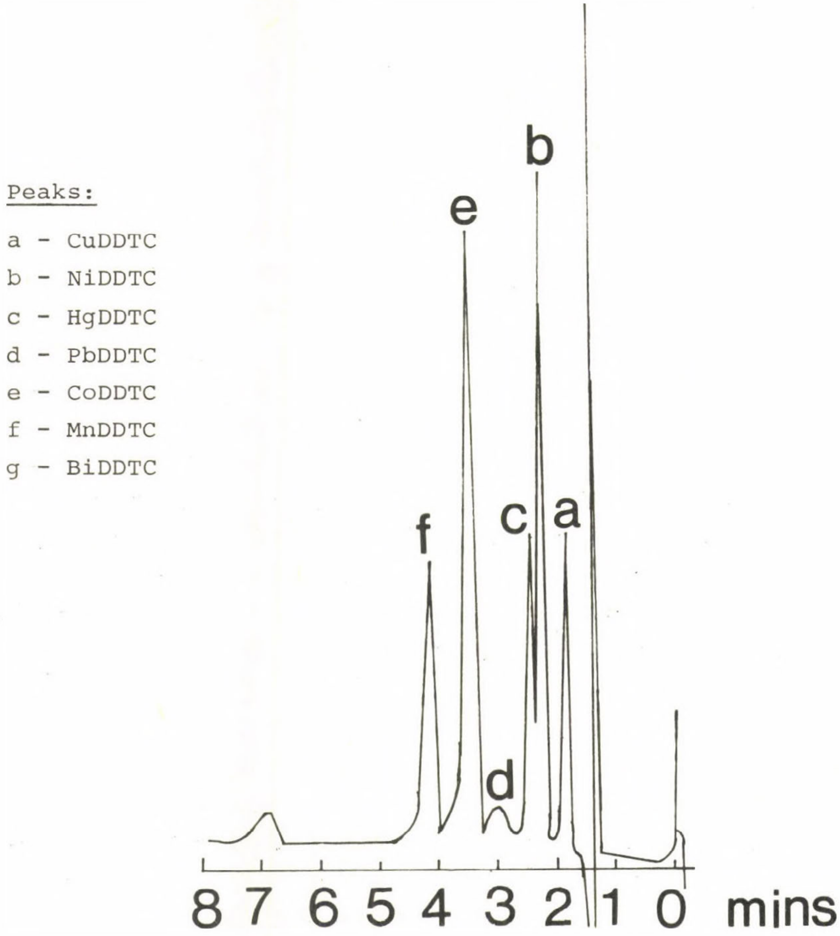


Fig. 1. Standard trace metal sample (Extracted with DDTC solution at pH 8.5)

injected as the DDTC complex. The chromatogram proved reproducible with retention times, retention volumes and peak heights remaining constant to better than ± 5% (coefficient of variation). Figure 2 shows calibration results for the peak heights of cobalt, copper, nickel, mercury, manganese and lead, extracted as the DDTC complexes. The number of theoretical plates in the 150-mm column ranged from 520 for lead to 4,000 for manganese and most of the other first-row transition elements.

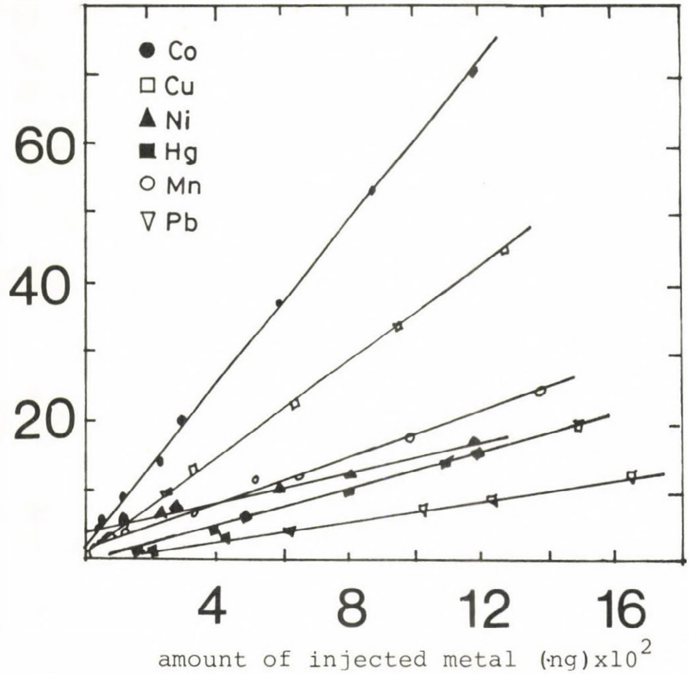


Fig. 2. Dependence of the peak height on the amount of metal injected for various metal-DDTC complexes

APPLICATION TO SOIL SAMPLES

The method of normal-mode HPLC using diethyldithiocarbonate was applied to the analysis of soil samples for various sources including the Gezira Board, Sudan, Bauchi State Agricultural Development Programme (Bauchi State, Nigeria/World Bank Project), Nigeria and Dr. M.M. Thomson, Imperial College of Science and Technology, England. The samples were used as soil extracts in 1M hydrochloric acid.

5 ml of each appropriate extract of fifteen samples were treated in four batches by solvent extraction following the procedure described above. 1 μl of these organic extracts (5 ml) were eluted on the Hypersil column, using benzene as eluent. Each sample was run in replicate and, with each batch of samples, standards of known quantities of individual elements were also extracted and their HPLC chromatograms run along with the samples.

Peak identification was carried out by reference to the retention volume (V_R) of standard metal chelate peaks under similar conditions. This was subsequently confirmed by spiking the sample solution with a measured amount of a standard solution of the metal in question before the extraction step.

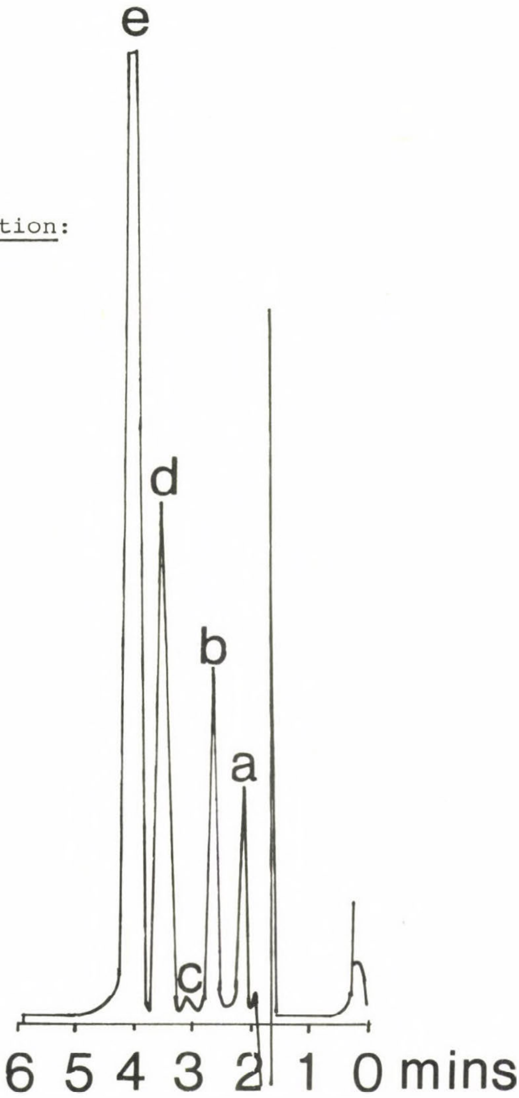
In some of the fifteen soil extracts examined in this study, it was found that the lead and zinc content was very high. This was as high as 600 to 900 ppm, and hence made the analysis of some trace metal difficult for a few of the samples.

However, the majority of the samples were found to contain low lead and zinc concentrations, hence do not interfere with the analysis of those samples. It was found that samples with up to 50 ppm each of lead and zinc in the soil extracts gave excellent results. Those with lead and zinc levels above 50 ppm each gave unreliable results. A typical chromatogram of one of the samples analysed is shown in Fig. 3. The analysis time for the determination of copper, cobalt, nickel and manganese in one of the samples is about thirty minutes using the prepared soil extracts in hydrochloric acid.

The results for the fifteen soil samples examined including the chromatographic conditions used are summarised in Table I. The samples were also analysed by atomic absorption spectroscopy (AAS) and the results were compared with those obtained by HPLC.

From the results shown in Table I, it is observed that there is good agreement between the HPLC results and those obtained by AAS. Owing to interference by broad lead and zinc dithiocarbamate peaks the determination of manganese in particular in samples SE10 and SE11 respectively are adversely affected.

In conclusion the application of the method (see Fig. 4) to the rapid analysis of soil sample extracts will be very useful particularly in developing countries where the careful characterization of soil samples for agricultural usage is fast becoming a priority, as the dwindling food situation in such countries becomes more serious. This method owing to its speed of execution will be useful for adoption on a routine basis, especially for large sample through laboratories in developing countries, as the skills needed to apply the method are not too demanding.



Peak identification:

- a – CuDDTC
- b – NiDDTC
- c – PbDDTC
- d – CoDDTC
- e – MnDDTC

Fig. 3. Typical soil sample (S.G. 4) extracted at pH 8.5 with DDTC solution in dry $CHCl_3$

Table I. Analysis of trace metals in soil samples
Column: 150 x 4.6 mm I.D., Hypersil 5 µm; eluent: HPLC grade benzene; flow rate: 1.53 ml/min; pressure: 287 p.s.i. (19.5 bar); detector: UV at 285 nm, 0.01 a.u.f.s.
Extraction was carried out at a buffer pH of 8.5 with 0.5% (w/w) DDTC in chloroform. A volume of 1 µl sample solution was injected (as metal dithiocarbamates)

Metal	Method	Sample No.														
		SG1	SG2	SG3	SG4	SG5	SN6	SN7	SN8	SN9	SN10^{+}	SK11^{+}	SK12	SK13	SK14	SK15
Copper, µg/ml^{-1}	HPLC	0.08	0.3	0.3	1.15	0.3	0.2	-*	1.0	0.2	1.2	0.6	0.65	0.3	0.15	0.75
	AAS	0.09	0.3	0.2	1.0	0.34	0.2	0.1	0.9	0.24	1.3	0.7	0.55	0.3	0.1	0.7
Nickel, µg/ml^{-1}	HPLC	-	0.6	0.3	0.4	0.46	2.5	0.3	1.1	0.8	-	-	0.3	0.1	-	-
	AAS	0.07	0.5	0.2	0.4	0.56	2.6	0.2	1.0	0.4	0.6	0.3	0.3	0.16	0.04	40.1
Cobalt, µg/ml^{-1}	HPLC	-	0.2	0.05	0.1	0.3	0.7	0.1	0.35	0.2	-	-	0.1	0.1	0.05	0.1
	AAS	0.04	0.22	0.1	0.13	0.3	0.6	0.13	0.3	0.2	0.2	0.2	0.12	0.1	0.02	0.07
Manganese, µg/ml^{-1}	HPLC	1.6	0.6	6.0	1.8	14.0	13.0	10.0	10.0	8.8	-	-	3.3	1.8	0.7	6.9
	AAS	1.3	0.5	5.9	2.2	14.5	13.8	9.5	10.3	8.6	36.3	35.2	3.7	2.0	0.8	7.0

*Dashes indicate not detected

$^{+}$Samples SN10 and SK11 showed serious interference possibly from lead and zinc. However, all other samples showed little or no interference effects during the determination of the trace metals

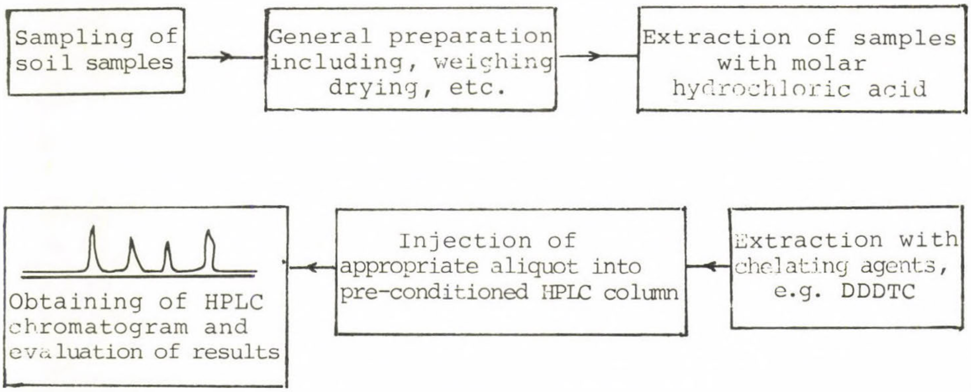


Fig. 4. Summary of HPLC procedure for trace metal analysis in soil samples

ACKNOWLEDGEMENT

Grateful acknowledgement is hereby made to the Gezira Board in the Sudan, the Bauchi State, Ministry of Agriculture and Forestry Resources, Bauchi, Nigeria and Dr. M. Thompson, of Imperial College of Science and Technology, London, England, for generous gift of soil samples.

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DETERMINATION OF URINARY GLYCOSAMINOGLYCANS IN CONNECTIVE TISSUE DISORDERS USING THIN-LAYER CHROMATOGRAPHY

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SUMMARY

Hereditary disorders exist in the metabolism of acid glycosaminoglycans (aGAG) accompanied by the abnormal excretion of urinary glycosaminoglycans (GAG). The exact knowledge of normal GAG excretion is important as a standard for comparison with possible abnormal GAG excretion in metabolic disturbances of connective tissues. Urinary aGAG were precipitated with cetyl trimethyl ammonium chloride and digested with hyaluronidase. Thin-layer chromatography can be employed to separate the mixtures obtained in aGAG precipitation. The method is simple, reproducible, shows a good recovery and may be used for the study of urinary GAG composition.

Our present information on the quantity and quality of acid glycosaminoglycans (aGAG) in connective tissue disorders is incomplete (2, 3, 5, 6, 8). It is now generally accepted that the metabolism of both collagen and proteoglycans is abnormal in connective tissue diseases (1, 4, 7). This paper deals with the determination of acid glycosaminoglycans in the urine in several connective tissue diseases. Thin-layer chromatography can be employed to separate the mixtures from aGAG precipitation. The method is simple, and reproducible with good recovery.

MATERIALS AND METHODS

Urines

Urines were collected separately from sixteen connective tissue disorder patients and three controls.

Materials

The aGAG reference standards (HA, HS, CS-4/6, KS and UA)* were generous gifts from the Pediatric Clinics of the Universities of Szeged and Pécs. Hyaluronidase (from bovine tests, activity ca.1,000 USP U/mg) was purchased from REANAL, Budapest. TLC aluminium sheets silica gel 60, Orcinol cryst. and cetyl trimetyl ammonium chloride were purchased from E. Merck, Darmstadt (F.R.G.). Alcian Blue 8GS was purchased from Chroma-Gesellschaft Schmidt Co., Stuttgart-Untertürkheim (FRG). Other materials were commercial products.

Methods

Glycosaminoglycans (GAG) were quantitated by Alcian Blue 8GS according to the procedure of Whiteman (10).

Separation of aGAG from urine. Urine specimens were kept frozen until analysis. Each urine sample was centrifuged and an aliquot of a 5% aqueous cetyl trimetyl ammonium chloride (CTAC) solution was added,with stirring, to the 20 ml urine to give a final concentration of 0.5% (pH 4.0 using acetic acid). The mixture was let to stand for 4 h at room temperature with occasional stirring, then kept overnight at 4°C, and finally centrifuged at 8,000 G for 10 min. The sediment was washed three times with 2 ml 95% ethanol containing 10% potassium acetate to remove CTAC. The residue was dissolved in 2 ml of 0.6 M NaCl. The mixture was left to stand overnight at 4°C, then centrifuged at 8,000 G for 10 min. The residue was dissolved in 100 μl of 0.6 M NaCl, then centrifuged at 8,000 G for 10 min.

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*Abbrevations used: HA = hyalorunic acid; HS = heparin sulfate; CS-4/6 = chondroitin sulfate A and C; DS = dermatan sulfate; KS = keratan sulfate; UA = uronic acid.

Thin-layer chromatography was performed with a 30 µl aliquot from the supernatant which was directly spotted [1.5 cm width on a 20 cm x 10 cm silica gel 60 precoated thin-layer chromatographic plate (Kieselgel 60, E. Merck, Darmstadt)].

10 µg each of HS, CS-4/6, DS, KS and UA were used as the standards. The chromatograms were developed for 2.5 h using the 40:60:5 (v/v) mixture of n-propanol-25% ammonia-water as the solvent. After drying, the plate was sprayed with a 2% solution of orcinol in ehtyl acetate and a few minutes later with 20% sulfuric acid. The plate is then heated to 100°C until a spot for aGAG appears.

The residue from the supernatant was digested with hyaluronidase (2 mg) for 8 h at 37°C. After digestion the mixture was centrifuged at 4,000 G for 20 min. Aliquots (30 µl) of the clear supernatant fluid were chromatographed by thin-layer chromatography on silica gel.

RESULTS

Table I indicates that normal urine contains chondroitin-4/6-sulfate and heparin sulfate. With the exception of heparin, all of GAG have been identified in the urine of patients with connective tissue diseases.

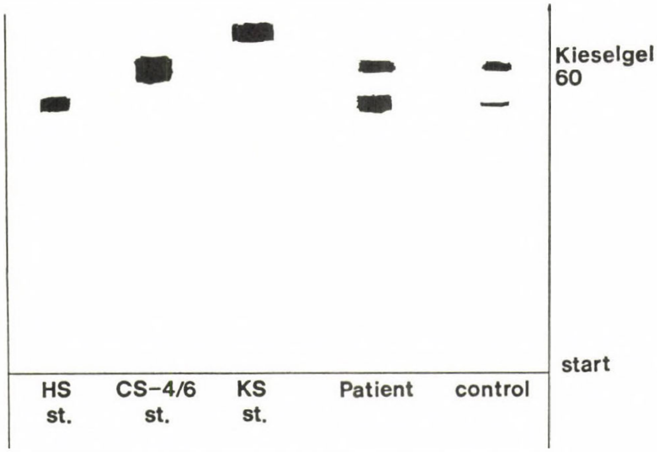

Fig. 1. Thin-layer chromatography of aGAG from patient, normal control and standards

Table I. Excretion pattern of urinary GAG from patients with CTD and mucopolysaccharidoses

No	Name	Age	Sex	Diagnosis*	Total GAG(mg) / creatinine (g)	Distribution of aGAG in bands**					UA
						HA	HS	CS-4/6	DS	KS	
1	Gy. M.	10	M	RA	190	10	10	20	15	15	30 %
2	T. Zs.	11	F	RA	120	15	15	25	8	15	22 %
3	Sz. I.	8	F	RA	70	20	7	40	5	7	
4	B. Gy.	8	F	RA	120	16	25	18	4	16	21 %
5	N. A.	8	M	RA	73	25	5	40	5	5	
6	Sz. Zs.	12	F	LED	160	3	12	68		5	
7	S. J.	6	M	Scleroderma	140	20	10	22	10	32	
8	B. Gy.	11	M	Scleroderma	90	15	16	38	12	15	
9	D. E.	0,5	F	O.I.C.	450	8	12	8	6	3	63 %
10	S. A.	0,2	M	O.I.C.	468	6	13	10	4	4	63 %
11	L. A.	0,6	F	O.I.C.	380	6	10	15	8	4	57 %
12	J. Sz.	5	M	O.I.A.	120	6	12	40	15	3	28 %
13	F. E.	12	F	Marfan syndrome	87	10	15	45	5	5	
14	S. M.	10	F	Marfan syndrome	98	8	20	52	5	5	
15	T. G.	5	M	Hunter syndrome	320	5	63	15	18	4	
16	H. B.	3	M	Morquio syndrome	250	2	62	20	2	60	
17	T. B.	5	M	Control	20		28	58			
18	Sz. E.	12	F	Control	15		20	60			
19	K. P.	1,5	M	Control	22		22	55			

*CTD: connective tissue diseases; RA: rheumatoid arthritis; LED: lupus erythematosus disseminatus; O.I.C.: osteogenesis imperfecta congenita; O.I.A.: osteogenesis imperfecta adulta; M: male; F: female

**Expressed as percent distribution in total GAG

A regular chromatogram of standard GAG (HS, CS-4/6, KS) control and abnormal excretion patterns of urinary GAG in Hunter syndrome before digestions with hyaluronidase is shown in Fig. 1. The testicular hyaluronidase-resistant aGAG were dermatan sulfate and heparin sulfate (and keratan sulfate).

DISCUSSION

The quantity and quality of GAG excreted with urine in five patients with proven rheumatoid arthritis indicates the diagnostic significance of the urine GAG analysis. In these cases the proportion of chondroitin-4/6-sulfates tended to increase.

Patients with osteogenesis imperfecta excreted a large amount of urinary GAG. The affected individuals showed a statistically significant increase in Alcian Blue binding over macromolecular uronic acid not seen in the normal group.

The Hunter syndrome urine contains approximately equal amounts of dermatan sulfate and heparin sulfate with excretion about 30 times greater than that excreted by a normal person. A large amount of GAG was excreted in the urine of Morquio syndrome. The proportion of keratan sulfate exceeded 60% and those of dermatan sulfate decreased remarkably.

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SEPARATION OF CONFORMATIONAL DIASTEREOMERS OF 2,3-BENZODIAZEPINES BY HPLC

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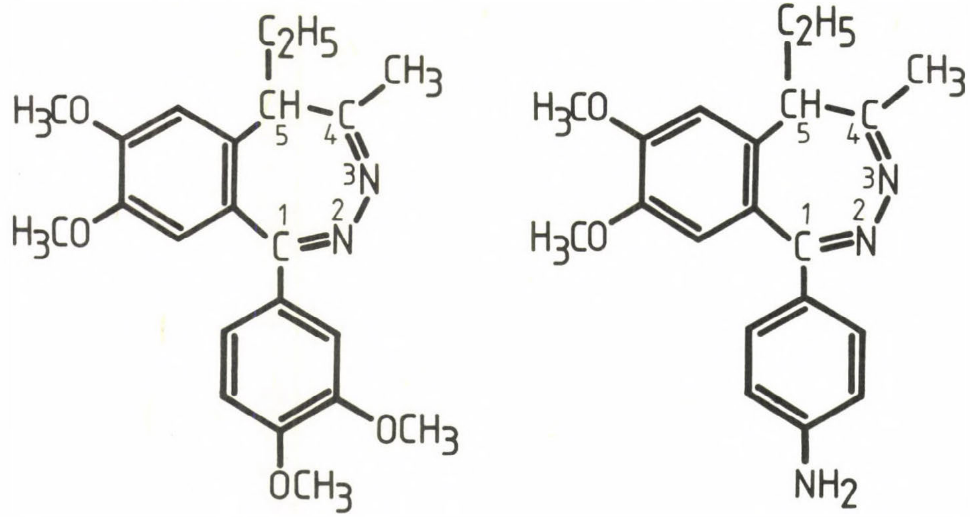
SUMMARY

An HPLC method has been developed for the separation and study of interconversion of 2,3-benzodiazepine conformers. It was found that tofizopam in ethanol prefers conformation in which the C-5 ethyl group is in pseudo-equatorial position. The equilibrium distribution in ethanol is attained after 12 hours. The separated conformers also showed interconversion if the fractions were dried within 2-3 minutes. The half-life of the interconversion observed by chromatography is found shorter for the other conformers. The conformer ratio of GYKI-52671 was independent of time.

INTRODUCTION

The conformational interconversion of 1-(3,4-dimethoxyphenyl)-4-methyl-5-ethyl-7,8-dimethoxy-5H-2,3-benzodiazepine), (Compound 1) and 1-(4-aminophenyl)-4-methyl-5-ethyl-7,8-dimethoxy-5H-2,3-benzodiazepine (Compound 2) has been studied in this work. The chemical structures of the investigated compounds can be seen in Fig. 1. The racemic form of Compound 1 is used as an anxiolytic agent (Tofisopam WHO, Grandaxin). The synthesis of racemic Tofizopam has already been described (1). NMR studies have indicated that this compound exists in two conformations. The majority of the molecules has a conformation in which the C-5 ethyl group is in pseudo-equatorial


Compound 1

Compound 2

Fig. 1. The chemical structures of the investigated compounds. Compound 1: Tofisopam, Compound 2: GYKI-52671

position (2). Figure 2 shows the two conformations of tofizopam. The interconversion between conformers has been detected by chiroptical investigations (3). The equilibrium distribution in chloroform was 15:85 %. Tofisopam conformers could be separated by reversed-phase HPLC and by TLC (4, 5). The investigation of Compound 1 with affinity chromatography using human serum albumin indicated the importance of the conformations (6). Sample 2 (GYKI-52671) is also a potentially active 2,3-benzodiazepine derivative. The study of the interconverting conformations of 2,3-benzodiazepines is important from the pharmacological activity point of view as different conformations show presumably different affinities to binding sites.

MATERIALS AND METHODS

The HPLC measurements were carried out on Separon SGX 10 µm silica gel packed in a 250 x 4 mm stainless steel column.

Mobile phases contained spectroscopic-grade cyclohexane, diethyl ether purchased from Merck (Darmstadt, FRG), and analytical grade ethanol and cc. NH_3 solution from Reanal Fine Chemical Works (Budapest, Hungary). The mobile phase composition for the investigations of Compound 1 was 70 % (v/v) cyclohexane, 20 % (v/v) diethyl ether and 10 % (v/v) ethanol. For Compound 2 25 % (v/v) cyclohexane, 60 % (v/v) diethyl ether, 14.8 % (v/v) ethanol and 0.2 % cc. ammonia solution was used as the mobile phase. Each measurement was carried out with isocratic elution with 1.5 ml/min flow rate R-, S-, and racemic Tofisopam was obtained from EGIS Pharmaceutical (Budapest, Hungary); Compound 2 was synthesized in Drug Research Institute (Budapest, Hungary) and signed as GYKI-52671. The samples were prepared by dissolving 5 mg of the compounds in 1 ml ethanol.

The HPLC equipment used consisted of Liquopump Model 312 (Labor MIM, Budapest, Hungary) with valves from Altex (Altex Inc., Berkeley, CA, USA). The Rheodyne Model 7125 injection valve (Rheodyne, Cotati, CA, USA) was assembled with 20 µl sample loop. Detection was carried out by a variable wavelength UV detector (Labor MIM, Budapest, Hungary) at 239 nm. The chromatograms were recorded by a Type OH-814/1 strip chart recorder (Radelkis, Budapest, Hungary).

Kinetic measurements were carried out with the solutions of R-, S-, and racemic forms of Compound 1 and racemic Compound 2. After dissolving the compounds in ethanol 20 µl of the solutions were immediately injected onto the column. The conformer ratios were measured after 1,2,3,4,12 and 14 hours. Investigations of conformational transition were carried out with separated conformers, each of which isolated by chromatography. Isolation was made by drying the fractions within 2-3 minutes by hot air. Fractionated conformers were dissolved in ethanol and the kinetic studies of interconversion were repeated. NMR spectra were run on a Varian XL-400 spectrometer in $CDCl_3$ at room temperature.

RESULTS AND DISCUSSIONS

R-, S-, and racemic forms of Compound 1 have quite similar kinetic properties. Having dissolved each form of Compound 1 in ethanol prefers conformation I (see Fig. 2). The chromatogram obtained can be seen in Fig. 3. The decrease of the peak area belonging to conformer I and the increase of conformer II indicate the advance of interconversion. The equilibrium distribution in ethanol was attained after 12 hours. The chromatogram obtained can be seen in Fig. 4.

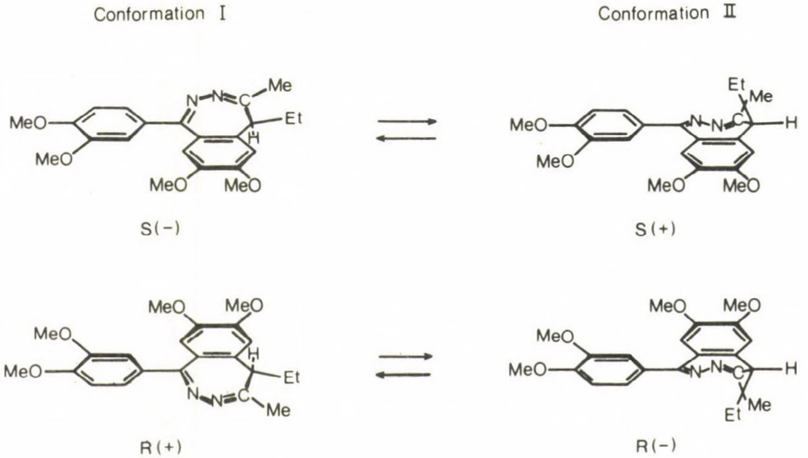


Fig. 2. The conformers of Tofisopam

When the kinetic measurements were repeated with separated conformer I the equilibrium settled again after 12 hours.

Conformer II presented unusual behaviour. The chromatogram made directly after dissolving the pure conformer (Fig. 5) shows that this conformer could exist in crystal-line form too. The equilibrium distribution was reached in 40 minutes (Fig. 6), however.

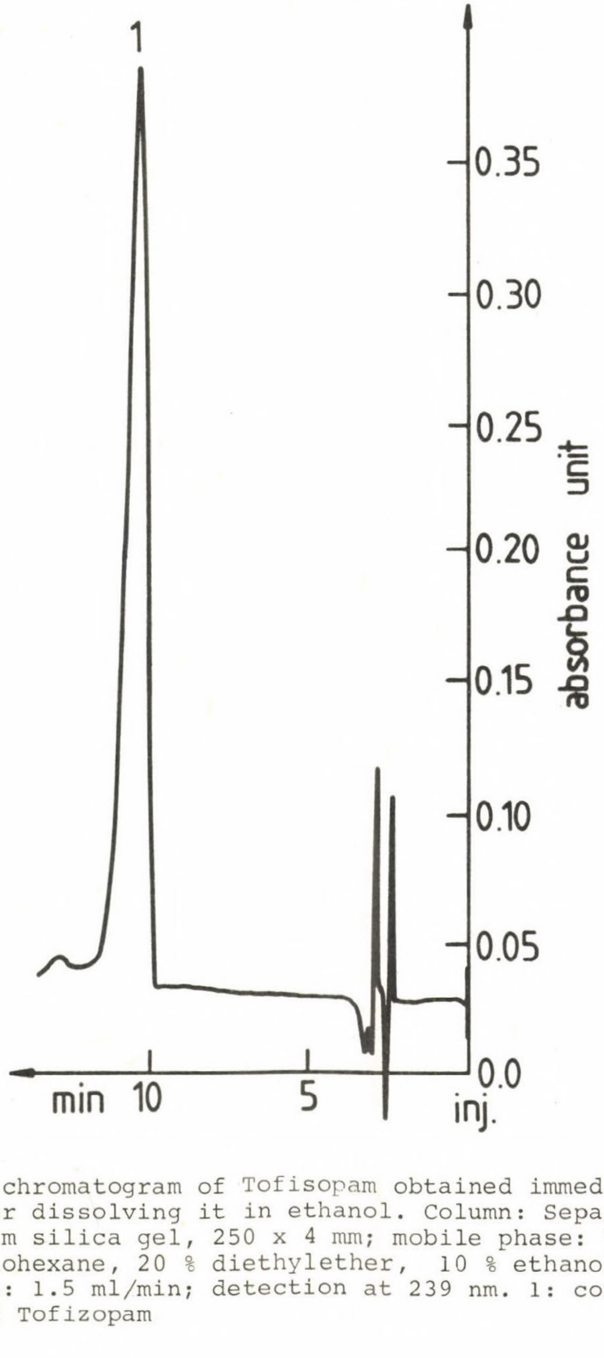


Fig. 3. The chromatogram of Tofisopam obtained immediately after dissolving it in ethanol. Column: Separon SGX 10 µm silica gel, 250 x 4 mm; mobile phase: 70 % cyclohexane, 20 % diethylether, 10 % ethanol; flow rate: 1.5 ml/min; detection at 239 nm. 1: conformation I of Tofizopam

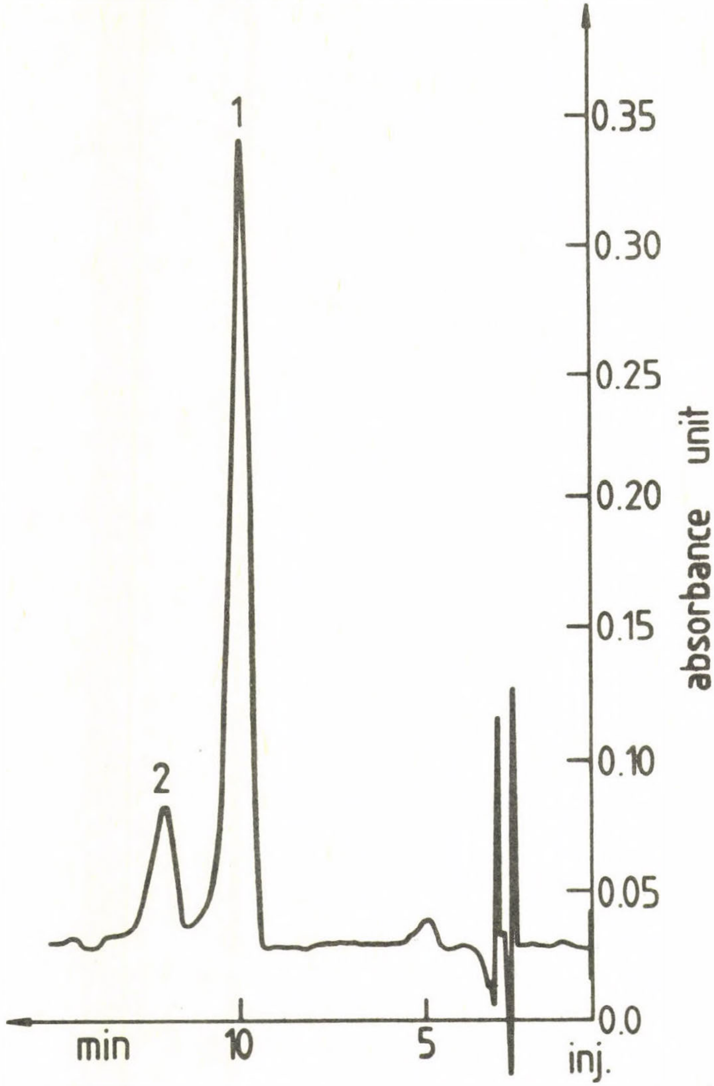


Fig. 4. The chromatogram of Tofisopam obtained 12 hours after dissolving it in ethanol. Chromatographic conditions: see Fig. 3. 1: conformation I; 2: conformation II

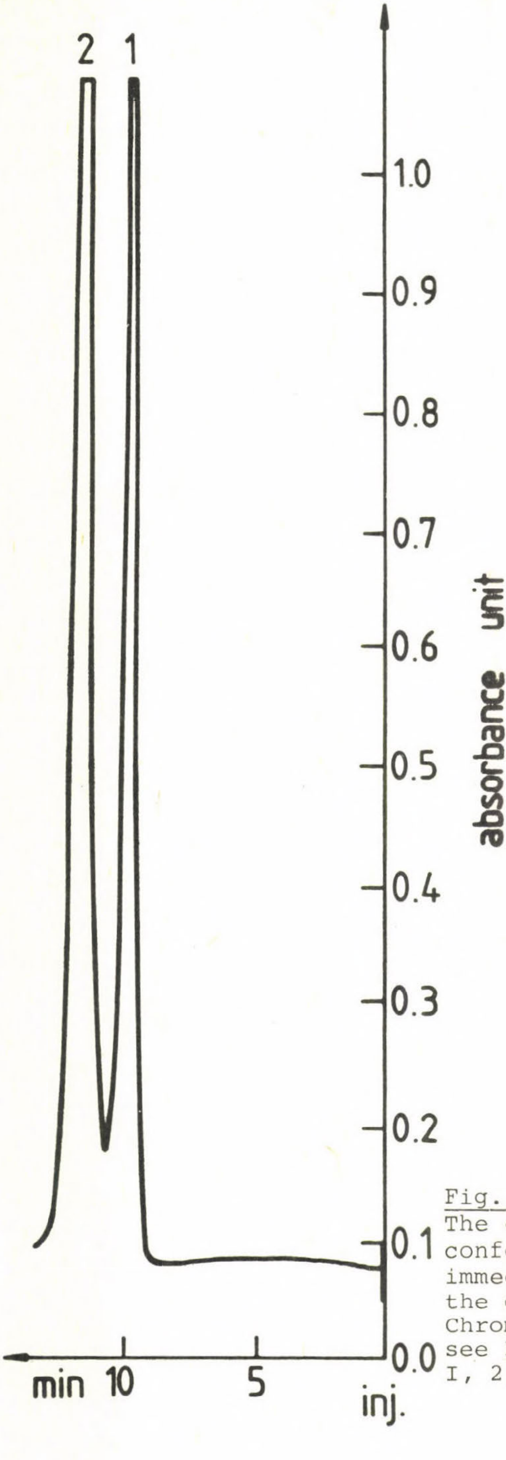


Fig. 5.
The chromatogram of separated conformation II obtained immediately after dissolving the dried fraction in ethanol. Chromatographic conditions: see Fig. 3. 1: conformation I, 2: conformation II

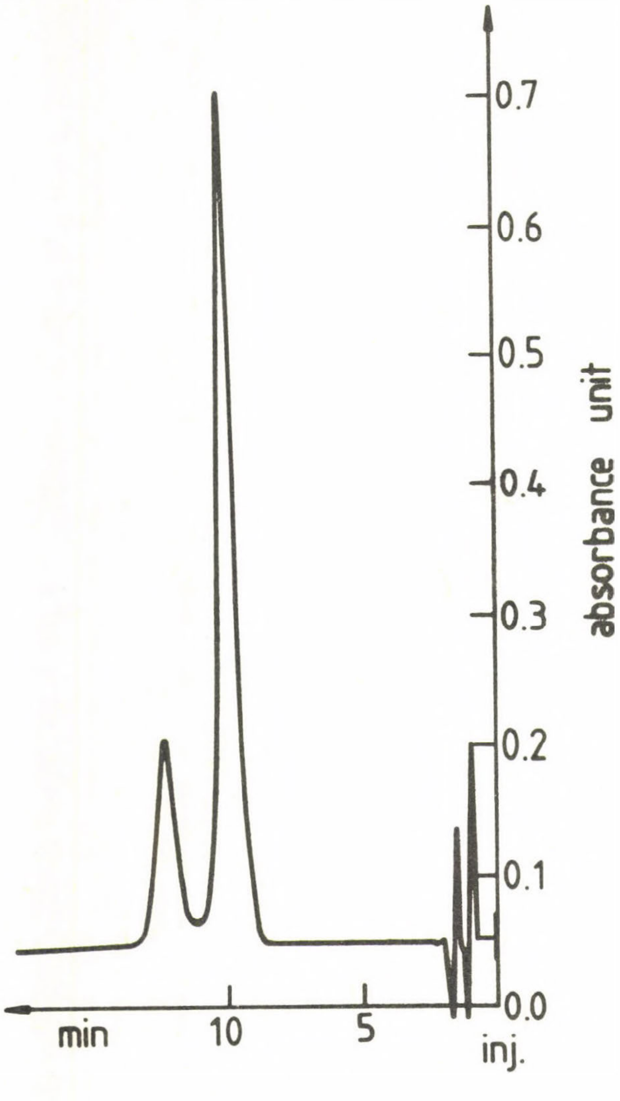


Fig. 6. The chromatogram of separated conformation II obtained 40 min after dissolving it in ethanol. Chromatographic conditions: see Fig. 3. 1: conformation I, 2: conformation II

Sample 2 was investigated with the same way. In the first minute, after dissolving of compound there were three peaks in the chromatogram (Fig. 7). The ratio of peak areas was inde-

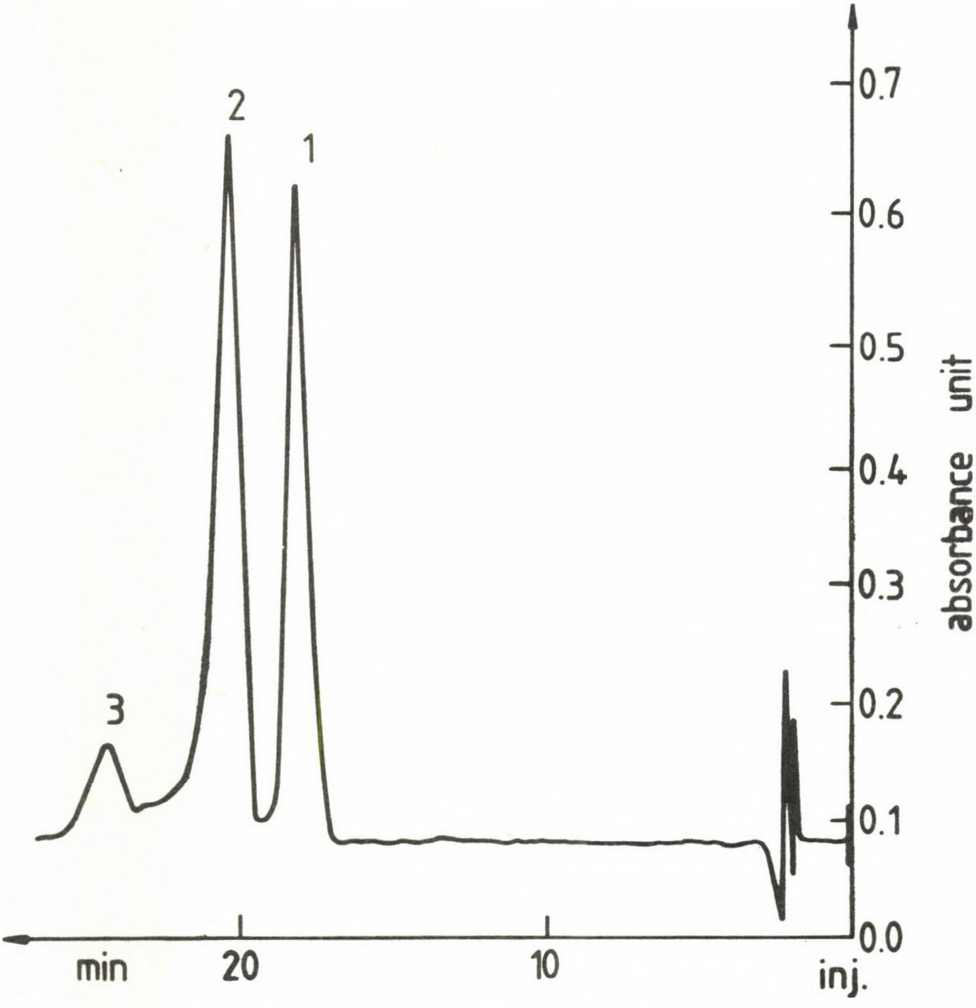


Fig. 7. The chromatogram of Compound 2 obtained immediately after dissolving it in ethanol. Chromatographic conditions: Separon SGX 10 μm silica gel, 250 x 4 mm; mobile phase: 25 % cyclohexane. 60 % diethyl ether, 14.8 % ethanol, 0.2 % cc. ammonia solution; flow rate: 1.5 ml/min; detection: 239 nm. 1: chemical structure see Fig. 8, 2 and 3: two conformers of Compound 2

pendent of time. Collecting the compounds of separated peaks and rechromatographed the first peak showed always the same retention time and no other peaks occurred in the chromatogram. Rechromatographing the second and third peaks each of them showed two peaks with the same ratio. By NMR studies the first peak proved to be the 3,4-dihydro derivative of Compound 2 shown in Fig. 8. Relevant NMR data: 4-Me: 1.01 ppm, doublet, J = 6.2 Hz; 4-H: 4.08 ppm, doublet of quartet, J = 6.2 Hz, J = 5.0 Hz; 3-H: 3.60 ppm, broad singlet, exchangeable. The second and the third peaks belonged to the two conformers of Compound 2. The equilibrium between them settled in a few seconds, could be seen on the chromatograms obtained after rechromatography of the two separated conformers.

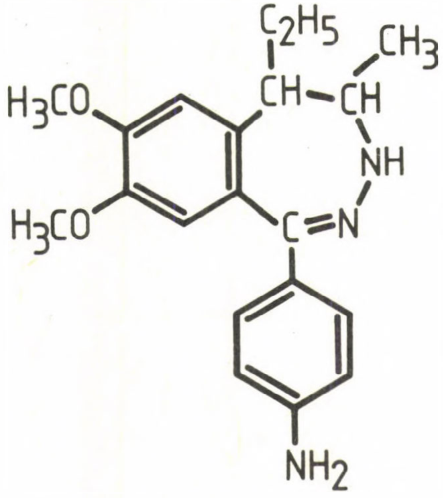


Fig. 8. The chemical structure of the compound found in a mixture with Compound 2 (GYKI-52671)

CONCLUSIONS

Compound 1 in ethanol prefers conformation I. The equilibrium distribution in ethanol is attained after 12 hours. The separated conformers also showed interconversion if the fractions were dried within 2-3 minutes. The half-life of the interconversion observed by chromatography is found shorter for conformer II.

The conformer ratio of Compound 2 in ethanol was independent of time.

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RESOLUTION OF WARFARIN VIA ENHANCED STEREOSELECTIVE BINDING TO HUMAN SERUM ALBUMIN INDUCED BY LORAZEPAM ACETATE

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SUMMARY

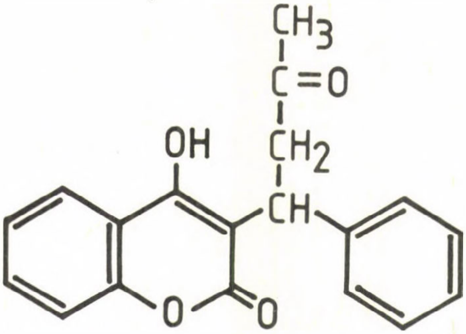
Binding of warfarin enantiomers to human serum albumin (HSA) was investigated in the presence of (RS)-lorazepam acetate (LAc) using affinity chromatographic technique. Since (S)-warfarin exhibited considerably enhanced binding in the presence of LAc, racemic warfarin could be resolved.

INTRODUCTION

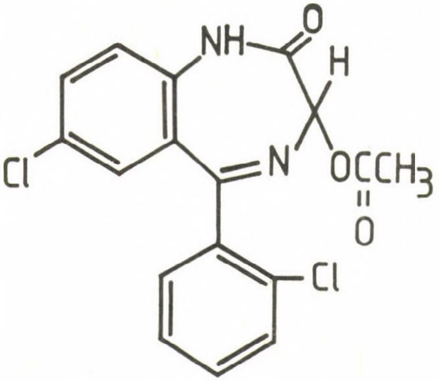
Binding studies performed by various methods proved that serum albumin has different affinity for the enantiomers of several chiral ligands. Liquid affinity chromatography is a sensitive technique to detect even small degrees of enantioselectivity and to perform resolution of racemates (1).

Warfarin is a chiral anticoagulant drug with stereoselective pharmacological action; in theraphy; nevertheless, the racemate is used (2) and radioactive enantiomers for tests are not easily available. The slightly stereoselective binding of warfarin to serum albumin could be applied for its chromatographic resolution in nanomolar quantities (3). A procedure is being presented here by which the sample size can be considerably enhanced.

Previously we have found (4) that simultaneous binding of (S)-warfarin and (S)-lorazepam acetate (LAc) to human serum albumin (HSA) resulted in enhanced binding of both ligands. Since this effect is enantioselective it can be utilized to get improved resolution of these ligands on immobilized HSA. While the chromatogram of racemic LAc obtained with buffer elution indicated a stereoselectivity factor (K_S/K_R) of two, in the presence of (S)-warfarin (S)-LAc was practically stuck to the column. Now the inverse experiment was performed studying the effect of LAc on the binding of warfarin enantiomers.



warfarin



lorazepam acetate

RESULTS AND DISCUSSION

Chromatograms of (R)- and (S)-warfarin as well as $\left[^{14}C\right]$ (RS)-warfarin (55 mCi/mmol, Amersham), applied as sodium salts, were taken both with buffer elution (Ringer pH: 7.4 with 0.1 % NaN_3) and in the presence of (RS)-LAc in the eluent. (LAc enantiomers were not available.) The column contained 1 % HSA immobilized on Sepharose 4B (Pharmacia). Detection was made by UV (308 nm) and liquid scintillation methods. Results are shown in Table I and Fig. 1.

According to data in Table I the following observations can be made:

(a) In buffer alone there is not much difference between the binding affinities of warfarin enantiomers ($K \sim 2 \times 10^5\ M^{-1}$),

(b) In the presence of (RS)-LAc the binding of (S)-warfarin is considerably enhanced. (This effect was brought about by the (S)-LAc only (4).)

Table I. Elution volumes of (R)- and (S)-warfarin (0.05 mg) on an HSA-Sepharose column (V_O~12 ml)

Sample	Eluent	
	buffer	10^{-4} M (RS)-LAc
(R)-warfarin	80 ml	80 ml
(S)-warfarin	60 ml	not until 300 ml eluted only by HSA solution

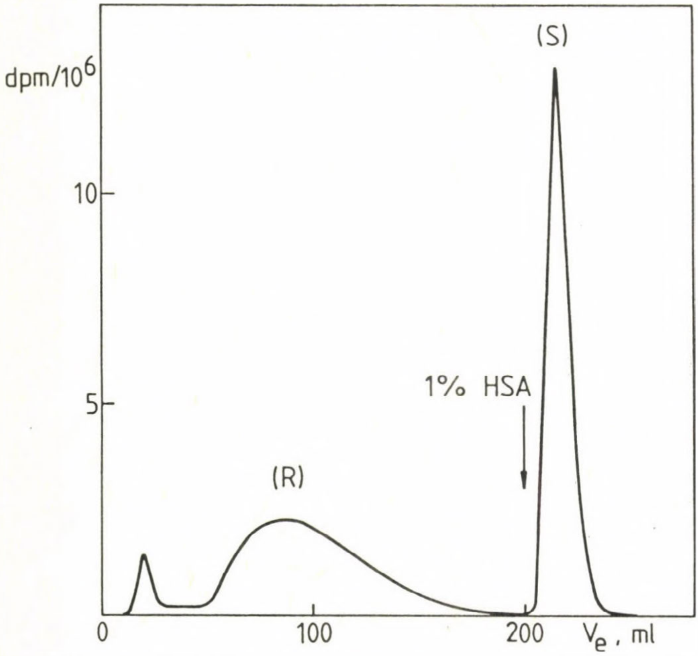


Fig. 1. Radiochromatogram of $[^{14}C]$ (RS)-warfarin (0.07 mg) on an HSA-Sepharose column (V_O~12 ml) using 10^{-4} (RS)-LAc eluent

Radiochromatogram of racemic warfarin (Fig. 1) shows that in the presence of (RS)-LAc half of the radioactivity could be eluted by albumin solution only, the total binding capacity of the column is available for resolution. On a 40 ml HSA-Sepharose column 1 mg of $[^{14}C]$ (RS)-warfarin could be conveniently resolved.

Materials corresponding to both peaks were purified, i.e. separated from LAc, HSA and buffer salts by the following procedure: acidification, extraction with chloroform, chromatography on silica column using 1:1 hexane:ethyl acetate solvent system. Identification of the enantiomers was made by rechromatography in the same way, proving optical purities of 100 % and 98 % for (R)- and (S)-warfarin, respectively. The radioactive warfarin enantiomers were used for biological experiments.

The above chromatographic technique is useful both in detection chiral binding interactions and in performing semi-preparative scale resolutions.

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Chromatography '87
H. Kalász and L.S. Ettre (Eds)

PERTURBATION ANALYSIS: A POWERFUL TOOL FOR PREDICTION OF THE DYNAMICS OF CHROMATOGRAPHIC COLUMNS

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ABSTRACT

We present here an approximate analytical solution of the general problem of nonlinear chromatography of a single species, in the presence of kinetic limitations and hydrodynamical axial dispersion. A numerical study shows that the perturbative solutions are very good approximations of the exact ones, a fact that is confirmed by an experimental study.

INTRODUCTION

The conception of a chromatographic separation operation involves a good knowledge of sorption column dynamics; in particular, a good comprehension of the influence of experimental conditions (geometry, concentrations, velocities, etc.) and of the model parameters (thermodynamics, hydrodynamics, physico-chemistry) on the throughput curve is essential. The mathematics of that type of nonlinear hyperbolic partial differential equations (P.D.E.) are generally complex, and, except for some particular cases (1), the P.D.E's of the general problem of nonlinear chromatography in presence of kinetic limitations and hydrodynamic axial dispersion are not solvable analytically. The classical attitude then consists in integrating numerically the problem (2), and everybody knows how expensive in computer time a parametric study of P.D.E's can be.

The approach we propose here consists in searching an approximate analytical solution, also called perturbative solution to the complete problem. The calculation consists in splitting the original problem in a succession of simpler ones, each of them being solvable exactly in terms of elementary functions.

GENERALITIES

Isothermal adsorption of a solute on a solid is characterized by a thermodynamical equilibrium function $q = f(c)$ (or isotherm), c and q being respectively the concentrations in the fluid and solid phase. When a sorption column is submitted to a positive concentration step (for $t \leq 0$, $c = 0$ and for $t > 0$ $c = c_0$, saturation step), several types of behaviour may be observed, depending on the isotherm's curvature on the interval $0 \leq c \leq c_0$ (Fig. 1):

- if $f'' \geq 0$ (unfavourable isotherm), the concentration front tends to spread out during its travel through the column: one speaks of a dispersive front.

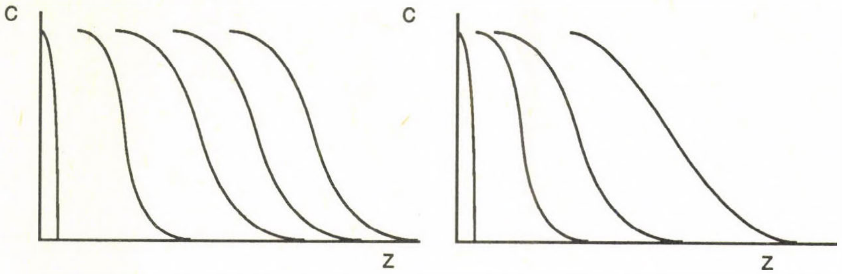
- if $f'' < 0$ (favourable isotherm), the front asymptotically approaches a stable front (or stationary front), which propagates in the column without deformation: one speaks of a compressive front.

- if f'' changes sign, a more complex behaviour is observed.

Moreover, the dispersive and compressive characters are reversed if the input is a negative step (regeneration step).

The compressive front is mathematically the most complicated one, and it is this case we will be dealing with in what follows. The description of the adsorption dynamics consists in a material balance over an infinitesimal column slice of thickness dz:

$$\frac{\partial X}{\partial t} + \lambda \frac{\partial Y}{\partial t} + \frac{\partial X}{\partial z} = \frac{1}{Pe} \frac{\partial^2 X}{\partial z^2}$$



compressive front dispersive front

Fig. 1. Propagation of the two types of fronts in the column

The sorption kinetics are described by a law of the following type:

$$\frac{\partial Y}{\partial t} = St\ F\ (X,\ Y)$$

In a reference frame propagating with the speed v of the stationary front the observer sees the compressive front spread out to its limit shape for t = +∞, and this suggests the change of variables Z = z-vt, T = t. Moreover, the theory of characteristics (3) allows simplification of the front's description in considering Z and Y as functions of X and T, rather than X and Y as functions of Z and T. After these transformations, the preceding equations become, with the notation $\Phi_\alpha = \partial\Phi/\partial\alpha$:

$$-\frac{Z_T}{Z_X} + \frac{(1-v)}{v}StF(X,\ Y) + \frac{(1-v)}{Z_X} = -\frac{1}{Pe}\,\frac{Z_{XX}}{(Z_X)^3}$$

$$Y_T - Y_X\,\frac{Z_T}{Z_X} - v\frac{Y_X}{Z_X} = StF(X,\ Y)$$

PERTURBATION ANALYSIS

The Stationary Front

The analytical resolution of the above system is impossible in the general case. For some particular types of kinetic laws F, and in the case of plug flow (Pe = ∞), analytical solutions in terms of Bessel functions exist (4). The main feature of perturbation analysis is to deal with the most general problem, but to expand the solution Z(X, T), Y(X, T) into a power series of the variable 1/T:

$$Z(X,\ T) = Z^{(0)}(X) + \sum_{n=1}^{+\infty} \frac{Z^{(n)}(X)}{T^n}$$

$$Y(X,\ T) = Y^{(0)}(X) + \sum_{n=1}^{+\infty} \frac{Y^{(n)}(X)}{T^n}$$

The method then consists in putting these expressions into the P.D.E's, and to equate the terms factors of the same power of 1/T on both sides of the equality sign. Doing this, one obtains at each order in 1/T a system of ordinary differential equations (O.D.E.) in the variable X. The solution of the O.D.E obtained at $O(1/T^n)$ then requires the knowledge of the solutions of the orders $O(1/T^p)$ with $p<n$. So, the idea is to split the initial problem into an infinite series of simpler ones. Obviously, this is interesting only if the series converge rapidly, which is the case in all following results.

The order $O(1/T^0)$ represents a front which propagates without deformation at speed v: this is the stationary front. At that order, the equations write:

$$Y^{(0)} = X - \frac{1}{Pe(1-v)} \frac{1}{Z_X^{(0)}}$$

$$-v \frac{Y_X^{(0)}}{Z_X^{(0)}} = St\ F(X,\ Y)$$

Unfortunately, this system remains unsolvable analytically, and we solve it by using perturbation theory, by expending it in powers of a small parameter. In a sorption column, the influence of axial dispersion is measured by the Peclet number. Except for very thin beds (L~1 cm) Pe is always much larger than unity, and so we expand Z^0 and Y^0 in powers of 1/Pe. Other choices are possible, but, although this one is mathematically the most complex, the 1/Pe expansion is the most rapidly converging one. The expansion writes:

$$Z^{(0)}(X) = \sum_{n=0}^{+\infty} \frac{Z^{(o,n)}(X)}{Pe^{n}}$$

The resolution is made step by step. The equations at $O(1/Pe^{0})$ are:

$$Y^{(0,0)} = X$$

$$\frac{1}{Z_X^{(0,0)}} = -\frac{St}{v} F(X, Y^{(0,0)})$$

and the solution is:

$$Z^{(0,0)}(X) = \frac{v}{St} \int_{X_{st}}^{X} \frac{dw}{-F(w,w)}$$

where Xst is the front's stoichiometric concentration.

The expression represents the stationary front in a system without axial dispersion, with a general kinetic law defined by F. When the expressions $F(X,Y) = f(X)-Y$ or $F(X,Y) = X-f^{-1}(Y)$ are taken for F, one recognizes the well-known formulae of the stationary front in the case of linear internal or external driving forces (5). So, leading order perturbation theory provides a generalization of this kind of expression to a more general type of kinetics.

The order $O(1/Pe)$ provides the leading order contribution of axial dispersion $Z^{01}(X)$ to the stationary front shape. The system reduces to a linear first order O.D.E, the solution of which being:

$$Z^{(0,1)}(X) = \int_{X_i}^{X} \frac{F_Y(w,w)}{F(w,w)} dw + Ln \frac{F(X_i,X_i)}{F(X,X)}$$

where X_i is the front's inflexion point. Replacing then $F(X,Y)$ by $f(X)-Y$, and adding Z^{00} and Z^{10}/Pe, we get:

$$Z^{(0)}(X) = \left(\frac{v}{St} + \frac{1}{Pe(1-v)}\right) \int_{X_{st}}^{X} \frac{dw}{w-f(w)} + \frac{1}{Pe(1-v)} Ln\frac{F(X_i,X_i)}{F(X,X)} +$$

$$+ \frac{1}{Pe(1-v)} \int_{X_i}^{X_{st}} \frac{dw}{w-f(w)} + O\left(\frac{1}{Pe^{2}}\right)$$

This expression allows calculation of the stationary front's width, as defined by Rhee and Amundson (6). These authors noticed on numerical simulations that this width was approximately given by the first term of the above expression. Perturbation analysis so allows validation of this conjecture and explicits the corrective terms to this approximation: we have observed a perfect agreement between our corrective terms and these authors' results.

In order to test the above approximate expressions, we have performed numerical integration of the stationary front's O.D.E's for $F(X,Y) = f(X) - Y$ and a Freundlich favourable isotherm $f(x) = x^{\alpha} (0<\alpha<1)$, and computed, for several Pe and St values, the maximum relative deviation between exact and perturbative solutions. The results are in the Table I below. E_1 is the deviation at order 1/Pe, and E_2 the one obtained at $O(1/Pe^2)$.

Table I.

Pe	St	E_1	$1/Pe^2$	E_2	$1/Pe^3$
120	0.01	1.7x10-3	6.9x10-5	2.4x10-4	5.8x10-7
120	0.1	7.3x10-5	6.9x10-5	4.9x10-5	5.8x10-7
120	1.0	1.2x10-4	6.9x10-5	2.1x10-5	5.8x10-7
120	10.0	9.8x10-5	6.9x10-5	1.0x10-6	5.8x10-7
120	100.	4.8x10-5	6.9x10-5	5.2x10-6	5.8x10-7
10	0.01	1.9x10-3	1.0x10-2	6.7x10-4	1.0x10-3
10	1.0	5.4x10-4	1.0x10-2	4.9x10-5	1.0x10-3
10	100.	5.1x10-5	1.0x10-2	3.6x10-6	1.0x10-3
1	1.0	2.2x10-2	1.0	1.8x10-4	1.0

One can expect the deviation to be of the same order of magnitude than the first term neglected in the deviation, and the table shows that E_1 (resp. E_2) is often much smaller than $1/Pe^2$ (resp. $1/Pe^3$).

Anyway, perturbation analysis always provides a very good approximation to the exact stationary front.

The Transient Front

We have performed the same work than above for the transient front, that is, we have expanded the general equations in powers of 1/T. The order 1/T then provides the leading contribution of the transient front.

Again, each order in 1/T requires a second expansion, which we choose to be in 1/Pe. For example, the result at order O(1/T) and for a kinetic law of type F(X,Y) = f(X) - Y is:

$$Z^{(1)}(X) = \left(\frac{v}{St} + \frac{1}{Pe(1-v)}\right)^2 \int_{X_i}^{X} \frac{dw}{\left[w-f(w)\right]^2} + O\left(\frac{1}{Pe^3}\right)$$

Again, we have performed the numerical integration of the complete set of P.D.E's, and computed for each couple (Pe, St) the maximum relative deviation between the approximate solutions of order O(1/T, 1/Pe) (that is E_1') and of order O(1/T, 1/Pe^2) (that is E_2') and the numerical exact solutions. The results are in the Table II:

Table II.

Pe	St	E_1'	$1/Pe^2$	E_2'	$1/Pe^3$
120	0.01	2.9x10-3	6.9x10-5	2.5x10-4	5.8x10-7
120	0.1	1.7x10-3	6.9x10-5	5.2x10-5	5.8x10-7
120	1.0	4.4x10-4	6.9x10-5	2.4x10-5	5.8x10-7
120	10.0	1.3x10-4	6.9x10-5	1.7x10-6	5.8x10-7
120	100.	5.5x10-5	6.9x10-5	5.5x10-6	5.8x10-7
10	0.01	3.1x10-3	1.0x10-2	7.0x10-4	1.0x10-3
10	1.0	9.1x10-4	1.0x10-2	5.5x10-5	1.0x10-3
10	100.	7.2x10-5	1.0x10-2	4.0x10-6	1.0x10-3
1	1.0	3.4x10-3	1.0	2.9x10-4	1.0

Here also the deviations are very small. Moreover one can expect the errors to increase with respect to the stationary case like $1/T^2$. For small concentrations, that is for small times, this deviation could consequently be expected to become very large. Fortunately, the convergence of our series is sufficiently rapid, and so, even for limit cases (Pe = 1, St = 1), the observed difference did not exceed a few percent.

EXPERIMENTAL RESULTS

In order to test the theory, we have performed a series of experiments, in a column of internal diameter 1.4 cm. The solution is an aqueous solution of phenyl-acetic acid (C_0 = 10 to 100 mmol/l). The resin is Duolite ES 861. The thermodynamical, hydrodynamical and kinetic parameters were determined by independent experiments, and put into our perturbative expressions of the transient fronts. The results have always been found satisfactory.

Figure 2 presents for example experimental points and the theoretical curve corresponding to one of our experimental runs.

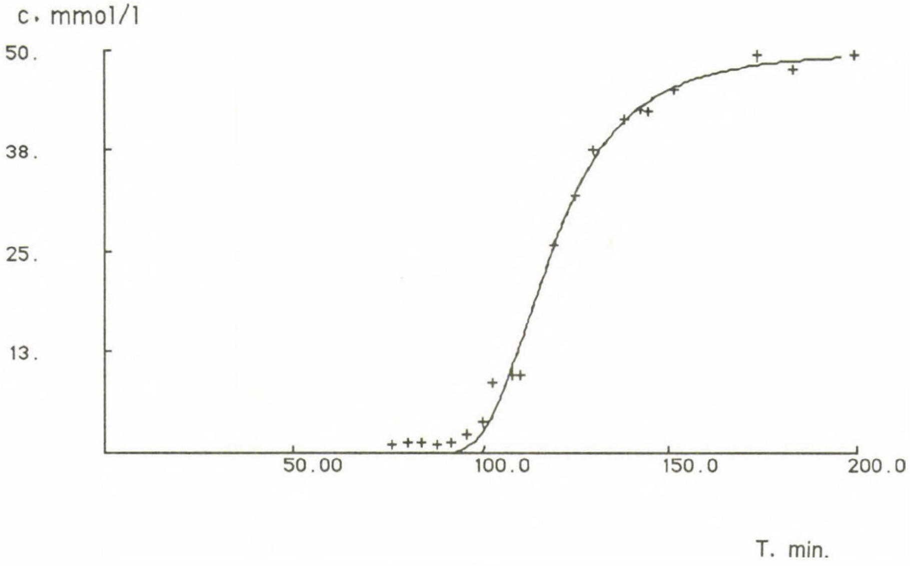


Fig. 2. Comparison between theory and experiment; the full curve is the perturbative solution at order O(1/T, 1/Pe)

As can be seen, the agreement is very good. Let us emphasize the fact that direct numerical simulation of such a curve requires a few CPU hours on a BULL/MINI6, but the computation based on our analytical expressions only requires a few seconds on the same machine.

CONCLUSION

The results presented here show that perturbation analysis provides very good approximations to the solutions of a nontriaval set of P.D.E's, and so, perturbation theory appears as a very powerful tool for chromatography modelisation.

NOTATIONS

-C_0: feed concentration (constant)
-c : fluid phase concentration
-D : axial dispersion coefficient
-f(X): equilibrium isotherm
-k : kinetic rate constant
-L : bed depth
-Q_0: $f(C_0)$
-q : solid phase concentration
-Pe: uL/D Peclet number
-St: kL/u Stanton number
-t : dimensionless time, measured in units $\tau = L/u$
-T : T = t
-u : constant intersticial fluid velocity
-v : $1/(1+\lambda)$ dimensionless propagation speed of the stationary front
-X : c/C_0
-Y : q/Q_0
-z : dimensionless abscissa, in units L
-Z : z-vt
-ε : bed porosity
-λ : $(1-\varepsilon)Q_0/(\varepsilon C_0) = (1 - v)/v$

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SEPARATION OF *CIS* AND *TRANS* ISOMERS OF CYCLIC COMPOUNDS. PART I. SEPARATION OF *CIS* AND *TRANS* 2-AMINO-1-CYCLOHEXANECARBOXAMIDE AND THEIR DERIVATIVES BY THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A method was developed for the separation of *cis* and *trans* 2-amino-1-cyclohexanecarboxamide and their methyl derivatives which were obtained from *cis* and *trans* hexahydroquinazolin-4-(3*H*)-one by catalytic hydrogenolysis. In this series of the *cis* isomers, the methyl-substituted carboxamide has a higher R_f value than the unsubstituted carboxamide. In the case of the *trans* isomers this holds only for the methyl-substituted carboxamide. In the separation of the *cis* and *trans* carboxamides and their methyl derivatives, the *cis* isomers have higher R_f values in most of the cases than the corresponding *trans* isomers.

INTRODUCTION

The hydrogenolysis of *cis* and *trans* hexahydroquinazolin-4(3H)-one (**1**-**2**) (1-3) was studied on various metal catalysts (4). It was assumed that the 2-amino-1-cyclo-hexanecarboxamides (1-3) and their derivatives (**3**-**8**) may be formed in the hydrogenolysis. The assumed substances were synthesized (5).

In this publication we report on the thin-layer chromatographic separation of prepared compounds **3**-**8** in various solvent systems. The chromatographic studies had two aims: to develop a separation method that permits a rapid qualitative evaluation of the results of the hydrogenolytic reaction; and to offer a possibility (through the separation of the *cis* and *trans* isomers) for the detection of any configurational isomerization that may occur during the hydrogenolysis. The separation of the starting substances 1 and 2 will be the subject of a later paper.

EXPERIMENTAL

Compounds 3-8 were separated using various solvent mixtures. The results of the chromatographic separations are listed in Tables 1 and 2.

Table 1. Separation of the hydrogenolysis products of *cis* and *trans* hexahydro-quinazolin-4(3*H*)-one (1, 2)*

Solvent system	R_f x 100 for compound					
	3	5	7	4	6	8
5:2:3 Butanol-acetic acid**-water	44	41	34	43	44	49
9:1 Ethanol-ammonium hydroxide	66	54	50	61	68	42

*Separation was carried out on DC-Alufolien Kieselgel 60 F_{254} (20x20 cm; 0.2 mm). Detection was carried out by ninhidrine or TMD methods.

**glacial

Table 1 relates to the separation of compounds 3, 5, 7 or of 4, 6, 8, presumably formed side by side during the hydrogenolysis of the given *cis* compound 1 or of its trans isomer 2. Naturally, the separation of the corresponding *cis* and *trans* isomeric pairs is also indicated by Table I. We have considered the separation of 5 and 6, and of 7 and 8, as the most important, these being the presumed primary hydrogenolysis products. If configurational isomerization occurs during the hydrogenolysis, the above-mentioned *cis* and *trans* isomers may

Table 2. Separation of cis and trans 2-amino-1-cyclohexanecarboxamides and their derivatives (3-8)*

Solvent system		R_f x 100 for compound			
		5	6	7	8
1:1	Dioxane-pyridine	25	8.5		
5:3:2	Dioxane-pyridine-methanol	29	19		
4:4:2	Dioxane-methanol-acetone	34	40		
9:1	Dioxane-ammonium hydroxide			37	30
4:4:2	Dioxane-methanol-ammonium hydroxide			70	61
6:35:3	Dioxane-methanol-ammonium hydroxide	57	61		
4:4:2	Dioxane-n-amylalcohol-ammonium hydroxide			59	48
9:1	Methanol-ammonium hydroxide			48	54
9:1	Isopropanol-ammonium hydroxide			57	39
9:1	n-Propanol-ammonium hydroxide			53	31
9:1	Methanol-acetic acid			53	45
7:3	Chloroform-methanol	30	22		
6:4	Chloroform-methanol	28	25		
4:4:2	Chloroform-methanol-acetone	30	34		
5:2:3	Chloroform-methanol-dioxane	21	16		
4:4:2	Chloroform-methanol-ammonium hydroxide			81	70
4:4:2	Chloroform-n-propanol-ammonium hydroxide			65	52

*Separation was carried out on DC-Alufolien Kieselgel 60 F_{254} (20x20 cm; 0.2 mm). Detection was carried out by ninhidrine or TMD methods.

be formed side by side. Accordingly, we evaluated the various solvent systems used for the separation of these isomers. These results are given in Table 2.

RESULTS AND DISCUSSION

As indicated by Table 1, the carboxamides 3, 5, 7 and 4, 6, 8 can be separated well from each other in either butanol-glacial acetic acid-water (5:2:3) or in ethanol-ammonia (9:1) solution.

Here primarily the separation of compounds **5** and **7**, or of compounds **6** and **8** is important because these are assumed to be formed side by side. Compounds **3** or **4** may be formed only secondarily. Butanol-glacial acetic acid-water (5:2:3) is more favourable for the rapid evaluation of the hydrogenolysis of the *cis* isomer **1**, while the ethanol-ammonia solution (9:1) solvent system is to be preferred for the corresponding *trans* isomer **2**: in the former system the difference between the R_f values is the highest for **5** and **7**, and in the latter system it is the highest for **6** and **8**.

The data in Table 1 demonstrate that for the *cis* acid amide and its derivatives, when the hydrogen of either the carboxamide N-H or the primary $-NH_2$ group attached to the cyclohexane skeleton is replaced by a methyl group, the R_f values of the methyl derivatives **5** and **7** are lower in both solvents than the R_f value of the unsubstituted acid amide **3**. The decrease is greater for compound **7** than for compound **5**.

In the *trans* isomers, however, the R_f value for the unsubstituted acid amide **4** is higher than the value for compound **8** in which the primary amino group is substituted, but lower than the R_f value of compound **6**.

As illustrated by Tables 1 and 2, numerous solvent systems were tested for the separation of the *cis* and *trans* isomers from each other. The data show that both butanol-glacial acetic acid-water (5:2:3) and ethanol-ammonia solution (9:1) are suitable for the separation of *cis* and *trans* isomers **3** and **4**, but because of the larger R_f differences the use of the latter solvent mixture is preferable.

The ethanol-ammonia solution (9:1) and dioxane-pyridine (1:1) solvent systems are most suitable for the separation of the *cis* and *trans* isomers **5** and **6** from each other. These are derivatives substituted on the acid amide nitrogen. Although the difference between the R_f values is larger in the latter solvent system, the low R_f value of the *trans* isomer means that it often gives a spot projecting from the start-point. Accordingly evaluation is simpler when the former solvent mixture is used.

Several solvent systems are suitable for the separation of the substituted amino acid amide isomers 7 and 8: for example, chloroform-n-propanol-ammonia solution (4:4:2), chloroform-methanol-ammonia solution (4:4:2) and isopropanol-ammonia solution (9:1). It should be noted, however, that, when the both isomers are present, the first of these three systems gives the best chromatogram.

The data in Table 2 further reveal that from the solvent mixtures used to separate the isomeric pair 5 and 6 the *cis* isomer (5) has the higher R_f value in six mixtures, while in three cases the R_f value is higher for the *trans* isomer (6).

For the isomeric pair 7 and 8, in eight of the solvent mixtures used, the R_f value was higher for the *cis* isomer (7). Only in the methanol-ammonia solution (9:1) system was the R_f value of the trans isomer (8) higher.

CONCLUSIONS

The hydrogenolysis products of *cis* and *trans* hexahydroquinazolin-4(3*H*)-one (1, 2) can be separated well in a butanol-glacial acetic acid-water (5:2:3) system. This provides a fast and convenient method for the qualitative determination of the composition of the mixture resulting from the hydrogenolysis reaction.

Comparing the results obtained for the unsubstituted acid amides (3, 4) with these for the methyl-substituted derivatives (5-8) it can be stated that, as a consequence of the substitution, the R_f value of the *cis* isomers (3, 5, 7) will decrease in the buthanol-glacial acetic acid-water (5:2:3) and ethanol-ammonia solution (9:1) systems, while in the case of the *trans* isomers (4, 6, 8) this is only true for the derivative with substitution on the primary amino group (8).

Regarding the separation of the *cis* and *trans* isomers of the acid amide and its derivatives, the most suitable solvent systems are ethanol-ammonia solution (9:1) for compounds 3 and 4, and also for compounds 5 and 6, and chloroform-n-propanol-ammonia solution (4:4:2) for compounds 7 and 8. For the *cis* and *trans* isomers of the acid amides (3, 4) and their derivatives (5-8) examined the *cis* isomers (3, 5, 7) have the higher values in most solvent systems.

It should be mentioned that an aqueous solvent system is not suitable for the separation of the hexahydroquinazolinones (1, 2) serving as the starting compounds in the hydrogenolysis reaction. These substances decompose on the action of water.

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THE DEVELOPMENT OF THE ANALYSIS OF TETRACYCLINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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Tetracycline (TC)* is an antibiotic produced by several degradation products which are formed following the reactions shown in Fig. 1. Chlortetracycline(CTC) which has a structure closely related to that of TC is another potential impurity of TC, as small amounts can be produced during fermentation. However, it appears that CTC is not present in detectable amounts in commercial samples of recent production.

It is not the intention of the authors to discuss all the high-performance liquid chromatography (HPLC) methods which have been mentioned for the analysis of tetracycline. The HPLC methods used for the identification of tetracyclines or for the determination of tetracycline in biological samples are not considered, only those which can be used for the purity control of TC are of interest.

Most official texts limit the amounts of 4-epitetracycline (ETC), 4-epianhydrotetracycline (EATC) and anhydrotetracycline (ATC), allowed to be present in TC. The European Pharmacopoeia (Ph. Eur.)(1) prescribes limits of 5% for ETC and 0.5% for EATC or ATC. The United States Pharmacopoeia (USP XXI)(2) limits EATC to 2%. The degradation products have no antibiotic activity; EATC is toxic and leads to a Fanconi-like syndrome.

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*For the meaning of the acronyms used to characterize the various tetracycline derivatives see Table I.

Table I. Acronyms used to characterize the different tetracycline derivatives

Acronym	Chemical name
ACTC	2-Acetyl-2-decarboxamidotetracycline
ATC	Anhydrotetracycline
CTC	Chlortetracycline
EATC	4-Epianhydrotetracycline
ETC	4-Epitetracycline
OTC	Oxytetracycline
TC	Tetracycline
UNK	Unknown

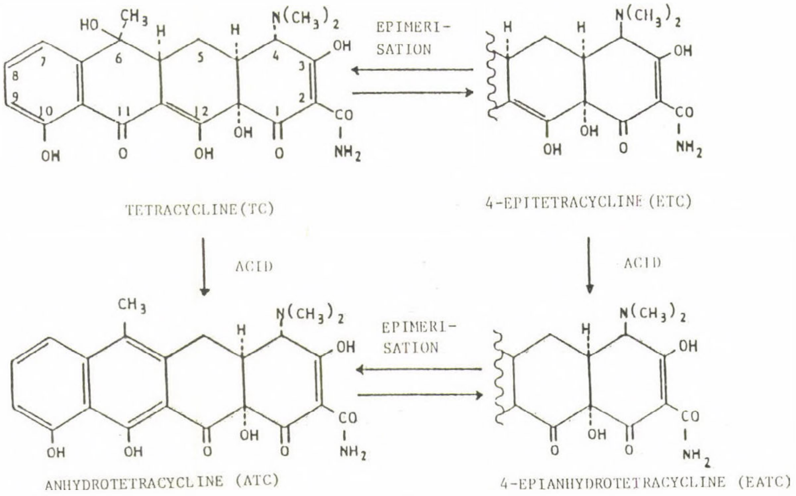

Fig. 1. Degradation of tetracycline

HPLC METHODS USING ION-EXCHANGE MATERIALS

The first HPLC method was published by a group from the Canadian Department of Health and Welfare and a chromatogram is shown in Fig. 2 (3). It is clear that the separation is not sufficient yet to allow proper quantitation of the degradation products. Figure 3 shows a chromatogram published by a group from Ohio State University (4). Complete separation of the four substances is obtained but it is probable that the resolution ETC-TC will decrease when the relative amount of TC is increased twenty times to obtain the TC:ETC ratio allowed for a commercial sample by the Ph. Eur. The TC:ETC ratio in Fig. 4, which was published by a researcher from the Laboratory of the Government Chemist in England, is also far too low, and should be increased twenty times as was mentioned for the previous examples (5). As the separation TC-ETC is very nice, this ratio increase was not expected to affect the separation very much and the method was tried out in the author's laboratory, using the same stationary phase, but the results obtained were not comparable at all. It was concluded that the published results were probably obtained with a particular column and were not checked on other columns of the same material before publication.

It regularly happens that a published separation cannot be reproduced. Very often this is due to the fact that the column used to obtain the published results has been used before for other chromatographic work and thereby is brought in an undetermined condition which cannot be reproduced by another laboratory. Another, not frequently occurring explanation is that a manufacturer sometimes changes the composition of the stationary phase but, for economical reasons, keeps selling the new material under the same label. Therefore, before publishing HPLC results an author should check whether the separation obtained can be reproduced on another, preferably new column. If the method is proposed as a general method for use in several laboratories, it is indicated to check the separation on several brands of the same type, e.g., several C-8 columns. A good HPLC method for general application should

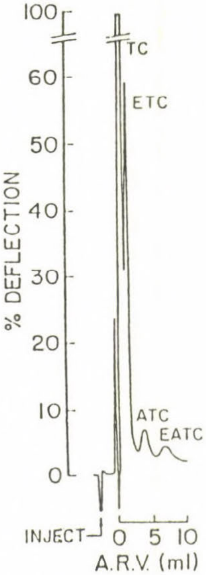

Fig. 2. First published analysis of TC and its metabolites on an ion-exchange column. From ref. 3. Column: 225 cm x 1.8 mm I.D., containing Pellionex CP-128. Mobile phase: ethanol-0.01 M Na^+ + 0.002 M $EDTA^{2-}$ (30:70), pH: 4.6. Flow rate: 1 ml/min. Detection: UV at 254 nm

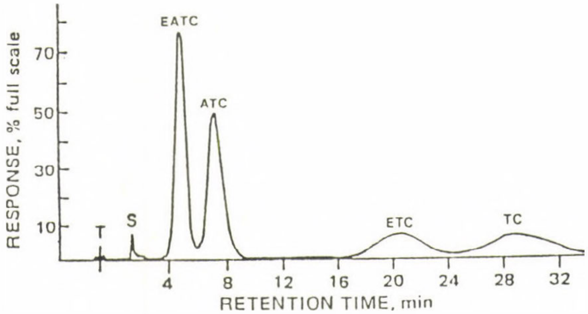

Fig. 3. Analysis of TC and its metabolites on an ion-exchange column. From ref. 4. Column: 10 cm x 3.2 mm I.D., containing a DuPont material (Zipax?), 25-37 µm. Mobile phase: 0.07 M sodium phosphate + 0.008 M Na_2EDTA, pH: 7. Flow rate: 1.1 ml/min. Detection: UV at 254 nm

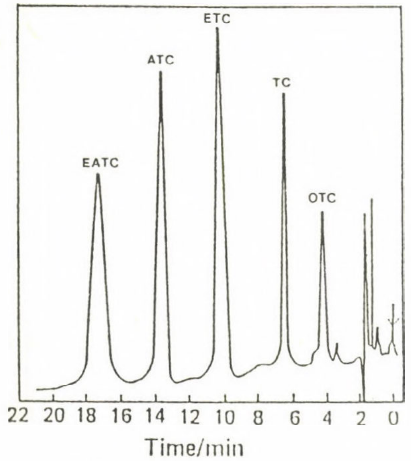

Fig. 4. Analysis of TC and its metabolites on an ion-exchange column. From ref. 5. Column: 15 cm x 4.6 mm I.D., containing LiChrosorb SCX 10 μm. Mobile phase: methanol-0.02 M Na_2EDTA + 0.05 M ammonium formate (30:70), pH: 5.8. Flow rate: 1 ml/min. Detection: UV at 270 nm

give comparable separations on different brands of stationary phase.

After 1979 no more results obtained on ion-exchange materials were published.

HPLC METHODS USING SILICA-BASED RESERVED-PHASE MATERIALS AND ACIDIC MOBILE PHASES

A comprehensive study on reversed-phase HPLC (RP-HPLC) of TC was published in 1975 by researchers from the University of Edinburgh, Scotland (6, 7). Chromatograms are shown in Fig. 5. They demonstrate the chromatographic problems encountered with RP-HPLC of TC. At relatively low concentrations of the organic modifier (chromatogram A) the pair of epimers ETC-TC is eluted fast and the separation is sufficient; the pair of epimers EATC-ATC is eluted too slowly, the separation is good but small amounts (<0.5%) cannot be detected. With increased

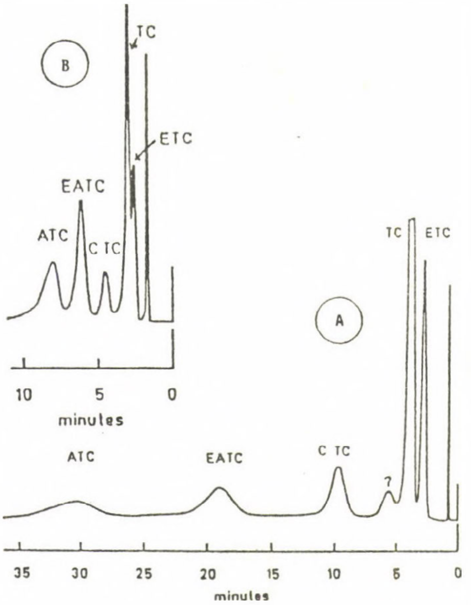

Fig. 5.
Analysis of TC and its metabolites by isocratic reversed-phase. From ref. 6. Column: 12.5 cm x 5 mm I.D., containing SC-TAS ("short-chain trialkylsilica"), 18µm. Mobile phase: acetonitrile-water, 0.1 M in $HClO_4$: (A) = 15:85; (B) = 25:75. Linear velocity: (A) 2.6 mm/sec; (B) 1.3 mm/sec. Detection: UV at 28o nm

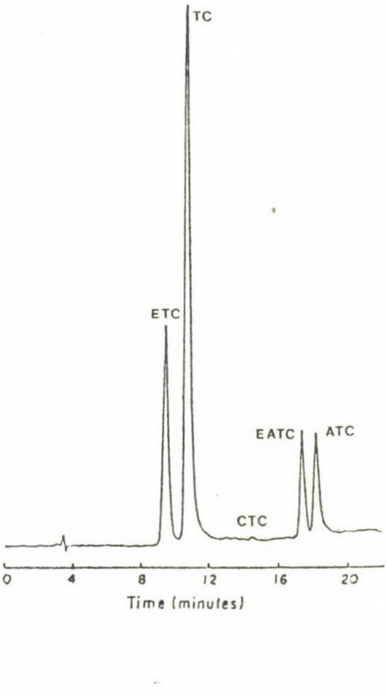

Fig. 6.
Analysis of TC and its metabolites by gradient elution reversed-phase HPLC. From ref. 9. Column: 30 cm x 3.9 mm I.D., containing µBondapak C-18, 10µm. Mobile phase: (A) methanol-water-0.2 M phosphate buffer pH: 2.5 (30:60:10); (B) methanol-acetonitrile-water-0.2 M phosphate buffer pH: 2.5 (50:20:20:10). Linear gradient in 15 minutes. Flow rate: 1 ml/min. Detection: UV at 280nm

concentrations of the organic modifier (chromatogram B) the detectability is improved but the ETC-TC separation declines. It should be noted that the TC:ETC ratio of 20:1 is not respected in the latter chromatogram. One year later a better separation was published by the same group, using a smaller particle size of the same stationary phase (8).

This kind of chromatographic problem is usually solved by gradient elution. The chromatogram shown in Fig. 6. was published by a group from the Upjohn Company, USA (9). The same group has already published earlier on RP-HPLC of TC (10, 11). Figure 6 shows complete separation of ETC, TC, EATC and ATC, but it is doubtful whether the TC:ETC ratio of 20:1 was respected. However, it is clear that gradient elution can be used for the quantitation of TC and its related substances but it requires more sophisticated equipment and is less suitable for routine work than isocratic elution.

A number of other papers mentioning isocratic RP-HPLC of TC with acidic mobile phases has been published (12-17). One of the chromatograms, obtained by an Australian group, is shown in Fig. 7. (15). Another, obtained by a Japanese group, is presented in Fig. 8. (16). As can be seen these methods do not yield substantial improvements. Several papers mentioned the use of one-step or continuous gradient elution RP-HPLC with acidic mobile phases (18-20). Although some improvements were achieved,the results were not substantially different from these obtained by Tsuji (Fig. 6.).

HPLC METHODS USING SILICA-BASED RESERVED-PHASE MATERIALS AND MOBILE PHASES AT NEUTRAL OR SLIGHTLY ALKALINE pH

A first important innovation in the HPLC of TC was published in 1980 by an Austrian group (21). A chromatogram is shown in Fig. 9. With a mobile phase at slightly alkaline pH the separation of the epimer pairs ETC-TC and EATC-ATC is much improved but the separation between TC and EATC is poor. Moreover, the method suffers from column instability due to the high pH (8.25) and temperature (50°C), which cause dissolution of the silica material. More recently a method using

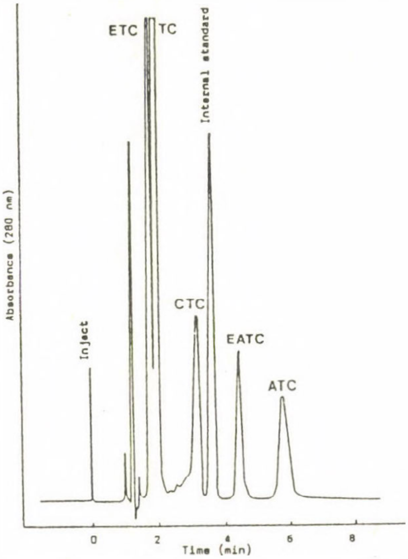

Fig. 7. Analysis of TC and its metabolites by isocratic reversed-phase HPLC. From ref. 15. Column: 30 cm x 3.9 mm I.D., containing µBondapak C-18, 10 µm. Mobile phase: water-ethanolamine-acetonitrile-dimethylformamide (76:0.5:24:6) + 5.2 g $(NH_4)_2HPO_4$ + H_3PO_4 to pH: 2.5. Flow rate: 2.5 ml/min. Detection: UV at 280 nm

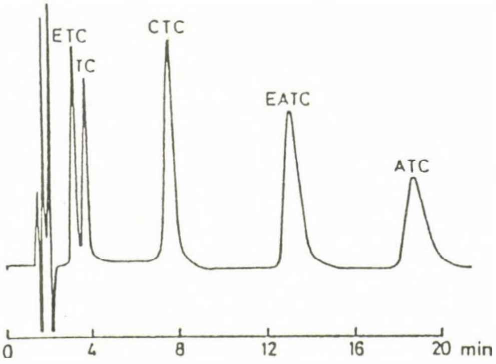

Fig. 8. Analysis of TC and its metabolites by isocratic reversed-phase HPLC. From ref. 16. Column: 15 cm x 4.6 mm I.D., containing Cosmosil 5 C-8, 5 µm. Mobile phase: methanol-acetonitrile-0.01 M oxalic acid (1:1:3.5), pH: 2.0. Flow rate: 1 ml/min. Detection: UV at 400 nm

a mobile phase at pH 6.6 and room temperature was published by researchers from the U.S. Food and Drug Administration (22). Although the column stability at this pH and temperature are improved, the separation is not substantially better, as can be seen in Fig. 10. The same year another variant was published by a Swedish group (23). The mobile phase, of pH 8.0, contains N,N-dimethyloctylamine, which is reported to improve the separation more than other amines. A chromatogram is shown in Fig. 11 ETC and TC, which are present in a normal ratio, are completely separated. Although EATC is eluted on the tail of the peak, it can be integrated without any problems. The stability of the packing material, used in the conditions described, has been mentioned to be satisfactory (24).

The introduction of a guard column between the pump and the injector improves the stability of the analytical column. An adaptation of this method will be introduced in the United States Pharmacopoeia for the analysis of doxycycline (25). More recently a group from Scotland published a method using a mobile phase at pH 7.0, containing cetrimide (17). The separation is comparable to those obtained with acidic mobile phases.

HPLC METHODS USING POLY(STYRENE-DIVINYLBENZENE) COPOLYMER AS THE STATIONARY PHASE

Poly(styrene-divinylbenzene) copolymer phases are more stable at extreme pH values than silica-based reversed phases. They behave as reversed-phase materials (26). The first method for chromatography of TC on polymeric material used a mobile phase at acidic pH. It was published by researchers from the Netherlands (27). The result is shown in Fig. 12. The separation is comparable to that obtained on silica-based reversed-phase materials with acidic mobile phases.

In the same period our laboratory published a method using polymeric material and an alkaline mobile phase (28). A chromatogram is shown in Fig. 13. ETC, TC, EATC and ATC are completely separated on a 10 cm column in about 15 min. The

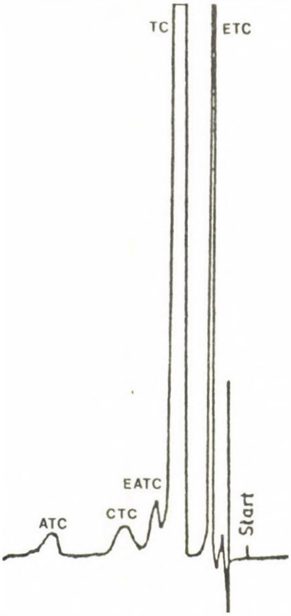

Fig. 9. Analysis of TC and its metabolites at alkaline pH. From ref. 21. Column: 25 cm x 3.2 mm I.D., containing LiChrosorb RP8, 5 µm. Mobile phase: n-butanol-methanol-mixture of 0.015 M phosphate buffer (pH: 7) and 0.001 M Na_2EDTA (2:8:90) to which triethylamine was added to adjust pH at 8.25. Flow rate: 1.5 ml/min. Temperature: 50°C. Detection: UV at 270 nm. Analysis time about 15 minutes

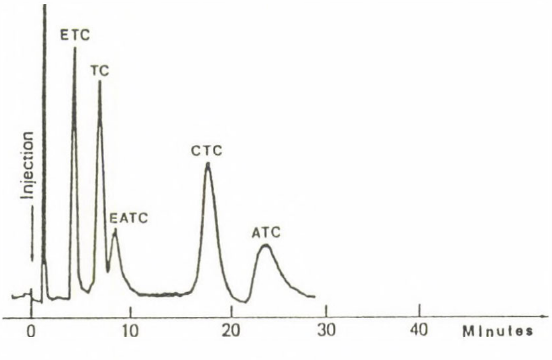

Fig. 10. Analysis of TC and its metabolites at neutral pH. From ref. 22. Column: 25 cm x 4.6 mm I.D., containing Vydac C-18, 10 µm. Mobile phase: methanol-0.001 M EDTA (18:82), pH: 6.6. Flow rate: 1.8 ml/min. Detection: UV at 380 nm

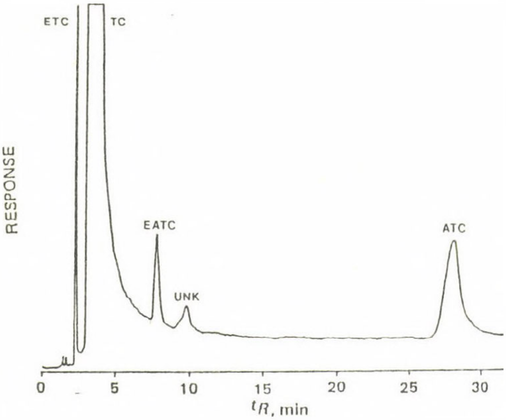

Fig. 11. Analysis of TC and its metabolites at alkaline pH. From ref. 23. Column: 15 cm x 4.5 mm I.D., containing LiChrosorb RP-8, 5 µm. Mobile phase: acetonitrile-0.1 M NaH_2PO_4-0.0194 M N,N-dimethyloctylamine (30:70) to which sodium hydroxide was added to adjust the pH at 8.0

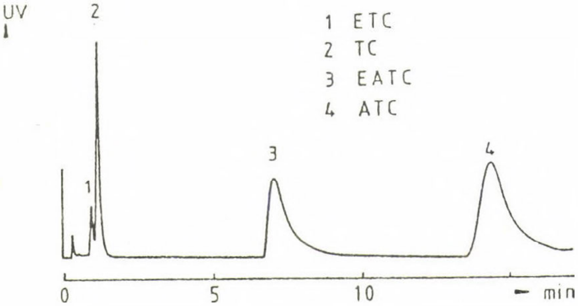

Fig. 12. Analysis of TC and its metabolites on a polymeric phase. From ref. 27. Column: 10 cm x 3 mm I.D., containing PRP-1 (Hamilton), 10 µm. Mobile phase: acetonitrile-dichloromethane-0.05 M acetate buffer (pH: 3.6) and 0.025 M EDTA (12.5:1:87.5). Flow rate: 0.8 ml/min. Detection: UV at 268 nm

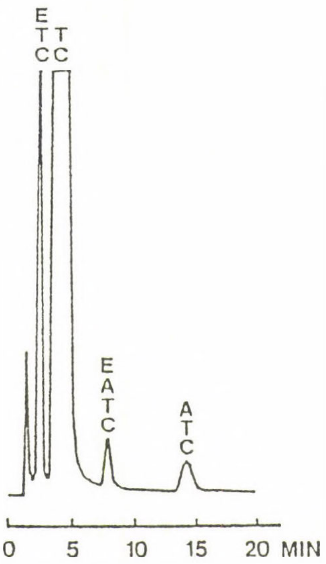

Fig. 13. Analysis of TC and its metabolites on a polymeric phase. From ref. 28. Column: 10 cm x 4.6 mm I.D., containing PLRP-S (Polymer Labs., Church Stretton, Shropshire, UK), 8 m. Mobile phase: tert. butanol-0.2 M phosphate buffer (pH: 9.0)-0.02 M tetrabutyl ammonium sulphate (pH: 9)- 0.1 M sodium acetate (pH: 9)-water (5.5:10:5:1:78.5). Flow rate: 1.0 ml/min. Temperature: 60°C. Detection: UV at 254 nm
Concentrations:

TC	tetracycline	
ETC	4-epitetracycline	4 % m/m
EATC	4-epianhydrotetracycline	0.5 % m/m
ATC	anhydrotetracycline	0.5 % m/m

quantitation of small amounts of impurities can easily be realised. The column can be used continuously for several months without stability problems. The method used here is based on a method previously published for doxycycline (29). Figure 14 shows a chromatogram obtained with the same mobile phase and a 25-cm long column containing the same polymeric material (30). Here an impurity, never isolated by HPLC before, is separated. The product was identified as 2-acetyl-2-decarboxamidotetra-

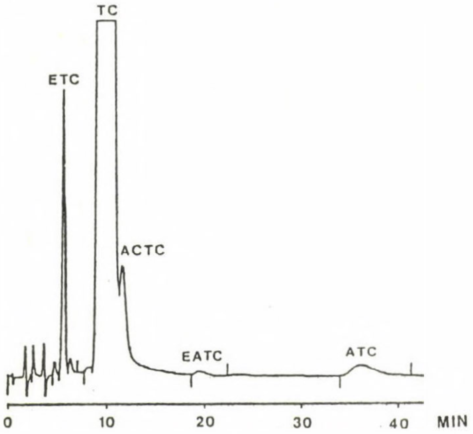

Fig. 14. Analysis of tetracycline and its metabolites on a polymeric phase. From ref. 30. Column, mobile phase and conditions as in Fig. 13. except that the column length was 25 cm.
Concentrations:

TC	tetracycline	
ETC	4-epitetracycline	1.1 % m/m
ACTC	2-acetyl-2-decarboxamidotetracycline	0.4 % m/m
EATC	4-epianhydrotetracycline	0.06% m/m
ATC	anhydrotetracycline	0.4 % m/m

cycline, which was previously separated from crude tetracycline by counter-current distribution (31). This impurity was found to be present in most commercial samples.

The method peresented in Figs 13 and 14 is very useful for the quantitative determination of tetracycline and its degradation products. The method can also be adapted for analysis of other tetracyclines such as oxytetracycline, doxycycline and demeclocycline.

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A SUBSTANCE ISOLATED FROM HUMAN AMNIOTIC FLUID THAT INHIBITS CONTRACTILE FUNCTION OF HUMAN MYOMETRIUM

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INTRODUCTION

Various chemical substances regulate the function of the smooth muscle. In recent years the investigators recognized the importance of endogenous peptides, their biological role and structure (1). Nevertheless, in spite of the active research on the mechanisms and substances maintaining the activity of the uterine smooth muscle at rest during pregnancy and initiating the onset parturition, the information obtained is far from being complete and is rather contradictive.

According to literature, amniotic fluid contains several substances affecting the activity of the uterine smooth muscle. Chemical structure of a variety of such substances is well defined (oxitocin, progesteron, estrogen, prostaglandines, angiotensin, catecholamines, serotonine, histamine, cAMP, prolaktine, ACTH, relaxin, VIP)(2-5). However, the structure of other smooth muscle active compounds in the amniotic fluid is not yet determined (6-17).

The results of our preliminary experiments - in accordance with literature - show that the substances found in the amniotic fluid at mid-pregnancy inhibit, while those at term stimulate the activity of the uterine smooth muscle. The aim of the present study was to isolate the inhibiting compound(s) from the amniotic fluid at mid-pregnancy and to assess their biological role and chemical structure.

EXPERIMENTAL PART

(1) Obtaining and storage of the samples:

Amniotic fluid obtained at the 16th-22nd weeks of pregnancy by amniocenthesis was centrifuged (4^{o}C, 10,000 rpm. 20 min). Supernatant was stored at -20^{o}C for further investigation.

(2) Chromatographic assay:

(a) Gel-chromatography: the column was filled with Sephadex G-25 fine. Column length: 75 cm; diameter: 1.3 cm; eluent: 0.9 % NaCl, at a flow rate of 2 ml/min; detection: 254 nm (LKB, Uvicord I); fraction volume: 8 ml; sample volume: 20 ml amniotic fluid. Biological activity of the efluent was determined on isolated rat uterus strip with biological detector operating in on-line manner (see ref. 19).

(b) Ion-exchange chromatography: fraction with inhibiting activity determined with biological detector which eluated from the SG-25 column at Kav values of 0.6-0.9 was further separated on a Whatmann CM32 column. Column length: 40 cm; diameter: 1.3 cm; eluents: 1. 0.2 M NaCl, pH=4.0 (180 ml).
2. 2.0 M NaCl, pH=4.0 (180 ml)
at a flow rate of 3 ml/min; detection: 254 nm (LKB, Uvicord S); fraction volume: 20 ml; sample volume: 50 ml. The fractions were desalinated with Dowex 50x8 gel, dissolved in Krebs-Ringer solution (19) and their biological activity on isolated rat uterus strip was determined with a biological detector operating in off-line manner (19). The active fraction was concentrated to one-half volume on Amicon UM 0.5 membrane.

(c) Reverse-phase HPLC: concentrated, biologically active fraction obtained in "b" was further purified with reversed-phase HPLC-chromatography.

For selecting the optimal separation parameters, the simplex grid optimization method has been applied (20); the separation parameters were as follows: column: Whatmann Magnum 9 ODS (9.4x500 mm); eluent: acetonitrile:n-propanol:water (4:20:76); flow rate: 5 ml/min; pump: Liquopump, 312 (Labor MIM, Hungary); detection: 254 nm (OE 308 UV detector, Labor MIM, Hungary); sample volume: 5 ml (Rheodyne M7125 injector). The obtained fractions were lyophilized and solved in Krebs-

-Ringer solution (19); their biological activity was determined on rat uterus strip with the biological detector operating in off-line manner (19, 21).

RESULTS

(a) Figure 1 shows the results of gel-chromatographic separation, the gel chromatographic pattern of the amniotic fluid and biological activity of the fractions. In our experimental set-up the efluent from the column passed the UV-detector and directly entered into the biological detector. The chromatogram shows the extinction at 254 nm; the upper part of the figure presents the pattern produced by the biological detector. Among the three fractions obtained from the column, the one eluting at Kav 0.6-0.7 inhibits contractions and enhances tone. The Kav 0.7-0.9 fraction completely but reversibly inhibits the contractions and decreases the tone.

(b) Figure 2 shows the results of ion-exchange chromatography (at 254 nm) and biodetection of the biologically active SG-25 fraction (Kav 0.7-0.9). The first fraction eluting with 0.2 M NaCl depresses the contractions of rat uterus and evokes a complete but reversible inhibiting and tone-enhancing effect. The biological activity of the second fraction eluted with 2.0 M NaCl will be discussed in another paper.

(c) The results of the reversed-phase HPLC separation with UV- and biodetection (254 nm) of the biologically active ion-exchange fraction (0.2 M NaCl) are summarized in Fig. 3. Among the six fractions detected at 254 nm those obtained at 32 minutes inhibit the contractions, while the peak obtained at 53 minutes elevates the tone.

DISCUSSION

Subsequent use of gel-, ion-exchange- and reversed-phase HPLC allowed us to purify a fraction from human amniotic fluid which reversibly inhibits the activity of both rat- and human uterus (27). Experimental results concerning the biological testing of this fraction (presented in this paper and in earlier

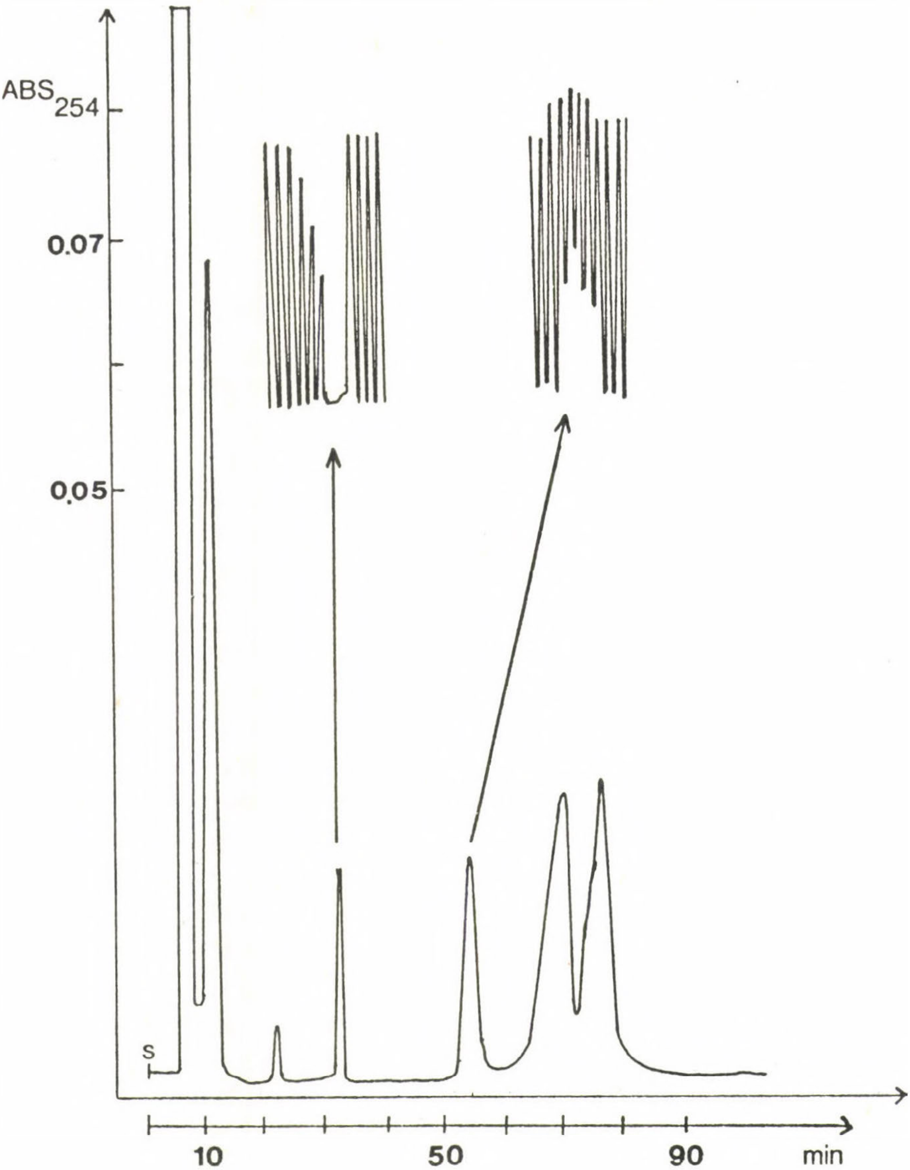

Fig. 1. Gel chromatography of human amniotic fluid and biological activity of the fractions on the isolated rat uterus. The lower part of the figure shows the extinction of the fractions at 254 nm; its upper part presents the biological activity. For experimental conditions see Experimental, Part 2a

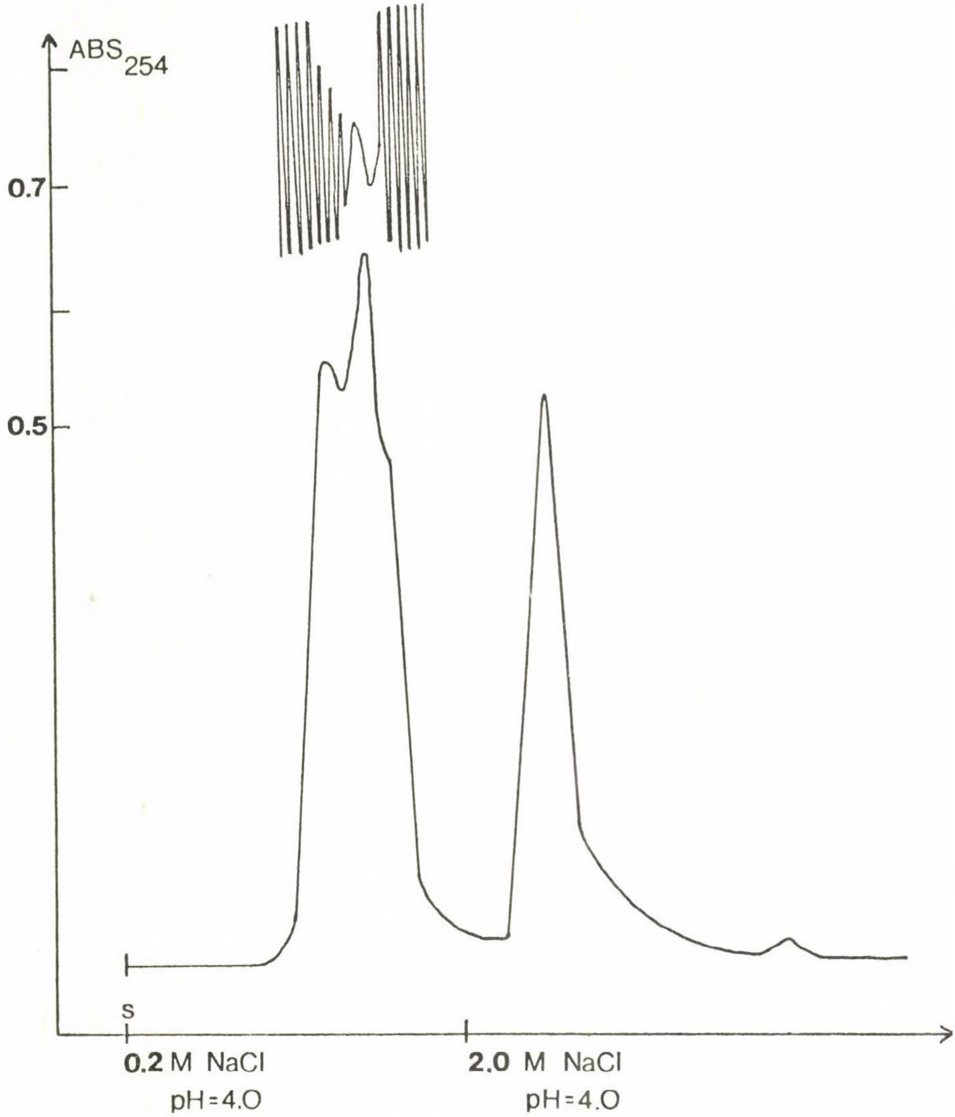

Fig. 2. Ion-exchange chromatography of the biologically active gel chromatographic fraction performed on Whatmann 32 gel. The lower part of the figure shows the extinction at 254 nm; its upper part presents the effect of the biologically active fraction on the rat uterus. For experimental conditions see Experimental, Part 2b

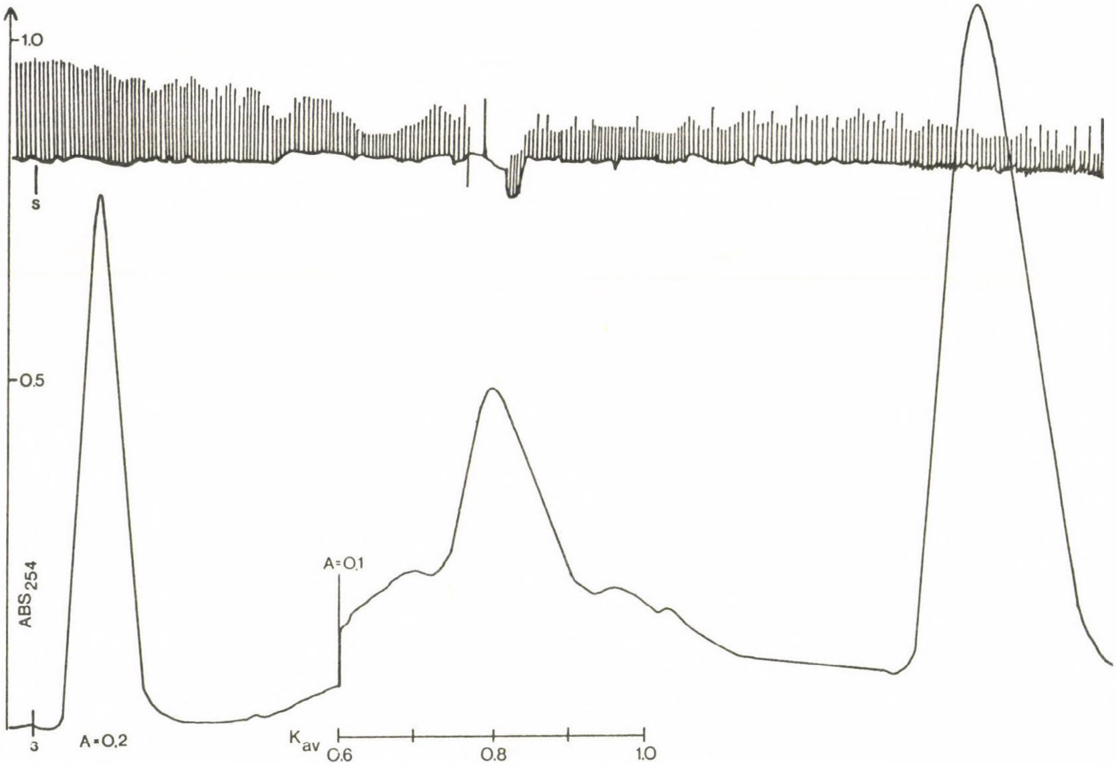

Fig. 3. Separation of the biologically active fraction obtained by ion-exchange chromatography with HPLC. The lower part of the figure shows the extinction at 254 nm; its upper part presents the results of biological detection of the two active fractions. For experimental conditions see Experimental, Part 2c

publications) show that this purified fraction distinct from substances of determined structure is able to affect function of uterine smooth muscle.

This fraction is not identical with: (i) relaxin (based on chromatographic differences: relaxin is eluated at Kav 0.1 from a SG-25 column); (ii) VIP (vasoactive intestinal peptide) also due to chromatographic differences. Besides, in contrast to our fraction, VIP relaxes rat aorta (23); (iii) prolactin (due to chromatographic differences); (iv) epinephrine (because the activity of our fraction cannot be blocked by Beta-receptor blocking compounds (23); (v) PGE2 and PGF relaxing human uterus, because these substances unlike our fraction stimulate the rat uterus (23).

Complete isolation of this factor, determination of its chemical structure and biological activity would help to better comprehend the molecular background of the functioning of the pregnant uterus and provide a possibility (using conventional and biological detection) to develop an analitical method for monitoring the temporary changes of the concentration of the active substances and for studying their origin and distribution in the organism.

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MEASUREMENT OF DNA-PROTEIN BINDING CONSTANTS BY A COMPUTERIZED CHROMATOGRAPH "MILICHROM"

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SUMMARY

DNA-protein binding constants were measured by gel chromatography. A system consisting of a MILICHROM microcolumn multiwavelength scanning liquid chromatograph and an ISKRA-226 computer was used for the measurement. The developed software permits to determine DNA and protein concentrations at any time. Data on the time dependencies of the RNA and protein concentrations are used to determine the DNA-protein binding constants. The method has been applied to the interaction of E. coli RNA polymerase with a number of plasmid DNAs and with four single-stranded oligodeoxyribonucleotides which are fragments of E. coli promoters.

INTRODUCTION

Many approaches are known to the measurement of DNA-protein binding constants through gel chromatography (1-4). Whatever the experimental set-up, the constants are calculated from the time dependencies of the DNA and protein concentrations. It is highly desirable to measure both dependencies. However, both cannot be obtained if the chromatogram is recorded only at one wavelength.

The MILICHROM multiwavelength scanning chromatograph (5) allows the two concentrations to be determined simultaneously. In previous investigations (3, 4) the chromatogram was recorded automatically at two wavelengths, so that the DNA/protein ratio could be followed. However, the procedure used did not permit to adequately estimate the measurement error, and the use of a large number of wavelengths was made extremely difficult.

If the data acquisition process involves a computer and the chromatogram is subsequently analysed, it is quite possible to determine the DNA and protein amounts with the maximum accuracy, allowing the measurements to be performed at any chosen number of wavelengths (6). The results can be presented in a convenient form for easy interpretation.

In the present paper we report on the study of the interaction of E. coli RNA polymerase with pAO3, pUC19 plasmid DNAs, pBR322 plasmid DNA with an inserted growth hormone gene, and with single-stranded oligodeoxyribonucleotide sequences which are fragments of the E. coli Spc and trpR gene promoters.

MATERIALS AND METHODS

DNA. The plasmid pAO3 (7) (strain C600), pUC19 (8)(JM83), and pBR322 with an inserted human growth hormone gene (HB101) were isolated from E. coli cells by alkali method followed by equilibrium centrifugation in a CsCl density gradient with ethidium bromide.

Oligonucleotides. We analysed the binding of E. coli RNA polymerase to the following single-stranded synthetic oligodeoxyribonucleotides:

No.1 5'-TATAATGCC -from the"-11-3" region of the Spc promoter;
No.2 5'-TATAATGCCGCG -from the"-11+1" region of the Spc promoter;
No.3 5'-TATCGTACTCTT -from the"-13-2" region of the trpR gene promoter;
No.4 3'-ATAGCATGAGAA -from the"-2-13" region of the trpR gene promoter;

Synthetic oligonucleotides No. 1 and No. 2 correspond to a section of transcribed DNA strand in the "-10" region of the Spc promoter of ribosomal genes of E. coli; No. 3 and No. 4 are complementary DNA fragments from the promoter of trpR repressor gene in E. coli oligonucleotides No. 1 and No. 2

were synthesized and kindly provided by N.V. Amerhanov, No. 3 and No. 4 - by L.V. Baranova (Institute of Cytology and Genetics, Sibirian branch of the Academy of Sciences). The oligonucleotide concentrations were spectrophotometrically determined during gel chromatography on SEPHADEX G-50S (Pharmacia).

RNA polymerase. The RNA polymerase holoenzyme was purified according to Burgess and Jendrisak (9). Its homogeneity was better than 95% according to electrophoresis in 7% polyacrilamide gel with SDS. The sigma subunit content was spectrophotometrically determined during its separation from the core enzyme on the BIO-REX 70 ion-exchange resin (Bio-Rad). The active enzyme concentration in the preparation was determined as described by Chamberlin (10). The RNA polymerase concentration was spectrophotometrically determined in the chromatographic run on SEPHADEX G-50S.

Binding and dissociation kinetics. RNA polymerase binding to DNA was performed under the following conditions: 50 μl of incubation mixture contained 20 mM TEA-HCl (pH 7.9), 100 mM $MgCl_2$, 0.2 mM DTT, 20 μg/ml DNA and different amounts of RNA polymerase (the enzyme/DNA molar ratio varied from 5 to 60). The mixture was incubated at 37°C for 30 minutes, then a stepwise formaldehyde fixation of specific DNA:RNA polymerase complexes was performed (11). Formaldehyde was added to 0.2%, two minutes later formaldehyde was added to 2%, and the solution was left for 10 min.

The RNA polymerase binding to oligonucleotides occurred in the course of enzyme chromatography in a column filled with oligonucleotide in the same buffer as for plasmid DNA binding at room temperature.

To examine the dissociation kinetics of the DNA:RNA polymerase complexes, we used heparin (Spofa) in concentrations from 5 to 100 g/ml for inactivating the free polymerase in solution. A specific time interval was allowed after adding heparin, then an aliquot of the incubation mixture was subjected to the stepwise formaldehyde fixation and gel chromatography.

Chromatography. Gel chromatography of formaldehyde-fixed DNA:RNA-polymerase complex was performed in 200-600 μl glass columns filled with TSK-gel HW 65 F (Toyo Soda). A solution containing 0.5 μg DNA and RNA polymerase was applied to the column by means of the pump of the MILICHROM chromatograph. The elution rate ranged from 10 to 20 μl/min. The eluent consisted of the buffer for RNA polymerase:DNA binding. Figure1 shows a typical chromatogram. The DNA and the complex were eluted in the excluded volume (peak A), while the free RNA polymerase was eluted about at the midrange of the column (peak B). To avoid any error associated with peak overlap, the DNA/protein ratio in the excluded peak volume was determined from the spectrum of its first half.

The RNA-polymerase:oligodeoxyribonucleotide binding isotherm was chromatographically determined by the pulse method (1). A 300-600 μl glass column filled with Sephadex G-50 S was balanced by a binding buffer with the oligonucleotide added in different concentrations (from 1 to 20 μg/ml). An RNA polymerase "pulse" (3-5 μg) was applied to the column. Chromatography was performed in a solution containing oligo-DNA in different concentrations (from 1 to 20 μg/ml). In the course of chromatography RNA polymerase overtakes the oligonucleotide, taking along the part bound to the enzyme. The RNA polymerase "pulse" is followed by a gap in oligonucleutide concentration due to the fact that the enzyme has removed part of the oligonucleotide out of the eluent. As a result we have a situation in which the RNA polymerase:oligonucleotide complex is in equilibrium with the dissolved oligonucleotide. The chromatogram is shown in Fig. 2. The RNA polymerase/oligonucleotide ratio in the complex is determined from the spectrum of the first part of peak A (Fig. 2).

Calculation of the RNA polymerase:DNA binding constants. These calculations are based on the ability of our software to decompose the spectrum into constituent substance spectra. The decomposition of a spectrum into the spectra of constituent substances provides an estimate of the contribution of each original substance to the absorbance of their mixture (or

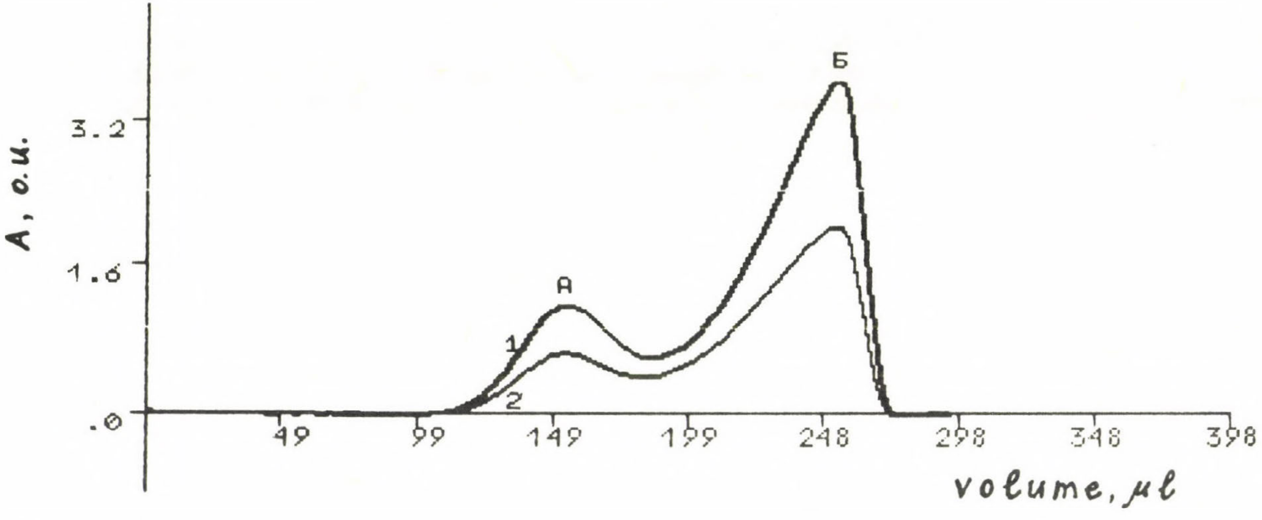

Fig. 1. Chromatographic elution profile of E. coli RNA polymerase:DNA complexes on TSK HW65 gel. Absorption A at 280 nm (curve 1) and 260 nm (curve 2). Peak A corresponds to DNA and the complex, peak B corresponds to free enzyme

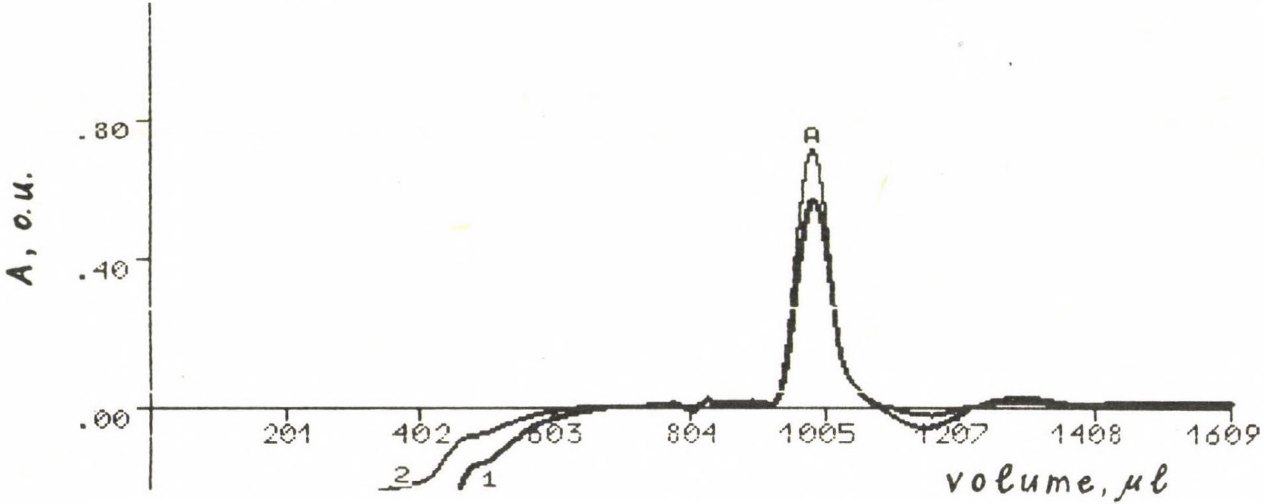

Fig. 2. Chromatographic elution profile of E. coli RNA polymerase:oligodeoxyribonucleotide complexes on SEPHADEX G-50 resin. Optical absorption at 260 nm (curve 1) and 280 nm (curve 2). Peak A corresponds to the complex

complex). Using the known extinction coefficients of the original substances and their molecular weights, one can determine the molar ratio of bound DNA and RNA polymerase in the chromatographic peak of their complex, which is necessary for estimating the binding constant.

The calculation of the binding constants is based on the assumption that there are two types of binding sites: strong binding sites and weak (nonspecific) binding sites. The strong binding is described by the equation

$$D + P \quad DP \tag{1}$$

where D is a strong binding site (all strong binding sites are considered to be identical), P is free RNA polymerase. DP is a strong binding site: protein complex. The specific binding constant

$$K = \frac{DP}{[D]\cdot[P]} = \frac{r}{(r_o - r)\cdot C_{DNA}\cdot R} \tag{2}$$

where C_{DNA} is the molar concentration of DNA in solution;
r is the RNA polymerase/DNA molar ratio in the complex,
i.e. $r = \frac{DP}{C_{DNA}}$;
r_o is the number of strong binding sites of DNA;
R is the RNA polymerase/DNA molar ratio in solution,

$$R = R_o - R_{ns} - r; \tag{3}$$

R_o is the molar ratio of total RNA polymerase and DNA concentrations;
R_{ns} is the molar ratio of the RNA polymerase involved in nonspecific binding to DNA.

The weak binding sites in our model are described by the equations of McGhee and von Hippel (12). The amount of free RNA polymerase in solution necessary to estimate the strong binding constant was determined from the equation of McGhee and von Hippel assuming that the nonspecific RNA-polymerase:DNA binding constant is equal to $5 \cdot 10^5$M (the DNA concentration here is measured in base pairs as moles per liter) at 0.05 M NaCl and 0.01 M $MgCl_2$ (13) and the size of binding site is 42 nucleotides (14).

In Eq. 2 C_{DNA} is known, R is found from the initial RNA polymerase concentration as described above (Eq. 3) and r is determined during the post-run processing of the chromatogram with the help of our software. Plotting the dependence of r on R makes the determination of the binding parameters K and R_o possible.

In the case of RNA polymerase:oligonucleotide binding we assume that there is only one type of binding sites, so that $r_o = 1$.

RESULTS AND DISCUSSION

Figure 3 shows the dependence of the mean number of RNA polymerase molecules specifically bound to DNA (r) on the initial number of RNA polymerase molecules per DNA base pair mole in solution (R_o). The binding isotherms were constructed for pBR322 DNA with a 800-bp-long insertion (human growth hormone gene)(≈5000 bp), pUC19 DNA (2686 bp) and pAO3 DNA (1683 bp). The initial slope of these curves is determined by the binding constant, while their saturation values determine the number of specific binding sites on the DNA concerned.

The number of binding sites is 20 ± 1 for pBR322 DNA with the insertion, 7 ± 1 for pUC19 DNA, 4 ± 1 for PaO3 DNA. The accuracy of these results is defined by the error in determining the protein/DNA ratio, which is mainly associated with the considerable overlap of the two chromatographic peaks (A and B in Fig. 1) at the saturating concentrations of RNA polymerase. Thus, for these plasmid DNAs, there is one enzyme binding site per 250, 380 and 420 bp, respectively. These data are in agreement with the subsequent electron-microscopic analysis of filtered complexes. All the binding isotherms reach saturation for $R_o > 0.05$, so the binding isotherms reach saturation, as the binding constants K are close for these DNAs. The dependencies of r on R were replotted in the r versus R coordi-

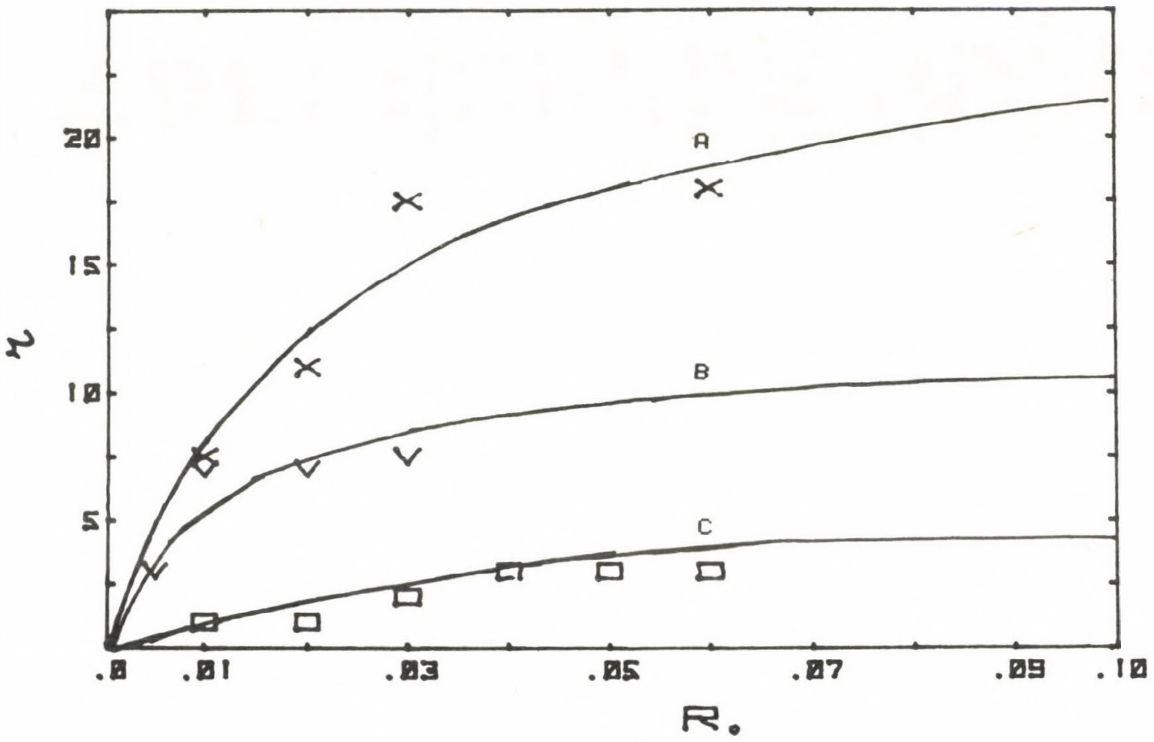

Fig. 3. Binding isotherms for E. Coli RNA polymerase with: (A) pBR322 plasmid DNA with the interaction (x); (B) pUC19 plasmid DNA (v); (C) pAO3 plasmid DNA (Π). r is the number of RNA polymerase molecules bound to DNA; R_0 is the number of RNA polymerase molecules in solution per nucleotide base pair

nates and the resulting relationships were used to determine the equilibrium constants of specific binding according to Eq. 2. The same value of $K = 3 \cdot 10^9 M$ was obtained for the three DNAs within the experimental accuracy (calculated per DNA base pair mole). This value accords with the data reported by Giacomoni et al. (15), where the binding constants for E. coli RNA polymerase and replicative fd phage DNA were determined by electron microscopy. It is also in agreement with the results of Seeburg et al. (17), who calculated the equilibrium constant for the interaction with five promoters of the fd replicative form on the basis of kinetic constant ratios determined by the filter binding technique. At 0.01 M $MgCl_2$, 0.12 M KCl, the value of K varied from $2 \cdot 10^8$ to $2 \cdot 10^9 M^{-1}$.

The binding constants for oligonucleotides can, in principle, be calculated from one point on the binding isotherm, since $r_o = 1$ and the number of unknown quantities in Eq. 2 is reduced to one. An equilibrium binding isotherm was obtained for preparation No. 1, and we simultaneously determined K and r_o. The maximum ratio of 1.28 obtained in this experiment may be due to the following reasons: (a) the lack of accurate information on the extinction coefficient for oligonucleotides; we assumed the extinction coefficient to be 35 o.u.·ml/mg for 260 nm; (b) a possible alteration of the oligonucleotide spectrum on RNA polymerase binding: the spectrum amplitude may change, though not its form; any substantial change in the RNA polymerase spectrum is less likely.

The experimental set-up imposes certain limitations on the maximum constant reading: the fragment concentrations must be sufficient for saturating RNA polymerase during chromatography. Therefore, under these conditions, we obtained the lower estimate of the binding constant with preparation No. 1:

$$K > 0.6 \cdot 10^6 M^{-1}.$$

No RNA polymerase binding was observed in the case of trpR promoter oligonucleotides (preparations No. 3 and No. 4) for oligonucleotide concentrations up to $1.2 \cdot 10^{-6} M$. While binding did occur in experiments with preparation No. 2, its parameters could not be determined because of insufficient amount of this oligonucleotide.

The binding of E. coli RNA polymerase to preparations from the "-10" region of the E. coli Spc promoter was also demonstrated by Knorre et al. (18), where the binding efficiency (10% for No. 1, 41% for No. 2) was measured by the labelled oligonucleotide:RNA polymerase complexes retained on filters.

Chromatography was also used to examine the dissociation kinetics of specific complexes of E. coli RNA polymerase and pBR322 DNA with the insertion. The formaldehyde fixation time must be much smaller than the average lifetime of a complex to make the formaldehyde fixation procedure adequate for the purpose of this study. It was found that a two minute formaldehyde treatment was sufficient for fixing all the specifically bound complexes. This is much less than the dissociation time of specific complexes, as reported in literature, e.g., by Giacomoni et al. (16).

Heparin, which is a strong inhibitor of RNA polymerase:DNA binding (19), was used to inactivate the free RNA polymerase in solution. In a chromatographic experiment heparin is to be preferred to tRNA,poly-d(A-T) or DNA, which also bind the RNA polymerase but, unlike heparin, make a sizable contribution to the optical absorption and can fail to separate from the complex peak in gel chromatography.

The dissociation kinetics for a given heparin concentration conforms to the exponential law

$$r(t) = r(0)\cdot\exp(-t/\tau) \qquad (4)$$

where t is time, τ is the average lifetime of a complex, which depends on the heparin concentration. All the RNA polymerase binding sites on a given DNA are assumed to be identical.

Figure 4 shows the dissociation kinetics of specific complexes of E. coli RNA polymerase and pBR322 DNA with the insertion for different concentrations. Since the r(0) value varied for different heparin concentrations, the plot shows the r(t)/r(0) ratio in a logarithmic scale. One can see that the dissociation rate of specific enzyme-DNA complexes depends

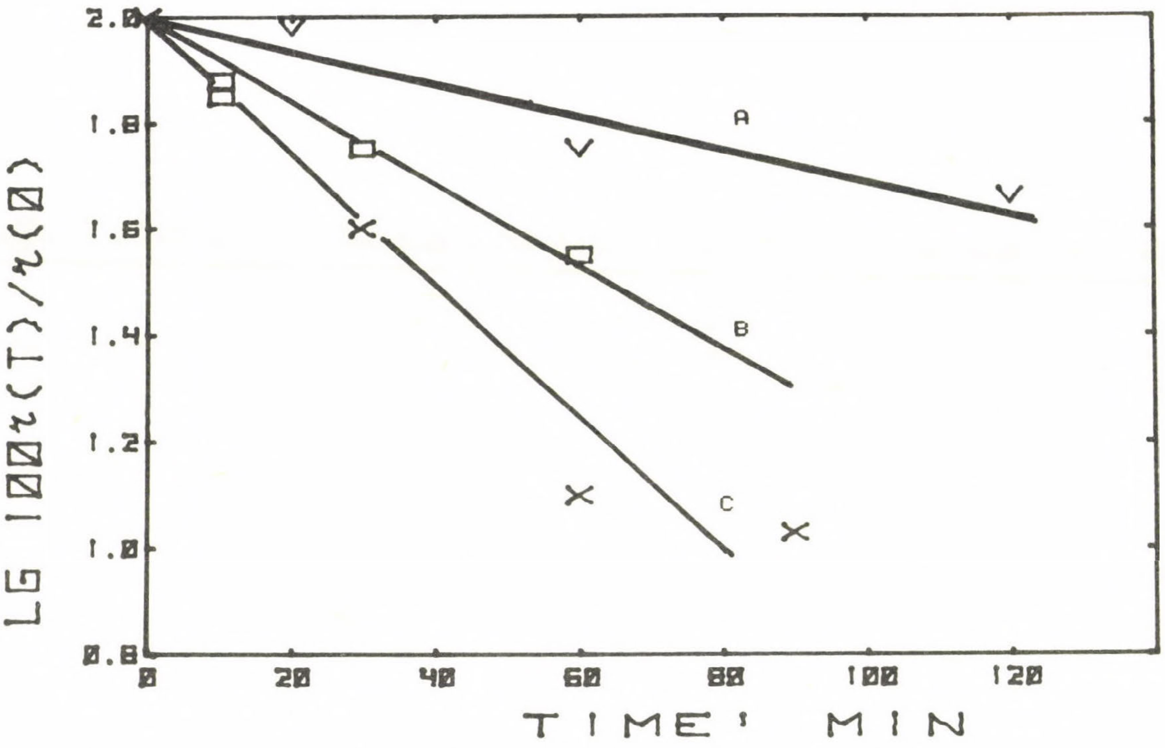

Fig. 4. Dissociation kinetics of specific complexes of E. coli RNA polymerase and pBR 322 RNA with the insertion for different heparin concentrations. The value 100 r(t)/r(0) plotted on the ordinate axis in the logarithmic scale; r(t) is the average number of enzyme molecules sepcifically bound to DNA at time t. (v) 5 μg/ml heparin; (Π) 50 μg/ml heparin; (x) 100 μg/ml heparin

on the heparin concentration in solution. For instance, for $C = 50\ \mu g/ml$ the dissociation constant is $K = 8\cdot 10^{-3} min^{-1}$, which corresponds to the average complex lifetime of 120 min.

Figure 5 plots the dependence of $1/\tau$ on C, the heparin concentration. In this range of heparin concentrations the dependence can be described by the linear equation:

$$1/\tau = K_a + K_t \cdot C \qquad (5)$$

obtained by Giacomoni et al. (16). Here K_a is the real complex dissociation rate constant and K_t is the rate constant for interaction of heparin and the specific complex. K_a can be obtained by extrapolating the values of $1/\tau$ to zero heparin concentration: in our case $K_a = 2\cdot 10^{-3} min^{-1}$, which corresponds to an average lifetime of 500 min at 0.05 M NaCl. The rate constant for the interaction of heparin and the specific complex has been estimated at $K_t = 23\cdot 10^{-4} l\cdot g^{-1}\cdot sec^{-1}$. The value of K_a is comparable to that of K for specific complexes of RNA polymerase and replicative fd DNA at 0.01 M NaCl (16). The value of K_t given by Giacomoni et al. (16) is less than ours by a factor of four. This may be due to the use of different commercial preparations of heparin, for heparin is a mixture of polymers with different molecular weights and we do not know the minimum polymer chain length required for binding RNA polymerase.

A ten-second treatment of complexes with 5 μg/ml heparin is known to result in a rapid and complete dissociation of nonspecific binding (20). We have demonstrated that such treatment of the complexes does not reduce the number of RNA polymerase molecules fixed on DNA by formaldehyde. This provides further evidence of the fact that formaldehyde does not fix nonspecific complexes.

CONCLUSIONS

In conclusion we would like to mention the advantages of the chromatographic approach. The averaged binding characteristics cited above can be further refined by electron micro-

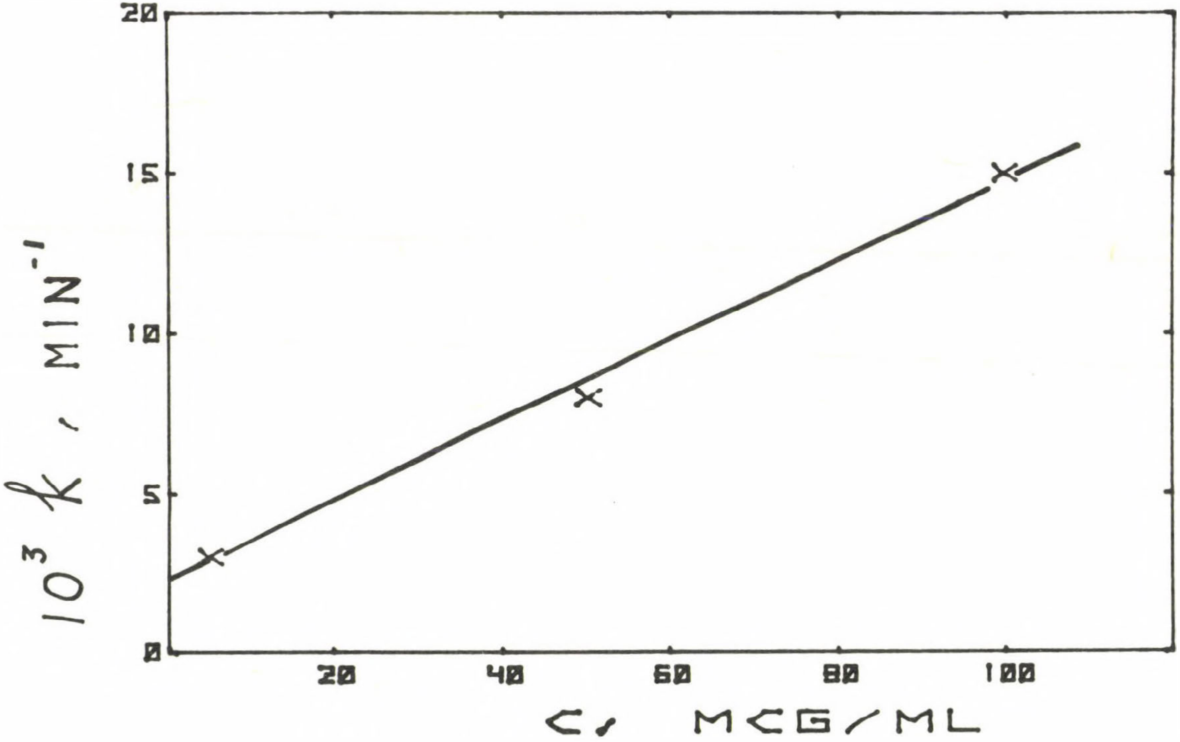

Fig. 5. Dependence of the dissociation rate constant on the heparin concentration in solution

scopy. The complexes fixed need no more purification after chromatography, before they can be subjected to electron microscopy.

It is interesting to compare chromatography with other methods. Binding on nitrocellulose filters is the most widely-used approach to the study of DNA:protein interaction. Supposedly, the strong binding of at least one protein molecule to DNA is sufficient for its retention on the filter. Hence this method cannot provide a complete binding isotherm if there is more than one binding site or if the binding is cooperative. Besides, some proteins cannot bind on nitrocellulose.

The chromatographic approach has a lot in common with centrifugation, but the detector used in MILICHROM microcolumn liquid chromatograph is much more sensitive, so that the chromatographic measurement of the equilibrium isotherm requires far smaller quantities of the investigated substance.

The MILICHROM chromatograph/ISKRA-226 computer system allowed to obtain the maximum information from the chromatographic curves, making the method more sensitive and less labor-intensive. The use of microcolumns and high sensitivity reduced the required sample quantities. As a further improvement the chromatographic experiment can be modified, for example ion-exchange or affinity chromatography may be used instead of gel chromatography.

*

APPENDIX

ASSESSMENT OF ACCURACY

Since in this study we used the maximum capability of our data processing system, we attached a particular importance to the accuracy of the spectral measurements and spectral decomposition.

Spectral measurement accuracy is determined under the "average spectrum" option of our software (6). The accuracy was assessed by the constancy of spectral ratios for different wavelengths along the peak and the reproducibility of spectra in different experiments. The accuracy is mainly affected by two factors: (a) the more or less correct subtraction of the baseline (which in turn depends on the system's chemical noise), and (b) the signal-to-noise ratio for a given peak ("peak height"). In the gel chromatography of E. coli RNA polymerase the integral signal to baselina noise ratio for the region of the chromatogram analysed is about 60 db. In a typical case (about 20 cycles to the region analysed) with such a signal-to--noise ratio the error associated with noise can be expected to come to 0.5 %. In some cases we reached this minimum, though in other cases the error was two or three times larger, which we think was due to the nonlinear character of the baseline. This assessment of accuracy is confirmed by the spectral reproducibility situation. The RNA polymerase spectra obtained in different experiments coincide to within 3 %, which should be regarded as the accuracy of the spectral measurement.

Before we examine the accuracy of spectral decomposition, we should make one important remark. Generally speaking, it is not correct to try and decompose the spectrum of a substance into its constituent spectra. Any spectrum, even one that has nothing to do with our components, can be in some optimal way decomposed into constituents. Therefore before one starts the decomposition of a substance spectrum, one must make sure that in this particular case the constituent spectra are going to be those of the actual components of the mixture.

The measure of the decomposition is the resultant error, which occurs after the spectrum is broken down into constituents. This error must be of the same order as or less than the spectral measurement error, which is equal to the spectra reproducibility error.

The spectral decomposition error was experimentally determined in the following way. The order of magnitude of the maximum admissible error of decomposition can be obtained if the spectrum of the original substance (RNA polymerase) is "decomposed" into one spectrum, namely that of the same substance obtained in another experiment. In this way the maximum error of decomposition was estimated at 4.5 %.

If a similar decomposition is performed for one of the spectra in which binding has been detected, then the "pure" polymerase content in the complex proves to be 93.8 %, with a decomposition error of 8.5 %, which exceeds the maximum admissible error. Thus the assumption that this peak contains only RNA polymerase does not work. If the DNA spectrum is added into the decomposition procedure, the polymerase contribution to the signal is reduced to 91 %, DNA accounts for 8.3 % of the total signal, and the decomposition error decreases to 3.4 %, i.e., to the admissible level.

It is assumed that the spectra are not affected by the interaction of the substances. For the case of RNA polymerase: oligoDNA interaction this assumption seems more valid for RNA polymerase than for the oligonucleotide. Supposedly, in this case changes in the oligonucleotide spectrum mainly occur because of the changed stacking interaction between bases. The changed interaction may alter the intensity of the absorption band (the hyper- or hypochrome effect) but should not affect the position of the nucleotide absorption band. It is the position of the absorption band that matters in spectral decomposition, while the hyper- or hypochrome effect should not have any crucial effect on the accuracy of decomposition, even though it might affect the estimated molar ratio. An error in the estimated DNA/enzyme molar ratio may alter the apparent maximum amount of bound enzyme, but not the binding constant.

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CHAMBER FOR PLANAR SUPERCRITICAL FLUID CHROMATOGRAPHY

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Introduction

Supercritical fluid chromatography (SFC) has been widely investigated and applied for the separation of various compounds (1-6). The basic procedure is to generate and keep the supercritical stage of the mobile phase at a definite temperature and over a certain pressure. The evolution of the method rapidly increased when capillary supercritical fluid chromatography (7) and new detection possibilities (8) had been introduced. A recent publication (9) also suggested the adaptation of the widely used HPLC equipment to SFC.

The employment of planar arrangement of the stationary phase for SFC has been surprisingly neglected. At the same time, there is a method called overpressured (forced-flow) thin-layer chromatography (OPLC) which has made thin-layer chromatography to be flexible even in the respects of the chromatographic conditions as pressure, temperature and detection. The method was invented about ten years ago, and the first experiments resulted in developments with circular front of the mobile phase (10). Further progress has made possible the generation of linear solvent front (11), optimization of the mobile phase flow velocity (12), the employment of multiple step gradient (13), working with reversed-phase HPTLC plates (14), possibilities for two-dimensional OPLC (15) (with the employement of diode-array detectors to monitor the effluent after the second dimensional development through the whole side of the system) and preparative-scale OPLC (16). Intensive investigations on forced-flow thin-layer chromatography resulted in high-pressure planar liquid chromatography (17) also showing the pressure limits of the method (18).

These findings have also given the basis for the construction of a planar SFC system and thereby to simultaneuously utilize the beneficial characteristics of both thin-layer and supercritical fluid chromatography at the same time. The construction of the set-up requires only relatively small modifications of the forced-flow thin-layer chromatography system.

Aims

The principal aim of the new system is to keep the features of TLC such as:

1. simultaneous separation of several samples or to perform two-dimensional separations;
2. to arrange subsequent detections after derivatization as direct visual observation in day-light, under UV light as well as both of them;
3. to keep the possibility of easy detection by post-chromatography derivatization;
4. to have the choice of "dried-on-the-sorbent" sampling;
5. to have the choice of "dry column" chromatography and thereby utilizing its spot concentrating effect.

An additional aim is to have the features of both column liquid and SFC chromatography such as:

1. to inject dissolved samples into the stream of the mobile phase;
2. to detect the separated components using a flow-through cell;
3. to have a wide choice of the flow velocity by using pump or pumps.

Thereby regulation and furthermore the optimization of the mobile phase flow velocity are possible.

Equipment for supercritical planar chromatography.

The set-up of forced-flow TLC has been modified taking three major points of view into considerations:

1. The cushion-like covering of the stationary phase was substituted by a plastic layer, which is able to resist to the pressure ranging from several ten to several hundred atmospheres. The sealing of the mobile phase inlet is also accomplished by using this layer.

2. The pressure drop is not finished at atmospheric pressure but the outlet of the system is kept under a definite backpressure.

3. The whole system is thermostated at an adequate temperature.

The cross-section of the set-up for supercritical planar chromatography is given in Fig. 1.

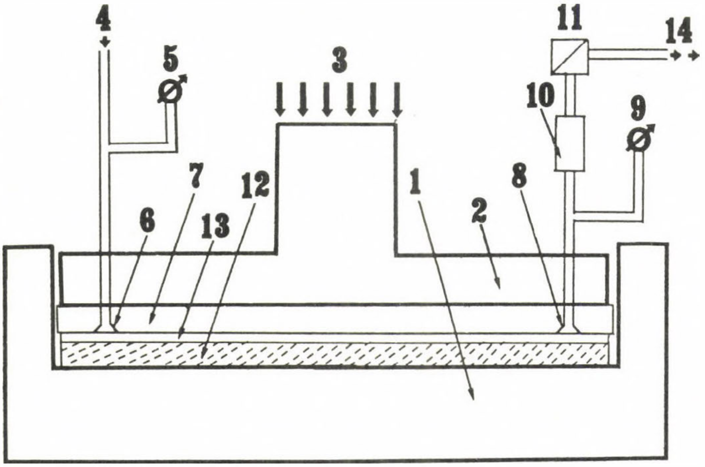

Figure 1.
Cross section of the chamber for planar SFC.
The sorbent (13) on the support layer (12) are between the two main parts of the set-up, namely the bottom support block (1) and the upper support block (2). The tubing is connected to the pump(s) (4) and the mobile phase pressure can be measured by the (inlet) pressure gauge (5). The mobile phase inlet (6) and outlet (8) are tightened to the stationary phase by the covering plastic layer (7). This layer is pressed onto the stationary phase by the help of mechanical pressure (3). The mobile phase outlet (8) is connected to the (outlet) pressure gauge (9) and the tubing goes through the detector (10) and the diaphragm valve (11) (adjusted to the adequate back-pressure) to the effluent collector (14). The bottom support block (1) and the upper support block (2) are thermostated while all other parts are cooled.

Changes in the mobile phase composition can be generated by using either gradient elution chromatography or displacement SFC on the planar arrangement of the stationary phase.

Planar SFC

The set-up for planar supercritical-fluid chromatography, i.e. for SFC with planar arrangement of the stationary phase gives a wide variety of application possibilities, such as development using

1. SFC with circular front of the mobile phase;
2. SFC with linear front of the mobile phase;
3. SFC with multistep gradient of the mobile phase;
4. SFC with the system thermostated at a definite; temperature;
5. preparative scale planar supercritical fluid chromatography with either off-line or on-line detection.

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STUDIES ON THE METABOLISM OF (-)DEPRENYL BY GAS CHROMATOGRAPHY MASS SPECTROMETRY

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Summary

The metabolism of (-)deprenyl was studied. The oxidative dealkylation of the substance resulted in propalgylanara, methamphetamine and amphetamine. The metabolites and the unaltered parent compound, present in the urine of rats pretreated with (-)deprenyl were identified by gas chromatography/mass spectrometry. Data obtained support our earlier findings and also contribute new knowledge to the metabolism of (-)deprenyl and some of its structural analogues.

Introduction

Johnston (1) found in 1968 that clorgyline inhibited the oxidative catabolism of 5-hydroxytryptamine (5-HT), which is catalysed by monoamine oxidase (MAO) enzyme, while it did not influence the oxidation of benzylamine (BA) and phenylethylamine (PEA). On the basis of these findings, two types of MAO enzymes were postulated, having specific substrates and inhibitors; MAO-A, which oxidizes 5-HT and noradrenaline (NA); and MAO-B, with high preference toward BA and PEA. However, some compounds are substrates for both types of enzymes, e.g. tyramine.

While clorgyline proved to be a specific inhibitor of MAO-A enzyme, Knoll et al.(2-5) discovered that the levorotatory form of a new MAO-B inhibitor (E-250) has a peculiar spectrum of activity, inhibiting BA and PEA oxidation by MAO-B enzyme.

E-250 later appeared as (-)deprenyl on the market under trade names JUMEX®, or ELDEPRYL®. Its potency for MAO inhibition was similar to that of the well-known MAO inhibitors, such as pargyline or tranylcypromine (5). At the same time, (-)deprenyl is a selective inhibitor of MAO-B enzyme.

The favourable pharmacological, clinico-pharmacological and clinical effects of (-)deprenyl stimulated interest in its mode of action, binding and metabolism. It was stated, that, in spite of repeated administration of (-)deprenyl, there is a selective inhibition pattern in the brain (6) of human patients and in the liver (7) of rats. Repeated administration of (-)deprenyl for three weeks both greatly increases the release of DA (dopamine) from the striatum and decreases the transformation of DA to 3,4-dihydroxyphenylacetic acid (DOPAC) (8). This explains the favourable therapeutic action of (-)deprenyl, as an adjuvant to the usual therapy of Parkinson's disease (e.g. levodopa) (9-10).

Reynolds et al.(11) have pointed out the presence of amphetamine and methamphetamine in the urine of human patients treated with (-)deprenyl, when urine samples were collected from male volunteers who were free of any other medication during the administration of (-)deprenyl.

These results were supported by some recent data. Thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) (12-13), as well as TLC and spacer-displacement thin-layer chromatography (SD-TLC) (14-15) were used in investigating the metabolism of (-)deprenyl in rats. Since that time several radioactive zones remained unidentified, the study of (-)deprenyl metabolism was supplemented by the application of gas chromatography/mass spectrometry (GC/MS).

Materials and methods

All chemicals were purchased from commercial sources. The standards, (-)deprenyl = N-methyl-N-propargyl-(2-phenyl-1-methyl)ethylammonium chloride; TZ-650 = N-methyl-N-propargyl-(2-phenyl)ethylammonium chloride and J-508 = N-methyl-N-propargyl-(-indanyl)ammonium chloride were kindly donated by Chinoin Pharmaceutical Company (Budapest, Hungary).

The gas chromatograph/mass spectrometer combination was a GC-MS HP-5985B instrument (Hewlett-Packard, Plao Alto, CA, USA), connected to the HP-2648A data analyser. The separation was achieved by temperature programming from 100 to 320 ^{0}C at 5 ^{0}C/min. Glass capillary columns 25 meter in length and 0.2 mm in ID were used. The stationary phase was methylsilicone and the flow rate of the helium carrier gas was 1 mL/min. The gas chromatograms and the mass spectra were recorded with a thermal printer.

The metabolic study was made by the administration of (-)deprenyl to rats. Wistar rats of both sexes, weighing 120 - 150 g, were used, solutions of (-)deprenyl, TZ-650 and J-508 were injected subcutaneously (s.c.) in the doses of 50 mg/kg. The urine was collected for 24 hours, and was immediately extracted or kept at -40 ^{0}C until analysis. The urine was adjusted to pH 11 with 0.5 mol/L sodium hydroxide, the basic metabolites were extracted with (3:1) mixture of chloroform and ethyl acetate. The organic layer was reextracted into buffer at pH = 1.5, and the extraction with chloroform and ethyl acetate mixture was repeated after the pH was adjusted to 11.

Results

Fig. 1 shows the gas chromatogram of basic compounds extracted from the urine of rats, pretreated with 50 mg/kg (-)deprenyl s.c. The peak with a retention time of 6.28 min is due to contamination from the organic solvents, used for extraction.

Fig. 2 shows the mass spectra of basic metabolites of (-)deprenyl. The peak with a retention time of 5.62 min is the unaltered deprenyl. In its mass spectrum (Fig. 2a) the molecular ion (187) is not present only its fragments, 91 and 96. Fig. 2b represents the mass spectrum of propargyl-anara, the product of N-demethylation of (-)deprenyl. It is discernible by the loss of 14 mass units from the original 96 mass fragments, yielding the main molecular ions 91 and 82. A remarkable peak at a retention time of 3.62 min is methamphetamine. It is due to the depropargylation of deprenyl, The mass spectrum of methamphetamine (Fig 2 c) shows the mass fragments 91 and 58, where the side chain (58) is the result of the loss of the benzyl group of N-methyl-(2-phenyl-1-methyl)ethylamine. The fourth main peak in the gas chromatogram is due to amphetamine (retention time 3.22 min); its mass spectrum is shown in Fig. 2d.

The extraction of rat urine also yielded some other compounds. At neutral or basic pH, two compounds can be extracted. Their mass spectra indicated them to be N-methyl-N-phenylisopropylglycine and N-phenylisopropylglycine , but their final identification will require the preparation of the standard substances and the comparison of their mass spectra.

Fig. 3 shows the gas chromatogram of (-)deprenyl metabolites, detected by the selected ion monitor. The mass fragment 91 is due to the benzyl group of the compounds. Therefore, any metabolite having the phenyl group (without substitution in the aromatic ring) and the neighbouring carbon will show this 91 mass fragment. The common occurrence of the 91 and 96, 82, 58, and 44 mass fragments indicates the presence of (unaltered) deprenyl, propargylanara, methamphetamine, and amphetamine, respectively.

Fig. 4 shows a similar pattern due to the metabolism of TZ-650, where the side chain differs from (-)deprenyl in the absence of 1-methyl substituent. The mass fragment of the benzyl group (91) is the same for the basic metabolites, but the fragments of the side chain should be smaller by 14 mass units. The main metabolites are characterized in Fig. 4 by the common occurrence of 91 and 82, 68, 44 and 30, due to TZ-650, N-propargylphenylethylamine, N-methylphenylethylamine, and phenylethylamine, respectively.

Fig. 5 shows the molecular ion of the metabolites of J-508. The whole, intact parent compound with a molecular weight of 185, its demethylated product (171), the depropargylated compound (147), and the bisdealkylated J-508 (133) can be detected by the selected ion monitor.

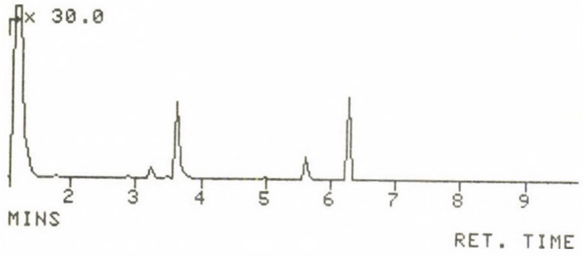

Fig. 1.

Gas chromatogram of (-)deprenyl metabolites extracted from urine of rats, s.c. treated with 50 mg/kg of (-)deprenyl

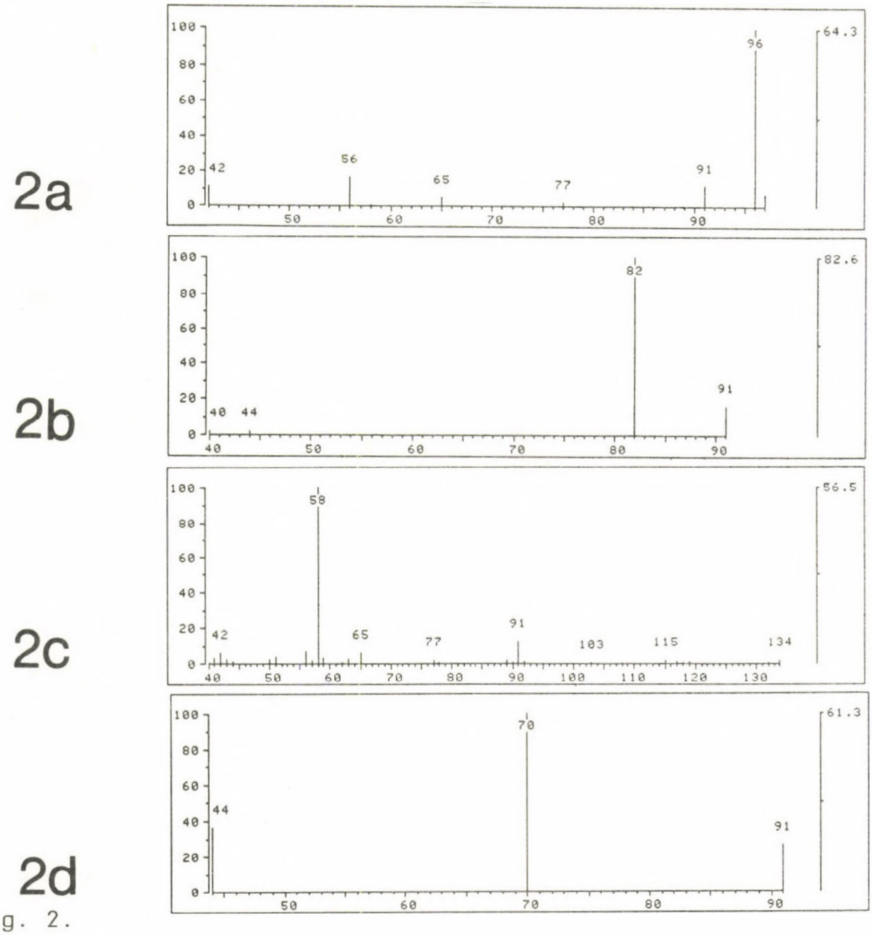

Fig. 2.

The mass spectra of (-)deprenyl and its metabolites. Figs 2a, 2b, 2c and 2d show mass spectra of (-)deprenyl, propargylanara, methamphetamine and amphetamine, obtained by GC/MS at retention times 5.62, 5.00, 3.62 and 3.22 respectively

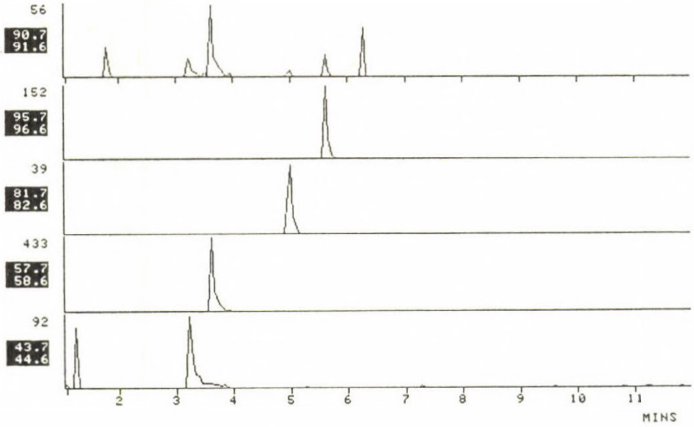

Fig. 3.

Gas chromatograms of (-)deprenyl metabolites, detected by a selected-ion monitor. The chromatogram at the top is due to the compounds containing the phenylalkyl group; the others were detected on the basis of ions with mass number of 96, 82, 58, and 44, characteristic of (-)deprenyl, propargylanara, methamphetamine, and amphetamine, respectively

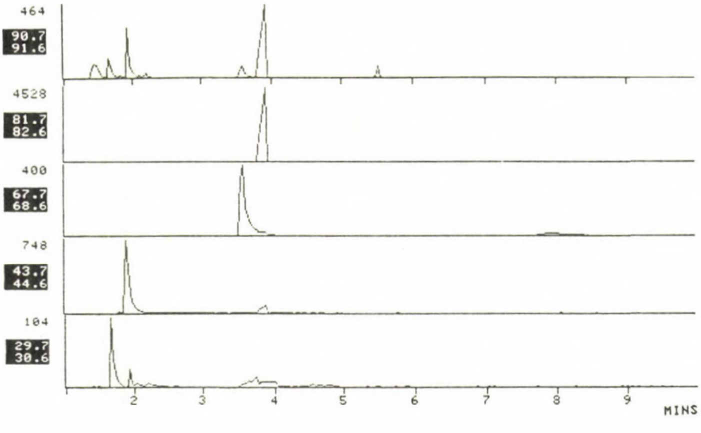

Fig. 4.

Gas chromatograms of TZ-650 metabolites, detected by a selected-ion monitor

Discussion

Our study on metabolism of (-)deprenyl has been restricted to the identification of basic metabolites by GC and TLC. Previous publications (11-13) have clearly shown that depropargylation as well as depropargylation and demethylation of (-)deprenyl yield methamphetamine and amphetamine, respectively. Some other investigators, using TLC method have suggested, that demethylation of (-)deprenyl may yield propargylanara. However, the lack of radiolabelled propargylanara standard precluded the reliable identification of propargylanara. The HPLC investigations of (-)deprenyl metabolism have never given definitive results either. SD-TLC data strongly support the contention of Magyar and Tóthfalusi (14) that one of the metabolites of (-)deprenyl is propargylanara, but final identification required GC/MS.

Fragments of the original molecule can be identified by their mass numbers: the loss of a methyl group results in a 96 - 14 = 82 fragment, the loss of a propargyl group (96 - 38) yields a 58 fragment, and the loss of propargyl + methyl (96 - 52) gives a 44 fragment. Finding of fragments with mass numbers of 82, 58 and 44 supports the notion of the existence of the corresponding metabolites (Fig. 3).

Similar scanning was carried out in the case of TZ-650, where the side chain is different from that of (-)deprenyl. Due to the absence of one methyl group, the benzyl group of TZ-650 produced the fragment from the phenylalkyl compound (91), while the tertiary amine fragment (N-methyl-N-methyl-N-propargyl) was represented by a fragment of 82 mass units. Certain metabolites, N-propargylphenylethylamine, N-methylphenylethylamine and phenylethylamine, i.e. the demethylated, depropargylated, and demethylated + depropargylated products, were characterized by the mass numbers of 68, 44 and 30, respectively.

When a compound is less fragmented than (-)deprenyl, its molecular ion can be detected (Fig. 5). This can be observed in the case of J-508, where the spectrum greatly facilitates the elucidation of the metabolism of J-508.

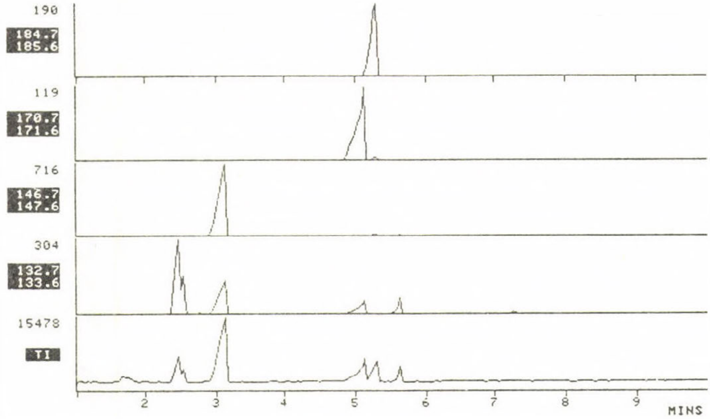

Fig. 5.

Metabolism of J-508. The molecular ion (185) with a loss of 14, 38 and 52 mass units indicate the parent compound, its demethylated, depropargylated and bisdealkylated metabolites

Another type of metabolic reaction is the oxidation of the triple bond of the propargyl group. This reaction may occur either alone or in combination with demethylation, resulting in different metabolites. The presence of these products has been detected, but standard substances for their final identification are still unavailable.

Very important problem is whether the oxidation of the aromatic ring (in para position) takes place. A recent publication suggested this type of metabolic alteration, Yoshida et al. (16) detected the presence of p-hydroxy-amphetamine and p-hydroxy-metamphetamine. The key-point of this result was the reaction of the basic metabolites with heptafluorobutyric acid anhydride (16). Our investigations confirmed the p-hydroxylation during the deprenyl metabolism, however, we detected substantial amount of p-hydroxy-propargylanara and a trace amount of p-hydroxy-methamphetamine only (17) when the basic metabolites were trimethylsilylated.

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INVESTIGATIONS ON THE STABILITY OF FENOTEROL HYDROBROMIDE IN INJECTION SOLUTIONS AND TABLETS BY HPLC METHOD

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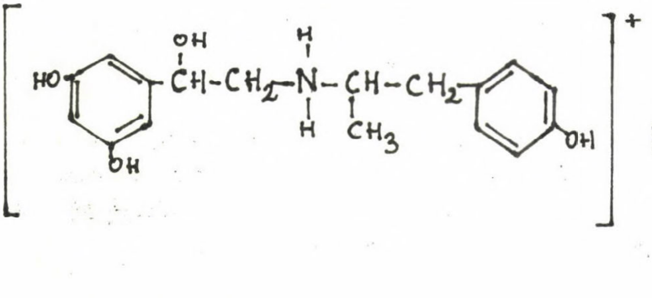
SUMMARY

The influence of the temperature, pH and the stabilizer used on the stability of drug forms of fenoterol hydrobromide (Hydroxyphenylorciprenaline hydrobromide) was investigated. The amount of the active substances was controlled in the presence of degradation products. LiChrosorb RP-8 (parcticle size 10 µm) and a mixture of methanol + 0.1 M NaH_2PO_4 (35 + 65 by vol.) were used as the stationary and mobile phases, respectively. The detection was carried out using a UV detector at 280 nm.

The external calibration method was used for quantitative measurements.

INTRODUCTION

Fenoterol hydrobromide (I), a derivative of orciprenaline (hydroxyphenylorfiprenaline)

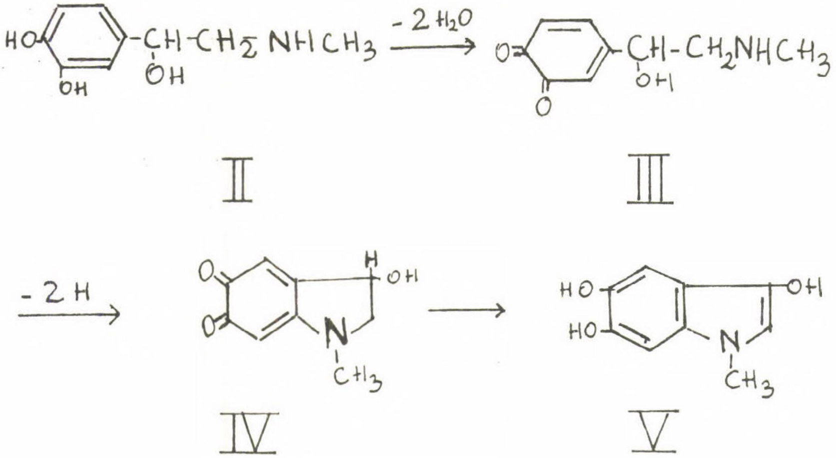


I

shows strong spasmolytical activity for the smooth muscles and decreases tension of the muscles of the uterus. It is used for the treatment of bronchial asthma as well as in gynaecology and obstetrics.

Although fenoterol hydrobromide is more stable than other substances of similar structure (II - V), its aqueous solutions used in pharmacy contain stabilizers.

The derivatives of phenylalcoholamines (e.g., adrenaline) are oxidized in a basic medium by atmospheric oxygen according to the following reactions:



The intermediate oxidation products of adrenaline (II) such as chinon (III), adrenochron (IV) and adrenolutyne (V) are toxic. This is the reason why it is important to prepare stable pharmaceutical forms of feneterol.

This paper reports on our investigations on the stability of feneterol hydrobromide in aqueous injection solutions and tablets. The injection solutions contained 0.5 mg of feneterol HBr in 10 ml of water; their isotonic effect was achieved using sodium chloride. Each tablet contained 5 mg of the active substance. We have investigated the influence of temperature, pH, atmospheric oxygen and the stabilizers used (sodium pyrosulfite, disodium versenate and nitrogen) on stability.

EXPERIMENTALS

In the preliminary investigations UV spectrophotometry was used for the determination of the stability of injection solutions and tablets.

Changes of colour, clarity and the pH of the solutions were also controlled.However, these methods appeared to be nonselective and thus, useless for the determination of feneterol HBr in the presence of the degradation products. Therefore, we have decided to check the usefulness of HPLC for this purpose. Until now the determination of feneterol HBr in pharmaceutical forms by HPLC has been not described in the literature. On the basis of some preliminary trials a satisfactory separation of feneterol HBr from its degradation products was obtained.

Our investigations were carried out using an LKB modular liquid chromatograph consisting of a Model 250 pump, a Model 2151 pump and a Model 2220 integrator. The following chromatographic conditions were used:

column	25 cm x 4 mm i.d., stainless steel, packed with LiChrosorb RP-18 (10 μm particles)
mobile phase	35:65 methanol-0.1 M aqueous $NaHPO_4$
flow rate	1 ml/min

100 μl of the injection solution or 100 μl of the extract of tablets was analyzed. The extract was prepared by extracting: a tablet containing 5 mg of feneterol HBr with 100 μl of water for 1 h, the mixture was then centrifuged. The determination of feneterol HBr was carried out by the external standard method at 280 nm. It should be noted that feneterol HBr has two UV absorption maxima, at 240 and 280 nm. The maximum at 280 nm appeared to be more convenient for the determination of feneterol HBr since one of the degradation products (containing probably -CO group) also absorbs at 240 nm.

However, the quantitative determination of the degradation products of feneterol should be carried out at 240 nm.

Table I gives the precision of the measurements. Figs 1-2 show the chromatograms obtained from injection solutions and extracts of tablets obtained under various conditions.

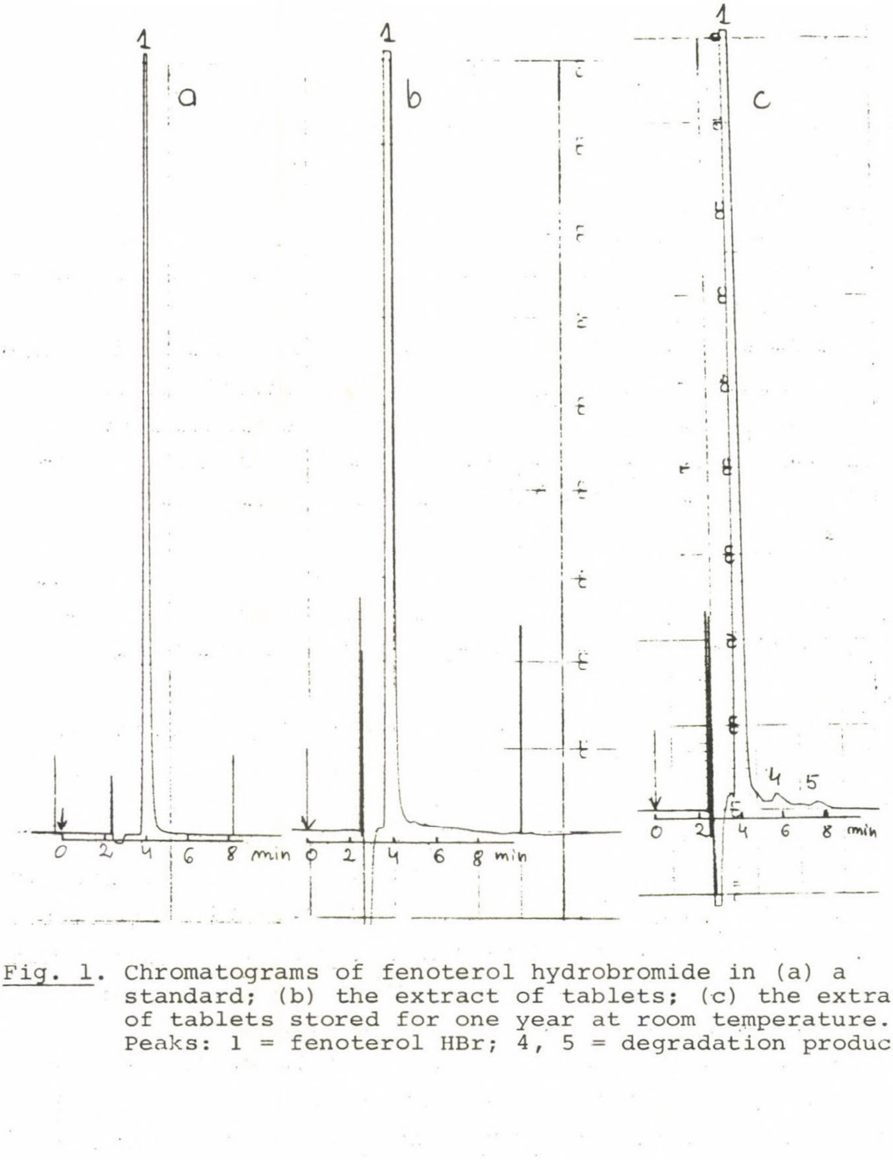


Fig. 1. Chromatograms of fenoterol hydrobromide in (a) a standard; (b) the extract of tablets; (c) the extract of tablets stored for one year at room temperature. Peaks: 1 = fenoterol HBr; 4, 5 = degradation products

Fig. 2. Chromatograms of fenoterol hydrobromide in injection solutions stored for one year at (a) room temperature; (b) 37°C; (c) 50°C. Peaks: 1 = fenoterol hydrobromide, 2-7 = degradation products →

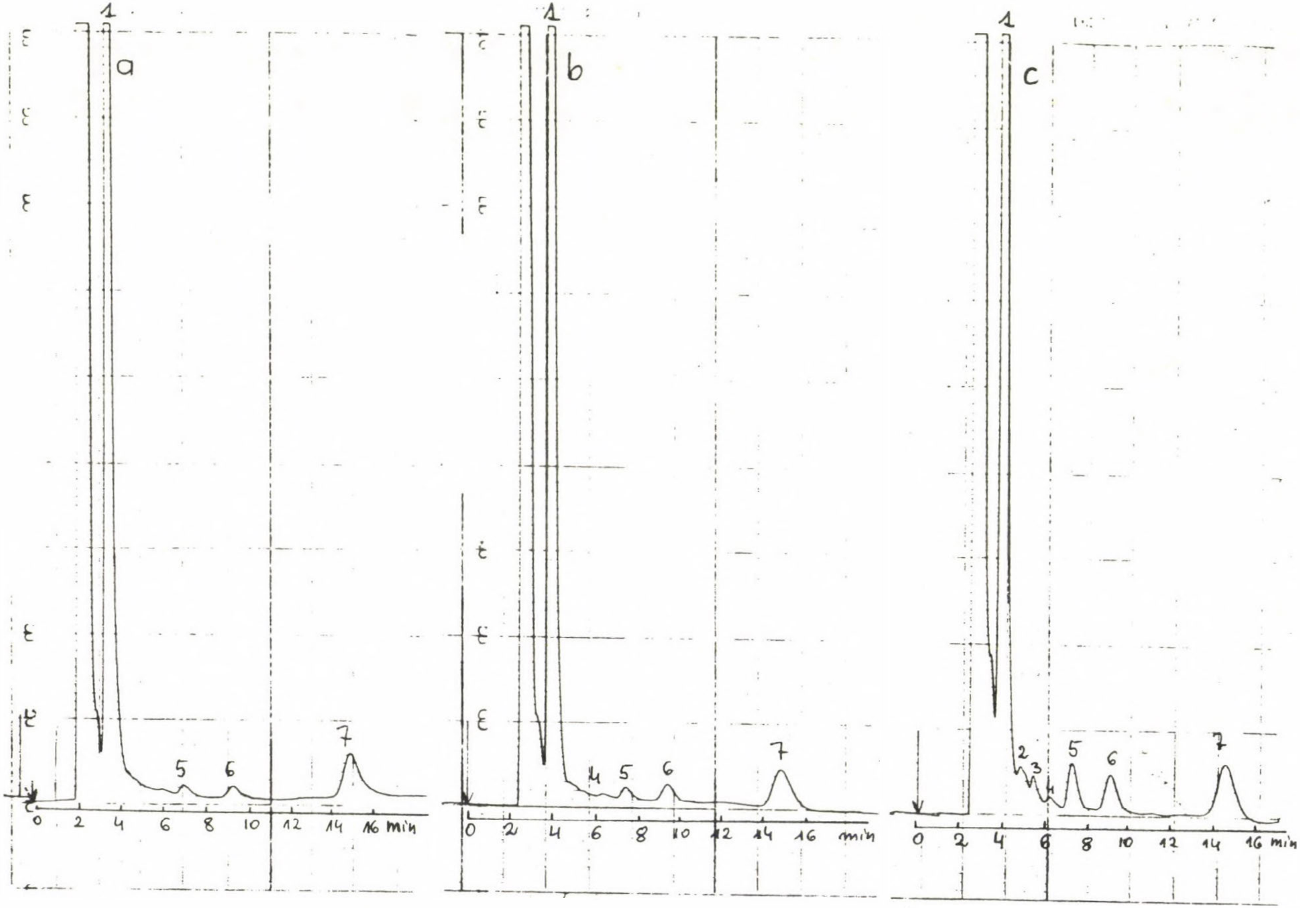

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Table I. The precision of the determinations

Concentration of feneterol HBr	0.495-0.505 mg/ml
Number of measurements	10
Mean concentration	0.499 mg/ml
Standard deviation	3.525×10^{-3}
Relative standard deviation	0.71%

DETERMINATION OF STABILIZERS

Disodium versenate was determined by colorimetry. A complex of bizmuth with thiourea was utilized as the colour reagent. This complex becomes colourless when disodium versenate is added.

Sodium pyrosulfite was determinated by polarography.

The colour, clarity and pH of the injection solutions were also controlled.

On the basis of preliminary tests it has been established that the degradation of the solutions is faster in basic and neutral media: in consequence of the degradation changes are observed in the colour and clarity of the solution and in the UV spectrum. The changes are especially visible in solutions stored at higher temperatures (37° and 50°C) but they are only small in acidic medium, at a pH of about 3.5.

The changes in the injection solutions were slower in ampoules filled with nitrogen.

Because the stability of the injection solutions was not satisfactory, sodium pyrosulfite in the amount of 0.01% was added as an additional antioxidant in addition to disodium versenate which is present in a concentration of 0.05%.

Table II summarizes the results of the investigation of acidic injection solutions containing sodium pyrosulfite and disodium versenate in ampoules filled with nitrogen.

Table II. Stability of the injection solutions containing fenoterol hydrobromide

Serial no. of the sample	Time of storage, month	Temperature °C	pH	Assay of fenoterol hydrobromide by HPLC mg/ml	Colour	Clarity	Assay of	
							sodium pyrosulfite mg/ml	disodium versenate mg/ml
140,285	0	20	3.55	0.050	Colourless	Clear	0.11	0.50
	3	20	3.55	0.049			-	-
	6	20	3.60	0.047			-	-
	9	20	3.65	0.047			0.10	0.50
	3	37	3.60	0.049			-	-
	6	37	3.65	0.047			-	-
	9	37	3.70	0.045			0.09	0.50
	3	50	3.65	0.048			-	-
	6	50	3.65	0.045			-	-
	9	50	3.70	0.043			0.06	0.50

INVESTIGATIONS OF THE STABILITY OF TABLETS

Tablets containing 5 mg of feneterol HBr were stored at room temperature, 37° and 50°C for 12 months. Determination of the active substance was carried out by HPLC. Simultaneously the appearance and colour of the tablets were also controlled (Table III summarizes the results).

CONCLUSIONS

Our investigations have shown the usefulness of HPLC for the analysis of pharmaceutical forms of fenoterol hydrobromide. HPLC permits the determination of fenoterol hydrobromide in its pharmaceutical forms, in the presence of degradation products.

When evaluating the results of the investigations it is obvious that the oxidation process has the most important

Table III. Stability of tablets containing fenoterol hydrobromide

Serial no. of sample	Time of storage month	Temperature °C	Assay of fenoterol hydrobromide by HPLC mg/tablet	Appearance	% part of degradation products
150,585	0	room	5.02	white	-
	6	room	4.95	cream-coloured	-
	12	room	4.70	pale yellow	1
	6	37	4.85	cream-coloured	-
	12	37	4.64	pale yellow	1
	6	45	4.84	cream-coloured	-
	12	45	4.54	pale yellow	1
270,585	0	room	4.90	white	-
	6	room	4.93	cream-coloured	-
	12	room	4.88	pale yellow	1
	6	37	4.79	cream-coloured	-
	12	37	4.80	pale yellow	1
	6	45	4.70	cream-coloured	-
	12	45	4.69	pale yellow	1

influence on the stability of injection solutions. This process is quicker at higher temperature. It was found that the presence of disodium versenate has no essential influence on oxidation.

The addition of 0.01% sodium pyrosulfite to the solutions reduces the rate of the oxidation process, however, it does not prevent completely the oxidation of fenoterol hydrobromide.

The tablets of fenoterol hydrobromide are more stable. In tablets stored for one year at room temperature the decrease in the amount of active substance was less than 1%.

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HPLC MONITORING OF THE METABOLIC CHANGES INDUCED BY ANTITUMOR AGENTS IN COMBINED CHEMOTHERAPY

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INTRODUCTION

Current interest in the application of liquid chromatographic (HPLC) methods to chemotherapeutic studies is primarily focussed on the determination of metabolite concentrations in different tissues (cells) and/or biological fluids (1, 2, 3). Considering the role and significance of biological response modifiers, such as polyamines, and the metabolism of nucleic acids in the malignant cell proliferation, the investigation of changes induced by some antitumour agents is of particular importance.

DL-α-difluoromethylornithine (DFMO), an enzyme-activated, irreversible inhibitor of ornithine decarboxylase (ODC, EC 4.1.1.17) has been shown to be a potent cytostatic by depletion of cellular polyamines in several types of cancer and in different experimental tumour systems, both in vivo and in vitro (4-9). In spite of some promising results concerning the synergic (potentiating) effect of DFMO in combination with nitrosoureas (10, 11, 12), alkylating and intercalating agents (13, 14) and interferon (15, 16, 17), there are only few observations regarding the metabolic interference with cyclophosphamide (18). Our preliminary investigations revealed a favourable therapeutic influence of the combined DFMO/cyclophosphamide treatment on P388 leukaemia-bearing mice (19). In the present work the applicability of some high-performance (reversed-phase and ion-exchange) liquid chromatographic methods is demonstrated for the monitoring of metabolic changes induced by single or combined drug treatments in the biosynthesis of polyamines and nucleotides.

MATERIALS AND METHODS

The purity of solvents purchased from commercial sources (LiChrosolv methanol and acetonitrile, Merck, Darmstadt, FRG) corresponded to HPLC standards. Ammonium phosphate buffers were prepared from Merck Suprapur reagents and dissolved in water distilled twice in glass. Reference compounds (polyamines, nucleotides) were from Serva (Heidelberg, FRG) and Reanal (Budapest). Acetyl putrescine (Ac-Pu) and spermidines (N^8- and N^1-Ac-Spd) were prepared according to the method of Tabor et al. (20). DL-α-difluoromethylornithine (DFMO, RMI 71.782) was kindly donated by the Centre de Recherche Merrell International (Strasbourg, France).

Tissue (cell) and urine polyamines were extracted and dansylated as described earlier (21, 22). Preparation of tumour cell nucleotides (adenosine-, cytidine-, uridine- and guanosine-5' mono-, di- and triphosphates, inositol-5' monophosphate; abbreviated as AMP, ADP, ATP, CMP, CDP, CTP, UMP, UDP, UTP, GMP, GDP, GTP and IMP) for HPLC analysis was carried out as reported previously (23).

In vivo Experiments

In an attempt to study the metabolic changes induced *in vivo* by DL-α-difluoromethylornithine (DFMO) and cyclophosphamide, P377 leukaemia cells ($5 \cdot 10^6$) were transplanted intraperitoneally into BDF_1 inbred male mice. Tumour-bearing animals (7-10 per group) were kept in metabolic cages (Techniplast, Buguggiate, Italy) and 24-hour urine samples were collected. Pelleted food (LATI, Gödöllő, Hungary) and water were given *ad libitum*. Experimental conditions and drug treatment schedules are summarized in Table I. DFMO was administered continuously to the tumorous mice in 2 % solution as sole drinking fluid. A single dose of 70 mg/kg cyclophosphamide (VEB Jenapharm, Rudolfstadt, GDR) was given intraperitoneally on the first day of tumour transplantation with or without DFMO treatment.

Table I. Effect of DL-α-difluoromethylornithine (DFMO) and cyclophosphamide treatments on the life span of P388 leukaemia-bearing mice

Groups	(n)	Treatment schedules and doses	Average life time, days ± SD	Increased life span ILS %
P388 CONTROL	25	-	9.8 ± 0.8	-
DFMO*	22	2 % DFMO in drinking water, continuously	13.5 ± 2.1	38
CYCLOPHOSPHAMIDE	20	cyclophosphamide 1 x 70 mg/kg, i.p. on the 1st day	29.7 ± 5.4	197
DFMO + CYCLOPHOSPHAMIDE	30	in combination as above	42.6 ± 15.4 with a total recovery of 20-30 % of animals	326

*70-80 mg DFMO intake per animal per day

Groups of 3-5 animals were daily sacrificed following tumour transplantation, and tissue (tumour cell, liver) homogenates were prepared and analysed. Particular groups of 30-50 tumour-bearing and drug-treated mice were kept for the determination of survival times.

Reversed-phase HPLC of Dansylated (Acetyl) Polyamines

Separation of putrescine (Pu), spermidine (Spd), spermine (Spn), acetyl-putrescine (Ac-Pu), N^8- and N^1-acetyl spermidines (N^8-, N^1-Ac-Spd) was performed in a Hewlett-Packard 1084B liquid chromatograph equipped with a 79850LC terminal and a Shimadzu RF-530 fluorescence detector (excitation: 340 nm, emission: 515 nm). Dansylated polyamines were chromatographed on a Hypersil-ODS column (250 x 4.6 mm, 5 μm, Shandon, Runcorn, U.K.) using the eluents A: 10 mM ammonium phosphate buffer (pH: 4.4) and B: acetonitrile:methanol (37:3, v/v), and a gradient program as indicated in Fig. 1. A mixture of dansylated polyamines and their acetyl derivatives containing

3.333 nmol of each per 10 µl was used for reference and quantitation. Urinary polyamine concentrations were expressed as nmol per mg of creatinine content.

Ion-exchange Liquid Chromatographic Separation of Nucleotide Phosphates on MonoQ HR 5/5 Column

A newly developed strong anion-exchanger column MonoQ HR 5/5 (50 x 5 mm, 10 µm, Pharmacia, Uppsala, Sweden) was applied for the separation of nucleotide phosphates of biological interest. Improvements in the shape of the concentration gradient of the ammonium phosphate buffer (pH: 7) necessary for the appropriate resolution of 13 nucleotides including inositol-5' monophosphate are shown in Fig. 5. The MonoQ HR 5/5 column was used in the Pharmacia FPLC System which consists of an LCC-500 gradient programmer controlling two P-500 reciprocating pumps, a MV-7 motor valve for the introduction of samples via 10-100 µl loops and a UV-M monitor for detection at 254 nm. Chromatograms and gradient shapes were simultaneously recorded either with a two-channel REC-482 recorder and/or printed out by the LCC-500 providing the separation diagrams (Figs 5 and 6), numerical values of retention times,peak areas or heights, programs, etc. Separations were quantitated using the individual calibration curves plotted by reference compounds as external standards.

RESULTS AND DISCUSSION

The growing knowledge of the role of natural polyamines in the growth and differentiation of normal and malignant cells has been built from research that relies on a wide range of liquid chromatographic techniques (1, 22). Recent results concerning the diagnostic value of polyamines have led to conclusion that identification and measuring of putrescine, spermidine, spermine and their acetyl derivatives (mainly Ac-Pu) originated from the urine of tissues of cancer patients, or of experimental animals may have importance either in the early recognition of malignant diseases or in the monitoring of different chemotherapeutic treatments (4, 7, 9).

In order to improve the separation and measuring of (acetyl)polyamines in biological samples we have modified a reversed-phase technique reported by Kabra et al. (24). Resolution of the dansylated Ac-Pu, N^8- and N^1-Ac-spermidines, putrescine, spermidine and spermine using a carefully selected gradient system of acetonitrile in ammonium phosphate buffer is illustrated in Fig. 1A. Increasing the selectivity of the separation a 2-3 % (v/v) methanol content in the mobile phase seemed to be necessary for the resolution of Ac-Pu and N^8/N^1-Ac-Spd isomers. Using this method elevated polyamine levels could be distinguished and measured in the urine of a cancer patient (Fig. 1B).

Similarly, the separation of dansyl polyamines using reversed-phase HPLC proved to be suitable for following the metabolic alterations induced by drug treatments. Metabolic studies concerning the natural polyamines were particularly advanced by exploring new cytostatic drugs such as DFMO (4, 9). Besides the well documented growth inhibitory effect of DFMO, by depleting the intracellular polyamines, little is known about its metabolic interactions in combination with other drugs. However, it seems most likely that polyamines, as biological response modifiers, and the metabolism of nucleic acids are also involved both in the mechanism of action of DFMO and of some alkylating and intercalating antitumour agents (6, 10, 13).

In our experiments some characteristic changes could be detected by HPLC in the polyamine metabolism of P388 leukaemia-bearing mice treated with DFMO and/or cyclophosphamide. Polyamine levels in P388 leukaemia cells during the tumour growth (A) and DFMO (B), cyclophosphamide (C) or combined treatments are compared in Fig. 2. It seems that DFMO alone caused a 50-70 % depletion of the Pu concentration on the fourth to seventh days of drug administration, while cyclophosphamide and DFMO/cyclophosphamide combination diminished the cellular polyamine contents from the beginning of the tumour growth. Leukaemia cells obtained from the surviving animals (days from 10 to 35) represented a polyamine distribu-

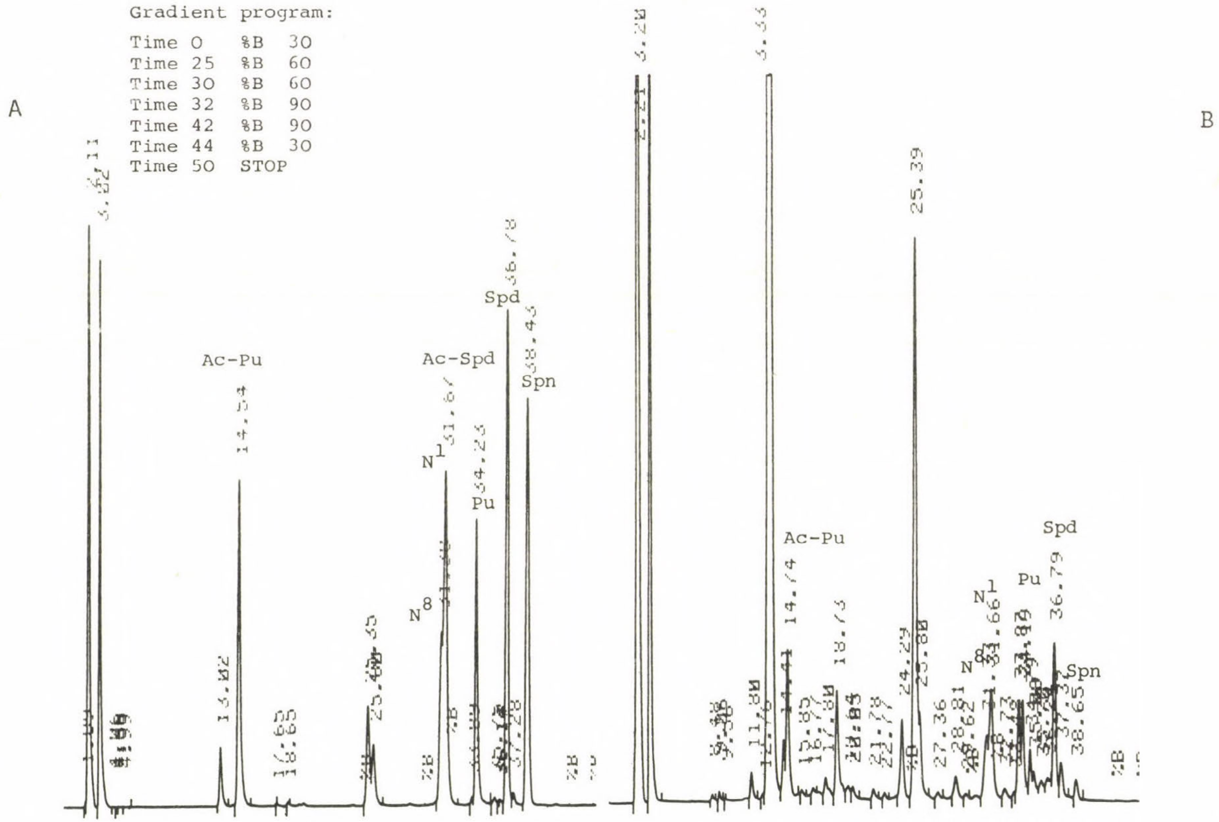


Fig. 1. (A) Reversed-phase HPLC separation of dansylated Ac-Pu, N^8- and N^1-Ac-Spd, Pu, Spd and Spn on a Hypersil-ODS column (0.46 x 25 cm, 5 µm, Shandon, U.K.) with ammonium phosphate (10 mM, pH: 4.4) - acetonitrile/methanol (37:3, v/v) gradient.
(B) Appearance of polyamines (Pu, Spd, Spn) and their acetyl derivatives (Ac-Pu, N^8- and N^1-Ac-Spd) in human urine. For abbrevations see Methods

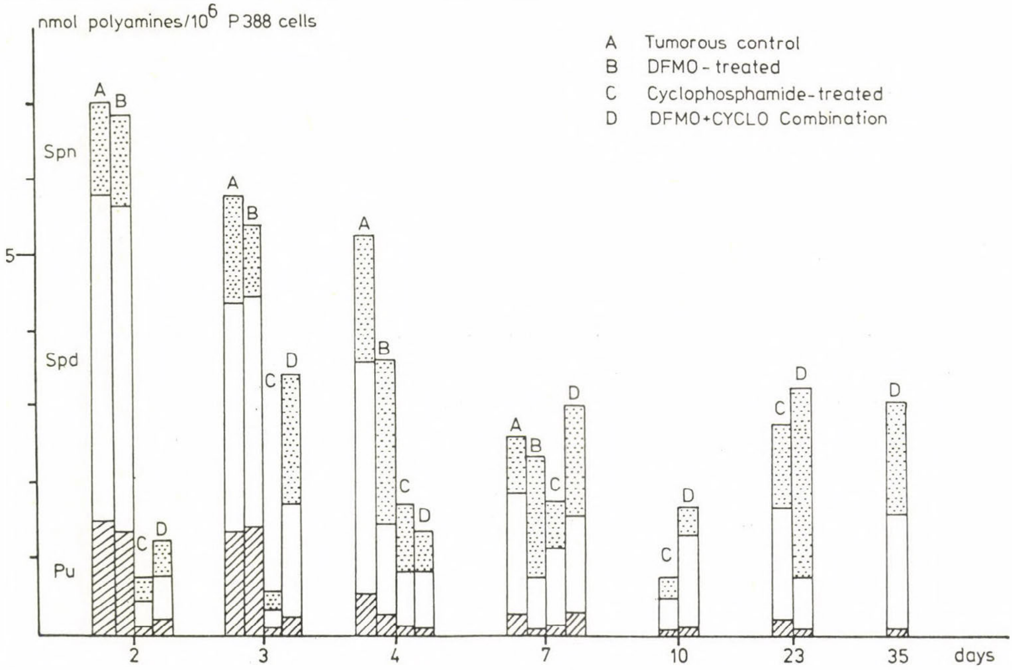


Fig. 2. Changes of polyamine concentrations in P388 leukaemia cells during the tumour growth and drug treatments

tion of low Pu and relatively high Spn content characteristic for a cell population in quiescent but viable state. Urinary excretion of polyamines in the same experiment is demonstrated in Fig. 3. It is to be noted that DFMO treatment significantly decreased the concentration of the polyamines, first of all the Pu concentration in the urine indicating obviously the inhibitory effect of DFMO on the biosynthesis of Pu. Relatively high level of Ac-Pu could be detected in the urine of tumour-bearing animals but it was only slightly affected by drug administrations. Particularly interesting changes could be observed in the urinary polyamine excretion of DFMO/cyclophosphamide-treated mice (Fig. 4). Combined therapy maintained the polyamine excretion at a level near to the normal, but some days later a tendency for separating into responsive and nonresponsive groups of animals occurred with the concomitant

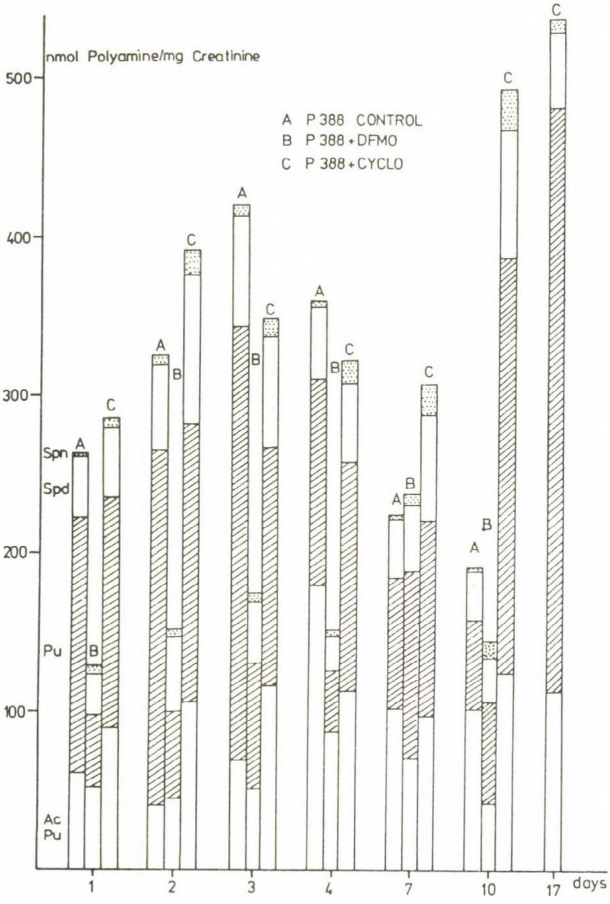


Fig. 3. Urinary excretion of polyamines in P388 leukaemia-bearing mice treated with DFMO and cyclophosphamide

alterations in their polyamine excretion. Putrescine and Ac-Pu concentrations were markedly lowered in the urine of surviving responders whose significant number (approx. 30 %, Table I) has recovered totally. On the contrary, nonresponders were going to die and their polyamine excretion elevated parallel with the growth of tumour. In general, it could be concluded that

(a) DFMO-induced depletion of cellular polyamines (Pu) potentiated the in vivo cytotoxicity of cyclophosphamide against P388 leukaemia cells;

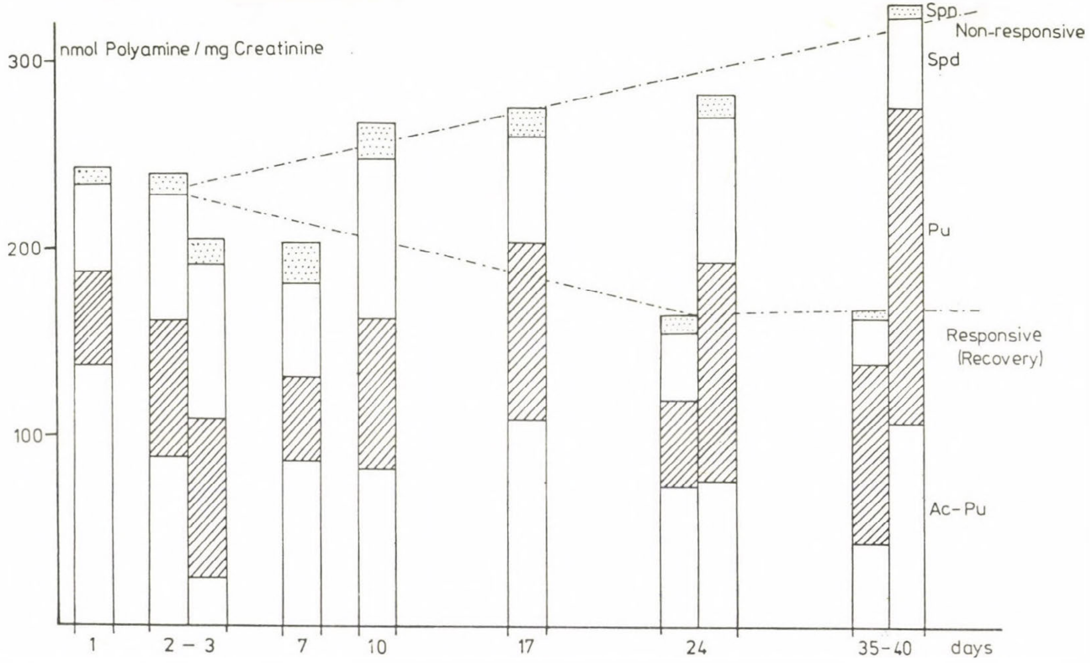
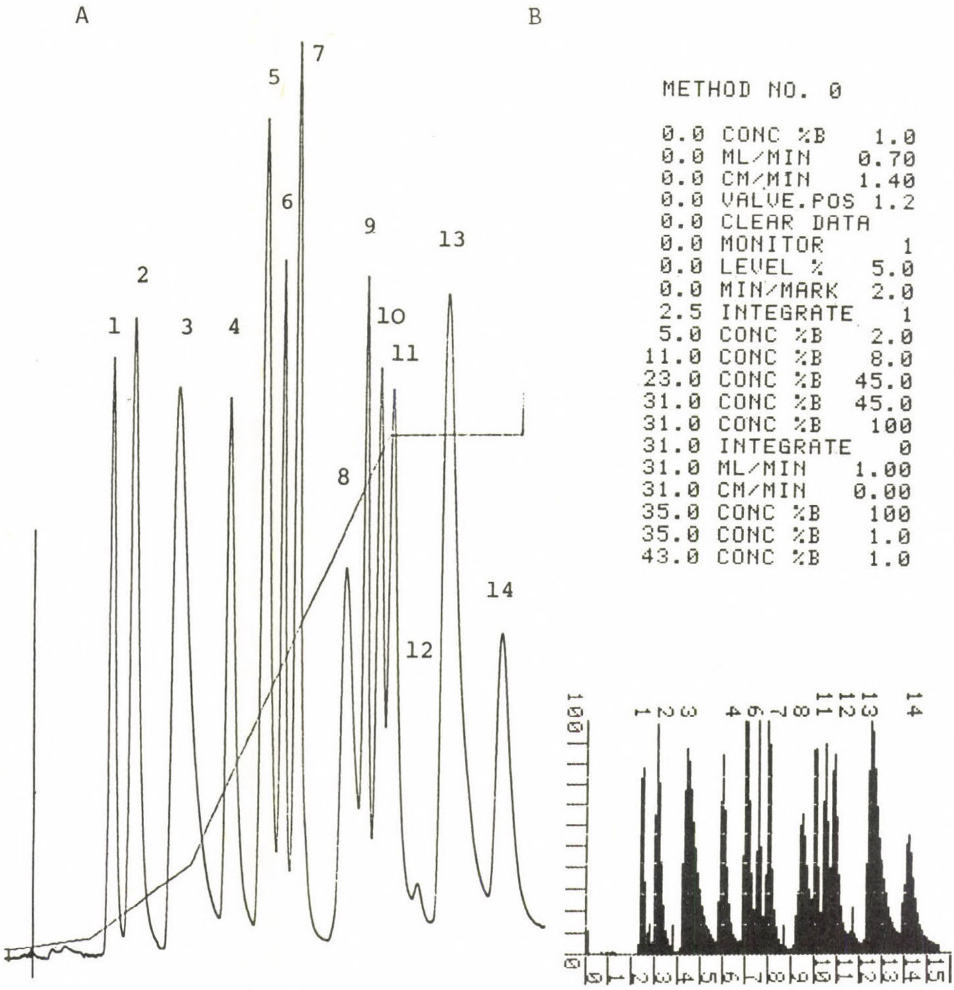


Fig. 4. Excretion of polyamines in the urine of responder and nonresponder P388 leukaemia-bearing mice treated with DFMO and cyclophosphamide in combination.



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METHOD NO. 0

 0.0 CONC %B      1.0
 0.0 ML/MIN      0.70
 0.0 CM/MIN      1.40
 0.0 VALVE.POS    1.2
 0.0 CLEAR DATA
 0.0 MONITOR        1
 0.0 LEVEL %      5.0
 0.0 MIN/MARK     2.0
 2.5 INTEGRATE      1
 5.0 CONC %B      2.0
11.0 CONC %B      8.0
23.0 CONC %B     45.0
31.0 CONC %B     45.0
31.0 CONC %B      100
31.0 INTEGRATE      0
31.0 ML/MIN      1.00
31.0 CM/MIN      0.00
35.0 CONC %B      100
35.0 CONC %B      1.0
43.0 CONC %B      1.0
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Fig. 5. Fractionation of nucleotide phosphates on a MonoQ HR 5/5 column (50 x 5 mm, 10 µm, Pharmacia, Uppsala) using a concentration gradient of ammonium phosphate buffer (from 0.01 to 0.5 M, pH: 7) and the Pharmacia FPLC System.

(A) Chromatogram of the reference compounds obtained by the REC-482 Recorder.

(B) Printing of the separation and elution program by the LCC-500 Liquid Chromatographic Controller of the FPLC System.

1 - CMP, 2 - UMP, 3 - AMP, 4 - IMP, 5 - GMP, 6 - CDP, 7 - UDP, 8 - ADP, 9 - CTP, 10 - UTP, 11 - GDP, 12 - unidentified, 13 - ATP, 14 - GTP

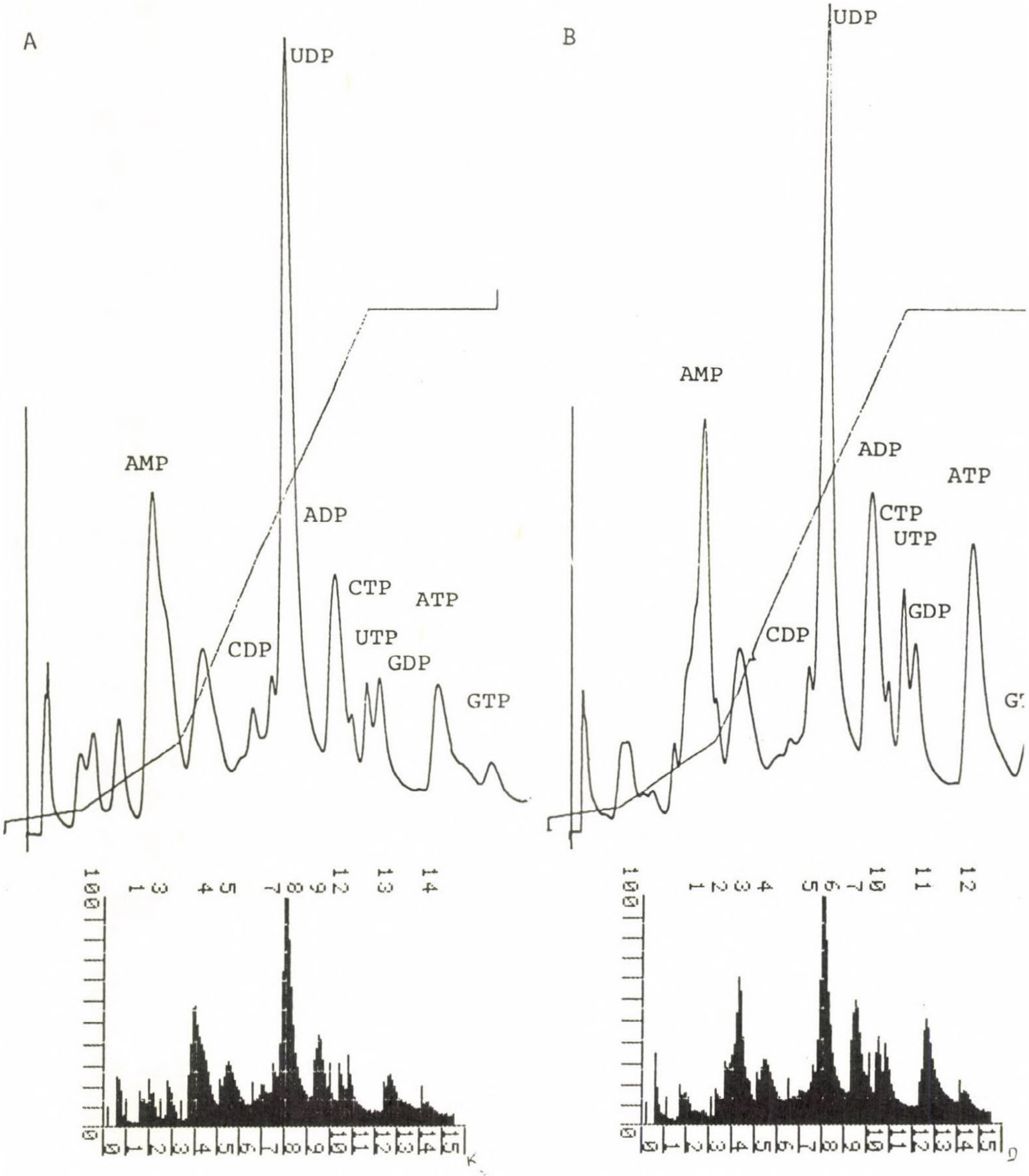

Fig. 6. Separation of nucleotide phosphates originated from control
(A) and
(B) DFMO-treated P388 leukaemia cells on a MonoQ HR 5/5 anion-exchanger column. Experimental conditions see in Fig. 5

(b) following the urinary excretion of polyamines it might be predictive in the efficacy of the combined chemotherapy of tumours.

In our previous work we have suggested that as a result of inhibition of key-enzyme activities (ODC; OCT, ornithine carbamoyltransferase, EC 2.1.3.3) in the urea cycle, and due to the channellization of carbamoyl phosphate and aspartate an enhanced formation of uridine- and cytidine-5' phosphates can be expected (8, 21). In order to investigate these metabolic changes in P388 leukaemia cells, ion-exchange and ion-pair liquid chromatographic methods were applied (23). In the present study we have worked out the ion-exchange separation of nucleotides on a new packing material, MonoQ HR 5/5, using an optimized elution program for ammonium phosphate buffer at PH:7 as shown in Fig. 5. Our results also demonstrate that a reasonable resolution of 13 nucleotide phosphates can be achieved using a simple linear concentration gradient and comparable chromatograms are obtained both by the REC-482 classic recorder and by the LCC-500 controller of the Pharmacia FPLC System. Retention times with a reproducibility of ± 2.76 % in average were obtained for all nucleotides tested. Linear correlations (r = 0.95) with individual slopes were found for nucleotides in a range from 1 to 15 nmoles plotted against the arbitrary peak areas. Quantitation of the individual fractions according to the peak areas given by the LCC-500 and compared to the reference standards was performed with an accuracy of 6.03 %. Alterations in the pH of phosphate buffer in a range from 5 to 8 did not improve the resolution. Importance of the purity of buffer substances concerning the baseline correction of gradient elution has been discussed earlier (23). Analytical grade reagents of Suprapur (Merck) quality were necessary to keep the appropriate conditions.

Determination of intracellular nucleotides in P388 leukaemia cells treated with DFMO demonstrated an enhanced biosynthesis of uridine- (UDP, UTP), adenosine- (ADP, ATP) and cytidine- (CTP) phosphates in comparison to the untreated controls (Figs 6A and B). Results obtained by ion-exchange chromatography using the MonoQ HR 5/5 column provided additional evidences to explain that DFMO produced metabolic imbalance of ornithine utilization in leukaemia cells contributing probably to the cytotoxic effect of cyclophosphamide. Increasing number of observations indicate the success of combined treatments in cancer chemotherapy simultaneously applying cytostatic and cytotoxic agents (11-18). DFMO as a potent inhibitor of polyamine biosynthesis exerts definite cytostatic effect on many human and experimental tumours. However, the synergic (potentiation) effect of DFMO seems to be the most promising field in its application by increasing the cytotoxicity of well known antitumour agents and giving a possibility for lowering the effective doses and toxicity. In spite of the obvious relationship between the urea cycle and the metabolism of nucleotides further investigations are needed to clarify the synergic effect of DFMO with alkylating agents.

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The authors thank the Centre de Recherche Merrell International (Strasbourg, France) and C.D. Houldsworth for DL-α-difluoromethylornithine, as well as Pharmacia Biotechnology International AB (Uppsala, Sweden) for the generous support and supply of MonoQ HR 5/5 columns. The skillful technical assistance of Mrs. Vera Scheuring, Mrs. Beata Akács and Mrs. Susan Németh is gratefully acknowledged.

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H. Kalász and L.S. Ettre (Eds)

USING VARIOUS CHROMATOGRAPHY TYPES AND ELECTROPHORESIS TO REVEAL MICROHETEROGENEITY IN CRYSTALLINE PROTEIN PREPARATIONS

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Highly specific ribonucleases with low molecular weight (M = 11,000-13,000 Da) find their use as regular objects in protein-nucleic recognition, and in research on molecular specificity mechanisms and on the ways of microorganism evolution. They are also widely applied in molecular biology to state the RNA structure and in organic synthesis to obtain nucleotides with assigned sequence (1).

Guanyl-specific RNase C_2 Aspergillus clavatus is one of the most thoroughly investigated fungus ribonucleases.

The main aim of our investigations was the study of highly pure, twice recrystallized guanyl-RNase C_2, marked by a high-quality index.

Ribonuclease (RNase) C_2 is a representative of the so-called small proteins. It has 104 amino acid residues, and its molecular weight is 11,242 Da. Concerning its molecular structure, the enzyme has been thoroughly investigated. The primary and tertiary structures of RNase were found with a resolution of 1.35 Å. A high level of homology was found in the structure of the well-known, guanyl-specific RNase T_1 Aspergillus oryzae (2, 3)(Fig. 1).

The highly purified guanyl-specific RNase C_2 enzyme preparations, when being lyophilized, are stable and retain their high activity for a long time. The presence of microheterogeneity in functionally homogeneous crystalline enzyme preparations is of both scientific and practical interest.

Fig. 1. Monocrystals of RNase C_2

Research in these fields is closely connected with the production of highly active and highly purified enzyme.

Crystallized guanyl-RNase C_2 was tested for the presence or absence of specific and contaminating activities. The N-terminal sequence of amino acids and the spectral characteristics were also determined (Table I, Fig. 2).

In spite of the high enzymatic purity of RNase C_2 preparations, with the help of electrophoresis some microheterogeneity was revealed as a minor band.

We tried to obtain some preliminary data about the possible nature of this phenomenon.

Table I. Parameters usually determined when evaluating the quality of RNase C_2 preparations, being purified

No.	Parameter	Value
1.	Specific activity	500,000-700,000 U/mg protein
2.	Contaminating activities:	absent
	- alkaline PMEase	
	- acid PMEase	
	- PDEase	
	- proteolytic activity	
3.	Specificity to dinucleotides decomposition GpC/ApC	more than 1×10^5
4.	Spectral characteristics A_{max}/A_{min} (A_{278}/A_{251})	more than 2.8
5.	N-terminal succession of amino acids (see also Fig. 2)	Asn-Thr-Ser-Glu-Pro-Gly-Ala-Cys-Val-Ile-Leu-Tyr-Phe-Hys-Arg-...

The nature of the enzyme microheterogeneity may result from the chemical individuality of the protein preparation or from genetically caused processes, such as translation errors, the formation of allele forms as well as from the reactions of post-translational modification. These are such reactions as methylation, deamidation and some others, occurring when enzymes are being separated, purified and stored. Microheterogeneity may also occur in the form of aggregation of protein molecules (4).

After being stored for some time the molecular weight of the enzyme was analysed. The analysis was made with the help of gel chromatography, applying Toyopearl HW-50 (Fig. 3).

The RNase activity was revealed to be in a single symmetric peak. There were no other peaks. The column was calibrated with the help of certain proteins with known molecular weight (Fig. 4).

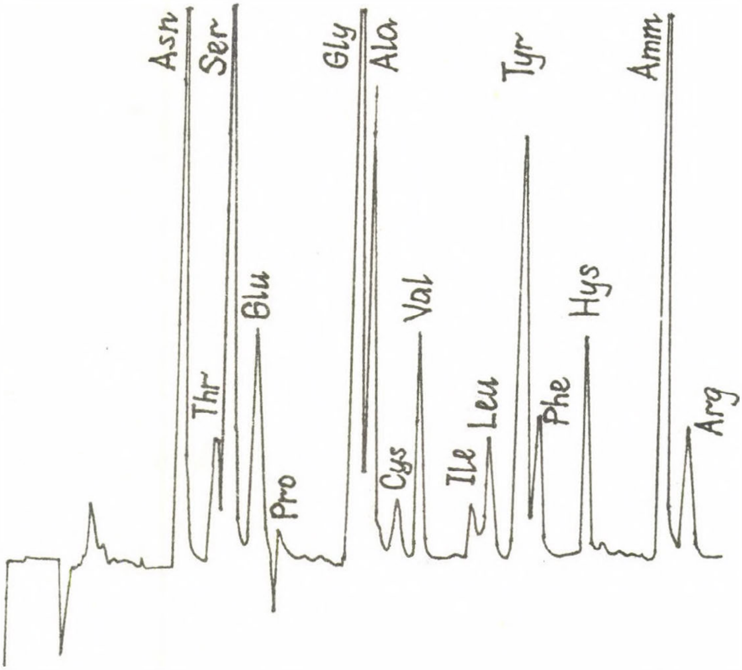

Fig. 2. RNase C_2 N-terminal amino acid sequence

However, it was found soon that RNase C_2 eluted from the column in volumes, characteristic of the dimer of RNase C_2, having a molecular weight 24,000 Da. We want to note that 0.2 M NaCl solution was used and this fact, most probably, led to the formation of the dimer.

With RNase C_2, the presence of a dimer or, in other words, the tendency to aggregation had also been found in the past (5). Therefore, the microheterogeneity of RNase C_2 preparations may be caused by the aggregation process.

The suggestion that the minor component can be slightly different from the basic one as to its charge was tested by using ion-exchange chromatography on Servacel 23 SH (Fig. 5).

RNase C_2, taken from the column, eluted as a single peak, with a small shoulder.

With the help of electrophoresis in native conditions it was discovered that the peak fraction contains a small amount

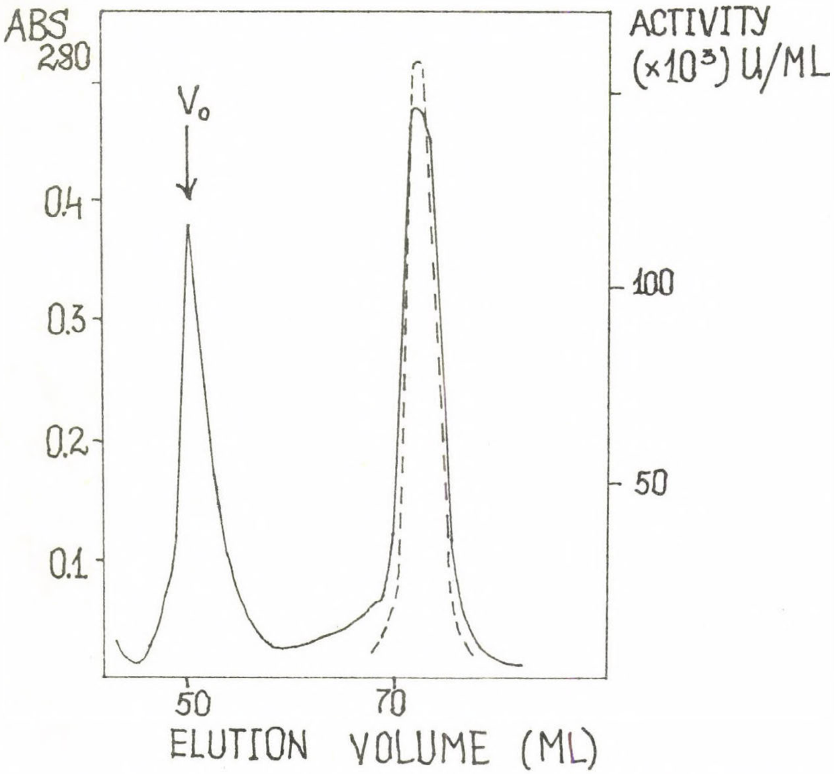

Fig. 3. Gel chromatography of RNase C_2 on Toyopearl HW-50. ——, A_{280}; ---, enzymatic activity. The avoid volume (V_o) of the column (75 cm x 1.5 cm) was estimated by the application of blue dextran (see "Experimental Procedure")

of the minor component, the quantity of which grows in the following eluates (see electrophoregram). Both bands had RNase activity by the so-called "sandwich" method. Electrophoresis in denaturating conditions resulted in a similar electrophoregram. Consequently, RNase C_2 also contains the minor component migrating faster under electrophoresis and eluting from the anion-exchange column later than the basic component. Thus, we can suggest that this minor component is an isoenzyme of RNase C_2. However, the attempt to separate these two isoenzymes on this column failed.

Earlier microheterogeneity was discovered for some other RNases of this family, such as RNase T_1 Aspergillus oryzae, RNase F_1 Fusarium monoliforme, RNase Ap_1 Aspergillus pallidus,

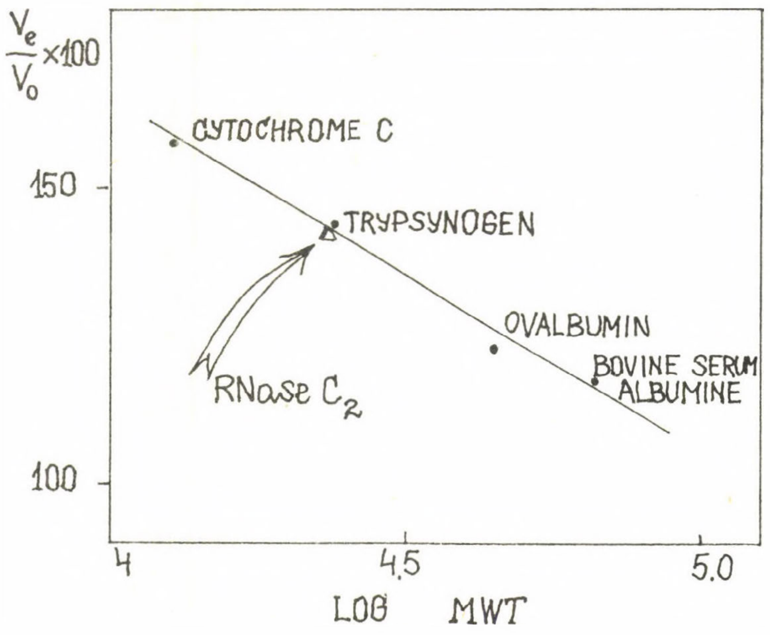

Fig. 4. The dependence of the volumetric output of proteins on their molecular size

and purine-specific RNase U_2 Ustilago sphaerogena, which also contains the more acidic isoenzyme. It had been suggested that the probable cause of this phenomenon is a certain difference in the composition of amino acids or amino acid sequences (6-9).

If we accepted the hypothesis, that microheterogeneity was the result of deamidation process, then it includes mainly the modification of the remnants of Asn and Gln. RNase C_2 comprises in its structure Asn_6 and Gln_4. In addition, Asn_4 and Gln_2 are situated on the very surface of the globule and may be subjected to deamidation. It is well known that deamidation is accompanied by the lowering of the isoelectric point.

The reasons for the existence of RNase C_2 isoforms are not fully clear and therefore should be investigated further.

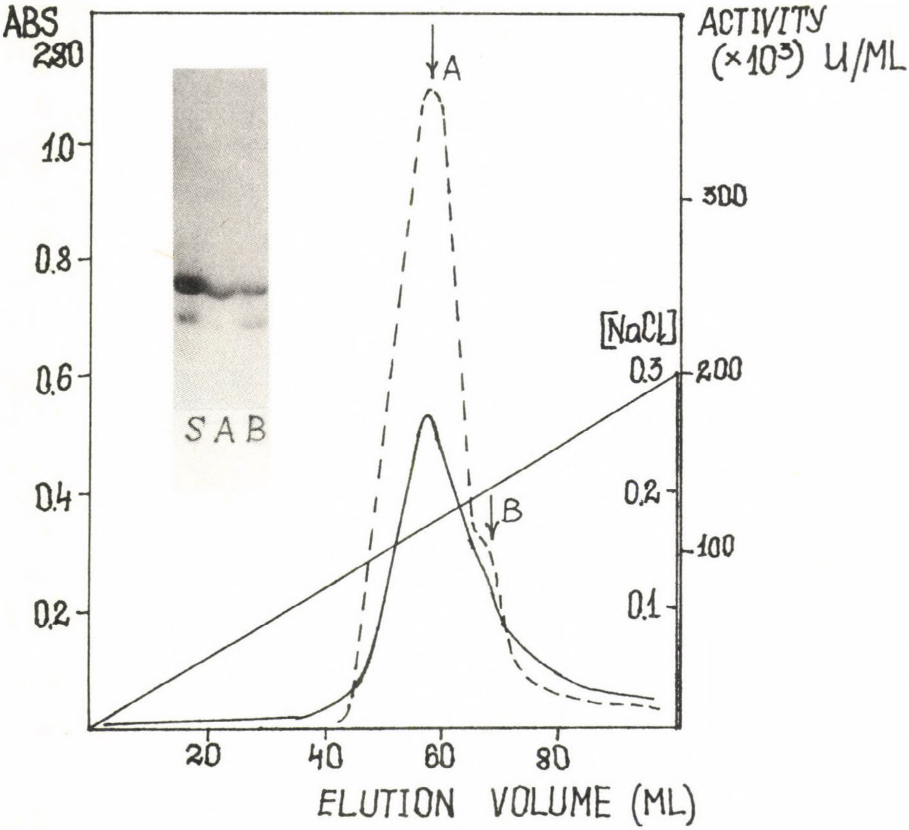

Fig. 5. Anion-exchange chromatography of RNase C_2 on Servacel 23 SH. ——, A_{280}; ---, enzymatic activity. Electrophoresis was performed in 7.5 % polyacrylamide gel, pH 8.9 (see "Experimental Procedure"). S - preparation of purified and crystallized RNase C_2, A - "peak" fraction from Servacel 23 SH, B - "shoulder" fraction from Servacel 23 SH

EXPERIMENTAL PROCEDURE

Purification of RNase C_2 was carried out according to the procedure given above (10). As a result a twice crystallized enzyme preparation was obtained.

Ion-exchange chromatography of crystalline RNase C_2 was performed on a 8 x 1 cm Servacel 23 SH column equilibrated by

0.02 M TRIS/HCl buffer, pH 7.5. Dissolved RNase sample 30 A_{280} was used. Elution was carried out using a linear gradient (0-0.3 M) NaCl in 0.05 M TRIS/HCl buffer, pH 7.5.

Gel chromatography was performed on Toyopearl HW-50 on a 75 x 1.5 cm column. Elution buffer was 0.2 M NaCl in 0.02 M TRIS/HCl, pH 7.1, flow rate 1.33 ml/min. Sample applied to the column contained 17 A_{280}. For the calibration of the column cytochrome C (M = 13,000 Da), trypsynogen (M = 24,000 Da), ovalbumin (M = 45,000 Da) and bovine serum albumine (M = 66,000 Da) were used, all from Sigma.

Servacel 23 SH was delivered by Reanal, Toyopearl HW-50 was purchased from Toyo Soda. Other chemicals were of reagent grade.

Enzyme assays were performed according to the method of Egami at pH 7.5, by measuring the acid soluble digestion products from yeast RNA at 260 nm. One enzyme unit is defined as a 1.0 increase in the optical density at 260 nm under the reaction conditions (37°C, 15 min)(11).

Determination of basic specificity was carried out by comparing the decomposition speed of two dinucleotides, GpC/ApC, registered on a Shimadzu spectrophotometer.

Polyacrylamide gel disc electrophoresis was performed on 7.5 % polyacrylamide gels, pH 8.9, according to the method of Davis (12). Electrophoresis was conducted for 3 h with a current of 1.7 mA per strip. The gels were strained by Commasie blue G-250. Activity of RNase after native electrophoresis was defined by the so-called "sandwich" method (13).

UV-spectra were registered with the help of a Shimadzu spectrophotometer.

The amino acid sequence was defined by using a Durrum D-500 automatic amino acid analyser, after hydrolysing of samples with 5.7 M HCl for 24 h at 105°C.

CONCLUSIONS

1. The presence of microheterogeneity was detected in guanyl-specific RNase C_2, a functionally homogeneous, highly purified and crystalline enzyme.

2. Ion-exchange chromatography on Servacel 23 SH and electrophoresis under native conditions were used to state the presence of the more acidic guanal-specific RNase C_2 minor component, possessing the activity.

3. The application of gel chromatography of guanyl-specific RNase C_2 on Toyopearl HW-50 and electrophoresis under denaturating conditions permitted to succeed, that in 0.2 M TRIS/HCl buffer, at pH 7.5, containing 0.2 ·M NaCl, the enzyme aggregates and is in its dimeric form.

4. It is suggested that the microheterogeneity of RNase C_2 may result from the reactions of post-translational modification, such as methylation, deamidation and several others, occurring at the time when the protein is separated, purified and stored: It may also be due to the process of aggregation, taking place under certain conditions.

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DETERMINATION OF HYDROCHLOROTHIAZIDE IN SERUM

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INTRODUCTION

Benzothiazine-type diuretics have been marketed for about 30 years, including Hypothiazid the preparation of Chinoin, Pharmaceutical and Chemical Works Ltd. (Budapest, Hungary) containing hydrochlorothiazide (HCTZ) as the active substance.

Recently combinations have also been extensively used. These are important because HCTZ increases potassium excretion. In order to eliminate this effect it is expedient to combine it with other diuretics such as e.g., triamterene (3), or amiloride (4) rather than to prescribe potassium preparations for the patients.

Our aim was to develop a HPLC method suitable for the routine measurement of the drug in rat and human sera. Numerous reports deal with kinetic studies from human blood (1) or urine (2).

Several authors (3, 5) use deproteinization with acetonitrile for obtaining HCTZ from plasma or serum. Others describe multiple extraction with ethyl acetate (6, 7), while Christophersen et al. (8) remove the disturbing proteins by gel filtration.

HPLC methods described in the literature use reversed-phase C_{18} packing and UV detection at 271 nm.

Most of the authors use a 20:80 mixture of acetonitrile and buffer as the eluent, the flow rate varying from 0.8 to 2.0 ml per minute. The pH varies within a wide range, and almost all values are mentioned between pH 3 and 8. For example, Upton et al. (3) used pH 3, Lin and Benet in Drug level monitoring (5) pH 3.7, Koopmans et al. (7) pH 5.5, Randolph et al. (9) pH 6.0, and Alton et al.(2) pH 7.15.

As internal standard a diuretic of similar structure, e.g., chlorothiazide (3) or one of the derivatives of hydrochlorothiazide, e.g., the bromo-derivative of hypothiazide (5) were described in the literature.

EXPERIMENTAL PROCEDURES

Materials and Equipments

The chemicals used were of analytical or chromatographic purity. Hypothiazide (batch No. 86031009) and N-methyl-hydrochlorothiazide (internal standard) were prepared in the Synthetic Research Laboratory of Chinoin.

Equipments

Centrifuge: Model K 70 Janetzky (Leipzig, GDR)
Vortex : (KUTESZ, Budapest, Hungary)
pH meter : Model OP 208 (Radelkis, Budapest, Hungary)
HPLC pump : Model BT 3020 (Biotronik, Maintal, FRG)
Detector : UV model BT 3030 (Biotronik, Maintal, FRG)
Recorder : Model 120 Servogor (Goertz, Wien, Austria)
Injector : Model 7125, 20 μl loop (Rheodyne Berkeley, California, USA)

Operating Conditions

Column : Nevisorb BST C-18, 250 x 4 mm, 10 μm particles (Bio-Separation-Technologies, Budapest)
Wavelength : 271 nm
Sensitivity: 0.02 AUFS
Flow rate : 1.0 ml/minute
Chart speed: 60 cm/hour
Eluent : 15:85 acetonitrile: 0.1 % acetic acid (pH 2.9)

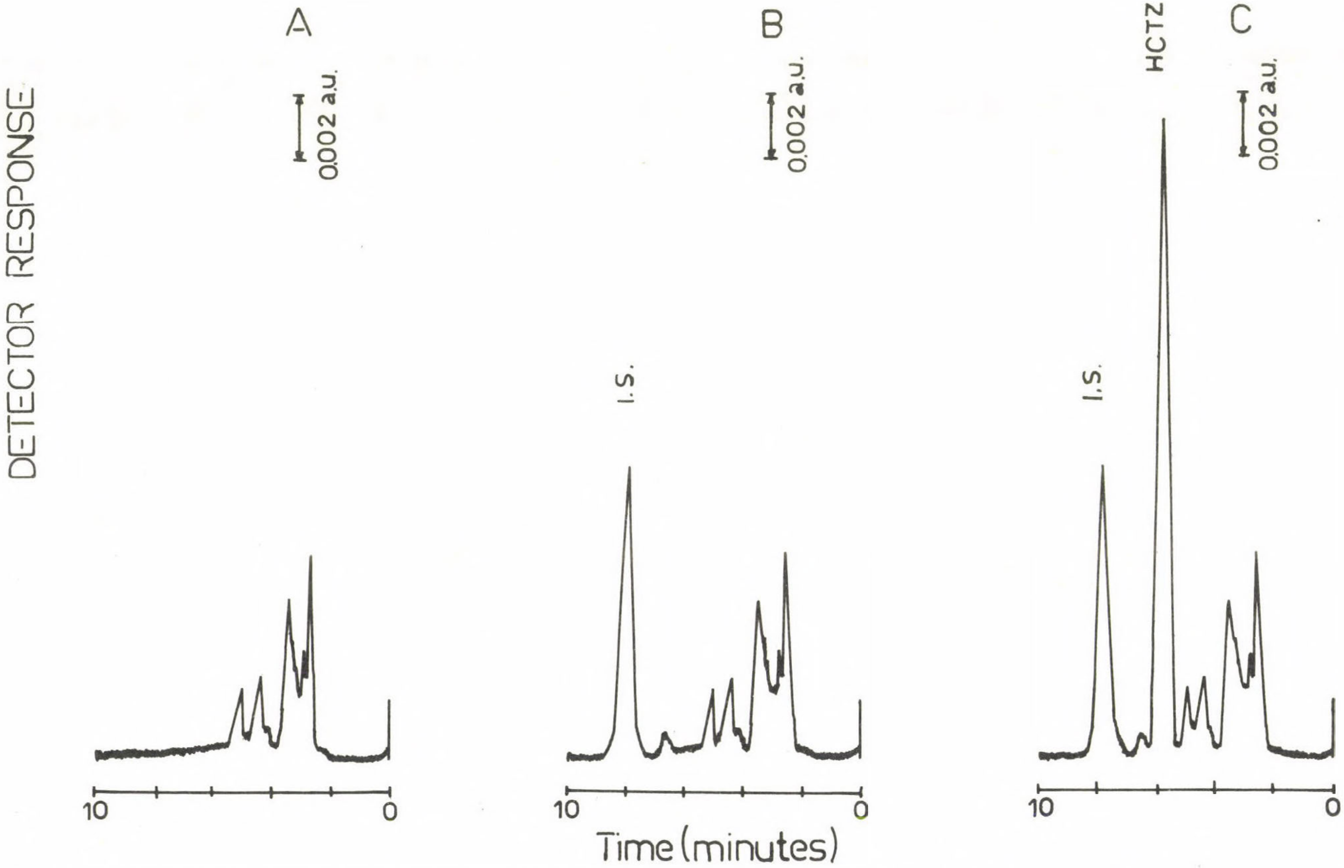

Fig. 1. Chromatograms: A - rat control serum; B - rat control serum containing internal standards; C - rat serum sample taken 30 min after treatment

Internal standard: N-methyl-hydrochlorothiazide
Retention time : HCTZ 6 min
I.S. 8 min

Extraction Method

0.5 ml rat or human serum containing 150 nm internal standard and 50, 100, 150 or 200 ng of HCTZ taken from a 10 μg/ml stock solution were applied onto 1.5 ml (0.27 g) extrelut (Merck Art. 11728). After 5 minutes it was washed twice with 1 ml of alkaline ethyl acetate (1.5 ml N NaOH in 50 ml ethyl acetate). The ethyl acetate was evaporated to dryness and the sample was dissolved in 0.5 ml eluent. A proper aliquot was injected into the liquid chromatograph.

Kinetic Experiment on Rat

Male albino rats of the CFY strain weighing 250-300 g were used in the experiment. HCTZ was given orally through a gastric tube in two doses (2.5 mg/kg and 5 mg/kg) in the form of a suspension prepared with Tween-20. Blood was taken from the sinus orbitalis of three animals in each group. The blood samples were pooled, and used for one measurement. Figure 1 shows a chromatogram of a blood sample taken at 30 minutes after treatment, while Fig. 2 shows the plot of the HCTZ concentrations of rat serum following oral treatment.

DISCUSSION

A simple, rapid and reliable method was developed for the extraction of HCTZ from serum. The extraction yield from serum (Table I) always exceeded 90 %.

The conditions, sensitivity and accuracy of the HPLC determination are the same as those described in the literature. N-methyl-hydrochlorothiazide applied as the internal standard can be considered optimal, its structure being very close to that of HCTZ; since its retention time is 8 minutes, it can be well distinguished from the 6-min retention time of HCTZ.

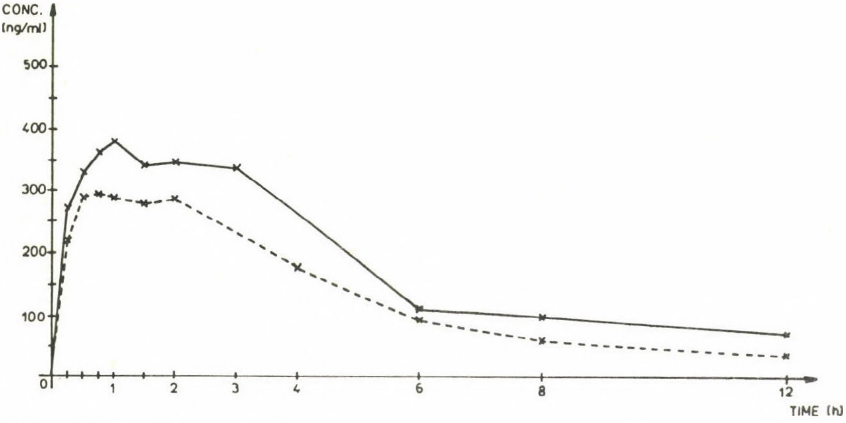

Fig. 2. It shows HCTZ concentrations in the serum of rat in the function of time after two oral administrations (2.5 mg/kg; (--); 5.0 mg/kg (—))

Table I. Reproducibility of HCTZ measurement

Concentration of HCTZ added to the serum ng/ml	Concentration mean ± S.D. ng/ml	Rel. S.D. %	Recovery %
100	101.9 ± 8.31	8.16	102.0
200	198.0 ± 13.62	6.88	99.0
300	273.8 ± 11.73	4.30	91.3
400	362.7 ± 5.37	1.48	90.7

The detection limit in serum is 10 ng/ml. The calibration curve is linear between 50 and 400 ng/ml.

The data of the kinetic studies were also evaluated mathematically, using a non-linear curve fitting program on an IBM PC computer. The data obtained (11) are summarized in Table II. A one-compartment oral model was fitted to the curves.

Table II. Curve fitted on kinetic data

Parameter	Dose 2.5 mg/kg body weight		Dose 5.0 mg/kg body weight	
	Fitted to the individual data	Fitted to the mean values	Fitted to the individual data	Fitted to the mean values
D/V_d	439.64 ± 17.58	461.30 ± 26.50	548.16 ± 46.13	542.50 ± 47.07
k_a h^{-1}	3.99 ± 1.10	3.44 ± 0.67	2.04 ± 0.44	2.08 ± 0.52
k_e h^{-1}	0.24 ± 0.02	0.24 ± 0.03	0.20 ± 0.03	0.19 ± 0.03
t_{lag} h	0.00 ± 0.07	0.00 ± 0.03	0.00 ± 0.05	0.00 ± 0.05
t_{max} h	0.75	0.83	1.25	1.26
C_{max} ng/ml	366.71	377.32	424.90	425.45
AUC_{calc} ng·h/ml	1813.32	1900.53	2701.20	2801.44
AUC_{mes} ng·h/ml	1690.37	1800.87	2446.70	2570.37
$t_{\frac{1}{2}(k_a)}$ h	0.17	0.20	0.34	0.33
$t_{\frac{1}{2}(k_e)}$ h	2.86	2.85	3.41	3.57

The lagtime (t_{lag}) was fixed and variance of that fixed value can be seen in this table.
Approximation of curves was done by equation:

$$V_{(t)} = \frac{D}{V_d}\left(k_a / (k_a - k_e)\right)\left(e^{-k_e t} - e^{-k_a t}\right)$$

AUC values calculated from the measured data and those obtained from the integration of the curve show good agreement. It can be seen in $t_{\frac{1}{2}}$ values that after a relatively rapid absorption the excretion is slow, and the blood contains detectable amounts of HCTZ, even after 12 hours. This is also evident from the AUC values of the fitted and calculated curves. The deviation of the parameters (Table II) is within 10 %, this also proves that the fitted model was adequate.

In the case of a higher dose (5 mg/kg) the combined study of the $t_{\frac{1}{2}}$ and k_a values indicates slower absorption.

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CHITIN-A NEW NATURAL STATIONARY PHASE IN CHROMATOGRAPHY

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Wide abundance of chitin in nature, its availibity, relatively simple isolation from biological materials (1) and also its physical and chemical similarity to cellulose, widely used in chromatography, were the reasons for investigations on the application of chitin as a stationary phase (2, 3-5). In nature chitin exists mainly in animal organisms and is not exhausted because some organism continuously produce it.

Among the polymorphic forms of chitin α-chitin was investigated as a prospective material for chromatography. This polymorphis form can be useful in chromatography because of the existence of interplate bonds and lack of swelling in water and organic solvents (6). In our investigations about the application of chitin as a stationary phase thin-layer chromatography was used because of its simplicity, speed, low cost and the possibility of its use as a pilot technique for HPLC.

With respect to its physical properties chitin (especially its α form) appears to be a good stationary phase in thin-layer chromatography. This assumption may be confirmed by following factors: possibilities of obtaining a mechanically stable chitin layer and the independence of the chromatographic retention values (R_F and R_M) on the preparation mode of the chitin suspension (Table I), the thickness of the chitin layer (Fig. 1), and the amount of the chromatographed substance (Fig. 2). The chromatographic retention values are dependent on the temperature (Fig. 3) and the time which elapses from the preparation of the thin layer to the measurements. This is

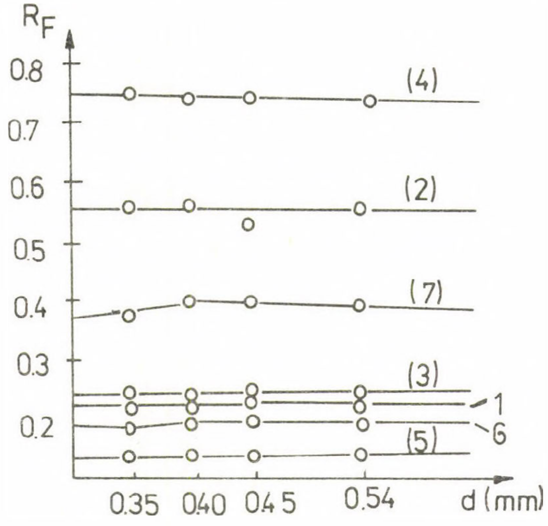

Fig. 1. R_F values of dye mixture of chitin with different thickness (d) of the layer. Mobile phase: acetone-water 1:4 v/v
1. methylene blue; 5. fuchsin;
2. p-aminoazobenzene; 6. methylene violet;
3. safranine; 7. malachite green
4. rhodamine;

Table I. Effect of the mode of plate preparation of the chromatographic retention values. Comparison of the R_F values obtained on chitin layers prepared with organic solvents (1) and from water suspension (2)

Substance	Mobile phase			
	Water		Water + Acetone (17:3 v/v)	
	1	2	1	2
o-cresol red	0.39	0.40	0.54	0.57
bromocresol green	0.11	0.12	0.16	0.16
o-bromocresol purple	0.25	0.25	0.33	0.30
tartrazine	0.03	0.03	0.03	0.03
green ligth	0.07	0.06	0.16	0.16
methylene blue	0.03	0.02	0.19	0.20
p-aminoazobenzene	0.12	0.14	0.37	0.36
safranine	0.15	0.15	0.24	0.23
rhodamine	0.19	0.18	0.51	0.54

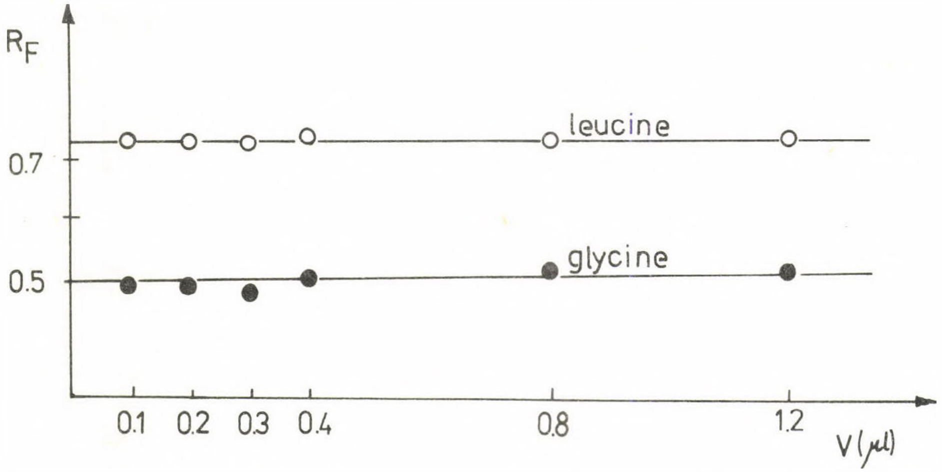

Fig. 2. Relationship between the R_F values and the sample volume (v). Mobile phase: methanol

important because the chitin layer stabilizes only in about 20 days (Fig. 4).

Because of the great variety of the functional groups existing on the chitin surface (OH groups, free or acetylated amino groups) chitin can be used for the separation of many types of compounds. In earlier papers the usefulness of chitin to the separation of amino acids in single-component (Fig. 5) and binary (Fig. 6) mobile phases has been shown. The next problem which has been examined was related to the separation of aqueous solutions of dyes using chitin as the stationary phase. Comparative investigations with cellulose as the stationary phase were also carried out. This problem is very important considering the presence of dyes in industrial wastes. It was found that the separation of dyes on chitin is in many cases better than on cellulose (Fig. 7). It was also seen that the shape of the separated spots is more regular and their size is smaller than in the case of cellulose. In general, separation of dyes does not occur in pure water because the elution power of water is too small for the separation of this type of mixtures; these mixtures can only be separated when a binary mobile phase is used. It has been observed that during

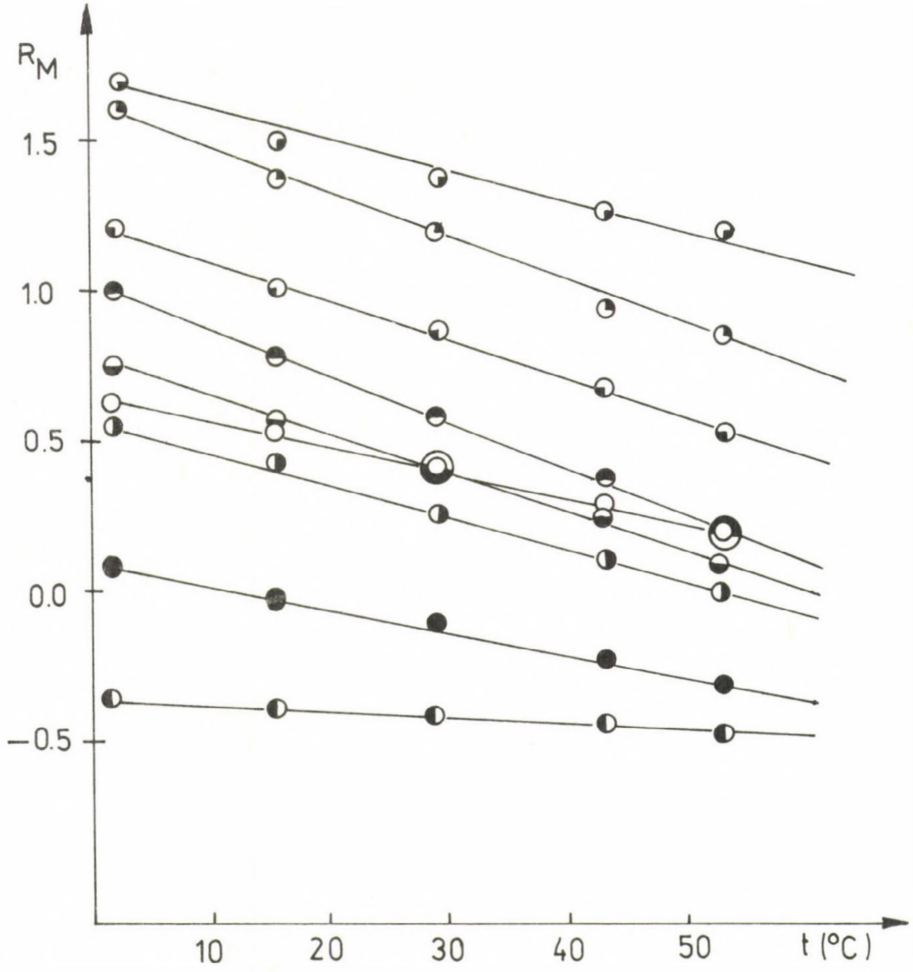

Fig. 3. Relationship between the R_M values and the temperature (t) of the chitin layers. Mobile phase: water-acetone 4:1 v/v

○ - methylene blue;
● - p-aminoazobenzene;
◑ - safranine;
◐ - rhodamine;
◓ - fuchsine;
◒ - methylene violet;
◔ - indigo carmine;
◕ - light green;
◔ - tartrazine

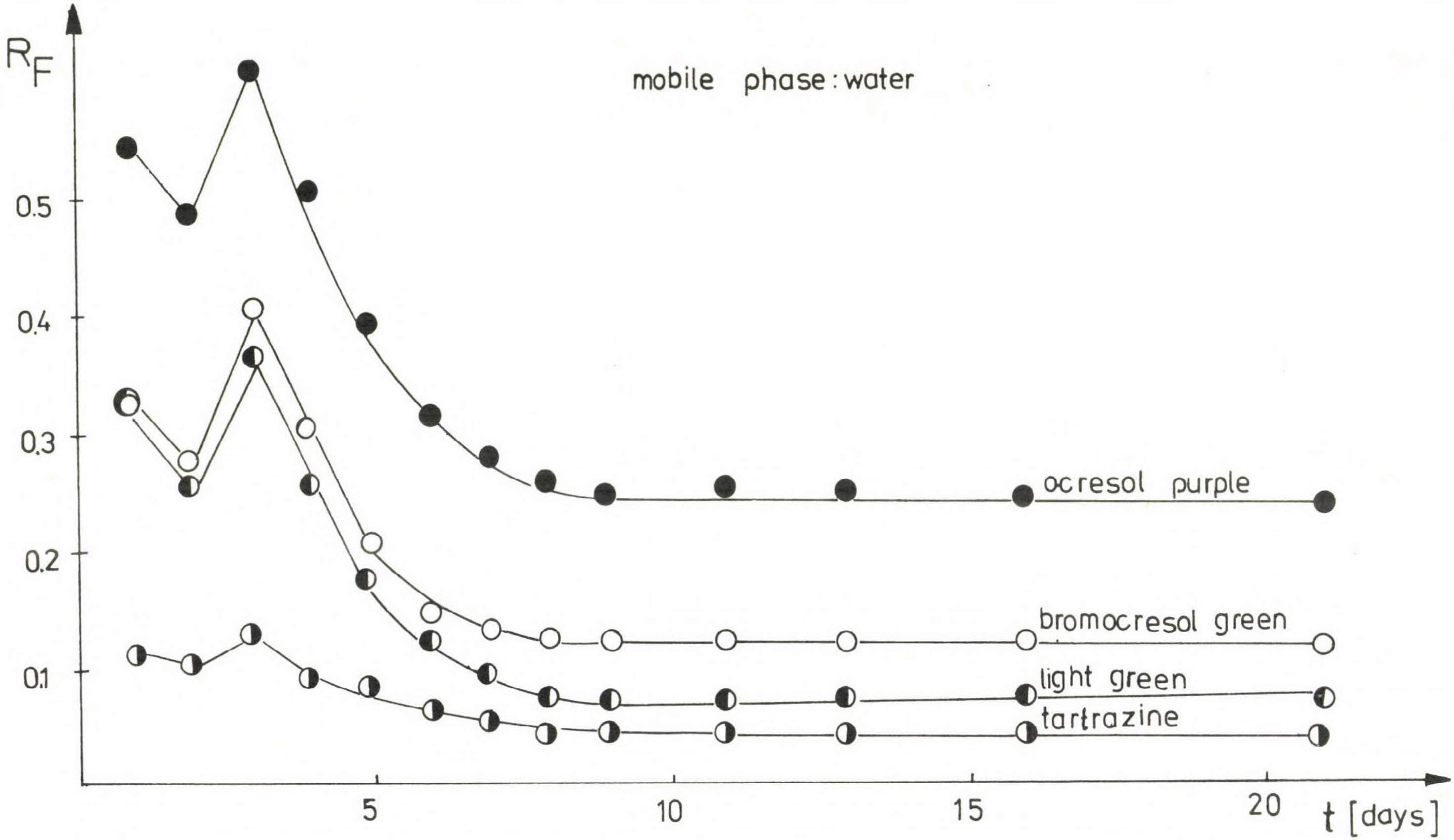

Fig. 4. R_F values as a function of the time (t) between plate preparation and plate development

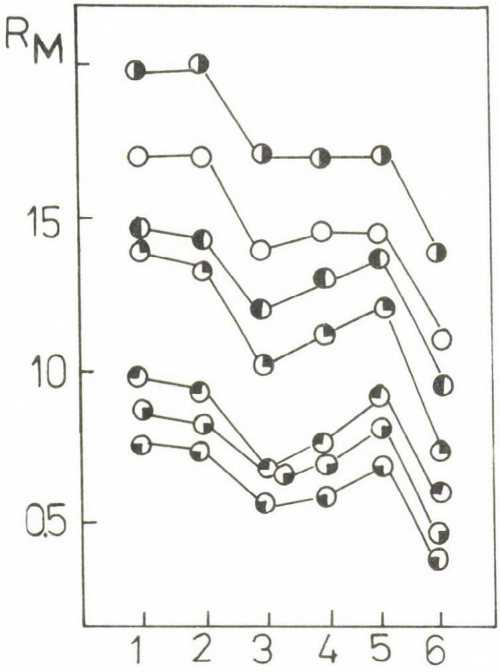

Fig. 5. Separation of amino acids on chitin bed using a single component mobile phase

○ - glycine;
◔ - alanine;
◒ - valine;
◕ - leucine;
◓ - phenylalanine;
◑ - seryne;
◐ - threonine

Mobile phase:

1 = pentanol-1;
2 = pentanol-2;
3 = butanol-1 ;
4 = butanol-2;
5 = 2-methylpropanol-2;
6 = propanol-1

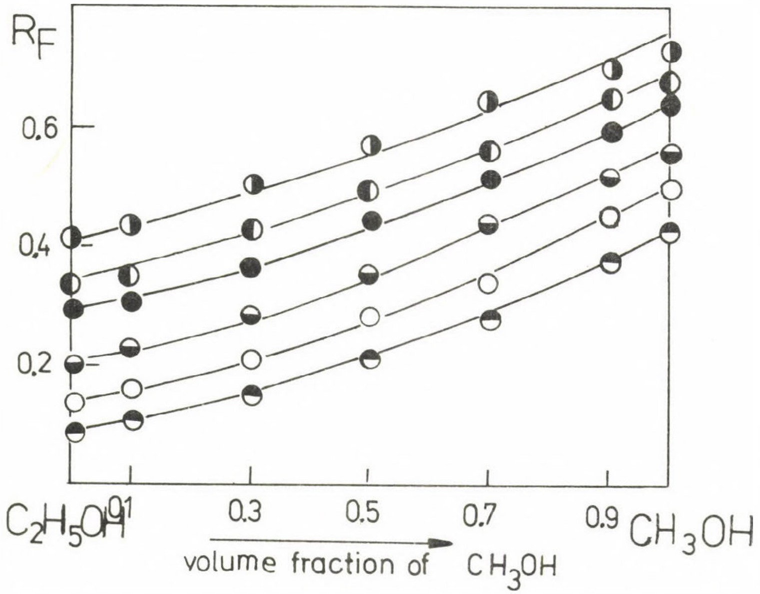

Fig. 6. Separation of amino acids on chitin bed in methanol-ethanol binary mobile phase

○ - glycine; ◐ - phenylalanine;
● - alanine; ◓ - serine;
◑ - valine; ◒ - threonine

the chromatographic process dyes behave differently than amino acids. In the case of amino acids addition of acetone as a more polar component of the mobile phase has caused an increase in the R_M values of the amino acids, indicating a decrease of the elution power of the mobile phase. At the same time, in the case of dyes addition of acetone causes a significant decrease in the R_M values in comparison to water, indicating that in the chitin-dye system acetone has a greater elution power than water.

Chitin also has chelating properties, because it does not show any affinity in relation to ions of metals of the I and II groups of the periodic table and interaction with metals capable of formation of coordinate bonds, i.e. with metals having closed d or f electronic subshells (7). Therefore chitin may be a good stationary phase for the separation of these

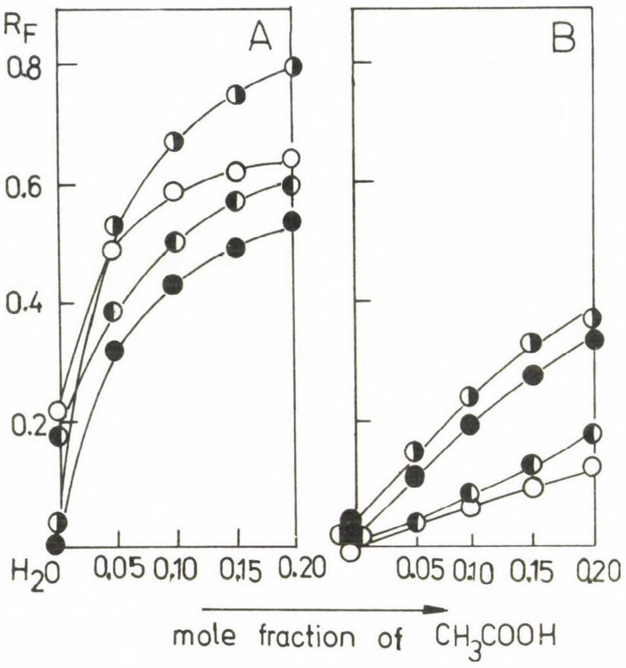

Fig. 7. Separation of dyes on (A)-chitin, (B)-cellulose in binary mobile phase: water-acetic acid

○ - methylene blue; ◑ - safranine;

● - p-aminobenzene; ◐ - rhodamine B

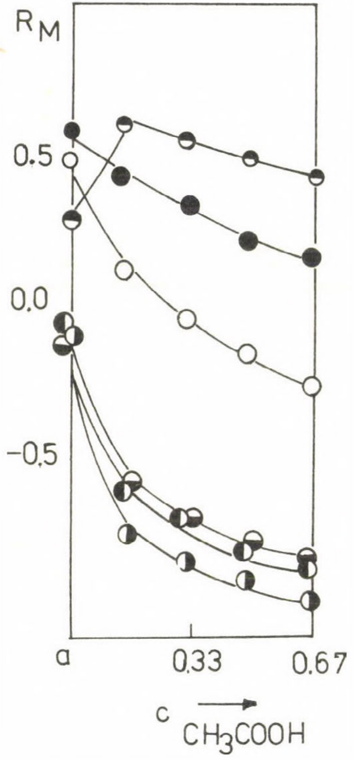

Fig. 8. Separation of inorganic ions on chitin bed. (a) 25 cm^3 CH_3OH + 70 cm^3 1 mol/dm^3 water solvent CH_3COONH_4

○ - Cu^{+2}; ◐ - Cd^{+2}; ◓ - Hg^{+2};

◑ - Co^{+2}; ◒ - Ni^{+2}; ● - Ag^{+1}

metal ions. Many metal ions can be separated on chitin even with very simple mobile phases. For example, we have separated Cu^{+2}, Co^{+2}, Cd^{+2}, Hg^{+2}, Ni^{+2} and Ag^{+1}. In this case when aqueous solutions are used as the mobile phase only the Ni^{+2}/C Ni^{+2}/Cd^{+2} and Cd^{+2}/Co^{+2} pairs cannot be separated (Fig. 8). Chitin also has the advantage that when inorganic salt solutions are used as the mobile phase the retention values are independent of the pH of the mobile phase.

Separation on chitin may be based on adsorption, ion exchange, ability of the mobile and stationary phase to complex formation or a combination of all these mechanisms (8). In this paper we would like to present the application of the chromatographic optimization theory for amino acid analysis in comparison to well-investigated use of silica. We test here two theories for the optimization of the thin-layer chromatographic process using binary mobile phases: the thermodynamic optimization theory (9) and the theory of Snyder (10).

The basic equation of the thermodynamic optimization theory of systems with binary mobile phases is Eq. 1 (9):

$$R_{M12} = x_1 \Delta R_{M12} + (x_1^s - x_1)\ (\Delta R_{M12} + A_z) + R_{M2} \qquad (1)$$

where R_{M12} is the R_M value of a given chromatographic solute in the binary mobile phase "1 + 2"; $\Delta R_{M12} = R_{M1} - R_{M2}$ is the difference between the R_M values for a given solute in pure solvents 1 and 2 (1 denotes the more polar solvent); x_1 is the volume or molar fraction of the more polar component of the binary mobile phase; $x_1^s - x_1$ is the adsorption excess of component "1" of the binary mobile phase and A_z is the parameter describing the intermolecular interactions between the solutes and the mobile phase components.

The fundamental equation of Snyder's theory for thin-layer systems with a binary mobile phase is Eq. 2 (10):

$$10^{-R_{M12}} = x_1 10^{-R_{M1}} + x_2 10^{-R_{M2}} \qquad (2)$$

The results can be presented in the form as a relationship between the experimental R_M values (R_{Me}) and the theoretical values (R_{Mc}) calculated from Eqs 1 and 2 for the investigated chromatographed systems. In a graphical presentation the $R_{Me} = f(R_{Mc})$ plot should be linear for both theories and for both stationary phases. In the case of an ideal agreement between the experimental and theoretical values the relationship $R_{Me} = f(R_{Mc})$ is represented by a straight line passing through the origin with a slope equal to unity. Because of deviations from the ideal relationship, a graphical documentation should be completed by a tabulation of the parameters of the

$$R_{Me} = aR_{Mc} + b \tag{3}$$

equation where a is the slope and b is the y-intercept.

For mobile phase systems containing members of the alcohol homologous series (ethanol-methanol and n-propanol-methanol) there is a good agreement between the experimental and theoretical data indicating the validity of the $R_{Me} = f(R_{Mc})$ relationship, and the relationship is linear. The value of the intercept is small. Both Eqs 1 and 2 are more valid for ethanol-methanol mixture, either on SiO_2 or on chitin (Fig. 9). This fact can be explained by the fact that the mobile phase consists of components having similar polarity, chemical structure and molecule size: they are the subsequent members of the homologous series. For mixtures containing solvents with more different polarity, e.g. n-propanol-methanol, the $R_{Me} = f(R_{Mc})$ relationship is also more differentiated in respect either to both optimization theories or to the stationary phase used. The differences between the values calculated from Eqs 1 and 2 are greater, especially when chitin is used as the stationary phase (Fig. 10). Significant differences are also observed in the case of an acetone-alcohol mixture used as the mobile phase. In spite of the fact that the $R_{Me} = f(R_{Mc})$ relationship is still linear, significantly greater deviations are observed between the theoretical and experimental values; also the deviation of the individual points from the linear plot is significant. Similar to the case of using n-propanol-methanol as the mobile phase the

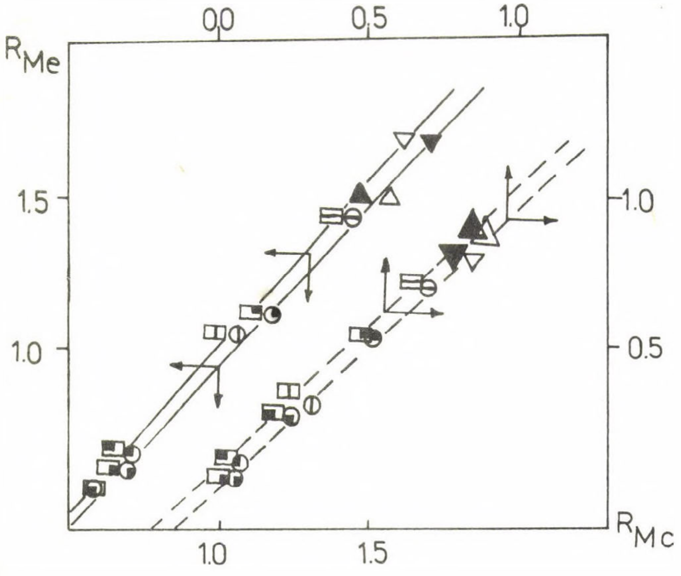

Fig. 9. Relationship between the experimental R_M values (R_{Me}) and the theoretical R_M values (R_{Mc}) calculated from Eq. 1 (○) and from Eq. 2 (□) on SiO_2 (solid lines) and chitin (dashed lines) layers. Mobile phase: 0.9 volume fraction C_2H_5OH + 0.1 volume fraction CH_3OH.

⊖ - glycine;	◐ - phenylalanine;
⦶ - alanine;	◕ - threonine;
◔ - valine;	△ - serine;
◑ - leucine;	▽ - histidine

Values of the slope (a) and intercept (b) of Eq. 3 in methanol-ethanol mobile phase; x_1 is the mole fraction of methanol

x_1	SiO_2				Chitin			
	Eq. 1		Eq. 2		Eq. 1		Eq. 2	
	a	b	a	b	a	b	a	b
0.1	1.02	0.02	1.00	0.00	1.00	0.00	1.00	-0.04
0.3	1.02	-0.15	0.82	0.38	1.00	0.00	1.00	-0.02
0.5	1.00	0.00	0.82	0.00	1.00	0.02	0.80	-0.06
0.7	1.00	0.00	1.00	-0.30	1.00	0.08	0.90	0.00
0.9	1.11	0.02	1.11	0.00	1.00	0.02	1.00	-0.02

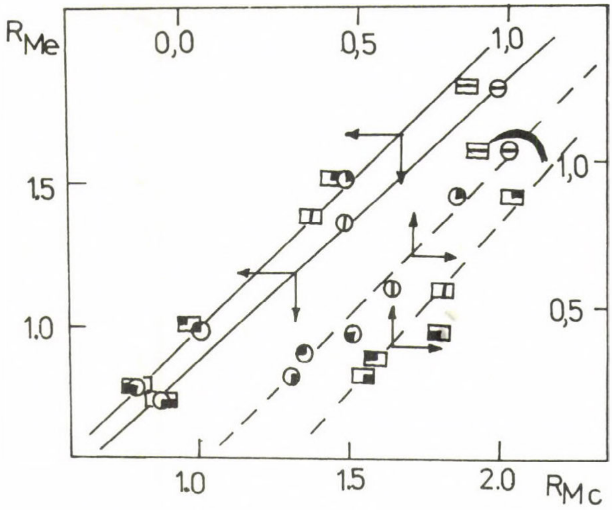

Fig. 10. Relationship between the experimental R_M values (R_{Me}) and the theoretical values (R_{Mc}) calculated from Eq. 1 (○) and Eq. 2 (□) on SiO_2 (solid lines) and chitin (dashed lines) layers. Mobile phase: 0.9 volume fraction of n-propanol + 0.1 volume fraction of metanol.

⊖ - glycine;	◔ - leucine;
⦶ - alanine;	◐ - phenylalanine;
◑ - valine;	◕ - threonine

Values of the slope (a) and intercept (b) of Eq. 3 in n-propanol-methanol mobile phase; x_1 is the mole fraction of methanol

x_1	SiO_2				Chitin			
	Eq. 1		Eq. 2		Eq. 1		Eq. 2	
	a	b	a	b	a	b	a	b
0.1	0.90	0.10	0.88	0.16	1.00	0.00	1.14	0.30
0.3	1.00	0.00	1.14	0.00	1.30	0.80	2.14	0.26
0.5	1.00	0.00	1.25	0.00	1.00	0.02	1.53	0.09
0.7	1.12	0.02	1.14	0.01	1.01	0.02	1.23	-0.02
0.9	1.06	0.07	1.00	0.00	1.00	0.10	0.89	-0.06

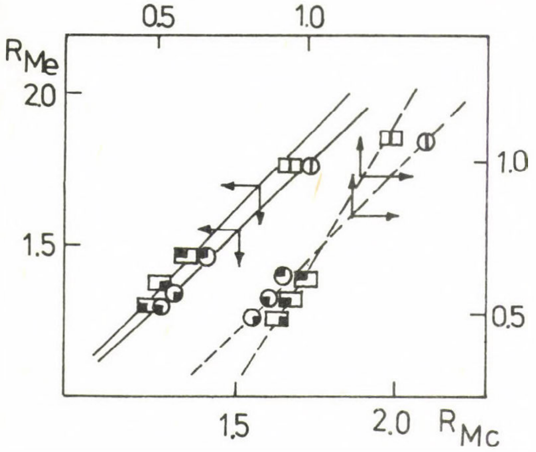

Fig. 11. Relationship between the experimental R_M values R_{Me} and the theoretical values R_{Mc} calculated from Eq. 1 (○) and Eq. 2 (□) on SiO_2 (solid lines) and chitin (dashed lines) layers. Mobile phase: 0.9 volume fraction of acetone + 0.1 volume fraction of methanol.

⦶ - alanine; ◕ - leucine;

◐ - valine; ◒ - phenylalanine

Values of the slope (a) and intercept (b) of Eq. 3 in acetone-methanol mobile phase; x_1 is the mole fraction of methanol

x_1	SiO_2				Chitin			
	Eq. 1		Eq. 2		Eq. 1		Eq. 2	
	a	b	a	b	a	b	a	b
0.1	0.92	0.20	1.00	0.14	1.17	-0.05	2.30	-1.16
0.3	1.00	0.00	1.50	0.26	1.27	-0.20	2.22	-0.51
0.5	1.00	0.00	1.00	0.20	1.27	0.08	2.50	0.00
0.7	1.00	-0.02	1.07	0.19	1.00	0.04	1.70	-0.11
0.9	1.00	0.08	1.20	0.02	2.50	0.02	1.19	-0.02

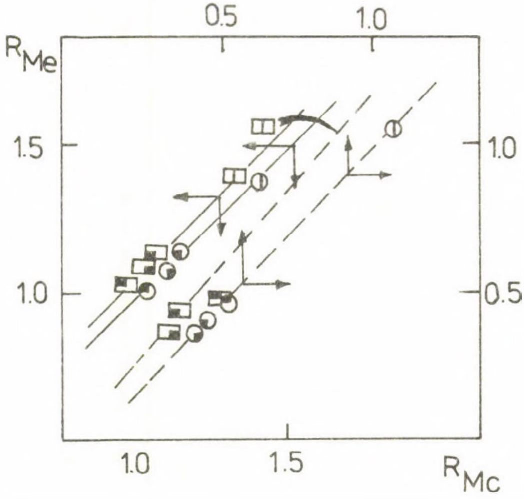

Fig. 12. Relationship between experimental R_M values R_{Me} and theoretical values R_{Mc} calculated from Eq. 1 (○) and from Eq. 2 (□) on SiO_2 (solid lines) and chitin (dashed lines) layers. Mobile phase: 0.7 volume fraction of aceton + 0.3 M volume fraction of ethanol.

⦶ - alanine; ◔ - leucine;

◕ - valine; ◒ - phenylalanine

Values of the slope (a) and intercept (b) of Eq. 3 in ethanol-acetone mobile phase; x_1 is mole fraction of acetone

x_1	SiO_2				Chitin			
	Eq. 1		Eq. 2		Eq. 1		Eq. 2	
	a	b	a	b	a	b	a	b
0.1	0.95	0.08	1.15	-0.16	1.05	-0.03	1.70	-0.05
0.3	1.10	-0.13	1.10	-0.05	1.15	-0.01	1.15	0.45
0.5	1.10	0.01	1.20	0.06	1.15	0.21	1.20	-0.03
0.7	1.10	-0.10	1.10	0.04	1.00	-0.01	1.20	-0.02
0.9	1.05	-0.08	1.05	0.00	1.10	-0.04	1.30	-0.02

differences are greater for the chromatographic systems containing chitin as the stationary phase. This is illustrated graphically in Figs 11 and 12.

The differences between the experimental and theoretical values calculated from Eqs 1 and 2 can be explained by the non-ideality of the investigated chromatographic system. Both theories assume a lack of specific intermolecular interactions in the chromatographic systems and an energetical homogeneity of the adsorbent surface; on the other hand, in the investigated system active mobile phases are used because the separated substances (amino acids) are very polar. From earlier investigations it is known that SiO_2 has an energetically heterogeneous surface (12) and chitin, because of the presence of various functional groups on its surface, also represents an energetically heterogeneous stationary phase. In spite of these inaccuracies, however, both theories can successfully be used for the preliminary determination of the separation conditions of amino acids on chitin layers.

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SOME PROBLEMS OF USING THIN-LAYER CHROMATOGRAPHY AS A PILOT TECHNIQUE FOR LIQUID COLUMN CHROMATOGRAPHY IN THE ANALYSIS OF ORGANIC COMPOUNDS

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INTRODUCTION

Due to the ease of the application of thin-layer chromatography to study the mechanism and selection of optimal conditions of the chromatographic process there is a growing interest in using this method as a pilot technique for high-performance liquid column chromatography. For this purpose correlations existing between the parameters describing the separation in both chromatographic techniques, i.e., between the R_F (R_M) value and the capacity factor k' of the chromatographed substance have to be defined.

In previous papers (1-3) we have shown that the type of mobile phase, its composition, the structures of the chromatographed substances and the types of chromatographic techniques compared significantly influence these correlations. The present paper deals with the efforts to utilize the thermodynamic description of the chromatographic process in the characterization of thin-layer chromatography as a pilot technique for column chromatography.

In the thermodynamic approach Os̀cik's equation (4-6) permits the calculation of the R_M values of the chromatographed substance:

$$R_{M12} = x_1 R_{M1} + x_2 R_{M2} + (x_1^s - x_1)(\Delta R_{M12} + A_{12}) \quad (1)$$

$$\Delta R_{M12} = R_{M1} - R_{M2}$$

where R_{M12}, R_{M1} and R_{M2} are the R_M values of the chromatographed substance obtained in a binary mobile phase and in pure solvents 1 and 2, respectively; x_1 and x_2 are the molar fractions of the mobile phase components; x_1^s is the molar fraction of the first component of the mobile phase in the surface phase; A_{12} is a constant characterizing the interactions between the chromatographed substance and the mobile phase components. The value of $(\Delta R_{M12} + A_{12})$ is constant for a given test substance in a given chromatographic system (adsorbent and mobile phase). Therefore, we can write that $R_{M12} + A_{12} = C$.

Assuming ideality of the bulk and surface phases and homogeneity of the adsorbent surface we can calculate the molar fraction of the more polar mobile phase component in the surface phase using Everett's equation (7):

$$x_1^s = \frac{K_{12}x_1}{x_2 + K_{12}x_1} \qquad (2)$$

where K_{12} denotes the adsorption equilibrium constant in the solid-binary solution system. After simple transformations (8) Eq. 1 assumes a linear form in which a function in the left-hand side is denoted as G:

$$G = \frac{x_1x_2}{R_{M12} - x_1R_{M1} - x_2R_{M2}} =$$

$$\frac{1}{C}\left(\frac{1}{K_{12} - 1} + x_1\right) = a\ x_1 + b \qquad (3)$$

where $a = \frac{1}{C}$ and

$$b = \frac{1}{C\ (K_{12} - 1)}$$

thus

$$K_{12} = \frac{a}{b} + 1 \qquad (4)$$

and $$A_{12} = \frac{1}{a} - \Delta R_{M12} \qquad (5)$$

From boundary conditions of Eq. 3 the K_{12} and A_{12} values can be calculated because:

for $x_1 = 0$ $G = b$
for $x_1 = 1$ $G = a + b$

The K_{12} values determined from chromatographic data according to Eq. 4 will be indicated below as K_{12G} (8).

Another way to calculate the value of K_{12} is from data obtained by liquid-adsorption chromatography (9):

$$K_{12} = 10^{-\Delta R_{M12}} \tag{6}$$

The K_{12} values determined according to Eq. 6 will be indicated as $K_{12\Delta}$ (8).

According to the equations presented above we have investigated the relationship between the $K_{12\Delta}$, K_{12G} and A_{12} values and the type of chromatographic technique. In addition we have tried to utilize the chromatographic parameters for the theoretical prediction of the separation in liquid column chromatography.

EXPERIMENTS

I. Thin-layer chromatography measurements. The R_M values of the investigated substances were determined by thin-layer adsorption chromatography. Silica gel 60 (Merck) was used as the adsorbent. Pure heptane and cyclohexane and their binary mixture served as the mobile phase. Chromatographed substances were naphthalene and its methyl derivatives and various aromatic hydrocarbons were used as the test substances. Two types of chromatographic chambers were used: Stahl's chamber and a sandwich chamber.

II. Liquid column chromatography measurements. The same mobile phases, adsorbent and test substances as mentioned above were also used in these measurements. The measurements were carried out in a liquid chromatograph Type 302 (PPDRP Techma-Robot, Warsaw, Poland), equipped with a UV detector.

A more detailed description of the measurements was presented in our previous papers (1-3).

RESULTS AND DISCUSSION

Figures 1 and 2 present the R_M vs. x_1 relationships obtained by TLC and liquid column chromatography. As seen the R_M vs. x_1 relationship obtained by TLC (in Stahl's chambers) and column chromatography is very similar for all investigated substances. This is particularly true for naphthalene and its derivatives (Fig. 1).

For aromatic hydrocarbons the difference in the R_M vs. x_1 relationships obtained by TLC and column chromatography is more pronounced (Fig. 2).

When using a sandwich chamber completely different R_M vs. x_1 relationships were obtained: in each case the R_M values obtained in sandwich chambers are significantly smaller than those obtained in Stahl's chambers or in the chromatographic column.

Figures 3 and 4 plot the G vas. x_1 relationships determined according to Eq. 3. In each case straight lines are obtained which is in good agreement with the theoretical prediction.

Equation 3 permits the calculation of the K_{12G} (Eq. 4) and A_{12} (Eq. 5) values characterizing a given chromatographic system.

Table I presents the $K_{12\Delta}$ values calculated according to Eq. 6 and the K_{12G} and A_{12} values calculated according to Eq. 4 and 5 for the three chromatographic techniques. As seen the $K_{12\Delta}$ values calculated for all three chromatographic techniques are practically constant. Only in the case of aromatic hydrocarbons are the K_{12} values calculated from data obtained by column chromatography differ from the K_{12G} values obtained from TLC measurements.

The K_{12G} and A_{12} values obtained by TLC have permitted the theoretical calculation of the capacity factors (k') of the individual substances. According to the theoretical relationship

$R_M = \log k'$

Eq. 1 assumes the following form:

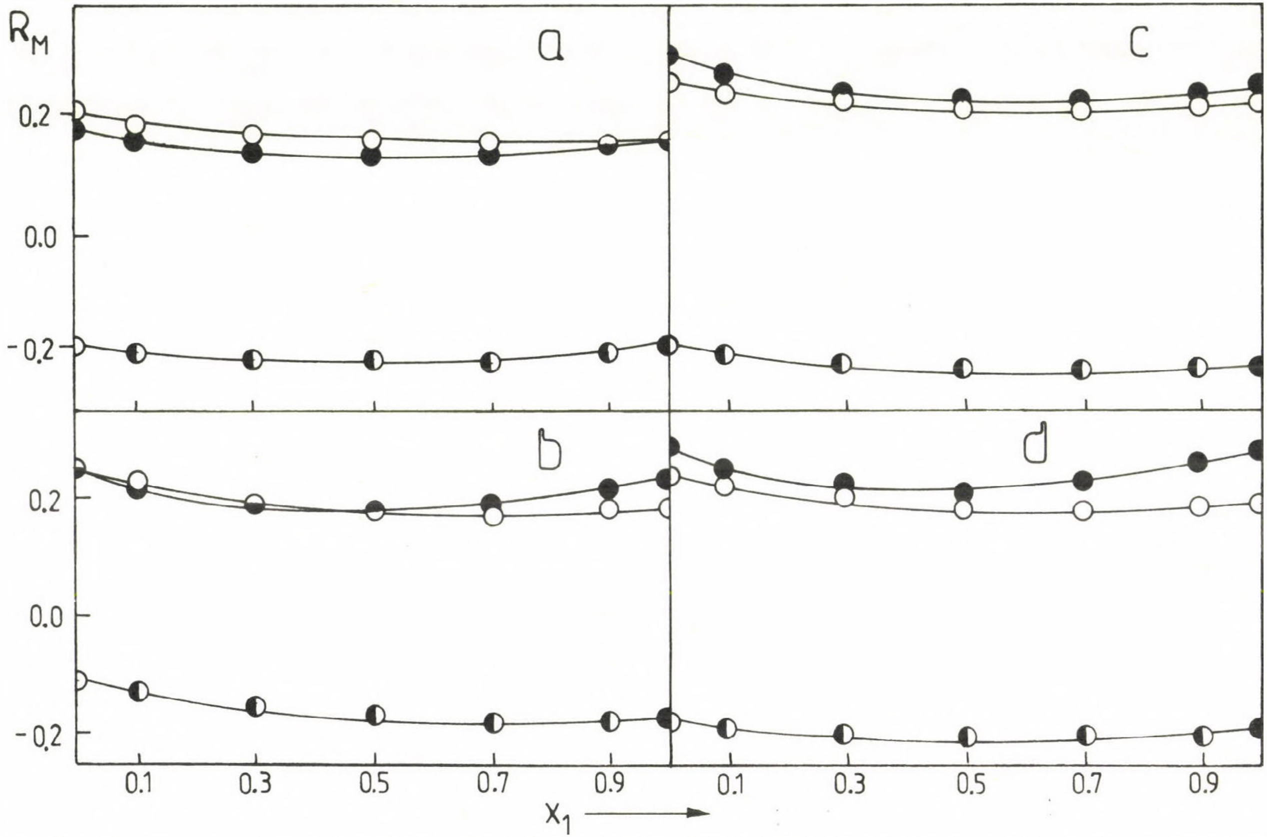

Fig. 1. R_M vs. x_1 relationships for (a) naphthalene, (b) 1-methylnaphthalene, (c) 1,3-dimethylnaphthalene, and (d) 1,4-dimethylnaphthalene. Chromatographic measurements: ○ TLC in Stahl's chamber; ◐ TLC in sandwich chamber; ● liquid column chromatography

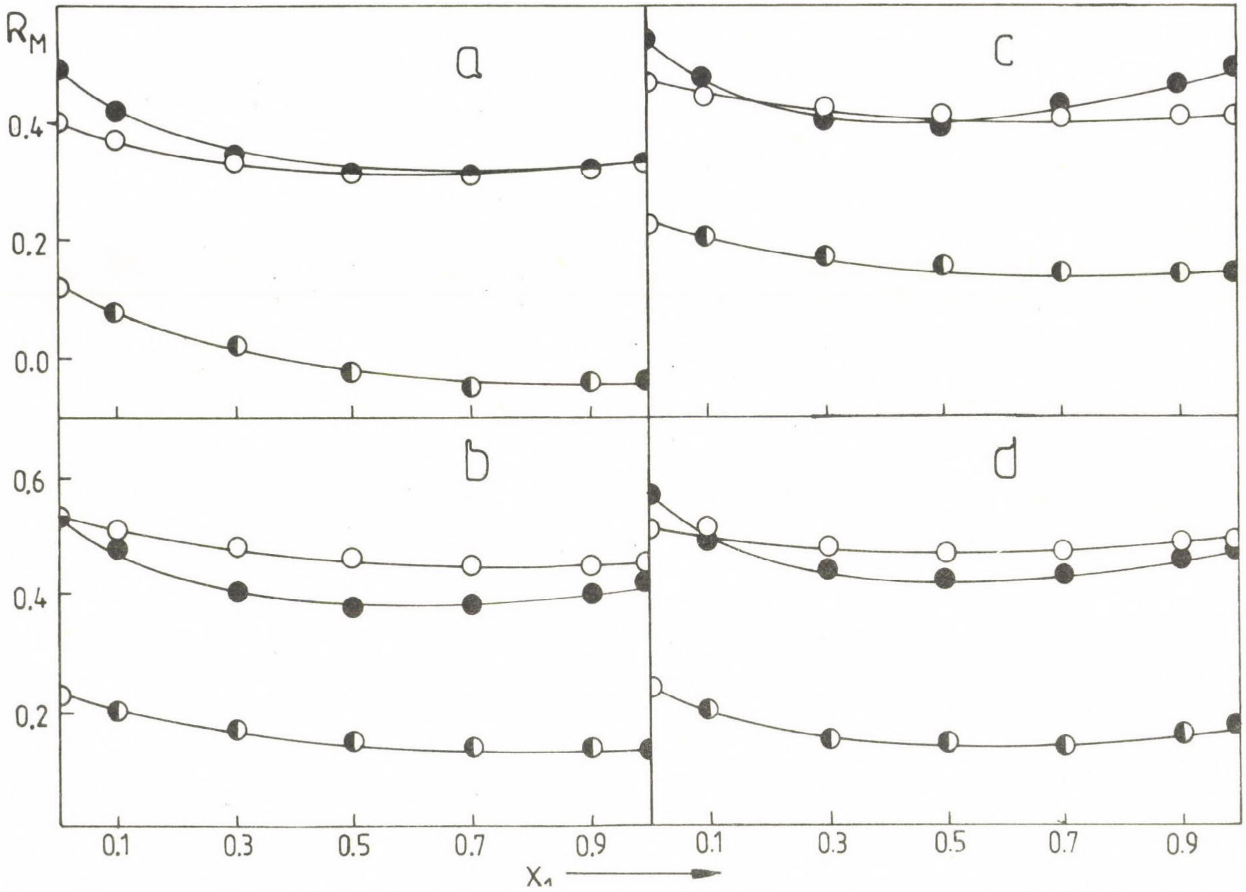

Fig. 2. R_M vs. x_1 relationships for (a) diphenyl, (b) anthracene, (c) fenanthrene, and (d) pyrene. Chromatographic measurements: ○ TLC in Stahl's chamber; ◐ TLC in sandwich chamber; ● liquid column chromatography

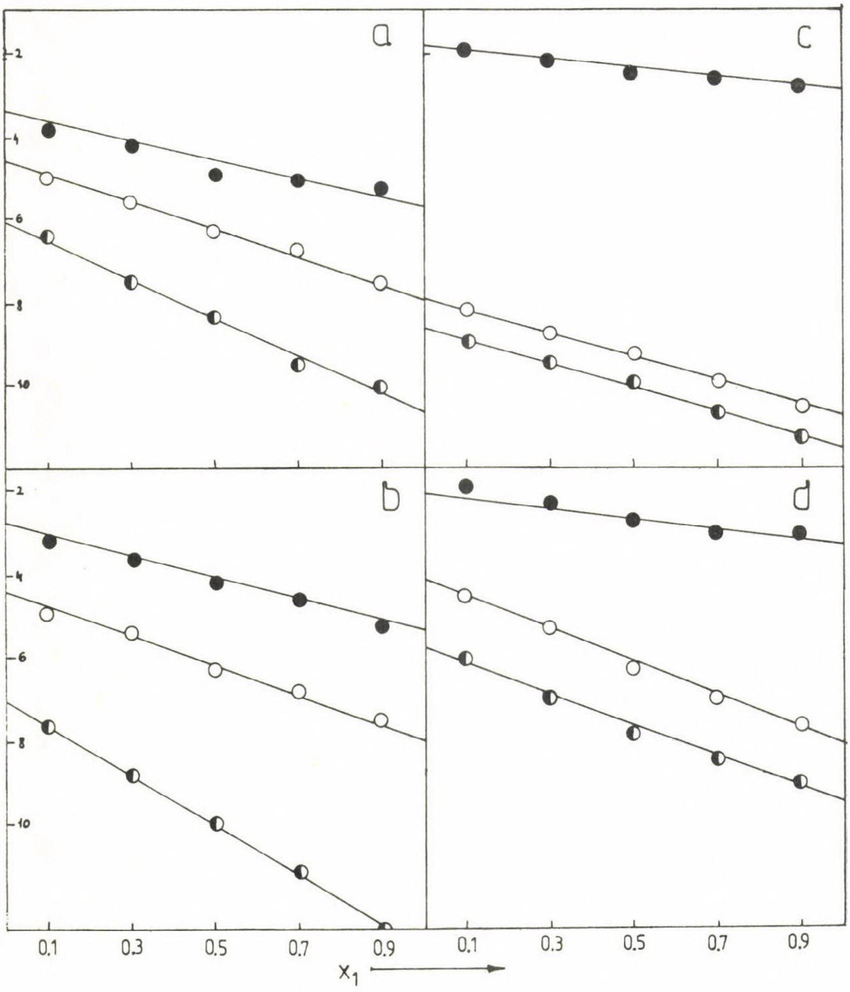

Fig. 3. G vs. x_1 relationships for (a) 1-methylnaphthalene, (b) 2-methylnaphthalene, (c) 2,3-dimethylnaphthalene, and (d) 2,6-dimethylnaphthalene. Chromatographic measurements: ○ TLC in Stahl's chamber; ◐ TLC in sandwich chamber; ● liquid column chromatography

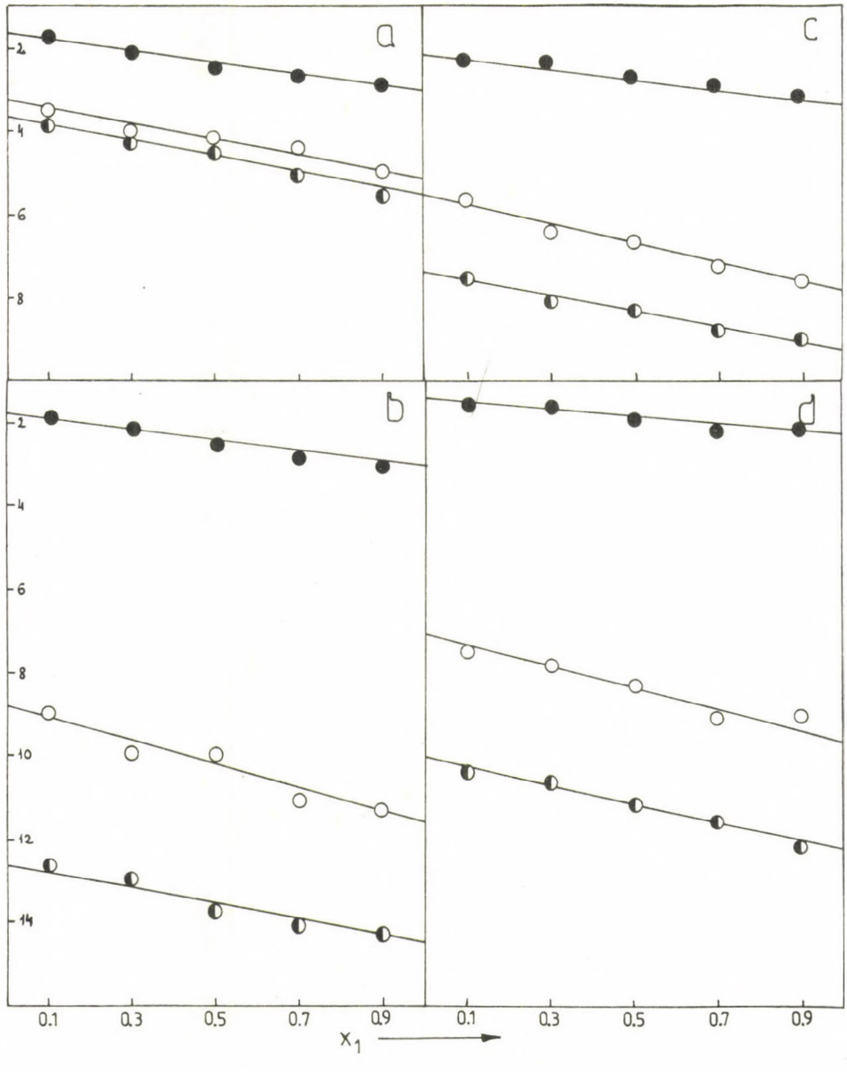

Fig. 4. G vs. x_1 relationships for (a) diphenyl, (b) pyrene, (c) anthracene, and (d) fluoranthene. Chromatographic measurements: ○ TLC in Stahl's chamber; ◐ TLC in sandwich chamber; ● liquid column chromatography

Table I. $K_{12\Delta}$, K_{12G} and A_{12} values obtained by (I) TLC measurements in Stahl's chamber; (II) TLC measurements in sandwich chamber; and (III) liquid column chromatography. Mobile phase: heptane - cyclohexane; adsorbent: silica gel 60

Test substances	I			II			III		
	$K_{12\Delta}$	K_{12G}	A_{12}	$K_{12\Delta}$	K_{12G}	A_{12}	$K_{12\Delta}$	K_{12G}	A_{12}
naphthalene	1.12	4.32	-0.02	1.00	2.24	-0.10	1.05	2.26	-0.16
1-methylnaphthalene	1.05	1.74	-0.28	1.10	1.75	-0.18	1.05	1.68	-0.27
2-methylnaphthalene	1.17	1.80	-0.20	1.17	1.87	-0.07	1.05	1.95	-0.36
1,3-dimethylnaphthalene	1.07	1.47	-0.33	1.09	1.25	-0.23	1.12	1.47	-0.58
1,4-dimethylnaphthalene	1.12	1.70	-0.21	1.02	1.70	-0.15	1.02	1.89	-0.48
1,5-dimethylnaphthalene	1.12	2.10	-0.09	1.07	1.63	-0.14	1.05	1.57	-0.35
2,3-dimethylnaphthalene	1.05	1.36	-0.33	1.07	1.33	-0.31	1.05	1.61	-0.89
2,6-dimethylnaphthalene	1.00	1.94	-0.26	1.12	1.65	-0.22	1.02	1.60	-0.82
diphenyl	1.17	1.51	-0.46	1.45	1.60	-0.35	1.45	1.76	-0.61
anthracene	1.20	1.27	-0.43	1.23	1.42	-0.34	1.29	1.53	-0.76
fenanthrene	1.15	1.46	-0.25	1.20	1.30	-0.37	1.12	1.69	-0.86
chrysene	1.02	1.45	-0.37	1.15	1.53	-0.71	1.41	1.52	-1.52
pyrene	1.07	1.32	-0.33	1.17	1.68	-0.49	1.26	1.61	-0.81
fluoranthene	1.02	1.39	-0.36	1.15	1.36	-0.39	1.32	1.52	-1.21

Table II. Experimentally measured (k'_{exp}) and theoretically calculated (k'_{th}) capacity factor values. Calculation of k'_{th} was carried out on the basis of TLC measurements in (I) Stahl's chamber [$K_{12G} = 1.77$, $A_{12} = -0.17$] ; and (II) sandwich chamber [$K_{12G} = 1.59$, $A_{12} = -0.175$]

Substances	x_1	k'_{exp}	k'_{th}(I)	k'_{th}(II)
naphthalene	0.1	1.41	1.48	1.48
	0.3	1.41	1.41	1.41
	0.5	1.45	1.38	1.41
	0.7	1.45	1.41	1.41
	0.9	1.45	1.41	1.45
1-methyl-naphthalene	0.1	1.58	1.74	1.74
	0.3	1.62	1.66	1.66
	0.5	1.58	1.62	1.66
	0.7	1.55	1.66	1.66
	0.9	1.58	1.66	1.70
2-methyl-naphthalene	0.1	1.58	1.74	1.74
	0.3	1.62	1.66	1.66
	0.5	1.55	1.62	1.66
	0.7	1.55	1.66	1.66
	0.9	1.58	1.66	1.70
1,3-dimethyl-naphthalene	0.1	1.86	1.95	1.95
	0.3	1.82	1.86	1.86
	0.5	1.78	1.78	1.82
	0.7	1.78	1.78	1.82
	0.9	1.78	1.82	1.82
1,4-dimethyl-naphthalene	0.1	1.74	1.91	1.91
	0.3	1.62	1.82	1.86
	0.5	1.62	1.78	1.82
	0.7	1.66	1.82	1.86
	0.9	1.78	1.86	1.86
1,5-dimethyl-naphthalene	0.1	1.78	2.00	2.00
	0.3	1.70	1.91	1.91
	0.5	1.78	1.86	1.91
	0.7	1.78	1.91	1.91
	0.9	1.82	1.91	1.95
2,3-dimethyl-naphthalene	0.1	2.00	2.29	2.29
	0.3	1.86	2.19	2.19
	0.5	1.86	2.13	2.19
	0.7	1.86	2.19	2.19
	0.9	2.00	2.19	2.14
2,6-dimethyl-naphthalene	0.1	1.86	2.09	2.09
	0.3	1.74	2.00	2.04
	0.5	1.78	2.00	2.00
	0.7	1.78	2.00	2.04
	0.9	2.00	2.04	2.04

Table III. Experimentally measured (k'_{exp}) and theoretically calculated (k'_{th}) capacity factor values. Calculation of k'_{th} was carried out on the basis of TLC measurements in (I) Stahl's chamber [$K_{12G} = 1.77$, $A_{12} = -0.367$]; and (II) sandwich chamber [$K_{12G} = 1.59$, $A_{12} = -0.44$]

Test substances	x_1	k'_{exp}	k'_{th}(I)	k'_{th}(II)
diphenyl	0.1	2.45	2.75	2.75
	0.3	2.19	2.40	2.40
	0.5	2.14	2.19	2.19
	0.7	2.04	2.09	2.14
	0.9	2.04	2.09	2.14
anthracene	0.1	2.82	3.09	3.09
	0.3	2.40	2.69	2.75
	0.5	2.40	2.57	2.57
	0.7	2.45	2.51	2.51
	0.9	2.51	2.57	2.57
fenanthrene	0.1	2.88	3.24	3.24
	0.3	2.51	2.95	2.95
	0.5	2.63	2.89	2.89
	0.7	2.63	2.89	2.89
	0.9	2.82	3.02	3.02
chrysene	0.1	5.50	6.46	6.60
	0.3	4.47	5.62	5.62
	0.5	4.57	5.12	5.25
	0.7	4.47	5.01	5.01
	0.9	4.68	5.01	5.01
pyrene	0.1	3.02	3.39	3.39
	0.3	2.75	3.02	3.02
	0.5	2.69	2.88	2.88
	0.7	2.69	2.82	2.82
	0.9	2.88	2.88	2.88
fluoranthene	0.1	4.17	4.90	4.90
	0.3	3.47	4.27	4.37
	0.5	3.63	3.98	4.07
	0.7	3.89	3.89	3.98
	0.9	3.89	3.98	3.98

$$\log k'_{12} = x_1 \log k'_1 + x_2 \log k'_2 + (x_1^s - x_1) \left(\log \frac{k'_1}{k'_2} + A_{12}\right) \qquad (7)$$

where

$$\log k'_1 = R_{M1} \quad \text{and} \quad \log k'_{12} = R_{M12}$$

Tables II and III present experimentally measured and theoretically calculated (according to Eq. 7) values of the capacity factors k' using K_{12G} and A_{12} values obtained from the TLC measurements in Stahl's chambers and in sandwich chambers. For all the investigated substances the k' values calculated from the two TLC measurements are almost identical. In the case of naphthalene and its methyl derivatives (Table II) very good agreement exists between the theoretically calculated and experimentally measured k' values in the whole concentration range of the binary mobile phase. In the case of aromatic hydrocarbons (Table III) significant differences exist between the theoretically calculated and experimentally measured k' values for low mobile phase concentrations, i.e., for $x_1 = 0.1$ and 0.3, but in all the other cases the agreement is very good.

These investigations show that the thermodynamic approach to the chromatographic process used so far mainly with respect to thin-layer chromatography may also be successfully used to column chromatography. The results presented in this paper indicate new possibilities in the application of thin-layer chromatography as a pilot technique for column chromatography, realized in systems with nonactive mobile phases.

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SPECIFIC SURFACE AREA OF THE ADSORBENT AS THE PARAMETER OF OPTIMIZATION IN ADSORPTION THIN-LAYER CHROMATOGRAPHY

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Adsorbents with different microporous structure have been used for a long time in thin-layer chromatography (TLC) for the separation of different substances (1-4). The microporous structure of adsorbents is an important parameter influencing the optimization of the chromatographic process. The role of the microporous structure of the adsorbent in TLC has been discussed in detail by Geiss (1) and Snyder (5). Practical possibilities for the regulation of the chromatographic process on the basis of different microporous structures have also been demonstrated by Rózyło (6,7). We have also tried to explain the physico-chemical meaning of the specific surface area of the adsorbent in TLC (8). There is an increasing interest in the role of the specific surface area of the adsorbent in TLC because of the increasing significance of this method as a pilot technique for the determination of the optimal separation conditions on both analytical and preparative scale. TLC is widely utilized for the recovery and purification of natural substances in research laboratories in the perfume and food industries as well in biochemistry (9).

It has been observed earlier that linear relationships exist between the differences in the R_M values and the specific surface area of the adsorbent. However, these relationships are not too convenient for routine use in the optimization of the separation conditions. Because the R_M values are linearly dependent on the adsorbent's surface area it has been investigated how the R_M values in single and binary mobile phases are dependent on the specific surface area of adsorbent. In this connection changes in the R_M values of the chromatographed substances on silica gel different specific surface area ranging from 50 to 100 m^2/g have been described (10, 11).

On the basis of the experimental data it was concluded that in the case of substances showing neither electron-acceptor nor electron-donor properties, the R_M values change in a regular manner, and a linear relationship exists between the R_M values and the specific surface area of the adsorbent. This relationship can be presented graphically in the form of a straight line, independently of the nature or the mobile phase (Figs 1 and 2).

$$R_M = as + b \tag{1}$$

where: s is the specific surface area of the adsorbent; and a, b are the parameters of the straight line.

Another relationship can be observed in the case substances which contain active substituents in their molecules (active substances)(11). In this case the relationships are parabolic. Because of specific properties of the investigated substances (phenol, acridine, quinoline and his methyl derivatives) only N - AB systems containing active solvents, according to the classification of Pimentel and Mc Clellan (12) can be used, because in N - N systems the R_M values were too high.

From a mathematical point of view the relationships can be presented in the following form:

$$R_M = ls^2 + as + b \tag{2}$$

where: l denotes the parameter describing the specific intermolecular interactions of the substance in the chromatographic system, a represents the parameter describing the non-specific intermolecular interactions between the chromatographed substance and other components of the chromatographic system, and b is the parameter describing the intermolecular interactions between the chromatographed substance and the mobile phase components if specific intermolecular interactions do

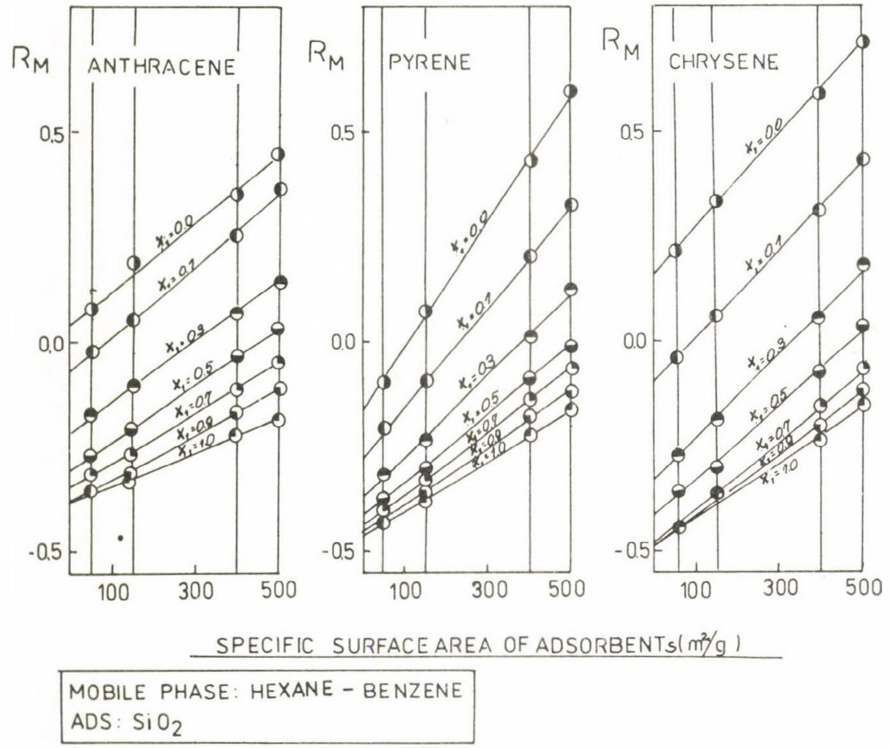

Fig. 1. The R_M values of selected substances as a function of the specific surface area (s) of the adsorbent (SiO_2). Mobile phase: hexane-benzene; volume fraction (x_1) of benzene in the mobile phase as indicated. Points represent measured values while the lines correspond to theoretically calculated values, using the equations given in the paper

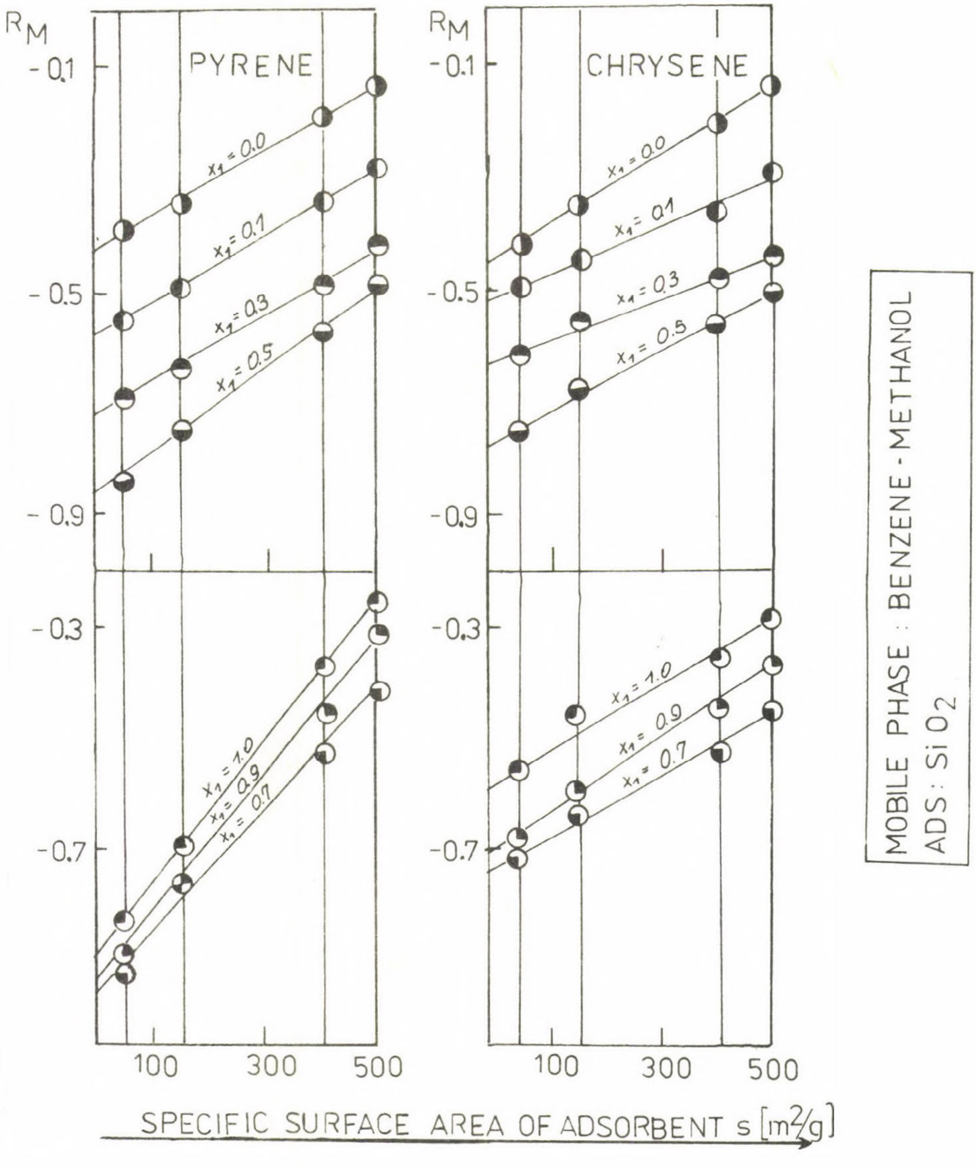

Fig. 2. The R_M values of selected substances as a function of the specific surface area (s) of the adsorbent (SiO_2). Mobile phase: benzene-methanol; volume fraction (x_1) of methanol in the mobile phase as indicated. Points represent measured values while the lines correspond to theoretically calculated values, using the equations given in the paper

not take place, then l = 0, and eq. (2) is simplified to eq. (1).

In our earlier papers (10, 11), we have tabulated a, b and l values for equations (1) and (2) and tried to establish correlations between these parameters and the physico-chemical characteristics of the components of the chromatographic system, i.e., of the chromatographed substance and the mobile phase components.

The main purpose of this paper is to verify the usefulness of earlier theoretical considerations for practical purposes.

In this connection we have attempted to define:

1. The R_M values of the chromatographed substances on the basis of the specific surface area of the adsorbent, using for non-active substances the parameters of equation (1) and for active substances the parameters of equation (2).
2. The specific surface area of the adsorbent, on the basis of the R_M values.

The following substances were tested:

(a) naphthalene, chrysene, and pyrene. They were chromatographed using hexane-benzene and benzene-methanol as the mobile phase.

(b) acridine, phenol, 6-methylquinoline and 8-methylquinoline. They were chromatographed using benzene-methanol and benzene-propanol as the mobile phase.

In all the systems investigated the volume fraction (x_1) of the more active component of the mobile phase was equal to 0.3, 0.5 and 0.7.

The R_M values were measured on three adsorbents with specific surface areas of 200, 230 and 355 m^2/g. The determination of the specific surface area and the chromatographic measurements have been described elsewhere (10, 11). The data obtained from these measurements were utilized for the verification of the theoretical considerations. Table I lists the a and b values which have been utilized in the derivation of the simple linear relationship for the selected non-active sub-

Table I. Comparison of the a and b coefficients of eq. 1 for some non-active substances

	Volume fraction (x_1) of benzene in hexane-benzene mobile phase		
	0.3	0.5	0.7
1-methylnaphthalene			
a x 10^2	0.08	0.05	0.02
b	-0.28	-0.37	-0.36
Chrysene			
a x 10^2	0.12	0.06	0.08
b	-0.21	-0.42	-0.47
Pyrene			
a x 10^2	0.12	0.11	0.10
b	-0.40	-0.55	-0.50
	Volume fraction (x_1) of methanol in benzene-methanol mobile phase		
	0.3	0.5	0.7
1-methylnaphthalene			
a x 10^2	0.05	0.07	0.10
b	-0.79	-0.86	-0.98
Pyrene			
a x 10^2	0.06	0.07	0.11
b	-0.72	-0.85	-0.85

stances while Table II lists the parabolic parameters for the selected active substances.

Figures 3-7 compare the experimental R_M values with the theoretical values obtained on the basis of the data listed in Table I and II. The lines present the theoretical relationships obtained from eq. 1 and Table I (10) while the points correspond to experimentally measured R_M values. Analogical relationships are presented in Figs 6 and 7, but now they correspond to the substances with a constant dipole moment (11).

In order to better illustrate the obtained results we have also tabulated the theoretical R_M values calculated from eqs 1 and 2 and the corresponding experimentally measured R_M values (Tables III and IV). From the data presented here it can be concluded that when the parameters of the $R_M = f(s)$ relationship are known one can predict with a relatively good

Table II. Comparison of the l, a and b coefficients of eq. 2 for some active substances

Substance	Volume fraction of the alcohol (x_1)								
	0.3			0.5			0.7		
	$l \cdot 10^4$	a	b	$l \cdot 10^4$	a	b	$l \cdot 10^4$	a	b
Mobile phase: benzene-methanol									
phenol	0.012	0.005	0.020	0.052	0.065	0.000	-0.730	-0.840	-0.837
acridine	0.014	0.010	0.010	0.066	0.024	0.020	-0.730	-0.750	-0.720
6-methylquinoline	0.011	0.013	0.022	0.046	0.023	-0.024	-0.670	-0.740	-0.720
8-methylquinoline	0.022	0.020	0.028	0.018	-0.015	-0.037	-0.740	-0.850	-0.850
Mobile phase: benzene-propanol									
phenol	0.015	0.016	0.022	0.002	0.001	-0.021	-0.610	-0.760	-0.760
acridine	0.012	0.019	0.026	0.017	0.000	-0.034	-0.560	-0.720	-0.720
6-methylquinoline	0.010	0.012	0.004	0.030	0.038	0.074	-0.460	-0.580	-0.570
8-methylquinoline	0.012	0.012	0.007	0.015	0.012	0.057	-0.640	-0.700	-0.720

Table III. Comparison of experimentally measured R_M values (R_{Me}) for non-active substances and R_M values calculated (R_{Mt}) from eq. 1, using the coefficients listed in Table I

Substance	Mobile phase	Specific surface area of SiO_2 m^2/g	Volume fraction (x_1) of the more polar component of the mobile phase					
			0.3		0.5		0.7	
			R_{Me}	R_{Mt}	R_{Me}	R_{Mt}	R_{Me}	R_{Mt}
1-methylnaphthalene	Hexane-benzene	200	-0.11	-0.12	-0.27	-0.27	-0.32	-0.32
chrysene			0.00	0.05	-0.30	-0.30	-0.34	-0.31
pyrene			-0.16	-0.20	-0.32	-0.33	-0.32	-0.30
1-methylnaphthalene		230	-0.13	-0.10	-0.26	-0.26	-0.32	-0.31
chrysene			-0.08	-0.07	-0.30	-0.28	-0.32	-0.29
pyrene			-0.12	-0.12	-0.30	-0.30	-0.30	-0.27
1-methylnaphthalene		355	0.00	-0.60	-0.20	-0.20	-0.30	-0.29
chrysene			0.20	0.21	-0.22	-0.21	-0.20	-0.19
pyrene			0.00	0.02	-0.16	-0.17	-0.16	-0.15
1-methylnaphthalene	Benzene-methanol	200	-0.71	-0.69	-0.72	-0.72	-0.80	-0.78
pyrene			-0.55	-0.60	-0.71	-0.71	-0.62	-0.63
1-methylnaphthalene		230	-0.68	-0.68	-0.72	-0.70	-0.74	-0.75
pyrene			-0.60	-0.59	-0.66	-0.69	-0.60	-0.60
1-methylnaphthalene		355	-0.62	-0.62	-0.64	-0.62	-0.60	-0.63
pyrene			-0.55	-0.51	-0.62	-0.61	-0.45	-0.47

Table IV. Comparison of experimentally measured R_M values (R_{Me}) for active substances and R_M values calculated (R_{Mt}) from eq. 2, using the coefficients listed in Table II

Substance	Mobile phase	Specific surface area of SiO_2 m^2/g	Volume fraction (x_1) of the alcohol in the mobile phase					
			0.3		0.5		0.7	
			R_{Me}	R_{Mt}	R_{Me}	R_{Mt}	R_{Me}	R_{Mt}
phenol	Benzene-methanol	200	-0.60	-0.58	-0.69	-0.69	-0.80	-0.68
acridine			-0.62	-0.63	-0.70	-0.67	-0.60	-0.64
6-methylquinoline			-0.55	-0.53	-0.62	-0.64	-0.60	-0.58
8-methylquinoline			-0.62	-0.62	-0.74	-0.74	-0.68	-0.66
phenol		230	-0.60	-0.55	-0.68	-0.66	-0.76	-0.78
acridine			-0.60	-0.60	-0.64	-0.65	-0.63	-0.62
6-methylquinoline			-0.50	-0.50	-0.62	-0.60	-0.66	-0.66
8-methylquinoline			-0.52	-0.51	-0.80	-0.78	-0.82	-0.79
phenol		355	-0.40	-0.40	-0.55	-0.55	-0.66	-0.65
acridine			-0.48	-0.47	-0.55	-0.56	-0.50	-0.53
6-methylquinoline			-0.36	-0.33	-0.52	-0.50	-0.62	-0.64
8-methylquinoline			-0.41	-0.40	-0.68	-0.66	-0.62	-0.64
phenol	Benzene-propanol	200	-0.55	-0.54	-0.72	-0.70	-0.70	-0.73
acridine			-0.50	-0.49	-0.62	-0.64	-0.66	-0.69
6-methylquinoline			-0.32	-0.36	-0.48	-0.44	-0.42	-0.40
8-methylquinoline			-0.56	-0.56	-0.60	-0.63	-0.60	-0.58
phenol		230	-0.52	-0.53	-0.66	-0.67	-0.71	-0.70
acridine			-0.48	-0.45	-0.62	-0.62	-0.66	-0.66
6-methylquinoline			-0.36	-0.34	-0.45	-0.42	-0.40	-0.38
8-methylquinoline			-0.55	-0.54	-0.58	-0.61	-0.55	-0.55
phenol		355	-0.42	-0.42	-0.58	-0.56	-0.62	-0.59
acridine			-0.36	-0.35	-0.50	-0.49	-0.52	-0.52
6-methylquinoline			-0.22	-0.23	-0.32	-0.33	-0.28	-0.26
8-methylquinoline			-0.44	-0.44	-0.50	-0.53	-0.36	-0.33

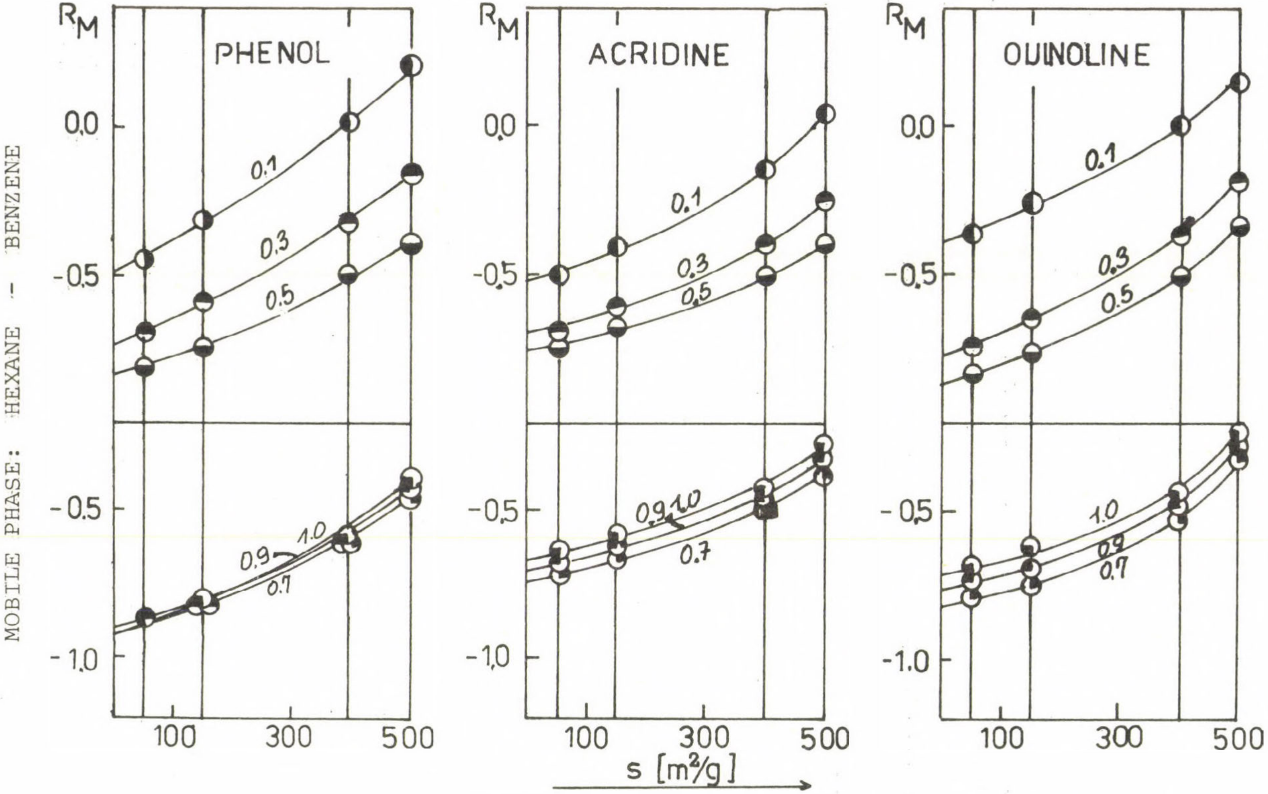

Fig. 3. The R_M values of selected substances as a function of the specific surface area (s) of the adsorbent (SiO_2). Mobile phase: benzene-methanol; volume fraction (x_1) of methanol in the mobile phase as indicated. Points represent measured values while the lines correspond to theoretically calculated values, using the equations given in the paper

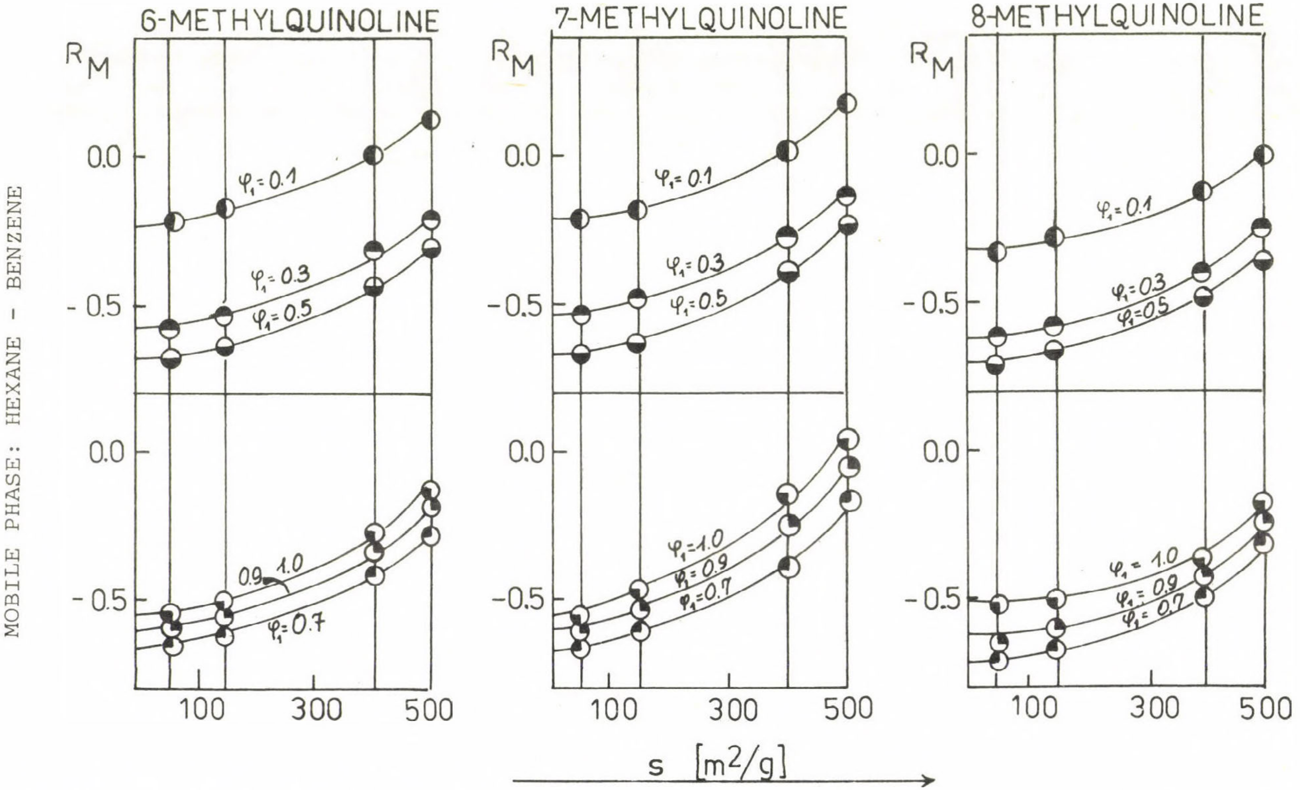

Fig. 4. The R_M values of selected substances as a function of the specific surface area (s) of the adsorbent (SiO_2). Mobile phase: hexane-benzene; volume fraction (x_1) of benzene in the mobile phase as indicated. Points represent measured values while the lines correspond to theoretically calculated values, using the equations given in the paper

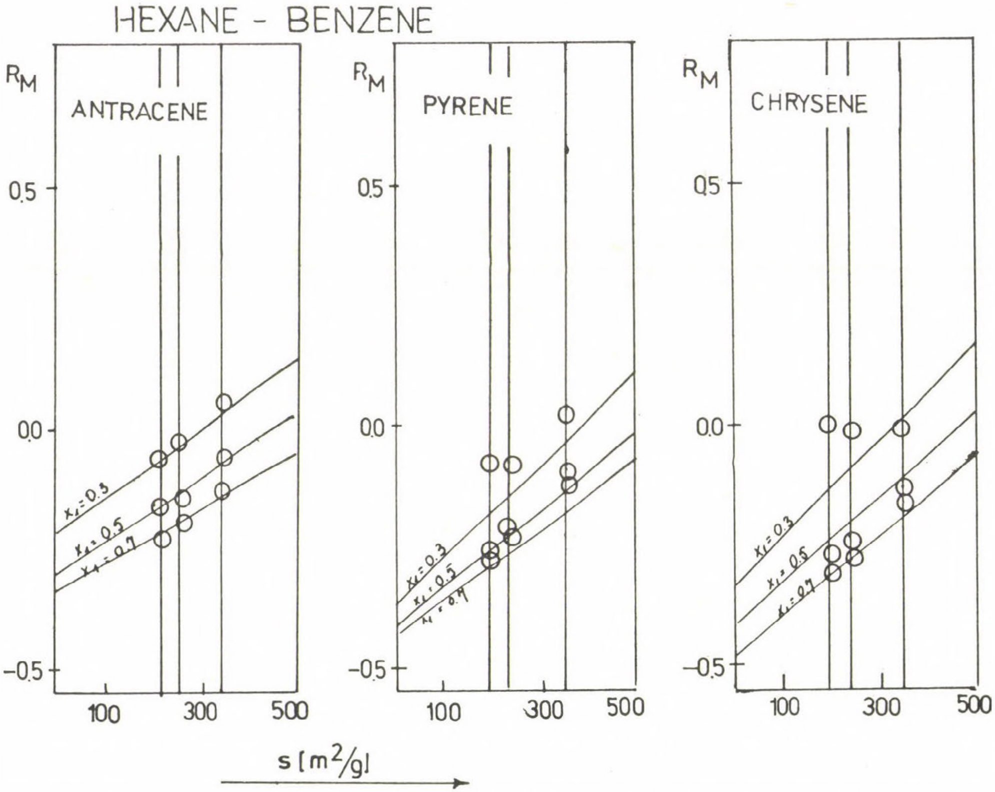

Fig. 5. The R_M values of selected substances as a function of the specific surface area (s) of the adsorbent (SiO_2). Mobile phase: hexane-benzene; volume fraction (x_1) of benzene in the mobile phase as indicated. Points represent measured values while the lines correspond to theoretically calculated values, using the equations given in the paper

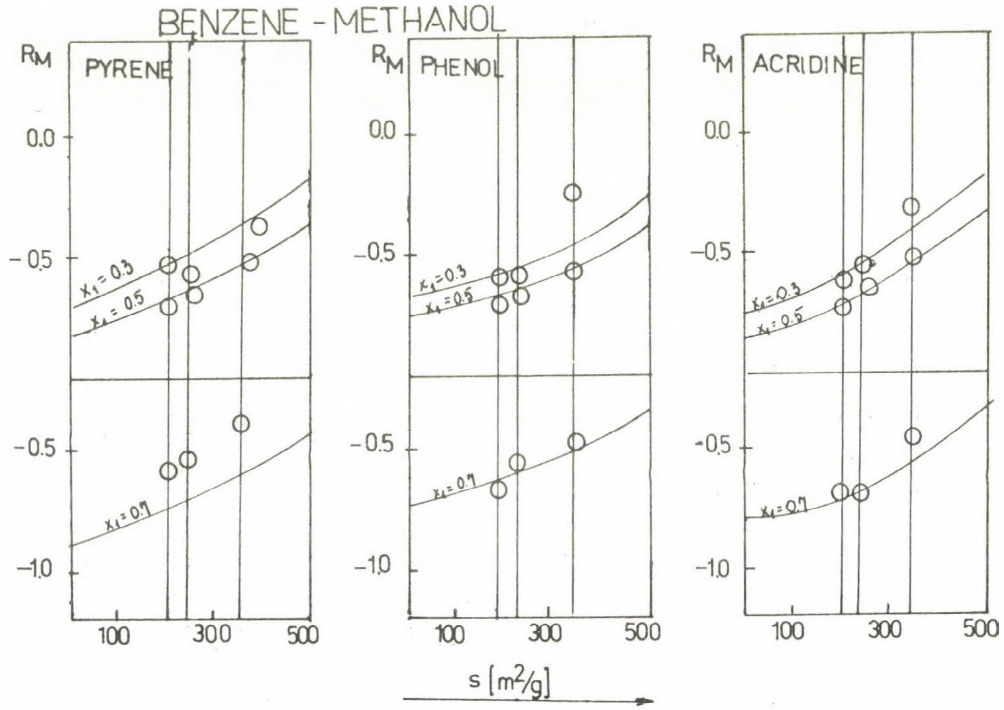

Fig. 6. The R_M values of selected substances as a function of the specific surface (s) of the adsorbent (SiO_2). Mobile phase: benzene-methanol; volume fraction (x_1) of methanol in the mobile phase as indicated. Points represent measured values while the lines correspond to theoretically calculated values, using the equations given in the paper

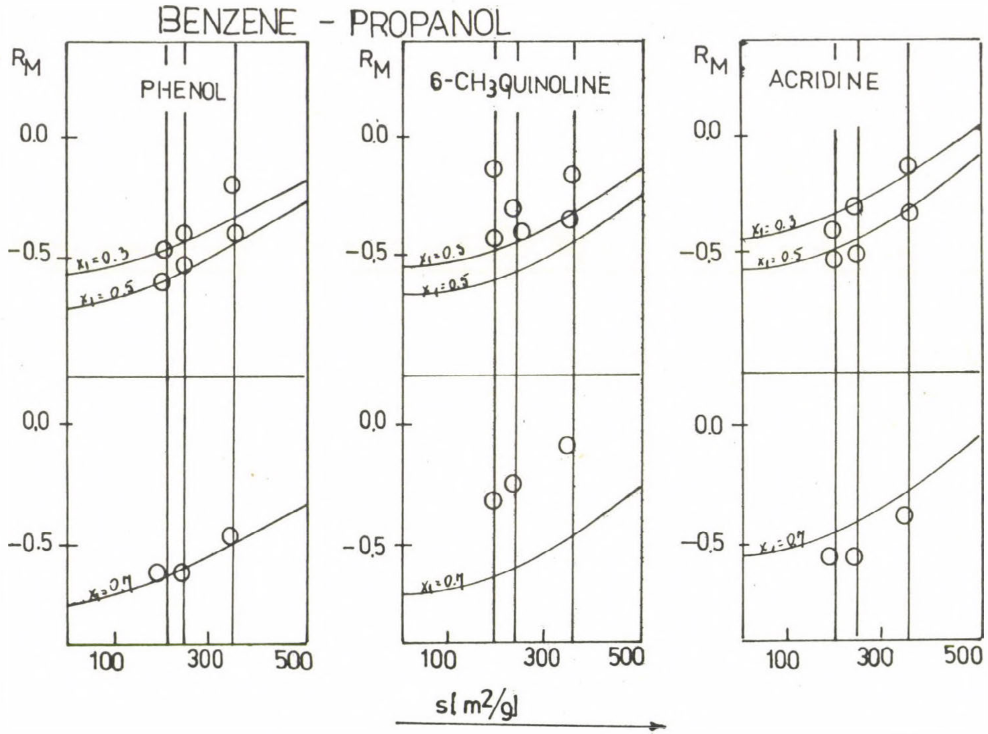

Fig. 7. The R_M values of selected substances as a function of the specific surface area (s) of the adsorbent (SiO_2). Mobile phase: benzene-propanol; volume fraction (x_1) of propanol in the mobile phase as indicated. Points represent measured values while the lines correspond to theoretically calculated values, using the equations given in the paper

accuracy the R_M values on silica gel with specific surface area ranging from 50 to 500 m^2/g.

This method can also be used for the approximate determination of the specific surface area. The solid lines in the figures correspond to the theoretical specific surface area values calculated on the basis of the data listed in Tables I and II, for eqs 1 and 2.

The relationships described here can be used for the calculation of the R_M values measured on silica having any specific surface area, as well as for the determination of specific surface area. It permits to eliminate a series of complicated and time-consuming measurements.

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MULTISTAGE TWO-DIMENSIONAL TLC AND ITS APPLICATION TO THE INVESTIGATION OF CATALYSTS FOR THE HYDROFORMYLATION OF OLEFINS

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ABSTRACT

This paper considers a new combined TLC technique, multistage two-dimensional chromatography, using volatile compounds and melts of organic compounds as the mobile phase for the analysis or rhodium carbonyls. The benefits of this technique, as compared with the singlestage and multistage, one-dimensional TLC techniques are outlined. The use of melts of organic compounds which "preserve" the chromatogram provides a way, at the final stage of a mixture separation, to apply this technique to the separation of mixtures of unstable compounds.

INTRODUCTION

A well-known catalyst for the hydroformylation of olefins - rhodium carbonyl - is distinguished from similar catalysts by its high activity. It is common knowledge (1) that several rhodium carbonyls may be present in the reaction mixture. A reliable technique for analyzing rhodium carbonyls and the determination of the catalyst's composition before and after the synthesis is, therefore, of definite interest. The problem is complicated by possible transformation, hydrolysis in particular, of the original carbonyl when synthesized by the known technique (2). Special methods are needed to guard against this phenomenon.

IR, UV, NMR spectroscopy and liquid chromatography (1), which are currently used to analyze metal carbonyls are all labour-consuming and require the sample to be prepared. To get around these drawbacks a new method was developed which is simple and easily available to any researcher. Preliminary investigations involving the separation of rhodium carbonyls by the known single-stage one-dimensional method did not, however, yield satisfactory results.

RESULTS AND DISCUSSION

The multistage two-dimensional TLC technique proposed by us preserves all the advantages of multistage one-dimensional TLC (3), the main ones of which are improvement in separation, and an increase in the speed and reproducibility of the method. The use of melts of organic compounds as the mobile phase "preserves" the compounds being separated (4, 5). This technique is used at the final stage of separation and helps to avoid the possible transformation of the rhodium carbonyls during subsequent determinations by the standard physico-chemical methods.

The final separation is performed in a direction perpendicular to the previous separations. This permits to considerably shorten the time needed for the separation, due to a reduction in the number of stages and a decrease in the duration of the last stage (the eluting agent travels 3 or 4 cm rather than 10-12 cm). Thus, the multistage two-dimensional TLC technique described here has obvious advantages over the single-stage and multistage one-dimensional TLC techniques.

The rhodium carbonyls were separated by multistage two-dimensional elution. At the first stage methylene chloride was used as the mobile phase and this made it possible to isolate $Rh_6(CO)_{16}$ (R_f = 0.84) from the mixture. The other compounds remain at the start (Fig. 1a). At the second stage two more complex substances, $Rh_4(CO)_{12}$ and $Rh_x(CO)_y^*$, (R_f = 0.81 and 0.70) were separated using a mixture of p-xylene-acetone in

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*Identification of this compound by IR spectroscopy could not specify its exact structure.

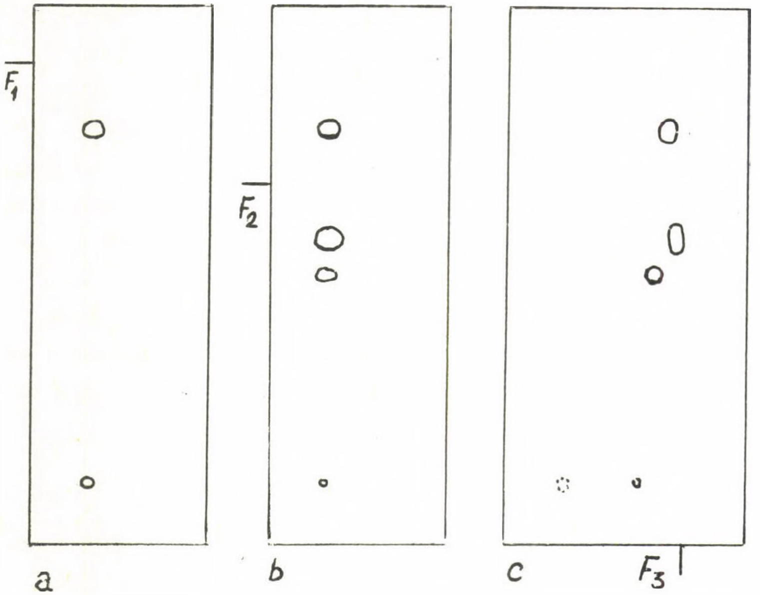

Figure 1. Stages of rhodium carbonyl separation. (a) Separation of $Rh_6(CO)_{16}$. IR data: Carbonyl frequencies: 2077 cm^{-1} (very strong); 2055 cm^{-1} (weak); 2044 cm^{-1} (weak; terminal); 1820 cm^{-1} (narrow; bridging). (b) Separation of $Rh_4(CO)_{12}$ and $Rh_x(CO)_y$. IR data: Carbonyl frequencies: $\underline{Rh_4(CO)_{12}}$: 2075 cm^{-1} (strong); 2069 cm^{-1} (strong); 2044 cm^{-1} (narrow; terminal); 1885 cm^{-1} (narrow; bridging). $\underline{Rh_x(CO)_y}$: 2088 cm^{-1} (narrow); 2016 cm^{-1} (narrow; terminal); 744 cm^{-1} (weak; bridging). (c) "Preservation" of the rhodium carbonyls

the ratio of 10:1 as the mobile phase. One more zone remains at the start (Fig. 1b). The third and final stage is accomplished using a phenol melt as the mobile phase. At this stage the chamber is installed in a thermostat and the separation is performed at 60°C. The eluting agent moves perpendicularly (Fig. 1c) and this makes it possible to coat all the separated compounds with a "preserving" layer. Simultaneously the last compound, $CuCl_2$ ($R_f = 0.6$) migrated from the start.

It was found that the individual compounds [$Rh_6(CO)_{16}$; $Rh_4(CO)_{12}$; $Rh_x(CO)_y$] in a fresh catalyst are in the ratio of 10:1000:1. On long standing the fraction of $Rh_6(CO)_{16}$ in the catalyst increases; a catalyst that has been used in a synthesis contains all the compounds in question in the ratio of 10:10:1. In a completely deactivated catalyst this ratio is 10:1:1.

Thus, the new technique for the separation of rhodium carbonyls enables the researcher to determine very quickly and reliably the composition of hydroformylation catalyst and to forecast its activity.

EXPERIMENTAL PART

Rhodium carbonyls are separated on Silufol-UF-254 TLC plates. A 1-μl sample is applied by a glass capillary. The concentration of the rhodium carbonyls in xylene solution was about 10^{-4} mole/l or 0.08 wt %.

Methylene chloride, a 1:10 mixture of acetone and xylene and phenol were used as the mobile phases. Before each stage the plate was dried in a thermostat at 45°C for three minutes. At the final stage the chromatographic chamber was placed in the thermostat and the separation was accomplished at 60°C.

IR-spectra were taken by a Spekord M80 instrument in the range of 2300 to 1500 cm^{-1}.

CONCLUSIONS

A new combined multistage two-dimensional chromatographic technique using volatile compounds and melts of organic substances as the mobile phase is described. The method has particular advantages in the separation of complex mixtures of unstable compounds such as rhodium carbonyls used as catalysts for the hydroformylation of olefins. Its particular advantages are the increase in the speed of analysis and reproducibility of the results, and an essential improvement in the separation.

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A NOVEL PROCEDURE FOR THE ACTIVATION OF SUPPORT MATERIALS

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INTRODUCTION

For several years, matrix-bound biologically active substances have been widely used in biosciences, biotechnology and medicine. Although a variety of activation procedures has been described for the binding of biologically active substances, in part also employed commercially (e.g., cyanogen bromide activation), many of these methods are afflicted with disadvantages, which considerably limit their applicability.

Generally, the choice of an activation procedure depends on many factors:

- functional groups of the matrix and of the ligand
- technological expense
- cost of procedure
- availability of the chemicals
- toxicity and biocompatibility of the chemicals
- chemical stability of the support material
- stability of the ligand
- reactivity of the functional groups
- coupling conditions
- non-specific adsorption to the modified support material
- stability of the covalent linkage between support and ligand
- storage possibilities of the activated matrix.

Therefore, it is not surprising that an ideal activation method that would fulfill all these requirements is still lacking.

We are presenting here a novel and simple activation procedure for hydroxyl-group containing surfaces.

MATERIAL AND METHODS

It is well established that the hydroxyl-groups on glass surfaces are the individual reaction centres with organosilanes (1). Thus, it would be logical that the hydroxyl-groups on other organic support materials react with organosilanes in a similar way (2). The principle of the activation method is shown in Figure 1.

The proposed method allows the simultaneous introduction of different functional groups into support materials by simple mixing of the organosilanes. This offers the possibility to couple biologically active ligands in parallel via different mechanisms.

We used this principle for the activation of different support materials, e.g., Sephadex®, Sepharose®, Separon®, Trisacryl®, etc. Especially we studied the suitability of beaded cellulose produced in the GDR as a matrix for immunoaffinity chromatography. The activation of cellulose by means of organosilanes is shown in Figure 2.

The binding of different biologically active substances to activated cellulose is shown in Figure 3.

Activated cellulose has been used both as beaded cellulose and as activated paper. The latter has been applied in immunoassay and electroblotting. The results of the binding of ^{125}I-labelled immunoglobulin G (^{125}I-IgG) as a model protein to activated paper are demonstrated in Table I.

These results prove, that substantial amounts of proteins could be bound to the activated paper, the grade of the paper used has almost no influence on the binding capacity, and after the initial three wash cycles, no leakage could be observed.

Table II compares the binding of ^{125}I-IgG to different beaded matrix materials.

In these experiments the protein was bound via the aldehyde-group of the glutaraldehyde or the epoxy-group, or both.

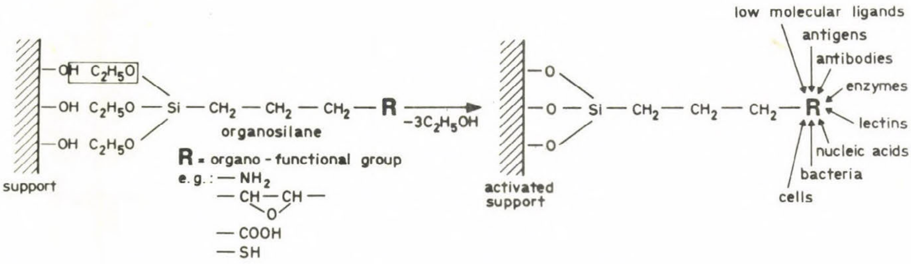


Fig. 1. Principle of the activation by means of organosilanes

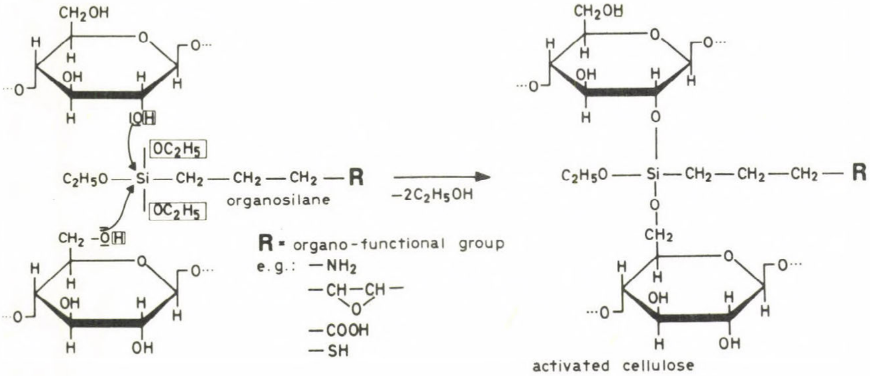


Fig. 2. Principle of the activation of cellulose by means of organosilanes

The amount of coupled protein is increased essentially (in most cases) by the introduction of two different functional groups into the gel. Up to 50 mg IgG/ml gel could be bound. As mentioned, we have tested the activated beaded cellulose as a support in immunoaffinity chromatography. For this purpose we bound human immunoglobulin G to the matrix and isolated the antibodies against human IgG from a rabbit antiserum and, vice versa, we bound the antibodies to the support and isolated the immunoglobulin G from human serum. To this end, the beaded cellulose was activated by two novel activation procedures (patents pending) - the represented

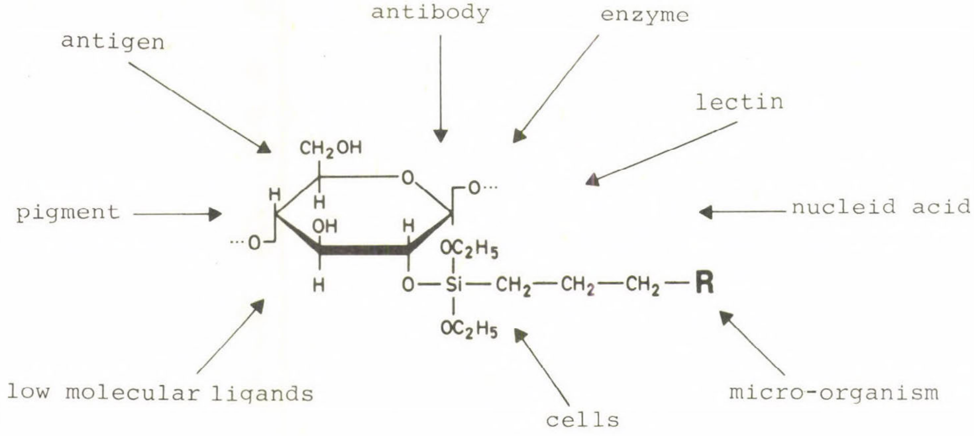


Fig. 3. Binding of biologically active substances to activated cellulose

Table I. Binding of^{125}I-IgG to activated papers (Aminopropyltriethoxysilane/glutaraldehyde + glycidoxypropyltriethoxysilane)

	Whatman 540	Filtrak 1389	Filtrak 380	FN 3
Bound protein after three wash cycles:				
10 g/ml	2,092 ng/cm^2	2,143 ng/cm^2	2,015 ng/cm^2	2,372 ng/cm^2
50 g/ml	5,536 ng/cm^2	6,122 ng/cm^2	5,867 ng/cm^2	5,867 ng/cm^2
Bound protein after further ten wash cycles:				
10 g/ml	1,888 ng/cm^2	1,939 ng/cm^2	1,786 ng/cm^2	2,092 ng/cm^2
50 g/ml	5,000 ng/cm^2	5,612 ng/cm	5,281 ng/cm^2	5,459 ng/cm^2
Bound protein after four weeks:				
10 g/ml	1,913 ng/cm^2	1,913 ng/cm^2	1,786 ng/cm^2	1,964 ng/cm^2
50 g/ml	5,102 ng/cm^2	5,612 ng/cm^2	5,306 ng/cm^2	5,281 ng/cm^2

Table II. Results of the binding of ^{125}I-IgG to different support materials

	Beaded cellulose	Separon Hema 1000^{R}	Trisacryl GF 2000^{R}	Sepharose 4B^{R}
Aminopropyltriethoxysilane + glutaraldehyde	12 mg/ml	23.6 gm/ml	46.7 mg/ml	5.6 mg/ml
Glycidoxypropyltriethoxysilane	7.2 mg/ml	-	32.7 mg/ml	-
Aminopropyltriethoxysilane/glutaraldehyde + glycioxypropyltriethoxysilane	26.4 mg/ml	53.9 mg/ml	34.2 mg/ml	15.2 mg/ml

silanization and the activation by means of chloroformates (3) (Fig. 4).

Both activated cellulose gels were compared with commercially available cyanogen bromide activated sepharose (Pharmacia, Sweden) - as reference support - under identical conditions. The results of the isolation of the antibodies by means of these three support materials are expressed as the activity of the antibodies determined by an enzyme immunoassay and are shown in Figure 5.

The results are expressed as the activity of the antibody per mg IgG/ml. These results clearly show that the two novel activation methods are at least comparable with the cyanogen bromide activation. The purity of the antibodies was tested, among other methods, by polyacrylamide gel electrophoresis (Fig. 6).

It is clearly shown that the antibody fractions are not contaminated by other serum proteins. This is a result of the low unspecific binding to the matrix.

SUMMARY

In summary, the comparison of the three different activated supports has clearly shown that all three activation methods give similar results in immunoaffinity chromatography.

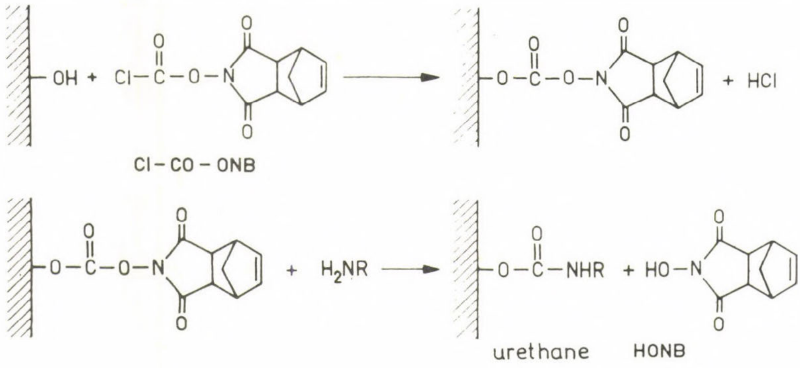


Fig. 4. Principle of the activation by means of a novel chloroformate and coupling of ligand

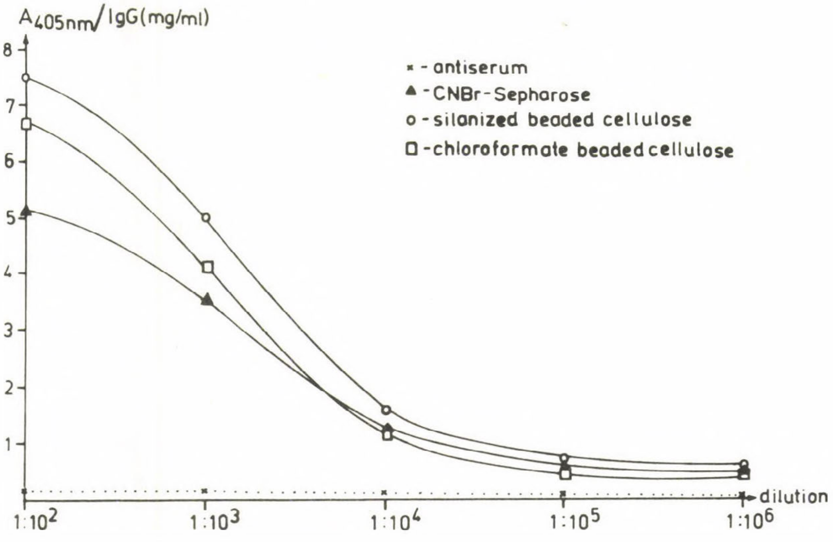
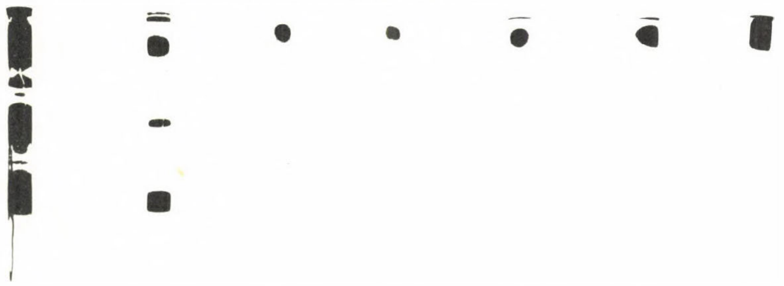


Fig. 5. Determination of antibody activity by enzyme immunoassay. Sample: 1 or 2 ml antiserum; eluates at pH 2.8

A: CNBr-Sepharose® B: silan. bead. cell.

C: chloroformate bead. cell.



1 antiserum

2 PBS washing fraction

3 eluate at pH 2.8 A

4 eluate at pH 2.8 B

5 eluate at pH 2.8 C

(2 ml antiserum)

6 eluate at pH 2.8 B

7 eluate at pH 2.8 C

(5 ml antiserum)

Fig. 6. Results of the polyacrylamide gel electrophoresis

In conclusion, the advantages of the activation of hydroxyl-groups containing support materials by means of organosilanes are as follows:

- technological simplicity
- low cost
- non-toxic chemicals
- high activation yield
- stability in the active state for several months
- possibility of simultaneous introduction of different functional groups
- mild (physiological) coupling conditions
- high coupling yield
- no leakage.

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A SIMPLIFIED HYDROPHOBICITY CRITERION FOR SOLUTES IN REVERSED-PHASE CHROMATOGRAPHY

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Organic solvent concentration in the mobile phase necessary for the elution of compounds with a given structure at desired capacity factor (k') values can be predicted with the help of retention models relating capacity factors, solute hidrophobicity and mobile phase composition:

$$\log k' = a_0 + a_1 \log C + a_2 h + a_3 h \log C \qquad (1)\ \text{or}$$

$$\log C = (\log k' - a_0 - a_2 h)/(a_1 + a_3 h) \qquad (2),$$

where C is the molar concentration of the organic solvent in the mobile phase and h is the hydrophobicity parameter.

It has been shown earlier, e.g. (1-4), that the calculated or experimentally found log P values* as well as the connectivity indices can be useful as hydrophobicity parameters in reversed-phase chromatography. However, in using this approach one is faced with several problems restricting the more extensive application of these parameters. For example, besides the knowledge of a fairly large number of structural increments, additive calculation of log P requires that correction be made for the various interactions between different parts of the solute molecule. On the other hand, the procedure for the experimental determination of log P is time-consuming and imposes stringent requirements on the purity of the solutes. Therefore, at present a simple and convenient description of hydrophobicity remains a much desired goal.

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*log P is the logarithm of the partition coefficient in an octanol-water system.

The proposed simplified criterion is based on the following assumptions:

- the size of the organic solute molecules depends primarily on the number of carbon atoms;
- the decrease in hydrophobicity observed when an additional polar functional group is introduced into the molecule of a polyfunctional organic compound is mainly due not to the type of the functional group, but merely to the fact the number of functional groups in the compound has been increased.

With these assumptions in mind one can arrive at a very simple hydrophobicity model that does not require a detailed set of increments. The total hydrophobicity (H) of a molecule containing a hydrocarbon skeleton and several (n_f) functional groups can be expressed by the simplified hydrophobicity criterion:

$$H = n_c + F(n_f) \qquad (3)$$

where n_c is the number of carbon atoms and F represents the functional groups.

An analysis of the behaviour of monofunctional polar solutes in reversed-phase chromatography shows that the introduction of a functional group in the molecule or the removal of four carbon atoms from it leads to a similar decrease in retention. Consequently, in its simplest form Eq. (3) should be rewritten as follows:

$$H = n_c - 4 \cdot n_f \qquad (4)$$

Unfortunately, such an oversimplified model becomes unsatisfactory at $n_f = 2$, because the effects of non-additivity in reversed-phase chromatography manifest even if the functional groups are spaced in the solute molecule. When additional functional groups are successively introduced into the solute molecule, non-additivity increases due to the increased number of pair interactions and to a decreased mean distance between the functional groups.

The following expression is proposed as an empirical function describing the non-additive effect of functional groups on retention:

$$F(n_f) = -4\sqrt{n_f} \tag{5}$$

The contribution of halogen and sulphur atoms to retention strongly depends on the nature of the functional group and on the remaining part of the molecule. As a rough approximation it can be claimed that

- in terms of its influence on retention each halogen atom is equivalent to one carbon atom;
- sulphur atoms in thiols, thioethers and thioesters fail to influence the net hydrophobicity of the solute.

Thus, finally term n_c in Eq. (3) should be replaced by n_H, i.e. the sum of primary hydrophobic fragments in the molecule (the sum of carbon and halogen atoms):

$$H = n_H - 4\sqrt{n_f} \tag{6}$$

It should be emphasized that the simplified criterion H does not appear to be an accurate descriptor of hydrophobicity for closely related compounds. It is rather a universal (but somewhat rough) measure of hydrophobicity for structurally diverse substances. Analysis of such compound sets reveals that criterion H is well correlated with log P parameters, which are calculated by a much more complex and less universal method. Such a comparison has been performed for 39 drugs belonging to different chemical classes. A linear relationship between H and log P was observed:

$$\log P = -1.08 + 0.42\ H \tag{7}$$
$$(r = 0.92)$$

The scattering of points around the straight line in Fig. 1 reflects mainly the different correction methods applied to the interaction of polar functional groups in the solute molecules. It follows from Eq. (7) that parameter H

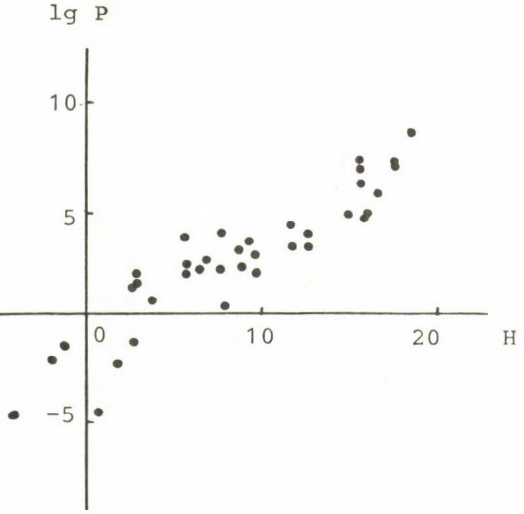

Fig. 1. Relationship between log P and the H values

can be used in retention models analogous to those described by Eqs (1) and (2). For instance, when C = const,

$$\log k' = a_0 + a_1 H \tag{8}$$

It will be shown now that various groups of organic compounds fit Eq. (8).

Homologous series. The number of functional groups and halogen atoms is constant within each homologous series. Hence, H is a linear function of n_C and can be used in various known retention models instead of n_C.

Compounds containing different hydrocarbon moieties. Molecules of chemically related drugs often share a common structural fragment containing a certain set of functional groups. Hydrocarbon substituents within these classes often vary in size and they may also differ in the degree of chain branching, saturation and cyclization. At the same time n_f in such classes is constant, but correlation between n_C and log k' is usually not as good as in homologous series. The chromatographic behaviour of such groups of substances can be described by Eq. (8). Figure 2a shows the log k' vs. H plots for barbiturates. Retention data were taken from Ref. (5). Retention indices (I) which are a linear function of log k' values can be used in models similar to Eqs (8) and (1). Figure 2b presents retention data of 3-propanylidonortropane analogues (6) in the coordinates of the equation

$$I = a_0 + a_1H \qquad (9)$$

In both cases it is clear that retention values and H are linearly correlated.

<u>Compounds containing different hydrocarbon moieties and different numbers or types of functional groups</u>. This type of compounds shares only a minor common fragment or none at all. In this case all the assumptions made earlier would adversely affect the accuracy of the model described by Eq. (8). Nevertheless, the correlation between H and the retention values of such substances is retained. Examples of such relationships are shown in Figs 3-6.

Figure 3 shows retention data for derivatives of 5-fluorouracil (a), 1,4-dihydropyridine (b) and cyclopentane (c) as a function of the H-parameters. For cyclopentanes parameter B* was used instead of log k'.

Retention data of polynuclear hydrocarbons and heterocycles containing sulphur, oxygen and nitrogen atoms (7) are shown in Figs 4a, b.

Capacity factors of more than 30 compounds, aromatic acids found in urine (8), including hydroxy- and methoxybenzoic acids, phenylacetic acids as well as derivatives of tryptophane, indole, cynnamic, mandelic and hyppuric acids, purines, pyrimidines and pyridines were utilized. The n_C values vary from 5 to 11 and the n_f values from 1 to 5. The appropriate

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*B is the log k' value extrapolated to an acetonitrile concentration 1 mole/liter with the aid of a linear relationship:

$$\log k' = B + B' \log C \qquad (10)$$

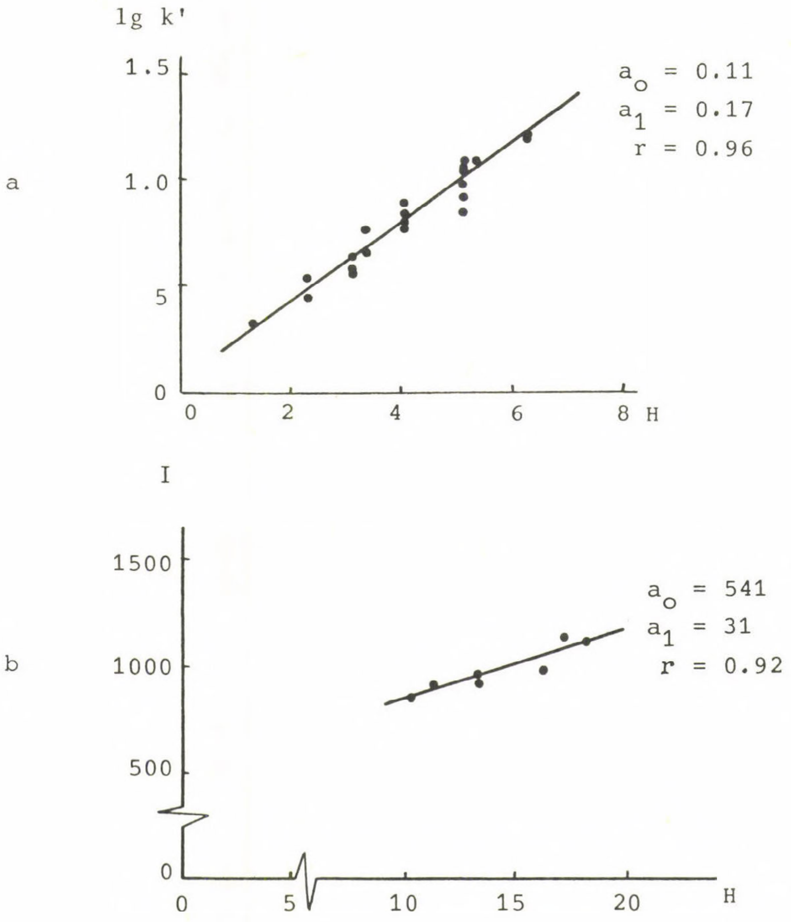

Fig. 2. Correlation between the retention values of barbiturates (a) and 3-propanilidonortropane analogues (b) and the simplified hydrophobicity criterion (H)

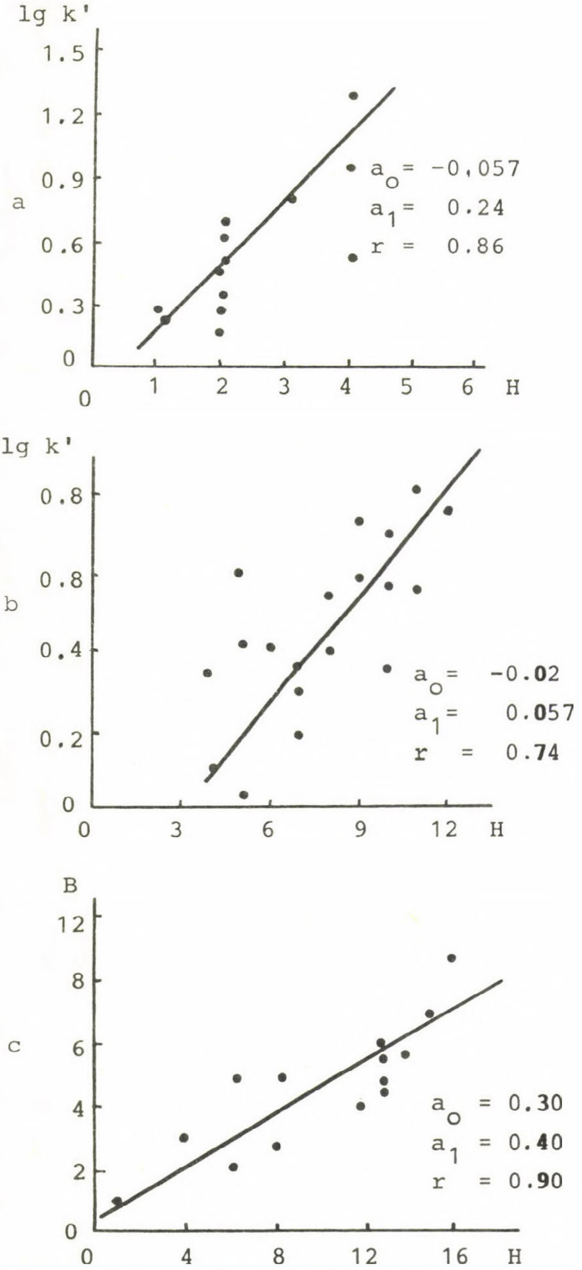

Fig. 3. Relationship between the retention parameters of 5-fluorouracil (a), 1,4-dihydropyridine (b), cyclopentane (c) derivatives and criterion H

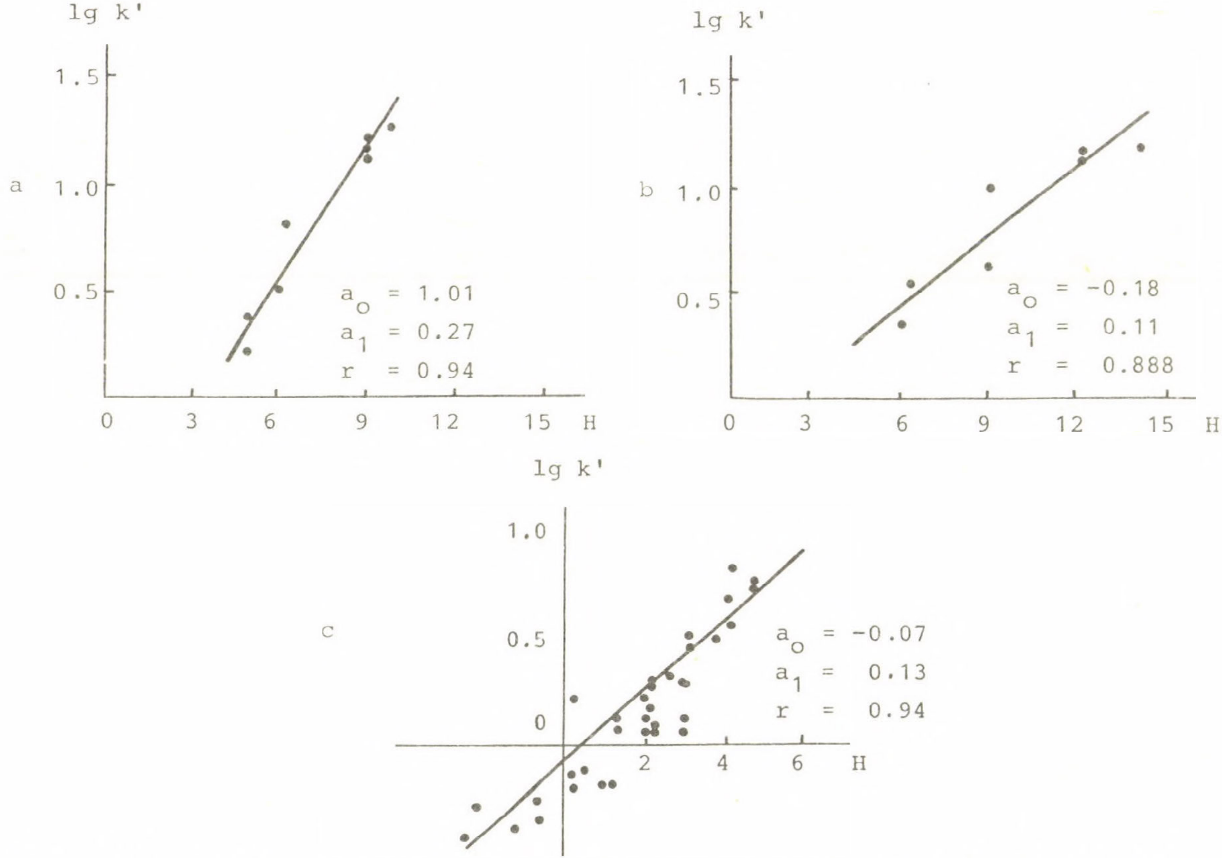

Fig. 4. Correlation between the logarithm of the capacity factors of polynuclear heterocycles (a and b) and aromatic carboxylic acids (c) and criterion H. Concentration of methanol in the mobile phase: (a) 50 vol-% and (b) 70 vol-%

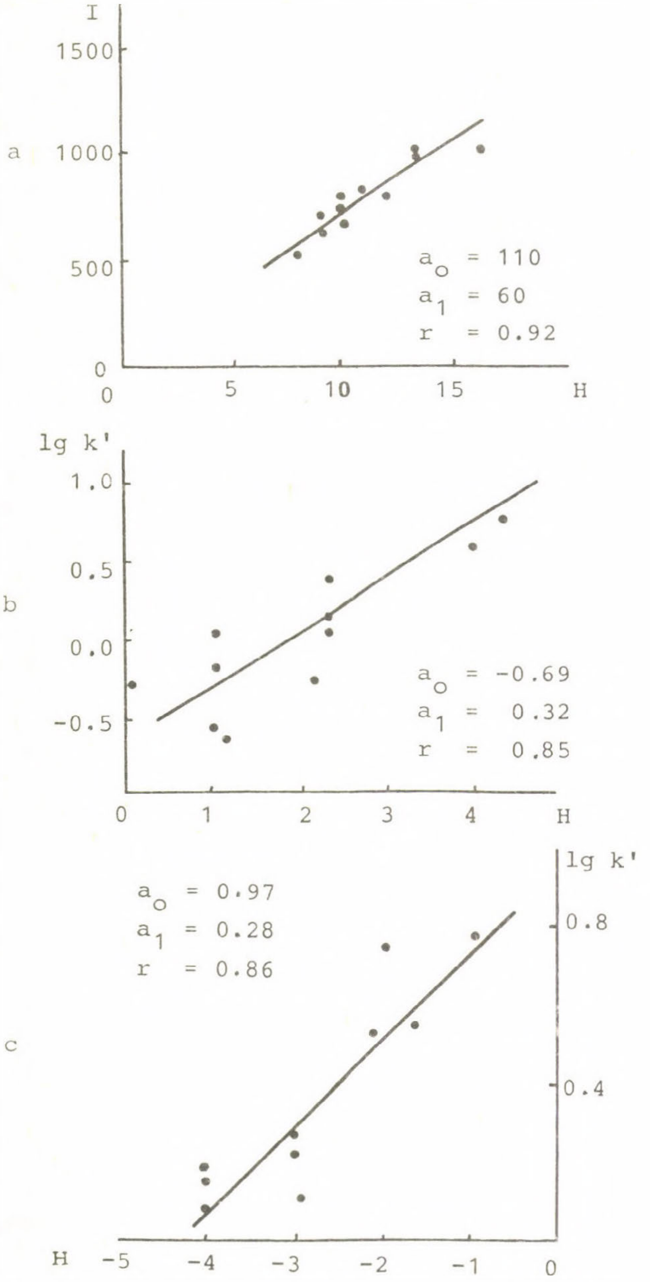

Fig. 5. Correlation between the retention of morphine analogues (a), polyfunctional aromatic amines (b) and pyrimidines (c) and the simplified hydrophobicity criterion (H)

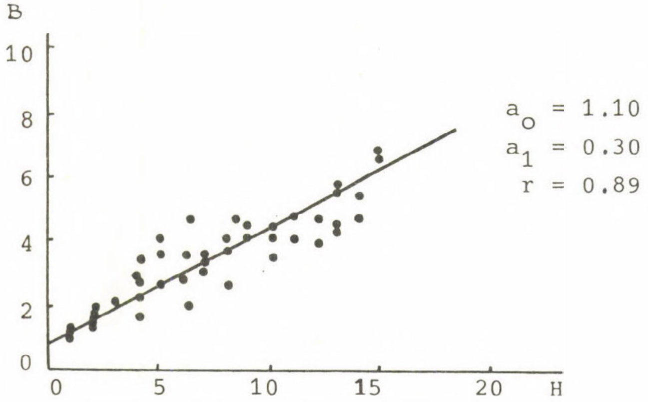

Fig. 6. Relationship between the capacity factors of 5-fluorouracils, 1,4-dihydropyridines and cylopentanes extrapolated to an acetonitrile concentration 1 mol/liter, and criterion H

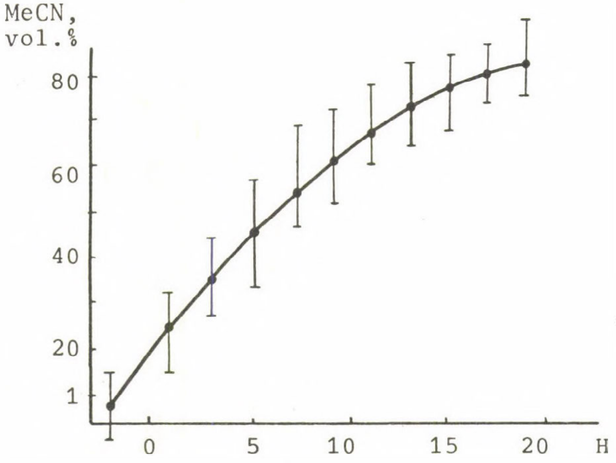

Fig. 7. Relationship between criterion H and the mean acetonitrile concentration necessary for elution at k' = 2

retention data are summarized in Fig. 4c.

Figure 5 shows retention data of morphine analogues on Bondapak C18 (6), polyfunctional aromatic amines on Lichrosorb RP18 (9), and pyrimidines on Separon C18 (10), as a function of the H parameter.

Relationship (8) permits the description of retention for different classes of compounds, even if they have been studied at different concentrations of the organic solvent in the mobile phase. In this case the first step involves the recalculation of the experimentally found log k' values for a common composition of the mobile phase. Thus, our retention data for 5-fluorouracils and 1,4-dihydropyridines are presented together with those for cyclopentanes in Fig. 6.

Parameter H can be used in Eq. (2) for evaluation of the organic solvent concentration necessary to elute a given substance at a desired k' value. A large amount of literature data concerning solvent conditions in reversed-phase HPLC can be represented as a function of the simplified hydrophobicity criterion. Retention data for more than 600 drugs analyzed in the reversed-phase HPLC mode have been used to construct Fig.7. The plots show rough estimates of the acetonitrile concentration required to elute these drugs at k' = 2.

It follows from the aforesaid that parameter H actually reflects the influence of solute structure on chromatographic behaviour. The existence of a relationship between H and log k' may serve as a basis for *a priori* calculations of retention values and for the prediction of the optimum mobile phase composition.

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CAPILLARY ISOTACHOPHORESIS IN PEPTIDE AND AMINO ACID CHEMISTRY - ANALYSIS OF CYST(E)INE-CONTAINING DI- AND TRIPEPTIDES

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INTRODUCTION

Isotachophoresis in capillary tubes (ITP) is nowadays recognized as a valuable analytical technique in peptide and amino acid analysis. The basic work of Kopwillem et al. (1-4) and Everaerts and van der Put (5) opened the way for new applications in this field, especially allowing the examination of the purity of synthetic (6-16) and naturally occurring (17-24) peptides as well as enabling the separation of various amino acid mixtures (25-27).

In the present study, analytical ITP was employed to control the synthesis and purification of cysteine/cystine-containing di- and tripeptides, especially L-alanyl-S(acetamidomethyl)-L-cysteine (Ala-(Acm)Cys) and bis-L-alanyl-L-cystine ($(\text{Ala-Cys})_2$). The main synthetic products, free amino acids and amino acid derivatives as well as contaminating salts could be analyzed in a single experiment. In addition to the qualitative approach, ITP affords information about the composition of the samples without the necessity to concomitantly analyze reference substances, thereby facilitating a simple subsequent quantification. Furthermore, the method allows an easy evaluation of the amino acid composition of the peptides after acid and enzymatic hydrolysis.

MATERIALS AND METHODS

Analytical Isotachophoresis

Anionic isotachophoretic analyses were performed by using a 2127 Tachophor (LKB, Bromma, Sweden) with an automatic control unit (13). Separations were carried out in a PTFE capillary (230 mm x 0.55 mm I.D.). Conductivity and UV (206 and 254 nm) signals were monitored by employing a two-channel recorder (Kipp and Zonen, Delft, The Netherlands) with a chart speed of 6 cm/min. For detection, the migration current was reduced from 210 to 60 μA. Depending on the chloride concentration of the sample and the chosen terminating electrolyte, the separations required 15-30 min. As leading electrolyte, a 10 mM solution of hydrochloric acid titrated to pH 9.1 with Ammediol was used. The terminating electrolyte buffer contained 10 mM glycine (electrolyte system I) or β-alanine (electrolyte system II), respectively, adjusted to pH 10 with saturated barium hydroxide solution. These electrolyte solutions were prepared from analytical grade chemicals provided by E. Merck, Sigma and Fluka as described in detail previously (11), and were stored at 4°C.

Peptide Synthesis and Purification

The dipeptide Ala-(Acm)Cys was synthesized by applying the N-carboxy anhydride (NCA)-method in aqueous phase (28-30) using alanine-NCA (produced by phosgen 31, 32) as carboxyl component and (Acm)Cys·HCl (obtained from BaChem, Bubendorf, Switzerland) as nucleophile in a molar ratio Ala-NCA: (Acm)Cys·HCl of 1.1:1.0. Removal of the Acm-group was performed after purification according to Veber et al. (33) with mercuric acetate at pH 4.0. After precipitation of excess Hg^{2+} with H_2S, the reaction mixture was air oxidized overnight at pH 7.0 in the presence of a small piece of copper wire, resulting in the formation of the tripeptide $(Ala-Cys)_2$.

Purification of the synthesized peptides was performed by gel filtration as described previously (23). Crude materials, intermediate and end products obtained during synthesis and purification were isotachophoretically analyzed in concentrations and amounts given in the figures.

Enzymatic and Acid Hydrolysis

By adding 0.1 mg aminopeptidase M (E.C. 3.4.11.2., E.Merck), enzymatic hydrolyses of Ala-(Acm)Cys and $(Ala\text{-}Cys)_2$ were performed at pH 7.2 and room temperature in 1 ml sodium phosphate buffer (12.5 mM) containing 1.5 mg of purified peptide material. Isotachophoretic analyses were carried out on aliquots obtained after 1 h (electrolyte system II).

For acid hydrolyses, 1.0 mg of the synthesized peptides were placed in glass tubes and dissolved in 0.5 ml of 5.7 M hydrochloric acid. Subsequently, the tubes were sealed *in vacuo* and incubated for 24 h at 110°C. The dried hydrolysates were resolved in distilled water and analyzed applying electrolyte system II.

RESULTS

Control of Peptide Synthesis and Purification

Applying conductivity detection and UV measurement at 206 nm, the crude material of Ala-(Acm)Cys was analyzed with electrolyte system II. As indicated by the conductivity signal, at least four zones were detectable (Fig. 1A). The main zone as well as two minor zones exhibited characteristic high UV absorption, suggesting the presence of peptide(amide) bonds.

One of the minor UV absorbing and the non-UV absorbing solutes present in the crude product could be identified with the aid of suitable reference substances. Analysis of an equimolar standard mixture of (Acm)Cys and alanine revealed one non-UV absorbing and one UV absorbing zone (Fig. 1B). It is evident, that the characteristic conductivity step heights and UV levels derived on one hand from the crude material and on the other hand from the standard substances are identical. Identification of the main synthetic product Ala-(Acm)Cys could be carried out by analyzing enzymatic and acid hydrolysates of the purified peptide (vide infra). As indicated by

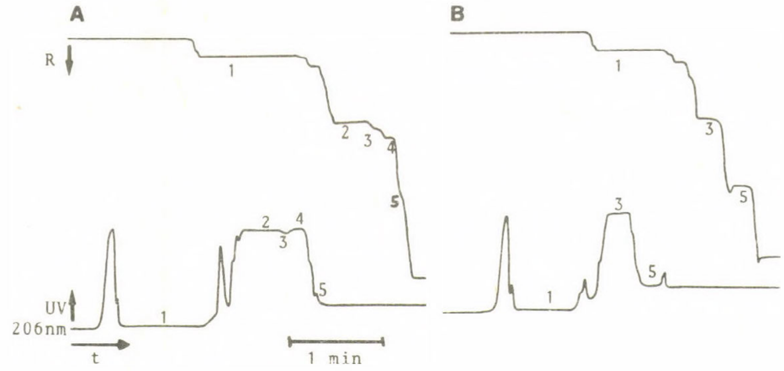

Fig. 1. Isotachophoretic analyses (electrolyte system II) of the crude material of Ala-(Acm)Cys (A) and a reference mixture (B). (A) 5 µl crude product corresponding to 2.63 µg peptide material; (B) 4 µl reference mixture corresponding to 0.77 µg of (Acm)Cys and 0.36 µg of alanine; Key: 1 = HCO_3^-; 2 = Ala(Acm)Cys; 3 = (Acm)Cys; 4 = unknown overreaction product; 5 = alanine

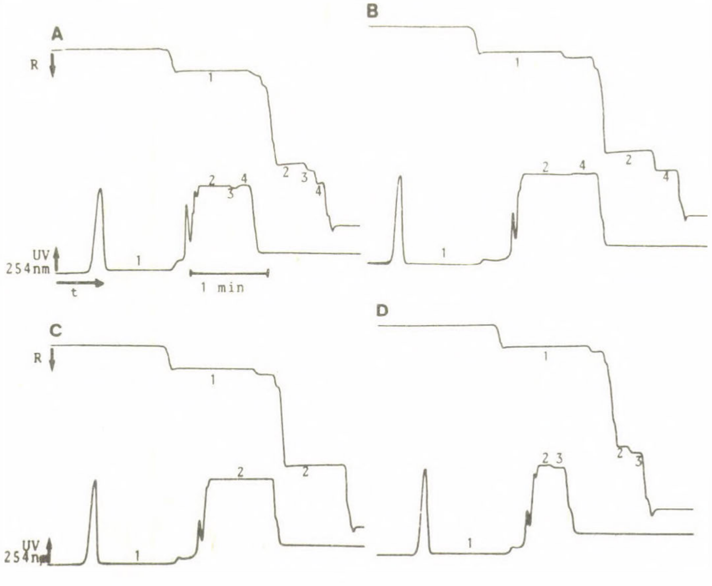

Fig. 2. Isotachophoretic analyses (electrolyte system I) of the crude material (A) and the different peptide fractions (B-D) obtained after gel chromatographic purification. Key as in Fig. 1. For each separation, 5 µl were injected, corresponding to 2.63 µg of material

the high UV absorption at 206 nm, the fourth zone corresponds to a minor synthetic peptide (presumably Ala-Ala[Acm]Cys).

The crude material (A) as well as the different peptide fractions (B-D) obtained after gel chromatographic purification were analyzed using electrolyte system I (Fig. 2). In addition to the peptide Ala-(Acm)Cys, the overreaction product Ala-Ala-(Acm)Cys and the starting amino acid (Acm)Cys could be detected in fraction 1 (Fig. 2B) and 3 (Fig. 2D), respectively. Obviously, fraction 2 contained only the main synthetic peptide (Fig. 2C).

Isotachophoretic analyses (electrolyte system II) of the crude peptide material after removal of the Acm-group and subsequent air oxidation (see Materials and Methods) revealed at least three zones as indicated by the conductivity signal (Fig. 3A). One of these zones showed characteristic UV absorption with a detection wavelength of 254 nm suggesting the presence of a cystine residue. By analyzing acid and enzymatic hydrolysates, this compound could be identified as the peptide $(\text{Ala-Cys})_2$ (see below). In addition, acetate (derived from mercuric acetate) and tiny amounts of starting Ala-(Acm)Cys were detectable (Fig. 3A). Evidently, one gel chromatographic purification step was sufficient to approach 100 % enrichment of $(\text{Ala-Cys})_2$(Fig. 3B).

Analysis of Acid and Enzymatic Hydrolysates

Analysis of the acid hydrolysate of Ala-(Acm)Cys revealed three distinct zones, one of them exhibiting characteristic low UV absorption at 254 nm (Fig. 4A). As demonstrated in Fig. 4B, enzymatic hydrolysis of Ala-(Acm)Cys resulted in the formation of two non-UV absorbing compounds, one of them also being formed during acid treatment.

Identical isotachopherograms were obtained after acid and enzymatic hydrolysis of $(\text{Ala-Cys})_2$(Fig. 5A and B, respectively).

Four of the solutes present in the peptide hydrolysates could be identified by analyzing possible hydrolysis products of Ala-(Acm)Cys and $(\text{Ala-Cys})_2$. As illustrated in Fig. 6, the

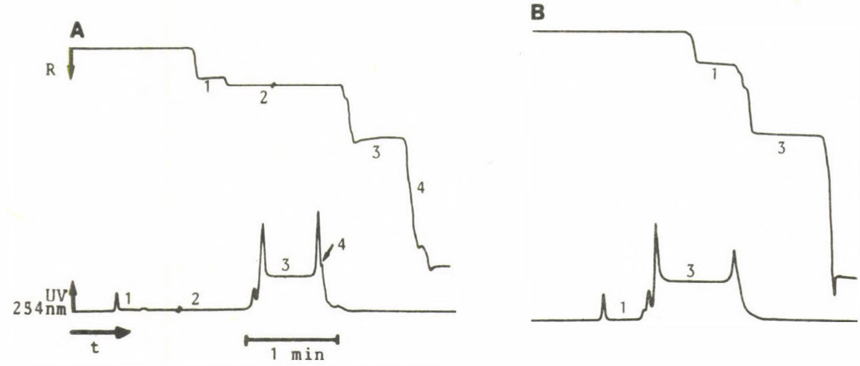

Fig. 3. Isotachophoretic analyses (electrolyte system II) of the peptide material after removal of the Acm group and subsequent air oxidation. (A) 10 µl crude material corresponding to 7.65 µg product; (B) 5 µl purified peptide corresponding to 3.83 µg material; Key: 1 = HCO_3^-; 2 = acetate; 3 = $(Ala\text{-}Cys)_2$; 4 = Ala-(Acm)Cys

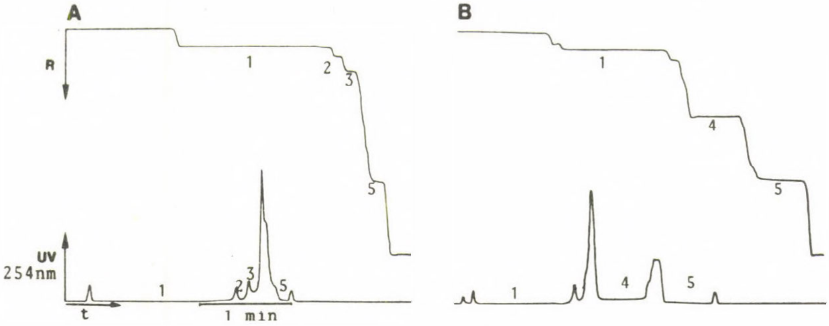

Fig. 4. Isotachophoretic analyses (electrolyte system II) of acid and enzymatic hydrolysates of Ala-(Acm)Cys. (A) 10 µl acid hydrolysate; (B) 2.5 µl enzymatic hydrolysates; Key: 1 = HCO_3^-; 2 = acetate; 3 = unknown hydrolysis product; 4 = (Acm)Cys; 5 = alanine

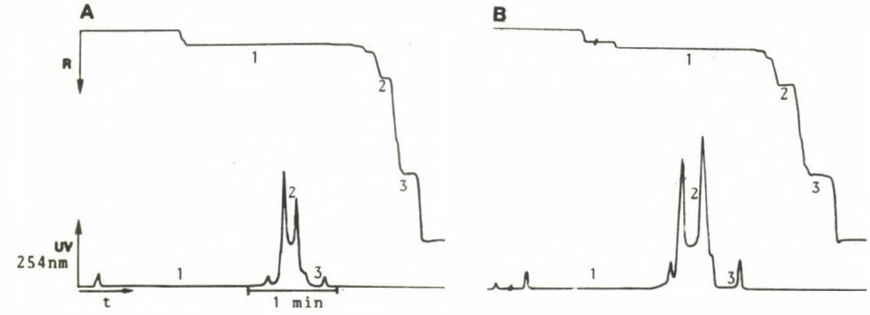

Fig. 5. Isotachophoretic analyses (electrolyte system II) of acid and enzymatic hydrolysates of $(\text{Ala-Cys})_2$. (A) 10 µl acid hydrolysate; (B) 5 µl enzymatic hydrolysate; Key: 1 = HCO_3^-; 2 = cystine; 3 = alanine

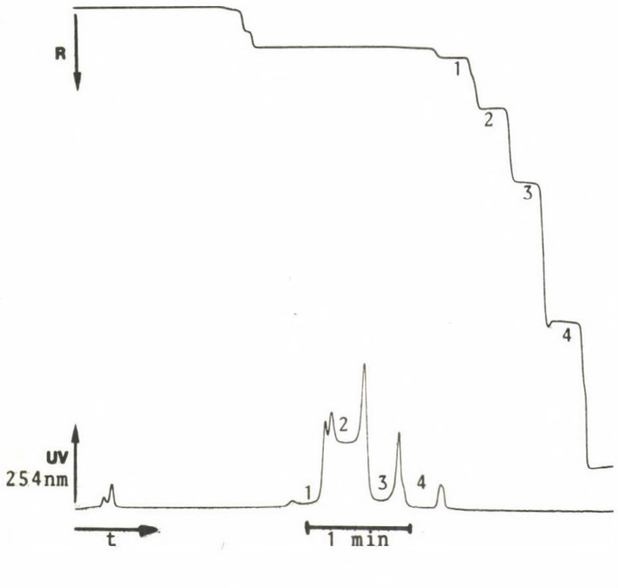

Fig. 6. Isotachopherogram of a reference mixture of acetate (1), cystine (2), (Acm)Cys (3) and alanine (4)

analysis of an equimolar standard mixture of acetate, cystine, (Acm)Cys and alanine provided convincing evidence that these substances are identical with four of the cleavage products detected in the hydrolysates (Figs 4 and 5). Owing to the lack of a suitable reference substance, one of the products formed during acid treatment of Ala-(Acm)Cys (Fig. 4A) could not be identified.

DISCUSSION

In numerous previous investigations, ITP was found to be a promising tool in controlling synthesis and subsequent purification of a variety of glutamine-, tyrosine-, alanine- and glycine-containing short chain peptides (8, 10-15). In the present study, we extended the use of ITP to monitor the course of synthesis and purification of two cyst(e)ine-containing dipeptides Ala-(Acm)Cys and $(\text{Ala-Cys})_2$, respectively.

Apparently, by applying anionic electrolyte systems at alkaline pH, ITP facilitates simultaneous analysis of the peptides Ala-(Acm)Cys, $(\text{Ala-Cys})_2$, free amino acids, amino acid derivatives and contaminating ions in the crude peptide materials as well as in the intermediate and end products during the purification procedures. This quality approach, exemplified in Figs 1-3, allows a direct consideration of both the reacton and purification conditions and, thus, facilitates the optimization of these procedures.

Obviously, the use of glycine instead of β-alanine as terminating ion obviously improved the separation of (Acm)Cys-containing compounds (Fig. 2). It is to note, that alanine cannot be detected with glycine as terminating electrolyte. Isotachophoretic analyses of the same purified peptide fractions applying electrolyte system II, however, clearly demonstrated that alanine could be completely removed under the chosen reaction conditions.

In contrast to the previous types of capillary plates, the use of a newly developed detector block (13, 15) enables UV measurements at 206 nm, especially suited for the detection of peptide(amide) bonds. Thus, a direct classification between free amino acids and unknown peptide(amide) bond-containing compounds is feasible. As illustrated in Figs 1 and 2, the main synthetic peptide Ala-(Acm)Cys as well as (Acm)Cys exhibited high UV levels at 206 nm, whereas for free alanine no UV absorption could be measured. Compared to Ala-(Acm)Cys, a slightly increased UV level was observed for the minor synthetic solute, strongly indicating that this zone corresponds to the "overreaction product" Ala-Ala-(Acm)Cys.

As outlined under Results, the successful extension of ITP to the determination of the composition of the synthesized peptides after acid and enzymatic hydrolysis facilitates their final identification (Figs 4-6). In this connection it is to emphasize that simultaneous detection of the observed cleavage products by applying alternative methods based on ion exchange and/or reversed phase chromatography is not possible.

As illustrated in Fig. 4A, enzymatic hydrolysis of Ala-(Acm)Cys resulted in the liberation of the respective amino acid components. In the acid hydrolysate, however, only free alanine, acetate and a slightly UV-absorbing unknown compound (presumably aminomethylcysteine) could be detected (Fig. 4B). This observation is in agreement with previous findings that the Acm protecting group is unstable under the usual conditions of acid hydrolysis, whereas peptide hydrolysis using aminopeptidase M leaves this group intact (33).

According to the basic principles of ITP (34), there is a linear relationship between the absolute amount of a sample ion injected and the corresponding zone length measured. This means, that under standardized analytical conditions ITP provides not only qualitative, but quantitative information about the composition of the sample. Generally, an absolute quantitative evaluation requires a calibration for all ionic species present in a sample. On the other hand, a relative quantification of sample ions with similar molecular weights and net charge is appropriate without the necessity to establish calibration curves or calibration constants. This is possible since the conductivity zone length is not influenced by substance-specific properties, e.g. absorption coefficients.

Based on these considerations, the ratios of (Acm)Cys/alanine and cystine/alanine formed during enzymatic hydrolysis of Ala-(Acm)Cys (Fig. 4B) and acid/enzymatic hydrolysis of $(\text{Ala-Cys})_2$ (Fig. 5) can be estimated to 0.95:1.00 and 1.93:1.00, respectively, thereby fully confirming the structure of the synthesized peptides.

Undoubtely, an adequate evaluation of ITP in analyzing Cyst(e)ine-containing peptides as well as free cystine and cysteine derivatives requires a comparison with other methods as by example ion-exchange and reversed-phase chromatography. Indeed, the major advantage of ITP over these chromatographic techniques lies in the fact that the mentioned solutes can be separated and detected without sample processing (Figs 1-6). Although free cystine and cystine-containing peptides are detectable using ion-exchange chromatography with post-column ninhydrin derivatization, low sensitivity and extremely prolonged analysis time (especially in the analysis of peptides) are further disadvantages of this method compared to ITP.

The determination of free cystine and cystinyl-peptides using reversed-phase HPLC is not feasible by means of established precolumn derivatization agents as by example o-Phthaldialdehyde (OPA) or 9-fluorenylmethycarbonylchloride (FMOC-Cl) (35, 36). Alternative precolumn derivatization using phenylisothiocyanate (PITC) revealed only poor recovery and linearity (37). Ongoing studies in our laboratory suggest that 5-dimethylaminonaphtalene-1-sulphonylchloride (DANSYL-Cl) may represent a useful derivatization agent for free cystine and cystinyl-peptides (37). Nevertheless, further studies are warranted to finally evaluate this latter technique as a routinely manageable method in peptide analytic including the afore mentioned substances.

Owing to the high sensitivity (0.5 nmol/injection) and resolution, the simultaneous separation/detection of short chain peptides, free amino acids, amino acid derivatives and inorganic ions and the possibility to directly classify the sample ions applying UV detection at 206 nm. ITP represents a suitable analytical method to control the synthesis and purification of cyst(e)ine-containing peptides. As a further clear benefit compared to alternative chromatographic methods, the use of ITP facilitates an easy identification of the synthesized peptides by directly analyzing acid and enzymatic hydrolysates.

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H. Kalász and L.S. Ettre (Eds)

PREPARATION OF DIFFERENT PHENYL PHASES FOR RP-HPLC

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SUMMARY

Three different phenyl-silica phases were prepared. The amount of organic moiety bound on the silica support was determined from thermogravimetric measurements. The specific surface areas of the gels were obtained from nitrogen sorption measurements at 77^{O}K. The selectivity of the phenyl-bonded silica gels was examined and compared with ODS-silica in reversed-phase liquid chromatography. Polar solutes are retained in greater extent on the phenyl-phases than on ODS gel. Phenyl-modified silica gels seem to be better stationary phases for hydroxy-aromatics than the ODS-phase. These phases improve the separation of the ortho, meta and para isomers of nitrophenols and dihydroxybenzene derivatives compared with the ODS-phase.

INTRODUCTION

Various bonded phases have been synthesized for use in liquid chromatography some of which are also commercially available. Among these the following bonded phases are widely used:

C_{18}	(octadecyl) (ODS)	silica
C_8	(octyl)	silica
C_6	(hexyl)	silica
C_2	(dimethyl)	silica
CN	(cyano)	silica

In comparison with the extensive literature on the reaction of alkylchlorosilanes and silica, only a limited number of papers have dealt with surface modification with phenyl-chlorosilanes (1-4).

A number of suppliers offer phenyl-bonded phases, but these have attracted little attention. These phases have been applied in the separation of peptides (5) and to some pharmaceuticals such as propanolol (6). Generally phenyl phases have been regarded as being more polar than ODS-silica and expected interactions on it (7, 8).

Our aim was to modify silica gels with three different organochlorosilanes: fully hydroxylated porous silica was treated with dichloromethylphenylsilane, dichlorodiphenylsilane and chlorotriphenylsilane. The retention behaviour of solutes on columns packed with these three types of phenyl-silica was examined in reversed-phase liquid chromatography. The chromatographic behaviour of hydroxy-aromatics was studied on phenyl- and octadecyl bonded silica gels.

EXPERIMENTAL

Apparatus

Chromatographic separations were carried out with a Varian 8500 solvent delivery system, a Model 7125 sample injector (Rheodyne, Berkeley California, USA) and a Varian Model 635 UV-Vis monitor. The columns were made of 250 x 4.0 mm I.D. stainless-steel tubes (Bio-Separation Technologies Co., Budapest, Hungary). The thermal studies were performed with a MOM Model 3427 Derivatograph (Budapest, Hungary). The B.E.T. surface area of silicas was determined by a sorptometer made at the Department of Physical Chemistry of the Technical University of Budapest (Hungary).

Materials

ODS-Hypersil 5 μm was obtained from Shandon (Cheshire, England) Separon SGX Si-100 5 μm was purchased from

Laboratorni Pristroje (Praha, Czechoslovakia). Dichloromethylphenylsilane (Wacker Silan), dichlorodiphenylsilane (Fluka), chlorotriphenylsilane (Fluka) and trimethylchlorosilane (Merck) were used as received. All other chemicals obtained from commercial sources were used without purification.

Chromatography

The test solutes were dissolved in the mobile phase at a concentration of 0.1 mg/mL by weight. Typically 20 μL was injected. Flow rate was 1 mL/min. Sample peaks were detected at 254 nm.

Phenyl-phase packing preparation

The preparation of phenyl packings was based on Unger's procedure (9). 10 g of dried silica gel was mixed with a 5 % solution of the chlorophenylsilane in anhydrous toluene and refluxed in a sealed flask. The reaction was complete within 8 h. The phenyl-silica gel was filtered and washed with toluene, methanol, water and methanol in this sequence. The solid phase was then kept at 80°C for several hours under vacuum. Residual hydroxyl groups were deactivated by treating the solid material with a 10 % solution of trimethylchlorsilane in toluene. These deactivated phenyl-bonded silica gels were high-pressure slurry-packed into 250 x 4.0 mm I.D. columns by Bio-Separation Technologies Co.

Analysis of the stationary phase

The amount of organic moiety bound on the silica supports was determined from thermogravimetric curves of the modified silica gel (10).

Data for the specific surface areas of the gels (S_{BET}) were obtained from nitrogen sorption measurements at -196°C.

RESULT AND DISCUSSION

Analysis of the stationary phase

The amount of organic moiety bound on the silica support was determined from the weight loss of the gel. The maximum surface concentration of the bonded Si-aryl groups are given in Table I: it decreases in the sequence phenylhydroxysilyl-diphenylhydroxysilyl-triphenylsilyl. The decrease in Si-aryl is a necessary consequence of the increase in the molecular volume of the modifier by subsequent replacement of chlorine atoms with phenyl groups.

S_{BET} values were obtained from nitrogen sorption measurements at -196°C. It was assumed that the starting silica support had a surface area of 535 m^2/g (information from Laboratorni Pristroje Praha, Czechoslovakia). These measurements (see Table I) demonstrate a considerable reduction in S_{BET}. The decrease in the S_{BET} values of packings containing different bonded phenylsilyl groups is about 25-30 % as compared to the starting silica gel.

Retention of test solutes

Table II shows the retention behaviour of test solutes on the different phenyl and ODS stationary phases in methanol-water (60:40 v/v). Comparison between phenyl and ODS phases reveal that the higher capacity factors of toluene, bromobenzene, p-xylene and propylbenzene on the ODS packing must be due to the octadecyl groups while the higher capacity factors of the polar aromatics as a phenol, cresol, acetophenon, anisol and methyl benzoate on the phenyl phases must be due to the phenyl groups. Again, selectivity factors show no significant difference between the different phenyl packings.

After comparing the capacity factors of the test solutes on phase A, B, C, and D, it is evident that the ODS phase is less polar than any of the phenyl phases and that the polarity of phenyl-silica decrease in the sequence phenylsilyl-diphenylsilyl-triphenylsilyl.

Table I. Characteristics of the prepared silica gels

Material	Particle size µm	Bonded functional group	Specific surface area S_{BET} m^2/g	Mean pore diameter Å	Surface conc. $\mu mol/m^2$	C%	Bonded phenyl group $\mu mol/m^2$
A	5	Phenyl methyl hydroxy silyl	397	100	3.6	13	3.6
B	5	Diphenyl hydroxy silyl	384	100	2.6	15	5.2
C	5	Triphenyl silyl	411	100	1.9	17.5	5.7
D**	5	Octadecyl dimethyl silyl	170*	100*	2.5*	12*	-

*Manufacturer's values
**Hypersil-ODS, Shandon, England

Table II. Capacity (k') and selectivity (α) factors of test solutes on phenyl-modified and C_{18} reversed-phase packings
Eluent, methanol-water (60:40 v/v); temperature, ambient. Column A, functional group = methylphenylsilyl: column B, functional group = diphenylsilyl, column C, functional group = triphenylsilyl, column D, functional group = octadecyldimetylsilyl

Sample	Column A		Column B		Column C		Column D	
	k'	α	k'	α	k'	α	k'	α
phenol	0.85		0.69		0.50		0.60	
		1.49		1.45		1.44		1.00
o-cresol	1.27		1.00		0.72		0.60	
		1.48		1.50		1.29		1.75
acetophenone	1.88		1.50		0.93		1.05	
		1.23		1.18		1.19		2.09
anisole	2.30		1.77		1.11		2.20	
		1.18		1.19		1.22		0.93
methyl benzoate	2.71		2.12		1.36		2.05	
		1.09		1.00		1.10		2.34
toluene	2.96		2.12		1.50		4.81	
		1.23		1.23		1.09		1.13
bromobenzene	3.62		2.62		1.64		5.45	
		1.22		1.18		1.31		1.70
p-xylene	4.42		3.08		2.14		9.30	
		1.56		1.51		1.48		1.78
propylbenzene	6.92		4.65		3.18		16.55	

Phenyl-modified silica gels seem to be better stationary phases for polar aromatics than the ODS phase.

Separation of hydroxy aromatics

Phenyl phases were assumed to be better phase for polar solutes than ODS packing. Therefore we examined the chromatographic behaviour of hydroxy-aromatics on both phenyl- and ODS-silica gel. Figure 1 shows the separation of hydroxy aromatics on the different stationary phases in methanol-water (60:40 v/v).

After comparing chromatograms A, B, C and D, it is evident that the ODS packing (chromatogram D) is too polar to separate these solutes with good efficiency. Figure 1A, B and C present the separation of the same compounds on the column packed with different phenyl supports. It can easily be seen that the separation of the investigated substances is the best

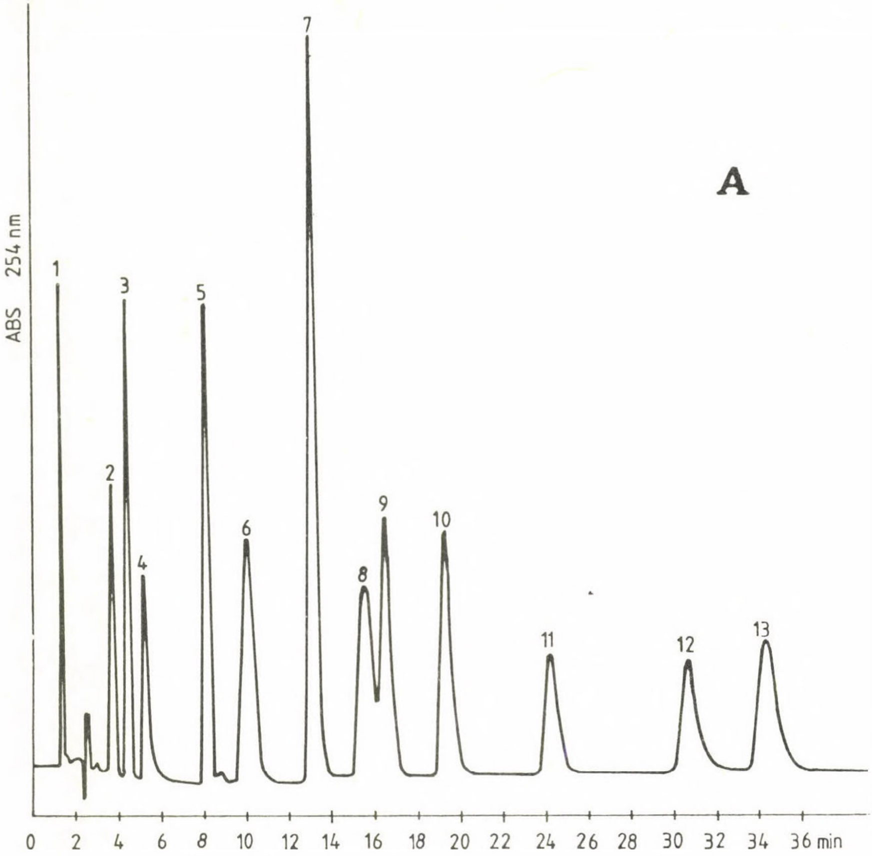

Fig. 1A. Separation of hydroxy-aromatics on methylphenyl phase. Peaks: (1) 3-amino-4-hydroxybenzoic acid, (2) hydroquinone, (3) resorcinol, (4) catechol, (5) phenol, (6) 4-nitrophenol, (7) 3-nitrophenol, (8) 2-nitrophenol, (9) methyl-4-hydroxybenzoate, (10) 2,4-dimethylphenol, (11) anisole, (12) 2-naphthol, (13) 1-naphthol, Mobile phase: 40:60 (v/v) methanol: :water. Flow rate: 1 mL/min. Detection: UV at 254 nm. Temperature: ambient

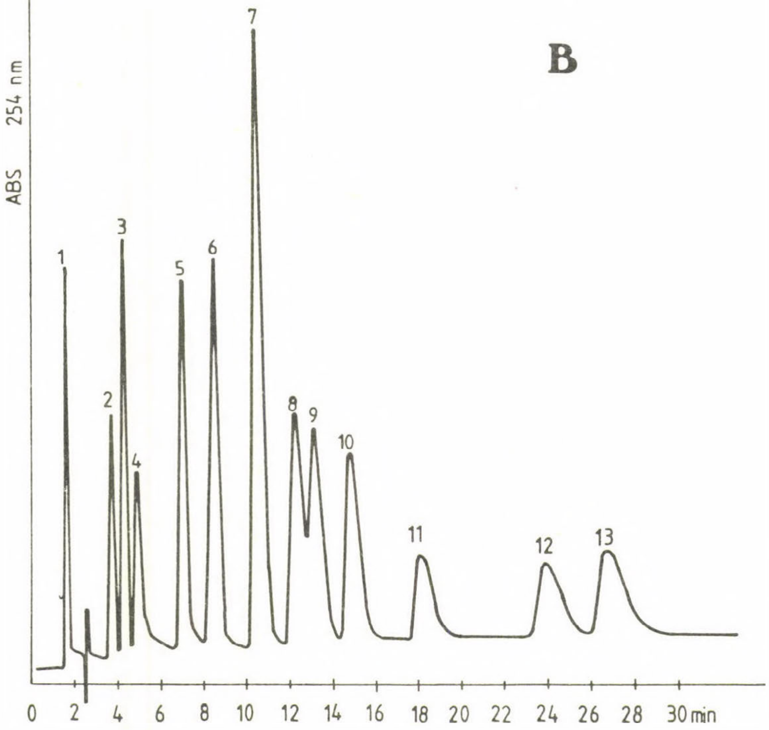

Fig. 1B. Separation of hydroxy-aromatics on diphenyl phase. Peaks: (1) 3-amino-4-hydroxybenzoic acid, (2) hydroquinone, (3) resorcinol, (4) catechol, (5) phenol, (6) 4-nitrophenol, (7) 3-nitrophenol, (8) 2-nitrophenol, (9) methyl-4-hydroxybenzoate, (10) 2,4-dimethylphenol, (11) anisole, (12) 2-naphthol, (13) 1-naphthol. Mobile phase: 40:60 (v/v) methanol::water. Flow rate: 1 mL/min. Detection UV at 254 nm. Temperature: ambient

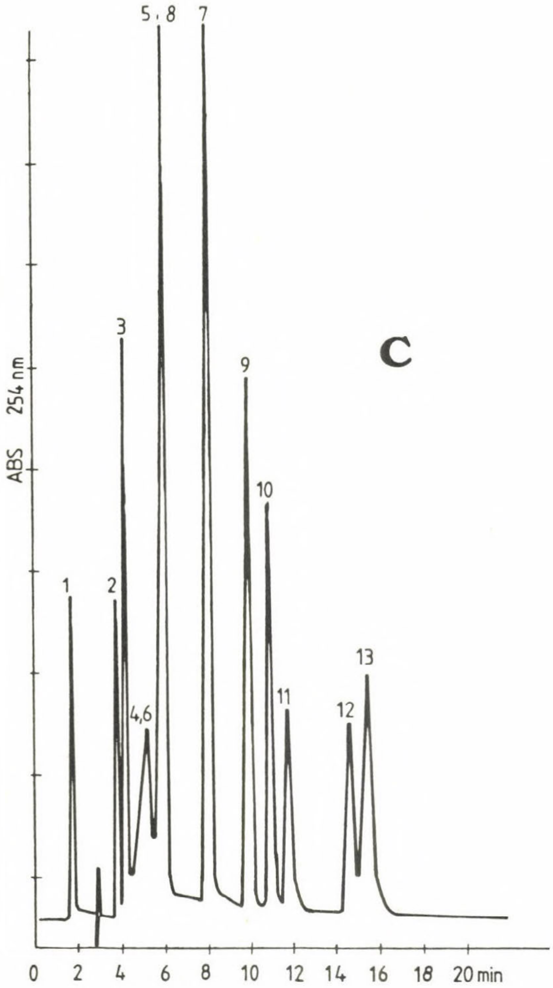

Fig. 1C. Separation of hydroxy-aromatics on triphenyl phase. Peaks: (1) 3-amino-4-hydroxybenzoic acid, (2) hydroquinone, (3) resorcinol, (4) catechol, (5) phenol, (6) 4-nitrophenol, (7) 3-nitrophenol, (8) 2-nitrophenol, (9) methyl-4-hydroxybenzoate, (10) 2,4-dimethylphenol, (11) anisole, (12) 2-naphthol, (13) 1-naphthol. Mobile phase: 40:60 (v/v) methanol: :water. Flow rate: 1 mL/min. Detection: UV at 254 nm. Temperature: ambient

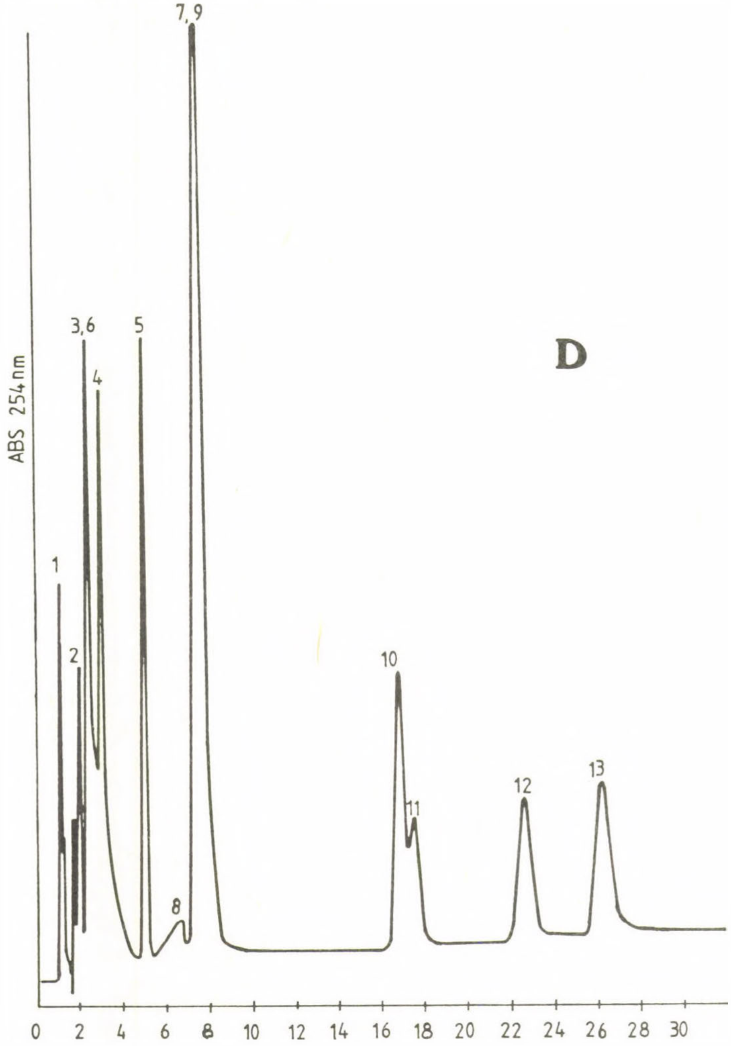

Fig. 1D. Separation of hydroxy-aromatics on ODS-phase. Peaks: (1) 3-amino-4-hydroxybenzoic acid, (2) hydroquinone, (3) resorcinol, (4) catechol, (5) phenol, (6) 4-nitrophenol, (7) 3-nitrophenol, (8) 2-nitrophenol, (9) methyl-4-hydroxybenzoate, (10) 2,4-dimethylphenol, (11) anisole, (12) 2-naphthol, (13) 1-naphthol. Mobile phase: 40:60 (v/v) methanol:water. Flow rate: 1 mL/min. Detection: UV at 254 nm. Temperature: ambient on the methylphenyl material (Fig. 1A), but both diphenyl (Fig. 1B), and triphenyl-silica (Fig. 1C) have been found to be well suited for this application.

Selectivity of the packings was observed for the chromatographic behaviour of dihydroxybenzene and nitrophenols. The order of elution of the dihydroxybenzenes from the ODS column was 1,4- 1,3- 1,2-dihydroxybenzenes and from the phenyl columns it was the same. The order of elution of the nitrophenols from the ODS phase was 1,4- 1,2- 1,3-nitrophenols and from phenyl phases it was 1,4- 1,3- 1,2-nitrophenols using methanol-water mixture as the mobile phase.

CONCLUSION

The main results of this study can be summarized as follows:

Polar solutes are retained in greater extent on phenyl phases than on ODS-silica gel.

Phenyl-modified silica gels seem to be better stationary phase for hydroxy-aromatics than ODS-phase.

The phenyl-phases improve the separation of the ortho, meta and para isomers of nitrophenols and dihydroxybenzene derivatives as compared with the ODS-phase.

On the phenyl-silicas the nitrophenol isomers are retained in the order para-meta-ortho. This is different from the para-ortho-meta order observed on ODS-silica.

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SIZE EXCLUSION SEPARATIONS OF PROTEINS ON STABILIZED DIOL PHASE

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SUMMARY

Silica gel particles stabilized by zirconium salt treatment and then covalently bonded with a layer of diol groups were investigated as a stationary phase for the high-performance liquid chromatography of proteins. A column packed with this material fractionated proteins by molecular weight (MW) in the range of 10,000 to 110,000 Daltons with a quasi-linear relationship between the logarithm of the MW and the elution volume. The zirconium-treated and the polymer-diol bonded surface permitted 500 h continuous use of buffered eluents at pH 8.2 and high ionic strength. With an appropriate mobile phase, all proteins eluted with a recovery of 90 % or greater.

INTRODUCTION

Particular interest is attached to size-exclusion separations of biological macromolecules. The porous inorganic particles used as column packing have the advantages of mechanical stability and permanent porosity over swelling carbohydrate gels such as cross-linked dextrans. The use of inorganic particle, however, is seriously limited by the fact that they either irreversibly adsorb or denature the sensitive biological substances.

In order to overcome these disadvantages the surface has to be chemically modified (1). Pioneering work on the size separation of biopolymers on inorganic materials was carried out by

Haller (2, 3) and Hiatt et al. (4) and then, Regnier and Noel (5) using controlled-porosity glass to which 1,2-epoxy-3-propoxy-propyltrimethoxysilane was chemically bonded, creating a glycophase-bonded material.

Other authors (6, 7) have also used chemical bonding of organo silanes to modify the silica surface. Silica and glass support matrices used in size-exclusion chromatography are soluble to some extent in buffered mobile phases. To overcome these disadvantages, several researchers (8, 9) introduced a coating technique using zirconium salts to deposit a continuous layer of metal oxide on the surface of this supports. Some suppliers such as Toyo-Soda (10) have employed polymers to help stabilize their packing materials. However, even polymer-coated silica packing exhibits this dissolution problem since the base can eventually penetrate the silica surface below the polymer allowing the formation of water-soluble organo silicates.

Stout and DeStefano (9) employed zirconium treatment and prepared monolayer diol phases to improve the water durability of the silica gel and demonstrated that the polymer-coated phases - such as Toyo-Soda TSK G 3000 SW - were not able to protect the packing materials against hydrolytic attack.

Our work is based on the assumption that coating the surface of silica with a zirconium salt and bonding the hydrophilic organo silane polymer can protect the packing materials from the dissolution problem much better than when only polymer coating or zirconium treatment and monolayer bonding procedures are used.

We also examined the usefulness of metal-coating and polymer-bonded support for protein separations. This paper reports on the results of these studies.

EXPERIMENTAL SECTION

Apparatus

Chromatographic separations were carried out with a Varian 8500 solvent delivery system, a Model 7125 sample injector (Rheodyne Berkeley, California) and a Varian Model 635

UV-Vis Monitor. The column was packed in 250 x 8 mm I.D. stainless-steel tube (Bio-Separation-Technologies Co., Budapest, Hungary). A 50 x 4.6 mm I.D. precolumn packed with 30 m, 200 Å, diol (Bio-Separation-Technologies Co.) was used to protect the analytical column. The thermal studies were performed with a MOM Model 3427 Derivatograph (Budapest, Hungary).

Materials

Proteins were obtained from different sources. Porous silica (Hypersil WP-300, 5 µm) was obtained from Shandon (England).

Zirconium oxychloride was purchased from Reanal (Budapest, Hungary). 3-Glycidoxypropyl-trimethoxysilane (Fluka) was used as received.

All other chemicals obtained from commercial sources were used without purification.

Chromatography

Protein samples were dissolved in the mobile phase at a concentration of 2 mg/mL. Typically 20 µl was injected. Flow rates were changed from 0.2 mL/min to 2 mL/min. Protein peaks were detected at 280 nm.

Preparation of continuous surface layer of zirconium oxide on silica gel

This preparation was based on the procedures of Tomb and Weetall (8) and Stout and DiStefano (9).

20 g Hypersil WP-300 and 200 mL aqueous 5 % (w/v) zirconium oxychloride solution were mixed and placed under a vacuum for about 30 min to remove air from the pores, and refluxed for 1 h. The slurry was cooled, filtered then dried overnight at 150°C. The zirconium-treated silica gel was then fired at about 800°C for 5 h.

Polymer-diol packing preparation

The preparation of diol packing was based on the procedures of Kirkland and Yates (10, 11)(see Fig. 1).

10 mL of 3-glycidoxypropyl-trimethoxysilane, 30 mL tetrahydrofuran and 1 mL 0.1 M sodium hydroxyde solution was added to a 100 mL round bottom flask. The mixture was then added to the zirconium-treated silica gel and refluxed for 8 h. The solvent was removed while stirring the mixture under vacuum. The resulting dry powder was then heated for 2 h at 150°C. When cooled, the solid support was washed with tetrahydrofuran, toluene, methanol and acetone, in this sequence. The solid phase was then kept at 80°C for several hours under vacuum.

These stabilized, hydrophilic, polymer-bonded phase packings were high-pressure slurry packed into 250 x 8 mm I.D. columns by Bio-Separation Technologies Co.

Analysis of the stationary phase

The epoxide content of the silica-based packing was determined by reaction with ionic halide (12).

The amount of organic moiety bound to the silica particles was determined by thermogravimetry, from 200°C to 1000°C (13).

Recovery studies

Protein recoveries were determined by comparing the peak area of the injected samples obtained through the chromatographic column with the area obtained in which the column was replaced with a stainless-steel capillary (14).

RESULT AND DISCUSSION

Analysis of the stationary phase

The amount of organic moiety bound on the silica support was determined from the weight loss of the gel. It was assumed that the silica support had a surface area of 60 m^2/g (Shandon

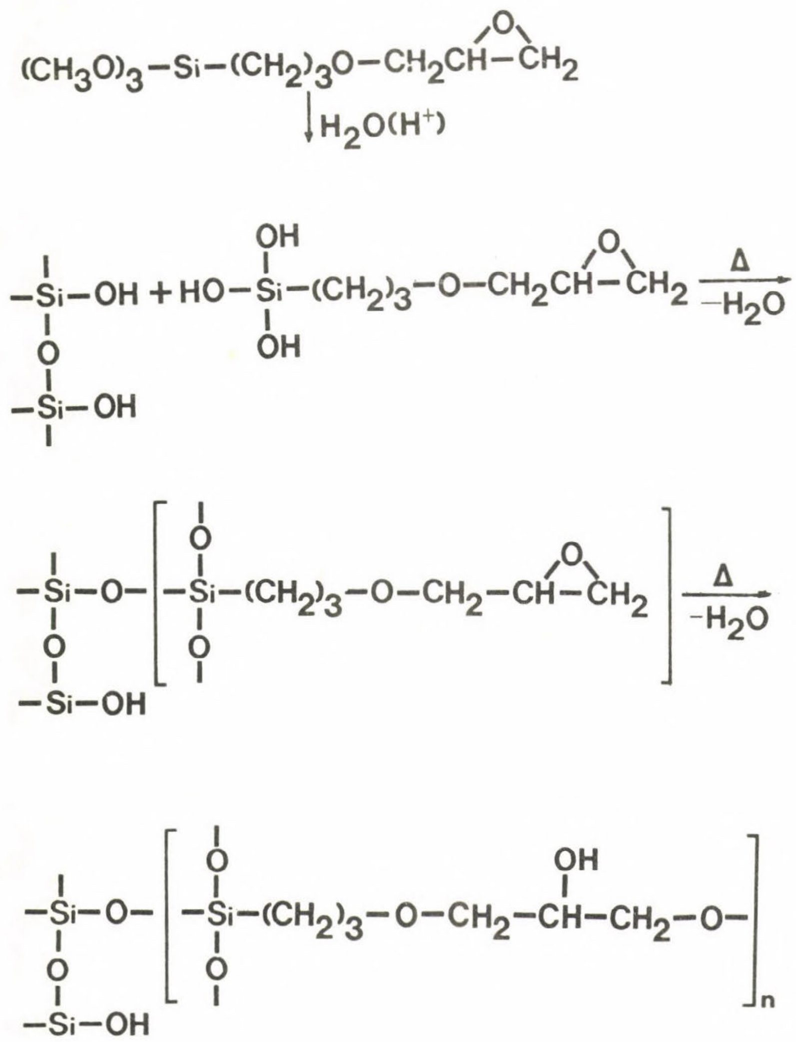

Fig. 1. Reaction scheme of the polymer-diol phase preparation

Suthern Product Ltd). The TGA weight loss of the silica gel indicated that the total organic content of the phase was 22 %. Analysis for epoxide content was negative.

Chromatography factors

If there is no interaction between a stationary phase and a series of proteins, then a linear relationship would be expected between log MW and the elution volume (V_R). According to some authors (14-16) chromatography does not separate the substances strictly by molecular weight. Other mechanisms such as hydrophobic and ion-exchange interaction and the structure of the proteins also affect the separation of proteins on gel columns. These interactions between the stationary phase and the proteins could be minimized, although not eliminated completely, by the appropriate choice of the mobile phase.

We investigated the plot of log MW vs. elution volume using a mobile phase consisting of 0.2 M sodium chloride and 0.01 M Tris (pH 7.7). The results are shown in Fig. 2. The elution volume of proteins are tabulated in Table I. The calibration plot of log MW vs. V_R demonstrates the quasi-linear relationship typical of gel cromatography separations of proteins. We have found that this packing support is suitable for the high-performance liquid chromatography of proteins.

In another series of experiments the chromatographic behaviour of this phase was examined as a function of the mobile phase and pH. These results are shown in Table II. The Table indicates that the elution volumes of some proteins are dependent on the ionic strength while for other proteins there appeared to be no effect. Consequently, we have found no correlation between V_R, pH, the mobile phase composition and the physical properties of proteins such as the pI.

Recovery

The recovery for a number of proteins from the Diol column was excellent with the mobile phase consisting of 0.2 M

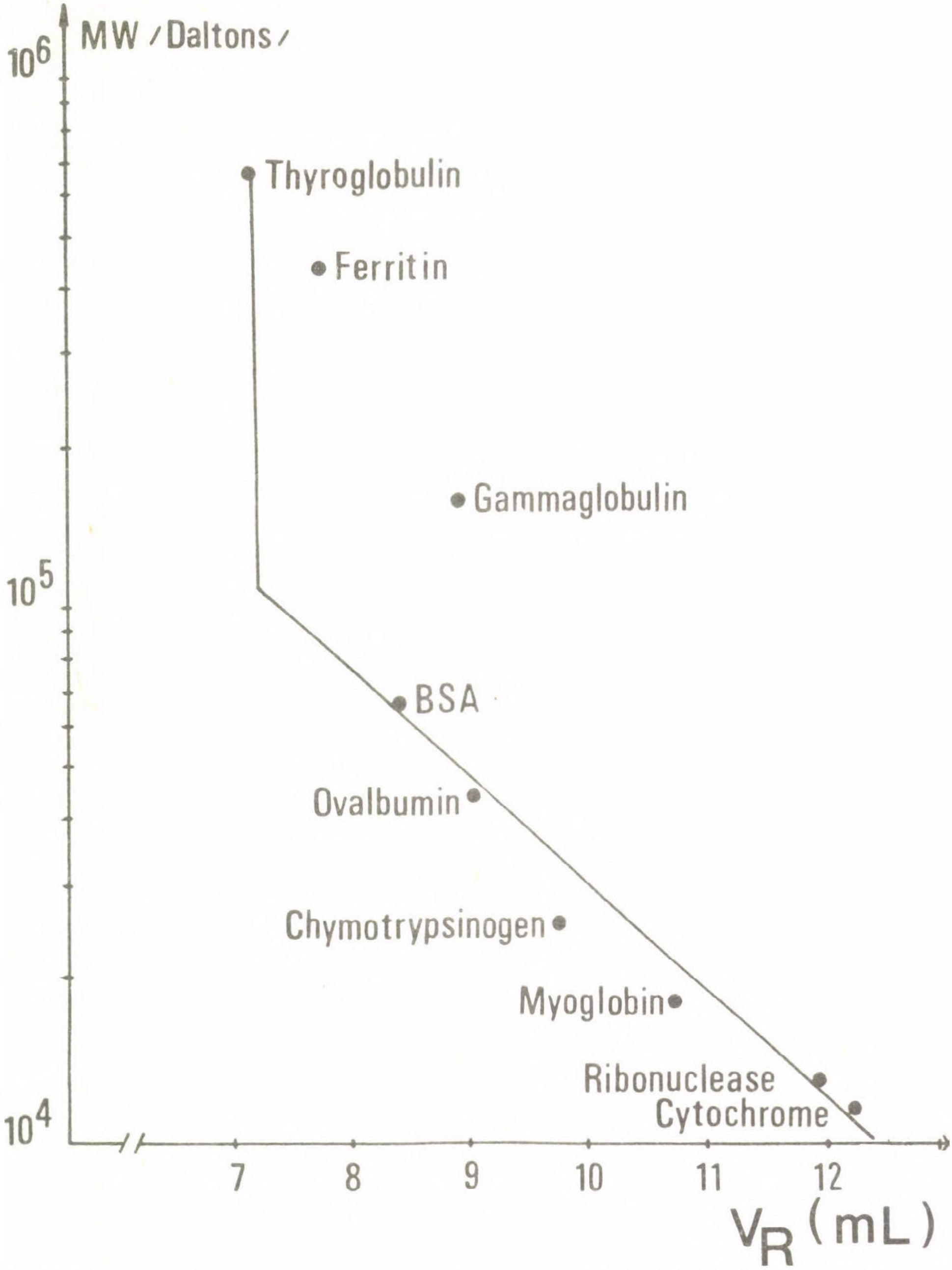

Fig. 2. Correlation between the elution volume and the logarithm of the molecular weight. Mobile phase: 0.2 M sodium chloride + 0.01 M Tris (pH 7.7)

Table I. Chromatographic parameters used for the gel filtration of proteins (Operating conditions are given in the legend of Fig. 3)

Sample component	MW (Daltons)	V_R (mL)	Recovery (%)
thyroglobulin	660 000	7.2	-
ferritin	440 000	7.8	-
gamma-immunoglobulin	160 000	9.0	85-87
bovine serum albumin	69 000	8.5	100
ovalbumin	43 500	9.1	95
chymotrypsinogen	25 000	9.8	101
myoglobin	17 800	10.8	100
ribonuclease A	13 000	12.0	101
chytochrome C	12 900	12.3	100
DNP-L-alanine	255	12.8	-

sodium chloride + 0.01 M Tris at pH 7.7 (Table I). Studies on the recovery were performed on a newly packed column. After three months the recoveries of the proteins were again determined under the same mobile phase conditions and found to be essentially the same as those given in Table I.

Chemical stability of the silica packing

All the experiments described in this report were performed with synthezized silica gel. This column was used over a period of three months, and the elution volumes of the proteins with a mobile phase consisting of 0.2 M sodium chloride plus 0.05 M Tris (pH 7.7) were nearly constant during this period.

In further stability tests this column was subjected to a flow rate of 0.5 mL/min for 100 h with a mobile phase of 0.2 M sodium chloride + 0.05 M Tris (pH 8.2). We selected these conditions so that we can compare our results with Stout's (9). After 100 h the column was re-equilibrated with the 0.2 M sodium chloride + 0.05 M Tris (pH 7.7) mobile phase and the elution volumes were determined. This process was repeated at 300 and 500 h.

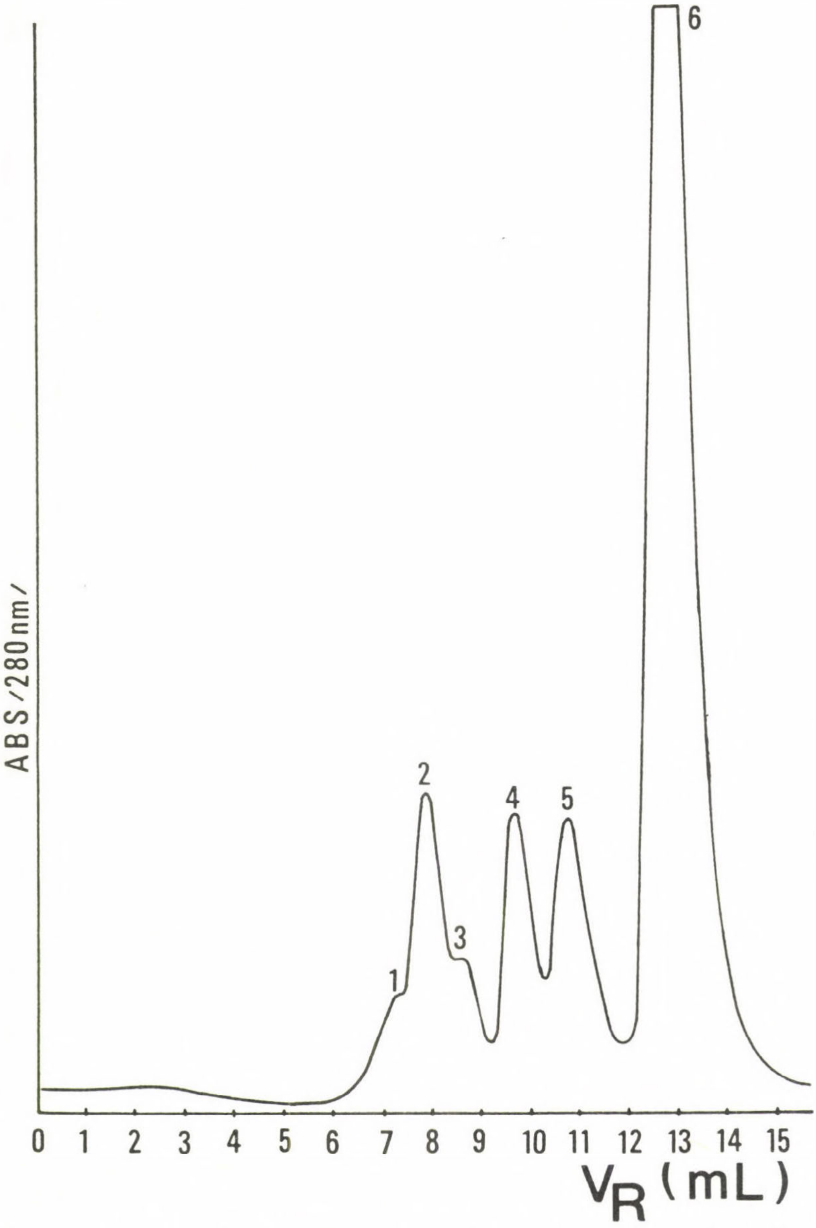

Fig. 3. Separation of proteins on a zirconium treated Diol phase I. The start of the test. Peaks (1) thyroglobulin (MW 660,000), (2) ferritin (MW 440,000), (3) bovine serum albumin (MW 69,000), (4) chymotrypsinogen (MW 25,000), (5) myoglobin (17,500), (6) DNP-L-alanine (MW 255). Mobile phase: 0.2 M sodium chloride + 0.05 M Tris (pH 7.7). Flow rate: 0.2 mL/min. Temperature, ambient. Detector: UV absorbance (280 nm)

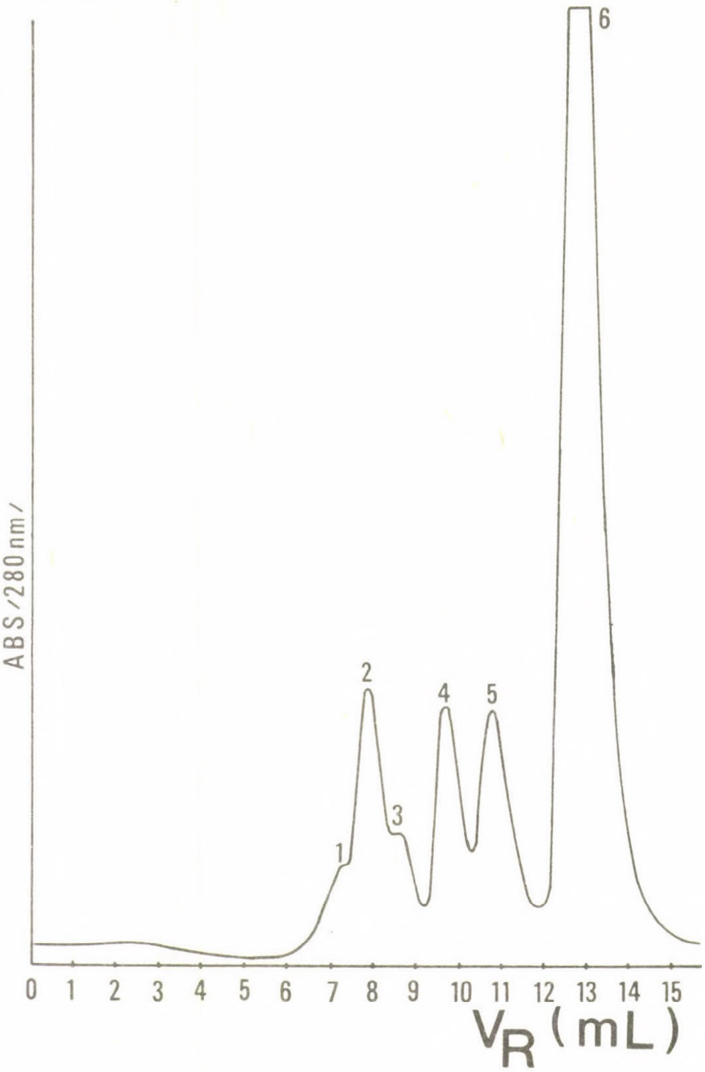

Fig. 4. Separation of proteins on zirconium treated Diol phase II. The end of the test (after 500 h). Peaks and conditions as in Fig. 3

Table II. Elution volume (V_R) as a function of mobile phase pH and ionic strength

Protein	0.2 M Na_2HPO_4 pH 7.5	0.05 M Na_2HPO_4 0.1 M $NaClO_4$ pH 7.6	0.2 M NaCl 0.05 M Tris pH 7.7	0.2 M NaCl 0.05 M Tris pH 8
thyroglobulin	-	-	7.2	7.5
ferritin	8	7.6	7.8	8.0
catalase	8.4	8.2	8.2	8.4
aldolase	8.5	8.3	8.3	8.5
gamma-immunoglobulin	8.8	8.5	9.0	8.5
bovine serum albumin	8.7	8.5	8.5	8.5
ovalbumin	8.8	9.0	9.1	8.8
chymotrypsinogen	9.2	8.6	9.8	9.3
myoglobin	10.8	10.8	10.8	10.2
α-amylase	-	-	9.4	8.9
ribonuclease	10.2	10.3	12.0	10.5
cytochrome-C	-	12.3	12.3	13.1
DNP-L-alanine	12.8	12.8	12.8	12.8

Figure 3 shows the separation of some commercial proteins on a freshly stabilized Diol column. Figure 4 shows the separation of the same proteins after 500 h on this column: this means that approximately 15 L of mobile phase (pH 8.2) were eluted through this column. Figures 3 and 4 show that the compounds appear to be eluting at a longer retention time except for myoglobin, but still have good peak shape. This can result from the slight degradation of the polymeric matrix itself which increase the pore volume of the synthesized packing. The peaks of chymotrypsinogen and myoglobin in Fig. 4 are smaller than in Fig. 3. This could result from the degradation of these proteins which were stored at 4°C in the solution for 3 months. Stout stated that the Toyo-Soda TSK G 3000 SW column lost 37 % of its bonded phase under the same conditions, and relates efficiency loss to the collapse of the packing material. We have found only slight degradation for our synthesized packing material.

In Stout's work (9) a pre-saturation column was used and the mobile phase was pumped through the column in a recirculation system. We achieved the same results with the difference that we have used fresh mobile phase and did not use a presaturation column for the stability test.

In summary we can state that the zirconium oxide blocks the sites on the surface which would be attacked by the hydroxide ions and the polymer-phase protects the silica-matrix better than a monolayer-phase.

ACKNOWLEDGEMENT

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CHROMATOGRAPHY OF HUMAN CYTOMEGALOVIRUS PROTEINS*

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SUMMARY

For the separation, isolation and structure determination of human cytomegalovirus envelope proteins which are responsible for immunological activity different chromatographic and electrophoretic methods were used: immune-affinity chromatography, size-exclusion chromatography, SDS-polyacrylamide-gel electrophoresis, and reversed-phase-HPLC. The methods producing single proteins and different fractions are compared to each other. The combination of different techniques led us to pure envelope proteins, in ready form and enough quantity for total characterization and sequence analysis. Gel chromatography was performed on Sephacryl-200 and -1000 column in the presence of SDS, with phosphate buffers. The reversed-phase column packing was Econosphere RP-300. Acetonitrile mobile phase containing 0.5 % trifluoroacetic acid was applied isocratically or using gradient. The immuno-adsorbents were prepared from AffiGel 10 with anti-HCMV-antibodies. The SDS-PAGE was used in both analytical and preparative form. In the latter case the isolation was performed by electro-elution.

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INTRODUCTION

Human cytomegalovirus (HCMV) is a member of the herpes virus family. HCMV infection is a major cause of virus-induced congenital disorder (1) and the virus is involved in severe complications in immuno-compromised individuals like organ transplant recipients or patients with malignant diseases (2).

An effective vaccine against the virus could prevent or eliminate the HCMV-associated diseases. Experimental live-attenuated viruses have been produced (3), but two characteristics of natural HCMV infection are troublesome with respect to their use as vaccine: the persistence of the virus in the infected host for life, and the oncogenic potential of the virus (4). A protein subunit vaccine, free of virus nucleic acid, might be more widely acceptable for human vaccination.

A first step towards the development of such a vaccine is the identification, separation, isolation and structure determination of proteins (glycoproteins) that confer protection. Thereafter, techniques such as cloning and expression or viral genes in alternative host systems or synthesis of immunologically active peptides could be applied.

Several studies have demonstrated that the viral envelope is able to induce virus-specific humoral and cellular immune response in animals (5, 6). A large number of glycoproteins with various molecular weights (58, 67, 86, 95, 116, 130 kD), protein complexes were identified as major antigenic components of the HCMV envelope (5-14, 17). However, the primary structure (amino acid composition and sequence) of these polypeptides has not been determined.

The need for pure HCMV protein preparations has stimulated our interest in the development of new effective procedures for separation and isolation of viral proteins. Polypeptides obtained by these methods are essential for the analysis of structure, biochemical and immunological characteristics of individual envelope proteins and ultimately provide a basis for the design of a subunit protein or peptide HCMV vaccine.

We summarize here our results in the isolation of envelope proteins from HCMV infected cell lysate, from purified virus or from its envelope fraction by the help of liquid chromatography and gel electrophoresis.

The main prerequisite for isolation of such viral membrane proteins is their solubility in the eluent used. They are hydrophobic compounds with strong tendency to aggregate, and are insoluble in water or aqueous buffer solutions. They can be solubilized by detergents, for example by mild, nonionic detergent, such as NP-40, or by strong ionic detergent such as SDS, or by high concentrations of denaturating agents such as urea or guanidine-hydrochloride.

I. ISOLATION OF HCMV PROTEINS BY LIQUID CHROMATOGRAPHY

I.1. *Size-exlusion chromatography*

The solvents containing additives mentioned earlier are useful in size-exclusion chromatography of viral proteins (22, 23).

The HCMV envelope proteins were extracted from the viral lipid bilayer membrane by nonionic detergent treatment (NP 40) and purified by centrifugation onto a 20 % sucrose cushion (5). This preparation was used for further chromatographic purification.

Based on the optimal molecular weight range Sephacryl 200 and 1000 column packings were selected for separation of HCMV envelope components. PBS containing sodium dodecyl sulfate (SDS) detergent and mercaptoethanol was used for elution (see Fig. 1). Mercaptoethanol assured the reducing condition to avoid the formation of artifacts due to complexation by S-S bridges. The compatible SDS-polyacrylamide gel electrophoresis (SDS-PAGE) proved to be very applicable to analyse the protein composition of the envelope preparation before and its fractions after the separation. Electrophoretic analysis of the flow-through fractions indicated that fractionation was achieved on the basis of differences in the molecular weight. All peak showed more than one protein band in SDS-PAGE.

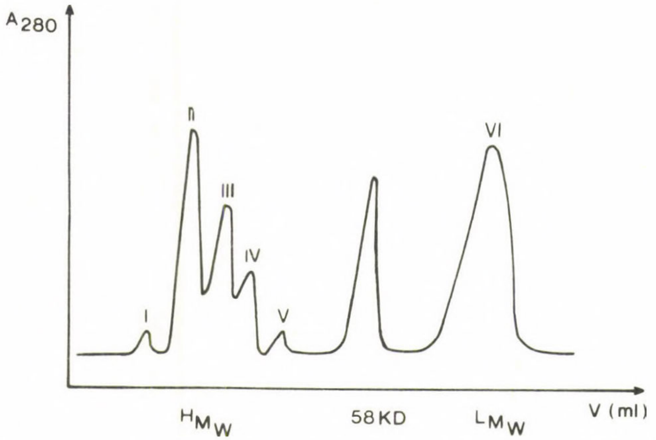


Fig. 1. GPC of HCMV envelope proteins. Column: 90x2 cm Shepacryl S-200; eluent: 0.08 M phosphate buffer pH 7.2, 0.15 M NaCl, cont. 0.1 % SDS and 10 mM mercaptoethanol; flow rate: 0.5 cm^3/min; fraction size: 1.5 cm^3/vial; detection: 280 nm. H_{M_W} : fractions with proteins of high M_W; L_{M_W}-fraction with low M_W

I.2. *High-performance liquid chromatography (HPLC)*

We have tried to utilize the excellent resolution of reversed-phase (RP)-HPLC (18) in order to obtain individual proteins (22, 23). Unfortunately, RP-HPLC of hydrophobic proteins is not feasible in solvents containing SDS or guanidine-HCl (24). Although 8M urea, which is also a widely used solubilizing agent, may be applied sometimes in RP-HPLC (24), there are some problems due to the high viscosity of 8M urea and the low solubility of urea in organic solvents.

Another possibility is to use trifluoroacetic acid or formic acid as additives, because they can promote the solubility of hydrophobic peptides and proteins (18). For example, trifluoroacetic acid is generally used in RP-HPLC solvents for separation and isolation of peptide hormones and their precursors. These aqueous solvent systems contain acetonitrile, methanol, propanol or THF as organic modifiers (18).

According to this approach Zaia (21) isolated the major matrix protein of HCMV with M_W 64 KD from a viral particles extract solubilized by 6M guanidium chloride. The proteins were eluted from a Synchropak C-18 reversed-phase column (300 nm pore size) by using an acetonitrile gradient in 0.1 % aqueous TFA (see Table I).

Epstein and coworkers (19, 20) isolated HCMV proteins on Brownlee RP-300 column using 20-70 % acetonitrile gradient. Chromatography in our laboratory was performed on Econosphere RP 18 column with wide pore size (300 nm) using acetonitrile--water-0.1 % TFA eluent in both isocratic and gradient profile (see Table I). Starting either from the whole viral extract or the capside, single protein with M_W 64 KD was obtained and its purity was verified by SDS-PAGE.

Experiences with various types of columns and gradients indicated that effective separation but no quantitative yields could be achieved under these circumstances. SDS-PAGE analysis of fractions collected from such gradients showed that most fractions contained multiple proteins. Thus, a second separation procedure should result in highly purified preparations of individual proteins from HCMV.

I.3. *Immunoaffinity chromatography*

The selectivity of separation and isolation can be increased by using immunoadsorbents as special column packings. In optimal case, it is required for the purification of an antigen protein to have a unique immunoadsorbent column containing immobilized, antigen-specific antibodies. Polyclonal or monoclonal antibodies raised against HCMV or its envelope antigen were used for immunoaffinity chromatography as biospecific ligands.

(a) Preparation of immunoadsorbent

Immunoglobulins were precipitated from sera or ascites fluid of guinea pigs immunized with purified HCMV or envelope proteins by saturated ammonium sulfate solution. IgG fraction was isolated by affinity chromatography on a protein-A Sepharose CL-4B column, using pH gradient (pH 8-3) during

Table I. Chromatographic data of RP-HPLC of HCMV proteins

	I. (21)	II. (19)	III.
Column	Synchropak C-18 (pore size 300 nm) 250 x 4 mm	Brownlee RP-300	Econosphere RP 18-300 or ODS-Hypersil-6 125 x 4 mm
Eluent	CH_3CN gradient in 0.1% aqueous TFA	CH_3CN gradient 20 → 70%	isocratic CH_3CN-H_2O 40:60 0.1 % TFA 35:65 gradient A: 80:20 H_2O-CH_3CN 0.1 % TFA B: CH_3CN 0.1 % TFA
Flow rate	1.0 ml/min	2.0 ml/min	1.4 ml/min
Detection	214 nm ^{35}S, ^{3}H	214 nm	220 nm
Sample	6M guanidinium chloride extract of HCMV	solubilized virus envelope or capside in 8M guanidine + 10 mM dithiothreitol	
Result	protein 64 KD	proteins with different MW	protein 64 KD t_R = 38.4 min

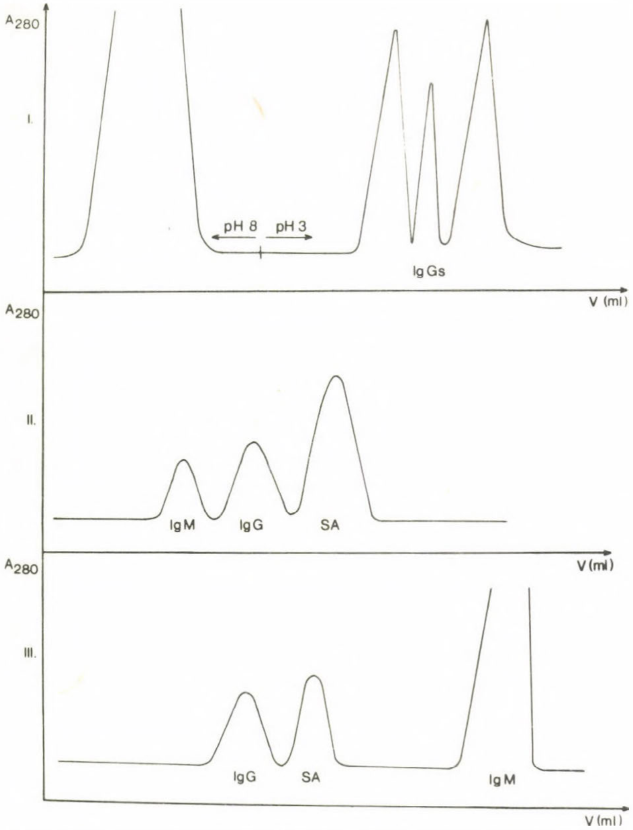


Fig. 2. Chromatography of immunoglobulins on protein A-Sepharose CL-4B (I) and on Sephacryl S-200 (II, III). SA-serum albumin; gel 10 % PA, electr. run at 12 mA

Table II. Chromatographic data of immunoglobulin preparations

Columns: 15 x 1 cm (I) 90 x 2 cm (II-III)
Protein A - Sepharose 4B

Packing: Sephacryl-S 200

Eluents: 1. Britton-Robinson buffer pH 8 and pH 3 (I)
2. 0.008M phosphate buffer pH 7.2 containing 0.15M NaCl (II)
3. 0.08M phosphate buffer pH 7.2 containing 1.5M NaCl (III)

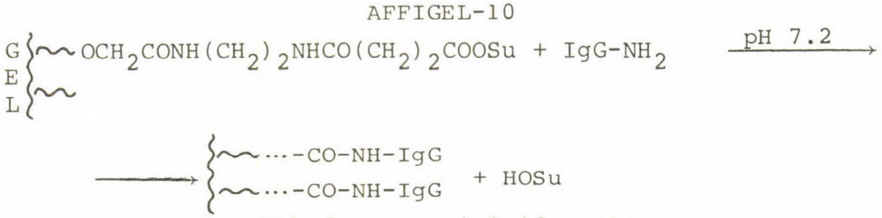
Detection: 280 nm

Flow rate: 0.5 cm^3/min

Fraction size: 1.5-3 ml/vial

Yields: 10-80 mg/15-20 ml serum or 5 ml ascites liquid elution (Fig. 2). IgM was purified from the flow-through fraction of the column and separated further on a Sephacryl-S-200 column. Single-step separation was achieved by gel-permeation chromatography on Sephacryl-S-200 column directly from the ammonium sulfate precipitated sera or ascites fluid. The impurities, like albumin, transferring or various proteases could be separated efficiently from the immunoglobulins using PBS elution. The IgM antibodies had higher separation factor if the phosphate buffer was used with significantly increased NaCl concentration (1.5 Mol/l)(see chromatograms and data in Fig. 2) (27).

The purified IgG fraction of anti-HCMV antibodies was coupled either to cyanogen bromide (CNBr)-activated Sepharose 4B or to Affigel-10, which is N-hydroxy-succinimide ester of a derivatized agarose gel. Characteristics of immunoadsorbents prepared by basic coupling procedures are summarized in Table III.



-COOSu-N-hydroxysuccinimide ester

SCHEME 1

Table III.

Immunoadsorbents	Method A	Method B
	anti gA-IgG- Sepharose 4B	anti gA-IgG- Affigel-10
Efficiency of couplings	55-60 %	60-75 %
Capacity of columns	6-7 mg IgG/ml/gel	8-12 mg IgG/ml/gel

(b) Isolation of viral proteins

These immunoadsorbents proved to be particularly useful in immunoaffinity chromatography, when monoclonal antibodies were immobilized to a matrix support for the purification of the HCMV envelope protein mixture called gA (7).

Example 1

^{35}S(Met) labelled HCMV infected cell lysate, 5-15 mg of purified virus, treated with 0.5 % Np40 or 1-1.5 mg of envelope were loaded to the columns and cycled over 3 times. The columns were washed extensively until the radioactivity reached the background level. The specifically bound proteins were eluted with 3M KSCN (pH 7.5). Peak fractions were pooled, dialysed and concentrated. Protein content was determined and the protein composition of this fraction was analysed by SDS-PAGE.

The preparation data are given in Table IV.

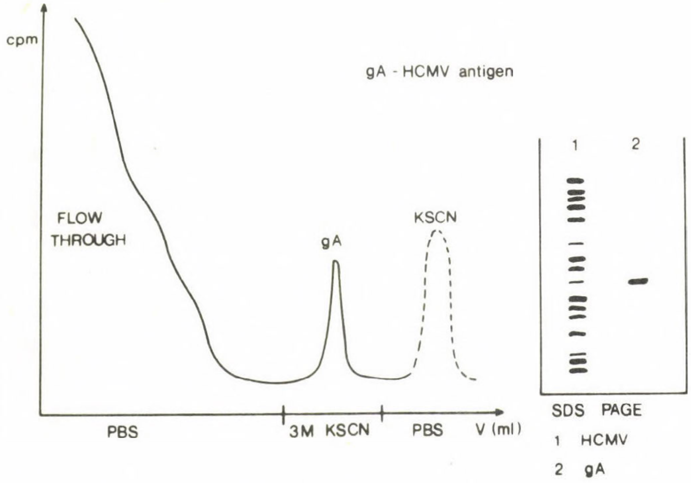


Fig. 3. A typical chromatogram and an electrophoretic pattern of purified gA-(HCMV antigen). PBS - 0.008 M phosphate buffer pH 7.2, containing 0.15 M NaCl

Table IV.

Starting material (amount)		Amount of isolated gA proteins (µg)	
		Method A	Method B
Cell lysate	(25 ml)	-	500
Purified virus	(12 mg)	1000	1200
Envelope	(8 mg)	600	-
	(2 mg)	-	600

We have previously shown that the HCMV envelope prepared by non-ionic detergent treatment contains cellular proteins as contaminants (15). Highly purified HCMV envelope was obtained by the application of pre-column methodology. Anti-cellular immunoglobulins coupled to CNBr activated Sepharose 4B were used as packing material in the immuno-affinitiy pre-column.

Example 2

Immunoglobulin G from sera or ascites fluid of guinea pigs immunized with gA protein was purified and coupled to CNBr-activated Sepharose 4B, as described I.3.a. The precolumn containing IgG from sera of guinea pigs immunized with unifected MRC-5 cells was prepared as described above.

^{35}S(Met) labelled and purified virus treated with NP40 HCMV envelope were applied to the anti-MRC-5 polyclonal - IgG precolumn and cycled over 3 times. The flow-through fraction of the last cycle was free of cellular proteins and loaded onto the anti-gA-IgG-Sepharose 4B column and cycled five times. The unbound protein was extensively washed from the column and the bound material was eluted with 0.1M diethylamine buffer (pH 11.5) containing 0.5 % (wt/vol) Nonidet P-40. Fractions were collected in tubes containing neutralizing buffer and radioactivity was determined by scintillation counting. Peak fractions were pooled and dialyzed. The protein content, determined by Lowry, was analyzed by SDS-PAGE. The electrophoretically separated proteins were visualized by silver staining.

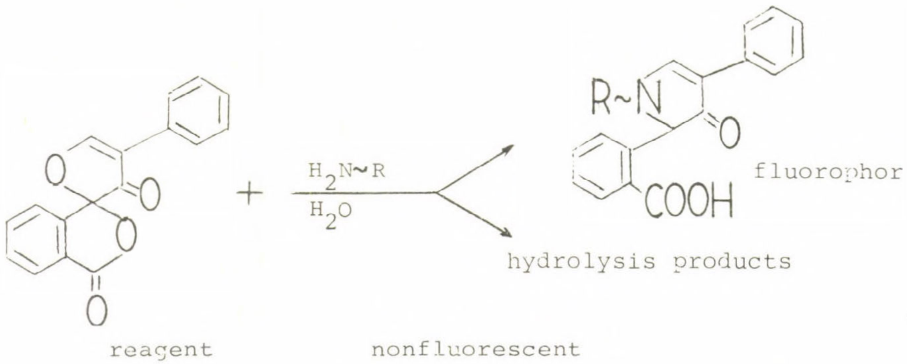
II. GEL ELECTROPHORESIS OF HCMV PROTEINS

Sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) analysis of immunoaffinity column fraction revealed the presence of a protein mixture. In order to obtain a homogeneous protein preparation, preparative separation of the eluted bound fraction was performed in a discontinuous buffer system (16) on slab gels. The method was optimized using 7.5 % separating gel. The proteins with molecular weight 58, 66, 130 and 200 kD were also separated very well on preparative loading (~200 µg). The visualized bands were sliced and collected separately. The pure proteins could be isolated from the gel slices by diffusion or by electroelution. Diffusion elusion is simpler but protein recoveries may be poor. Electrophoretic elution is generally more satisfactory because of its speed and efficiency. Proteins eluted were lyophilized.

The need for sensitivity enhancement of detection and for visualization of the bands and the availability of fluorescamine (FLURAM®) as an unique labelling reagent for studies of proteins and peptides led us to use it as a fluorescent label and staining method for analytical and preparative gel electrophoresis of viral proteins.

Fluorescamine reacts with primary amino groups to yield fluorescent products but fluorescamine and its hydrolysis products are nonfluorescent. The labelling procedure is simple and rapid. The excitation maximum is at 390 nm and the emission maximum is at 475 nm. Fluorescamine labels only the free N-terminal and lysine ε-amino groups of proteins (25, 26). It is a very useful reagent for fluorimetric detection and quantitation of primary amines, amino acids, peptides and proteins in the picomole range.

Generally, viral proteins can also be easily labelled with fluorescamine.



R~NH_2 viral protein

SCHEME 2

The application of fluorescamine in SDS-PAGE has many advantages. The procedure of labelling is fast, selective and results in stabile derivatives without fluorescent hydrolysis byproducts leaving the labelled protein as the only fluorescent component. The major advantage of fluorescamine is the ability to monitor the progress of electrophoretic separation simply by exposing the gel to UV light at 390 nm.

We developed the application of fluorescamine for staining analytical gels (Example 3) and for the localization of viral proteins in preparative slab gels (Example 3). In the case of preparative use, 10 % of a HCMV protein sample was derivatized and mixed with the remaining portion before electrophoretic run (Fig. 4). The running bands were visualized in UV light. The visualized bands could be cut and collected separately. The single proteins were isolated from the SDS-gel pieces by electroelution.

<u>Example 3</u>

The protein sample (~0.5 nmol) was dissolved in basic borate buffer (pH 9.2). It was reacted with 0.5 % fluorescamine solution in dimethylsulfoxide. The excess of the reagent was neutralized by ethanolamine. After complete reactions (10 and

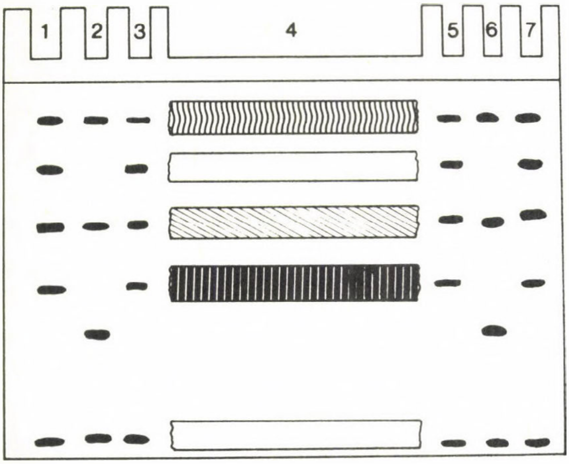


Fig. 4. Prepartive SDS-PAGE pattern of HCMV antigens using FLURAM derivatives. Lane 1, 3, 5, 7: HCMV proteins, anal. loading; 4: preparative loading; 2, 6: M_W standards. Staining:1, 7: silver, the others FLURAM labelling (or staining); gel 7.5 % pA; run at 30 mA

20 min, respectively) the sample was loaded into the slab gel. The separated bands were visualized in UV light. After electrophoresis an analytical gel could be stained by 1 % fluorescamine solution in acetone, if the gel was prepared for staining by washing at first with methanol-water, then with borate buffer (pH 9.2), finally with acetone-water mixtures.

Envelope antigen proteins with molecular weight 58 and 130 kD were isolated by the methods described above.

Our studies demonstrate that some of the HCMV proteins isolated by procedures summarized in this paper can induce virus-specific immune responses including neutralizing antibody production and T-cell response (6). Further studies are in progress to determine the primary structure of these antigenic proteins.

ACKNOWLEDGEMENTS

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SIMPLE HPLC METHOD FOR THE DETERMINATION OF INDOMETHACIN IN RAT SERUM

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SUMMARY

A simple and sensitive high-performance liquid chromatographic method was developed for the determination of indomethacin in rat serum. The method involves rapid quantitative extraction using ethyl acetate without any precipitation or centrifugation. The recoveries were better than 90 %.

The separation was carried out on a C-18 reversed-phase column with a methanol - 0.05 M phosphate buffer pH = 3 (80-20) mobile phase at a flow rate of 1 ml/min. Indomethacin was measured with UV detection at 300 nm over a linear range of 0.25-16 μg/ml. An internal standard of glibenclamide was used for quantitation. Inter- and intra-day precision were smaller than 6 %. The limit of sensitivity for indomethacin in serum was 0.1 μg/ml.

INTRODUCTION

Indomethacin (I) is a widely used anti-inflammatory drug with complex therapeutic effect. Recently, some combinations were created to reduce toxicity. Methods for the quantitation of I in physiologic fluids have included spectrofluorometry (1, 2), gas chromatography (3-6) and radioisotope dilution (7). Most of these methods lack of specificity or require derivatization.

Several high-performance liquid chromatographic assays had been reported from human plasma (8-12) but unfortunately they were unsuitable for rat serum.

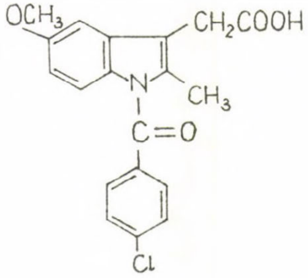
In order to facilitate routine monitoring of indomethacin concentrations, a simple specific HPLC procedure was developed which involves a rapid quantitative extraction on Extrelut column using ethyl acetate.

EXPERIMENTAL

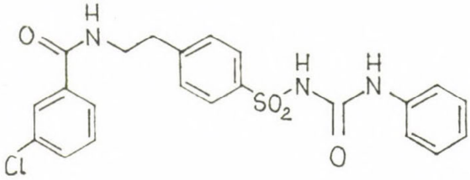
Reagents

Analytical reagent grade chemicals were used without further purification. All solutions were prepared by using distilled and deionized water.

Indomethacin (I) and glibenclamide (G), used as internal standard, were furnished by Chinoin.



I



G

For the standard samples I and G were dissolved in acetonitrile and the stock solutions (0.2 mg/ml) were stored at 4°C in darkness up to one month.

Extraction Procedure

To 250 μl of rat serum, 25 μl internal standard solution (2.5 μg G) and 250 μl 0.05 M phosphate buffer (pH = 3) were added.

After vortex mixing the sample was transferred to the top of a little column filled with 1.3 ml (~0.23 g) Extrelut (Merck). The solution was allowed to soak for 15 minutes then was extracted with 2 ml of ethyl acetate. The organic phase was evaporated under a nitrogen stream. The residue was taken up with 250 μl mobile phase and 50-μl aliquots were injected into the system.

Plasma standard curves were prepared from a stock solution of I by serial dilution.

Chromatography

The HPLC system consisted of a solvent delivery system (BT3020, Biotronik, GFR) and a 50-μl loop injector (Rheodyne 7125, USA). A 25 x 0.4 cm I.D. reversed-phase column (BST, Hungary) was packed with 10 μm LiChrosorb C18 (Merck). A variable-wavelength UV detector (UVIDEC-100II, Jasco, Japan) was used at 300 nm. A recorder (Servogor 120, BBC, Austria) was linked to the detector and a chart speed of 1 cm/min was used.

The mobile phase for isocratic chromatography was a 80-20 (v/v) mixture of methanol - 0.05 M phosphate buffer (pH = 3). The chromatographic system operated at room temperature at a flow-rate of 1 ml/min. The mobile phase was degassed by ultrasonic treatment.

RESULTS

The internal standard and indomethacin gave sharp symmetrical peaks under the described conditions with retention times of 4.6 min and 5.3 min, respectively.

Figure 1A shows a chromatogram of 250 μl extract of blank serum which was processed as described above. Figure 1B shows a chromatogram, obtained from spiked serum containing 2 μg/ml of I and 10 μg/ml of the internal standard G. It is evident that no interference from endogenous serum constituents was observed.

The linearity of the method was evaluated in serum in the concentration range of 0.25-16 μg/ml. Standard curves were prepared by adding known amounts of drug to blank serum samples and determining the peak height ratios (I/G). The data are best described by a linear equation $Y = 0.2544X +$

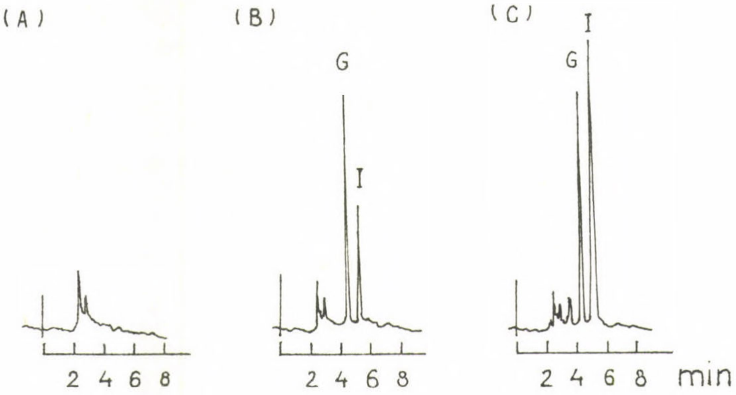


Fig. 1. Chromatograms of (A) a blank serum sample; (B) a serum sample spiked with indomethacin (2 µg/ml serum) and internal standard (10 µg/ml G); (C) a rat serum sample 2 h after administration of 2.5 mg/kg indomethacin. Calculated I concentration = 4.69 µg/ml

0.0102 where X is the concentration of indomethacin in µg/ml, and Y is the peak height ratio of indomethacin to glibenclamide. A mean correlation coefficient of 0.9989 ± 0.0010 was obtained, indicating a high degree of linearity (n = 9).

The extraction efficiency was estimated by comparing the peak heights of chromatograms obtained from extracted and directly injected samples. (Results are presented in Table I) Recoveries (mean ± S.D.) of I and the internal standard G were 91.4 ± 4.8 % and 92.3 ± 4.6 %, respectively.

The accuracy and precision of the assay are demonstrated in Table II, the results of which are based on nine determinations of calibration curves. Good reproducibilitiy was found and the average coefficient of variaton was 3.8 %.

In the present analytical conditions, the minimum concentration that could be accurately measured was about 0.1 µg/ml (signal-to-noise ratio = 5) with 250 µl serum sample. Higher sensitivity may be possible by increasing the serum volume.

In conclusion a new simple HPLC method has been developed which is applicable to the specific routine determination of indomethacin in rat serum.

Table I. Extraction efficiency for indomethacin (n = 9 at each concentration)

Concentration (μg/ml)	Recovery (%; mean ± S.D.)
0.25	89.1 ± 7.1
0.5	91.0 ± 6.6
1	90.8 ± 3.2
2	92.0 ± 3.6
4	91.6 ± 4.4
8	92.9 ± 3.9
16	92.2 ± 4.1

Table II. Reproducibility of indomethacin determination in serum

Indomethacin added (μg/ml)	Mean peak height ratio I/G	± S.D.	C.V. (%)
0.25	0.0691	0.0040	5.8
0.5	0.1313	0.0062	4.7
1	0.2565	0.0091	3.6
2	0.5264	0.0117	2.2
4	1.0363	0.0437	4.2
8	2.0766	0.0550	2.6
16	4.0631	0.1397	3.4
Mean C.V. = 3.8 %			

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RAPID DETERMINATION OF TOCOPHEROLS BY HPLC

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Isomeric forms of tocopherols as natural antioxidants stipulate the stability of unsaturated lipids against oxidation. The α-, β-, γ- and δ-tocopherols differ not only in their biological activity, but also in their antioxidation power. Therefore it is possible to determine the stability of vegetable oils by their tocopherol level during refining and storage. The method of vitamin E control is to ensure rapid and effective determination of all tocopherol isomers.

Traditional chemical and electrochemical methods for vitamin E analysis only permit the determination of the total tocopherol content but do not give information about the individual isomers. Chromatography is the most effective technique for this purpose. However, thin-layer (TLC), gas-liquid (GLC) and classical column chromatography are rather labour-consuming and prolonged, as they require the pretreatment of the oils involving saponification, extraction and derivatisation of the tocopherols before the analysis. In addition, TLC and GLC do not eliminate the possibility of tocopherol oxidation during detection.

High-performance liquid chromatography (HPLC), which is widely used for the analysis of complicated natural compounds, makes possible the rapid analysis of tocopherols at ambient temperature without derivatisation (1-3). However, even in this case many authors carry out a pretreatment of the oils involving saponification and extraction of the tocopherols (4-6). There are some papers in the literature devoted to the direct anlysis of tocopherols in oils, however, this method has no application in the USSR. It was therefore of great interest to evaluate the capabilities of the microcolumn liquid chromatograph "Milichrom" produced in the USSR for the direct analysis of tocopherol isomers in oils.

A rapid and selective HPLC method for the determination of α-, β-, γ- and δ-tocopherols in different vegetable oils (sunflower, soybean, cottonseed, sea-buckthorn, wheat germ) and partially in hydrogenated oil is presented in the present paper. The samples were directly dissolved in the eluent without any pretreatment (saponification and extraction) and the solution injected into the chromatographic column. The analysis was carried out on both an imported (A) and a home-made (B) equipment:

(A) The chromatographic system consisted of a Beckman 110A pump; a Kratos SF-757 variable-wavelength spectrophotometer (detection at 295 nm), a column consisting Ultraspher Si 5 μm (250 x 4.6 mm) and a Rheodyne 7125 100 μl loop injection valve. The mobile phase was a 1-1.5 % solution of propanol-2 in hexane at a flow rate of 1-1.5 ml/min. A Shimadzu C-R3A integrator was used for data reduction. The concentration in the analysed oils was about 0.01 g/ml.

(B) Microcolumn chromatograph "Milichrom" (USSR), a double refracting scanning spectrophotometer (detection at 294 nm), a column containing Silasorb 600 (Lachema, Brno) 5 μm (62 x 2 mm). The same eluent was used as in (A), at a flow rate of 0.1 ml/min. Injection was carried out with the eluent stop flow; the sample volume injected into the chromatographic column was 3-15 μl.

It is possible to selectively determine the tocopherols in oils at 294 nm because the main oil components do not absorb UV-light at the wavelength used in our work. Thus these compounds do not interfere with the direct determination of tocopherols in oils. In order to remove the polar components adsorbed on the silica during the analysis it is necessary to clean the column with methanol and then with the eluent for 30 minutes after every 8-10 hours of use.

The results of the chromatographic analysis of sunflower, cottonseed, soybean and wheat germ oils are shown in Fig. 1. The sample of D,L-α-tocopherol (Serva, FRG) and a mixture containing 93 % of δ-, 6 % of γ- and 0.7 % of α-tocopherol were used for peak identification. For the qualitative determination of tocopherol isomers we have used the relative retention times (relative to α-tocopherol). In normal-phase chromatography on silica the retention times vary depending on many factors such as the quality of hexane, its moisture content, etc. We have found linear relationship between the logarithm of the relative retention time and the atomic number of carbon atoms in the homological series of tocopherols which can be used for the identification of isomers. As seen in Fig. 1, complete separation of α-, γ- and δ-tocopherols is possible under the conditions used.

According to literature data the most difficult step in vitamin E analysis is the separation of the positional isomers β- and γ-tocopherols. For the identification of β-tocopherol we used wheat germ oil. The β-tocopherol eluted before the γ-isomer (Fig. 1), which corresponds to literature data (2, 5).

A calibration method was used for the quantitative determination of tocopherol isomers. Standard solutions of α-tocopherol in hexane were prepared by successive dilution. Linear relationship was observed between the α-tocopherol concentration and the detector response in concentrations ranging from 0.01 to 10 μg/ml (A) and from 5 to 50 g/ml (B).

Since the extinction factors of all tocopherol isomers are nearly the same at 295 nm (1, 7) quantitative determination of β-, γ- and δ-tocopherols could be accomplished according to the calibration plot made for α-tocopherol.

The proposed method has been tested in terms of its reliability of the method of standard addition. The average recovery of the tocopherols was 98.5-99.8 %. Experimental data on the analysis of tocopherol isomers and of the total vitamin E content in different oils and partially hydrogenated oil are presented in Table I. The standard deviation was 1.3-2.6 at a

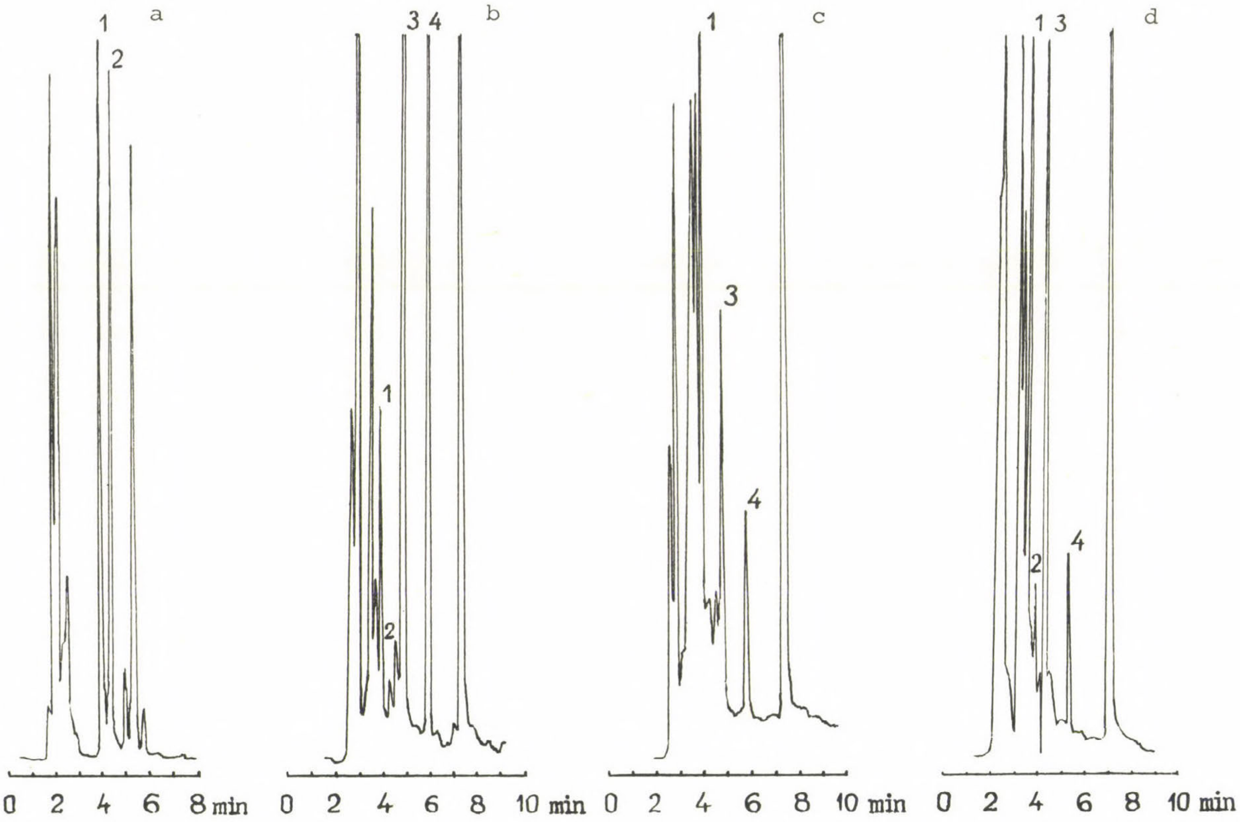

Fig. 1. Analysis of tocopherol isomers in oils: a) wheat germ; b) soybean; c) sunflower; d) cottonseed. Peaks: 1 = α-tocopherol; 2 = β-tocopherol; 3 = γ-tocopherol; 4 = δ-tocopherol. Analysis conditions: detection at 295 nm; silica column; mobile phase consisting of 1.5 % propanol-2 in hexane, at a flow rate of 1 ml/min

Table I. Tocopherol content present in different oils and partially hydrogenated oil

Sample	Analysis conditions*	Tocopherol content, mg per 100 g of oil (in parentheses: %)				
		α	β	γ	δ	Total
Sunflower oil	A	39.3 (92.9)	-	2.4 (5.7)	0.6 (1.4)	42.3
	B	41.5 (82.3)	-	8.9 (17.7)	-	50.4
Soybean oil	A	8.2 (7.8)	1.3 (1.2)	69.8 (66.2)	26.2 (24.8)	105.5
	B	11.4 (10.9)	-	68.4 (65.2)	25.1 (23.9)	104.9
Cottonseed oil	A	29.0 (41.4)	4.3 (6.1)	33.2 (47.4)	3.6 (5.1)	70.1
Sea-buckthorn oil	A	75.8 (90.2)	-	10.2 (10.8)	8.5 (9.0)	94.5
Wheat germ oil	A	209 (66.1)	83 (26.3)	24 (7.6)	-	316
Partially hydrogenated oil	A	21.0 (35.5)	-	27.9 (47.1)	10.3 (17.4)	59.2

*Instrument and analytical conditions used (see text)

tocopherol content of 30-70 mg per 100 g of oil, and 0.2-1.8 at a tocopherol content of 1-25 mg per 100 g of oil. Tables I-II illustrate the fact that the obtained results on the qualitative and quantitative content of tocopherol isomers are in good agreement with reference data.

Table II. Tocopherol content present in different oils according to literature data

Oil	Tocopherol content, mg per 100 g of oil (in parentheses: %)					Reference
	α-	β-	γ-	δ-	Total	
Sunflower	57.0	-	3.9	1.0	61.0	(1)
	(93.4)		(6.4)	(1.6)		
	61.6	-	2.0*	3.4	67.0	(8)
	(92.0)		(3.0)	(5.0)		
Soybean	7.0	-	92.4	37.0	136.0	(1)
	(5.1)		(67.9)	(27.2)		
	7.5	1.2	77.6	25.7	112.0	(2)
	(7.0)	(1.0)	(69.0)	(23.0)		
	10.2	-	67.3*	36.5	114.0	(8)
	(9.0)		(59.0)	(32.0)		
Cottonseed	57.0	4.6	31.7	1.0	89.0	(1)
	(64.0)	(4.5)	(35.6)	(1.1)		
	49.5	-	47.5*	2.0	99.0	(8)
	(50.0)		(48.0)	(2.0)		
Wheat germ	184.5	73.9	21.3	<1	279.7	(2)
	(66)	(26)	(8)			

*- total β- + γ-tocopherol content

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GROUP SEPARATION OF DIFFERENT PETROLEUM FRACTIONS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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Progress in petroleum production and processing is impossible without the development of methods for the rapid determination of group and individual composition of crude petroleum and of petrochemical products. Various physico-chemical techniques and routine analytical methods are usually applied together for the solution of this rather complex problem.

For example petroleum middle distillates are known from the evidence of capillary gas chromatography (CGC) to contain more than 200 components and naturally their unambiguous resolution and identification is time consuming. Even 100 metre long capillary columns are usually insufficient to achieve full separation of such mixtures. Another serious shortcoming of CGC is the poor reproducibility of capillary column parameters; therefore each time a new preliminary standardization is necessary with multicomponent mixtures of known compositions. In order to make the determinations more convenient, rapid and distinct it is advantageous to use CGC in combination with HPLC and spectrophotometric methods.

GROUP SEPARATION BY HPLC

We have carried out the group separation of middle and heavy petroleum distillates with HPLC. A stainless steel column, 100 mm long x 4 mm i.d., packed with silica gel KCK, modified by alumina and calcium cations was used. The use of cation-modified silica gels resulted in an essential increase of the selectivity of group separation of petroleum hydrocarbons.

Table I lists data on the selectivity (α_c) and capacity factors (k') obtained on the adsorbents prepared in our laboratory.

Table I.

No.	Adsorbent	k' for			α_c for	
		benzene	naphthalene	phenanthrene	naphthalene	phenanthrene
1	silica gel KCK	0.3	0.5	0.8	1.7	2.7
2	Ca^{2+}-modified silica gel	0.35	0.70	1.85	2.0	5.3
3	Al^{3+}-modified silica gel	0.48	1.00	2.70	2.1	5.7

The columns were packed with adsorbents having small particle size (5 μm); their efficiency measured for benzene was 50-55,000 theoretical plates per metre. Dry hexane was used as the mobile phase at a flow rate of 1 ml/min. The instrument was equipped with a UV detector operated at 254 nm. The saturated hydrocarbon (SH) fraction was monitored by added carbon tetrachloride which had been shown in control runs with a dielectric constant detector to be co-eluting with this fraction.

Preparative liquid chromatography of gasoline distillates was carried out using 300 mm long x 15 mm i.d., stainless steel columns packed with silica gel KCK.Column efficiency was 30,000 theoretical plates per metre.

Figures 1a-c represent separation of a reference blend consisting of CCl_4 and aromatic hydrocarbons (AH), and of gasoline fractions (80-200°C) with varying AH content, accomplished on an analytical column packed with aluminized silica. It is apparent that due to the sharp peak corresponding to saturated hydrocarbons, the benzene, naphthalene and diphenyl fractions are well resolved. The high column selectivity also ensured the separation of xylene isomers.

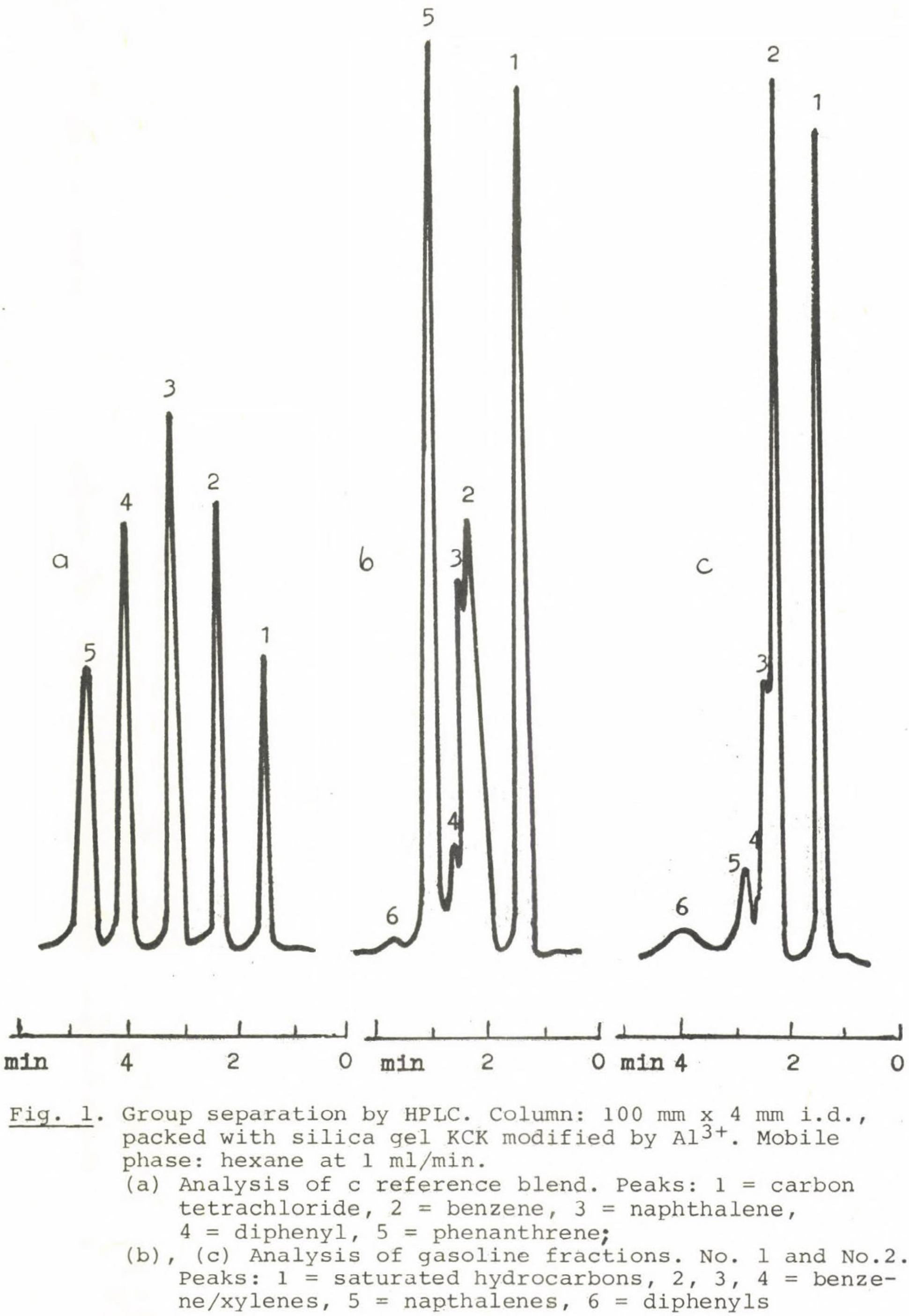


Fig. 1. Group separation by HPLC. Column: 100 mm x 4 mm i.d., packed with silica gel KCK modified by Al^{3+}. Mobile phase: hexane at 1 ml/min.

(a) Analysis of c reference blend. Peaks: 1 = carbon tetrachloride, 2 = benzene, 3 = naphthalene, 4 = diphenyl, 5 = phenanthrene;

(b), (c) Analysis of gasoline fractions. No. 1 and No.2. Peaks: 1 = saturated hydrocarbons, 2, 3, 4 = benzene/xylenes, 5 = napthalenes, 6 = diphenyls

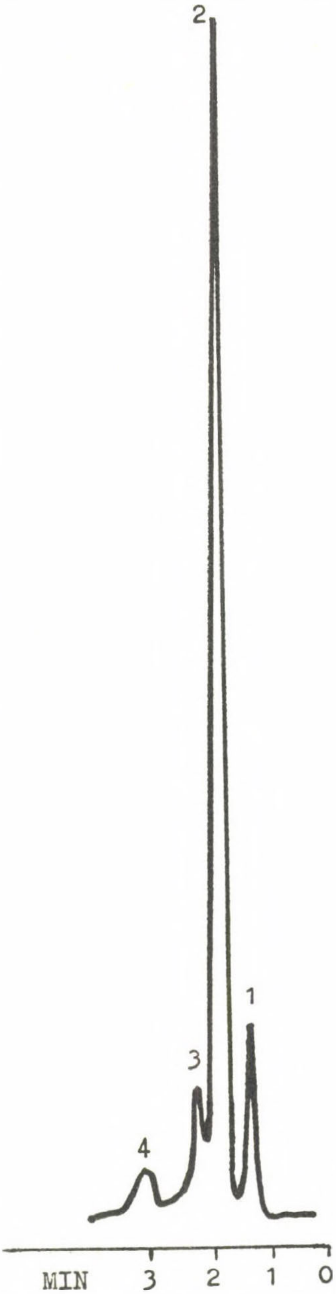


Fig. 2. Group separation of a gasoline on silica gel KCK modified by Ca^{2+}. Column dimensions: 100 mm x 4 mm i.d. Mobile phase: hexane at 1 ml/min. Peaks: 1 = saturated hydrocarbons, 2 = benzene/xylenes, 3 = naphthalenes, 4 = diphenyls

Spectrophotometric investigation of preparatively obtained narrow cuts proved the absence of monocyclic aromatic hydrocarbons in the saturated hydrocarbon fraction; similarly, the monocyclic aromatic hydrocarbon fraction was clean of naphthalenes and diphenyls.

Figure 2 shows the group separation of gasoline distillates using a Ca^{2+}-modified silica column. It is noteworthy that a reasonable separation took only a few minutes.

Figures 3a, b show the group separation of heavy Diesel fuel distillates (180-320°C), and a liquid paraffin fraction (250-340°C). Because of the carcinogenic properties of some polycyclic aromatics there is an urgent need for the development of methods to monitoring the aromatic hydrocarbon content of these distillates. Complexity of the composition and likely overlap of the methyl- and alkyl-substituted aromatic hydrocarbons are the reason of the much poorer separation of the heavy fractions. For example the alkylnaphthalene peaks could coelute with the polymethylbenzenes, whereas alkylphenanthrenes may be expected to have a similar retention as polymethylnaphthalenes. Consequently, it is advantageous to separate narrower cuts, e.g., those boiling within a 50° range.

CAPILLARY GC OF LC FRACTIONS

Separation of the individual components present in gasoline distillates, after group separation of saturated and aromatic hydrocarbons by preparative LC has been achieved by capillary GC, using the following columns:

- 100 m long column coated with the weakly polar phase OV-17; and
- 15 m long support-coated open-tubular column coated with the highly polar phase Carbowax 20M.

Figure 4 gives an example for the separation of the monocyclic aromatic fraction on the Carbowax 20M column.

Identification by retention index revealed that the gasoline distillates contain about 20 aromatic hydrocarbons.

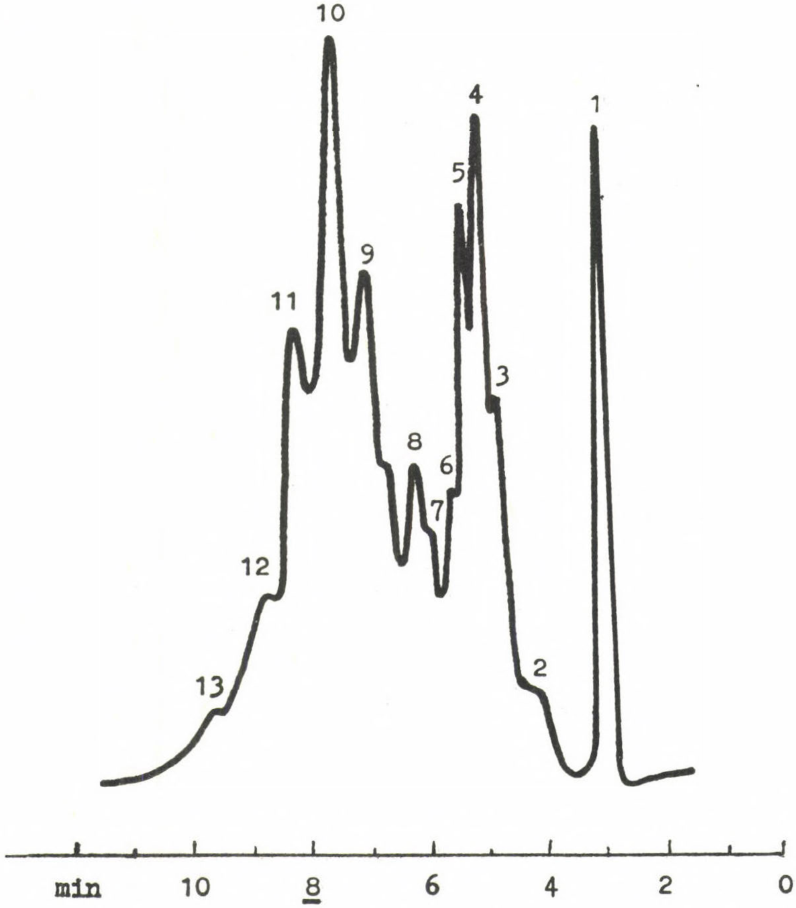
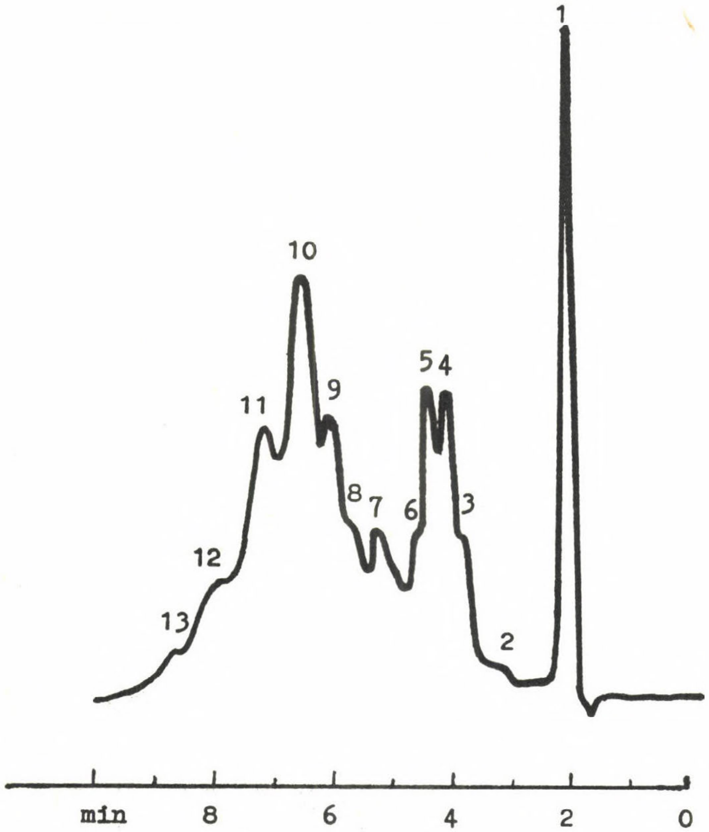


Fig. 3. Group separation by HPLC, on silica gel KCK modified by Al^{3+} column dimensions: 100 mm x 4 mm i.d.
(a) Analysis of a Diesel fuel;
(b) Analysis of a liquid paraffin sample ⟶

QUANTITATIVE MEASUREMENTS

Quantitative analysis of aromatic hydrocarbons in gasoline distillates has been carried out by HPLC and spectrophotometric methods. Alkylbenzenes, naphthalenes and diphenyls were calibrated against the typical members of their groups such as benzene, α-methylnaphthalene and diphenyl. Calibration plots of peak heights (areas) versus amounts of injected samples



(an example is given in Fig. 5) showed good linearity, while their slopes provide the required calibration coefficients. For the calibration, solutions of gasoline distillates No. 1 and No. 2 of known concentrations were injected into the liquid chromatograph. Based on the calibration coefficients for benzene, α-methylnaphthalene and diphenyl and the known concentrations of these standards, we were able to estimate the concentrations of alkylbenzenes, naphthalenes and diphenyls present in the analysed distillates.

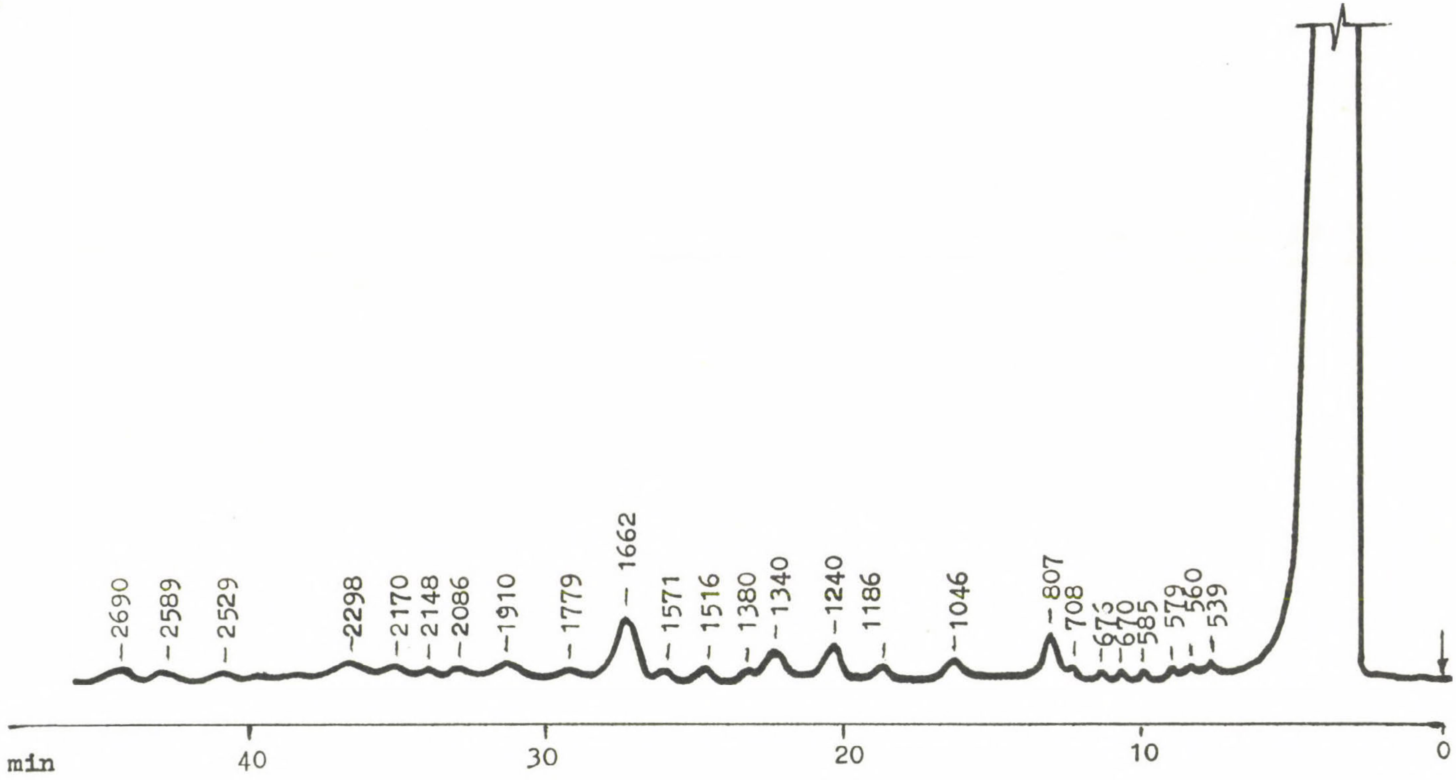


Fig. 4. Separation of the individual components present in the monocyclic aromatic fraction of a gasoline sample. Column: 15 m x 0.5 mm i.d., SCOT, coated with Carbowax 20M. Carrier gas: helium

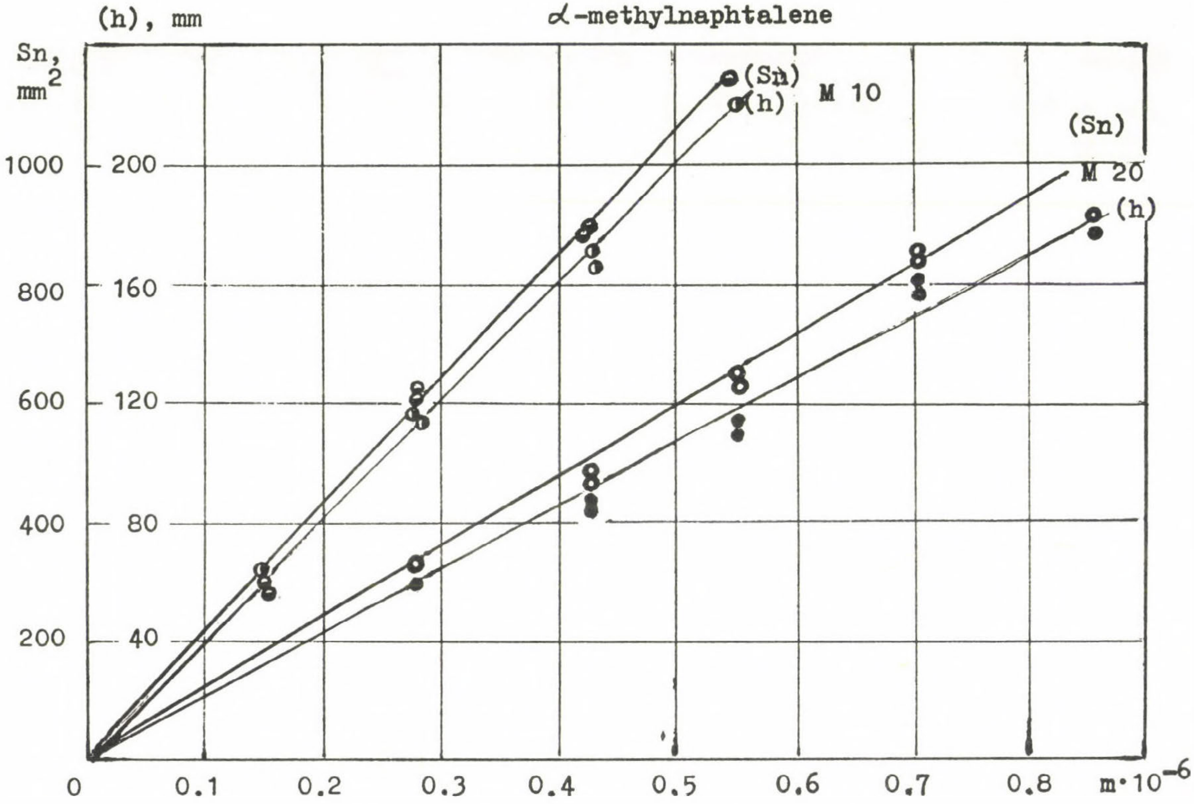


Fig. 5. Calibration plots of peak height (h) or area (Sn) vs. amount of α-methylnaphthalene

The precision and sensitivity of quantitative determinations of aromatic hydrocarbons in petroleum distillates could be further enhanced by the use of a variable wavelength UV-VIS detector which permits the selective monitoring of benzenes at 200 nm, naphthalenes at 230 nm, and phenanthrenes at 255 nm.

Spectrophotometric quantitative analysis of gasoline distillates has been accomplished on a Perkin-Elmer Model 402 instrument in hexane or pentane solutions. The results of HPLC and spectrophotometric determination were in a fair agreement with the time-consuming, laborious and poorly sensitive chemical analysis.

CONCLUSIONS

The combination of HPLC, capillary GC and spectrophotometric techniques, along with the utilization of highly selective HPLC stationary phases based on small particle size silica proved well its usefulness for the group separation and the determination of individual constituents in petroleum distillates, providing high precision and reproducibility at shorter analysis times. Quantitative analysis is further facilitated by a preliminary group separation of complex mixtures by means of high-performance preparative liquid chromatography.

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HPLC ANALYSIS OF CANNABINOIDS USING AMINO BONDED STATIONARY PHASE COLUMN

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INTRODUCTION

Narcotic addiction is one of the main social problems of humanity. To resolve this problem it is indispensable to apply modern chemical analytical methods for the identification and quantitation of the narcotic drugs.

This paper deals with the investigation of neutral cannabinoids originated from hashish or marihuana type drugs of abuse, applying high-performance liquid chromatography (HPLC).

In connection with the analysis of cannabinoids a large number of reports have been published, but there are some contradictions in the literature, especially in the field of quantitation. The uncertainty of the quantitative results can be attributed to the simultaneous presence of both neutral and acidic cannabinoids in the sample. The acidic cannabinoids are highly labile compounds, easily undergoing decomposition to the corresponding neutral compounds. The gas chromatographic analysis (GC) will give the amounts of the "total" cannabinoids, i.e. the neutral cannabinoids being originally in the sample together with those formed by the decomposition of cannabinoid acids upon the injection (1-2).

For the characterization of the questioned samples Baker et al. (3) suggested to determine the neutral and acidic cannabinoids separately using GC and HPLC methods, respectively. For this reason both the normal (4, 5) and the reversed phase (3, 6-9) HPLC methods have been published. Hendrix et al. (10) reported the decomposition of the cannabinoid acids during

HPLC separation applying silica gel or aluminium-oxide columns. The application of bonded-phase chromatography in reversed-phase mode eliminates the problem mentioned above, but it leads to the following difficulty: the application of reversed-phase HPLC demands the injection of the sample in a polar solvent because of the miscibility of the solvent with the mobile phase. However, using a polar solvent for the extraction of cannabinoids, the extract contains too many "ballasts", i.e. non-cannabinoid compounds, which may cause an irreversible binding of these components to the support of the analytical column. Moreover, the too many non-cannabinoids may disturb the separation and the quantitation of the components of interest.

Elsohly et al. (11) described a method for the analysis of marihuana and hashish samples, applying chloroform for the extraction, followed by redissolving into ethanol prior to the chromatographic separation. However, the redissolving is not advisable because evaporation of the cannabinoids was observed during the heating process (5). Harvey (12) prefers the non-polar solvents for the extraction of cannabinoids because obtaining a "cleaner" extract than in the case when using a polar solvent. Cannabinoids seem to be more stable in non-polar solvents for the storage, than in polar ones (13).

Taking into consideration the standpoints described above, we have developed a polar bonded-phase HPLC procedure for the determination of neutral cannabinoids, using amino-bonded phase column. This procedure allows the application of a non-polar solvent for the extraction and eliminates the possibilities of decomposition and the wasting of the sample components. In order to evaluate the new method quantitative results obtained by the amino-bonded and the reversed-phase (14) methods were compared.

EXPERIMENTAL

Materials, Equipments

The extraction and the polar phase HPLC separation were performed with analytical-grade n-hexane and ethanol (Reanal, Budapest, Hungary). For the reversed-phase HPLC and sample preparation Lichrosolv methanol (Merck, Darmstadt, FRG) and bidistilled water purified according to Gurkin's method (15) were used. The extraction was performed in a KLN G40.41 ultrasonic bath (KLN Ultraschall GmbH, Heppenheim, FRG). The extracts were filtrated through Whatman GF/D glass microfibre filters (Whatman Ltd., Whatford, UK) and 2-μm porosity filtertips (Supelco, S.A., Gland, Switzerland), respectively. For the sample preparation, prior to the reversed-phase HPLC analysis silica and C_{18} SEP-PAK cartridges (Waters Associates, Milford, Massachusetts, USA) were applied. The HPLC separation was carried out on a Hewlett Packard Model 1084B liquid chromatograph equipped with a HP-79875A variable-wavelength UV detector. For data collection and evaluation the HP 79850 B computing-integrator was used. All of the HP marked instruments were purchased from Hewlett-Packard (Palo Alto, California, USA).

Samples

The cannabinoid standards such as delta-8-tetrahydrocannabinol (delta-8-THC), delta-9-tetrahydrocannabinol (delta--9-THC), cannabinol (CBN) and cannabidiol (CBD), were received from the UN Narcotic Laboratory Section, Vienna.

The marihuana sample used for the demonstration was confiscated by the Hungarian Customs Service at the border.

Extraction

1 gramme of accurately weighted powdered marihuana was extracted three times with 10 ml n-hexane by sonication for 10 minutes. The filtered extracts were combined in a volumetric flask and diluted to 100 ml with n-hexane. The efficiency of the extraction was controlled by thin-layer chromatography (16). In the case of the polar bonded-phase HPLC the neutral cannabinoids were determined by the direct injection of the n-hexanic extract without any preseparation. In the case of reversed-phase HPLC a sample clean-up (14) was carried out applying silica and C_{18} SEP-PAK cartridges prior to the HPLC analysis.

HPLC analysis

Conditions of the polar bonded phase separation:

Mobile phase: n-hexane - ethanol (98:2 v/v)
Analytical column: Ultrasil-NH_2, 25 cm x 4.6 mm I.D., particle size 10 μm (Beckman Instruments, Berkeley, California, USA)
Guard column: Brownlee Amino cartridge packed with Lichrosorb 10 Amino support, 3 cm x 4.6 mm I.D. (Pierce Eurochemie B.V., Rotterdam, The Netherlands)
Flow rate: 2 ml/min
Temperature: 40°C
Detection: absorbance at 215 nm (Ref. 430 nm)
Injection: 20 μl

Conditions of the reversed-phase separation:

Mobile phase: methanol - water (80:20 v/v)
Analytical column: Ultrasphere-Octyl, 25 cm x 4.6 mm I.D., particle size 5 μm, (Beckman Instruments, Berkeley, California, USA)
Flow rate: 1 ml/min
Temperature: 60°C
Detection: absorbance at 220 nm (Ref. 430 nm)
Injection: 20 μl

Calibration

Quantitative determination by the external standard method was performed in both HPLC separation modes. The calibration curves were constructed for the cannabinoids using injection of standard solutions containing different amounts of standards in the range of 0.5-2.6 μg. A good linearity has been found for each studied compound. The slope and the intercept of the calibration curves is given in Table I. The correlation coefficients are also indicated in the table.

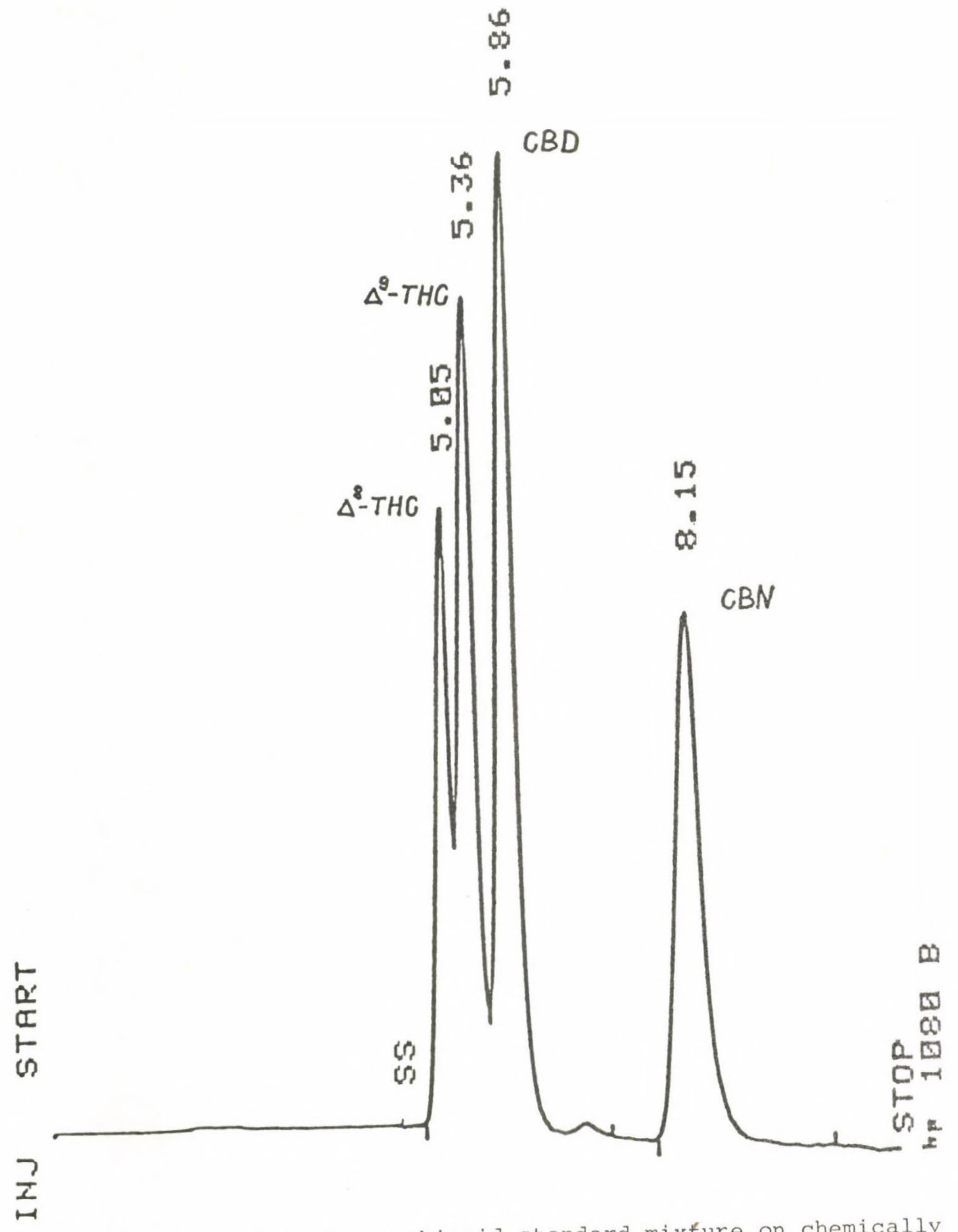

Fig. 1. Separation of cannabinoid standard mixture on chemically bonded amino stationary phase (See experimental conditions in the text)

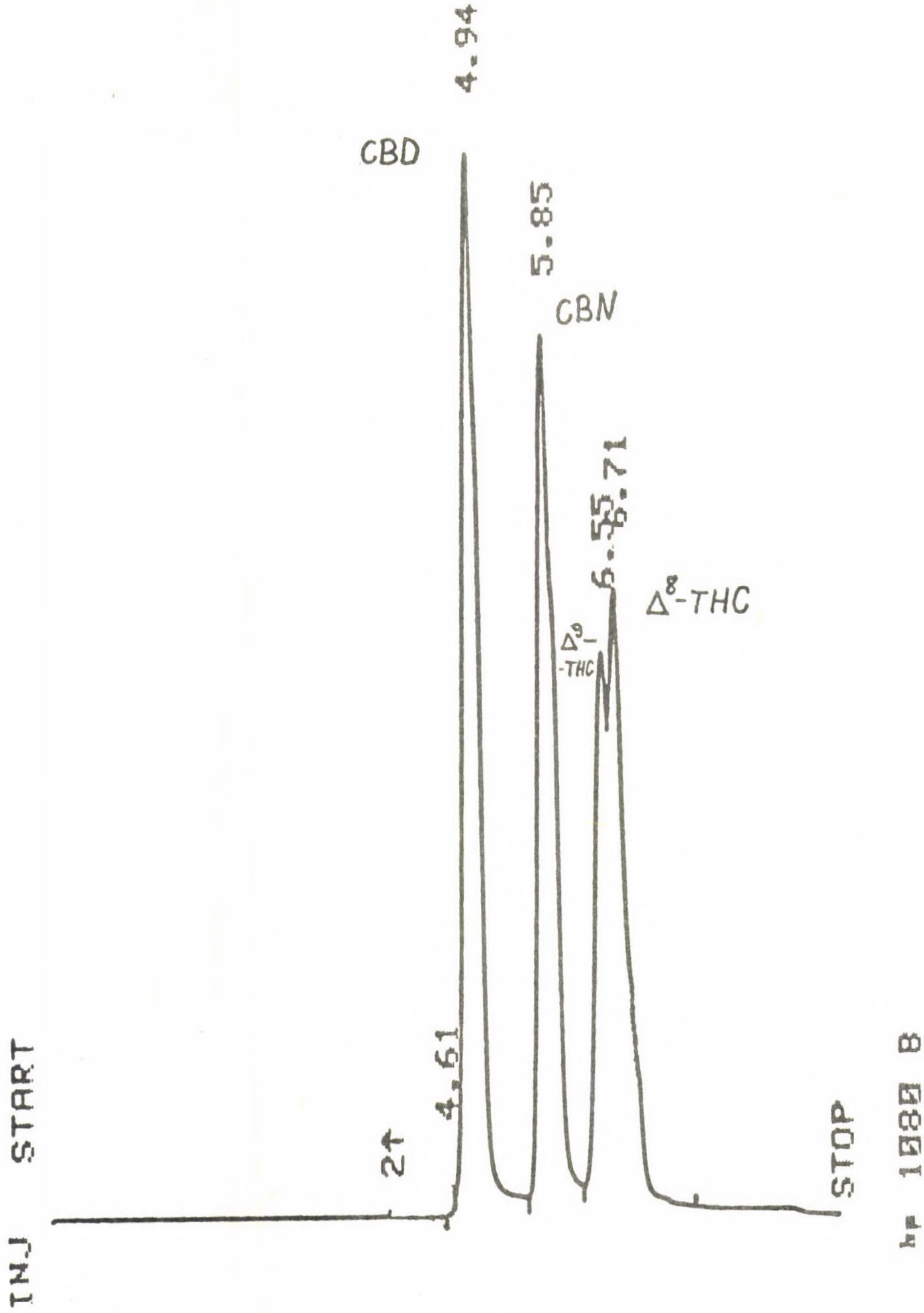

Fig. 2. Separation of cannabinoid standard mixture on chemically bonded octyl stationary phase (See experimental conditions in the text)

RESULTS AND DISCUSSION

In Figs 1 and 2 the separation of a model mixture can be seen, obtained by amino and octyl bonded-phase HPLC, respectively. There is a better resolution between the two THC isomers on the amino phase than on the octyl phase. The latter phase, however, gives better resolution for the peak pair of delta-9--THC and CBD than does the amino phase. It should be noted that in most of the cases, the non-baseline separation between the two THC isomers does not disturb the quantitative determination of delta-9-THC, since the delta-8-THC isomer practically does not occur in real samples.

Separation of the components of a marihuana extract on amino and octyl stationary phases are shown in the Figs 3 and 4, respectively. In the sample investigated the delta-8-THC isomer could not be detected.

The quantitative results obtained by the two methods are listed in Table II. In this table the results of statistical tests ("t" and "F"; p = 1 %) are also indicated for the mean values and standard deviations. A significant difference was found between the mean values of THC and CBD, obtained by the two HPLC methods. We have also found significant differences in the standard deviation values for the same compounds. The results obtained by amino bonded-phase HPLC have smaller standard deviations and the amounts determined are regularly higher than those obtained by reversed-phase HPLC. These discrepancies are caused by the pretreatment wasting of the sample components.

The time of the HPLC separation is about 9 minutes in each method, but the whole analysis time is nearly twice as long in the case of the reversed-phase method, due to the time-consuming clean-up procedure prior to separation.

In Table III the detection limits achieved by the two methods are listed, considering the signal-to-noise ratio being equal to three. As seen there is no considerable difference between the detection limits obtained by the two methods.

The dynamic characteristics of the reversed-phase column have not changed at all after the analysis of as much as 100

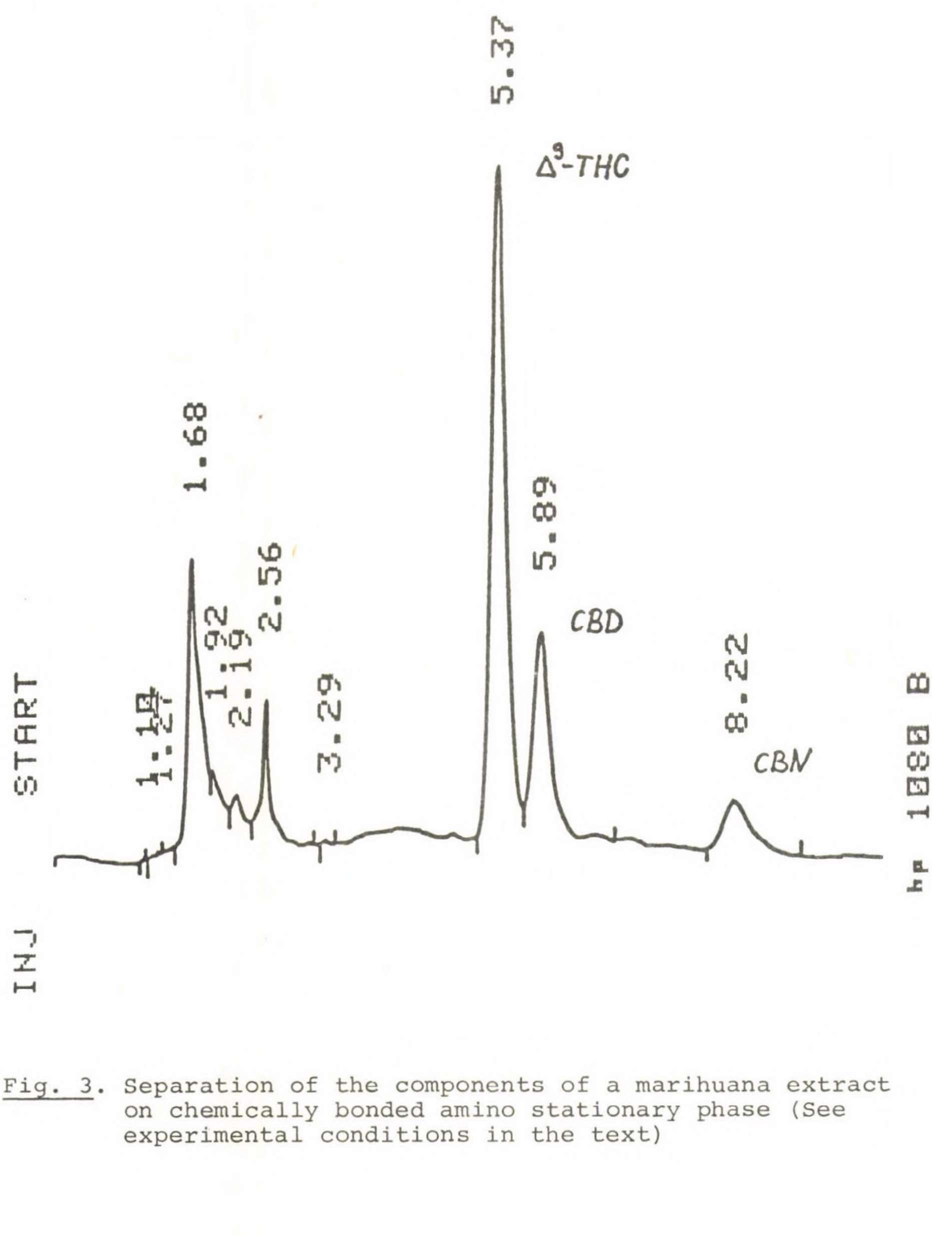

Fig. 3. Separation of the components of a marihuana extract on chemically bonded amino stationary phase (See experimental conditions in the text)

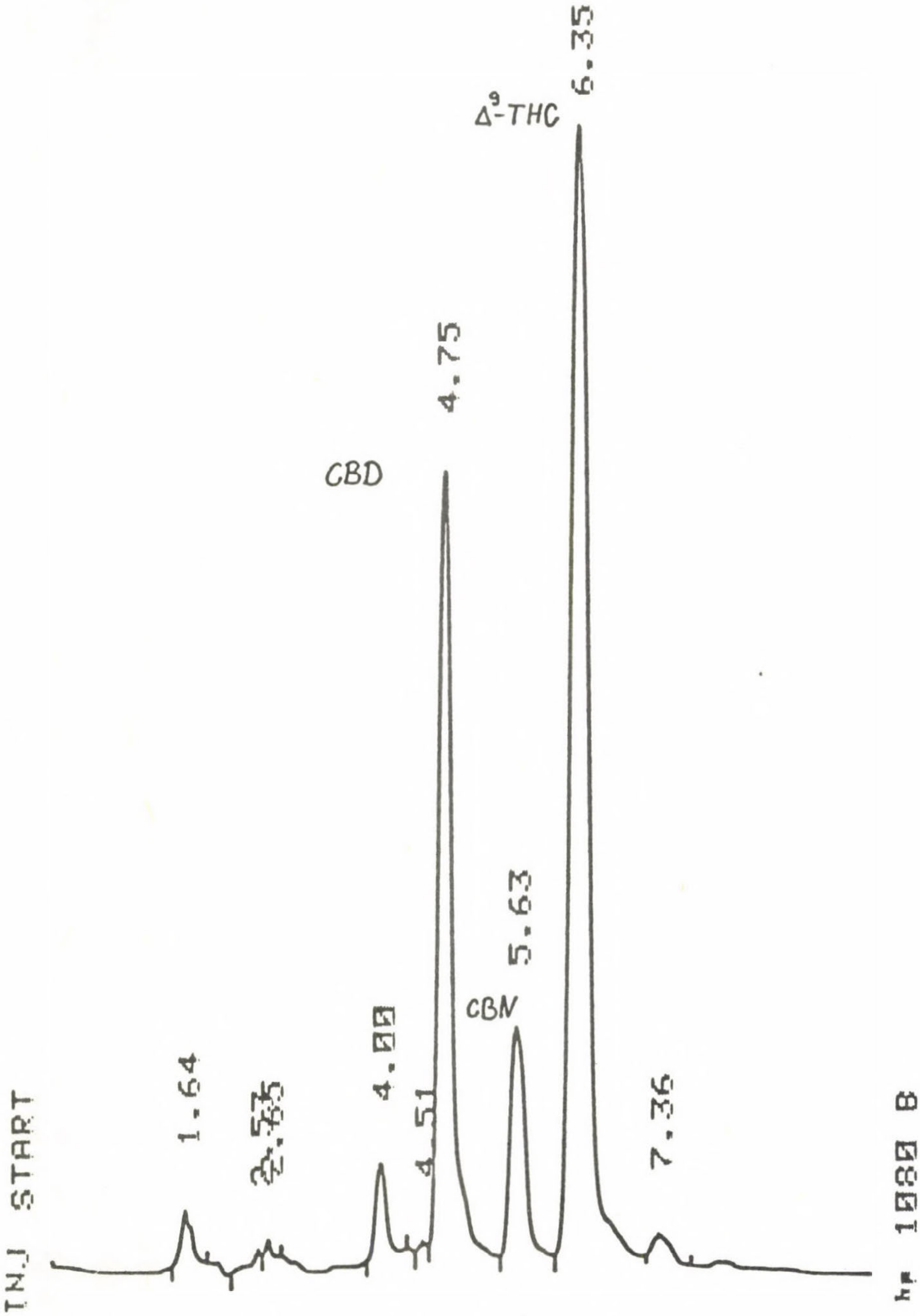

Fig. 4. Separation of the components of a marihuana extract on chemically bonded octyl stationary phase (See experimental conditions in the text)

Table I. Results of the calibration of neutral cannabinoids, obtained by the external standard method HPLC separation: normal (NP-HPLC) and reversed (RP-HPLC) phase mode.
A: the slope of the calibration curve;
B: the intercept of the calibration curve;
r: correlation coefficient

Cannabinoid	NP-HPLC			RP-HPLC		
	A (rel. area unit/µg)	B (rel. area unit)	r	A (rel. area unit/µg)	B (rel.area unit)	r
delta-9-THC	286,339	11,579	0.9993	507,845	2292	0.9992
CBD	217,435	13,419	0.9998	452,362	15,134	0.9993
CBN	274,880	10,380	0.9998	720,714	8027	0.9998

Table II. Quantitative and statistical results of the determination of the neutral cannabinoids from a marihuana sample, obtained by normal (NP-HPLC) and reversed phase (RP-HPLC) separation modes
S.D. - standard deviation;
R.S.D. - relative standard deviation;
No. of parallel measurements: 10

Cannabinoid	HPLC system					
	NP-HPLC			RP-HPLC		
	average cannabinoid content (mg/g)	S.D. (mg/g)	R.S.D. (%)	average cannabinoid content (mg/g)	S.D. (mg/g)	R.S.D. (%)
delta-9-THC	42.41	0.23	0.54	36.61	2.17	5.92
CBD	22.07	0.35	1.61	21.93	1.10	5.01
CBN	7.02	0.28	3.96	5.01	0.39	7.86

Table III. Detection limits at a signal-to-noise ratio of 3, obtained by normal (NP-HPLC) and reversed-phase (RP-HPLC) separation modes

Separation mode	Detection limits of cannabinoids (ng)		
	delta-9-THC	CBD	CBN
NP-HPLC	1.44	1.90	1.50
RP-HPLC	0.81	0.91	0.57

samples, which is due to the efficiency of the clean-up procedure using the SEP-PAK cartridges. The dynamic characteristics of the amino bonded-phase column system (consisting of the guard and analytical columns) changed remarkably after injection of approximately 50 samples. The dynamic characteristics were restorable by flushing the guard column cartridge with 8-10 column-volumes of ethanol.

Concluding the results of the investigations, the amino bonded-phase HPLC procedure is more favourable for the quantitative determination of the neutral cannabinoids being originally in the plants or of their derivatives.

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SEPARATION OF NUCLEOTIDES IN CELLS OF DIFFERENT METABOLIC COMPLEXITY BY USE OF REVERSED-PHASE AND ION-PAIR HPLC

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SUMMARY

Results on concentration of nucleotides, nucleosides, and nucleobases in cells of different metabolic complexity determined by reversed-phase and ion-pair chromatography are presented in this paper. Concentration profiles were examined on rabbit red blood cells of different maturity, on Ehrlich ascites tumour cells, and on solid sarcomas at different stages of proliferation.

The maturation of reticulocytes to erythrocytes is accompanied by loss of organelles and energy-requiring processes as well as the switch from aerobic to anaerobic ATP production. The concentrations of purine mono- and triphosphates are two- to fourfold in reticulocytes in comparison with erythrocytes. Still greater differences exist for nucelosides and nucleobases. The reticulocytes show also higher ratios between purine tri- and diphosphates. Negligible or no differences were found for oxidized pyridine dinucleotides. These observations indicate the higher metabolic activity and diversity of reticulocytes.

Mouse ascites cells show profound differences of nucleotides when analyzed at day seven or eleven after inoculation representing the periods of proliferation or plateau. It was shown that these cells lose major portions of purine mono-, di- and triphosphates between the seventh and eleventh day after inoculation. The rate of uptake of ^{14}C-adenine, ^{14}C-hypoxanthine, and ^{14}C-adenosine into Ehrlich ascites cells was studied. It decreased between the 5th and 12th day. A relationship between nucleotide concentration of solid sarcoma tumours of rats (MV 202 Ner) and their growth rate was observed.

INTRODUCTION

For discovery of purine metabolism high-performance liquid chromatography is considered to be the best method for the determination of purine metabolites. Free nucleotides, nucleosides, and nucleobases play a central role in the regulation of cell metabolism. They are of importance for the nucleic acid metabolism and the regulation of the energy state of the cell. The methods for the separation and quantitative determination of purine and pyrimidine compounds have been intensively improved in recent years. Therefore, various modes are available including anion-exchange chromatography very frequently used for nucleotides, reversed-phase HPLC for nucleosides as well as ion-pair HPLC for the separation of all interesting purine compounds in a single run.

Ion-exchange chromatography is widely applied although it possesses the disadvantages of using highly concentrated eluent buffers and requiring long run times (1). The separation of nucleosides and nucleobases by the ion-exchange mode was improved by using ODS columns with phosphate buffer/methanol eluents (2-5).

Nowadays reversed-phase and ion-pair chromatography became the method of choice for the quantification of purine and pyrimidine compounds (6-9). Ion-pair HPLC enables the analyst to simultaneously determine the nucleotides and their derivatives.

For the selection of an appropriate HPLC procedure for the analysis of biological samples one has to consider besides the particular topic the complexity of the nucleotide metabolism, e.g. the variety of the enzymatic pathways, the turnover rates and the concentration profiles.

This paper deals with the variety of nucleotide metabolites in cells of different tissues and developmental stages detected by the application of reversed-phase and ion-pair HPLC.

The cells chosen were: rabbit red blood cells of different maturity, Ehrlich ascites tumour cells at two stages of proliferation, and solid sarcoma (MV 202 Ner) of different growth rates. Additionally, the changes in the uptake of nucleosides and nucleobases during the proliferation of ascites cells were also investigated.

Changes in the nucleotide profile as well as morphological alterations characterized by electron microscopy in relationship to the growth rate of solid sarcoma were also studied.

Three separation techniques will be presented: separation of purine metabolites and their derivatives by reversed-phase HPLC, and analysis of nucleotides and their derivatives by ion-pair reversed-phase HPLC.

EXPERIMENTAL PART

Chemicals

All reference standards (purine bases, purine and pyridine compounds) of the highest analytical grade available were purchased from Boehringer (Mannheim, FRG), Fluka (Buchs, Switzerland), Sigma (St. Louis, MO, USA). KH_2PO_4 and Na_2HPO_4 were from Ferak (Berlin-West), TRA (triethanolamine) from Boehringer, and $NH_4H_2PO_4$ was from Fisher Scientific Co. (Fairlown, NJ,USA). Methanol and acetonitrile were obtained from Merck (Darmstadt, FRG). TBA (tetrabutyl ammonium phosphate, PIC Reagent A) was from Waters Assoc. (USA). Water was glass-distilled twice to get it sufficiently pure for HPLC analysis. Dibutyl phtalate came from Laborchemie (Apolda, GDR). The ^{14}C-labeled adenosine, adenine, and hypoxanthine were from Amersham (Great Britain).

HPLC

For the HPLC analysis three equipments were used: (A) Du Pont (Wilmington, DE, USA), (B) Waters Associates (Milford, MA, USA), (C) Beckman (San Ramon, CA, USA).

System A: The chromatographic system was assembled from 8800 Series gradient liquid chromatographic system, 850 column compartment, 8800 pump module, absorbance detector (254 nm) and a variable wavelength detector, all of Du Pont, and an SP 4100 integrator from Sepctra-Physics (Santa Clara, CA, USA) which performed peak area calculations. The following columns were applied: Zorbax ODS, 7 µm, 250 x 4.6 mm i.d.; stainless-steel guard column 50 x 4.6 mm i.d. The mobile phase consisted of 0.15 M KH_2PO_4/Na_2HPO_4 buffer, pH 6.85 (Buffer A) and the same phosphate buffer containing 20% methanol (v/v %, Buffer B). Gradient: 7 min 100% Buffer A, 5 min 0-20% B, 5 min 20-50% B, 20 min 50% B. The flow rate was 1 ml/min. At the end of gradient the system was switched back by pumping Buffer A for 15 min. The temperature was 18-22°C.

The peaks were identified in the following manner:

(i) Some of the biological samples were divided, one of each of the reference compounds was added to the aliquot, and subsequently the HPLC analysis was performed.

(ii) The UV-absorbance of biological samples as well as of a mixture containing all the reference compounds was measured at two wavelengths (254 nm and 280 nm).

For quantitation the columns were calibrated with several diluted mixtures containing constant proportions of all reference compounds as well as with mixtures of variable proportions of these compounds. The mixture containing the reference compounds was treated with perchloric acid, neutralized and analyzed in the same manner as the biological sample. Buffer solutions were filtered through a 0.45 µm HA filter and acetonitrile and methanol through a 0.45 µm HF filter of Millipore Intertech (France). Before use the buffer solutions were degassed with helium.

System B: It consisted of a M 510 and a M 45 solvent delivery system, Model 441, and a Lamda-Max Model 481 absorbance detector, U6K universal liquid chromatograph injector, Model 660 solvent programmer and a Z-module RCSS (Radial Compression Separation System). The absorbance was registered at 254 nm and 280 nm and the peaks were integrated by a 730 date module (Waters Associates, Milford, MA, USA). The column was a NovaPak C_{18} plastic cartridge (100 x 8 mm i.d.). (Waters) and flow rate was 2.50 ml/min. A 0.01 M $NH_4H_2PO_4$ buffer containing 2 mM PIC A and acetonitrile (15 v/v %) was used as the mobile phase. The separation was carried out in the isocratic mode.

System C: It consisted of a Beckman Model 450 data system, two Beckman Model 112 HPLC-pumps, a Model 421 gradient programmer, a Model 165 rapid scanning UV-Vis detector, a Model 210 sample injector and a Z-module RCSS. The absorbance was registered at 254 nm and 280 nm, and the peaks were integrated by the data system. In order to check the purity of the peaks scans of all detected peaks were taken. The column was a NovaPak C_{18} plastic cartridge. The mobile phase was 10 mM $NH_4H_2PO_4$ containing 2 mM PIC A, pH 5 (Buffer C) and 85% C + 15% acetonitrile (Buffer D). Gradient: 10 min 0-100% D, 2 min 100-0% D. The flow rate was 2 ml/min.

Cell preparation

Blood of rabbits was taken from ear vein. Reticulocytosis was induced by daily bleeding of 50-60 ml. Reticulocyte counts up to 70% have achieved by centrifugation for 20 min at 500 g. The supernatant was removed, the top layer of the cells with high percentage of reticulocytes was taken and the cells suspended in cold isotonic saline.

The Ehrlich tumour was propagated in female mice of the ICR strain by peritoneal transplantation. Tumour cells were obtained by peritoneal puncture 6-12 days after inoculation. They were washed once with 0.9% NaCl at 4°C and then suspended in the medium (mixture of Eagls basal medium and Borsook medium) (10, 11).

Fast growth of solid sarcoma (MV 202 Ner) was accomplished by transplantation of 1 x 10^6 cells into the hind-quarter of rats. A slower tumour growth rate was obtained by repeated transplantations of an increasing number of cells.

Extraction procedure and sample preparation

The cell suspensions were deproteinized with chilled 6 % perchloric acid, centrifuged for 10 min at 1,200 g and neutralized with TRA/K_2CO_3. After filtration 20 or 50 µl of the supernatant were analyzed by HPLC. Pieces of about 200-500 mg of sarcoma were taken and immediately introduced into liquid nitrogen.

For the analysis of ^{14}C-hypoxanthine uptake into ascites cells (2% cytocrit) incubated at 37°C after 2-minute time intervals 200 µl aliquots of the suspension were taken, given on a dibutyl phtalate layer, centrifuged, and employed for liquid scintillation counting (12).

RESULTS AND DISCUSSION

Reversed-phase HPLC of extracts of rabbit reticulocytes

The maturation of reticulocytes to erythrocytes is accompanied by the loss of organelles and energy-requiring processes as well by the switch from aerobic to anaerobic ATP production (13, 14). Little is known about the changes in the nucleotide metabolism during this period. Figure 1 demonstrates the chromatograms of perchloric acid extracts from rabbit red blood cell suspensions containing 5 or 66.7% reticulocytes. Values estimated in blood of different reticulocyte count were taken to extrapolate for pure erythrocytes or reticulocytes, resp. In some experiments further enrichment of reticulocytes was reached by centrifugation. Table I gives a summary of these data.

The concentration of purine mono- and triphosphates are two- to fourfold in reticulocytes comparing with erythrocytes. Still greater differences exist for nucleosides and nucleobases. The reticulocytes also show higher ratios between purine tri- and diphosphates. Negligible or no differences are seen for the oxidized pyridine dinucleotides. These observations indicate the higher metabolic activity and diversity of reticulocytes. Comparable data for the concentrations of

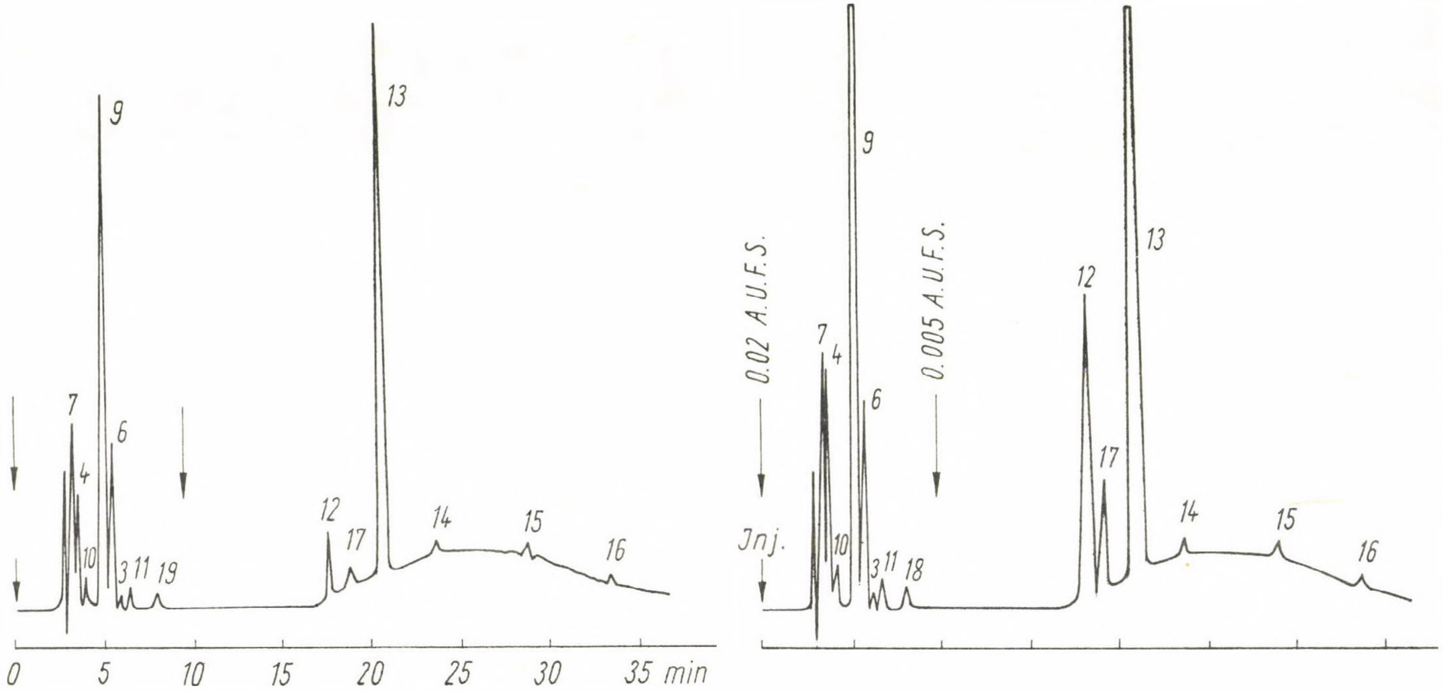

Fig. 1. Separation of purine and pyridine compounds in extracts of rabbit red blood cells containing 5 % (lower panel) or 66.7 % (upper panel) reticulocytes. For conditions see the experimental section (HPLC-instrument A).
Peaks: 7 = GTP; 4 = GDP; 10 = IMP; 9 = ATP; 6 = ADP; 3 = AMP; 11 = NADP$^+$; 18 = hypoxanthine; 12 = NADPH; 17 = inosine; 13 = NAD$^+$; 14 = NADH; 15 = adenosine; 16 = adenine

Table I Concentrations of metabolites in mature and immature rabbit red blood cells (μmole/cell)

Metabolite	Erythrocytes	Reticulocytes
ATP	1,395	3,580
ADP	310	395
AMP	40	161
adenosine	0.6	3.6
adenine	0.5	3.3
GTP	216	680
GDP	63	95
IMP	50	110
inosine	2.5	41
hypoxanthine	12	68
$NADP^+$	48	51
NAD^+	82	110

adenine nucleotides were found in rabbit red cell suspensions containing 90% reticulocytes (15).

Differences in the nucleotide profile of animal species may be dependent on their different enzyme activities (13).

Ion-pair chromatography of extracts of Ehrlich ascites tumour cells

Figure 2 presents the chromatographic peak pattern of acidic extracts of ascites tumour cells taken at day 6 and 12 after inoculation. The retention characteristic of purine and pyrimidine compounds is changed with respect to the reversed-phase mode. At first monophosphates are eluted followed by di- and triphosphates. The complete run is performed within 20 minutes and it is therefore appreciably faster than in the reversed-phase system. Table II shows profound differences when cells are analyzed at the seventh or eleventh day after inoculation which represent the periods of proliferation or plateau, resp. Comparable data for adenine nucleotides at days 7 to 9 after inoculation were published (16).

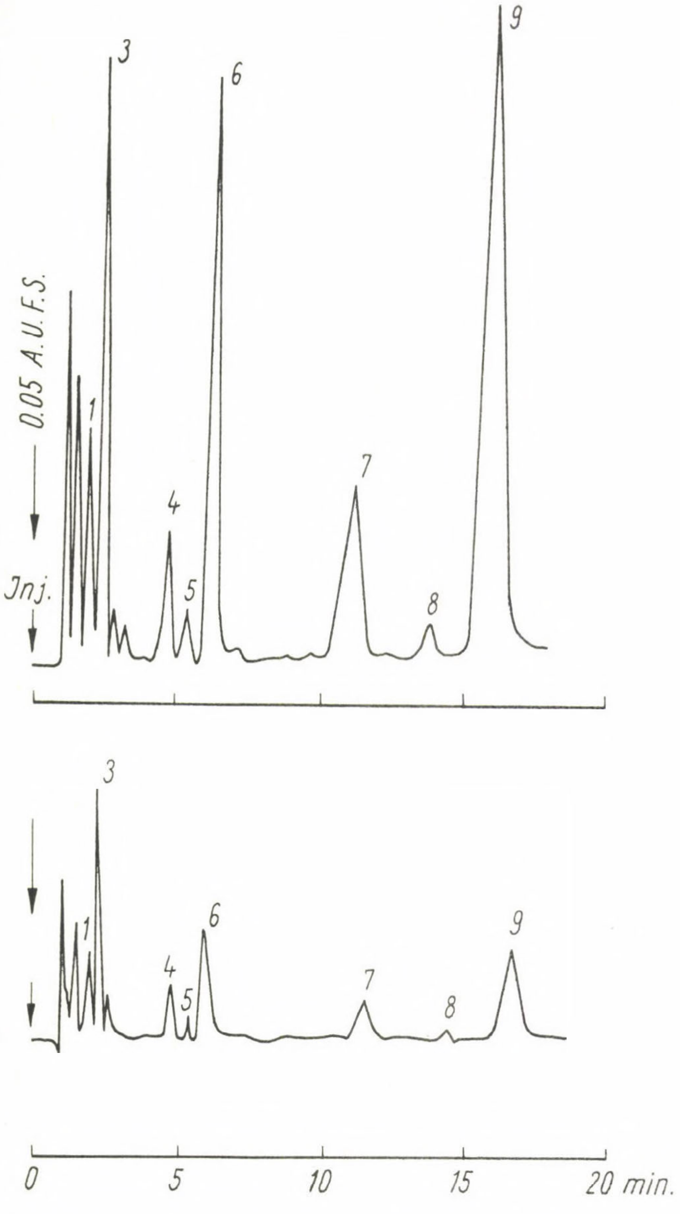

Fig. 2. Chromatographic peak pattern of extracts of Ehrlich ascites cells at day 6 (upper panel) and 12 (lower panel) after inoculation. For conditions see the experimental section (HPLC instrument B).
Peaks: 1 = GMP; 3 = AMP; 4 = GDP; 5 = UDP; 6 = ADP; 7 = GTP; 8 = UTP; 9 = ATP

Table II Nucleotide levels in Ehrlich ascites tumour cells (μmole/cell ± s.d; n = 4)

Metabolite	7th day	11th day
ATP	2,512 ± 373	270 ± 42
ADP	881 ± 297	214 ± 48
AMP	166 ± 52	112 ± 62
GTP	639 ± 225	124 ± 31
GDP	201 ± 16	57 ± 13
GMP	118 ± 22	92 ± 14
UTP	99 ± 60	10.8 ± 0.2
UDP	32 ± 17	1 ± 0.1
UMP	0	0
ΣAdN	3,559 ± 480	596 ± 89
ΣGN	953 ± 227	273 ± 36
ΣUN	131 ± 62	12 ± 0.2

Table III ^{14}C-hypoxanthine uptake in Ehrlich ascites tumour cells (pmol ^{14}C-hypoxanthine x h^{-1} x ml $cells^{-1}$)

P_i (mM)	5/6 days (n=6)	11/12 days (n=5) after inoculation
0.35	36 ± 8	14 ± 5
10.0	41 ± 12	22 ± 3

In incubation experiments with ^{14}C-hypoxanthine it was found that the incorporation rate decreases between day 6 and 12 after inoculation (Table III). This could diminish the salvage pathway and, therefore, cellular nucleotide concentrations. The uptake of hypoxanthine was reduced to 50%. Similar results were obtained by incorporation of ^{14}C-adenosine and ^{14}C-adenine (not shown here). At the higher concentration of inorganic phosphate the incorporation of ^{14}C-hypoxanthine was increased due to the function of phosphate for maintenance of the PRPP-level (12).

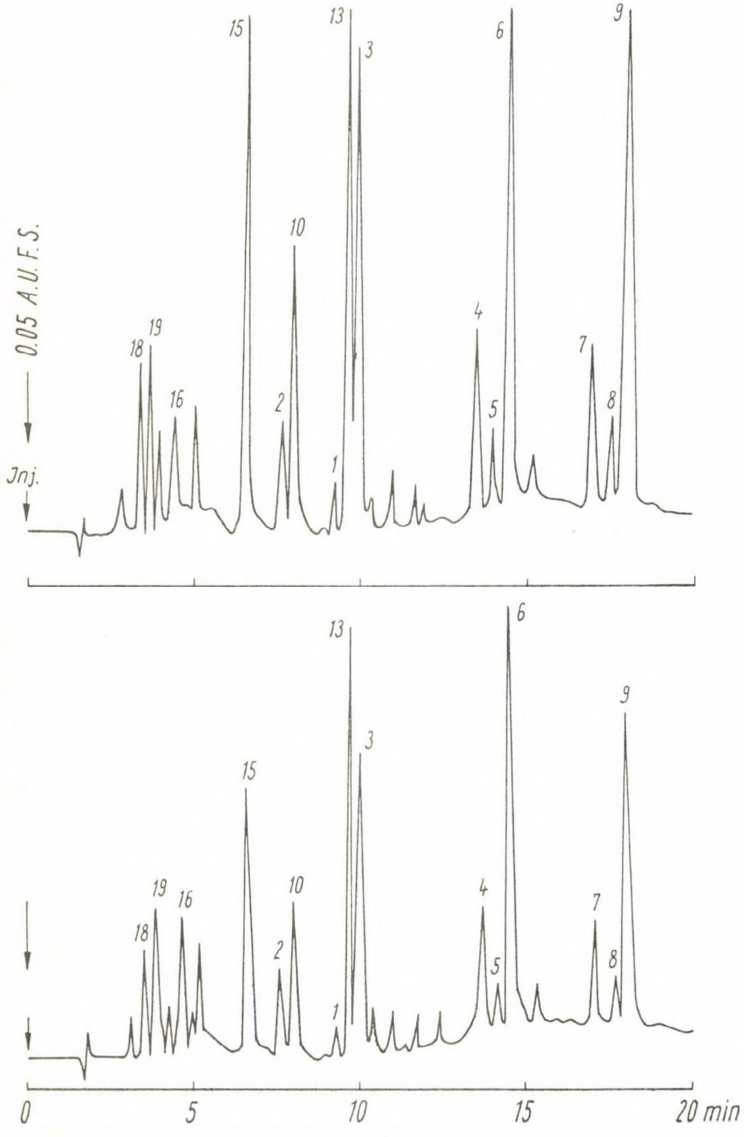

Fig. 3. Chromatograms of extracts of solid sarcoma with 14 days (upper panel) and 45 days (lower panel) growth rate. For conditions see the experimental section (HPLC-instrument C).
Peaks: 18 = hypoxanthine; 19 = xanthine; 16 = adenine; 15 = adenosine; 2 = UMP; 10 = IMP; 1 = GMP; 13 = NAD$^+$; 3 = AMP; 4 = GDP; 5 = UDP; 6 = ADP; 7 = GTP; 8 = UTP; 9 = ATP

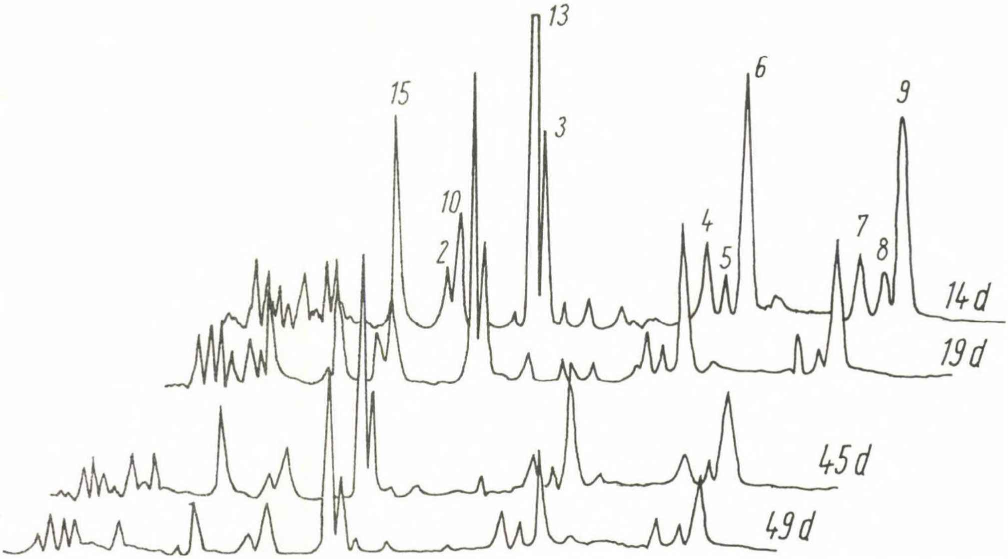

Fig. 4. Chromatographic peak pattern of extracts of solid sarcoma with different growth rates (14, 19, 45, 49 days), documenting the changes in metabolite concentrations. For conditions see the experimental section (HPLC-instrument C). Peaks as described in Fig. 3.

Table IV Nucleotide levels in sarcoma cells (MV 202 Ner) (pmole/mg protein, ± S.E.M; n = 4)

Metabolite	Fast growth rate (14 days)	Slow growth rate (45 days)
ATP	838 ± 82	535 ± 27
ADP	500 ± 40	365 ± 28
AMP	395 ± 27	208 ± 18
adenosine	348 ± 45	222 ± 12
GTP	234 ± 26	185 ± 23
GDP	211 ± 17	161 ± 3
UTP	98 ± 32	73 ± 12
UDP	77 ± 2	49 ± 11
UMP	153 ± 1	99 ± 33
IMP	537 ± 23	291 ± 22
hypoxanthine	113 ± 15	83 ± 17
xanthine	115 ± 79	25 ± 3
inosine	58 ± 5	52 ± 7

Ion-pair chromatography of extracts of solid sarcoma (MV 202 Ner)

Sarcomas with different growth rate had differences in nucleotide, nucleoside and nucleobase concentrations; the faster growing tumour was characterized by higher metabolite concentrations (Table IV, Figs 3 and 4).

The ultrastructural preparations were evaluated morphometrically. Faster growing sarcomas possessed a higher number of mitochondria, and the nucleus/cytoplasma ratio was almost identical. The higher amount of dinucleotides, mononucleotides, nucleosides, and nucleobases may be explained by the necrosis of 25% of the tissue.

ACKNOWLEDGEMENT

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Chromatography '87
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CHROMATOGRAPHIC PURIFICATION OF ANTIBODIES AND ANTIBODY-ENZYME CONJUGATES

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The enzyme-linked immunosorbent assay (ELISA) has proved to be a simple and sensitive method suitable for screening large numbers of samples and meristem tissues for virus infection (1, 2, 3).

Successful applications of the immunoenzymatic assay are related to the availability of highly purified reagents, such as antibody-enzyme conjugates and antibodies.

The objective of the present study was to compare various chromatographic purification techniques used for antibodies and antibody-enzyme conjugates while investigating virus diseases of decorative plants by means of the double antibody "sandwich" form of ELISA.

TESTING

- of IgG: (a) concentration of IgG, c = A_{280}:1.4 (mg/ml) in PBS (15 mM phosphate buffer pH 7.4, containing 0.1 M NaCl); (b) purity of IgG - A_{280}/A_{250}; ratio A_{280}/A_{250} should exceed 2.5 for pure IgG; (c) immunoelectrophoresis against sheep anti-rabbit serum; (d) Immunospecificity-Ouchterlony double diffusion in agarose.
- of conjugates: (a) activity of enzyme - AP - conversion substrate - p-nitrophenyl phosphate - in diethanolamine buffer pH 9.8 at A_{405}; HRP - conversion substrate - 4-aminoantipyrin-phenol-H_2O_2 reagent - in phosphate buffer pH 7.0 at A_{510}; (b) concentration

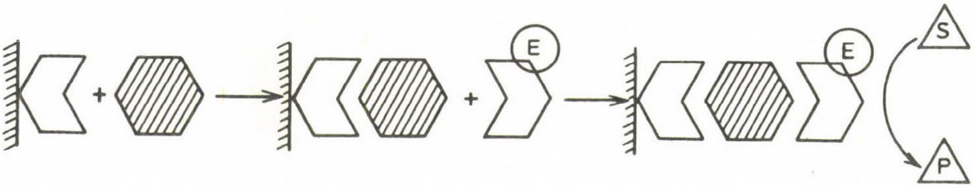


Fig. 1. Method for the measurement of the antigen consisting of the double antibody "sandwich" ELISA.

⬢ :antigens - Carnation mottle virus (CarMV); Tomato aspermy virus (ToAV); Crysanthemum B virus (CrBV) e.a.;

〈 :antibodies - immunoglobulins G (IgG); IgG^A - anti-ToAV antibody; IgG^B - anti-CrBV antibody; IgG^M - anti-CarMV antibody e.a.;

Ⓔ〈 :Enzyme - IgG conjugate;

○ :Enzyme - horse-radish peroxidase (HRP), Biolar, $R_Z > 2.7$, activity>650 U/mg protein; Enzyme - alkaline phosphatase (AP), Biolar, activity - 500 U/mg protein;

S :substrate;

P :product

of conjugate IgG-HRP, $c = A_{403} x 0.44$ (mg/ml); (c) "working dilution" - tested by double antibody "sandwich" method of ELISA: (1) coating of plates with IgG (5 μg/ml) in carbonate buffer pH 9, 6, 3 h at 37°C, wash with PBS-T (PBS, containing 0.05 % Tween); (2) incubating with 1 % solution of albumine 15' at 37°C wash with PBS-T; (3) incubating with virus (0.5-1 μg/ml), 3 h at 37°C, wash with PBS-T; (4) incubating with antibody conjugated to enzyme (conjugate dilution) 3 h at 37°C, wash with PBS-T; (5) incubating with enzyme substrate 30' at 37°C, measured photometrically substrate conversion: AP - ΔA_{405} and HRP - ΔA_{510}. "Working dilution" is generally defined as the dilution of conjugate that gives in double antibody sandwich method of ELISA minimum substrate conversion (ΔA_{405} or ΔA_{510}) at least 1.0 in 30' at 37°C.

PURIFICATION OF ANTIBODIES

Antibodies (IgG) have been obtained from a rabbit antiserum against purified viruses of plants with the titre 1:64 to 1:1,024. The main steps of IgG purification process are represented in Fig. 2. Precipitation with inorganic salt or with polyethylene glycol (PEG) allowed to obtain γ-globulin fraction, which, in addition to IgG, contained also other globulins and impurities of albumins.

Further purification of IgG involved anion-exchange chromatography (Table I). The IgG did not adsorb on the anion exchangers under the conditions used and was eluted in the void volume while other proteins were adsorbed by the anion exchanger (Fig. 3). The yield was 18-30 % of the total serum protein.

Under the same conditions, aminopropylsilochrome bonded 80-90 % of the protein, including IgG. Sorption of IgG was realized in lower values of pH (Table II). Elution by increasing ionic strength (up to 1 M NaCl) or lowered pH (down to pH 5.5) did not desorb IgG, but elution by increased concentration of Tris-HCl buffer caused desorption of IgG with an approximately 60 % yield.

Methods of immunoelectrophoresis, electrophoresis and double precipitation in gel were used to show that purification on various DEAE-ion exchangers resulted in highly purified IgG fraction. None of these sorbents has special advantages. The results of IgG obtained from crude antisera on an affinity column, prepared by coupling sheep anti-rabbit IgG to CNBr--agarose by using the method of Boorsma D.(4), are presented in Fig. 4 and Table III. Fraction 2 contains highly purified IgG but yields in only 4.5 %, fraction 1 contains albumin impurities (see Fig. 4B).

PREPARATION AND PURIFICATION OF CONJUGATES

IgG was coupled to HRP by the periodate method and its modifications, see Fig. 5A (5, 6, 7) and to AP by the one-step glutaraldehyde method, see Fig. 5B (8, 9).

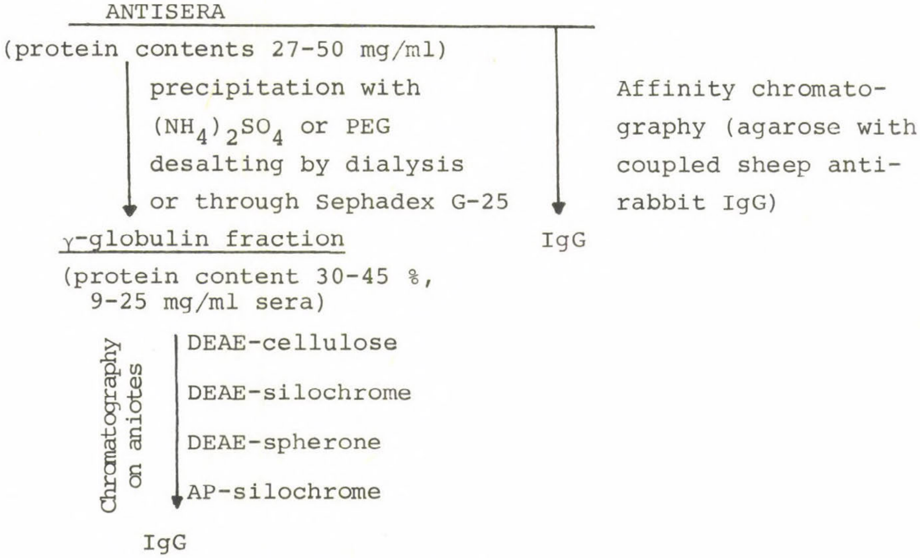


Fig. 2. IgG purification from sera

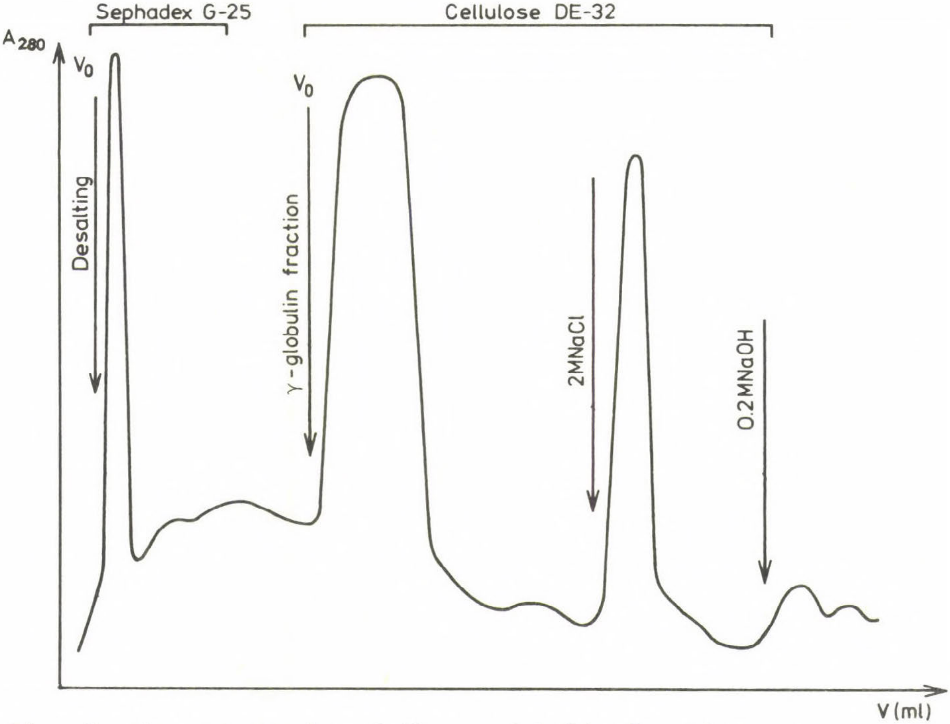


Fig. 3. Chromatography of the γ-globulin fraction on DEAE cellulose

Table I. Chromatography IgG on anionites

Sorbent	Antisera, titre	Conditions of chromatography	Flow rate (ml/min)	Sample before chromatography/void volume					Content of IgG mg/ml sera
				V ml	Total protein mg	$\frac{A_{280}}{A_{250}}$	Percent of sera	total protein by chromatography	
Cellulose DE-32, Whatman, 0.9-1.1 meqv/g	CarMV 1:1,024	15 mM phosphate buffer, containing 0.05 M NaCl, pH 7.0, R = 800-850, column dia. 1.3 : 2.0 x 10 : 20 cm	0.5	10	318	2.52	44	100	
				18	206	2.58	29	65	11.5
DEAE cellulose, fibrous, Biolar, 0.9 meqv/g	CarMV 1:512		0.5	9	302	2.52	44	100	
				31	151	2.72	22	50	8.8
DEAE cellulose, microcrystaline, Biolar, 0.55 meqv/g	Cr BV 1:512		0.83	14	94	2.3	36	100	
				18	53	2.5	23	57	10.5
DEAE-spheron, Lachema, 1.5 meqv/g	ToAV 1:64		0.83	22	229	2.3	37	100	
				33	108	2.4	17	45	5.0
DEAE-silochrome, Biolar, 0.056 meqv/g	Cr BV 1:512		1.67	21	221	2.3	36	100	
				29	142	2.4	20	58	14.2
Aminopropylsilochrome (AP -), Biolar, 0.2 meqv/g	CarMV 1:1,024		1.17	22	216	2.1	44	100	
				23	37	1.1	8	7	-

Table II. Chromatography of IgG on aminopropylsilochrome

Conditions of chromatography	pH	1. before chromatography 2. Void volume	Total protein (mg)	$\frac{A_{280}}{A_{250}}$	% of total protein
Sorption in 0.015 M phosphate buffer, containing 0.05 M NaCl R = 800-850 Ω, $m_{sorbent}$ = 1 g	7.0	1	159	2.52	100
		2	23.6	1.2	14
	6.8	1	46.1	2.09	100
		2	10.1	1.27	22
	6.4	1	47.3	2.05	100
		2	15.5	1.50	33
	6.0	1	50.5	1.85	100
		2	23.4	1.39	54
Elution with phosphate buffer, containing NaCl 0.05 → 1 M			0	-	-
Elution with buffer tris-HCl pH 7.0, 0.05 → 0.5 M Max elution ~ 0.4 M tris-HCl			93.8	2.66	59 (10.4 mg/ml sera)

Table III. Chromatography of antisera on affinity column

Preparation	Total protein (mg)	$\frac{A_{280}}{A_{250}}$	% of total protein	IgG content of sera (mg/ml)
Antisera (anti-ToAV), titre 1:64	120	2.0	100	-
Void volume	100	-	-	-
Fraction F_1	15	2.63	9	5.1
Fraction F_2	4.8	2.8	4.5	1.5

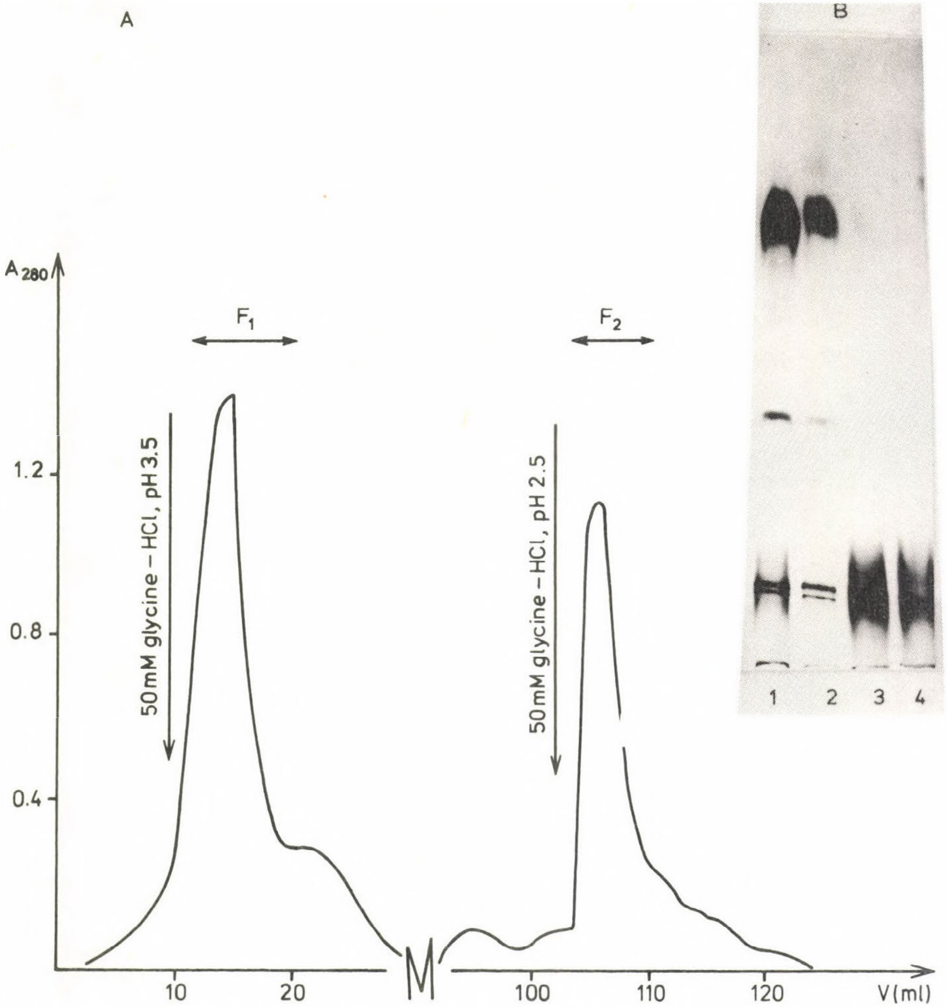


Fig. 4. A: Chromatography of the antisera on the affinity column, prepared by coupling sheep anti-rabbit IgG to CNBr-agarose (10 mg IgG/ml sorbent);
B: Separation of serum proteins by 7.5 % PAGE
1: antisera; 2: void volume fraction; 3: F_1; 4: F_2

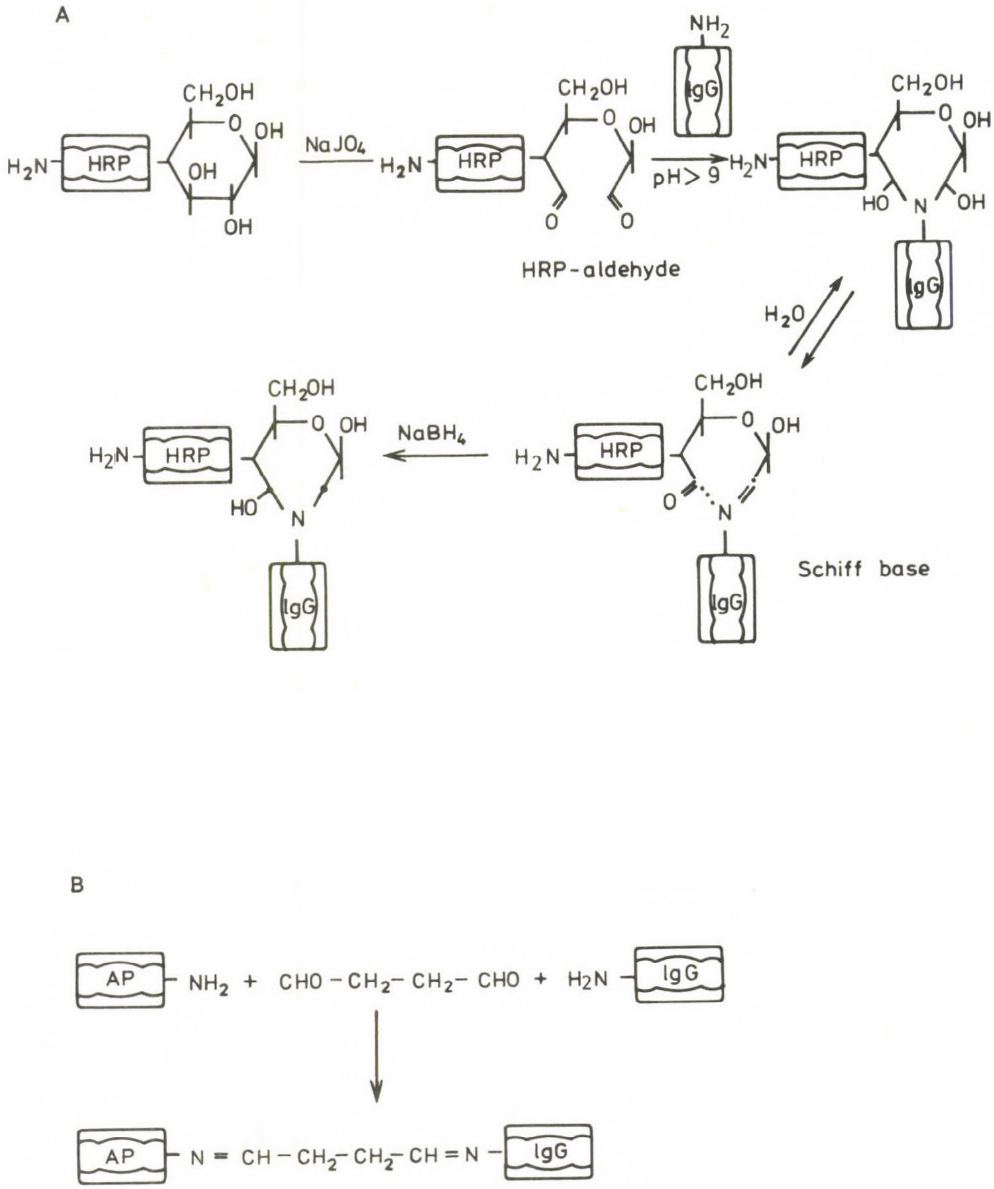


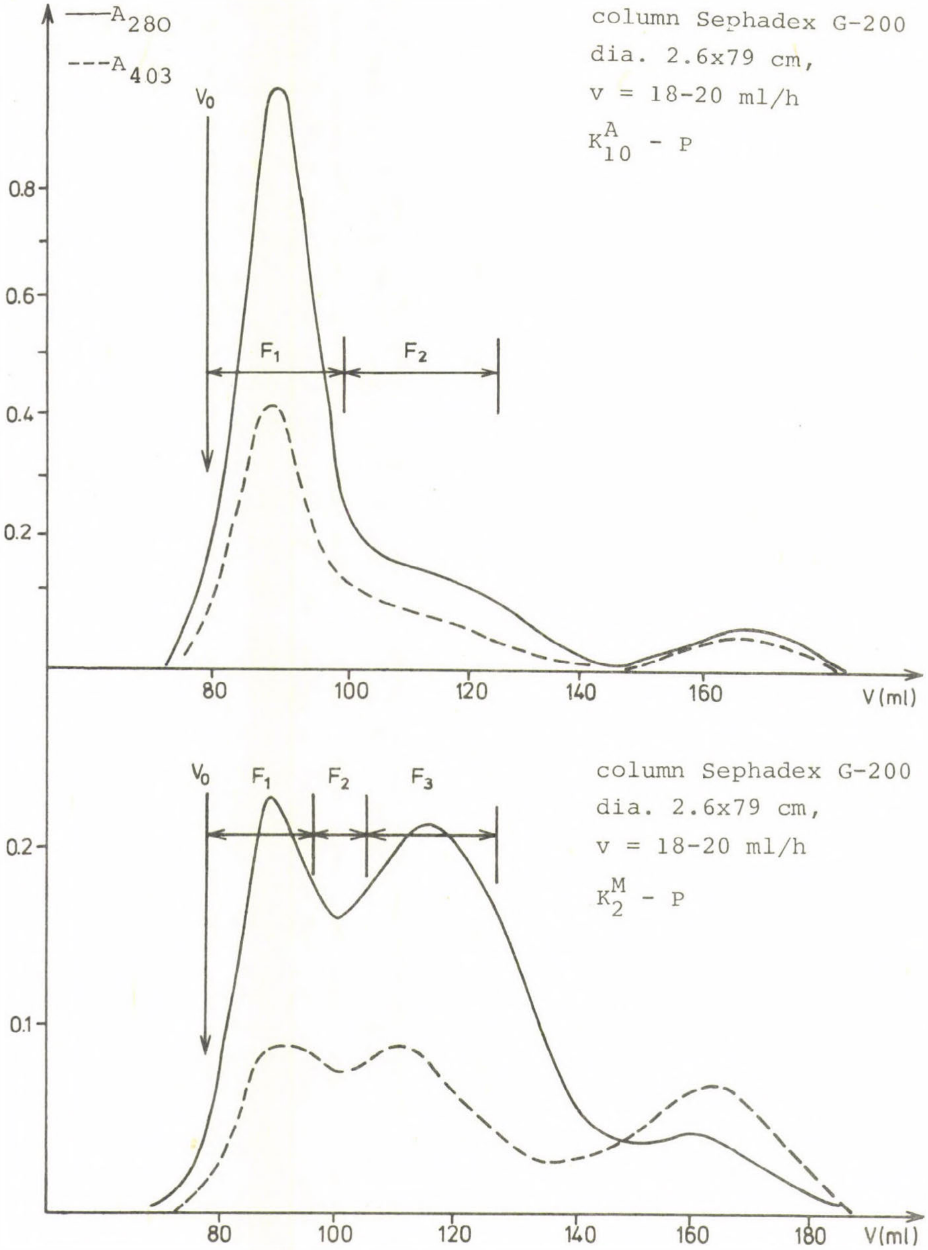
Fig. 5. Principle of the conjugating methods. A: Principle of the periodate method of conjugating HRP to protein - IgG; B: Principle of the one-step glutaraldehyde method of conjugating AP to protein - IgG

The conjugation process yields a complicated reaction mixture which consists of a free enzyme, IgG and their associates. We have separated this mixture by gel-permeation chromatography on Sephadex G-200 and Toypearl HW 55F. Typical results of conjugation of IgG with HRP are shown in Table IV and Fig. 6. The elution pattern depends on the conjugation conditions: periodate concentration, ratio IgG:HRP, and other factors. The elution pattern K_2^M - P is characteristic of the conjugation by the method of Nakane (5) at a IgG:HRP ratio of 1:2 : 1. The amount of bound peroxidase does not exceed 21 %. Conjugation by the method of Tijssen (7) (oxidation is provided by 8 mM $NaJO_4$ and reaction is stopped by adding Sephadex G-25 to the reaction mixture, ratio IgG:HRP is 1:3) yields an increased percent of HRP in fraction 1 up to 50-70 % and in fraction 2 to 9-26 % (see Fig. 6) K_{10}^A - P and K_6^M - P. Fraction 2 contains the conjugate and free IgG, while fraction 3 contains free HRP.

The same conjugation mixture was separated on Toypearl HW 55F, which has larger exclusion limits than Sephadex G-200 (see Fig. 6) K_7^M - P. A certain portion of conjugate is eluted in void volume in both cases - on Sephadex G-200 - 40-50 % and on Toypearl HW 55F - 20 %. It shows that more than 20 % of the conjugate is in the form of associates. Fraction 2 contains 30 % of HRP and is very heterogeneous in molecular weights. Fraction 3, like fraction 2, when chromatographed on Sephadex G-200 contains free IgG.

Conjugate K_8^M - P is an example of separating of reaction mixture of unsuccessive conjugation, see Fig. 6 and Table IV.

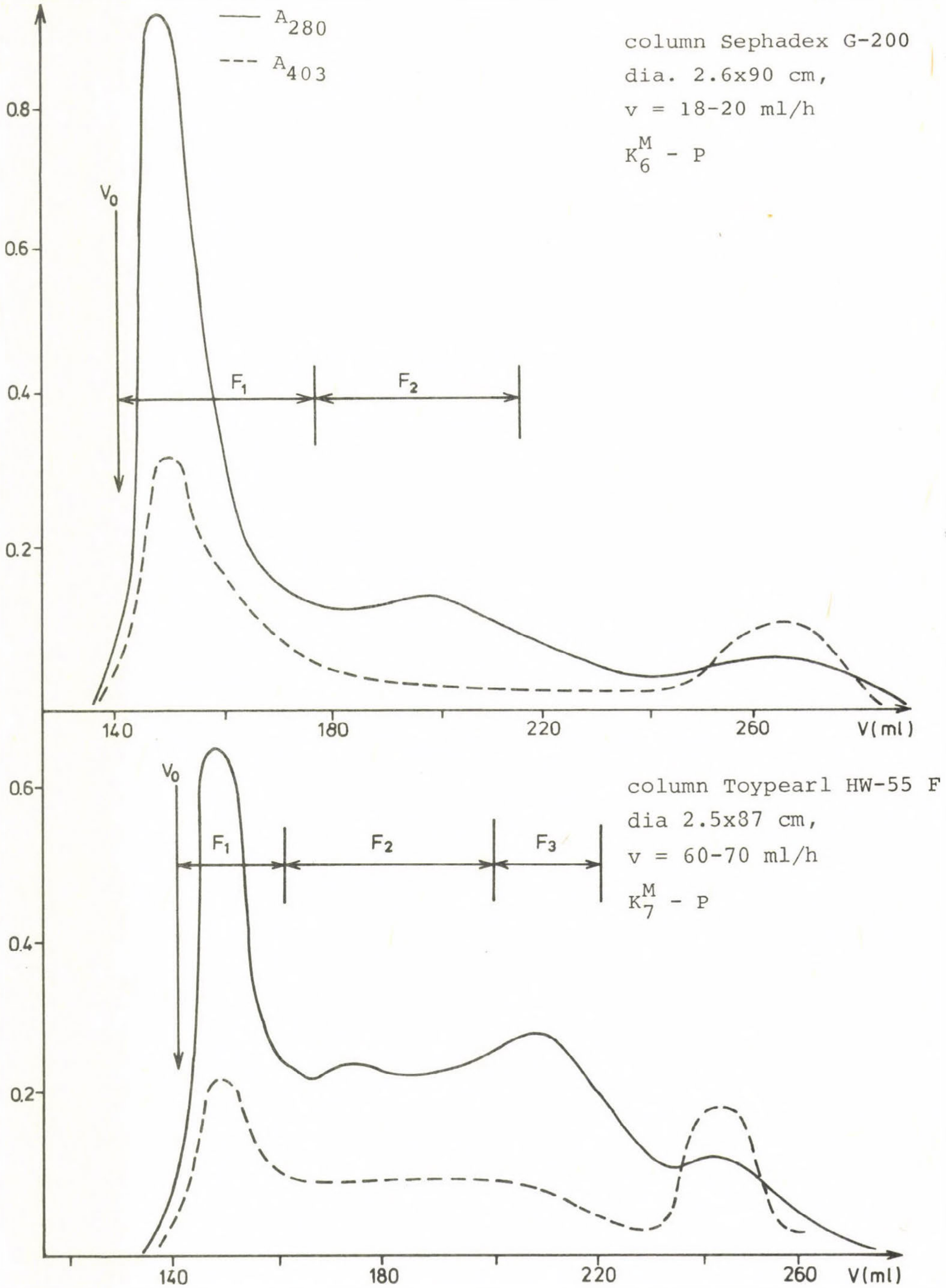
Typical results of chromatography of conjugate IgG-AP are given Fig. 7 and Table V.

—A_{280}
---A_{403}
column Sephadex G-200
dia. 2.6x79 cm,
v = 18-20 ml/h
K^{A}_{10} - P
V_0
F_1
F_2
0.8
0.6
0.4
0.2
80
100
120
140
160
V(ml)
column Sephadex G-200
dia. 2.6x79 cm,
v = 18-20 ml/h
K^{M}_{2} - P
V_0
F_1
F_2
F_3
0.2
0.1
80
100
120
140
160
180
V(ml)

(A)

Fig. 6.

→



column Sephadex G-200
dia. 2.6x90 cm,
v = 18-20 ml/h
K_6^M - P

column Toypearl HW-55 F
dia 2.5x87 cm,
v = 60-70 ml/h
K_7^M - P

(B)

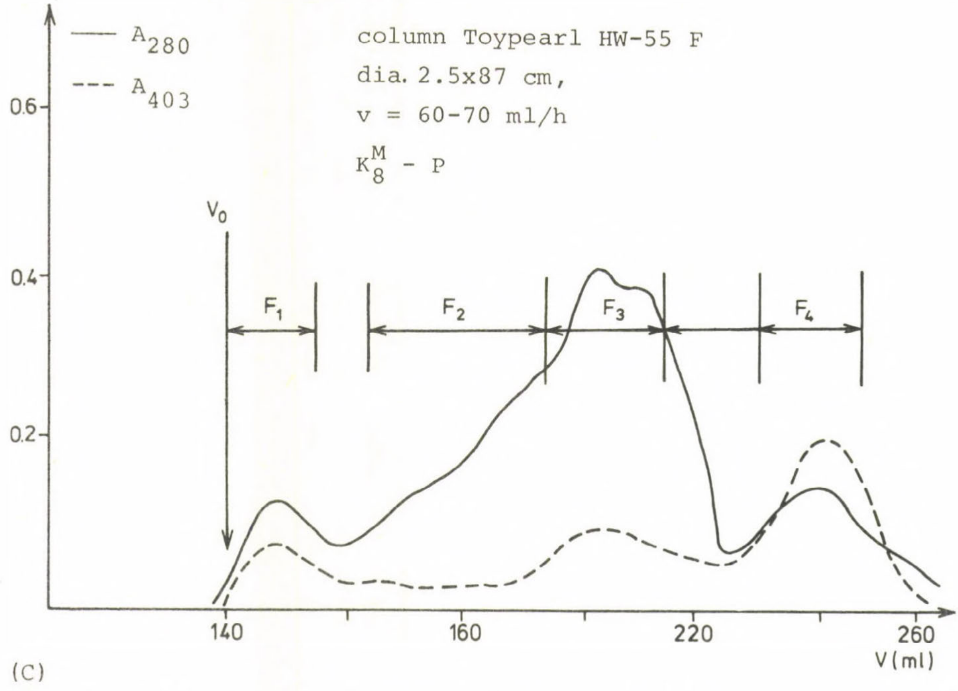


Fig. 6. Chromatography of the conjugate reaction mixture on Sephadex G-200 and Toypearl HW 55 F[(A)(B)(C)]

Table IV. Chromatography of conjugate reaction mixture prepared by periodate method

Characteristics of conjugate	Preparation	V (ml)	Amount of HRP (mg)	Total protein A_{280}	$\frac{A_{403}}{A_{280}}$	% of initial HRP
K_2^M - P	before chromatogr.	1.7	3.7	9.9	0.51	75
IgG^M + HRP (2:1) method of Nakane, Sephadex G-200	F_1	10	0.34	2.0	0.39	7
	F_2	7.6	0.25	1.3	0.44	5
	F_3	13	0.46	2.5	0.42	9
	$F_1+F_2+F_3$	-	1.05	5.8	-	21
K_{10}^A - P	before chromatogr.	3.5	3.9	17	0.5	78
IgG^A + HRP (3:1), method of Tijssen, Sephadex G-200	F_1	17.5	2.5	12	0.47	50
	F_2	27	0.9	5	0.47	18
	F_1+F_2	-	3.4	17	-	68
K_6^M - P	before chromatogr.	8	9.3	49	0.42	100
IgG^M + HRP (3:1), method of Tijssen, Sephadex G-200	F_1	32	4.4	27.5	0.36	47
	F_2	17	0.64	4.0	0.35	9
	F_1+F_2	-	5.14	31.5	-	56
K_7^M - P	before chromatogr.	5	5.8	30	0.42	100
IgG^M + HRP (3:1), method of Tijssen, Toypearl HW 55F	F_1	20	1.2	4.7	0.39	20
	F_2	45	1.8	4.0	0.32	30
	F_3	15	0.44	3.0	0.27	7.5
	F_1+F_2	65	3.0	8.7	-	50
K_8^M - P	before chromatogr.	4	2.6	17	0.35	50
IgG^M + HRP (3:1), method of Tijssen, Toypearl HW 55F	F_1	12	0.19	1.6	0.35	3.5
	F_2	20	0.86	7.0	0.26	6
	F_3	20	0.57	6.8	0.28	10
	F_4	25	0.9	2.7	0.20	15

Table V. Chromatography of conjugate reaction mixture IgG-AP prepared by one-step glutaraldehyde method on Sephadex G-200

Characteristics of conjugates	Preparations	V (ml)	Total protein A_{280}	% of total protein	% of AP activity	V of "working dilution" (ml)
K_2^B - AP	before chr.	4	20	100	100	-
IgG^B + AP (1:1.5)	F_1	19	11	55	25	200
	F_2	22	3.5	17	20	-

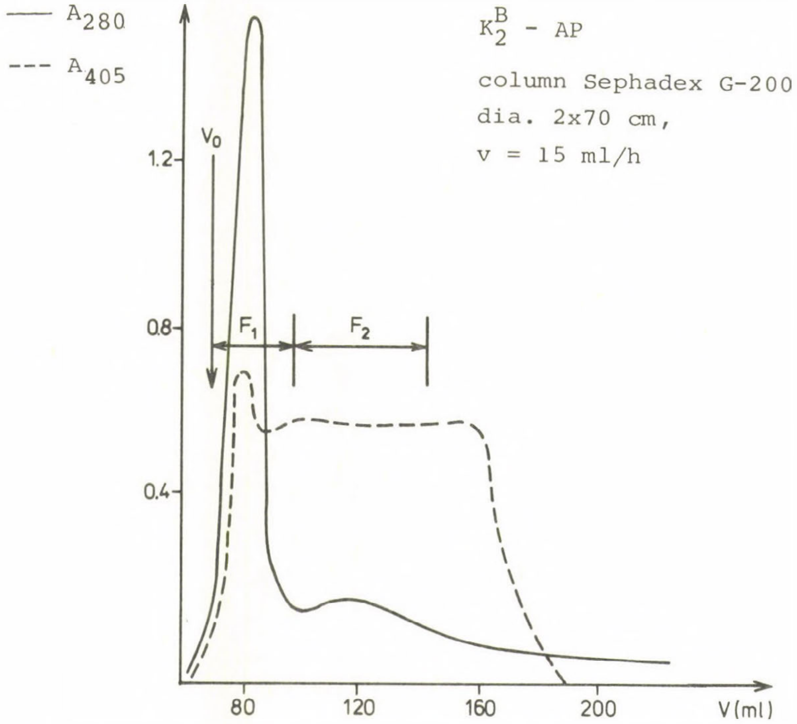


Fig. 7. Chromatography of the conjugate reaction mixture IgG - AP. ——: A_{280}; ---: activity of AP

CONCLUSIONS

Antibodies were obtained from rabbit serum against various plant viruses, such as carnation mottle virus, chrysanthemum virus B, tomato aspermy virus and some others, with the titre of 1:64 to 1:1,024. Specific IgG was isolated from crude serum by affinity chromatography on sheep anti-rabbit IgG immobilized on agarose or from serum-globulin fraction by anion-exchange chromatography. The best results were obtained by purification of IgG by desalting with ammonium sulphate and chromatography on DEAE-sorbents. The amount of purified IgG is 8-13 mg per ml of antiserum. Characteristics of the preparations were determined by means of immunoelectrophoresis, PAGE and double gel precipitation techniques.

Horse-radish peroxidase and intestine alkaline phosphatase were used as enzyme labels. The HRP conjugates were prepared by the several modifications of two step periodate oxidation process. The maximal amount of coupled HRP (~60 %) was obtained by using the method of Tijssen (7). The elution pattern on Sephadex G-200 and Toypearl HW 55F revealed that conjugates are heterogeneous in molecular weights; negligible amount of the enzyme label remained uncoupled to IgG and practically no antibody remained unreacted.

Alkaline phosphatase conjugate was prepared by one-step glutaraldehyde method. The purification of the conjugate on Sephadex G-200 showed that 25 % of the enzyme activity was coupled. The conjugate thus obtained was tested for enzymatic and immunobiological activity.

Antibodies and conjugates were applied in determination of virus infection of chrysanthemums and carnations both in mother plants or meristems.

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