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JOURNAL OF CHROMATOGRAPHY

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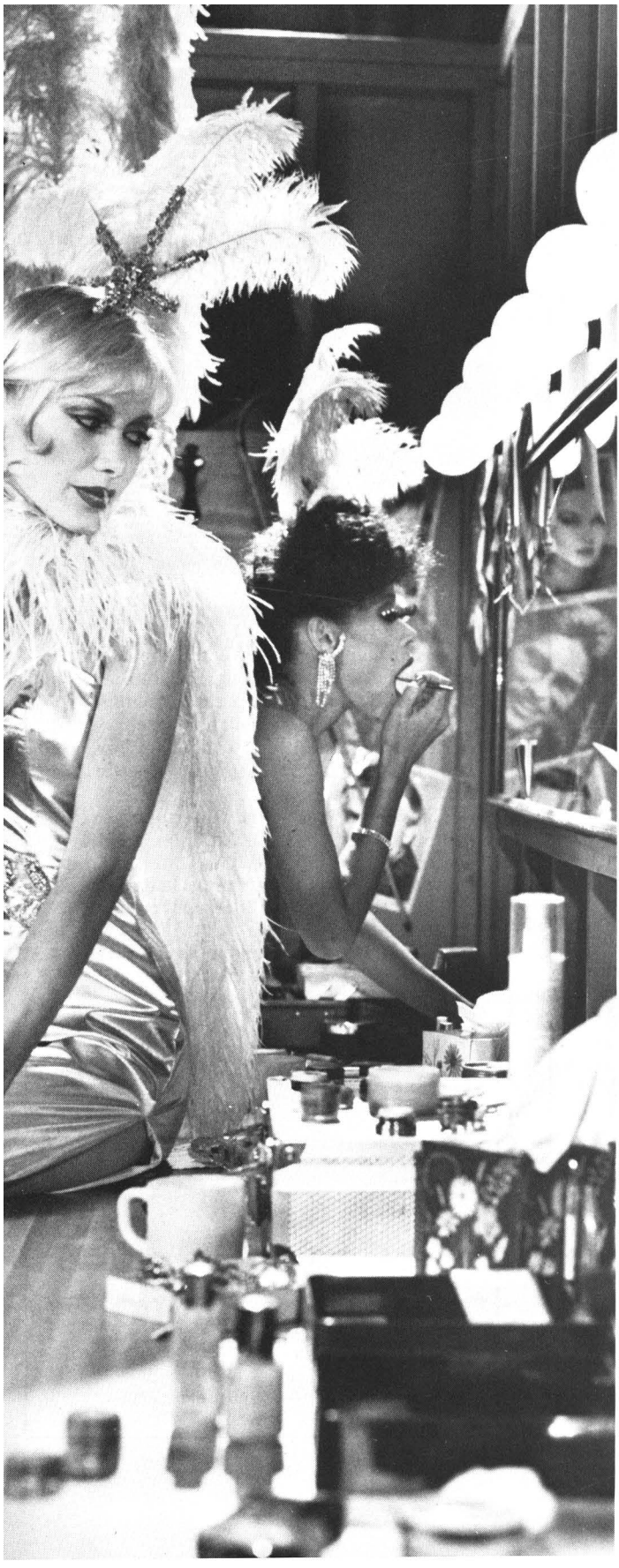

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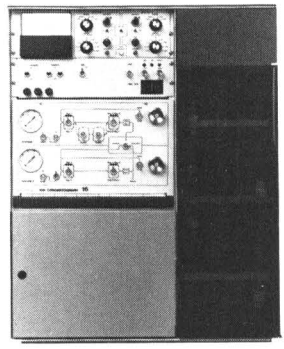

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STATISTICAL TREATMENT OF EXPERIMENTAL DATA

By J.R. GREEN, *Lecturer in Computational and Statistical Science, University of Liverpool, U.K. and* D. MARGERISON, *Senior Lecturer in Inorganic, Physical and Industrial Chemistry, University of Liverpool, U.K.*

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Obituary

Karl Hans Georg Wunderly

Charlie Wunderly-Böhme, who died on November 14th, 1979, was born in Zürich as the son of Hans Wunderly and Nanny Volkart. He began his studies in the chemistry department of the Eidgenössische Technische Hochschule (ETH) in 1918. One year later he had the opportunity to accompany his father as a member of the first Swiss Mission of Economic Studies to the U.S.A., Canada and England. He terminated his studies at the ETH as the best of his class, specialising in electrochemistry. He then travelled extensively with his uncle in Brasil and the Argentine, and in 1924 he received his doctorate at the Physico-Chemical Institute of the ETH, with a thesis on the aminolysis of various amino acids.

His first job was in Buenos Aires with the tanneries of Bally Limitada. In the summer of 1927 he joined his father's firm, the tanneries Wunderly & Co., in Meilen near Zürich.

His many friends will always remember him in the garden of the family house, that he and his family continued to inhabit and where he and his wife, Madeleine Böhme, built the marvellous Park Theatre.

In 1931 he returned to the Institute of Physical Chemistry of the ETH and later continued his research work at the Medical Clinic of the University of Zürich.

The technique of paper electrophoresis and the first electropherograms had been reported in 1949 by Durrum and then by Kunkel and Tiselius, and it was the merit of Dr. Wunderly that he applied this method clinically, examining thousands of pathological sera and reporting his findings first in a series of papers, as well as in numerous lectures, and later in a small monograph, *Die Papierelektrophorese*, which was translated into numerous languages; and later in the volume *Die Bluteiweisskörper des Menschen* (Wuhrmann/Wunderly, Verlag Benno Schwabe & Co.).

If one looks today at the numerous photos of paper electropherograms one is immediately struck by the fact that all modern technical modifications of zone paper electrophoresis may have simplified the technique, but they have not improved upon the actual separations obtained so long ago by Charlie Wunderly.

But he did not dedicate his life only to scientific research. He lived for and with the community in which he was born, Meilen, founding and building the open-air theatre of Meilen in his own garden, in which on his 80th birthday, he had the joy of attending a performance of the Zürich Chamber Orchestra under Edmonde de Stoutz, who played for him and his friends. He was a keen sailor all his life, and as a patron of the arts, the statues which decorate Meilen as gifts from him to his town bear witness to his eclecticism and generosity.

He is survived by his wife and two children.

M. LEDERER

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NOVEL POROUS CARBON PACKINGS IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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(First received April 24th, 1980; revised manuscript received July 17th, 1980)

SUMMARY

Porous carbon packings of particle size 5–20 μm were prepared by calcination of purified active carbons and cokes having narrow size ranges. Highly efficient columns packed with 10-μm particles were obtained by means of the high-viscosity slurry technique, and exhibited about 3000 theoretical plates at 100 mm length and optimal eluent flow-rate. The loading of columns, $\theta_{0.1}$, was determined to be 25–50 μg of solute per gram of packing. Eluotropic series of solvents were found to be similar to those on pyrocarbon-modified carbons and silicas. The log k' values for homologous compounds increased linearly with the hydrocarbonaceous surface area at constant eluent composition. Studies with polar solutes indicated that the volume polarizability rather than dipole moment was the decisive molecular property of the solute with respect to retention. The successful application of carbon packings in aqueous organic eluents having a pH $>$ 8.5 was demonstrated.

INTRODUCTION

In classical column liquid chromatography, polar adsorbents such as silica and alumina are commonly employed. The reversed-phase mode of modern high-performance liquid chromatography (HPLC), however, requires non-polar adsorbents. Such packings are made by silanization of appropriate silicas with *n*-alkylsilanes. By bonding of *n*-octyl and *n*-octadecyl functional groups to the silica surface its hydrophilic character is greatly reduced. However, the materials still do not behave in a totally hydrophobic manner because their hydrophobic character depends on the type of *n*-alkyl chain bonded and on the surface coverage. The most striking illustration of this fact is the observed chemical instability of reversed-phase packings towards aqueous eluents, which can be attributed to the reactivity of siloxane groups anchoring the *n*-alkyl groups and to the presence of residual silanol groups.

* Presented in part at the 12th International Symposium on Chromatography, Baden-Baden, September 1978.

** Present address: Hewlett-Packard GmbH, Waldbronn, G.F.R.

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In contrast to silica-based adsorbents, active carbon, pyrolytic and graphitized carbon offer a pronounced lipophilic surface. The extent of lipophilic nature can vary widely, depending on the bulk structure and the types of surface functional groups[1,2]. Pure graphite alone possesses a truly non-polar and homogeneous surface due to its structure, and thus can be considered as a model for non-polar adsorbents. Support for this view has been provided by gas adsorption studies and by calculations of adsorbent–adsorbate interactions on graphite[3,4]. Graphite is a soft and non-porous material and is therefore unsuitable for reversed-phase columns which demand mechanically stable packings and a sufficiently large surface area for retention. Some separations in classical LC on active carbon species have been reported[5–8]. The use of these species in HPLC, however, appears highly problematical due to their complex surface composition, the large specific surface area and the presence of micropores.

The first thorough attempts to prepare suitable carbon packings for HPLC were made by Guiochon and co-workers[9–11] by hardening of thermal carbon black agglomerates through pyrolytic decomposition of benzene followed by graphitization. Although extensive retention studies were carried out on these packings, severe problems remained in obtaining sufficient mechanical stability in the 5–10 μm size range. For this reason, these workers performed the pyrolysis of benzene on silica instead of carbon black[12]. A quite different procedure was recently introduced by Plzák *et al.*[13], who reduced polytetrafluoroethylene with alkali-metal amalgams (see also ref. 14). Controlled thermal degradation of polymer beads is another way of producing carbon species[15]. The materials obtained, however, are microporous and small particles in the 5–10 μm size range are expensive to make.

The previously described procedures all require extremely elaborate techniques such as pyrolysis, thermal degradation, etc. Therefore, the primary objective of our study was to find a simpler way of production. Purification and heat treatment of mechanically hard cokes and active carbon species appears to be a promising method. Although our studies are not yet complete and much more research will have to be done to fully characterize the properties of the materials, we present here the first results. After synthesizing suitable materials, retention studies were carried out under reversed-phase conditions to elucidate the specificities in the retention of solutes and to establish the eluotropic strength of solvents. Finally, efforts were made to examine the stability of carbon packings in highly alkaline water–methanol mixtures.

EXPERIMENTAL

Processing of carbon

The starting materials were ground to the desired particle size with a Type Pulverisette 5/2 ball-mill (Fritzsch, Idar-Oberstein, G.F.R.). The two zirconia milling beakers (250 ml volume) were filled with 60 g of carbon material together with six milling beads of 30 mm diameter, also made of zirconia. Optimum milling time for grinding to 5–10 μm particle size was found to be 10 min.

Sizing into narrow 5-μm and 10-μm cuts was performed by a Type Multiplex A 100 MZR zigzag siever (Alpine, Augsburg, G.F.R.). The speed of the rotating wheel was varied in the range of 10,000–18,000 rpm and at a corresponding air flow velocity of 45–37 Nm^3/h. Particle size distribution of the batch was controlled by means of photosedimentation[16].

In order to extract the inorganic impurities the sized carbon materials were subjected to several treatments in the following sequence:

(i) 350 g of material were heated under reflux for 72 h in 1.5 l of hydrochloric acid solution (20%, w/w), then washed with deionized water to remove chloride ions

(ii) 40 g of the resulting material were treated at 430°K with 100 ml of hydrofluoric acid (40%, w/w) in a beaker made of glassy carbon (Sigradur KAZ 26); Sigri, Meitingen, G.F.R.)

(iii) the residue was washed with a hot sodium hydroxide solution to remove ions, washed with deionized water to neutrality and then dried at 473°K.

For heat treatment, 20-g amounts of the purified or native material were placed into cylinders (22 × 120 mm) composed of pure graphite and fitted with a porous screw cap. The graphite cylinders were kindly supplied by Dr. Delle, Kernforschungsanlage, Jülich, G.F.R. The samples were first heated to 473°K for 8 h and then flushed with dry helium. Calcination was carried out at Sigri Elektrographit, Frankfurt/M, G.F.R., using a high-temperature oven (Balzers, Liechtenstein). The oven was first flushed with dry helium and heated at the desired temperature for 2 h. The temperature was varied between 2073 and 3073°K, and the length of treatment was between 5 min and 3 h. After cooling, the samples were removed from the graphite cylinders and stored in glass bottles over helium.

The chemical composition and surface properties of the starting materials and of the intermediate and final products were characterized by the following quantities: elemental analysis of C, H, N and O; ash content; crystallinity as determined by X-ray spectroscopy; particle size distribution by photosedimentation; apparent density due to helium, apparent density due to mercury; specific surface area according to BET[17]; pore volume distribution by mercury porosimetry[17].

Chromatographic measurements

Two types of liquid chromatographs were used, a DuPont Model 830 and a Hewlett-Packard 1084A. Both were operated with a UV detector at 254 nm. Injection volume was 5 μl. The column dimensions were 100 × 4.2 mm, 250 × 4.2 mm and 100 × 10 mm. Column performance was characterized by the dependence of reduced plate height on reduced velocity, the number of theoretical plates, N, obtained under optimal velocity conditions, column resistance factor, φ, and by the peak asymmetry factor, A_s. Quantities measured were calculated using standard equations[18].

RESULTS AND DISCUSSION

Processing and properties of porous carbon

The primary requirement of a packing in HPLC is sufficient hardness, which enables stable packings to be obtained. The previously described pure carbons only partially meet this condition. To overcome this problem we chose hard cokes and active carbons. The refinement of these products involved the following consecutive steps:

(i) milling of starting materials to the desired particle size range

(ii) sizing of ground materials into narrow cuts in the range of 5–10 μm

(iii) purification to remove the non-carbonaceous impurities

(iv) calcination at temperatures above 2073°K to produce an essentially non-polar and homogeneous surface.

Six cokes and active carbons, were selected as starting materials (see Table I) as all have a large bulk crushing strength. The specific surface area spanned more than one order of magnitude from 20 to 869 m^2/g. All products are typical microporous solids except product 5 which additionally possesses some mesopores. Depending on the degree of activation, the carbon content varied between 81 and 91%, w/w. The extraction step described in the Experimental section was thought to remove basic and acidic constituents as well as silica from the crude product when they were accessible to the extracting solvent. Indeed, for product 2 this extraction resulted in an increase of the carbon content from 81.8% to 90.6% whereas the ash content diminished from 10.7% to 3.8%. However, this treatment was not so effective for product 2 (*cf.*, native product 6).

TABLE I

CHARACTERISTICS OF STARTING CARBONACEOUS MATERIALS

No.	*Type*	*Supplier*	S_{BET} *(m^2/g)*	V_p *(ml/g)*	$d_{app(He)}$ *(g/ml)*	*Carbon content (%)*	*Ash content (%)*
1	Coke	Bergbauforschung, Essen, G.F.R.	20	0.41	—	87.3	7.35
2	Coke	Bergbauforschung	72	0.26	1.83	81.8	10.70
3	Coke	Bergbauforschung	131	0.57	—	81.4	7.10
4	Active carbon	Lurgi, Frankfurt/M, G.F.R.	277	—	—	89.0	1.90
5	Active carbon	Degussa, Hanau, G.F.R.	572	0.94	2.11	90.2	3.30
6	Active carbon	Bergbauforschung	869	0.34	2.02	91.1	2.0

Chromatographic tests with a series of solvent compositions and solutes showed that the extracted carbon products still behave as slightly polar adsorbents. Until now, graphitization or calcination was thought to be the only possible means of creating an inert and non-polar surface. It is well known that not all cokes and active carbons are suitable for graphitization, *i.e.*, the extent of crystallinity of the final product depends on the degree of microcrystallinity of the starting materials[19]. The inorganic impurities may also influence graphitization through the intermediate formation of carbides. This type of catalytic graphitization is often observed but difficult to explain. The rate of graphitization usually becomes noticeably higher above 2200°K, whereas catalytic graphitization generally occurs at lower temperatures. The degree of graphitization, g, can be calculated by

$$g = (3.440 - \bar{d})/0.086$$

where $\bar{d}$ is the mean interlayer spacing of the material derived from X-ray diffraction measurements. $\bar{d}$ is "known to decrease monotonically with the development of the structure from $\bar{d} \geqslant 0.344$ nm in a disordered carbon to 0.3354 nm for near-perfect graphite at room temperature"[20].

All extracted carbons were subjected to graphitization but most work was centered on product 2. Treatment of a batch of extracted carbon 2 having 19.5 μm particle size was carried out at two temperatures, 2673°K and 3073°K. Length of treatment was 2 h. Both products obtained were grey and had a carbon content of 99.5%. Neither oxygen nor nitrogen could be detected by elemental analysis. The ash content was smaller than 0.1%, w/w. The helium density was raised to 2.10 g/ml (3073°K) from 1.83–2.08 g/ml (2673°K). The g value of the extracted product 2 treated at 2073°K for 2 h was calculated to be 0.45. According to this low degree of graphitization the structure of the heat-treated carbon packing resembles more a two-dimensional, disordered structure than a three-dimensionally ordered graphite structure. Therefore it is more precise to designate the products as heat-treated carbons rather than graphitized carbons. Extended calcination studies were also performed on extracted and native active carbon 6. Again the carbon content was above 99.0%. Surprisingly, the apparent helium density after calcination was found to be lower than that of the starting material. Depending on the temperature and length of exposure during calcination, $d_{app(He)}$ ranged from 1.46 to 1.79 g/ml compared to the 2.02 g/ml of the native product 6. This observation is only consistent with the formation of closed pores during calcination which are not accessible to helium.

Heat treatment resulted in a small reduction of mean particle size. For batches of product 6 having $d_{p50} = 9.80$ μm the final particle size decreased to $d_{p50} = 8.9$ μm. The scanning electron micrograph of the product in Fig. 1 shows angular particles with rounded edges. Pore size analysis by means of nitrogen sorption and mercury penetration indicated the presence of mesopores and macropores. No micropores could be detected. The specific surface area of all calcined products ranged between 1–5 m^2/g even at the low calcination temperature of 2073°K. In principle, calcination of extracted or native hard cokes and active carbons yields microparticulate carbon packings with high mechanical stability.

Performance characteristics of carbon columns

Stable and efficient carbon columns could be packed by means of the high-viscosity slurry technique employing a slurry of 17.5% w/w dioxan–paraffin oil (21:79, v/v). The suspension in the packing autoclave was covered with a layer of dry n-heptane and forced downwards into the column (100 × 10 mm) at a constant flow-rate of about 10 ml/min. Column performance was represented by plots of reduced plate height, h, *vs.* reduced linear velocity[20]. An example is given in Fig. 2. The shape of the curves is consistent with those obtained on common HPLC packings including reversed-phase materials[21–23]. The minimum value of h occurs at a reduced velocity range $1 < v < 10$. Values of the reduced plate height at optimum velocities range from 3.5 to 10 depending on the capacity factor, *i.e.*, the plate height, H, corresponds to 3.5–10 times the mean particle diameter of the packing. Analysis of the log h–log v dependences of good packed columns in terms of the equation[20]

$$h = B/v + A\,v^{0.33} + C\,v$$

gives values $A = 1.0$, $B = 2.0$ and $C = 0.1$. By curve-fitting using a program kindly supplied by A. Cooke and N. Nelson, Institute of Chemical Analysis and Forensic Science, Northeastern University, Boston, MA, U.S.A., the following values were ob-

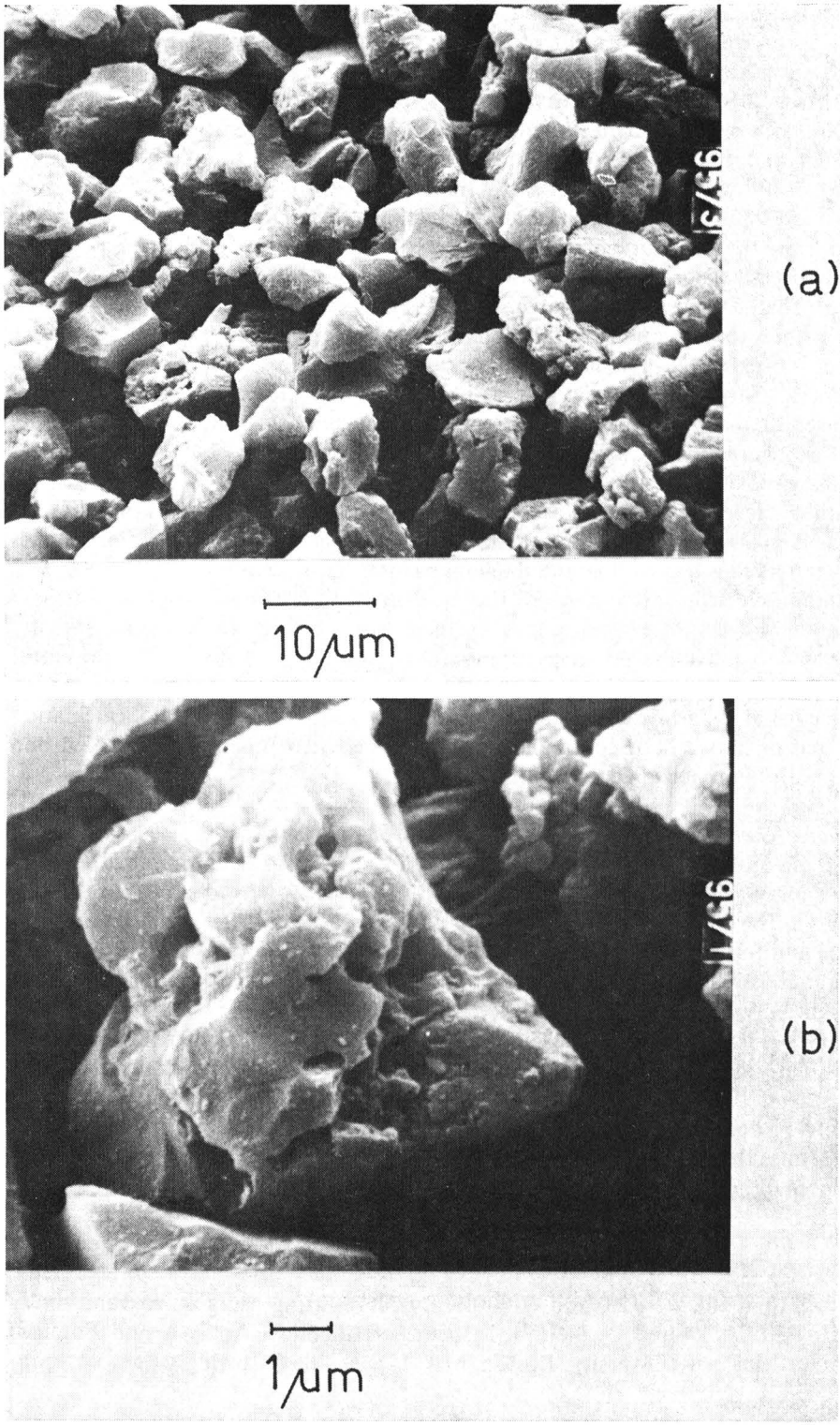

Fig. 1. Scanning electron micrograph of native (a) and calcined (b) (T_c = 2073°K) product 6 having d_p =9.80 μm and 8.90 μm, respectively. Magnification: × 1500 (a); × 7900 (b).

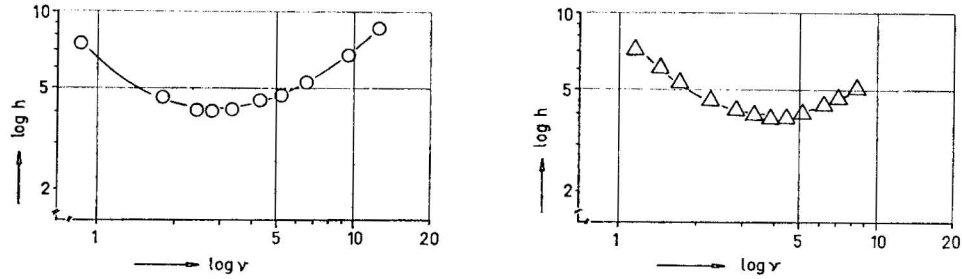

Fig. 2. Dependence of reduced plate height *vs.* reduced velocity on carbon column (100 × 10 mm). Packing: product 6 treated at 2023°K for 5 min; $d_p = 8.9\,\mu$m. Eluent: pure methanol. Solutes: ○, phthalic acid diethyl ester, $k' = 0.22$; △, phthalic acid dibutyl ester, $k' = 0.66$.

tained from Fig. 2: $k' = 0.22$: $A = 0.31$, $B = 5.80$, $C = 0.57$; $k' = 0.66$: $A = 0.09$, $B = 7.48$, $C = 0.48$.

On the 100 × 10 mm column, 2800 (3180) theoretical plates were obtained for the solute having $k' = 0.22$ (0.66) at the minimum in the h *vs.* v curve at a reduced velocity, v, of 2.5 (3.9), which corresponds to a linear flow-rate of $u = 0.37$ (0.44) mm/sec and a volume flow-rate of $f_v = 0.70$ ml/min. The eluent was pure methanol. Column permeability, expressed by the column resistance factor, Φ (ref. 20), which also reflects the quality of the column packing, was 1000–2000 which is at the upper limit of the acknowledge Φ drange of 500–1000. Peak shape was described by the asymmetry factor, A_s, which is defined as the peak width at 0.13 of the peak height divided by the peak width at 0.6 of peak height. Ideal Gaussian peaks have $A_s = 2.0$; for the test solutes, A_s ranged from 2.05 to 2.50.

Particular emphasis was placed on the assessment of the maximum possible loading of carbon columns. On the basis of Snyder's[24] concept of the linear sample capacity, $\theta_{0.1}$, of adsorbents, this quantity is affected by the adsorbent type, the heterogeneity of the adsorbent surface and the specific surface area. $\theta_{0.1}$ is defined as the mass of solute injected per gram of packing that produces a 10% decrease of k'. This amounts to 100–1000 μg/g on bare silica[17] and reversed-phase silica packings[22]. As the specific surface area of the carbon packings is about two orders of magnitude smaller than that of silica packings (3 compared to 300 m²/g), a $\theta_{0.1}$ value of 10–50 μg/g would be expected. Another frequently used relative measure of maximum column loading is $\theta_{50\%}$, this being the mass of solute per gram of packing which results in a 50% increase in plate height from the extrapolated "zero mass injection"[25]. Colin *et al.*[25] reported values of $\theta_{50\%}$ of 10–350 μg/g on reversed-phase packings and of 10–60 μg/g on pyrocarbon-modified silica packings, the latter having S_{BET} values of 40–80 m²/g (ref. 26).

Mass loading measurements were carried out on a column (100 × 10 mm) packed with material 6, calcinated at 2073°K for 5 min with $d_p = 8.9\,\mu$m. The eluent was methanol, and 5 μl of solutions of phthalic acid diethyl ester (PEE) and of dibutyl ester (PBE) were injected. The results were: PEE, $k' = 0.22$: $\theta_{0.1} = 50.0\,\mu$g/g, $\theta_{50\%} = 12.5\,\mu$g/g; PBE, $k' = 0.66$: $\theta_{0.1} = 25.0\,\mu$g/g, $\theta_{50\%} = 6.25\,\mu$g/g. The data indicate that the two values of $\theta_{0.1}$ and $\theta_{50\%}$ do not coincide, but that the more retarded compounds exhibit a lower loading compared to the more weakly retarded

compounds, which is consistent with theory. The $\theta_{50\%}$ value is smaller by a factor of 5 compared to pyrocarbon-modified silica and by a factor of 25 in the case of reversed-phase silica packings. This implies that in practical work with carbon packings there must be careful control of the mass of injected solute as well as the use of sensitive detectors.

Retention and selectivity of solutes on carbon packings in reversed-phase chromatography

Assessment of eluotropic series of solvents. The solvent strength parameter, ε^0, as well as the order of solvent strengths (eluotropic series) are useful in selecting a suitable solvent for an analysis in adsorption chromatography.

Neglecting secondary adsorbent effects, the eluotropic strength is defined as[10,28]

$$\log (k'_{i,j}/k'_{i,k}) = A_i(\varepsilon^0{}_k - \varepsilon^0{}_j)$$

where k'_i is the capacity factor, A_i the molecular cross-sectional area of solute i and $\varepsilon^0{}_k$ and $\varepsilon^0{}_j$ the solvent strength parameters of solvents k and j. A_i is obtained by

$$A_i = V_i^{2/3} \cdot N^{1/3}$$

where V_i is the molar volume of solute and N the Avogadro number. Using methanol as reference solvent ($\varepsilon^0 = 0$), the solvent strengths of various solvents were calculated and are listed in Table II. The data indicate that the eluotropic series spans only a small range of ε^0. As expected, aromatics such as benzene and xylene appear to be the strongest solvents. The solvent sequence of the two polynuclear aromatics in Table II is consistent with the findings of Colin *et al.*[10] on pyrocarbon-modified thermal carbon blacks. Large deviations are observed between the eluotropic strength series of non-polar and polar molecules. For instance, tetrachloromethane and methyl sulphoxide act as much stronger solvents for the two polar solutes. The set of data on ε^0 is too limited to develop general guidelines and more work will be carried out on this subject.

The above discussion concerned single solvents as eluents. Much higher selectivity is expected to be provided by binary solvent mixtures. Of these, only water–methanol mixtures up to about 45:55 (v/v) were investigated. By applying the procedure suggested by Scott and Kucera[28] it was found that above this composition the eluent ceases to wet the packing sufficiently. The dependence of the capacity factor of various solutes on the composition of the water–methanol eluent was measured. For all solutes, $\log k'$ rises linearly with the water content, in agreement with the results of Colin *et al.*[25] on pyrocarbon-modified silica packings.

Correlation between retention parameters and molecular properties of solutes. Having established the solvent strength series on carbon packings, it is of particular interest to explain the retention of solutes in terms of their molecular properties. It is known for reversed-phase chromatography on silanized silicas that the logarithm of the capacity factor increases linearly with the number of carbon atoms per molecule, or more precisely, with the hydrocarbonaceous surface area of solute, TSA_S, in a homologous series at constant eluent composition[29,30]. A similar behaviour was observed on pyrocarbon-modified silicas and carbon black[25]. The results obtained on our carbon packings (Fig. 3) are consistent with these findings. The compounds studied differ in the number of methyl groups, *e.g.*, methylbenzenes and methyl-

TABLE II

ELUOTROPIC SERIES OF SOLVENTS ON PRODUCT 6 CALCINED AT 2073°K FOR 5 min
d_{p50} = 8.9 μm; classification in the order of decreasing capacity factor.

Solvent	ε^0 *for solute*			
	Naphthalene	*Phenanthrene*	*Phthalic acid dibutyl ester*	*2-Naphthaldehyde*
Methanol	—	—	—	—
n-Hexane	0.003	0.018	0.049	0.018
Tetrachloromethane	0.012	0.021	0.038	0.026
Acetonitrile	0.022	−0.006	0.020	0.010
Methyl sulphoxide	0.024	0.031	0.040	0.034
Tetrahydrofuran	0.028	0.030	0.018	0.025
Trichloromethane	0.028	−0.003	−0.004	0.040
Dichloromethane	0.038	0.020	—	0.034
Dioxan	—	0.034	—	0.036
Benzene	—	—	—	—
Xylene	—	—	—	—

naphthalenes, in the number of methylene groups, *e.g.*, phthalic acid esters, and in the number of conjugated aromatic ring systems, *e.g.*, polynuclear aromatics, with the exception of fluorene and fluoranthene. The linearity of the plots for each homologous series is satisfactory, even when positional isomers are included.

The greater slope of the funtion log k' *vs.* TSA_S for carbon packings compared to silanized silicas can be attributed to their more homogeneous and more hydrophobic surface, lacking polar sites. It should be noted in this context that the specific surface area of carbon packings is smaller by a factor of about 100 than that of silanized silica packings. Under these conditions, the retention of solutes is largely controlled by non-specific dispersion interactions. Thus, the relevant molecular parameter of the solute should be the volume polarizibility rather than the molecular dipole moment. The enhanced retention of aromatic solutes on carbon packings reflects the π-systems of the aromatic rings which permit a high degree of dispersion interaction, particularly when they are favourably positioned at the surface. Polynuclear aromatics composed of more than four conjugated aromatic rings are so retarded in methanol that stronger solvents have to be employed for their elution.

Limited systematic retention studies were carried out with polar aromatic solutes. Table III collects some data obtained for pairs of positional isomers. The *ortho*- and *meta*-derivatives differ in their molecular dipole moments, whereas the volume polarizibilities are approximately equal. The results suggest that there is no obvious relationship between the capacity factor and the molecular dipole moment of the solutes, as observed on reversed-phase silica packings[30]. In the latter case, log k' decreases linearly with the square of the molecular dipole moment at constant volume polarizibility of solute. Further studies, however, are needed to shed more light on the retention of polar solutes on carbon.

Selectivity studies on carbon packings

Carbon packings are well suited for separations of members of homologous series of aromatic compounds due to their pronounced hydrophobic selectivity.

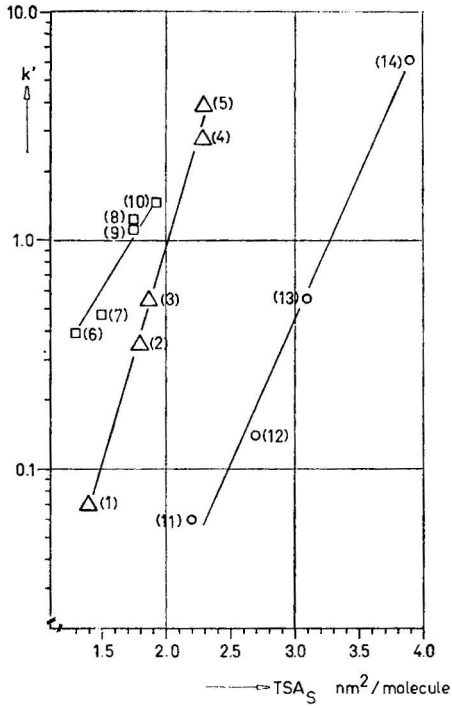

Fig. 3. Logarithm of the capacity factor of solute *vs.* the total hydrocarbonaceous surface area, TSA_S. Column: 250 × 4 mm I.D. Packing: product 2, extracted with HCl and HF, calcined at 3073°K for 3 h; d_p = 6.2 μm. Eluents: methanol for polynuclear aromatics; methanol–water (55:45, v/v) for other solutes. Solutes: 1 = naphthalene; 2 = fluorene; 3 = phenanthrene; 4 = fluoranthene; 5 = pyrene; 6 = toluene; 7 = 1,4-xylene; 8 = 1,2,4-trimethylbenzene; 9 = 1,3,5-trimethylbenzene; 10 = 1,2,4,5-tetramethylbenzene; 11 = phthalic acid methyl ester; 12 = ethyl ester, 13 = butyl ester; 14 = cyclohexyl ester.

Separations were also carried out for aromatic solutes carrying polar functional groups. In the application of carbon packings, particular emphasis was placed on the operation of columns at high eluent pH. A series of alkaloids was employed as test substances in methanol–water mixtures as eluents. The pH of the aqueous phase was adjusted using appropriate buffers. The alkaloids tested were mainly basic substances. Their capacity factors were observed to decrease with increasing pH of the buffer solution. Nevertheless, the selectivity of the carbon packing at pH around 7 was not sufficient to resolve the substances, but improved at pH 11.0–12.0 (Fig. 4). It should be added that this particular column was tested over a period of several months without incurring noticeable loss in efficiency and selectivity.

CONCLUSIONS

The structure of carbon packings varies widely from microcrystalline as in glassy carbons to fibrous and graphite-like, depending on the origin and mode of treatment. Glassy carbons are superior for two reasons: first, the microparticles provide a sufficiently high degree of hardness and mechanical stability; secondly, the

TABLE III

CAPACITY FACTORS AND MOLECULAR PROPERTIES OF SOLUTES

Column: 250 × 4 mm. Packings: a = HCl,HF-extracted product 2 calcined at 3073°K for 3 h, d_p = 6.2 μm; b = as in (a) but d_p = 19.5 μm; c = as in (a) but calcined at 2673°K for 3 h, d_p = 19.5 μm; d = as in (a) but d_p = 5.0 μm. Eluent: water–methanol (45:55, v/v); flow-rate: 1 ml/min. Detector: UV, 254 nm.

Solute	*Volume polariz-ibility, α* (10^{24} μm³)*	*Molecular dipole moment*[31], *μ (D)*	*k' on column*			
			a	*b*	*c*	*d*
2-Xylene	14.20	0.45	1.40	0.60	0.33	2.86
4-Xylene	14.28	—	1.42	0.62	0.36	3.29
2-Bromotoluene	15.35	1.45	2.74	1.03	0.59	—
4-Bromotoluene	15.40	1.95	2.90	1.17	0.57	—
2-Chlorophenol	16.70	1.43	0.25	0.11	0.09	0.98
4-Chlorophenol	16.81	2.70	0.52	0.18	0.10	1.25
Naphthalene	17.61	—	4.62	2.02	0.70	—
1-Naphthol	18.36	1.39	4.56	1.30	0.26	—
2-Naphthol	—	1.41	3.77	1.00	0.23	—
1-Methylnaphthalene	19.32	0.51	8.59	3.65	1.56	—
1-Nitronaphthalene	20.16	3.87	14.30	4.35	0.82	—
1-Bromonaphthalene	20.37	1.59	—	—	4.03	—

* Calculated by the formula

$$\alpha = \frac{3}{4} \cdot \frac{n_D^2 - 1}{n_D^2 + 2} \cdot \frac{M}{\varrho A}$$

where n_D is the refractive index, M the molar mass and ϱ the density of the solute molecule.

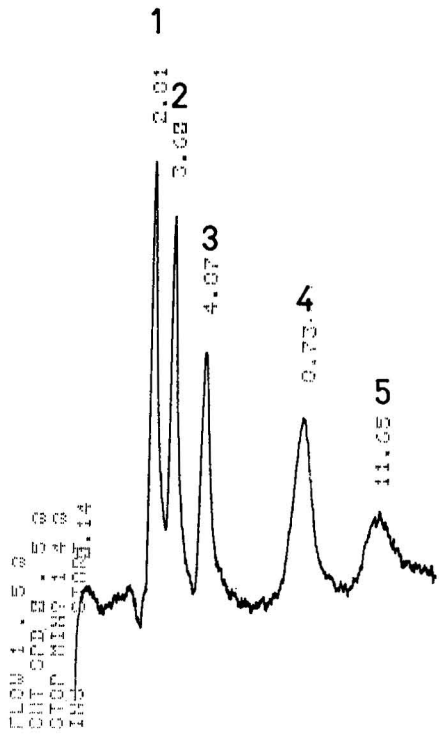

Fig. 4. Separation of alkaloids on porous carbon. Column: 100 × 10 mm. Packing: product 6 calcined at 2073°K for 5 min; d_p = 8.9 μm. Eluent: methanol–water (80:20, v/v) buffered to pH 11.2; flow-rate: 1.5 ml/min. Detector: UV, 254 nm. Injection volume: 25 μl. Solutes: 1 = ephedrine; 2 = codeine + anaestesin; 3 = theobromine; 4 = caffeine; 5 = strychnine.

number of surface defect sites is reduced due to the lower surface area, rendering less likely the formation of polar functional groups on exposure to oxygen.

With regard to their application in HPLC, it is considered unlikely that carbon packings will replace silica-based supports in reversed-phase chromatography and related techniques in the near future. Their chemical inertness and resistance to alkali are, however, advantageous when the particular separation requires an aqueous organic solvent of pH >7.0 as eluent.

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THE R_F VALUE AS A CONSTANT IN THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A comparison has been made of the results of a number of experiments concerning R_F values in thin-layer chromatography randomly chosen from the literature. The study led to the conclusion that in thin-layer chromatography a constant R_F value can be obtained.

INTRODUCTION

There are two fields in which the reproducibility of R_F values might be of special interest, *viz.*, in the relationship between chemical structure and chromatographic behaviour and in systematic qualitative analysis.

In 1968, a symposium was held on "Reproducibility of R_F values in thin-layer and paper chromatography". Most of the contributions dealt with questions concerning the rigid standardization of operating conditions. Lederer[1], in his introduction to the symposium, disagreed with this concept because simplicity is in fact the great advantage of thin-layer and paper chromatography. He also wondered whether "when compiling chromatographic data one can combine R_F values by various authors into one table". During the same symposium, Hais[2] expressed the opinion that "no serious worker would consider it more than a dream to pool R_F values obtained in different laboratories".

Although it is still often questioned whether published R_F values can be reproduced, extensive inter-laboratory experiments have shown that these values can be made constant with the aid of a simple correction equation[3–5].

In this paper, the Galanos and Kapoulas equation[6] is compared with the equation recently developed by Van Wendel de Joode *et al.*[7]. The data used in the calculations have been taken from published tables.

CORRECTION EQUATIONS

One of the first attempts to arrive at a constant R_F value was the introduction of the relative R_F value, defined as[8]

$$R_X = \frac{R_F \text{ (compound)}}{R_F \text{ (reference compound)}} \quad (1)$$

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The drawback of this procedure is that it is based on the supposition that the line representing the variations which cause the R_F value to deviate can be expressed by a straight line passing through the origin of the coordinate system, whereas this seldom occurs in practice.

Williams[9] formulated the following equation, containing factors that would correct R_F values for different experimental conditions:

$$R_{cp} = R_c + \alpha_p + \beta_p R_c + \varepsilon \tag{2}$$

where R_{cp} is the observed R_M of compound c on plate p, α_p and β_p are constants characterizing plate p, ε is the residual variation and R_c is the "ideal" R_M value of compound c in the system that one is trying to reproduce. This equation shows the additive character of the constants describing the variable conditions in practical chromatography and contains only extramolecular factors.

An important move towards the solution of the problem of obtaining constant R_F values was made by Galanos and Kapoulas[6]. They classified the variations caused by experimental conditions according to the mobility of compounds on chromatograms into the following groups:

(1) Variations that do not change the R_F values themselves, but the distances of the spots from the starting point. This type of variation is not contradictory to the postulate of a constant R_F value, *i.e.*

$$R_F^0 = R_F$$

where R_F^0 and R_F represent "tabular" and measured R_F values, respectively. The term "tabular" R_F values defines a set of R_F values, either given in the literature or determined in the lbaoratory, that are taken as models to be reproduced.

(2) Variations that change the R_F values but not the ratios of the distances of the spots from the starting point. Variations of this type can be expressed as follows:

$$R_F^0 = aR_F$$

(3) Variations that change both the individual R_F values and the ratios of the distances of the spots from the starting point.

Galanos and Kapoulas found the following equation to hold in practice:

$$R_F^0 = aR_F + b \tag{3}$$

One of the postulates on which the equation is based is that all R_F variations are additive properties, while the constants a and b are the sums of a number of individual constants $a^1, a^2, a^3, \ldots, a^n$ and $b^1, b^2, b^3, \ldots, b^n$, respectively. The constants a and b can be calculated by simple analytical geometry, provided that experimental R_F values are determined from authentic samples:

$$a = \frac{R_{FA}^0 - R_{FB}^0}{R_{FA} - R_{FB}}$$

$$b = R_{FA}^0 - aR_{FA}$$

where R^0_{FA} and R^0_{FB} are the tabular R_F values and R_{FA} and R_{FB} the measured R_F values of two reference compounds A and B. The basis of this equation is, in fact, very similar to that of eqn. 1. The equation of Galanos and Kapoulas has been tested in detail in inter-laboratory investigations[3–5].

Recently, Van Wendel de Joode *et al.*[7] derived an equation from thermodynamic principles:

$$\frac{1}{R^0_F} = \frac{a}{R_F} + b \tag{4}$$

The correction procedure is analogous to that of eqn. 3:

$$a = \left(\frac{1}{R^0_{FA}} - \frac{1}{R^0_{FB}}\right) \Big/ \left(\frac{1}{R_{FA}} - \frac{1}{R_{FB}}\right)$$

$$b = \frac{1}{R^0_{FA}} - \frac{a}{R_{FA}}$$

In this paper the results obtained with eqns. 3 and 4 are compared with the tabular R_F values.

RESULTS AND DISCUSSION

Geiss[10] published R_F values of some dyes on silica gels of different origin and under conditions of different relative humidity (63 and 79%). The R_F values obtained in the experiments were recalculated with the aid of eqns. 3 and 4 and by arbitrarily taking one set of R_F values obtained on silica gel G (Merck, Darmstadt, G.F.R.) as the R^0_F values. The R^0_F values are indicated as tabular and the calculated ones as corrected (R^c_F) values. The R^0_F values of butter yellow and *p*-hydroxyazobenzene were applied as reference values for the calculation of the constants a and b in the equations. As these values were taken from the graph in the paper by Geiss[10], a slightly greater error might be expected than that occurring when tabular values are used. Table I shows the results [note: in the tables, (p.d.) stands for "per definition", R_{F3} for calculated R_F values from eqn. 3 and R_{F4} for those obtained when using eqn. 4].

TABLE I

R^0_F VALUES, MEAN R_F VALUES AND STANDARD DEVIATIONS (s) FOR SOME DYES

Dye	$\overline{R_F}$*	s	R^c_{F3}	s	R^c_{F4}	s	R^0_F
Butter yellow	0.778	0.098	0.730	0(p.d.)	0.730	0(p.d.)	0.73
Impurity	0.692	0.143	0.649	0.057	0.639	0.031	0.63
Sudan red	0.508	0.184	0.465	0.097	0.437	0.047	0.40
Indophenol	0.377	0.175	0.332	0.077	0.301	0.031	0.29
Sudan black	0.265	0.169	0.221	0.065	0.196	0.023	0.19
p-Hydroxyazobenzene	0.185	0.119	0.13	0(p.d.)	0.13	0(p.d.)	0.13

* Mean R_F values taken from Geiss's paper[10].

The absolute differences between the tabular and the calculated R_F values are given in Table II.

TABLE II

ABSOLUTE DIFFERENCES BETWEEN TABULAR AND CALCULATED R_F VALUES

Dye	$R_F^0 - R_{F3}^c$	$R_F^0 - R_{F4}^c$
Butter yellow	0	0
Impurity	0.019	0.009
Sudan red	0.065	0.037
Indophenol	0.042	0.01
Sudan black	0.031	0.006
p-Hydroxyazobenzene	0	0

Tables I and II show that the application of eqn. 4 results in smaller deviations from the tabular values than that of eqn. 3, standard deviations for eqn. 4 are smaller than for eqn. 3, and R_F values can be reproduced to within 0.03 R_F unit, even under the widely different conditions of Geiss's[10] experiments.

Różyło[11] investigated the change in R_F values on layers of silica gel of different porosity, with mixtures of carbon tetrachloride and chloroform as eluents. The mean R_F values and the standard deviations are given in Table III.

TABLE III

MEANS AND STANDARD DEVIATIONS (s) OF ORIGINAL AND CALCULATED R_F VALUES OBTAINED ON SILICA GEL LAYERS OF DIFFERENT POROSITY

Solvent: 0.3 molar fraction $CHCl_3$ in CCl_4.

Compound	$\overline{R_F}$	s	$\overline{R_{F3}^c}$	s	$\overline{R_{F4}^c}$	s	R_F^0
1-Naphthol	0.373	0.200	0.347	0.036	0.341	0.015	0.36
2-Naphthol	0.25	0.221	0.250	0(p.d.)	0.250	0(p.d.)	0.25
Quinoline	0.230	0.104	0.275	0.047	0.262	0.043	0.29
8-Hydroxyquinoline	0.473	0.221	0.473	0.089	0.469	0.48	0.41
8-Methylquinoline	0.433	0.198	0.420	0.042	0.414	0.011	0.42
Carbazole	0.583	0.160	0.600	0.025	0.590	0.026	0.57
Fluorenone	0.620	0.089	0.620	0(p.d.)	0.620	0(p.d.)	0.62

The R_F values obtained on a silica gel of porosity 200 Å were arbitrarily used as the R_F^0 values. The reference values $R_{F(\text{2-naphthol})}^0 = 0.25$ and $R_{F(\text{fluorenone})}^0 = 0.62$ were applied to calculate R_{F3}^c and R_{F4}^c. All original data show large deviations from the mean. Satisfactory results, however, are obtained with eqn. 4. The standard deviations of R_{F4}^c are smaller than those of R_{F3}^c. Table IV shows the absolute differences between the tabular and the calculated R_F values.

TABLE IV

ABSOLUTE DIFFERENCES BETWEEN TABULAR AND CALCULATED R_F VALUES

Compound	$R_F^0 - R_{F3}^c$	$R_F^0 - R_{F4}^c$
1-Naphthol	0.013	0.019
2-Naphthol	0(p.d.)	0(p.d.)
Quinoline	0.015	0.028
8-Hydroxyquinoline	0.063	0.059
8-Methylquinoline	0	0.006
Carbazole	0.030	0.020
Fluorenone	0(p.d.)	0(p.d.)

Again, in both instances a large increase in reproducibility was achieved. In this experiment both equations give the same tabular values, within certain limits.

Changes in R_F values caused by varying amounts of stationary phase in thin-layer partition chromatography can also be corrected by using eqn. 3 or 4. Graham *et al.*[12] prepared plates from cellulose slurries containing different amounts of formamide. In Table V some of the results they obtained on plates containing 0.5, 3.0 and 6.0 *M* formamide are recorded.

TABLE V

R_F VALUES FOR SOME PHENOLS ON CELLULOSE PLATES CONTAINING DIFFERENT AMOUNTS OF FORMAMIDE

Phenol	*Formamide concentration in slurry (M)*		
	0.5	*3.0*	*6.0*
2-Methylphenol	0.40	0.13	0.08
3,4-Dimethylphenol	0.38	0.125	0.06
2,6-Dimethylphenol	0.78	0.53	0.36
2,5-Dimethylphenol	0.63	0.265	0.14
2,3,4,6-Tetramethylphenol	0.92	0.80	0.72

When the values found on 3.0 *M* formamide are taken as the R_F^0 values and $R^0_{F(\text{2-methylphenol})} = 0.13$ and $R^0_{F(\text{2,3,4,6-tetramethylphenol})} = 0.80$ as the reference values, the results given in Table VI are obtained.

TABLE VI

MEANS AND STANDARD DEVIATIONS (s) OF ORIGINAL AND CALCULATED R_F VALUES

Compound	$\overline{R_F}^*$	s	$\overline{R^c_{F3}}$	s	$\overline{R^c_{F4}}$	s
2-Methylphenol	0.203	0.172	0.13	0(p.d.)	0.13	0(p.d.)
3,4-Dimethylphenol	0.188	0.196	0.113	0.011	0.115	0.01
2,6-Dimethylphenol	0.557	0.211	0.495	0.032	0.524	0.006
2,5-Dimethylphenol	0.345	0.255	0.296	0.031	0.255	0.034
2,3,4,6-Tetramethylphenol	0.813	0.103	0.800	0(p.d.)	0.800	0(p.d.)

* Calculated from Table V.

The deviations of the calculated R_F values (Table VI) from the tabular values are given in Table VII.

TABLE VII

ABSOLUTE DIFFERENCES BETWEEN TABULAR AND CALCULATED R_F VALUES

Compound	$R_F^0 - R^c_{F3}$	$R_F^0 - R^c_{F4}$
2-Methylphenol	0	0
3,4-Dimethylphenol	0.012	0.01
2,6-Dimethylphenol	0.035	0.006
2,5-Dimethylphenol	0.031	0.010
2,3,4,6-Tetramethylphenol	0	0

CONCLUSION

The problem of the inter-laboratory comparison of R_F values in TLC would seem to have been solved[3–5]. Compounds can be (tentatively) identified by a set of determined or already published R_F values. It is not necessary to run authentic samples along with the compounds to be identified. The R_F value can be handled as a constant, which is expecially useful when the compounds are difficult to obtain.

In this paper, both eqn. 3 and eqn. 4 have been shown to give reasonably reliable results, even on layers of different porosity, on layers containing different amounts of stationary phase or under conditions of different relative humidity. Eqn. 4 seems to give the smallest deviations.

In order to arrive at constant R_F values, the following precautions have to be taken: use solvents of known composition; run at least two easily obtainable reference compounds on every chromatogram, preferably with R_F values of about 0.1 and 0.9; run a "pilot" compound* as a control on every chromatogram, the R_F value of which should lie between *ca.* 0.3 and 0.8; the chromatogram should not be taken into consideration for the recording of R_F values if the R_F value of this compound differs by more than 0.03 R_F unit from its tabular value.

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* A pilot compound is used in order to detect irregularities in the thin-layer chromatogram that might influence the accuracy of the R_F measurement.

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GAS CHROMATOGRAPHY WITH A VAPOUR MOBILE PHASE AT THE FINITE CONCENTRATION OF SORBATE

I. THE SORPTION EFFECT

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SUMMARY

The characteristic features of the sorption effect in chromatography with a vapour mobile phase are examined. A peak having a pronounced leading edge is usually formed under these conditions.

INTRODUCTION

The use of water vapour as the mobile phase[1] in gas chromatography expands the possibile range of applications, leading in a number of cases to more symmetrical peaks and better separations. Additional advantages arise in preparative chromatography, where sophisticated devices to circulate the carrier gas can be avoided, and, sometimes, the recovery of separated fractions is simplified.

Few studies have been made of the peculiarities of band diffusion at high sorbent concentrations in chromatography with a vapour mobile phase (VPC). The present work examines the sorption effect; the temperature effects associated with sorption–desorption of a component in VPC will be described later.

THEORETICAL

Asymmetry of a peak at high sample concentrations is usually explained by the non-linearity of the sorption isotherm. Recently[2–5] it has been shown that a high concentration of the eluted substance can itself result in peak asymmetry as a consequence of the flow-rate variation along the column: in the section of the sorbent occupied by the eluted substance the flow is made up of the carrier gas and the substance itself, and the flow-rate here is higher and varies with concentration. This effect, called the "sorption effect", results in asymmetric peaks with tailing. A more complicated situation occurs in VPC. In this case, high sample concentrations disturb the previously established sorption equilibrium with respect to the vapour eluent, which is then desorbed from the packing.

Let us first consider the physical picture of the band movement in conventional elution chromatography and in chromatography with a vapour mobile phase. We shall proceed from the assumption that sorption equilibrium is established instan-

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taneously and the boundaries of the band are sharp. The theory is valid at constant mass flow-rate of the eluent.

Let the band of the eluted substance, under the conditions of elution chromatography, occupy the sorbent section whose boundaries are delineated in Fig. 1 by solid lines. Sorption equilibrium is established between the gaseous and the stationary phases inside the band. The part of the band located in the gaseous phase will be displaced over a small distance, dx, by the flow of the carrier gas and will then occupy the position shown in Fig. 1 by dashed lines. The sorption equilibrium at the edges of the band will have been disturbed too: the portion of the sorbent at the left margin of the band will come into contact with the pure carrier gas, and here the sorbate will be displaced by the carrier gas flow into the band; the portion of the sorbent at the right margin of the band, previously free of the sorbate, will now be in contact with the carrier gas containing the substance which will be adsorbed and removed from the carrier gas flow.

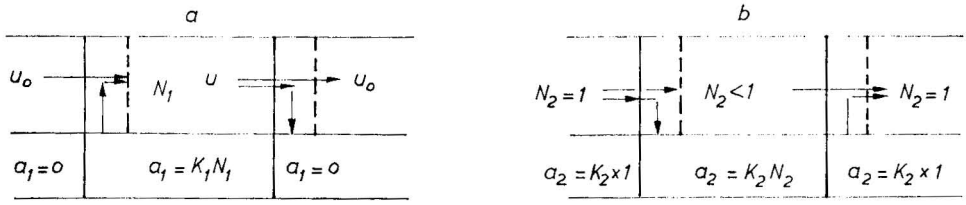

Fig. 1. Mechanism of band displacement with nitrogen (a) and water vapour (b) as the carrier gas.

As a result the flow of the substance suddenly increases very sharply at the left boundary of the band and then decreases again at the right boundary to its initial value. This effect will be more pronounced for readily adsorbed components. At high sample concentrations the increase in the flow-rate inside the band will be greater. Therefore, a chromatographic peak can be visualized as resulting from a large number of bands with sharp boundaries, with the highest flow-rate corresponding to the maximum of the peak; tailing of the peak is thus observed.

In chromatography with vapour eluents the situation is more complicated since, in this case, not only the substance but also the eluent itself undergoes sorption–desorption. Outside the chromatographic band the sorbent is in equilibrium with the pure water vapour, the mole fraction of which in the gaseous phase is $N_2 = 1$. Since the gaseous phase inside the band contains a certain amount of the sorbate, $N_2 < 1$, and the quantity of water in the sorbent is also smaller than outside the band. When the part of the band located in the gaseous phase is displaced over a small distance, dx, and occupies the position designated in Fig. 1b by the dashed line, a section of the sorbent with a decreased water content will appear at the left boundary of the band. An additional quantity of the eluent will then be sorbed here, and, therefore, a smaller amount of it will enter the band: the eluent flow inside the band will become lower than to the left of it. At the right boundary of the band the portion of the sorbent previously in equilibrium with pure water will come into contact with the gaseous phase containing a smaller amount of it. The eluent will be desorbed from this portion of the sorbent, and its flow to the right of the band will become greater than inside it

and equal to the flow to the left of the band. The flow-rate of the vapour eluent inside the band is thus smaller than outside it. The higher is the sorbate concentration the stronger is this effect. Therefore, inside the band with the usual Gaussian concentration profile the eluent flow is minimal in the region of themaximum sorbate concentration. Such a variation of the eluent flow inside the band must result in a chromatographic peak with a prominent leading edge.

For substances which are not adsorbed the above effect of the eluent flow-rate variation will be the only one, whereas in the case of adsorbed substances the overall effect will be a combination of those of the eluent flow-rate variation and the sorbate flow-rate variation. Depending on the extents of adsorption of the substance and the eluent, the flow-rate inside the band can become either higher or lower than outside it. If the substance is more strongly adsorbed than the eluent, the amount of desorbed substance at the left boundary of the band will exceed that of the adsorbed eluent, and the overall flow inside the band will be greater than outside it, causing tailing of the peak. The opposite occurs if the substance is less strongly adsorbed than the eluent.

Let us now examine quantitatively the dependence of the rate of displacement of the sorbate on its concentration. This problem, at high sorbate concentrations and taking account of eluent sorption, has been considered in terms of the "theory of chromatography without a carrier gas"[6,7].

Proceeding from the set of equations

$$\frac{\partial a_i}{\partial x} + \frac{\partial (uN_i)}{\partial x} = \frac{\partial a_i}{\partial t}$$

where N_i is the mole fraction in the gaseous phase, a_i is the packing concentration in mol/cm^3, u is the flow-rate, x is the longitudinal coordinate, t is the time and indices $i = 1, 2$ refer to the substance and the eluent, general relationships were obtained[6,7] for the flow-rate, u, and the rate of the band, v, which can be written as*

$$u = u_0 \, e^F \tag{1}$$

where

$$F = \int_0^{N_1} \frac{\partial N_1}{r - N_1}; \qquad r = \frac{\partial a_1/\partial N_1}{\partial a_1/\partial N_1 - \partial a_2/\partial N_2} \tag{2}$$

$$v = \frac{\partial (uN_1)/\partial N_1}{\partial a_1/\partial N_1} \tag{3}$$

Eqns. 1–3 are easily solved if the sorption isotherm is linear, when $\partial a_i/\partial N_i = \varepsilon + \mu K_i$, where ε, μ are the fractions of packing volume occupied by the gas and the stationary phase and K_i is the distribution coefficient. In this case r is constant and:

$$F = \ln r/(r - N_1) \tag{4}$$

$$u = u_0 r/(r - N_1) \tag{5}$$

* A brief derivation of eqns. 1–3 was given in ref. 5.

$$v = \frac{u_0}{\partial a_1/\partial N_1}\left(\frac{r}{r - N_1}\right)^2 \tag{6}$$

The quantity before the parentheses in eqn. 6 is equal to the rate of substance displacement at $N_1 \to 0$, and that in the parentheses is the correction for the sorption effect. It is seen that the rate of substance displacement depends on N_1, which results in the peak asymmetry. Let us examine two limiting cases.

(1) The substance is not sorbed but the (vapour) eluent is sorbed. Then $\partial a_1/\partial N_1 = \varepsilon$, $\partial a_2/\partial N_2 = \varepsilon + \mu K_2$, $r = -\varepsilon/\mu K_2$ and:

$$v = u_0\varepsilon/(\varepsilon + \mu K_2 N_1)^2 \tag{7}$$

From this expression it is seen that, with increasing N_1, v decreases, *i.e.*, when unsorbed substance is introduced into the vapour eluent flow, a peak with leading must be formed. At $N_1 \to 0$, $v = u_0/\varepsilon$

(2) The substance is sorbed, but the eluent is not sorbed (conventional elution chromatography). Then $\partial a_1/\partial N_1 = \varepsilon + \mu K_1$, $\partial a_2/\partial N_2 = \varepsilon$, $r = \varepsilon/\mu K_1$, + 1 and

$$u = u_0\Gamma_1/(\Gamma_1 - \mu K_1 N_1) \tag{8}$$

$$v = u_0\Gamma_1/(\Gamma_1 - N_1)^2 \tag{9}$$

where:

$$\Gamma_1 = \varepsilon + \mu K_1$$

In this case, with increasing N_1, v increases, *i.e.*, a peak with a pronounced tailing edge is formed. At $N_1 \to 0$ we get the usual expression:

$$v = u_0/\Gamma_1$$

If both the substance and the eluent are sorbed:

$$v = u_0\Gamma/[\Gamma_1 + (\Gamma_2 - \Gamma_1)N_1]^2 \tag{10}$$

In this case, the nature of the peak asymmetry depends on the relative strengths of the eluent and substance adsorptions. If the substance is sorbed more strongly than the eluent ($\Gamma_1 > \Gamma_2$), the sorption effect facilitates the formation of a peak with tailing. At $\Gamma_1 < \Gamma_2$ the opposite peak asymmetry occurs.

Isotherm non-linearity, superimposed on the sorption effect, produces a radical effect on peak asymmetry. In the case of conventional elution chromatography, $r = \varepsilon/\mu K_1 + 1$. In most cases of strongly adsorbed substances, $\varepsilon \ll \mu K_1$, which is also true when the isotherm is not linear. In packed layers, $\varepsilon \approx 0.5$, $\mu \approx 0.1$ and for instance for $K_1 > 50$, $\varepsilon/(\varepsilon + \mu K_1) < 0.1$. Then $r \approx 1$ and:

$$v = \frac{u_0}{\partial a_1/\partial N_1}\left(\frac{1}{1 - N_1}\right)^2 \tag{11}$$

This means that in conventional elution chromatography the nature of the peak distortion, associated with the sorption effect, depends only little, if at all, on the shape of the sorption isotherm. If the latter is approximated by the polynomial

$$c_i = K_1 N_1 + b_1 N_1^2 \tag{12}$$

then:

$$v = \frac{u_0}{\varepsilon + \mu (K_1 + 2b_1 N_1)} \left(\frac{1}{1 - N_1}\right)^2 \tag{13}$$

When the isotherm is convex to the ordinate axis, $b_1 < 0$, and the isotherm effect enhances the sorption effect; when $b_1 > 0$ the two effects are partially compensated. By multiplying the terms in the denominator and combining those with the same power of N_1, it is, however, easy to show that complete compensation, *i.e.*, the equality of all the coefficients in N_1, is impossible. If K_1 is small, in eqn. 13 $1 + \varepsilon/\mu(K_1 + 2b_2 N_1)$ must be used instead of 1.

When the unsorbed substance is introduced into a flow of vapour eluent the sorption isotherm of which is approximated by eqn. 12, we obtain:

$$r = -\varepsilon/\mu(K_2 + 2b_2 - 2b_2 N_1) \tag{14}$$

After substituting this expression into eqn. 2 we get:

$$F = \int_{N_1}^{0} \frac{\mu (K_2 + 2b_2 - 2b_2 N_1)}{\varepsilon + \mu (K_2 + 2b_2 - 2b_2 N_1) N_1} \, \mathrm{d}N_1^2 \tag{15}$$

which, after rearrangement and integration, yields

$$F = \ln \frac{\varepsilon}{\varepsilon + \mu (K_2 + 2b_2 - 2b_2 N_1) N_1} + \int_{N_1}^{0} \frac{2\mu b_2 N_1}{\varepsilon + \mu (K_2 + 2b_2 - 2b_2 N_1) N_1} \, \mathrm{d}N_1 \tag{16}$$

If we restrict ourselves to the case of only slightly curved isotherms, when $b_2 \ll K_2$, the second integral will become merely a correction to the whole expression for F, and it can be simplified*. Specifically, for strongly adsorbed components, ε can be disregarded. After integrating we get:

$$F = \ln \frac{\varepsilon}{\varepsilon + \mu (K_2 + 2b_2 - 2b_2 N_1) N_1} + \ln \frac{K_2 + 2b_2 - 2b_2 N_1}{K_2 + 2b_2} \tag{17}$$

Substitution of this expression into eqn. 1 then yields:

$$u = u_0 \frac{\varepsilon (K_2 + 2b_2 - 2b_2 N_1)}{[\varepsilon + \mu N_1 (K_2 + 2b_2 - 2b_2 N_1)] (K_2 + 2b_2)} \tag{18}$$

* In gas–liquid chromatography the partition isotherm is usually almost linear and the assumption $b_2 < k_2$ is valid.

Taking $N_1 \ll 1$ and disregarding the $2b_2N_1$ term, we get:

$$u = u_0\varepsilon/[\varepsilon + \mu N_1(K_2 + 2b_2)] \tag{19}$$

The corresponding expression for v will have the form

$$v = u_0\varepsilon/[\varepsilon + \mu N_1(K_2 + 2b_2)]^2 \tag{20}$$

which becomes eqn. 7 as $b_2 \to 0$. When $b_2 < 0$ (convex isotherm) the sorption effect is weakened, and when $b_2 > 0$ (concave isotherm) it is correspondingly increased compared with the sorption effect at a linear isotherm.

Finally, let us examine the case where the adsorptions of the eluent and the substance are of comparable strengths. In this case

$$r = \frac{\varepsilon + \mu\,(K_1 + 2b_1N_1)}{\mu\,(K_1 - K_2) + 2\mu\,[N_1(b_1 + b_2) - b_2]} \tag{21}$$

and, correspondingly:

$$F = \int_0^N \frac{\mu\,(K_1 - K_2) + 2\mu\,[N_1(b_1 + b_2) - b_2]}{\varepsilon + \mu K_1 + \mu N_1\,[2(b_1 + b_2)\,(1 - N_1) - (K_1 - K_2)]}\,\mathrm{d}N_1 \tag{22}$$

Restricting ourselves to the case of relatively small N_1 values and disregarding the $N_1(b_1 + b_2)$ terms, after integration we get:

$$F = \frac{(K_1 - K_2) - 2b_2}{(K_1 - K_2) - 2(b_1 + b_2)} \ln \frac{\Gamma_1}{\Gamma_1 + N_1(\Gamma_2 - \Gamma_1) + 2\mu(b_1 + b_2)} \tag{23}$$

For a small isotherm curvature, when b_2 is a minor correction to the K_1 value, the factor before the logarithm can be taken as approximately equal to 1. We then obtain

$$u = \frac{u_0\Gamma_1}{\Gamma_1 + N_1[(\Gamma_2 - \Gamma_1) + 2\mu(b_1 + b_2)]} \tag{24}$$

and:

$$v = \frac{u_0\Gamma_1^2}{(\Gamma_1 + 2\mu b_1N_1)\,\{\Gamma_1 + N_1[(\Gamma_2 - \Gamma_1) + 2\mu(b_1 + b_2)]\}^2} \tag{25}$$

The expression in parentheses in the denominator represents the direct effect of the sorption isotherm on the rate of band displacement, whereas the expression in braces characterizes the influence of the sorption effect. At b_1, $b_2 < 0$ the leading edge of the peak decreases due to the sorption effect (at $\Gamma_1 < \Gamma_2$), and at b_1, $b_2 > 0$ it increases.

RESULTS AND DISCUSSION

The influence of the sorption effect on the form of chromatographic bands may be confirmed by the observation of asymmetric peaks for unsorbed substances in VPC Experiments were conducted on previously described apparatus[8], using 20% polyethylene glycol adipate (PEGA) on polychrome as sorbent.

Fig. 2 shows the output curves for methane in nitrogen and in water vapour at different sample volumes. At small volumes (25–30 μl) the methane peak has the same form in water vapour and in nitrogen and is characterized by slight tailing. An increase in volume to $\geqslant$100 μl results in opposite form of peak asymmetry in the water vapour flow, whereas in nitrogen only an increase of the methane concentration in the band and a slight expansion of the band are observed.

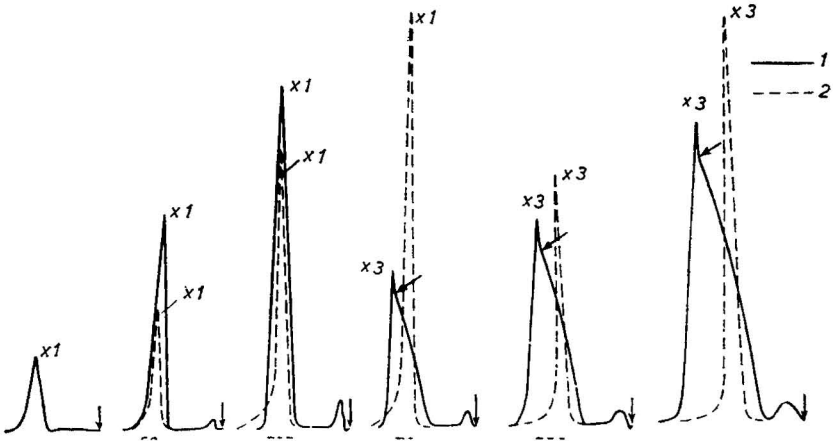

Fig. 2. The peaks of methane in a flow of water vapour (———) or nitrogen (- - -). Column: 1 m × 4 mm. Temperature: 150°C. Sorbent: 20% PEGA on polychrome.

The same behaviour is observed when the peak is recorded with a katharometer or a flame-ionization detector. Similar results have been obtained on other sorbents with high capacities for water, *e.g.*, on 1,2,3-tris(β-cyanatoxypropane). Hydrophobic phases of the E-301 type and an increase in column temperature result in a reduction or complete disappearance of the above effect.

Similar peak asymmetry is also observed for sorbed substances on stationary phases and adsorbents having high capacities for water such as PEGA, polyethylene glycol and silochrome. The formation of asymmetric peaks with prominent leading edges is more pronounced for the lower members of the homologous series of paraffins, alcohols and aromatic hydrocarbons. When nitrogen is used as the carrier gas, peaks with tailing are generally formed.

The change in the nature of peak asymmetry from tailing to leading when nitrogen carrier gas is replaced by water vapour has been noted previously[9,10], and explained in terms of the change in the shape of the sorption isotherm. However, it is also necessary to take account of the sorption effect. The asymmetry of methane peaks can only be explained in terms of the latter effect. In a number of cases the changes in peak asymmetry can be accounted for by the joint influence of the sorption effect and the shape of the isotherm.

Fig. 3 shows the relationship between the asymmetry coefficient of nonane and undecane peaks and the unit loading obtained on a column of 16.7% PEGA on polychrome at 120°C. The asymmetry coefficient was calculated from

$$A = 2(a_1 - a_2)/(a_1 + a_2)$$

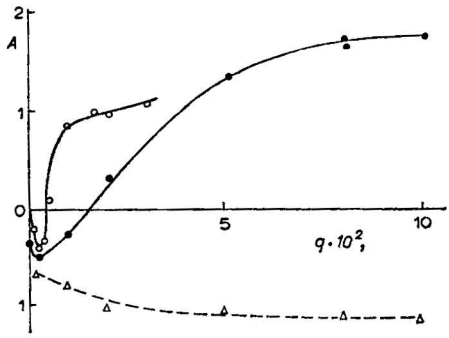

Fig. 3. Relationship between asymmetry coefficient and specific loading* in a flow of nitrogen (- - -) or water vapour (———). ○ = Nonane; △, ● = undecane. Sorbent: 16.7% PEGA on polychrome. Temperature: 120°C.

where a_1 and a_2 are the half-widths of the peaks at the leading and tailing edges. For peaks with a prominent leading edge, $A < 0$; for those with tailing $A > 0$ and for symmetric peaks $A = 0$. It can be seen that, with increasing specific loading*, A undergoes complex changes: initially symmetric peaks gradually acquire tailing, but then the nature of the asymmetry changes and peaks with pronounced leading edges are formed. It is impossible to explain such changes only in terms of the influence of the sorption isotherm, unless one assumes that the isotherms have a sigmoidal shape, which is not usually observed in the case of dissolution. All the experimental results are, however, simply explained by assuming that at least two factors affect the asymmetry: thus at moderate loadings the influence of the isotherm predominates, and at higher loadings that of the sorption effect predominates. It is a characteristic of the weakly sorbed nonane that the change to peaks with pronounced leading edges takes place at small loadings, in agreement with the above reasoning.

The present results thus confirm the significant role of the sorption effect in the formation of chromatographic bands at high sorbate concentrations.

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* The specific loading is the sample size (cm^3) divided by the cross-sectional area (cm^2) of the column.

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GAS CHROMATOGRAPHY WITH A VAPOUR MOBILE PHASE AT THE FINITE CONCENTRATION OF SORBATE

II. TEMPERATURE EFFECTS AND THE CHARACTERISTICS OF PREPARATIVE SEPARATIONS

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SUMMARY

Temperature variations caused by the passage of the chromatographic band through the sorbent have been measured with water vapour as the carrier gas. With unsorbed and weakly sorbed substances, the passage of the band results in cooling of the packing owing to water desorption; the heating of the packing during elution of readily sorbed substances is not as large as in the case of conventional elution chromatography.

INTRODUCTION

The process of gas chromatography is usually regarded as taking place under isothermal conditions. However, when the sample concentration in the column is sufficiently high, the isotherm is disturbed because of the evolution and absorption of sorption heat ("temperature effect"). A detailed analysis of this phenomenon and its influence on band formation in conventional elution chromatography has been made by Scott[1].

In the case of vapour mobile phase chromatography (VPC), the displacement of the band of a sorbate over a given section of the sorbent decreases the water content in the gaseous phase above this section and disturbs the sorption equilibrium with regard to water: a certain amount of water is desorbed from the stationary phase, with absorption of heat. The reverse occurs at the rear edge of the band: water is absorbed in the stationary phase, and the heat of absorption is evolved. The above processes must have a substantial influence on the temperature effect.

THEORETICAL

According to Scott[1], the amount of heat evolved upon sorption of a substance (or absorbed upon its desorption) on a theoretical plate is equal to

$$Q_1 = K_1 h_1 (\partial x/\partial v)\, \partial v$$

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where h_1 is the heat of sorption, K_1 is the distribution coefficient, x is the concentration of substance in the gaseous phase and v is the gas volume expressed in terms of the theoretical plate capacity

$$v = w\tau/(v_g + v_l K_1)$$

where w is the flow-rate of carrier gas, τ is the time and v_g, v_l are the volumes of the gaseous and the liquid phase on the theoretical plate.

If x is replaced by the mole fraction, N, the thermal effect associated with water desorption caused by the change in the water content in the gaseous phase will be expressed by

$$Q_2 = -K_2 h_2 (\partial N/\partial v)\, \partial v$$

and the overall effect of heat evolution (absorption) will be

$$Q = (K_1 h_1 - K_2 h_2)(\partial x/\partial v)\, \partial v$$

where indices 1 and 2 refer to the sorbate and the vapour eluent.

The equation of thermal balance for the theoretical plate can be written as

$$(v_l \varrho_l c_l + v_s \varrho_s c_s)\, \partial\theta = (K_1 h_1 - K_2 h_2)(\partial x/\partial v)\, \partial v - (AZ/w)(v_g + K_1 v_l)\, \theta \qquad (1)$$

where A is the plate surface area, Z is the thermal conductivity of the packing, v, ϱ, c are the volume, density and heat capacity of the packing, θ is the excess temperature and indices l and s stand for the stationary phase and the support. Denoting the coefficient of $d\theta$ by a we get Scott's equation

$$\frac{\partial\theta}{\partial v} + \beta\theta = \alpha \frac{\partial x}{\partial v} \qquad (2)$$

where $\beta = AZ(v_g + K_1 v_l)/aw$ characterizes the loss of heat to the environment and $a(K_1 h_1 - K_2 h_2)/a$ is the thermal effect resulting from the sorption and desorption of the substance and the water.

Under adiabatic conditions, $\beta = 0$, and the solution of eqn. 2 has the form

$$\theta = \alpha x_0 \, e^{-\mu^2/2n}/\sqrt{2\pi n}$$

where n is the number of theoretical plates and μ is the band dispersion. In this case the shape of the temperature curve and that of the chromatographic peak are the same.

Although the obtained equations are only applicable to the case of a linear isotherm, they can be used for a qualitative discussion of the experimental data. Three important conclusions follow from the analysis of these expressions.

(1) The introduction of an unsorbed substance into the vapour eluent flow must result in cooling of the packing in the region of this substance's band. In this case we have $K_1 h_1 = 0$ and $\alpha = -K_2 h_2/a < 0$.

(2) The sign and the magnitude of the temperature signal resulting from the introduction of a sorbed component into the vapour eluent flow will be determined by the relationship between K_1h_1 and K_2h_2.

(3) Even for well-sorbed components, when $K_1h_1 > K_2h_2$, the temperature signal in nitrogen must be greater than in water vapour.

RESULTS AND DISCUSSION

The experiments were conducted on a Tsvet-I chromatograph with an attachment to create the vapour eluent flow[2]. Six openings were made in the wall of the column (850 × 18 mm), at different distances from the column inlet. Copper–constantan thermocouples on porcelain insulation were inserted into the openings which were then sealed with epoxide cement. Twelve thermocouples were used, connected in pairs in a differential circuit: one thermocouple was placed inside the packing on the column axis, and the second was fixed with epoxide cement on the column wall adjacent to the point of entry of the first thermocouple. All the six pairs of thermocouples were connected through a switch to an EZ-9 recording potentiometer. The distances from the column inlet to the thermocouples were 20, 120, 220, 370, 570 and 770 mm. The column was filled with Chromatone N AW DMCS with 15% of Carbowax 6000 which is a good sorbent for water.

In accordance with the theoretical analysis, when an unsorbed substance is introduced into the water vapour flow a negative temperature signal is observed which indicates that water is desorbed from the stationary phase. It is to be noted that the higher is the dose of unsorbed substance the greater is this effect. The dependence of θ for the first thermocouple on the dose of the unsorbed substance is linear, passing through the origin with a slope of 0.3°C/ml. When the water vapour flow passes through a column operated in the frontal chromatography mode, it was found that $\theta = +6.0$°C. From a comparison of these values, it can be concluded that the presence of 10 ml of the unsorbed substance in the zone of thermocouple 1 results in the desorption of half the water dissolved in the stationary phase.

Results of the measurement of thermal effects for sorbed substances in a flow of nitrogen and water vapour are given in Fig. 1. In nitrogen all the six thermocouples recorded a positive temperature signal on passage of the sorbate band, and in water vapour a negative signal was recorded for ethanol and propanol. In the case of butanol the signal is approximately zero; for this compound, probably, $K_1h_1 \approx K_2h_2$. When hexanol is eluted the signal is positive but is much (2–3 times) smaller than in the nitrogen flow.

The above trends are clearly seen in Fig. 2 where the magnitude of the temperature signal for the first thermocouple is presented as a function of the number of carbon atoms, n_c, in the alcohol molecule. When nitrogen is used as the carrier gas the dependence of the magnitude of the temperature signal on n_c lies in the region of positive temperatures; on water vapour it passes into the negative region.

It is also of interest to examine the shape of the temperature curves. In the nitrogen flow (Fig. 3) the temperature curve usually coincides in shape with that of the concentration curve in elution chromatography. This is explained by the low magnitude of the temperature signal, which determines the size of the second term on the right-hand side of eqn. 1. At sufficiently high values of θ the adiabatic conditions

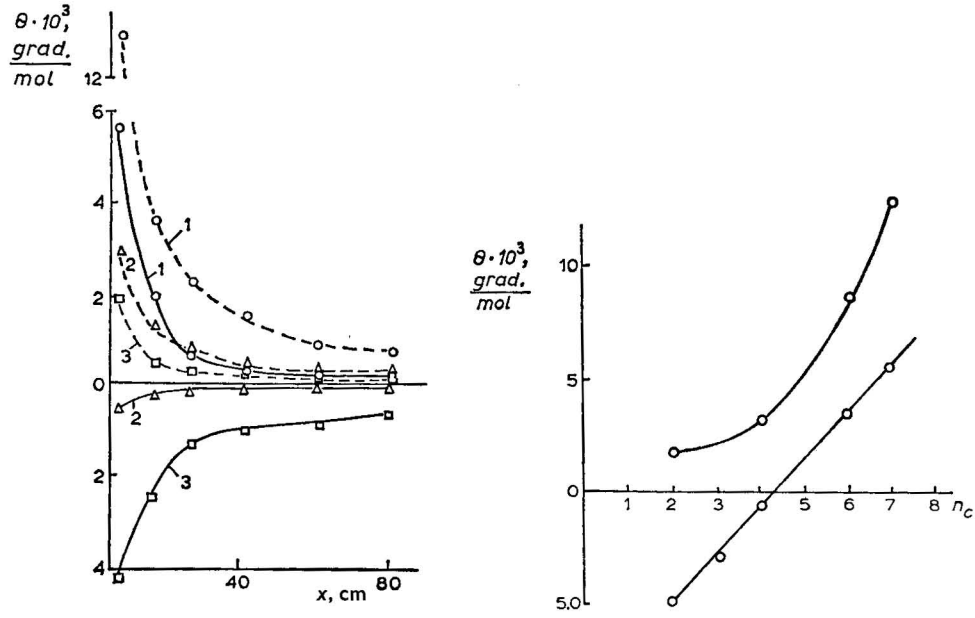

Fig. 1. Magnitude of the temperature signal at different sections of the sorbent layer in a flow of nitrogen (- - -) or water vapour (—) for hexanol (1), butanol (2) and ethanol (3).

Fig. 2. Dependence of the magnitude of the temperature signal on the number of carbon atoms in *n*-alcohols in a flow of nitrogen (upper curve) or water vapour (lower curve).

of the process are not met. In these cases, at the rear edge of the temperature curve, we see a negative "tumble", associated with the partial removal of the heat evolved during the passage of the band.

When an unsorbed (or weakly sorbed) substance was introduced into the flow of water vapour the temperature response was negative, and on its sorption branch (*i.e.*, the water vapour sorption) a positive "tumble" is observed. In this case it would be natural to associate the "tumble", as above, with a non-adiabatic process, but here resulting from the heat absorbed on displacement of the band of the substance. When a strongly sorbed component, *e.g.*, hexanol, is introduced into the water vapour flow, a positive temperature response is obtained. Its shape may be exactly the same as that observed in nitrogen, but can also be different. At temperatures below 125°C a negative "tumble" is seen at the leading edge. This is probably associated with the fact that at the moment of entry of the band into the sorbent section saturated with water the extent of water desorption is greater than the sorption of the substance.

From these experiments it can be concluded that the temperature effect in chromatography with a vapour mobile phase is quite different from the temperature effect in conventional elution chromatography. Scott[1] showed that in elution chromatography this effect produces an additional tailing of the peak. In chromatography with a vapour mobile phase the temperature effect result in more pronounced leading edges for unsorbed and weakly sorbed substances, and in a decrease in peak

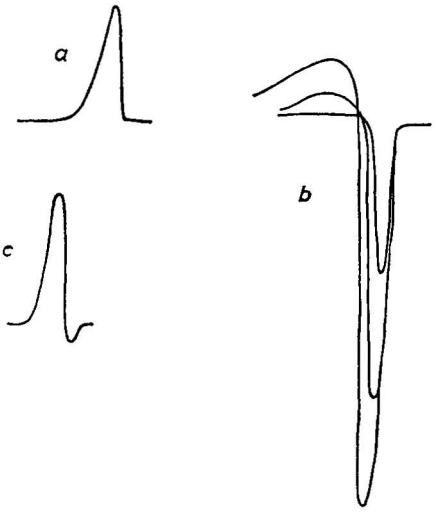

Fig. 3. Shape of the temperature signal for the elution of butanol by nitrogen (a) and water vapour (b), and of hexanol by water vapour (c).

tailing for strongly sorbed substances. It is thus seen that on the whole the temperature effect increases the influence of the sorption effect[3] on the shape of the peak.

Let us now examine the effect of replacement of nitrogen by water vapour on the main separation parameters in preparative chromatography, using benzene–toluene, ethanol–propanol and octane–decane as sorbates. The stationary phases, 20% PEGA and Apiezon N, were applied on refractory brick (grain size 0.4–0.6 mm) and on Chromosorb W. When calculating the resolution, R, only the two adjacent half-widths of the peaks (Fig. 4) were taken into account:

$$R = \Delta l/(\Delta S_1 + \Delta S_2)$$

In this way the resolution for asymmetric peaks correctly reflects their separation and is directly correlated with the purity of fractions.

From Fig. 5 it is seen that when PEGA, which is a good sorbent for water, is used as the stationary phase, R increases for ethanol–propanol on changing from nitrogen to water vapour, and decreases for the other pairs of substances. When Apiezon N is used the resolution does not depend significantly on the type of mobile phase.

We showed previously[4] that, for members of an homologous series, the replacement of nitrogen by water vapour has little effect on the separation coefficient, α. The present variation of R can therefore be explained mostly by the variation of the distribution coefficient. It is known[5] that, with an increase in the latter, R usually increases under conditions of overloading. We have also shown[4] that, on changing from nitrogen to water vapour, the distribution coefficient increases for ethanol and propanol and decreases for benzene, toluene and paraffins, in accordance with the present results.

A most important characteristic of separation in industrial preparative chromatography is the throughput, $P = q/\tau$, where q is the dose introduced. There are

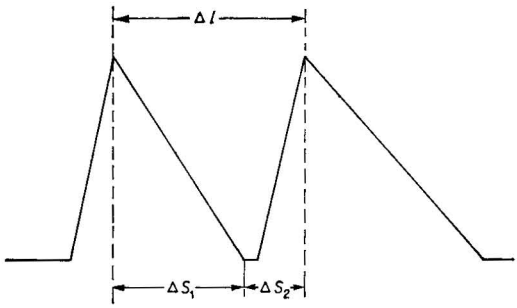

Fig. 4. Calculation of R for asymmetric peaks.

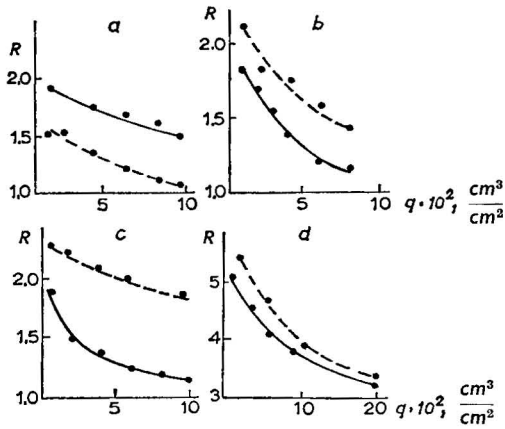

Fig. 5. Dependence of R on specific loading in aflow of nitrogen (- - -) or water vapour (—) at 120°C: a, ethanol–propanol on PEGA; b, benzene–toluene on PEGA; c, octane–decane on PEGA; d, octane–decane on Apiezon L.

two possible modes of operation of a preparative installation. In the first mode the next dose is introduced after the complete elution of all the components of the previous batch, then $\tau = \tau_p$ is the time between the input and the output of all the components of the batch. In the second mode the next dose is introduced before all the components of the previous mixture have been completely eluted, in this case $\tau = \tau_a$ is the time from the start of the elution of the first component of the mixture to the end of the elution of its last component. The method of calculation of P is described in ref. 6.

Table I shows the experimental values of P determined for both modes of operation (P_p and P_a). The throughput in preparative chromatography for all the studied mixtures is higher in water vapour than in nitrogen, despite the fact that the size of the dose at which $R = 1$ is lower for the benzene–toluene and octane–decane mixtures in water vapour. The throughput is increased by shortening the separation time, τ_p and τ_a.

It should be noted that the formation in the water vapour flow of peaks with a steep rear edge produces a favourable effect when the main component is free from heavy impurities. In a number of cases, the use of water vapour can thus improve the characteristics of preparative separation.

TABLE I

THROUGHPUT IN NITROGEN AND WATER VAPOUR FLOWS AT 120°C

Mixture	*Eluent*	τ_p *(sec)*	τ_a *(sec)*	$P_p \cdot 10^3$ *($cm^3/cm^2 \cdot sec$)*	$P_a \cdot 10^3$ *($cm^3/cm^2 \cdot sec$)*
Ethanol–propanol	Nitrogen	115	81	0.87	1.66
	Steam	90	49	1.45	2.63
Benzene–toluene	Nitrogen	150	70	0.80	1.72
	Steam	82.5	57	1.21	1.75
Octane–decane	Nitrogen	135	105	1.41	1.81
	Steam	60.5	45.5	1.65	2.20

CONCLUSIONS

This study has shown that in chromatography with vapour eluents the shape of the peak at high sorbate concentrations is strongly affected by the sorption and temperature effects which facilitate the formation of peaks with predominant leading edges. In a number of cases, the characteristics of preparative separation in a flow of water vapour are better than in flows of the conventional carrier gases.

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POTENTIOMETRIC DETECTOR WITH A GLASS ELECTRODE FOR THE ION-EXCHANGE CHROMATOGRAPHY OF CARBOXYLIC ACIDS

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SUMMARY

A method for the detection of carboxylic acids in the effluent from ion-exchange columns, based on the deviation of the pH of a buffer solution on the addition of Brønsted bases, is described. A high-sensitivity flow-through glass electrode pH meter was designed for this purpose. From 1 to 10 μequiv. of carboxylic acids or their salts in the effluent can be detected.

INTRODUCTION

Ion-exchange chromatography is a powerful method for the separation or analysis of carboxylic acids, but difficulties with detection have prevented its wider application.

A colorimetric method with an indicator has been described previously[1], but the reproducibility was unsatisfactory becasue of non-linearity of the colour development, instability of the indicator and contamination of the photometer cell by oxidized pigment.

A simple method for the detection of carboxylic acids or their salts in the effluent from the ion-exchange columns by direct measurement of pH with a glass electrode is presented in this paper. The principle is based on the deviation of the pH of a buffer solution on the addition of strong Brønsted bases. The effluent containing carboxylate ions is mixed with a standard buffer solution, and the pH is measured with a high-sensitivity pH meter. The deviation of the pH of the mixture from the original value of the standard buffer is proportional to the content of carboxylate in the effluent.

PRINCIPLES AND THEORETICAL

The pH of buffer solution containing a weak acid HA is represented by

$$\mathrm{pH} = \mathrm{p}K_a + \log \frac{q_{A^-}}{q_{HA}} \quad (1)$$

where $\mathrm{p}K_a$ is the exponent of the dissociation constant of the acid and q_{HA} and

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q_{A-} are the amounts of HA and A^-, respectively, in the solution. When a base B^- is added to the solution, which has a sufficiently high pK_a value compared with HA, the following reaction would occur:

$$HA + B^- \rightarrow A^- + HB$$

The pH of the mixture then becomes

$$pH = pK_a + \log \frac{q_{A-} + q_{B-}}{q_{HA} - q_{B-}} \tag{2}$$

If we plot $pH - pK_a$ ($= \Delta pH$) against q_{B-}/q_{A-}, the curve shown in Fig. 1 is obtained.

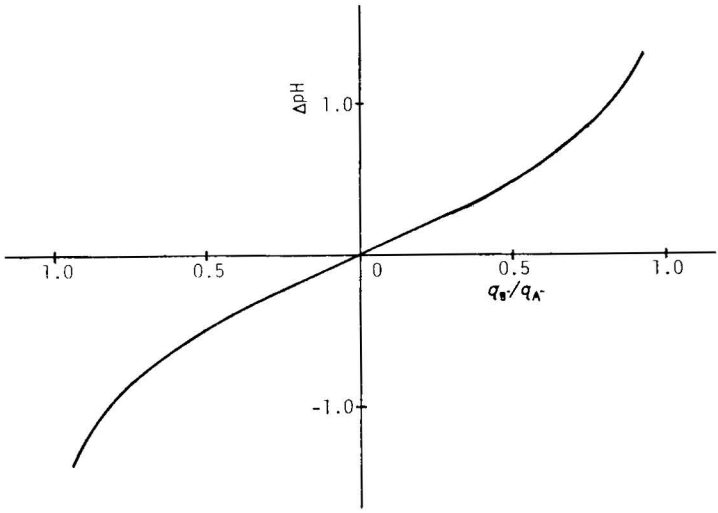

Fig. 1. Deviation of the pH of buffer solution on addition of strong base.

If we assume that $q_{A-} = q_{HA} = 1/2\ q_0$, and differentiate eqn. 2 with respect to q_{B-} in the neibourhood of $q_{B-} = 0$, then we obtain

$$dpH = 1.737 \cdot \frac{dq_{B-}}{q_0} \tag{3}$$

If we use concentration instead of amount, and replaced pH by Δ pH and dq_{B-} by b, the following equation is obtained:

$$\Delta pH = 1.737 \cdot \frac{b}{c} \tag{4}$$

where $c = q_0/V$, $b = q_{B-}/V$ and V is the final volume of the mixture. Eqn. 4 holds in the range $pH = pK_a \pm 0.2$ with an error of $\pm 0.1\%$.

A schematic diagram of the chromatographic system is shown in Fig. 2.

Eluent (1) is supplied by a pump (2) through a sample injector (3) to the main column (4), which is maintained at a constant temperature with a circulating thermostat bath (5). Samples are injected into the stream of eluent with the sample injector. Effluent from the main column containing carboxylate ions flows through an after-treatment column, "pH control column" (6), which is packed with a weak acid-type cation-exchange resin. The resin works as a solid-phase buffering agent, and the effluent is neutralized and maintained at pH 8.5–9.5, in which range most carboxylic acids exist as carboxylate ions. Then the effluent is led into the electrode system (9) and mixed with equal volume of standard buffer solution (7). As the carboxylate ions act as a strong base, the pH of the mixture deviates from the original value of the standard buffer. The pH of the mixture is measured with a high-sensitivity pH meter (11) and recorded on a recorder (12). A pH meter (10) is used to monitor the pH of the effluent from the pH control column.

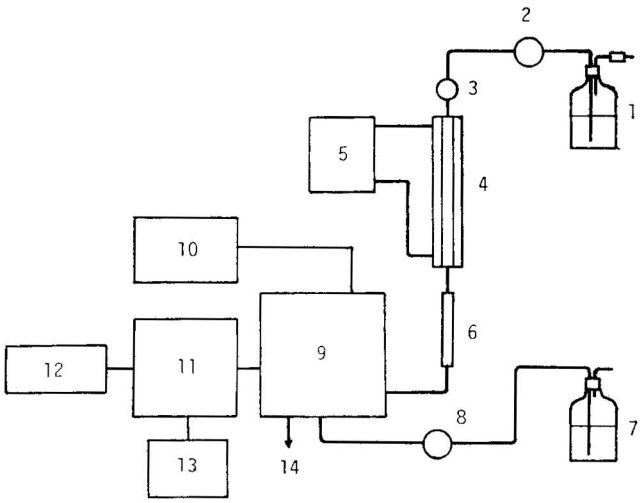

Fig. 2. Schematic diagram of the chromatographic system. 1 = Eluent; 2 = pump; 3 = sample injector; 4 = main column; 5 = circulating thermostat bath; 6 = pH control column; 7 = standard buffer solution; 8 = pump; 9 = electrode system; 10 = monitoring pH meter; 11 = high-sensitivity pH meter; 12 = recorder; 13 = digital voltmeter; 14 = waste.

EXPERIMENTAL

Columns

A strongly basic anion-exchange resin (Hitachi custom resin 2630, Cl^-, 16–20 μm) was packed into a glass column (180 × 5 mm I.D.) and used as the main chromatographic column. The column was jacketed and circulated with water from the thermostat bath to maintain a temperature of 30°C.

The pH control column (100 × 5 mm I.D., glass) was packed to half its height with a weakly acidic cation-exchange resin (Amberlite XE-64, K^+, 44 μm) and the H^+ form of the same resin was packed on top of it.

Solutions

Potassium chloride solution (0.2 and 0.3 *M*) containing 0.01 *M* potassium

hydroxide was used as the eluent. A mixed solution of 0.01 *M* phthalic acid and 0.01 *M* potassium hydrogen phthalate was used as the standard buffer solution in potentiometric detection. These two solutions were degassed under reduced pressure and stored in an incubator at 50°C.

Buffer solutions for calibration of the pH meters were supplied by Horiba (Kyoto, Japan).

Detector

Two flow-through glass electrodes (Hitachi–Horiba Model 6901–25T) were used in the detection system as shown in Fig. 3 (5, 12). As a reference electrode, silver chloride electrodes (Hitachi–Horiba Model 2401-15T) (7, 14) were connected to the respective glass electrodes. The effluent from the column system was passed into the detection system from the right-hand side of Fig. 3. The solution first contacted with the effluent monitor electrode (5) which connected with the pH meter (Hitachi–Horiba Model F-7DE). Then the solution was mixed with the standard buffer solution, and driven into the second glass electrode (12) through a dropping insulator (11), which also worked as a mixer of the solutions. The glass electrode was connected to the high-sensitivity pH meter. The waste solution from the detector electrode was drained off through another dropping insulator (16). The whole assembly of the electrode system was placed in a thermostat chamber maintained at 25°C.

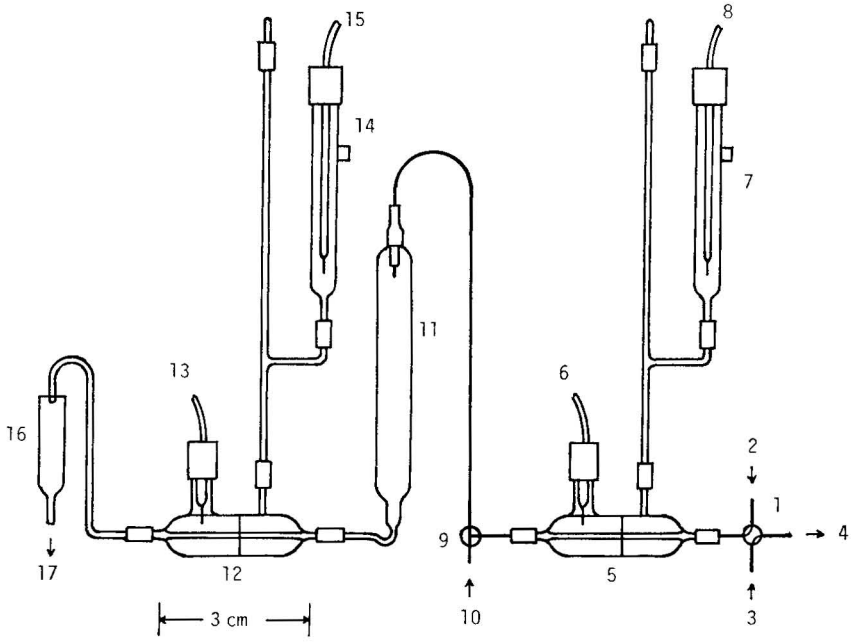

Fig. 3. Electrode system. 1 = Four-port valve; 2 = effluent from the column system; 3 =calibration buffers; 4 = waste; 5 = monitor electrode; 6 = output wiring of the monitor electrode; 7 = reference electrode for the monitor electrode; 8 = output wiring of the reference electrode; 9 = mixing joint; 10 = standard buffer solution; 11 = dropping insulator; 12 = detector electrode; 13 = output wiring of the detector electrode; 14 = reference electrode for the detector electrode; 15 = output wiring of the reference electrode; 16 = dropping insulator to waste; 17 = waste.

The high-sensitivity pH meter was specially designed for the potentiometric determination of pH deviation. The electrical circuit is shown in Fig. 4. The output of the glass electrode is converted into alternating current with a vibrating-reed condenser (VLC), and amplified with an amplifier with a junction-type field-effect transistor on its first stage. The amplified signal is rectified with a syncronous rectifier and output to a pen recorder (Hitachi Model QPD 33) and a digital voltmeter (Yokogawa Type 2805) through an integrating amplifier. Part of output signal was fed back to another terminal of the VLC. The integrating amplifier smoothed the signal and stabilized the feedback loop.

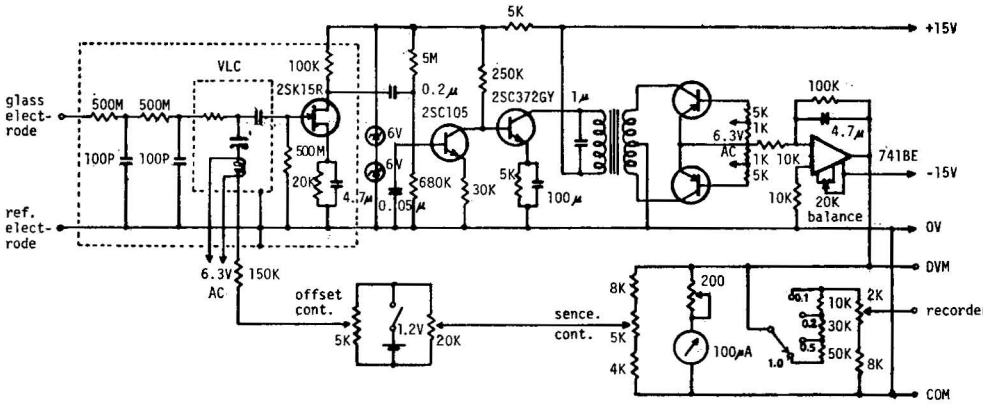

Fig. 4. Circuit diagram of the high-sensitivity pH meter. K = kΩ; M = MΩ; μ = μF; P = pF.

Pumps and valves

A plunger pump (Kyowa Seimitsu, Model KHU-W52) was used for pumping the eluent. A reciprocal pump (produced by Hitachi for an amino acid analyser) and a peristaltic pump (Tokyo Rikakikai, Type MP-2) were used to supply the standard buffer and the calibrating buffers, respectively.

A double-four-port valve (Kyowa Seimitsu, Model KMM-4V-2) was used as a loop injector to add samples to the chromatographic column. A four-port valve (Kyowa Seimitsu, Model KMM-4V) was inserted prior to the inlet of the electrode system to supply the calibration buffers to the glass electrodes.

Methods

The monitoring pH meter was calibrated with buffer solution of pH 7 and 4 by the usual method. The high-sensitivity pH meter was adjusted with buffer of pH 4 and the standard buffer for detection itself so that the reading on the digital voltmeter was 100 mV/pH.

Samples were injected with a loop injector into the stream of eluent. The eluent and the standard buffer solution were supplied at the rate of 60 ml/h.

The resins used for the pH control column were regenerated after several experiments by washing with three volumes of 2 *M* hydrochloric acid, and converted into the appropriate forms if necessary.

RESULTS AND DISCUSSION

Example of chromatogram

A typical example of the separation of aliphatic acids by the method described is shown in Fig. 5. Sample solution (500 μl) containing 5 μmoles of each acids was injected; 0.2 *M* solution was used as the starting eluent, and changed to 0.3 *M* after 24 min.

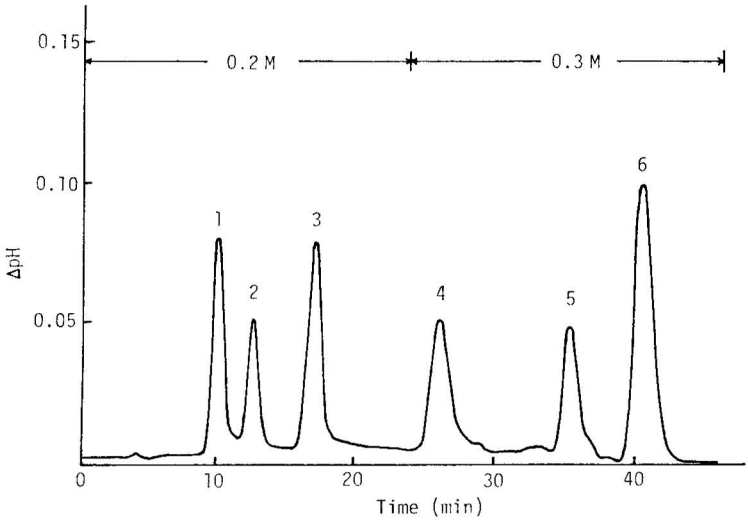

Fig. 5. Chromatography of aliphatic acids. Main column: 180 × 5 mm I.D., Hitachi custom resin 2630 (Cl^-). pH control column: 100 × 5 mm I.D., Amberlite XE-64 (H^+ 50 mm + K^+ 50 mm). Eluent: 0.2 *M* KCl (0–24 min) and 0.3 *M* KCl (24–45 min), both containing 0.01 *M* KOH. Temperature, 30°C, flow-rate, 60 ml/h; pressure, 3.5 MPa. Peaks: 1 = acetic acid; 2 = formic acid; 3 = CO_3^{2-}; 4 = succinic acid; 5 = oxalic acid; 6 = citric acid.

Effect of variation of flow-rates of solutions

It can be predicted from eqn. 4 that variation of the flow-rates of the effluent and the standard buffer solution affects the response of the detector and leads to noise or drift on the chromatogram. Constancy of the flow-rate should, therefore, be more strictly maintained in this method than in other methods. However, the short-period pulses from the plunger pump did not have any observable effect on the detection. Rather long-period changes in flow-rate led to a small amount of noise on the chromatogram, but this could be overcome by using an air damper.

Effect of temperature

The electromotive force of the glass electrode varies with temperature according to the Nernst equation. When the detector electrode was placed in a thermostat chamber, however, variations in room temperature had no effect on the output.

Noise from electrical circuits

Because the thermal noise from high-impedance circuits around the glass electrode is estimated to be of the order of microvolts, it can be neglected. Noise from

the transistor circuits can be depressed to levels below the sensitivity of the detector system by use of a low-noise junction-type field-effect transistor on the first stage of the amplifier.

Life of the pH control column

About 250 ml of the effluent could be treated with one batch of the above column. As significant drift was observed in the initial stage of the life of the column, conditioning for 30 min or more is desirable. The exhausted resins were regenerated and recycled as described previously.

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THERMALLY STABILIZED VERY THIN (0.02–0.3 mm) POLYACRYLAMIDE GELS FOR ELECTROPHORESIS

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SUMMARY

A simple technique and device for the preparation of gels are described that complement the conventional "pouring" technique, which fails in the preparation of bubble-free gels with thicknesses less than about 0.4 mm. Included are methods for processing and manipulation, design of a thermostating platen and gel casting device, and chemical treatment of glass and polyester substrates to which the polyacrylamide gel adheres during processing. The gel is cast directly between the glass cover plate and the thermostating platen, and this assembly provides isothermal conditions in the gel layer during the electrophoresis. Further, techniques for introducing spacers, stacking gel, sample slot forming and sample application have been developed and applied to DNA sequencing and protein sodium dodecyl sulphate electrophoresis. The results obtained indicate that the technique is simple to use, has many analytical applications (DNA sequencing, sodium dodecyl sulphate–polyacrylamide gel electrophoresis, isoelectric focusing) and has various advantages.

INTRODUCTION

The theoretical justification for the use of very thin gels for zone electrophoresis can be deduced from simple models and equations. The main advantages of thinner gels are as follows: (1) small temperature gradient across the gel thickness; the efficient heat transfer, in combination with gel thermostating, eliminates distortions caused by thermal effects; (2) lower Joule heating per unit electrical field and surface area, permitting the application of higher field strengths, resulting in improved resolution and substantially shorter running times; (3) significantly shorter staining (fixing) and de-staining times; and (4) less sample is needed (higher sensitivity).

The use of very thin (< 0.2 mm) polyacrylamide gel has been mentioned previously[1], but its wider application has been limited by problems in the preparation of larger bubble-free gels and in their handling.

In this paper, a simple technique and device for the preparation of bubble-free gels less than 0.3 mm thick are described that complement the conventional "pouring" technique, which fails in the preparation of thinner gels, because the elimination of gas bubbles trapped in the thin layer is very difficult. Thin gels up to

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1000 mm long, 300 mm wide and 1.5 mm thick had been prepared using this technique. A procedure for the treatment of polyester and glass surface was developed, which makes it possible to polymerize the gel on a transparent support to which it adheres during all subsequent processing steps, including scanning and storage. An inexpensive glass thermostating platen was designed that maintains the gel layer under isothermal conditions during the electrophoresis.

Techniques for introducing spacers, stacking gel, sample slot forming and sample application have been developed for thin gels and applied to DNA sequencing and protein sodium dodecyl sulphate (SDS) electrophoresis in a vertical system.

MATERIALS AND EQUIPMENT

Gel electrophoresis

Polyacrylamide solutions for protein separation were prepared as described elsewhere[2,3]. A modified Laemmli system was used in the discontinuous and phosphate buffer in the continuous method, in both instances varying the ionic strength. The gels and buffers for DNA sequencing will be described elsewhere[4].

Spacers were made of PTFE and sample combs of PTFE, polyester or stainless steel treated so as to be hydrophobic. Sample application was performed by means of thin-walled glass capillaries for X-ray analysis (A. Müller Glas- und Vakuumtechnik, Berlin, G.F.R.). Power supplies with a range of 0–2000 V are suitable.

Device for gel casting*

A sliding technique was developed for preparing very thin gels. It consists of a flat, horizontal supporting bed provided with guiding studs on each side, between which a flat plate, to be coated with the gel, is laid down. Next to this plate, leaving a gap, a dummy plate having the same thickness is placed. PTFE spacer strips are laid over the plates next to their outer edges, and a third plate is placed on the spacers, so that it covers the dummy plate and a few millimetres of the plate that is to be coated. The cover plate can slide over the spacers; this sliding motion is constrained to one direction by the guiding studs. A slow horizontal sliding motion permits the gel solution to be spread evenly between the bottom and the top plate. Between the guiding studs, the top and bottom plates are accessible and can be clamped firmly together after the space between them has been filled with gel solution. A minimum of manipulation is required. On the device shown in Fig. 1, 200 mm wide gels up to 600 mm long can be cast. Plates of standard width that fit between the guiders are used, but otherwise they can be of any desired length or thickness. The top plate may have one or more cut-outs of any shape at its sides for bringing the gel into contact with buffer solutions.

Hydrophobic treatment (gel repel coat) of at least one of the surfaces in contact with gel solution speeds up the sliding step and decreases the risk of trapping an air bubble.

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* Available soon commercially.

Thermostating

An inexpensive glass thermostating platen was designed and constructed. For efficient transfer of heat from the gel the platen is used in place of one of the two glass plates in the gel mould. In protein separation the platen cools the gel, whereas in DNA sequencing it heats it.

The water circulating and thermostating unit was a Thermomix–Frigomix combination from B. Braun, Melsungen, G.F.R.

Gel supports

To make handling possible, thin gels need a suitable support (desirable properties: electrical insulator, high thermal conductivity, transparent, inert to staining dyes, resistant to chemicals, dimensional stability, flexibility and the possibility of cutting out certain parts of the gel). The following materials were found to be suitable supports: nylon net, cellophane, polyester, polystyrene and glass. A process for surface treatment of the last three materials was developed by which the polymerized gel adheres covalently to them during all subsequent processing steps. Thin polyester sheets and glass plates were found to be the most convenient materials for gel supports.

For the surface treatment, the relatively non-hazardous γ-methacryloxypropyltrimethoxysilane (Wacker Chemie, Munich, G.F.R.) was found to be a suitable coupling agent amongst many tested, as used in the semiconductor industry.

The following procedure is applied to glass and polyester surfaces (Hostaphan BN 100 or BN 180 polyester sheet from Kalle Wiesbaden, G.F.R.) to enhance adhesion of the polyacrylamide gel:

(1) The surface is washed (glass with acetone, polyester with ethanol or isopropanol) and dried.

(2) Solutions are prepared: (A) 3 ml of water + 0.3 ml of glacial acetic acid; (B) 100 ml of ethanol + 0.3 ml of the silane, stirring well; (C) A and B are mixed. The whole of step 2 should not take longer than 2 min and solution C should be used immediately because the silane is quickly hydrolysed.

(3) Solution C is applied to the surface to be treated (dip, spray or paint on with lint-free paper) which, after standing for 2 min, is dried with lint-free paper.

(4) The plate is baked at 120°C (100°C with polyester) for 15 min, resulting in a dry and spotless surface.

(5) The treated polyester or glass plates can be used immediately or stored. Prior to use the surface is wiped with lint-free paper soaked in ethanol to remove excess of the silane and dried.

Prior etching of the polyester sheet in NaOH (as was reported to be necessary, in the process described in ref. 1) is not needed in the above process.

With the longer running times (*ca.* 3 h) and higher gel temperatures (50–60°C) encountered in DNA sequencing, the gel adhered to the polyester sheet less strongly.

This inconvenience was not observed with glass plates as the gel support.

METHODS

Preparation of very thin gels

The method is shown schematically in Fig. 1. First, a few drops of 5% glycerine solution are deposited on one face of the thermostating platen and a polyester sheet, treated on the top side, is rolled on to it using a rubber roller (the sheet can also be placed on the glass cover, resulting in even better heat transfer from the gel). Two PTFE spacers are placed on the polyester. Located next to the platen, the glass cover plate rests on a dummy block, the thickness of which is very nearly the same as that of the platen. The glass cover extends about 5 mm over the platen edge. About two thirds of the prepared gel solution (roughly three times the calculated ideally minimum amount of the solution needed should be prepared) is poured on to the polyester in the cut-out of the cover plate. The solution is drawn by capillary forces below the cover plate, quickly filling the 5 mm wide strip. The cover is then carefully slid over the thermostating platen, forming a thin layer of solution between the polyester sheet and the glass cover. More gel solution is poured in front of the cover when needed. Any trapped bubbles are removed by sliding the cover plate slowly backwards, until the bubbles are released. When a bubble appears at the edge of the cover, it can be removed with the help of a thin stainless-steel strip.

The sample comb is inserted below the cover plate either at the start or at the end of the sliding motion. The plates are then firmly clamped and transferred to a location for horizontal polymerization. If difficulties with comb insertion are feared, additional narrow PTFE spacers (about 0.1 mm thicker than the desired gel thickness determined by the normal spacers) are placed on the outside next to the normal spacers. The additional spacers are removed after sliding and comb insertion.

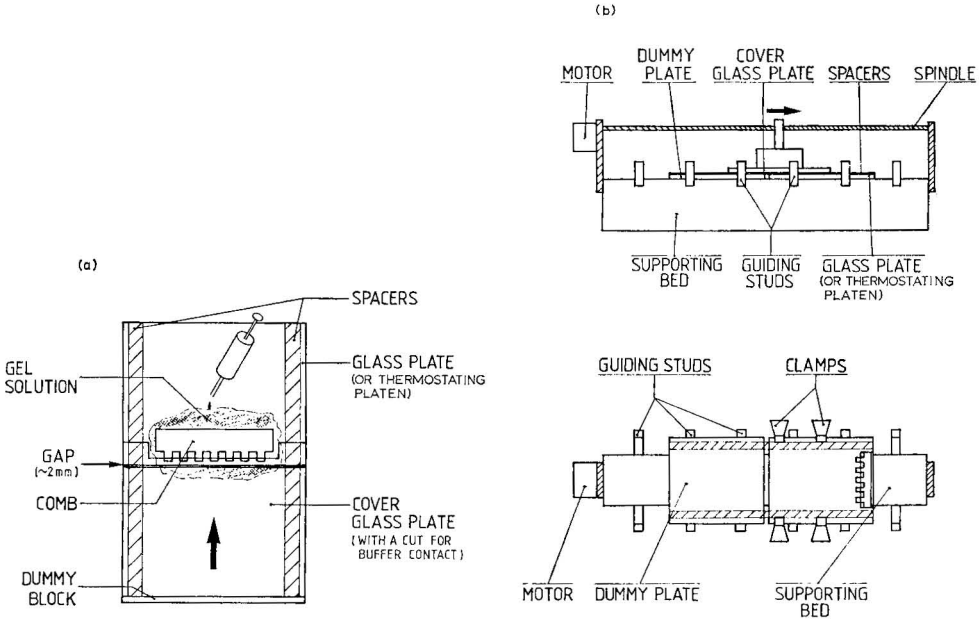

Fig. 1. Preparation of gels by sliding technique (schematic): (a) principle; (b) gel casting device.

The gel solution is held in place by capillarity and does not leak out. To prepare a gel 200 × 200 mm with this technique takes about 1 min.

A simple device for the preparation of thin gels was designed (see above), providing for guiding of the cover plate and an easy means of rapid clamping, limiting the manipulation to the minimum.

To cast a stacking gel, a cone-shaped stacking PTFE spacer is carefully inserted (at an angle, to avoid introducing bubbles) in the separation gel solution, instead of the comb above, and clamped. After polymerization, the stacking spacer is removed and stacking gel solution poured using, *e.g.*, a Raven Pipetman. If a bubble appears, it is removed with the aid of a thin stainless-steel strip. The sample comb is inserted, the plates are re-clamped and the stacking gel is polymerized in either a vertical or horizontal position. After removal of the stacking spacer, the stacking space may be flushed with buffer and any remnants of gel removed with the aid of the stainless-steel strip. After flushing, the buffer is sucked out of the stacking space with a filter-paper.

The gel preparation is facilitated when one of the surfaces in contact with the gel solution is treated to make it hydrophobic. The usual gel repellant agents give satisfactory results.

The thin gels polymerized on the polyester foil (or glass plate) rest directly on the thermostating platen and their temperature can be optimally regulated during the run, in either a vertical or a horizontal running system.

Safety precautions for work with the acrylamide monomer must be observed during the whole process.

Vertical system

As described in the previous section, the gel is cast directly between the thermostating platen and the glass cover plate. This assembly can be clamped in any conventional vertical system of appropriate size and the platen connected to a thermostating bath unit. After filling the buffer in the buffer chambers, the sample comb is carefully removed and remnant gel rim left behing the comb on the cover plate is scratched away using a spatula. The sample slots are flushed with the buffer (using a 1-ml Raven Pipetman or Pasteur pipette).

The sample is most conveniently applied by means of glass capillaries (*e.g.*, thin-walled glass capillaries for X-ray analysis from A. Müller), which can be washed and re-used many times. Very thin metal capillaries on syringes have been developed for this purpose, but proved to be two orders of magnitude more expensive, the inner diameter is smaller and their maintenance requirements are greater.

To ensure uniform sample deposition, the filled capillary is placed in a solid holder prior to insertion into the sample slot. The sample is dispensed by regulation of air pressure, using a syringe with micrometer control or an air pump[4] and silicone-rubber connecting tubes, although the "mouthpiece method" works as well with less viscous samples. Samples of 0.2–6 μl have been applied in this way.

Although 0.05 mm thick gels were used for protein separation, it is the step of sample application (its viscosity and the availability of thin-walled glass capillaries) that limits at present the practical thickness range of the very thin gels to 0.1–0.2 mm in the vertical method.

In protein separations, the voltage applied to a 10% gel (200 × 200 mm) was

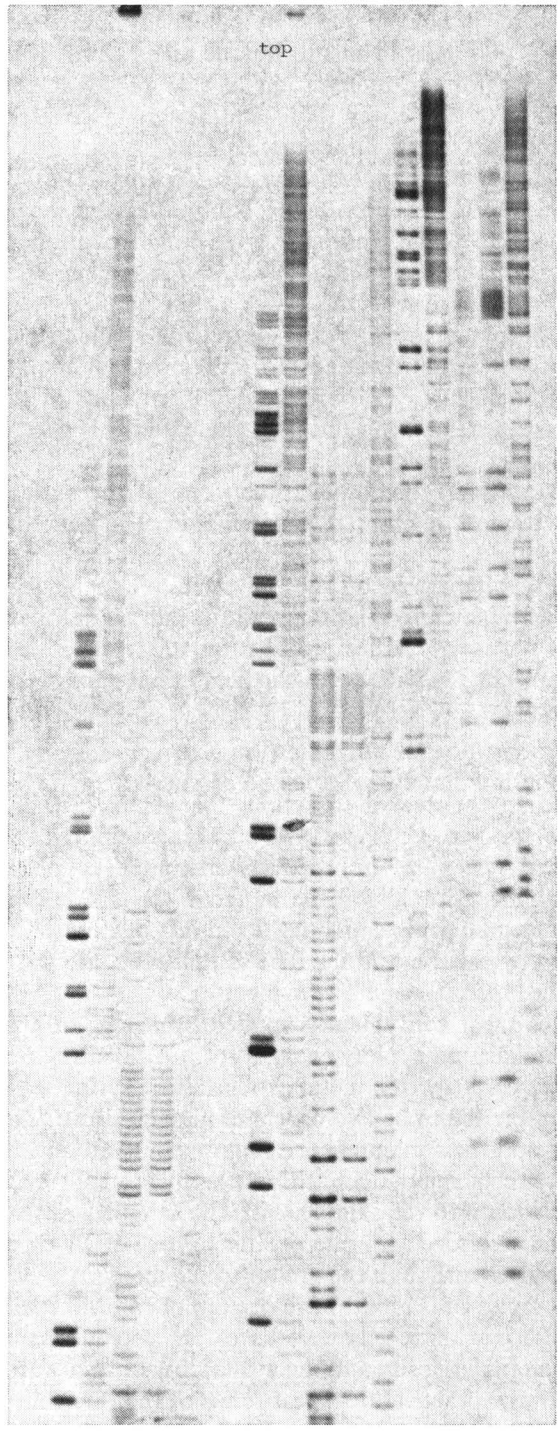

Fig. 2. Thermostated (55°C) DNA sequencing gel, thickness 0.2 mm, size 200 × 400 mm, E = 50 V/cm, I = 12 mA (from ref. 4).

in the range 300–1000 V, the applied power was 3–20 W and the running time was 20–60 min at a gel temperature of 10°C. For gels (200 × 400 mm) used in DNA sequencing[4], the applied voltage was 1800–2000 V, the power was 20–30 W and the running time was about 50 min at a gel temperature of 55°C.

Horizontal system

The horizontal system offers the advantage of simple application of the sample, and therefore gels thinner than 0.1 mm are practicable. Tests have been made in our laboratory on 0.05–0.1 mm thick gels with very encouraging results. The voltage applied to a 10% gel (100 × 100 mm) was in the range 400–500 V, the applied power was 5–10 W and the running time was 30–80 min at a gel temperature of 6°C. The forming of sample slots is at present the limiting factor in our laboratory for the use of the very thin gels in a horizontal system. Isoelectric focusing, in which sample slots are not needed, is relatively easy on thin gels[1].

RESULTS AND DISCUSSION

DNA sequencing

A DNA-sequencing autoradiograph obtained on a 0.2 mm thick thermostated gel (200 × 400 mm) is shown in Fig. 2. Gels of thickness down to 0.14 mm were tested with similar results. The bands are straight in both the vertical and horizontal directions and the autoradiograph is suitable for analysis by computer. The band sharpness and the number of resolved bases are higher than those with the sequencing pattern of the same sample obtained by conventional techniques.

The method and results of the sequencing will be reported elsewhere[4].

Protein separation

Standard protein markers (low-molecular-weight range) from Bio-Rad Labs. (Richmond, CA, U.S.A.), separated in a SDS continuous phosphate buffer system on a 0.13 mm thin gel (200 × 200 mm) are shown in Fig. 3. The gel and sample pockets were formed as described above. A 2-μl volume of sample solution (0.15 μg of each protein) was loaded into the 5 mm wide sample pocket.

Good results were obtained in the discontinuous system with Laemmli-type buffers. With 3 μl of sample solution (0.15 μg of each protein) the observed band width was comparable to that in the continuous system above, in agreement with observations by other workers[5–7].

Discussion

The results obtained indicate that the technique of using thermally stabilised very thin (0.1–0.2 mm) gels is very simple in practice, has many analytical applications (DNA sequencing, SDS–polyacrylamide gel electrophoresis (PAGE), isoelectric focusing), and brings all the expected advantages as listed in the Introduction. Manipulations during gel preparation are easier and take less time than in the conventional techniques. Gels of 3–40% concentration were prepared without difficulty using the technique described.

In DNA sequencing, the application of thermostated very thin gels[4] improves

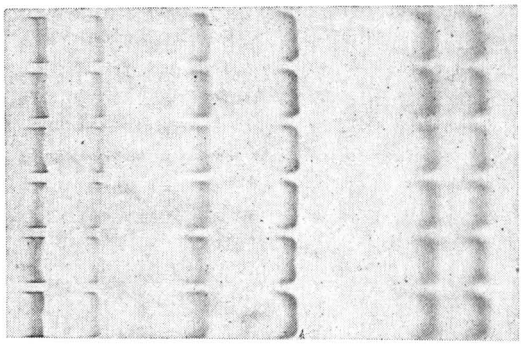

Fig. 3. SDS–PAGE continuous phosphate buffer, gel thickness 0.13 mm, size 200 × 200 mm, Bio-Rad low-molecular-weight protein standard, temperature 10°C, $E = 40$ V/cm, $I = 20$ mA.

the resolution, as predicted by theoretical considerations, and virtually eliminates thermal pattern distortions.

Typical running, staining and destaining times for protein separation on a conventional 1 mm thick gel are compared in Table I with those for a 0.1–0.15 mm thin thermostated gel. The total time requirement is an order of magnitude shorter for the very thin gel.

TABLE I

COMPARISON OF TYPICAL RUNNING, STAINING AND DESTAINING TIMES FOR SDS–PAGE ON A CONVENTIONAL (1–1.5 mm THICK) AND A VERY THIN (0.15 mm) POLYACRYLAMIDE GEL (SIZE 200 × 200 mm)

Gel thickness (mm)	*Time (min)*			
	Run	*Fix + stain*	*Destain*	*Total*
1–1.5	360	120	180	660
0.15	60	10	15	85

The lowest protein load applied in our test runs was 0.01 μg of protein per band, clearly visible after destaining on the 0.1 mm thin gel used in the discontinuous SDS separation run.

In several tests broad protein bands were observed when the thin gels were run at a high field strength ($\gtrsim$ 50 V/cm) without previous optimization of ionic strength, sample load or stacking voltage. Further study of the factors (temperature, sample load, field strength, ionic strength, current density, gel concentration) that affect the resolution and band width with very thin gels will be necessary in order to optimize the technique for different applications, and to extend similar investigations made with thicker gels. According to our experience the geometric scaling effects agree very well with physico-chemical predictions for a uniform layer, neglecting surface effects.

Variation of pore size and thickness on the thin gels will be further evaluated, although no effect attributable to a variation in thickness has been observed during the tests with standard glass plates.

There is interest in applying more sensitive detection methods to the very thin gel technique, and to investigate gel supporting structures further.

CONCLUSION

Very thin polyacrylamide gels (0.02–0.2 mm thick) are prepared with the aid of a gel casting device. The gel is cast directly between the glass cover plate and a thermostating platen, which maintains the gel layer under isothermal conditions during electrophoresis. To make handling possible, a chemical procedure for the treatment of glass and polyester surfaces was developed that makes it possible to polymerize the gel on a transparent support to which it adheres during subsequent processing. The sample is most conveniently applied by means of glass capillaries which are simply washed and re-used many times. To ensure uniform sample deposition, a regulating sample dispensing system is used. Gels of 3–40% concentration can be prepared without difficulty using the technique described. The results obtained indicate that the technique is simple to use, has many analytical applications (DNA sequencing, SDS–PAGE, isoelectric focusing) and has various advantages.

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PREPARATION AND EVALUATION OF 3-AMINOPROPYLTRIETHOXYSILANE-TREATED SILICA FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF LOW-MOLECULAR-WEIGHT ALDEHYDES AS THEIR LUTIDINE DERIVATIVES

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SUMMARY

Formaldehyde and other low-molecular-weight aldehydes were studied as lutidine derivatives by high-performance liquid chromatography using an NH_2-chemically bonded stationary phase, prepared from silica gel treated with benzene solutions containing 5–30% 3-aminopropyltriethoxysilane. From elemental analysis data for nitrogen and carbon, the maximum number of accessible NH_2 surface groups per gram of silica gel was estimated to be 0.45×10^{21}.

INTRODUCTION

Free formaldehyde (HCHO) remaining in underwear has been reported to be one cause of skin trouble[1,2]. Acetylacetone (AA) reacts with HCHO under mild conditions to form the corresponding lutidine, and colorimetric[3,4] and fluorometric methods[5] using this reaction have been widely applied for the determination of free HCHO in underwear. However, AA reacts similarly with other aldehydes, necessitating the chromatographic separation of lutidines.

On the other hand, Papa and Turner[6] and Carey and Persinger[7] reported on the high-performance liquid chromatography (HPLC) of carbonyl compounds as their 2,4-dinitrophenylhydrazone derivatives. This method was not successful in the determination of trace amounts of HCHO in underwear, because the high temperatures and high concentrations of acid required to transform low-molecular-weight aldehydes into hydrazones, also caused the decomposition of resins contained in underwear producing an error in the HCHO determination.

I have examined the separation of HCHO–AA and lutidine derivatives by HPLC using various columns such as ODS, styrene–divinylbenzene copolymer, silica gel ion-exchange resin and silica gel treated with 3-aminopropyltriethoxysilane (3APTS), but could not obtain satisfactory results except with the last column. Thus, I have now studied the optimal conditions for the HPLC determination and separation of HCHO in underwear as the lutidine derivative on silica gel treated with various 3APTS–benzene solutions. The separation of other lutidine derivatives of various aldehydes was also examined.

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EXPERIMENTAL

Reagents

HCHO (aqueous), acetaldehyde, propionaldehyde, *n*-butyraldehyde, *n*-valeraldehyde, *n*-capronaldehyde, *n*-heptanal and AA were obtained from Wako (Osaka, Japan). 3APTS was purchased from Aldrich (Milwaukee, WI, U.S.A.). A highly microporous spherical silica gel [mean pore diameter 95 Å, surface area (BET) 380 m^2/g, particle size distribution 5.5 μm] was obtained from Fuji-Davison (Aichi, Japan). Hexane and ethanol were used after distillation. The other reagents and organic solvents were reagent grade.

Apparatus

Two liquid chromatographs were employed: a Hitachi 635 T equipped with a visible spectrophotometer and a Hitachi 204 S fluorescence spectrophotometer; and a Type KHU 16 of Kyowa Seimitsu Mini Micro Pump equipped with a Type KLC-200 Kyowa Seimitsu variable-wavelength detector.

Stationary phase and elemental analysis

A 5-g amount of dried silica gel was added to 50 ml of a 0.5, 1.0, 1.5, 2.0, 2.5 or 3.0% benzene solution of 3APTS. After stirring for 24 h at room temperature, the silica gel was filtered with a glass filter, washed several times with benzene and acetone and then dried *in vacuo* at 70°C for 2 days. Nitrogen and carbon contained in the silica gel treated with 3APTS (3APTS-5 to 3APTS-30, shown in Table I) were determined by elemental analysis using a Perkin-Elmer Type 240 Elemental Analyzer.

TABLE I

SURFACE TREATMENTS AND ELEMENTAL ANALYSES

Mono = Monofunctional; Bi = bifunctional; for the meaning of these terms, see text.

Sample	*Concn. of 3APTS in benzene (%)*	*N Calc. (%)*		*N Found (%)*	*C Calc. (%)*		*C Found (%)*
		Mono	*Bi*		*Mono*	*Bi*	
SiO_2	0 (original)	0	0	0	0	0	0
3APTS-5	0.5	0.30	0.31	0.27	1.82	1.36	2.79
3APTS-10	1.0	0.58	0.60	0.58	3.52	2.56	3.66
3APTS-15	1.5	0.84	0.87	0.82	5.09	3.74	5.43
3APTS-20	2.0	1.09	1.13	1.03	6.55	4.85	6.85
3APTS-25	2.5	1.32	1.38	1.06	7.91	5.91	7.69
3APTS-30	3.0	1.53	1.61	1.13	9.19	6.91	6.56

Column preparation

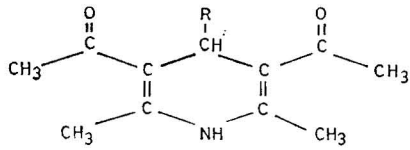
Silica gel treated with 3APTS was packed was packed into stainless-steel columns (50, 75, 125, 150, 200, 225, 250, 300, 400, 450 or 475 mm × 4 mm I.D.) using a balance density method and a 10-ml stainless-steel packer at a rate of 500 kg/cm^2 (Kyowa Seimitsu Type KHW-20 Ultra High Pressure Pump).

Preparation of lutidine derivatives

A 2-ml volume of HCHO (aq.) or other aldehydes (Table II), 2 ml of AA, 3 ml of acetic acid and 15 g of ammonium acetate were added to 100 ml of water. For HCHO, the mixture was warmed to 40°C for 60 min and for other aldehydes to 60°C for 30 min. After cooling, the product was filtered off, washed with cold water and then cold ethanol, dried at room temperature and recrystallized from diethyl ether.

TABLE II

PREPARATION OF HCHO-AA AND OTHER LUTIDINE DERIVATIVES OF ALDEHYDES



Parent compound	*R*	*M.p.* (°C)	*Formula*	*Analysis: Calc. (Found) (%)*		
				C	*H*	*N*
HCHO	H	200–204	$C_{11}H_{15}NO_2$ (HCHO-AA)	68.35 (68.02)	7.83 (7.85)	7.25 (6.97)
CH_3CHO	CH_3	157–159	$C_{12}H_{17}NO_2$	69.53 (69.47)	8.27 (8.51)	6.76 (6.73)
CH_3CH_2CHO	CH_3CH_2	162–164	$C_{13}H_{19}NO_2$	70.54 (70.42)	8.66 (8.82)	6.33 (6.37)
$CH_3(CH_2)_2CHO$	$CH_3(CH_2)_2$	130–132	$C_{14}H_{21}NO_2$	71.44 (71.69)	9.00 (9.25)	5.96 (6.14)
$CH_3(CH_2)_3CHO$	$CH_3(CH_2)_3$	110–113	$C_{15}H_{23}NO_2$	72.24 (72.12)	9.30 (9.25)	5.62 (5.40)
$CH_3(CH_2)_4CHO$	$CH_3(CH_2)_4$	120–122	$C_{16}H_{25}NO_2$	72.95 (72.76)	9.57 (9.86)	5.32 (5.16)
$CH_3(CH_2)_5CHO$	$CH_3(CH_2)_5$	141–143.5	$C_{17}H_{27}NO_2$	73.60 (73.80)	9.82 (10.01)	5.05 (5.01)

Procedure

A 1-g amount of chopped sample (underwear) was immersed in 100 ml of water for 60 min at 40 °C, and the extract was filtered through a glass filter (G2) by the method of Kojima and Ohba[4]. To a 5-ml aliquot of the filtrate, a mixture of 5 ml of 2 *M* ammonium acetate, 0.05 *M* acetic acid and 0.02 *M* AA was added. The reaction mixture was warmed in a water-bath at 40°C for 30 min as described by Kojima and Ohba[4]. After cooling, 5 ml of internal standard solution (30 μg of the lutidine derivative of propionaldehyde in 1 ml of chloroform) were added. The mixture was shaken well and allowed to stand for some minutes. The aqueous phase was then discarded and the organic phase dried over anhydrous sodium sulphate. A 50-μl volume of the resulting solution was subjected to HPLC. The operating conditions are given in the legend to Fig. 3.

RESULTS AND DISCUSSION

Fig. 1 shows the correlation between the capacity factor (k') of each lutidine derivative and the concentration of the 3APTS solution with which the silica gel was treated. A plateau in k' was observed from 3APTS-15 to 3APTS-30, which means that almost all reactive OH groups on the silica gel surface were substituted with 3APTS.

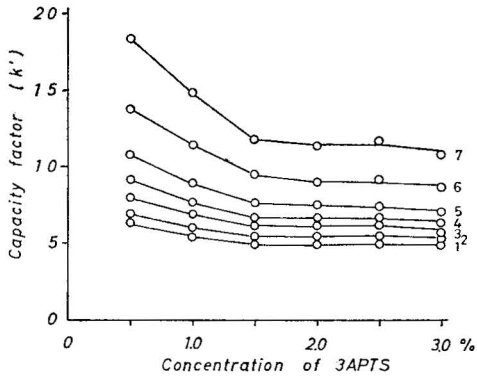


Fig. 1. Relationship between the capacity factor, k', of lutidine derivatives and the concentration of 3APTS solution. Column: 250 × 4 mm I.D. Mobile phase: hexane–ethanol (25:1). Curves: 1 = formaldehyde; 2 = acetaldehyde; 3 = propionaldehyde; 4 = *n*-butyraldehyde; 5 = *n*-valeraldehyde; 6 = *n*-capronaldehyde; 7 = heptanal.

From the elemental analysis of silica gel treated with 3APTS solutions of various concentrations, the number of accessible NH_2 surface groups per gram of silica gel can be estimated by the following procedure. If 3APTS is substituted monofunctionally on silica gel, the surface structure of silica gel can be written as:

$$\begin{array}{l} \quad OC_2H_5 \\ \quad | \\ -Si-(CH_2)_3-NH_2 \\ \quad | \\ \quad OC_2H_5 \end{array}$$

The number of accessible NH_2 surface groups per gram is then given by

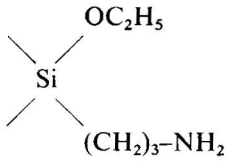
$$[(N/100)/14.0067 \times 1] \times 6.022 \times 10^{23} \quad (1)$$

or

$$[(C/100)/12.011 \times 7] \times 6.022 \times 10^{23} \quad (2)$$

where N = nitrogen weight percentage, C = carbon weight percentage and 6.022×10^{23} = Avogadro's number.

If 3APTS is substituted bifunctionally on silica gel, the surface structure of silica gel can be written as:



The number of accessible NH_2 surface groups per gram is then given by:

$$[(N/100)/14.0067 \times 1] \times 6.022 \times 10^{23} \quad (3)$$

or

$$[(C/100)/12.011 \times 5] \times 6.022 \times 10^{23} \quad (4)$$

Substitution of the values of N and C found by elemental analysis (Table I) into eqns. 1, 2, 3 or 4 gives the number of accessible NH_2 surface groups per gram of silica gel (Table III). As is seen from the data in Tables I and III, increase of the 3APTS concentration in benzene increases the surface modification of silica gel, but approaches saturation at greater than 1.5% 3APTS. This tendency explains well the plateau in k' from 3APTS-15 to 3APTS-30 in Fig. 1. The agreement between the values calculated according to the monofunctional reaction mechanism seems to be better than that for those according to the bifunctional reaction mechanism. It is therefore suggested that the reaction between silica gel and 3APTS takes place monofunctionally. The nitrogen and carbon percentages and the number of accessible NH_2 surface groups according to eqns. 1, 2, 3 or 4 were also calculated with the assumption that all 3APTS molecules react with silica gel (calc. values in Tables I and III). The comparison between these values and the corresponding "found" values again shows that saturation of 3APTS on silica gel takes place with about 1.5% 3APTS in benzene. Using the data for 3APTS-15 to 3APTS-30 in Table III, the number of accessible NH_2 surface groups per gram of silica gel was 0.45×10^{21}.

TABLE III

SURFACE TREATMENTS AND THE NUMBER OF ACCESSIBLE NH_2 SURFACE GROUPS PER GRAM

Sample	*No. of accessible NH_2 surface groups per gram ($\times 10^{21}$)*							
	Calc. Mono		*Found Mono*		*Calc. Bi*		*Found Bi*	
	eqn. 1	*eqn. 2*	*eqn. 1*	*eqn. 2*	*eqn. 3*	*eqn. 4*	*eqn. 3*	*eqn. 4*
SiO_2	0	0	0	0	0	0	0	0
3APTS-5	0.13	0.13	0.12	0.20	0.13	0.14	0.12	0.28
3APTS-10	0.25	0.25	0.25	0.26	0.26	0.26	0.25	0.37
3APTS-15	0.36	0.37	0.35	0.39	0.37	0.38	0.35	0.55
3APTS-20	0.47	0.47	0.44	0.49	0.49	0.49	0.44	0.69
3APTS-25	0.57	0.57	0.46	0.55	0.59	0.59	0.46	0.77
3APTS-30	0.66	0.66	0.49	0.47	0.69	0.69	0.49	0.66

It was found that HCHO–AA could be easily extracted with organic solvents, such as dichloromethane, chloroform and ethyl acetate. Of these solvents, chloroform was chosen as the most suitable since it can be clearly separated from the aqueous

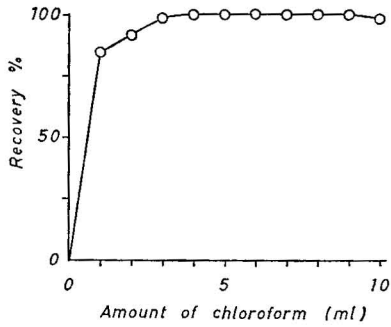


Fig. 2. Effect of chloroform on the extraction of HCHO-AA (20 μg).

phase as lower layer, which is convenient for the separation procedure. Fig. 2 shows the efficiencies of HCHO–AA (20 μg) extraction with chloroform from aqueous solvents. A 5-ml volume of chloroform was required.

The chromatographic behaviour of HCHO–AA was studied on columns of various lengths of silica gel treated with 3APTS in mixed solvents such as hexane–methanol, –ethanol, –*n*-propanol, –isopropanol and –*n*-butanol. The hexane–ethanol solvent (25:1) and 250 × 4 mm I.D. column gave the best separation, and with a suitable flow-rate owing to the low viscosity. Fig. 3 shows a liquid chromatogram of

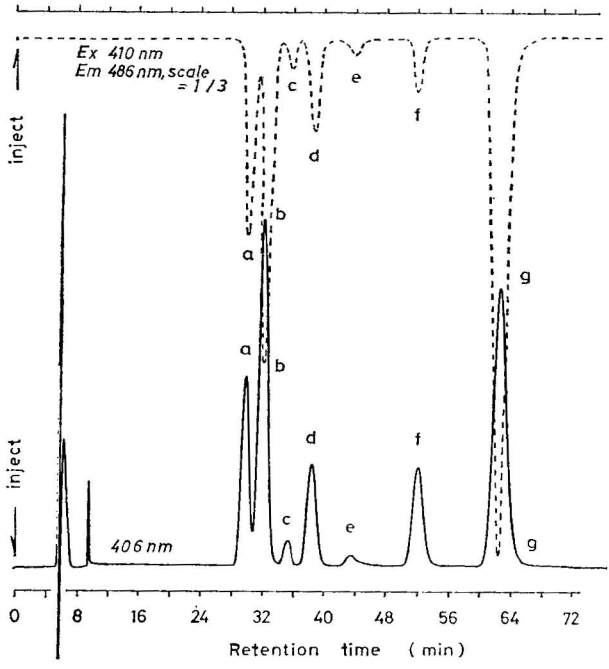


Fig. 3. Separation of HCHO-AA and other lutidine derivatives. Stationary phase: silica gel treated with 2.0% 3APTS. Mobile phase: hexane–ethanol (25:1), flow-rate 0.8 ml/min. Detection: ———, 406 nm, 0.04 a.u.f.s.; - - -, excitation 410 nm (slit 10 nm), emission 486 nm (slit 10 nm). Peaks: a = *n*-heptanal (6.1 μg); b = *n*-capronaldehyde (9.2 μg); c = *n*-valderaldehyde (1.3 μg); d = *n*-butyraldehyde (5.2 μg); e = propionaldehyde (0.2 μg); f = acetaldehyde (2.1 μg); g = formaldehyde (3.6 μg).

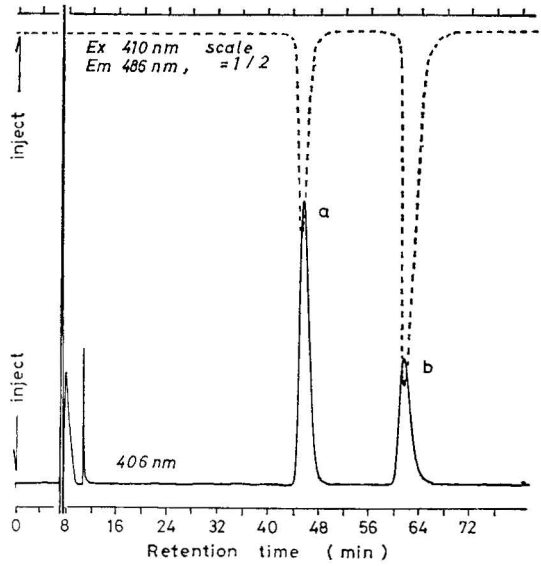


Fig. 4. Liquid chromatogram of formaldehyde (b) in underwear and propionaldehyde as internal standard (a, 1.5 μg). Stationary phase: silica gel treated with 2.5% APTS. Other conditions as in Fig. 3.

HCHO–AA and lutidine derivatives of some parent compounds in hexane–ethanol (25:1). From this result, propionaldehyde was chosen as internal standard for HCHO.

Fig. 4 shows a typical liquid chromatogram obtained from HCHO in underwear and propionaldehyde as internal standard.

A calibration graph constructed by plotting the ratio of the peak area of HCHO to that of the internal standard was linear and passed through the origin for HCHO in the range, 0.02–1.0 μg and 0.05–6.0 μg in 1 ml of aqueous solution, using fluorescence and visible spectrophotometers respectively. Nine replicate determinations on a test solution containing HCHO (1 μg) gave a standard deviation of 1.83%, and the limit of detection was 100 pg.

ACKNOWLEDGEMENT

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USE OF SOLUTE PARTITION FOR COMPARATIVE CHARACTERIZATION OF SEVERAL AQUEOUS BIPHASIC POLYMERIC SYSTEMS

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SUMMARY

Eight biphasic ficoll–dextran systems differing in the molecular weight of the latter polymer and in the polymer composition were studied. The effect of the salt composition on the phase diagrams for all systems was examined and it is shown that salts present in the polymer mixture should be considered as essential components of the mixture involved in the separation of phases.

The partition behaviour of a number of proteins and dinitrophenyl-amino acids in the systems was studied. It is shown that the partition coefficients obtained in different phase systems can be correlated using the relation $\ln K_i = a \ln K_0 + b$, where K_i and K_0 are the partition coefficients of a solute in the system in question and in the system chosen as the reference, respectively, and a and b are the scaling factors independent of the chemical nature of the solutes partitioned.

The difference in the relative hydrophobicities between the two phases was determined experimentally in two phase systems and calculated for six other systems. It is shown that it is possible to compare the partition results obtained in various polymer phase systems using any solutes chosen as "partition markers".

INTRODUCTION

The partition of biological macromolecules and particles in aqueous biphasic polymeric systems is commonly used as an extremely sensitive method for the separation of cells and biopolymers[1,2]. It has been shown that the partition technique can be used as an analytical method for determining the relative hydrophobicities of the distributed macromolecule or particle surface[3–7]. We previously proposed[8] a ficoll–dextran phase system which has several advantages over the common dextran–polyethylene glycol (PEG) system, particularly for analytical studies of both macromolecule and cell surface properties.

The analytical application of the method, however, appears to be limited owing to minor variations in polymer composition and batch variations in polymer characteristics, which are known to have large effects on the partition behaviour of a

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given substance in the dextran–PEG phase system[9–11]. Similar effects have been observed by us when using the ficoll–dextran phase system.

It is obvious that the analytical application of the partition technique depends greatly on the possibility of comparing different data obtained by various workers generally using polymers of different batches and manufacture. Some factors are required for scaling of the partition values obtained in phase systems with different polymer compositions and formed from various polymer samples.

It was the purpose of this work to find the factors for scaling of the partition values obtained in the phase systems formed by ficoll and various dextran batches.

EXPERIMENTAL

Materials

Polymers. Ficoll 400 (lots 3004, 6594, 7868 and 11069) and dextran T 40 (lot 2771) were purchased from Pharmacia (Uppsala, Sweden). Dextrans of molecular weight about 40,000 were obtained from Polfa (Kutno, Poland) (lot 325-71), Loba (Vienna, Austria), Ferak (Berlin, G.F.R.) and under the trade-name Reopolyglucinum (lot 190677) from Minmedprom (Moscow, U.S.S.R.). Dextrans of molecular weight about 70,000 under the trade-name Polyglucinum (lots 310670, 390476 and 580870) were obtained from Minmedprom.

Proteins. Equine heart cytochrome *c* and horse skeletal muscle myoglobin were purchased from Calbiochem (Los Angeles, CA, U.S.A.). Human serum albumin, Cohn fraction V, was obtained from ICN Pharmaceuticals (Cleveland, OH, U.S.A.). Human plasma was obtained as described earlier[7].

Amino acids. Dinitrophenylated amino acids (DNP-L-Ala, DNP-L-Phe, DNP-Gly) were obtained from Serva (Heidelberg, G.F.R.). 2,4-Dinitrofluorobenzene was purchased from Calbiochem. L-Norleucine and DL-norvaline were obtained from Reanal (Budapest, Hungary) and DL-2-amino-*n*-octanoic acid from BDH (Poole, Great Britain). The amino acids were dinitrophenylated as described in ref. 12.

Chemicals. Sodium alkyl sulphates, R_nOSO_3Na ($n = 8, 10, 12$), were prepared by the method of Dreger *et al.*[13]. All salts and chemicals used were of analytical-reagent grade.

Determination of molecular weight distribution and other properties of dextran samples

The molecular weight distribution of the dextran samples was measured by high-performance liquid chromatography (HPLC) in the size-exclusion mode. A DuPont Model 830 liquid chromatograph provided with a refractive index detector (LDC, Model 1107) and a Rheodyne fixed-volume loop injector (20 μl) was used. Two DuPont high-performance size-exclusion columns (SE-100, SE-500; 25 cm × 6.2 mm I.D.) loaded with small (8-μm) porous silica particles were connected in series. Ethanol (2%) filtered through MF-Millipore filters (0.45 μm) was used as the eluent in the solvent delivery system. The solvent flow-rate was 0.9 ml/min and the recorder speed was set at 1.5 cm/min. A 1% solution of the sample in the mobile phase was used for injection.

Dextran standards and glucose were used for column calibration. The dextran standards used (T 10, T 20, T 40, T 70, T 110, T 150, T 250, T 500 and T 2000) were obtained from Pharmacia with weight-average molecular weights ($\bar{M}_w$), number-

average molecular weights ($\overline{M}_n$) and molecular weight distribution (MWD) curves supplied by the manufacturer. As the dextran standards have wide MWD [dispersity (1.4–3.0)], a calibration graph was obtained from the geometric mean of the $\overline{M}_w$ and $\overline{M}_n$ values and peak elution volumes[14]. Using the calibration graph the $\overline{M}_w$ and $\overline{M}_n$ values of the dextran samples were determined as described elsewhere[14,15]. The dextran samples were analysed 2–3 times, always together with all of the standards.

The degree of branching characteristic for the dextran samples was estimated by use of ^{13}C NMR spectroscopy as described by Seymour *et al.*[16]. A Brucker WP-80 spectrometer was employed in the Fourier-transform mode.

Near-infrared measurements on the dextran samples in water (10% solutions) were carried out as described by Aizawa *et al.*[17].

Preparation of phase systems

Stock solutions of ficoll (*ca.* 40%, w/w) and dextran (*ca.* 35%, w/w) in water were prepared. A mixture containing the amounts of polymers indicated below was prepared by weighing appropriate amounts of the stock polymer solutions. The appropriate amounts of 0.44 *M* sodium phosphate buffer (pH 7.4) and 0.6 *M* sodium chloride in 0.04 *M* sodium phosphate buffer (pH 7.4) were added so as to achieve the required ionic composition (ionic strength) and the polymer composition of the phase system. The amounts of sodium chloride and sodium phosphate buffer (pH 7.4) in a given phase system can be calculated from the equations $C_{NaCl} = (0.288 - I)/0.75$ and $C_{buffer} = 0.11 - 0.67C_{NaCl}$, where C_{NaCl} and C_{buffer} are the sodium chloride and sodium phosphate buffer concentrations, respectively, and I is the ionic strength in the phase system.

The phase diagrams were constructed as described by Albertsson[1].

Phase systems. The final phase systems used had the following polymer compositions (all in weight-percent):

System I: 14% ficoll (lot 6594), 12% Reopolyglucinum;
System II: 14% ficoll (lot 6594), 12% dextran 40 (Polfa);
System III: 14% ficoll (lot 6594), 12% dextran T 40 (Pharmacia);
System IV: 14% ficoll (lot 11069), 12% dextran 40 (Loba);
System V: 14% ficoll (lot 6594), 12% Polyglucinum (lot 390476);
System VI: 11.5% ficoll (lot 6594), 10% Polyglucinum (lot 390476);
System VII: 12.5% ficoll (lot 7868), 10.8% Polyglucinum (lot 310670);
System VIII: 12.5% ficoll (lot 11069), 10.8% Polyglucinum (lot 580870).

Determination of partition coefficients of solutes

As the top phase was ficoll-rich in systems I–V and dextran-rich in systems VI–VIII, the partition coefficient, K, in this work is defined as the ratio of sample concentration in the ficoll-rich phase to sample concentration in the dextran-rich phase. After settling of the phases (settling time 24 h) aliquots of both phases (0.1–0.2 ml) were carefully pipetted from the phase system, each was diluted by addition of an appropriate volume of water and the concentrations of the partitioned solute were determined.

The concentrations of sodium alkyl sulphates were measured by the method described by Hayashi[18]. The serum albumin concentrations were determined using the Coomassie G-250 technique[19]. The cytochrome *c* and myoglobin concentrations

were determined by absorbance measurements at 410 nm, and those of the dinitrophenylated amino acids were determined at 360 nm. In all instances the correspondingly diluted pure phases were used as blank solutions.

The partition coefficient for each solute was determined at six ionic strengths as the mean of two measurements on two or more dilutions from each partition carried out 2–4 times at a given ionic strength. The deviation from the mean K value did not exceed 3% for any of the solutes studied.

RESULTS

Polymer characteristics and phase diagrams

Fig. 1 shows the phase diagrams for the ficoll–dextran systems with different dextran samples in the presence of 0.15 M sodium chloride in 0.01 M sodium phosphate buffer (pH 7.4). It can be seen that the higher the weight-average molecular weight of dextran (see Table I), the lower is the total polymer concentration required for phase separation. It is evident that the previously used polymer composition of 14% (w/w) ficoll and 10% (w/w) dextran 40^{4-8} does not provide phase separation in all of the polymer mixtures under study. In particular, the dextran batch from Polfa (lot 325-71) did not give phase separation at the above polymer composition, in contrast to the dextran sample from the same manufacturer used previously[7]. Hence, the polymer composition of 14% ficoll and 12% dextran was chosen for most of the

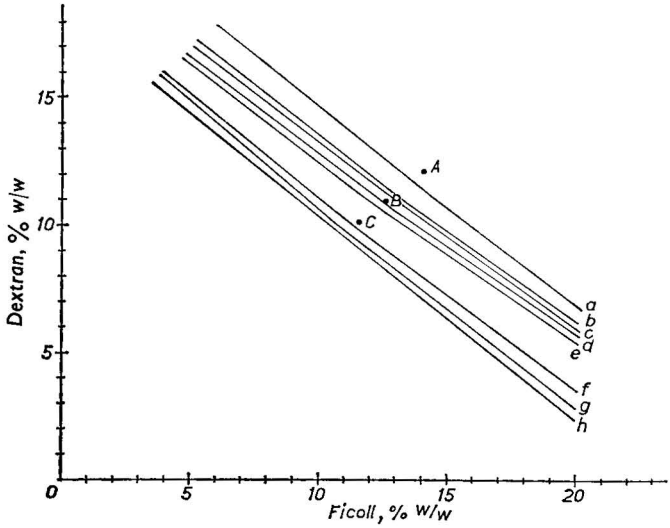

Fig. 1. Phase diagrams for biphasic ficoll–dextran systems in the presence of 0.15 M NaCl in 0.01 M sodium phosphate buffer (pH 7.4). (a) Ficoll (lot 3004 or 6594), Reopolyglucinum; (b) ficoll (lot 6594), dextran 40 (Polfa, lot 325-71); (c) ficoll (lot 3004 or 6594), dextran T 40 (Pharmacia, lot 2771); (d) ficoll (lot 11069), dextran 40 (Loba); (e) ficoll (lot 3004), dextran 40 (Ferak); (f) ficoll (lot 7868), Polyglucinum (lot 310670); (g) ficoll (lot 11069), Polyglucinum (lot 580870); (h) ficoll (lot 3004 or 6594), Polyglucinum (lot 390476). Point A corresponds to the polymer composition 14% (w/w) ficoll, 12% (w/w) dextran, point B to 12.5% (w/w) ficoll and 10.8% (w/w) dextran and point C to 11.5% (w/w) ficoll, 10% (w/w) dextran.

systems as this composition gives rise to phase systems with similar volumes of both phases. It should be noted, however, that the densities of the two phases in systems I–IV with the above composition are very close and one obtains the so-called isopycnic phases[1], which hinders the partition experiments.

In order to characterize the phase systems under study, the systems with the above polymer composition in the presence of 0.15 *M* sodium chloride in 0.01 *M* sodium phosphate buffer (pH 7.4) and in the presence of 0.11 *M* sodium phosphate buffer (pH 7.4) were transformed into a one-phase system and the polymer compositions at the binodial (corresponding to a ficoll to dextran ratio of 1.16) were determined. The total polymer concentrations characteristic of these compositions are given in Table I together with the molecular weight characteristics of the dextran samples. The molecular weights of the ficoll samples were not determined. However, the phase diagrams and the binodial polymer compositions were compared for the systems formed by Reopolyglucinum and Polyglucinum (lot 390476) with two ficoll batches (lots 3004 and 6594), and no difference could be detected.

TABLE I

MOLECULAR WEIGHT DISTRIBUTION CHARACTERISTICS OF DEXTRAN SAMPLES AND TOTAL CONCENTRATIONS OF FICOLL 400 AND DEXTRAN REQUIRED FOR SEPARATION OF PHASES IN POLYMER MIXTURES

The total polymer concentrations required for separation of phases in the polymer mixtures were determined as characteristic for the polymer compositions at the corresponding binodial (corresponding to a ficoll/dextran ratio of 1.16). The polymer concentrations are given in weight-percent.

Dextran	$\bar{M}_w \cdot 10^{-3}$*	$\bar{M}_n \cdot 10^{-3}$	$\bar{M}_w/\bar{M}_n$	*C*	
				*Ionic strength 0.176 M***	*Ionic strength 0.288 M****
Reopolyglucinum	34.5	18.3	1.89	25.4	23.2
Dextran 40 (Polfa, lot 325-71)	39.4	28.9	1.37	24.1	21.7
Dextran T 40 (Pharmacia, lot 2771)	41.5	28.8	1.44	23.8	21.4
Dextran 40 (Loba)	41.4	25.0	1.66	23.4	21.4
Dextran 40 (Ferak)	44.8	29.6	1.51	23.1	20.5
Polyglucinum (lot 310670)	58.6	23.2	2.52	21.2	19.9
Polyglucinum (lot 580870)	64.5	22.7	2.84	20.7	19.4
Polyglucinum (lot 390476)	68.8	23.5	2.93	20.5	19.1

* The deviation from the mean $\bar{M}_w$ value is *ca.* 5%.

** In the presence of 0.15 *M* NaCl in 0.01 *M* sodium phosphate buffer (pH 7.4).

*** In the presence of 0.11 *M* sodium phosphate buffer (pH 7.4).

The degree of dextran branching as detected by the ^{13}C NMR technique was 3–6% for all of the dextran samples examined, and cannot be regarded as a property that affects phase separation in the polymer mixtures or governs the partition properties of the phase systems. Therefore, this characteristic will not be considered further.

An attempt to detect any differences in the dextran solutions using near-infrared measurements[17] failed completely.

Partition of proteins

It has been shown earlier[4,7] that the relationship between the logarithm of the

protein partition coefficient and the ionic strength of the phase system can be described by

$$\ln K = A + BI \tag{1}$$

where I is the ionic strength and A and B are constants (the ionic strength was varied as indicated above from 0.176 to 0.288 M).

A least-squares treatment of the partition data led to the A and B values listed in Table II for the proteins examined. It can be seen that the A and B values are different and depend on the phase system used in the partition experiments. It should be noted that the ratio B/A appears to be specific for a given protein, independent of the system used. This seems reasonable according to the physical meaning of A and B[4–7]. The B/A value is -3.78 ± 0.17 for human serum albumin and -12.88 ± 0.90 for cytochrome *c*. The myoglobin partition appears to be independent of the ionic strength, and hence the B/A value for this protein is zero. The observed differences in the partition behaviour of the proteins require separate discussion, which is beyond the scope of this paper.

TABLE II

CHARACTERISTICS OF THE PARTITION BEHAVIOUR OF PROTEINS IN AQUEOUS FICOLL–DEXTRAN BIPHASIC SYSTEMS

The logarithm of the partition coefficient of a given protein ($\ln K$) depends on the ionic strength of the phase system (I) as $\ln K = A + BI$. Parameters A and B calculated from the experimental K values are presented as the means ± standard deviation with the correlation coefficient values given in parentheses. The ionic strength was varied from 0.176 to 0.288 M as described in the text. Polymer compositions of the phase systems are indicated in the text.

Phase system	*Human serum albumin*		*Myoglobin**	*Cytochrome c*	
	$-A$	B	$-A$	A	$-B$
I	1.64 ± 0.04 (0.998)	6.0 ± 0.19	0.32 ± 0.04 (0.998)	0.28 ± 0.25 (0.892)	3.44 ± 1.0
II	2.86 ± 0.04 (0.999)	10.38 ± 0.19	0.33 ± 0.02 (0.989)	0.22 ± 0.04 (0.996)	2.83 ± 0.16
III	2.92 ± 0.10 (0.997)	11.4 ± 0.41	0.34 ± 0.02 (0.997)	0.24 ± 0.14 (0.958)	3.39 ± 0.59
IV	2.28 ± 0.09 (0.996)	8.2 ± 0.37	0.38 ± 0.01 (0.999)	0.30 ± 0.26 (0.892)	4.18 ± 1.06
V	1.97 ± 0.17 (0.980)	7.3 ± 0.74	0.35 ± 0.04 (0.997)	0.28 ± 0.06 (0.994)	3.75 ± 0.25
VI	1.13 ± 0.09 (0.987)	4.62 ± 0.38	0.22 ± 0.03 (0.989)	0.20 ± 0.04 (0.990)	2.28 ± 0.18
VII	2.01 ± 0.06 (0.998)	7.72 ± 0.25	0.27 ± 0.04 (0.999)	0.20 ± 0.08 (0.974)	2.50 ± 0.34
VIII	2.28 ± 0.09 (0.996)	8.81 ± 0.38	0.27 ± 0.03 (0.999)	0.21 ± 0.04 (0.971)	2.63 ± 0.16

* Partition of myoglobin is independent of the ionic strength in the I range used, hence the B value is zero in all phase systems examined.

The total plasma proteins, being a complex multi-component system, were distributed in phase systems VII and VIII. The A values are -1.61 ± 0.11 and -1.70 ± 0.12, respectively, and the B values are 6.88 ± 0.45 and 7.73 ± 0.51, respectively.

Partition of sodium alkyl sulphates

The partition behaviour of sodium alkyl sulphates as a function of the ionic strength of the system was studied in phase systems I–VI. It has been shown previ-

ously[4] that the effect of ionic strength and alkyl chain length on the partition of surfactants can be expressed as

$$\ln K = C + BI + Em \tag{2}$$

where m is the number of CH_2 groups in the alkyl chain of the solute molecule, E characterizes the difference in the relative hydrophobicity between the two phases of a given system and C and B are constants.

The main purpose of these experiments was to establish the E values needed to characterize the phase systems under study. Unfortunately, the indirect method for the measurement of surfactant concentration[18] did not allow us to obtain reliable results. We have just managed to find that the E values are different and vary from 0.03 to 0.06 in phase systems I–VI.

Partition of dinitrophenylated amino acids

The concentrations of DNP-amino acids can be measured directly. Hence these compounds can be used for evaluating the relationship between hydrophobic character and partition coefficient in biphasic polymeric systems. Unfortunately, this method for determining the E values came to our attention when the polymer batches needed for systems I–VI had been used up. Therefore, the DNP-amino acids were partitioned in phase systems VII and VIII only.

The relationship between the logarithm of the partition coefficient and the ionic strength of the phase system can be described by eqn. 1. The corresponding A and B values are given in Table III. The A value as a function of the number of CH_2 groups in the amino acid aliphatic side-chain is presented in Fig. 2.

TABLE III

CHARACTERISTICS OF THE PARTITION BEHAVIOUR OF DNP-AMINO ACIDS IN TWO FICOLL–DEXTRAN PHASE SYSTEMS

Logarithm of the partition coefficient depends on the ionic strength (I) of the system as $\ln K = A + BI$ (for details see text). The B values for the DNP-amino acids are 1.624 ± 0.01 and 1.500 ± 0.050 in phase systems VIII and VII, respectively. The polymer compositions of systems VIII and VII are given in the text.

DNP derivative	$-A$	
	Phase system VIII	*Phase system VII*
Glycine	0.190	0.194
Alanine	0.152	0.163
Norvaline	0.107	0.128
Norleucine	0.087	0.106
2-Amino-*n*-octanoic acid	0.019	0.056
Phenylalanine	−0.001	0.039

DISCUSSION

Phase separation in ficoll–dextran mixtures

The data in Fig. 1 and Table I indicate that the ionic composition and/or ionic strength of the polymer mixture and the molecular weight of dextran influence phase

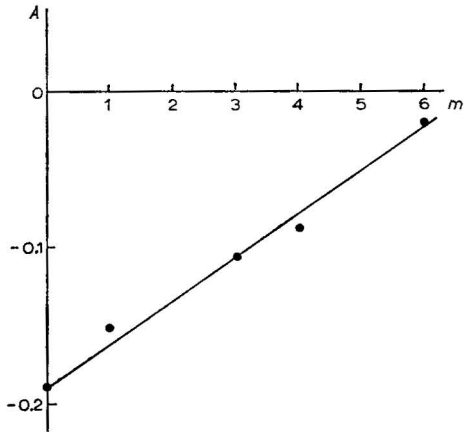

Fig. 2. Value of parameter A as a function of the aliphatic side-chain length (m) of dinitrophenylated glycine, alanine, norvaline, norleucine and 2-amino-*n*-octanoic acid.

separation in the polymer mixtures studied. The dependence of the total polymer concentration required for phase separation on the dextran weight-average molecular weight ($\overline{M}_w$) can be described as

$$C_\Sigma = \alpha + \beta \log \overline{M}_w \tag{3}$$

where C_Σ represents the total concentration of both polymers needed for phase separation and α and β are constants depending on the ionic composition and/or ionic strength of the polymer mixture. α and β are 97.8 ± 4.1 and -16.0 ± 0.9, respectively, in the presence of 0.15 M sodium chloride in 0.01 M sodium phosphate buffer (pH 7.4) (correlation coefficient $r^2 = 0.991$). In the presence of 0.11 M sodium phosphate buffer (pH 7.4), the corresponding α and β values are 76.4 ± 6.8 and -11.9 ± 1.5, respectively ($r^2 = 0.958$). It should be noted that eqn. 3 is valid only for the dextran $\overline{M}_w$ range $30 \cdot 10^3$–$70 \cdot 10^3$ and it may be invalid for dextrans of other molecular weights.

Our data indicate that salts present in a given polymer mixture and their concentration cannot be considered as factors that affect solely the partition behaviour of solutes and particles in the phase systems formed. The salts appear to be the essential components of the system, governing its formation and its separation properties. This seems to be at variance with the accepted point of view[1] that phase separation in mixtures containing only non-ionic polymers occurs independently of the salt composition and concentration. Nevertheless, the data obtained in this study seem to justify the above conclusion.

Partition of solutes and factors of scaling

In order to compare the partition coefficients of solutes determined in two different solvent pairs, the following equation has to be used[20,21]:

$$\ln K_i = a \ln K_0 + b \tag{4}$$

where K_i and K_0 denote the partition coefficient in the two phase systems and a and b are constants.

In order to correlate the partition coefficients obtained in various phase systems, the results should be scaled by selection of any system as a reference. System VIII was selected here as large amounts of the polymer batches needed for this system were available.

As parameter A in eqn. 1 is the ln K value at zero ionic strength in the medium, eqn. 4 can be rewritten as

$$A_i = aA_0 + b \tag{5}$$

where A_i is the A value for a given solute in the ith phase system, A_0 is the A value for the same solute in system VIII and a and b are constants.

It is clear that the values of a and b can be calculated according to eqn. 5 from the ln K values for any two solutes partitioned in phase systems i and VIII. The A values obtained for albumin and myoglobin were treated according to eqn. 5 to determine a and b for the phase systems studied. The a and b values are listed in Table IV. The A values for cytochrome c (A_i^{cyt}) in all of the phase systems were calculated from the corresponding a and b values and the A_o^{cyt} value for cytochrome c in phase system VIII. The calculated A_i^{cyt} values are listed in Table IV together with those found experimentally, and it can be seen that they agree well. The data for the total plasma proteins given above indicate that the parameters a and b can be used for complex protein mixtures as well as for individual proteins.

TABLE IV

FACTORS FOR SCALING THE PARTITION VALUES OBTAINED IN VARIOUS PHASE SYSTEMS (a AND b), CHARACTERISTICS OF THE DIFFERENCE IN THE RELATIVE HYDROPHOBICITIES BETWEEN THE TWO PHASES OF THE SYSTEMS (E) AND THE RELATIVE HYDROPHOBICITIES OF PROTEINS EXPRESSED IN TERMS OF EQUIVALENT CH_2 GROUPS (n)

Phase system	a	b	$A_{calc.}^{cyt}$*	$A_{exp.}^{cyt}$**	E***	$n = (A - b)/E$		
						Albumin	*Myoglobin*	*Cyto-chrome c*
I	0.66	−0.143	0.28	0.28 ± 0.25	0.018	−83.2	−9.83	7.61
II	1.27	0.010	0.26	0.22 ± 0.04	0.034	−84.4	−10.00	6.76
III	1.28	0.007	0.26	0.24 ± 0.14	0.035	−83.6	−9.91	7.06
IV	0.94	−0.125	0.32	0.30 ± 0.26	0.025	−86.2	−10.20	7.00
V	0.81	−0.132	0.30	0.28 ± 0.06	0.022	−83.5	−9.91	6.73
VI	0.45	−0.098	0.19	0.20 ± 0.04	0.012	−86.0	−10.17	8.50
VII	0.81	−0.041	0.22	0.20 ± 0.08	0.022	−84.4	−10.00	7.78
VIII§	—	—	—	0.21 ± 0.04	0.027	−85.6	−9.96	6.91

* A values for cytochrome c calculated as described in the text from the experimental A value found in phase system VIII and the corresponding a and b values.

** Experimental A values found for cytochrome c.

*** E values were determined experimentally in phase systems VII and VIII and calculated for the other phase systems (for details see text).

§ Phase system VIII was chosen as the reference system.

It has been suggested previously[5] that the factor a in eqn. 4 reflects the difference in the relative hydrophobicities between the two phases of a given phase system with respect to that specific for the reference phase system. In order to check this assumption, the partition behaviour of the DNP-amino acids was examined in phase systems VII and VIII.

The A and B values for the amino acid derivatives are given in Table III. The results show that the a and b values calculated from the protein partition coefficients can be used for comparison of the partition behaviour of the DNP-amino acids in systems VII and VIII. This fact indicates that a and b can be used for scaling the partition results obtained in various phase systems for any solutes independent of their chemical nature. The effect of an additional CH_2 group on the ln K value, *i.e.*, parameter E in eqn. 2, can be determined by comparison of the A values for the DNP derivatives of glycine, alanine, norvaline, norleucine and 2-amino-*n*-octanoic acid. The E values are 0.022 and 0.027 in phase systems VII and VIII, respectively.

It can be seen that parameter a in eqn. 4 is related to E_{VII} and E_0 specific for the systems VII and VIII, respectively, by

$$a_{VII} = E_{VII}/E_0 \tag{6}$$

Hence, the E_i values for phase systems I–VI can be calculated according to eqn. 6 in the following form:

$$E_i = a_i E_0 \tag{7}$$

The calculated E_i values for the phase systems studied are listed in Table IV.

It was assumed earlier[4–7] that the relative hydrophobicity of any solute or particle can be expressed in terms of the equivalent number of CH_2 groups (experimental proof will be published later) using the equation

$$n = A/E \tag{8}$$

where n is the equivalent number of CH_2 groups the total hydrophobicity of which is equal to that produced by the compound in question at zero ionic strength in the medium and A and E are as defined above.

Thus, the hydrophobic character of the proteins can be determined from the A_0 and E_0 values found in phase system VIII, and from the corrected A_i values, *i.e.*, $A_i - b_i$, and E_i values obtained in phase systems I–VII. The relative hydrophobicity of cytochrome *c* at zero ionic strength in the medium is equivalent to that of 7.29 ± 0.6 CH_2 groups, human serum albumin is characterized by -84.6 ± 1.2 CH_2 groups and myoglobin by -10.0 ± 0.13 CH_2 groups.

The data reported seem to justify the conclusion that it is possible to compare the partition coefficients obtained in various aqueous polymer biphasic systems. For this correlation any solutes can be chosen as "partition markers" for calibration of the phase system under study relative to that selected as the reference system. This fact allows one to conclude that the partition technique can be used as an analytical tool in various fields of biochemical and biomedical research.

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GAS–LIQUID CHROMATOGRAPHIC SEPARATION OF METHOXY-SUBSTITUTED QUINONES ON NEMATIC LIQUID CRYSTALS

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SUMMARY

The use of liquid crystals N,N′-bis(*p*-butoxybenzylidene)-α,α′-bi-*p*-toluidine and N,N′-bis(*p*-phenylbenzylidene)-α,α′-bi-*p*-toluidine (BPhBT) as stationary phases for gas–liquid chromatographic (GLC) separations of methoxyquinones is reported. The chromatographic profiles of mono- and dimethoxyquinones having 2–5 aromatic rings are presented. The retention ratios for benzanthraquinone/1-CH_3O-/2-CH_3O-/3-CH_3O-/4-CH_3O- at 272°C on BPhBT were 1.00/1.19/1.81/3.63/2.53, and the respective resolutions were $R_{1,2} = 6$, $R_{2,3} = 5$, and $R_{3,4} = 6$. The liquid crystal columns gave better resolution with shorter elution times when compared with OV-17 columns. The orders of elution of the isomers on the liquid crystal columns were predictable from the molecular shape and substitution patterns. This should make it possible to predict molecular structure (isomer position) from the GLC retention data.

INTRODUCTION

During our development of new methods for the synthesis of polycyclic aromatic hydrocarbons (PAHs)[1–3] it has become apparent that the work would be greatly helped if the method used for analyzing the reaction products were also selective enough to give information about the structure. The order of separation obtained with nematic liquid crystal gas–liquid chromatographic (GLC) phases has been attributed to the ordered structure of the liquid crystal mesophase. These ordered structures affect the ability of solute molecules to fit or solubilize into the liquid phase, and this property is the basis of their selectivity. Molecules of nematic liquid crystals while in the nematic state align themselves in a parallel head-to-tail orientation. This orientation contributes to the separation of molecules on the basis of their length-to-breadth ratios and molecular planarity, in addition to, boiling point and polarity differences. The previous success in our laboratory using liquid crystals for the separations of PAHs[4,5] led us to investigate these phases for methoxyquinone separations.

EXPERIMENTAL

A Varian Model 1440 gas–liquid chromatograph equipped with a flame-ionization detector and a linear temperature programmer was used for this study. Chromatograms were generated on a 1-mV f.s. strip-chart recorder using an electrometer setting of 10^{-11} A/mV. The nitrogen carrier gas flow-rate was 60 ml/min, air flow-rate was maintained at 200 ml/min and hydrogen flow-rate was 30 ml/min as measured by a soap-bubbler flow-meter. Sample injection volumes were 1–2 μl. The chromatographic columns were either a 5% OV-17, 2.5% N,N′-bis(*p*-butoxybenzylidene)α,α-bi-*p*-toluidine (BBBT) or 2.5% N,N′-bis(*p*-phenylbenzylidine)α,α'-bi-*p*-toluidine (BPhBT) on 100–120 mesh Chromosorb HP, packed in 6 ft. and 3 ft. × 2 mm I.D. glass columns. The liquid crystal column packings were prepared by the solvent slurry technique and fluidized drying with nitrogen[4,5]. The liquid crystals were carefully purified and glass-distilled chloroform was used for preparing the packing by the solvent slurry technique. Oxygen and water traps were used to remove these substances from the carrier gas. Approximately 2–3 in. at the inlet/outlet ends of each column was packed with a 3–5% Dexsil 300 or SE-30 on 100–120 mesh Chromosorb W HP as terminal buffers. The addition of the conventional phase regions at the ends of each column is recommended if the columns are inserted into the injection port for on-column injection and into the detectors for reduced dead volumes.

We prepared the substituted naphthoquinones (NTQs), anthraquinones (ATQs)[6], phenanthraquinones (PhTQs)[7], benzanthraquinones (BATQs)[1–3] and dibenzanthraquinones (DBATQs)[8] as intermediates in the synthesis of substituted PAHs. Their structures were deduced from the route of synthesis, the spectral and physical data, and by chemical conversion to known compounds.

RESULTS AND DISCUSSION

We studied the separation of quinones and their methoxy isomers on the nematic liquid crystals BBBT and BPhBT and compared the results with those obtained with an OV-17 column (Table I).

The order of elution of the isomers on the liquid crystal columns always followed the same pattern. The isomers having the larger length-to-breadth ratios were retained longer. For example, the elution order BATQ < 1-CH_3O- < 2-CH_3O- < 4-CH_3O- < 3-CH_3O-BATQ was consistent with increasing length-to-breadth ratios. The elution order of BATQ < 11-CH_3O- < 8-CH_3O- = 10 CH_3O- < 9-CH_3O-BATQ was also consistent. The OV-17 column gave an elution order of BATQ < 9-CH_3O- = 10-CH_3O- = 11-CH_3- < 8-CH_3O-BATQ. The elution order for the disubstituted CH_3O-BATQs using a BPhBT phase was 3,11- < 3,8- = 3,10- < 3,9-di-CH_3O-BATQs. The elution order for the disubstituted ATQs was 2,7-diCH_3O-ATQ < 2,6-diCH_3O-ATQ using a BBBT column; no separation was obtained with the OV-17 column. The magnitude of the separations, and the order of elution, was as expected from the differences in molecular shape and the length-to-breadth ratios. The order of elution for the isomers of a particular quinone (*e.g.* 1-, 2-, 3-, and 4-CH_3O-BATQ) and for unsubstituted quinones (*e.g.* ATQ, BATQ, DB[*a*,*j*]ATQ and DB[*a*,*h*]ATQ) were predictable from length-to-breath considerations. The larger the

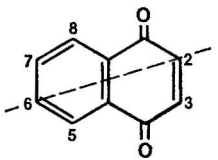
TABLE I

GLC RETENTION DATA FOR METHOXYQUINONES USING BBBT, BPhBT AND OV-17 LIQUID PHASES

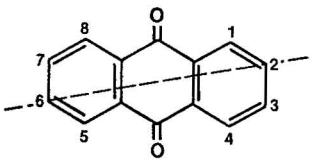
NTQ = Naphthoquinone, ATQ = anthraquinone, PhTQ = phenanthraquinone, BATQ = benzanthraquinone, DB[*a,h*]ATQ = dibenz[*a,h*]anthraquinone, DB[*a,j*]ATQ = dibenz[*a,j*]anthraquinone.

Compound	*Retention Times (min)*				
	BBBT	*BBBT*	*BPhBT*	*BPhBT*	*OV-17*
1,4-NTQ	1.27*	0.97***	—	—	—
6-CH_3O-NTQ	3.82				
5-CH_3O-NTQ	4.65				
1,4-PhTQ	4.13**	10.75	—	—	8.21**
5-CH_3O-PhTQ	4.13				17.91
6-CH_3O-PhTQ	9.64				17.91
7-CH_3O-PhTQ	14.62				22.49
8-CH_3O-PhTQ	10.70				21.40
9,10-ATQ	3.80**	9.93	—	—	4.75§§§
2-CH_3O-ATQ	12.03				11.93
2,7-diCH_3O-ATQ	36.66				31.32
2,6-diCH_3O-ATQ	45.45				31.32
BATQ	—	44.48	3.59§,+	3.48§,+	10.98§§
1-CH_3O-BATQ			4.27	4.16	18.61
2-CH_3O-BATQ			6.48	6.37	20.52
3-CH_3O-BATQ			13.02	12.91	24.95
4-CH_3O-BATQ			9.07	8.96	23.73
8-CH_3O-BATQ				8.59	26.11
9-CH_3O-BATQ				11.82	24.34
10-CH_3O-BATQ				8.59	24.34
11-CH_3O-BATQ				6.21	24.34
3,11-diCH_3O-			17.95+		
3,8-diCH_3O-			26.90		
3,10-diCH_3O-			30.60		
3,9-diCH_3O-			50.52		
DB[*a,h*]ATQ	—	—	32.67§	—	58.0§
1-CH_3O-ATQ			32.67		—
2-CH_3O-ATQ			62.04		110.0
3-CH_3O-ATQ			131.04		138.5
4-CH_3O-ATQ			75.54		131.0
DB[*a,j*]ATQ	—	—	19.56§	—	58.0§
1-CH_3O-ATQ			—		—
2-CH_3O-ATQ			35.85		110.0
3-CH_3O-ATQ			77.06		138.5
4-CH_3O-ATQ			47.35		131.0

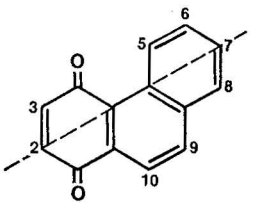
* Column temperature, 190°C.
** Column temperature, 225°C.
*** Column temperature programmed from 192 to 242°C at 4°C /min with a 15-min initial delay.
§ Column temperature, 262°C.
§§ Column temperature, 210°C.
§§§ Column temperature, 226°C.
\+ Separate mixtures of isomers



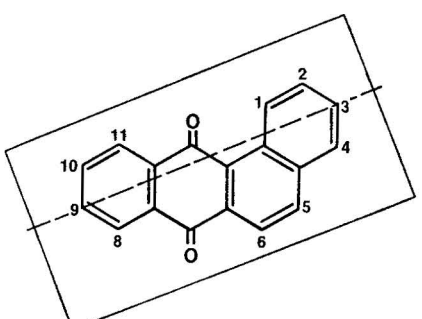
1,4-NTQ



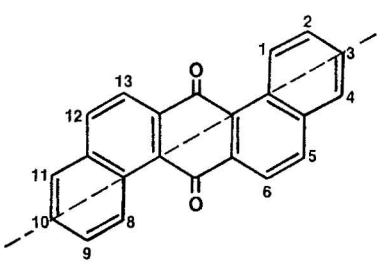
9,10-ATQ



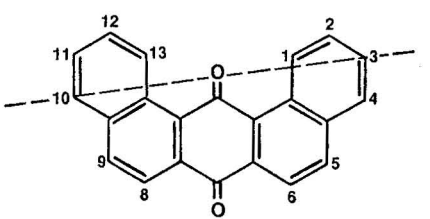
1,4-PhTQ



7,12-BATQ



7,14-DB[a,h]ATQ



7,14-DB[a,j]ATQ

Fig. 1. Structures and numbering used for the methoxyquinones studied.

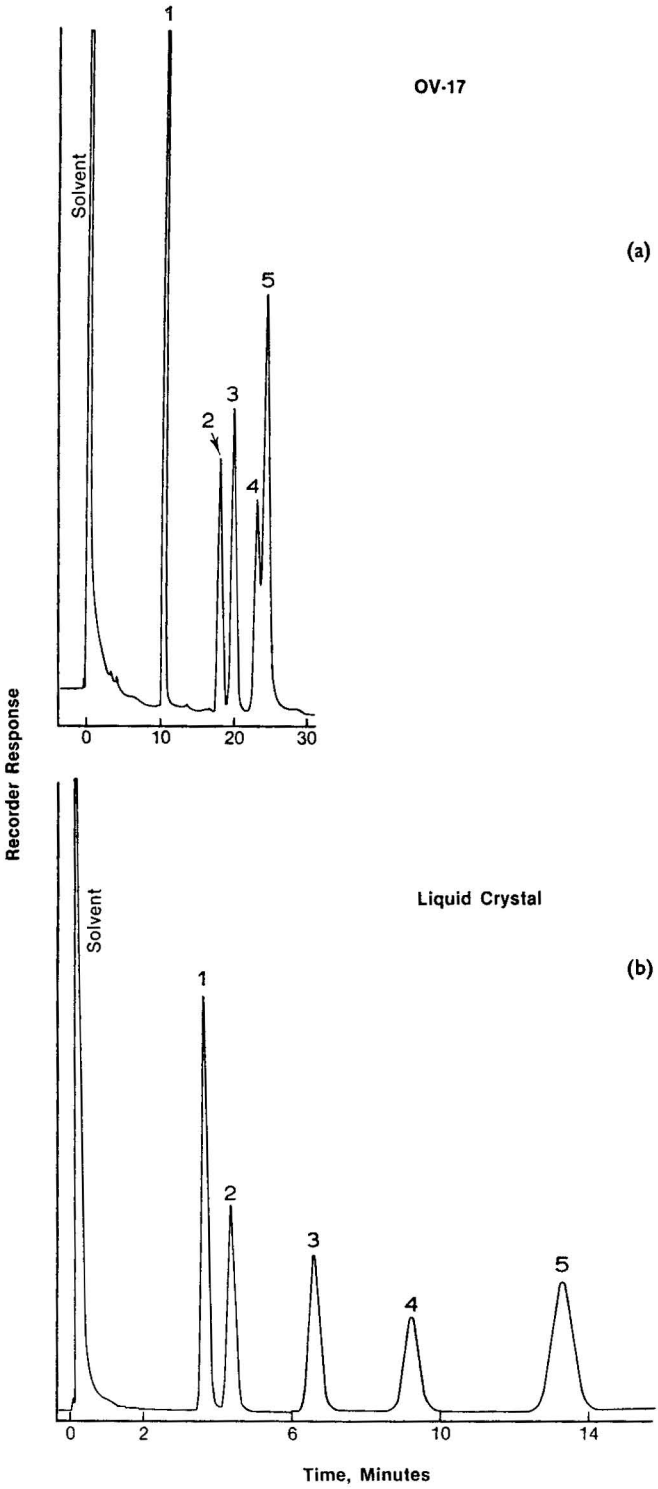


Fig. 2. Chromatograms of 1-, 2-, 3- and 4-methoxybenzanthraquinones. (a) Column: 6 ft. × 2 mm I.D.; packing: 5% OV-17. Conditions: oven 262°C; flow-rate 60 ml/min. (b) Column: 6 ft. × 2 mm I.D.; packing: 2.5% BPhBT. Conditions: oven 262°C; flow-rate 60 ml/min. Peaks: 1 = 7,12-BATQ; 2 = 1-CH_3O-BATQ; 3 = 2-CH_3O-BATQ; 3 = 4-CH_3O-BATQ; 5 = 3-CH_3O-BATQ.

contribution from the methoxy substituent to increasing the length instead of the breadth of this imaginary rectangle (Fig. 1 and Table I, 7,12-BATQ example) the longer the retention time. When the methoxy substituent contributed to the breadth of the molecule, the retention times were shorter. The line drawn between the 3- and 9-positions of BATQ connects the position with the longest retention times (Table I) and should divide this imaginary rectangle. The same exercise can be done with all the methoxyquinones in Table I. This method of predicting elution order holds for the unsubstituted quinones as well as those that are substituted. If 9,10-ATQ is considered to be the parent compound (Fig. 1) then extending its length with the addition of aromatic rings should increase the retention time in a predictable manner. The observed order of elution is ATQ < BATQ < DB[*a*,*j*]ATQ < DB[*a*,*h*]ATQ as expected. This type of consistent elution is potentially useful in predicting structural information of closely related isomeric compounds.

The retention ratios and resolutions of the mono- and dimethoxy isomers were considerably better using the liquid crystal columns than with OV-17. This is illustrated by the separation of the 1-, 2-, 3-, and 4-CH_3O-BATQ in Fig. 2. The retention ratios for these isomers using BPhBT were 1.00/1.52/3.05/2.12 compared

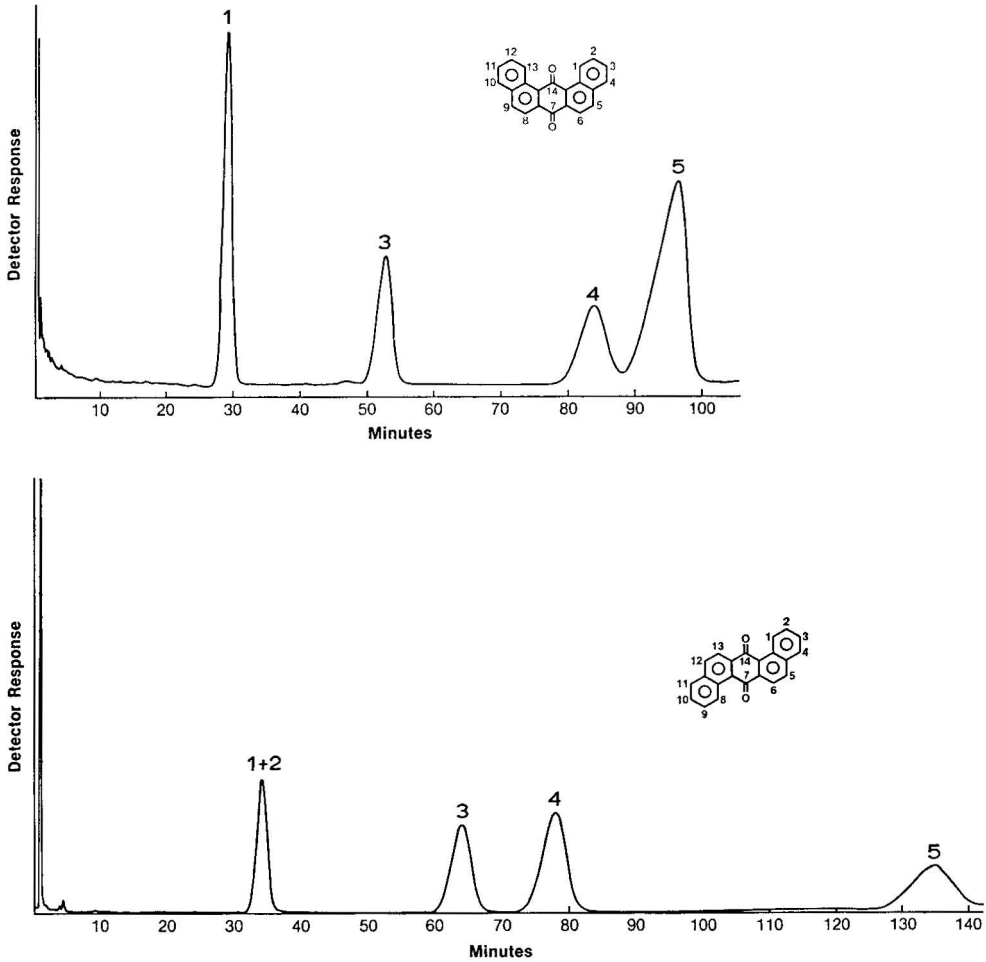


Fig. 3. Chromatograms of monomethoxydibenz[*a*,*h*] and [*a*,*j*]anthraquinones. Column: 3 ft. × 2 mm I.D.; packing: 2.5% BPhBT. Conditions: oven 262°C; flow-rate 60 ml/min. Peaks: 1 = unsubstituted; 2 = 1-CH_3O-; 3 = 2-CH_3O-; 4 = 4-CH_3O-; 5 = 3-CH_3O-.

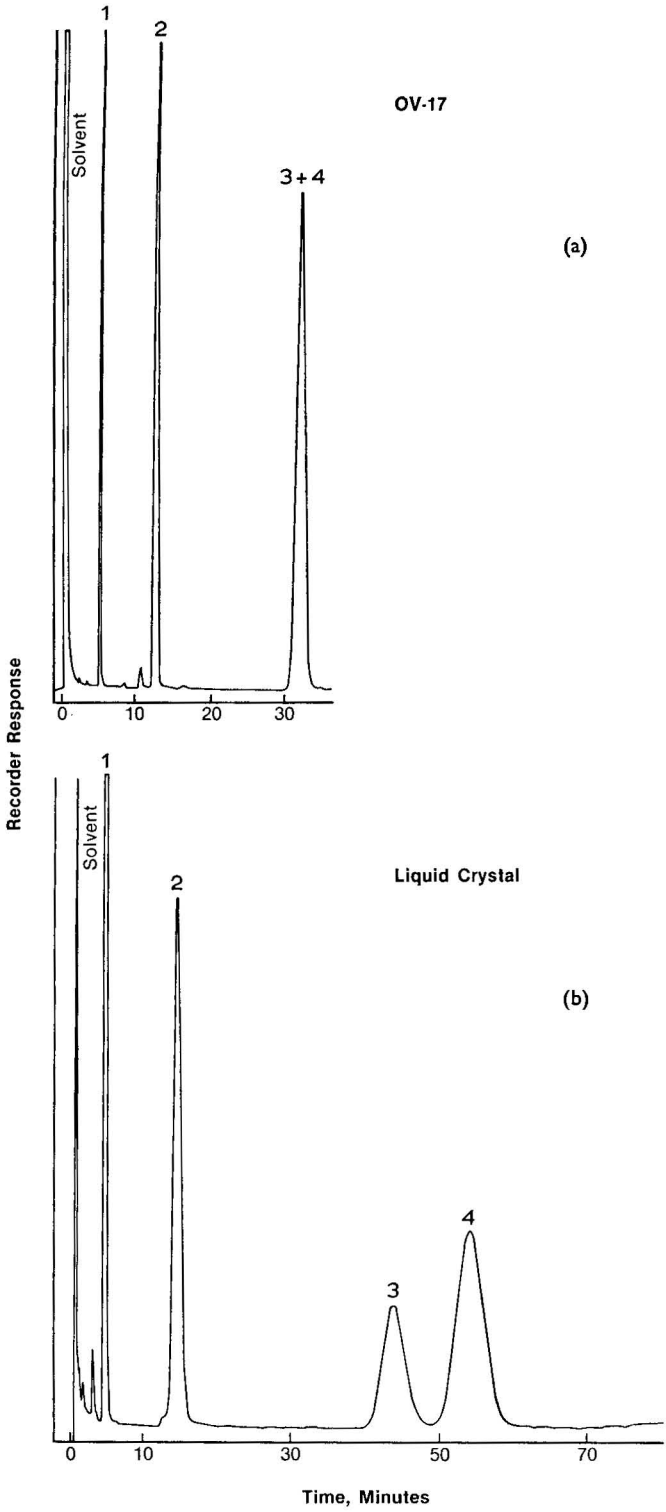


Fig. 4. Chromatograms of mono- and dimethoxyanthraquinones. (a) Column: 6 ft. × 2 mm I.D.; packing: 5% OV-17. Conditions: oven 262°C; flow-rate 60 ml/min. (b) Column: 6 ft. × 2 mm I.D.; packing: 2.5% BBBT. Conditions: oven 225°C; flow-rate 60 ml/min. Peaks: 1 = 9,10-ATQ; 2 = 2-CH_3O-ATQ; 3 = 2,7-diCH_3O-ATQ; 4 = 2,6-diCH_3O-ATQ.

with 1.00/1.10/1.34/1.28 using OV-17. The respective resolutions were $R_{1,2} = 6$; $R_{2,3} = 5$; $R_{3,4} = 6$ for the CH_3O-BATQ on BPhBT, superior to the resolutions obtained with OV-17. The retention ratios for the 5-, 6-, 7-, and 8-CH_3O-PhTQ were 1.00/2.33/3.54/2.60 using BBBT *versus* 1.00/1.00/1.29/1.24 using OV-17. The same improvement in the retention ratio and resolution was also noted for the CH_3O-DBATQ isomers (Table 1 and Fig. 3). Some of the mono- and dimethoxyquinones, which were not resolved on the OV-17 column, were easily resolved on the BBBT and BPhBT columns. The 5- and 6-CH_3O-PhTQ retention ratio on OV-17 was 1.00/1.00, but on BBBT it was 1.00/2.33 with a resolution of 3. Likewise, 2,7-diCH_3O-ATQ and 2,6- diCH_3O-ATQ were not resolved on OV-17 but were resolved with a retention ratio of 1.00/1.24 on BBBT as shown in Fig. 4. The DB[*a,h*]ATQ and DB[*a,j*]ATQ and their positional isomers (*e.g.* 2-CH_3O-DB[*a,j*]ATQ and 2-CH_3O-DB[*a,h*]ATQ) were not separated on OV-17. However, these compounds were easily separated on the liquid crystal columns (Fig. 4) with a retention ratio of 1.00/1.67 for the unsubstituted quinones with a resolution of 3.1.

An additional advantage of liquid crystal compared with OV-17 columns is the shorter elution time. For example, 3-CH_3O-BATQ eluted from BPhBT in 13.02 min *versus* 24.95 min from the OV-17 column. A disadvantage of the BBBT column was the tailing and non-Gaussian peaks observed for the terminal quinones, *e.g.* 1,4-NTQ, 6-CH_3O-NTQ, and 5-CH_3O-NTQ and the 1,4-PhTQ isomers. This was not observed using the OV-17 column. The peak shapes for the internal quinones using the liquid crystal columns were Gaussian as shown in Figs. 2–4.

The isomers with the larger length-to-breath ratios were consistently retained longer on the liquid crystal columns. These columns gave the best separations with the shortest elution times and were consistently better able to separate the closely related isomers. These results enabled us to assign tentative structures to isomers obtained from synthesis reactions from which two isomers were possible due to molecular symmetry or bond rotation. For example, the 1-CH_3O-BATQ and 3-CH_3O-BATQ were obtained from the reaction of naphthoquinone with *meta*-methoxystyrene. The ratio (10:1) of these two isomers in the isolated product mixture[1] was determined and tentative structures were assigned based on the data from the BPhBT and BBBT columns. The resolution of these two isomers was 17 using the BPhBT column. A mixture containing a 10:1 isomer ratio was easily integrated when $R = 17$. The detection, isomer identification, and relative amounts of 2,6- and 2,7-dimethoxyphenanthracene-9,10-diones, from the reaction of 6-methoxynaphthoquinone and 1-methoxy-3-trimethylsilyloxybutadiene, were also determined[6] using the BBBT column (Fig. 3). These columns have also been useful for the detection and quantitation of other isomeric products from synthetic reactions which could not be resolved by thin-layer chromatography or conventional GLC. The liquid crystal columns showed consistent separations based on length-to-breadth ratios which hold promise for predicting molecular structure of closely related isomers.

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MEASUREMENT OF Δ^1-TETRAHYDROCANNABINOL IN PLASMA TO THE LOW PICOGRAM RANGE BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY USING METASTABLE ION DETECTION

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SUMMARY

A method for the assay of Δ^1-tetrahydrocannabinol (Δ^1-THC) in plasma using combined gas chromatography–mass spectrometry with metastable ion monitoring is described. Δ^1-THC was extracted with hexane and the extracts were methylated with diazomethane to shift the peaks produced by endogenous plasma constituents away from the cannabinoid region. The Δ^1-THC was then converted into its trimethylsilyl derivative and quantitated using the metastable ion at m/z 371 formed in in the $M^+ \rightarrow [M - CH_3]^+$ transition with [1″,1″,2″,2″-$^2H_4$]cannabinol as the internal standard. Δ^1-THC could be measured to 5 pg/ml in plasma. This assay is 20–100 times more sensitive than existing assays and has the advantage of not needing the usual extensive purification step.

INTRODUCTION

Plasma levels of Δ^1-tetrahydrocannabinol (Δ^1-THC) during cannabis intoxication are usually in the nanogram range[1] and because of the high lipophilicity of the drug, the levels reached after administration decline very rapidly to the low nanogram or upper picogram region within a few hours of administration. Elimination of the drug from the body is slow but because of the very low plasma levels there have been few direct pharmacokinetic studies. Estimates based on radioactivity measurement suggest a half-life of 1–2 days[2], whereas in the dog a value of 8 days has been found[3]. Current assays of Δ^1-THC in plasma[4,5] do not provide the necessary sensitivity either to obtain accurate pharmacokinetic data for more than a few hours or to confirm the presence of Δ^1-THC for forensic purposes after this time.

Several methods for Δ^1-THC measurement based on gas–liquid chromatography (GLC)[6–10], thin-layer chromatography (TLC)[11,12], gas chromatography–mass spectrometry (GC–MS)[1,13–15] and radioimmunoassay[16–18] have been reported in the recent literature and in two symposia devoted to cannabinoid assays[4,5] and, in addition, combination techniques such as high-performance liquid chromatography (HPLC) coupled with radioimmunoassay[19] or MS[20] have been developed. However, the best of these only provide for measurements to the 100–500 pg/ml range with

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detection limits *ca.* 100–200 pg. Furthermore, except for some radioimmunoassay techniques, considerable clean-up of the extracted drug is required before assay, particularly by GC–MS. Methods based on Sephadex LH-20 chromatography[13,15] or solvent partition[7–9,12,14] are the most common but result in an analysis taking several hours. Radioimmunoassay techniques, although sensitive also detect many cross-reactive THC metabolites. However, an HPLC–radioimmunoassay technique has recently been described[19] which overcomes this problem; this has a reported detection limit of 100 pg/ml.

This paper describes a new assay for Δ^1-THC in plasma, based on GC–MS and using metastable ion monitoring. It is capable of measuring Δ^1-THC in 1 ml of plasma at the 5 pg/ml level and requires no clean-up of the sample before GC–MS. Using this method we have been able to follow the plasma levels of Δ^1-THC in the rabbit for 7 days after a single intravenous dose of 1 mg/kg.

EXPERIMENTAL

Materials

Δ^1-THC was obtained from the National Institute on Drug Abuse (Rockville, MD, U.S.A.) and was found by GC–MS to be 98% pure. [1″,1″,2″,2″-$^2H_4$]cannabinol ([$^2H_4$]CBN) was synthesized from [1″,1″,2″,2″-$^2H_4$]Δ^1-THC by dehydrogenation with chloranil[21,22]; the labelled Δ^1-THC was prepared from the condensation of [1′,1′,2′,2′-$^2H_4$]olivetol with (+)-*trans*-metha-2,8-dien-1-ol as described previously[23]. 3β-Hydroxypregna-5, 16-dien-20-one was obtained from Sigma (London, Great Britain) and N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Aldrich (Milwaukee, WI, U.S.A.). An ethereal solution of diazomethane was prepared from Diazald (Aldrich) by distillation. Plasma was obtained from John Radcliffe Infirmary (Oxford, Great Britain).

All solvents were distilled twice before use and all glassware was silanized with a 5% solution of dichlorodimethylsilane followed by methanol.

Preparation of standard Δ^1-THC samples

Δ^1-THC (100 μg) was converted into its trimethylsilyl (TMS) derivative by reacting it with BSTFA (100 μl) for 10 min at 60°C. Serial dilutions with BSTFA were made to give solutions containing 100 ng, 10 ng, 1 ng, 100 pg, 10 pg and 1 pg/ml.

Solutions containing, 2, 5, 10, 20, 30 and 50 pg of Δ^1-THC with 10 ng of 3β-hydroxypregna-5,16-dien-20-one, and 100, 200, 500 and 1000 pg of Δ^1-THC with 100 ng of the steroid were prepared in BSTFA (100 μl per sample).

For experiments with [$^2H_4$]CBN as the internal standard, 100 μl solutions of Δ^1-THC and [$^2H_4$]CBN were prepared containing 2, 5, 10, 20 and 30 pg of Δ^1-THC with 20 pg of [$^2H_4$]CBN; 50, 100 and 200 pg of Δ^1-THC with 200 pg of [$^2H_4$]CBN; and 500 and 1000 pg of Δ^1-THC with 2000 pg of [$^2H_4$]CBN.

Determination of detection limits and instrumental precision

Samples (1 μl) of derivatized Δ^1-THC were injected at increasing dilution until the peak could no longer be detected. The internal standards were then evaluated in a similar way. Concentration *versus* peak ratio curves were drawn using peak height measurements, and two samples containing 7 pg of Δ^1-THC with the appropriate amounts of each internal standard were injected several times to check the precision of

the peak height measurements in the presence of background noise and as a check on instrumental reproducibility.

Quantitation of Δ^1-THC in plasma

Samples were run in triplicate and extractions were performed in 10-ml screw-cap sample vials. For the calibration curve, Δ^1-THC and [$^2H_4$]CBN in ethanol (up to 20 μl) were added to plasma in the concentrations shown in Table I and left to equilibrate with plasma proteins for 30–60 min. Sodium bicarbonate (*ca.* 1 g) was added and the drugs were extracted with 2 ml of hexane, each sample being agitated for 2 min on a vortex mixer. The samples were then centrifuged for *ca.* 10 min at 1500 *g* to break the emulsion. The extraction was repeated twice and the combined hexane extracts were blown to dryness with a nitrogen stream in a 0.3 ml conical vial. The residue was taken up in methanol (50 μl) and an excess of ethereal diazomethane was added. After 2 min the solution was again blown to dryness with a nitrogen stream and 20 μl of BSTFA were added. The mixture was then heated at 60°C for 10 min. Aliquots (0.1–5 μl) were examined by GC–MS.

TABLE I

CALIBRATION OF Δ^1-THC IN PLASMA

Volume of plasma (ml)	*Δ^1-THC/ml*	*[$^2H_4$]CBN/ml*	*Steroid/ml*
1	1 μg	2 μg	—
1	100 ng	200 ng	—
1	50 ng, 10 ng, 5 ng	20 ng	500 ng
1	1 ng, 500 pg	2 ng	50 ng
1	100 pg, 50 pg, 40 pg	200 pg	10 ng
4	20 pg	20 pg	—
5	10 pg	20 pg	—

Recovery

For measurements of recovery, 3β-hydroxypregna-5,16-dien-20-one was added to the above samples before extraction in the concentrations shown in Table I.

Measurement of plasma Δ^1-THC levels in the rabbit

One female New Zealand white rabbit (2.21 kg) was treated intraveneously (marginal ear vein) with a suspension (2.21 ml) of Δ^1-THC in Tween-80 and physiological saline at a dose of 1 mg/kg. Blood samples were collected into heparinized glass tubes from the marginal ear vein of the other ear periodically for 7 days under procaine local anaesthesia. The plasma was separated by centrifugation and stored at −20°C until required. Samples (1 ml) were used for analysis of the early fractions; 2–3-ml samples were used for later time periods. Δ^1-THC was extracted as described above using [$^2H_4$]CBN as the internal standard; 200 ng of [$^2H_4$]CBN were added to samples taken to 20 min, 20 ng to samples taken to 4 h, 2 ng to samples taken to 12 h, and 200 pg to samples taken to 7 days.

Gas chromatography–mass spectrometry

GC–MS measurements were made with a V.G. Micromass 70/70 F mass spectrometer interfaced via a glass jet separator to a Varian 2400 GLC instrument.

The chromatograph was fitted with a 2 m × 2 mm I.D. glass column packed with 1% SE-30 on 100–120 mesh Gas-Chrom Q (Applied Science Labs., State College, PA, U.S.A.) and operated at 220°C. The injector and separator temperatures were 300°C and 280°C, respectively. Helium at 30 ml/min was used as the carrier gas. The mass spectrometer was operated at 70 eV with a trap current of 1 mA and an ion source temperature of 260°C. Tuning of the metastable peak was achieved as follows: the instrument was tuned to *m/z* 371 with the accelerating voltage at 4 kV and the collector slit was opened to give a flat-top peak (resolution *ca.* 700). The accelerating voltage was then raised to 4.16 kV to bring the metastable ion into focus, and the source focusing controls were adjusted to give maximum sensitivity.

RESULTS AND DISCUSSION

The major factor limiting the sensitivity of most reported assays for Δ^1-THC is interference by endogenous compounds having similar extractive and analytical properties to Δ^1-THC. This results in limits of detection of the drug in plasma being several orders of magnitude higher than can be achieved with the pure compound. Using GC–MS for example, we have observed a 5-pg limit for the TMS derivative of pure Δ^1-THC but find that this is increased to *ca.* 1 ng when the drug is assayed in plasma. It would appear, therefore, that the best way to improve sensitivity would be to reduce the interference from endogenous compounds. Most assays have attempted this by the use of an extensive purification step between the extraction and assay stages. However, by using a low polarity solvent such as hexane[15] to reduce the amount of endogenous material extracted and by improving the selectivity of the GC–MS stage by the use of metastable ion monitoring, we have been able to dispense with the purification stage and to achieve an almost clean background from a blank plasma. Against this, Δ^1-THC can be assayed to the detection limit of the instrument.

Metastable ion monitoring[24] on a Nier–Johnson-type double focusing mass spectrometer involves raising the accelerating voltage to the point where daughter ions formed in a metastable transition in the first field free region acquire sufficient energy to be transmitted through the electrostatic and magnetic analysers. Other fragment ions, most of which arise from the endogenous contaminants by different fragmentation mechanisms, are trapped by the electrostatic analyser leaving an almost clean background.

For the present assay the metastable ion at *m/z* 371 from the $M^+ \rightarrow [M-CH_3]^+$ transition in the spectrum of the TMS derivative was monitored; previous work has indicated that this derivative gives the best GC–MS characteristics[25]. The detection limit for the TMS derivative of pure Δ^1-THC was determined by injecting the compound into the instrument at increasing dilutions. The concentration *versus* peak height ratio was plotted and the response was found to be linear from 1 μg to the detection limit of 500 fg (3:1 signal-to-noise ratio).

The best type of standard for the assay was considered to be one giving a common ion which could be monitored by single ion recording. This method has been reported to give greater precision than the use of deuterated standards with multiple ion monitoring[26] and in any case multiple ion monitoring would involve independent variation of accelerating and electrostatic analyser voltages. An available steroid, 3β-hydroxypregna-5,16-dien-20-one, whose TMS derivative also contained

an ion at m/z 371 formed in a metastable transition from m/z 386 was evaluated as a suitable standard. It had a suitable retention time (6 min 15 sec on SE-30 compared with 2 min 20 sec for Δ^1-THC) but the calibration curve was linear only for samples containing the same amount of the steroid standard injected onto the column. This was attributed to the unequal responses of the compounds and the different slopes of their concentration response lines. Even so, with samples giving a linear calibration from 1 to 50 pg, 7 pg of Δ^1-THC could be measured fairly accurately (found 7.1 pg, coefficient of variation (C.V.) 4.07% ($n = 3$); and 7.1 pg, C.V. = 12.06%, $n = 3$).

The best standard would be another cannabinoid whose spectrum contained a metastable ion at m/z 371 formed from m/z 386 as it should exhibit similar extractive and GC–MS properties to Δ^1-THC. Isomeric THCs (Δ^6- and Δ^7-THC) were investigated, but they did not separate from Δ^1-THC under GLC conditions appropriate for the assay. Consequently, CBN labelled with four deuterium atoms in the side-chain was synthesized from the available [1″,1″,2″,2″-$^2H_4$]Δ^1-THC[23]; the deuterium label increased the molecular weight of its TMS derivative to 386 and the spectrum contained a prominent $[M-CH_3]^+$ ion at m/z 371. It separated from Δ^1-THC on 1% SE-30 (retention time Δ^1-THC = 2 min 20 sec; retention time [$^2H_4$]CBN = 3.0 min) and gave a similar metastable response. Fig. 1 shows the trace recorded for the TMS derivatives of 10 pg of Δ^1-THC and 20 pg of [$^2H_4$]CBN injected onto the column. Using this standard, a linear calibration was obtained from 1 ng to 2 pg; 7 pg was measured as follows: 7.0 pg, C.V. = 3.9% ($n = 7$) and 7.05 pg, C.V. = 4.4% ($n = 4$).

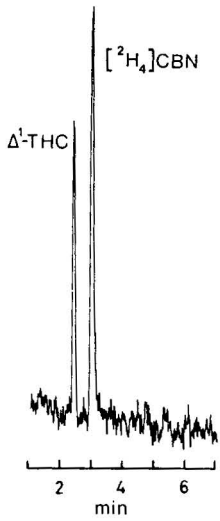

Fig. 1. Metastable ion chromatogram (m/z 386 → m/z 371) of 10 pg of Δ^1-THC and 20 pg of [1″, 1″, 2″, 2″-$^2H_4$]CBN separated as their TMS derivatives on a 1% SE-30 packed column at 220°C.

Δ^1-THC and [$^2H_4$]CBN were extracted from plasma with hexane in the presence of sodium bicarbonate. When the samples as their TMS derivatives were examined by GC–MS, additional peaks were present (Fig. 2) which interfered with the measurement of Δ^1-THC. These endogenous compounds were also extracted from

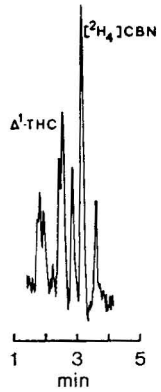

Fig. 2. Metastable ion chromatogram (m/z 386 → m/z 371) of 10 pg of Δ^1-THC and the [$^2H_4$]CBN standard extracted from plasma and separated as their TMS derivatives (1% SE-30, 220°C).

blank plasma and could not be separated from Δ^1-THC on either SE-30 or OV-17 columns. From their GLC characteristics they appeared to be fatty acids. The plasma extracts were therefore methylated with diazomethane before TMS ether formation in an attempt to achieve selective methylation of the endogenous compounds. Under the conditions used no reaction was observed for Δ^1-THC but the endogenous compounds were completely methylated and disappeared from the chromatogram. Chromatograms from blank plasma samples then contained no peaks in the cannabinoid region and against this Δ^1-THC could be measured to 5 pg/ml. In addition, no differences were found either in the background or the extractive properties of Δ^1-THC and [$^2H_4$]CBN from rabbit, guinea-pig or human plasma.

Recovery of Δ^1-THC and [$^2H_4$]CBN was measured using 3β-hydroxypregna-5,16-dien-20-one as the internal standard and were in the range 70–80% for all concentrations tested (Table I). It was essential that all glassware was silanized before use to achieve high recovery. It was then well washed with redistilled ethyl acetate before use. The calibration curve using [$^2H_4$]CBN as the internal standard was linear over the range tested (1 μg to 10 pg/ml) and is shown in Fig. 3. The chromatogram for one of the 10 pg/ml samples is shown in Fig. 4. Accuracy and precision measurements are shown in Table II. The average time for a complete assay was *ca.* 1 h. Care was taken to ensure that, during repetitive injections onto the GLC column, the cannabinoid peaks did not co-elute with cholesterol from a previous injection (retention time = 19 min). This compound appeared to be the most concentrated residual plasma component and its spectrum contained a weak metastable ion at m/z 371. When co-elution did occur the metastable ion abundance from the cannabinoid was increased considerably thus upsetting the assay. No other interactions of this type were observed. In no case did we find sufficient CBN levels in the plasma, either with the calibration samples or in subsequent experiments involving treatment of animals, to interfere with the peak height of the [$^2H_4$]CBN standard; earlier reports that CBN is a major intermediary metabolite of Δ^1-THC[27] appear to have been discounted[28].

To evaluate the assay under experimental conditions, a female New Zealand white rabbit was treated intravenously with Δ^1-THC at a dose of 1 mg/kg and plasma

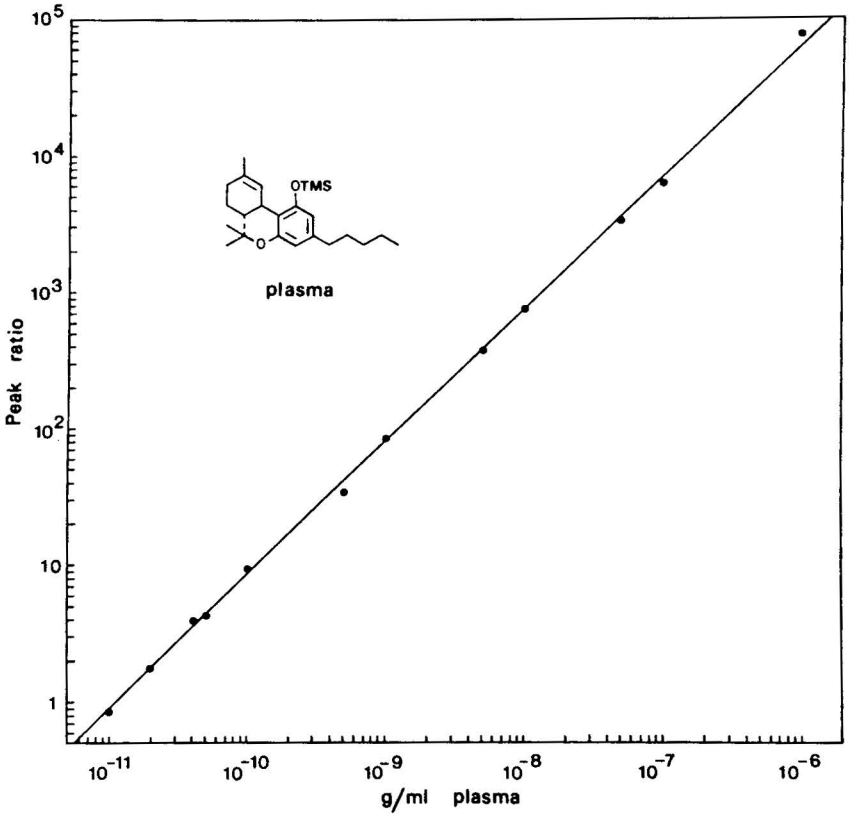

Fig. 3. Calibration curve for Δ^1-THC and [$^2H_4$]CBN extracted from plasma.

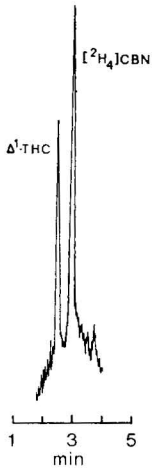

Fig. 4. Metastable ion chromatogram (m/z 386 → m/z 371) of 10 pg of Δ^1-THC and the [$^2H_4$]CBN standard separated on a 1% SE-30 packed column at 220°C following methylation and trimethylsilylation of the extract.

TABLE II

ACCURACY AND PRECISION FOR THE DETERMINATION OF Δ^1-THC IN PLASMA

Actual concn.	*Observed concn.*	*C.V. (%)*	*n*
30 pg	26.5 pg	12.1	5
70 pg	74.0 pg	10.3	5
300 pg	258 pg	0.74	3
3 ng	3.01 ng	9.1	3
30 ng	28.7 ng	3.2	3
300 ng	293 ng	6.4	2

levels were followed to the detection limit of the assay. Δ^1-THC could be measured for 7 days to a level of 10 pg/ml (Fig. 5). A chromatogram from the sample taken on day 7 is shown in Fig. 6.

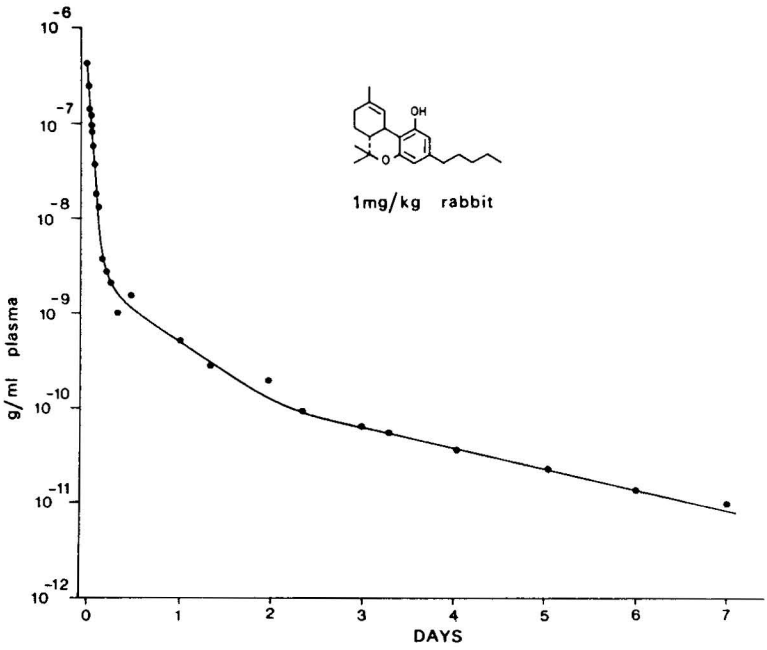

Fig. 5. Plasma levels of Δ^1-THC in the rabbit following a 1 mg/kg intravenous injection.

CONCLUSION

This assay, based on metastable ion monitoring, is more sensitive than existing assays and can detect Δ^1-THC to 5 pg/ml in plasma using 1-ml plasma samples. In addition it requires no purification step between extraction and assay stages; the time taken for a single assay is thus reduced to *ca.* 1 h. Using the method it is possible to follow Δ^1-THC elimination in plasma for times in excess of one week and it should thus be possible to obtain accurate pharmacokinetic data. As metastable transitions

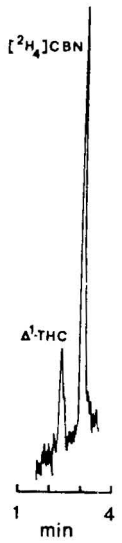

Fig. 6. Metastable ion chromatogram of Δ^1-THC and the [$^2H_4$]CBN standard from the sample taken on day 7 from the rabbit treated with Δ^1-THC.

can be promoted by the use of a collision cell[29,30] it is hoped that in the near future the sensitivity of this assay can be improved still further.

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CHROM. 13,223

GAS CHROMATOGRAPHIC ANALYSIS OF α-KETO ACIDS IN AQUEOUS SOLUTION AS THE O-(2,3,4,5,6-PENTAFLUOROBENZYL)OXIMES OF THEIR METHYL ESTERS

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(First received May 20th, 1980; revised manuscript received August 5th, 1980)

SUMMARY

As an extension of previous work on carbonyl compounds, a method is described for the gas chromatographic analysis of α-keto acids in aqueous solution. The keto acids were derivatized with pentafluorobenzyloxylamine to yield the oximes. After extraction with ethyl acetate from acidified, salt-saturated solution, these compounds were esterified with diazomethane and separated by gas chromatography.

INTRODUCTION

An elevated excretion of α-keto acids has proved to be of great value as a biochemical marker of hereditary metabolic diseases such as maple syrup urine disease and phenylketonuria. For gas chromatographic (GC) analysis, α-keto acids have to be converted into various volatile and/or stable derivatives, such as methyl esters[1], methyloximes of trimethylsilyl esters[2], trimethylsilyloximes of trimethylsilyl esters[3], O-trimethylsilylquinoxalinol derivatives[4–8] and 2,4-dinitrophenylhydrazone methyl esters[9]. The 2,4-dinitrophenylhydrazones of α-keto acids have been used successfully for the isolation of these acids. Kallio and Linko[9] carried out the GC of the α-keto acid hydrazones after converting them into methyl esters. Recently, we reported that pentafluorophenylhydrazones[10] or O-pentafluorobenzyloximes (O-PFBO)[11] of carbonyl compounds were much more volatile than the corresponding 2,4-dinitrophenylhydrazones, and therefore their GC separation could be performed at much lower temperatures. As an extension of our work on carbonyl compounds, this paper describes a method for the separation and quantification of α-keto acids in aqueous solution by GC as the O-PFBO derivatives of their methyl esters.

EXPERIMENTAL

Reagents

O-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine (pentafluorobenzyloxylamine, PFBOA) hydrochloride was synthesized from pentafluorobenzyl bromide (Aldrich,

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Milwaukee, WI, U.S.A.) and N-hydroxyphthalimide (Tokyo Kasei, Tokyo, Japan)[11]. α-Keto acids (pyruvate, α-ketobutyric acid, α-ketovaleric acid, α-ketoisovaleric acid, α-ketocaproic acid, α-ketoisocaproic acid, α-keto-β-methyl-*n*-valeric acid and α-ketooctanoic acid) were obtained from Sigma (St. Louis, MO, U.S.A.).

Sample solutions were aqueous solutions containing 0.5 μmol/ml of each α-keto acid. The internal standard (IS) was a 0.5 μmol/ml solution of *p*-xylylene dichloride in ethyl acetate.

Apparatus and conditions

A Shimadzu Model GC-4APF gas chromatograph equipped with a hydrogen flame-ionization detector (FID) and a GC-4APE gas chromatograph equipped with a 10-mCi Ni-63 electron-capture detector (ECD) were used. Separations were carried out by using a glass column, 2.0 m × 3 mm I.D., packed with 3% XE-60 on 80–100-mesh Celite 545 (AW DMCS). The column temperature was 130°C, the detector temperature 150°C, the injection temperature 150°C and the chart speed 0.25 cm/min.

Standard procedure

To a 1.0-ml aliquot of sample solution containing α-keto acids in a 10-ml centrifuge tube was added 0.5 ml of PFBOA solution (1.0 mg/ml; *ca.* $4.0 \cdot 10^{-6}$ *M*). The mixture was shaken well and allowed to stand for 30 min at room temperature. After saturation with sodium chloride and acidification with 1 drop of 18 *N* sulphuric acid, the O-PFBO derivatives of the α-keto acids were extracted with 1.0 ml of ethyl acetate containing *p*-xylylene dichloride (0.5 μmol/ml) as internal standard. Excess of sodium chloride and the aqueous layer were removed with the aid of a syringe with a long needle. After evaporation of the ethyl acetate extract in another small vessel, the oximes were converted into their methyl esters with freshly prepared diazomethane in diethyl ether. The reaction mixture was evaporated to dryness again and the residue of methyl esters was dissolved in 0.3 ml of ethyl acetate for GC analysis. Quantitation was carried out using calibration graphs obtained from known amounts of α-keto acids.

RESULTS AND DISCUSSION

The retention times of the O-PFBO derivatives of eight α-keto acid methyl esters relative to that of pyruvate obtained on different columns are given in Table I. In the chromatograms of the O-PFBO derivatives of α-keto acid methyl esters, the formation of a double peak was observed, corresponding to *syn* and *anti* isomers. The appearance of similar double peaks caused by isomeric forms of O-PFBO derivatives of carbonyl compounds has previously been observed on gas chromatograms[11]. This stereoisomerism seemed to make qualitative and quantitative work with α-keto acids difficult and has, in fact, been indicated as a disadvantage of hydrazones or oximes derivatives for the GC of carbonyl compounds. However, PFBOA has the following merits as a derivatization agent for their GC. The reaction of carbonyl compounds with PFBOA proceeded readily in weakly acidic media (pH 2–5) at room temperature to yield compounds extractable from aqueous solution with organic solvents, and the complete removal of unreacted reagent was

TABLE I

RELATIVE RETENTION TIMES OF O-PENTAFLUOROBENZYLOXIMES OF α-KETO ACID METHYL ESTERS

α-Keto acid	*Stationary phase and column temperature*			
	3% XE-60 (130°C)	*1.5% SE-30 (110°C)*	*1.5% OV-17 (100°C)*	*2% OV-1 (120°C)*
Pyruvic acid	0.73, 1.00* (1:17.7)	0.72, 1.00	0.72, 1.00	0.75, 1.00
α-Ketobutyric acid	0.95, 1.15 (1:4.3)	1.05, 1.32	0.99, 1.27	1.05, 1.26
α-Ketovaleric acid	1.32, 1.54 (1:1.5)	1.52, 1.87	1.46, 1.83	1.53, 1.91
α-Ketoisovaleric acid	1.02, 1.20 (6.5:1)	1.25, 1.52	1.06, 1.39	1.17, 1.40
α-Ketocaproic acid	1.95, 2.27 (1:2.3)	2.51, 3.06	2.42, 3.05	2.30, 2.79
α-Ketoisocaproic acid	1.51, 1.73 (1:1.4)	1.97, 2.39	1.69, 2.14	1.78, 2.15
α-Keto-β-methyl-*n*-valeric acid	1.44, 1.56 (8.8:1)	1.83, 2.13	1.63, 1.99	1.74, 2.10
α-Ketooctanoic acid	3.76, 4.02 (1:6.8)	6.03, 6.94	6.36, 7.65	5.99, 7.05

* The retention time of pyruvate was taken as unity; all α-keto acids showed double peaks corresponding to *syn* and *anti* isomers resulting from condensation reactions with PFBOA. Values in parentheses show the ratio of the areas of the two peaks on the chromatogram.

easily achieved. The resulting derivatives were highly stable in organic solvents, much more volatile than other derivatives, and therefore their GC separation could be carried out at much lower temperatures. Also, the O-PFBO derivatives were extremely sensitive to the electron-capture detector.

O-Trimethylsilyl-quinoxalinols are very useful derivatives for the GC of α-keto acids because of the absence of stereoisomerism[5–8]. They are eluted as a single peak on the chromatogram. However, with regard to rapid reaction, volatility of the derivatives, sensitivity to the electron-capture detector and simplicity of procedure, PFBOA is a much better derivatization agent. In addition, the double peaks consisted of a main peak and a smaller peak (shown in parentheses in Table I) and under constant reaction conditions the main peak was sufficiently stable for determination. All measurements were performed using main peaks. The smaller peak usually came before the main peak, but the ratio of the peak area of the second one (with the longer retention time) to that of the first became smaller with increasing bulk of the R group in the α-keto acid (R-CO-COOH), and with α-ketoisovaleric acid and α-keto-β-methyl-*n*-valeric acid the ratio was reversed, presumably owing to steric hindrance by a methyl group in position 3. The above phenomena suggest that the first of the double peaks is the *syn*- form.

Typical GC separations of some α-keto acids as their O-PFBO methyl derivatives are illustrated in Fig. 1, in which the smaller "secondary peaks" of each α-keto acid are indicated as 1′, 2′ and 3′. The O-PFBO methyl derivatives were about 1000 times more sensitive to the ECD than to the FID. A stable response was

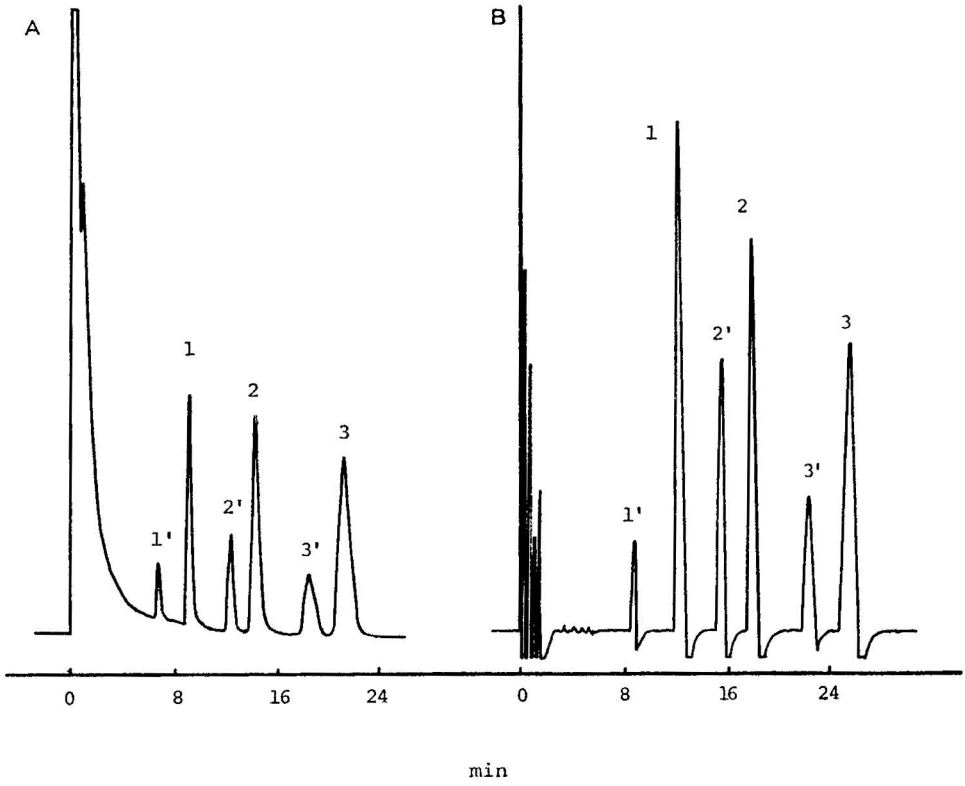

Fig. 1. Gas chromatograms of a mixture of three α-keto acids as their pentafluorobenzyloxime methyl esters on a 3% GE XE-60, 2.0-m glass column, 130°C. 1 = Pyruvic acid; 2 = α-ketovaleric acid; 3 = α-ketocaproic acid. The secondary peaks of the compounds are indicated by 1′, 2′ and 3′, respectively. (A) Analysis with an FID; 0.5 μg of each α-keto acid was injected. (B) Analysis with an ECD; 0.5 ng of each α-keto acid was injected.

observed on the chromatogram even for the injection of 10 pg of α-keto acids as their O-PFBO derivatives.

A series of preliminary investigations was carried out in order to find suitable conditions for reaction and extraction. The formation of the O-PFBO derivatives in aqueous solution was easily achieved with low concentrations of PFBOA. The reagent concentration was made about four times greater than those of the α-keto acids. Fig. 2 shows the effect of variation of pH on the extent of the condensation reaction with 1.0 ml of 0.5 μmol/ml of α-ketobutyric acid according to the procedure described under Experimental. The measured values were constant at pH 2–5 (see Fig. 2).

The effects of variations in the reaction temperature and reaction period on the extent of the condensation reaction were investigated. Fig. 3 shows the results with 1.0 ml of 0.5 μmol/ml of pyruvate. As can be seen, the condensation reaction was complete in 30 min at room temperature, after which the measured values were constant for up to 120 min. The reaction period for α-keto acids was therefore fixed at 30 min at room temperature in order to obtain constant extents of reaction. Ethyl

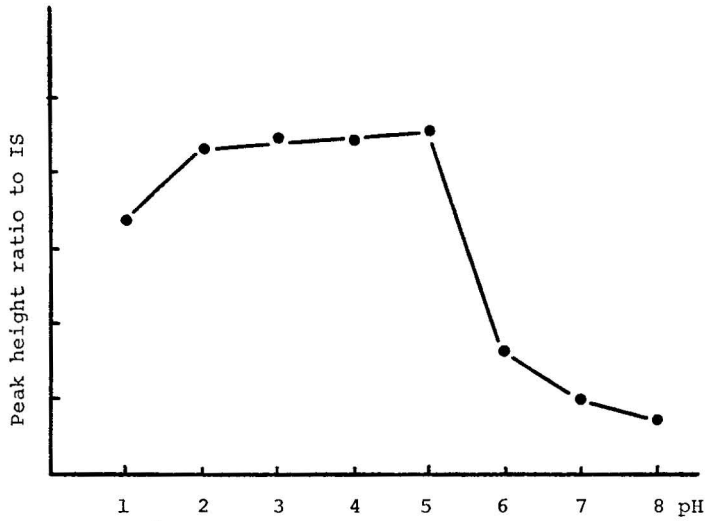

Fig. 2. Effect of pH on the condensation reaction of α-ketobutyric acid with PFBOA in aqueous solution.

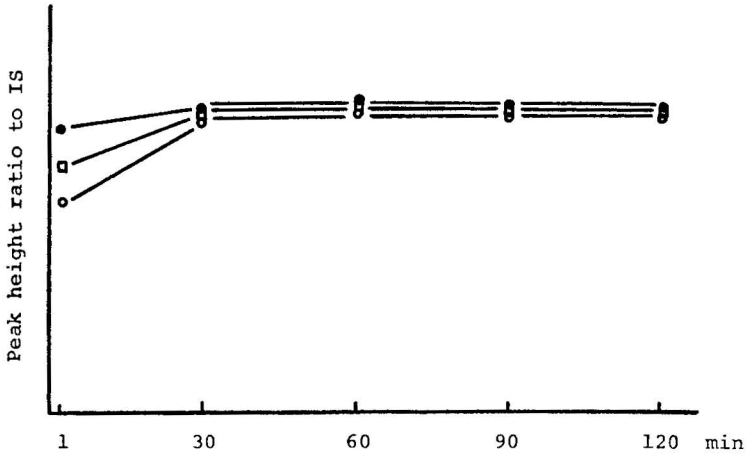

Fig. 3. Effects of variations in the reaction temperature and reaction period on the extent of the condensation reaction of pyruvate with PFBOA in aqueous solution. ○, 0°C; □, room temperature; ●, 60°C.

acetate was a suitable solvent for the extraction of the oximes. Salting-out improved the extent of extraction.

The effect of pH of the solution on the extent of extraction was examined. With the O-PFBO derivative of pyruvate, the extent of extraction increased with increasing acidity of the medium. In addition, in order to prevent an excess of PFBOA from being extracted, extraction was carried out in acidic media. A sample solution containing individual α-keto acids was measured according to the procedure described and linear relative response graphs passing through the origin were obtained with all of the α-keto acids studied for concentrations in the range 0.2–1.0 μmole in 1.0 ml of aqueous solution (Fig. 4).

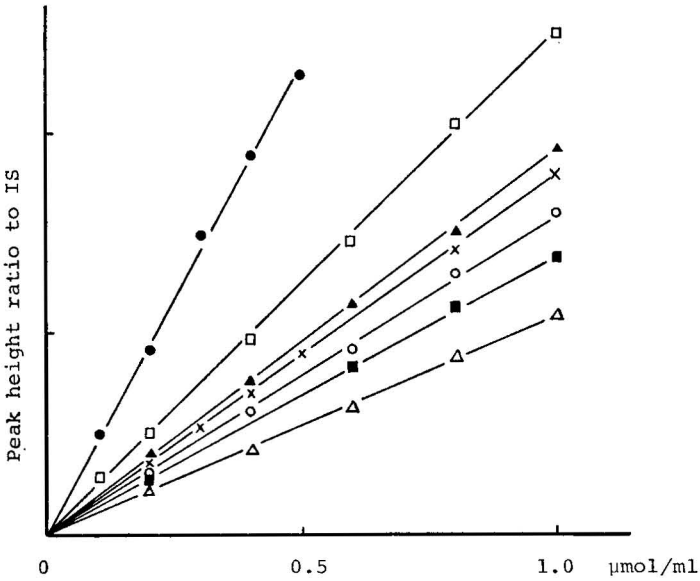

Fig. 4. Calibration graphs for some α-keto acids. ■, Pyruvic acid; △, α-ketobutyric acid; ●, α-ketovaleric acid; □, α-ketoisovaleric acid; ▲, α-ketocaproic acid; ×, α-ketoisocaproic acid; ○, α-keto-β-methyl-*n*-valeric acid. Conditions: 3% XE-60, 2.0-m glass column, FID. *p*-Xylylene dichloride was used as an internal standard.

On an identical sample solution containing 0.5 μmol/ml of α-keto acid in aqueous solution, the coefficient of variation after repeated derivatizations was 4.5% for pyruvate ($n = 7$) and 1.9% for α-ketobutyric acid ($n = 5$).

From the above results, it is concluded that PFBOA is an excellent derivatization agent for the GC determination of α-keto acids in aqueous solution and therefore the method should be of use for the determination of very small amounts of α-keto acids in biological fluids.

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CHROM. 13,181

DETERMINATION OF CLINDAMYCIN IN PHARMACEUTICALS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING ION-PAIR FORMATION

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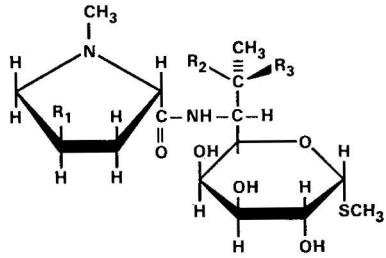
SUMMARY

A reversed-phase ion-pairing high-performance liquid chromatographic procedure with refractive index or UV 214 nm detection was developed for the separation of clindamycin, clindamycin B, and 7-epiclindamycin. The chromatographic retention behavior of these compounds on an octadecylsilane column was investigated as a function of pairing-ion, mobile phase composition, and pH. The method was applied to the determination of clindamycin in bulk drug and in a number of pharmaceutical formulations. The relative standard deviations for all assays was in the 0.5–2% range.

INTRODUCTION

Clindamycin (V, Fig. 1) is a highly effective antibiotic against Gram positive aerobes and both Gram negative and Gram positive anaerobic pathogens[1]. It is synthesized from Lincomycin (I, Fig. 1), an antibiotic produced by microbial fermentation, by substituting chlorine for the hydroxyl group in the 7 position which results in an increase in biological activity.



I	LINCOMYCIN	R_1 = $CH_3-CH_2-CH_2-$	R_2 =	OH	R_3 =	H
II	LINCOMYCIN B	R_1 = CH_3-CH_2-	R_2 =	OH	R_3 =	H
III	CLINDAMYCIN B	R_1 = CH_3-CH_2	R_2 =	H	R_3 =	Cl
IV	7-EPICLINDAMYCIN	R_1 = $CH_3-CH_2-CH_2-$	R_2 =	Cl	R_3 =	H
V	CLINDAMYCIN	R_1 = $CH_3-CH_2-CH_2-$	R_2 =	H	R_3 =	Cl

Fig. 1. Structures of clindamycin and related compounds.

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Common impurities in clindamycin bulk drug are clindamycin B (III), 7-epiclindamycin (IV), and a small amount of the lincomycin starting material (Fig. 1). Clindamycin B is formed from the small amount of lincomycin B (II) which is a normal by-product produced during fermentation; 7-epiclindamycin is produced during the synthesis of clindamycin, presumably by attack of chloride ion under acid conditions on an oxazoline intermediate[2].

Several methods have been reported for the determination of clindamycin in bulk drug and dosage forms including a microbiological method[3], gas–liquid chromatography (GLC)[4], and high-performance liquid chromatography (HPLC)[5]. The microbiological method is time-consuming and only measures total activity. The GLC method requires an extraction and a derivatization step. The HPLC method, although rapid, does not separate the epimers, clindamycin and 7-epiclindamycin.

The present study was undertaken to develop a rapid HPLC method capable of separating lincomycin, clindamycin, 7-epiclindamycin, and clindamycin B. The effects of mobile phase composition, pH, and pairing-ion were studied to find conditions where all compounds are separated.

EXPERIMENTAL

Apparatus

A modular HPLC was used for this work consisting of a single-piston pulse-dampened Altex pump (Model 110A, Berkeley, CA, U.S.A.); a syringe-loaded six-port injection valve fitted with a 10- or a 25-μl loop (Model 7120, Rheodyne, Berkeley, CA, U.S.A.); a 30 cm $\times$ 3.9 mm I.D. μBondapak® C_{18} column (No. 27324, Waters Assoc., Milford, MA, U.S.A.); a differential refractometer detector (Model R401, Waters Assoc.); a 214-nm fixed-wavelength UV detector with a Zn-line source (Model 1203, Laboratory Data Control, Riviera Beach, FL, U.S.A.); and a 10-mV recorder operated at 0.5 cm min^{-1} (Model XKR, Sargent Welch, Skokie, IL, U.S.A.). Data were collected and processed using the computer system developed in house[6]. pH values were measured on the final methanol–water mobile phase so only "apparent" values are measured.

Reagents

Sterile, double-distilled water; acetic acid (No. 2504, Mallinckrodt, St. Louis, MO, U.S.A.); distilled-in-glass UV grade methanol (Burdick & Jackson, Muskegon, MI, U.S.A.); and internal standard phenethyl alcohol (No. 313, Eastman, Rochester, NY, U.S.A.) were used as received. All the ion-pairing reagents were from Eastman: ethane sulfonic acid (No. 6217), sodium butane sulfonate (S035), sodium pentane sulfonate (No. 10178), sodium hexane sulfonate (No. 10263), sodium octane sulfonate (No. 10265), and D,L-10-camphor sulfonate (No. 5057).

Samples of clindamycin B, 7-epiclindamycin, clindamycin, lincomycin, and lincomycin B all as hydrochloride salts were supplied by the Fine Chemical Division of The Upjohn Company. The purity of the clindamycin reference standard was determined by the Control Division of The Upjohn Company using several complementary techniques.

Mobile phase

All mobile phases contained 0.005 *M* pairing-ion in methanol–water mixtures. After mixing, the mobile phases were adjusted to the desired pH with 1 *M* sodium hydroxide or hydrochloric acid, filtered through a 5-μm filter, and degassed by sonication and vacuum.

Two mobile phases were used for analysis of samples. When the differential refractometer was used as the detector, the mobile phase was composed of methanol–water (60:40), 2 ml glacial acetic acid per liter (0.035 *M*), and 0.005 *M* D,L-10-sodium camphor sulfonate, adjusted to pH 6.0. When 214 nm UV detection was used, the mobile phase was composed of methanol–water (60:40), 0.01 *M* phosphate buffer, and 0.005 *M* sodium pentane sulfonate.

The column was operated at 900 p.s.i.g. at a flow-rate of 1.0 ml min^{-1} for all experiments.

Sample preparation and analysis

Bulk drug samples were prepared by dissolving accurately weighed samples (*ca.* 15 mg) in 1.0 ml of internal standard solution (0.5 ml of phenethyl alcohol in 100 ml of mobile phase). Aliquots (25 μl) of the samples were chromatographed, corresponding to 375 μg on column, at a detector setting of 16 $\times$, $3.8 \cdot 10^{-4}$ refractive index units full scale, when the differential refractometer was used as a detector. Amounts of clindamycin were determined by comparison of peak height or peak area ratios from the sample preparation to those obtained from a standard prepared in an analogous manner.

Hard-filled capsules (HFCs) were prepared by emptying the contents of 10 capsules, mixing, weighing an amount equivalent to *ca.* 75 mg of clindamycin base, extracting for 30 min with mobile phase containing internal stadard, filtering, and chromatographing.

Syrups were prepared by pipetting an amount equivalent to 50 mg of clindamycin base, adding 5 ml of internal standard, and chromatographing.

When added sensitivity is required, a 214 nm UV detector is employed using a mobile phase containing pentane sulfonate. In this case, the amount of clindamycin injected on column can be reduced by a factor of four to obtain an equivalent signal-to-noise ratio with the differential refractometer detector.

RESULTS AND DISCUSSION

The most difficult compounds to separate in developing this procedure were the epimers, clindamycin (V) and 7-epiclindamycin (IV). They differ structurally only in the configuration of the chlorine and hydrogen in the 7 position. Since clindamycin is extremely water-soluble (800 g l^{-1}) and is protonated at the normal operating range of most HPLC columns (pK_a 7.6), a reversed-phase ion-pairing system was used.

Chromatography was optimized by varying the methanol–water ratio, pH, and the ion-pairing reagent in the mobile phase.

A methanol–water ratio of 60:40 (v/v) gave a capacity factor for clindamycin in the 1–4 range. Under these conditions, clindamycin and 7-epiclindamycin coeluted. Addition of D,L-10-camphor sulfonate to the mobile phase sharpened the peaks and greatly reduced tailing by ion-pairing with the charged tertiary amine.

The greatest improvement in the selectivity of the mobile phase was obtained by varying the pH. The dependence of the capacity factors on the mobile phase pH in the range 2.5–6.5 for compounds I–V are given in Fig. 2. Lincomycin and lincomycin B eluted close to the solvent front independent of the mobile phase pH, demonstrating the importance of the substituted propyl group on retention. Clindamycin and 7-epiclindamycin are more non-polar than clindamycin B, propyl group *vs.* ethyl group on the pyrrolidine ring, and elute later at all pH values. At low pH values, clindamycin and 7-epiclindamycin coelute. In the pH range 5–6.2, clindamycin and 7-epiclindamycin are baseline resolved. With further increase in basicity, the retention of clindamycin becomes longer and 7-epiclindamycin and clindamycin B coelute.

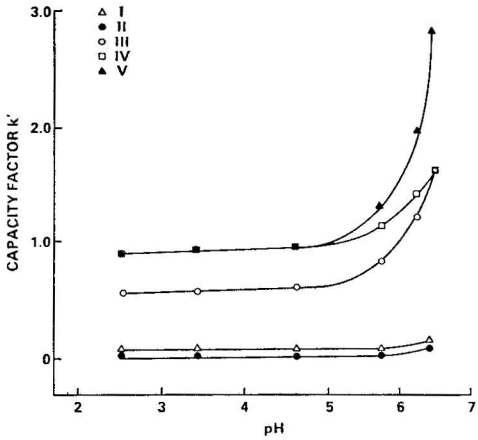

Fig. 2. Dependence of the capacity factors (k') of compounds I–V on the apparent pH of the mobile phase. Chromatography was performed on a 10-μm μ-Bondapak C_{18} column with a mobile phase of methanol–water (60:40), 0.01 *M* ammonium nitrate, 0.005 *M* camphor sulfonate, and 0.035 *M* acetic acid. The pH was adjusted with 0.1 *M* HCl or NaOH.

The effect of chain length of the pairing-ion on the capacity factors of compounds I–V was studied. Fig. 3 shows a plot of capacity factor *vs.* the length of straight-chain alkyl sulfonates C_2–C_8. The length of the chain had no effect on the retention of lincomycins and little effect on the clindamycins with pairing ions less than five carbons. Capacity factors markedly increased with pairing ions above five carbons. The greatest resolution between clindamycin B, 7-epiclindamycin and clindamycin was with five- and six-carbon pairing ions. Compounds I–V had similar retention time with camphor sulfonate and pentane sulfonate.

Typical chromatography of a synthetic mixture of compounds I–V is given in Fig. 4 and the capacity factors and retention times are given in Table I. Under the optimum conditions of the method, the clindamycins were baseline resolved and the lincomycins coeluted near the solvent front. Use of more polar columns, cyano or phenyl, and less polar mobile phases failed to increase the retention of the polar lincomycins.

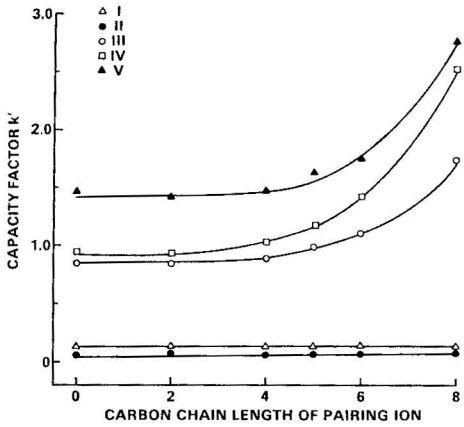

Fig. 3. Dependence of the capacity factors (k') of compounds I–V on the carbon chain length of the pairing ion. All pairing ions were 0.005 M straight-chain alkyl sulfonates. Mobile phase pH 6, all other conditions as given in Fig. 2.

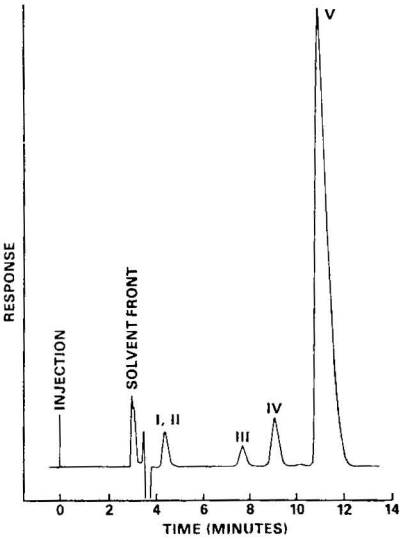

Fig. 4. HPLC chromatogram of a synthetic mixture of compounds I–V. Chromatography was performed on a 10-μm μBondapak C_{18} column with a mobile phase of methanol–water (60:40), 0.035 M acetic acid, and 0.005 M camphor sulfonate at pH 6. The flow-rate was 1.0 ml min^{-1} at 900 p.s.i.g. Compounds were detected with a differential refractometer calibrated to $3.8 \cdot 10^{-4}$ refractive index units full scale.

The chromatographic system developed is useful for quality control and stability testing and has been applied to the determination of clindamycin in pharmaceutical formulations, HFCs, and syrup.

A series of HFC and syrup placebos were spiked with amounts of clindamycin reference standard from 50–150% of label to demonstrate the recovery and precision

TABLE I

CHROMATOGRAPHIC CHARACTERISTICS OF CLINDAMYCIN, POTENTIAL IMPURITIES, OR DEGRADATION PRODUCTS

Chromatography conditions are given in Fig. 4.

Compound	*Retention volume (ml)* *	*Relative retention volume*	*Capacity factor*
Lincomycin B	4.41	0.39	0.12
Lincomycin	4.50	0.40	0.14
Phenethyl alcohol (internal standard)	5.90	0.53	0.49
Clindamycin B	7.69	0.68	0.95
7-Epiclindamycin	9.15	0.81	1.31
Clindamycin	11.3	1.00	1.86

* The column void volume was 3.95 ml.

TABLE II

DATA FOR THE ANALYSIS OF CLINDAMYCIN HYDROCHLORIDE HARD-FILLED CAPSULES*

(a) Recovery data (mg/HFC)

Amount added (mg)	*Amount found (mg)*	*Recovery (%)*
25.1	25.5	101.0
50.4	49.4	98.0
75.4	76.2	101.0
100.5	100.4	99.9
150.5	150.1	99.7
220.3	219.6	99.7
		Average: 99.9%
		Rel. S.D.: 1.10%

(b) Precision data

Theory 25 mg/HFC	*Theory 75 mg/HFC*	*Theory 150 mg/HFC*
24.9	77.7	150.0
25.1	76.1	149.0
25.2	74.6	147.0
24.8	75.2	152.0
24.9	75.1	151.0
25.0	75.0	153.0
Average: 25.0	Average: 75.6	Average: 150.0
Rel. S.D.: 0.60%	Rel. S.D.: 1.50%	Rel. S.D.: 1.43%

* Placebo tablets contain clindamycin 25–150 mg; lactose, corn starch, talc, and magnesium stearate.

of the procedure. Recovery was quantitative by peak height and by peak area responses for both formulations (Tables IIa and IIIa). Precision was 1.5% and 2.6% relative standard deviation for the HFC and syrup formulations, respectively (Tables IIb and IIIb). A typical chromatogram of the HFC formulation spiked with clindamycin B and 7-epiclindamycin is given in Fig. 5. Total analysis time is *ca.* 13 min. Chromatography for the syrup formulation was similar. Data for the analysis of typical production lots of HFC and syrup are given in Table IV.

TABLE III

DATA FOR THE ANALYSIS OF CLINDAMYCIN HYDROCHLORIDE SYRUP

(a) Recovery data (mg/ml syrup)			*(b) Precision data (theory 25 mg/ml)*
Amount added (mg)	*Amount found (mg)*	*Recovery (%)*	
34.3	34.9	101.7	26.3
44.4	45.2	101.8	25.1
54.5	55.1	101.1	26.2
60.5	61.2	101.2	26.2
71.2	70.2	98.6	25.0
86.0	86.0	100.0	24.9
		Average: 100.7%	24.9
		Rel. S.D.: 1.23%	Average: 25.4
			Rel. S.D.: 2.60%

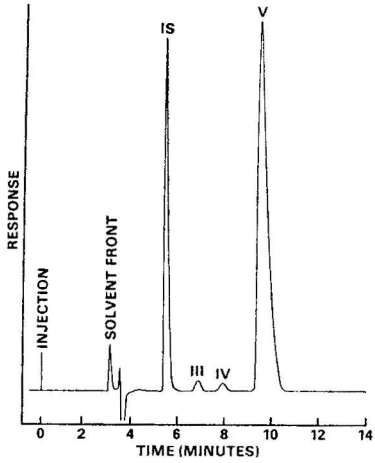

Fig. 5. HPLC chromatogram of a typical lot of 75 mg HFC spiked with clindamycin B and 7-epiclindamycin containing internal standard, phenethyl alcohol. Conditions as given in Fig. 4.

TABLE IV

ANALYSES OF CLINDAMYCIN HFCs AND SYRUP

Sample	*Theory (mg/dose)*	*Found (mg/dose)*
HFC	25	25.3
HFC	25	26.1
HFC	75	75.6
HFC	75	74.2
HFC	150	150.0
HFC	150	149.0
Syrup	25	24.9
Syrup	25	25.2

This system was also used for the analysis of bulk drug samples. Here, the amount of clindamycin B and epiclindamycin was quantitated by peak area response. The response factors of clindamycin, clindamycin B, and 7-epiclindamycin were equal within experimental error using refractive index and UV 214 nm detection, so the percentage of impurity present could be calculated by summing the area of all peaks and calculating the amount by peak area per cent. The detection limit for clindamycin B and 7-epiclindamycin was *ca.* 0.2% using this method. Clindamycin was quantitated by comparison to a reference standard. The results of the analysis of bulk drug are given in Table V showing excellent recovery and precision.

TABLE V

DATA FOR THE ANALYSIS OF CLINDAMYCIN HYDROCHLORIDE BULK DRUG

Amount added (mg)	*Amount found (mg)*	*Recovery (%)*
4.91	4.92	100.2
9.86	9.88	100.2
13.25	13.15	99.2
22.18	22.2	100.0
32.17	32.3	100.4
44.26	43.98	99.4
		Average: 99.9
		Rel. S.D.: 0.49%

If additional sensitivity is required, the camphor sulfonate can be replaced by pentane sulfonate and UV 214 nm detection can be employed.

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FLUORIMETRIC ASSAY FOR ORNITHINE DECARBOXYLASE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A highly sensitive method for the assay of ornithine decarboxylase in sample solutions prepared from rat tissue homogenate is described which employs high-performance liquid chromatography with fluorescence detection. Putrescine formed from ornithine under the optimal conditions for the enzyme reaction is treated by Cellex P column chromatography for clean-up and converted into the fluorescamine derivative in the presence of cupric ion which inhibits the reaction of interfering amines with fluorescamine. The derivative is separated by reversed-phase chromatography on LiChrosorb RP-18 with linear gradient elution. The lower limit of detection for putrescine formed enzymatically is 5 pmol.

INTRODUCTION

Ornithine decarboxylase (ODC; E.C. 4.1.1.17), one of the enzymes in the polyamine biosynthetic pathway in mammalian tissues, catalyzes the decarboxylation of ornithine to putrescine (Put), and appears to perform an important regulatory function in cell division and growth[1–4]. The activity of ODC in preparations from tissue homogenates is normally very low and so has been assayed radiochemically using [1-^{14}C]ornithine as substrate, the amount of [^{14}C]carbon dioxide evolved enzymatically being measured[5–8]. The method is very sensitive, although any loss of the carbon dioxide formed should be carefully avoided, but the radioactive substrate is expensive and not easy to prepare. The assay of ODC may be achieved by estimation of the Put formed enzymatically. In this case, methods for the determination of di- and polyamines (Put, cadaverine, spermidine and spermine) in biological samples are applicable if their sensitivities are sufficiently high. Many methods have been reported [*e.g.*, enzymatic and immunological methods, ion-exchange chromatography, gas chromatography, gas chromatography–mass spectrometry, thin-layer chromatography and high-performance liquid chromatography (HPLC)], and have been reviewed by Seiler[9] and Bachrach[10]. Among these, HPLC with fluorescence detection seems to be suitable for selective and rapid determination of Put at the picomole level in the presence of many biogenic amines other than the polyamines; here the polyamines are converted into the corresponding 5-dimethylaminonaphthalene-1-sul-

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phonyl[11–13] or fluorescamine[14,15] derivatives before the HPLC separations. HPLC of the fluorescamine derivatives was improved previously in our laboratory to give a simple, simultaneous and highly sensitive determination of the amines in serum[16].

Recently, we found that the reaction of many biogenic amines other than Put, cadaverine and 1,6-hexanediamine (Hda) with fluorescamine is inhibited considerably by cupric ion, which may serve to minimize the interference from those amines. This paper describes a highly sensitive method for the assay of ODC in sample solutions prepared from tissue homogenates. The method is based on the determination of Put, formed from the substrate under the optimum conditions for the enzyme reaction, as its fluorescamine derivative by means of the previously improved HPLC[16] with some modifications and utilizing the above observation on Cu^{2+}. An ODC preparation from rat intestinal mucosa homogenate was employed to establish the assay procedure, and Hda was used as an internal standard.

EXPERIMENTAL

Materials

All solvents and chemicals were of reagent grade.

ODC was prepared from rat intestinal mucosa as follows. Sprague–Dawley male rats (8–10 weeks old) were killed by cervical dislocation. The intestines were quickly removed and placed in saline. The intestinal mucosa was scraped off with a glass-slide, placed in about five volumes of homogenation buffer (20 m*M* phosphate buffer (pH 7.3), containing 0.1 m*M* dithiothreitol, 10 m*M* β-mercaptoethanol and 0.1 m*M* disodium ethylenediaminetetraacetate) and then homogenized with an Omega electric homogenizer at 1200 *g* for 15 min. After centrifugation at 20,000 *g* for 60 min, the supernatant was subjected to protein fractionation with ammonium sulphate. The fraction precipitating between 20 and 80% saturation with ammonium sulphate was collected, dissolved in 10 ml of the buffer and dialized against 2 l of the buffer for 14 h. The above subsequent operations were carried out at 0–4°C. The protein concentration was adjusted to 5 mg per 0.3 ml or less with the buffer, and measured by the method of Lowry *et al.*[17] using bovine serum albumin as a standard protein.

High-performance liquid chromatography

A Mitsumi liquid chromatograph was equipped with a 7120 syringe-loading sample injector, a Hitachi gradient device and a Hitachi 203 spectrofluorimeter fitted with a Hitachi flow-cell unit (cell volume, 20 μl) operating at the emission wavelength of 490 nm and the excitation wavelength of 390 nm. The column (150 × 4 mm I.D.) was packed with LiChrosorb RP-18 (particle size 5 μm; Japan Merck, Tokyo, Japan). The column temperature was 30 ± 0.5°C. A linear gradient elution was carried out between 45 and 80% methanol containing 35 m*M* sodium benzenesulphonate, 0.1 *M* ammonium chloride and 7 m*M* acetate buffer (pH 4.0) during 25 min at a constant flow-rate of 1 ml/min.

Procedure

The incubation mixture consisted of 0.3 ml of the ODC preparation, 0.1 ml of 2 m*M* pyridoxal phosphate, 0.2 ml of 12.5 m*M* dithiothreitol, 0.2 ml of 0.1 *M* phosphate buffer (pH 7.3) and 0.2 ml of 10 m*M* ornithine. It was incubated at 37°C

for 1 h, then 0.1 ml each of 2.0 nmol/ml Hda (as an internal standard) and 3 *M* perchloric acid (to stop the enzyme reaction) were successively added. The resulting mixture was centrifuged at 1200 *g* for 10 min. The supernatant was neutralized with *ca.* 0.2 ml of 1.5 *M* potassium hydroxide, mixed with 0.5 ml of chloroform and 0.2 ml of methanol and then centrifuged at 1200 *g* for 5 min. The aqueous layer (*ca.* 1.6 ml) was applied to a Cellex P column (15 × 0.5 cm I.D.) prepared as described previously[16], and the column was washed successively with 2 ml of 0.01 *M* phosphate buffer (pH 6.0), 1.0 ml of water and 1.0 ml of 0.1 *M* sodium chloride. Then, both Put and Hda were eluted from the column with 1 ml of 0.5 *M* sodium chloride. To the eluate, 0.5 ml of 0.4 *M* borate buffer (pH 9.2) and 0.2 ml of 20 m*M* cupric acetate were added, followed by 0.5 ml of 1.0 m*M* fluorescamine solution in anhydrous acetone with vigorous mixing. After adding 1.0 ml of 0.3 *M* succinic acid, the fluorescamine derivatives were extracted with 1 ml of diethyl ether by shaking for *ca.* 2 min on a Vortex mixer. The mixture was allowed to stand for 2 min. The ether layer was then transferred to a test-tube and mixed with 0.15 ml of 0.4 *M* borate buffer (pH 10). An aliquot (100 μl) of the resulting aqueous layer was injected into the chromatograph.

For the blank, the same procedure was carried out except that incubation was omitted. The amount of Put formed was obtained from the ratio of the net peak height due to Put to that due to the internal standard. ODC activity was expressed as pmol of Put formed per 30 min per mg of protein.

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram obtained according to the procedure. Put is completely separated from Hda (internal standard). The small peak 1 is due to the substrate ornithine remaining even after the treatments with the Cellex P column and cupric ion. When the former treatment is omitted, a large peak due to the substrate, which masks that due to Put, is observed. The recoveries of Put and Hda

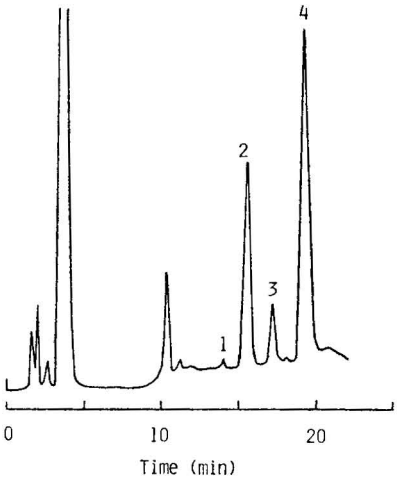

Fig. 1. Chromatogram obtained according to the procedure. Peaks: 1 = ornithine; 2 = putrescine; 3 = cadaverine; 4 = 1,6-hexanediamine (internal standard).

from the Cellex P column were both 96%. Cupric ion has a useful inhibitory effect on the derivatization of the interfering amines with fluorescamine, but not on that of Put, cadaverine and Hda under the present conditions (Table I).

TABLE I

INHIBITORY EFFECT OF CUPRIC ION ON THE REACTION OF BIOLOGICAL AMINES WITH FLUORESCAMINE

To 10 nmol of the amine in a mixture of 1.0 ml of 0.5 *M* sodium chloride and 0.5 ml of 0.4 *M* borate buffer (pH 9.2) mixed with 0.2 ml of 20 m*M* cupric acetate solution or water, 0.5 ml of 1 m*M* fluorescamine solution in acetone were added. The fluorescence intensity was measured with a Hitachi MPF-4 spectrofluorimeter in a 1 × 1 cm cell at 480 nm with the excitation at 394 nm.

Amine	*Inhibition (%)*	*Amine*	*Inhibition (%)*
Tryptamine	100	Lysine	54
Histidine	100	Isoleucine	40
Histamine	98	Spermidine	25
Phenylalanine	98	Agmatine	55
Leucine	95	1,3-Diaminopropane	80
Spermine	95	Cadaverine	0
Ornithine	87	1,6-Hexanediamine	0
Arginine	87	Putrescine	0

Extraction of the fluorescamine derivatives with diethyl ether and the back-extraction of the extract into the borate buffer serve to concentrate the derivatives approximately ten-fold. This gives a sufficient sensitivity to the assay of a low ODC activity. The lower limit of detection for Put formed enzymatically is 5 pmol. The sensitivity is comparable to that of the radiochemical method.

A linear relationship was obtained between the ratio of the peak height of Put to that of the internal standard and the amount of Put added in the range of 5–200 pmol to the assay mixture without incubation. The recovery of Put added to the incubated mixture in amounts of 15–200 pmol was 95 ± 4% (mean ± S.D.).

Substantially all of the ODC (*ca.* 90%) in the supernatant of the intestinal mucosa homogenate is precipitated between 20 and 80% saturation with ammonium sulphate. This fraction was used as the ODC preparation.

ODC is most active at pH 7.0–7.5 in 10–80 m*M* phosphate buffer (Fig. 2); 20 m*M* phosphate buffer (pH 7.3) was used. A maximum and constant activity is obtained in the presence of 0.5–3 m*M* ornithine with the Michaelis constant (K_m) at 0.1 m*M* (Fig. 3); 2 m*M* ornithine was employed in the incubation mixture as saturating concentration for the enzyme reaction. Pyridoxal phosphate, the coenzyme of ODC, at a concentration above 0.1 m*M* in the incubation mixture results in an activity of ODC approximately eight times higher than that in its absence; a concentration of 0.2 m*M* was used in our assay procedure. Dithiothreitol stabilizes the enzyme at a concentration greater than 2 m*M* in the incubation mixture; 2.5 m*M* was used as the optimum. Hda (internal standard) has a weak inhibitory effect on the amount of Put formed when added to the incubation mixture. It was therefore added at the end of the enzyme reaction. The enzyme activity is linear with the time up to at least 60 min, when the mixture containing 5 mg or less protein is incubated at 37°C (Fig. 4).

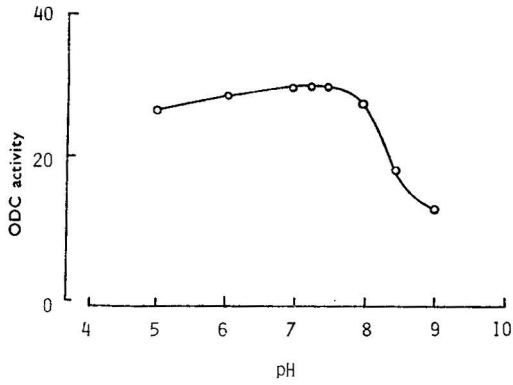

Fig. 2. Effect of pH on ODC activity.

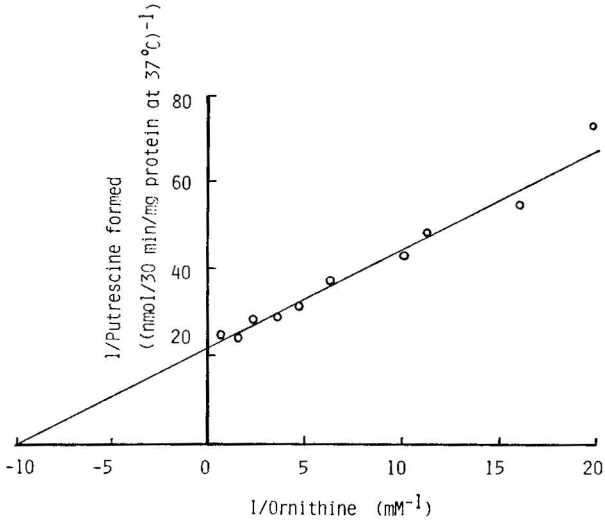

Fig. 3. Lineweaver–Burk plot of the dependence of ODC activity on ornithine concentration.

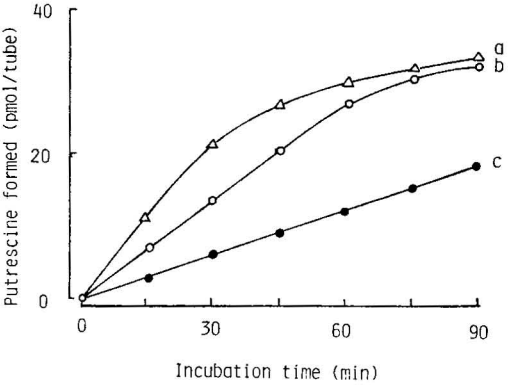

Fig. 4. Effect of incubation time and protein concentration on the amount of putrescine formed. Amount of protein in the incubation mixture: (a) 8; (b) 5; (c) 3 mg.

The precision was established with respect to repeatability. The standard deviation was 1.3 ($n = 20$) for a mean activity of 32 pmol per 30 min per mg protein.

This method also permits the assay of ODC in preparations obtained from other rat tissues, *e.g.*, liver, lung, prostate and brain. This study provides the first HPLC method for the assay of ODC. The method is very sensitive and can be used in place of the radiochemical method.

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ESTRADIOL AFFINITY CHROMATOGRAPHY

APPLICATION TO PURIFICATION OF MURINE ALPHA-FETOPROTEIN

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SUMMARY

A one-step batch procedure is described for purification of murine alpha-fetoprotein (AFP) by estradiol affinity chromatography. Various ratios of carbodiimide (C), diaminononame (D) and estradiol hemisuccinate (E) were tested to determine optimal conditions for AFP purification. Although yields of AFP ranged from 15 to 44% depending on the reagent ratio employed, AFP isolates free of other protein contaminants were achieved at C:D:E ratios of 10:10:1 with a 29% yield. Both estrone and estradiol proved efficient as elution agents to free AFP bound to the estradiol–Sepharose beads, but higher yields were produced with estrone. After isolation the estrogen-eluted AFP preparations were analyzed by (1) estradiol-binding assays, (2) third-party radiocoprecipitation, (3) inhibition of radioimmunoassay for estrone and estradiol and (4) exchange of unlabeled for radiolabeled estradiol. These results indicated that the steroid remained attached to the eluted AFP molecule.

INTRODUCTION

A major difficulty in the purification of alpha-fetoprotein (AFP) has been its separation from albumin due to similarities in size, charge and isoelectric point. Only partial success has been achieved by combining such techniques as gel filtration, ion-exchange, isofocusing, and lectin and antibody affinity chromatography[1,2]. Until recently only cibacron blue–agarose chromatography had shown promise as a possible method for separating albumin from AFP in mammalian biological fluids[3]. However, since mouse and rat AFP have been shown to possess high binding affinity for estrogens[4,5], Uriel *et al.*[6] and Hassoux *et al.*[7] have used these properties to develop an estradiol affinity chromatographic technique for the purification of rodent AFP. While attempting to apply their methods, we have devised a one-step batch procedure for isolating murine AFP devoid of albumin. Our results also show a previously unreported property of the AFP isolated by estradiol-affinity chromatography.

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MATERIALS AND METHODS

Amniotic fluid

Amniotic fluid was drawn from Nya:NYLAR mice (15 to 18 days pregnant) with a tuberculin syringe fitted with a 26-gauge needle. The mouse amniotic fluid (MAF) was pooled, centrifuged and stored at −20°C.

Immunologic procedures

An AFP standard prepared as previously described for hepatoma fluids[8] was used to quantitate AFP and to provide antigen for immunization. AFP content was quantitated by the radial immunodiffusion method of Mancini *et al.*[9]. The production of rabbit antiserum to AFP, either from amniotic fluid or hepatoma derived, has been described[10,11]. Double diffusion in agar was performed by the method of Ouchterlony[12] and immunoelectrophoresis by the method of Scheidigger[13]. The minimum detection level of these agar procedures is 10 μg/ml. Protein was determined by the folin–phenol method of Lowry *et al.*[14]. Rabbit anti-mouse transferrin, γ-globulin, and albumin were purchased from Cappel Laboratories (Downington, PA, U.S.A.).

Gel electrophoresis

Analytical polyacrylamide disc gel electrophoresis (PAGE) was performed at 4°C with constant current (2 mA/tube) for 4 h or until the dye front was 1 cm from the end of the gel. The samples had been layered onto gel columns of 4, 6 and 8% acrylamide by the procedure of Joshi and Ebert[15]. The buffer was 0.15 *M* glycine, 0.02 *M* trizma base, at pH 8.6, and the gel stain was amido black. For maximal analytical sensitivity the gel tubes were loaded with 200 μl of the concentrated test samples to detect impurities.

Biochemicals

Sepharose 4B was purchased from Pharmacia (Uppsala, Sweden); diaminononane (D) from Aldrich (Milwaukee, WI, U.S.A.); [2,4,6,7N-^{3}H]17β-estradiol (100 μCi/mmol) and [2,4,6,7N-^{3}H]17β-estrone (85 μCi/mmol) from Amersham-Searle (Chicago, IL, U.S.A.); 17β-estradiol-monohemiscuccinate (E) and unlabeled estrone (E_1) and estradiol (E_2) from Sigma (St. Louis, MO, U.S.A.); and 1-ethyl-3-(3-diethylaminepropyl)carbodiimide (C) from Ott Chemicals (Muskegon, MI, U.S.A.).

Binding of estradiol to Sepharose

E_2 was bound to Sepharose beads by a procedure modified from Arnon *et al.*[16]. Cyanogen–bromide-activated Sepharose 4B (10 g), swollen in 1.0 m*M* HCl (pH 3.0), was prehydrolyzed in 0.1 *M* $NaHCO_3$–0.5 *M* NaCl (pH 8.3) for 2 h. After centrifugation beads were added to the 9-carbon spacer (100 mg D) at a concentration of 2 mg of spacer/ml of basic buffer (pH 8.3; see above) and incubated at 4°C for 16 h. The beads were washed with alternating cycles of basic buffer and 0.1 *M* sodium acetate–1.0 *M* NaCl (pH 4.0). Using the trinitrobenesulfonic acid determination for amines, it was calculated that 72% of the spacer was attached to the beads.

The D–Sepharose complex was then linked to an E_2 ligand via the carboxylic group. C (500 mg), dissolved in 30 ml of distilled water (pH 4.5) and 30 ml of dioxane, was mixed with 30 mg of E in 30 ml of dioxane–water (pH 4.5) for 30 min at ambient

temperatures. The D–Sepharose beads were washed once with 50% dioxane–water (pH 4.5). After centrifugation the supernatant was removed from the packed beads, and 30 ml of E_2–C was added to 10 ml of packed beads. The reagents were mixed overnight at 4°C with gentle agitation. After centrifugation the supernatant was removed, and the packed beads were washed exhaustively with 50% dioxane–water until monitoring at 280 nm showed zero absorbance. The amount of estradiol bound to D–Sepharose, determined by monitoring 280 nm absorbance of the solution before and after the binding procedure, was calculated to be 30% (1.0 mg E/1.0 g dry sepharose). The E_2–Sepharose beads were washed and stored in phosphate-buffered saline (PBS; pH 7.0).

AFP isolation on E_2-affinity beads

Equal volumes of E_2–Sepharose beads in PBS and Millipore-filtered MAF were mixed and gently agitated overnight at 4°C. After centrifugation the supernatant was removed, and the packed beads were washed with PBS until a zero absorbance was attained. To remove the bound protein (AFP) from the beads, 6 ml of saturated E_2 (or E_1) in 15% dioxane–PBS was added, and the mixture was gently agitated for 2 h at ambient temperatures. The entire mixture was then transferred to a syringe, Millipore-filtered into dialysis tubing, dialyzed exhaustively against PBS to remove all traces of dioxane, and then analyzed for AFP. The maximal binding of AFP to the beads prepared in the study ranged from 500 to 1000 μg per ml of swollen E_2-linked Sepharose beads. We used the E_2–Sepharose beads only once and could not regenerate them.

Estrogen-binding assay

Estrogen binding of MAF and purified AFP was examined by a dextran-coated charcoal procedure similar to that used in previous studies of steroid hormone binding[17,18]. For each assay 0.05 ml of MAF or purified AFP was added to an appropriate number of ice-chilled 12 × 75 mm disposable glass culture tubes, followed by 0.40 ml of TED buffer (10 m*M* Tris, 0.5 m*M* EDTA, 0.5 m*M* DTT, pH 7.4) and 0.05 ml of [^{3}H]E_2 (80 nmol) in the same buffer. In inhibitor studies 0.05 ml of unlabeled E_2 (10 μmol) was substituted for buffer, and the mixture was incubated for 30 min before the [^{3}H]E_2 incubation. After incubation for 1.0 h in an ice bath 1.0 ml of dextran-coated charcoal particles (0.5%) suspended in TED buffer was added to the incubation mixture to adsorb free (unbound) [^{3}H]E_{23} After 15 min the charcoal was sedimented at 1800 *g* for 10 min in a refrigerated centrifuge. The supernatant was decanted into a Scintiverse cocktail (Fisher Scientific, Pittsburgh, PA, U.S.A.), and radioactivity was determined in three 2-min counting cycles in a liquid scintillation counter (40% efficiency). In previous studies Scatchard analysis of data on binding of AFP to [^{3}H]E_2 showed an association constant of $0.16 \cdot 10^9$ M^{-1} and a binding-site concentration of $2.2 \cdot 10^5$ fmol/ml MAF[18]. E_2 bound to AFP was immunoprecipitated using a double-antibody radioimmunoassay called third-party radiocoprecipitation as previously described[18].

RESULTS

Effect of reagent ratios on chromatographic yield

To determine the most efficient yields from E_2 affinity chromatography, various ratios of C, D and E ligand were tested in four sepharose experiments (Table I). Maximal yields resulted when 500 mg of C were combined with 100 mg of D and 30 mg of E_2 ligand (Table I, Seph 3) in a ratio of 16.6:3.3:1. The quantity of 9-carbon side-chain reagent (D) was kept constant in three of the four experiments. Moreover, yields of 20–30% could be consistently obtained when reagent ratios were employed at minimal concentrations (Table I, Seph 1). Excessively high amounts of reagents (Seph 4) proved inefficient due to increased binding of the beads for all protein moieties.

TABLE I

ESTRADIOL AFFINITY CHROMATOGRAPHIC YIELDS OF AFP FOR VARIOUS AMOUNTS AND RATIOS OF CARBODIIMIDE (C), DIAMINONONANE (D) AND ESTRADIOL HEMISUCCINATE (E)

Experiment	*C:D:E reagents*		*MAF–AFP total protein (mg/ml)*	*AFP Isolated (mg/ml)*	*Yield (%)*
	Amounts (mg)	*Ratio*			
Seph 1	100:100:10	10:10:1	0.974	0.300	29.00
Seph 2	200:100:20	10:5:1	0.974	0.150	15.40
Seph 3	500:100:30	16.6:3.3:1	1.120	0.500	44.60
Seph 4	2000:200:150	13:1.3:1	1.462	0.030	2.05

The impurities in the AFP isolates from the four Sepharose experiments are listed in Table II. The AFP isolated with Seph 1 (29% yield) was devoid of all impurities, including albumin. Seph 3 gave a higher AFP yield (44%), but the isolate contained traces of other protein moieties, mainly albumin. Subsequent immunization of rabbits with AFP isolated by Seph 1 resulted in the production of monospecific antisera.

TABLE II

ISOLATION PRODUCTS FROM ELUTION WITH ESTRONE (E_1) OR ESTRADIOL (E_2)
Agar techniques = immunodiffusion, immunoelectrophoresis; PAGE = polyacrylamide gel electrophoresis; ND = Not done.

Experiment	*C:D:E ratio*	*Elution steroid*	*Albumin*		*AFP*		*Other**	
			Agar	*PAGE*	*Agar*	*PAGE*	*Agar*	*PAGE*
Seph 1	10:10:1	E_1	−	−	+	+	−	−
		E_2	ND	ND	ND	ND	ND	ND
Seph 2	10:5:1	E_1	−	−	+	+	+	+
		E_2	−	−	+	+	−	−
Seph 3	16.6:3.3:1	E_1	+	+	+	+	−	−
		E_2	+	+	+	+	−	−
Seph 4	13:1.3:1	E_1	−	+	+	+	−	−
		E_2	−	+	+	+	−	−

* Serum or amniotic fluid component other than albumin or AFP.

Effect of elution steroid on chromatographic yield

Six pools of MAF with various C:D:E ratios were eluted with either E_1 or E_2. Saturated solutions of E_1 proved mostly superior or equal to E_2 (Table III). Yields of AFP with E_2 were predominantly 15–30% of the starting material depending on the C:D:E ratio. Yields with E_1 ranged generally from 15 to 45%.

TABLE III

ESTRADIOL AFFINITY CHROMATOGRAPHIC YIELDS WITH EITHER E_1 OR E_2 AS THE ELUTION STEROID

Different pools of MAF were used as starting material.

MAF pool	*C:D:E ratios*	*Elution steroid*	*MAF–AFP total protein (mg/ml)*	*AFP isolated (mg/ml)*	*Yield (%)*
MAF-A	10:5:1	E_1	0.974	0.150	15.4
		E_2	0.974	0.150	15.4
MAF-B	13:1.3:1	E_1	1.462	0.030	2.1
		E_2	1.462	0.025	1.7
MAF-C	10:10:1	E_1	0.701	0.160	22.8
		E_2	0.701	0.130	18.5
MAF-D	10:10:1	E_1	0.842	0.145	17.2
		E_2	0.842	0.159	18.8
MAF-E	16.6:3.3:1	E_1	1.34	0.475	35.4
		E_2	0.736	0.225	30.5
MAF-F	16.6:3.3:1	E_1	1.117	0.500	44.7
		E_2	1.130	0.350	30.9

Chromatographic yields from individual MAF samples

When various individual samples of MAF were chromatographed with C:D:E ratios of 10:10:1 (from Table I, Seph 1) followed by E_1 elution, the yields were strinkingly similar, ranging from 19.2 to 24.8% (Table IV). AFP concentrations of approximately 0.2 mg/ml were consistently obtained with an average yield of 21%.

TABLE IV

ESTRADIOL AFFINITY CHROMATOGRAPHIC YIELDS FOR INDIVIDUAL MAF SAMPLES (C:D:E RATIO, 10:10:1)

MAF sample	*Total protein (mg/ml)*		*AFP isolated (mg/ml)*	*Yield (%)*
	MAF	*AFP*		
MAF 1	3.245	1.070	0.220	20.4
MAF 2	1.510	0.498	0.125	24.8
MAF 3	3.346	1.115	0.235	21.0
MAF 4	3.050	1.006	0.195	19.2
MAF 5	2.210	0.736	0.150	20.4

E_2 binding assay of E_1-eluted AFP

The MAF and the E_1-eluted AFP were subjected to estrogen-binding assays, using the dextran-coated charcoal procedure. After adjustments for total AFP protein, the specific bound counts (cpm) in native MAF were 20 to 45% higher than

those in the E_1-eluted AFP isolates (Table V, experiments 1–5). Since the E_1-eluted AFP was partially inhibited from binding to the $[^3H]E_2$, some steroid apparently remained bound to the eluted AFP molecule.

In an attempt to mimic the experimental conditions which produced E_1-eluted AFP, native MAF was dialyzed against PBS, E_1 and E_2 (Table V). Dialysis against either E_1 or E_2 resulted in 30–45% less $[^3H]E_2$ binding than dialysis against PBS.

TABLE V

SPECIFIC E_2 BINDING OF NATIVE MAF, AFP PURIFIED BY E_2 AFFINITY CHROMATOGRAPHY, AND MAF DIALYZED AGAINST PBS OR STEROIDS

Native MAF was adjusted to AFP total protein content for comparison to radioactive counts obtained with E_1-eluted AFP. Specific $[^3H]E_2$ bound to native or PBS-dialyzed MAF was considered to be 100%.

Experiment number	*Dialyzed against*	*Specific $[^3H]E_2$ bound*			
		Native MAF		*E_1-isolated AFP*	
		*cpm**	*%*	*cpm**	*%*
1	None	6301	100	3850	62
2	None	5593	100	3078	55
3	None	7258	100	5760	80
4	None	6610	100	4750	70
5	None	6541	100	4600	70
6	PBS	36,663	100	—	—
7	E_1	19,516	53	—	—
8	E_2	24,859	67	—	—

* Specific count (cpm) = total bound cpm minus cpm inhibitable by estrone.

Tests on estrogen-eluted AFP

The results to this point suggested that estrogen becomes attached to the AFP molecule during elution with saturated steroid solutions. To test this hypothesis, 50 μl of $[^3H]E_2$ (120 nmol) was added to the saturated E_2 solution just prior to sepharose E_2–MAF exposure. After elution and subsequent washing procedures, the eluted AFP was subjected to third-party radiocoprecipitation[18] using antisera monospecific to murine AFP, and 1160 cpm of radioactivity were detected. Since no other $[^3H]E_2$ was added, this radioactivity had to originate from solutions during the elution procedures. In non-specific binding studies using non-immune rabbit serum in lieu of anti-AFP serum, 197 cpm was precipitated.

Radioimmunoassays (RIAs) specific for either E_1 or E_2 were used to further investigate whether the steroid remained bound to the eluted AFP. Native MAF and steroid-eluted AFP were used as inhibitors in these assays. While native MAF did not inhibit the formation of complexes, E_2-eluted AFP inhibited 55% of the anti-E_2 counts (576 *versus* 1286 cpm), and E_1-eluted AFP inhibited 22% of the anti-E_1 counts (992 *versus* 1280 cpm). To further demonstrate the presence of steroid on AFP derived from E_2–Sepharose beads, an exchange of unlabeled for radiolabeled steroid was performed (Fig. 1). When E_2-eluted AFP was incubated at 37°C in the presence of $[^3H]E_2$ (80 nmol), after a slight initial decrease in binding, an increase in the counts occurred from 4 to 8 h incubation. Native MAF previously unexposed to estrogens

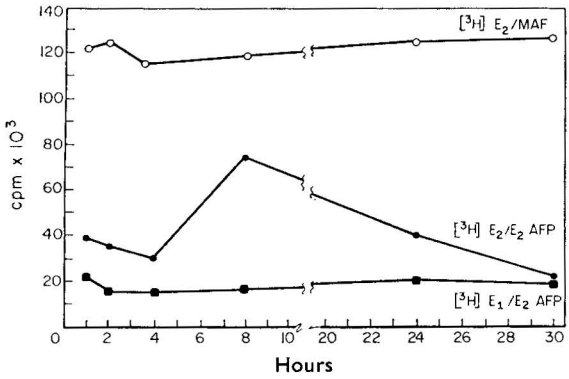

Fig. 1. Exchange of unlabeled for radiolabeled estrogen ([^{3}H]E_1 or [^{3}H]E_2) on native murine amniotic fluid (MAF), estradiol-eluted alpha-fetoprotein (E_1AFP) and estrone-eluted alpha-fetoprotein (E_2AFP). Samples were incubated at 37°C in a water bath with [^{3}H]E_2 or [^{3}H]E_1 for 1, 2, 4, 8, 24 or 30 h, then with dextran-coated charcoal, and assayed as described in the text.

showed a similar decrease at 2 to 3.5 h incubation, but remained essentially stable for 30 h thereafter.

Attempts to exchange unlabeled E_2 for radiolabeled E_1 with E_2-eluted AFP proved unsuccessful. After an initial decrease, the counts remained unchanged for the subsequent 28 h of incubation.

DISCUSSION

Our demonstration of a one-step batch procedure for purification of mouse AFP by E_2-affinity chromatography differs in several ways from the work by Arnon *et al.*[16]. (a) Various combinations of reagents were tested to determine the best C:D:E ratios for optimal AFP yields; (b) E_1 and E_2 were used to elute AFP bound to the sepharose beads, AFP yields proved E_1 to be the better eluting agent; (c) when the eluent (saturated E_2) was spiked with [^{3}H]E_2-bound AFP was precipitated; (d) for our one-step procedure no columns were necessary; (e) AFP was eluted at room temperature rather than 4°C; (f) the entire mixture, including the Sepharose beads was transferred to a syringe and Millipore-filtered directly into dialysis tubing. This step removed all protein attached to the beads and minimized protein loss during transfer.

Our yields were compatible with those in previous studies with E_2-linked Sepharose beads. Uriel *et al.*[6] reported yields of 42% for mouse AFP and 25% for rat AFP. However, these investigators achieved higher binding capacities (70 g/0.3 ml of beads) than we did (40 g/1.0 ml beads); differential E_2 binding in various rodent species may exist, and/or procedural differences in Sepharose ligand bead preparation may account for this disparity. Later studies by Hassoux *et al.*[7] found absorbent binding capacities of 0.5–0.6 mg/g of swollen beads, but their procedure follows that of Cuatrecases *et al.*[19] in using AH-Sepharose 4B and so is not directly comparable to ours.

We have demonstrated that some elution steroid remains bound to the AFP molecule after estrogen chromatography. The AFP isolated with E_2–Sepharose beads

consistently bound less [^{3}H]E_2 than equivalent amounts of AFP in MAF. AFP was eluted with saturated steroid solutions spiked with [^{3}H]E_2 and assayed for specific immunoprecipitation of the isolated AFP bound to the radiolabeled steroid. In these precipitates, free of exogenous [^{3}H]E_2, radiolabeled E_2 was present. Further studies indicated that AFP isolated by E_2 chromatography could inhibit complexing in E_2 RIAs; the AFP prepared by our procedure could neutralize up to 55% of the antibodies directed against either E_1 or E_2. If estrogen remained attached to the eluted AFP, an exchange of unlabeled for radiolabeled estrogen should be demonstrable. Such an exchange occurred after 4–8 h incubation at 37°C. Thus the AFP molecule did not readily exchange bound estrogen. Finally cross-exchange experiments showed that unlabeled E_1 was not readily exchangeable with labeled E_2.

Nevertheless, elution steroid remains demonstrably bound to at least some of the eluted AFP. Further support for this observation is provided by the findings of Keller *et al.*[20], who compared AFP preparations isolated by various E_2 affinity procedures. In their cell-culture studies involving immunoregulation, the various AFP isolates appeared to contain E_2 bound to the AFP molecule. In contrast, Hassoux *et al.*[7] found that AFP isolated by E_2 affinity chromatography has an affinity constant of 0.5 to $0.6 \cdot 10^8$ M^{-1} and a molecular combining ratio of less than unity (0.30–0.64). No mention was made of elution steroid attachment or suppression of [^{3}H]E_2 binding to the AFP molecule. However, their exchange procedure involved equilibrium dialysis at 4°C for 24 h, whereas our non-equilibrium dextran-coated charcoal procedure took place at 37°C for 30 h with the exchange at 4–8 h. It is conceivable that unlabeled E_2 was exchanged for [^{3}H]E_2 inadvertently during the equilibrium dialysis, as its incubation period would provide sufficient time for completion of steroidal exchange.

E_2-linked Sepharose chromatography is a valuable tool for rapid one-step purification of AFP with yields of 20–40% of the starting material. Our yields of rat AFP (15–20%) are lower than mouse AFP, in agreement with the studies of Uriel *et al.*[6]. We have also used this procedure to purify AFP from hepatoma-bearing mice, which have high concentrations of serum AFP[21]. The yields to date have been low (10–15%), possibly due to the heavy protein concentrations of the tumor sera, which may compete for binding sites on the Sepharose beads. In addition, we have performed E_2–Sepharose chromatography of human AFP to provide an injection vehicle for animal immunization, as described by Arnon *et al.*[16]. The immunizations produce antisera suitable for use in immunologic assays, provided the antisera are first absorbed with normal adult human serum.

Finally, the E_2 affinity beads can be used to remove specific anti-AFP immunoglobulin G from total immunoglobulin G. After AFP is attached to the beads and prior to elution with E_1, rabbit anti-AFP immunoglobulin G can be applied to the beads and incubated. The mixture is centrifuged to remove non-binding protein components. The remaining solution decanted from the beads contains immunoglobulin G devoid of anti-AFP antibodies.

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Note

Assessment of quantitative relationships between Kováts' retention index and chemical structure: prediction for pyrido-pyrimidine derivatives

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Quantitative relationships between chemical structure and gas chromatographic behaviour of organic compounds have been discussed in several papers[1–12]. Most of these reports concerned relatively simple compounds, such as alkanes, alkenes and aromatic hydrocarbons. The present paper investigates similar relationships for a heteroaromatic group of compounds, pyrido-pyrimidine derivatives, where specific interactions between the ring and heteroatoms may occur (Fig. 1). Pyrido-pyrimidine derivatives, prepared by Mészáros and Hermecz[13], show strong analgesic activities, and one of them is marketed as Probonz®. The results of their gas chromatographic (GC) investigations have already been published[14], and even the relationships between structure and retention indices have been studied.

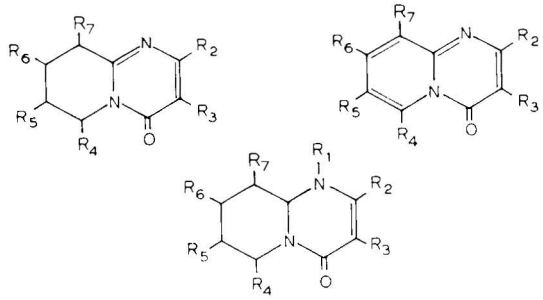

Fig. 1. General structures of investigated compounds. R_1 = H, CH_3, C_2H_5, $COCH_3$, C_4H_9; R_2 = H, CH_3, C_2H_5, C_3H_7; R_3 = CH_3, C_2H_5, $COOC_2H_5$, $COOC_3H_7$, $COOC_4H_9$, COO-*tert*.-butyl, $OCH(CH_3)_2$, $CONH_2$, C_6H_5, CN; R_4 = H, CH_3; R_5 = H, CH_3; R_6 = H, CH_3; R_7 = H.

The starting point of our investigations was the assumption that models of the quantitative structure–activity relationship (QSAR) are also applicable to quantitative structure–retention index relationship (QSRR) studies. According to the simplest

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models of Free and Wilson[15] and BEL–Free[16], the biological activity (or in our case, the retention index) can be considered as a sum of increments assigned to the substituents of the derivatives within a congeneric series[15,16].

METHODS

The model proposed by Free and Wilson[15] for QSAR can be adapted for retention indices as follows.

The measured retention indices, I, for all molecules are regarded as the sum of the index increments of the structural part, Q, common to each compound and of the substituents, x_z, attached at z positions in Q. Let us denote by j_z the serial number of an arbitrary substituent in the position z. Then, the measured retention index, I, of a compound can be expressed as follows

$$I = i_0 + \sum_{z=1}^{n} \sum_{j_z=1}^{m} i_{jz} x_{jz} \tag{1}$$

where n is the number of substitution positions, m is the number of substituents in position z, x_{jz} is a structural parameter, assuming a value of 0 or 1 depending on whether substituent j_z is absent or present in the molecule investigated, i_0 is the increment of the skeleton, *i.e.*, Q, and i_{jz} is the retention index increment. Our purpose is to calculate the i_0 and i_{jz} values.

For the values of i_{jz} the following relationship is assumed:

$$\sum_{j_z=1}^{m} i_{jz} = 0 \tag{2}$$

If q molecules are investigated, q equations of the above type can be set up, according to the model. Since the number of equations is greater than the number of variables, the values of i_{jz} in eqn. 1 can be calculated by regression analysis. Eqn. 1 can then be applied for prediction of retention indices for new compounds.

The results can more easily be interpreted if the index increments of the substituents are related to the unsubstituted case, *i.e.*, to the atom, by subtraction. The δi_{jz} increments can be obtained, for substituent w and substituent position z, as follows:

$$\delta i_{wz} = i_{wz} - i_{Hz} \tag{3}$$

($i_{H_z} = i$ value with a H atom in position z).

The BEL–Free method is a specific procedure for improving the predictive ability of the Free–Wilson method. It is based essentially on steptwise regression technique, omitting variables which disturb the prediction. First, eqn. 1 is solved as in the Free–Wilson method, but, in our case, without the restriction of eqn. 2. The retention index of the compound selected for prediction (and the corresponding confidence interval) is also estimated. Then the equation is refined by a trial-and-error procedure, variables which increase the prediction confidence interval being gradually eliminated. This process is continued until a minimal confidence interval is found by removing variables or re-entering previously eliminated variables.

The final equation is used for the prediction of the retention index for the selected compound. Since this process eliminates the unnecesary variables from the Free–Wilson equation in "backward" steps, it is called backward elimination from the Free–Wilson equation (BEL–Free method).

EXPERIMENTAL

The Free–Wilson and BEL-Free calculations were carried out by means of our interactive program system, written in FORTRAN, on the Siemens 7755 computer at the Institute for Coordination of Computer Techniques, Budapest. The GC conditions are shown in Table I.

TABLE I

GAS CHROMATOGRAPHIC CONDITIONS

Chromatograph	Hewlett-Packard 5710A, Chinoin Digint 21 Integrator
Column	6 ft. × 1/4 in., glass
packing	Chromosorb W CMDS
stationary phases	3% OV-17, 3% OV-1
Carrier gas	nitrogen, 30 ml/min
Temperatures	
column	240°C
detector	300°C
injector	300°C
Sample	1 μl, applied with Hamilton syringes
solvent	chloroform
Attenuator	128 × 10

RESULTS AND DISCUSSION

Table II contains the statistics of the calculations. Table III compares the calculated and measured retention indices for the two new molecules. The differences between the measured and calculated values can be considered as acceptable, bearing in mind that the compounds are fairly polar and contain three heteroatoms and regarding the conditions of measurement.

TABLE II

STATISTICS OF CALCULATIONS

	OV-1	*OV-17*
Multiple correlation coefficient, R	0.9921	0.9965
Lower 95% confidence interval for R	0.9784	0.9905
R^2	0.9842	0.9931
Amount of explained variance	0.9703	0.9876
Significance of regression, F ratio (%)	0.0000	0.0000
Standard error of the dependent variable	307.0688	351.4068
Standard error of the estimate	52.9056	39.0522

TABLE III

MEASURED AND CALCULATED INDEX VALUES OF THE TWO NEW COMPOUNDS

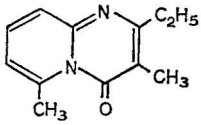
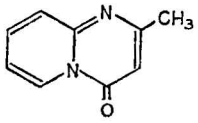
Compound	*OV-1*		*OV-17*	
	Meas.	*Calc.*	*Meas.*	*Calc.*
	1874	1886	2170	2162
	1694	1698	2048	2060

TABLE IV

INDEX INCREMENTS OF SUBSTITUENTS ON OV-1 AND OV-17 STATIONARY PHASES

Substituent		*Index increment*	
		OV-1	*OV-17*
R_1	–	−58.36	−60.25
	Ethyl	224.20	254.89
	n-Butyl	375.20	362.89
	Methyl	295.82	298.01
	Acetyl	88.92	114.98
R_2	Methyl	45.89	−4.47
	n-Propyl	131.24	96.21
	H	−19.97	−6.25
	Ethyl	158.49	88.76
R_3	H	−388.47	−394.41
	Ethyl	−212.21	−278.86
	Phenyl	336.97	349.51
	$CONH_2$	223.89	255.35
	CO-Ethyl	167.66	186.11
	CO-*n*-propyl	198.46	245.49
	CO-*n*-butyl	295.46	342.49
	Methyl	−332.03	−393.49
	CN	26.46	40.49
	CO-*tert.*-butyl	127.46	150.49
R_4	Methyl	4.34	2.10
	H	−12.14	−5.74
	Axial methyl	−107.27	−44.02
	Ethyl	120.94	49.34
R_5	H	−1.19	−1.43
	Methyl	38.09	45.79
R_6	H	−4.63	−4.63
	Methyl	71.69	71.82
R_7	H	−20.14	−20.88
		46.33	48.01
Skeleton		2073.48	2483.85

The relative contributions of the substituents to the retention indices, *i.e.* the δi_{jz} values of eqn. 3, are listed in Table IV. Since δi_{jz} values are analogous to δI values (obtained by simple subtraction), the retention index contributions of Table IV can formally be compared with δI values published in our previous paper[14]. The results indicate that the mathematical methods used for the investigation of chemical structure–biological activity relationships can be applied with acceptable accuracy for the prediction of retention indices.

The Free–Wilson and BEL–Free methods seem to be suitable for the investigation of structure–retention index relationships and for the prediction of the retention indices of new compounds after measurements on a few members in a congeneric series.

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Note

Semipreparative high-performance liquid chromatographic separation of singlet oxygen derived limonene hydroperoxides

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(Received August 12th, 1980)

The reaction of singlet oxygen ($^1O_2$) with (+)-limonene (1) is well known[1,2], but the hydroperoxides (3a–8a) that are formed, as evidenced by iodometric titration and infrared, have never been isolated and characterized. Instead, their structures have been inferred indirectly by reduction to the corresponding alcohols (3b–8b). A previous report on the TLC separation of limonene hydroperoxides[3] was of limited value for our purposes since their mixture was poorly resolved and little characterization work was done. High-performance liquid chromatographic (HPLC) techniques have recently been used with success in the separation of some alkyl hydroperoxides[4] by reversed phase with acetonitrile–water, and in the separation of lipid[5,6] and cholesterol[7] hydroperoxides employing both normal- and reversed-phase columns.

We wish to report the complete HPLC resolution of the limonene hydroperoxides (3a–8a) employing columns with Whatman Partisil 10 and Partisil 5, and also with these two columns in tandem. Characterization of the hydroperoxides by direct spectroscopic techniques, reduction to known alcohols, and gas–liquid chromatographic (GLC) thermograms will be published elsewhere.

EXPERIMENTAL

The hydroperoxides were prepared from (+)-limonene by the rose bengal sensitized singlet oxygen reaction of Schenck *et al.*[1]. The solvents were from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.), "distilled in glass," and were all degassed under aspirator vacuum for 1 min prior to use.

The HPLC system was a Waters Model ALC/GPC 201 which included a M-6000 pumping system, a M-U6K universal injector and a M-R 401 differential refractometer. The columns were Whatman Partisil-PXS consisting of 25 cm × 4.6 mm I.D. stainless steel tubing packed with 10- or 5-μm microparticulate silica. The Partisil 10 column was placed before the Partisil 5 column when they were used in tandem. Two Partisil 5 columns were less useful because of the resulting operative higher pressure. A guard column, consisting of 7 cm × 2.1 mm I.D. stainless-steel tubing and packed with Whatman HC-Pellosil, was used in all cases. A flow-rate of 2 ml/min was employed for all separations.

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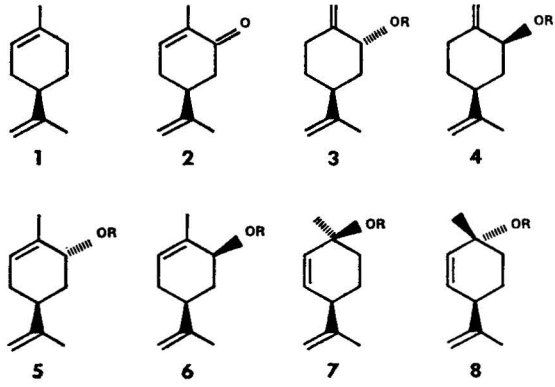

RESULTS AND DISCUSSION

The best solvent system previously found[8] for the separation of a complex mixture of monoterpene alcohols, ethyl acetate–methylene chloride (2.5:97.5), was utilized initially for the elution of the hydroperoxides (3a–8a). The results showed that they eluted much faster than their respective alcohols (3b–8b), with a retention time similar to that of carvone (2). In order to increase the capacity factor (k')* of the hydroperoxides, the polarity of the solvent was reduced to ethyl acetate–methylene chloride (0.25:99.75), and a considerably better separation was obtained (Fig. 1A). Reducing the polarity further to 100% methylene chloride was slightly better overall

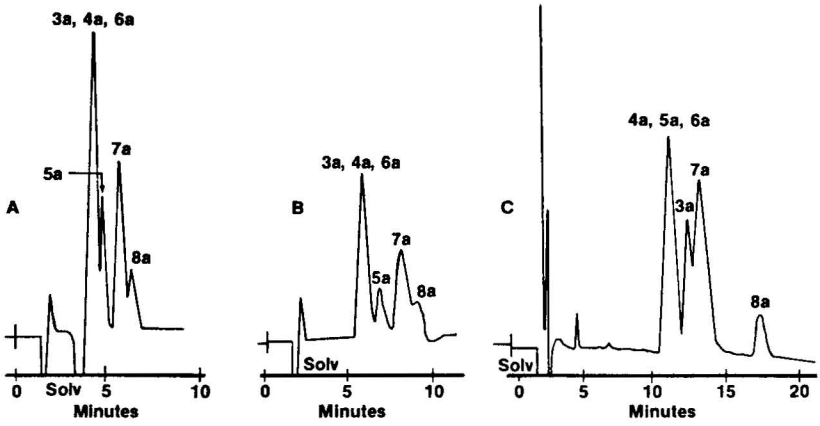

Fig. 1. HPLC separation of limonene hydroperoxides, 3–4 μl. A, ethyl acetate–methylene chloride (0.25:99.75); B, 100% methylene chloride; C, ethyl acetate–hexane (4:96).

* The capacity factor is defined here as $(V_x - V_0)/V_0$ where V_0 is the void volume and V_x is the elution volume of the peak of interest.

(Fig. 1B); in this case, 5a separated from 3a, 4a and 6a. Next, various levels of ethyl acetate–hexane were tried and the best separation in the series was obtained with 4% ethyl acetate. As shown in Fig. 1C, a much better separation of 7a and 8a was achieved and also, 3a was separated from 4a, 5a and 6a. Levels of chloroform in toluene were also employed, and the system chloroform–toluene (10:90) allowed the separation of 4a from 3a, 5a, and 6a. The best overall separation was obtained with ethyl acetate–toluene (0.5:99.5) (Fig. 2). In this case, 4a and 6a were separated from each other and from 3a and 5a. Conditions previously employed by Chan and Levett[5] for lipid hydroperoxide separation, ethanol–hexane (0.75:99.25), proved useless for the limonene hydroperoxides.

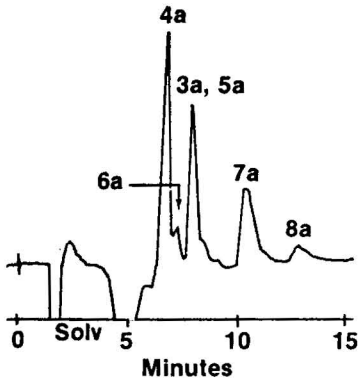

Fig. 2. HPLC separation of 4 μl of limonene hydroperoxides with ethyl acetate–toluene (0.5:99.5).

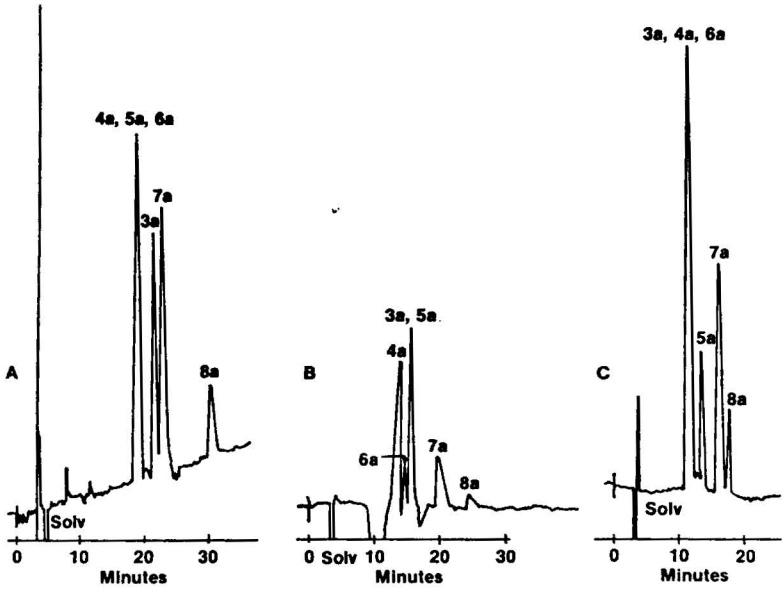

Fig. 3. Hydroperoxide separations on two columns of Partisil 10 and 5 in tandem. A, ethyl acetate–hexane (4:96), 2.5 μl; B, ethyl acetate–toluene (0.5:99.5), 10 μl; C, 100% methylene chloride, 5 μl.

Column packing size and length were also investigated. It was found that Partisil 5 gave better resolutions than Partisil 10, and that the use of Partisil 10 and 5 columns in tandem improved the separations over those achievable on them individually. Chromatograms for separations using ethyl acetate–hexane (4:96), ethyl acetate–toluene (0.5:99.5) and 100% methylene chloride with the tandem columns are shown in Fig. 3A, B and C.

For preparative purposes, all the solvents except toluene could readily be removed by rotary evaporation at 28°C, full aspirator vacuum, without affecting the hydroperoxides. Removal of toluene, however, required a temperature of 33°C which resulted in a rearrangement of the hydroperoxides if volumes were reduced below 0.5 ml. Consequently, when toluene was used, isolation of the materials first required concentration of each fraction to 0.5 ml followed by rechromatography twice with a different solvent, resulting in removal of most of the toluene. All the compounds were separated with the mixture of ethyl acetate–toluene (0.5:99.5) except hydroperoxides 3a and 5a (Fig. 3B). However, these two were separated upon rechromatography of this fraction using 100% methylene chloride (Fig. 3C) or ethyl acetate–hexane (4:96) (Fig. 3A).

The order of elution for each *trans*/*cis* pair of the alcohols (3b–8b) is *trans* before *cis* both by HPLC and GLC[8]. However, for the hydroperoxides, the HPLC order of elution for the first two pairs, 3a:4a and 5a:6a, is *cis* before *trans* and for the last pair, 7a:8a, it is still *trans* before *cis*. This order is found for all the hydroperoxide runs in which resolution is achieved between any of the isomeric pairs.

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Note

Reversed-phase high-performance liquid chromatographic detection of pemoline in doping control

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(Received July 22nd, 1980)

Pemoline (2-imino-4-oxo-5-phenyloxazolidine) is a psycho-stimulant drug used in many pharmaceutical preparations (Deadyn, Didascon, Stimul, Tradon, etc.), and some cases of drug abuse in athletes have been found.

The determination of pemoline in urine or other biological liquids can be carried out by gas chromatography only after hydrolysis and derivatization. The compound can be detected by a flame ionization detector and identified by mass spectrometry[1], by using a nitrogen-selective detector[2,3] or electron-capture detector[4]. All these procedures are time consuming and not very reliable for routine doping control.

In a previous paper[5] we described a simple method for the determination of pemoline in urine by high-performance liquid chromatography (HPLC) without any chemical transformation. Here we report an improvement that was obtained for this analysis using the recent advances in HPLC, *e.g.*, the use of reversed-phase columns, gradient elution and variable-wavelength detectors, and also a more simple and rapid procedure for sample extraction using XAD-2 macroreticular resin[6].

EXPERIMENTAL

Chromatography

A Hewlett-Packard Model 1084-B liquid chromatograph was used equipped with automatic injection and scanning of the spectra of eluted peaks. The column (25 cm × 4.6 mm I.D.) was packed with 10 μm LiChrosorb RP-18 (or RP-8) and connected to a short (5 cm) pre-column packed with the same phase. In this way the chromatographic column is protected from the many impurities in the biological samples. Other conditions: mobile phase, water–methanol (85:15), flow-rate 2 ml/min; column temperature 40°C; water temperature 70°C; methanol temperature 40°C; detector wavelength, 220 nm.

After the elution of pemoline the solvent composition was raised to 100% methanol with a fast gradient and maintained for 10 min, to clean the column of other retained compounds.

The instrument was programmed to return, after this time, to the initial con-

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ditions and be ready for another analysis. Standard solutions (1 μg/ml) of pemoline in methanol were injected as reference. Pemoline has a retention time of about 8 min.

Sample extraction

Volumes (10 ml) of urine were passed through a column containing 1 g of XAD-2 resin followed by 20 ml of distilled water. The column was allowed to run dry, the effluent discarded and pemoline eluted with 3 ml of methanol. A 40–50 μl volume of this solution was directly injected in the liquid chromatograph. In the case of a suspected peak having a retention time within ± 5% that of pemoline, the sample was injected again, a spectrum recorded at the top of the eluted peak in the wavelength range 190–350 nm and compared with reference spectra.

RESULTS AND DISCUSSION

Pemoline is strongly adsorbed by XAD-2 resin from urine, and a recovery of 80–85% is obtained after elution with methanol. This drug is largely excreted unchanged in urine and a maximum is reached 2–4 h after administration. A urine concentration of 1–2 μg/ml is obtained after administration of a single therapeutical dose (10 mg).

In our method, 10 ng of pemoline injected in the chromatograph show a well detectable peak. This amount corresponds to a minimum detectable concentration in urine of 0.1–0.05 μg/ml.

Fig. 1A shows the chromatogram of a standard solution and Fig. 1B the corresponding UV spectrum of eluted peak. Pemoline has a strong absorption maximum at 220 nm; since this maximum occurs at short wavelengths the best solvents to be used in a mixture with water are methanol or acetonitrile. We prefer the first for routine analysis because it is less expensive and safer.

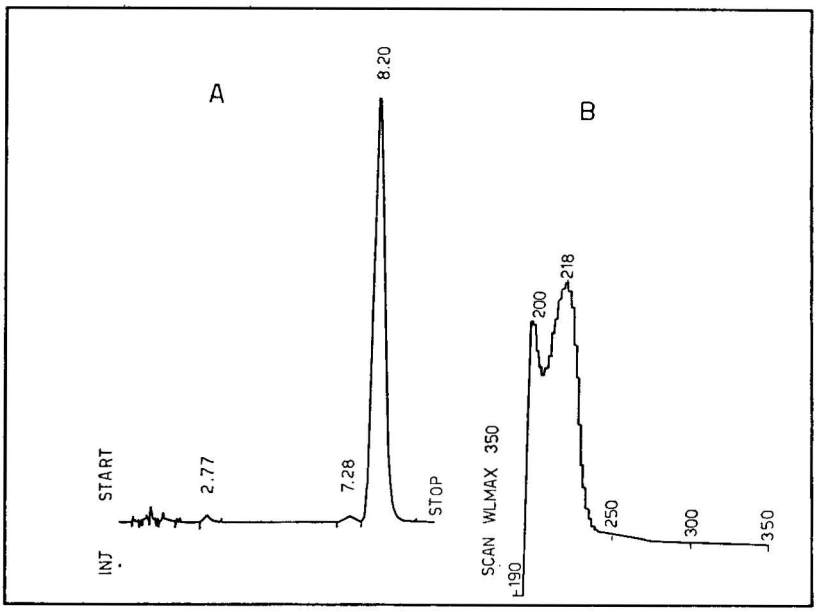

Fig. 1. A, Chromatogram of a standard solution of pemoline. Column: LiChrosorb RP-8 (25 cm × 4.6 mm I.D.). Mobile phase: water–methanol (85:15); flow-rate 2 ml/min. Retention times in minutes. B, UV spectrum recorded on the eluted peak of pemoline.

Fig. 2A shows the chromatogram of a urine blank and Fig. 2B that of the urine of the same subject 4 h after ingestion of a single therapeutical dose (10 mg) of pemoline. As can be observed, in reversed-phase HPLC, many compounds are eluted well before pemoline as non-interfering peaks. At the end of each analysis it is important to clean the column by increasing the concentration of methanol to 100% to eliminate interferences of late eluted peaks from the previous injections.

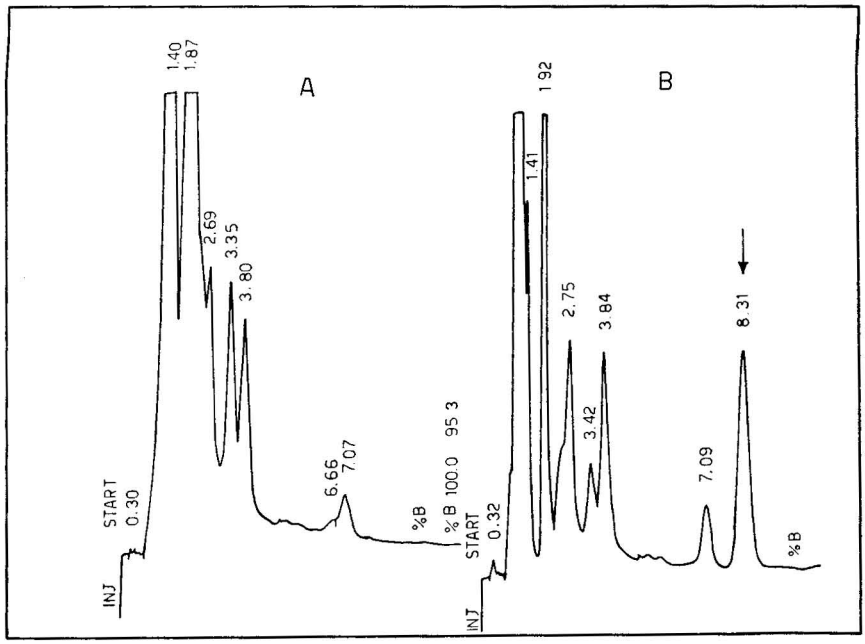

Fig. 2. Chromatogram of a urine blank (A) and of urine 4 h after the administration of 10 mg of pemoline. Conditions as in Fig. 1.

The remaining part of the sample extracts can be utilized for other confirmatory tests; the suspected peak can be easily collected and examined by HPLC on a different column, by gas–liquid chromatography after hydrolysis and esterification and by mass spectrometry either by direct injection of the collected peak or in combination with gas chromatography.

Following this procedure we have carried out numerous analyses for the detection of pemoline in doping control with a good selectivity and sensitivity, in a short time and without any complicated sample treatment.

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CHROM. 13,165

Note

Separation of vitamin B_6 by reversed-phase ion-pair high-performance liquid chromatography

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(Received July 10th, 1980)

In animals, vitamin B_6 occurs in the form of pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM), their corresponding phosphates and their metabolite 4′-pyridoxic acid (PIC). The determination of these different forms in biological materials is not only important in connection with nutritional problems[1], but also for problems of clinical chemistry[2]. Recently, high-performance liquid chromatography (HPLC) has been used for the analysis of B_6 compounds, and several workers[3–6] have reported the use of an ion-exchange column for the separation of such compounds. Ion-pair chromatography can, in many cases, be used instead of ion-exchange for ionic or ionizable compounds and has some advantages such as high selectivity, column performance and simplicity of the mobile phase[7]. PN, PL and PM can be separated by this technique using 1-heptanesulphonic acid as the pairing agent[8].

The present study employed ion-pair chromatography with cetyltrimethylammonium bromide (CTAB) for the separation of B_6 compounds, particularly phosphates and PIC, and for the determination of PIC in urine.

EXPERIMENTAL

Chemicals

Pyridoxamine 5′-phosphate (PMP), pyridoxal 5′-phosphate (PLP), PN, PL, PM and CTAB were purchased from Nakarai Chemicals (Tokyo, Japan) and PIC from Sigma (St. Louis, MO, U.S.A.). Pyridoxine 5′-phosphate (PNP) was synthesized by the procedure of Kuroda[9]. 4′-Propoxypyridoxine 5′-phosphate (4′-PPNP) used as the internal standard, was synthesized by phosphorylating 4′-propoxypyridoxine[10] with a H_3PO_4–P_2O_5 mixture. All other chemicals were of analytical grade.

Chromatography

A Hitachi Model 635-T high-performance liquid chromatograph was used for the analyses. The wavelength of the UV detector was set at 292 nm. The sensitivity was kept on 0.08 a.u.f.s. throughout.

All mobile phases contained 0.002 *M* CTAB in methanol–phosphate buffer solution (1:1). CTAB was used as the pairing agent. Phosphate buffer solution was prepared from 0.1% NaH_2PO_4 solution and the pH values (3.4–4.2) were adjusted with 0.1% H_3PO_4 solution. The mobile phases were filtered through a 0.45-μm

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membrane filter (Toyo Roshi, Tokyo, Japan) and degassed in an ultrasonic bath prior to use. The analyses were carried out at room temperature on a stainless-steel column (15 cm × 4 mm I.D.) packed with LiChrosorb RP-8 (5-μm particles) (E. Merck, Darmstadt, G.F.R.). The flow-rate was 0.5 ml/min.

Sample preparation

Standard solutions of B_6 compounds were prepared in distilled water and stored at 5°C. Methanol–water (1:1) was used as the diluent. Urine samples were filtered with a 0.45-μm membrane filter, diluted with methanol–water (1:1) and a 20-μl sample was injected onto the column.

Calibration

Peak height ratios were calculated by dividing the heights of the PNP, PIC and PLP peaks by the height of the internal standard and plotted against the amounts of PNP, PIC and PLP.

RESULTS AND DISCUSSION

In order to find the optimal chromatographic conditions, the influence of CTAB concentration and of the pH of the mobile phase on the retention time was investigated. The influence of the pH of the mobile phase was studied using phosphate-buffered mobile phases of different pH values (range 3.4–4.2) (Fig. 1). The retention

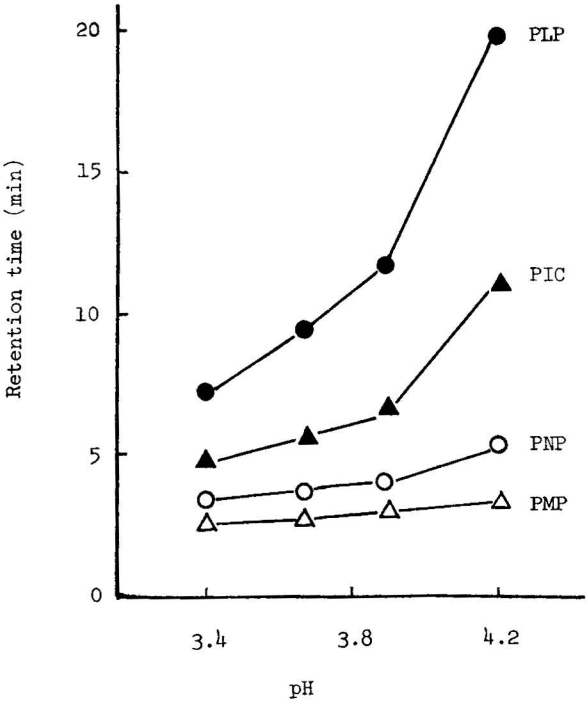

Fig. 1. Relationship between retention times and pH of the mobile phase for B_6 compounds.

times of B_6 compounds increased and could be resolved better as the pH was increased. However, at pH 4.2, poor reproducibility of the retention time was observed. Therefore, the optimal pH seemed to be 3.89. PLP and PNP showed slight peak tailing when the pH of the mobile phase was adjusted with acetate buffer solution instead of phosphate buffer solution. The retention times of B_6 compounds were little affected by the CTAB concentration ($5 \cdot 10^{-4}$–$5 \cdot 10^{-3}$ *M*).

A typical chromatogram is shown in Fig. 2. Separation of PNP, PIC and PLP was complete in about 12 min. PMP was eluted with the solvent front and not retained under any of the conditions.

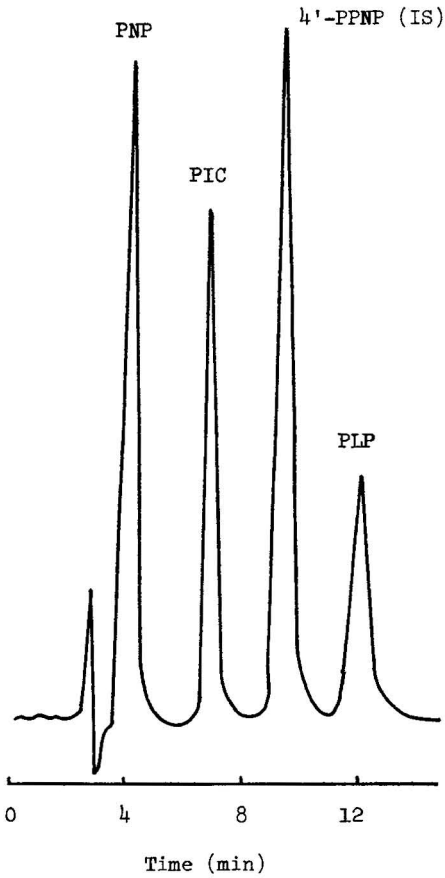

Fig. 2. Chromatogram of B_6 compounds. Mobile phase: phosphate buffer solution–methanol (1:1) with CTAB (0.002 *M*), pH 3.89.

The calibration curves were linear over the range of 0.2–1.5 nmoles of PNP and 0.2–4.0 nmoles of PIC and PLP (Fig. 3). The correlation coefficient for each B_6 compound was greater than 0.99. In the range of 1.0–4.0 nmoles, the mean PIC recovery from control urine was 98.7 $\pm$ 7% ($n = 6$).

The main metabolite of vitamin B_6 in human urine is PIC and its determination is important in studies of the bioavailability[11] of vitamin B_6 in various dosage

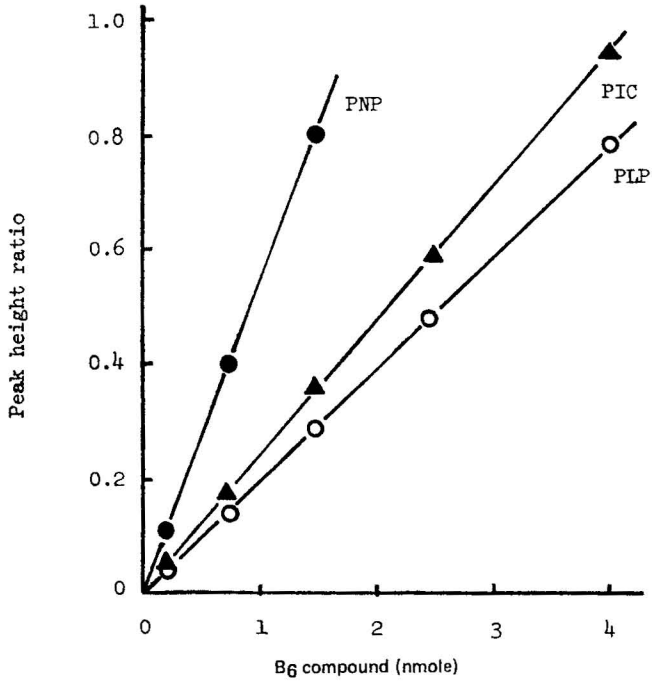

Fig. 3. Calibration curves of B_6 compounds.

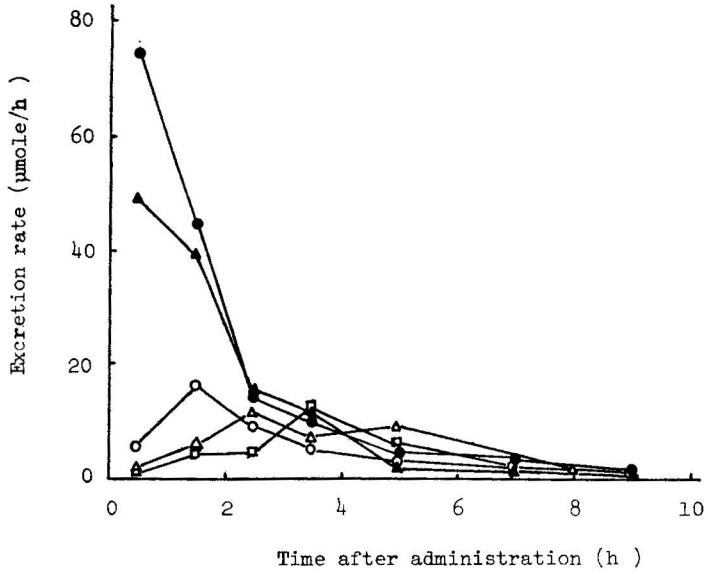

Fig. 4. Urinary excretion of PIC following oral administration of various forms of vitamin B_6: ○—○, PN; ●—●, PL; △—△, PM; ▲—▲, PLP; □—□, PMP.

forms and its metabolism in humans[1,12]. We used the procedure described above to determine the PIC content in human urine. Urinary excretion of PIC for an orally administered dose of 243.14 μmoles of B_6 compounds was determined (Fig. 4). Following oral administration of PL and PLP, the urinary excretion rate of PIC was rapid compared with the other B_6 compounds. The reason for this is that PL[13] and PLP are more easily metabolized to PIC in the human body than other B_6 compounds. The data obtained were similar to the results published by Rabinowitz and Snell[13].

Major advantages of our chromatographic technique are that PNP, PLP and PIC can be assayed at the same time in a simple manner, and the degree of accuracy and reproducibility are excellent. However, the sensitivity is not adequate for blood determination. We are now studying more sensitive methods which use other detection techniques, such as the fluorometric method.

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The authors wish to thank Miss R. Kotake and Miss Y. Shimamoto for their help during this study.

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Note

Separation of digitalis glycosides by micro high-performance liquid chromatography

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The cardiac glycosides are therapeutically important substances which are widely used in the treatment of heart disease. Methods published so far for the determination of digitalis glycosides are based on paper, thin-layer and gas–liquid chromatography. High-performance liquid chromatography (HPLC) has been also employed[1–7] but it is not always satisfactory with respect to the determination of low concentrations of digitalis glycosides. In recent years, micro high-performance liquid chromatography (MHPLC) has been developed for measuring micro-scale amounts by Ishii and co-workers[8–12].

This paper reports the separation and quantitation of digitalis glycosides by MHPLC. The study was concentrated on the investigation of the chromatographic behaviour of nanogram amounts using a reversed-phase system and ultraviolet (UV) detection (220 nm) based on the α,β-unsaturated lactone ring. The MHPLC of digitoxin, gitoxin, digoxin, lanatoside A and lanatoside B was performed on a 5-μm ODS column, using acetonitrile–methanol–water (1:1:1) as the mobile phase. Various mixtures of methanol and water were used as the mobile phase for the separation of digitoxin and its metabolites, gitoxin and its metabolites and digoxin and its metabolites. Detection limits were as low as 1 ng for a 0.1-μl injection and the separation times varied between 30 and 45 min.

EXPERIMENTAL

Instruments

The apparatus was a Familic-100 micro high-performance liquid chromatograph (Japan Spectroscopic Co., Tokyo, Japan) equipped with a Uvidec-100 UV spectrophotometer monitoring the absorbance at 220 nm. The micro flow-through cell consisted of a quartz tube with a volume of 0.3 μl. The micro column used throughout was a PTFE tube packed with SC-01 (Japan Spectroscopic Co.). This was a reversed-phase column (0.5 mm I.D.) containing 5-μm porous silica particles covalently linked with ODS groups.

Materials

The chromatographic solvents were of analytical-reagent grade from Wako (Osaka, Japan). The cardiac glycosides were purchased from E. Merck (Darmstadt,

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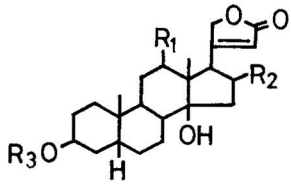
G.F.R.). The mono- and bisdigitoxosides of digitoxigenin, gitoxigenin and digoxigenin were prepared by hydrolysis of the secondary glycosides according to the methods of Kaiser and co-workers[13,14]. All of these materials were checked for homogeneity by thin-layer chromatography and their structures are given in Table I.

TABLE I

STRUCTURES OF THE DIGITALIS GLYCOSIDES AND AGLYCONES INVESTIGATED



Compound	R_1	R_2	R_3*
Digitoxigenin	H	H	H
Digitoxigenin monodigitoxoside	H	H	-D
Digitoxigenin bisdigitoxoside	H	H	-D-D
Digitoxin	H	H	-D-D-D
Gitoxigenin	H	OH	H
Gitoxigenin monodigitoxoside	H	OH	-D
Gitoxigenin bisdigitoxoside	H	OH	-D-D
Gitoxin	H	OH	-D-D-D
Digoxigenin	OH	H	H
Digoxigenin monodigitoxoside	OH	H	-D
Digoxigenin bisdigitoxoside	OH	H	-D-D
Digoxin	OH	H	-D-D-D
Lanatoside A	H	H	-D-D-AcD-G
Lanatoside B	H	OH	-D-D-AcD-G

* D = digitoxose; AcD = acetyldigitoxose; G = glucose.

Procedure

Solvents were prepared immediately before use. The chromatographic conditions for each separation are presented with the chromatograms. Separate solutions of each cardiac glycoside or aglycone were carefully prepared by weighing the compound and dissolving it in methanol. Chromatographic retention times of standards were determined, and compared with peaks with similar retention times in mixture of cardenolides. Calibration graphs were constructed using the average peak areas from three chromatograms.

RESULTS AND DISCUSSION

The chromatographic separation of representative cardiac glycosides was carried out under a variety of conditions. For the reversed-phase chromatography, the selection of a suitable eluent was studied by investigating combinations of the organic solvent and water; the UV absorption of the chromatographic solvents has an influence on the sensitivity of detection at 220 nm.

Initial work was directed towards the separation of digitoxin, gitoxin, digoxin,

lanatoside A and lanatoside B. As shown in Fig. 1, these substances were separated satisfactorily on an ODS bonded silica column (SC-01) when acetonitrile–methanol–water (1:1:1) was employed as the mobile phase at a flow-rate of 4 μl/min. The PTFE column (165 × 0.5 mm I.D.) used in this technique was much smaller than those used in ordinary HPLC. A detection wavelength of 220 nm was employed on the basis of the butenolide ring.

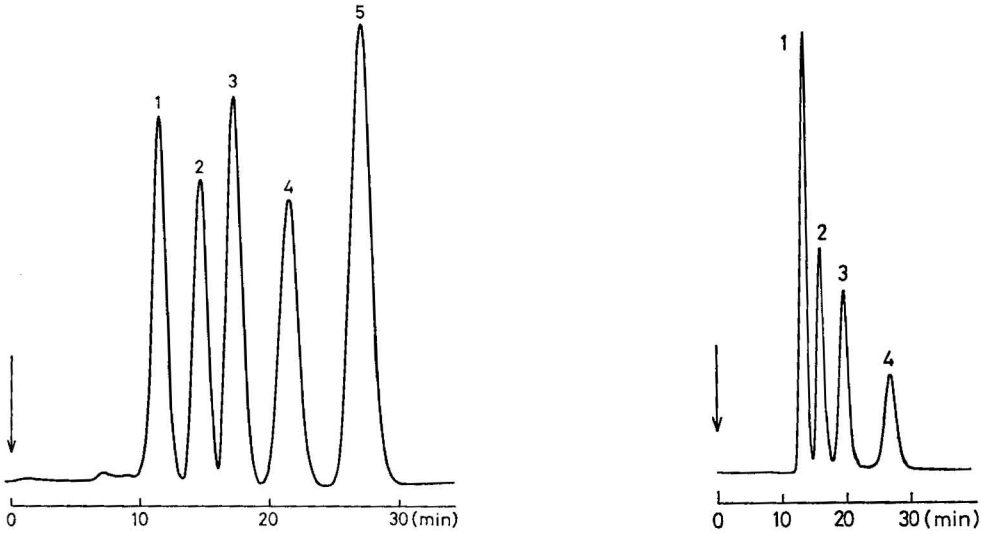
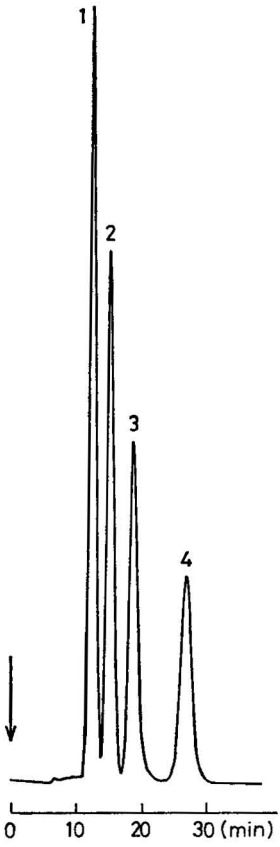


Fig. 1. Separation of a mixture of digitalis glycosides. Peaks: 1 = digoxin; 2 = lanatoside B; 3 = gitoxin; 4 = lanatoside A; 5 = digitoxin. Conditions: Jasco SC-01 column (165 × 0.5 mm I.D.); mobile phase, acetonitrile–methanol–water (1:1:1); flow-rate, 4 μl/min; UV monitor at 220 nm; sample volume, 0.1 μl.

Fig. 2. Separation of digitoxin and its metabolites. Peaks: 1 = digitoxigenin; 2 = digitoxigenin monodigitoxoside; 3 = digitoxigenin bisdigitoxoside; 4 = digitoxin. Conditions: Jasco SC-01 column (151 × 0.5 mm I.D.); mobile phase, methanol–water (5:2); flow-rate, 4 μl/min; UV monitor at 220 nm; sample volume, 0.1 μl.

Fig. 2 shows the separation of a mixture of digitoxin and its metabolites (digitoxigenin bisdigitoxoside, digitoxigenin monodigitoxoside and digitoxigenin) eluted with methanol–water (5:2). Similarly, gitoxigenin, its mono- and bisdigitoxosides and gitoxin were separated into four peaks when methanol–water (2:1) was employed, as illustrated in Fig. 3. Fig. 4 shows the results of the chromatography of a mixture of digoxin and its metabolites using methanol–water (6:5). The separations in all examples are sufficiently good and reproducible to permit quantitative work.

The calibration graphs in Fig. 5 were constructed by plotting the peak areas of digitoxin and gitoxin obtained by MHPLC using acetonitrile–methanol–water (1:1:1) as the mobile phase against amount of sample. Detection with a Uvidec-100 UV spectrophotometer (220 nm) showed a linear response to each cardiac glycoside in the range 5–25 ng. Similarly, Fig. 6 shows the calibration graphs for the MHPLC of digitoxigenin, its mono- and bisdigitoxosides and digitoxin using methanol–water (5:2) as the mobile phase. Peak areas for each compound were measured for various



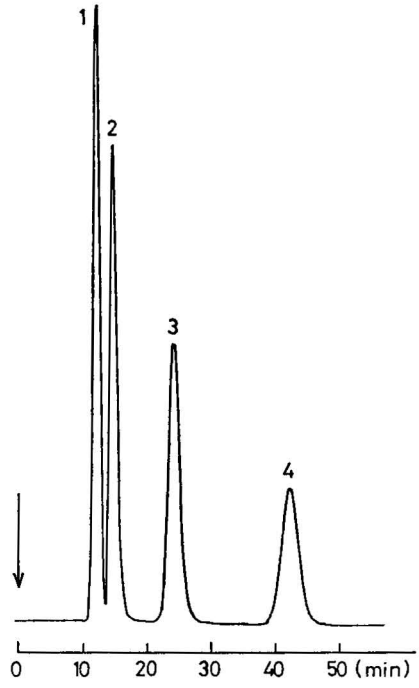


Fig. 3. Separation of gitoxin and its metabolites. Peaks: 1 = gitoxigenin; 2 = gitoxigenin monodigitoxoside; 3 = gitoxigenin bisdigitoxoside; 4 = gitoxin. Conditions: mobile phase, methanol–water (2:1); other conditions as in Fig. 2.

Fig. 4. Separation of digoxin and its metabolites. Peaks: 1 = digoxigenin; 2 = digoxigenin monodigitoxoside; 3 = digoxigenin bisdigitoxoside; 4 = digoxin. Conditions: mobile phase, methanol–water (6:5); other conditions as in Fig. 2.

amounts from 5 to 40 ng by UV detection (220 nm). The limit of accurate quantitative measurement of these steroids was about 5 ng and the detection limit at a signal-to-noise ratio of 3:1 was less than 1 ng.

The method presented appears to be better than previous methods, as MHPLC of digitalis glycosides is more sensitive, convenient and inexpensive than ordinary HPLC. On the basis of the separation efficiency and sensitivity of the proposed procedure, it seems feasible to determine digitalis glycosides and their metabolites in urine and other biological fluids. Moreover, this work suggests that MHPLC can be a valuable method for the direct coupling of liquid chromatography and mass spectrometry. Quantitation of major cardiac glycosides in *Digitalis purpurea* leaf by MHPLC is being conducted and the details will be reported elsewhere.

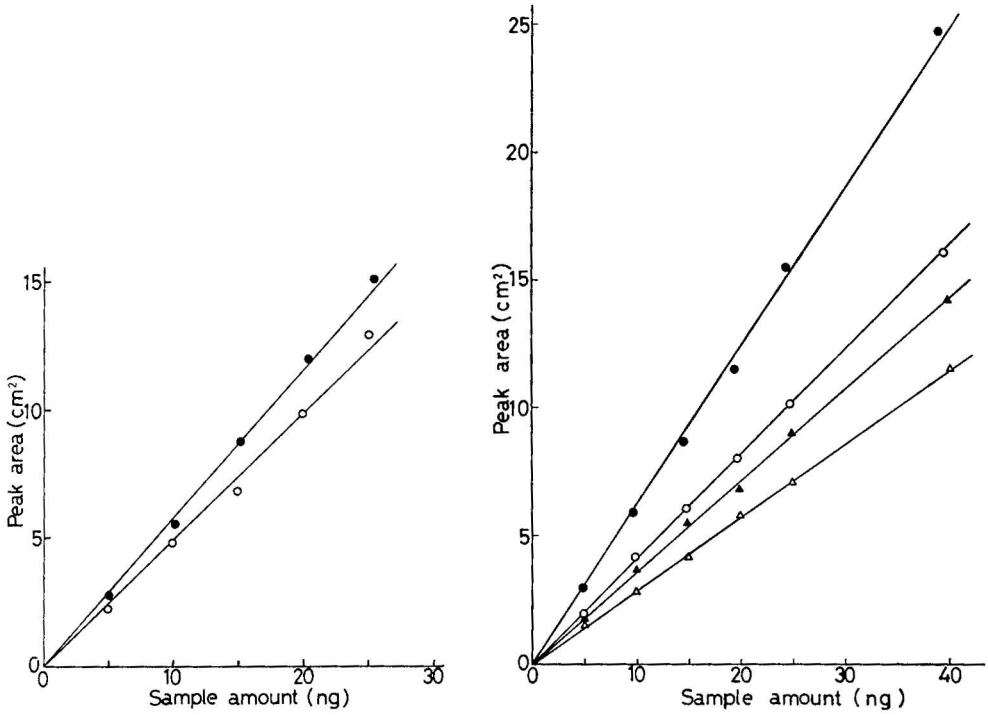


Fig. 5. Calibration graphs for digitoxin (●) and gitoxin (○).

Fig. 6. Calibration graphs for digitoxin and its metabolites. ●, Digitoxigenin; ○, digitoxigenin monodigitoxoside; ▲, digitoxigenin bisdigitoxoside; △, digitoxin.

ACKNOWLEDGEMENT

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Note

High-performance liquid chromatographic determination of amoxycillin in pharmaceutical dosage forms

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(Received June 27th, 1980)

Recently, several chemical methods for the determination of amoxycillin in biological fluids have been reported. Miyazaki *et al*[1]. described the determination of amoxycillin based on the formation of a fluorescent derivative. The high-performance liquid chromatographic (HPLC) determination of amoxycillin and ampicillin in biological fluids was reported by Vree *et al.*[2]. Subsequently, Lee *et al.*[3], and Carlqvist and Westerlund[4] described more sensitive HPLC methods involving post-column derivatization.

To date, HPLC methods have not been directed to the analysis of amoxycillin in pharmaceutical dosage forms. The present official assay method of the British Pharmacopoeia[5] involves a colourimetric determination based on the copper(II) ion catalysed formation of the penicillenic acid. This method was recommended previously for ampicillin but has been replaced by the method of Bundgaard[6] involving colourimetric determination of penicillins by reaction in imidazole–mercury solution. The iodometric method is official in the Code of Federal Regulations[7].

Canadian drug quality assessment programs require reliable and time-saving methods for the analysis of large numbers of samples. Due to the lack of specificity of the official methods, a reversed-phase HPLC method for the determination of amoxycillin in bulk drug substance and oral preparations was developed.

EXPERIMENTAL

An SP 8000 liquid chromatograph (Spectra-Physics, Santa Clara, CA, U.S.A.), equipped with a SP 8300 fixed-wavelength detector (254 nm) and with a data system, was employed during the study. A reversed-phase column (RP-8, 10 μm, 25 cm $\times$ 4.6 mm I.D., Brownlee Labs., Santa Clara, CA, U.S.A.) was employed at 30°C with a flow-rate of 1.0 ml/min. Injections of 10 μl were used for all solutions to be analysed.

Mobile phase

The mobile phase consisted of 6% (v/v) methanol in 0.05 *M* phosphate buffer (pH 5.0).

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Solutions

Internal standard stock solution. Phenoxyacetic acid was dissolved in water, 4 g/l.

Bulk drug substance and capsule solutions. To an accurately weighed amount of bulk drug substance or homogeneous capsule contents equivalent to 20 mg of amoxycillin, was added 2.0 ml of internal standard solution and the volume made to 10.0 ml with water. A small stirring bar was then added and the contents of the flask stirred until dissolution was complete (0.5–2 h). The amoxycillin standard (USP Amoxycillin Reference) solution was prepared in a similar manner.

RESULTS AND DISCUSSION

A typical chromatogram of an amoxycillin capsule formulation is shown in Fig. 1. Excipients from capsule formulations did not interfere.

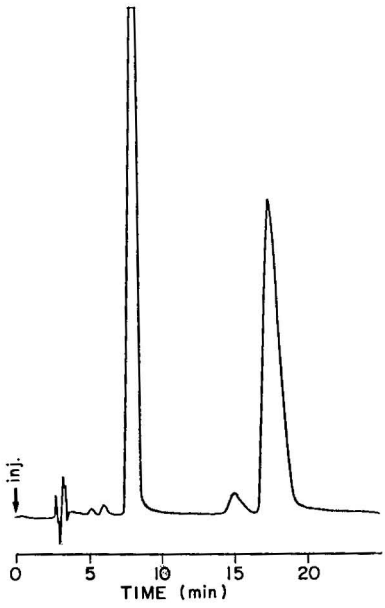

Fig. 1. Chromatogram of amoxycillin capsule contents on a reversed-phase column (RP-8) with a mobile phase of 6% methanol in 0.05 *M* phosphate (pH 5.0).

Table I shows the relative retention times of amoxycillin and other related compounds.

The linearity of the chromatography system was verified by injection of six solutions containing amoxycillin from 0.5 to 2.5 mg/ml and 0.8 mg/ml internal standard. A straight line, coefficient of correlation = 0.9998 ($y = 0.877x - 0.0197$), was obtained when the ratios of the area counts of the amoxycillin divided by the area counts of the internal standard were plotted *versus* concentration of amoxycillin.

TABLE I

RELATIVE RETENTION TIMES OF AMOXYCILLIN AND OTHER RELATED COMPOUNDS

Compound	*Relative retention time**
D(−) *p*-Hydroxyphenylglycine	0.38
Amoxycilloic acid	0.76
6-Aminopenicillanic acid	0.83
Amoxycillin	1.00
Methyl paraben	1.93
Phenoxyacetic acid (internal standard)	2.22
Ethyl paraben	3.97
Propyl paraben	7.65

* See text for chromatographic conditions.

Ten consecutive injections of a solution of amoxycillin (2 mg/ml) resulted in a relative standard deviation of the area ratio of the amoxycillin peak to that of the internal standard of 0.72%.

A number of samples of bulk drug substance were analysed for amyxocillin content by HPLC. These samples were also determined by the colourimetric method of Bundgaard[6] based on the reaction of penicillins with imidazole and mercuric chloride. The results are shown in Table II. Good correlation between the HPLC and chemical assay was obtained. Additionally the water content of each bulk was determined by the standard Karl Fischer techniques. Generally, the bulk drugs having a higher potency, *e.g.* USP and BP standards, also showed a higher water content.

TABLE II

HPLC AND CHEMICAL ASSAY OF AMOXYCILLIN BULK DRUG SUBSTANCE

Sample	*Potency* (μg/mg)*		*Water (%)*
	HPLC	*Chemical*	
USP standard	850**	850**	13.97
BP Standard	853	855	14.49
Manufacturer 1 A	885	885	14.45
B	819	791	12.70
Manufacturer 2 A	806	799	11.98
B	806	824	12.41
Manufacturer 3 A	806	825	12.02
B	830	839	11.77
Manufacturer 4 A	852	850	12.20
B	842	848	11.60
C	819	816	12.30
Manufacturer 5	833	836	12.34
Manufacturer 6	815	829	12.11

* Average of duplicate determinations. All values based on USP Standard.
** Label claim.

Capsule formulations from four manufacturers were also assayed by both the HPLC and chemical assay methods. The results are presented in Table III. Again good correlation between the two methods was obtained. Ten replicate determinations of sample A from manufacturer number 3 gave a mean of 93.5% and relative standard deviation of 0.58%.

TABLE III

HPLC AND CHEMICAL ASSAY OF AMOXYCILLIN CAPSULE FORMULATIONS

Sample	*Label claim (%)**	
	HPLC	*Chemical*
Manufacturer 1 A	96.8	97.3
B	101.6	99.5
Manufacturer 2 A	95.6	95.9
B	97.3	96.1
Manufacturer 3 A	94.0	—
B	97.1	97.9
Manufacturer 4	92.4	89.1

* Average of duplicate determinations. All results relative to USP Standard.

Most chromatograms of bulk drug samples and formulations contained a small peak eluting prior to amoxycillin, relative retention time, 0.76. Degradative studies showed this to be the penicilloic acid of amoxycillin. Relative to the amoxycillin peak and based on relative peak areas,there was *ca.* 0.2–0.6% of the penicilloic acid present in the sample solutions. 6-Aminopenicillanic acid is also eluted prior to amoxycillin; however, it is only partially resolved from the penicilloic acid of amoxycillin.

The HPLC system described is a rapid, precise and accurate method for the specific analysis of amoxycillin bulk drug substance and solid oral dosage forms.

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Note

Liquid chromatographic determination of submicrogram amounts of ipsenol and ipsdienol, pheromone components of *Ips paraconfusus* Lanier

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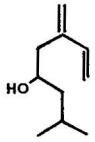
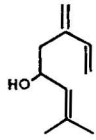
(First received June 19th, 1980; revised manuscript received July 31st, 1980)

Ipsenol (I, 2-methyl-6-methylene-7-octen-4-ol) and ipsdienol (II, 2-methyl-6-methylene-2, 7-octadien-4-ol) are components of the aggregation pheromone of the bark beetle *Ips paraconfusus* Lanier[1]. These pheromone components were dispersed into the forest environment from slow release devices in bark beetle control experiments.



(I)



(II)

This paper describes an analytical method developed to assess the quantity and quality of ipsenol and ipsdienol remaining after their elution in field experiments.

Gas chromatography was used previously to analyze *Ips* pheromone components[2,3]; however, certain *Ips* pheromone degradation products, such as their polymers, are not volatile and will not readily elute from a gas chromatographic column. I found that a liquid chromatograph with a variable-wavelength ultraviolet detector can be used to determine purity of the pheromone components.

EXPERIMENTAL*

Apparatus

Ultraviolet spectra were determined in acetonitrile in a Unicam SP800 spectrophotometer. Chromatograms were run with a Waters Assoc. (Milford, MA, U.S.A.) Series ALC-200 chromatograph fitted with a U6K injector, a Tracor Model 970 detector, and a Linear Model 555, 10-in. recorder.

* Trade names and commercial products or enterprises are mentioned solely for information. No endorsement by the U.S. Department of Agriculture is implied.

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Column. A Waters Assoc. 30 cm × 3.9 mm I.D. column packed with 10-μm Porasil was used.

Mobile phase. Acetonitrile (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) was the mobile phase.

Reagents

Racemic ipsenol and ipsdienol were from the Chemical Samples Co. (Columbus, OH, U.S.A.) and were freshly distilled under reduced pressure.

Procedure

The pheromone components were dissolved in acetonitrile before injection. The solvent was degassed under vacuum before using. Calibration of standard curves were determined by triplicate injections of known quantities of ipsenol and ipsdienol at several absorption units full scale (a.u.f.s.).

RESULTS AND DISCUSSION

A typical chromatogram of ipsdienol exposed to the forest environment is shown in Fig. 1. Exposed ipsenol except for an earlier elution time has almost an identical chromatogram. Analyses by this method showed degradation of ipsenol from 5 to 10% and of ipsdienol from 1 to 2% when these pheromones components were exposed to the environment. There was also no cross contamination of one

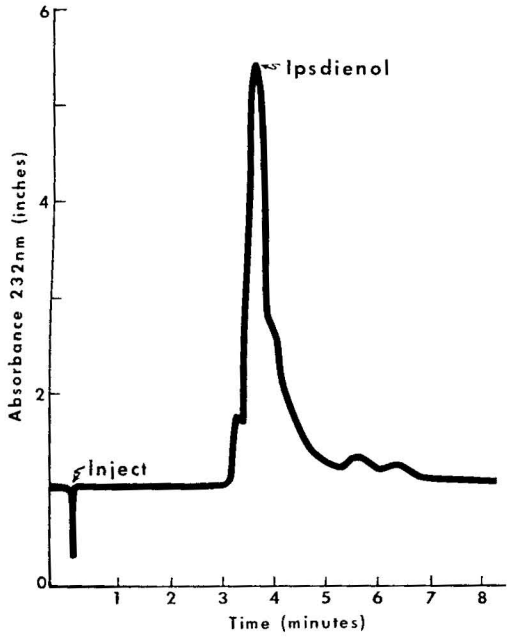

Fig. 1. Typical chromatogram of ipsdienol after exposure to forest environment. Separation on a μPorasil column (30 cm × 3.9 mm I.D.) at ambient temperature with acetonitrile at 1.1 ml/min.

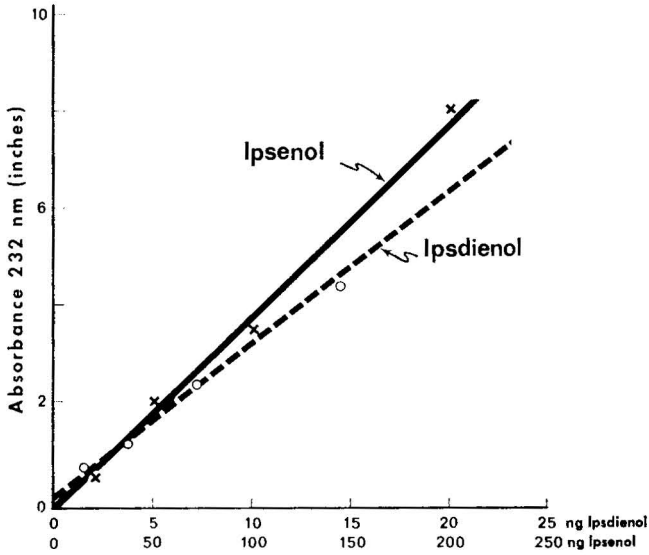

Fig. 2. Peak height *vs.* quantity of pheromone at 232 nm on 10 mV, 10-in. recorder. Ipsenol: 0.04 a.u.f.s.; ipsdienol: 0.01 a.u.f.s.

component into the other in our slow release devices. The two components can be detected at the submicrogram level (0.01–0.005 μg) if necessary.

The λ_{max} of both ipsenol and ipsdienol in acetonitrile is at 225 nm with log ε_{max} at 3.97 and 4.38, respectively. Almost no absorption occurs at 254 nm. The UV absorbance was monitored at 232 nm as a compromise between solvent impurities and pheromone absorbancy. With an eluent flow-rate of 1.1 ml/min and an average pump pressure of 300 p.s.i., the retention time was 3.3 min for ipsenol and 3.6 min for ipsdienol. Calibration curves show that the minimal detectable amount of ipsenol at 0.01 a.u.f.s. is about 5–19 ng, and for ipsdienol it is about 2–4 ng. At the ranges shown, the detector response is linear (Fig. 2). These sensitivities may prove useful in measuring release rates of the pheromones in slow release devices and residual amounts in the environment.

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Note

Combination of infrared spectroscopy and thin-layer chromatography for the identification of slightly soluble substances

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(Received July 16th, 1980)

In thin-layer chromatographic (TLC) investigations the R_F values obtained are often used for identification of substances. However, these values are not sufficiently specific for the identification of unknown compounds, although selective colour reactions may be applied.

Substances can be identified with a high degree of reliability by a combination of chromatographic separation and spectroscopic characterization methods, *e.g.*, the Wick-Stick method of Garner and Parker[1]. Thus, a wide range of substances amenable to direct infrared (IR) spectroscopic investigation have been separated with a suitable TLC system, purified and concentrated on potassium bromide which serves as a carrier material for IR spectroscopy. However, this method can be used only for substances with solubilities sufficiently high for chromatography.

Recently Székely and Baumgartner[2] reported that substances with low solubilities can be chromatographed at temperatures up to 170°C using solvents of low volatility. We have combined this method with the Wick-Stick technique mentioned above and obtained good results with substances thermally stable over the range of temperature employed.

EXPERIMENTAL

Small 10 × 10 cm thin-layer plates were obtained by cutting commercially available 20 × 20 cm silica gel pre-coated plates[3]. The TLC separation was performed with high boiling eluents at temperatures of *ca.* 150°C (according to ref. 2). The Wick-Stick, a pre-pressed KBr triangle[1] (purchased from Harshaw, Cleveland, OH, U.S.A.), was inserted into a metal holder and placed in a cylindrical glass vial (Fig. 1). The spot to be analyzed was scraped off the TLC plate and transferred into this vial. The spot should contain at least 10 μg of material. Better results were obtained if the corresponding by-spots* of several chromatograms were collected, combined and rechromatographed to yield a single spot.

About 200 μl of high boiling solvent, *e.g.*, 1-bromonaphthalene, were added to the Wick-Stick and the vial heated in an oven to 170°C. The solution rose in the KBr triangle because of capillary forces. The solvent evaporated in the upper part

* "By-spot" = chromatographic zone with lower concentration and intensity than the main zone (see Fig. 3).

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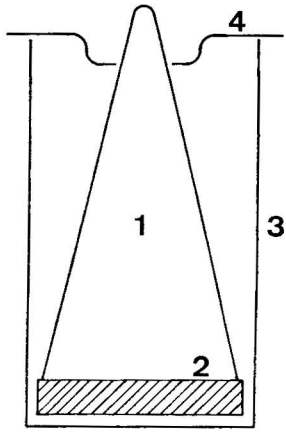


Fig. 1. Wick-Stick. 1 = KBr triangle; 2 = triangle holder; 3 = glass vial; 4 = metal cap. The length of the vial has been somewhat reduced in order to allow the top of the pyramid to extend beyond the upper end of the vial. This greatly facilitates the evaporation of the solvent.

of the triangle and the component to be analyzed was accumulated in the tip. It was necessary to repeat this procedure two or three times. After a few hours, even colourless compounds could be detected on the tip of the Wick-Stick as light yellow spots. The tip of the triangle corresponding to about 20 mg of KBr was cut off, dried in a vacuum at 50°C for several hours and used to make a micro-pellet.

The tip was ground in an agate mortar and placed in the 5 × 1 mm opening of a lead mask (see Fig. 2). The mask was pressed in a conventional press for KBr pellets (diameter, 13 mm; applied pressure, $p = \approx 15$ tons). Then the resulting embedded micro-pellet was focused in the beam of the IR spectrophotometer. It should be noted that only older models of spectrophotometers need a beam condenser accessory. The lead mask also obviates very delicate handling of a micropress.

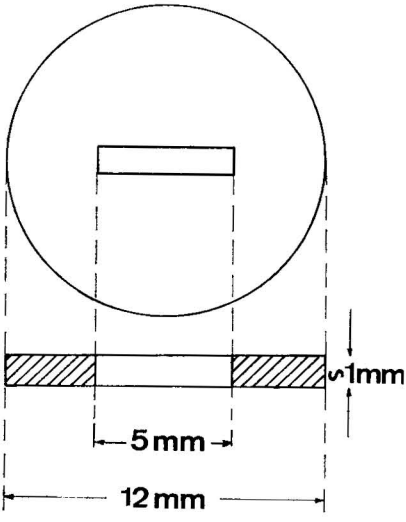


Fig. 2. Lead mask pressed in a conventional press for KBr pellets (diameter, 13 mm; applied pressure, $p = 15$ tons). The resulting embedded micropellet was then focused in the beam of the IR spectrophotometer.

RESULTS

The method described was tested on some dyestuffs and their intermediates which are virtually insoluble at room temperature. The TLC separation of indigo (Fig. 3) at 150°C serves as an example: a blue main spot (R_F 0.70) and a reddish by-spot (R_F 0.60) were observed[2]. The main-spot was purified directly by the Wick-Stick method using 1-bromonaphthalene as eluent, whereas the by-spots from several chromatograms had to be collected first and then purified using the same method.

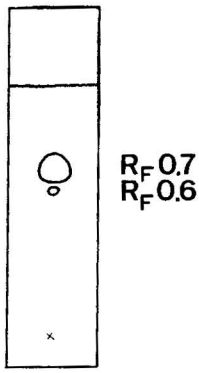
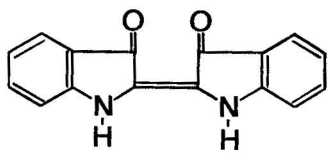


Fig. 3. Separation of indigo at 150°C Layer: Merck silica gel F_{254} pre-coated plate. Solvent: di-*n*-butyl phthalate–N-methylpyrrolidone (9:1). Application: 5 μl of 0.1% sample solutions in 1-bromonaphthalene. Separation time: 15 min for a separation distance of 7 cm Wick-Stick: 200 μl 1-bromonaphthalene at 170°C, followed by two 100-μl portions. Time: *ca.* 5 h.

IR spectra

The IR spectrum of the main spot exhibits specific absorption bands at 1628, 1615, 1483, 1463, 1316, 1298, 1200, 1172, 1127, 1070, 879, 858, 763, 754, 745, 712, 698 and 668 cm^{-1}. With the aid of these absorptions the substance has been identified as indigotin (I).

The chemical structure of the by-spot could be determined as indirubin (II) by its characteristic absorptions at 1667, 1618, 1595, 1482, 1464, 1321, 1300, 1258, 1208, 1178, 1146, 1098, 1019, 1002, 962, 802, 783, 754/749 and 709 cm^{-1}.



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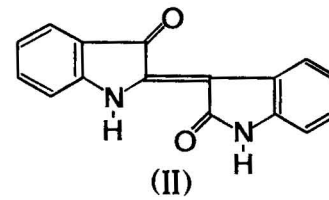

(II)

ACKNOWLEDGEMENTS

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Note

Rapid preparative separation of natural products by centrifugal thin-layer chromatography

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Among the commonly used preparative-scale separation techniques, centrifugal chromatography has so far played only a minor role. However, attempts to increase the separation speed by acceleration of the flow-rate of the mobile phase using centrifugal force were reported many years ago by Hopf[1] and Caronna[2]. Numerous papers dealing with this topic have appeared and were reviewed by Deyl *et al.*[3]. An apparatus called a chromatofuge has been developed and its performance illustrated by the separation of four closely related purines: caffeine, theobromine, theophylline and xanthine[4]. Recently ,an instrument for preparative, centrifugally accelerated, radial, thin-layer chromatography (TLC), the Chromatotron, became commercially available. In this system, the TLC plate (rotor) is not horizontal but inclined, and thus allows more efficient collection of the eluate.

The efficiency of this method was demonstrated by Derguini *et al.*[5], who separated 100-mg amounts of *cis/trans* isomeric esters on silica with *n*-hexane–diethyl ether (99:1). The recovery of pure esters from the Chromatotron was 90% whereas it was only 80% in preparative liquid chromatography.

In connection with our work on the isolation of constituents of higher plants, we have evaluated the Chromatotron for its suitability to achieve rapid preparative separations of various classes of natural products. This paper describes the application of centrifugal TLC to the isolation of xanthones, triterpenes and saponins. Some advantages and limitations of the method are discussed.

EXPERIMENTAL

All of the separations were carried out on a Chromatotron Model 7924 (Harrison Research, Palo Alto, CA, U.S.A.). The rotors were coated with silica gel GF_{254} for TLC (E. Merck, Darmstadt, G.F.R.); the layer thickness was 2 mm (prepared from a slurry formed of 60 g of silica gel, 2.4 g of calcium sulphate hemihydrate and 120 ml of water). Solvent was delivered by the pump at a flow-rate of 4–6 ml/min. UV detection was carried out at 254 or 366 nm. Fractions of 2–4 ml were collected. In the separation of triterpenes, the fractions were monitored by TLC on pre-coated aluminium sheets (Merck) with chloroform–methanol (9:1); detection was effected with Godin reagent[6]. Prior to sample application, the plates were pre-washed with solvent to remove impurities from the silica gel.

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RESULTS AND DISCUSSION

The principle of the operation of the Chromatotron is simple, as shown in Fig. 1. The mixture to be separated is applied as a solution near the centre of a rotor coated with a thin-layer of adsorbent (layer thickness 1–4 mm). Elution with a solvent gives concentric bands of the components, which are spun off from the edge of the rotor together with solvent. A collection system brings the eluate to a single output tube. UV-active compounds can be observed directly through a quartz lid during the separation.

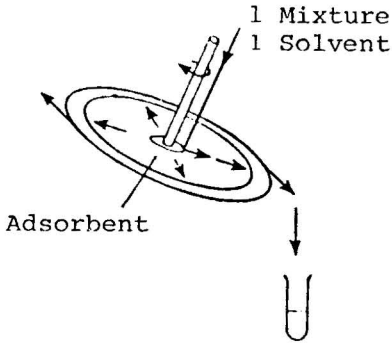
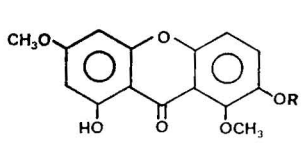


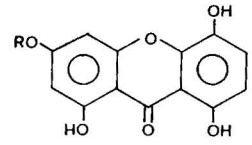
Fig. 1. Principle of centrifugal TLC.

Isolation of xanthone aglycones, which are strong inhibitors of monoamino oxidase[7], from American *Gentiana* species[8] was carried out by centrifugal TLC. A crude chloroform extract of *Gentiana detonsa* Fröl. (400 mg) chromatographed with chloroform as eluent furnished a first fraction of the least polar constituents (fatty acids, pigments, etc.), followed by decussatin (1) (7 mg) and gentiacaulein (2) (12 mg) within 20 min.

A mixture (120 mg) of bellidifolin (3) and desmethylbellidifolin (4) obtained after acid hydrolysis of a methanol extract of *Gentiana strictiflora* (Rydb.) A. Nels afforded pure compound 3 (55 mg) and pure compound 4 (48 mg) in less than 30 min. Chloroform with increasing amounts of methanol was used as the solvent.



1 : R = CH_3: decussatin
2 : R = H: gentiacaulein



3 : R = CH_3: bellidifolin
4 : R = H: desmethylbellidifolin

Separation of xanthone aglycones from *Gentiana* species was previously carried out[9] by polyamide open-column chromatography with methanol–water–acetic acid (90:5:5) and required at least one full day (column packing not included).

We also employed centrifugal TLC for the purification of saponin hydrolysates. A mixture (80 mg) of oleanolic acid and hederagenin could easily be separated. Chloroform–methanol (99.5:0.5) was used to obtain oleanolic acid, whereas for the

elution of the more polar hederagenin, we switched to chloroform–methanol (98:2). The separation was completed in less than 20 min and about 30 mg of each sapogenin was obtained in pure form.

From a column chromatographic fraction (359 mg) of a ginseng extract, it was possible to isolate 50 mg of ginsenoside Rg_1 and 75 mg of ginsenoside Rd with chloroform-methanol-water (100:30:3). However, no baseline separation could be achieved. Preliminary assays showed that centrifugal TLC is probably more suitable for the separation of less polar saponins possessing only one or two sugars, such as *Hedera* saponins[10].

Selection of the solvent is based on the TLC behaviour of the sample to be separated, but R_F values should be less than 0.5. We noticed that if the R_F values are higher than 0.5 on analytical TLC plates, and even with $\Delta R_F = 0.1$–0.2, the sample was eluted too quickly from the centrifugal TLC instrument and no separation occurred. One should start with a relatively weakly polar solvent systems (R_F *ca.* 0.1) and increase the polarity during the separation.

CONCLUSION

Centrifugal TLC is a simple and very rapid technique for the preparative separation or purification of natural products in the range from several milligrams to 0.5 g. It can replace preparative TLC (no scraping of bands to recover the sample) and, in some instances, column chromatography. The method is economical as the consumption of solvent is very small (generally less than 150 ml) and the coated rotors can be regenerated for re-use. However, the resolution is limited and cannot be compared with that in HPLC. There is also a restriction in the choice of stationary phases. Recently, a procedure has been developed for converting silica gel layers with starch binder into octadecyl reversed-phase layers[11]. The method is based on that described by Gilpin and Sisco[12] for the preparation of chemically bonded TLC plates. This will greatly increase the versatility of the technique and make it more suitable for the separation of polar substances. When used in combination with other chromatographic methods, preparative centrifugal TLC can be of great help for the isolation of natural products.

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