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Journal of Chromatography (incorporating *Biomedical Applications* and *Chromatographic Reviews*) In the course of 1977, also the cumulative indexes for Vols. 121–130 and 131–140 will appear.

MONTH	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	130 131	132/1 132/2 132/3	133/1 133/2	134/1 134/2	135/1 135/2	136/1 136/2 136/3	137/1 137/2	138/1 138/2	139/1 139/2	140/1 140/2 140/3	142 144/1	144/2 144/3
Biomedical Applications	143/1		143/2		143/3		143/4		143/5		143/6	
Chromatographic Reviews				141/1				141/2				141/3

Scope. The *Journal of Chromatography* publishes papers on all aspects of chromatography, electrophoresis and related methods. Contributions consist mainly of research papers dealing with chromatographic theory, instrumental development and their applications. The section *Biomedical Applications*, which is under separate editorship, deals with the following aspects: developments in and applications of chromatographic and electrophoretic techniques related to clinical diagnosis (including the publication of normal values); screening and profiling procedures with special reference to metabolic disorders; results from basic medical research with direct consequences in clinical practice; combinations of chromatographic and electrophoretic methods with other physicochemical techniques such as mass spectrometry. In *Chromatographic Reviews*, reviews on all aspects of chromatography, electrophoresis and related methods are published.

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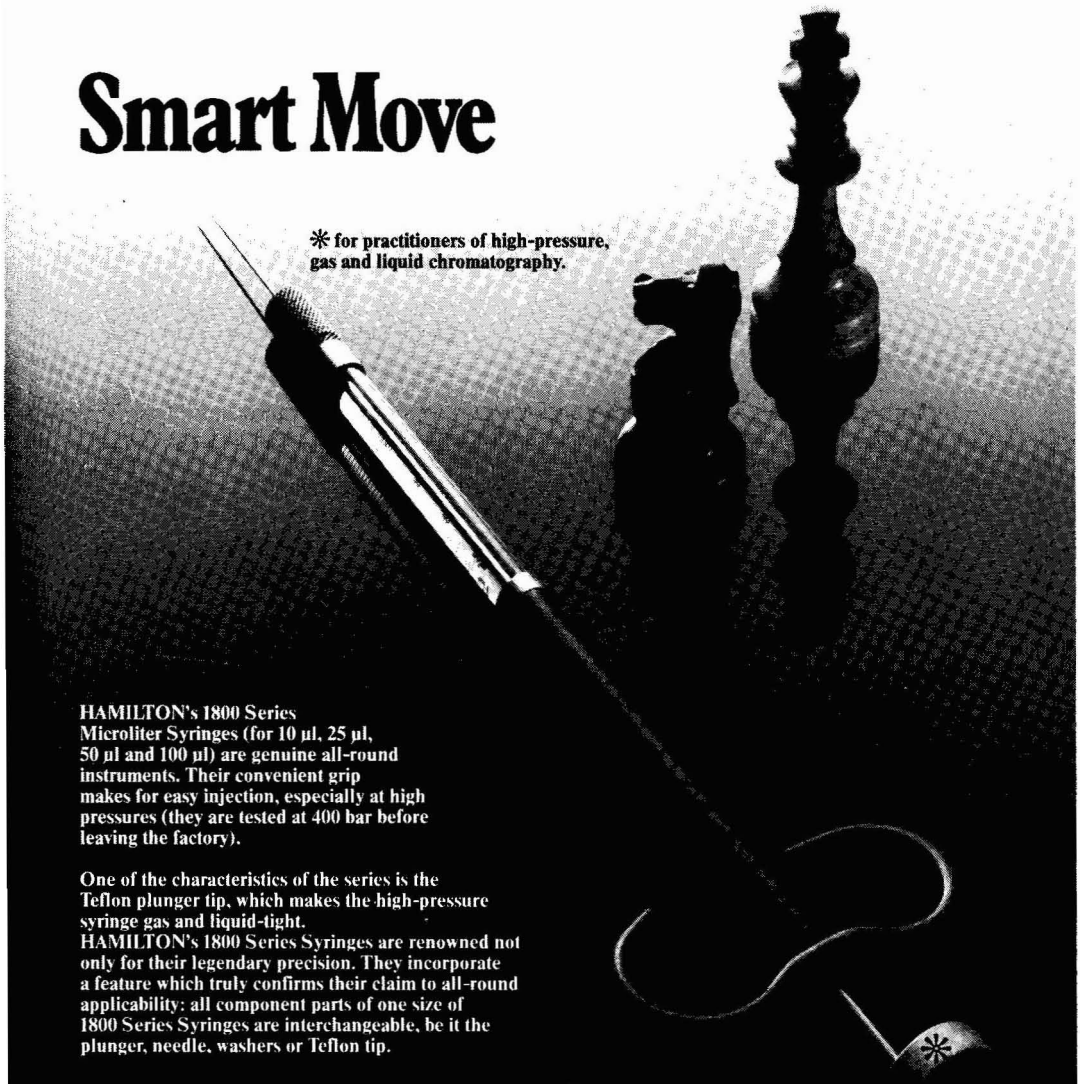

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Vapor-Liquid Equilibria Using UNIFAC

A Group-Contribution Method

by AAGE FREDENSLUND, *The Technical University of Denmark,* JÜRGEN GMEHLING, *University of Dortmund, and* PETER RASMUSSEN, *The Technical University of Denmark.*

The UNIFAC group-contribution method for predicting vapor-liquid equilibria in non-electrolyte mixtures at normal pressures, was first developed by Aage Fredenslund, R. L. Jones and J. M. Prausnitz at the University of California, Berkeley, in 1974. Since then it has been considerably revised and extended and incorporated into computer programs for multicomponent distillation design.

In this book, the UNIFAC method and its background are described in detail and extensive information provided for its use. With relatively few parameters (about 300) the method covers 75% of the published vapor-liquid equilibria data for non-electrolytes at normal pressures. On the basis of these parameters, vapor-liquid equilibria may be predicted in a large number of binary and multicomponent mixtures for which no data exist. The book gives all the parameters needed to implement the method. Easy-to-understand computer programs for the prediction of phase equilibria and for multicomponent distillation column design using UNIFAC are listed, the programs being written in such a manner that the reader may easily incorporate them into his own design programs.

For the process design engineer, the book will serve as a manual which will enable him to use UNIFAC with confidence. It will also be of interest to scientists and students concerned with models for vapor-liquid equilibria and their application.

CONTENTS: 1. Introduction. 2. Vapor Phase Nonideality. 3. Liquid Phase Nonideality. 4. The UNIFAC Group-Contribution Method. 5. Determination of UNIFAC Parameters. 6. Prediction of Vapor-Liquid Equilibria in Binary Systems. 7. Prediction of Vapor-Liquid Equilibria in Multicomponent Systems. 8. Prediction of Phase-Splitting and Excess Enthalpy. 9. Application of UNIFAC to Distillation Column Design. Appendix. 1. Calculation of Fugacity and Activity Coefficients. The Consistency Test. 2. UNIFAC Programs. 3. Parameter Estimation Program. 4. Distillation Programs. 5. List of Phase Equilibrium Data used in the Determination of UNIFAC Parameters. Nomenclature.

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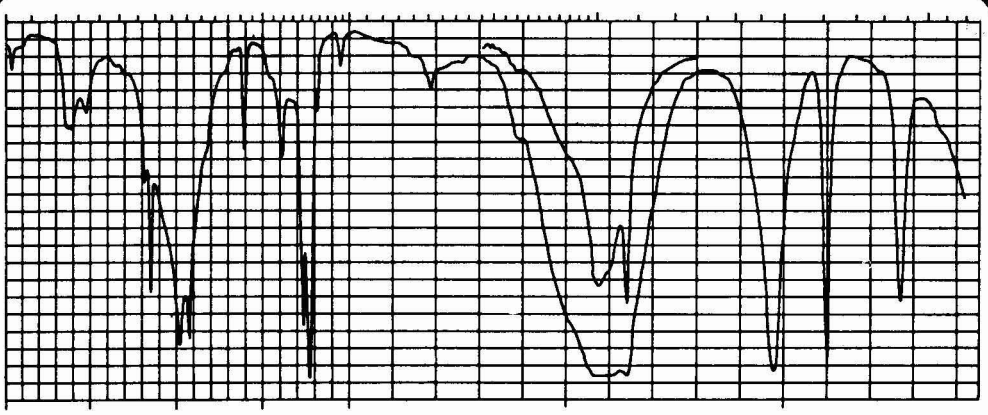

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A STUDY OF MICRO-HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

I. DEVELOPMENT OF TECHNIQUE FOR MINIATURIZATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

D. ISHII, K. ASAI, K. HIBI, T. JONOKUCHI and M. NAGAYA

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(Received April 28th, 1977)

SUMMARY

Several technical problems such as the following have been solved in order to perform micro-high-performance liquid chromatography (MHPLC): (1) a method for packing a narrow column with stationary phase and selection of suitable column materials; (2) preparation of micro-flow cells suitable for a micro-column; (3) improvement of the detector system; (4) methods for the pressurized passage of mobile phase at low flow-rates and for injection of a micro-amount of sample solution; and (5) gradient elution methods suitable for micro-columns.

The theory of the spreading of sample components in narrow tubes for column connections is considered and examples of experiments using MHPLC are presented.

INTRODUCTION

The columns for most commercial high-performance liquid chromatographic (HPLC) instruments are too large to be used for analytical purposes alone, and many advantages should result from a reduction of the column cross-section. The relationship between column diameter and column efficiency was investigated by Wolf[1], who found that as the column diameter was reduced the column efficiency also decreased, and the practical minimum column diameter was about 2 mm. These phenomena have been sometimes said to result from "wall effects". However, we consider the phenomena to be the effects resulting from a relative increase in the extra-column dead volume, and that miniaturization of HPLC would be feasible by decreasing the extra-column dead volume.

EXPERIMENTAL

Preparation of micro-columns

PTFE tubing of 0.5 mm I.D. and 1.0 mm O.D. was selected as the main micro-

column material, as it is readily available commercially, chemically stable and easy to machine. This tubing has a cross-section about one-fifteenth of the *ca.* 2 mm I.D. tubing that is widely used in ordinary high-performance liquid chromatographs. Investigations were carried out mainly with columns 5–30 cm long.

The slurry packing technique was used for packing the stationary phase. A tube several times longer than that finally required was first prepared and the stationary phase was suspended in a suitable solvent as a slurry, which was placed in a small bottle. An air-tight syringe (*ca.* 250 μl) was connected with the tube and they were filled with the solvent that was used to prepare the slurry. Then the lower end of the tube was dipped into the slurry, the syringe was attached to a micro-feeder and the slurry was sucked up to the upper end of the tube by manual or electrical operation of the feeder. The lower end of the tube was plugged tightly with a small amount of quartz-wool (less than *ca.* 2 mm thick) so as to prevent the packing material from leaking out, and the micro-feeder was operated manually or electrically, thereby discharging the solvent. In this way, a dense packing of the stationary phase was achieved. This method easily gave a good packing ratio of 60:40 (particles:vacancies).

The condition of packing during the delivery of the solvent under pressure could be observed through the semi-transparent PTFE tube. If a loose packing condition was observed, the syringe was detached from the tube, filled with the solvent and re-connected with the tube to feed the solvent again under pressure.

The column thus packed was cut to the required length and a small plug of quartz-wool was placed on the packing at the upper part of the tube, leaving a space 1–2 cm long for connection of the mobile phase delivery tube.

The method described can be used to prepare any micro-column with a volume 1–2% of that of columns used in ordinary HPLC. The same method is applicable to packing a 0.25 mm I.D. tube with stationary phase.

It has often been stated that if the column diameter is decreased below *ca.* 2 mm, the HETP would increase, and this effect has been described as a "wall effect". However, the decreased "wall effect" for the PTFE tubing may be considered to result from the fact that as the PTFE tubing has a softer wall surface than that of stainless steel tubing, the stationary phase under pressure adheres to the wall surface with a resultant increase in surface coverage.

Preparation of micro-flow cell and improvement of spectrophotometric detector

An investigation was made of the necessary structure of micro-flow cells for UV absorption that are suitable for use with such micro-columns. In the initial stage, two parallel quartz plates and a PTFE spacer were used to form a Z-type cell, which was found to be unsuitable. In the method currently being used a quartz tube 0.3–1 mm I.D. is connected directly with the micro-column, as shown in Fig. 1, and the dead space at the joint is made as small as possible. This method permits the simple preparation of flow cells of various sizes by using quartz tubes of different inner diameters. The volume of such a micro-flow cell is about 0.1–0.3 μl.

A photometer or a UV/visible spectrophotometer (UVIDEC-1 or UVIDEC-100, from Japan Spectroscop. Co., Tokyo, Japan) can be used as the detector. The path length of a micro-flow cell is relatively short, which results in a low sensitivity, but this can be increased by using a light source with a higher output or a photomultiplier with a higher output and sensitivity. A high-sensitivity UV detector (SPD-1, from Shimadzu

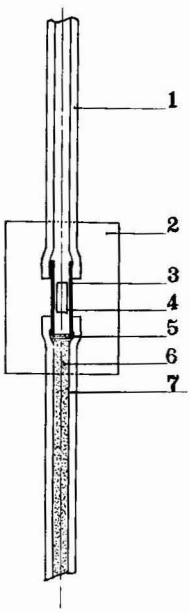


Fig. 1. Micro-flow cell. 1 = Outlet (PTFE tube); 2 = focused plate; 3 = micro-flow cell (quartz tube); 4 = optical window; 5 = quartz-wool; 6 = stationary phase; 7 = separation column.

Seisakusho, Kyoto, Japan) for HPLC is capable of giving good results even when connected directly with a micro-flow cell.

Methods for pressurized passage of mobile phase at low flow-rate and for injecting a micro-amount of sample solution

If the same linear flow-rate as that used in ordinary HPLC is used in micro-high-performance liquid chromatography (MHPLC), similar retention times are obtained. Thus, a flow-rate of *ca.* 10 μl/min is adequate with a 0.5 mm I.D. column in MHPLC. If one run of a chromatographic experiment is completed within 20 min with such a flow-rate, a total volume of about 200 μl of mobile phase will be required, which can be accommodated in a small syringe. As the diameter of the syringe plunger is reduced, less pushing power is required and the accuracy of the flow-rate is increased. An air-tight syringe of volume 50–250 μl was used to feed the mobile phase, fitted with a commercial micro-feeder that consisted of a small synchronous motor, gears and screws in order to push the plunger at a constant rate. Variable flow-rates could be obtained with this micro-feeder and these syringes. This system could also be used to charge the packing materials, as described under *Preparation of micro-columns*. The cross-section of the plunger of a 250-μl syringe is about 4 mm^2, so that, even if a pressure of 100 kg/cm^2 is required to feed the mobile phase, a plunger force of only 4 kg is needed. This estimate indicates that the pump for the pressurized feeding of mobile phase in MHPLC requires a very small force compared with that needed with large-bore columns for fractionation.

The sample volume to be injected must be reduced in proportion to the column size, and can be expected to be about 0.05–0.5 μl in MHPLC. The practical applicability of MHPLC depends on the exact injection of such a small volume of sample into

the column inlet without being spread or disturbed. In the initial stage of development, injection using a micro-syringe was tried. However, when the syringe needle was withdrawn after a sample injection the injected sample solution was spread and disturbed, with unfavourable effects, as the diameter of the micro-syringe needle was not negligible in comparison with the inner diameter of 0.5 mm of the micro-column. In MHPLC, the dead volume must be as small as possible, so that an on-column system for sample injection is preferable.

We therefore employed a new sampling method, in which the air tight syringe used for pressurized feeding of the mobiele phase, was available and this technique was successful. The method adopted is illustrated in Fig. 2. First, the mobile phase is sucked into the stainless-steel tube (*ca.* 0.3 mm I.D.) by operating the micro-feeder manually, its lower end is then dipped into the sample solution and the micro-feeder (equipped with a magnified micrometer) is slightly rotated manually so as to place a micro-amount of the sample solution into the tube. The tip of the stainless-steel tube is immediately dipped into a vessel containing mobile phase in order to wash the outside wall of the tube, and then a microamount of mobile phase is sucked in. Hence a sandwich sampling operation is applied (this sequence of washing and sucking the mobile phase can be omitted, when the sample solution is relatively diluted and the contamination from the outside wall of stainless tube gives no undesirable influences to chromatographic operation.) The stainlesssteel tube is then connected to the end of the micro-column and finally the microfeeder is electrically operated to effect a chromatographic elution.

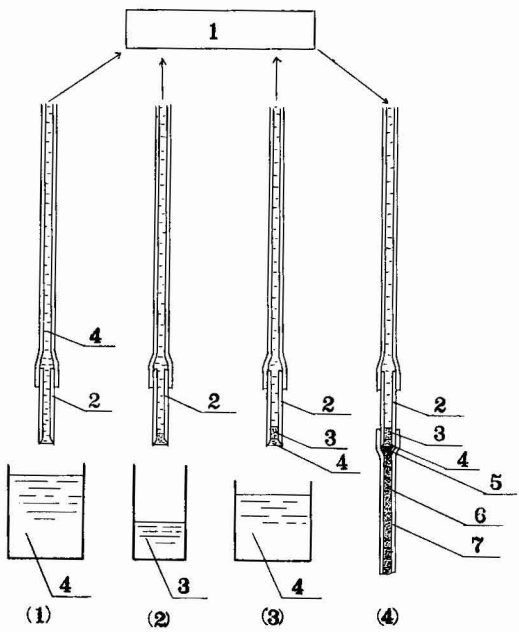


Fig. 2. Sample introduction. 1 = Micro-feeder; 2 = connection tube; 3 = sample solution; 4 = mobile phase; 5 = quartz-wool; 6 = packing material; 7 = micro-column. Operations: (1) suck up carrier liquid; (2) suck up sample solution; (3) suck up mobile phase; (4) connection.

Gradient elution

In HPLC, the gradient elution method is very useful as it allows the composition of the mobile phase to be varied continuously, and various gradient elution systems are commercially available. The gradient elution system is also suitable for MHPLC. Unlike ordinary HPLC, a total amount of only *ca.* 200 μl of mobile phase is required for one gradient elution chromatographic run in MHPLC. A continuous supply of such an amount of gradient mobile phase, although apparently difficult, can actually be achieved very easily, as follows. The gradient solution is sucked from a mixing vessel and stored in a fine tube (0.5 mm I.D. and *ca.* 1 m long), which is connected with a micro-column and, finally, the gradient solution is dispensed under pressure. The scheme of this method is shown in Fig. 3.

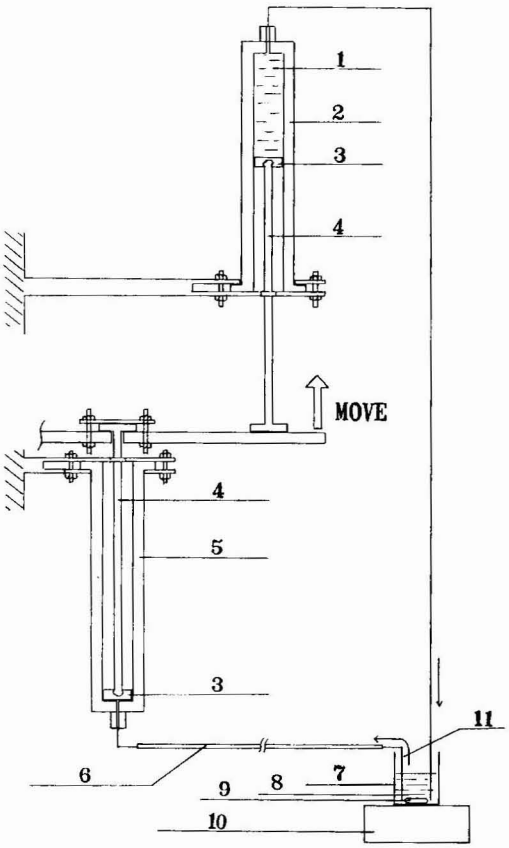


Fig. 3. System for preparation of gradient solution. 1 = Adding liquid; 2 = gas-tight micro-syringe; 3 = PTFE; 4 = syringe plunger; 5 = gas-tight micro-syringe; 6 = stock tube for gradient solution; 7 = mixing vessel; 8 = mixture; 9 = magnet; 10 = magnetic stirrer; 11 = connection tube.

RESULTS AND DISCUSSION

Examples of experiments using micro-high-performance liquid chromatography

In MHPLC, good baseline stability was obtained over a wide range of wavelengths and good resolution was achieved, as shown in Fig. 4. Fig. 5 shows the effect

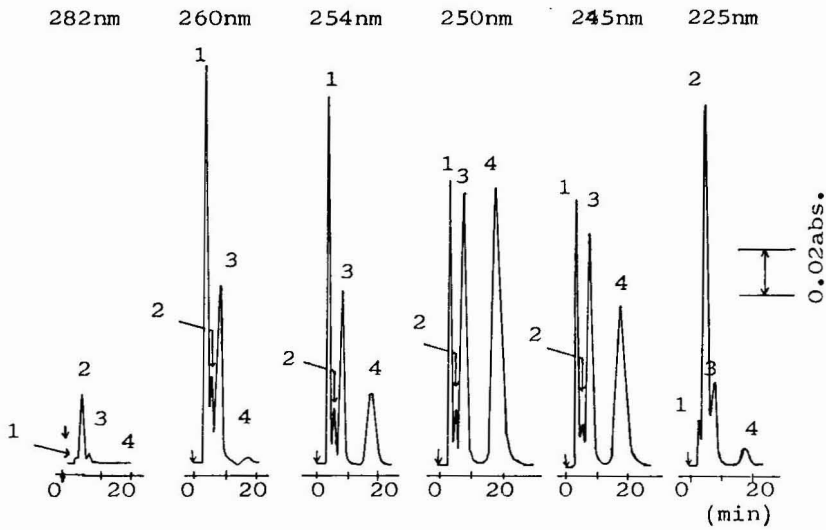


Fig. 4. Difference of peak response according to wavelength. Peaks: 1 = benzene; 2 = naphthalene; 3 = biphenyl; 4 = anthracene. Sample: mixture of 2% of benzene, 0.02% of naphthalene, 0.02% of biphenyl and 0.004% of anthracene in methanol. Sample size: 0.05 μl. Column temperature: 15°. Column: 15 cm × 0.5 mm I.D. PTFE tube packed with Permaphase ODS (30 μm). Mobile phase: 60% methanol in water. Flow-rate: 8 μl/min.

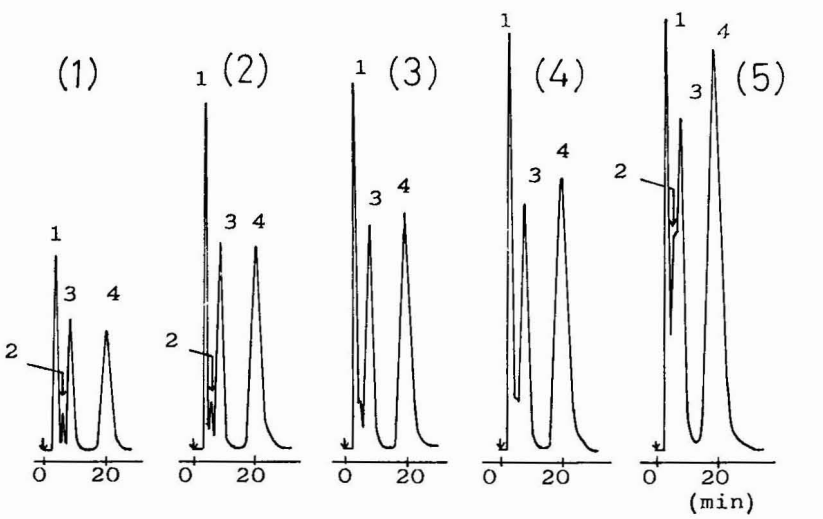


Fig. 5. Effect of sample loading on column efficiency. Peaks: 1 = benzene; 2 = naphthalene; 3 = biphenyl; 4 = anthracene. Sample size: (1) 0.04 μl; (2) 0.08 μl; (3) 0.12 μl; (4) 0.17 μl; (5) 0.33 μl. Sample: mixture of 2% of benzene, 0.02% of naphthalene, 0.02% of biphenyl and 0.004% of anthracene in methanol. Column: 15 cm × 0.5 mm I.D. PTFE tube packed with Permaphase ODS (30 μm). Column temperature: 15°. Mobile phase: 60% methanol in water. Flow-rate: 8 μl/min. Wavelength: 250 nm.

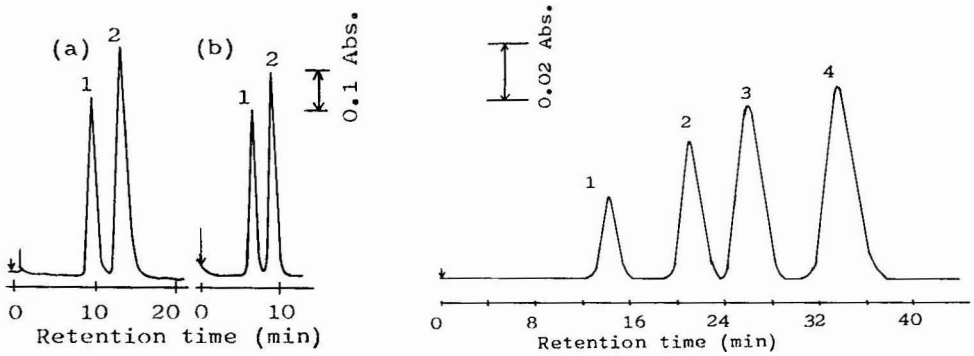


Fig 6 Typical separation on porous silica phase. Peaks: 1 = dioctyl phthalate; 2 = dibutyl phthalate. Sample size: 0.5 μl. Sample: mixture of 0.5% of dioctyl phthalate and 0.5% of dibutyl phthalate in *n*-hexane. Column: PTFE tube (8 cm × 0.5 mm I.D.) packed with silica beads (5 μm). Mobile phase: water-saturated dichloromethane. Flow-rate: (a) 3.2 μl/min; (b) 4.6 μl/min. Column temperature: ambient.

Fig. 7. Separation of aromatic hydrocarbons on Zorbax ODS. Peaks: 1 = naphthalene; 2 = biphenyl; 3 = fluorene; 4 = anthracene. Sample: mixture of 0.033% of naphthalene, 0.017% of biphenyl, 0.033% of fluorene and 0.0033% of anthracene in methanol. Sample size: 0.25 μl. Column temperature: 50.5°. Column: PTFE tube (12 cm × 0.5 mm I.D.) packed with Zorbax ODS. Mobile phase: 60% methanol in water. Flow-rate: 8 μl/min.

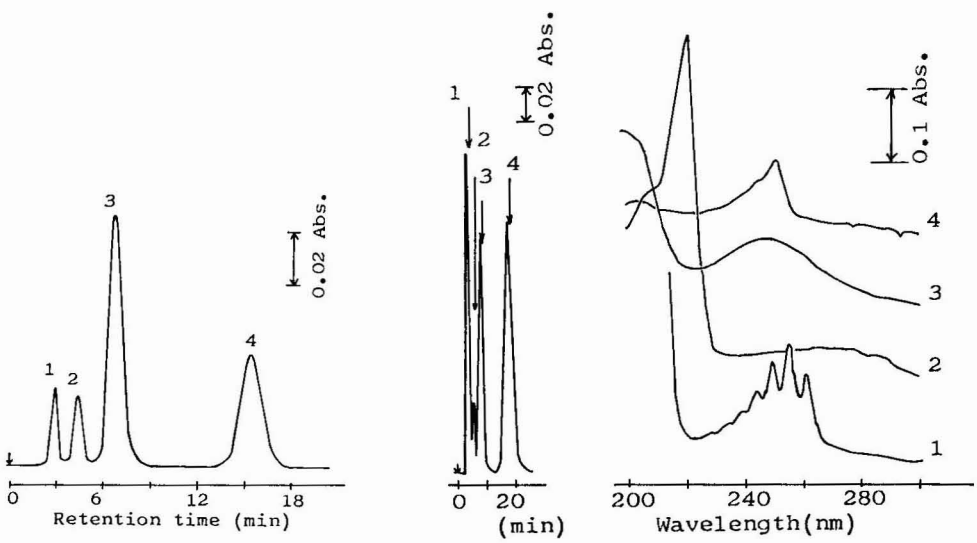


Fig. 8. Separation of aromatic hydrocarbons on a column of 0.25 mm I.D. Peaks: 1 = benzene; 2 = naphthalene; 3 = biphenyl; 4 = anthracene. Sample: mixture of 0.5% of benzene, 0.02% of naphthalene, 0.02% of biphenyl and 0.004% of anthracene in methanol. Sample size: 0.06 μl. Column: PTFE tube (20 cm × 0.25 mm I.D.) packed with Permaphase ODS (30 μm). Mobile phase: 50% methanol in water. Flow-rate: 4 μl/min.

Fig. 9. Separation of aromatic compounds and measurement of UV spectra at the peak maximum. Peaks: 1 = benzene; 2 = naphthalene; 3 = biphenyl; 4 = anthracene. Sample size: 0.05 μl. Sample: mixture of 2% of benzene, 0.02% of naphthalene, 0.02% of biphenyl and 0.04% of anthracene in methanol. Column: PTFE tube (15 cm × 0.5 mm I.D.) packed with Permaphase ODS. Column temperature: 15°. Mobile phase: 60% methanol in water. Flow-rate: 8 μl/min.

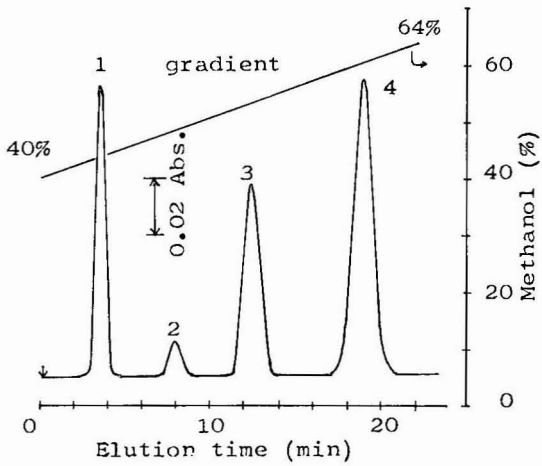


Fig. 10. Typical example of gradient elution. Peaks: 1 = benzene; 2 = naphthalene; 3 = biphenyl; 4 = anthracene. Sample: mixture of benzene, naphthalene, biphenyl and anthracene in methanol. Sample size: 0.05 μl. Column: PTFE tube (15 cm × 0.5 mm I.D.) packed with Permaphase ODS (30 μm). Mobile phase: 40–60% methanol in water. Flow-rate: 8 μl/min.

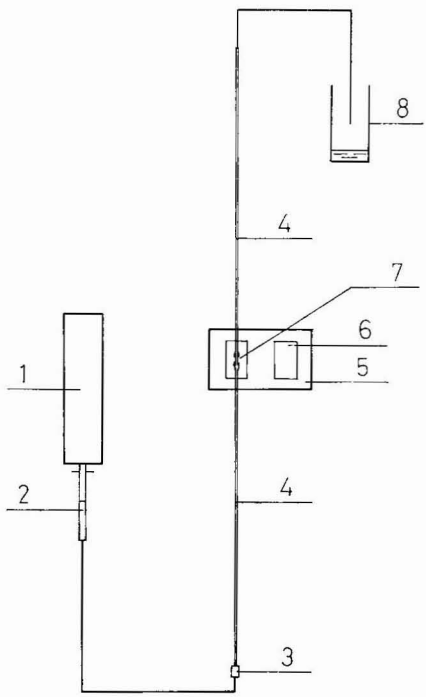


Fig. 11. Schematic diagram of apparatus for measuring the dispersion of a solute in a stream of solvent. 1 = Micro-feeder; 2 = gas-tight micro-syringe; 3 = sample inlet; 4 = PTFE tube; 5 = detector (UV spectrophotometer); 6 = reference cell; 7 = micro-flow cell; 8 = reservoir.

of sample loading on the column efficiency. It was found that the smaller was the sample size, the better was the resolution, and a sample size below 0.2 μl was preferable. Fig. 6 shows a typical separation of dioctyl and dibutyl phthalate on porous silica and Fig. 7 shows separation of aromatic hydrocarbons on a Zorbax ODS column. Fig. 8 shows a typical separation of aromatic hydrocarbons on a 0.25 mm I.D. column.

Spectra with good signal-to-noise ratios could be obtained at wavelengths from 200 to 300 nm using a micro-flow cell, and an example is shown in Fig. 9. Measurements of UV absorption spectra were made by temporarily stopping the pressurized delivery of mobile phase at a suitable position. This experiment showed that a mixture containing 1 μg of benzene, 10 ng of naphthalene, 10 ng of biphenyl and 2 ng of anthracene can be separated and that the measurement of UV absorption spectra is also possible together with component separation. Such separations, which are based on a rerversed-phase distribution provided by Permaphase ODS, give HETP values of 0.4–0.8 mm. The gradient elution method described under *Gradient elution* was applied to these mixed aromatic hydrocarbons and yielded a good separation (Fig. 10).

Dispersion of a solute in a stream of solvent through empty tubes

In a high-performance liquid chromatograph, at least two connecting tubes have generally been used, one at a point between the injection site and the column and the other at a point between the column and the detector. Even if the diameters of these tubes are small enough, the sample solute is dispersed in a stream of mobile phase through these tubes. In ordinary HPLC, the extra-column dispersion of solute in these connecting tubes may be estimated to be *ca.* 30–60 μl.

Such an extra-column dispersion in MHPLC may be less than 1 μl and it may be negligible, as "on-column" and "on-cell" systems are adopted in MHPLC. However, the peak dispersion in MHPLC is comparatively small and therefore dispersion

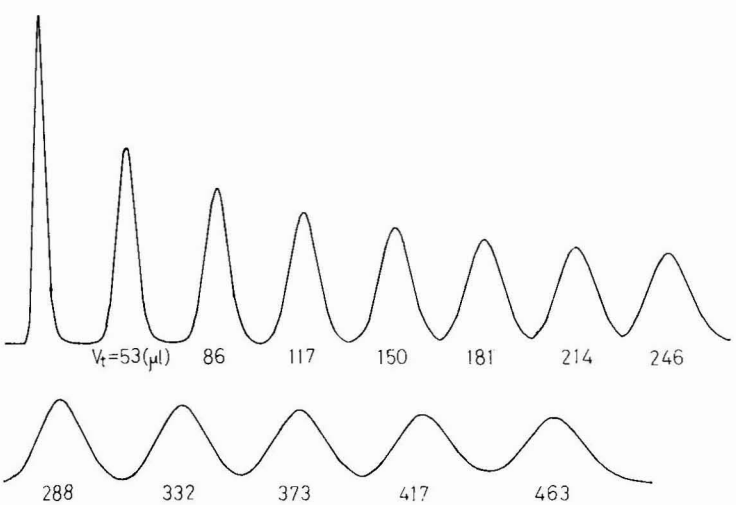


Fig. 12. Dispersion of a solute in a stream of solvent. V_t (μl) = amount of mobile phase required from injection until the peak maximum emerges. Column: 0.5 mm I.D. empty PTFE tube. Mobile phase: methanol. Flow-rate: 8 μl/min. Sample: monochlorobenzene. Sample size: 0.2 μl.

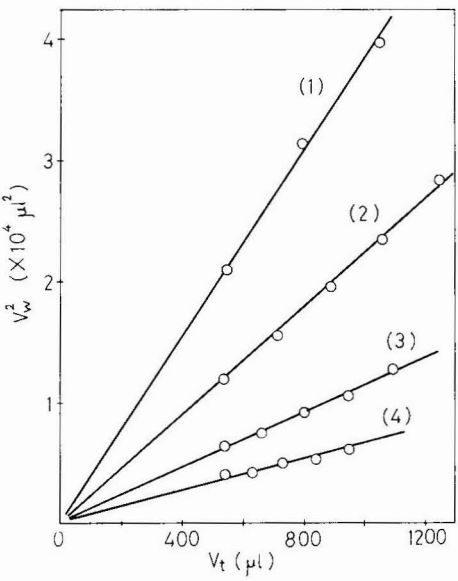
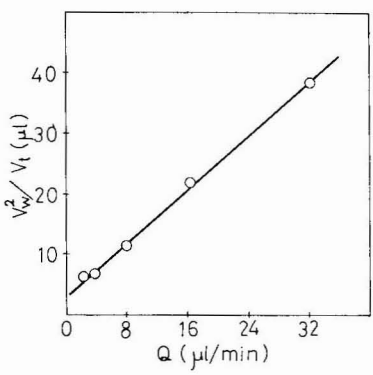


Fig. 13. Relationship between V_w^2 and V_t. V_w (μl) = dispersion of a solute in a stream of solvent; V_t (μl) = retention volume. Column: 70 cm × 1.0 mm I.D. PTFE tube. Mobile phase: methanol. Flow-rate: (1) 32 μl/min; (2) 16 μl/min; (3) 8 μl/min; (4) 4 μl/min. Sample: monochlorobenzene.

near the joints may be significant in comparison with the total peak dispersion, so this dispersion is considered below.

An apparatus for measuring this dispersion is shown in Fig. 11. Two empty tubes are connected to the front and rear of the micro-flow cell. Once the sample has been injected, the sample is detected repeatedly by changing the direction of flow of the mobile phase. The variation of the amount of mobile phase required until the peak



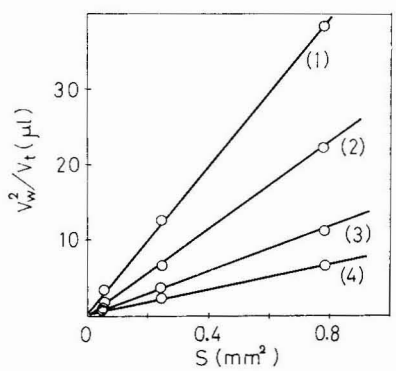


Fig. 14. Relationship between V_w^2/V_t and Q. V_w (μl) = dispersion of a solute in a stream of solvent; V_t (μl) = retention volume; Q (μl/min) = flow-rate of mobile phase. Column: 70 cm × 1.0 mm I.D. PTFE tube. Mobile phase: methanol. Sample: monochlorobenzene. Temperature: ambient.

Fig. 15. Relationship between V_w^2/V_t and S. V_w (μl) = dispersion of a solute in a stream of solvent; V_t (μl) = retention volume; S (mm^2) = cross-section of the column. Column: PTFE tube, I.D. 0.25, 0.5 and 1.0 mm. Mobile phase: methanol. Flow-rate: (1) 32 μl/min; (2) 16 μl/min; (3) 8 μl/min; (4) 4 μl/min. Temperature: ambient. Sample: monochlorobenzene.

TABLE I

DISPERSION OF A SOLUTE IN A STREAM OF SOLVENT

V_t (μl)	V_w (μl)	V_w^2/V_t (μl)
22.1	9.28	3.90
52.8	15.47	4.53
86.3	19.41	4.37
117.3	23.03	4.52
149.8	25.70	4.41
181.2	28.80	4.58
213.7	31.14	4.54
245.7	33.60	4.59
288.4	36.37	4.59
331.5	39.14	4.62
373.1	41.38	4.59
416.8	44.37	4.72
462.6	46.93	4.76
	Mean:	4.52

maximum is detected (V_t) was obtained by the above method. The inner diameter of the tubing was 0.25–1.0 mm and the volume of the micro-flow cell was about 1 μl. A commercial UV spectrophotometer was used as a detector. The results are shown in Figs. 12–15 and Table I.

The following equation can be derived from the results in Fig. 13:

$$V_w^2 = k_1 V_t \tag{1}$$

where V_w (μl) is the dispersion of a solute in a stream of solvent. Similarly, from the results in Fig. 14, we have

$$V_w^2 = k_2 Q \tag{2}$$

where Q (μl/min) is the flow-rate of the mobile phase. From the results in Fig. 15, we obtain

$$V_w^2 = k_3 S \tag{3}$$

where S (mm^2) is the cross-section of the tubing. Finally, from eqns. 1, 2 and 3, we find

$$V_w^2 = k V_t Q S \tag{4}$$

Taylor[2] dealt with the dispersion of a solute in a stream of solvent quantitatively, and Ouano[3] introduced an equation for calculating the coefficient of molecular diffusion:

$$D = 0.212 (Q/L) (V_t/V_w)^2 \tag{5}$$

which can be rewritten as

$$V_w^2 = \frac{0.212}{D} \cdot V_t Q S \tag{6}$$

where D is the coefficient of molecular diffusion and L is the length of the tubing.

It was found that the empirical eqn. 4 obtained by MHPLC agreed with eqn. 6. Further, we found that V_w^2/V_t (4.52 from Table I) agreed with the calculated value (4.49 from eqn. 5.)

The dispersion in the joints used in MHPLC is calculated to be less than 0.5 μl from eqn. 6, which is very small compared with the total peak dispersion (several microlitres in MHPLC) and could be neglected. Moreover, it is expected that the technique described will be useful for the simple measurement of D values.

CONCLUSION

It has been demonstrated that in HPLC the volume of the column can be reduced to 1–2% of its normal value without any deleterious effects on its performance. This reduction results not only in a great decrease in the amount of sample required but also in a great decrease in the consumption of mobile phase and expensive packings. Micro columns can be easily prepared by investigators themselves and may be applied to various separation by selecting the appropriate packing materials. and is expected to be useful when applied to preparatory experiments in order to select suitable conditions for fractionation by means of PHLC.

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PREPARATION, OPTIMISATION AND SLURRY PACKING OF AN AMINO BONDED PHASE FOR THE ANALYSIS OF SUGARS IN FOOD BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Optimisation of the separation of fructose, glucose, sucrose, maltose and lactose on an amino bonded phase is described. The factors affecting support loading have been investigated with a view to simplifying and shortening the phase preparation procedure. Columns were packed using different slurry media to determine the effect on peak shape and column efficiency. It is shown that careful control of support loading improves sugar separations thus permitting a faster analysis time.

INTRODUCTION

The analysis of saccharides in food by high-performance liquid chromatography (HPLC) has become an attractive proposition owing to recent advances in bonded phase columns. Separation times are shorter than for gas–liquid chromatography[1]. which also suffers from the problems of derivatisation and extensive sample clean-up. Although several papers[2–5] have demonstrated the HPLC separation of a wide range of saccharides on bonded phases, only one author[6] has defined the nature of the phase and given a method of preparation. There is no information concerning investigation of phase synthesis and the effect of support loading on chromatography. As will be demonstrated, a full understanding of these factors is of fundamental importance if the full potential of the amino alkyl phase is to be realised for the analysis of saccharides in food.

EXPERIMENTAL

Apparatus

The detector was a differential refractometer model R401 from Waters Assoc. (Hertford, Great Britain) and was used in conjunction with a Model 750/03 reciprocating pump from Applied Chromatography Systems (Luton, Great Britain). Sample injection was achieved on a Specac 2,500-p.s.i. valve (Sidcup, Great Britain) fitted with a 30-μl loop. All columns were made from 316 grade seamless stainless-steel tube 6.35 mm O.D. $\times$ 4.6 mm I.D. obtained from Tubesales (Southampton, Great Britain).

An Orlita pump model DMP 1515 (A. J. G. Waters, Slough, Great Britain) was used for column packing. Stainless-steel unions were obtained from H. S. Chromatography Packings (Bourne End, Great Britain) and stainless-steel mesh from Sankey Green Wire Weaving (Warrington, Great Britain).

Chemicals and reagents

Acetonitrile and hexane were both HPLC grade from Rathburn (Walkerborn, Great Britain). All other solvents were analytical reagent grade. Solvents that were used for phase synthesis were stored over molecular sieve 4A in order to remove residual water. The chromatographic support was Partisil 5 (the same batch was used throughout) from Whatman (Maidstone, Great Britain) and was modified with γ-aminopropyltriethoxysilane from Aldrich (Wembley, Great Britain).

Evaluation of bonding procedure

General technique and determination of support loading. Silica used for bonding was stored over a saturated solution of lithium chloride (relative humidity 12%, 20–35°) for 24 h prior to its reaction with silane. The silica was then either shaken at room temperature or refluxed in a dry non-polar solvent with γ-aminopropyltriethoxysilane. After filtering through a No. 4 sintered glass funnel and washing with solvent, the bonded phase was dried in an oven at 120°.

Support loading was determined by combusting approximately 1 g of phase over a bunsen burner at 600°. The percentage support loading was then expressed as:

$$\% \text{ Loading} = \frac{\text{Weight loss after combustion} \times 100}{\text{Weight of phase before combustion}}$$

Factors affecting support loading. The effect of silica:silane ratio, reaction temperature and reaction time on support loading were investigated with a view to obtaining a simple rapid procedure for phase preparation.

Electron microscopy. All phases were examined under the electron microscope for evidence of silane polymerization. Since this has an important effect on chromatography, it was felt that the technique might provide valuable information on the interpretation of the chromatographic performance.

Column packing

General technique. The packing equipment used was very similar to that described by other authors[7,8] and consisted of a slurry reservoir and analytical column connected by a $^1/_4$-in. stainless-steel union. The analytical column was terminated in a zero dead volume $^1/_4$–$^1/_{16}$ in. reducing union fitted with a 8-μm mesh disc. Three grams of phase were dispersed in a slurry medium and poured into the packing assembly. The slurry was compressed to a compact bed by pumping solvent through the assembly at 5000 p.s.i. The column was then rapidly equilibrated to the conditions required for sugar analysis with 50 ml of acetonitrile–water (4:1, v/v). After disconnecting from the reservoir the top of the column was fitted with a 8-μm disc and a $^1/_4$–$^1/_{16}$ in. zero dead volume reducing union.

Slurry packing investigation. All columns used in the chromatographic evaluation were slurried in acetonitrile–water (4:1) and the slurry compressed by pumping

through with hexane. Subsequently however, a slurry packing investigation was undertaken to determine the effect of different slurry media on column efficiency, pressure and peak shape. The effect of using hexane and acetonitrile–water (4:1) to compress the slurry was investigated. Each column was evaluated in an identical manner using a 30-μl standard containing fructose, glucose and sucrose. Column efficiency was determined using the sucrose peak (3 separate determinations).

Chromatographic evaluation of phases and application to sugar analysis

Phases with a wide range of support loadings were evaluated chromatographically using a sugar standard containing fructose, glucose, sucrose, maltose, and lactose. Column efficiency (N) and capacity ratio's (k') were calculated and the optimum support loading for the separation of the above five sugars determined.

RESULTS AND DISCUSSION

Factors affecting support loading

Silica:silane ratio. The effect of silica:silane ratio on support loading is shown graphically in Fig. 1. At high silane concentrations a small decrease in concentration produced a large reduction in support loading. This trend is reversed at low concentrations, where for instance ratio's of 20:1 and 10:1 gave a difference of only 0.8%.

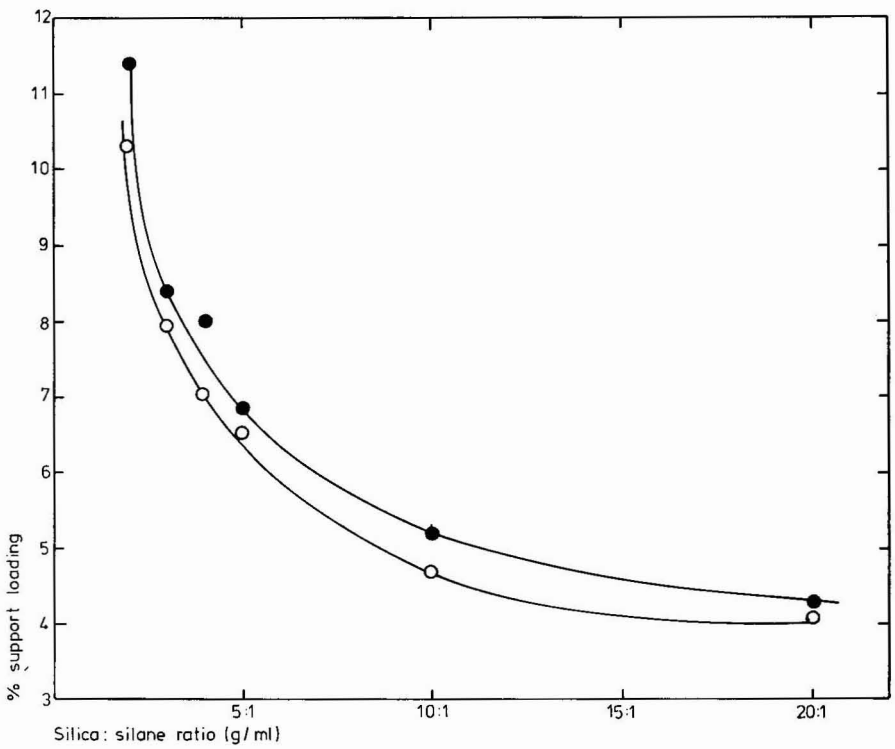


Fig. 1. Graph of percentage support loading against silica:silane ratio. ○, 5-min shake; ●, 2-h reflux.

TABLE I

EFFECT OF TEMPERATURE ON SUPPORT LOADING

Solvent	*Reaction temperature (°C)*	*Phase loading (%)*
Hexane	25	10.2
Hexane	68	11.4
Toluene	110	11.2
Xylene	142	12.4

TABLE II

EFFECT OF REACTION TIME AND TEMPERATURE ON SUPPORT LOADING

Reaction time (min)	*Support loading (%)*
90	8.0
60	8.0
30	8.0
15	8.0
5*	7.0

* At room temperature.

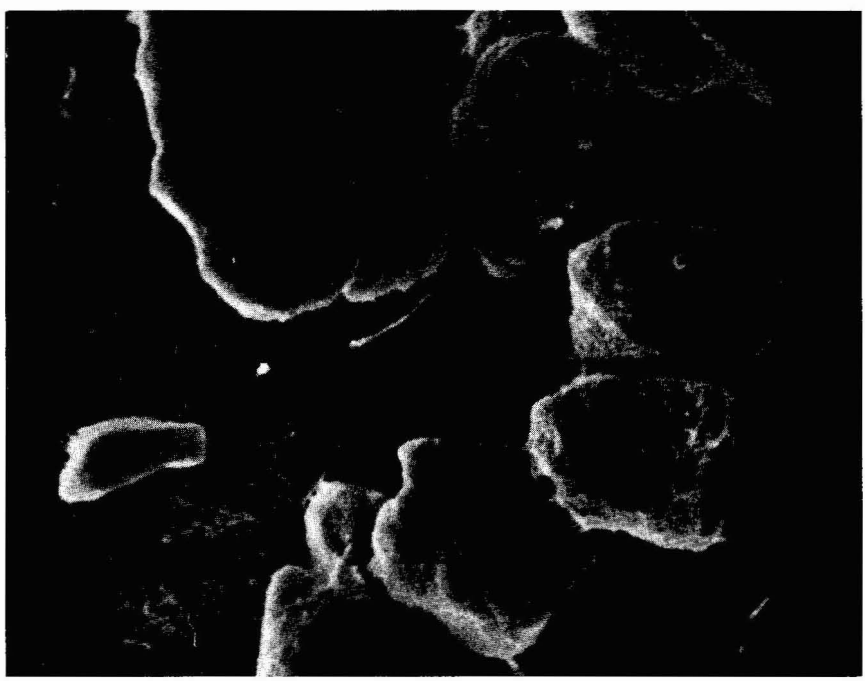


Fig. 2. "Webbing" effects on Partisil 5. Magnification, ×10,000; scale, 1 μm = 10 mm.

Reaction temperature and time. Table I shows that reaction temperature has very little effect on support loading. It required a 117° rise in temperature to effect an increase in loading from 10.2 to 12.4%. Further evidence of the ease with which the ethoxysilane reacts with the surface silanols came from the investigation of the effect of reaction time on loading (Table II). Identical loadings were recorded for silica–silane reactions taking place in refluxing hexane from 90 min down to 15 min. Indeed, shaking for 5 min at room temperature still resulted in a 7% loading. A graph of silica:silane ratio against loading using the "5-min shake" procedure gave a similar curve to that obtained for the 2-h reflux and is shown in Fig. 1. This curve was used to obtain specific support loadings for chromatographic evaluation.

Electron microscopy studies

Electron microscopy on the high amino phase loadings showed silica particles that were either covered in a "web" or connected by long "strands" (see Fig. 2 and 3). These effects were still apparent after the phase had been combusted at 600°. The strands in Fig. 3 are 500–1000 Å thick, although much of this is due to the gold film with which the slide was made up. It is highly improbable that a large number of

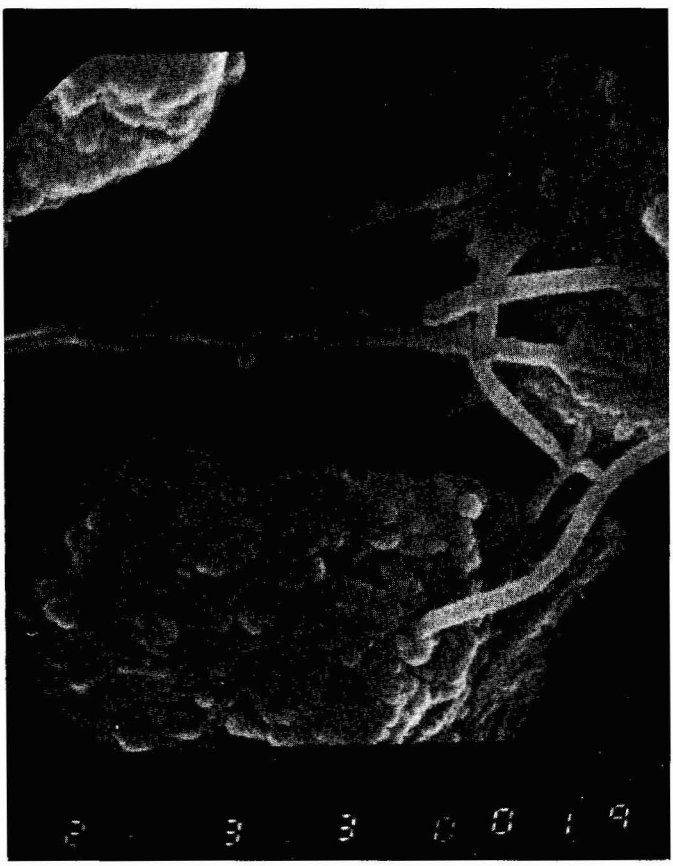


Fig. 3. High amino phase loading on 5μm silica. Magnification × 15,000.

small particles of silica are present prior to "bonding"[9]. Therefore the strand thickness cannot be represented by small silica particles bonded to a siloxane chain as in Fig. 4. This would therefore imply a considerable degree of polymerisation. The later batches of phase were devoid of any "strands" or "webbing" effects. It therefore seems likely that these were caused either by water entering the reaction mixture or impure silane.

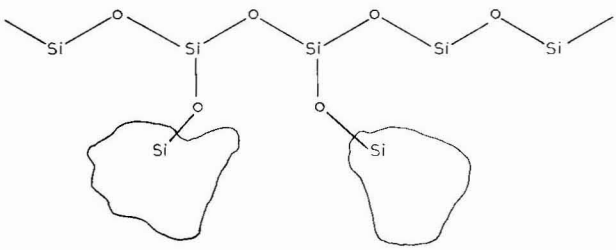


Fig. 4. Small silica particles bonded to a siloxane chain.

Phases that exhibited "strands" also proved very difficult to pack. Although the packing pressure was in excess of that used for routine analysis, the level of the phase always dropped by approximately $^1/_8$ in. below the top of the column after a short period of use. This is probably caused by the continual shearing of the strands under continuous mechanical pressure. The net result is a decrease in resolution as a consequence of the dead volume left at the top of the column. We have also observed "strands" with other bonded phases that exhibited "packing down".

Chromatographic evaluation of phases and implications for sugar analysis

The effect of support loading on sugar capacity ratio's is shown in Fig. 5. The graph may be divided into two specific regions. Above a support loading of 8.5%, k' is a constant for a particular sugar. Below this figure, k' decreases in an approximately linear manner. Since water on the silica surface can also cause silane condensation, the increase in loading without an accompanying increase in k' is interpreted as representing phase polymerisation. The fact that the more heavily loaded supports yielded columns with lower efficiencies (Table III) favours this argument, as the lower efficiencies would be anticipated on the grounds of poor mass transfer resulting from the polymeric matrix[10,11]. Initially, the predominant reaction probably occurs between the silica surface silanols and the silane monomers, with only a small degree of polymerisation. Once, however, all the available surface hydroxyls have reacted, *i.e.* a monolayer of aminopropyl groups exists, the polymerisation of the silane monomers undoubtedly becomes the predominant reaction. An 8.6% loading, therefore, probably represents an amino-phase monolayer.

Slurry packing investigation

A summary of the effect of different slurry media on column pressure, efficiency and peak shape is given in Table IV. Column number 145 was the only one where hexane was used to compress the slurry, acetonitrile–water (4:1) being used for the rest. The best column efficiency was obtained with a water slurry and pumping through with acetonitrile–water. Using a water slurry but pumping through with

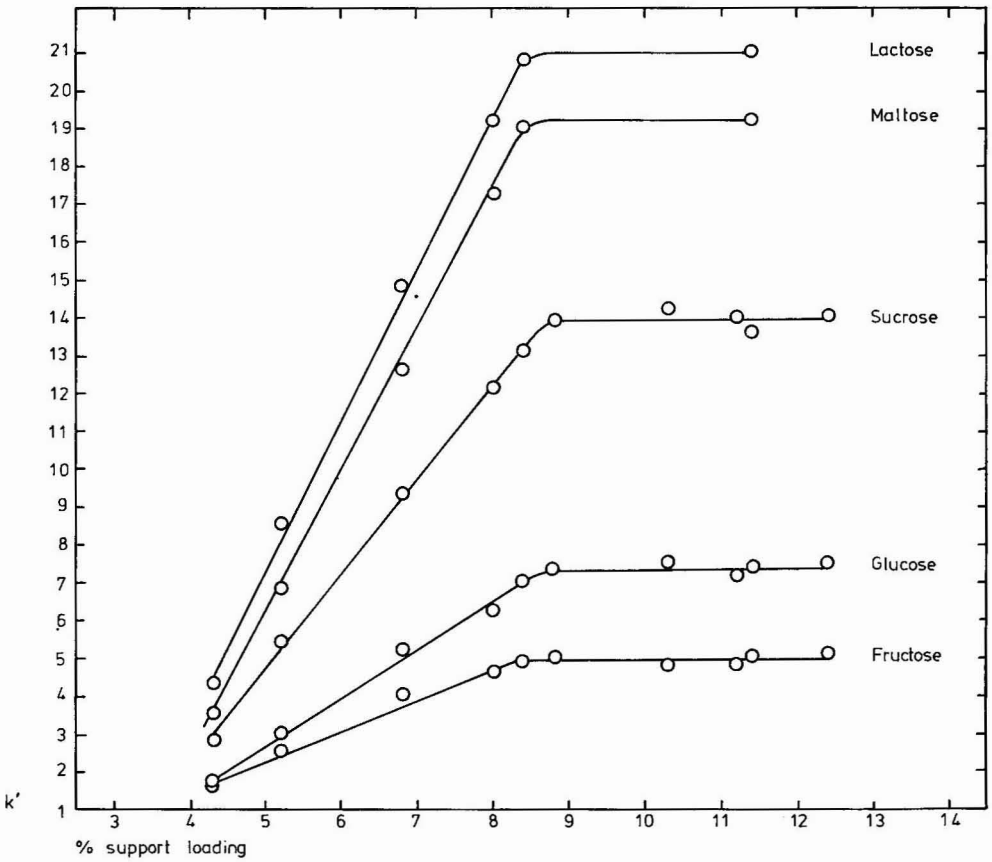


Fig. 5. Graph of k' against support loading for fructose, glucose, sucrose, maltose and lactose.

hexane gave a lower column pressure and poorer efficiency. The acetonitrile and acetonitrile–water slurries both gave columns with low back pressures and poor efficiencies. This could be interpreted as due to a less compact bed. Both columns exhibited badly tailing peaks. The tetrabromoethane slurry gave the column with the highest pressure, suggesting a compact bed, however, it also yielded the lowest efficiency. This might at first appear to present an anomaly. However, the sugar k' values

TABLE III

EFFECT OF SUPPORT LOADING ON COLUMN EFFICIENCY

Column efficiency (plates per metre)	*Support loading (%)*
4200	12.4
7500	11.2
6300	11.4
9200	10.2

TABLE IV

EFFECT OF SLURRY MEDIA ON COLUMN PRESSURE, EFFICIENCY AND PEAK SHAPE

Column no.	*Slurry media*	*Pressure (p.s.i.)*	*Column efficiency (plates per metre)*			*Average efficiency*	*Peak shape**
			1	*2*	*3*		
137	Acetonitrile–water (4:1)	1510	9600	10,700	11,300	10,500	T
138	Water	1720	12,400	12,100	11,300	12,100	G
146	Water	1720	13,500	12,300	11,800	12,500	G
140	Acetonitrile	1570	9300	9100	10,100	9500	T
141	Carbon tetrachloride	1760	9000	9100	8600	8900	VG
142	Tetrabromoethane	1980	9200	8600	8500	8800	L
145	Water	1620	10,000	9200	10,100	9800	G

* T = tail, G = good, VG = very good, L = leading edge.

on this system were considerably lower than expected and it is probable that this effect was caused by the retention of the tetrabromoethane within the phase. High column pressure could then be attributed to the increased viscosity and the lower k' values to the more hydrophobic nature of the phase. Peak shape on this system was characterised by a leading edge. The column packed with carbon tetrachloride slurry exhibited similar characteristics to the tetrabromoethane slurry.

Optimisation of analysis time for sugars

Resolution[12] is a function of α (separation factor), N (the column efficiency) and k' (the column capacity ratio):

$$R_s = \tfrac{1}{4}\left(\frac{\alpha - 1}{\alpha}\right)\sqrt{N}\left(\frac{k'}{1 + k'}\right)$$

The resolution between maltose and lactose ($R_{m/l}$) and between fructose and glucose ($R_{f/g}$) were plotted against support loading to determine the value for which $R_{m/l}$ and $R_{f/g}$ are at a maximum. Sucrose was omitted from this study as it is always well separated from the other four sugars and therefore not a limiting factor in the analysis time. The graph obtained is given in Fig. 6. As might be anticipated $R_{f/g}$ increased with increasing phase loading and consequently 8.5% represents the optimum for fructose–glucose separation. Surprisingly $R_{m/l}$ peaks at 6.3% which consequently represents the best value for maltose–lactose separation. The optimum loading for all five sugars was chosen as 7.0%. Although $R_{f/g} > R_{m/l}$ at this point the fructose peak tailed in practice and consequently 7.0% and not 6.3% was the loading at which equal resolution was achieved between the maltose–lactose and fructose–glucose peaks. If the resolution between adjacent peaks exceeds baseline separation, analysis time is wasted. Where this occurs the column efficiency is greater than required since, at a fixed percentage loading, resolution is a function of N. The efficiency may be reduced by an increase in the linear velocity or a reduction in column length. High linear velocities are commonly used with microparticulate silica owing to the relatively flat curve obtained for the plot of HETP (height equivalent to a theoretical plate) vs. u (linear velocity). Fig. 7 shows this plot for lactose using 6.2% loading and flow-rates from 1 to 7 ml/

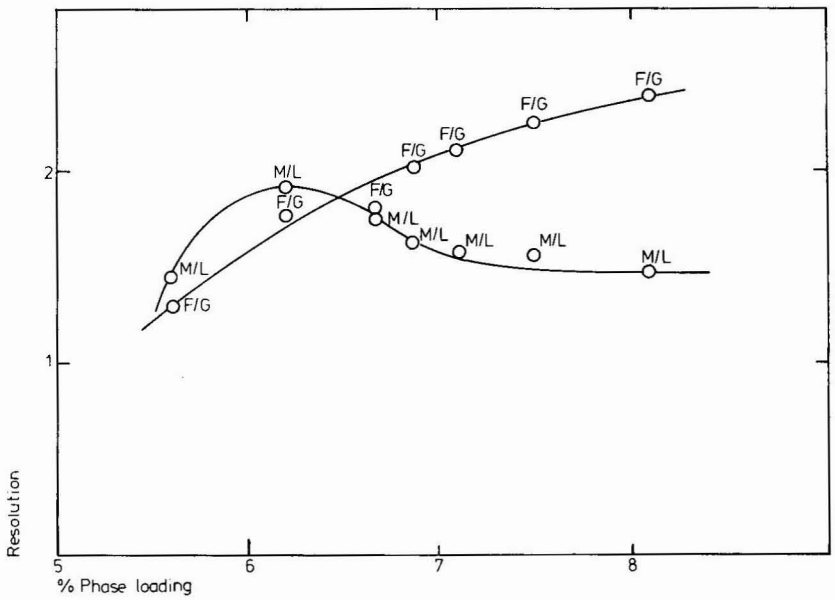


Fig. 6. Graph of resolution against percentage support loading for fructose–glucose (F/G) and maltose–lactose (M/L).

min. From this it can be seen that a six-fold increase in flow-rate, for lactose, produces only a two-fold increase in HETP. Unfortunately a high linear velocity generates a high column back pressure and consequently a compromise was reached by reducing the column length and increasing the flow-rate to give a rapid analysis time with a reasonable back pressure. Fig. 8 demonstrates the separation of all five sugars in 10 min on a 16 × 0.46 cm I.D. column at a flow-rate of 3 ml/min and a pressure of 1900 p.s.i.

Application to food analysis

We have been analysing sugars in a wide range of foodstuffs for several months. Over this period of time a general extraction procedure has evolved: (1) Sample maceration (2) cold water extraction, (3) protein precipitation, (4) filtration, (5) freeze drying of filtrate.

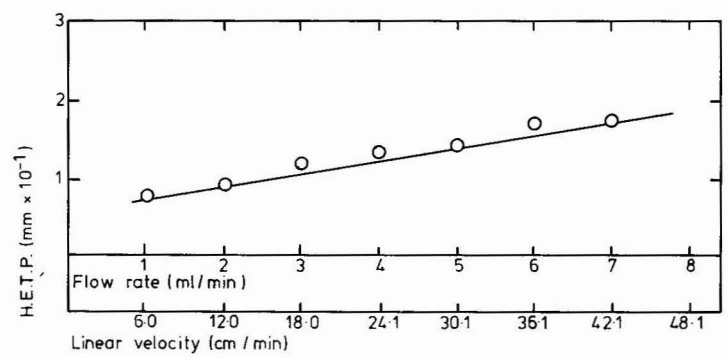


Fig. 7. Graph of HETP against flow-rate and linear velocity for lactose.

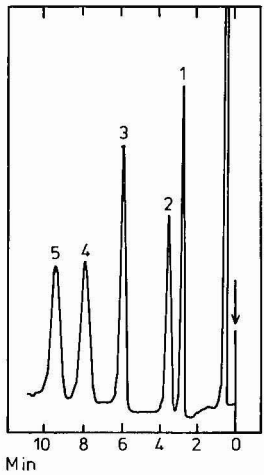
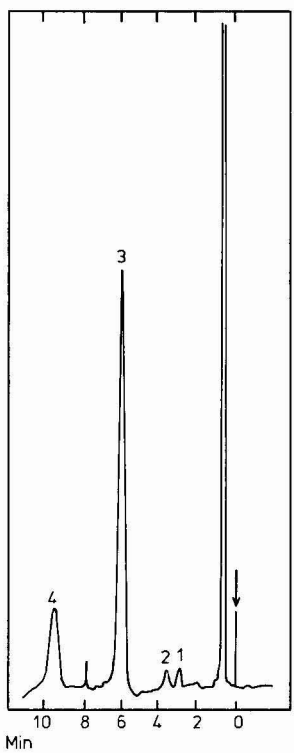


Fig. 8. HPLC separation of sugars. 1 = Fructose, 2 = glucose, 3 = sucrose, 4 = maltose, 5 = lactose. Solvent, acetonitrile–water (4:1). Column. Partisil 5, 7% NH_2 phase, 16 × 0.46 cm I.D. Flow-rate, 3 ml/min.



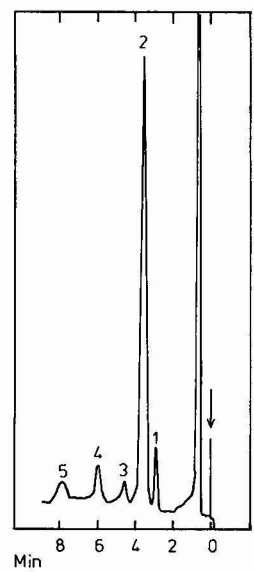


Fig. 9. Ice cream extract. 1 = Fructose, 2 = glucose, 3 = sucrose, 4 = lactose. Solvent, acetonitrile–water (4:1). Column, Partisil 5, 7% NH_2 phase, 16 × 0.46 cm I.D. Flow-rate, 3 ml/min.

Fig. 10. Enzyme-treated soy extract. 1 = Fructose, 2 = glucose, 3 = unknown, 4 = sucrose, 5 = maltose. Other conditions as in Fig. 9.

Obviously some food samples need not be taken through all the steps (*e.g.* sweets, fruit drinks), but conversely we have not as yet been presented with a food sample that we could not analyse by this procedure. Samples are taken through steps 1–5 (where necessary) as soon as they are received for analysis. In this manner the possibility of sample degradation is eliminated. The freeze dried extract is then dissolved in acetonitrile–water (3:7) just prior to chromatography. Three examples of food analyses are given in Fig. 9, 10 and 11.

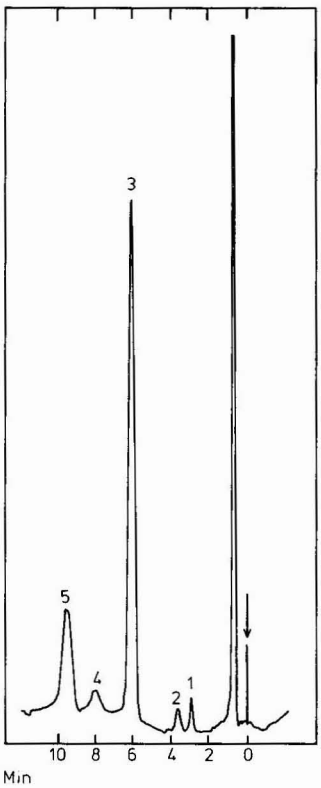


Fig. 11. Breakfast cereal extract. 1 = Fructose, 2 = glucose, 3 = sucrose, 4 = maltose, 5 = lactose. Other conditions as in Fig. 9.

CONCLUSIONS

A wide range of support loadings may be prepared in 5–10 min by shaking Partisil in varying concentrations of γ-aminopropyltriethoxysilane in hexane at room temperature.

The best column efficiencies were obtained using a water slurry and pumping acetonitrile–water (4:1) through the column to compress the phase to a compact bed. This also serves to equilibrate the column to the conditions required for sugar analysis. By using a 7% support loading the separation of fructose, glucose, sucrose, maltose, and lactose may be achieved in only 10 min on a 16 $\times$ 0.46 cm I.D. column at a pressure of 1900 p.s.i.

The application of the amino column to food analysis is not difficult. Some sample extracts may be chromatographed directly. However, large quantities of co-extractable material should, as far as possible, be removed in order to maintain a reasonable column lifetime.

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SIMULTANEOUS GAS CHROMATOGRAPHIC ANALYSIS OF LOWER FATTY ACIDS, PHENOLS AND INDOLES USING A GLASS CAPILLARY COLUMN

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SUMMARY

The simultaneous gas chromatographic separation of a mixture of 14 lower fatty acids, 11 phenols and 7 indoles has been effected by using a glass capillary column. Complete separation of the mixture has been obtained, except for phenol and *o*-cresol and *o*-ethylphenol and 3,5-xylenol whose peaks overlapped, and 2- and 3-methylindoles which were poorly separated. The optimum conditions are as follows: stationary phase, FFAP or PEG-20M; column (20 m × 0.28 mm I.D.) temperature, held for 1 min at 145° (FFAP) or 130° (PEG-20M); column oven, heated at 4°/min from 145 or 130 to 180° (FFAP) or 165° (PEG-20M), maintained at 180 or 165° for 30 min; carrier gas (helium) flow-rate, 0.6 ml/min (FFAP), 1.3 ml/min (PEG-20M); flame ionization detector. The method has been applied to the analysis of lower fatty acids, phenols and indoles in Japanese cigarette smoke, where the peaks were identified by the disappearance method using an alkaline pre-column.

INTRODUCTION

The identification and quantitation of lower fatty acids, phenols and indoles is often required in the organic analysis of foods, cigarettes and cigarette smoke, drugs and automobile exhaust gases, especially in odour pollution analysis. Because these compounds have low odour threshold values, at levels below 1 part per 10^9 in air, the simultaneous analysis of these compounds presents an interesting problem. Direct gas chromatographic (GC) analysis of these compounds at low concentrations has been limited by adsorption and decomposition in the column, ghosting phenomena, the tailing of elution peaks and the separability of many isomeric compounds.

Recently, Robinson[1] reported the GC separation of C_2–C_8 fatty acids on Chromosorb 101, with short analyses times. Okabayashi *et al.*[2] obtained good separations of C_2–C_5 fatty acids by using a FAL-M*, and Di Corcia and co-workers[3–14] separated C_2–C_5 lower fatty acids, phenol isomers, lower aliphatic amines, alcohols and other compounds at the ng level by using gas–liquid–solid chromatography, without adsorption and tailing. Hrivňák and co-workers[15,16] and Barber *et al.*[17] reported the separation of lower fatty acids and phenols by using open-tubular columns

* Polyoxyethylenesorbitan ester + PEG-20M.

containing tricresyl phosphates; capillary columns containing Emulphor O (refs. 18 and 19), squalane[18], Ucon[20] and SF-96 (ref. 21) have also been used for the analysis of lower fatty acids, phenols and indoles, and packed columns of silicone oil DC-550 (ref. 22), SE-30 and 52 (refs. 23 and 24) and XE-60 (ref. 25) and Reoplex 400 (ref. 26) have been used for the analysis of indoles. Unfortunately, when packed columns are employed, the simultaneous GC separation of mixtures of lower fatty acids, phenols and indoles is difficult, because the fatty acids exhibit relatively low boiling points whereas the indoles have higher boiling points and many isomers. In general, the identification of lower fatty acids, phenols and indoles from a complex mixture has been carried out by means of gas chromatography–mass spectrometry (GC–MS). This technique is costly, requires complex instrumentation and is not convenient for on-site environmental pollution analysis.

In this study, glass capillary columns of high resolution were used to achieve the simultaneous GC analysis of low concentrations of lower fatty acids, phenols and indoles, and the identification of these compounds was carried out by the disappearance method using an alkaline pre-column, since these compounds are acidic.

EXPERIMENTAL

Reagents

Fourteen lower fatty acids, 11 phenols, 7 indoles and ethanol were obtained from PolyScience (Niles, Ill., U.S.A.), Aldrich (Milwaukee, Wisc., U.S.A.), Katayama (Osaka, Japan), Tokyo Kasei Kogyo (Tokyo, Japan) and Wako (Osaka, Japan). All of the reagents used were guaranteed or reagent grade chemicals.

Apparatus

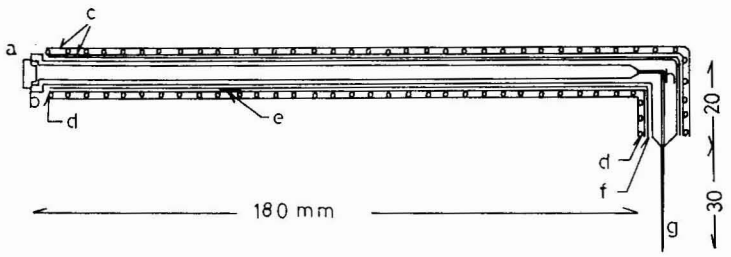
The gas chromatograph used was a Shimadzu Model GC5AP$_5$F (dual columns system) equipped with on-column injection, flame ionization detector (FID) and a digital integrator (Shimadzu Model ITG-2A) for the determination of retention time and quantitative analysis. Two support-coated open-tubular glass capillary (G-SCOT) columns (20 m $\times$ 0.28 mm I.D.) containing FFAP and PEG-20M, respectively, were obtained from Gasukuro Kogyo (Tokyo, Japan).

Chromatographic conditions

The two glass capillary columns were preconditioned at 180° (FFAP) for 3 h, or at 165° (PEG-20M) for 3 h, with a constant flow of helium (*ca.* 1 ml/min) before being connected to the FID. The chromatographic conditions were: (a) stationary phase, FFAP; column temperature, held for 1 min at 145°; column oven, heated at 4°/min from 145 to 180°, maintained at 180° for 30 min and then cooled to 145°; injection port and detector temperatures, 180°; carrier gas (helium) flow-rate; 0.6 ml/min; purge gas (helium) flow-rate, 60 ml/min; splitting ratio, 1:230; air and hydrogen flow-rates for the FID, 1.0 l/min and 50 ml/min, respectively; (b) stationary phase, PEG-20M; column temperature held for 1 min at 130°; column oven, heated at 4°/min from 130 to 165°, maintained at 165° for 30 min and then cooled to 130°; injection port and detector temperatures, 180°; carrier gas (helium) flow-rate, 1.3 ml/min; purge gas (helium) flow-rate, 60 ml/min; splitting ratio, 1:100; air and hydrogen flow-rates for the FID, 1.0 l/min and 50 ml/min, respectively.

Pre-column

Fig. 1 shows a pre-column used for the trapping of samples such as cigarette smoke. It consists of Pyrex glass (18 cm × 4 mm I.D.) packed with Tenax-GC (60–80 mesh)[27–34] and equipped with a nichrome wire for heating and a thermistor for determining the temperature. An alkaline pre-column consisted of 2% potassium hydroxide on glass beads (30–60 mesh): front port, 1.5 cm × 4 mm I.D. plus Tenax-GC (60–80 mesh); needle port, 15.5 cm × 4 mm I.D. The pre-columns were pre-conditioned for 3 h at 220° with a nitrogen flow-rate of 60 ml/min.



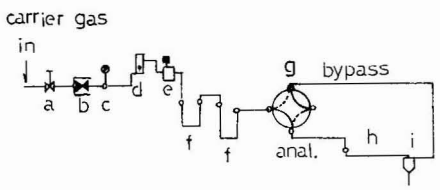


Fig. 1. The trapping pre-column: a = fitted GC injection port septum; b = Pyrex glass tube (4 mm I.D., 6 mm O.D.); c = glass fibre cloth; d = nichrome wire (0.25 mm); e = thermistor; f = aluminium foil; g = syringe needle (0.6 mm I.D.); h = capillary (1.5 mm I.D.). The syringe needle and Pyrex glass tube were fitted by heat treatment.

Fig. 2. Diagram of pre-column injection: a = stop valve; b = pressure controller; c = pressure gauge; d = flow meter; e = mass flow controller; f = molecular sieve 5 A (40 cm × 6 mm I.D. stainless steel); g = four-way cock (one-way stop); h = pre-column; i = GC injection port.

The method of trapping and injecting sample gas was as follows. In the case of standard solutions, the standard solution was injected into the pre-column by use of a microsyringe, and then let into the carrier gas line of the gas chromatograph (Fig. 2). In the trapping of a sample gas (cigarette smoke), the cigarette smoke was trapped in the pre-column at a flow-rate of 0.2 l/min using a vacuum pump. The cigarette used was a Japanese filter-less cigarette (70 mm). After *ca.* 15 min (necessary for the elution of bulky solvent or lower hydrocarbons) the trapped sample was injected into the pre-column, which was set into the carrier gas line of the gas chromatograph, by heating the pre-column with a nichrome wire for 30 sec from room temperature to 200°, maintaining this temperature for 30 min and then cooling to room temperature.

The standard solutions of the reagents were dissolved in water or ethanol to give 10^{-4} mol per 10 ml of solvent. The volume injected was usually 1–6 μl.

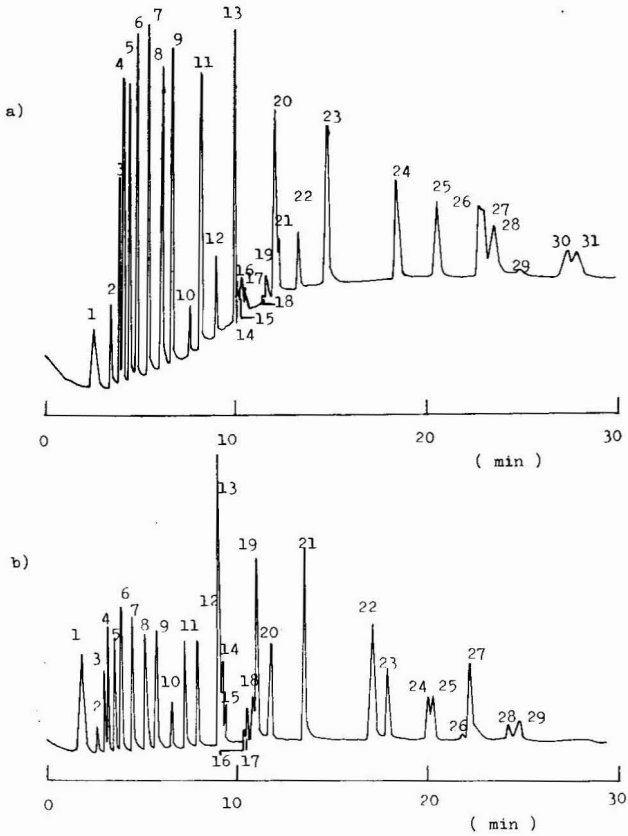


Fig. 3. Typical gas chromatograms of a mixture of 14 lower fatty acids, 11 phenols and 7 indoles obtained by the pre-column injection method using Tenax-GC (18 cm × 4 mm I.D.). (a) G-SCOT glass capillary column (20 m × 0.28 mm I.D.) containing FFAP; other GC conditions as in Experimental, except for FID range 2 (× 0.01 V) and sensitivity 10^3 (× MΩ). Peaks: 1 = solvent (water–ethanol); 2 = CH_3COOH (1.2 μg); 3 = C_2H_5COOH (1.5 μg); 4 = iso-C_3H_7COOH (1.7 μg); 5 = n-C_3H_7COOH (1.7 μg); 6 = iso-C_4H_9COOH (2.0 μg); 7 = n-C_4H_9COOH (2.0 μg); 8 = iso-C_5H_{11}-COOH (2.4 μg); 9 = n-$C_5H_{11}COOH$ (2.4 μg); 10 = 2,6-$C_6H_3(CH_3)_2OH$ (0.5 μg); 11 = n-C_6H_{13}-COOH (2.6 μg); 12 = C_6H_5OH (0.3 μg) + o-$C_6H_4(CH_3)OH$ (0.9 μg); 13 = n-$C_7H_{15}COOH$ (1.4 μg); 14 = p-$C_6H_4(C_2H_5)OH$ (0.3 μg); 15 = 2,5-$C_6H_3(CH_3)_2OH$ (0.2 μg); 16 = p-$C_6H_4(CH_3)OH$ (0.3 μg); 17 = m-$C_6H_4(CH_3)OH$ (0.4 μg); 18 = 2,3-$C_6H_3(CH_3)_2OH$ (0.2 μg); 19 = 1,2-$C_8H_5(CH_3)_2N$ (1.0 μg); 20 = n-$C_8H_{17}COOH$ (1.6 μg); 21 = o-$C_6H_4(C_2H_5)OH$ (0.4 μg) + 3,5-$C_6H_3(CH_3)_2OH$ (0.3 μg); 22 = 3,4-$C_6H_3(CH_3)_2OH$ (0.9 μg); 23 = n-$C_9H_{19}COOH$ (1.7 μg); 24 = n-$C_{10}H_{21}COOH$ (1.9 μg); 25 = C_8H_7N (1.9 μg); 26 = 3-$C_8H_6(CH_3)N$ (1.6 μg); 27 = 2-$C_8H_6(CH_3)N$ (1.5 μg); 28 = n-$C_{11}H_{23}COOH$ (2.0 μg); 29 = 5-$C_8H_6(CH_3)N$ (0.1 μg); 30 = 2,3-$C_8H_5(CH_3)_2N$ (0.9 μg); 31 = 2,5-$C_8H_5(CH_3)_2N$ (1.2 μg). (b) G-SCOT glass capillary column (20 m × 0.28 mm I.D.) containing PEG-20M; other GC conditions as in Experimental, except for FID range 16 (× 0.01 V) and sensitivity 10^3 (× MΩ). Peaks: 1–5, 7–9 and 11 as in (a); 6 = iso-C_4H_9COOH (2.4 μg); 10 = 2,6-$C_6H_3(CH_3)_2OH$ (1.0 μg); 12 = C_6H_5OH (0.6 μg) + o-$C_6H_4(CH_3)OH$ (1.8 μg); 13 = n-$C_7H_{15}COOH$ (2.4 μg); 14 = p-$C_6H_4(C_2H_5)OH$ (0.6 μg) + 2,5-$C_6H_3(CH_3)_2OH$ (0.4 μg); 15 = p-$C_6H_4(CH_3)OH$ (0.6 μg) + m-$C_6H_4(CH_3)OH$ (0.8 μg); 16 = 1,2-$C_8H_5(CH_3)_2N$ (1.0 μg); 17 = 2,3-$C_6H_3(CH_3)_2OH$ (0.4 μg); 18 = 3,5-$C_6H_3(CH_3)_2OH$ (0.6 μg); 19 = n-$C_8H_{17}COOH$ (3.2 μg); 20 = o-$C_6H_4(C_2H_5)OH$ (0.8 μg) + 3,4-$C_6H_3(CH_3)_2OH$ (1.8 μg); 21 = n-$C_9H_{19}COOH$ (3.4 μg); 22 = n-$C_{10}H_{21}COOH$ (3.8 μg); 23 = C_8H_7N (1.9 μg); 24 = 3-$C_8H_6(CH_3)N$ (1.6 μg); 25 = 2-$C_8H_6(CH_3)N$ (1.5 μg); 26 = 5-$C_8H_6(CH_3)N$ (0.1 μg); 27 = n-$C_{11}H_{23}COOH$ (4.0 μg); 28 = 2,3-$C_8H_5(CH_3)_2N$ (0.9 μg); 29 = 2,5-$C_8H_5(CH_3)_2N$ (1.2 μg).

RESULTS AND DISCUSSION

Typical gas chromatograms

Typical gas chromatograms of a mixture of 14 lower fatty acids, 11 phenols and 7 indoles obtained by use of the Tenax–GC pre-column (18 cm × 4 mm I.D., 60–80 mesh) injection method are shown in Figs. 3–5. Fig. 3 shows typical gas chromatograms of a mixture of 32 compounds obtained using FFAP (a) and PEG-20M (b) glass capillary columns. The analysis time of each chromatogram was less than 30 min. The separation of the 14 lower fatty acids was complete in the two liquid phases, but that of the 11 phenols was incomplete, especially for pairs of compounds such as phenol and *o*-cresol and *o*-ethylphenol and 3,5-xylenol. The separation of the 7 indoles was complete except for 2- and 3-methylindole whose peaks were poorly separated. The separation of the two indoles was better on the PEG-20M column than on the FFAP column, but the separation of propionic and isobutyric acids was better on the FFAP column than on the PEG-20M column.

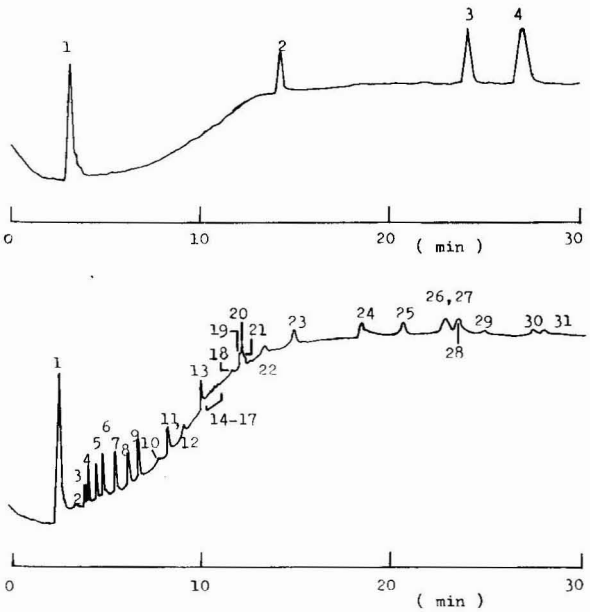


Fig. 4. Typical gas chromatogram of a mixture of 14 lower fatty acids, 11 phenols and 7 indoles obtained by the pre-column injection method using columns of 2% potassium hydroxide on glass beads (1.5 cm × 4 mm I.D.) + Tenax-GC (15.5 cm × 4 mm I.D.). GC conditions and sample size as in Fig. 3a.

Fig. 5. Typical gas chromatogram of a mixture of 14 lower fatty acids, 11 phenols and 7 indoles obtained by the pre-column injection method using a Tenax-GC (18 cm × 4 mm I.D.) column. GC conditions as in Fig. 3a. Peaks as in Fig. 3a but all amounts are one tenth those previously given.

Fig. 4 shows a typical gas chromatogram of a mixture of 14 lower fatty acids, 11 phenols and 7 indoles obtained by use of the pre-column injection method using 2% potassium hydroxide on glass beads (1.5 cm × 4 mm I.D.) plus Tenax-GC (15.5 cm × 4 mm I.D.) and the FFAP glass capillary column. The peaks of 32 compounds

disappeared completely, although three unknown peaks were produced which did not overlap with those of the 32 compounds. It is important that the peaks of the 32 compounds, especially those of the 7 indoles, as well as those of lower fatty acids and phenols, disappeared when the Tenax-GC–2% potassium hydroxide system was used. This system is therefore superior for the identification of the 32 compounds.

Fig. 5 shows a typical gas chromatogram of a mixture of 14 lower fatty acids, 11 phenols and 7 indoles at the 0.1-μg level obtained by use of the Tenax-GC pre-column (18 cm $\times$ 4 mm I.D.). The chromatogram represents at the minimum detectable level of each compound by the present method. As shown in Fig. 5, *n*-butyric acid (a representative odorant of the lower fatty acids) and indole were still detected in the lower limits of 0.17 and 0.19 μg, respectively. Therefore, the minimum detectable quantity of the 32 compounds was *ca.* 0.05 μg, and this method may be applicable to the trace analysis of these compounds.

Fig. 6 shows typical calibration curves for the quantitative analysis of representative compounds, *i.e.*, isobutyric acid, 2,6-xylenol and indoles, obtained using the FFAP glass capillary column with the Tenax-GC (18 cm $\times$ 4 mm I.D.) pre-column. The detector response produced a linear relation in the ranges 0.5–5 μg for 2,6-xylenol, 0.4–20 μg for indoles and 0.5–20 μg for isobutyric acid.

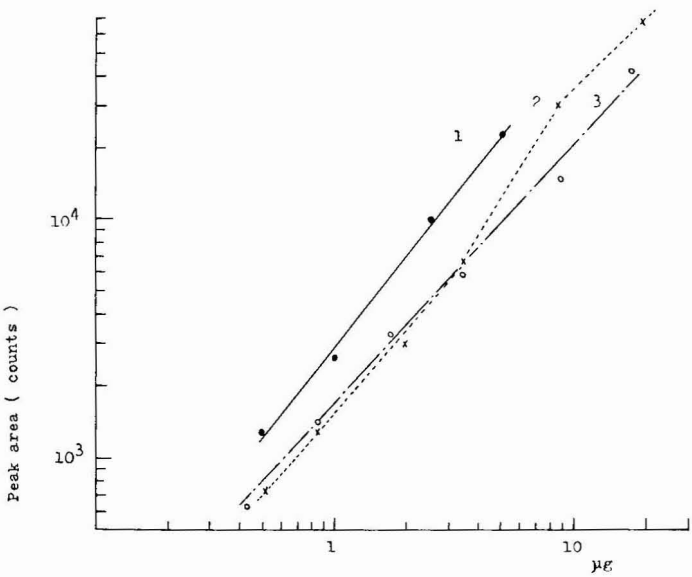


Fig. 6. Typical calibration curves for isobutyric acid (3), 2,6-xylenol (1) and indole (2) obtained using a Tenax-GC pre-column (18 cm $\times$ 4 mm I.D.). GC conditions as in Fig. 3a.

Application to cigarette smoke

Fig. 7 shows a typical gas chromatogram of lower fatty acids, phenols and indoles in the smoke obtained from a Japanese cigarette (10-mm portion of a 70-mm filter-less cigarette). The pretreatment procedures were as described in Experimental. As shown in Fig. 7, at least 75 peaks were recognized and 25 compounds were identified as 10 lower fatty acids, 11 phenols and 4 indoles, *i.e.*, iso- and *n*-butyric, iso- and *n*-valeric, iso- and *n*-caproic, *n*-caprylic, *n*-pelargonic, *n*-capric and *n*-undecanoic

acids, and phenol, *o*-, *m*- and *p*-cresol, *o*- and *p*-ethylphenol, 2,3-, 2,5-, 2,6-, 3,4- and 3,5-xylenol, indole, 2- and 3-methylindole and 1,2-dimethylindole. The quantitative results for the representative compounds expressed in milligrams per cigarette, were as follows: *n*-butyric acid, 0.075; isovaleric acid, 0.09; phenol, 0.13; 2,6-xylenol, 0.01; indole, 0.01; 3-methylindole, 0.05. These results are in reasonable agreement with literature values[35] for the lower fatty acids, but for the phenols were lower than the results of Guerin *et al.*[36], while, the results for the indoles were greater than the values of Hoffmann and Rathkamp[25].

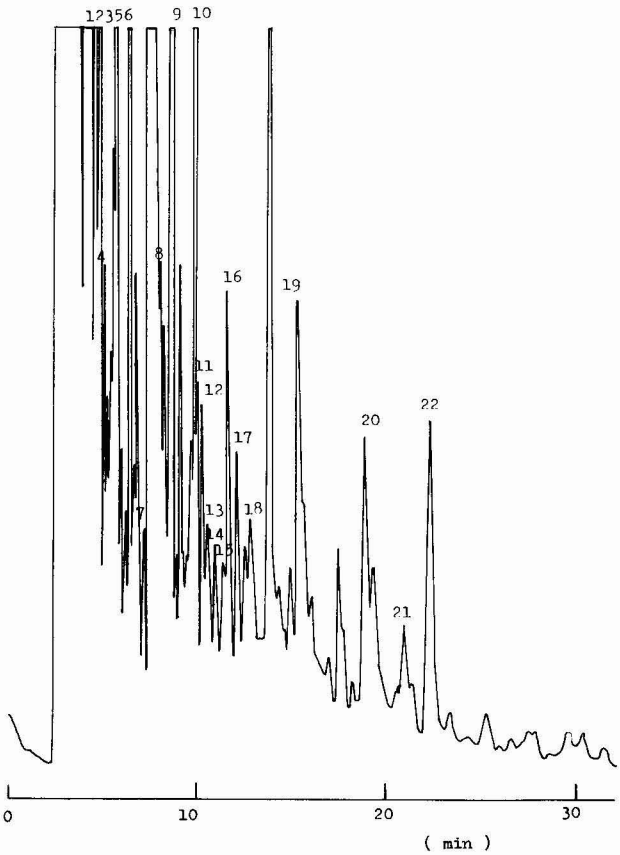


Fig. 7. Typical gas chromatogram of lower fatty acids, phenols and indoles in the smoke obtained from a Japanese cigarette using a Tenax-GC (18 cm × 4 mm I.D.) column. GC conditions as in Fig. 3a. Peaks: 1 = iso-C_3H_7COOH; 2 = *n*-C_3H_7COOH; 3 = iso-C_4H_9COOH; 4 = *n*-C_4H_9COOH; 5 = iso-$C_5H_{11}COOH$; 6 = *n*-$C_5H_{11}COOH$; 7 = 2,6-$C_6H_3(CH_3)_2OH$; 8 = *n*-$C_6H_{13}COOH$; 9 = C_6H_5OH + *o*-$C_6H_4(CH_3)OH$; 10 = *n*-$C_7H_{15}COOH$; 11 = *p*-$C_6H_4(C_2H_5)OH$; 12 = 2,5-$C_6H_3(CH_3)_2$-OH + *p*-$C_6H_4(CH_3)OH$; 13 = *m*-$C_6H_4(CH_3)OH$; 14 = 2,3-$C_6H_3(CH_3)_2OH$; 15 = 1,2-$C_8H_5(CH_3)_2$-N; 16 = *n*-$C_8H_{17}COOH$; 17 = *o*-$C_6H_4(C_2H_5)OH$ + 3,5-$C_6H_3(CH_3)_2OH$; 18 = 3,4-$C_6H_3(CH_3)_2OH$; 19 = *n*-$C_9H_{19}COOH$; 20 = *n*-$C_{10}H_{21}COOH$; 21 = C_8H_7N; 22 = 2-$C_8H_6(CH_3)N$ + 3-$C_8H_6(CH_3)N$.

Fig. 8 shows a typical gas chromatogram of cigarette smoke obtained by use of the column containing 2% potassium hydroxide on glass beads (1.5 cm × 4 mm I.D.) plus Tenax-GC (15.5 cm × 4 mm I.D.). The peaks of the acidic compounds

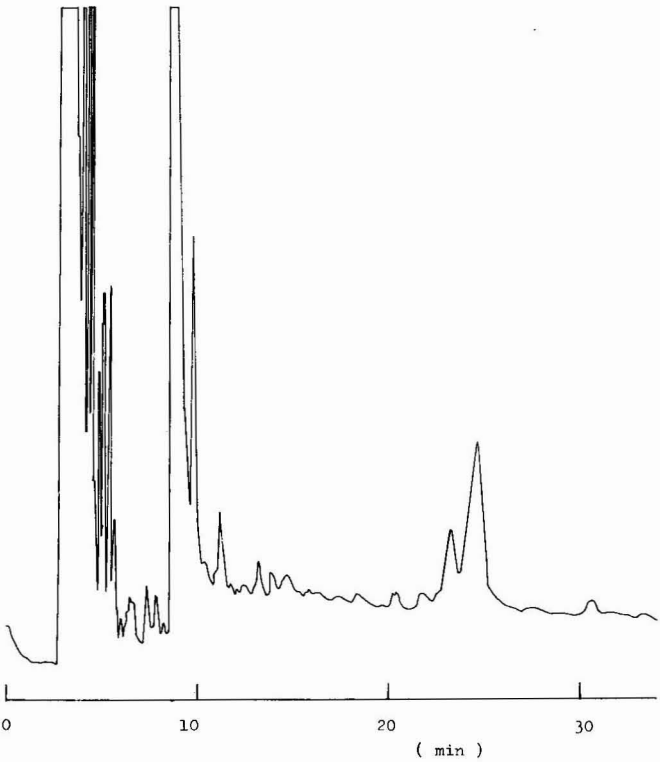


Fig. 8. Typical gas chromatogram of lower fatty acids, phenols and indoles from the smoke of a Japanese cigarette obtained by the pre-column injection method using a column of 2% potassium hydroxide on glass beads (1.5 cm × 4 mm I.D.) + Tenax-GC (15.5 cm × 4 mm I.D.). GC conditions as in Fig. 3a.

in Fig. 7, such as the lower fatty acids, phenols and indoles, respectively, disappeared completely; however, the remaining peaks had longer retention times than on the column containing only Tenax-GC (Fig. 7), *e.g.*, 9 compared with 8 min. The presence of the lower fatty acids, phenols and indoles was confirmed by the disappearance of their peaks on the alkaline column containing Tenax-GC.

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GAS CHROMATOGRAPHIC ANALYSIS OF ACETOPHENONE OXIME AND ITS METABOLITES

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SUMMARY

A gas–liquid chromatographic (GLC) method has been developed for monitoring the metabolic reduction of acetophenone oxime or oxidative metabolism of the corresponding amine, α-methylbenzylamine in liver homogenates. The oxime, amine, N-hydroxy-α-methylbenzylamine and acetophenone are quantitatively determined after GLC separation of components with temperature programming on an SP-2401-DB-coated column. The first three compounds were silylated with N,O-bis(trimethylsilyl)-acetamide prior to chromatographic analysis to enhance the stability and improve the chromatographic properties of these components. The effluent gas was monitored with flame ionization detection, and permitted quantitation of components at sub-μg/ml levels with reproducibility between injections of $\pm 2\%$. The optimal composition of enantiomeric mixtures of (*R,S*)-α-methylbenzylamines formed during metabolic reduction of acetophenone oximes were determined by conversion to diastereomeric amides and subsequent GLC analysis.

INTRODUCTION

Interest in the metabolism of oximes was generated by the discovery that primary aliphatic amines (*e.g.* amphetamine) are oxidatively metabolized to oximes which can then be converted to the corresponding ketone by enzymatic or chemical hydrolysis. The oxidation proceeds by initial N-oxidation to yield hydroxylamines[1–3] which are dehydrogenated to yield the oxime, or alternatively via C-oxidation to generate a transient carbinolamine[4–6] which eliminate water to form an imine which is N-hydroxylated to provide the oxime. The metabolism of the resultant oximes has been similarly studied[7,8]. The oxime is reduced by hepatic enzymes to produce the corresponding hydroxylamine and amine.

Several gas and thin-layer chromatographic methods have been reported for monitoring these reactions and have been reviewed by Beckett and Haya[9]. The hydroxylamine has been shown to be quite unstable both in solution and when subject-

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ed to the thermal stresses of gas–liquid chromatographic (GLC) analysis. The various factors for handling these compounds in solution have been described recently by Beckett *et al.*[10]. Breakdown of the hydroxylamine during GLC analysis has required prior derivatization of the hydroxylamine. Similarly, the amine and oxime have proven to require derivatization to enhance their stability for GLC analysis as well as to reduce the severe tailing observed during chromatography of these compounds. None of these methods is capable of simultaneously analyzing for the ketone, oxime, hydroxylamine and amine. Furthermore, reduction of the oxime generates an asymmetric carbon atom; therefore, a method capable of determining the optical purity of the metabolically derived hydroxylamine and amine was desired.

A GLC method is described for the simultaneous analysis of acetophenone oxime and its metabolites N-hydroxy-α-methylbenzylamine, α-methylbenzylamine and acetophenone from biological fluids. A means for resolving the enantiomeric amine metabilotes is presented using a procedure similar to that previously described by Martin *et al.*[11].

EXPERIMENTAL

Apparatus

Gas chromatography was carried out on a Varian 2100 chromatograph equipped with flame ionization detector (FID). High-performance liquid chromatography (HPLC) was performed on a component system consisting of a Waters Assoc. (Milford, Mass., U.S.A.) Model 6000-A solvent delivery system, Model U-6K septumless injector and Model 440 dual-channel absorbance detector operated at 254 nm.

Reagents

Acetonitrile and methanol were purchased from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.) N,O-bis(trimethylsilyl)acetamide (BSA) was obtained from Pierce (Rockford, Ill., U.S.A.). N-trifluoroacetyl-(*S*)-prolyl chloride (TPC), 0.1 *M* solution in chloroform containing 6.95% (*R*)-isomer, was purchased from Regis (Chicago, Ill., U.S.A.). (*R*)-(+)-, (*S*)-(−)- and (*R,S*)-(±)-α-methylbenzylamine were obtained from Aldrich (Milwaukee, Wis., U.S.A.) and acetophenone and acetophenone oxime were obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.). N-Hydroxy-α-methylbenzylamine was synthesized by reduction of acetophenone oxime with diborane[12].

Extraction

Microsomal suspensions (to which 0.001% EDTA had been added) containing oxime, hydroxylamine, amine and ketone were adjusted to pH 4, deaerated with nitrogen, and extracted with an equal volume of chloroform. The organic phase was retained for further analysis. The aqueous layer was adjusted to pH 11.5, and extracted with an equal volume of chloroform.The organic phase was removed and either combined with the chloroform solution obtained from the pH 4 extraction or processed separately.

To determine the extraction efficiency for partitioning of amine and hydroxylamine into chloroform, both compounds were dissolved in acetate buffer (0.2 *M*; pH 4, containing 0.001% EDTA) to the extent of 0.75 mg/ml. The solutions were then

carried through the 2-step extraction just described. 4-Fluorophenol (0.5 mg/ml) was added to each separated phase as internal standard and the mixtures analyzed by HPLC, Separation of α-methylbenzylamine, acetophenone oxime and N-hydroxy-α-methylbenzylamine utilized a μBondapak C_{18} column (4 mm O.D. $\times$ 30 cm) (Waters Assoc.) operating at 2.0 ml/min with methanol–water containing potassium hydrogen phosphate (7.1 g/l) adjusted to pH 4.2 (30:70) as mobile phase. With these conditions the retention volumes of amine, hydroxylamine, and oxime were 5.6, 8.4 and 30.8 ml, respectively. Calculation of the amount of amine or hydroxylamine in either aqueous or organic phase was made by drawing an aliquot from either extraction phase, injecting it into the chromatograph and comparing the peak height (relative to internal standard) for the component of interest with standard curves constructed for the amine and hydroxylamine. These standard curves were generated from HPLC analysis of solutions of known concentration of amine and hydroxylamine (in the concentration range 0.25–1.0 mg/ml) prepared both in chloroform and 0.2 *M* acetate (pH 4) buffer. All measurements were made relative to a constant amount of 4-fluorophenol (internal standard) and made in duplicate.

Silylation procedure

The chloroform layers obtained after extraction of amine, hydroxylamine, oxime and ketone were evaporated to dryness. An approximate 60-fold molar excess of BSA (20-fold excess for each derivatizable compound) was added to the mixture, followed by 3 parts of dry acetonitrile (for each part of BSA). The mixture was shaken vigorously for 1 min, and then heated at 60–70° for 30 min. N,N-Dimethylaniline was added as internal standard and the mixture diluted to volume with dry acetonitrile.

Gas chromatographic analysis

Mixtures containing ketone and silylated amine, hydroxylamine and oxime were separated on a 6 ft. $\times$ $^1/_4$ in. glass column packed with GP 5% SP-2401-DB coated on Supelcoport (100–120 mesh). Nitrogen, used as carrier gas, flowed at a rate of 30 ml/min. Mixtures were separated with the aid of a temperature program: 75° for 2 min, then heated at 10°/min to 125° where the temperature was maintained for 1 min. The injector and detector temperatures were 140° and 160°, respectively. The amount of each component in a mixture was determined as the ratio of its peak height relative to a known amount of N,N-dimethylaniline (internal standard). Concentrations were determined from calibration curves constructed for each compound, prepared by GLC analysis of standard solutions of silylated amine, hydroxylamine, and oxime and underivatized ketone, each at 13 different concentrations in the range of 0.3–500 μg/ml. Curves were prepared for each compound by plotting concentration *vs.* peak height ratio of material. All measurements were made in triplicate and subjected to linear regression analysis (Table I).

Determination of the optical composition of (R,S)-α-methylbenzylamine mixtures

The chloroform layer obtained from the pH 11.5 extraction was evaporated to dryness and the residue dissolved in 250 μl chloroform. Twenty-five microliters (2.5 μmoles) of TPC solution were added to the solution (containing *ca.* 1.25 μmole of acylable compound), followed in 1 min by the addition of 2.5 μmoles of triethylamine.

TABLE I

STANDARD CURVES FOR GLC ANALYSIS OF ACETOPHENONE OXIME METABOLITES

Each curve represents triplicate analysis of each compound at 10 or 13 concentrations in the range of 0.3–500 μg/ml. Analysis performed as described in Experimental. Sensitivity is based on 1-μl injections.

Compound	*Retention Time*	*Sensitivity limit (μg/μl)*	*Linear regression parameters**		
			Slope	*Intercept*	*Correlation coefficient*
α-Methylbenzylamine	4.9	8.0	2.030	−0.001	0.999
Acetophenone	5.5	0.3	1.650	−0.002	0.998
N-Hydroxy-α-methylbenzylamine	6.3	0.3	2.259	0.002	0.999
Acetophenone oxime	6.7	0.3	2.018	0.004	0.999

* Amount of each compound determined as ratio of peak height of compound relative to internal standard N,N-dimethylaniline.

One minute later, 250 μl of sodium chloride-saturated 1 *N* HCl solution were added and the mixture was shaken vigorously for 1 min. After phase sepatation, 2.5 μl of the chloroform solution were subjected to GLC analysis. The resulting diastereomeric amides were chromatographed on a 4 ft. × $^1/_8$ in. glass column packed with 3% OV-17 coated on Gas-Chrom Q (100–120 mesh). Components were separated (with nitrogen carrier gas flowing at 30 ml/min) isothermally (temperatures: injector, 250°; column, 200°; FID, 260°).

RESULTS

The analysis of acetophenone oxime, its metabolites, N-hydroxy-α-methylbenzylamine and α-methylbenzylamine, and degradation product, acetophenone, was accomplished in three stages: (1) extraction from biological fluid (in the present study rat liver homogenate), (2) chemical derivatization of the oxime, amine and hydroxylamine, and (3) GLC analysis of the mixture with flame ionization detection of the effluent gas.

Extraction

Extraction from biological fluid was carried out in two steps. The ketone, oxime and hydroxylamine were extracted into chloroform after adjustment of the pH to 4. At this pH the amine ($pK_a \approx 9.4$) exists completely in the protonated form and remains in the aqueous phase. No amine was detected in the organic phase after extraction; while at this pH the hydroxylamine ($pK_a \approx 4.8$) was quantitatively (100%) extracted into chloroform. After readjustment of the pH to 11.5, the amine was quantitatively extracted into chloroform. The chloroform extracts were then either combined or analyzed separately. Solvent was removed prior to the next stage in the analysis sequence. At pH 4, only 0.8% of the hydroxylamine was converted to oxime in 4 h. At pH 11.5, however, the hydroxylamine proved unstable, being converted to oxime. Similarly, the presence of EDTA (0.001%) was required to maximize the stability of the hydroxylamine[10]. Extraction efficiency was determined by analyzing the organic phase by a HPLC method capable of discriminating among amine, hydro-

xylamine and oxime, a requirement not met by simple spectrophotometric monitoring of reaction mixtures. The amount present in either phase was determined by comparison of peak height ratio with curves constructed from HPLC analysis of known amounts of each component dissolved in chloroform or in pH 4 acetate buffer (Fig. 1).

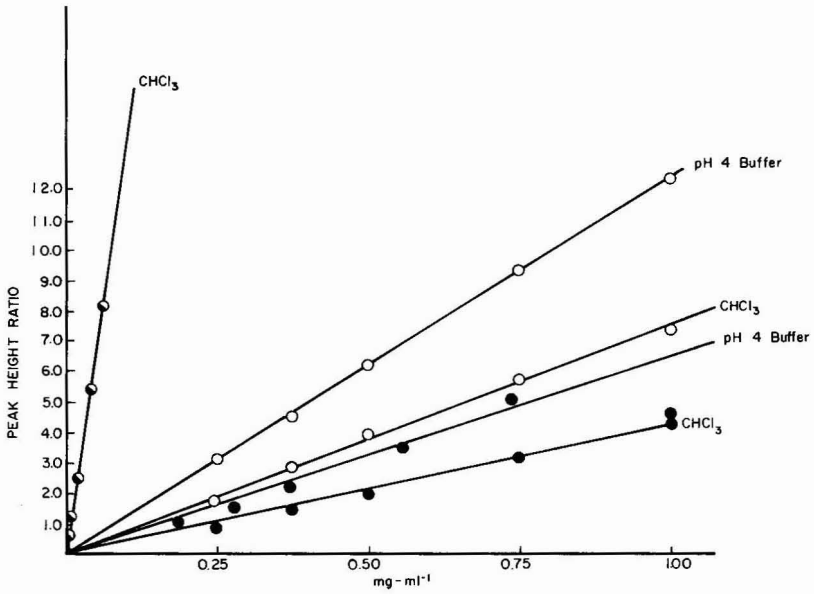

Fig. 1. Calibration curves for determining the extraction of efficiency of acetophenone oxime (◑), N-hydroxy-α-methylbenzylamine (●) and α-methylbenzylamine (○) from acetate buffer (pH 4) into chloroform.

Attempts were also made to extract the amine and hydroxylamine into chloroform over a pH range of 6.0–8.0, as an ion pair with heptafluorobutyric acid. This procedure, however, provided poorer extraction selectivity than extracting the components as the free base.

Derivatization

Derivatization of the hydroxylamine, amine and oxime was required prior to analysis. These components proved unstable to the stresses imposed by gas chromatographic analysis. The hydroxylamine disproportionates to oxime and amine. Instability of the amine and oxime was determined by the observation that GLC peak areas for the components were not linearly related to concentration. Furthermore, tailing of peaks was observed with non-derivatized molecules, necessitating their conversion to more chromatographable compounds.

The oxime, hydroxylamine and amine were converted to trimethylsilyl derivatives by reaction with BSA. This was the only silylating reagent (of the six tried) capable of reacting with all three compounds. Silylation of the oxime and hydroxylamine occurred easily and reaction could be completed within 5 min at room temperature. Among the silylating reagents tried [trimethylsilylchloride, BSA, hexamethyldisilazane, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), trimethylsilylimidazole and

tri-sil®], only BSA was capable of derivatizing the amine. Silylation of amine with this reagent, however, still required somewhat strenuous conditions. The reaction was allowed to proceed in dry acetonitrile with agitation at 60–70 % for 30 min. Maximum yield of silylated products was obtained when the molar ratio of silylating reagent to analyte was 50–60:1. Under these conditions, 100 % conversion of oxime, hydroxylamine and amine to the corresponding silyl derivative was observed, *i.e.* GLC analysis revealed no underivatized compound. When reaction time was shortened or the temperature lowered, less than quantitative silylation of amine was observed.

If provisions were made to exclude moisture from solutions containing these derivatives, they were stable for 48–72 h. At 72 h, only 3 % loss of the hydroxylamine and 2 % loss of oxime and amine were detected. All derivatives were stable for 24 h when no such precautions were taken.

Gas chromatographic analysis

The ketone and silylated derivatives of the amine, oxime and hydroxylamine were separated on a GP 5 % SP-2401-DB-coated column with temperature programming. As shown in Fig. 2, this approach gives sharp well-defined peaks with good resolution of all components; while total analysis time for the four compounds was still only 7 min. The retention times for each of the components is shown in Table I. Quantitative analysis of the components was based on calibration curves generated for each compound. The amount was determined as a ratio of peak height of compound relative to N,N-dimethylaniline (retention time 4.5 min), present as internal standard. Standard curves were constructed with mixtures containing the four compounds of interest at thirteen different concentrations distributed over a concentration range from 0.3 to 500 μg/ml. All measurements were made in triplicate and plots of peak height ratio *vs.* concentration were constructed through linear regression analysis. Regression constants for all components are shown in Table I. Calibration curves were repeated on three consecutive days and were highly reproducible. Sensitivity limits for the method are *ca.* 0.3 μg/ml for each component (8 μg/ml for the amine) from 1-μl on-column injections. Precision between injections is ± 2 %.

Determination of the optical composition of (R)- and (S)-α-methylbenzylamine mixtures

Resolution of optical mixtures of (*R*)- and (*S*)-forms of α-methylbenzylamine formed during enzymatic reduction of acetophenone oxime was accomplished by reaction of the enantiomeric amines with TPC[13] which converts them to diastereomeric amides separable by GLC on an OV-17 column. GLC analysis produced two peaks with retention times of 3.0 and 3.6 min (Fig. 3). The first peak corresponds to the amide formed from the (*R*)-amine, as determined by subsequent injection of an authentic sample of appropriately derivatized optically pure amine. This peak also contains small amount of the amide formed by reaction of the (*S*)-amine and (*R*)-acid chloride (present as a minor impurity in the resolving reagent), since (*R*,*S*)- and (*S*,*R*)-stereoisomers are, in fact, enantiomers and not resolvable. Quantitation of optical composition was made from peak ratio measurements. Percent optical composition was calculated making correction for the amount of (*R*)-TPC present as impurity in the resolving reagent using the equation[14]

$$R = \frac{50\,(R_{app} - x)}{50 - x}$$

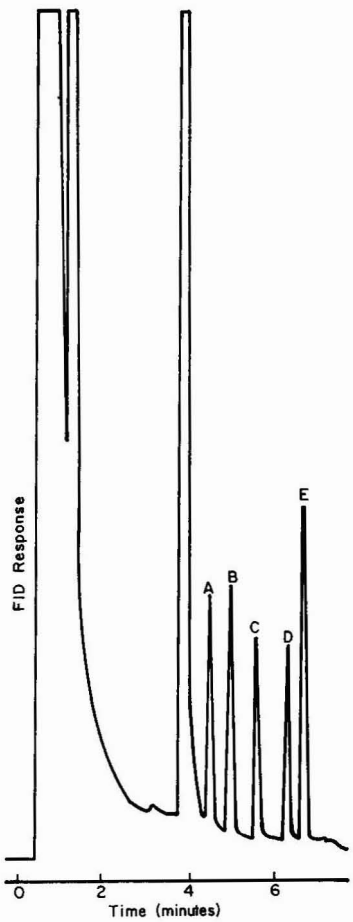

Fig. 2. Gas–liquid chromatogram of (A) N,N-dimethylaniline (internal standard), (B) silylated α-methylbenzylamine, (C) acetophenone, (D) silylated N-hydroxy-α-methylbenzylamine and (E) silylated acetophenone oxime, using the separation system described in the text.

where R is the calculated percentage of (R)-amine, R_{app} is the apparent percentage of (R)-amine determined from peak ratio measurements, and x is the percentage (R)-isomer in the TPC solution. Using mixtures of known optical composition, the percentage of (R)-amine in mixtures could be determined with an accuracy of $\pm 1\%$, over the complete enantiomeric composition range (*i.e.* 0–100% (R)-amine in the mixture).

DISCUSSION

A rapid, simple and sensitive GLC analysis for acetophenone oxime and its major metabolites and breakdown product is described. Acetophenone oxime serves only as a model compound. Similar metabolic products have been isolated from both reductive oxime metabolism and oxidative metabolism of aliphatic primary amines (*e.g.* amphetamine). The method should serve as a general procedure for monitoring metabolic reactions involving such compounds.

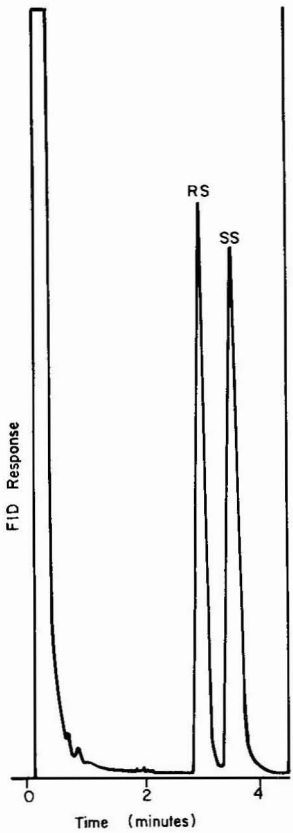

Fig. 3. Gas chromatogram showing the resolution of a racemic modification of (*RS*)-(±)-α-methylbenzylamine as diastereomeric trifluoroacetyl-(*S*)-prolyl amides.

Considerable care must be exercised in handling the aliphatic hydroxylamine. Beckett *et al.*[10] have recently shown the lability of such compounds at higher pH values and in the presence of many heavy metals. To preclude metal-catalyzed breakdown, EDTA or 8-hydroxyquinone must be added to the mixture. The hydroxylamine begins to show significant instability at pH > 7.5. Therefore, extraction must be carried out in two steps since the amine only exists to an appreciable extent as the free base above pH 10.5. The pH of the mixture was thus first adjusted to 4 to maximize hydroxylamine stability, where all components except the amine were extracted. At this pH, only 0.8 % of the hydroxylamine was lost in 4 h. Once the hydroxylamine had been extracted, the mixture could safely be made alkaline so that the amine could be extracted as its free base. This initial separation of amine from hydroxylamine was also necessary in instances where the optical composition of enantiomeric amine mixtures was to be determined, since both the amine and hydroxylamine are converted to the same amide after reaction with TPC.

Whereas the extraction efficiency of amine, oxime or ketone could be monitored spectrophotometrically, hydroxylamine extractability could only be monitored with its prior chromatographic separation. The hydroxylamine is readily oxidized under

numerous conditions to the oxime[10], which has a UV spectrum which overlaps with that of the hydroxylamine. The molar absorptivity of the oxime is, however, two orders of magnitude greater than that for the hydroxylamine; so that formation of a small amount of oxime would give exceedingly high results for the extraction efficiency of hydroxylamine determined by simple spectrophotometric analysis. Although the LC method used, clearly resolved all components, it is unacceptable for monitor-metabolic levels, because of the poor sensitivity of the method, when the effluent is monitored spectrophotometrically (UV), *i.e.* the molar absorptivity of amine and hydroxylamine are quite low ($\varepsilon \approx 200$ at 254 nm).

Quantitative derivatization of the oxime, amine and hydroxylamine was attained using BSA as silylating agent. The amine was resistant to derivatization and could only be silylated with this reagent and only under strenuous conditions. Silylation, however, enhanced the stability and reduced tailing allowing the components to be rapidly analyzed at submicrogram levels. The standard error of regression given in Table I indicate the goodness of fit of the regression lines generated from 39 data points at 13 different concentrations for each analyte. Derivatization of the hydroxylamine has alternatively been reported using BSTFA[9,15] or trifluoroacetic anhydride[16]. However, the other methods do not provide a none-step analysis of all four components of interest. BSTFA, furthermore, did not effectively silylate the amine and was therefore not a suitable reagent.

Reductive metabolism of oximes proceeds with the generation of an asymmetric center. To determine the optical composition of the enantiomeric amine mixture formed during microsomal oxime reduction, the amines were converted to diastereomeric amides with a chiral acid chloride; the amides were then separated by GLC. The (*R*)-isomer eluted prior to the (*S*)-amine. This is the same order of elution found by Gordis[13] for amphetamine reacted with (*S*)-TPC and by Martin *et al.*[11] who reacted various β-phenylisopropyl amines with N-pentafluorobenzoyl-(*S*)-(−)-prolyl-l-imidazole to effect GLC resolution.

Attempts to resolve the enantiomeric N-hydroxy-α-methylbenzylamines by reaction with (*S*)-TPC to form diastereomeric hydroxamic acids were unsuccessful. GLC of the "derivatized" mixtures indicated that the hydroxylamines are converted to the same products which form on reaction with α-methylbenzylamine. This reaction has not been investigated, but since it may proceed with loss of chirality, it is inappropriate for analysis of the hydroxylamines. Since the hydroxylamine and amine are quantitatively separated by extraction prior to derivatization, the optical composition of the amine can still be determined without interference from the hydroxylamine.

Although other methods are available for analysis of such mixtures, they(1) require multiple-column analysis, (2) require long analysis times, and (3) lack the precision and simplicity of this method.

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SENSITIVE AND SPECIFIC GAS CHROMATOGRAPHIC AND EXTRACTION METHOD FOR THE DETERMINATION OF ORPHENADRINE IN HUMAN BODY FLUIDS

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SUMMARY

A gas chromatographic and extraction method for the assay of orphenadrine in plasma and urine has been developed, in which diphenhydramine is used as the internal standard. The procedure involves extraction with isopentane and alkali flame ionization (nitrogen) detection. Orphenadrine N-oxide and N-dealkylated orphenadrine did not interfere with the analysis. Orphenadrine concentrations down to 1 ng/ml can be determined. Application in a pharmacokinetic/bioavailability study is reported.

INTRODUCTION

Orphenadrine hydrochloride (trade names: Disipal, Brocadisipal, Brocasipal) is frequently used in the therapy of Parkinson's disease, or drug-induced parkinsonism. Although it has been used for many years and studies of its metabolic fate in the rat[1,2], dog[2], monkey [2] and man[3,4] have been reported, detailed information on its pharmacokinetics and therapeutically active blood levels is scarce. The extensive study by Khan[5] on orphenadrine pharmacokinetics, based on urine data, involved volunteers on a special dietary regimen (high water-loading; oral dosages of ammonium chloride to acidify the urine) intended to increase renal clearance, hence cannot be regarded as representative of normal clinical conditions.

The pharmacokinetic profile of orphenadrine hydrochloride in man and the relationship between action and blood levels are ill-defined largely because a sufficiently sensitive and specific method for its determination in blood (plasma) is unavailable.

In their description of the determination of diphenhydramine at the nanogram level in human plasma with the aid of a nitrogen-specific gas chromatographic (GC) technique, Bilzer and Gundert-Remy[6] noted that orphenadrine might be measured analogously. However, the method is not sufficiently specific, for two reasons: (i) the orphenadrine metabolite orphenadrine N-oxide, which can be recovered from human urine for *ca.* 5% of the orphenadrine dose[4,5], may interfere; and (ii) the GC separation of orphenadrine from its metabolite N-demethylorphenadrine, which occurs in human plasma and urine in significant quantities, is unsatisfactory.

This paper reports an extraction and GC method by which a kinetic plasma

(and urine) study can be performed in human subjects medicated with orphenadrine.

The application of a sensitive nitrogen detector makes it possible to determine orphenadrine to the desired level of 1 ng/ml. The extraction and GC procedure is such that interference due to the metabolites orphenadrine N-oxide and N-dealkylorphenadrine can be avoided, and it even permits simultaneous quantitative determination of orphenadrine and N-demethylorphenadrine.

MATERIALS AND METHODS

All chemicals were of analytical grade. Orphenadrine, N-demethylorphenadrine, N,N-didemethylorphenadrine and their hydrochlorides and orphenadrine N-oxide were prepared in our laboratory. On the thin-layer chromatograms, all compounds produced single spots at different retention times in different solvent systems. All solvents were distilled twice in glass prior to use. The glassware was treated with nitric acid and carefully boiled out with ethanol. The phosphate buffer of pH 12 was prepared by dissolving 22.25 g of $Na_2HPO_4 \cdot 2H_2O$ and 4.46 g of NaOH in 500 ml of distilled water.

Isolation procedure

To 0.5–2 ml of blood plasma or urine in a glass-stoppered centrifuge tube were added 10–100 ng of internal standard, dissolved in distilled water. Diphenhydramine hydrochloride was chosen as internal standard because of its structural similarity to orphenadrine. The pH was adjusted to 12 by addition of 5 *M* NaOH and 0.5 ml of 0.25 *M* phosphate buffer, and the solution was made up to 3 ml with water. After equilibration for *ca.* 15 min, 3 ml of isopentane were added, and the mixture was extracted for 30 min on a Heidolph apparatus. After centrifugation at 2500 *g* for 10 min, the phases were separated and the aqueous phase was re-extracted with 3 ml of isopentane. The organic phase was collected, and shaken with 1 ml of 0.5 *M* HCl in a clean centrifuge tube for 15 min. The acidic aqueous phase was then washed out with 3 ml of isopentane for 5 min.

The organic solvent was discarded and the aqueous phase made alkaline (pH 12) with 5 *M* NaOH and 0.5 ml of phosphate buffer. Subsequently, a 15-min extraction with 3 ml of isopentane and centrifugation were carried out. The organic phase was transferred to a clean tapered tube, the inner wall of which had been carefully rinsed with ethanol. Finally, the sample was evaporated to dryness under a stream of nitrogen, and the residue dissolved in 10 μl of ethanol. A 1–2 μl sample was injected onto the GC column.

Reduction of orphenadrine N-oxide with $TiCl_3$

To 1 ml of a solution of orphenadrine N-oxide in water (100 ng/ml), or to 1 ml of plasma, were added 1 ml of 2 *M* HCl and 0.2 ml of $TiCl_3$ (15%, w/v), and the mixture was kept in a glass-stoppered tube in the dark at room temperature for 10 min. Immediately after the reaction, 100 ng of the internal standard diphenhydramine were added, and the mixture was made alkaline and extracted for quantitative analysis, as described above.

Gas chromatography

Equipment. A Hewlett-Packard gas chromatograph Type HP 5750, fitted with a sensitive nitrogen-flame ionization detector (rubidium bromide crystal) Model 14161B plus a selective detector to improve the signal to noise ratio were used[7].

Very recently we have also used the dual nitrogen–phosphorus flame ionization detector Model HP 18789 A, installed in a gas chromatograph Type HP5730A. This detector, with a new collector design, enables highly selective (it produces virtually no response to hydrocarbons) and highly sensitive detection of a wide range of compounds containing nitrogen and phosphorus atoms[8].

Column. Glass columns (1.3 and 1.5 m × 2.3 mm I.D.), packed with 3% KOH + 3% Carbowax 20 M on 100–120 mesh Gas-Chrom Q, were used.

Operating conditions. The carrier gas had a flow-rate of 30 ml/min. For optimal operation of the HP 14161B detector auxiliary helium gas (16 ml/min) had to be introduced directly into the detector system. The hydrogen flow was adjusted very carefully to 28 ± 0.5 ml/min. The air flow-rate was 200 ml/min. Oven, detector and injection port temperatures are indicated in the legends to the Figs. 1 and 3.

A high sensitivity and selectivity require optimal ionization current and, therefore, the distance between the flame and the collector containing the RbBr crystal had to be adjusted carefully[7,9]. The response of the detector varied with the cleanliness of the crystal.

The hydrogen flow-rate in the HP 18789A detector was 3 ml/min, and the air flow-rate was 50 ml/min. Voltage control was set at 16 V.

Calibration graphs and recovery. Quantitative data were derived from calibration curves, obtained by the addition to blank plasma of diphenhydramine hydrochloride as internal standard in constant concentration and orphenadrine hydrochloride in different concentrations. After extraction of the plasma as outlined above, the ratio of the GC peak areas of orphenadrine and diphenhydramine was plotted against their weight ratio. The same procedure was followed for N-demethylorphenadrine. The recovery at different concentrations was measured by extraction of plasma to which known amounts of orphenadrine hydrochloride had been added; following extraction, a known amount of diphenhydramine was added as external standard, and the ratio of the GC peak areas of the two bases was calculated and compared with those of corresponding amounts from standard solutions.

RESULTS

Fig. 1 shows typical gas chromatograms obtained by injection of part of a control human plasma extract and an extract of plasma to which, before extraction, known amounts of diphenhydramine, orphenadrine, N-demethylorphenadrine and N,N-didemethylorphenadrine had been added. The column used had 1800 plates. As can be seen from the chromatogram, there was sufficient separation between the internal standard, orphenadrine and its two metabolites.

When the method is used in pharmacokinetic studies with orphenadrine, it is of great importance to find out whether the metabolite orphenadrine N-oxide interferes with the determination of orphenadrine. It was established that orphenadrine N-oxide decomposes to orphenadrine and other products at the ambient temperature in the injection port of the gas chromatograph (unpublished results). Thus, without

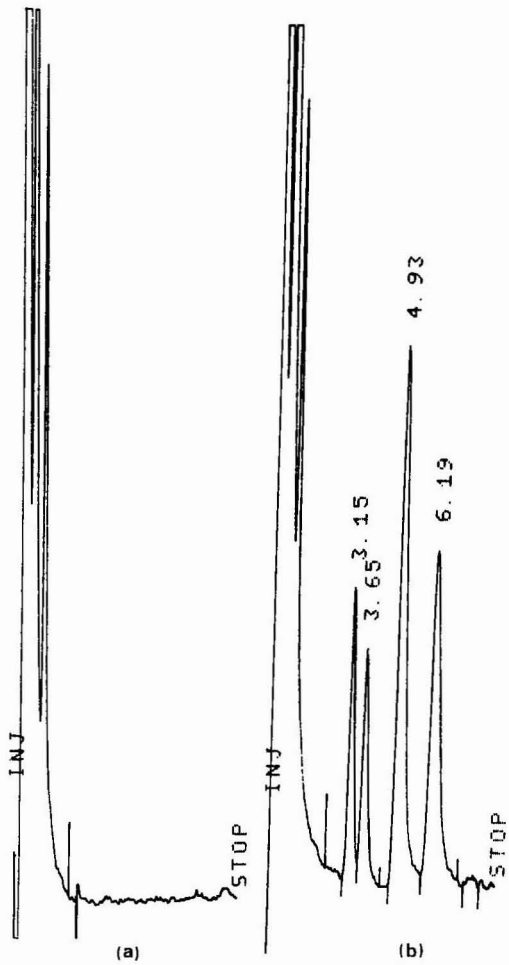

Fig. 1. Typical gas chromatograms of blank plasma extract (a) and an extract of 1 ml of plasma (b), to which 5 ng of diphenhydramine (t_R 3.15 min), 4.8 ng of orphenadrine (t_R 3.65 min), 21.5 ng of N-demethylorphenadrine (t_R 4.93 min) and 36.4 ng of N,N-didemethylorphenadrine (t_R 6.19 min) had been added. Nitrogen detector HP 18789A. Integrator HP 3380. Electrometer: $5 \cdot 10^{-12}$ A/mV. Attenuation, 1. Column length, 1.3 m. The temperatures of the oven, the detector and the injection port were 200°, 300° and 250°, respectively. Samples were injected with an automatic sampler HP 7671A; injection volume: 2 μl.

further precautions, orphenadrine N-oxide would partly be determined as orphenadrine and thus affect the specificity. By appropriate choice of extraction solvent, this risk can be avoided.

Orphenadrine N-oxide can be extracted from plasma and urine with polar organic solvents such as chloroform or, to a lesser extent, with diethyl ether. On the other hand, no detectable amounts of the N-oxide are extracted when isopentane is used as described in the isolation procedure. Even the addition of a 100-fold excess of orphenadrine N-oxide does not contribute significantly to the orphenadrine level in

the 10–500 ng/ml concentration range. Such an excess is not observed in practice (see applications), and thus isopentane is the solvent of choice.

A representative calibration graph is shown in Fig. 2. The lower detection limit, defined as the amount giving a signal three times greater than the noise at the maximal sensitivity of the nitrogen detector, is 0.05 ng orphenadrine or *ca.* 1 ng in 1 ml of plasma (see Fig. 1). The method has a good precision. The standard deviations of the analysis are $\pm 6\%$ ($n = 4$) in the concentration range 10–200 ng/ml of plasma. The average total recovery of orphenadrine from human plasma in the 20–200 ng/ml concentration range is 77%, with a standard deviation *ca.* 5% ($n = 5$). At lower concentrations (down to 1 ng/ml), recovery is lower (*ca.* 60%).

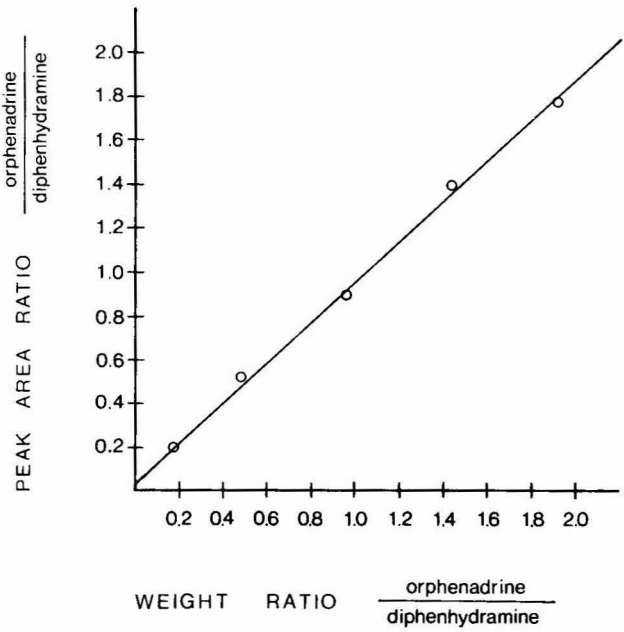

Fig. 2. Standard curve for the determination of orphenadrine concentrations.

Applications

The applicability of the analytical method was demonstrated by measuring as a function of time the concentrations in plasma from a volunteer given a single oral dose of 100 mg of orphenadrine. Fig. 3 shows a gas chromatogram of plasma extracted 24 h after administration. Blood was collected in heparinized tubes, and centrifuged at 2500 *g*. The samples were separated and frozen until analysis.

Fig. 4 shows the variation with time of the orphenadrine and N-demethylorphenadrine plasma concentrations. There appears to be a considerable rise in orphenadrine concentration *ca.* 1 h after administration, indicating rapid absorption. The linear time course on a log scale during the elimination phase (monitored until 48 h after dosage) indicates a first-order kinetic process. In this example, the biological half-life was calculated as *ca.* 16 h, consistent with the results of radio-tracer studies in man[3]. N-Demethylorphenadrine has a longer half-life. When orphenadrine N-

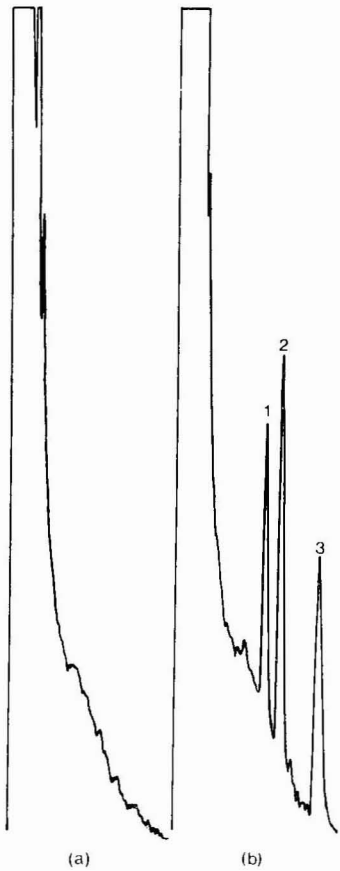

Fig. 3. Gas chromatogram of a control plasma extract (a) and a plasma extract from a volunteer's blood 24 h after treatment with 100 mg of orphenadrine hydrochloride (b). Peak 1, diphenhydramine (internal standard); peak 2, orphenadrine; peak 3, N-demethylorphenadrine. N,N-didemethylorphenadrine was not found. Nitrogen detector: HP 15161B. Electrometer: 10^{-11} A/mV. Attenuation, 4. Column length, 1.5 m. The temperatures of the oven, detector and injection port were 180°, 400° and 220°, respectively. Recorder chart, 0.5 cm/min.

oxide is added to human plasma, it is reduced quantitatively to orphenadrine within 10 min by $TiCl_3$ in hydrochloric acid, a medium in which orphenadrine itself is not converted to a measurable degree.

In various plasma samples, including some from patients treated with a daily 300-mg oral dose of orphenadrine over periods up to a few years, reduction with $TiCl_3$ never increased the orphenadrine concentration by more than 7%. Since a 100-fold excess of N-oxide (added to plasma) failed to increase the response of orphenadrine significantly on application of our isolation procedure, it can be concluded that the amount of N-oxide present in the plasma as a metabolite does not interfere with the determination of orphenadrine concentrations.

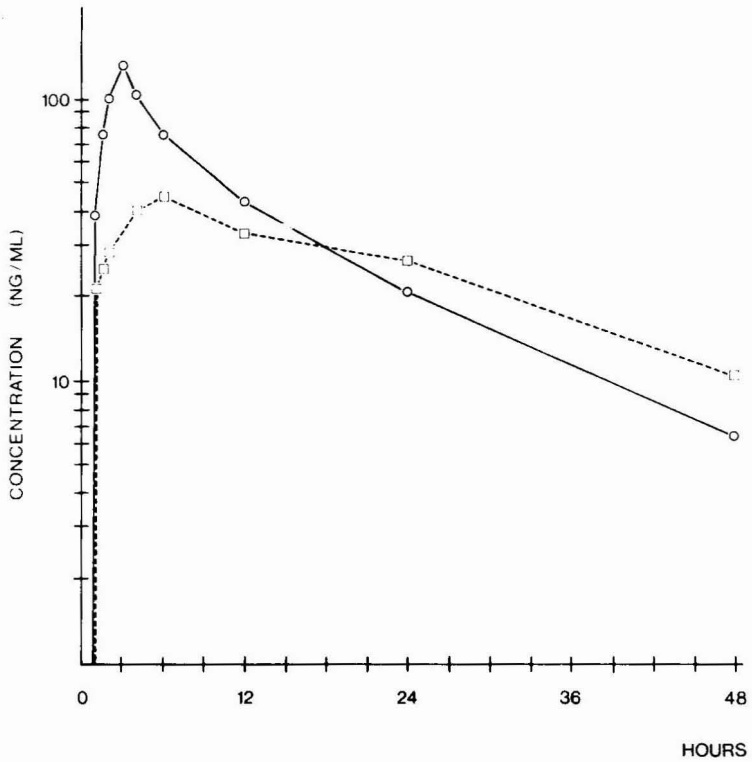

Fig. 4. Plasma concentration–time curves of orphenadrine (○) and N-demethylorphenadrine (□) after a single oral dose of orphenadrine (100 mg as a tablet) to a healthy volunteer.

DISCUSSION

As Fig. 4 shows, the maximal plasma concentration after an oral 100 mg dose of orphenadrine (in clinical practice, such a dose is applied for multimedication) is *ca.* 150 ng/ml. For a study of the pharmacokinetic profile of orphenadrine the concentration should be followed for at least three times the half-life. This implies accurate measurement in the 5–150 ng range, which is made possible by an isolation method based on extraction with isopentane and sensitive nitrogen detection after separation on a KOH–Carbowax column. The method is selective and specific enough to separate orphenadrine from its N-dealkylated metabolites and its N-oxide*.

Although with normal flame ionization it is possible to detect concentrations down to 5 ng/ml, the technique requires a thorough clean-up procedure to eliminate contaminants from blood plasma, extraction solvents, glassware, and rubber stoppers as supplied together with vacutainer tubes. When a nitrogen detector is used, re-extraction at pH 3 and washing with isopentane (it is essential that all the washing liquid should be removed) suffices. Interfering peaks in the gas chromatogram are not

* Other metabolites containing nitrogen and having similar chromatographic properties were not found, in agreement with current knowledge about metabolic pathways.

observed, and the time elapsing between two individual injections can be limited to *ca.* 10 min. The method is, therefore, suitable for routine purposes.

An important factor that may influence the reliability and accuracy of the method, especially at low concentrations, is adsorption of orphenadrine onto the glass walls. Such adsorption can be reduced considerably by careful rinsing of the inner wall of the glass centrifuge tube with ethanol just before the final isopentane extract is introduced.

In this manner, an acceptable recovery (50–60%) is possible even at the lowest concentrations. Adsorption on the column can be minimized by periodic injection of some extra base (*e.g.* 1 μg). It is recommended that solutions in water, plasma, alcohol, etc., should be stored for the shortest possible time, as loss on standing due to adsorption cannot be avoided. The above considerations also hold for N-demethylorphenadrine if determined quantitatively, alone or simultaneously with orphenadrine.

CONCLUSION

The method reported is suitable for routine determinations of low orphenadrine concentrations in human plasma and urine. It separates orphenadrine from its N-dealkylated metabolites and its N-oxide, thus providing a useful tool in pharmacokinetic and bio-availability experiments.

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IDENTIFICATION OF HYDROXY ACIDS BY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

In an aqueous solution of lactone-forming acids, several forms of the acids exist, *e.g.* lactone forms and open-chain forms. The different forms of such acids were separated as trimethylsilyl (TMS) derivatives by gas–liquid chromatography (GLC) on OV-17. At pH 10 the lactone-forming acids used in this investigation were entirely converted in the open-chain form, and under these conditions each acid gave one peak by GLC of the corresponding TMS derivative. Acids derived from carbohydrates were reduced in mixtures with other acids to their corresponding alditols and separated as acetates by GLC on OV-225.

The following acids can be separated by the above-mentioned methods: citric, erythronic, D-galacturonic, D-glucaric, DL-isocitric, DL-malic, D-ribonic, and *meso*-tartaric acids.

INTRODUCTION

A mixture of acids derived from plants may contain different types of lactone-forming hydroxy acids: lactone-forming acids with few hydroxy groups, *e.g.* isocitric acid[1], or acids of carbohydrate derivation, *e.g.* D-glucaric[2], D-galactaric[3] and D-galacturonic acid[4].

Gas–liquid chromatography (GLC) of the open-chain trimethylsilyl (TMS) derivatives is a convenient method for the separation and identification of lactone-forming acids[5,6]. It has been shown that silylation of the lactonized acids, followed by GLC, often gives multiple peaks on the gas chromatogram[7].

Co-chromatography with reference substances is necessary to ensure a reliable identification. Most acids of carbohydrate derivation are difficult to obtain, but a number of alditols are readily available. The configuration of the acids can be confirmed by reduction to the corresponding alditols which, after derivatization, can be analysed by GLC[8,9]. The differences between the mass spectra of the diastereomeric acids of carbohydrate derivation are due to differences in the relative intensities of their peaks[10,11]. Thus identification of such acids by use of gas chromatography–mass spectrometry (GC–MS) depends on the availability of reference compounds.

In an investigation of lactone-forming acids in succulent plants[12] the identifi-

cation of such acids was greatly influenced by the methods for separation of the acid mixtures, and the availability of reference substances. The results presented here show that different methods of derivatization, combined with different conditions for the GLC analysis, give information that is of great importance for the identification of different types of hydroxy acid in mixtures, especially when reference acids are not available. All methods for GLC separation described in this paper can be applied to GC–MS analysis. Two common plant acids, malic and citric acid, were included in the investigation, which was carried out with pure acids.

EXPERIMENTAL

Chemicals

The following substances were derivatizated and chromatographed: citric acid (Merck, Darmstadt, G.F.R.), erythronolactone (supplied by Dr. F. Wold, University of Illinois, Urbana, Ill., U.S.A.), D-galacturonic acid monohydrate (Schuchardt, München, G.F.R.), DL-isocitric acid lactone (Type III, Sigma, St. Louis, Mo., U.S.A.), DL-malic acid (BDH, Poole, Great Britain), D-ribonolactone (Sigma), D-glucaric acid 1,4-lactone (Sigma) and *meso*-tartaric acid monohydrate (Fluka, Buchs, Switzerland).

The silylation was performed with trimethylchlorosilane (pure, Koch-Light, Colnbrook, Great Britain) and 1,1,1,3,3,3-hexamethyldisilazane (Merck) and pyridine (Merck) which, before use, was distilled and kept over pellets of sodium hydroxide (Elektrokemiska Aktiebolaget, Bohus, Sweden).

The chemicals used for the preparation of the alcohol acetates were: Dowex 50W-X8 (H^+), 20–50 mesh (Fluka), pretreated and kept in anhydrous methanol (Merck)[13], sodium borohydride (for synthesis, Merck–Schuchardt), The acetic acid, acetic anhydride and chloroform were all from Merck and of analytical reagent grade.

Apparatus

A Varian 1400 gas chromatograph with a flame-ionization detector was used. Of the two columns applied, one column (glass coil, 3 m × 2 mm I.D.) was filled with 10% OV-17 on Gas-Chrom Q (80–100 mesh), the other (glass coil, 2 m × 2 mm I.D.) with 3% OV-225 on Varaport 30 (100–120 mesh). The OV-17, the Gas-Chrom Q and the Varaport 30 were purchased from Supelco (Bellefonte, Pa., U.S.A.), while the OV-225 was from Applied Science Labs. (State College, Pa., U.S.A.). The detector temperature was 300° for the OV-17 column and 280° for the OV-225 column. The injector temperature was 250°, and the carrier gas (nitrogen) was used at a flow-rate of 40 ml/min. The temperature was programmed from 120° at a rate of 1°/min (OV-17), and from 150° at a rate of 2°/min (OV-225).

The retention times were recorded by an Autolab minigrator (Spectra Physics) and the chromatograms printed out by an OmniScribe recorder (Houston Instruments).

Acid mixture

Samples of 5 mg of each acid were dissolved in water (40 ml) and kept at room temperature for 3 days. Aliquots of 10 ml of this solution were used for derivatization.

Sodium salts

The acid (10 mg) was dissolved in water (10 ml) and sodium hydroxide (0.1 *N*) added until pH 10 was reached[5]. The neutralized solution was heated for 30 min at 60° and the procedure repeated until pH remained at 10. The acid mixture was treated in the same way.

TMS derivatives

The TMS derivatives were prepared directly from each reference acid, from the dried acid mixture, from the sodium salt of each acid and from the sodium salts prepared from the acid mixture. The silylation reagent was the same as that used by Raunhardt *et al.*[7].

Reduction of the acids

Each acid (10 mg) and the dried mixture were separately esterified with methanol and Dowex 50W-X8 (H^+)[2]. The methyl esters, dissolved in water (10 ml), were reduced by adding sodium borohydride (10 mg) twice, at an interval of 2 h.

After a total reduction time of 20 h, the excess of borohydride was neutralized with acetic acid (8 *M*) and removed by repeated evaporation following addition of acetic acid (0.8 *M* in methanol). Finally the alcohols were dissolved in anhydrous methanol and evaporated to dryness.

Acetylation of the reduced acids

To the dried alcohols acetic anhydride (1 ml) was added and the mixture heated for 1 h at 100°. After removal of excess acetic anhydride by evaporation, the acetates were dissolved in water (2 ml) and extracted with chloroform (2 ml). Prior to GLC analysis the acetates were dissolved in methanol.

RESULTS AND DISCUSSION

The GC retention data of the substances, after different methods of derivatization, are listed in Table I. The retention times were always measured relative to the corresponding derivative of the *meso*-tartaric acid. Typical chromatograms of the different derivatives of the acid mixture are shown in Figs. 1—3.

TMS derivatives

The lactone-forming acids usually exist in aqueous solution as an equilibrium between the different forms. In most cases, evaporation and desiccation of a solution of a lactone-forming acid does not lead to complete lactonization of the acid, hence several peaks appear on the gas chromatogram (Fig. 1). For all of the lactone-forming acids used in this investigation, adjustment to pH 10 was sufficient to convert the acid entirely into the open-chain form (Fig. 2). Under the conditions used two peaks were obtained for galacturonic acid, originating from the α- and β-anomeric forms.

The retention time for the open-chain TMS derivative is shorter than for the corresponding derivative of the same acid in lactone form. Acids that cannot form lactones always give peaks with the same retention times. A comparison between the gas chromatogram of the TMS derivatives directly prepared from an unknown acid mixture and the chromatogram of the TMS derivatives prepared from the sodium

TABLE I

RETENTION TIMES OF THE DIFFERENT ACID DERIVATIVES RELATIVE TO THE CORRESPONDING DERIVATIVES OF *MESO*-TARTARIC ACID

For the chromatographic conditions, see under *Apparatus*.

Substances	*TMS derivatives of the substances chromatographed on the OV-17 column*	*Acetates prepared of the reduced substances chromatographed on the OV 225 column*
Citric acid	1.83	2.13
Erythronic acid	0.78	1.00
Erythronolactone	0.86	1.00
α-Galacturonic acid	2.26	2.31
β-Galacturonic acid	2.39	2.31
Isocitric acid	1.90	2.13
Isocitric acid lactone	1.94	2.13
Malic acid	0.78	0.48
Ribonic acid	1.37	2.01
Ribonolactone	1.45	2.01
Glucaric acid	2.19	3.22
Glucaric acid 1,4-lactone	2.33	3.22
meso-Tartaric acid	1.00	1.00

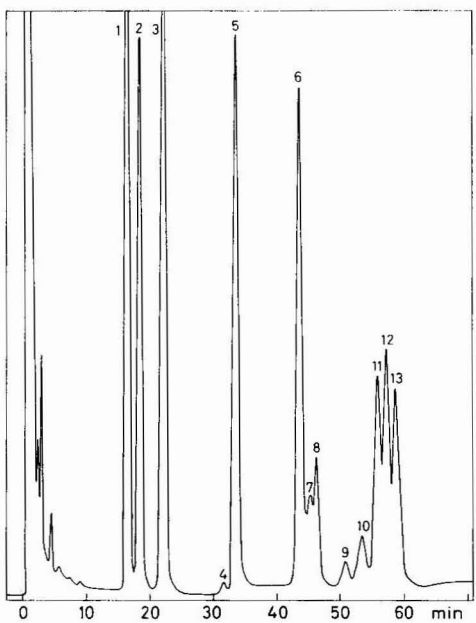

Fig. 1. Chromatogram of the TMS derivatives of the acid mixture. Column: OV-17. Peaks: 1, malic acid; 2, erythronolactone; 3, *meso*-tartaric acid; 4, ribonolactone; 5, ribonic acid; 6, citric acid; 7, isocitric acid; 8, isocitric acid lactone; 9, unknown; 10, glucaric acid; 11, α-galacturonic acid; 12, glucaric acid 1,4-lactone; 13, β-galacturonic acid.

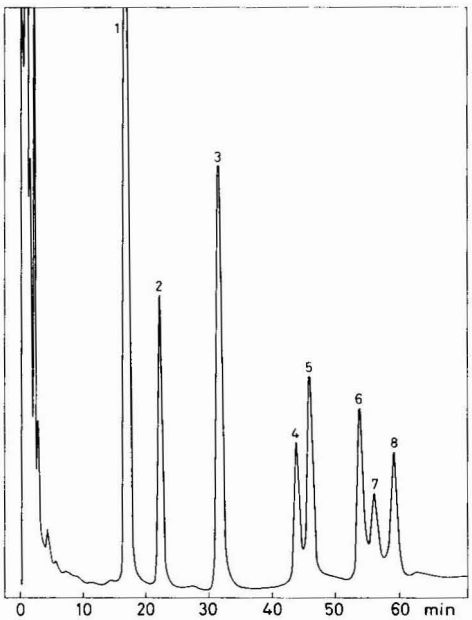

Fig. 2. Chromatogram of the TMS derivatives prepared from the sodium salts of the acid mixture. Column: OV-17. Peaks: 1, erythronic acid, malic acid; 2, *meso*-tartaric acid; 3, ribonic acid; 4, citric acid; 5, isocitric acid; 6, glucaric acid; 7, α-galacturonic acid; 8, β-galacturonic acid.

salts of the same mixture, provides an important basis for identification of the individual acids (Figs. 1 and 2). In this way not only could the lactone-forming acids be located on the chromatograms, but also the number of the different lactones could be evaluated.

The mass spectra of the TMS derivatives gave additional information. For the acids derived from carbohydrates, the type of acid (-uronic, -onic, -aric) and the number of carbon atoms were readily established[10,11,14].

Acetates of the reduced acids

The configurations of the acids derived from carbohydrates were confirmed by GLC of the acetates of the reduced acids. Many alditols, not commercially available, can easily be prepared by reduction of the corresponding aldoses and can be used as reference compounds.

The acetate of the reduced malic acid gave a single peak whereas the corresponding derivatives of citric and isocitric acid gave a number of small peaks together with the major ones (Fig. 3). The two last-mentioned acids were not separated under the chromatographic conditions used. As expected, erythronolactone and *meso*-tartaric acid gave peaks with the same retention time. Like the TMS derivatives, the acetates can be identified by means of GC–MS.

These results show that the acids derived from carbohydrates can be reduced to alditols and chromatographed as acetates in the presence of normally occurring plant acids.

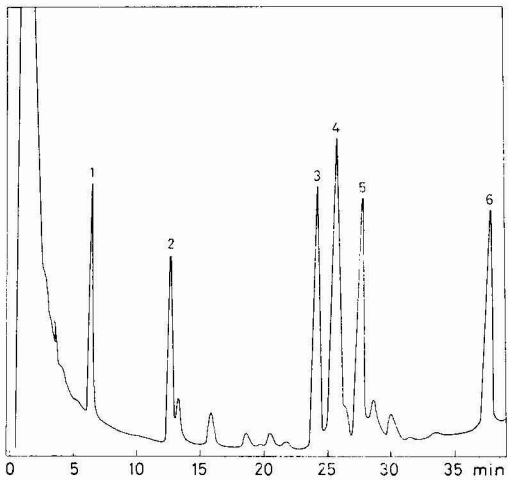

Fig. 3. Chromatogram of the acetates of the reduced acid mixture. Column: OV-225. Peaks: 1, malic acid; 2, *meso*-tartaric acid (= erythritol), erythronic acid (= erythritol); 3, ribonic acid (= ribitol); 4, citric acid, isocitric acid; 5, galacturonic acid (= galactitol); 6, glucaric acid (= glucitol). Most unnumbered peaks are due to citric and isocitric acid.

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GAS–LIQUID CHROMATOGRAPHIC DETERMINATION OF CLONIDINE AND SOME ANALOGUES IN RAT BRAIN TISSUE

BRAIN CONCENTRATIONS AND HYPOTENSIVE ACTIVITY

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SUMMARY

A simple and sensitive gas–liquid chromatographic method has been developed for the quantitative determination of clonidine and some structurally related imidazolidines in rat brain tissue. The aqueous brain homogenates are first purified and then extracted into benzene. Samples are injected directly into the gas chromatograph. The extraction procedure is selective, and the use of a phosphorus–nitrogen detector enables accurate determinations corresponding to brain concentrations down to at least 10 ng/g. The rat brain concentrations of clonidine and its derivatives achieved at the moment of maximal decrease in arterial pressure are proportional to the doses administered intravenously, and are not influenced by the effect of the compounds on the blood pressure or by the method of anaesthesia employed. It is concluded that, for the linear part of the dose–response curves for these compounds, the brain concentration is a measure of the hypotensive effect.

INTRODUCTION

The antihypertensive drug clonidine (Catapresan®; 2-[2,6-dichlorophenylimino]imidazolidine hydrochloride) manifests its action via a central mechanism. This central hypotensive effect is presumably brought about by the excitation of central α-adrenergic receptors located at medullary sites[1–3]. The therapeutic action of clonidine is exerted after doses in the microgram range and, consequently, plasma concentrations and tissue contents are low. It has been possible to measure plasma and tissue levels after the administration of rather high doses of radiolabelled clonidine to humans and animals[4–7], by gas–liquid chromatography (GLC)[8–10] and by GLC–mass spectrometry (MS)[11]. These reports mainly aimed at a study of the fundamental pharmacokinetics of clonidine, and the GLC methods used were rather elaborate. Hitherto, the relation between the brain concentration and depressor effect of clonidine has been ignored, in spite of the fact that the therapeutic (hypotensive) action of this drug is of central nervous origin.

The present paper describes a convenient sensitive GLC method for the quantitative determination of clonidine and some of its structurally related imidazolidines in rat brain tissue. The compounds were administered intravenously, and brain concentrations were established at the moment of maximal decrease in blood pressure, in order to relate them directly to the hypotensive effect.

MATERIALS AND METHODS

Administration of the drugs; extraction from brain tissue

Male Wistar rats (weight 190–220 g) were anaesthetized with diethyl ether and surgically prepared in order to allow artificial respiration, injection of drugs via a jugular vein and measurement of arterial pressure via a carotid artery. Pressure was recorded via a Statham P23 Db transducer connected to a Hellige HE-19 recorder. During the operation, and throughout the experiment, the animal was kept under anaesthesia by continuous administration of diethyl ether ($6.1 \pm 0.2\%$, v/v, $n = 41$; for determination see *Gas–liquid chromatography*) to the inspiration air by means of a "Vapor" evaporator device (Drägerwerk, Lübeck, G.F.R.). After an equilibrium period of *ca.* 20 min, the blood pressure and heart rate had usually reached a constant level.

The imidazolidine of which the brain content was to be established was injected intravenously at a dose listed in Table I. At a preselected time after administration (see Table I), the animal was decapitated and the entire brain was removed immediately. The tissue was rinsed in saline to remove blood, gently blotted on filter paper and weighed. The brain was then homogenized in an aqueous solution of 0.02 *N* hydrochloric acid. The homogenate was transferred quantitatively to a 50-ml stoppered glass centrifuge tube and the volume was made up to 20 ml with 0.02 *N* HCl. Sodium chloride (1 g), benzene (10 ml) and ethyl acetate (10 ml, containing the internal standard) were then added. The tube was stoppered and shaken by hand for 5 min. After centrifugation for 10 min at 1000 *g*, the clear aqueous layer was isolated and again shaken with benzene (20 ml) for 5 min followed by centrifugation (10 min, 1000 *g*). 1 ml of an aqueous solution of 1 *N* sodium hydroxide was added to the isolated aqueous layer which was then extracted with benzene (20 ml). After centrifugation (10 min, 1000 *g*), the organic phase was pipetted off and evaporated to dryness under reduced pressure in a 10-ml conical glass tube. The residue was dissolved in 100 μl of benzene, and 1–10-μl samples were analysed by GLC.

Standards

Standard brain extracts were prepared by homogenation of the entire brains of untreated control rats in 0.02 *N* hydrochloric acid containing a known amount of the particular imidazolidine hydrochloride. The standards were then treated as described above.

Gas–liquid chromatography

Diethyl ether in the inspiration air was determined on a Packard Series 149 Becker gas chromatograph equipped with a flame ionization detector (temperature, 180°; hydrogen flow-rate, 30 ml/min; air flow-rate, 300 ml/min) and 1-mV Kipp

TABLE I

BRAIN CONCENTRATIONS (MEAN ± S.E.) OF TZ-1, TZ-13 AND TZ-18 AFTER INTRAVENOUS ADMINISTRATION TO ETHER-ANAESTHETIZED RATS

Assays of brain contents were performed at the moment of maximal decrease in blood pressure (t). The results for TZ-18 obtained under pentobarbital anaesthesia (see text) are marked with an asterisk (*).

Imidazolidine derivative	*Dose (μg/kg)*	*Number of determinations*	*t (min)*	*Brain concentration (ng/g)*
TZ-1	10	4	8	18.0 ± 2.6
(Clonidine)	20	4	10	39.7 ± 4.8
	35	4	15	64.2 ± 4.9
	50	3	20	91.5 ± 2.8
TZ-13	50	4	10	10.6 ± 2.0
(2-Cl,4-Me)	100	5	15	22.2 ± 2.1
	200	4	20	44.6 ± 4.2
TZ-18	20	3	4	60.6 ± 8.9
(2,4,6-tri-Cl)	20	3	4	57.1 ± 6.1*
	45	4	6	131 ± 7
	70	3	8	198 ± 12
	100	3	10	298 ± 8
	100	3	10	293 ± 10*

recorder. A glass column (2 m × 2 mm I.D.) packed with Porapak Q (80–100 mesh) was used at an oven temperature of 160° and an injector temperature of 180°. The carrier gas was nitrogen; flow-rate, 30 ml/min. Samples of inspiration air were injected directly. The gas chromatograms were evaluated by an integrating system from Spectra-Physics (Autolab system IV). The diethyl ether concentration (%, v/v) was determined by comparison with a standard solution in isooctane.

Extracts of rat-brain homogenates were analyzed on a Perkin-Elmer Series 3920 gas chromatograph equipped with a phosphorus–nitrogen detector (PND) and a 1-mV Kipp recorder. The setting and optimizing of the detector for this particular analytical problem was performed according to the procedure outlined by Kolb *et al.*[12] and by Hartigan *et al.*[13], which resulted in the following operation parameters: temperature, 280°; hydrogen flow-rate, 2 ml/min; air flow-rate, 100 ml/min. A glass column (2 m × 2 mm I.D.) packed with 3% OV-17 on Chromosorb 750 (80–100 mesh) was used at an oven temperature of 200–270° (depending on the derivative to be analyzed) and an injector temperature of 280°. The carrier gas was helium; flow-rate, 30 ml/min. Brain extracts obtained as described above were injected in benzene. The gas chromatograms were evaluated by an integrating system from Spectra-Physics (Autolab system IV).

Chemicals and drugs used

Chromosorb 750 (80–100 mesh) (Chrompack, Middelburg, The Netherlands); OV-17 (Packard, Delft, The Netherlands); Porapack Q (80–100 mesh) (Waters Assoc., Milford, Mass., U.S.A.); sodium chloride (Merck, Darmstadt, G.F.R.), benzene (Merck), diethyl ether (Brocacef, Maarssen, The Netherlands) and ethyl acetate (Merck) were distilled through a 100-cm Vigreux column before use. Clonidine

(Catapresan®; Boehringer, Ingelheim, G.F.R.), indicated by the code TZ-1; 2-(2,3-dichlorophenylimino)imidazolidine free base (TZ-9), 2-(2-chloro-4-methylphenylimino)imidazolidine hydrochloride (TZ-13), 2-(2,4,6-trichlorophenylimino)imidazolidine hydrochloride (TZ-18) and 2-(4-bromo-2,6-dichlorophenylimino)imidazolidine free base (TZ-21) were obtained by synthesis[14].

RESULTS

Standard curves were constructed by analyzing benzene samples to which the free bases of clonidine (TZ-1; 2,6-di-Cl), TZ-13 (2-Cl,4-Me) and TZ-18 (2,4,6-tri-Cl) had been added in different amounts. These three compounds were selected because they represent the major classes of phenyl-substituted imidazolidines. The standard curves passed through the origin and were linear up to at least 500 ng of the derivative injected. This finding indicated that there were no adsorption losses on the column and also demonstrated the linearity of the recording with the PND. Standard curves were also prepared from brain homogenates to which known amounts of the hydrochlorides of these three substances (25–400 ng) had been added. The internal standards employed were 2-(arylimino)imidazolidine bases selected on account of their retention times in order to ensure a convenient peak separation. TZ-9 (2,3-di-Cl) was used as internal standard for the determination of clonidine and TZ-13, and TZ-21 (2,6-di-Cl, 4-Br) was used as the internal standard for TZ-18.

The GLC peaks of these derivatives after extraction from brain homogenates were well-defined; no interfering peaks were observed when comparing the chromatograms with those obtained from extracts of blank homogenates. The reproducibility of the determinations was characterized by a relative standard deviation of *ca.* 3% when injecting the same sample repeatedly. The standard curve of clonidine is illustrated in Fig. 1. The relation between the relative peak area and the amount of clonidine added to rat brain homogenates is linear up to at least 400 ng. The same holds true for the curves of TZ-13 and TZ-18. This indicates that the extraction procedure is free from disturbances.

The concentration of the same three imidazolidine derivatives achieved in rat brain tissue after intravenous administration of different amounts was established. The procedure prior to isolation of the brain (see Materials and methods) was identical to that followed in order to quantify the hypotensive effect of these particular compounds[15]. These experiments were carried out on anaesthetized rats. However, due to the interference of the pentobarbital peak in the chromatograms, diethyl ether (*ca.* 6%, v/v; for determination see Materials and methods) was employed as the anaesthetic instead of pentobarbital. The doses chosen for the intravenous administration of the imidazolidines spanned the range of the dose–response characteristics corresponding to the hypotensive effect[15]. In order to relate the brain concentration directly to the hypotensive effect of the substances, the moment of maximal decrease in blood pressure was taken as the time at which these assays of brain content were performed. The particular preselected times were deduced from the response–time curves, established separately[15].

Clonidine (TZ-1; 10, 20, 35 and 50 μg/kg), TZ-13 (50, 100 and 200 μg/kg) and TZ-18 (20, 45, 70 and 100 μg/kg) were injected intravenously into rats and the brain contents of these derivatives, reached at the moment of maximal decrease in blood

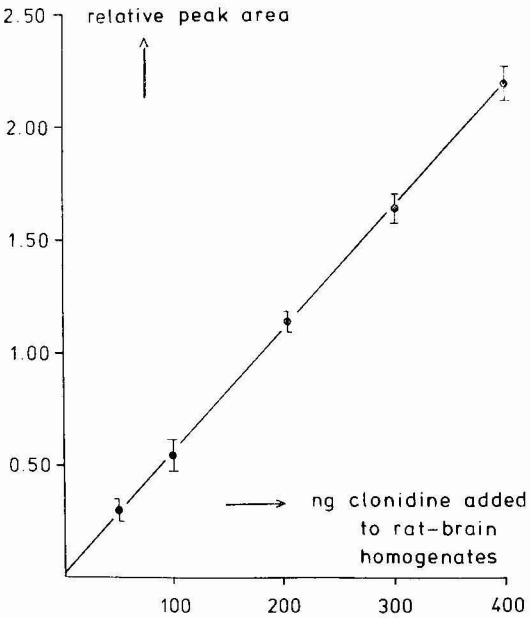

Fig. 1. Standard curve of clonidine. The compound was added in different amounts to rat brain homogenates with TZ-9 as an internal standard. Each point represents the mean value (± S.E.) from four experiments.

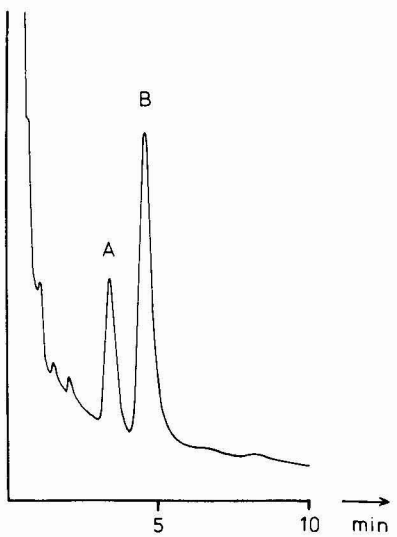

Fig. 2. Typical gas–liquid chromatogram obtained from an extract of rat brain homogenate. Peaks: A = clonidine; B = TZ-9 as an internal standard. The animal had received 10 μg/kg clonidine intravenously and the entire brain was removed 8 min after injection. The relative peak area of clonidine corresponded to 17.5 ng/g brain tissue (*cf.* Table I for the mean value from four experiments).

pressure, were established. The results are listed in Table I and are expressed as ng per g of brain tissue, calculated with the aid of the standard curves. Fig. 2 shows a typical gas–liquid chromatogram obtained from an extract of a brain homogenate of a rat which had received 10 μg/kg of clonidine (TZ-1) intravenously. This figure illustrates the selectivity of the extraction procedure and the sensitivity of the PND.

As can be seen from Table I, the method offers the possibility of the accurate determination of imidazolidines from extracts of brain homogenates corresponding to brain concentration levels down to at least *ca.* 10 ng/g. In principle, concentrations ten times lower than this should be easily detectable.

The brain concentrations of clonidine (TZ-1), TZ-13 and TZ-18, listed in Table I, are plotted in Fig. 3 against their corresponding intravenously administered doses. The curves pass through the origin and are linear. It may be concluded that the brain concentration reached at the moment of maximal decrease in blood pressure is proportional to the dose administered intravenously.

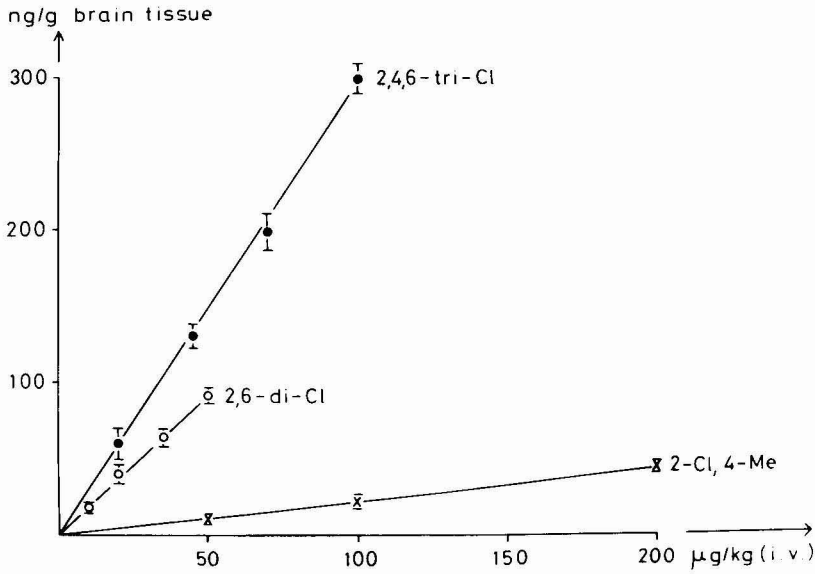

Fig. 3. Relation between brain concentration (ng/g, mean ± S.E.) of ether-anaesthetized rats and dose (μg/kg) administered intravenously for clonidine (2,6-di-Cl), TZ-13 (2-Cl,4-Me) and TZ-18 (2,4,6-tri-Cl). Brain contents were established at the moment of maximal decrease in arterial pressure. For numerical values see Table I.

The effects on the blood pressure of ether-anaesthetized rats after intravenous administration of clonidine and the two derivatives were considerably less than the responses measured when pentobarbital was used as the anaesthetic*. Thus the question arose as to whether the use of this volatile compound influences the disposition of the imidazolidines in the brain. This was investigated for TZ-18 which possesses a sufficiently long GLC retention time to enable an accurate determination of the

* It is outside the scope of this paper to go into a detailed discussion of these results, which are the subject of forthcoming investigations.

brain content without interference from pentobarbital in the chromatogram. In addition to the experiments described above, TZ-18 was given intravenously to rats anaesthetized with pentobarbital (75 mg/kg, intraperitoneal) at doses of 20 and 100 μg/kg. The brain concentrations of TZ-18 at the moment of maximal decrease in blood pressure are reported in Table I (marked with an asterisk), and are compared with the results obtained previously (ether anaesthesia) in Fig. 4. The results clearly demonstrate that the brain concentrations achieved by TZ-18 were virtually the same in ether and pentobarbital-anaesthetized rats. Furthermore, these data indicate that the results obtained with diethyl ether are directly comparable to those which would have been encountered if pentobarbital had been used.

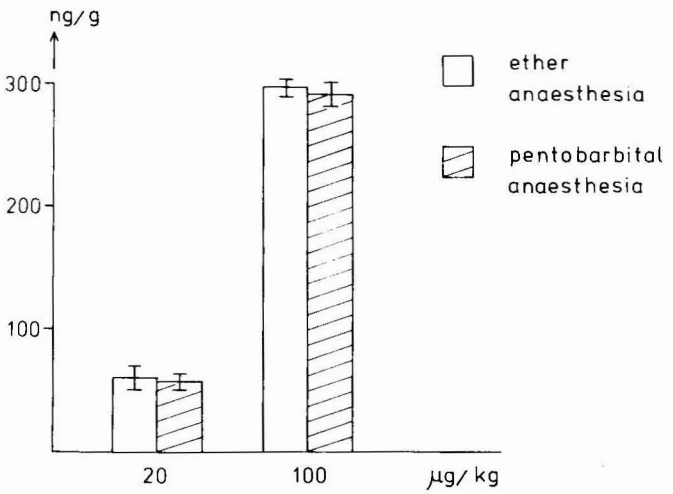

Fig. 4. Brain concentrations (ng/g, mean ± S.E.) of TZ-18 after intravenous application of 20 and 100 μg/kg to rats under pentobarbital (75 mg/kg, intraperitoneal) or ether anaesthesia (*ca.* 6%, v/v). Assays of brain contents were performed at the moment of maximal decrease in arterial pressure. For numerical values see Table I.

DISCUSSION

Apart from analytical procedures, which enabled radiolabelled clonidine to be determined directly from tissue and plasma[4–7], only a few GLC methods have been reported for the evaluation of clonidine. Cho and Curry[8] employed electron-capture GLC (GLC–ECD) after extraction. However, this method is not sufficiently sensitive to permit accurate measurements and tailed peaks are observed on different stationary phases. Concentrations of clonidine in plasma have been determined by selective ion monitoring after GLC–MS[11]. A GLC–ECD method has also been developed for the monotrifluoroacetyl derivative of clonidine[9]. However, in spite of the high sensitivity, this procedure is elaborate and impurities in the internal standard are also detected. Recently, Edlund and Paalzow[10] described a GLC–ECD procedure for clonidine in plasma, in which the drug was assayed as the pentafluorobenzyl derivative. This method is highly sensitive for clonidine, but is probably not generally applicable to the determination of related derivatives.

The procedure reported in the present paper is simple, the extraction from

brain homogenates is selective and the use of the PND enables accurate determinations of brain concentrations down to 10 ng/g.

The concentrations of TZ-1, TZ-13 (2-Cl, 4-Me) and TZ-18 (2,4,6-tri-Cl) in rat brain at the moment of maximal decrease in blood pressure were found to be proportional to the dose administered intravenously (Fig. 3). It therefore seems that the brain concentrations are not influenced by the effect of the compounds on the blood pressure. In addition, these results illustrate the importance of lipophilicity as a measure of the tendency of the three substances to penetrate into the brain. The lipophilicity, as determined by the partition coefficients (log P') between octanol and buffer (pH = 7.4), decreases from TZ-18 (log $P' = 1.47$) via clonidine (log $P' = 0.62$) to TZ-13 (log $P' = -0.48$) and is paralleled by the decrease in the slopes of the lines in Fig. 3. Approximately 1.4% of the amount of clonidine administered intravenously is found in the brain, compared with *ca.* 2.3% for the more lipophilic TZ-18 and only 0.2% of the hydrophilic TZ-13. The use of diethyl ether as the anaesthetic instead of pentobarbital did not affect the brain content, as demonstrated for TZ-18.

The hypotensive effects of clonidine, TZ-13 and TZ-18 were quantified with the aid of dose–response curves following intravenous application to pentobarbital-anaesthetized rats[15]. The depressor activity of clonidine is very pronounced, whereas TZ-13 and TZ-18 are less potent. The dose–response characteristics have a sigmoid shape. For the linear part of the curves the logarithm of the dose administered intravenously is directly proportional to the depressor response. A linear relation also exists between the brain concentration and dose. Consequently, we submit that, for the linear part of the dose–response characteristics of clonidine and its derivatives, the logarithm of the brain concentration, assayed at the moment of maximal decrease in blood pressure, is directly related to the hypotensive effect.

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SPECIFIC THIN-LAYER CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF QUINIDINE IN BIOLOGICAL FLUIDS

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SUMMARY

A sensitive, accurate and specific spectrodensitometric method has been developed for the determination of quinidine in biological fluids. It involves extraction of quinidine, dihydroquinidine and metabolites, their separation on thin layers and quantitation of the corresponding spots by direct scanning in a densitometer at 278 nm. A linear relationship was obtained between the ratio of the peak area of an unknown sample to that of the standard and the concentration of the compounds at 0.4–4 μg/ml. The recovery from plasma was from 96 to 103% for quinidine and from 93.5 to 98.5% for dihydroquinidine. A comparison was made between this thin-layer chromatographic method and the fluorimetric assay frequently used for the determination of quinidine in plasma at present. The method is recommended for clinical assays and pharmacokinetics studies.

INTRODUCTION

Variations in the plasma levels after oral and intravenous administration of quinidine, an effective atrial and ventricular cardiac antiarrhythmic agent, have prompted the Food and Drug Administration to require evidence of drug bioavailability for all marketed oral quinidine pharmaceutical preparations[1].

The reasons for substantial differences in bioavailability of quinidine are not quite certain. Several studies have indicated that variations in both rate and the extent of quinidine absorption from the gastrointestinal tract may be responsible for the differences in quinidine plasma levels in subjects ingesting equivalent oral doses of this drug. Another contributing factor may be weaknesses in the analytical procedures used to evaluate quinidine plasma levels, many of which are neither specific nor sensitive enough for the low concentrations observed.

Most of the analytical methods make use of the ability of quinidine to fluoresce in acid and involve either precipitation of plasma proteins and direct fluorimetric détermination of the filtrate[2,3] or extraction of the drug into an organic solvent and fluorimetric determination after acidification or transfer to sulfuric acid[4–7]. A photofluorimetric method of this type[8] is the most frequently used assay for the determination of quinidine in plasma at present. In this case the drug is extracted from plasma

with benzene and from the benzene into sulfuric acid. Fluorescence of the sulfuric acid solution is then measured[5]. Extraction of the drug from benzene into acid is said to separate quinidine from its more polar metabolites and other interfering substances from plasma[9]. In another similar spectrofluorimetric assay[10] the benzene extracts are washed with sodium hydroxide solution to remove some of the fluorescent metabolites. Consequently, the extraction methods produce lower results than the methods not using extraction, but they have greater specificity. None of these fluorimetric measurements, however, differentiates between quinidine and dihydroquinidine, a known impurity in medicinal grade quinidine.

More selective methods, including gas chromatography[11–13], high-speed liquid chromatography combined with thin-layer chromatography (TLC)[14] and chemical ionization mass spectrometry[15], have been used for determination of quinidine from pharmaceutical preparations and biological fluids, but most of these are time consuming procedures and require unusual instrumentation.

The use of TLC has been proposed and a method developed for the separation and quantitation of quinidine and other alkaloids in the extracts obtained from Cinchona bark on thin layers of silica gel using direct reflection and fluorescence measurements[16]. Other TLC methods have consisted of separation of quinidine on the plates, elution of the corresponding quinidine bands into a suitable solvent followed by measurements of the drug using an appropriate physicochemical assay procedure[17,18]. These methods require special care to avoid loss of the silica gel during transfer from the plates and to achieve complete extraction from the adsorbent. Direct quantitation of quinidine on chromatoplates has been reported for estimation of quinidine in serum[19]. Small volumes of deproteinated sera were spotted directly onto the chromatoplates and the fluorescence of the spots of quinidine was measured after development. In another TLC assay[20] the serum or plasma containing quinidine and salicylic acid was applied directly to silica gel layers without extraction, the proteins being precipitated on the plate with ethanol. The chromatograms were then developed and quantitated by fluorescence scanning. These TLC methods indicate the potential of TLC for quinidine measurements, but their failure to separate the drug and metabolites from serum proteins before applying to the plate constitutes a troublesome weakness, and their failure to demonstrate separation of dihydroquinidine and metabolites gives them no distinct advantage over other methods.

In this communication a method is described which is accurate for levels of quinidine in biological fluids found in single-dose studies. It involves extraction of quinidine, dihydroquinidine and metabolites, their separation on thin layers and quantitation of the corresponding spots by direct scanning in a densitometer. This procedure provides greater precision and sensitivity, its technique is simple and it is time saving. The major advantage is its specificity and the provision for simultaneous quantitation of quinidine, dihydroquinidine and metabolites on the same chromatoplate. The deficiency of the fluorimetric assay was demonstrated by chromatographic separation of the fluorescing components in the extracts obtained by the fluorimetric procedure[8] applied to samples of plasma spiked with quinidine sulfate, and to rabbit plasma and urine samples. Application of the TLC assay was demonstrated by measuring rabbit plasma and urine following an intravenous dose of quinidine gluconate.

EXPERIMENTAL

Materials and reagents

Silica gel 60 precoated plates 20 × 20 cm were used (E. Merck, Darmstadt, G.F.R.). A 50-μl Hamilton syringe was used for dissolving the evaporated extracted samples and 10-μl Hamilton syringes for spotting. The chromatograms were developed in glass tanks, 7 × 22 × 22 cm, containing 100 ml of solvent. The developing liquid was methanol–acetone (5:1).

All solvents were certified A.C.S. spectranalyzed grade.

Samples of quinidine sulfate and dihydroquinidine were received from Eli Lilly (Indianapolis, Ind., U.S.A.). Quinidine gluconate injections USP 80 mg/ml (Eli Lilly) were obtained commercially.

Authentic standard solutions of quinidine and dihydroquinidine were prepared from the stock solutions in chloroform containing 0.1 mg/ml, calculated as the free base.

Apparatus

Absorbance was measured with a UV–VIS-2 chromatograph scanner (Farrand, Valhalla, N.Y., U.S.A.) and recorded as a peak on a Farrand Model 100 strip chart recorder. Simultaneously, the area under each peak was computed and recorded with an integrator (CDS 101 Chromatography Data System, Varian, Palo Alto, Calif., U.S.A.).

Fluorescence was measured with an Aminco-Bowman spectrofluorimeter (American Instrument, Silver Spring, Md., U.S.A.).

Extraction procedure

The extraction of quinidine from plasma and urine was done in the following manner, which is similar to that applied to other basic drugs[21,22].

To 0.5–1.0 ml of plasma or urine in a 15-ml glass centrifuge tube is added 0.5 ml of 0.1 *N* sodium hydroxide and 4 ml of dichloromethane, and the mixture is mechanically shaken at room temperature for 15 min. The tube is then centrifuged for 10 min and the organic layer transferred to a second tube. The aqueous layer is re-extracted in the same manner with another 4 ml of dichloromethane and the combined organic layers are evaporated to dryness at 45° under a stream of nitrogen. The residue is dissolved in 50 μl of chloroform and an aliquot of 10 μl spotted on a TLC plate. On each plate three 10-μl spots of standard solutions are placed to serve as control for the particular plate. The plate is developed in the methanol–acetone solvent in a saturated tank. After the plate is developed, it is allowed to air dry and the absorbance intensity is measured at 278 nm in the spectrodensitometer.

The appropriate peak area corresponding to the quinidine in each unknown sample (A_u) is then divided by the area of the standard (A_s) on the same plate to obtain the ratio A_u/A_s.

Using standard solutions of quinidine and dihydroquinidine in chloroform, standard curves of concentration *vs.* area ratio were prepared and they were used for determination of quinidine (and if needed dihydroquinidine) in plasma and urine specimens.

Recovery

The recovery of quinidine and dihydroquinidine was determined by adding known amounts of the drugs to plasma (concentration 0.5–5 μg/ml) and comparing the absorbance peak areas obtained after extraction from these spiked plasma samples with the respective authentic standards scanned on the same plate.

Animal experiment

A 10-mg/kg dose of quinidine gluconate solution was injected over a period of 1 min into the ear vein of a New Zealand albino rabbit. Blood and urine specimens were collected and they were analyzed by the fluorimetric assay[8] and this TLC method.

Procedure for chromatographic analysis of the extracts from the fluorimetric method

Human plasma spiked with quinidine and collected rabbit plasma and urine samples were analyzed according to the fluorescence method[8]. After reading the fluorescence on the spectrofluorimeter, the 3-ml sulfuric acid extracts were made alkaline with sodium hydroxide and re-extracted with dichloromethane and chromatographed on a thin-layer plate as described in the above TLC procedure. The developed chromatoplate was observed under UV radiation and it was scanned with the spectrodensitometer.

RESULTS AND DISCUSSION

Standard curves for quinidine and dihydroquinidine are illustrated in Fig. 1. A linear relationship between the area ratios and quinidine and dihydroquinidine concentrations was observed in the range between 0.4 and 4 μg/ml. A greater dilution or a larger aliquot of the final solution may be readily used to bring the concentration of the drug in the sample applied to the plate within the desired range.

Recoveries of quinidine and dihydroquinidine from spiked human plasma samples were calculated by comparison of the areas under the peaks with those from standard solutions which had been spotted directly on the plates. These data, pre-

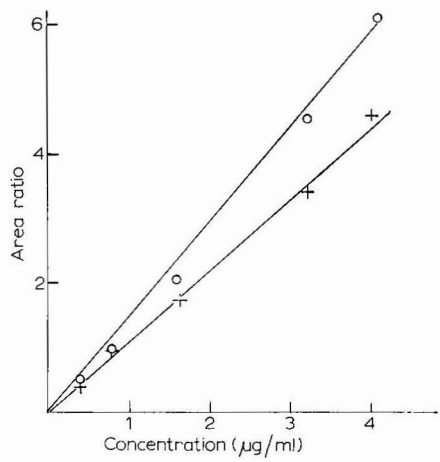

Fig. 1. Relationship of area ratio, A_u/A_s, to concentration, quinidine (○) and dihydroquinidine (×).

TABLE I

RECOVERY OF QUINIDINE AND DIHYDROQUINIDINE FROM SPIKED HUMAN PLASMA

Amounts added to plasma (μg/ml)		*Number of samples*	*Recovered (μg/ml)*		*Percent recovery ± coeff. variation*	
Quinidine	*Dihydro-quinidine*		*Quinidine*	*Dihydro-quinidine*	*Quinidine*	*Dihydro-quinidine*
0.5	—	5	0.49	—	97.5 ± 3.30	—
1.0	—	5	0.96	—	96.1 ± 2.40	—
2.0	—	5	2.06	—	103.4 ± 0.61	—
4.0	—	5	4.08	—	101.7 ± 9.70	—
5.0	—	5	5.00	—	100.3 ± 0.70	—
—	0.5	2	—	0.47	—	94.0 ± 0.00
—	1.0	2	—	0.94	—	93.9 ± 1.60
—	2.0	2	—	1.94	—	96.9 ± 4.00
—	4.0	2	—	3.81	—	93.5 ± 1.20
—	5.0	2	—	4.93	—	98.4 ± 0.20

sented in Table I, show recovery of 96–103% for quinidine and 93.5–98.5% for dihydroquinidine.

The effect of quinidine metabolites and dihydroquinidine on the fluorimetric assay was demonstrated on several samples of human plasma spiked with quinidine,

TABLE II

FRACTIONATION BY TLC OF THE EXTRACTS FROM THE FLUORIMETRIC ASSAY

Amount added to plasma (μg/ml)	*Per cent distribution of each fraction found by TLC in the assay extracts*			
	Quinidine	*Dihydroquinidine*	*Endogenous fluorescent compound*	*Metabolites*
Spiked human plasma				
0.5	20.8	0	79.2	0
1.0	39.4	8.6	52.0	0
2.0	54.1	13.5	32.4	0
4.0	65.4	15.6	19.0	0
5.0	67.6	15.5	16.9	0
Time after dosage (min)				
Rabbit plasma				
15	33.8	0	0	66.2
120	18.2	0	0	81.8
300	17.7	0	0	82.3
Rabbit urine				
15	100.0	0	0	0
30	70.8	0	0	29.2
60	100.0	0	0	0
90	86.7	5.7	0	7.6
120	61.0	24.6	0	14.4
180	43.9	6.0	0	50.1
240	40.8	9.2	0	50.0
300	35.9	16.1	0	48.0

and plasma and urine specimens collected after an intravenous injection of quinidine gluconate to a rabbit. These samples, measured by the fluorimetric method, were chromatographed and they usually contained several fluorescent compounds other than quinidine, including dihydroquinidine, metabolites and sometimes an endogenous compound extracted from human plasma. The results of these measurements are presented in Table II. It was noted that the amounts of the fluorescent compounds, other than quinidine, varied between different samples from the same animal. Such variations would constitute a substantial contribution to the difference commonly observed with the fluorimetric method.

Fig. 2 shows a typical recording of a plate scan from a sample of rabbit urine extract. When examined under UV radiation, quinidine (spot 5 with R_F 0.45), dihydroquinidine (spot 4 with R_F 0.26) and six additional fluorescent spots were observed on the developed chromatoplate.

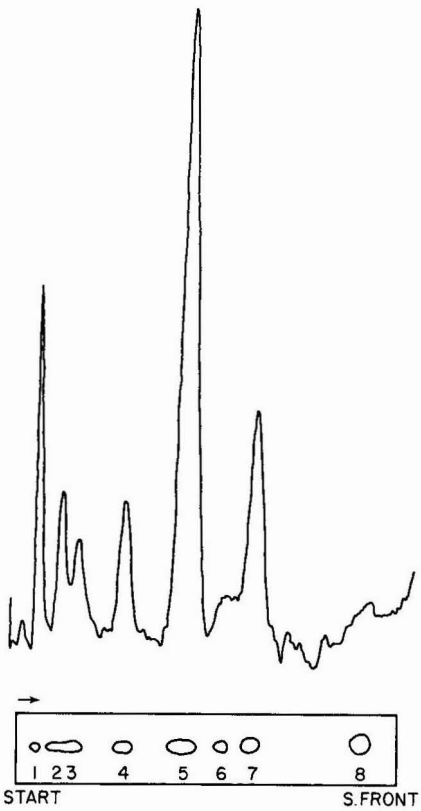

Fig. 2. Thin-layer chromatogram and densitometric scan of quinidine (5), dihydroquinidine (4) and metabolites (1, 2, 3, 6, 7, 8) isolated from rabbit urine.

The differences between the results obtained by the fluorimetric method and this TLC method are presented in Table III. Several plasma and urine samples from a rabbit, after quinidine gluconate injection, were measured by the two methods sepa-

TABLE III

COMPARISON OF THE RESULTS OF RABBIT PLASMA AND URINE SAMPLES USING THE FLUORIMETRIC AND TLC METHODS

Sample number	*Quinidine in plasma (μg/ml)*		*Quinidine in urine (μg/ml)*	
	Fluorimetric assay	*TLC*	*Fluorimetric assay*	*TLC*
1	0.15	0.10	0.23	0.22
2	0.08	0.05	0.44	0.30
3	0.04	0	0.22	0.20
4	–	–	1.32	1.02
5	–	–	2.06	1.20
6	–	–	3.40	1.52
7	–	–	2.60	1.20
8	–	–	3.00	1.05

rately. As expected, the fluorimetric method gave higher concentrations than the TLC assay, since it expresses all extractable fluorescing compounds as quinidine. In the TLC assay, metabolites and other fluorescing substances are separated from quinidine (Fig. 2).

Quinidine is metabolized in the organism and these metabolites are present in serum and urine in addition to unchanged quinidine (Table II). Quinidine metabolites show substantially less of the antiarrhythmic activity of quinidine and to control quinidine therapy the actual quinidine concentration must be determined, especially since the relative amount of quinidine metabolites in serum may vary considerably between individuals. The described TLC method is the most specific in this respect. It also enables the codetermination of dihydroquinidine, a compound said to have antiarrhythmic activity of the same order as that of quinidine or even greater and invariably present in the commercial quinidine preparations.

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SYNTHESIS AND ION-EXCHANGE PROPERTIES OF A SEMICRYSTALLINE, MAGNESIUM-SELECTIVE NIOBIUM ANTIMONATE

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SUMMARY

Semicrystalline niobium antimonate is shown to be suitable for use as an ion exchanger from 40° to 300°. It has high chemical stability and can be used to separate Mg^{2+} quantitatively from Sr^{2+}, Ba^{2+}, Hg^{2+}, Al^{3+}, Ga^{3+}, Fe^{3+}, La^{3+} and Th^{4+}. Mg^{2+} is eluted with 0.01 *M* nitric acid and the other ions are elute dwith a solution of 2 *M* in nitric acid and 0.25 *M* in ammonium nitrate. The results of X-ray, infrared thermogravimetric and differential-scanning calorimetric studies of the crystalline' sample are presented and discussed.

INTRODUCTION

Synthetic inorganic ion exchangers are being increasingly used in the field of inorganic, nuclear and organic chemistry owing to their resistance to heat and to radiation, and may prove to be important in biological and medicinal chemistry because of their high selectivity. Although considerable work has been done on these materials, some aspects of them need elucidation; for example, amorphous materials sometimes show a lack of reproducibility of composition, dissolve significantly in the eluents and are unstable at high temperatures.

The oxides of niobium and tantalum are noted for their chemical and thermal inertness, and their salts may also prove to be superior in these respects. We have therefore attempted to make a systematic study of the ion-exchange behaviour of these substances; our work on niobium arsenate[1], tantalum antimonate[2] and tantalum arsenate[3,4] has been published. Here, we describe the ion-exchange behaviour of semicrystalline niobium antimonate, with particular regard to its reproducibility, chemical dissolution, thermal stability and analytical utility.

EXPERIMENTAL

Reagents

Niobium pentoxide and antimony pentachloride were obtained from BDH (Poole, Great Britain); all other reagents were of AnalaR grade. Niobium pentoxide (20.00 g) was heated with 400 ml of concentrated sulphuric acid containing 200 g of ammonium sulphate, and the clear solution was diluted with water to 750 ml to give a solution 0.1 *M* in niobium.

Apparatus

The following instruments were used: a Bausch & Lomb Spectronic 20 colorimeter, an Elico pH meter (model Li-10), a Philips X-ray unit, a Perkin-Elmer spectrophotometer, Dupont 900 and 950 thermal analyzers, and an electric Sico shaker.

Syntheses of niobium antimonate

The samples were prepared by mixing acid solutions of 0.1 *M* niobium and 0.1 *M* antimony in the volume ratio 1:2 at room temperature; sodium hydroxide solution was added to the mixture, with constant shaking, until the pH was nearly 1. The resulting precipitate was divided into two parts; one part was set aside at room temperature for 24 h (sample AT_1), and the other part was boiled under reflux with the mother liquor for 20 h (sample AT_2). Sample AT_1 was dried at 40°, then boiled under reflux in 3 *M* sulphuric acid for 24 to 30 h (sample AT_3); sample AT_4 was prepared by similarly refluxing sample AT_1 (dried at 40°) with 3 *M* sulphuric acid for *ca.* 100 h. To prepare sample AT_5, the precitate of niobium antimonate was washed with water until

TABLE I

SYNTHESIS, ION-EXCHANGE CAPACITY, COMPOSITION AND REPRODUCIBILITY OF NIOBIUM ANTIMONATE

All samples were white in colour.

Sample No.	*Ion-exchange capacity for Na^+ (mequiv./g)*	*Ratio of Nb to Sb in product*	*Distribution coeff. in demineralized water*		
			Mg^{2+}	*Al^{3+}*	*Th^{4+}*
AT_1	0.86	1.836	1067	1400	4400
	0.84	1.845	1078	1420	4400
	0.86	1.839	1076	1400	4400
AT_2	0.76	0 892	800	550	4250
	0.74	0.889	810	550	4250
	0.78	0.881	800	550	4250
AT_3	1.10	1.408	20	272	900
	1.10	1.410	20	275	870
	1.12	1.406	24	275	870
AT_4	1.16	1.234	2.85	62	385
	1.16	1.228	2.80	62	390
	1.14	1.232	2.85	62	390
AT_5	1.20	1.469	180	173	125
	1.20	1.438	186	173	125
	1.16	1.452	184	173	138

the pH of the washings was 6, then the material was heated under reflux in 3 *M* sulphuric acid for 24 to 30 h. Samples AT_3, AT_4 and AT_5 were successively washed with 1 *M* and 0.25 *M* sulphuric acid and with demineralized water, then dried at 40° and converted into the H^+ form with dilute nitric acid.

Synthesis of hydrated antimony pentoxide

A concentrated solution of sodium hydroxide was added to 0.1 *M* antimony pentachloride solution to bring the pH to 1, and the precipitate so obtained was washed, filtered and dried at 40°. The dried product was heated under reflux with 3 *M* sulphuric acid for 24 to 30 h, then washed successively with 1 *M* and 0.25 *M* sulphuric acid and demineralized water.

Synthesis of hydrated niobium pentoxide

Concentrated sodium hydroxide solution was added to the 0.1 *M* niobium solution until the pH was 1; the subsequent treatment was as in the synthesis of hydrated antimony pentoxide.

Ion-exchange capacity

The ion-exchange capacities of the various samples of niobium antimonate were determined by the column method; the results are summarized in Tables I and II. The capacities of hydrated antimony pentoxide and hydrated niobium pentoxide were 1.80 and 0.68 mequiv./g, respectively.

TABLE II

ION-EXCHANGE CAPACITIES FOR VARIOUS CATIONS

Cation	*Capacity (mequiv./g) of sample*		
	AT_1	AT_3	AT_5
Li^+	0.36	0.44	0.50
Na^+	0.84	1.10	1.20
K^+	0.96	1.66	1.20
Mg^{2+}	0.44	0.46	0.40
Ca^{2+}	0.66	0.70	0.56
Sr^{2+}	0.86	0.76	0.80
Ba^{2+}	1.10	1.00	1.20

Composition

A 200-mg portion of well-powdered niobium antimonate was dissolved in 25 ml of hot concentrated sulphuric acid, and antimony was precipitated from the solution as sulphide and estimated volumetrically[5]; the niobium was determined gravimetrically with cupferron[6] as described previously[1]. The results are presented in Table I.

Dissolution

To study its chemical stability, 200 mg of the material was shaken with 25 ml of the appropriate solution for 6 h; after removal of undissolved material, niobium and antimony were determined in the filtrate by methods involving use of potassium thiocyanate (with diethyl ether extraction)[7] and Rhodamine B[8], respectively. The results are shown in Table III.

TABLE III

DISSOLUTION OF NIOBIUM ANTIMONATE

All results are given in mg/l.

Solvent	*Sample* AT_1		*Sample* AT_2		*Sample* AT_3		*Sample* AT_4		*Sample* AT_5	
	Nb	*Sb*	*Nb*	*Sb*	*Nb*	*Sb*	*Nb*	*Sb*	*Nb*	*Sb*
Demineralized water	0.0	8.60	0.0	4.80	0.0	4.20	0.0	3.80	0.0	4.80
4 *M* Nitric acid	1.75	30.40	0.0	0.0	0.0	0.00	0.0	0.0	2.0	18.30
4 *M* Hydrochloric acid	5.40	1522	2.60	288	2.0	255	2.0	240	1.50	212
4 *M* Sulphuric acid	88	38	62	36	0.0	2.0	0.0	2.0	0.0	0.0
0.1 *M* Sodium hydroxide	680	730.50	680	730	460	560	460	560	640	838
1 *M* Ammonia	360	659	352	659	352	659	—	—	—	—
1 *M* Ammonium nitrate	0.0	60.81	0.0	66	0.0	49.50	0.0	43	0.0	56.0
1 *M* Acetic acid	1.50	109.50	1.0	36	—	—	—	—	—	—
1 *M* Sodium acetate	1.00	60.81	1.0	60.81	—	—	—	—	—	—
1 *M* Oxalic acid	—	730.50	—	96	—	—	—	—	—	—
1 *M* Citric acid	31.50	67	33	72	—	—	—	—	—	—
1 *M* Tartaric acid	—	97.40	—	—	—	—	—	—	—	—

Drying of the sample

The sample was dried in an electric furnace in air for 1 h at each selected temperature.

X-ray studies

X-Ray diffractograms were taken with use of Cu K_α radiation (nickel filter); the results are shown in Table IV.

TABLE IV

COMPARISON OF d^0 SPACINGS (Å)

I/IO = percent transmittance.

*Sample AT_3**		*Sample AT_2**		*Antimony pentoxide***		*Antimonic acid****		*Antimonic acid*§*	
d^0	I/IO	d^0	I/IO	d^0	I/IO	d^0	I/IO	d^0	I/IO
5.97	42	5.975	45	6.00	100	6.00	100	5.83	100
3.13	55	3.13	36	3.10	80	3.128	80	3.09	62–70
3.01	54	3.01	37	2.97	80	2.995	75	2.957	62–70
2.60	16	2.59	11	—	—	2.594	15	2.569	14–17
2.40	17	2.40	8	—	—	—	—	—	—
2.04	16	—	—	—	—	1.998	17	1.981	14–18
1.84	24	1.76	12	1.82	50	1.835	31	1.822	31–36
1.76	19	—	—	—	—	1.754	22	1.741	22–23
1.57	18	1.57	12	1.550	36	1.565	14	1.565	22–29
1.50	18	—	—	—	—	—	—	—	—
1.467	12	1.467	8	—	—	—	—	—	—
1.350	10	—	—	1.15	4	—	—	—	—

* This work on niobium antimonate, with Cu radiation.

** With Mo radiation (see ref. 9).

*** With Cu radiation (see ref. 10).

§ With Cu radiation (see ref. 11).

Infrared studies

The infrared (IR) spectra were recorded by the Nujol method and are shown in Figs. 1 and 2.

Thermogravimetric and differential-calorimetric analysis

Thermogravimetric analysis (TGA) was performed under an atmosphere of

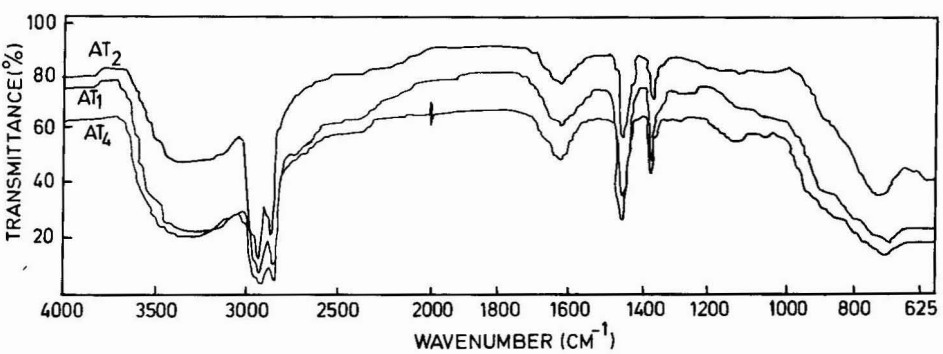

Fig. 1. IR spectra of niobium antimonate samples.

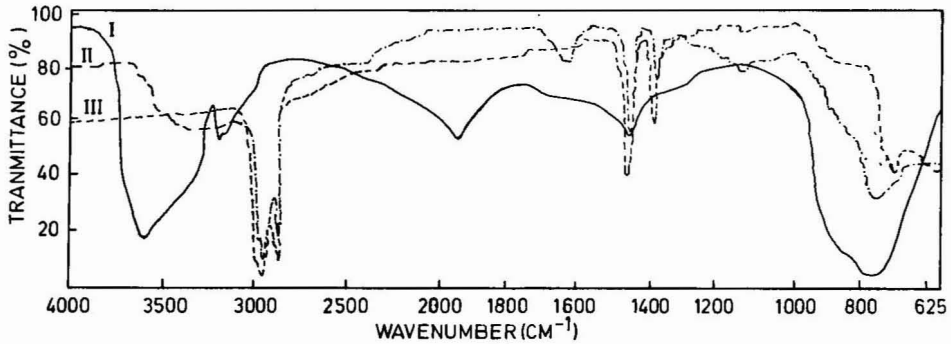

Fig. 2. IR spectra of niobium antimonate samples and antimonic acid. I = Antimonic acid (crystalline); II = NbSb (sample AT_3); III = NbSb (sample AT_3) heated at 1000°.

nitrogen flowing at 400 ml/min; the heating rate was 5°/min and the time constant was 1 sec Differential scanning calorimetry was carried out in an atmosphere of nitrogen as well as in air; the results are shown in Figs. 3–5.

Determination of distribution coefficients

A 250-mg portion of the exchanger (H^+ form) was allowed to stand in 25 ml of the cation solution in the appropriate solvent, the amount of cation being 3% of the total ion-exchange capacity of the exchanger. The mixture was shaken intermittently, and portions of the supernatant liquid were withdrawn and titrated with 2 m*M* EDTA. The results are shown in Figs. 6–8 and in Table V.

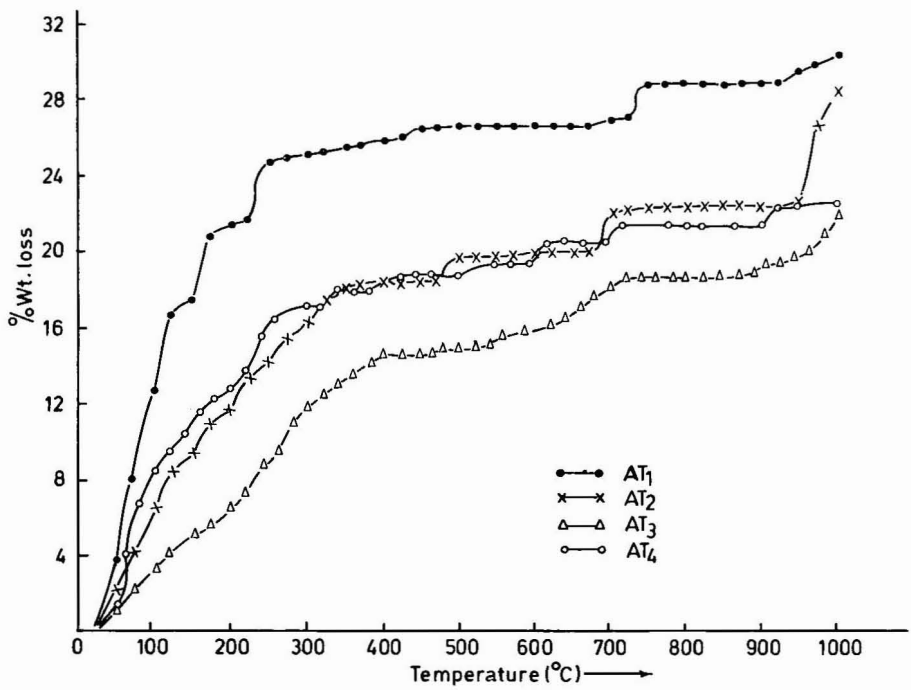

Fig. 3. TGA curves for niobium antimonate samples.

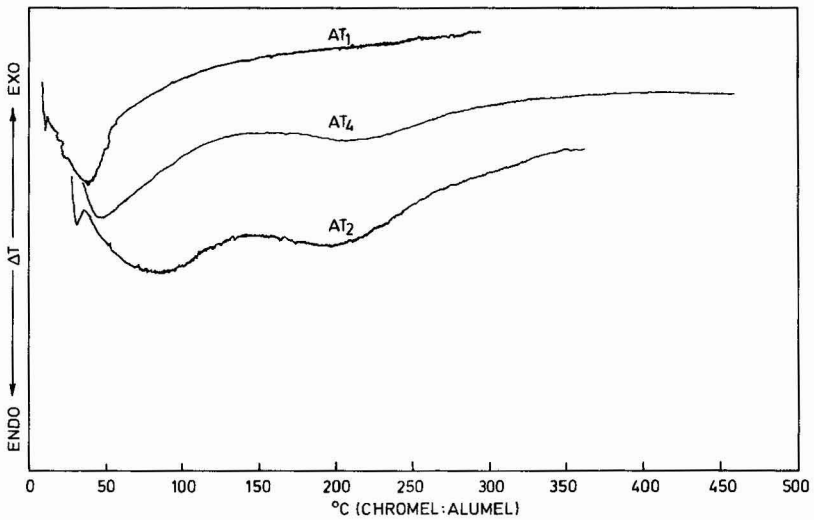

Fig. 4. Differential-scanning calorimetric curves for niobium antimonate samples.

Separation

For separation studies, a glass column was filled with 1.5 g of niobium antimonate (sample AT_3), in the H^+ form, on a glass-wool support. The flow-rate in all the separations was 0.6–0.8 ml/min. Mg^{2+} was eluted with 0.01 *M* nitric acid and the other ions were eluted with a solution which is 2 *M* in nitric acid and 0.25 *M* in ammonium nitrate. The results are shown in Table VI.

For separation studies on sample AT_3 dried at 300°, Cd^{2+}, Cu^{2+} and Ni^{2+} were

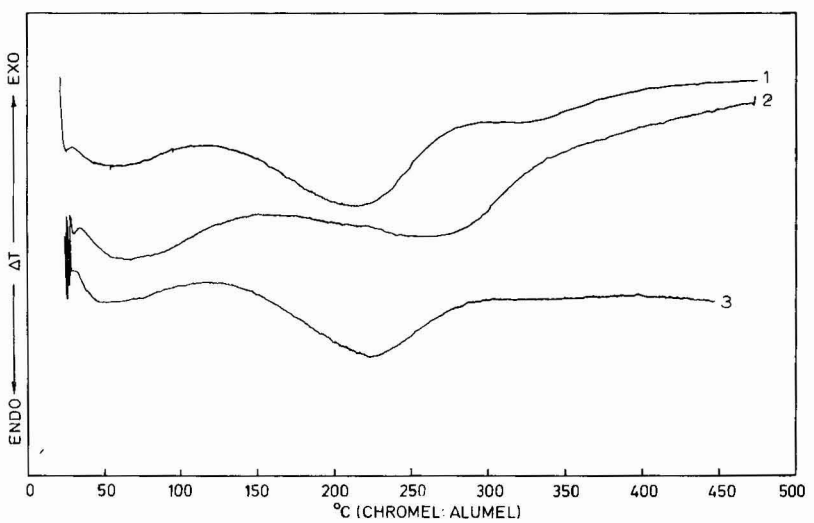

Fig. 5. Differential-scanning calorimetric curves for sample AT_3. 1 = Under nitrogen (400 ml/min); 2 = in static air; 3 = dynamic air (400 ml/min).

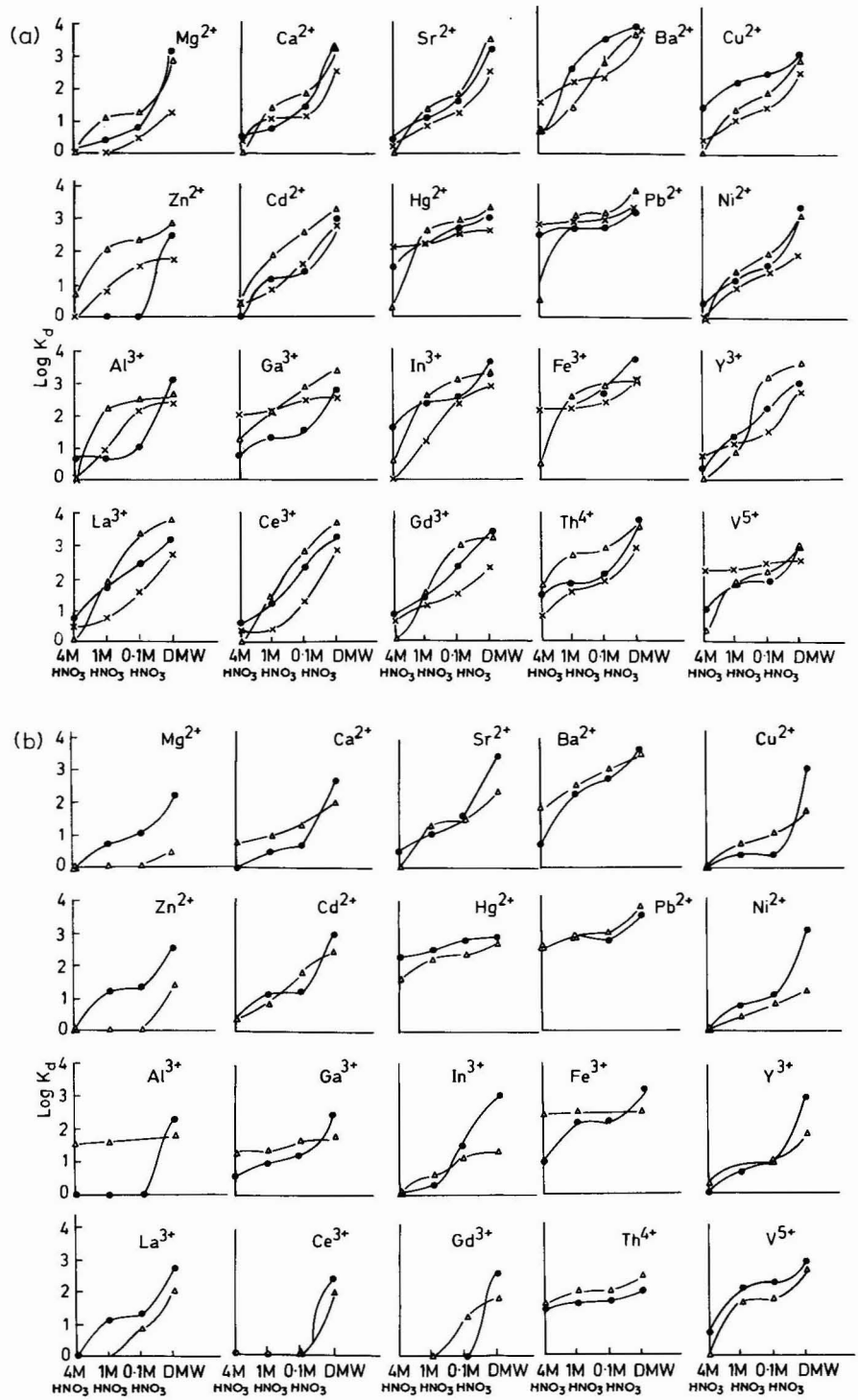

Fig. 6. K_d values on niobium antimonate samples. (a), ● – AT_1; △ – AT_2; × = AT_3. (b), △ = AT_4; ● = AT_5. DMW = Demineralized water.

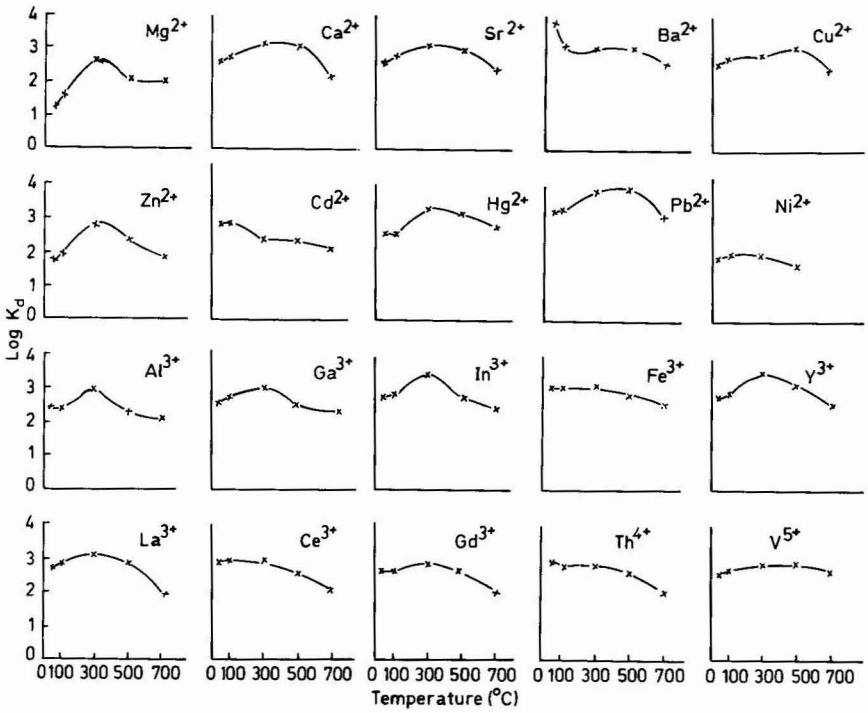

Fig. 7. K_d values on sample AT_3 at various temperatures.

eluted with a solution 0.1 *M* in nitric acid and 0.2% in ammonium nitrate, and Hg^{2+} and Pb^{2+} were eluted with a solution 2 *M* in nitric acid and 1.5 *M* in ammonium nitrate. The other conditions were the same. The results are shown in Table VII.

RESULTS AND DISCUSSION

It is clear from Table I that, even although the mixing ratio of the two reagents is the same for all five samples of niobium antimonate, the ratio of niobium to antimony ratio in the samples varies from 0.889 to 1.839; this is due to the different procedures used in the syntheses. Refluxing with 3 *M* sulphuric acid improves the reproducibility of the material (sample AT_3), probably because the material becomes more homogeneous. The reproducible behaviour of the samples was checked by determining the distribution coefficients of Mg^{2+}, Al^{3+} and Th^{4+}. The results, which showed that the deviation was within the limits of experimental error, are included in Table I.

The results of tests on the chemical dissolution of niobium antimonate are shown in Table III; all five samples were chemically stable. The stability in demineralized water and in mineral acids is decreased in the order AT_4, AT_5, AT_3, AT_1, AT_2, which indicates that refluxing with sulphuric acid improves the stability. Niobium antimonate is less stable in alkaline medium due to its conversion into niobates and in 4 *M* hydrochloric acid due to formation of chloro-complexes of antimony.

The ion-exchange capacities of samples AT_1, AT_3 and AT_5 for various cations are listed in Table II, from which it is apparent that the capacity for a cation increases

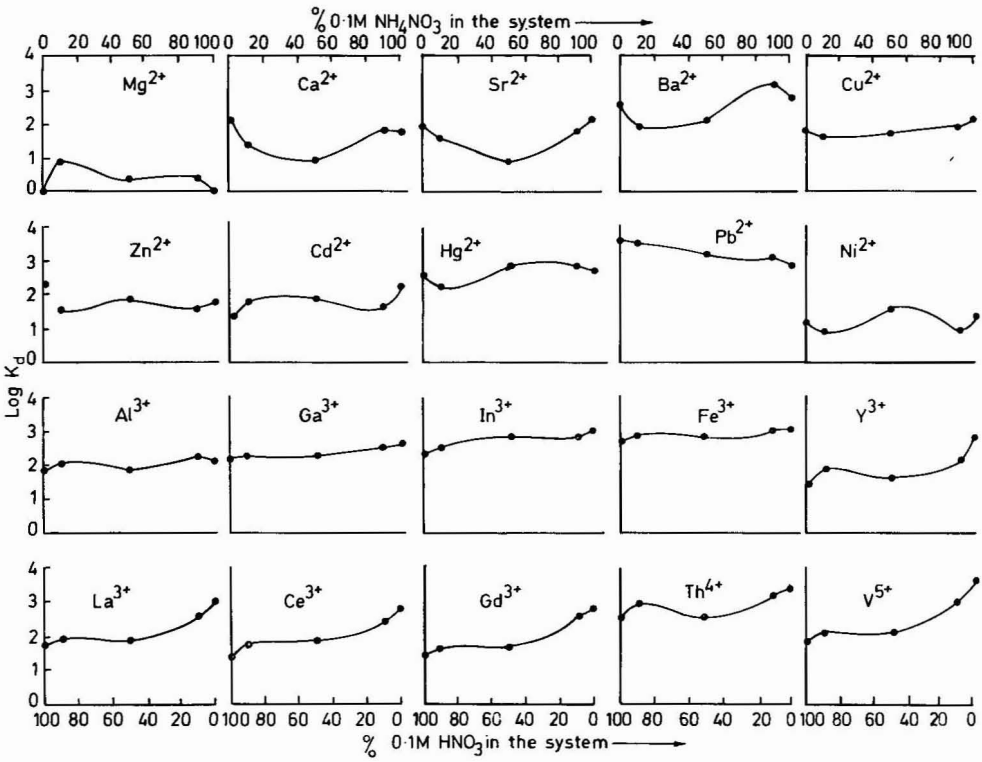

Fig. 8. K_d values on sample AT_3 dried at 300°.

TABLE V

K_d VALUES AT pH 1 (0.1 *M* NITRIC ACID)

Cation	*Niobium antimonate (AT_3)*	*Hydrated Sb_2O_5*	*Hydrated Nb_2O_5*
Mg^{2+}	2.85	1.40	0.00
Ca^{2+}	17.50	126.67	0.00
Sr^{2+}	23.30	196.00	4.62
Ba^{2+}	280.00	470.00	62.20
Cu^{2+}	25.86	—	2.88
Zn^{2+}	37.50	—	2.95
Cd^{2+}	39.24	—	0.00
Hg^{2+}	410.00	683.34	370.00
Pb^{2+}	1066.67	1450.00	425.00
Ni^{2+}	26.67	218.20	12.90
Al^{3+}	160.00	140.00	0.00
Ga^{3+}	325.00	—	17.50
In^{3+}	271.45	—	4.45
Fe^{3+}	285.72	308.00	22.08
Y^{3+}	30.00	—	10.76
La^{3+}	50.00	—	2.04
Ce^{3+}	20.00	176.80	0.00
Gd^{3+}	34.48	—	0.00
Th^{4+}	95.00	350.00	50.00
V^{5+}	260.00	50.11	142.86

TABLE VI

SEPARATIONS ACHIEVED ON NIOBIUM ANTIMONATE SAMPLE AT_3

Experiment No.	*Mixture loaded*	*Cation eluted*	*Eluent**	*Total volume of effluent (ml)*	*Amount loaded (μg)*	*Amound found (μg)*	*Error (%)*
1	Mg^{2+}–Sr^{2+}	Mg^{2+}	A	120	172.80	174.72	+1.10
		Sr^{2+}	B	60	319.20	319.20	+0.18
2	Mg^{2+}–Ba^{2+}	Mg^{2+}	A	120	172.80	173.76	+0.55
		Ba^{2+}	B	60	414.00	397.44	−4.00
3	Mg^{2+}–Al^{3+}	Mg^{2+}	A	120	172.80	173.76	+0.55
		Al^{3+}	B	70	135.20	132.60	−1.924
4	Mg^{2+}–Hg^{2+}	Mg^{2+}	A	120	172.80	168.96	−2.22
		Hg^{2+}	B	80	800.00	784.00	−2.00
5	Mg^{2+}–Ga^{3+}	Mg^{2+}	A	120	172.80	168.96	−2.22
		Ga^{3+}	B	40	154.00	157.60	+1.92
6	Mg^{2+}–Fe^{3+}	Mg^{2+}	A	120	172.80	173.76	+0.55
		Fe^{3+}	B	80	296.80	280.00	−5.67
7	Mg^{2+}–La^{3+}	Mg^{2+}	A	120	172.80	174.68	+2.20
		La^{3+}	B	60	583.80	583.80	0.00
8	Mg^{2+}–Th^{4+}	Mg^{2+}	A	120	172.80	173.76	+0.55
		Th^{4+}	B	80	881.60	844.48	−4.21

* A = 0.01 *M* nitric acid; B = 2 *M* nitric acid–0.25 *M* ammonium nitrate.

as the radius of the hydrated ion decreases. This shows that ion exchange takes place with the hydrated form of the cation.

X-Ray diffractograms (not shown) of samples AT_1, AT_2 and AT_3 showed that AT_2 and AT_3 were semicrystalline, whereas AT_1 was amorphous. Hydrated niobium pentoxide is also amorphous, but hydrated antimony pentoxide[9] shows some *d* spacing (*d*, spacing between adjacent planes in the crystal). Antimonic acid samples prepared by Ito and Abe[10] and by Baetsle and Huys[11] also showed crystalline characteristics. A comparison of the *d* values of niobium antimonate, hydrated antimony pentoxide and different antimonic acids is made inTable IV. The *d* spacings of niobium anti-

TABLE VII

SEPARATIONS ACHIEVED ON NIOBIUM ANTIMONATE SAMPLE AT_3 DRIED AT 300°

Experiment No.	*Mixture loaded*	*Cation eluted*	*Eluent**	*Total volume of effluent (ml)*	*Amount loaded (μg)*	*Amount found (μg)*	*Error (%)*
1	Cd^{2+}–Hg^{2+}	Cd^{2+}	C	100	414.40	403.20	−2.70
		Hg^{2+}	D	60	262.60	272.70	+3.66
2	Cu^{2+}–Pb^{2+}	Cu^{2+}	C	80	390.60	378.00	−3.01
		Pb^{2+}	D	60	724.50	701.28	−2.94
3	Ni^{2+}–Pb^{2+}	Ni^{2+}	C	90	318.60	323.50	+1.84
		Pb^{2+}	D	60	724.50	701.28	−2.94

* C = 0.1 *M* nitric acid–0.2% ammonium nitrate; D = 2 *M* nitric acid–1.5% ammonium nitrate.

monate resemble those reported for hydrated antimony pentoxide and antimonic acid. The maximum inter-layer distances in AT_2 and AT_3 are 5.95 and 5.97 Å, respectively.

The IR spectra of samples AT_1, AT_2, AT_3 and AT_4 are shown in Figs. 1 and 2; all four samples have peaks at *ca.* 1600 cm^{-1} and 750 cm^{-1}, and samples AT_3 and AT_4 show peaks at 1150 cm^{-1}. The other maxima in the spectra represent Nujol peaks. The maximum at 1600 cm^{-1} corresponds to the deformation vibrations of interstitial water and of the hydroxyl groups [$\delta_1(H_2O)$ and (OH)] and the maximum at *ca.* 750 cm^{-1} is due to the stretching vibration of the M–O bonds [ν_1(Nb–O, Sb–O)]. Earlier IR studies on crystalline antimonic acid[12] showed that the maxima between 1100 and 1330 cm^{-1} corresponded to the deformation of Sb–OH groups [δ_2(Sb–OH)]. Crystalline antimonic acid shows a peak at *ca.* 1280 cm^{-1}, but niobium antimonate (samples AT_3 and AT_4) shows one at *ca.* 1150 cm^{-1}. Thus, we interpret this maximum as arising only from deformation of Sb–OH groups. This also suggests that sample AT_3 is crystalline, the shift in band frequency may be due to differences in the crystal structures of the two materials.

The peak maxima at 1600 cm^{-1} and 1150 cm^{-1} are heat-sensitive and disappear when the material is heated for 30 min at 1000°. The peak at 1600 cm^{-1} is lost due to the removal of the interstitial water, and that at 1150 cm^{-1} due to conversion of $Sb(OH)_5$ into Sb_2O_5 and finally Sb_2O_3.

The results of the thermogravimetric analysis are shown in Fig. 3, from which it can be seen that the thermal stability of the samples decreases in the order AT_3, AT_4, AT_2, AT_1. Sample AT_1 loses more weight up to 400°, and, after this, its weight remains constant up to 700°. Thus, this sample contains a greater number of external water molecules, and the weight loss is due to the removal of these molecules and to condensation of hydroxyl groups (the condensation takes place between 450° and 700°). The weight losses (%) between 450° and 700° as calculated from the curves are 0.42, 3.80, 3.30 and 1.68 for samples AT_1, AT_2, AT_3 and AT_4, respectively. Of these samples, the total weight loss on heating to 400° is the least for AT_3, so that this material can be safely used at temperatures up to 400°. There is one inflexion point on the TGA curves between 900° and 1000°. The weight loss in this range is due only to conversion of Sb_2O_5 into Sb_2O_3, the optimum temperature for this reaction being 930°. The weight losses (%) in this range are 1.27, 4.05, 4.84 and 0.86 for AT_1, AT_2, AT_3 and AT_4, respectively. Our contention that samples AT_2 and AT_3 lose more weight owing to conversion of Sb_2O_5 into Sb_2O_3 is supported by the fact that these samples contain more antimony than do samples AT_1 and AT_4.

The results of differential scanning calorimetry are shown in Figs. 4 and 5 and suggest that sample AT_3 is probably the best of all the materials studied. Removal of water of crystallization begins at 45, 85, 75 and 50° for AT_1, AT_2, AT_3 and AT_4, respectively, and condensation starts at 200, 250 and 200° for AT_2, AT_3 and AT_4, respectively. This confirms the greater stability of sample AT_3 with respect to condensation.

The ion-exchange capacity of AT_3 on drying in air decreases with increase in temperature. The results are plotted in Fig. 9; for comparison, data on other antimonates, *i.e.*, those of tin[13], titanium[14], tantalum[2], chromium[15], cerium[16], aluminium[17] and iron[18], are included. The curves show that sample AT_3 is second only to ferric antimonate in this respect. The fall in ion-exchange capacity is due to condensation of hydroxyl groups at higher temperatures.

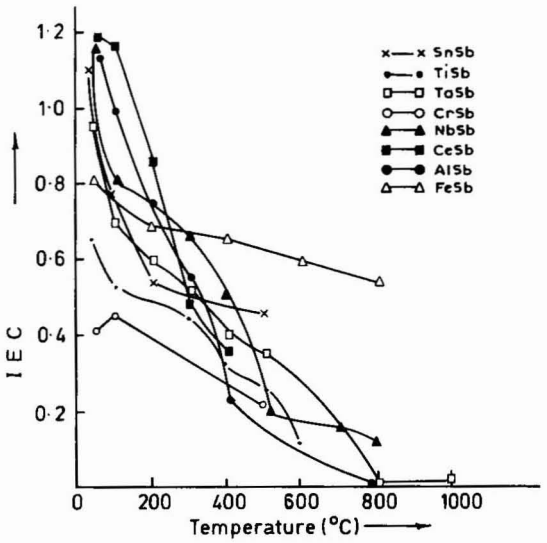

Fig. 9. Loss of ion-exchange capacity (IEC, mequiv./g) of various antimonates.

The distribution coefficients of metal ions in demineralised water are so high that one cannot decide whether or not niobium antimonate is selective for any cation. In order to investigate its selectivity for metal ions distribution coefficients (K_d) were determined in 0.1 *M*, 1 *M* and 4 *M* nitric acid; the results are shown in Fig. 6. The K_d values at pH 1 indicate the selectivity of the material. The selectivity sequence is almost the same for all samples, but the K_d values differ between samples. This difference is probably attributable to differences in composition and in the method of synthesis. The material having the greatest antimony content has the greatest uptake of ions. To find out whether niobium antimonate is a new phase or only a mixture of the oxides of niobium and antimony, the K_d values of some metal ions were determined on hydrated antimony pentoxide and hydrated niobium pentoxide at pH 1. The results for these materials and for sample AT_3 are given in Table V and show that the uptake of ions is different for the three materials; thus, AT_3 is not a mixture of oxides, but is a new phase. This conclusion is not confirmed by the X-ray data, since the *d* values for AT_3 are the same as those for antimonic acid.

The distribution coefficients for the dried samples of AT_3 show a regular trend, *i.e.*, the K_d values of most cations increase on drying the samples from 40° to 300°, and then decrease on drying to a higher temperature. The results are plotted in Fig. 7. This increase in K_d value may be due to a decrease in hydroxyl groups, as the oxides have a greater adsorptive power than the hydroxides. The dried material was selective towards Hg^{2+}, Pb^{2+}, Fe^{3+} and Th^{4+} throughout the temperature range studied. The K_d values on AT_3 dried at 300° were also determined in 0.1 *M* nitric acid, 0.1 *M* ammonium nitrate and a mixture of the two; the results are given in Fig. 8, which shows that the K_d values decreases as the content of 0.1 *M* nitric acid in the system increases.

Niobium antimonate sample AT_3 appears to be specific for separating Mg^{2+} from numerous metal ions; Mg^{2+} is only slightly adsorbed and hence can be separated

quantitatively from Sr^{2+}, Ba^{2+}, Hg^{2+}, Al^{3+}, Ga^{3+}, Fe^{3+}, La^{3+} and Th^{4+} (see Table VI). The K_d values of Hg^{2+} and Pb^{2+} differ from those of Cu^{2+}, Ni^{2+} and Cd^{2+} on sample AT_3 (dried at 300°) in 0.1 *M* nitric acid as well as in 0.1 *M* nitric acid–0.1 *M* ammonium nitrate (1:1) hence Cd^{2+}, Cu^{2+} and Ni^{2+} have been separated from Hg^{2+} and Pb^{2+} on columns of AT_3 so dried (see Table VII).

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CHROMATOGRAPHIC STUDY OF SOME INORGANIC IONS ON SEPHADEX GEL IN THIOCYANATE MEDIA

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SUMMARY

The adsorption of thiocyanate ion on a Sephadex G-15 gel has been studied. The chromatographic separation of thiocyanate, chloride, bromide and iodide on a Sephadex G-15 column was achieved by elution with 0.25 *M* sodium sulphate solution. Distribution ratios of alkaline earth metal, Zn^{2+}, Cd^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+} on Sephadex G-15 gel were determined. The chromatographic separation of the above metal ions on Sephadex G-15 gel was investigated.

INTRODUCTION

Gel chromatography is one of the useful analytical techniques in liquid chromatography. The mechanism of separation in gel chromatography is based on the molecular sieve effect, and this technique has been applied extensively in biochemistry and organic polymer chemistry.

Since 1966, gel chromatographic studies of inorganic compounds have been undertaken by several workers[1–3]. It has been reported that side-effects such as adsorption play an important role in the gel chromatographic separation of inorganic compounds[4–6], but little information is available on these side-effects.

In this study, we have found that thiocyanate ion is adsorbed on Sephadex gels and have investigated some chromatographic applications of this phenomenon. It has been clarified that the adsorption occurs reversibly and that a relatively short time is required for the establishment of the adsorption equilibrium. The adsorption isotherm of thiocyanate ion on Sephadex G-15 is linear, which explains the symmetric elution curve of thiocyanate ion on a gel column using water as the eluent.

The separation between thiocyanate and halide ions other than fluoride has been examined. As many metal ions form thiocyanate complexes, it was worthwhile investigating the chromatographic behaviour of metal ions on Sephadex gel with adsorbed thiocyanate ion in an aqueous thiocyanate medium in order to ascertain whether any useful separations could be achieved[7]. The metal ions studied were alkaline earth metal ions and several bivalent transition metal ions. Distribution ratios for these metal ions were determined at various metal or thiocyanate ion concentrations.

EXPERIMENTAL

All of the batch and elution experiments were carried out at 20 ± 1°.

Materials

All reagents used were of commercially available analytical grade, unless otherwise stated.

Sodium trimetaphosphate was prepared by the method described in the literature[8].

Gel

Sephadex G-15 gel from Pharmacia (Uppsala, Sweden) was used.

Adsorption of thiocyanate ion on Sephadex G-15

Five grams of Sephadex G-15 gel were placed in a conical flask fitted with a rubber stopper and 40 ml of 0.1 *M* thiocyanate solution were added. The flask was shaken mechanically for various time periods, then the gel was allowed to settle and the thiocyanate ion in the supernatant solution was analysed by the Volhard method.

Solvent uptake by the Sephadex G-15 was determined by a batch method using Blue Dextran 2000, which is considered not to be able to diffuse in the gel phase.

Effect of the presence of electrolytes on the adsorption of thiocyanate ion

The extent of adsorption of thiocyanate ion was determined by similar experiments to those mentioned above. The concentrations of co-existing electrolytes were varied from 0.05 to 1 *M*, while the initial concentration of thiocyanate ion was kept constant at 0.1 *M*.

The concentration of thiocyanate ion was determined by the Volhard method or colorimetry using iron(III) ion.

Measurement of distribution ratios of metal ions

Distribution ratios of a metal ion in the Sephadex G-15–thiocyanate system were measured by changing the metal ion concentration at a constant thiocyanate concentration. About 5 g of gel and 40 ml of solution containing thiocyanate and the metal ion were used. The pH of the aqueous phase in these systems was adjusted to about 2.0 with hydrochloric acid.

Determination of metal ions

Metal ion concentrations in the thiocyanate media were determined by atomic-absorption or atomic-emission spectroscopy using a Nippon Jarrel-Ash Model AA781 instrument.

Elution procedure

A Sephadex G-15 column was prepared as described in the literature[9]. A column of length 60 cm and I.D. 1.0 cm was used for elution with Sephadex G-15 gel. In each elution, the volume of sample solution delivered to the column was 1 ml and the eluents were changed stepwise in order to obtain satisfactory separations. Each

fraction of the effluent was collected with an automatic fraction collector and analysed for each component. The flow-rates in each elution were about 25 ml/h.

RESULTS AND DISCUSSION

The rate of adsorption of thiocyanate ion on Sephadex G-15 gel is shown in Fig. 1. Adsorption equilibrium is attained in the first 1–2 h. Although detailed data are omitted here, preliminary experiments indicated that the rate of desorption of thiocyanate ion on Sephadex G-15 is as fast as the rate of adsorption. In batch experiments, therefore, the shaking time of reaction flask was set at 4 h.

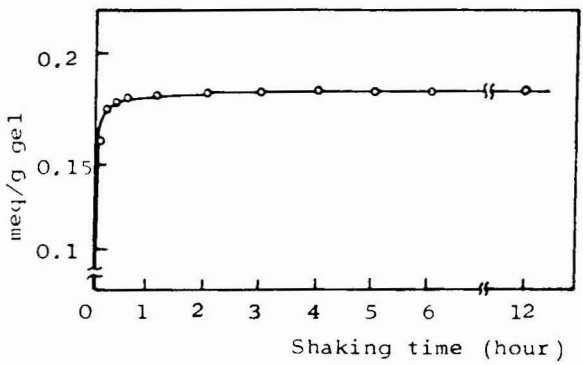

Fig. 1. Rate of adsorption of thiocyanate ion on Sephadex G-15.

In batch experiments, the amounts of thiocyanate ion adsorbed on the gel were determined as a function of equilibrium concentrations of thiocyanate ion in a solution phase. In Fig. 2, the results at 20° are plotted, with milliequivalents of adsorbed thiocyanate ion per gram of the gel on the ordinate and milliequivalents of thiocyanate ion per millilitre of equilibrium solution on the abscissa. A linear adsorption isotherm was obtained for a range of thiocyanate concentration of about 1.5 *M*. The slope of the isotherm is then equal to the distribution ratio, *D*, where

$$D = \frac{\text{mequiv. of } SCN^- \text{ per gram of gel}}{\text{mequiv. of } SCN^- \text{ per ml of solution}}$$

This straight line indicates that the distribution ratio is independent of the solute concentration in the range examined and that the adsorption is not of the chemisorption type, which entails strong, covalent bonding between solute and adsorbent and slow rates of desorption[10]. A pH dependence of the amount of thiocyanate adsorbed was not observed.

Sephadex G-15 gel equilibrated with 10 *M* aqueous thiocyanate solution adsorbed 14.8 mequiv. of thiocyanate per gram of gel. Di Gregorio and Sinibaldi[11] suggested that the adsorption of thiocyanate ion on Sephadex gel can be attributed to a "hydrophobic interaction".

We investigated the adsorption of thiocyanate ion on Sephadex gel in the presence of several anions such as perchlorate, chloride, nitrate, sulphate and tri-

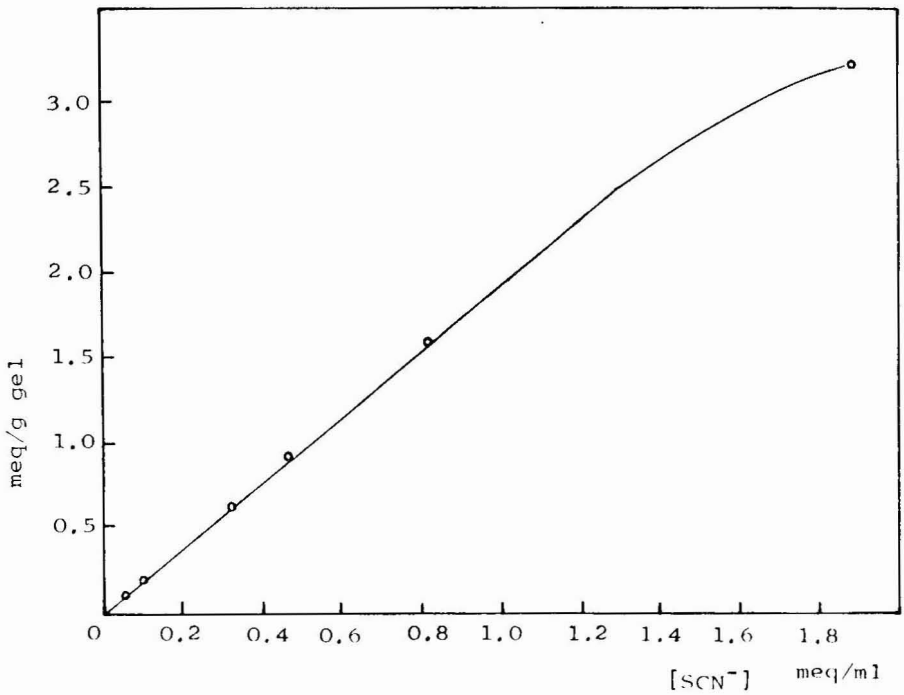

Fig. 2. Adsorption isotherm of thiocyanate ion on Sephadex G-15 at 20°.

metaphosphate. The initial concentration of sodium thiocyanate was kept constant at 0.1 *M* and the concentrations of the above co-existing electrolytes, except trimetaphosphate, were varied from 0.05 to 1 *M*; for trimetaphosphate, the concentration was varied from 0.05 to 0.5 *M*. In Fig. 3, the amounts of adsorbed thiocyanate ion are

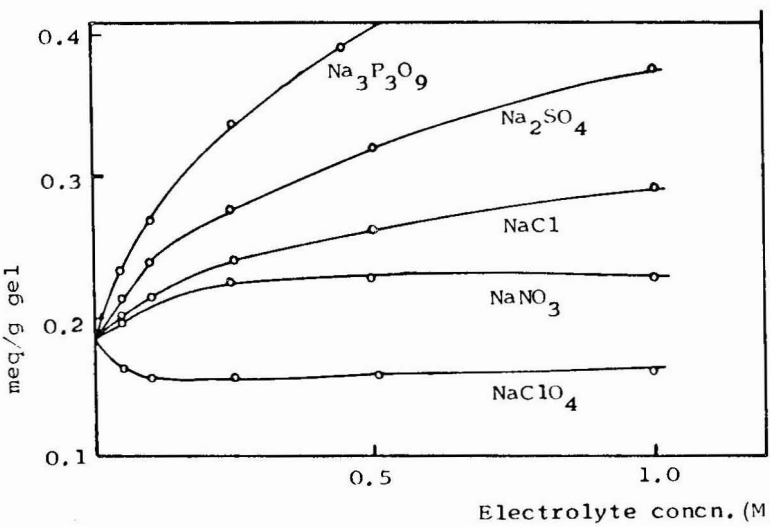

Fig. 3. Effect of various anions on the adsorption of thiocyanate on Sephadex G-15.

plotted as a function of the concentration of electrolyte present. It can be seen that the amount of thiocyanate ion adsorbed decreased when 0.05 *M* of perchlorate was added. However, further addition of perchlorate caused no change in the amount of thiocyanate adsorbed. On the contrary to the above result, the addition of amions other than perchlorate gave an increase of the amount of thiocyanate ion adsorbed on the gel. In every instance, the amount of thiocyanate ion adsorbed increased with increase in the electrolyte concentration, in the order nitrate < chloride < sulphate < trimetaphosphate. This trend is related to the affinity of these anions for Sephadex gel; the anions that interact more strongly with the gel cause a greater decrease in the amount of thiocyanate ion adsorbed.

In adsorption chromatography, when the adsorption isotherm is linear, the band migration rate is independent of the solute concentration and the band shape is of a symmetrical Gaussian type[10]. A chromatographic run of thiocyanate ion with Sephadex G-15 using water as the eluent gave a symmetrical elution curve, as expected.

We applied this characteristic adsorption of thiocyanate ion on Sephadex gel to effect the separation between thiocyanate and halide ions (except fluoride), which interfere in the argentimetric determination of thiocyanate ion. Chloride and bromide ions have been considered not to be adsorbed strongly on Sephadex G-15 gel, but it has been pointed out by Deguchi[4] that iodide ion is adsorbed on the gel. An elution diagram for a mixture of chloride, bromide, iodide and thiocyanate ions using water as the eluent is shown in Fig. 4a. It can be seen that iodide ion is adsorbed on the gel more weakly than thiocyanate ion. The elution volume of Blue Dextran 2000 coincides with the void volume of the gel column. Fig. 4a shows that the separation of thiocyanate ion and chloride and/or bromide ions is almost complete. However, overlapping of the elution curves of iodide and thiocyanate ions also occurs, and we

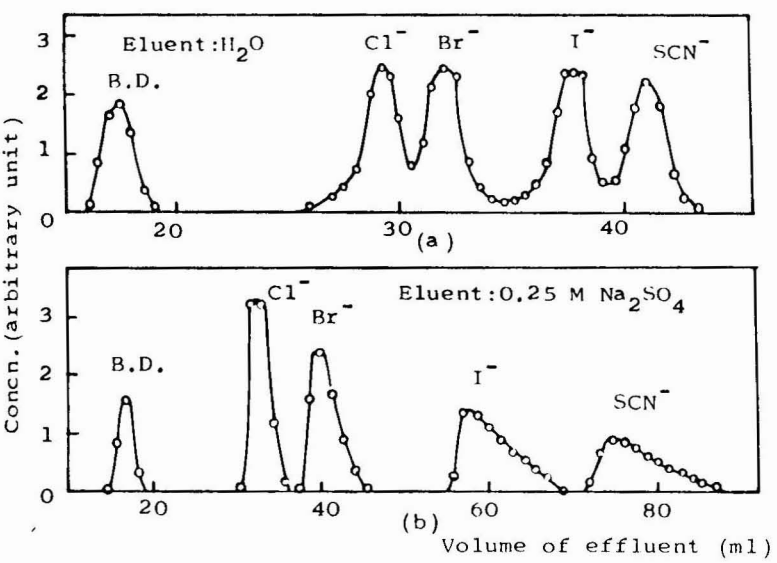

Fig. 4. Gel chromatographic separation of thiocyanate, iodide, bromide and chloride ions by use of a Sephadex G-15 gel column. B.D. = Blue Dextran 2000. Sample concentration: 0.1 *M*. Eluent: (a) water; (b) 0.25 *M* sodium sulphate solution.

used 0.25 *M* sodium sulphate solution as the eluent in order to improve this separation (Fig. 4b). As expected from the data shown in Fig. 3, the elution volume of each ion increased, but the separation of chloride, bromide, iodide and thiocyanate ions could be achieved completely by this procedure.

Sephadex G-15 gel with adsorbed thiocyanate is applicable to the separation of metal ions that have different affinities for thiocyanate in the gel phase. To determine the affinity quantitatively we have defined the distribution ratio for a metal ion, *D*, as (milliequivalents of a metal ion per gram of the gel)/(milliequivalents of a metal ion per millilitre of solution). The distribution ratio would be expected to depend on both the ratio of the concentration of a metal ion to that of thiocyanate ion in the gel or solution phase and the absolute concentrations of the metal or thiocyanate ion in the solution (gel) phase. We then examined the change in the distribution ratio with the metal ion concentration when the initial concentration of thiocyanate ion in solution was kept at 0.1 *M*. The results are shown in Fig. 5. The value of *D* increases with a decrease in the metal ion concentration and reaches a nearly constant value when the metal ion concentration falls below 10^{-3} *M*.

The dependence of the distribution ratios of metal ions on thiocyanate concentration was measured and the results are shown in Fig. 6. The concentrations of the metal ions in this experiment were about 10^{-4} *M*. The distribution ratios tend to increase with increase in the concentration of thiocyanate ion present in the aqueous phase. However, for most of the metal ions, other than Co^{2+} and Zn^{2+}, the magnitude of the increase is relatively small. Although the distribution ratios for Zn^{2+} are not

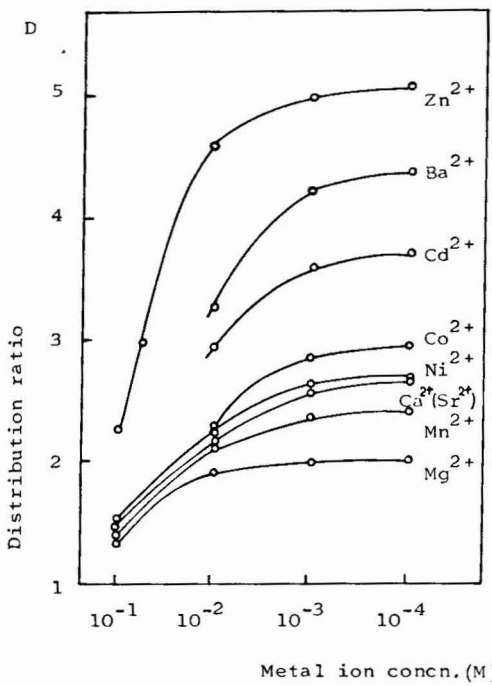

Fig. 5. Distribution ratio of metal ions on Sephadex G-15 at various metal ion concentrations. Ammonium thiocyanate concentration, 0.1 *M*; pH = 2.

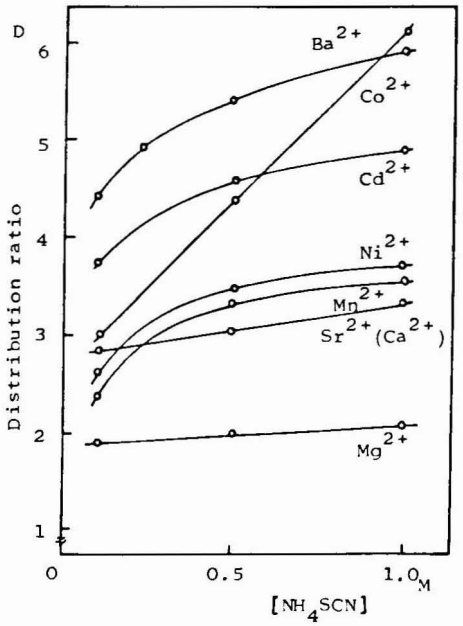

Fig. 6. Distribution ratios of metal ions on Sephadex G-15 at various thiocyanate concentrations. Metal ion concentration = 10^{-4} *M*; pH = 2.

shown in Fig. 6, the values are 7.3, 84 and 114 for thiocyanate ion concentrations of 0.1, 0.5 and 1 *M*, respectively. The distribution ratio of Zn^{2+} depends markedly on the thiocyanate ion concentration.

The separation of mixtures of alkaline earth metal and several transition metal

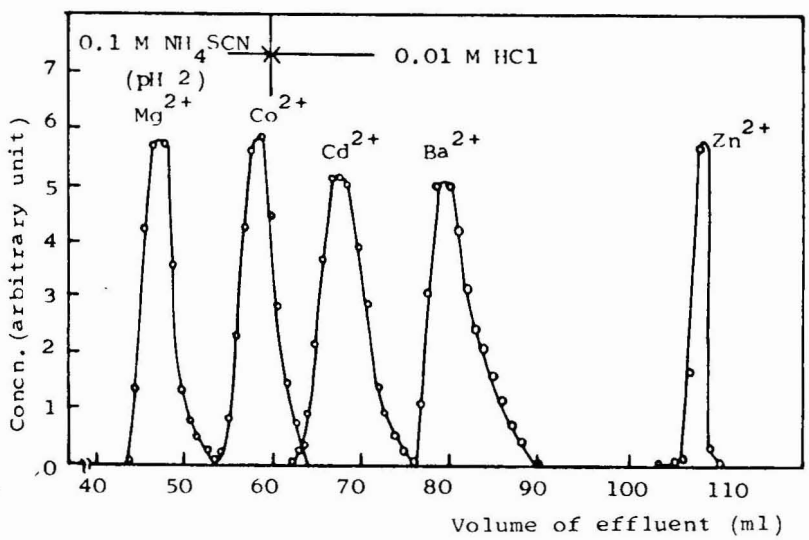

Fig. 7. Separation of several metal ions by use of a Sephadex G-15 column. Sample concentration = 10^{-3} *M*.

ions was examined with the Sephadex column using 0.1 *M* ammonium thiocyanate solution of pH 2 as the eluent. This eluent was considered to give a better resolution. Zn^{2+} was expected from the equilibrium data to be adsorbed strongly on the gel column, the eluent was changed to 0.01 *M* hydrochloric acid solution at an effluent volume of 60 ml. A typical elution diagram is shown in Fig. 7. Mg^{2+}, Co^{2+}, Cd^{2+}, Ba^{2+} and Zn^{2+} can be separated almost completely from each other. However, Mn^{2+}, Ni^{2+}, Ca^{2+} and Sr^{2+} were eluted together at the same elution position as that of Co^{2+}.

Although the strong affinity of Ba^{2+} for thiocyanate ion has not been reported, barium is strongly adsorbed on the gel with adsorbed thiocyanate ion. Furthermore, extensive analytical applications using Sephadex G-15 gel with adsorbed thiocyanate ion are to be expected.

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Note

Deactivation of glass open-tubular columns with PEG 20M via the gas phase

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Much progress has been made in the development of glass open-tubular columns, especially for apolar stationary phases. However, current methods of deactivation of the column wall are open to criticism, particularly for high-temperature analyses (above 200°) and trace analyses at the picogram level. As a workable alternative, we advocated the use of relatively thick films of the stationary phase, so as to mask rather than eliminate the activity of the column wall[1]. However, the separation times on these columns are quite long and the need for well-deactivated columns of high-phase ratio ($\geqslant$ 500) is obvious.

Aue *et al.*[2] described the formation of an ultra-thin film of chemically bonded PEG 20M on diatomaceous earth supports, and in a subsequent paper[3] they showed that these surfaces were highly inert. Cronin[4] applied this method to glass open-tubular columns in order to achieve a compatible surface for subsequent coating with PEG 20M. Blomberg[5], Schomburg *et al.*[6] and Grob and Grob[7] used variants of the method primarily to effect a deactivation, rather than to obtain a retentive film of chemically bonded phase. In all of these variants the PEG 20M was applied as a solution in dichloromethane. However, in 1970 Ives and Giuffrida[8] showed that vapours of PEG 20M, bled from a short pre-column on to a ready-to-use column, were considerably more effective in reducing tailing than was treatment of the support with solutions of PEG 20M.

We have examined the procedure of Ives and Giuffrida for the deactivation of non-coated glass open-tubular columns.

EXPERIMENTAL

A glass tube (8 × 0.25 in O.D., 2 mm I.D.), packed over a length of 3 in. with 5% PEG 20M on Chromosorb W AW, was inserted in the hot zone of the injection port of a gas chromatograph. In the oven compartment the exit end of this pre-column tapered off to 1.2 mm to match the outer diameter of the Duran 50 glass capillary

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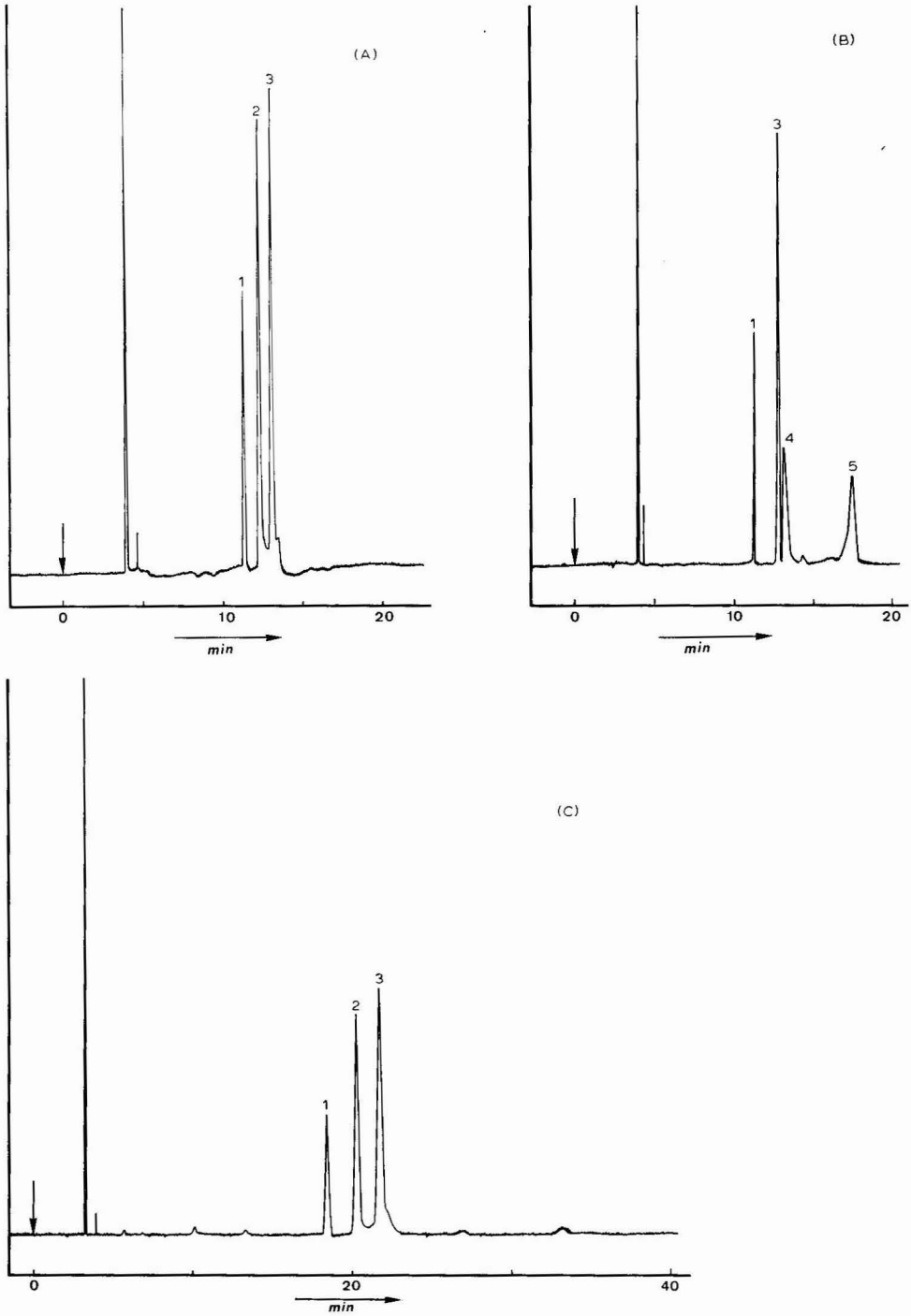

Fig. 1. (A) Test mixture (1 μl) on a thin film PEG 20M deactivated column coated with SE-30. Peaks: 1 = dieldrin; 2 = endrin; 3 = *p,p'*-DDD. (B) Test mixture (1 μl) on a thin film non-deactivated column coated with SE-30. Peaks: 1 = dieldrin; 3 = *p,p'*-DDD; 4 = endrin decomposition product 1; 5 = endrin decomposition product 2. (C) Test mixture (1 μl) on a thick-film non-deactivated SE-30 column. Peaks as in (A). For conditions and compositions of the test mixtures, see text.

column. The connection between the two columns was made with shrinkable PTFE tubing. The temperature of the pre-column was *ca.* 5–10° higher than that of the capillary column. The exact temperatures do not seem to be very critical and were *ca.* 250°. The PEG 20M was allowed to bleed through the capillary column overnight, at a nitrogen flow-rate of 1–3 ml/min. In order to test the deactivation of the resulting column wall, a deactivated Duran column (30 m × 0.4 mm) was coated with a film of 0.2 μm SE-30 (phase ratio 500) according to the static method of Bouche and Verzele[9]. For comparative purposes, a second similar column was prepared but without deactivation. SE-30 was chosen as the stationary liquid, because a thin layer of this apolar phase does not contribute substantially to the deactivation of the column wall[1], whereas a polar phase exerts a deactivating effect of its own.

Both columns were conditioned overnight at 235° and then kept at 220° for 4 weeks. An aliquot of 1 μl of a test mixture, containing 1.5 pg of dieldrin and 7.5 pg each of endrin and *p,p'*-DDD, was injected using a solids injection system[10]. The gas chromatograph was a Pye Series 104 Model 84, equipped with a 10-mCi ^{63}Ni electron capture detector (ECD). The oven temperature was 217°, and the detector was kept at 300°. Carrier gas, argon–methane (95:5); pre-pressure, 0.2 atm. Detector purge gas, argon–methane (95:5); flow-rate, 25 ml/min.

RESULTS AND DISCUSSION

Endrin is known to be very sensitive to the adsorptive and catalytic activity of the column wall. Fig. 1a shows the elution pattern of the test mixture on a PEG 20M deactivated column. Endrin is eluted between dieldrin and *p,p'*-DDD, as is the case on a thick film (phase ratio 190) non-deactivated SE-30 column (Fig. 1c). Hence, the elution sequence in Fig. 1a is not affected by the presence of the deactivating agent. As shown in Fig. 1b, endrin is not eluted from a thin film non-deactivated column. The compound decomposes to yield two products, which elute after *p,p'*-DDD and show reduced plate numbers. We presume that complete decomposition takes place in the first few meters of the column, since a gradual breakdown over the entire column length would give rise to an increased noise level, rather than to two distinct peaks.

The absence of decomposition products in the chromatograms a and c shows that the decomposition is indeed due to the column wall activity and not to decomposition in the injection system.

CONCLUSIONS

We have described a new simple method for deactivation of the column wall. The surfaces obtained even allowed the analysis of labile (*e.g.*, endrin) compounds at the picogram level. A more detailed study on the properties of the surfaces obtained will be published in a subsequent paper. A comparison will be made between the thermal stability of columns deactivated by the above method and by other methods involving PEG 20M. The compatibility of these surfaces with polar stationary phases will also be discussed. Preliminary results suggest that the gas-phase PEG 20M deactivation method will be generally applicable.

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Note

Simple and reliable gas chromatographic assay for the determination of carbamazepine in plasma

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Therapeutic levels of carbamazepine in plasma are now determined routinely in many laboratories, and gas–liquid chromatography (GLC) is the technique generally employed[1–5]. Unfortunately, carbamazepine has low thermal stability and in many GLC systems readily undergoes acid-catalysed hydrolysis to iminostilbene and rearrangement to 9-methylacridine[6], resulting in multiple peaks and poor reproducibility. To overcome this problem the use of derivatives, which may have greater thermal stability, has been suggested[7–9] but this introduces into the procedure an additional step which may be difficult to control.

There is therefore a need for a simple and reliable GLC procedure in which carbamazepine can be chromatographed underivatised and give a single reproducible peak. In this paper, a system that fulfils these criteria is described.

EXPERIMENTAL

Materials and equipment

Carbamazepine, iminostilbene and 9-methylacridine were kindly supplied by Geigy (Macclesfield, Great Britain) and amitriptyline was obtained from Roche (London, Great-Britain). Diethyl ether and acetone were redistilled before use. GLC analyses were carried out with a Pye Unicam Series 104 chromatograph equipped with an alkali flame ionization detector, and infrared (IR) spectra were measured in chloroform with a Pye Unicam SP 4000 spectrophotometer using NaCl optics. Mass spectra were obtained with a VG Micromass MM16B mass spectrometer *via* a direct inlet system.

Method

Carbamazepine together with added internal standard (amitriptyline, 0.02 μmol) was extracted from plasma (1 ml) by shaking with diethyl ether (8 ml) for 5 min. After the addition of ammonium sulphate (2 g, diethyl ether-washed) and further shaking for 30 sec, the organic phase was decanted into a conical centrifuge tube and evaporated to dryness under nitrogen at 40°. The residue was dissolved in acetone (50 μl)

and an aliquot (3 μl) injected into the chromatograph. Chromatograms were run at 235° with a glass column (1.0 m × 4 mm i.d.) packed with 5% Apiezon L–1% KOH on diatomite CLQ (JJ's Chromatography, King's Lynn, Great Britain) and a carrier gas (argon) flow-rate of 45 ml/min. The injection port temperature was 250° and the detector temperature was 260°. The concentration of carbamazepine was calculated by measuring the peak height ratio of carbamazepine to internal standard in each chromatogram and relating it to a calibration graph (Table I).

TABLE I

PREPARATION OF CALIBRATION GRAPH

The drugs were added to 1-ml aliquots of drug-free plasma and then assayed as detailed in the Method section

Tube no.	*Carbamazepine (μmol/l)*	*Amitriptyline (μmol/l)*
1	2	20
2	5	20
3	10	20
4	20	20
5	40	20
6	60	20
7	80	20
8	100	20

Reproducibility

Within-batch precision was determined from replicate ($n = 20$) analyses made simultaneously on a plasma pool to which carbamazepine (64.0 μmol/l) had been added. Between-batch precision was determined from serial analyses ($n = 20$) of the plasma pool made over three months. The coefficient of variation (standard deviation divided by the mean) was then calculated in each case.

Stability of carbamazepine during GLC

The GLC column was connected to an effluent splitter (ratio 100:1) and the effluent containing the carbamazepine peak condensed in a glass trap at room temperature. The product thus collected was examined by mass spectrometry (ionization beam energy 70 eV, ion source temperature 220°, accelerating voltage 4 kV) and IR spectroscopy and the results compared with spectra obtained from authentic carbamazepine, iminostilbene and 9-methylacridine.

RESULTS

Typical chromatograms obtained with this GLC system are shown in Fig. 1. Fig. 1A is an extract of drug-free plasma and Fig. 1B is an extract of plasma from a patient undergoing anti-epileptic therapy with carbamazepine. It can be seen that carbamazepine (peak 1) gives a single, sharp and symmetrical peak (retention time 3.2 min) which is adequately separated from the internal standard (amitriptyline, peak 2, retention time 4.6 min.). No interfering plasma peaks occur in the same region of the chromatogram (2–6 min) as carbamazepine and amitriptyline and no subsequent components are eluted when the chromatogram is run for up to 3 h.

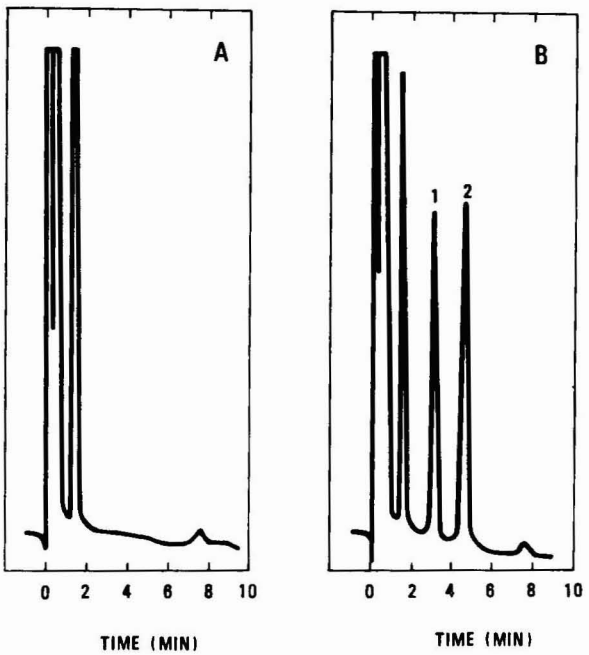

Fig. 1. Chromatograms (A) from an extract of drug-free plasma and (B) from plasma containing 17 μmol/l carbamazepine (peak 1) and 20 μmol/l amitriptyline (peak 2).

Fig. 2, which is a calibration graph prepared as detailed in Table I, shows that the method is linear for carbamazepine concentrations in the range 2–100 μmol/l and that the standard curve passes through the origin. The within-batch precision as determined from the plasma pool (theoretical value 64.0 μmol/l) was 63.5 $\pm$ 2.2 μmol/l (coefficient of variation 3.5%) and the between-batch precision was 63.7 $\pm$ 4.2

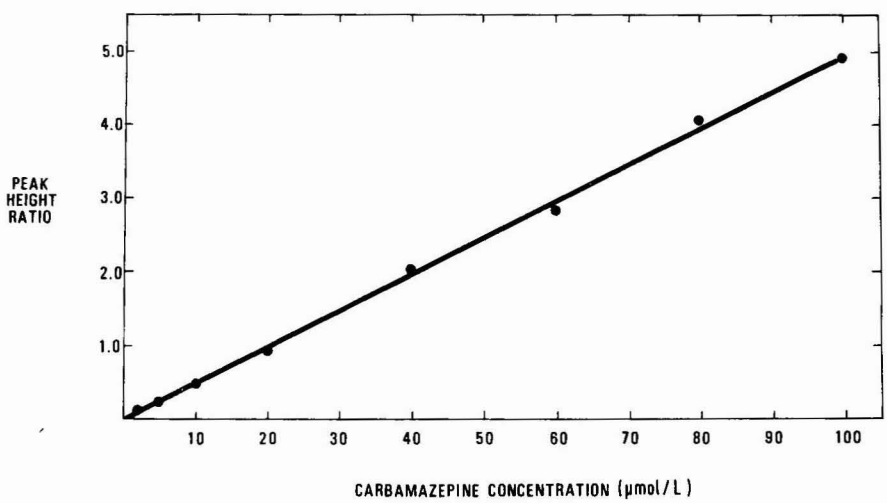

Fig. 2. Calibration graph obtained by plotting the peak height ratio of carbamazepine to internal standard against carbamazepine concentration. Each point represents a single estimation.

μmol/l (coefficient of variation 6.6%). The recovery of carbamazepine and amitriptyline from plasma was 80% and 85%, respectively.

Fig. 3 displays the IR spectra for the ranges 4000–2900 cm^{-1} and 1800–400 cm^{-1} of the three standard compounds (i) carbamazepine, (ii) 9-methylacridine and (iii) iminostilbene, together with the single column effluent (iv).

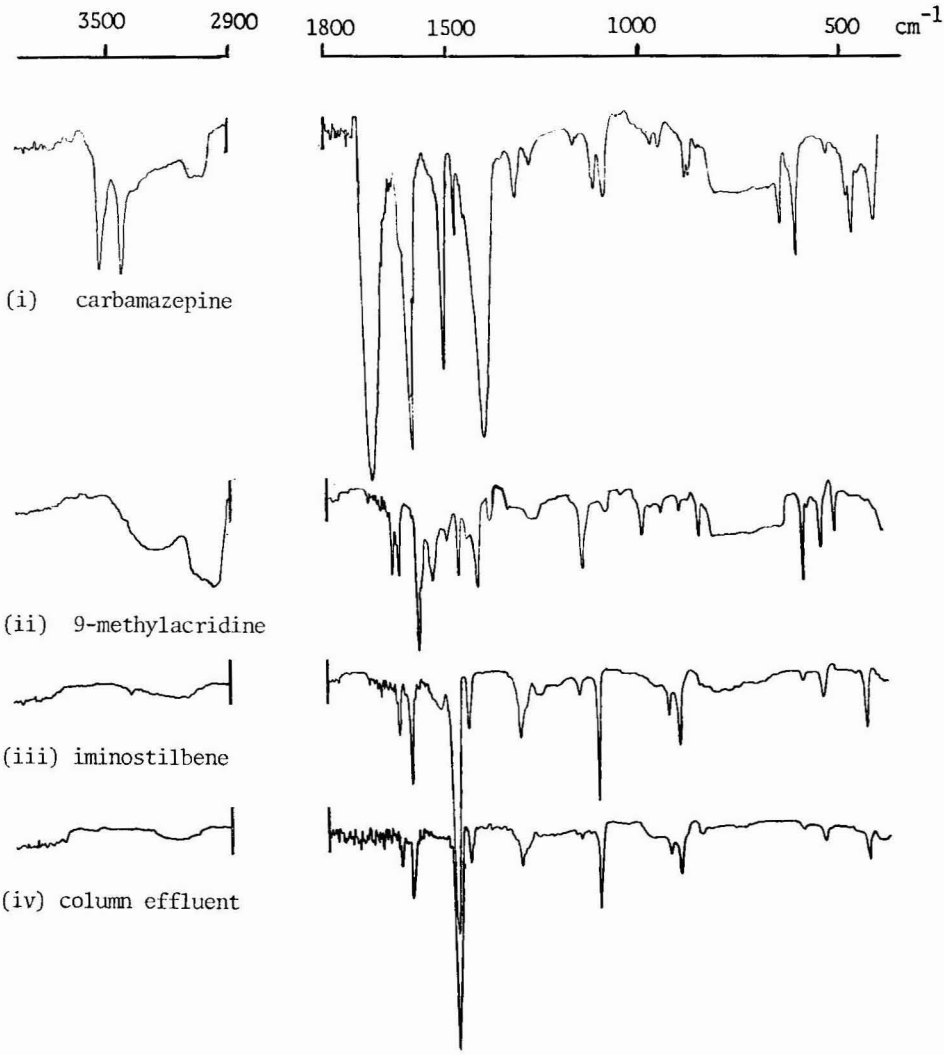

Fig. 3. IR spectra of (i) carbamazepine, (ii) 9-methylacridine, (iii) iminostilbene and (iv) column effluent measured in chloroform with NaCl windows.

DISCUSSION

The major problem associated with the GLC determination of therapeutic levels of carbamazepine in plasma is the weak thermal stability of the drug and the ease with which it undergoes on-column, acid-catalysed degradation and rearrange-

ment to multiple products[6]. Because the degree of decomposition is not constant, systems in which it occurs tend to suffer from poor precision. Although it is possible to stabilise carbamazepine prior to GLC by making the trimethylsilyl[7] or cyano[9] derivatives, or by reacting it with dimethylformamide dimethyl acetal[8], we have found that a more simple alternative is to chromatograph the underivatised drug on a column of Apiezon L incorporating KOH. Under such alkaline conditions, carbamazepine emerges as a single peak (Fig. 1B) which is sharp, symmetrical and reproducible from one injection to another. The mass spectrum of the column effluent shows a major ion at $m/e = 193$ corresponding to $M^+ - 43$, *i.e.* loss of the amide moiety from carbamazepine accompanied by a hydrogen migration. The mass spectrum of the carbamazepine standard, under the same conditions, shows no parent ion at $m/e = 236$ and variation of the ionising voltage down to 25 eV produces no significant effect. The major ion recorded occurs at $m/e = 193$. The mass spectra of both iminostilbene and 9-methylacridine display parent ions at $m/e = 193$ and the fragmentation pattern for all three compounds is essentially the same though some minor variations in intensity are noticable.

The IR spectra of carbamazepine, iminostilbene and 9-methylacridine together with that of the column effluent are shown in Fig. 3. Carbamazepine is characterised by the bands at 3543 and 3435 cm^{-1} which are assigned to ν_{N-H} and the very strong band at 1680 cm^{-1} indicating the presence of a carbonyl group. The spectrum of 9-methylacridine is more complex and displays significant strong bands at 1563, 1418 and 600 cm^{-1}. Strong bands at 1473, 1118 and 448 cm^{-1} characterise the spectrum of iminostilbene. The IR spectrum of the column effluent is clearly identified as that of iminostilbene and the absence of bands at 1563, 1418 and 600 cm^{-1} suggests that no significant amount of 9-methylacridine is produced. Neither does any carbamazepine remain. In this system therefore carbamazepine undergoes complete hydrolysis to yield a single stable product identified as iminostilbene. Subsequent reactions such as rearrangement to 9-methylacridine do not occur. Thus the method is entirely suitable for the routine measurement of carbamazepine. Not only is it linear (Fig. 2) over and beyond the therapeutic range (upper limit approximately 60 μmol/l), but also it is accurate and precise. Excellent agreement was obtained between the observed values of the serum pool (63.5 $\pm$ 2.2 μmol/l within-batch, coefficient of variation 3.5%; 63.7 $\pm$ 4.2 μmol/l between-batch, coefficient of variation 6.6%) and the weighed-in value (64.0 μmol/l). In addition, the method is simple to perform, involving only a single ether extraction followed by direct GLC analysis of the extract. This is made possible by the use of an alkali flame ionization detector which eliminates any interference that might otherwise occur from non-nitrogen containing, diethyl ether-soluble plasma constituents such as lipids and cholesterol. Similarly, interference from other anti-epileptic drugs such as phenobarbitone, primidone and diphenylhydantoin, one or more of which may be given simultaneously with carbamazepine, does not occur. Although these drugs are extracted by diethyl ether, being acidic in nature they are retained by the alkaline column and do not produce peaks on the chromatogram.

The method has now been used successfully for several months to monitor plasma carbamazepine levels, thereby assisting in the control of dosage. It has also been used to compare and correlate levels of carbamazepine in plasma with those in saliva[10].

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Note

Separation of isomeric (hydroxyphenyl)acetic acids by reversed-phase high-performance liquid chromatography

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(Received May 5th, 1977)

The separation of *ortho*, *meta*, and *para* isomers by high-performance liquid chromatography (HPLC) is desirable for resaons of speed and quantitation. The problems encountered with paper and thin-layer chromatography, such as lack of precise quantitation, or with gas–liquid chromatography (GLC), which often requires derivatization and sometimes leads to thermal degradation, are not common to HPLC[1].

Wulf and Nagel[1] have demonstrated the separation of *o*- and *p*-hydroxybenzoic acid isomers on μBondapak C_{18}®*. Waters Assoc. (Milford, Mass., U.S.A.) has also indicated the separation of *o*-, *m*-, and *p*-benzenedicarboxylic acid isomers on μBondapak C_{18}.

Excessive excretion of (*p*-hydroxyphenyl)acetic acid occurs in disorders such as cystic fibrosis, scurvy, steatorrhoea, macrocytic anemia, and tyrosinosis of the newborn[2]. Improved methods of analysis for this metabolite could be of great importance. Methods for the determination of (*o*-hydroxyphenyl)acetic acid in disorders of phenylalaninemia[3] and phenylketonuria[4] are also important. In our research, a separation of *o*-, *m*-, and *p*-isomers of (hydroxyphenyl)acetic acid was needed for some plant isolation work. It is the purpose of this note to report a facile separation of these three isomers.

MATERIALS AND METHODS

Apparatus

A Waters Assoc. ALC-100 liquid chromatograph equipped with an M-6000 pump, a U6K injector, and a Model 440 absorbance detector (12.5 μl volume) was

* Mention of a trade name or proprietary product does not constitute an endorsement by the U.S. Department of Agriculture.

used for all separations. A Waters Assoc. μBondapak C_{18} column (30 $\times$ 0.4 cm I.D.), particle size 10 μm, was selected for the separations.

Reagents

Phenylacetic acid and *o*-, *m*-, and *p*-isomers of (hydroxyphenyl)acetic acid were obtained from Aldrich (Milwaukee, Wisc., U.S.A.). Distilled water, methanol distilled-in-glass from Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.), and analyzed reagent acetic acid from J. T. Baker (Phillipsburg, N.J., U.S.A.) were used as eluents for the liquid chromatography.

Column chromatographic procedure

Solutions of (*o*-hydroxyphenyl)acetic acid (1.0%), (*m*-hydroxyphenyl)acetic acid (0.85%), (*p*-hydroxyphenyl)acetic acid (1.0%) and phenylacetic acid (4%) in methanol were prepared for injection onto the μBondapak C_{18} column (volumes of solutions are indicated in the figures). Detection was by ultraviolet absorption at 254 nm.

First, the retention volume (R_v) of phenylacetic acid was determined with the following eluents: methanol, methanol–water (9:1), methanol–water (3:1), methanol–water (1:1), methanol–5% acetic acid (1:1), and methanol–5% acetic acid (1:3). Then the retention volumes of each of the three (hydroxyphenyl)acetic acids were determined individually with methanol–5% acetic acid (1:3) as the eluent. A mixture of these three isomers and phenylacetic acid then was injected with the same eluent to visualize the separation.

Finally, a mixture of the three (hydroxyphenyl)acetic acids was injected with the following eluents: methanol–5% acetic acid (3:17), methanol–5% acetic acid (1:9), and 5% acetic acid.

RESULTS AND DISCUSSION

The R_v value of phenylacetic acid was found to vary little with changes in the eluent concentration from straight methanol to methanol–water (1:1). The polarity of the molecule due to its ionization caused it to elute near the void volume. In order to increase the R_v of phenylacetic acid, one must suppress the ionization of the molecule to decrease its polarity by the addition of acetic acid to the eluent. The use of methanol–5% acetic acid (1:3) gave a satisfactory R_v for phenylacetic acid and served as a starting point for the resolution of the three (hydroxyphenyl)acetic acids.

The optimal separation of *o*-, *m*-, and *p*-isomers of (hydroxyphenyl)acetic acid with reference to phenylacetic acid is illustrated in Fig. 1. The small unidentified peak following the *ortho* isomer is an impurity in the *meta* isomer. The separation shown in Fig. 1 occurred because of the differences in reduced polarity of the isomers in the presence of acetic acid.

The effect of reducing the methanol concentration on the elution of the isomeric (hydroxyphenyl)acetic acids is shown in Fig. 2. The retention volumes of the isomers increase as the concentration of methanol in the eluent decreases. The separation between the *meta* and *ortho* isomers almost disappears; however, the separation

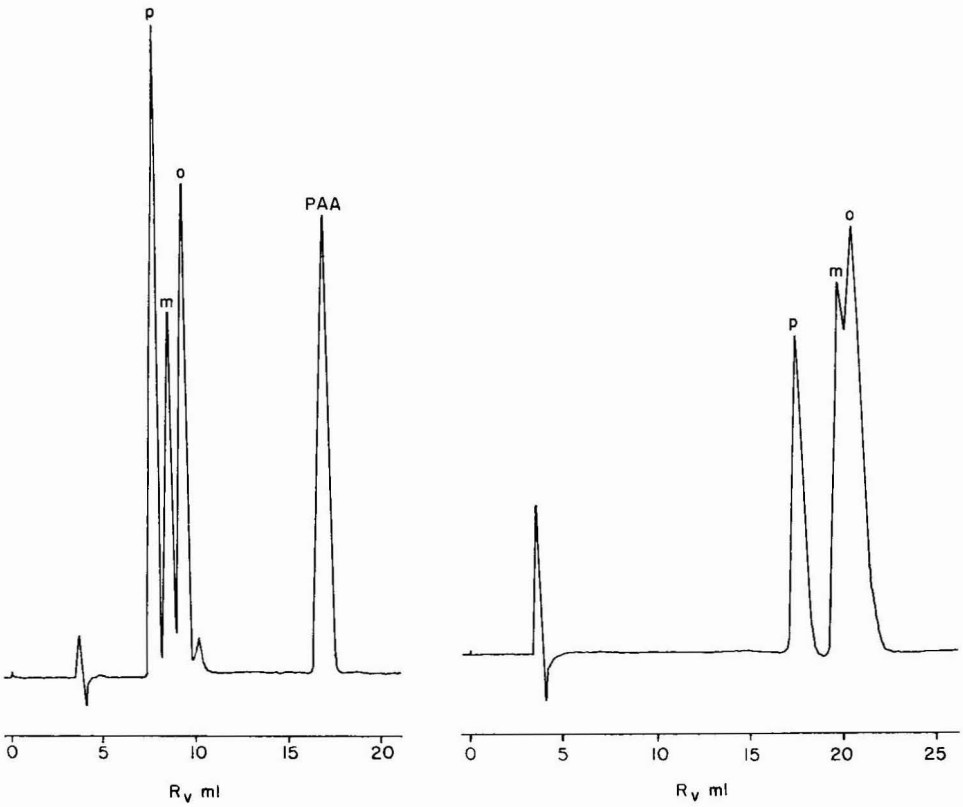

Fig. 1. Optimal separation of *o*-, *m*-, and *p*-isomers of (hydroxyphenyl)acetic acid with reference to phenylacetic acid (PAA). Column, μBondapak C_{18} (30 × 0.4 cm. I.D.), particle size, 10 μm. Eluent, methanol–5% acetic acid (1:3). Detection, 254 nm; attenuation, 0.5 absorbance units. Sample size, 20 μl (5 μl of each component solution). Flow-rate, 1 ml/min. Temperature, ambient.

Fig. 2. Effect of reducing the methanol concentration on the elution of (hydroxyphenyl)acetic acids. Same conditions as in Fig. 1 except: eluent, 5% acetic acid; sample size, 25 μl (5 μl of *o*-isomer solution and 10 μl of each of the *m*- and *p*-isomer solutions).

of the *para* and *meta* isomers is maintained. The differences in retention volumes again are attributable to differences in reduced polarity of the isomers in the presence of acetic acid.

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CHROM. 10,177

Note

Determination of anticoagulants in serum by column liquid chromatography

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(Received May 5th, 1977)

A rapid and reliable determination of anticoagulants in blood serum is urgently needed for the control of the concentration of these drugs during the treatment of heart and arterial diseases. In the past mainly fluorimetric procedures have been used, which failed to be specific unless combined with thin-layer chromatography[1]; more recently, gas chromatographic methods have been described. Mundy *et al.*[2] pointed out that these gas chromatographic determinations were not suitable and this has been confirmed by experiments in our laboratory.

The first liquid chromatographic investigations were performed by Vesell and Shively[3] using a pellicular packing material, and similar work was carried out later by Mundy *et al.*[2]. Fasco *et al.*[4] and Vanhaelen-Fastré and Vanhaelen[5] presented a method based on reversed-phase liquid chromatography.

The aim of our study was to achieve an efficient separation and a rapid quantification of some commonly used anticoagulants using a versatile selective mobile phase system in liquid–solid chromatography on silica. The procedure presented here may serve as a basis for the development of a routine analytical method.

EXPERIMENTAL

The anticoagulants used in this study are listed in Table I.

All solvents were of pro analysi grade and obtained from Merck (Darmstadt, G.F.R.). A Kipp 771 liquid chromatograph (Kipp & Sons, Delft, The Netherlands) equipped with a Zeiss PM2A variable-wavelength UV detector (Carl Zeiss, Oberkochen, G.F.R.) operated at 281 nm has been used throughout this study. The packing material was LiChrosorb SI 60 (Merck) with a mean particle diameter of 5 μm.

The extraction procedure was similar to the procedure described by De Wolff and Van Kempen[1] and is outlined in Table II.

The columns (300 mm $\times$ 4.6 mm I.D.) were packed by means of a balanced-density slurry technique. Plate numbers achieved with these columns ranged from 10,000 to 15,000.

The composition of the mobile phase, which consisted of dichloromethane, ethanol and water, was varied in order to find optimal separation conditions, as has been described elsewhere[6]. Capacity ratios (k) were calculated from the retention

* To whom requests for reprints should be addressed.

TABLE I

ANTICOAGULANTS USED

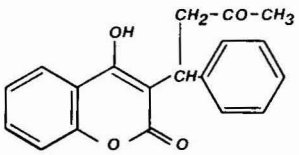
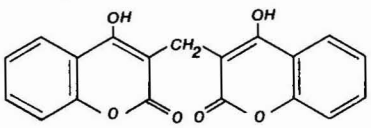
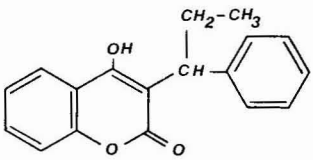
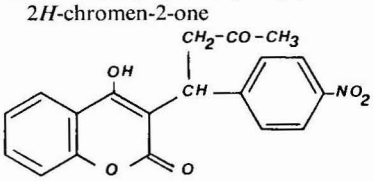
Common name	*Manufacturer*	*Systematic name and structure*
Warfarin	Sigma (St. Louis, Mo., U.S.A.)	4-Hydroxy-3-(3-oxo-1-phenylbutyl)-2*H*-chromen-2-one 
Dicoumarol	Sigma	4,4′-Dihydroxy-3,3′-methylenedi-(2*H*-chromen-2-one) 
Marcoumar	Hoffmann-La Roche (Nutley, N.J., U.S.A.)	4-Hydroxy-3-(1-phenylpropyl)-2*H*-chromen-2-one 
Sintrom	Ciba-Geigy (Summit, N.J., U.S.A.)	4-Hydroxy-3-[1-(4-nitrophenyl)-3-oxobutyl]-2*H*-chromen-2-one 

TABLE II

SCHEME FOR EXTRACTION OF ANTICOAGULANTS FROM SERUM

Stage	*Operations*
Extraction	(1) Mix 2 ml of serum with 0.2 ml of 5 *N* hydrochloric acid and homogenize. (2) Add 10 ml of trichloromethane and homogenize for 3 min. (3) Centrifuge for 10 min at 2000 rpm.
Concentration	(4) Collect 8 ml of the organic phase (lower layer). (5) Evaporate the solvent to dryness in a stream of nitrogen at 60°.
Sample preparation	(6) Dissolve the residue in 0.5 ml of eluent and ultrasonicate for 1 min.

times of the components and an unretarded compound (benzene). Samples were injected by means of an injection valve with a 50-μl loop.

RESULTS AND DISCUSSION

The optimal eluent composition for the separation of a test mixture containing all four anticoagulants in Table I was found to be dichloromethane–ethanol–water (98.8:1.0:0.2, v/v/v).

A chromatogram of the test mixture is shown in Fig. 1. As far as the eluent composition is concerned, it is necessary to work outside the miscibility gap of the dichloromethane–ethanol–water phase diagram[6]. In order to ensure stable separation conditions, it is also necessary to control carefully the water and ethanol contents.

The overall extraction efficiency is determined by, among other factors, the pH and the protein content of the aqueous phase, the type of organic phase and the mixing conditions. The composition of the aqueous phase also determines whether only the free drugs or also the originally protein-bound drugs will show up in the

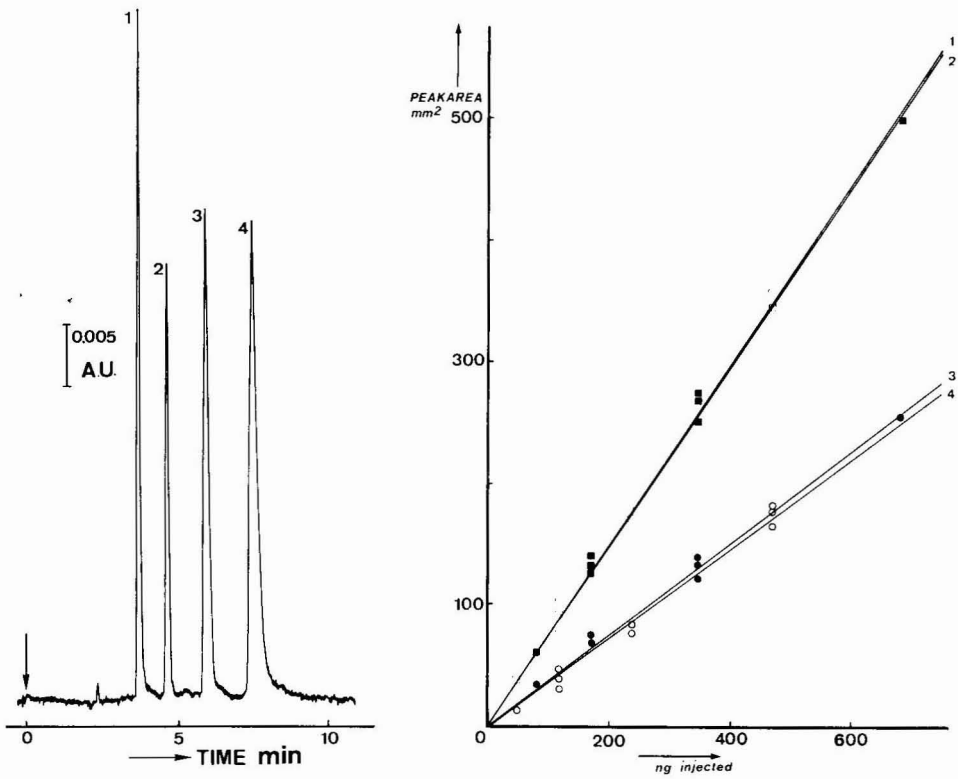


Fig. 1. Chromatogram of a test mixture of anticoagulants. Column: 300 × 4.6 mm I.D., LiChrosorb SI 60, $\bar{d}_p = 5\,\mu$m. Eluent: dichloromethane–ethanol–water (98.8:1.0:0.2, v/v/v). Flow-rate: 1.5 $cm^3 \cdot min^{-1}$. Detection: UV, 281 nm. Components: 1, Marcoumar ($k = 0.5$); 2, warfarin ($k = 0.9$); 3, sintrom ($k = 1.5$); 4, dicoumarol ($k = 2.1$). Volume injected: 50 μl.

Fig. 2. Calibration graphs. 1, Sintrom serum extract (■), correlation coefficient 99.6%; 2, sintrom test mixture (□), correlation coefficient 99.7%; 3, marcoumar serum extract (●), correlation coefficient 99.5%; 4, marcoumar test mixture (○), correlation coefficient 99.5%.

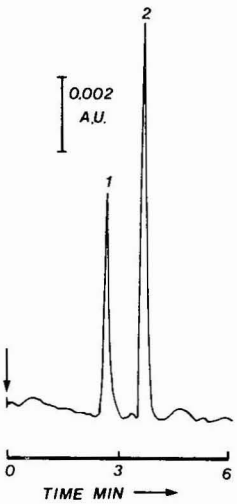


Fig. 3. Chromatogram of bovine serum extract. Conditions as in Fig. 1. Components: 1, marcoumar, 185 ng injected, 1.54 μg/ml in serum; 2, sintrom, 267 ng injected, 2.03 μg/ml in serum.

organic phase. The calibration graphs in Fig. 2 show that the overall recoveries of marcoumar and sintrom, dissolved in human serum and extracted as described in Table II, were 100 ± 3% and 102 ± 3%, respectively. The correlation coefficients for the calibration graphs were better than 99.5%.

We used the serum calibration graphs in Fig. 2 to determine the concentrations of sintrom and marcoumar in bovine serum samples. A typical chromatogram is shown in Fig. 3.

Anticoagulant concentrations ranged from 0.5 to 2.5 μg/ml in serum. The reproducibility of our analysis was within 3%, derived from the standard deviation of the recovery experiments. This method can be used for concentrations down to 0.1 μg/ml in serum. The minimal detectable amount for each of the four drugs studied was 2 ng. The presence of acetylsalicylic acid did not interfere with our anticoagulant determination[1].

ACKNOWLEDGEMENT

Prof. Dr. H. C. Hemker of the State University of Limburg, Maastricht, The Netherlands, is thanked for the gift of bovine serum samples.

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Note

Thin-layer chromatographic identity and purity test for rifampin

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The rifamycins (Fig. 1) are a family of antibiotics obtained by fermentation and chemical modification whose structures consist of a naphthoquinone moiety spanned by an aliphatic ansa bridge[1]. Rifamycin B (the major fermentation product) has the unusual property that in aqueous solutions containing dissolved oxygen it changes spontaneously into microbiologically more active substances such as rifamycin O and rifamycin S[2]. Rifamycin SV, obtained from rifamycin S by mild reduction, has been employed as a parenteral anti-tubercular drug since 1962. 3-Formylrifamycin SV, prepared by oxidizing Mannich bases of rifamycin SV, has yielded several derivatives with remarkable biological activity. The most therapeutically important of these is the 3-(4-methylpiperazinoiminomethyl) derivative, rifampin[3], a broad-spectrum antibiotic used orally in the treatment of tuberculosis.

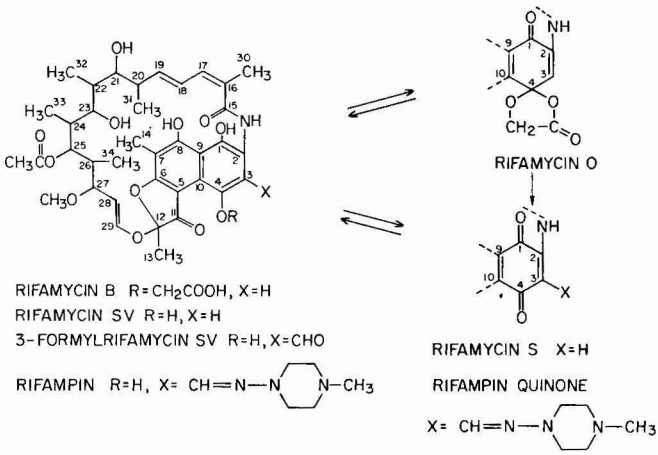

Fig. 1. Structures of rifamycins.

Impurities in rifampin can arise from incomplete separation of the drug from its precursors and from degradative changes during formulation and storage[3]. Under acidic conditions, rifampin is hydrolyzed to form 3-formylrifamycin SV. At pH values greater than 7 and in the presence of atmospheric oxygen, rifampin under-

goes oxidation to rifampin quinone and to an N-oxide. The major metabolite in man[4] 25-desacetylrifampin, can also form 25-desacetylrifampin quinone.

Rifampin is available in Canada from a variety of commercial sources. It is listed in the USP[5] and governed by regulations published in the Code of Federal Regulations[6]. USP specifications include identity controls for rifampin bulk drug material by infrared spectroscopy. No identity or purity tests are stipulated for finished products. In Canada, rifampin products are controlled under Division 8 of the Food and Drug Regulations and quality standards for raw materials and finished dosage forms are contained in the New Drug Submission[7].

Under drug quality assessment programs operating in this country, large numbers of samples are tested for identity, potency and purity, necessitating the use of reliable and time-saving methods. The present paper describes a thin-layer chromatographic (TLC) method for the identification of rifampin and for its resolution from the above-mentioned possible degradative and synthetic by-products. Direct comparison of spot intensities with known quantities allows the semi-quantitative determination of the more common impurities, rifampin quinone, 3-formylrifamycin SV and rifampin N-oxide.

EXPERIMENTAL

Chemicals and equipment

Authentic samples of rifamycin B, rifamycin O, rifamycin S, 3-formylrifamycin SV, 25-desacetylrifampin, 25-desacetylrifampin quinone, rifampin, rifampin quinone and rifampin N-oxide were generously provided by Ciba (Dorval, Canada) and Dow Chemical (Richmond Hill, Canada). USP rifampin reference standard, Lot F, was used as the working standard for purity comparisons. 1-Amino-4-methylpiperazine was purchased from ICN Pharmaceuticals (Plainview, N.Y., U.S.A.).

Commercially available precoated silica gel 60F (Merck, Elmsford, N. Y., U.S.A.) plates (EM Brand 20 × 20 cm, 0.25 mm thickness) were used. The solvent system consisted of chloroform–methanol–water (80:20:2.5).

Solutions for spotting

Standard solutions. Solutions (1.5 mg/ml) of each of the above rifamycins (Table I) were prepared in chloroform. An additional more concentrated solution of the USP rifampin reference standard (15.0 mg/ml) was prepared in chloroform.

Sample solutions. For the rifampin bulk drug substance and capsules, solutions containing 15 mg/ml were prepared by placing an accurately weighed portion of powder equivalent to 150 mg rifampin in a 10-ml volumetric flask, shaking thoroughly, then diluting with chloroform to volume.

Chromatographic procedure

Identity test. For identification purposes, standard solutions (1 μl) of all the rifamycins were spotted on the plate. In addition, a 10-μl aliquot of the sample solution representing 150 μg of rifampin was also applied. The plate was inserted into a filter paper-lined chromatographic chamber which had been saturated with solvent vapour for 1 h prior to use. The plate was developed to a height of 15 cm (*ca.* 45 min), removed from the chamber and allowed to dry at room temperature. The rifamycins

appear as coloured spots ranging from yellow to purple on a white background. Visualization may also be obtained by viewing the plate under short-wavelength ultraviolet light. In this case, the rifamycins appear as dark spots on a bright green background. The identity test is positive when the major spot in the sample solution appears at the same R_F as that of the spot from the USP rifampin reference standard solution.

Purity test. For a semi-quantitative determination of the amounts of impurities present in the sample solution, 1- and 2-μl aliquots of the impurity in question (as identified by the above TLC test), representing 1.5 and 3.0 μg, respectively, were applied to the plate. Ten-microliter aliquots of the sample solutions (equivalent to 150 μg of rifampin) were also spotted. For comparison purposes, a 10-μl aliquot of the more concentrated USP rifampin reference standard solution (15 mg/ml) was spotted as well. The plates were developed as described above and the level of impurity was evaluated by comparing the intensity of the impurity spot in the sample with that of the standards.

RESULTS AND DISCUSSION

In Table I are listed the R_F values, minimum detectable amounts and colours of the spots of the various rifamycins when chromatographed in this system. All are resolved except for rifamycin S and O; however, these two can be distinguished by their different colours. Other TLC systems have been published, but these usually dealt only with rifampin alone[3], its metabolites[4] or other rifamycins[8,9]. Therefore, this report includes TLC data on all the rifamycins listed in Table I. The 1-amino-4-methylpiperazine was not visible unless sprayed with a reagent such as ninhydrin, but all other compounds exhibited coloured spots without spraying. The minimum detectable quantities listed in Table I are the amounts of these coloured spots visible without any further treatment. Increased sensitivity is possible by fluorescence quenching when the spots are viewed under short-wavelength ultraviolet light.

TABLE I

R_F VALUES OF RIFAMYCINS ON SILICA GEL 60

Solvent system: chloroform–methanol–water (80:20:2.5).

Compound	*Minimum detectable amount (μg)*	*R_F Value**	*Spot colour*
Rifamycin O	0.75	0.83	yellow
Rifamycin S	0.75	0.81	brown
Rifampin quinone	0.30	0.75	purple
25-Desacetylrifampin quinone	0.15	0.66	purple
Rifampin	0.30	0.60	orange
25-Desacetylrifampin	0.30	0.41	orange
3-Formylrifamycin SV	0.75	0.27	pink
Rifamycin B	0.75	0.15	yellow
Rifampin N-oxide	0.15	0.09	yellow
1-Amino-4-methylpiperazine	0.15**	0.05**	brown

* Average of five plates.

** Visible only after spraying with ninhydrin.

Fig. 2 shows a chromatogram of commercial formulations, representative of those available on the Canadian market, in which the levels of rifampin quinone, 3-formylrifamycin SV and rifampin N-oxide are quantitated. From a comparison of the spot intensities these impurities generally were found to be present at less than 1 % levels except in the case of product C (an expired lot, spot 5) where the rifampin quinone level was about 2 %. The USP rifampin reference standard (spot 6), which has a labelled potency of 98.8 %, was shown to contain rifampin quinone and N-oxide but at less than the 1 % impurity level.

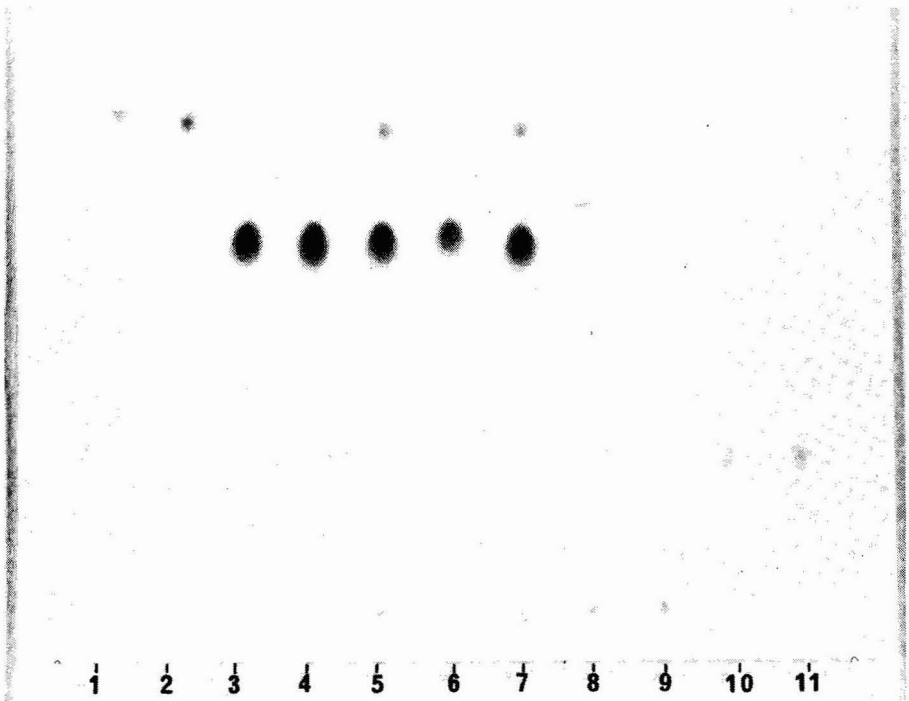

Fig. 2. TLC chromatogram of rifampin formulations and impurities on silica gel. 1 = 1.5 μg of rifampin quinone; 2 = 3.0 μg of rifampin quinone; 3 = 150 μg of product A; 4 = 150 μg of product B; 5 = 150 μg of product C; 6 = 150 μg of USP rifampin reference standard; 7 = 150 μg of product D; 8 = 1.5 μg of rifampin N-oxide; 9 = 3.0 μg of rifampin N-oxide; 10 = 1.5 μg of 3-formylrifamycin SV; 11 = 3.0 μg of 3-formylrifamycin SV.

Although amounts of rifampin equivalent to 150 μg were spotted, levels of up to 300 μg could be chromatographed without spot distortion or streaking. With the minimum detectable amount of rifampin N-oxide being 0.15 μg, the application of 300 μg of rifampin would allow the detection of 0.05 % levels of this impurity by this TLC procedure.

The proposed TLC method is a simple procedure suitable for the identification of rifampin and as a limit test for the known related impurities.

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CHROM. 10,211

Letter to the Readers

Flat-bed ion-exchange materials

Flat-bed techniques using ion exchangers have been employed for 23 years. Amongst the early types there were home-made resin-impregnated filter-papers which were soon superseded by a range of Amberlite ion-exchange papers containing various resins. Unfortunately, the most useful paper for investigations in inorganic chemistry, namely that with the strongly basic anion-exchange resin, Amberlite SB-2 paper, is no longer available in Europe.

We tried to replace this paper with a ready-made thin layer which is marketed by Macherey, Nagel & Co. (Düren, G.F.R.), Polygram Ionex-25 SB-Ac, and which, according to the label, has a layer of "0.25 mm strongly basic ion-exchange resin, acetate form".

As it is virtually impossible to convert such layers into another anionic form by washing, we felt that the acetate form was well chosen as it would not interfere much with eluents such as hydrochloric acid. We had published a survey of the behaviour of metal ions in nitrite media using these layers[1] and the results were confirmed with other media[2].

Recently, we started to re-examine the anion-exchange behaviour of some cobalt(III) complexes of the type $Co(en)_3^{3+}$ on these layers as there have been some reports that anionic outer-sphere complexes could be studied in equilibrium experiments with anion-exchange resins[3]. We were therefore pleased when some preliminary experiments with Polygram Ionex-25 SB-Ac layers showed rather strong adsorption for this type of complex. Typical results are shown in Fig. 1.

We were less enthusiastic, and even perplexed, when even stronger adsorption was found under conditions where no outer-sphere complexation was likely (Fig. 2). We thought at first of phenomena associated with some particular property of the resin, mainly because no adsorption could be observed either on Amberlite SB-2 papers or on Dowex-1 columns. As the label does not indicate the degree of cross-linking of the strongly basic ion-exchange resin, we wrote to the manufacturer for further information.

We were informed that the layer is made of "Kieselgel for thin layers ion-exchange resin, strongly basic, 8% DVB with quaternary ammonium groups, acetate form, binder as usual for the preparation of thin-layer plates or sheets". This reply explained the unusually strong adsorption of the cationic complexes: Kieselgel is a very efficient cation exchanger for these complexes, as we had reported previously[4].

The composition and the manufacturer's reply pose a rather serious problem. Much of the chromatographic work carried out nowadays is not performed merely in order to obtain a separation, but usually some chemical problem or discussion concerning the mechanism of such a separation is involved, with conclusions based on what one thinks the sorbent–solute interactions are about. This is impossible, however, if the label for the thin-layer material is misleading or incomplete. I consider

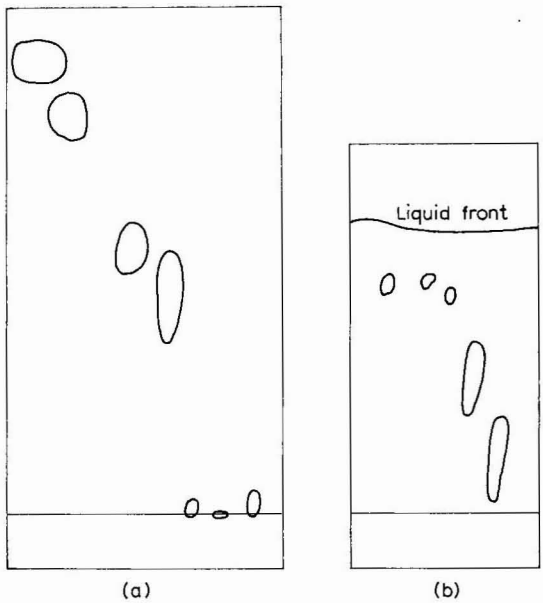

Fig. 1. (a) Movement (from left to right) of Ni^{2+}, $Co(en)_3^{3+}$, $Co(tn)_3^{3+}$, $Co(pn)_3^{3+}$, $Co(dip)_3^{3+}$, $Co(o\text{-phen})_3^{3+}$ and CrO_4^{2-} on Polygram Ionex-25 SB-Ac developed with 4 *N* ammonium sulphate solution. (b) Movement (from left to right) of $Co(en)_3^{3+}$, $Co(tn)_3^{3+}$, $Co(pn)_3^{3+}$, $Co(dip)_3^{3+}$ and $Co(o\text{-phen})_3^{3+}$ on Polygram Ionex-25 SB-Ac developed with 5 *N* perchloric acid.

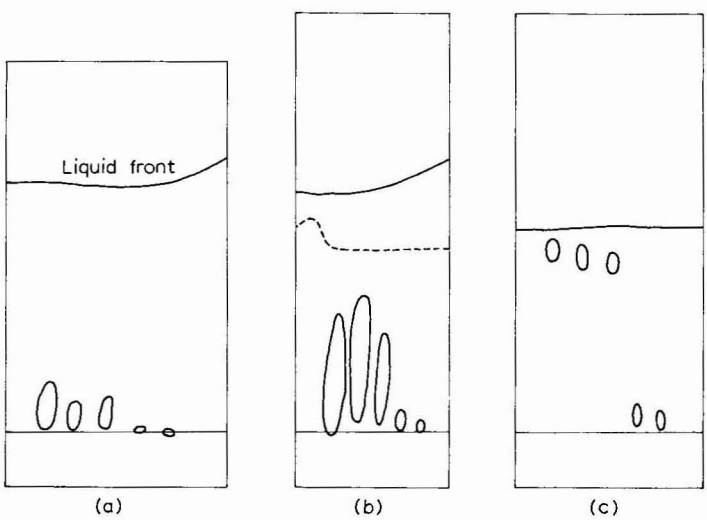

Fig. 2. Chromatogram of cobalt complexes on Polygram Ionex-25 SB-Ac. The complexes (from left to right) are $Co(en)_3^{3+}$, $Co(tn)_3^{3+}$, $Co(pn)_3^{3+}$, $Co(dip)_3^{3+}$ and $Co(o\text{-phen})_3^{3+}$. Developing solvent: (a) 0.5 *N* sodium acetate solution; (b) 1 *N* hydrochloric acid; (c) 6 *N* hydrochloric acid.

it undesirable for a manufacturer to market an anion exchanger that has a cation exchanger added without mentioning the fact. Furthermore, the reply from the manufacturer in this instance also mentions a "binder as usual for the preparation of thin-layer plates of sheets", which seems to be unnecessarily vague information. It will certainly add a factor of uncertainty to any speculation based on the chromatographic behaviour on such "secret" layers.

In describing this problem I am aware that it is only an example and that similar problems exist nowadays with column supports for both gas and liquid chromatography as well as for most other ready-made thin layers.

I conclude thus this letter with a plea to all manufacturers to label their chromatographic products correctly and completely, and with a further plea to all authors who encounter similar problems to report them promptly in the literature and, when using unusual supports, to describe them as fully as possible.

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CHROM. 10,204

Letter to the Editor

Computer processing of fatty acid analysis data

Sir,

Recently, Aston[1] presented a Fortran program for processing fatty acid data which computes the concentrations of each fatty acid, the percentage of glycerol, the theoretical iodine value, etc. It allows processing of a large amount of data and may be of value to those who are not familiar with computer programming. However, we noticed that the program does not take into account the amount of water lost during esterification of the fatty acids with glycerol, and thus the amount of fat formed from 100 g of fatty acids is overestimated. This gives rise to an appreciable underestimation of the "theoretical iodine value" and weight % of glycerol.

Since 1 mole of fat is formed by the esterification of 1 mole of glycerol with 3 moles of fatty acids and the elimination of 3 moles of water, the amount of fat (WF) from 100 g of fatty acids (f.a.) may be calculated as:

$$WF = 100 + \sum_{1}^{n}\left(\frac{\text{Wt. \% of f.a.}}{\text{Mol. wt. of f.a.}}\right)_i \left(\frac{92.09}{3} - 18.02\right) \text{ or}$$

$$WF = 100 + (\text{moles of glycerol associated with 100 g of f.a.}) \times \times (92.09 - 18.02 \times 3) \qquad (1)$$

The addition of iodine to 100 g of fatty acids is correctly represented by ii (ref. 1, p. 123, line 42) if the term "iodine equivalent" (= the number of gram atoms of iodine bound to 100 g of fatty acids) replaces "iodine mole equivalent". The iodine value (IV_T) may then be estimated from gas chromatographic analysis (ref. 1, p. 123, iv, line 48) as:

$$IV_T = \text{"theoretical iodine value"} = = \frac{\text{Weight of iodine associated with 100 g of f.a.'s}}{WF} \times 100 \qquad (2)$$

Accordingly, the wt.% of glycerol in fat should be given by (*cf.* ref. 1, p. 123, vi, line 30):

$$\frac{\text{Weight of glycerol residues associated with 100 g of f.a.'s}}{WF} \times 100 \qquad (3)$$

Neglect of the amount of water eliminated during esterification gives rise to an underestimation of the "theoretical iodine value" of 5–7% depending on the mean molecular weight of the fatty acids ($\overline{\text{Mol. wt.}}$) in the fat. The deviation (ΔIV_T) between the true "theoretical iodine value" and that presented by Aston[1] may be estimated from the expression:

$$\text{Error \%} = \frac{\Delta IV_T}{IV_T} \times 100 = 100\left(1 - \frac{\overline{\text{(Mol. wt.)}} + 12.683}{\overline{\text{(Mol. wt.)}} + 30.699}\right) \qquad (4)$$

Taking butter fat ($\overline{\text{Mol.wt.}} = 230$) and horse fat ($\overline{\text{Mol.wt.}} = 280$) as typical examples, errors of 6.9 and 5.8% respectively are calculated.

The program may be corrected according to eqns. 3 and 2 by changing statement numbers 76 and 77 (ref. 1, p. 128, lines 6 and 10):

```
C   GLCW = WEIGHT OF FAT DERIVED FROM 100 GM OF FATTY
    ACIDS
(75) GLMT = GLCM + GLMN
(76) GLCW = GLMT * 38.049
C
C   COGL = PERCENT GLYCEROL RESIDUES IN FAT
(77) COGL = GLMT * 89.071 * 100/(100 + GLCW)
```

As part of graduate training in fat analysis[2], students of the faculty have to check "theoretical iodine values", calculated from gas chromatograms, against the values obtained by direct titrimetry of the fat[3]. We have found that for dry fats there is generally close agreement between the experimental and calculated iodine values. However, significantly lower "theoretical iodine values" than titrimetric values are calculated if higher unsaturated fatty acids are missing from the chromatogram.

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H. F. DE BRABANDER
R. VERBEKE

1 J. W. Aston, *J. Chromatogr.*, 131 (1977) 121.
2 R. Verbeke, *Scheikundige analyse der eetwaren van dierlijke oorsprong*, Graduate course, Veterinary Faculty, Univ. Ghent, 1973.
3 H. Pardun, *Handbuch der Lebensmittelchemie, Bd. IV, Lipids*, Springer, Berlin, 1969, p. 573.

(Received April 4th, 1977)

Reply

Sir,

The alteration to the equation for the calculation of the theoretical iodine value in the above paper by Drs. De Brabander and Verbeke is correct. The failure to account for the loss of water of esterification was an oversight on my part. I will enclose correction notes with all future reprint requests.

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J. W. ASTON

(Received May 12th, 1977)

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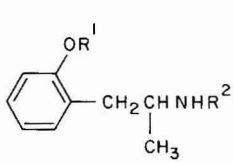
CHROM. 10,371A

Letter to the Editor

Human metabolism of methoxyphenamine

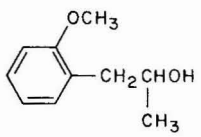
Sir,

Our initial studies of the *in vivo* metabolism of methoxyphenamine (Orthoxine®) in primates were complete[1] before we became aware of a preliminary study of Chundela and Slechtova[2] describing the excretion of methoxyphenamine and two of its metabolites in the urine of man. These workers examined concentrated ether extracts of urine, the pH of which ranged between 5.5 and 6.9, by means of thin-layer chromatography (TLC), gas–liquid chromatography (GLC) and combined gas–liquid chromatography–mass spectrometry (GLC–MS), and showed that three products were present; the major one was unchanged methoxyphenamine (Ia).

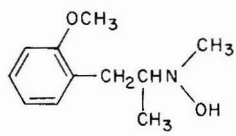


I

	R^1	R^2
a)	CH_3	CH_3
b)	CH_3	H
c)	H	CH_3



II



III

Metabolite 1 was identified correctly by interpretation of its mass spectrum as 2-amino-1-(*o*-methoxyphenyl)propane (Ib) although Chundela and Slechtova[2] named it wrongly as a dimethyl derivative of methoxyphenamine (I) rather than as the N-demethylated derivative of I. They did not compare the TLC, GLC and GLC–MS behaviour of metabolite 1 with an authentic synthetic sample of Ib. In our study[1], such a comparison was made of trifluoroacetylated metabolite 1 and synthetic Ib. The compounds were identical.

Chundela and Slechtova[2] did not identify their metabolite 2 but suggested that it could be either N-hydroxylated methoxyphenamine (III) or a metabolite of the latter, namely 1-(*o*-methoxyphenyl)-2-propanol (II), though they do not interpret their mass spectrum of this metabolite in terms of II or III. We have synthesized II and III to assist us in identifying *in vitro* metabolites of methoxyphenamine[3]. The mass spectra of these compounds (Fig. 1A and B) were clearly different from the reported[2]

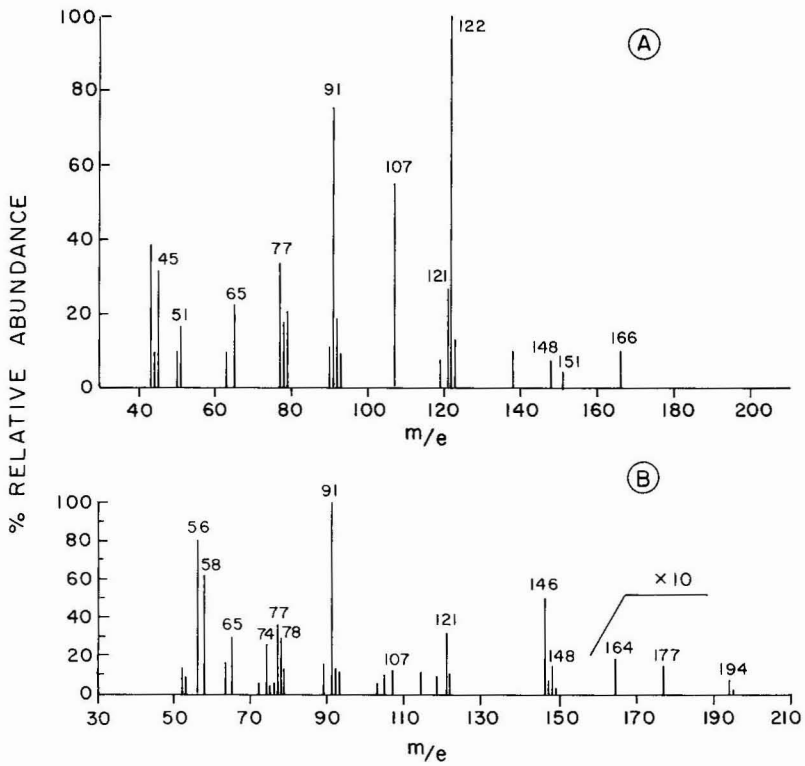


Fig. 1. Normalised mass spectra of II (A) and III (B).

spectrum of metabolite 2. Diagnostic ions observed in the mass spectra of II and III (Fig. 1) are rationalized in Figs. 2 and 3.

A comparison of their spectrum of metabolite 2 with that of our[1] synthetic and metabolically produced 1-(*o*-hydroxyphenyl)-2-(methylamino)propane (Ic) revealed that these spectra were identical, apart from the minor differences expected when different instrumentation is used. Metabolite 2 described by Chundela and Slechtova[2] is therefore the O-demethylated derivative of methoxyphenamine which we found to be the major metabolite of methoxyphenamine in man.

SYNTHESIS OF REFERENCE COMPOUNDS II AND III

Detailed syntheses will be described elsewhere; condensed descriptions are given here.

Preparation of 1-(o-methoxyphenyl)-2-propanol (II)

Reduction of 1-(*o*-methoxyphenyl)-2-propanone with sodium borohydride in methanol gave II as a colorless oil which had infrared, proton magnetic resonance and mass spectra (Fig. 1A) consistent with its structure. GLC analysis on a glass column, 1.8 m × 0.3 cm I.D., packed with 5% OV-225 on Chromosorb W AW DMCS, 100–120 mesh, with nitrogen (60 ml/min) as carrier gas and at oven temperature of 140° gave a single peak, t_r = 2.9 min.

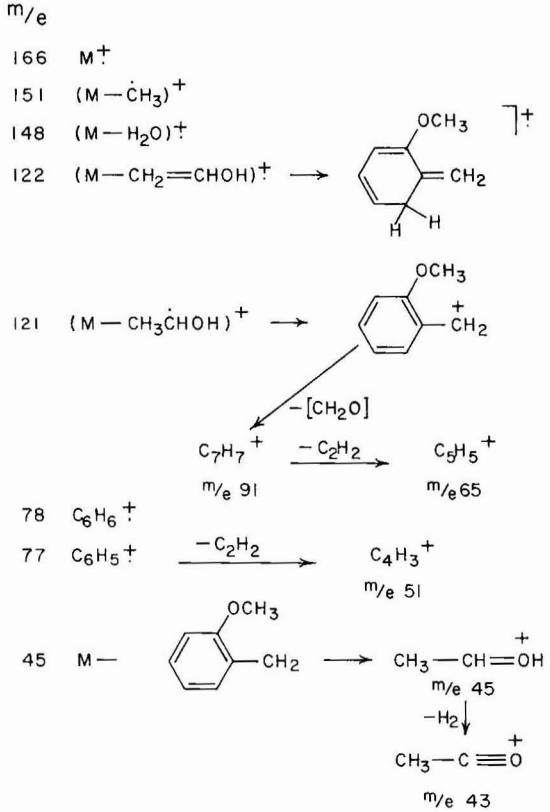
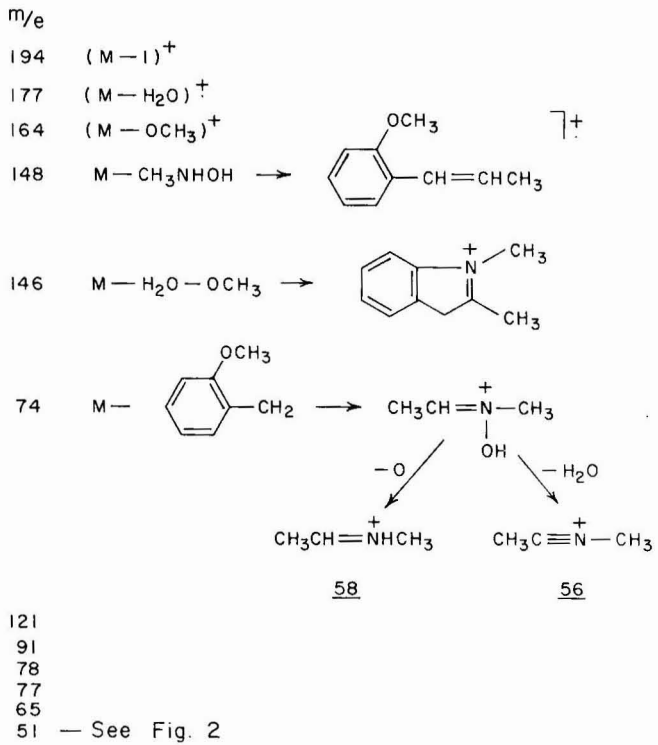


Fig. 2. Postulated mass fragment ions of II.

Fig. 3. Postulated mass fragment ions of III.



Preparation of 1-(o-methoxyphenyl)-2-propylhydroxylamine (III)

III was prepared by reductive N-methylhydroxylamination of 1-(*o*-methoxyphenyl)-2-propanone according to the method of Morgan and Beckett[4]. The oxalate salt with m.p. 148–149° analyzed (C,H,N) satisfactorily for the structure. Infrared, proton magnetic resonance and mass spectra (Fig. 1B) were consistent with the structure.

ACKNOWLEDGEMENTS

The authors thank S.-F. Liu, G. R. Jones (Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta) and J. K. Cooper (Drug Research Laboratories, Health Protection Branch) for technical assistance and G. Morris for drafting.

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CHROM. 10,371B

Letter to the Editor

Human metabolism of methoxyphenamine

Sir,

K. K. Midha and R. T. Coutts have rightly shown our error in naming the first metabolite isolated from urine after administering methoxyphenamine. This was, in fact, an error introduced during printing as the original manuscript and the inaugural dissertation of the first author, presented in October 1975 (pp. 117–123) at the Medical School, Charles University, Prague, name the first metabolite as the N-demethylated derivative of methoxyphenamine. The habilitation paper describes its parameters in both TLC and GLC, including measured retention indices. We apologize for our lack of attention in correcting our preliminary note. We are also sorry that we did not have at our disposal an authentic synthetic sample of metabolite 2 for the interpretation of the mass spectrum. After the preliminary note was published we obtained a reagent for methylation and thus we were able to contradict our suggestion that metabolite 2 is not N-hydroxylated methoxyphenamine but the O-demethylated derivative of methoxyphenamine.

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CHROM. 10,503

Book Review

The hydrophobic fragmental constant; Its derivation and application; A means of characterizing membrane systems (Pharmacochemistry Library, Vol. 1), by R. F. Rekker, Elsevier, Amsterdam, Oxford, New York, 1977, XIX + 389 pp., price Dfl. 97.50, US$ 38.95, ISBN 0-444-41548-3.

Had it not been for the spectacular ascent of "reversed-phase" chromatography as the leading technique in high-performance liquid chromatography, this book could be of little interest to the readers of the journal. The magic of "hydrophobic" or "solvophobic" interactions, which are responsible for solute retention on non-polar stationary phases, however, is increasingly used by chromatographers to solve a wide range of separation problems very conveniently and efficiently. Therefore, the extensive literature in medical chemistry dealing with the hydrophobic properties of various biologically important compounds suddenly has become interesting for the potential of predicting retention behaviour in "reversed-phase" chromatography from "hydrophobicity" of solutes.

What the hydrophobic effect actually is still remains controversial as far as a rigorous thermodynamic treatment is concerned. Indisputably, it is a kind of non-covalent interaction which is literally caused by the hatred of water. This makes the thought of a hydrophobic bound *outré* with regard to orthodox textbook chemistry, whereas other non-covalent molecular interactions such as coulombic and hydrogen bonding, which are due to attraction between the species, have been long understood. Attraction and repulsion are easy to appreciate, they are ingrained in human nature. This may be the reason why scientists have had a relatively easy task in quantifying the corresponding forces on the molecular level and adequately describing the phenomena.

To deal with the world of biological molecules and to understand their interactions entirely we have to reach beyond the simplistic notions of love and hatred. The enlightment has come from the realization that not only governments and people but also molecules can act on the principle "the enemy of my enemy is my friend". Hydrophobic or more generally lyophobic interactions, which play an extremely important role in determining the architecture and dynamic behaviour of biological substances, arise from such a molecular attitude with the solvent, in particular water, playing the role of the enemy. As such "friendship" is not easy to understand, it is no wonder that the quantitative description of the phenomenon has been difficult and the progress is slow. Not having anything better to characterize the pertinent molecular properties, a relatively modest amount of partition coefficient data obtained in systems composed of water and a non-polar solvent have been subjected to a monumental statistical analysis in order to factor out some numbers on the hydrophobicity of the structural elements of the solute molecules.

Rekker's book is the latest comprehensive treatment of the subject which has been popularized by Hansch. It is an expansion of the author's work on hydrophobic fragmental constants which measure the hydrophobicity of structural elements in chemical compounds. Like the π-values introduced by Hansch and used widely, they are derived from partition coefficients obtained in suitable organic solvent–water systems. Although the subtitle promises a characterization of membrane systems, the accomplishments of the book in this regard are less noteworthy.

It is not easy to read the book for many reasons. At the beginning the uninitiated reader finds the *lingua franca* of this type of literature somewhat strange. The first terms encountered are SAR and QSAR, the acronyms for structure–activity relationship and quantitative SAR, respectively. The use of such terms is so widespread that in the preface feelings are expressed "in SAR-like terms". Nevertheless with some efforts the reader can acquire the skill to follow the text and it is a worthy undertaking.

The book presents a large amount of data distilled via correlation analysis but a cursory reading does not make it clear to a chromatographer how to use the material. To draw maximum benefits from the book, further reading of the pertinent literature may be required. It would have been desirable to have a more detailed general introduction to linear free energy relationships, the *point d'appui* of the whole approach.

What lies behind the vast data analysis and sometimes quodlibetic attention to minor details

is clear. Medicinal chemists and chromatographers both want to know how the chemical structure of a substance determines its behaviour in their respective systems. Some time ago, the center of such activities was in the realm of chromatography due to the need to identify the spots or peaks on the chromatogram. In the recent years, however, the main emphasis is shifted to predict the biological activity of a substance from its chemical structure. The reason for this is the high cost of biological testing of potential drugs as well as toxic or carcinogenic substances. In fact, by employing sophisticated statistical analysis with high speed computers, medicinal chemistry has made great advances in this regard while chromatographers have been preoccupied with the development of high-performance liquid chromatography.

Both chromatographic retention and biological activity can be described by linear free energy relationships which have been used by physical chemists to interpret the effect of structural parameters on chemical rates and equilibris. With biological compounds the so-called hydrophobic properties are of particular interest because an unambiguous definition of a hydrophobic free energy is difficult. Here, reversed-phase chromatography in its new form as practiced today can fill a gap.

For this reason anyone familiar with the accomplishments and potential of high-performance liquid chromatography would feel that the technique does not receive due attention in this book. This is probably owing to the fact that most data came from partition coefficients measured in octanol–water. Yet, the excellent treatise on the use and relevance of chromatographic measurements by Tomlinson in *Chromatographic Reviews*[1] should have been mentioned. This reviewer is convinced that modern liquid chromatography as a versatile, rapid microanalytical tool will play a significant role in collecting data for QSAR studies. The present gap can be eliminated when the QSAR people become acquainted with the potential and methodology of modern liquid chromatography and chromatographers supply enough evidence that their technique is superior to the classical methods.

With this in mind, Rekker's book can be not only a useful source of hydrophobic parameters to be used for quantitative structure–retention relationships, QSRR if you don't mind, but also a stimulant for the development of more refined chromatographic methods and means for collecting and analysis of data for use in QSAR.

New Haven, Conn. (U.S.A.) CSABA HORVÁTH

1 E. Tomlinson, *J. Chromatogr.*, 113 (1975) 1.

Author Index

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2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
3 H. C. S. Wood and R. Wrigglesworth, in S. Coffey (Editor), *Rodd's Chemistry of Carbon Compounds*, *Vol. IV, Heterocyclic Compounds*, Part B, Elsevier, Amsterdam, Oxford, New York, 2nd ed., 1977, Ch. 11, p. 201.
4 E. C. Horning, J.-P. Thenot and M. G. Horning, in A. P. De Leenheer and R. R. Roncucci (Editors), *Proc. 1st Int. Symp. Quantitative Mass Spectrometry in Life Sciences, Ghent, June 16–18, 1976*, Elsevier, Amsterdam, Oxford, New York, 1977, p. 1.

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