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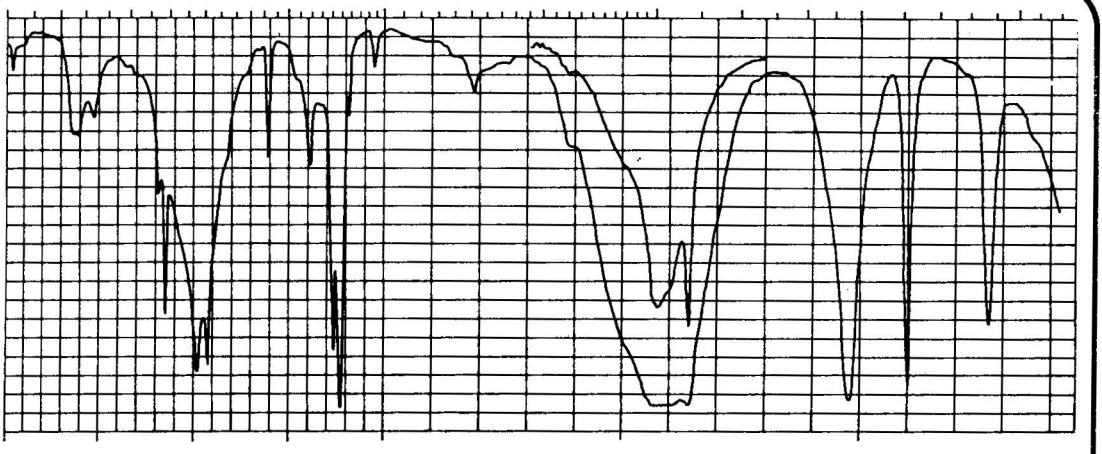

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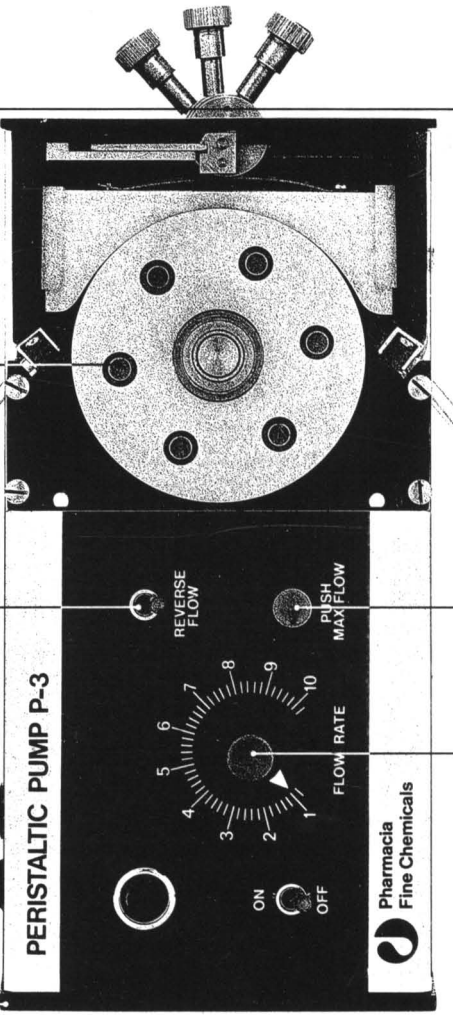

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LARGE-BORE COATED COLUMNS FOR SAMPLING AND CONCENTRATION OF ORGANIC VOLATILES IN AIR, HEADSPACE AND WATER ANALYSIS

D. A. M. MACKAY and M. M. HUSSEIN
Life Savers, Inc., Port Chester, N.Y. (U.S.A.)
(Received February 21st, 1979)

SUMMARY

Large-bore coated (LBC) columns were used as sampling and concentrating traps in analyses for traces of organic volatiles in air and water. This simple technique utilizes long metal columns thinly coated with SE-30 for direct trapping of the organics. The sample is simply passed through the LBC column; the trapped organics are then thermally desorbed onto a conventional porous polymer pre-column or onto a second LBC column. If desired, this can be shorter or narrower bore than the initial LBC sampling column. The sample is finally desorbed onto the gas chromatographic column for analysis. Multiple transfers between LBC columns are possible, with increased concentration at each transfer, resulting in a "concentration pump" effect. The technique offers the advantages of great simplicity, efficiency and ease of sample transfer. Samples are obtained with low back-pressure and minimal interfering artifacts. The system shows almost complete imperturbability to moisture.

Indifference to moisture and the low back-pressure enable direct sampling of very large volumes of air and even breath. Direct sampling of aqueous systems was also possible. The latter area was not fully investigated but offers potential for water pollution analysis and in direct examination of biological fluids and aqueous flavor extracts where heat sensitivity is a problem. With LBC columns the sampling and concentration sequence exposes the substances sought to no more drastic conditions than those they will be subjected to in the process of gas chromatographic analysis.

INTRODUCTION

An increasing interest has been shown within the past few years in headspace and air analyses. The interest in the field is amply demonstrated by the large numbers of publications and symposia, and the numerous techniques devised for sampling, concentration, and analysis of volatiles.

The advent of gas chromatography (GC) has rendered headspace analysis a valuable technique in determination of volatiles at the sub-threshold levels.

* Presented at the fifth annual FACSS meeting on November 1, 1978, in Boston, Mass., U.S.A.

The techniques employed in sampling and concentrating of volatiles in the air and headspace varied with ingenuity of the researchers. The methods included direct sampling and injection on the column of up to 10 ml (refs. 1, 2), concentrating the volatiles on either pre-cooled open tubing[3], or short pre-columns packed with porous polymers[4–9], or with very small amounts of activated charcoal[10,11].

The introduction of porous polymers as trapping devices has facilitated the sampling for volatiles and enhanced the interest in headspace analysis. Environmental awareness and demands have also played a significant role.

In spite of the wide acceptance and use of porous polymers, they are not of absolute efficiency and have certain disadvantages, depending on the amount and type of polymer used, such as artifact peak formation and loss in the more volatile components especially when large volumes are sampled on short pre-columns. Butler and Burke[12] reported on the capacity and efficiency of the various polymers and concluded that each has its own merits, depending on the problem at hand, and none has universal applicability. Mackay and Hussein[13] reported briefly on some of the problems encountered in sampling on porous polymers.

This work deals with a novel technique which involves sampling of air and headspace volatiles on large-bore coated (LBC) columns. The column is thinly coated with SE-30. Other coating materials are possible.

The use of wide bore columns for sampling was suggested[14] in 1977 by using Tygon tubing to sample breath for sulfur-containing onion compounds. The results obtained from the Tygon tubing, although not spectacular, led to the idea that coated metal columns can achieve good results because of the better control in their coating and their less impermeable nature. The use of LBC metal columns was recently reported by the same authors[13]. They illustrated the advantages of sampling on LBC columns and compared them to porous polymer columns.

The predominant uses of coated open-tubular columns are in analysis rather than sampling. These, of course, are the well known Golay columns and their modifications, support-coated open-tubular (SCOT) capillary metal and glass columns. The work on the subject is too extensive to cite. However, the books of Jennings[15] and Ettre[16] give excellent background and coverage. Self *et al.*[17] utilized a few inches of coated capillary Nylon tubing as an enrichment trap for organic volatiles from headspace prior to their analysis on a coated Nylon capillary column. However, the trap was cooled, during sampling, by immersion in liquid oxygen. The flow-rate (<1 ml/min) and the amount of sample were low. Larger volume samples were possible if the concentration of water vapor was low.

Success in GC headspace analysis of very diluted mixtures depends on optimal concentration and transfer of the trace volatiles onto the analytical column. This requires that all components are at maximum concentration in a short single band at the head of the analytical column. This concentration effect is achieved after sampling on LBC column by transfer of the trapped volatiles to a short Tenax-GC pre-column. The use of Tenax-GC pre-column, however, can be avoided by using another shorter, narrower bore LBC column than the sampling column for the transfer trap. The sample is then eluted thermally off the transfer trap onto the analytical column, and the analysis is initiated concurrently with the final desorption.

Due to ease of elution, a single transfer of the trapped sample off the LBC column onto the receiving trap is usually sufficient to achieve the narrow concentra-

tion band on the analytical column. However, multiple transfers which give a "concentration pump" effect can be done, giving a concentration factor of about 10 times at each transfer.

This work demonstrates the applicability of LBC columns to sampling of organic volatiles. The factors affecting the trapping efficiency of LBC columns, such as column length, thickness of coating, sample volume, and sampling flow-rate are investigated. The use of stationary phases other than SE-30 as wall coating materials will be explored in future publications, and also the effect of greatly increased diameter.

Although the main utility of LBC columns is in sampling for and concentration of organic volatiles, some separation can be achieved if used as GC columns. A 50 ft. $\times$ $\frac{1}{4}$ in. O.D. (0.186 in. I.D.) with 0.014 mm SE-30 wall coating has approximately 4000 theoretical plates.

The use of LBC columns offers the advantages of efficiency, low back-pressure, minimal interfering artifacts, ease of sample elution, and indifference to moisture.

The low back-pressure and indifference to moisture are very useful features in breath analysis whereby large samples can be collected by directly blowing on the LBC column with minimal precaution to prevent introduction of saliva onto the column. To minimize moisture condensation, the column was maintained at 50° while sampling.

As a demonstration of the effectiveness of LBC columns in entrapment of organic compounds, flavorants were successfully analyzed for by direct sampling of their extremely dilute aqueous solutions on the LBC column. Flavor profiles were also obtained for soft beverages and beers.

Liquid sampling on LBC columns has a potential in many areas especially in analysis for water pollutants, fingerprinting of oil spills for identification of the source, and in quality control of beverages where a fingerprint of the flavor in the finished product can be easily obtained. This topic is currently being pursued and will be detailed further.

LBC columns are extremely efficient in retention of organic volatiles. However, losses are encountered with the very volatile components. The extent of losses depends on the sampling volume, sampling flow-rate, length and diameter of LBC column, and amount of wall coating.

The utility of LBC columns in sampling for organic volatiles in gaseous media and the factors affecting the efficiency of trapping are demonstrated in this work by a simulated headspace system giving consistently identical samples. A 0.1-μl volume of peppermint oil was deposited in the inlet side of a cold finger-condenser which was maintained at 40°. The effluent side of the condenser was connected to the LBC column with a short PTFE tube. The inlet side of the condenser was connected to a high-purity nitrogen tank. Sampling was initiated by starting the flow of nitrogen, which was previously set at 100 ml/min, and continued until the desired volume was reached.

Upon collection of the sample, the LBC column is heated to desorb the sample onto a short Tenax-GC pre-column or onto another LBC column, which is in turn thermally eluted for analysis onto the analytical GC column.

EXPERIMENTAL

Gas chromatographic conditions

A Perkin-Elmer Model 3920 gas chromatograph with flame-ionization detector was used. The column used was a single aluminum 8 ft. × $\frac{1}{4}$ in. O.D. (0.186 in. I.D.) packed with 10% Carbowax 20M on Chromosorb W 80–100 mesh (acid washed and DMCS treated). The carrier gas was nitrogen at a flow-rate of 50 ml/min, measured at ambient temperature. The column oven temperature was programmed at 4°/min from 70 to 230°. Injector and interface temperatures were 250°. Recorder: 5 mV at 0.5 in./min chart speed. The integrator used was a Vidar Model 6230.

Materials

Pre-column packing and LBC coating materials were (a) Tenax-GC (60–80 mesh; Applied Science Labs., State College, Pa., U.S.A.) and (b) SE-30 silicon rubber (100% methyl) (Analabs, North Haven, Conn., U.S.A.).

The pre-columns used were short lengths of Pyrex glass tubing 15 cm × 0.6 cm O.D. (0.38 cm I.D.). The material was packed between two glass wool plugs in the pre-column so that the packing is within the hot zone of the tube heating oven which is used for sample desorption.

Large-bore coated columns

Large-bore aluminum ($\frac{1}{4}$ in. O.D.; 0.186 in. I.D.) and stainless-steel ($\frac{1}{8}$ in. O.D.; 0.08 in. I.D.) tubings of the desired length were coiled to fit in the oven of a gas chromatograph which was used for desorption of the sample. The column was cleaned by repetitive aspiration of benzene and dried. Coating of the column with SE-30 was done following the procedure previously described[13]. The weight of coating in a 50 ft. × 0.186 in. I.D. column ranged from 1.8 to 4.5 g. Thicker coating may be achieved by repetitive application of the SE-30 solution and drying. Thinner coating may also be achieved by quick passage of the solution through the column. Each gram of SE-30 coating in a 50 ft. × 0.186 in. I.D. results in a coating thickness of 0.0046 mm, on the assumption of a uniform coating.

Sampling

A 0.1-μl volume of peppermint oil was deposited onto a small glass wool plug placed in the inlet of a cold-finger condenser. A 0.1-μl syringe fitted with a Chaney adaptor was used to minimize variation in injection size. The condenser, which was maintained at 40° in a water bath prior to deposition of the sample, was connected with a PTFE tube to a purified nitrogen cylinder. The sampling column was connected to the effluent side of the sampling system and the nitrogen flow, which was previously adjusted to the desired rate, was initiated and continued until the desired sampling volume was attained.

The method previously described by Hussein and Mackay[13] was followed in collection of actual air and/or headspace samples.

Sample desorption

The sample was eluted off the sampling LBC column onto a short Tenax-GC

pre-column, or onto a shorter, narrower bore LBC column than the one used for sampling. The sampling column was heated at 250° for 12 min while 50 ml/min nitrogen was flowing through it. The receiving pre-column or short LBC column was outside the heating oven and connected to the sampling column with a short 1/16-in. O.D. stainless-steel tube. The receiving pre-column or LBC column was at ambient temperature.

The sample was eluted for analysis off the receiving Tenax-GC pre-column onto the analytical column as described in the previous work[13,14]. The receiving LBC column was heated to 225° for 6 min, with a heating tape controlled with a thermostat, for desorption of the sample. The nitrogen flow of 50 ml/min of the analytical column was redirected through the LBC column during elution with a Toggle valve.

The LBC elution process was repeated when multiple sample transfer (concentration pump) was desired.

Liquid sampling

A 20-ml aliquot was poured through a 25 ft. × 0.25 in. O.D. (0.186 in. I.D.) LBC column (0.019-mm SE-30 wall coating). The effluent sample was collected in a beaker and recirculated 4 times through the LBC column. The LBC column was rinsed with 2 × 20 ml distilled water to eliminate or minimize any non-volatile residuals remaining on the column.

The column was then purged with nitrogen at 100 ml/min for 5 min to eliminate the bulk of the moisture. The sample was eluted off the LBC column by heating at 250° for 12 min with a 50 ml/min nitrogen flow and trapped onto a Tenax-GC pre-column connected (if desired) to a back-up charcoal pre-column containing 5 mg charcoal, 90–100 mesh. Both pre-columns were maintained at ambient temperature. The use of the charcoal is to trap escaping volatiles off the Tenax-GC pre-column.

The extent of moisture on the pre-column depends on the nature of the sample. If the moisture is excessive, the pre-columns, in tandem, are purged with nitrogen at 100 ml/min until no visible moisture is noted. The direction of nitrogen flow should be the same as that of sample elution onto the pre-columns. The time required varied from 15 to 50 min.

An alternative procedure for minimizing moisture condensation on the pre-column is to maintain their temperature at 50° during elution of the sample off the LBC column. An infrared lamp can be used for this procedure.

The final analyses were performed by desorption of the volatiles at 250° in the case of the Tenax-GC pre-column, and at 300° for the charcoal pre-column.

RESULTS AND DISCUSSION

Peppermint oil was chosen for the simulated headspace sampling method because of its complexity and the diversity in volatility of its constituents, and because it is widely used and identities of its components are well established.

The simulated headspace sampling method is similar to real situations of volatile stripping. However, continuation of sampling beyond 3 l of the stripping gas, when the sample has already been effectively transferred onto the LBC column, is a severe test of the trapping efficiency of the sampling device, since, contrary to usual headspace sampling, the organic volatiles introduced into the column in the first few

TABLE I

COMPARISON OF SAMPLING EFFICIENCY OF IDENTICAL AMOUNT OF PEPPERMINT OIL USING VARYING SAMPLING VOLUMES ON LBC COLUMN AND A SHORT TENAX-GC PRE-COLUMN

LBC column: 50 ft. × 0.186 in. I.D.; 0.014 mm coating. Tenax-GC pre-column: 200 mg Tenax.

Compound	*Amount in 0.1-µl sample (µg)*	*Sampling efficiency (%)**			
		LBC column			*Tenax-GC pre-column; 3 l*
		3 l	*24 l*	*100 l*	
α-Pinene	0.9	101.9	8.2	0	18.1
β-Pinene	1.4	98.2	19.7	0	32.6
Limonene	1.4	108.3	149.7	147.0	109.1
Eucalyptol	5.3	95.7	25.1	14.4	47.4
Menthone	18.9	91.8	84.1	69.4	91.5
Menthofuran	3.9	101.3	87.7	73.8	86.1
Isomenthone	3.1	106.4	217.8	207.4	83.5
Menthyl acetate	5.0	94.3	92.7	76.1	98.4
Neomenthol	4.8	96.7	76.0	38.0	91.8
Menthol	41.3	96.1	88.5	62.3	96.3

* Expressed as percentage of amount found in direct injection of same amount of oil.

liters, are now followed by very large volumes of pure carrier gas which tends to the volatiles right through the trap.

The data in Table I compare the retention of peppermint oil components on

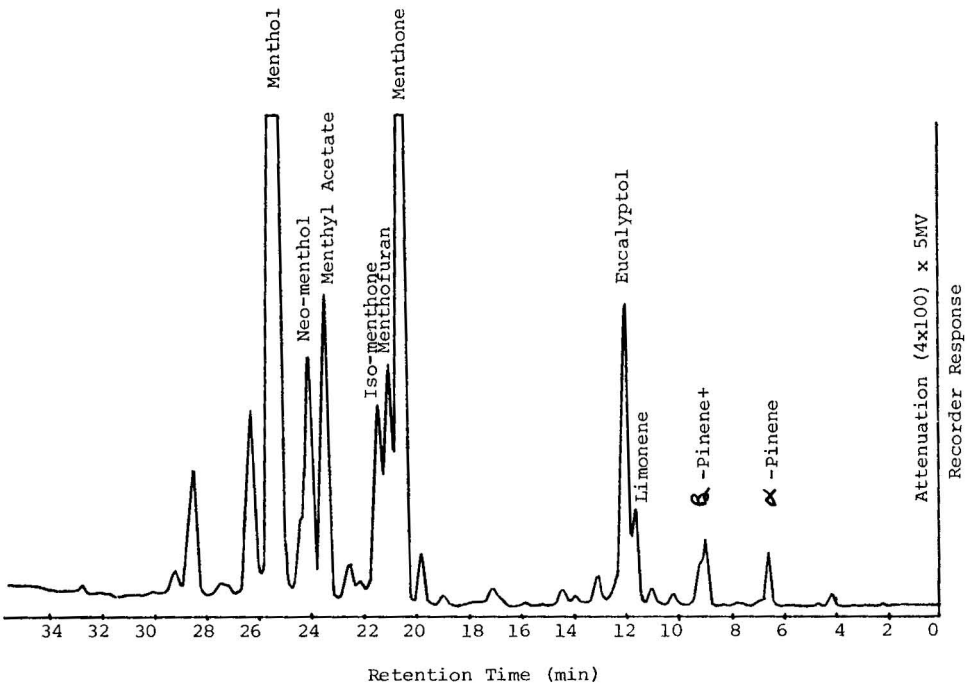

Fig. 1. Analysis of peppermint oil; 0.1-µl sample injected directly into the gas chromatograph.

a 50 ft. × 0.186 in. I.D. LBC column and a wall coating of 0.014 mm SE-30 with various sampling volumes from 3 to 100 l, and retention of the same components on a short Tenax-GC pre-column with 3 l of flushing gas. Retention of the more volatile components, α- and β-pinene, with this sampling volume is better on the LBC column than the Tenax pre-column.

The higher the volume of gas passed through the column, the higher the loss, especially of the more volatile compounds. The high value of limonene can not be explained except for the possibility of an artifact that is more evident with the large flush volume. The high value of isomenthone is also hard to explain except for the possibility of isomerization of some menthone. Such isomerization with heat is possible as has been reported by De Mayo[18].

Figs. 1 and 2 show GC analyses of 0.1 μl peppermint oil by direct injection and by sampling on a 50 ft. × 0.186 in. I.D. LBC column (0.014 mm SE-30 coating) with 3 l nitrogen. The LBC column sample was in turn eluted onto a Tenax-GC pre-column. Aside from the two artifact peaks in the LBC analysis, the chromatograms are similar. The encountered artifacts are generated from the SE-30 coating. The presence of these artifacts will interfere in the analysis only of highly volatile constituents.

The volatile organic compounds are retained by the LBC column due to absorption by the wall coating. The extent of retention of the sample components is, therefore, affected by dimensions of the LBC column, amount of wall coating, and

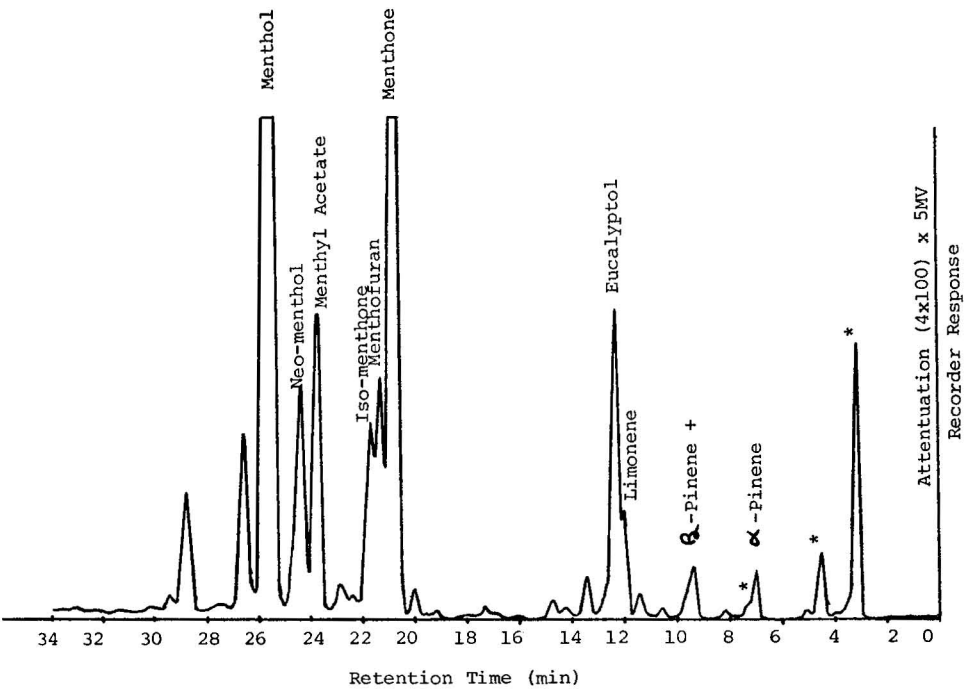

Fig. 2. Analysis of 0.1 μl peppermint oil after sampling on LBC column by sweeping with 3 l nitrogen. * = Artifact from LBC column.

volume of sample or sweep gas. Table II shows differences in retention of components of the identical amount of peppermint oil when sampled with 3 l nitrogen on LBC columns with various wall coating thickness, length, and internal diameter.

TABLE II

COMPARISON OF SAMPLING EFFICIENCY OF LBC COLUMNS OF VARIOUS DIMENSIONS AND WALL COATING WITH IDENTICAL AMOUNTS OF PEPPERMINT OILS AND SAME SWEEP VOLUMES

Column dimensions: A, 5 ft. × 0.186 in. I.D. (wall coating, 0.104 mm); B, 5 ft. × 0.186 in. I.D. (wall coating, 0.025 mm); C, 10 ft. × 0.186 in. I.D. (wall coating, 0.030 mm); D, 3 ft. ×0.085 in. I.D. (wall coating, 0.066 mm); E, 5 ft. × 0.085 in. I.D. (wall coating, 0.055 mm).

Compound	*Sampling efficiency (%)* *				
	A	*B*	*C*	*D*	*E*
α-Pinene	120.1**	11.9**	44.1	0	0
β-Pinene	90.9	0.5	84.1	0	0
Limonene	109.9	84.3	126.7	20.2	30.0
Eucalyptol	104.3	33.8	102.8	3.3	4.1
Menthone	99.7	80.9	86.6	81.2	87.6
Menthofuran	101.7	95.6	89.7	86.3	92.0
Isomenthone	99.6	101.7	112.8	129.2	145.7
Menthyl acetate	110.5	83.7	92.3	90.1	99.3
Neomenthol	112.0	91.0	98.8	98.0	105.0
Menthol	102.4	88.3	93.8	92.1	102.3

* Expressed as percentage of amount found in direct injection of same amount of 0.1 μl.
** Some artifact interference.

The effect of the coating thickness is noted in columns A and B of Table II when the same dimension columns were coated with 0.104 and 0.025 mm. The higher thickness resulted in full retention of all components, while appreciable losses are noted in α- and β-pinenes from the lower thickness of wall coating, but when the internal diameters are equivalent, the longer column is more retentive as can be seen from columns B and C of Table II.

The wider bore LBC columns, unexpectedly, are more efficient than the narrower bore ones even if the wall coating is thicker in the latter, as seen from columns B and E of the table.

In all cases the less volatile components, menthol, neomenthol, and menthyl acetate are fully retained.

The flow-rate of sampling onto the LBC column also affects the extent of retention of the organic volatiles by the column. Table III shows a comparison in retention when the identical samples are swept on the column with 12 l nitrogen at 100 and 500 ml/min. While full retention of all components was obtained with the 100 ml/min flow-rate, appreciable losses occurred in the more volatile components (the pinenes, limonene, and eucalyptol) with the high flow-rate.

As an illustration of the utility of LBC columns in real situations, volatile organics were determined in the air of a confectionery plant by sampling 6 l at 100 ml/min on a 50 ft. × 0.186 in. I.D. column coated with a 0.014-mm layer of SE-30. The analysis is shown in Fig. 3. In addition to sampling for volatiles, solids

TABLE III

COMPARISON OF SAMPLING EFFICIENCY OF IDENTICAL AMOUNTS OF PEPPERMINT OIL ON SAME LBC COLUMN USING SAME SAMPLING VOLUME (12 l) BUT VARYING SAMPLING FLOW-RATE

Compound	*Sampling efficiency (%)* *	
	100 ml/min	*500 ml/min*
α-Pinene	96.4	23.0**
β-Pinene	97.7	13.7
Limonene	109.5	86.0
Eucalyptol	104.6	59.7
Menthone	99.7	82.7
Menthofuran	101.8	83.6
Isomenthone	112.6	95.3
Menthyl acetate	87.3	107.2
Neomenthol	107.3	101.0
Menthol	94.8	80.3

* Expressed as percentage of amount found in direct injection of same amount of oil.
** Some artifact interference.

were also sampled and analyzed for simultaneously in the same sample by attaching a short pyrex glass tubing containing a small wad of pre-cleaned glass wool to the inlet of the LBC column. The volatile organics were trapped on the LBC column while the solids (sucrose and glucose) were retained by the glass wool pre-filter. The glass-wool filter was extracted with 10 ml distilled water and sucrose and glucose were determined as silyl ether derivatives by GC on an OV-17 column[19]. The amounts of sucrose and glucose were 12.4 and 0.6 μg/l air, respectively. The total organic volatiles in the air sample was 1.58 mg, *i.e.* $2.6 \cdot 10^{-4}$ g/l.

Identities of the components indicate that peppermint, spearmint, and wintergreen oils are in the sampled air. Candies were being manufactured with the three products at the time.

In a previous work by the authors[13] comparisons of sampling large air volumes on LBC and long Tenax-GC columns were shown. Sampling on the latter was reported to have the disadvantage of large interfering artifacts. However, retention of the extremely volatile components, excluding alcohols, was found to be better on the long Tenax column. The ideal system is simultaneous sampling on both LBC and long Tenax columns. The sample from the Tenax column is utilized for analysis of the highly volatile components, using the LBC sample for the remaining components.

Sub-threshold levels of air odorants were previously[13] determined through sampling large air volumes on LBC columns outside a confections manufacturing plant.

Since the artifact interference from the Tenax-GC is not significant when a short pre-column with a small amount of Tenax is used, the final sample elution was made onto this type of pre-column. The short pre-columns were used for convenience. The use of porous polymers can be avoided by carrying out the final elution onto a shorter and narrower LBC column than the one used for sampling. This was successfully carried out with comparable results to those obtained when the Tenax-GC pre-columns were used as the final elution traps.

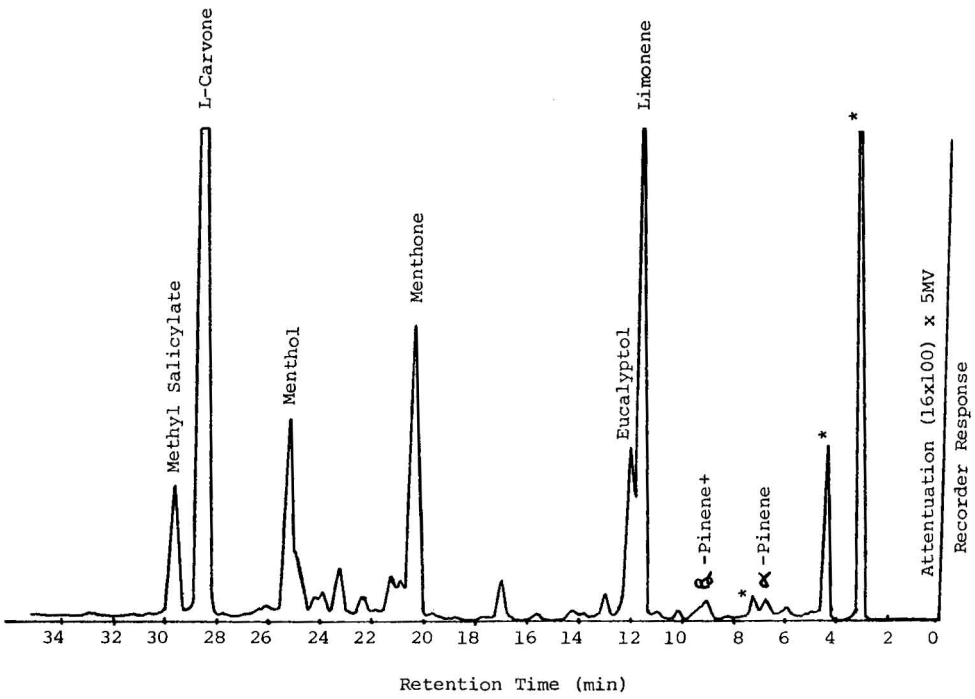

Fig. 3. Analysis of 6 l air in confections plant; sampled directly onto LBC column at a flow-rate of 100 ml/min, desorbed onto a short Tenax-GC pre-column. * = Artifact from LBC column.

TABLE IV

COMPARISON OF MULTIPLE SAMPLE TRANSFERS (CONCENTRATION PUMP) BETWEEN TWO LBC COLUMNS

A 0.1-μl volume of peppermint oil was swept onto the first column with 3 l nitrogen.
A, Sample trapped on a 50 ft. × 0.186 in. I.D. LBC column (0.014 mm coating) and transferred onto another 5 ft. × 0.186 in. I.D. column (0.104 mm coating) followed by elution onto Tenax-GC precolumn and final analysis. B, Sampling and transfer columns are same as in A. Sample was cyclically transferred between the two columns 4 times prior to final elution onto Tenax-GC pre-column and analysis.

Component	*Recovery (%)**	
	A, 1 transfer	*B, 4 transfers*
α-Pinene	**	**
β-Pinene	76.1	20.8
Limonene	127.4	67.7
Eucalyptal	111.0	100.5
Menthone	76.2	54.6
Menthofuran	96.7	60.2
Isomenthone	101.7	203.2
Menthyl acetate	133.7	89.1
Neomenthol	109.6	64.3
Menthol	103.8	58.2

* Compared to amount found in direct injection 0.1 μl peppermint oil.
** Not determined due to an interfering artifact.

Multiple transfer of the trapped sample between two LBC columns was carried out to demonstrate its feasibility. However, the ease of sample elution off the LBC column usually renders the process of multiple transfer or "concentration pump" unnecessary except for extremely dilute concentrations.

The data presented in Table IV illustrate the feasibility of the principle with relatively little sample loss. Recovery of trapped components after two and four multiple transfers between the 50-ft. LBC column and another 5-ft. one shows the higher the number of transfers the higher the loss of sample. However, achieving 60% or higher recovery of the sample after four transfers, although not ideal, is satisfactory considering the small sample (90 μg).

Although the concentration ratio between the original sample volume and volume of carrier gas used for the final transfer is 5, the extent of concentration can be much larger depending on the initial sampling volume, the number of transfers, and types of columns.

Indifference of the LBC columns to moisture and their low back-pressure feature enables sampling and analysis of large volumes of breath for organic volatiles. The sample was collected by directly blowing into the column through a PTFE mouthpiece which contained a glass-wool plug to inhibit introduction of saliva onto the column. The column was maintained at 50° during sampling to minimize condensation of moisture. Fig. 4 shows analysis for sulfur components in 5 l of breath after the mouth was rinsed with dilute onion oil solution. The procedure for mouth

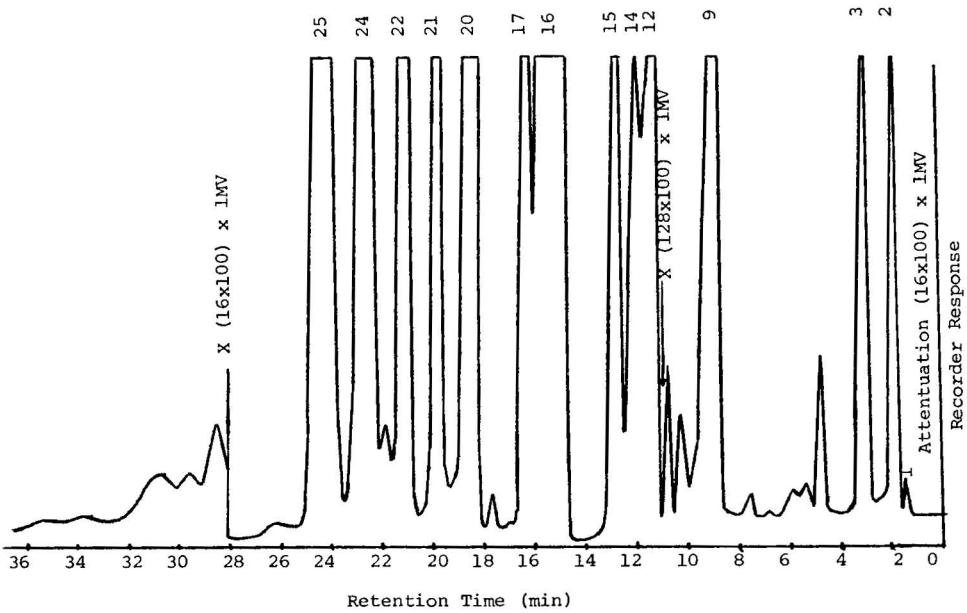

Fig. 4. Analysis for sulfur compounds in breath following mouth rinse with 0.005% onion oil solution; 10 large breaths sampled directly on LBC column (50 ft. × 0.186 in. I.D.; 0.014 mm SE-30 coating). Sample was desorbed onto short Tenax-GC pre-column and eluted on GC column for analysis. FPD detector was used; other GC conditions are same as previously reported[14]. Tentative peak identity: 1 = methyl mercaptan; 2 = dimethyl sulfide; 3 = propyl mercaptan; 9 = dimethyl disulfide; 16 = dipropyl disulfide.

odorization and analysis are the same as reported in the previous work[14], The work should be consulted for further reference on onion breath. Except for a slight methyl mercaptan odor, no onion odor was noted in the LBC column effluent upon sampling of the breath. Analysis of the breath on a LBC column for peppermint oil components after ingestion of peppermint candy was reported in a previous work[13].

Effectiveness of the LBC columns in retaining organic volatiles is dramatically illustrated by their use in entrapment and analysis of trace peppermint oil constituents in an aqueous solution containing a trace amount of peppermint oil. Fig. 5 shows analysis for peppermint oil components after a 20-ml aliquot of a 10-ppm solution was recirculated through a 25 ft. × 0.186 in. I.D. LBC column, coated with 2.1 g SE-30 at approximately 0.019 mm thickness.

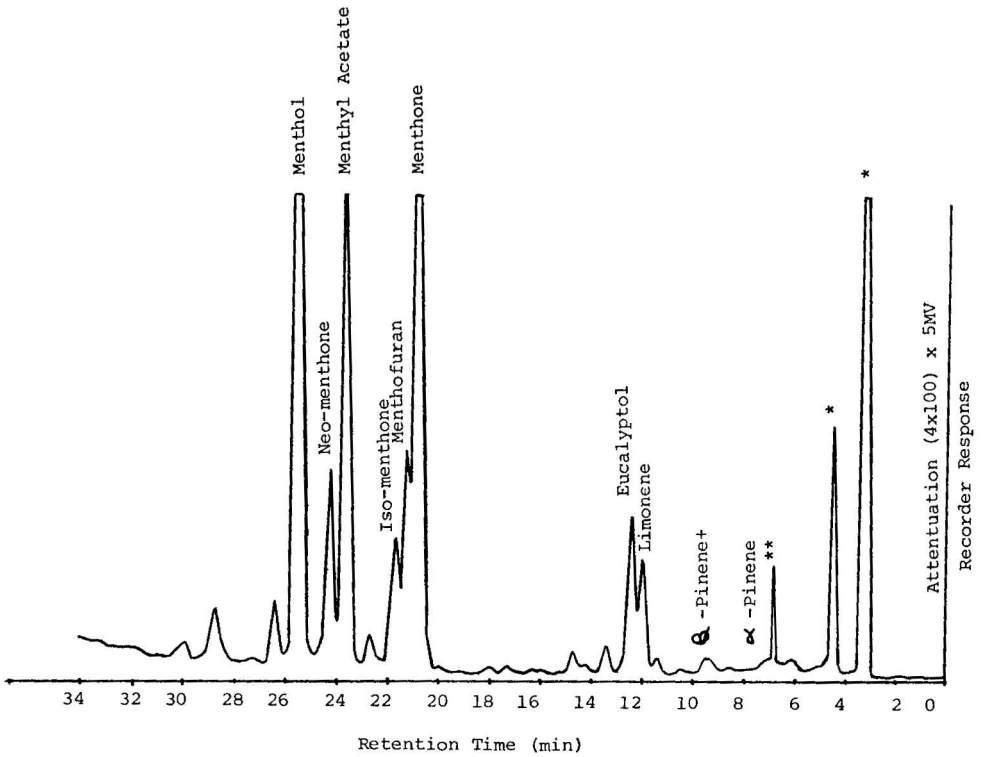

Fig. 5. Analysis of dilute aqueous peppermint oil solution (0.001 % peppermint oil) by direct sampling onto 25 ft. × 0.186 in. I.D. LBC column; 20-ml aliquot was recirculated 5 times through the column. * = Artifact from LBC column; ** = quench effect of residual moisture.

The recirculated aliquot contained 0.2 μl peppermint oil. Recovery of the individual components varied. While 68 % of the menthyl acetate and 60 % of the menthone in the aliquot was trapped and accounted for, only 23 % of menthol was recovered. The wide difference in recoveries from the solution points to preferential partition by the SE-30 coating.

Although the profile of the peppermint oil components recovered by the LBC

column from the aqueous solution differs from that of direct analysis of the oil (Fig. 1), it still is unmistakably that of peppermint.

CONCLUSIONS

The LBC columns have been proven effective in concentration of volatiles from air. The features of low back-pressure and the indifference to moisture render the technique extremely useful in analysis for organic volatiles in the breath and large air samples.

Direct sampling of liquids on LBC columns is a promising area especially for water pollution analysis and quality control of flavors in beverages.

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REFERENCES

1 J. Tonzetich, *Arch. Oral Biol.*, 16 (1971) 587.
2 B. T. Larsson, *Acta Chem. Scand.*, 19 (1965) 159.
3 R. Teranishi, T. R. Mon, A. B. Robinson, P. Cary and L. Pauling, *Anal. Chem.*, 44 (1972) 18.
4 A. Dravnieks, B. K. Krotszynski, J. Whitfield, A. O.Donnell and T. Burgwald, *Environ. Sci. Tech.*, 5 (1971) 1220.
5 W. G. Jennings, R. H. Wohleb and M. J. Lewis, *J. Food Sci.*, 37 (1972) 69.
6 A. Zlatkis, W. Bertsch, H. A. Lichtenstein, A. Tishbee, F. Shunbo, H. M. Liebich, A. M. Cocia and N. Fleischer, *Anal. Chem.*, 45 (1973) 763.
7 M. Novotny, M. L. Lee and K. D. Bartle, *Chromatographia*, 7 (1974) 333.
8 J. P. Mieure and M. W. Dietrich, *J. Chromatogr. Sci.*, 11 (1973) 559.
9 J. W. Russell, *Environ. Sci. Tech.*, 9 (1975) 1175.
10 R. G. Clark and D. A. Cronin, *J. Sci. Food Agr.*, 25 (1975) 1615.
11 K. Grob and G. Grob, *J. Chromatogr.*, 62 (1971) 1.
12 L. D. Butler and M. F. Burke, *J. Chromatogr. Sci.*, 14 (1976) 117.
13 M. M. Hussein and D. A. M. Mackay, in G. Charalambous and G. Inglett (Editors), *Flavor of Foods and Beverages—Chemistry and Technology*, Academic Press, New York, 1978, p. 209.
14 D. A. M. Mackay and M. M. Hussein, in G. Charalambous (Editor), *Analysis of Foods and Beverages—Headspace Techniques*, Academic Press, New York, 1978, p. 283.
15 W. Jennings, *Gas Chromatography with Glass Capillary Columns*, Academic Press, New York, 1978.
16 L. S. Ettre, *Open Tubular Columns In Gas Chromatography*, Plenum Press, New York, 1965.
17 R. Self, D. G. Land and J. C. Casey, *J. Sci. Food Agr.*, 14 (1963) 209.
18 P. de Mayo, in K. W. Bentley (Editor), *The Chemistry of Natural Products, Vol. II, Mono- and Sesqueterpenoids*, Interscience Publishers, New York, 1959, p. 76.
19 G. D. Brittain, J. E. Sullivan and L. R. Shewe, in I. Domsky and G. Perry (Editors), *Recent Advances in Gas Chromatography*, Marcel Dekker, New York, 1971, p. 223.

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RETENTION OF FLUORINATED TRANSITION-METAL β-DIKETONATES IN GAS CHROMATOGRAPHIC COLUMNS

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SUMMARY

Retention of the Al(III), V(III), Cr(III), Fe(III), Mn(III), Co(III) and Cu(II) derivatives of the β-diketones, 1,1,1-trifluoropentane-2,4-dione, 1,1,1,5,5,5-hexafluoropentane-2,4-dione, 1,1,1-trifluoro-5-methylhexane-2,4-dione, 1,1,1-trifluoro-5,5-dimethylhexane-2,4-dione, 1,1,1,2,2-pentafluoro-6,6-dimethylheptane-3,5-dione and 1,1,1,2,2,3,3-heptafluoro-7,7-dimethyloctane-4,6-dione in chromatographic columns with various supports has been studied. Chemical effects due to the nature of the support, ligand and the metal ion probably contribute to the observed magnitude of the retention. Irreversibility of the phenomenon is interpreted as arising from dissociation in the stationary phase and retention therein or to reaction at active sites on the support. Several mechanisms for these reactions are proposed.

INTRODUCTION

There is now ample evidence[1–3] that many chelates (involving different metals and ligand systems) exhibit the volatility and thermal stability necessary for gas chromatography (GC). Yet, due to the *hetero-molecular* interactions which can occur when these compounds are introduced into a chromatographic column (in contrast to the essentially *homo-molecular* environment prevailing during the evaluation of thermal properties), numerous problems become apparent. Inevitably, the result has been a delay in the application of the GC of metal chelates to a wider range of analyses.

For the most widely studied class of chelates, the β-diketonates, many have been shown to be too reactive for chromatographic analysis. Even in solution reactions unfavourable to chromatographic separation are known to occur. For example, derivatives of lanthanide ions, including those of ligands with sterically hindering substituents, associate[4] in solution forming binuclear and trinuclear clusters. Another peculiarity of β-diketonate reactivity, which has been demonstrated by GC[5], is their participation in "scrambling" reactions[5–8] in solution. Furthermore, among the ab-

normalities reported[9–11] for β-diketonates as evidence of their on-column reactions have been "adsorption" and displacement effects, extraneous peaks and shoulders, unusual peak shapes and elevated baselines. Additional on-column reactions are the "olation" reaction occurring with monohydrates of various lanthanide acetylacetonates[12], and reaction of the β-diketonates of the alkali and alkaline-earth metals[13–15] leading to the formation of heteronuclear species. On-column redox transformations[5,16] have also been observed with the β-diketonates of both vanadium(III) and oxovanadium(IV), and may even occur with chelates of other transition metal ions such as cobalt, iron and manganese.

From these observations, it is possible to envisage two different phenomena occurring inside the packed GC column. In the first, which ultimately sets the chromatographic limits of detection, the mainly *physical* effect includes true adsorption and perhaps, displacement effects displayed by the support. Since weak forces are generally involved, this process is reversible and provides a plausible explanation of the regular deactivation of columns of the type reported in the earlier and successful[17–20] gas chromatographic studies of the less reactive β-diketonates. Those which do not show obvious reactivity, such as the trifluoroacetylacetonates of Al(III)[17,18], Ga(III)[18], In(III)[18] and Rh(III)[19], or the still more favourable derivatives of Be(II)[20] and Cr (III)[21] which have been determined at sub-nanogram levels, probably fall into this category. The second group of phenomena includes the diverse reactions of a *chemical* nature such as those dealing with the chelates *per se*, or their reactivity towards a stationary phase or, indeed, impurities therein or in the support. Into this group would be placed those instances of marked retention* in the column irrespective of the chemical reaction involved. In fact, many of these reactions are not well understood and some, at least, may be no more than the predictable consequence of reactions, at elevated temperatures, of impurities present within the column or introduced with the chelates (especially traces of water, oxygen or solvents[22]). An example is the retention of the stable chromium(III) chelate of trifluoroacetylacetone in columns containing diatomite supports, and this has been attributed[23] to surface silanol (–Si–OH) groups. As is shown later, improvements in column performance achieved with silylated supports are enhanced by further on-column silylation before use. In other reactions, column performance may be expected to deteriorate under the influence of chelates capable of functioning as Lewis acids in the chemical degradation[22] of polysiloxane stationary phases, and there is evidence[24] for this when dealing with fluorinated alkyl and the more volatile of the fluorinated aryl β-diketonates.

Within this context, this paper presents data illustrating several features of the two effects, originating from the chelates of various transition metal ions and column support materials, upon the column behaviour of a group of β-diketonates. Inasmuch as the chemical effect leads to irreversible chemical reactions in the column, we refer to this retention of chelates as *absorption* in preference to, and by way of distinction from, adsorption. The chelates used for the study comprise the aluminium(III),vanadium(III), chromium(III), iron(III), manganese(III), cobalt(III) and copper(II) complexes of 1,1,1-trifluoropentane-2,4-dione (HTfa), 1,1,1,5,5,5-hexafluoropentane-2,4-dione (HHfa), 1,1,1-trifluoro-5-methylhexane-2,4-dione (HTbm), 1,1,1-trifluoro-5,5-

* Retention, here, is not to be confused with the partitioning process occurring in the GC column.

TABLE I

INSTRUMENTAL CONDITIONS

Parameters	*Descriptions*
GC column	Packed glass, 8 ft. × 2 mm I.D. 3% Dexil 300 on Supelcoport (100–200 mesh)
Oven temperature	175–300° at 4°/min
Injection temperature	300°
Detector temperature	300°
Auxiliary temperature	300°
Carrier gas	Helium, 33 ml/min
Sample size	1 μl
GC detector	FID
Ionization mode	Electron impact
Electron energy	70 eV
Emission	200 μA
Ion source pressure	3×10^{-6} Torr
Ion source manifold	270°
Analyzer manifold	130°

RESULTS AND DISCUSSIONS

The gas chromatogram of the PAHs in Charles River sediment is shown in Fig. 1. The major PAHs are determined and tabulated in Table II. Obviously, a pyrolytic origin is indicated by the predominant parent PAHs. Some of the components are not determined because of the lack of standard compounds. However, their structures are proposed based on retention times and mass spectra. They are also indicated in Fig. 1.

Sulfur-containing and alkylated PAHs are searched for by spiking with dibenzothiophene and 1-methylpyrene. The spiked dibenzothiophene eluted right before phenanthrene, but further confirmation with mass spectra showed that the GC peak of Charles River sediment in the region of the dibenzothiophene peak contains a mixture of compounds. Dibenzothiophene was then detected using the data system with selected ion monitoring at the mass of its molecular ion; the response indicates a level of less than 500 ng/g. The spiked 1-methylpyrene showed up in the methylated fluoranthene and pyrene region without a precise superimposition on any of the peaks in that region. The level of these methylated PAHs is 0.9–1.1 μg/g based on their FID signals and abundances of their molecular ions. Dibenz[*a*,*c*]anthracene, dibenz[*a*,*h*]anthracene and *o*-phenylenepyrene all eluted together. Their determination is based only on molecular ion abundances.

The recoveries in Table II are calculated based on the differences in FID signals between the spiked and unspiked samples against the FID signals of the standards. The recoveries are lower for dibenzothiophene and for larger PAHs. In order to trace out the steps which cause these lower recoveries, the following experiments were performed. (a) The sediment was ultrasonically extracted with two additional portions of 60 ml of cyclohexane after the usual two extractions. (b) Standard compounds were partitioned between 20 ml volumes of cyclohexane and cyclohexane-saturated nitromethane and their partition coefficients determined by GC. (c) Standard compounds

TABLE II

MAJOR PAHs IN CHARLES RIVER SEDIMENT

PAH	*Concentration ± S.D. (μg/g)*	*Recovery (%)*
Phenanthrene/anthracene	2.9 ± 0.4	96.1
Fluoranthene	7.8 ± 0.3	99.1
Pyrene	7.1 ± 0.2	99.2
Benz[*a*]anthracene	3.4 ± 0.5	89.4
Chrysene/triphenylene	3.8 ± 0.5	89.7
Benzo[*b*/*k*]fluoranthene	6.0 ± 0.3	88.1
Benzo[*a*/*e*]pyrene	5.7 ± 0.7	71.5
Perylene	0.9 ± 0.2	85.2
Dibenz[*a*,*c*/*a*,*h*]anthracene	0.7 ± 0.2	80.0
o-Phenylenepyrene	2.3 ± 0.5	82.2
Benzo[*g*,*h*,*i*]perylene	2.8 ± 0.3	85.3
Coronene	0.5 ± 0.1	78.1
Dibenzothiophene	<0.5	85.1
1-Methylpyrene		92.1
Methyl-(pyrene/fluoranthene)	0.9–1.1	

were deposited onto the silica gel column. Then they were washed, eluted and analyzed exactly as the real samples.

No improvement in recovery is obtained with two additional extractions. Also, Soxhlet extraction for 18 h yielded the same results. Both observations indicated that losses due to incomplete extraction of the spikes are very unlikely.

The partition coefficients of the PAHs of interest between nitromethane and cyclohexane are listed in Table III. They range from 1.2 to 2.8. In general, the coefficient is lower for alkylated PAHs and higher for larger PAHs. Comparison of the coefficients between fluoranthene and pyrene, benzo[*a*]pyrene and perylene, dibenz-[*a*,*c*]anthracene and benzo[*g*,*h*,*i*]perylene indicates that PAHs of the same molecular weight but with more compact molecular structures have lower coefficients. However, even with the lowest partition coefficient (1.2 for 1-methylpyrene) less than 1 % is left in the discarded cyclohexane layer after six equal-volume partitions. This loss is within the uncertainty of the final results and will not cause the variation in recoveries. This conclusion agrees with the observation that the final recovery of 1-methylpyrene is not lower than all the others.

The recoveries of standard compounds from a silica gel column are listed in Table IV. They vary from 78.6 to 100.1 %. This variation in recovery has a similar trend as that of the whole analysis but much less in magnitude. It appears that irreversible adsorption on the silica gel column partially accounts for the low recoveries.

Since the steps in the sample preparation tested with standard compounds do not show as wide a variation in recovery as the whole analysis, there are two factors left to be considered: stability and matrix effect. It has been suggested that some PAHs are less stable than the others, for example, BaP is less stable than pyrene and the BaA skeleton structure can undergo ring isomerization[8]. These instabilities will show up more in the whole analysis which takes a longer period of time than the single steps. BaP has consistently shown lower recovery than its isomer perylene which elutes quite close to it on the GC.

TABLE III

PARTITION COEFFICIENTS BETWEEN NITROMETHANE AND CYCLOHEXANE

PAH	*Partition coefficient*
Dibenzothiophene	1.8
Phenanthrene	1.9
Fluoranthene	1.9
Pyrene	1.5
1-Methylpyrene	1.2
Benz[*a*]anthracene	2.0
Chrysene	2.0
Benzo[*a*]pyrene	2.0
Perylene	2.5
Dibenz[*a*,*c*]anthracene	2.8
Benzo[*g*,*h*,*i*]perylene	2.2
Coronene	2.2

Another difference between single-step analysis and the whole analysis is the matrix effect. In the total analysis, PAHs are much more complex in composition and are presented together with various foreign compounds. The matrix effect is most obviously shown with dibenzothiophene and phenanthrene. Their recoveries are lower from a silica gel column when alone, than from the whole analysis. It seems that the foreign compounds, mostly aliphatics, being selectively washed out by pentane, have helped dibenzothiophene and phenanthrene to remain on the column. The matrix might also affect the sample transfer from one step to the next. When the nitromethane-extracted residue was transferred onto the silica gel column, only a very small amount of solvent was used and the larger PAHs with lower solubilities might not be completely transferred. This loss is usually enhanced by the solubility suppression by co-solubles in a complex matrix.

Both freeze-dried and desiccator-dried sediments were analyzed. The desiccator-dried material has a 20–25% lower content of PAHs. The discrepancy might be attributed to the difference in moisture content which resulted from the two different drying processes. Also, since desiccation only removes water while freeze drying removes all volatiles indiscriminately, some volatiles which remained in

TABLE IV

RECOVERIES FROM SILICA GEL COLUMN CHROMATOGRAPHY

PAH	*Recovery (%)*
Dibenzothiophene	78.6
Phenanthrene	91.4
Fluoranthene	99.9
Pyrene	100.1
1-Methylpyrene	100.0
Benz[*a*]anthracene	98.5
Chrysene	98.5
Benzo[*a*]pyrene	93.0
Perylene	90.4
Dibenz[*a*,*c*]anthracene	94.9
Benzo[*g*,*h*,*i*]perylene	90.7
Coronene	87.0

sediment in the desiccator might be pulled out in the freeze dryer. Another important factor could be the degradation of PAHs during drying in the desiccator at room temperature for prolonged periods of time. It took 2 months to dry totally 1 in. thick of wet sediment in a desiccator. Although the desiccator was covered with yellow plastic to cut down the photooxidation under UV light, oxidation and other chemical degradation might not have been completely prevented.

To assure that the procedure is free from contamination, blanks of cyclohexane with activated copper were analyzed. Clean gas chromatograms were obtained at highest GC sensitivity.

When pentane and methylene chloride come into contact during column chromatography, heat of mixing is generated which causes the low boiling solvents to evaporate and form bubbles inside the column. This problem has been solved by circulating cooling water in the water jacket outside the column.

Although other solvents, such as benzene–methanol[2] and methylene chloride[3], have been used as extraction solvents for PAHs in sediments, it was found in this experiment, that cyclohexane is just as efficient but more selective.

Soxhlet extraction with cyclohexane for 18 h has been compared to the described ultrasonic extraction. No significant difference is observed. Ultrasonic extraction is a much faster procedure and it avoids the prolonged exposure which can lead to contamination. Also, by adding Cu powder into the sediment to be extracted, the coextracted sulfur simultaneously forms black CuS precipitate wich is filtered off together with the extracted sediment. Therefore, another distinct advantage of this procedure is that it combines the sulfur-removal step which is usually required for the analysis of PAHs in sediments by GC–MS.

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REFERENCES

1 J. B. Andelman and J. E. Snodgrass, *CRC Critical Review Environ. Control*, 4 (1974) 69.
2 R. Freudenthal and P. W. Jones (Editors), *Polynuclear Aromatic Hydrocarbons: Chemistry, Metabolism, and Carcinogenesis*, Raven Press, New York, N.Y., 1976.
3 D. J. Tilgner and H. Daun, *Residue Rev.*, 27 (1969) 19.
4 M. Popl, M. Stejskal and J. Mostedky, *Anal. Chem.*, 47 (1975) 1947.
5 J.W. Howard, T. Fazio, R. H. White and B. A. Klimeck, *J. Ass. Offic. Anal. Chem.*, 51 (1968) 122.
6 W. Gräf, H. Eff and S. Schormair, *Zentralbl. Bakt. Hyg., I. Abt. Orig.*, B161 (1975) 85.
7 J. S. Warner, *Anal. Chem.*, 48 (1976) 578.
8 R. C. Lao, R. S. Thomas H. Oja and L. Dubois *Anal. Chem.*, 45 (1973) 908.
9 R. C. Lao, R. S. Thomas and J. L. Monkman, *J. Chromatogr.*, 112 (1975) 681.
10 M. L. Lee, M. Novotny and K. D. Bartte, *Anal. Chem.*, 48 (1976) 1566.
11 M. Dong, D. C. Locke and E. Ferrand, *Anal. Chem.*, 48 (1976) 368.
12 A. Bjorseth, *Anal. Chim. Acta*, 94 (1977) 21.
13 W. Cautreels and K. Van Cauwenberghe, *J. Chromatogr.*, 131 (1977) 253.
14 D. Brocco, V. Cantuti and G. P. Cartoni, *J. Chromatogr.*, 49 (1970) 66.

15 J. D. Butler, *Chem. Ber.*, 11 (1975) 358.
16 A. M. Krstulovic, D. M. Rosie and P. R. Brown, *Amer. Lab.*, 9 (1977) 11.
17 D. F. S. Natusch and B. A. Tomkins, *Anal. Chem.*, 50 (1978) 1429.
18 M. L. Lee, G. P. Prado, J. B. Howard and R. A. Hites, *Biomed. Mass Spectrom.*, 4 (1977) 182.
19 W. Giger and M. Blumer, *Anal. Chem.*, 46 (1974) 1663.
20 W. Giger and C. Schaffner, *Anal. Chem.*, 50 (1978) 243.
21 R. A. Brown and P. K. Starnes, *Mar. Pollut. Bull.*, 9 (1978) 162.
22 A. Hase and R. A. Hites, *Geochim. Cosmochim. Acta*, 40 (1976) 1141.
23 R. E. Laflamme and R. A. Hites, *Geochim. Cosmochim. Acta*, 42 (1978) 289.
24 Z. Alizenshtat, *Geochim. Cosmochim. Acta*, 37 (1973) 559.
25 G. Müller, G. Grimmer and H. Böhnke, *Naturwissenschaften*, 64 (1977) 427.
26 G. Grimmer and H. Bohnke, *Cancer Lett.*, 1 (1975) 75.
27 E. B. Overton, J. Bracken and J. L. Laseter, *J. Chromatogr. Sci.*, 15 (1977) 169.

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CHROM. 11,964

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF ISOMERIC DIHYDRODIOLS OF POLYCYCLIC HYDROCARBONS

THE EFFECT OF CONFORMATION ON ELUTION ORDER

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SUMMARY

The orders in which K-region and non-K-region dihydrodiols derived from benz[*a*]anthracene, 7-methylbenz[*a*]anthracene, 7,12-dimethylbenz[*a*]anthracene, 3-methylcholanthrene, dibenz[*a*,*h*]anthracene and benzo[*a*]pyrene elute from a Partisil 5 column in cyclohexane–ethanol (98:2) have been examined using high-performance liquid chromatography. The elution profiles obtained show that, within a series of dihydrodiols derived from any one hydrocarbon, those *trans*-dihydrodiols that are known to exist predominantly in a preferred quasi-diequatorial conformation elute much earlier, presumably because of intramolecular hydrogen bonding, than do those that for steric reasons, are known to exist in a predominantly quasi-diaxial conformation. These differences appear to be sufficiently large to permit the conformation of an uncharacterized dihydrodiol to be predicted from its relative retention time on high-performance liquid chromatography.

INTRODUCTION

Interest in the preparation and properties of dihydrodiol derivatives of polycyclic hydrocarbons increased following reports that certain non-K-region dihydro-

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diols appear to be involved in the metabolic activation of carcinogenic polycyclic hydrocarbons through their conversion into reactive vicinal diol-epoxides[1–4]. The preparation of mixtures of isomeric dihydrodiols derived from several different polycyclic hydrocarbons by the use of an ascorbic acid–ferrous sulphate–EDTA system has been described[5–7]. The dihydrodiols were separated and purified using high-performance liquid chromatography (HPLC) on a column of Partisil 5 eluted with cyclohexane–ethanol prior to characterization and nuclear magnetic resonance (NMR) spectrometry and it became apparent that the order in which the different dihydrodiols derived from a single hydrocarbon eluted from a column of Partisil 5 was dependent upon their conformation. This paper describes the results obtained in experiments in which the chromatographic characteristics of dihydrodiols derived from six polycyclic hydrocarbons were examined on HPLC under our chromatographic conditions and were then considered in relation to their known conformations.

EXPERIMENTAL

Materials

Samples of the *trans*-1,2-, 3,4-, 5,6-, 8,9- and 10,11-dihydrodiols derived from benz[*a*]anthracene[7], the *trans*-1,2-, 3,4-, 5,6-, 8,9- and 10,11-dihydrodiols derived from 7-methylbenz[*a*]anthracene[5], the *trans*-3,4-, 5,6-, 8,9- and 10,11-dihydrodiols derived from 7,12-dimethylbenz[*a*]anthracene[7], the *trans*-4,5-, 7,8-, 9,10- and 11,12-dihydrodiols and the *cis*-2a,3-diol derived from 3-methylcholanthrene[6], the *trans*-1,2-, 3,4- and 5,6-dihydrodiols derived from dibenz[*a,h*]anthracene[8] and the *trans*-4,5-, 7,8-, 9,10- and 11,12-dihydrodiols derived from benzo[*a*]pyrene[9] were obtained from the parent hydrocarbons using an ascorbic acid–ferrous sulphate–EDTA oxidation system as previously described and were characterized by examination of their UV, mass and ^{1}H-NMR spectral properties.

High-performance liquid chromatography

Mixtures of dihydrodiols derived from each polycyclic hydrocarbon were separated using a DuPont 830 instrument fitted with a Model 837 variable wavelength UV detector (DuPont, Hitchin, Great Britain) and a Partisil 5 silica column (250 mm × 5 mm I.D.) (Whatman, Maidstone, Great Britain), which was eluted with HPLC grade cyclohexane (Rathburn Chemicals, Walkerburn, Great Britain)–ethanol (dried and redistilled before use) (98:2). Samples (0.5 ml) were applied as solutions in cyclohexane–tetrahydrofuran (3:1), the eluting solvent was passed at a flow-rate of 1.6 ml/min at 40° and the eluent monitored for UV absorption at an appropriate wavelength.

RESULTS AND DISCUSSION

trans-Dihydrodiols derived from polycyclic aromatic hydrocarbons are known to adopt a preferred quasi-diequatorial conformation in the absence of other factors[10–13] and dihydrodiols adopting this conformation elute earlier on HPLC under our chromatographic conditions, presumably because of intra-molecular hydrogen bonding which reduces their polarity, than do dihydrodiols adopting a predominantly quasi-diaxial conformation. The results obtained, when the behaviour of dihydrodiols

related to benz[*a*]anthracene was examined, are shown in Fig. 1. The four dihydrodiols known to exist in a predominantly diequatorial conformation[11,13], the *trans*-3,4-, 5,6-, 8,9- and 10,11-diols, eluted earlier than the related *trans*-1,2-diol, which, as a "bay-region" diol, is hindered and exists in a quasi-diaxial conformation[13]. When

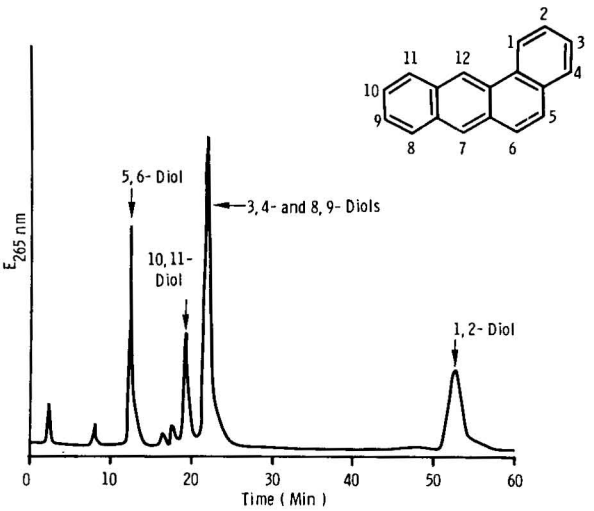

Fig. 1. The elution profile obtained when a mixture of the *trans*-dihydrodiols derived from benz[*a*]anthracene were subjected to HPLC using a Partisil 5 silica column (250 mm × 5 mm I.D.) eluted with cyclohexane–ethanol (98:2). The sample (0.5 ml) was applied as a solution in cyclohexane–tetrahydrofuran (3:1) the eluting solvent was passed at a flow-rate of 1.6 ml/min at 40° and the eluate monitored for UV absorption.

the HPLC elution characteristics, under our chromatographic conditions, of dihydrodiols derived from 7-methylbenz[*a*]anthracene were examined (Fig. 2), the main change that was found in the elution order, compared with that for the diols derived from the unsubstituted hydrocarbon, concerned the delayed elution of the 5,6- and 8,9-dihydrodiols. This was not unexpected since these two diols are known to exist predominantly in a quasi-diaxial conformation[5,14], presumably because of steric interactions between the hydroxyl groups on either the 6- or the 8-positions and the adjacent methyl substituent at the 7-position.

Results in agreement with those obtained with 7-methylbenz[*a*]anthracene and with NMR data[7] were obtained when the elution order of the *trans*-dihydrodiols derived from 7,12-dimethylbenz[*a*]anthracene was examined (Fig. 3). Here the 10,11-diol also eluted in a position consistent with its existence in a predominantly quasi-diaxial conformation[7], presumably because of interaction with the adjacent 12-methyl substituent. The 1,2-dihydrodiol derived from 7,12-dimethylbenz[*a*]anthracene has not been detected either as a metabolite[7,15] or as a major product of oxidation of the hydrocarbon[7], possibly because of strong steric hindrance, and therefore has not been examined, but it would be expected to exist in a diaxial conformation. The presence of a methyl group in the 12-position is known to distort the benz[*a*]anthracene molecule so that the 1,2,3,4 ring no longer lies in the same plane as the anthracene

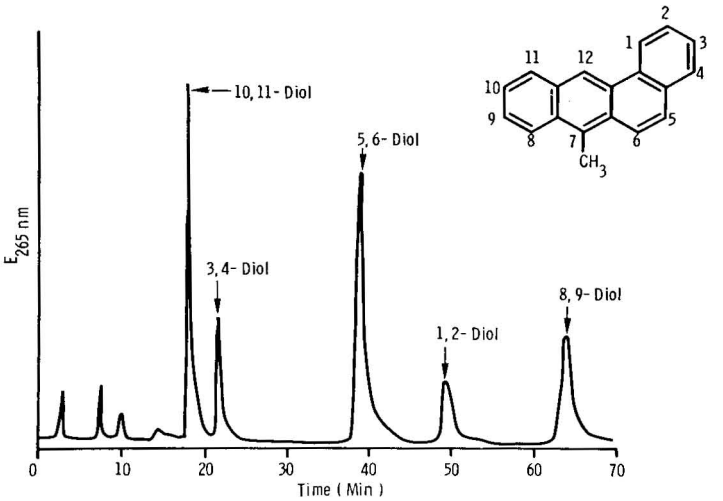

Fig. 2. The elution profile obtained when a mixture of the *trans*-dihydrodiols derived from 7-methylbenz[*a*]anthracene was subjected to HPLC exactly as described in the text and in the legend to Fig. 1.

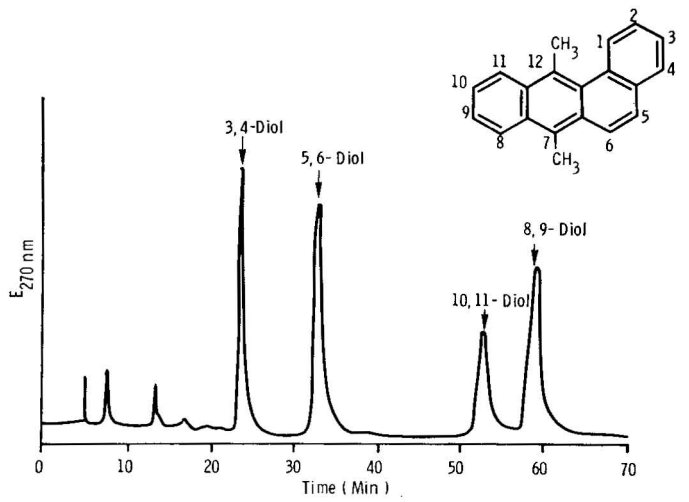

Fig. 3. The elution profile obtained when four *trans*-dihydrodiols derived from 7,12-dimethylbenz[*a*]-anthracene were subjected to HPLC exactly as described in the text and in the legend to Fig. 1.

nucleus[16,17]. Fig. 4 shows the HPLC elution profile obtained with the dihydrodiols derived from 3-methylcholanthrene, which is also in agreement with NMR data[6,18]. The *trans*-4,5- and 9,10-dihydrodiols exist in the preferred quasi-diequatorial conformation and elute early, whilst the "bay-region" 7,8-diol elutes much later because of its quasi-diaxial conformation. The *trans*-11,12-dihydrodiol also elutes early in a position that is consistent with its quasi-diequatorial conformation and it is evident from comparisons between both the NMR data on, and the chromatographic

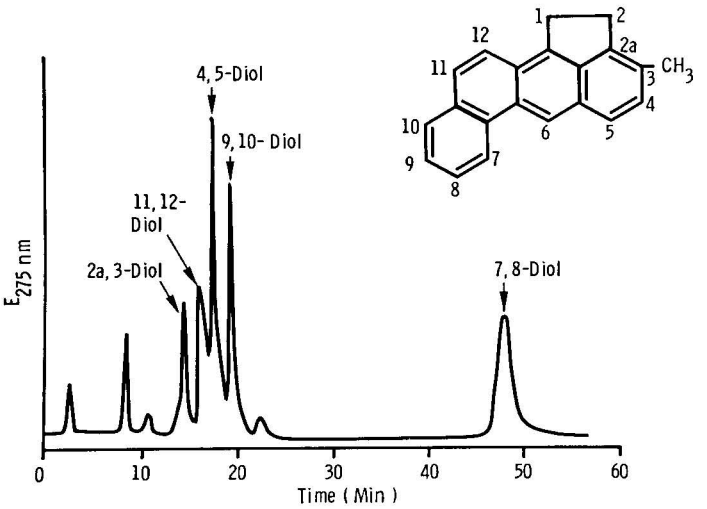

Fig. 4. The elution profile obtained when a mixture of dihydrodiols derived from 3-methylcholanthrene was subjected to HPLC exactly as described in the text and in the legend to Fig. 1.

behaviour of, the K-region dihydrodiols of 3-methylcholanthrene and 7-methylbenz-[*a*]anthracene that, whilst methyl substitution at the 7-position of 7-methylbenz[*a*]-anthracene results in the adoption of a predominantly quasi-diaxial conformation, the presence of the 1,2-methylene bridge in 3-methylcholanthrene has a much smaller effect on the conformation of the corresponding K-region 11,12-diol. The conformation of the *cis*-2a,3-diol has not been investigated.

The relationships between HPLC elution order, under our chromatographic conditions, and conformation for the dihydrodiols derived from the other two hydrocarbons studied, dibenz[*a*,*h*]anthracene (Fig. 5) and benzo[*a*]pyrene (Fig. 6), are simpler since these hydrocarbons are unsubstituted. The "bay-region" 1,2-dihydrodiol of dibenz[*a*,*h*]anthracene exists predominantly in the quasi-diaxial conformation[8] and elutes considerably later than the other two quasi-diequatorial diols, the 3,4- and 5,6-derivatives. Within the series of dihydrodiols related to benzo[*a*]pyrene, the quasi-diequatorial K-region 4,5-diol[14] elutes first and is followed by the other diol, the 7,8-diol, that can adopt this preferred conformation[10,11]. The second K-region diol, the 11,12-derivative elutes later than these two diequatorial diols and presumably exists in a predominantly quasi-diaxial conformation because of its position adjacent to a "bay-region" in the molecule; there do not appear to be any NMR data available for the 11,12-diol that might support this conclusion. The other "bay-region" diol derived from benzo[*a*]pyrene, the 9,10-diol, exists in a predominantly quasi-diaxial conformation[10,11] and elutes considerably later than the other *trans*-diols (Fig. 6).

The data that have been obtained for the different *trans*-dihydrodiols derived from six polycyclic hydrocarbons that are presented here and that relate their elution order to their conformation appear to be consistent. Within any series of related *trans*-dihydrodiols, it appears that K-region diols in the preferred quasi-diequatorial conformation are the least polar and elute first under our chromatographic conditions and that these are followed by any non-K-region diols that are also predominantly

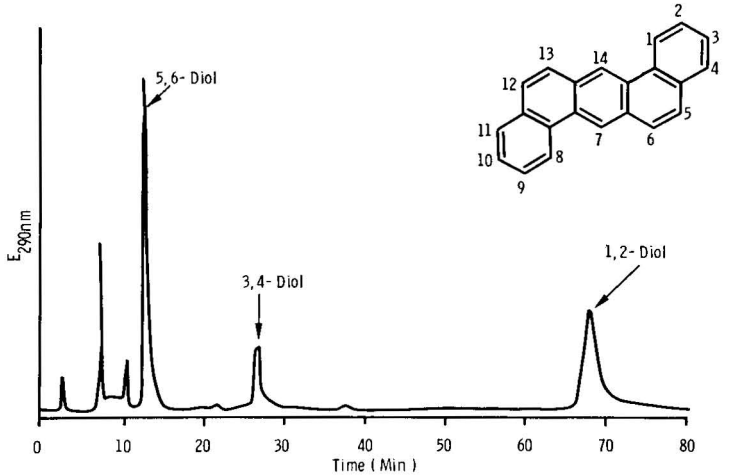

Fig. 5. The separation of three *trans*-dihydrodiols of dibenz[*a*, *h*]anthracene using HPLC as described in the text and in the legend to Fig. 1.

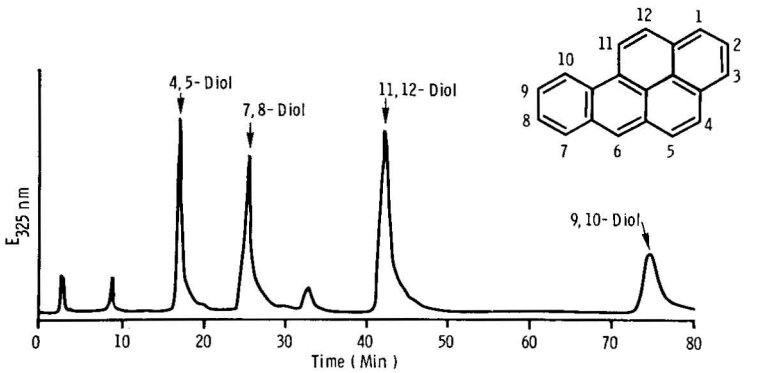

Fig. 6. The elution profile obtained when a mixture of four *trans*-dihydrodiols derived from benzo[*a*]-pyrene were subjected to HPLC as described in the text and in the legend to Fig. 1.

quasi-diequatorial. K-Region diols existing, because of steric interactions, in a predominantly quasi-diaxial conformation elute next and are followed by the more polar non-K-region diols that are in a predominantly quasi-diaxial conformation. Those non-K-region diols that have one hydroxyl group adjacent to a methyl substituent are the most polar of all and it appears that the presence of an adjacent methyl group can have a greater effect on diol conformation than an adjacent "bay-region", a point that is illustrated by the elution characteristics of the 8,9- and 1,2-dihydrodiols of 7-methylbenz[*a*]anthracene (Fig. 2). The results reported here are in good general agreement with others that have been obtained for dihydrodiols derived from unsubstituted hydrocarbons[19]. Those differences that do exist between the two sets of data may well be due to the distinctly different column adsorbents and eluting solvents that were used. The reason why K-region diols in a particular conformation

invariably appear to elute before any non-K-region diols that are also in that conformation is not clear at present, however. The generalization mentioned above may be useful in permitting predictions to be made about the identity of uncharacterized dihydrodiol metabolites of a polycyclic hydrocarbon from comparative HPLC elution data in so far as they may be tentatively classified as K- or non-K-region dihydrodiols existing in predominantly quasi-diequatorial or diaxial conformations. The factors that affect the order in which dihydrodiols elute on HPLC under any particular chromatographic conditions are not sufficiently well understood at present to permit predictions to be made about the order in which related diols of a similar type that exist in a similar conformation will elute.

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REFERENCES

1 P. Sims, P. L. Grover, A. Swaisland, K. Pal and A. Hewer, *Nature (London)*, 252 (1974) 326.
2 B. Tierney, A. Hewer, C. Walsh, P. L. Grover and P. Sims, *Chem.-Biol. Interactions*, 18 (1977) 179.
3 W. Levin, A. W. Wood, R. L. Chang, H. Yagi, H. D. Mah, D. M. Jerina and A. H. Conney, *Cancer Res.*, 38 (1978) 1831.
4 A. W. Wood, W. Levin, P. E. Thomas, D. Ryan, J. M. Karle, H. Yagi, D. M. Jerina and A. H. Conney, *Cancer Res.*, 38 (1978) 1967.
5 B. Tierney, B. Abercrombie, C. Walsh, A. Hewer, P. L. Grover and P. Sims, *Chem.-Biol. Interactions*, 21 (1978) 289.
6 B. Tierney, A. Hewer, H. Rattle, P. L. Grover and P. Sims, *Chem.-Biol. Interactions*, 23 (1978) 121.
7 B. Tierney, A. Hewer, A. D. MacNicoll, P. G. Gervasi, H. Rattle, C. Walsh, P. L. Grover and P. Sims, *Chem.-Biol. Interactions*, 23 (1978) 243.
8 A. D. MacNicoll, P. M. Burden, H. Rattle, P. L. Grover and P. Sims, *Chem.-Biol. Interactions*, in press.
9 A. Hewer, O. Ribeiro, C. Walsh, P. L. Grover and P. Sims, *Chem.-Biol. Interactions*, in press.
10 H. Yagi, O. Hernandez and D. M. Jerina, *J. Amer. Chem. Soc.*, 97 (1975) 6881.
11 R. E. Lehr, M. Schaeffer-Ridder and D. M. Jerina, *J. Org. Chem.*, 42 (1977) 738.
12 D. R. Thakker, H. Yagi, R. E. Lehr, W. Levin, M. Buening, A. Y. H. Lu, R. L. Chang, A. W. Wood, A. H. Conney and D. M. Jerina, *Mol. Pharmacol.*, 14 (1978) 502.
13 D. M. Jerina, H. Selander, H. Yagi, M. C. Wells, J. F. Davey, V. Mahadevan and D. T. Gibson, *J. Amer. Chem. Soc.*, 98 (1976) 5988.
14 D. E. Zacharias, J. P. Glusker, R. G. Harvey and P. P. Fu, *Cancer Res.*, 37 (1977) 775.
15 M. W. Chou and S. K. Yang, *Proc. Nat. Acad. Sci., U.S.A.*, 75 (1978) 5468.
16 D. Sayre and P. H. Friedlander, *Nature (London)*, 187 (1960) 139.
17 J. A. Iball, *Nature (London)*, 201 (1964) 916.
18 D. R. Thakker, W. Levin, A. W. Wood, A. H. Conney, T. A. Stoming and D. M. Jerina, *J. Amer. Chem. Soc.*, 100 (1978) 645.
19 D. R. Thakker, H. Yagi and D. M. Jerina, *Methods Enzymol.*, 51C (1978) 279.

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PURIFICATION DU BENZO[*a*]PYRÈNE COMMERCIAL

ISOLEMENT ET DÉTERMINATION DES IMPURETÉS MAJEURES

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(Reçu le 15 mars 1979; manuscrit modifié reçu le 9 avril 1979)

SUMMARY

Purification of commercial benzo[a]pyrene. Isolation and determination of major impurities

Commercially available benzo[*a*]pyrene preparations contain several impurities with a structure similar to that of benzo[*a*]pyrene. In this work a chromatographic method is described for the purification of gram quantities of benzo[*a*]pyrene. The three major impurities have been isolated in weighable quantities and their structures have been identified by spectroscopic methods, showing two sulphur-containing isomers $C_{18}H_{10}S$ and one tetrahydrobenzopyrene $C_{20}H_{16}$.

INTRODUCTION

L'accroissement du nombre d'analyses effectuées sur les hydrocarbures polyaromatiques dans les problèmes relatifs à l'environnement, ainsi que les études du mécanisme cancérigène de ces produits[1] rend de plus en plus nécessaire l'obtention de produits de référence certifiés. Dans ce but, le Bureau Communautaire des Produits de Référence des Communautés Européennes met à la disposition des chercheurs et des industriels des hydrocarbures polyaromatiques de référence nantis d'un certificat de pureté et continue d'accroître la variété de ses produits.

Ce travail présente une contribution du Centre Commun de Recherche à l'établissement d'un stock de benzo[*a*]pyrène certifié. Il a pour but d'établir une méthode permettant la préparation de quelques dizaines de grammes de benzo[*a*]-pyrène purifié à plus de 99.5% au départ de produits commerciaux.

Les impuretés principales rencontrées au cours de ce travail, sont isolées et caractérisées.

PARTIE EXPÉRIMENTALE

Conditions analytiques et expérimentales

Chromatographie en phase gazeuse. Les déterminations chromatographiques sont effectuées en isotherme à 250° à l'aide d'un appareil Hewlett-Packard 5730A.

Le détecteur à ionisation de flamme travaille sous un débit d'hydrogène de 40 ml/min et un débit d'air de 240 ml/min. La colonne en acier inoxydable a une longueur de 1 m et un diamètre de 1/8 in. La phase fixe est constituée de 1.5% de BBBT [N,N′-bis(*p*-butoxybenzylidène)-α,α′-bi-*p*-toluidine] imprégnée sur Chromosorb W HP (100–120 mesh). L'hélium est utilisé comme gaz vecteur.

Chromatographie liquide à haute performance (HPLC). L'appareil utilisé est constitué d'une pompe 6000A de Waters Assoc. et d'un détecteur UV 440 A, de la même firme, travaillant à 254 nm. Les fonctions analytiques et semi-préparatives sont assurées par deux colonnes en série de Partisil 5 de 25 cm de longueur et 0.9 cm de diamètre intérieur éluées à l'hexane sous un débit de 7 ml/min. La pression d'entrée est de 3000 p.s.i. L'ensemble fonctionne à température ambiante.

Combinaison chromatographie en phase gazeuse (GC)–spectrométrie de masse (MS). Un spectromètre quadrupolaire SQ 156 de la firme Ribermag equipé d'un système d'informatique S 150 est associé à un chromatographe Carbo Erba type Fractovap 2350.

Les conditions chromatographiques sont identiques à celles décrites dans la partie chromatographie gazeuse, excepté le fait que la phase de BBBT est suivie sur une longueur de 10 cm d'une phase de Dexsil 300. Cette dernière phase limite l'important "bleeding" du BBBT à la température de travail.

À la sortie de la colonne, une moitié de l'effluent est prélevé par un tube capillaire en Pt–Ir et envoyé au séparateur du spectromètre de masse, l'autre moitié est envoyée au détecteur à ionisation de flamme (FID). La source à bombardement électronique travaille à 150°, le potentiel des électrons est de 70 eV et le courant de 0.3 mA. La pression de travail de la source est de 10^{-6} Torr.

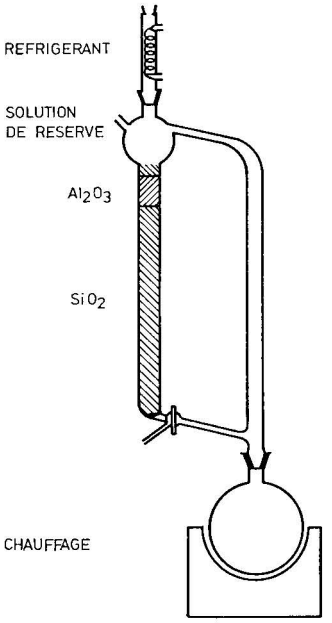


Fig. 1. Colonne d'élution. Eluant: 3 à 5% de benzène dans l'hexane.

Chromatographie sur colonne. La purification chromatographique du benzo-[*a*]pyrène est effectuée par une colonne en verre de 50 cm de longueur et de 2.5 cm de diamètre intérieur (Fig. 1) avec recyclage de l'éluant par distillation. L'éluant est une solution de 3 à 5% de benzène dans l'hexane. La colonne est remplie sur 30 cm de silica gel (No. 923; W. R. Grace & Co., Baltimore, Md., U.S.A.) chauffé durant une nuit à 170° et refroidi à température ambiante sous exsiccateur. Le silica gel est précédé de 5 cm de bauxite (Porocel, 20–60 mesh) intimement mélangé à 5 g de benzo[*a*]pyrène commercial. La durée de l'élution est de 10 h et son avancement est suivi par fluorescence UV.

Quatre fractions sont receuillies. Les deux premières contiennent principalement les impuretés (moins de 1% de la charge). La troisième fraction est recyclée (40 à 50% de la charge). La quatrième fraction contient le benzo[*a*]pyrène de pureté > à 99.5%.

Recristallisation. Le benzo[*a*]pyrène (1 g) à 98.7% de pureté (Fig. 2) en solution benzénique saturée est ajouté à température ambiante à 300 ml d'hexane. Après homogénéisation, la solution est abandonnée sous refroidissement d'eau courante à 13°. La cristallisation s'amorce quasi-immédiatement. Après 4 h environ, 50% du produit est cristallisé.

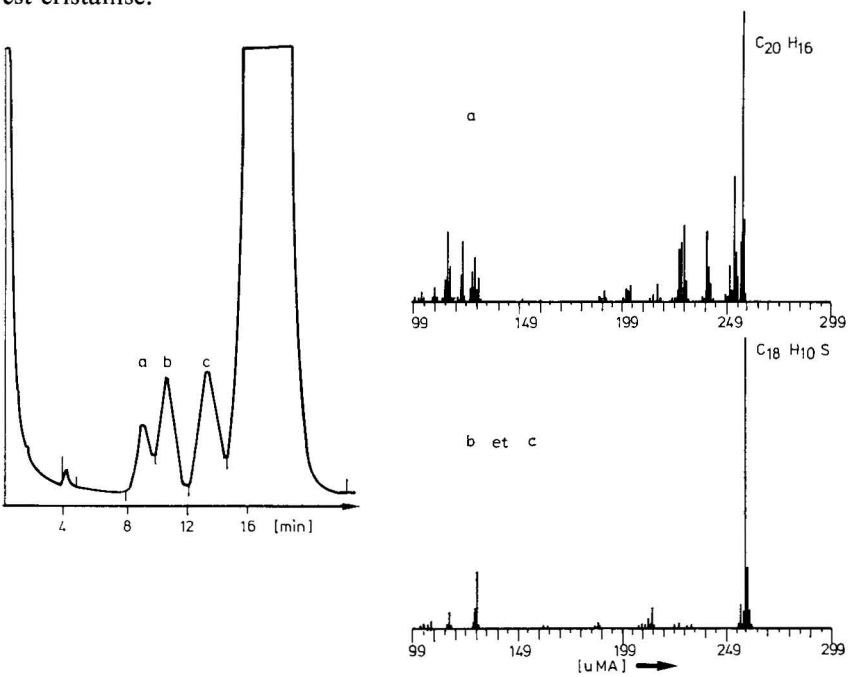


Fig. 2. GC et GC–MS du lot expérimental de benzo[*a*]pyrène. Pics: a, tétrahydrobenzo[*a*]pyrène; b et c, benzanthracéno- et chrysénothiophène.

La pureté du produit cristallisé est alors de 98.5%, la solution résiduelle après évaporation sous vide du solvant fournit un benzo[*a*]pyrène à 98.85% de pureté. La pureté est déterminée par chromatographie en phase gazeuse.

Sulfonation des impuretés. Une solution benzénique saturée de 1 g de benzo-[*a*]pyrène commercial est étendue de deux fois son volume d'hexane et portée à la

température d'un bain de glace. L'acide sulfurique concentré (10 cm^3) est ajouté peu à peu en agitant la solution. On laisse ensuite le bain revenir à température ambiante et on dilue la solution acide par 200 ml d'eau. La solution benzénique est decantée, lavée par une solution alcaline diluée et séchée sur Na_2SO_4 anhydre. Après passage sur une colonne de 10 cm de silicagel, on obtient environ 0.6 g de benzo[*a*]pyrène à 99.4% de pureté.

Spectrométrie ultra-violette. Les analyses UV sont réalisées à l'aide du spectromètre à double faisceau Modèle 25 de la firme Beckmann couvrant la gamme de 190 à 700 nm par deux sources de radiation; une lampe au tungstène et une lampe au deuterium. Toutes les analyses ont été effectuées en solution dans l'hexane.

Spectrométrie de résonance magnétique nucléaire (RMN). Ces analyses sont réalisées par un appareil Jeol Type PS 100. Les échantillons sont mis en solution dans le CS_2.

Mesure des points de fusion. Les points de fusion sont relevés à l'aide de l'appareil Mark I de la firme Electrothermal Engineering.

Produits chimiques

Solvants. Tous les solvants utilisés pour ce travail proviennent de la firme Baker Chemicals (Deventer, Pays-Bas) et sont d'une pureté >99%.

Benzo[a]pyrène. Le lot de benzo[*a*]pyrène utilisé au cours de ces travaux a été choisi après examens chromatographiques (GC, HPLC et GC–MS) parmi les benzo[*a*]pyrènes commerciaux alors disponibles en quantité suffisante (Fig. 3). L'analyse chromatographique montre 3 impuretés principales et donne une pureté de 98.7% au lot utilisé dans ce travail (Fig. 2).

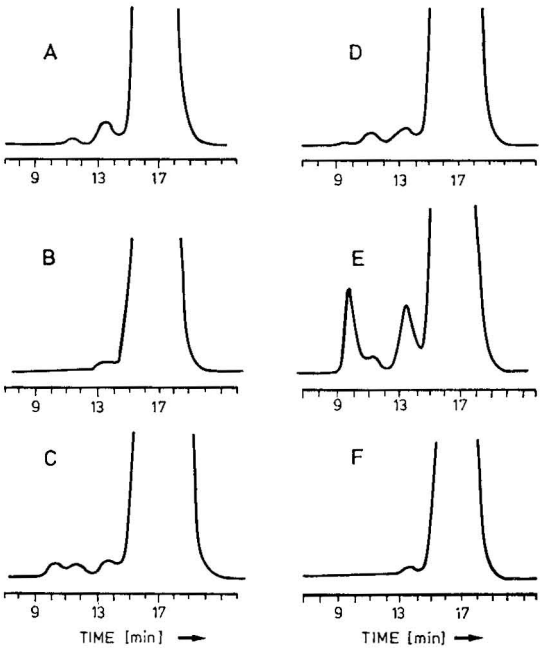


Fig. 3. GC de 6 benzo[*a*]pyrène commerciaux (A–F). Colonne 1 m × 3 mm I.D. 1.5% BBBT sur Chromosorb. Température de colonne 250°.

RÉSULTATS ET DISCUSSION

L'examen préliminaire (Fig. 3) montre que tous les échantillons commerciaux analysés présentent principalement les mêmes impuretés.

L'analyse par GC–MS identifie deux isomères $C_{18}H_{10}S$ de masse moléculaire 258 de structure benzophénanthrothiophène (Fig. 2) et un composé $C_{20}H_{16}$ de structure tétrahydrobenzopyrène.

Purification du benzo[a]pyrène

Par recristallisation. Le benzopyrène est peu soluble dans les solvants usuels et par conséquent nécessite l'emploi de grands volumes de solvant lorsque l'on traite plusieurs grammes de produit. L'opération est longue, car le taux de purification du benzopyrène est faible et l'oxydation est un problème important encore aggravé si l'on utilise des solvants oxygénés tels que le méthanol et le tétrahydrofurane. Dans ce travail, les meilleurs résultats ont été obtenus en cristallisant une solution benzénique saturée de benzo[*a*]pyrène, dans l'hexane.

La recristallisation provoque un enrichissement du composé cristallisé en impuretés soufrées tandis que la solution résiduelle s'enrichit en autres impuretés.

En reprenant alternativement la solution et le produit cristallisé, on obtient après quatre opérations, un benzopyrène à 99.3 % de pureté.

L'oxydation, inévitable lors des manipulations, est aisément éliminée par l'élution du benzo[*a*]pyrène oxydé, en solution benzénique, sur une colonne de 10 cm de silica gel. Le rendement est voisin de 30 %.

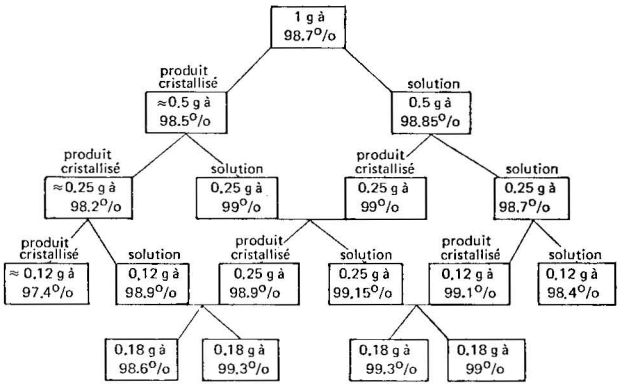


Schéma de recristallisation.

Par voie chimique. Se basant sur le fait que la sulfonation du benzo[*a*]pyrène est une réaction lente à basse température en présence d'acide sulfurique concentré[2], il est possible de se débarrasser des composés soufrés en dissolvant le benzopyrène à froid dans l'acide sulphurique. Les composés soufrés, légèrement plus réactifs que le benzo[*a*]pyrène sont sulfonés préférentiellement.

Par chromatographie sur colonne. Le silica gel et l'alumine permettent tous deux de séparer les impuretés du benzo[*a*]pyrène[3]. L'élution de ce dernier par une solution d'hexane contenant 3 à 5 % de benzène sur une colonne de silica gel activé

permet d'obtenir un benzopyrène d'une pureté voisine de 100% avec un rendement approchant l'unité. L'élution est cependant très longue (8 à 10 h) et les volumes d'éluants engagés sont importants.

Afin d'éviter ces volumes d'éluants qui deviennent gênants, surtout lors de la concentration des impuretés, il est préférable de recycler l'éluant en le distillant grossièrement après passage sur la colonne chromatographique. Le distillat est condensé en tête de colonne par un réfrigérant, comme l'indique la Fig. 1.

Il nous est loisible de recueillir les éluants, soit en changeant de ballon après passage de chaque impureté, soit en recueillant celles-ci par le robinet inférieur et en compensant, d'une quantité égale de la solution de réserve, le volume d'éluant extrait.

Les 4 fractions recueillies, examinées par chromatographie et spectrométrie de masse donnent:

(a) une première fraction contenant principalement le premier, dans l'ordre GC, des isomères soufrés et le tétrahydrobenzopyrène;

(b) Une deuxième fraction contenant principalement le deuxième isomère soufré et du benzopyrène;

(c) une troisième fraction contenant principalement du benzopyrène de pureté voisine de 98% que l'on recycle;

(d) une fraction de benzo[*a*]pyrène (50 à 60% de la charge) de pureté supérieure à 99.5%.

Isolement des impuretés

Les impuretés extraites des fractions enrichies obtenues dans la chromatographie sur colonne sont isolées par HPLC (Fig. 4). On recueille séparément les trois impuretés principales que l'on désire analyser en détail.

Les impuretés mineures sont rassemblées en deux fractions et identifiées par GC–MS. Elles sont essentiellement constituées de dihydrobenzopyrène, de methyl-

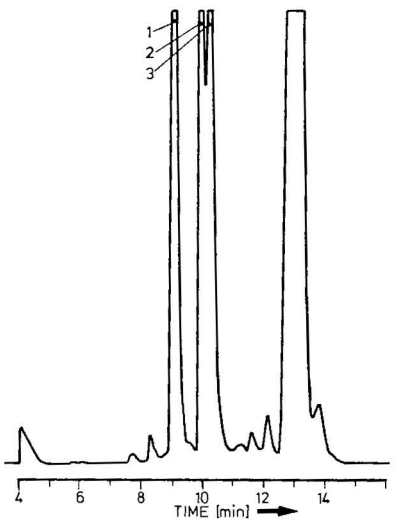


Fig. 4. HPLC des fractions enrichies en impuretés. 1 = Benz[*a*]anthracéno[1,12b,12a,12-*bcd*]-thiophène; 2 = tetrahydrobenzo[*a*]pyrène; 3 = chryséno[4,4a,4b,5-*bcd*] thiophène.

benzo[*a*]pyrène, de pyrène, de méthylpyrène, de chrysène et de tétrahydrobenzothiophenanthrène.

Il convient de noter que l'ordre d'élution des impuretés en GC est différent de celui réalisé par HPLC. Ainsi le tétrahydrobenzopyrène qui apparaît en premier lieu en chromatographie gazeuse apparaît en second lieu en chromatographie HPLC.

Analyses des impuretés

Chaque impureté principale isolée par HPLC est examinée par spectrométrie UV, MS et RMN. Le point de fusion de chaque impureté est également relevé.

Analyse par MS

Les impuretés 1 et 3 du chromatogramme HPLC présentent le même spectre de masse (spectre b et c, Fig. 2). L'identification des composés soufrés est basée sur le caractère aromatique de ces impuretés, qui même à potentiel élevé (70 eV) assure une partie importante de l'ionisation au pic moléculaire.

Les deux introductions, chromatographique et directe, qui ont été utilisées confirment mutuellement le rapport isotopique de leurs groupes parents ($P+1 = 21\%$, $P+2 = 6\%$) permettant, sans ambiguïté, d'attribuer la formule brute $C_{18}H_{10}S$ aux composés soufrés.

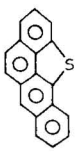
Le pic P−45, déjà rencontré dans l'analyse de phénanthrothiophène[4] tend à indiquer une structure condensée thiophénique du type benzophénanthrothiophène pour les composés soufrés.

Les rapports isotopiques et la fragmentation de l'impureté 2 (spectre a, Fig. 2) indique un composé aromatique hydrogéné de formule brute $C_{20}H_{16}$.

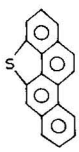
Examen UV

Le profil des spectres UV des hydrocarbures polyaromatiques[5–8] est peu affecté par le remplacement d'un cycle aromatique par un cycle thiophénique. La Fig. 5 montre les spectres UV du thiophénanthrène et du pyrène. Ce dernier n'accuse qu'un léger décalage vers le rouge par rapport au premier.

L'impureté No. 1 montre un spectre, très voisin du benzo[*a*]pyrène (Fig. 6) qui autorise les deux structures suivantes:

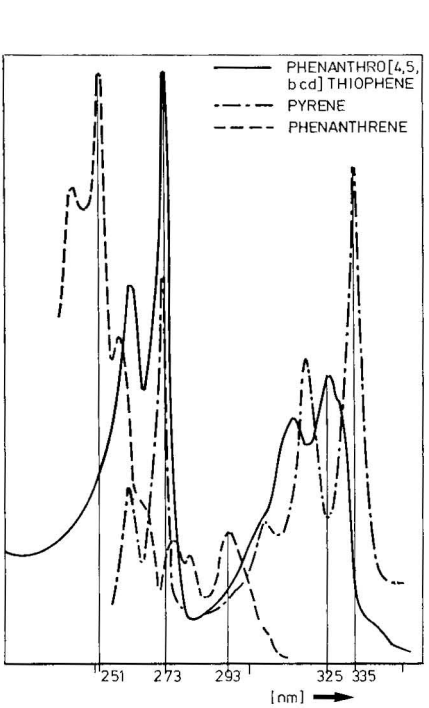


(a)



(b)

Par analogie avec le déplacement vers le rouge provoqué par l'adjonction au phénanthrène, d'un cycle thiophénique, en position ortho et péri condensée entraînant le déplacement vers le rouge de 22 nm pour la bande K et de 40 nm pour la bande B, on remarque que la structure (a) proviendrait de l'adjonction d'un cycle thiophénique en position *ortho* et *péri*, au benzo[*a*]anthracène tandis que la structure (b) provien-



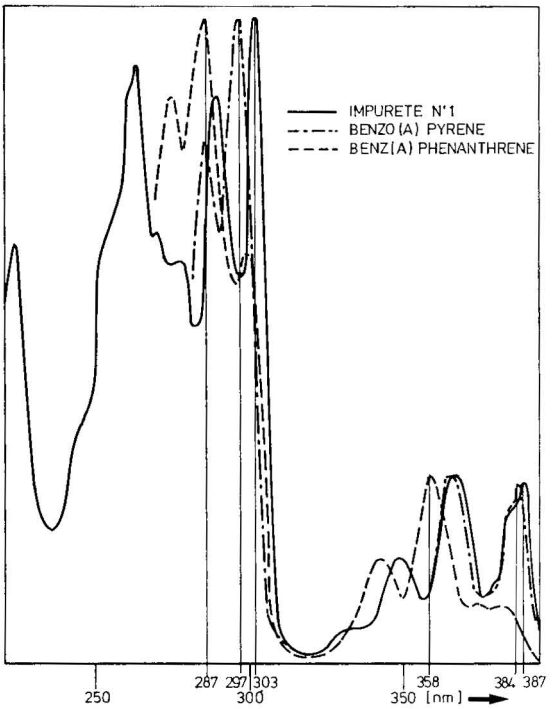


Fig. 5. Spectres UV des phénanthrène, pyrène et phénantro[4,5-*b*,*c*,*d*]thiophène dans l'hexane.

Fig. 6. Spectres UV de l'impureté HPLC No. 1, du benzo[*a*]pyrène et du benzo[*a*]phénanthrène dans l'hexane.

drait de l'adjonction d'un cycle thiophénique au chrysène. En ôtant 40 nm à la $\lambda_{max.}$ de bande B et 20 nm à la $\lambda_{max.}$ de la bande K, on trouve, pour le spectre de l'impureté No. 1 du chromatogramme HPLC, les $\lambda_{max.}$ du benz[*a*]anthracene, alors que la même opération sur l'impureté No. 3 (Fig. 7) restitue les $\lambda_{max.}$ du chrysène. On en conclut que l'impureté No. 1 correspondrait à la structure (a) et l'impureté No. 3 à la structure (b).

L'impureté No. 2 présente un profil analogue au pyrène avec un léger décalage vers le rouge. Ceci indique que les quatre CH_2 se trouvent dans un même cycle en position *a* ou *e* du pyrène.

Analyse par RMN (Fig. 8)

L'attribution des raies d'adsorption du benzo[*a*]pyrène par Martin *et al.*[9] ainsi que par Cavalieri et Calvin[10] met en évidence l'absence de proton H_1 et H_2 dans le spectre de l'impureté No. 1.

L'impureté No. 3 montre par contre, la présence de protons H_1 et H_2 et l'absence de protons H_6 et H_7.

Relevé des points de fusion

Les points de fusion relevés à l'aide de l'appareil Mark 1 de la firme Electrothermal engineering nous donnent les valeurs ci-dessous:

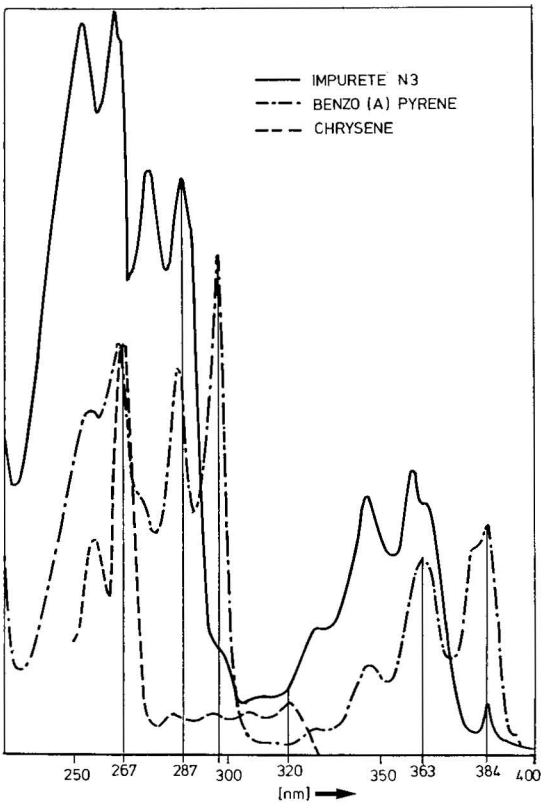


Fig. 7. Spectres UV de l'impureté HPLC No. 3, du benzo[*a*]pyrène et du chrysène dans l'hexane.

No. impureté	*Couleur*	*Point de fusion (°C)*
1	Jaune-clair	153.5–154
2	Blanc	111.5–112
3	Blanc	172.5–173

CONCLUSIONS

La chromatographie est le moyen de purification le plus attrayant, non seulement parce que le rendement est excellent, mais aussi parce qu'il nécessite peu de moyens et permet la récupération aisée des impuretés.

Ces impuretés présentes à haute concentration dans les premières fractions chromatographiques autorisent l'utilisation de colonnes semi-préparatives en HPLC analytique. Sans cette préconcentration, le temps requis pour isoler des quantités pondérables par ces colonnes serait prohibitif. Tel est le cas de la recristallisation où les fractions "enrichies" en impuretés présent encore un taux d'impuretés trop faible par rapport au benzo[*a*]pyrène.

Dans tous les cas, il a été constaté une oxydation du benzo[*a*]pyrène en solution. Particulièrement rapide à la lumière, elle est moins importante dans l'obscurité

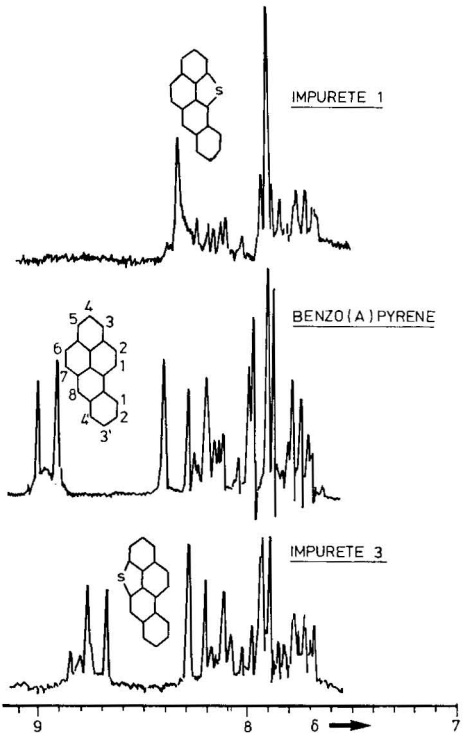


Fig. 8. Spectre RMN des impuretés HPLC No. 1, no. 3 et du benzo[*a*]pyrène en solution dans le CS_2.

et négligeable pour un produit cristallisé conservé dans un flacon coloré. Heureusement, cette oxydation est aisément éliminée par passage du produit en solution benzénique sur 10 cm de silica gel.

L'isolement et l'identification de composés soufrés dans certains hydrocarbures polyaromatiques avaient déjà été décrits dans une publication précédente[4]. Des propositions de structure pour une impureté soufrée isolée par HPLC dans le benzo[*a*]pyrène avaient même été avancées.

Ce travail démontre l'existence de deux isomères soufrés de points de fusion sensiblement différents. Leurs structures établies par RMN sur la base de l'existence des protons H_1 et H_2 sont confirmées par l'analyse ultra-violette.

REMERCIEMENTS

Je remercie tout particulièrement M. Ph. Glaude dont les analyses par résonance magnétique nucléaire ont permis de confirmer les structures des impuretés isolées.

RÉSUMÉ

Le benzo[*a*]pyrène délivré commercialement possède des impuretés communes, présentant toutes des structures voisines du benzo[*a*]pyrène. Ce travail propose

une méthode chromatographique permettant la purification du benzo[*a*]pyrène en quantité de l'ordre du gramme. Les impuretés majeures ont été isolées en quantités pondérables et leurs structures élucidées par méthodes spectroscopiques, montrent la résence de deux isomères soufrés $C_{18}H_{10}S$ et d'un tétrahydrobenzopyrène $C_{20}H_{16}$.

BIBLIOGRAPHIE

1 M. Novotny, M. L. Lee et K. D. Bartle, *J. Chromatogr. Sci.*, 12 (1974) 606.
2 I. Berenblum, *Nature (London)*, 156 (1945) 601.
3 L. Zechmeister et L. Cholnoky, *Principles and Practice of Chromatography*, Chapman and Hall, London, 1950.
4 W. Karcher, R. Depaus, J. Van Eijk et J. Jacobs, *Carcinogenesis*, 4 (1979).
5 G. Chatot, M. Castegnaro, J. L. Roche et R. Fontanges, *Chromatographia*, 3 (1970) 507.
6 G. Grimmer et H. Böhnke, *Erdöl Kohle, Erdgas, Petrochem. Brenst.-Chem.*, 31 (1978)272.
7 E. Sawicki, T. R. Hauser et T. W. Stanley, *Int. J. Air Poll.*, 2 (1960) 253.
8 E. Sawicki, W. Elbert, T. W. Stanley, T. R. Hauser et F. T. Fox, *Anal. Chem.*, 32 (1960)810.
9 H. Martin, N. Defay et F. Geerts-Evrerol, *Tetrahedron*, 20 (1964) 1073.
10 E. Cavalieri et M. Calvin, *J. Chem. Soc., Perkin Trans. I*, 1 (1972) 1253.

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CHROM. 11,920

IDENTIFICATION OF ERGOT ALKALOIDS WITH A PHOTOCHEMICAL REACTION DETECTOR IN LIQUID CHROMATOGRAPHY

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SUMMARY

An on-line photochemical reaction detector is described for the identification of ergot alkaloids by high-performance liquid chromatography. The fluorescence signal of alkaloids decreases within about 20 sec of irradiation and disappears selectively from complex chromatograms. The application of this principle to urine samples is described.

INTRODUCTION

Ergot alkaloids are an important group of pharmaceutically active substances. The identification of ergot alkaloids in a high-performance liquid chromatography (HPLC) chromatogram is usually difficult, but especially useful and necessary in complex matrices, such as biological materials. Blake *et al.*[1] noticed this difficulty in the quantitative analysis of lysergic acid dimethylamide (LSD). For HPLC, UV and fluorescence spectroscopy can be used for on-line identification. To enhance the reliability of this approach, an additional specific identification method is therefore desirable.

Recently, Twitchett *et al.*[2] have shown the feasibility of a photochemical reactor as an on-line detection system in liquid chromatography (LC). They mentioned very briefly the decrease in fluorescence of LSD on irradiation and the use of this phenomenon in the identification of LSD. In the present study we have investigated the application of a photochemical reaction detector in the qualitative determination of a wide range of ergot alkaloids.

Stoll and Schlientz[3] have investigated the photochemistry of ergot alkaloids and proposed the reaction shown in Fig. 1, whereby no fluorescence is observed for the lumi derivatives of ergot alkaloids. This loss of the fluorescence on irradiation with UV light has been investigated for ergot and 9,10-dihydroergot alkaloids and seems to be specific to this group of compounds. It can therefore be used for identification purposes. From these investigations it appears that the scheme in Fig. 1

* On a summer research assistantship at Sandoz, Basle, Switzerland.

does probably not apply to the reaction occurring in the photochemical reactor. This belief is based on the observation that the dihydroergot alkaloids also show a fluorescence decrease on irradiation, even though this group has already a saturated bond in the 9–10 position.

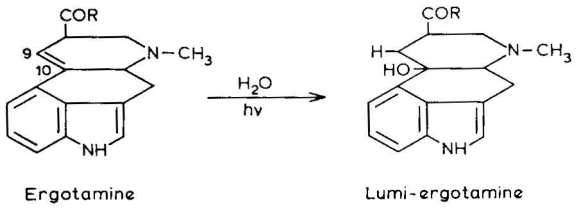

Fig. 1. A possible photochemical reaction of ergot alkaloids.

EXPERIMENTAL

Reagents

All ergot alkaloids were obtained from Sandoz (Basle, Switzerland). The water was doubly distilled. The acetonitrile was HPLC grade (Rathbury, Walkerburn, Great Britain) and the sodium hydrogen carbonate was analytical grade (Merck, Darmstadt, G.F.R.).

Instrumental

All experiments were carried out with the HPLC system described in Fig. 2

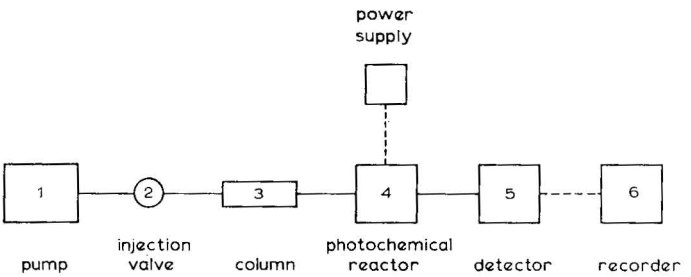

Fig. 2. Instrumental setup for HPLC with a photochemical reaction detector. 1, Waters M-6000 pump (Waters Assoc., Milford, Mass., U.S.A.); 2, Valco injection system (Valco, Houston, Texas, U.S.A.) with a 100-μl loop; 3, pre-packed column RP-18, 5-μm particle size (Knauer, Oberursel, Switzerland) 120 mm × 4.6 mm I.D.; 4, photochemical reactor; 5, spectrofluorometer SFM-22 (Kontron, Zurich, Switzerland) with a 50-μl flow-cell and operating at λ_{ex} = 327 nm and λ_{em} = 410 nm, for the dihydroergot alkaloids at λ_{ex} = 280 nm and λ_{em} = 340 nm; 6, recorder W + W 1100 (Kontron). Peaks were integrated with the Hewlett-Packard Laboratory Data System HF 3354.

Photochemical reactor

The reactor (Fig. 3) consists of a lamphouse and power supply (Siemens, 5 NS 1102) with a XBO 150W/1 Xenon high-pressure lamp. Around this lamp a coiled quartz capillary (1 m × 0.5 mm I.D.) (Felber, Basle, Switzerland) with a coil diameter of 6 cm is placed. Under the lamphouse, aluminium foil enhances the reflection of the emitted light. Cooling is achieved with pressurized air, precooled by solid CO_2 in a Dewar vessel.

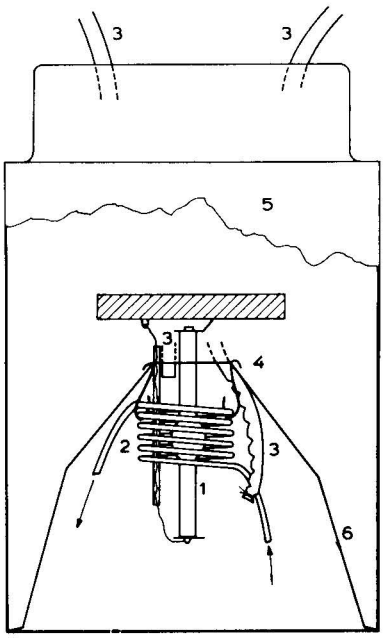

Fig. 3. The photochemical reactor. 1, Xenon high pressure lamp; 2, quartz capillary; 3, cooling; 4, clip; 5, lamphouse; 6, aluminium reflection shield.

RESULTS AND DISCUSSION

Band broadening in the reactor

The band broadening in the reactor, σ_v, when measured on non-irradiated peaks (with k' 1–3) was *ca.* 200 μl at a flow-rate of 1.5 ml/min. The band broadening could be reduced by fitting the quartz capillary with connectors of zero dead volume leading to the column and the detector, instead of PTFE and silicone tubes used in our experiments. An even smaller band broadening in the system can also be realized by using flow-segmentation techniques[4].

Effect of different parameters on the photochemical reaction

Influence of the flow. The lower the flow-rate, φ, the longer is the reaction time t_r (for $\varphi = 0.5$ ml/min $t_r = 53$ sec; for $\varphi = 1.0$ ml/min $t_r = 27$ sec; for $\varphi = 1.5$ ml/min $t_r = 18$ sec, *etc.*). Fig. 4 shows the influence of flow variation on the signal decrease, S_{irr}/S, where S_{irr} is the signal of an irradiated compound (peak areas or peak height) and S the signal of the non-irradiated compound. For positive identification of compounds, very low S_{irr}/S values, obtained at low flow-rates, are especially important. The flow variation has the same effect on the fluorescence signal of the 9,10-dihydroergot alkaloids.

Variation of concentration. The influence of the concentration on the signal decrease of five ergot alkaloids is shown in Fig. 5: the lower the concentration, the larger the decrease. At concentrations of *ca.* 10 ng/100 μl injection a plateau is reached, which means that the signal decrease is no longer dependent on the concentration. The same effect is observed with the dihydroergot alkaloids.

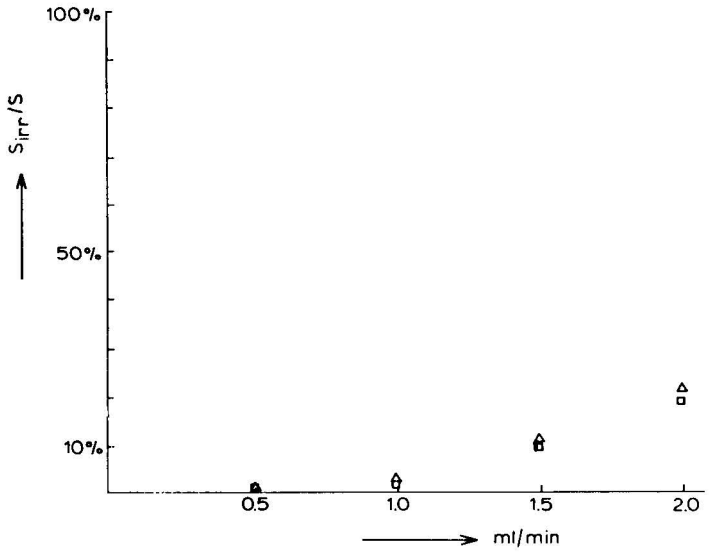

Fig. 4. The dependence of the signal decrease, S_{irr}/S, on the flow-rate. Conditions: eluent, 0.01 *M* $NcHCO_3$-acetonitrile (58:42, v/v) pH 2.1; sensitivity control medium, fine 50. Aci-ergotamine (0.14 μg/ml); aci-ergotaminine (0.19 μg/ml); ergotamine (0.19 μg/ml); ergocornine (0.19 μg/ml); and ergokryptine (0.23 μg/ml). △, Aci-ergotamine; □, other compounds.

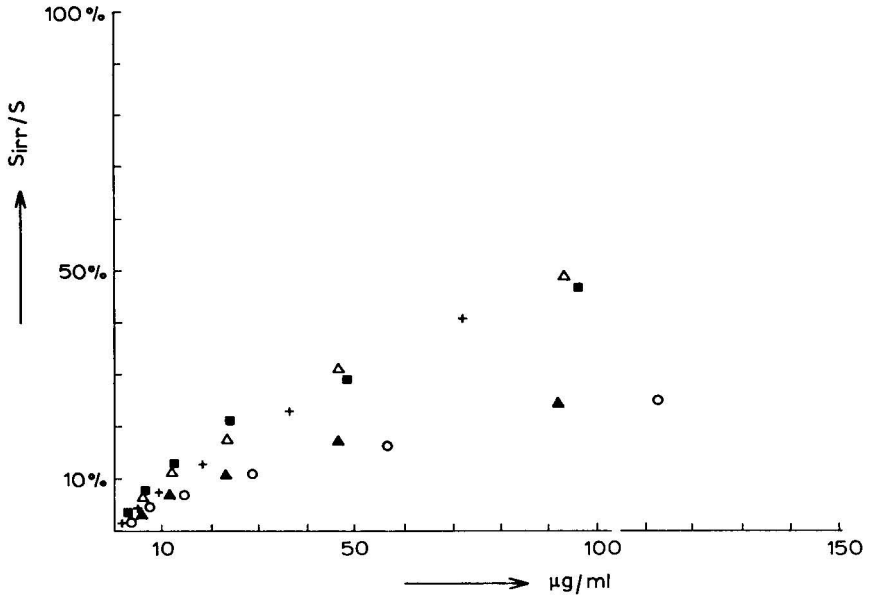

Fig. 5. Dependence of the signal decrease of five ergot alkaloids on their concentrations. Conditions: eluent 0.01 *M* $NaHCO_3$–acetonitrile (58:42, v/v); pH 8.5; sensitivity control low, fine 50; flow-rate 1.5 ml/min. △, Aci-ergotamine; ■, ergotamine; +, aci-ergotaminine; ▲, ergocornine; ○, ergokryptine.

Influence of the composition of the mobile phase. Changing the percentage of acetonitrile in the eluent by 4%, from 58:42 (v/v) to 62:38 (v/v) 0.01 *M* $NaHCO_3$–acetonitrile at pH 8.5, results in an increase of k' values by *ca.* 100% (see Table I); the signal decrease S_{irr}/S also becomes higher. The increase of the S_{irr}/S value at higher percentages of acetonitrile is due to a higher value of S_{irr}, resulting from the lower polarity of acetonitrile in the mobile phase. The same effect is noticeable in batch experiments with dihydroergotamine.

TABLE I

SIGNAL RATIOS (S_{irr}/S) AND RETENTION DATA OF VARIOUS ALKALOIDS AS A FUNCTION OF DIFFERENT ELUENT COMPOSITIONS

*Eluent**	*pH*	*Compound***	*k'*	*Concentration**** *(μg/ml)*	*Measured* S_{irr}/S
58:42 (v/v)	8.5	A-E	0.8	23.3	0.314
		A-Erm	2.3	11.2	0.230
		E	2.9	11.1	0.291
		E-C	4.6	8.4	0.175
		E-K	6.5	8.4	0.170
62:38 (v/v)	8.5	A-E	1.5	16.8	0.344
		A-Erm	4.4	6.9	0.196
		E	5.5	6.7	0.277
		E-C	8.7	4.9	0.169
		E-K	12.6	4.6	0.171
58:42 (v/v)	2.2	A-E	1.2	12.8	0.483
		A-Erm	1.6	8.9	0.487
		E	1.8	11.7	0.521
		E-C	2.2	9.7	0.459
		E-K	3.0	9.4	0.475
62:38 (v/v)	2.2	A-E	1.3	12.7	0.383
		A-Erm	1.8	8.2	0.396
		E	2.3	10.1	0.422
		E-C	2.7	8.2	0.396
		E-K	3.9	7.7	0.376

* 0.01 *M* $NaHCO_3$–acetonitrile.

** A-E = aci-ergotamine; A-Erm = aci-ergotaminine; E = ergotamine; E-C = ergocornine; E-K = ergokryptine.

*** For correction of these values, see eqn. 1.

An increase in absolute signal of *ca.* 300% was observed when the percentage of acetonitrile was varied from 30 to 50%. It was noticeable that the measured S_{irr}/S values were lower for compounds with a higher k'. This could be due to dilution in the column and the reactor. The concentrations in Table I are therefore corrected for this effect according to eqn. 1:

$$\frac{C_{max}}{C_0} = \frac{V_0}{AL\sqrt{2\pi^3}} \cdot \frac{N^{1/2}}{(1+k')} \tag{1}$$

where C_{max} is the concentration at the peak maximum, C_0 the injected concentration,

V_0 the injection volume, A the cross-section of the separation column, L the length of the column, N the plate number and k' the capacity factor. When the percentage of acetonitrile at pH 8.5 was changed, ergokryptine, for instance, gives the same S_{irr}/S value for two very different concentrations (see Table I). Plotting S_{irr}/S as a function of these corrected concentrations, we found that the decrease in fluorescence (S_{irr}/S) was almost the same for all the measured compounds.

A drop of the pH in the mobile phase from 8.5 to 2.2 produced a pronounced change in the k' values, which can be attributed to the ionic form in which the alkaloids exist at this low pH. After correction again for dilution in the column and the reactor, it becomes obvious that the S_{irr}/S values in an acidic medium are much higher. Possibly the 9–10 position in the molecule is stabilized and attack by a H_3O^+ ion becomes more difficult. An increase in the signal of *ca.* 30% was observed when the pH was lowered to 1.5 in batch experiments with dihydroergotamine at eluent compositions of 30–50% acetonitrile.

Effect of irradiation on band broadening

An interesting observation is that band broadening in the reactor is substantially reduced following irradiation. This is due to the fact that at lower ergot alkaloid concentrations a greater signal decrease is obtained. The improved resolution after irradiation is seen in Fig. 6. This is typical for this ergot system. The following

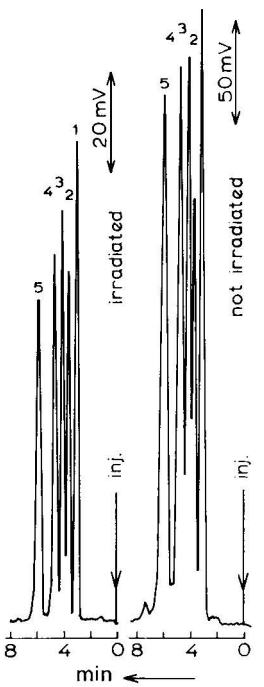

Fig. 6. Separation of five ergot alkaloids. Conditions: eluent, 0.01 *M* $NaHCO_3$–acetonitrile (58:42, v/v); sensitivity control low, fine 50; flow-rate 1.5 ml/min. 1, aci-ergotamine (11.7 μg/ml); 2, aci-ergotaminine (9.1 μg/ml); 3, ergotamine (12.0 μg/ml); 4, ergocornine (11.6 μg/ml); 5, ergokryptine (14.2 μg/ml).

explanation can be given. Let us assume that the peaks, entering the detector, are approximately Gaussian:

$$C = C_{max} \exp [-(t - t_R)^2/2\sigma^2] \quad (2)$$

and the concentration dependence of S_{irr}/S is expressed as

$$S_{irr}/S = a \times c + b \quad (3)$$

with $a = 0.008$ and $b = 0.08$ for aci-ergotamine, where a and b were calculated from experimental data shown in Fig. 5. It can be seen that the combination of eqns. 2 and 3 leads to smaller σ values for irradiated peaks. This effect is shown in Fig. 7.

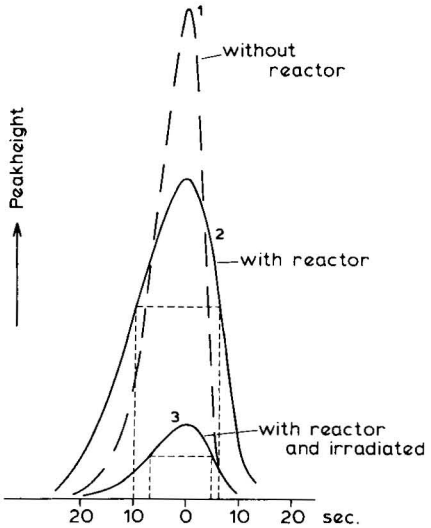

Fig. 7. The influence of reactor and irradiation on peak shape. Compound, aci-ergotamine; eluent, 0.01 M $NaHCO_3$–acetonitrile (58:42, v/v); pH 8.5, flow-rate 1.5 ml/min. 1, Without reactor (2σ = 9 sec); 2, with reactor (2σ = 16 sec); 3, with reactor and irradiated (2σ = 11.5 sec).

Selective identification of ergot alkaloids

For selective identification of ergot alkaloids a decrease of at least 90% in the fluorescence signal is necessary. This is best achieved with a low flow-rate (*e.g.* 0.5 ml/min), a low percentage of acetonitrile and a high pH. However, the amount of acetonitrile and the pH strongly influence the separation (see Table I). Therefore the possible variations are limited. The use of more polar chemically bonded surfaces might improve this situation. The use of a high pH shortens the column life and decreases its separation power[5]. Therefore the following conditions represent a good compromise: 0.01 M $NaHCO_3$–acetonitrile (58:42, v/v) at pH 2.2 at a flow-rate of 0.5 ml/min. Under these conditions the fluorescence decrease was 90–99% for 17 ergot alkaloids and dihydroergot alkaloids.

In a spiked urine sample one can see the decrease of the signals of dihydroergotoxine (Fig. 8) and various other ergot alkaloids in relation to a blank urine (Fig. 9).

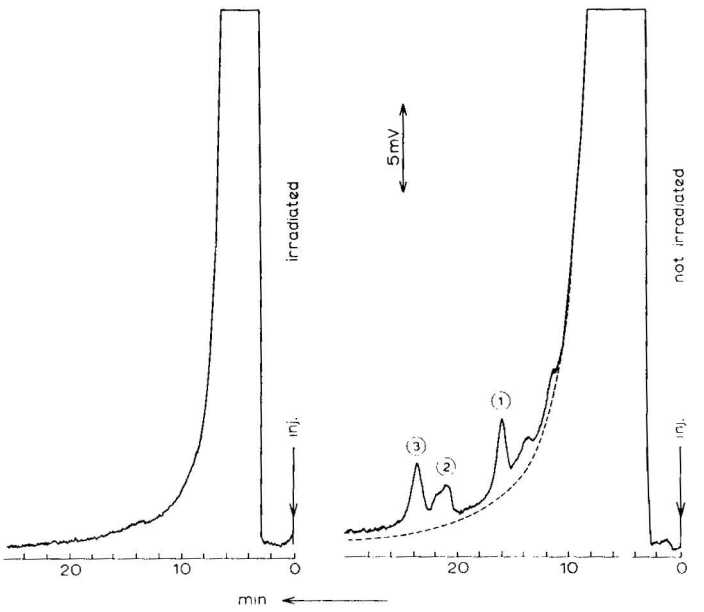

Fig. 8. Urine sample, spiked with dihydroergotoxine. $\lambda_{em} = 280$ nm, $\lambda_{em} = 340$ nm; sensitivity control medium, fine 50; flow-rate 0.5 ml/min; $v_p = 30$ cm/h; recorder 5 mV; inj. 100 μl. 1, Dihydroergocornine (46 ng/ml); 2, $\alpha + \beta$ dihydroergokryptine (46 ng/ml); 3, dihydroergocristine (46 ng/ml). – – –, blank urine.

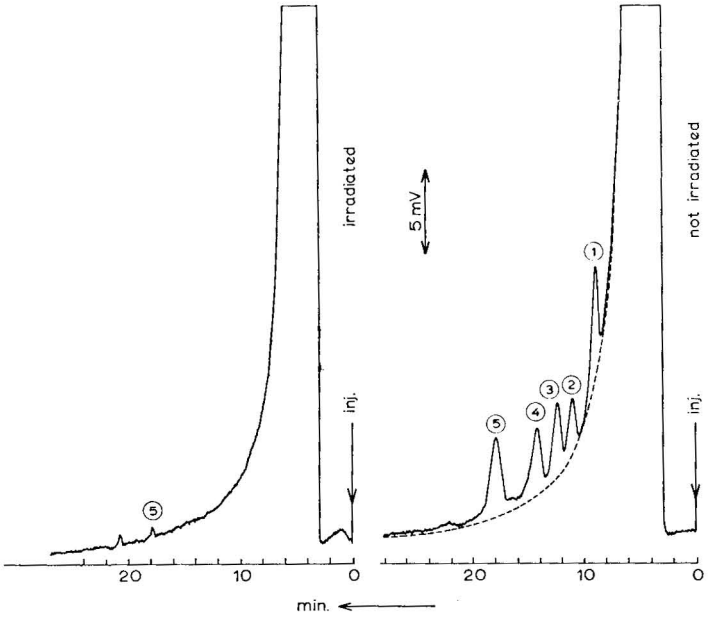

Fig. 9. Urine sample, spiked with 5 ergot alkaloids. $\lambda_{ex} = 327$ nm, $\lambda_{em} = 415$ nm; sensitivity control medium, fine 50; $v_p = 30$ cm/h; recorder 5 mV; inj. 100 μl. 1, Aci-ergotamine; 2, aci-ergotaminine; 3, ergotamine; 4, ergocornine; 5, ergokryptine; each 0.2 μg/ml. – – –, blank urine.

CONCLUSIONS

The photochemical detection technique in HPLC offers a selective identification for all the ergot alkaloids tested. Although it is suspected that the mechanism in Fig. 1 is not necessarily followed under the high-density irradiation conditions used here, one can conclude that the technique is of general applicability to ergot alkaloids. Preliminary results[6] suggest that the drastic fluorescence decrease may be at least partly due to a change in the indole ring, since indole exhibits the same behaviour on irradiation. The technique is suitable for qualitative work in complex samples.

The present study also indicates some of the general possibilities and the potential use of photochemical reactions for post-column reaction detectors in HPLC.

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REFERENCES

1 E. T. Blake, P. J. Cashman and J. I. Thornton, *Anal. Chem.*, 45 (1973) 394.
2 P. J. Twitchett, P. L. Williams and A. C. Moffat, *J. Chromatogr.*, 149 (1978) 683.
3 A. Stoll and W. Schlientz, *Helv. Chim. Acta*, 38 (1955) 585.
4 L. R. Snyder, *J. Chromatogr.*, 125 (1976) 287.
5 A. Wehrli, J. C. Hildenbrand, H. P. Keller, R. Stampfli and R. W. Frei, *J. Chromatogr.*, 149 (1978) 199.
6 A. H. M. T. Scholten, unpublished work.

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USE OF 2,6-DINITRO-4-TRIFLUOROMETHYLBENZENESULFONIC ACID AS A NOVEL DERIVATIZING REAGENT FOR THE ANALYSIS OF CATECHOLAMINES, HISTAMINES AND RELATED AMINES BY GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

We have found that 2,6-dinitro-4-trifluoromethylbenzenesulfonic acid reacts rapidly and specifically with primary amines at room temperature. We have used this reagent for derivatizing phenylethylamines, including catecholamines, and histamine and 1-methylhistamine. After the reaction, hydroxyl groups were derivatized to form the corresponding trimethylsilyl ethers, and the final derivatives were analyzed by gas chromatography with electron-capture detection. These derivatives are stable, possess excellent gas chromatographic properties and are detected with high sensitivity. We have applied this method to the analysis of histamine and 1-methylhistamine in human urine.

INTRODUCTION

Amines, such as the catecholamines, dopamine and norepinephrine, and histamine, have key physiological roles in organisms from lower invertebrates to man, even though they are present in very low concentration. Other amines, such as phenylethylamine and octopamine, are usually present in even lower concentration, and have been referred to as "trace amines"[1]. Attempts to elucidate the possible physiological function of these compounds have required the use of extremely sensitive and specific analytical techniques. One approach has been to convert the amines into volatile derivatives which can be separated by gas chromatography (GC) and detected by electron-capture detection (ECD). Several derivatives, including trifluoroacetyl (TFA)[2–6], pentafluoropropionyl (PFP)[6–8], heptafluorobutyryl (HFB)[8–10], O-trimethylsilyl (TMS) ether N-pentafluorobenzylimine[11], and dinitrophenyl (DNP)[12,13], derivatives have been found to be suitable for the analysis of amines by GC–ECD.

The DNP derivatives are particularly advantageous for the analysis of amines in tissues, since they are stable to solvent extraction and/or chromatographic proce-

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dures which can be used to isolate them from the bulk of the tissue components. Day *et al.*[12] used 2,4-dinitrofluorobenzene (DNFB) to dinitrophenylate several aliphatic amines and to separate them by GC. However, since DNFB reacts with hydroxyl groups in addition to amino groups, compounds which contain both functional groups would incorporate two or more DNP groups and would therefore be too non-volatile to be separated by GC. In order to avoid this problem, Edwards and Blau[13] used 2,4-dinitrobenzenesulfonic acid (DNBS), which reacts relatively specifically with primary amino groups, and to subsequently derivatize the hydroxyl groups with a trimethylsilylating reagent. These procedures have been successfully used for the analysis of several phenylethylamines in tissues by GC combined with either ECD[13] or chemical-ionization mass spectrometry (CI-MS)[14].

However, we have found that the DNP derivatives of histamine and related compounds are unsuitable for GC analysis, since they exhibited excessive tailing. In an attempt to avoid this problem, we have studied the chromatographic properties of the corresponding 2,6-dinitro-4-trifluoromethyl (DNT) derivatives. Crosby and Bowers[15] originally used α,α,α-trifluoro-3,5-dinitro-4-chlorotoluene to form the DNT derivatives of amines released by the hydrolysis of carbamate pesticides. These derivatives could be detected by ECD in amounts as low as 50 pg. We also observed[13] that the DNT derivatives were more volatile, exhibited less tailing, and could be detected in lower amounts than could the corresponding DNP-phenylethylamines by GC–ECD. However, the reagent which Crosby and Bowers[15] used suffers from the same lack of specificity as DNFB in that it reacts not only with primary amino groups but also with secondary amino groups and hydroxyl groups. In the present report, we have used 2,6-dinitro-4-trifluoromethylbenzenesulfonic acid (DNTS) to derivatize various amines. This reagent has the advantages of introducing the DNT-group, but, as expected, it had the specificity towards primary amino groups as did DNBS. The DNT derivatives of histamine and 1-methylhistamine, unlike the DNP derivatives, had good GC properties and could be used for the analysis of these compounds as well as of other amines in biological fluids. An additional advantage of DNTS is that it has a much greater reactivity than does DNBS and completely derivatizes primary amines in 10 min at room temperature. As a result, catecholamines, which are destroyed under the conditions needed to react with DNBS[13], may be reacted with DNTS to form stable derivatives.

MATERIALS AND METHODS

Reagents and solvents

All standard compounds were obtained in the highest purity available from commercial sources and used without further purification. The following amines were obtained as the hydrochloride salts: benzylamine and phenylethylamine (K & K Labs., Plainview, N.Y., U.S.A.), phenylethanolamine, DL-normetanephrine and 4-hydroxy-3-methoxyphenylethylamine (3-methoxytyramine) (Regis, Chicago, Ill., U.S.A.), *m*-octopamine (norphenylephrine), *p*-octopamine, *p*-tyramine, phenylpropanolamine (norephedrine) and piperidine (Aldrich, Milwaukee, Wisc., U.S.A.), and *m*-tyramine (Vega-Fox, Tucson, Ariz., U.S.A.). *o*-Tyramine hydrogen bromide was synthesized by Dr B. L. Goodwin (Queen Charlotte's Maternity Hospital, London, Great Britain). L-Epinephrine and the hydrochloride salts of L-norepinephrine, do-

pamine and tryptamine were obtained from Sigma (St. Louis, Mo., U.S.A.). Histamine dihydrochloride, 1-methylhistamine dihydrochloride and 3-methylhistamine dihydrochloride were purchased from Calbiochem (San Diego, Calif., U.S.A.). D-Amphetamine sulfate was purchased from Sigma, L-metaraminol-D-bitartrate from Regis, and serotonin creatinine sulfate monohydrate from Aldrich. The hydrochloride salt of *p*-chlorophenylethylamine was prepared from the free base (Aldrich). Putrescine, cadaverine, 1,7-diaminoheptane, spermidine and spermine were obtained from Aldrich. N,O-bis-(trimethylsilyl)acetamine (BSA) was obtained from Pierce (Rockford, Ill., U.S.A.). Nanograde benzene and ethyl acetate were purchased from Mallinckrodt (St. Louis, Mo., U.S.A.). Reagent grade 1-butanol and chloroform were obtained from Fisher (Pittsburgh, Pa., U.S.A.). 4-Chloro-3,5-dinitrobenzotrifluoride was obtained from K & K Labs.

Derivative formation

The sodium salt of DNTS was synthesized by the nucleophilic substitution reaction in which the sulfite ion acting as a nucleophile displaces the halide on the activated aromatic ring of 4-chloro-3,5-dinitrobenzotrifluoride[16].

Stock solutions of each amine were prepared in 0.001 *N* HCl (except 0.1 *N* HCl for epinephrine) at a concentration of 1 mg/ml of the free base. The standard solutions were stored at −20°. A 10-μl amine solution was reacted with 100 μl of 0.11 *M* DNTS (in 50% saturated sodium borate) for 10 min at room temperature. The reaction mixture immediately turned yellow on addition of DNTS. The DNT-amines were extracted twice with 400 μl of benzene (or ethyl acetate in the case of the dopamine, norepinephrine, diamine and polyamine derivatives). After centrifugation 750 *g* for 5 min), the organic layers were transferred to acid-washed, silanized 0.3-ml Reacti-Vials (Pierce) and evaporated to dryness under nitrogen. Hydroxylated amine derivatives were converted into the corresponding TMS-ethers by addition of 10 μl of BSA and 40 μl of benzene (or ethyl acetate) and heating to 60° for 15 min. N-DNT, O-TMS derivatives were evaporated to dryness under nitrogen. Appropriate dilutions of the derivatives were made before injection into the gas chromatograph.

Gas–liquid chromatography

A Model 7400 Packard gas chromatograph equipped with a ^{63}Ni electron-capture detector was used. Glass columns, either 6 ft. or 3 ft. in length and 2 mm I.D. were silanized and respectively packed with 3% OV-1 or 3% SP-2250 on 80–100 mesh Supelcoport (Supelco, Bellefonte, Pa., U.S A.).

GC–ECD analysis of histamine and 1-methylhistamine in human urine

The extraction of histamine and 1-methylhistamine from the urine of a human subject was carried out following essentially the method of Fram and Green[17]. A 4-ml urine sample (in duplicate) was placed in a 15-ml conical centrifuge tube and its pH adjusted to 13 with 10 *N* NaOH. The sample was saturated with NaCl and shaken for 15 min with 4 ml of a mixture of 1-butanol and chloroform (3:2, v/v). After centrifugation at 1650 *g* for 15 min, the organic layer was transferred to a 15-ml centrifuge tube containing 6 ml of *n*-heptane and 0.5 ml of 0.2 *N* HCl. The mixture was shaken for 15 min and then centrifuged at 750 *g* for 15 min. After the upper phase had been aspirated off, the aqueous phase was transferred to a 5-ml conical centrifuge

tube and lyophilized. The residue was then derivatized using 1 ml of 0.11 *M* DNTS as described above. The DNT derivatives were extracted four times with 400 μl of benzene. After centrifugation, the combined organic phase was evaporated to dryness. The residue was taken up in benzene and injected into the gas chromatograph.

Gas chromatography–mass spectrometry

The chemical-ionization mass spectra of the derivatives were obtained on a Finnigan Model 3200F combined gas chromatograph–mass spectrometer. The gas chromatograph was equipped with a 3 ft. × 2 mm I.D. silanized U-column packed with 3% SP-2250 on 80–100 mesh on Supelcoport. The GC–CI-MS conditions were: column and injection port temperature, 230°; transfer line temperature, 265°; analyzer, 70°; electron energy, 120 eV; emission current, 0.49 mA; electron multiplier voltage, 1800 V; ion source pressure, 0.9 Torr (methane); methane flow-rate, 12 ml/min.

TABLE I

CHROMATOGRAPHIC RETENTION DATA OF N-DNT, O-TMS AMINES

n.d. = not determined; t_R = retention time (min); Rel. t_R = relative retention time, phenylethylamine = 1.0. No peaks were observed for the secondary amines, piperidine and epinephrine. At 230°, 3-methylhistamine eluted with a t_R = 6.69 min, using 3% SP-2250.

Amine	*Column*					
	*3% OV-1**		*3% SP-2250***			
	250°		*220°*		*230°*	
	t_R	*Rel.* t_R	t_R	*Rel.* t_R	t_R	*Rel.* t_R
Benzylamine	0.89	0.75	1.38	0.70	0.98	0.71
Amphetamine	0.98	0.83	1.18	0.60	0.89	0.64
Phenylethylamine	1.18	1.00	1.97	1.00	1.38	1.00
Phenylpropanolamine	1.38	1.17	1.38	0.70	0.98	0.71
Phenylethanolamine	1.58	1.34	1.97	1.00	1.38	1.00
1-Methylhistamine	1.77	1.50	4.92	2.47	3.35	2.43
o-Tyramine	1.87	1.58	2.76	1.40	1.77	1.28
p-Chlorophenylethylamine	2.17	1.84	4.13	2.10	2.76	2.00
m-Tyramine	2.36	2.00	3.94	2.00	2.56	1.86
Histamine	2.56	2.17	9.15	4.64	5.91	4.28
p-Tyramine	2.56	2.17	4.63	2.35	2.95	2.14
Metaraminol	2.56	2.17	2.46	1.25	1.77	1.28
m-Octopamine	2.76	2.34	3.64	1.85	2.36	1.71
p-Octopamine	3.25	2.75	4.33	2.12	2.76	2.00
3-Methoxytyramine	3.94	3.34	7.28	3.70	4.92	3.57
Dopamine	4.53	3.84	6.99	3.55	4.53	3.28
Normetanephrine	4.53	3.84	6.00	3.05	3.94	2.85
Norepinephrine	5.12	4.33	5.91	3.00	3.74	2.71
Tryptamine	5.71	4.84	n.d.	n.d.	n.d.	n.d.
Serotonin	10.83	9.18	n.d.	n.d.	n.d.	n.d.

* A 6 ft. × 2 mm I.D. glass column was used with nitrogen flow-rate of 32 ml/min. Injection port and detector: 275°.

** A 3 ft. × 2 mm I.D. glass column was used with a nitrogen flow-rate of 44 ml/min. Injection port and detector: 245°.

RESULTS

The GC characteristics of the derivatives of 20 amines on 3% OV-1 and 3% SP-2250 are shown in Table I. Although neither stationary phase is able to separate all of the amine derivatives, those not separated on one phase may be separated on the other. Thus, phenylethylamine and phenylethanolamine have identical retention times on 3% SP-2250, but they are well separated on 3% OV-1. On the other hand, histamine, *p*-tyramine, and metaraminol elute with retention times which are identical on 3% OV-1 but which greatly differ on 3% SP-2250. In cases where the identity of a peak is ambiguous, the use of two different columns may aid in an unequivocal identification. Alternatively, the sample may be injected with and without trimethylsilylation, since DNT-amines which contain hydroxyl groups (*e.g.* phenylethanolamine and *p*-tyramine) will elute from the column only when the hydroxyl groups have been derivatized. Peaks corresponding to non-hydroxylated DNT-amines (*e.g.*, phenylethylamine and histamine) will be unaltered by trimethylsilylation.

Fig. 1 illustrates the separation of phenylethylamine, phenylethanolamine, the *ortho*, *meta*, and *para* isomers of tyramine, dopamine, norepinephrine and tryptamine on 3% OV-1. A mixture containing 1-methylhistamine and histamine was separated on 3% SP-2250 (Fig. 2). The DNT derivatives of diamines, except for putrescine (*e.g.*, cadaverine and 1,7-diaminoheptane) and polyamines (*e.g.*, spermidine and spermine) exhibited poor GC properties and low sensitivity towards the ECD. Putrescine gave a sharp peak which appeared before phenylethylamine on both stationary phases.

We have obtained preliminary results for the determination of histamine and 1-methylhistamine in human urine (Fig. 3). The excretion of these two compounds was estimated to be 55 and 285 μg/24 h, respectively.

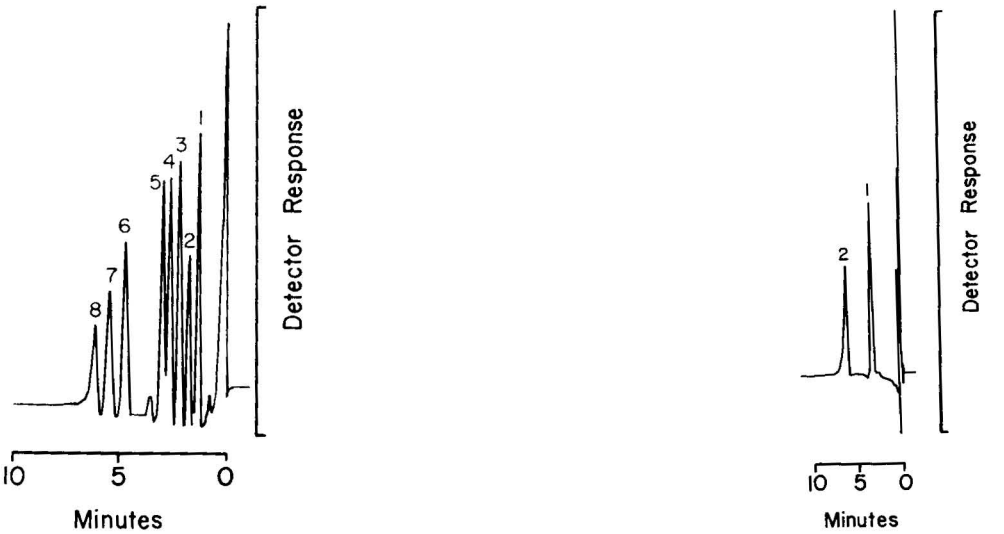


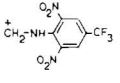
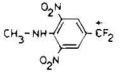
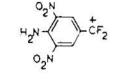
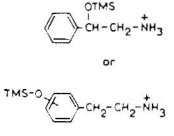
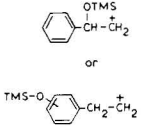
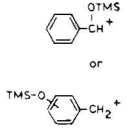
Fig. 1. Gas chromatographic separation of N-DNT, O-TMS derivatives of 0.2 ng each of (1) phenylethylamine, (2) phenylethanolamine, (3) *o*-tyramine, (4) *m*-tyramine, (5) *p*-tyramine, (6) dopamine, (7) norepinephrine, and (8) tryptamine. Conditions: 6 ft. × 2 mm I.D. glass column packed with 3% OV-1 on Supelcoport (80–100 mesh); column, 250°; injection port and detector, 275°; flow-rate, 30 ml/min.

Fig. 2. Gas chromatographic separation of N-DNT derivatives of 1-methylhistamine (5 ng) and histamine (4 ng). Conditions: 3 ft. × 2 mm I.D. glass column packed with 3% SP-2250 on Supelcoport (80–100 mesh); column 230°; injection port and detector, 245°; flow-rate, 44 ml/min. Peaks: (1) 1-methylhistamine, (2) histamine.

TABLE II

PARTIAL METHANE CI MASS SPECTRA OF REPRESENTATIVE N-DNT, O-TMS AMINES

Data are *m/e* (relative abundance). Probable structures of the ion fragments are shown.

*Amine**	MH^+	$MH^+ - HF$	$MH^+ -$ *TMSOH*						
Bz	342 (11)	322 (100)	—	264 (19)	—	—	—	—	—
PE	356 (63)	336 (100)	—	264 (21)	246 (11)	232 (16)	—	—	—
PEOH	444 (3)	424 (23)	354 (100)	264 (6)	246 (22)	232 (13)	210 (6)	193 (18)	179 (66)
m-Tyr	444 (17)	424 (23)	—	264 (5)	246 (3)	232 (12)	210 (23)	193 (100)	179 (27)
p-Tyr	444 (2)	424 (6)	—	264 (4)	246 (2)	232 (8)	210 (25)	193 (100)	179 (33)
p-Oct	—	512 (2)	442 (28)	264 (57)	246 (50)	232 (21)	210 (9)	193 (100)	179 (14)
HA	346 (100)	326 (13)	—	—	—	232 (44)	—	—	—
1-MHA	360 (100)	340 (33)	—	—	—	232 (19)	—	—	—

* Bz, benzylamine; PE, phenylethylamine; PEOH, phenylethanolamine; *m*-Tyr, *m*-tyramine; *p*-Tyr, *p*-tyramine; *p*-Oct, *p*-octopamine; HA, histamine; 1-MHA, 1-methylhistamine.

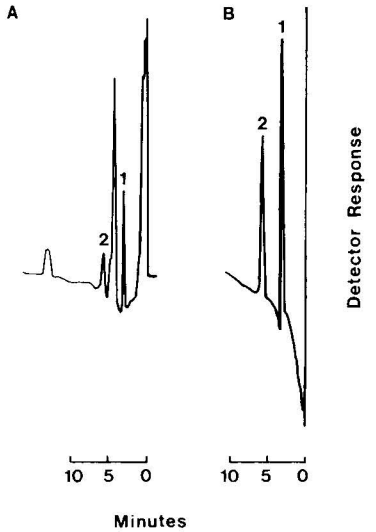


Fig. 3. GC–ECD analysis of histamine and 1-methylhistamine in human urine. (B) Standard: 10 μg of each amine in 4 ml of water and extracted as described in the text. (A) Sample: 4 ml of a urine specimen carried through the extraction procedure. Peaks: (1) 1-methylhistamine, (2) histamine. Conditions were the same as in Fig. 2.

The partial methane CI mass spectra of the N-DNT, O-TMS derivatives of some representative amines are given in Table II. All of the amines except octopamine have an M + 1 peak. However, this represents the base peak in only the spectra of the histamine derivatives. The derivatives of phenylethylamine and benzylamine have base peaks resulting from the loss of HF, while the derivatives of hydroxylated phenylethylamines have base peaks (*m/e* 193) corresponding to the O-TMS phenylethyl fragment.

DISCUSSION

Our results suggest that DNTS is an important new reagent which may effectively replace DNBS in derivatizing amines for their analysis by GC–ECD. The DNT derivatives are more volatile, exhibit improved GC properties and are more sensitive to ECD than are the corresponding DNP-amines. An important advantage of DNTS is that it is extremely reactive compared with DNBS and can form the DNT-amines under very mild conditions (*i.e.* 10 min at room temperature). This is apparently due to the fact that the electron-withdrawing trifluoromethyl group of DNTS facilitates the removal of the sulfonic acid group during the reaction with an amine. Consequently, whereas catecholamines and serotonin are destroyed during their reaction with DNBS[13], they can be easily derivatized with DNTS to form stable derivatives.

In spite of the high reactivity of DNTS, it nevertheless has a specificity towards primary amino groups that is comparable to that of DNBS. None of the secondary amines tested produced any color change in the reaction mixture nor did they result in any peaks in the gas chromatograms. That hydroxyl groups did not react with DNTS is shown by the fact that the DNT derivatives of hydroxylated amines failed to produce a peak unless they were trimethylsilylated. Moreover, the mass spectra (Table II) indicated that all of the derivatized compounds had the structures expected

The utility of DNTS is illustrated by the fact that it forms derivatives with histamine and 1-methylhistamine which have excellent GC properties. Presumably owing to the polar nature of the imidazole ring, previous attempts have largely been unsuccessful in analyzing these amines by GC. For example, Cancalon and Klingman[18] failed to observe any GC peaks for either the TFA or TMS derivatives of histamine. Although Navert[19] reported that the TFA, HFB and TMS derivatives of histidine, histamine and various N-methylhistamines could be analyzed by GC with flame ionization detection, we could not detect either the TFA or the HFB derivatives of histamine and 1-methylhistamine by GC–ECD.

GC–ECD analysis of the DNT derivatives formed from human urine extracts gave values of histamine and 1-methylhistamine excretion of 55 and 285 μg/day, respectively. These values are in good agreement with earlier results of 16–53 μg/day of histamine and 140–480 μg/day of 1-methylhistamine reported by Fram and Green[17], using a spectrophotometric technique.

Our results also suggest that DNTderivatives could be used to determine concentrations of catecholamines and other related amines in biological samples by GC–ECD. In addition, the high molar extinction coefficient of these derivatives makes them amenable to analysis by high performance liquid chromatography. Finally, it should be possible to analyze the DNT derivatives by GC–MS by procedures similar to those used for the analysis of DNP derivatives[14].

ACKNOWLEDGEMENTS

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REFERENCES

1 E. Usdin and M. Sandler (Editors), *Trace Amines and the Brain*, Vol. 1, Marcel Dekker, New York, 1976.
2 D. D. Clarke, S. Wilk, S. E. Gitlow and M. J. Franklin, *J. Gas Chromatogr.*, 5 (1967) 307.
3 S. Kawai and Z. Tamura, *Chem. Pharm. Bull.*, 16 (1968) 699.
4 L. M. Bertani, S. W. Dziedzic, D. D. Clarke and S. E. Gitlow, *Clin. Chim. Acta*, 30 (1970) 227.
5 I. L. Martin and G. B. Baker, *Biochem. Pharmacol.*, 26 (1977) 1513.
6 E. Ånggard and G. Sedvall, *Anal. Chem.*, 41 (1969) 1250.
7 K. P. Wong, C. R. J. Ruthven and M. Sandler, *Clin. Chim. Acta*, 47 (1973) 215.
8 F. Karoum, F. Cattabeni and E. Costa, *Anal. Biochem.*, 47 (1972) 550.
9 L. M. Cummins and M. J. Fourier, *Anal. Lett.*, 2 (1969) 403.
10 S. Wilk, S. E. Gitlow, M. J. Franklin and H. E. Carr, *Clin. Chim. Acta*, 10 (1964) 193.
11 A. C. Moffat and E. G. Horning, *Biochim. Biophys. Acta*, 222 (1970) 248.
12 E. Day, T. Golab and J. Koons, *Anal. Chem.*, 38 (1966) 1053.
13 D. J. Edwards and K. Blau, *Anal. Biochem.*, 45 (1972) 387.
14 D. J. Edwards, P. S. Doshi and I. Hanin, *Anal. Biochem.*, 96 (1979) 308.
15 D. G. Crosby and J. B. Bowers, *J. Agric. Food Chem.*, 16 (1968) 839.
16 J. T. Gerig and J. D. Reinheimer, *J. Amer. Chem. Soc.*, 97 (1975) 168.
17 D. H. Fram and J. P. Green, *J. Biol. Chem.*, 240 (1965) 2036.
18 P. Cancalon and J. D. Klingman, *J. Chromatogr. Sci.*, 10 (1972) 253.
19 H. Navert, *J. Chromatogr.*, 106 (1975) 218.

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CHROM. 11,917

CONVENIENT METHOD FOR THE GAS CHROMATOGRAPHIC ANALYSIS OF HEXOSAMINES IN THE PRESENCE OF NEUTRAL MONOSACCHARIDES AND URONIC ACIDS

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(Received April 20th, 1979)

SUMMARY

A convenient gas chromatographic method has been devised for the analysis of hexosamines in the presence of neutral and acidic sugars, which involves sequential derivatization reactions of nitrous acid deamination, mercaptalation, and trimethylsilylation. This method allows rapid, simultaneous determination of 0.1–1 μmole samples of hexosamines with coefficients of variation of less than 3%.

INTRODUCTION

Hexosamines are basic constituents of a variety of carbohydrate materials, including glycolipids, glycoproteins and proteoglycans, and their determination is of primary importance in the elucidation of the structures of these materials. The gas chromatographic determination of hexosamines in carbohydrate materials is most frequently performed on the trimethylsilyl derivatives of the N-acetylated methanolysates[1], for complete separation of their peaks from those of accompanying neutral and acidic monosaccharides. However, this method gives multiple peaks of hexosamine derivatives owing to different anomeric configurations, and is laborious. Borohydride reduction of the acid hydrolysates, followed by trifluoroacetylation[2], may convert hexosamines into trifluoroacetylated hexosaminitols, which give single peaks, but this procedure necessitates purification of the reduction products on a column of Sephadex prior to trifluoroacetylation. Recently a novel derivatization method[3] was reported, which involves sequential reactions of nitrous acid deamination, oximation, and acetylation. Nitrous acid deamination is a rapid reaction, but the other two are time-consuming. Therefore, we replaced these reactions by mercaptalation and trimethylsilylation[4]. In this paper we describe this simple and rapid 2,5-anhydrohexose dithioacetal trimethylsilylate method for the determination of hexosamines in the presence of neutral and acidic monosaccharides.

EXPERIMENTAL

Materials

2-Amino-2-deoxy-D-glucose and -D-galactose hydrochlorides (Wako, Osaka,

Japan) were used as samples of hexosamines. Chondroitin sulfate (whale cartilarge), hyaturonic acid (human umbilical cord), and porcine mucine were purchased from Seikagaku Kogyo (Tokyo, Japan), Tokyo Kasei (Tokyo, Japan), and Sigma (St. Louis, Mo., U.S.A.), respectively. The urinary carbohydrate materials were obtained by dialysis of urine samples for 24 h against distilled water, followed by freeze-drying of the non-dialyzable fractions. Barium nitrite was purchased from Nakarai (Kyoto, Japan). Other chemicals were also obtained from commercial sources. They were of the highest grade available.

Apparatus

Gas chromatography was performed on a Shimadzu 4BMPF instrument equipped with a hydrogen flame ionization detector. A sodium chloride-treated capillary column (50 m × 0.28 mm I.D.) coated with SF-96 was used at 225° throughout the work. This column was supplied by Gasurkuro Kogyo (Tokyo, Japan). The flow-rate of the carrier gas (nitrogen) was regulated at 1 ml/min by use of a 100:1 splitter. The eluate was continuously mixed with the scavenger gas (nitrogen), 50 ml/min, and the mixture was introduced into the detector. Peaks were integrated by a Shimadzu Chromatopak E1A integrator.

Hydrolysis of carbohydrate materials

A sample of a carbohydrate material (1–2 mg) or a mixture of carbohydrate materials contained in an ampoule was dissolved in 4 *M* hydrochloric acid (200 μl), and the ampoule was flushed with nitrogen, sealed, heated for 6 h on a boiling water-bath, and then opened. The solution was transferred with water washings to a reaction tube (5 cm × 5 mm I.D.), and evaporated to dryness under reduced pressure in a desiccator containing sodium hydroxide.

Analysis of hexosamines

A 0.1 *M* aqueous solution of 3-O-methyl-D-glucose (internal standard, 10–100 μl) was added to a sample of a hexosamine hydrochloride (0.1–1 μmole) or a mixture of hexosamine hydrochlorides contained in a reaction tube, or a hydrolysate obtained as above, and the mixture was evaporated to dryness under reduced pressure in a desiccator containing sodium hydroxide. To the residue were added a freshly prepared cold solution (100 μl) of 0.1 *M* barium nitrite and cold 0.1 *M* sulfuric acid (75 μl), and the mixture was kept for 1 h in an ice-bath. The mixture was evaporated in the same manner, and 20 μl of ethanethiol–trifluoroacetic acid (2:1, v/v) was added to the residue. The reaction tube was closed tightly with a polyethylene stopper, and kept for 10 min at 25°. Then pyridine (50 μl), hexamethyldisilazane (100 μl), and trimethylchlorosilane (50 μl) were added, in that order, and the mixture was incubated for 30 min at 50° with occasional shaking. The mixture was centrifuged, and the 1-μl sample of the supernatant was analysed by gas chromatography. A mixture of authentic specimens of hexosamines was treated in the same manner, and the amounts of hexosamines in the sample were calculated by comparing the peak areas of hexosamine derivatives for the sample, relative to the internal standard, with those for the authentic specimens.

RESULTS AND DISCUSSION

Nitrous acid deamination of hexosamines is usually performed with a large excess of sodium nitrite in hydrochloric acid. However, the results indicated that the bulky crystals of resultant sodium chloride hampered the following mercaptalation process. The use of *n*-butyl nitrite in the presence of acid catalysts, such as hydrochloric acid and trifluoroacetic acid, resolved this problem, but the rate of deamination was slow even at high temperatures. Eventually, we adopted the combination of barium nitrite and sulfuric acid. The resultant precipitates of barium sulfate could be removed easily by centrifugation, but this operation could be omitted by minimizing the amounts of the nitrite and the acid used, as the loss of products was insignificant under such conditions.

Both 2,5-anhydro-D-mannose and -D-talose derivatives, formed respectively from 2-amino-2-deoxy-D-glucose and -D-galactose, gave single gas chromatographic peaks in the pentose region, and their separation was complete (Fig. 1), when they were chromatographed under the same conditions as described for the analysis of neutral monosaccharides and uronic acids (Scot SF-96 capillary column, 50 m, 225°). The resolution of 2,5-anhydro-D-mannose from D-xylose and/or D-ribose was poorer but satisfactory, unless too much of the sample was applied to the column. The use of inositol as the internal standard was inappropriate, because the peak of its derivative was superimposed on that of 2,5-anhydro-D-talose. Therefore, 3-O-methyl-D-glucose was chosen for the internal standard; its derivative appeared between the 6-deoxyhexose and uronic acid regions.

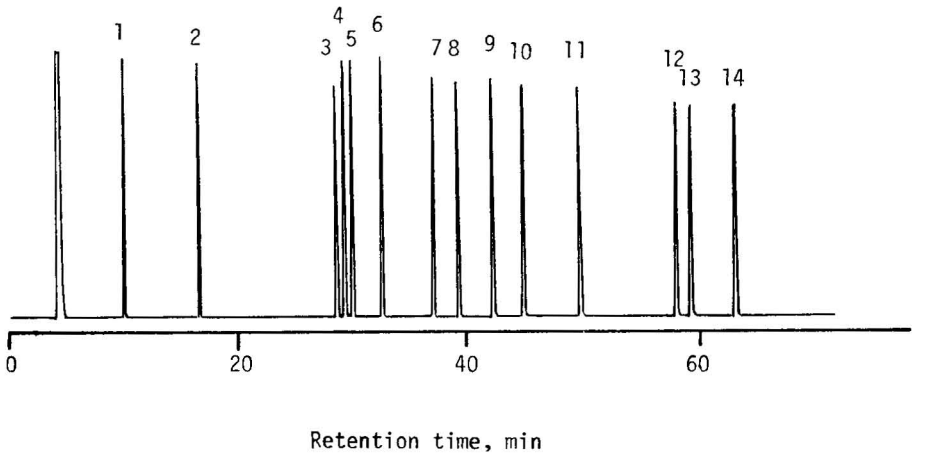

Fig. 1. Gas chromatogram of a mixture of the derivatives of hexosamines, neutral monosaccharides and uronic acids. Peaks are assigned to the trimethylsilylated diethyl dithioacetal derivatives of D-glyceraldehyde (1), D-erythrose (2), 2,5-anhydro-D-mannose derived from 2-amino-2-deoxy-D-glucose (3), D-xylose (4), L-arabinose (5), 2,5-anhydro-D-talose derived from 2-amino-2-deoxy-D-galactose (6), L-rhamnose (7), L-fucose (8), 3-O-methyl-D-glucose (internal standard) 9, D-galacturonic acid (10), D-glucuronic acid (11), D-glucose (12), D-mannose (13) and D-galactose (14).

Table I gives the comparative yields of the 2,5-anhydro-D-mannose derivative formed on deamination of 2-amino-2-deoxy-D-glucose with barium nitrite and sulfuric acid under various conditions. The maximal yield was obtained when the hexosamine

TABLE I

OPTIMIZATION OF REACTION CONDITIONS FOR DEAMINATION OF 2-AMINO-2-DEOXY-D-GLUCOSE WITH BARIUM NITRITE AND SULFURIC ACID

Barium nitrite		*Sulfuric acid*		*Relative yield of 2,5-anhydro-D-mannose*
Concentration (M)	*Volume (μl)*	*Concentration (M)*	*Volume (μl)*	
0.1	100	0.1	25	93
0.1	100	0.1	50	97
0.1	100	0.1	75	100
0.1	100	0.1	100	23
0.1	100	0.1	125	11
0.2	100	0.2	25	70
0.2	100	0.2	50	89
0.2	100	0.2	75	86
0.2	100	0.2	100	14
0.2	100	0.2	125	11
0.3	100	0.3	25	62
0.3	100	0.3	50	36
0.3	100	0.3	75	32
0.3	100	0.3	100	10
0.3	100	0.3	125	7

was treated with 0.1 *M* barium nitrite (100 μl) and a slightly smaller volume (75 μl) of 0.1 *M* sulfuric acid at 0°. The use of larger volumes of sulfuric acid caused a marked decrease of yield. With nitrite and acid solutions of higher concentration, the yield of

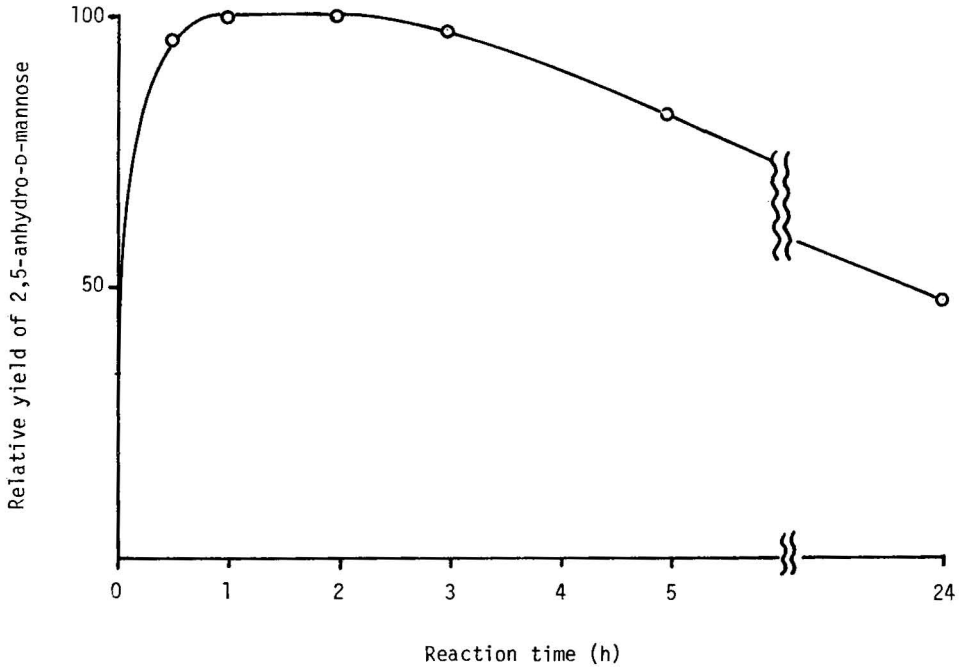

Fig. 2. Course of the deamination of 2-amino-2-deoxy-D-glucose.

the 2,5-anhydro-D-mannose derivative was lower, probably owing to some of the product being adsorbed on the surface of the precipitates.

Fig. 2 shows the course of the deamination of 2-amino-2-deoxy-D-glucose under the optimum conditions mentioned above. The reaction was very rapid, and a plateau was reached in 1 h. After 5 h the product decomposed gradually, and the yield of 2,5-anhydro-D-mannose was reduced to 48% after 24 h. No remarkable difference in yield was observed between the reactions in open and capped vessels at 0°, but higher reaction temperatures were unprofitable. The conditions for mercaptalation and trimethylsilylation have been already established[4,5].

On the basis of the results obtained above, a procedure was devised for the analysis of hexosamines, which are simpler and more rapid than any other reported methods[1–3]. Although the authentic specimens of 2,5-anhydrohexoses were unavailable and their absolute yields were unknown, the gas chromatogram obtained under the optimum conditions indicated that there were no peaks other than those of 2,5-anhydrohexose derivatives for both hexosamines. Both hexosamines gave their 2,5-anhydrohexose derivatives, which had the same molar response factor (0.73)

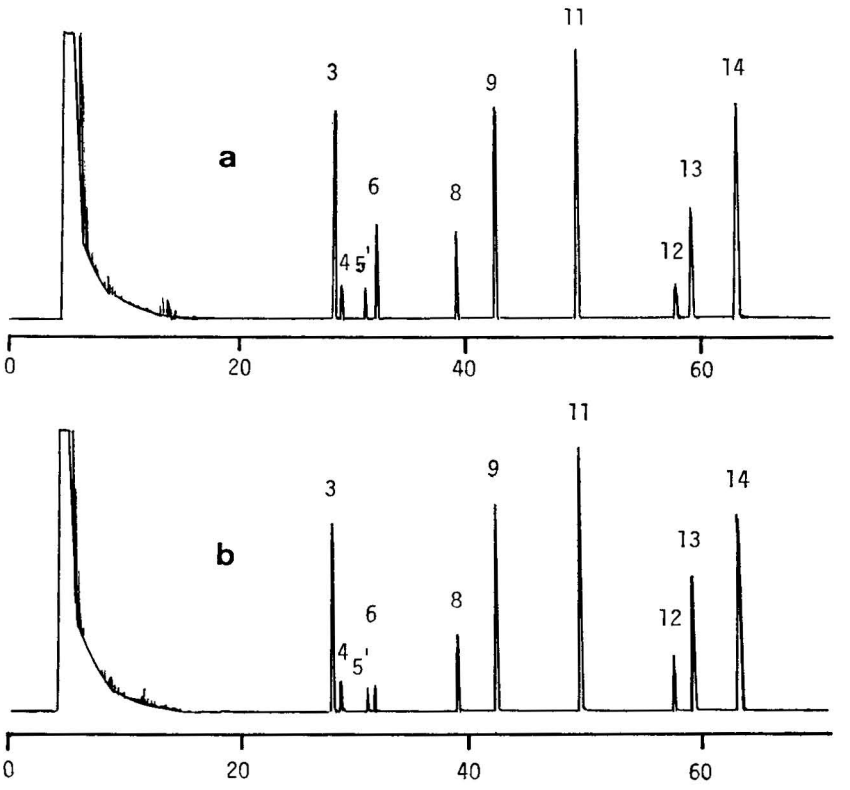

Fig. 3. Gas chromatogram for the hydrolysate of the non-dialysable fraction of the urine sample from a patient bearing breast cancer (a) and that from normal female adult (b). The numbering is the same as in Fig. 1 except for 5′ (unknown peak).

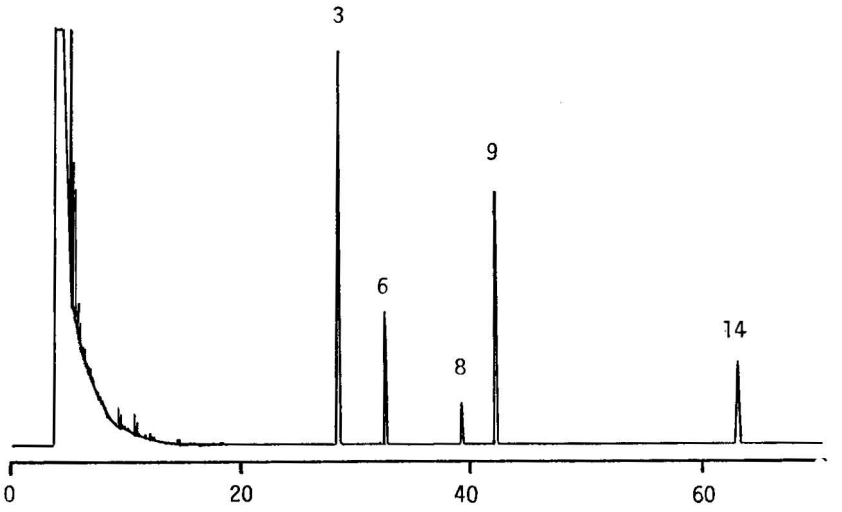

Fig. 4. Gas chromatogram for the hydrolysate of porcine mucine. The numbering is the same as in Fig. 1.

relative to 3-O-methyl-D-glucose, with coefficients of variation less than 3% for 1-μmole samples. The presence of neutral monosaccharides and uronic acids had no influence on the determination of hexosamines. On the other hand, the determination of neutral monosaccharides and uronic acids was not affected by pretreatment with the deaminating reagents.

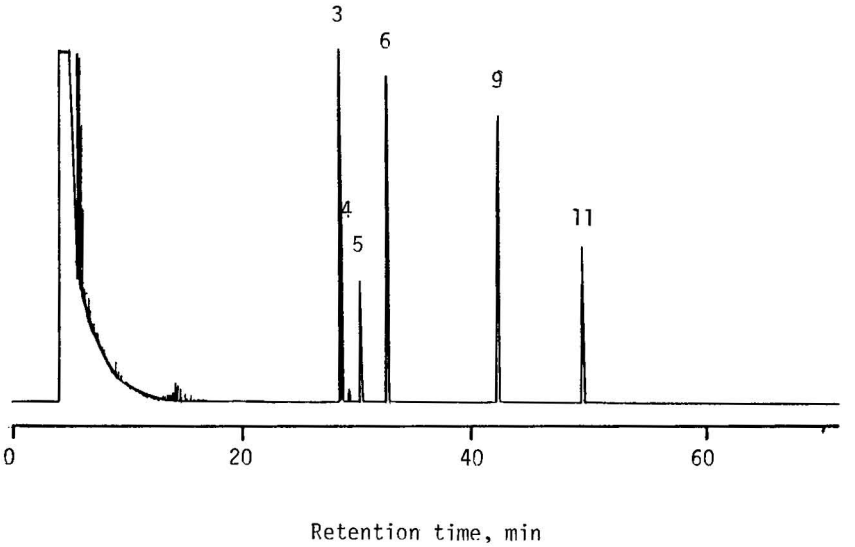

Fig. 5. Gas chromatogram for the hydrolysate of an artificial mixture of chondroitin sulfate A from whale cartilage and hyaluronic acid from human umbilical cord. The numbering is the same as in Fig. 1.

Fig. 3 shows an example of the application of the method to the determination of hexosamines in the non-dialysable fractions of human urines. The samples were hydrolysed for 6 h in 4 *M* hydrochloric acid at 100° in a nitrogen atmosphere. Under these conditions the amounts of hexosamines released reached the maximal values. It was noticeable that the molar ratio of 2-amino-2-deoxy-D-galactose to 2-amino-2-deoxy-D-glucose for the urine sample of a patient bearing breast cancer (a) was considerably higher than that for a normal urine sample (b). This may indicate possible diagnostic value.

Fig. 4 shows the chromatogram for the hydrolysate of porcine mucine. Both the 2,5-anhydro-D-mannose and -D-talose derivatives were detected, together with the derivatives of neutral monosaccharides. Fig. 5 shows the chromatogram for the hydrolysate of an artificial mixture of chondroitin sulfate A and hyaluronic acid. A considerable part of D-glucuronic acid, the common component uronic acid, decomposed during hydrolysis to yield D-arabinose, but approximately equal amounts of 2-amino-2-deoxy-D-glucose in hyaluronic acid and 2-amino-2-deoxy-D-galactose in chondroitin sulfate A were detected as the corresponding 2,5-anhydrohexose derivatives. A small amount of D-xylose, resulting from the carbohydrate–peptide interfacial positions in these proteoglycans, was also detected.

The foregoing examples demonstrate the usefulness of this procedure for the determination of the constituent hexosamines in carbohydrate materials.

REFERENCES

1 J. R. Clamp, G. Dawson and L. Hough, *Biochim. Biophys. Acta*, 148 (1967) 342.
2 Z. Tamura, T. Imanari and Y. Arakawa, *Chem. Pharm. Bull.*, 16 (1968) 1864.
3 R. Varma and R. S. Varma, *J. Chromatogr.*, 139 (1977) 303.
4 S. Honda, N. Yamauchi and K. Kakehi, *J. Chromatogr.*, 169 (1979) 287.
5 S. Honda, Y. Fukuhara and K. Kakehi, *Anal. Chem.*, 55 (1978) 55.

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CHROM. 11,908

DETERMINATION OF UNCHANGED HYDRALAZINE IN PLASMA BY GAS–LIQUID CHROMATOGRAPHY USING NITROGEN-SPECIFIC DETECTION

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SUMMARY

A gas chromatographic method is described for the specific determination of unchanged hydralazine in plasma. On treatment with 2,4-pentanedione, hydralazine is converted into 1-(3,5-dimethyl-1-pyrazolyl)phthalazine, a stable compound which is easily extracted from biological material and determined quantitatively by gas chromatography with nitrogen-specific detection. 4-Methylhydralazine is used as an internal standard. The sensitivity of the method is *ca.* 10 ng/ml. Hydrazones of hydralazine do not interfere with the assay.

INTRODUCTION

A number of analytical procedures have been developed for the determination of hydralazine (1-hydrazinophthalazine, active ingredient of Apresoline®) in biological material: (1) Perry[1], 1953 (ninhydrin complex formation and spectrophotometry); (2) Schulert[2], 1961 (*p*-hydroxybenzaldehyde hydrazone formation and spectrophotometry); (3) Zacest and Koch-Weser[3], 1972 (modification of procedure 2); (4) Zak *et al.*[4], 1974 (*p*-methoxybenzaldehyde hydrazone formation and spectrophotometry); (5) Jack *et al.*[5], 1975 (tetrazolophthalazine formation and gas chromatography); (6) Talseth[6], 1976 (modification of procedure 5); (7) Haegele *et al.*[7], 1976 (modification of procedure 6); (8) Zak *et al.*[8], 1977 (modification of procedure 7); (9) Haegele *et al.*[9], 1977 (cyclohexanone hydrazone formation and gas chromatography–mass spectrometry).

However, almost all of these methods measure both unchanged hydralazine and its hydrazone derivatives. Moreover, the hydrazones present in the plasma are susceptible to hydrolysis at low pH and regenerate hydralazine[10].

The first gas chromatographic (GC) method published[5], which is based on the formation of a tetrazolophthalazine, does not differentiate between "free" and "bound" hydralazine. It was therefore modified by Zak *et al.*[8]. In the modified procedure a pH range of 2.85–3.90 is employed to ensure derivatization of only the free hydralazine. Following oxidative degradation of the free hydralazine with

MnO_2, the hydrazones are hydrolyzed and derivatized at pH 0.9 in the presence of nitric acid.

In a recent study by Schneck *et al.*[11] free hydralazine was estimated from the difference between assays of acid-labile conjugates and of total hydralazine. This procedure was applied to plasma samples from subjects of slow or fast acetylator phenotype receiving repeated doses of hydralazine. The levels of free hydralazine reported were considerably higher than those of the acid-labile hydrazones. Since this finding is in conflict with previously reported data found in single dose experiments, where the free hydralazine was measured directly[8,12], it is necessary to confirm the pharmacokinetics of free hydralazine in human plasma by use of chemical analytical principles different from those employed to date.

A procedure previously reported by Smith *et al.*[13] for the specific determination of unchanged hydralazine in Apresoline® tablets was therefore modified for its application to biological samples. The assay is based on the formation of 1-(3,5-dimethyl-1-pyrazolyl)phthalazine after addition of 2,4-pentanedione directly to plasma (Fig. 1). This method has several advantages over all previous ones. First, the reaction can be carried out under conditions which do not affect the labile hydrazones. Secondly, the derivative formed has favourable properties for GC and detection by a nitrogen-specific detector.

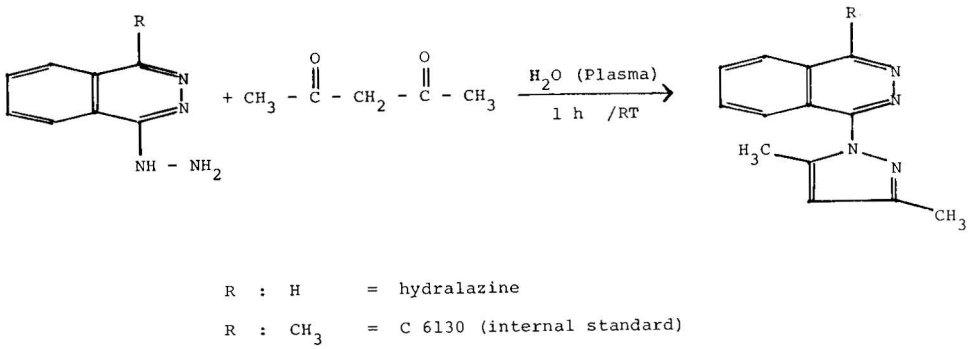

Fig. 1. Formation of the pyrazole derivative of hydralazine and of the internal standard, 4-methylhydralazine. RT = Room temperature.

The stability of hydralazine in native plasma is poor. It is degraded to phthalazine, especially in the presence of oxygen at pH 7.0. In native plasma at a pH of 7.4 a loss of *ca.* 20% of the hydralazine was reported by Reidenberg *et al.*[14] after incubation for 4 h at 37°. At pH 6.4, which is the pH employed after addition of the internal standard solution in the method reported here, hydralazine is stable sufficiently to react completely with 2,4-pentanedione within *ca.* 40–60 min (Fig. 2). Thus a reaction time of 1 h was chosen for the standard procedure. The overall yield (using 1 ml plasma and 300 ng hydralazine) is *ca.* 40% (Fig. 2).

EXPERIMENTAL AND RESULTS

Chemicals and reagents

The following chemicals and reagents were used: Hydralazine hydrochloride

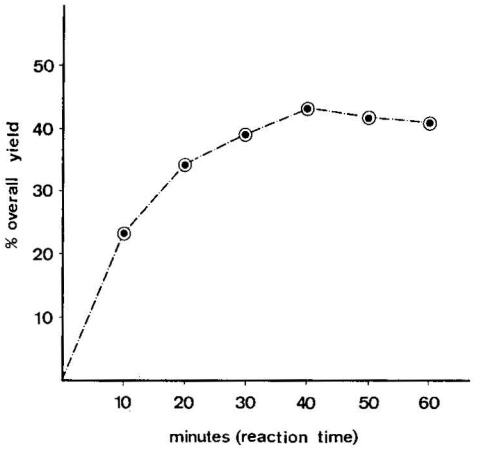

Fig. 2. Reaction kinetics of pyrazole formation in plasma. Percent yield of 1-(3,5-dimethyl-1-pyrazolyl)phthalazine and its dependence on the reaction time.

(Ciba-Geigy, Basle, Switzerland), solutions in 0.1 *N* HCl, freshly prepared; hydralazine pyruvic acid hydrazone (Ciba-Geigy); 4-methylhydralazine hydrochloride (Ciba-Geigy), solutions in 0.1 *N* HCl, freshly prepared; 2,4-pentanedione (EGA-Chemie, Steinheim, G.F.R.; puriss. 99%); *n*-hexane, distilled.

Procedure

A 1-ml volume of plasma, 0.1 ml internal standard solution (294 ng/0.1 ml)* and 0.2 ml 2,4-pentanedione (pH of the reaction mixture, *ca.* 6.4) were thoroughly mixed and shaken for 1 h at 200 rpm on a mechanical (horizontal) rotary shaker. Hexane (2 ml) was then added and the mixture shaken for 10 min at 200 rpm. The organic phase was transferred to a clean vial and evaporated under a stream of nitrogen at 40°. (The vials should not be left in the water-bath longer than necessary.) The residue was redissolved in 0.2 ml hexane and aliquots of 5 μl were injected into the gas chromatograph.

Gas–liquid chromatography

The instrument used was a Pye-104 gas chromatograph equipped with a Perkin-Elmer nitrogen-specific detector. The column was of Pyrex glass (2 m × 2 mm I.D.), packed with 3% OV-17 on Chromosorb W HP (80–100 mesh). Other conditions: carrier gas (helium) flow-rate, 35 ml/min; temperature of column oven, detector and injector, 230°. The retention times under the given conditions were 3.4 min for the hydralazine derivative and 4.6 min for the internal standard derivative.

The structure of the derivative of hydralazine was ascertained by mass spectrometry (Department of Spectroscopy, Ciba-Geigy; spectrum No. 7270).

Specificity for unchanged hydralazine

Blank plasma was spiked with hydralazine in concentrations ranging from 9.5 to 240 ng/ml. The samples were split into two equal parts. To one part were added

* For calibration curves, hydralazine is added in the same manner.

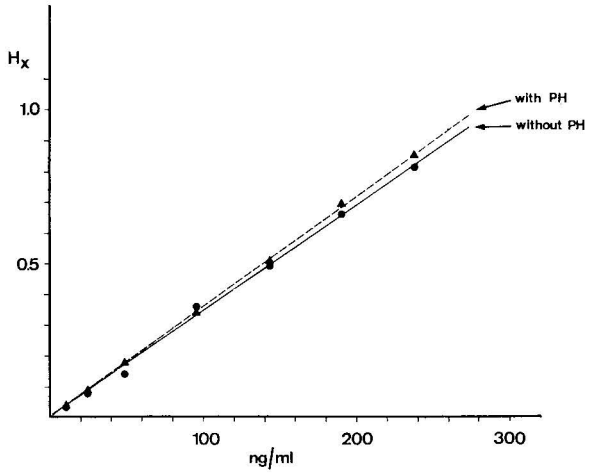

Fig. 3. Specificity test. Calibration curves with and without addition of pyruvic acid hydrazone (PH). Internal standard: 294 ng/ml. Sample volume: 1 ml plasma. ▲, Each sample + 680 ng PH; ●, without PH. H_x = peak height of the derivative of hydralazine/peak height of the derivative of the internal standard.

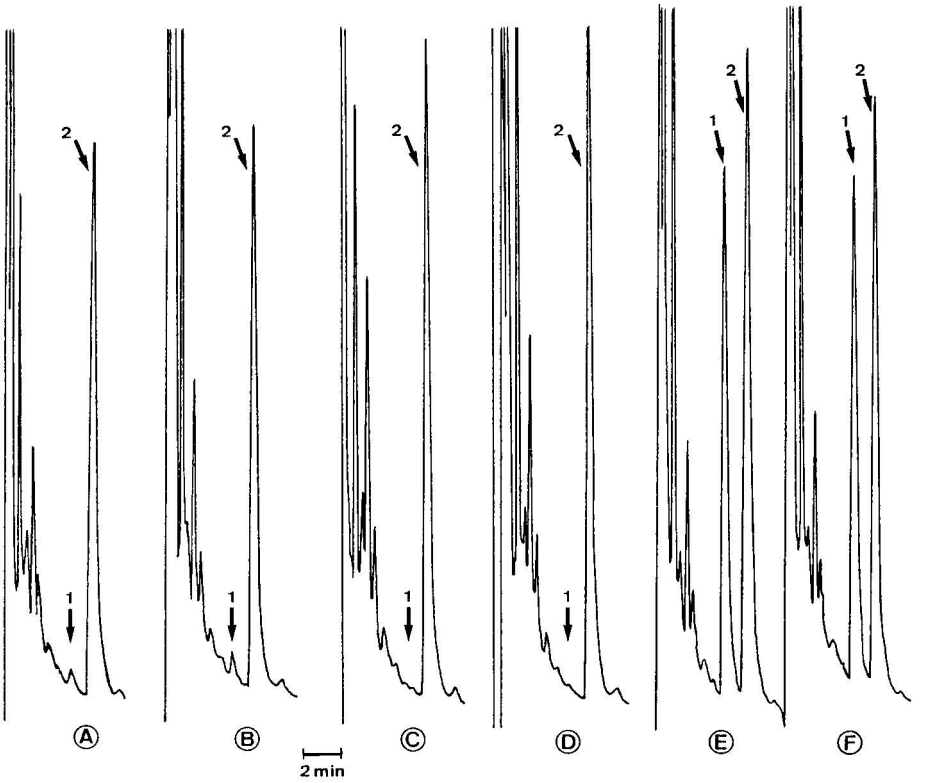

Fig. 4. Chromatograms of extracts of spiked plasma (1 ml) samples. Extracts: A = 9.5 ng hydralazine; B = 9.5 ng hydralazine and 680 ng pyruvic acid hydrazone; C = blank; D = 680 ng pyruvic acid hydrazone. E = 238 ng hydralazine; F = 238 ng hydralazine and 680 ng pyruvic acid hydrazone. A–F also contained 294 ng internal standard. Peaks: 1 = hydralazine derivative; 2 = internal standard derivative. 5-μl aliquots of 0.2 ml were injected.

TABLE I

DETERMINATION OF HYDRALAZINE IN SPIKED PLASMA SAMPLES BY TWO DIFFERENT METHODS

A = Specific method for unchanged hydralazine (reaction with 2,4-pentanedione at pH 6.4); B = method for apparent hydralazine, described by Jack *et al.*[5] (reaction with nitrite at pH 0.2).

Hydralazine in plasma (ng/ml)		
Initial	*Found (method A) ($n = 3$): $\bar{x} \pm s(x)$*	*Found (method B) ($n = 3$): $\bar{x} \pm s(x)$*
236	235 ± 15.56	255.5 ± 3.54
47.2	51 ± 0	52 ± 1.41
147.5	154.5 ± 3.53	148 ± 0
20	19.0 ± 0	20.7 ± 1.2
10	9.7 ± 0.6	9.3 ± 0.8
30	28.0 ± 0	30.0 ± 0

680 ng of the pyruvic acid hydrazone of hydralazine. Both parts were then analyzed as described.

It was found that the hydrazone did not interfere with the analysis of unchanged hydralazine. No difference could be seen between the samples containing hydrazone and the samples containing only unchanged hydralazine (Fig. 3).

The blank plasma sample containing only the 680 ng of the pyruvic acid hydrazone of hydralazine showed no peak in the chromatogram at the position where the hydralazine derivative would appear (Fig. 4).

A comparison between the method reported previously for the determination of apparent hydralazine[5] and the method reported here for unchanged hydralazine has been carried out using spiked plasma samples. Six different pools were prepared. The results (Table I) show good agreement between the two methods. The sensitivity of both procedures is about the same (*ca.* 10 ng/ml).

Application of both GC methods to the analysis of biological samples

Twenty-one male rats (200 g) were treated by gavage with 12 mg/kg doses of hydralazine. Three animals were sacrificed at each of the following times: 0, 0.5, 1,

TABLE II

PLASMA LEVELS OF APPARENT AND UNCHANGED HYDRALAZINE IN RATS TREATED WITH A SINGLE ORAL DOSE OF HYDRALAZINE (12 mg/kg)

0.5–1.0 ml plasma were used for both assays.

Time after administration (h)	*Apparent hydralazine* (ng/ml) ($n = 3$): $\bar{x} \pm s(x)$*	*Unchanged hydralazine** (ng/ml) ($n = 3$): $\bar{x} \pm s(x)$*
0	0	0
0.5	1310 ± 198	98.0 ± 6.9
1	373 ± 32	39.7 ± 0.6
2	264 ± 2.8	29.0 ± 0
3	222 ± 6.4	30.0 ± 4.2
6	82 ± 11	10.0 ± 1.4
24	10 ± 0	0

* GC assay for apparent hydralazine[5].

** GC assay for unchanged hydralazine described in this paper.

2, 3, 6 and 24 h after administration. The plasma samples for each time point were pooled and analysed both for apparent hydralazine according to the method of ref. 5 and for unchanged hydralazine by the present method. The results in Table II demonstrate that between 7.5 and 13.5% of the apparent hydralazine in the plasma of rats can be accounted for as unchanged hydralazine.

The analytical principle described in this paper has been found applicable to the assay of dihydralazine (1,4-dihydrazinophthalazine) in biological fluids. Its detailed description will be given in a subsequent paper.

ACKNOWLEDGEMENTS

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REFERENCES

1 H. M. Perry, *J. Lab. Clin. Med.*, 41 (1953) 566–573.
2 A. R. Schulert, *Arch. Int. Pharmacodyn.*, 132 (1961) 1–15.
3 R. Zacest and J. Koch-Weser, *Clin. Pharmacol. Ther.*, 13 (1972) 420–425.
4 S. B. Zak, M. F. Bartlett, W. E. Wagner, T. G. Gilleran and G. Lukas, *J. Pharm. Sci.*, 63 (1974 225–229.
5 D. B. Jack, S. Brechbühler, P. H. Degen, P. Zbinden and W. Riess, *J. Chromatogr.*, 115 (1975) 87–92.
6 T. Talseth, *Clin. Pharmacol. Ther.*, 21 (1976) 715–720.
7 K. D. Haegele, H. B. Skrdlant, N. W. Robie, D. Lalka and J. L. McNay, Jr., *J. Chromatogr.*, 126 (1976) 517–534.
8 S. B. Zak, G. Lukas and T. G. Gilleran, *Drug. Metab. Dispos.*, 5 (1977) 116–121.
9 K. D. Haegele, A. J. McLean, P. du Souich, H. B. Skrdlant, B. Werckle and J. L. McNay, *Clin. Res.*, 25 (1977) 460A.
10 P. A. Reece, P. E. Stanley and R. Zacest, *J. Pharm. Sci.*, 67 (1978) 1150.
11 D. W. Schneck, J. S. Sprouse, K. Miller, J. E. Vary, P. O. DeWitt and A. H. Hayes, *Clin. Pharmacol. Ther.*, 24 (1978) 714–719.
12 A. Melander, A. Hanson, H. Liedholm and E. Waehlin, *Abstracts of the VIII World Congress of Cardiology, Tokyo, 1978*, p. 367.
13 K. M. Smith, R. N. Johnson and B. T. Kho, *J. Chromatogr.*, 137 (1977) 431–437.
14 M. M. Reidenberg, D. Drayer, A. L. De Marco and C. T. Bello, *Clin. Pharmacol. Ther.*, 14 (1973) 970.

Journal of Chromatography, 176 (1979) 381–390

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF EMETINE AFTER OXIDATIVE ACTIVATION TO A FLUORESCENT PRODUCT

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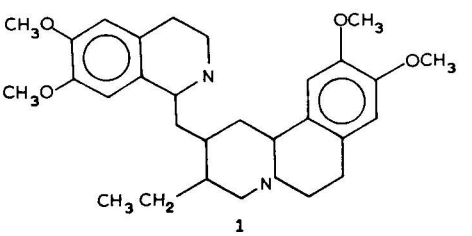
SUMMARY

A clinically useful analytical method is described for monitoring plasma levels of emetine. The drug is initially extracted from plasma with dichloromethane (0.3 volumes). The extract can be analyzed directly by paired-ion reversed-phase high-performance liquid chromatography to levels of 500 ng/ml of plasma by spectrophotometric monitoring of column effluent. For analysis of emetine at lower concentrations, the dichloromethane extracts are subjected to mild mercuric acetate oxidation prior to separation, thereby converting emetine to a fluorescent product. Spectrofluorometric monitoring of the column effluent readily extends the sensitivity of the assay to 10 ng of emetine/ml of plasma. At these levels measurements can be made with a precision of $\pm$ 4%.

INTRODUCTION

Emetine (1), an alkaloid of *Cephaelis ipercacuanha*, has been used in the therapy of a wide variety of disorders since the seventeenth century. Its usefulness in amebiasis has been well established[1]. At the cellular level emetine inhibits protein biosynthesis[2]



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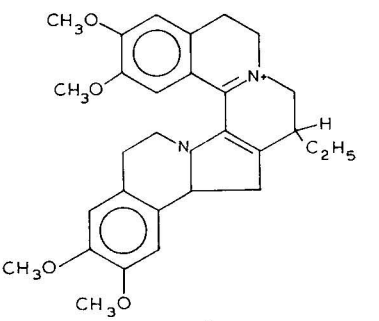

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and interferes with oxidative phosphorylation[3], suggesting its possible role in cancer chemotherapy. Lewisohn[4] and Van Hoosen[5] described clinical tumor regressions in a series of patients with a variety of malignancies after emitine therapy. These findings, reported prior to 1920, have more recently been corroborated by several clinical studies[6–8]. Emetine's important clinical advantage as an antineoplastic agent is its non-myelosuppressive behavior[6] and the disadvantages are the severe myopathy and cardiac arrhythmias[6–10] which occur in association with the dosage schedules used to date.

In an effort to utilize more effectively the chemotherapeutic properties of the drug while minimizing its toxic effects, the pharmacokinetics of emetine will be studied in man.

A variety of methods have been described for the analysis of emetine in crude plant material and pharmaceutical formulations. These techniques include aqueous[11] and nonaqueous[12] titrimetry, spectrophotometry[13–15], thin-layer chromatography (TLC)[16], and normal-phase high-performance liquid chromatography (HPLC) of the underivatized drug[17] and its Dns derivative[18]. In previous studies carried out in biological matrices the method of choice has been the oxidation of emetine to produce fluorescence and the measurement of total solution fluorescence. Mercuric acetate has been used as the oxidant under strenuous conditions[19] to insure the production of a single fluorescent product, rubremetine (2).

This report describes alternative conditions for the molecular modification of emetine to a fluorescent product and subsequent ion-pair reversed-phase HPLC analysis. Emetine may be quantitated in plasma to levels of 10 ng/ml.

EXPERIMENTAL

Apparatus

Chromatography was performed on a component system consisting of a Waters Assoc. (Milford, Mass., U.S.A.) Model 6000 A pump, Model U6K injector, and Model 440 absorbance detector operated at 280 nm. Fluorescence detection was done with a Schoeffel Instr. (Westwood, N.J., U.S.A.) FS 970 L.C. fluorometer. Mobile phase optimization was accomplished with the addition of a second pump and a Waters 660 solvent programmer. The column used was a Waters μBondapak C_{18} (30 cm $\times$ 3.9 mm I.D.).

UV-Vis spectra were recorded using a Cary (Palo Alto, Calif., U.S.A.) Model 118 spectrophotometer and 1-cm quartz cells. Fluorescent emission and excitation spectra were recorded with a Hitachi–Perkin-Elmer (Norwalk, Conn., U.S.A.) Model MPF-2As spectrophotofluorimeter.

Materials

Emetine hydrochloride was obtained from Sigma (St. Louis, Mo., U.S.A.). Sodium pentane, hexane, heptane, and octane sulfonates were purchased from Eastman (Rochester, N.Y., U.S.A.), and were used as received. Naphthalene was purchased from Aldrich (Milwaukee, Wisc., U.S.A.) and mercuric acetate was purchased from Fisher Chemical (Fair Lawn, N.J., U.S.A.). Methanol and dichloromethane were Fisher HPLC grade. Chloroform was Fisher spectral grade and 1,2-dichloroethane was ACS reagent grade from Matheson, Coleman and Bell Manu-

facturing Chemists (Norwood, Ohio, U.S.A.). Ethanol, USP (95%) was used and all water was distilled in glass following mixed bed deionization.

Plasma was either purchased (Community Blood Center, Kansas City, Mo., U.S.A.) as recovered human plasma containing citrate-phosphate–dextrose anticoagulant, or was obtained from blood freshly drawn from healthy human volunteers using heparinized collection tubes. Plasma was stored at 5° and was used within one week of collection.

Extraction

Emetine was extracted from plasma (9 ml) or phosphate buffer (pH 7.4, 0.1 *M*) with 0.3 volumes of dichloromethane by shaking for 3 min in sealed conical centrifuge tubes. Phase separation was accomplished by centrifugation at 1200 *g* for 15 min.

Oxidation

A measured volume (7.5 ml) of dichloromethane extract was removed and evaporated to dryness in a conical centrifuge tube with a gentle stream of nitrogen. An ethanol solution (500 μl) of mercuric acetate [10 g $Hg(CH_3COO)_2$ per 100 ml of acetic acid–35% aqueous sodium hydroxide (73:27) diluted with 95% ethanol to 1 l] was added to the residue, vortex mixed and sealed for 1 h at room temperature (20–24°) prior to HPLC analysis.

Chromatography of underivatized emetine from plasma and buffer extracts

The mobile phase consisted of 2.5 m*M* octane sulfonate and 0.5% glacial acetic acid in methanol–water (56:44). The flow-rate was maintained at 2.0 ml/min. Injections of buffer were as large as 100 μl but no more than 25 μl of organic extracts were injected. Column effluent was monitored spectrofluorometrically at 280 nm.

Chromatography of mercuric acetate reaction mixtures

The mobile phase was as described above but the methanol concentration was increased to 60%. The flow-rate was 2 ml/min. Injections (5 μl) of the ethanol mixture were made on-column. Column effluent was monitored spectrophotometrically [λ_{ex} = 225 nm, cutoff filter λ_{em} = 418 nm].

RESULTS AND DISCUSSION

Initially a chromatographic system was developed to monitor unchanged emetine in buffer and plasma extracts. Emetine was chromatographed as an ion aggregate on an octadecylsilane bonded phase column using C_5–C_8 alkyl sulfonates as the hetaerons and UV absorbance detection at 280 nm. Fig. 1 shows the effect on retention of the addition of methanol to the mobile phase. In each case the alkyl sulfonate concentration was 2.5 m*M* and the concentration of acetic acid was 0.5%. Table I shows the system efficiency achieved with each of the alkyl sulfonates. In addition to greater efficiency the use of the more hydrophobic hetaeron and higher methanol concentration resulted in a simpler chromatogram in the vicinity of the emetine peak. This is probably due to the more rapid elution of neutral species relative to the cationic emetine. Sodium octane sulfonate was chosen as the counter ion at a methanol concentration of 56%. Under these conditions emetine was well

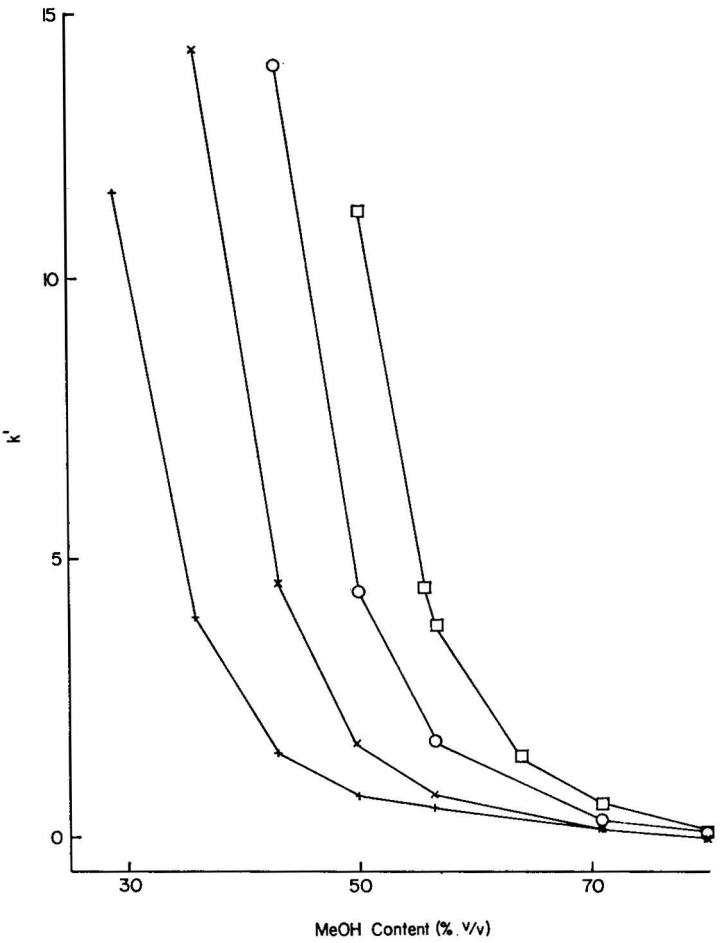


Fig. 1. Capacity factor (k') for emetine, *vs.* methanol concentration in mobile phase containing 0.5% (v/v) acetic acid and 2.5 m*M* sodium alkyl sulfonate. Pentane (+); hexane (×); heptane (○), octane (□) sulfonate.

TABLE I

NUMBER OF THEORETICAL PLATES (N) FOR INDIVIDUAL CHROMATOGRAPHIC SYSTEMS AT $k' = 4.2$ WITH $CH_3(CH_2)_nSO_3Na$ HETAERONS

$N = 16[t_r/t_w]^2$; t_r = retention time, t_w = peak width at baseline. $k' = (t_r - t_u)/t_u$; t_u = time of elution of unretained species (water). 25 μl of 10^{-4} *M* emetine hydrochloride were injected onto C_{18} column with mobile phase 2.5 m*M* alkyl sulfonate, 0.5% acetic acid in methanol–water of composition to yield k' (emetine) = 4.2.

n	N
4	1450
5	1600
6	1850
7	2200

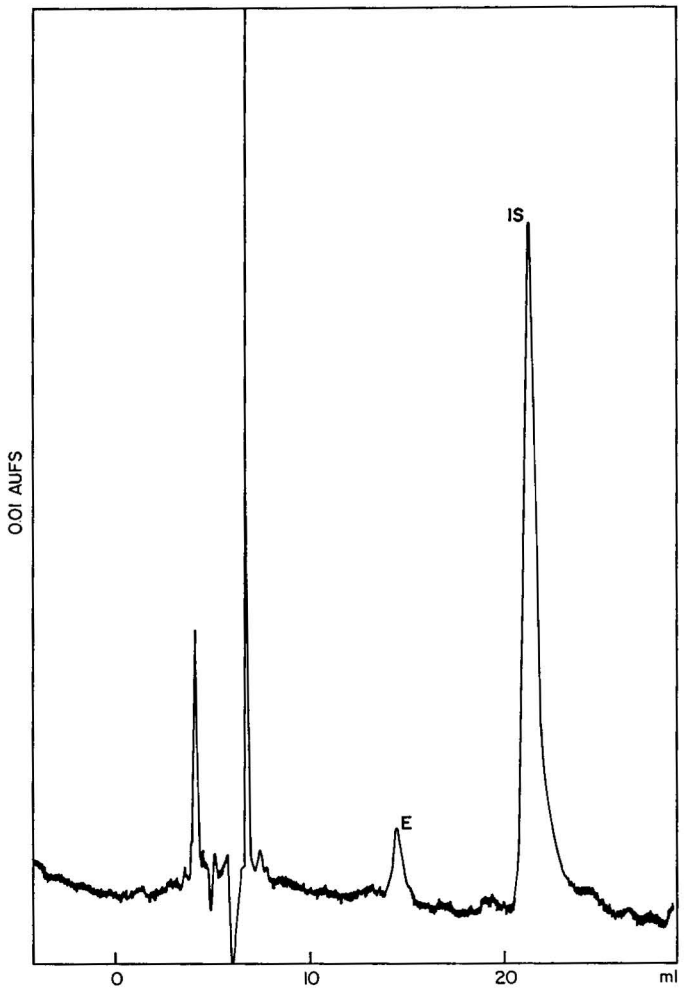


Fig. 2. Chromatogram of underivatized emetine extracted with dichloromethane from plasma. Emetine (E, $V_r = 14.5$); naphthalene (IS, $V_r = 21.2$). Octadecylsilane bonded phase column with 0.5% acetic acid and 2.5 mM sodium octane sulfonate in methanol–water (56:44) as the mobile phase.

resolved from coextracted plasma constituents as seen in Figs. 2 and 3, and rapidly eluted from the column (retention volume, $V_r = 14.5$ ml).

The efficiency of emetine extraction was determined with chloroform, dichloromethane, and 1,2-dichloroethane from phosphate buffer. The buffer containing emetine was analyzed before and after single-batch extraction. The extract was analyzed after extraction. Emetine was quantitatively extracted when the phase-volume ratio (org:aq) was 1:10 with all solvents used. Analyses were subsequently carried out by extraction with 0.1–0.3 volumes of dichloromethane.

Fresh plasma was spiked with emetine at concentrations of 1–100 μg/ml. Samples (3 ml) were extracted with 1 ml of dichloromethane containing 10^{-4} M naphthalene present as the internal standard. HPLC analysis and plotting of peak

height ratios (emetine to naphthalene) yielded a straight line of slope 0.101 $(\mu g/ml)^{-1}$, intercept of −0.056 with a correlation coefficient of 0.999. This system had a practical detection limit of 0.5 $\mu g/ml$, making it adequate for monitoring emetine administered as a bolus injection or from oral dosage forms, but inadequate for use in clinical investigations of slow i.v. infusions of the drug.

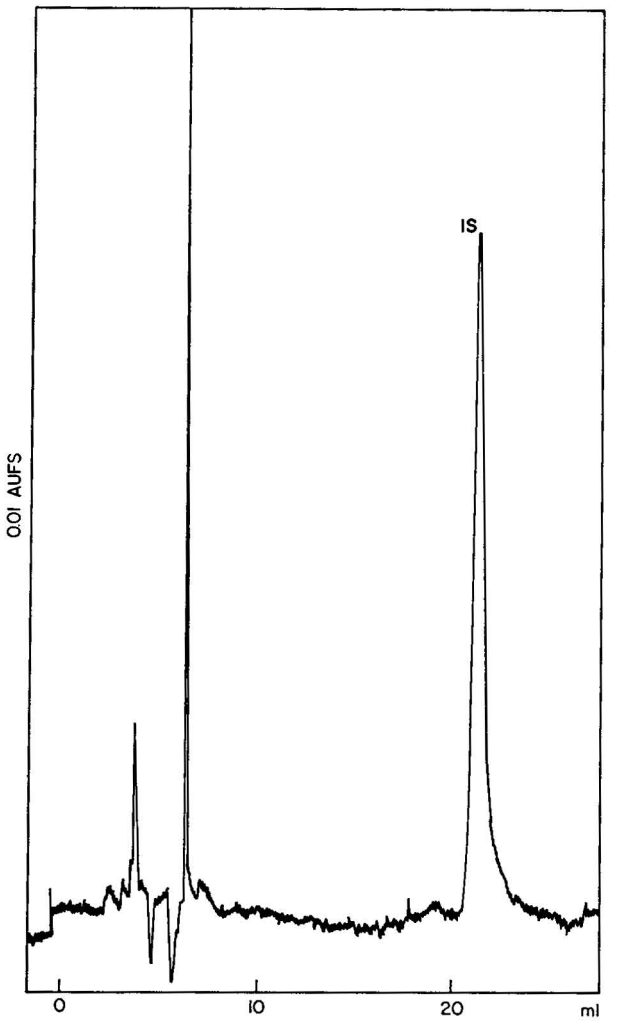


Fig. 3. Chromatogram of a dichloromethane extract of plasma in the absence of emetine. Chromatographic system as in Fig. 2.

To improve the sensitivity of the method, emetine was subjected to pre-column oxidative molecular modification to a fluorescent species which was then detected spectrofluorometrically in the column effluent. Using the chromatographic system just described, the oxidation of emetine was monitored under a variety of conditions.

Choosing only mercuric acetate as the oxidant, temperature and solvent were varied, seeking conditions that would yield a single product of enhanced detectability in a short period of time, under conditions convenient for large sample through-put.

When emetine (160 μg/ml) was refluxed in 5% (v/v) acetic acid containing 1% (w/v) mercuric acetate, the emetine peak (E) (capacity factor, $k' = 4.2$) disappeared with apparent first-order behavior ($t_{1/2} = 12$–14 min) and a new peak, B, ($k' = 4.6$) concomitantly appeared. This product was fluorescent ($\lambda_{max,ex} = 355$ nm; $\lambda_{max,em} = 450$ nm) but the peak corresponding to the product began to decrease in intensity after the reaction had proceeded for 40 min ($t_{1/2}$ 100–120 min). Two other peaks, C

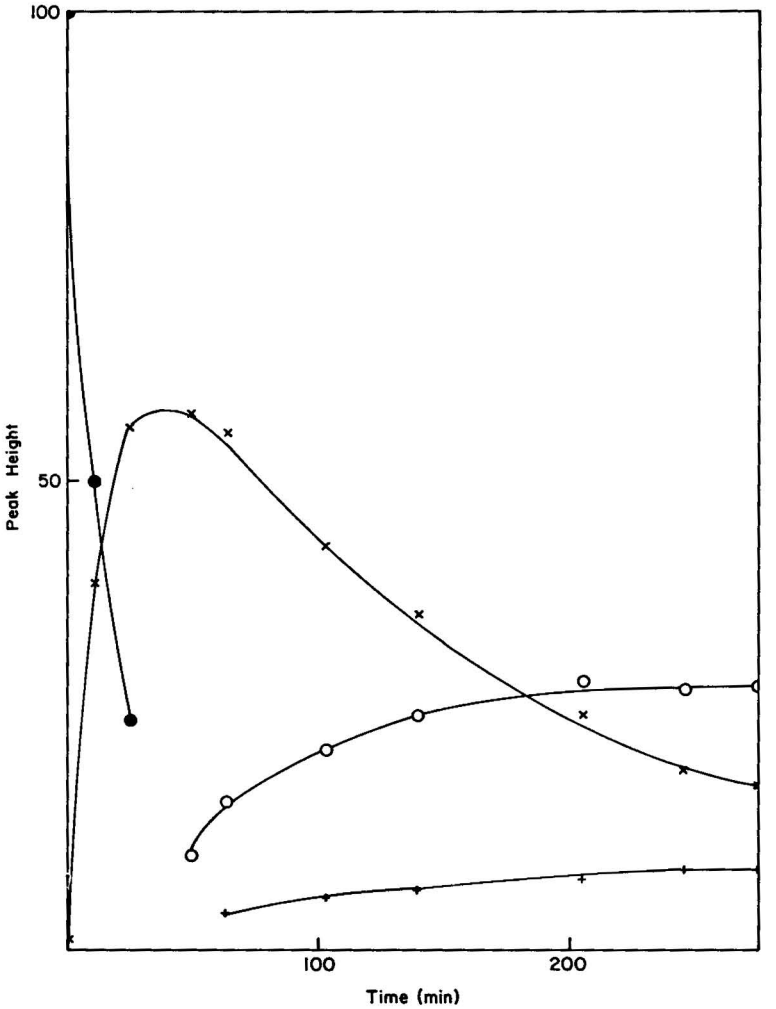
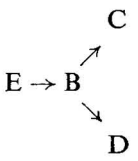


Fig. 4. Reaction course observed when emetine is refluxed in 5% aqueous acetic acid made 1% in mercuric acetate. Reaction followed by HPLC (octadecylsilane bonded phase column; 0.5% (v/v) acetic acid and 2.5 mM sodium octane sulfonate in methanol–water (56:44) mobile phase). E (●); B (×); C (○); D (+).

and D ($k' = 3.9$, $k' = 14.4$) appeared at a rate similar to the loss of peak B, suggesting:



as a possible reaction pathway. Fig. 4 shows the peak height *vs.* time data for this reaction scheme. Attempts were made to alter reaction conditions to preserve B, since both C and D seemed to be ultimate reaction products and therefore not suitable as analytical derivatives.

Reduction of the reaction temperature did little to improve the reaction course except to effectively displace the time axis of Fig. 4. At no point did it appear that the emetine was quantitatively converted to B.

The more strenuous conditions employed in the procedure of Schwartz and Rieder[19] (1 h at 130°) may produce a single product, or alternatively the fluorescence of a combination of oxidation products may be measured.

The reaction could be controlled by solvent modification. Addition of ethanol to the reaction medium facilitated oxidation of emetine and stabilized B. At an optimum level of 70% ethanol, reaction to form B was complete at room temperature in 60 min and B was stable for several hours. Under these reaction conditions formation of C and D was not observed.

Plasma extracts subjected to this treatment were then analyzed by paired-ion reversed-phase chromatography on a C_{18} column using methanol–water (60:40) containing 0.5% acetic acid made 2.5 m*M* in sodium octanesulfonate as a mobile phase. The derivatized product eluted with a retention volume of 8.6 ml [underivatized emetine, $V_r = 8.1$ ml] and was monitored spectrofluorometrically [$\lambda_{ex} = 225$ nm; $\lambda_{em,cutoff} = 418$ nm]. The resulting chromatogram is shown in Fig. 5a and a blank in Fig. 5b.

To validate the pre-column derivatization method a calibration curve was constructed by subjecting plasma sample containing emetine (20–1050 ng/ml) to the analysis scheme presented. Regression analysis of the data for this curve generates the line $y = 9.7 \cdot 10^{-3}\, x + 3.7 \cdot 10^{-2}$ with a correlation coefficient of 0.998. Chromatographic analysis must be carried out within 2 h after completion of oxidative derivatization to eliminate interfering peaks corresponding to slowly formed fluorescent derivatives of plasma constituents. The methods, as described, have a practical detection limit of 10 ng emetine/ml of plasma.

Thus, two methods are presented for the analysis of emetine in human plasma. After extraction, emetine can be analyzed directly by paired-ion reversed-phase chromatography when levels are $\geqslant$ 500 ng/ml; by introducing an oxidation step between extraction and chromatography, emetine can be detected spectrofluorometrically to levels of 10 ng/ml of plasma.

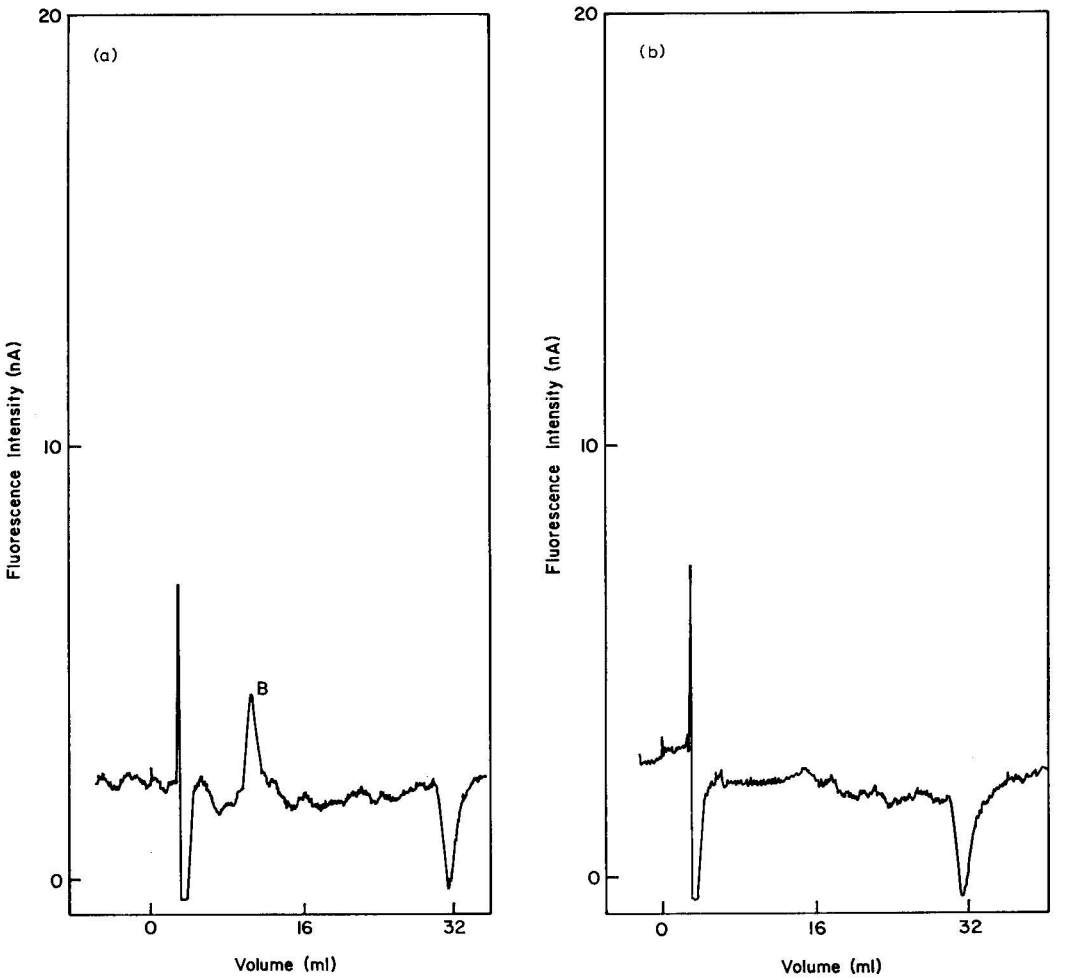


Fig. 5. (a), Chromatogram of oxidation product (B, $V_r = 8.6$) formed as described in text from emetine (20 ng/ml) extracted with dichloromethane from plasma. (Octadecylsilane bonded phase column; 0.5% (v/v) acetic acid and 2.5 m*M* sodium octane sulfonate in methanol–water (60:40) mobile phase). (b), Chromatogram of reaction mixture of dichloromethane extract of plasma not containing emetine. Reaction and chromatographic conditions as in (a).

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REFERENCES

1 I. M. Rollo in L. S. Goodman and A. Gilman (Editors) *The Pharmacological Basis of Therapeutics*, Macmillan, New York, 3rd ed., 1966, Ch. 53.
2 A. P. Grollman, *J. Biol. Chem.*, 243 (1968) 4089.

3 A. J. Brink, J. C. N. Katae, S. P. Muller and A. Lochner, *J. Pharmacol. Exp. Ther.*, 165 (1969) 251.
4 R. Lewisohn, *J. Amer. Med. Ass.*, 70 (1918) 9.
5 B. Van Hoosen, *Women's Med. J.*, 29 (1919) 102.
6 F. Panettiere and C. A. Coltman, Jr., *Cancer*, 27 (1971) 835.
7 C. G. Moertel, A. J. Schutt, R. G. Han and R. J. Reitemeir, *Cancer Chemother. Rep.*, 58 (1974) 229
8 R. C. Kane, M. H. Cohen, L. E. Broder, M. I. Bull, P. J. Creaven and B. E. Fossieck, Jr., *Cancer Treatment Rep.*, 59 (1975) 1171.
9 S. Siddiqui, D. Firat, S. Olshin, *Cancer Treatment Rep.*, 57 (1973) 423.
10 B. R. Manno and J. E. Manno, *Clin. Tox.*, 221 (1977) .
11 G. N. Thomis and A. Z. Kotionis, *Anal. Chim. Acta.*, 14 (1956) 457.
12 K. Acel, M. M. Tuckerman, *J. Pharm. Sci.*, 59 (1970) 1644.
13 N. L. Alport and N. R. Jones, *Quart. J. Pharm., Pharmacol.*, 15 (1942) 238.
14 F. J. Bandelin, *J. Amer. Pharm. Ass., Sci. Ed.*, 39 (1950) 493.
15 A. D. Thomas, *J. Pharm. Pharmacol.*, 28 (1976) 838.
16 M. S. Habib and K. J. Harkiss, *J. Pharm. Pharmacol.*, 21 (1969) 575.
17 R. Verpoorte and A. B. Svendsen, *J. Chromatogr.*, 100 (1974) 227.
18 R. W. Frei, W. Santi and M. Thomas, *J. Chromatogr.*, 116 (1976) 365.
19 D. E. Schwartz and J. Rieder, *Clin. Chim. Acta*, 6 (1961) 453.

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CHROM. 11,944

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF INTERMEDIATES IN THE OXYTOCIN SYNTHESIS*

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(First received March 22nd, 1979; revised manuscript received April 25th, 1979)

SUMMARY

A high-performance liquid chromatographic method for the determination of peptides formed during the synthesis of oxytocin, is described. No derivatization is necessary; all peptides are detected in UV at 215 nm. The limits of detection are in the ng range, both for free and protected peptides. Reproducibilities of 2–3% relative standard deviation were obtained. The simplicity, speed and selectivity of the separations render this technique suitable to in-process control of oxytocin synthesis.

INTRODUCTION

In peptide synthesis, many steps must be taken to protect functional groups before the coupling of amino acids, as well as for their subsequent liberation. However, in the absence of a 100% yield for these reactions, impurities in peptides, similar to the main product, will occur. There is a need for chromatographic techniques capable of determining qualitatively and quantitatively closely related peptides. A review of all techniques available up until 1972, such as thin-layer chromatography (TLC), paper chromatography (PC), gas chromatography (GC) or electrophoresis, was published by Rosmus and Deyl[1]. TLC and PC permit rapid qualitative results, but exact quantitative determinations are hardly possible. GC can only be applied after derivatization, and is therefore not suitable for routine analysis. Thus, many attempts in column liquid chromatography have been made during the last few years. Selective peptide separations are possible with classical anion exchangers: however, this a very time-consuming method[2,3]. Important improvements have been made since the development of chemically modified stationary phases for high-performance liquid chromatography (HPLC). Different authors describe separations of peptides and proteins without previous derivatization[4–19]; most of these separations are carried out by reversed-phase HPLC.

In this paper it is shown that reversed-phase HPLC is a powerful tool for the determination of free, as well as, of protected peptides that are formed during the

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synthesis of oxytocin. This is the basis for the optimization and in-process control of the complete synthesis.

EXPERIMENTAL

Materials

All solvents used were of analytical grade quality (E. Merck, Darmstadt, G.F.R.). The peptides were provided by Sanabo (Vienna, Austria). Their structures are given in Table I. As a stationary phase for the HPLC separations LiChrosorb RP-8 (E. Merck), 5 μm particle size, was used. Steel columns, 15 cm $\times$ 3.2 mm I.D., were packed by a slurry technique[20].

TABLE I

STRUCTURE OF THE INVESTIGATED SUBSTANCES

CbO = Carbobenzoxy.

Structure	*Compound*
CbO-Pro	CbO-proline
CbO-Leu-Gly-ethyl ester	CbO-dipeptide ester
CbO-Pro-Leu-Gly-ethyl ester	CbO-tripeptide ester
CbO-S-benzyl-Cys-Pro-Leu-Gly-NH_2	CbO-tetrapeptideamide
CbO-Asn-S-benzyl-Cys-Pro-Leu-Gly-NH_2	CbO-pentapeptideamide
Asn-S-benzyl-Cys-Pro-Leu-Gly-NH_2	Pentapeptideamide
CbO-Gln-Asn-S-benzyl-Cys-Pro-Leu-Gly-NH_2	CbO-hexapeptideamide
Gln-Asn-S-benzyl-Cys-Pro-Leu-Gly-NH_2	Hexapeptideamide
PyroGln-Asn-S-benzyl-Cys-Pro-Leu-Gly-NH_2	Pyroglutamine-hexapeptide amide
CbO-Gln-ONP	CbO-Gln-nitrophenyl ester
Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2	Nonapeptideamide
Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2	Oxytocin

Apparatus

A Hewlett-Packard 1010B liquid chromatograph, equipped with a Rheodyne 905-42 loop injector (injection volume 20 μl) was used for all separations. For detection a Perkin-Elmer LC 55 and a Schoeffel SF 770 spectrophotometer were used. The detection wavelength was 215 nm. All separations were performed isocratically at room temperature (20–22°).

A Laboratory Data System 3353 (Hewlett-Packard) was used for the evaluation of the data. Before injection, solid samples were dissolved in the mobile phase or methanol. Liquid samples (*e.g.* reaction solutions, mother liquors) can be injected after dilution to a suitable concentration with the mobile phase.

RESULTS AND DISCUSSION

The aim of the investigation was to develop a simple method for the determination of the most important intermediates in oxytocin synthesis. Therefore only one column was used and only isocratic elution was applied for all analyses. A column, length 15 cm, filled with the smallest particles commercially available (5 μm) was sufficient for an optimal separation of the relevant peptides.

Qualitative separations

For peptide separations reversed-phase materials with a carbon chain of 8 or 18 carbon atoms, proved to be suitable[7,8,16–18]. Our experiments showed no significant difference in the selectivity of LiChrosorb RP-8 (C_8 carbon chain) and LiChrosorb RP-18 (C_{18} carbon chain). All further investigations were performed with LiChrosorb RP-8.

For the mobile phase, mixtures of sodium phosphate buffer and acetonitrile

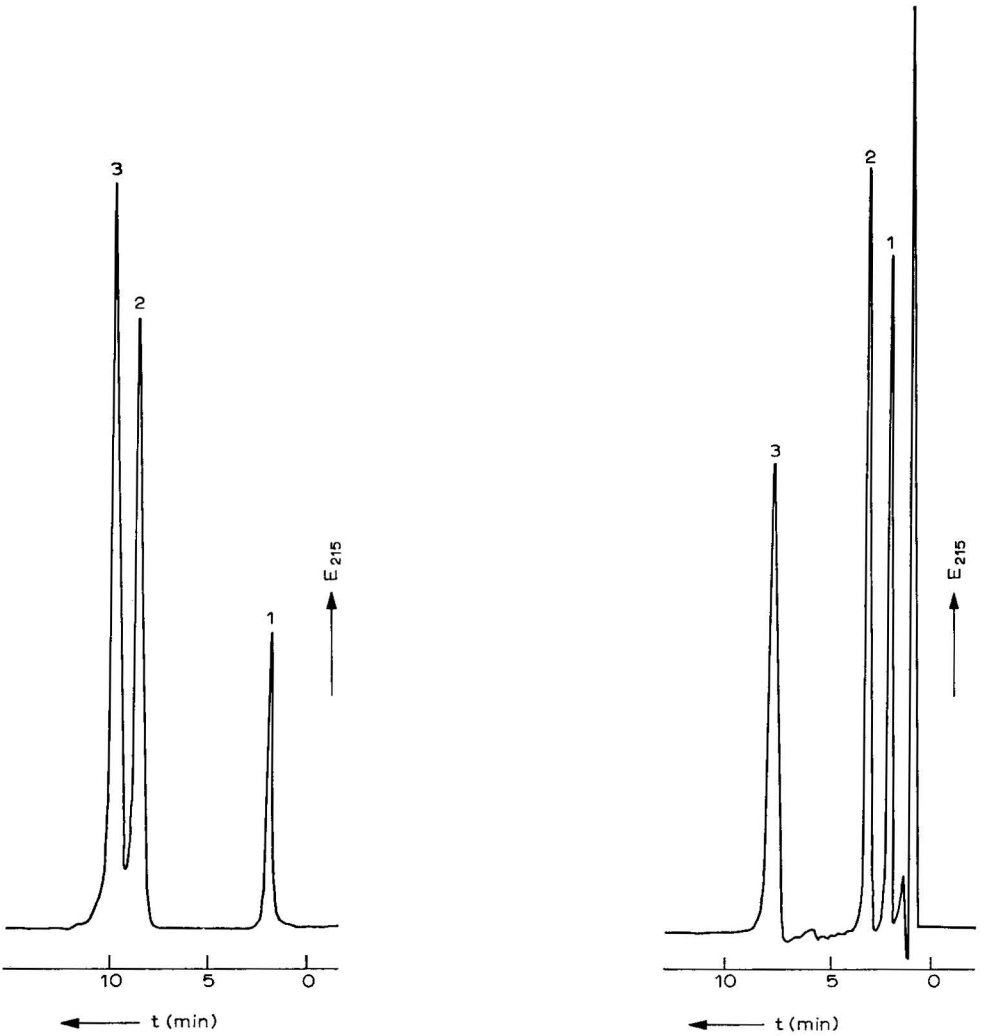

Fig. 1. Separation of CbO-Pro (1), CbO-Leu-Gly-ethyl ester (2) and CbO-Pro-Leu-Gly-ethyl ester (3). Column: LiChrosorb RP-8, 5 μm, 15 cm × 3.2 mm I.D. Mobile phase: phosphate buffer (0.015 *M*, pH 7.0)–acetonitrile (60:40); pressure, 120 bar; flow-rate, 0.7 ml/min. Detector: Perkin-Elmer LC 55.

Fig. 2. Separation of CbO-hexapeptideamide (1), CbO-pentapeptideamide (2) and CbO-tetrapeptideamide (3). Column, mobile phase and detector as in Fig. 1. Pressure, 200 bar; flow-rate, 1.5 ml/min.

were found to be the most suitable. The use of triethylammonium phosphate[17], or other ion-pairing agents, were of no advantage to the following separations.

Fig. 1 shows the separation of a mixture containing a carbobenzoxy-protected amino acid and a protected dipeptide ester. They are the basis for the synthesis of the third component, the protected tripeptide ester. The three compounds are eluted in order of their molecular weight. Unmarked peaks are solvent peaks or unknown impurities.

In the following synthesis a CbO-tetrapeptideamide, a CbO-pentapeptideamide and a CbO-hexapeptideamide are produced. These three peptides can be separated without difficulties, as shown in Fig. 2. The elution order, compared to Fig. 1, has changed. The molecule with the highest molecular weight elutes first, the smallest molecule (CbO-tetrapeptideamide) last.

The lengthening of the peptide chain by one amino acid can be studied using a similar mobile phase. An example is given in Fig. 3. Free pentapeptideamide is reacted with CbO-glutamine-nitrophenyl ester to give CbO-hexapeptideamide. For optimal control of the reaction it should be possible to monitor the concentration of all reactants in the same run. As can be seen from Fig. 3, a separation of the relevant compounds is possible within a few minutes. Additionally, CbO-pentapeptideamide that may be present as an impurity, is detectable. For a quantitative determination the substances must be dissolved in the mobile phase, because the free pentapeptideamide is eluted shortly after the dead volume time. In practice, signals from reaction solvents overlap with the signal of the free pentapeptideamide. In most cases, how-

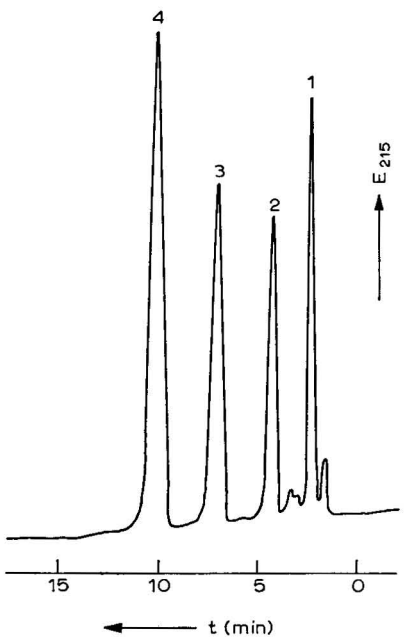

Fig. 3. Separation of pentapeptideamide (1), CbO-hexapeptideamide (2), CbO-pentapeptideamide (3) and CbO-Gln-ONP (4). Column: LiChrosorb RP-8, 5 μm, 15 cm × 3.2 mm I.D. Mobile phase: phosphate buffer (0.015 *M*, pH 7.0)–acetonitrile (70:40); pressure, 130 bar; flow-rate, 0.6 ml/min. Detector: Perkin-Elmer LC 55.

ever, it proved to be sufficient to monitor the increase of the amount of CbO-hexapeptideamide that is parallel to the decrease of the amount of free pentapeptideamide.

Peptides containing glutamine easily form pyroglutamine peptides by an intramolecular cyclisation of glutamine, especially in acidic media. Therefore a determination of pyroglutamine peptides is of great interest. An example of such a separation is shown in Fig. 4. Free pentapeptideamide, free hexapeptideamide, which is synthesized after coupling of glutamine, and pyroglutamine-hexapeptideamide, that can be formed as a by-product, are clearly separated.

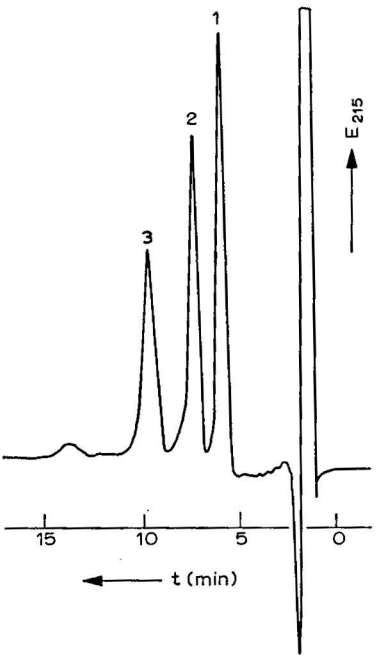

Fig. 4. Separation of hexapeptideamide (1), pentapeptideamide (2) and pyroglutamine-hexapeptideamide (3). Column: LiChrosorb RP-8, 5 μm, 15 cm × 3.2 mm I.D. Mobile phase: phosphate buffer (0.015 *M*, pH 3.0)–acetonitrile (78:22); pressure, 175 bar; flow-rate, 0.9 ml/min. Detector: Schoeffel SF 770.

The good selectivity of the technique is demonstrated by the separation of oxytocin and its preliminary stage, the reduced nonapeptideamide (Fig. 5). The two compounds differ only in their oxidation stage. The cysteine groups of the reduced nonapeptideamide are oxidized to cystine in oxytocin (Table I). As can be seen in Fig. 5, the pH value has a strong influence on the selectvity of this separation. Whereas at pH 3.0 a baseline separation is possible (A), at pH 7.0 no suitable separation can be achieved (B). Some precautions must be taken to allow for the detection of nonapeptideamide in its reduced form; all solvents necessary in preparing the sample solution or the mobile phase, must be treated with nitrogen before use. Nonapeptideamide must be stored under nitrogen; otherwise oxidation takes place.

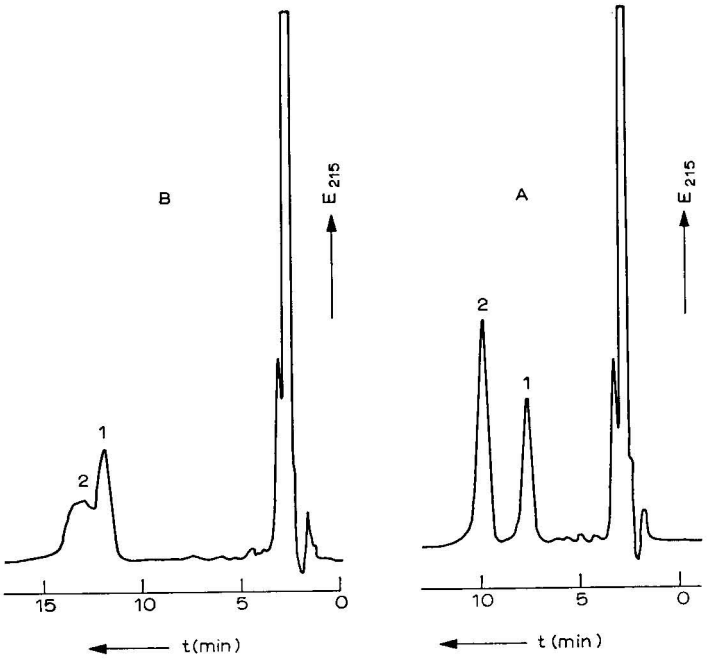

Fig. 5. Separation of oxytocin (1) and nonapeptideamide (2). Column: LiChrosorb RP-8, 5 μm, 15 cm × 3.2 mm I.D. Mobile phase: (A) phosphate buffer (0.015 *M*, pH 3.0)–acetonitrile (82:18); (B) phosphate buffer (0.015 *M*, pH 7.0)–acetonitrile (82:18); pressure, 140 bar; flow-rate, 0.7 ml/min. Detector: Perkin-Elmer LC 55.

Quantitative determinations

As detection wavelength, 215 nm was chosen. This is near the UV-absorption maximum of all tested peptides and CbO-peptides, and thus extends the detection limits to the ng range. The results of a study of 4 peptides are summarized in Table II. All values are based on the evaluation of peak areas. The correlation coefficients were determined for 5 injections in a concentration range of 3–300 μg peptide/ml. For this range, a linear calibration curve is obtained. The reproducibilities were calculated for 7 determinations at the concentrations given in Table II. The higher standard deviation of CbO-tetrapeptideamide is caused by its high retention time (Fig. 2). The form of the eluted peak is not ideal under these conditions, so that peak integration

TABLE II

QUANTITATIVE DETERMINATION OF 4 PEPTIDES

Peptide	*r**	*Relative S.D. (%)*	*Detection limit*		*Conditions*
			µg/ml	*ng*	
CbO-tetrapeptideamide	0.999	3.7 (33 µg/ml)	0.84	42	Fig. 2
CbO-pentapeptideamide	0.999	1.8 (18 µg/ml)	0.42	21	Fig. 2
CbO-hexapeptideamide	0.999	1.9 (13 µg/ml)	0.34	17	Fig. 2
Hexapeptideamide	0.998	2.2 (50 µg/ml)	1.00	50	Fig. 4

* Regression coefficient.

is difficult. A less polar mobile phase gives a lower retention time, and leads to a standard deviation comparable to those for the other compounds (Table II).

The main reasons for the different detection limits of CbO-tetrapeptideamide, CbO-pentapeptideamide and CbO-hexapeptideamide can also be found in the different retention times of the substances. The detection limits of CbO-peptides and free peptides do not differ significantly ,as can be seen by comparing the values of CbO-hexapeptideamide and free hexapeptideamide.

CONCLUSIONS

With the example of oxytocin synthesis, it has been shown that free and protected peptides can be determined simultaneously, simply and quickly by using isocratic reversed-phase liquid chromatography. The method has been applied in routine analysis for more than half a year. The use of this technique for controlling the synthesis of other peptides of similar form, should be possible.

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REFERENCES

1 J. Rosmus and Z. Deyl, *J. Chromatogr.*, 70 (1972) 221–239.
2 C. Haworth, *J. Chromatogr.*, 67 (1972) 315–323.
3 H. Frank and H. Hagenmaier, *J. Chromatogr.*, 106 (1975) 461–464.
4 S. Ho Chang, K. M. Gooding and F. E. Regnier, *J. Chromatogr.*, 125 (1976) 103–114.
5 R. Burgus and J. Rivier, *Pept., Proc. Eur. Pept. Symp., 14th*, (1976) 85–94.
6 W. S. Hancock, C. A. Bishop and M. T. W. Hearn, *FEBS Lett.*, 72 (1976) 139–141.
7 K. Krummen and R. W. Frei, *J. Chromatogr.*, 132 (1977) 27–36.
8 K. Krummen and R. W. Frei, *J. Chromatogr.*, 132 (1977) 429–436.
9 J. J. Hansen, T. Greibrokk, B. L. Currie, K. N.-G. Johansson and K. Folkers, *J. Chromatogr.*, 135 (1977) 155–164.
10 E. J. Kikta, Jr. and E. Grushka, *J. Chromatogr.*, 135 (1977) 367–376.
11 W. Mönch and W. Dehnen, *J. Chromatogr.*, 140 (1977) 260–262.
12 G. W.-K. Fong and E. Grushka, *J. Chromatogr.*, 142 (1977) 299–309.
13 J. Molnár and C. Horváth, *J. Chromatogr.*, 142 (1977) 623–640.
14 J. T. M. Bakkum, H. C. Beyerman, P. Hoogerhout, C. Olieman and D. Voskamp, *Rec. Trav. Chim. Pays-Bas*, 96 (1977) 301–306.
15 W. Mönch and W. Dehnen, *J. Chromatogr.*, 147 (1978) 415–418.
16 E. Lundanes and T. Greibrokk, *J. Chromatogr.*, 149 (1978) 241–254.
17 J. E. Rivier, *J. Liquid Chromatogr.*, 1 (1978) 343–366.
18 B. Larsen, V. Viswanatha, S. Y. Chang and J. Hruby, *J. Chromatogr. Sci.*, 16 (1978) 207–210.
19 W. S. Hancock, C. A. Bishop, J. E. Battersby, D. R. K. Harding and M. T. W. Hearn, *J. Chromatogr.*, 168 (1979) 377–384.
20 M. Martin and G. Guiochon, *Chromatographia*, 10 (1977) 194–204.

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CHROM. 11,962

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF BETAMETHASONE 17-VALERATE AND ITS DEGRADATION PRODUCTS

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SUMMARY

A high-performance liquid chromatographic assay of betamethasone 17-valerate is described. The procedure may be use for quantitative assay of the degradation products, betamethasone 21-valerate and betamethasone, and the application to the analysis of ointments is described. The method is also suitable for the determination of the kinetics of decomposition from one experimental run, and the determination of rate constants from a four-compartment sequential reaction is described.

The procedure is also applicable to other corticosteroids, and hydrocortisone 17-butyrate, hydrocortisone 21-butyrate, and hydrocortisone may similarly be determined without modification to the method.

INTRODUCTION

The esters of corticosteroids are a widely prescribed group of drugs. Esterification usually involves the C_{21} hydroxyl group, but betamethasone is unusual, being esterified instead at C_{17}. The derivative most widely used for topical application is betamethasone 17-valerate (Fig. 1, I), which has fifteen times the activity of the 21-isomer (II)[1]. The 21-valerate derivative is the thermodynamically more stable compound, and although stable in properly formulated dosage forms, the 17-valerate may rapidly rearrange to this less active form under non-ideal conditions. Further decomposition to the parent steroid (III) may also occur[2]. One possible source for non-ideal conditions existing in a formulated system is the widespread use of dilutions of proprietary systems[3]. We have recently shown that such preparations may have half-lives as short as 40 min for the active isomer[4]. Traditionally, assays have been based upon the tetrazolium blue colour reaction[5], and although degradation may be followed by thin-layer chromatography (TLC) and densitometry[4], the procedure is tedious and needs a large number of standards for acceptable results. Several reports have now appeared on the application of high-performance liquid chromatography (HPLC) to corticosteroid analysis[6–8], including a recent paper on betamethasone sodium phosphate[9]. In view of the advantages of these techniques over the colorimetric procedures, we wish to report an HPLC procedure for the assay of betamethasone

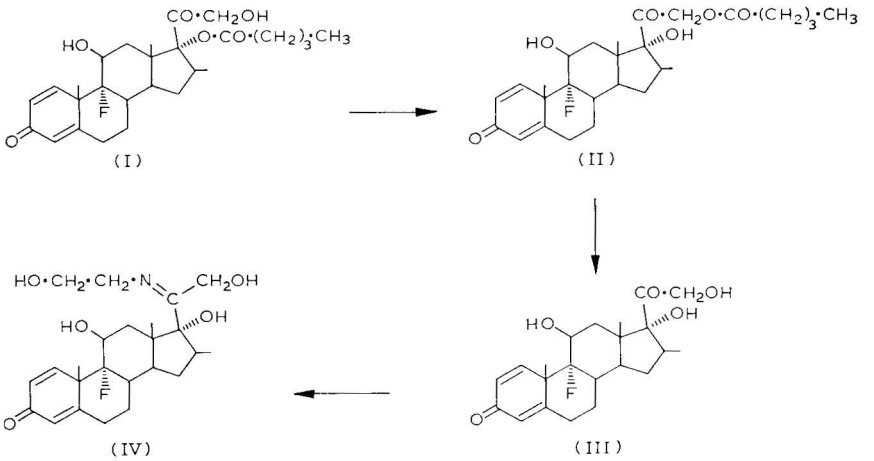

Fig. 1. Decomposition of betamethasone 17-valerate with ethanolamine. I = betamethasone, 17-valerate; II = betamethasone 21-valerate; III = betamethasone; IV = betamethasone 21-hydroxyethylimine.

17-valerate. This method may also be used to follow the rearrangement (betamethasone 21-valerate) and hydrolysis (betamethasone) products and to obtain a full kinetic profile of the degradation of betamethasone 17-valerate. The method is suitable for other corticosteroids and may be used for the analysis of the hydrocortisone 17-butyrate→hydrocortisone 21-butyrate→hydrocritisone system without modification.

EXPERIMENTAL

Apparatus and conditions

Analyses were performed with a high-performance liquid chromatograph, constructed from an Altex 100A constant-flow solvent-metering pump, a Rheodyne 7120 injector fitted with a 20-μl loop, and a Pye LC3 variable-wavelength ultraviolet monitor, equipped with an 8-μl flow-cell and operated at 260 nm with a sensitivity of 0.32 a.u.f.s. Chromatography was performed with a 25 cm × 4.6 mm I.D. column of Spherisorb (5 μm spherical, totally porous silica) and a mobile phase of ethyl acetate–chloroform–methanol (71:28:1) saturated with water and delivered at a flow-rate of 1 ml/min under a pressure of 60 bar.

Materials and methods

Calibration. Standard solutions of betamethasone 17-valerate, betamethasone 21-valerate and betamethasone were prepared, either separately or in mixture, in dimethyl sulphoxide over a concentration range of 20–120 ng ml^{-1}. The standard solutions (20 μl) were chromatographed and calibration lines were constructed on the basis of peak-height measurement.

Ointments. Synthetic ointments containing 0.1% (w/w) of betamethasone 17-valerate were prepared by dissolving the steroid in propylene glycol (5%) ultrasonically and dispersing this into a white soft paraffin base (95%) by trituration on a glass slab. Commercial Betnovate ointment (Glazo, Greenford, Great Britain) nomi-

nally containing 0.1 % (w/w) of betamethasone 17-valarate, was also used. The steroid content of these ointments was determined by weighing 1 g into glass-stoppered test tubes and partitioning the mixture between hexane (10 ml) and dimethyl sulphoxide (10 ml). The dimethyl sulphoxide extract (20 μl) was chromatographed and the peak heights were used for quantitative analysis.

Kinetics. A solution of betamethasone 17-valerate (0.01 % w/v) was prepared in propylene glycol containing ethanolamine (0.128 %). The solution was maintained at 60°, and 20-μl aliquots were injected directly into the chromatograph at intervals of up to 80 h.

Hydrocortisone. Hydrocortisone 17-butyrate, hydrocortisone 21-butyrate and hydrocortisone were dissolved in dimethyl sulphoxide (20–100 ng ml^{-1}) and were treated in a manner identical to that of the betamethasone solutions.

RESULTS AND DISCUSSION

The chromatograms obtained from betamethasone and its 17- and 21-valerate derivatives are in Fig. 2 and those from hydrocortisone and its 17- and 21-butyrate esters are shown in Fig. 3. The retention parameters are recorded in Table I. In each case the order of elution is 21-ester, 17-ester and parent alcohol. This, and the relative retention of betamethasone and hydrocortisone, are paralleled by the TLC behaviour of these compounds[4].

Quantitative analysis was readily achieved. A convenient range of concentrations for the assay of betamethasone 17-valerate in pharmaceuticals is 20–120 ng ml^{-1} and over this range both the 17- and 21-esters and betamethasone yielded linear calibration plots. Reproducibility was satisfactorily controlled by the loop injector, and an internal standard was not required. Peak-height measurements allowed rapid and accurate analysis and yielded the following regression equations (x in ng ml^{-1}, y in mm):

betamethasone 17-valerate: $y = 376.7x - 0.2$ ($r^2 = 99.90\%$)
betamethasone 21-valerate: $y = 413.6x + 0.1$ ($r^2 = 99.95\%$)
betamethasone: $y = 292.7x$ ($r^2 = 99.92\%$)

The difference in slope mainly reflects the increasing half-peak width with longer retention times. These data may be used directly to assay solid or solution forms of the three compounds, either alone, upon admixture, or as degraded samples, by dilution to approximately 100 ng ml^{-1}, followed by immediate chromatography.

The analysis of pharmaceutical preparations is more complex. Betamethasone 17-valerate is extensively used in dermatology, formulated as an ointment or a cream. In these systems a large quantity of excipient is present, which must be removed before a reliable assay may be undertaken. Typically, an ointment consists of propylene glycol (5 %), sufficient to dissolve the steroid, dispersed in a soft paraffin base (95 %). A useful preliminary treatment of these systems involves the partitioning of the ointment between two immiscible organic solvents[10]. The partition coefficient for betamethasone 17-valerate between dimethyl sulphoxide and hexane exceeds 1500 suggesting that this solvent system should yield adequate recoveries for assay purposes. Partitioning of synthetic ointments between dimethyl sulphoxide and hexane enables

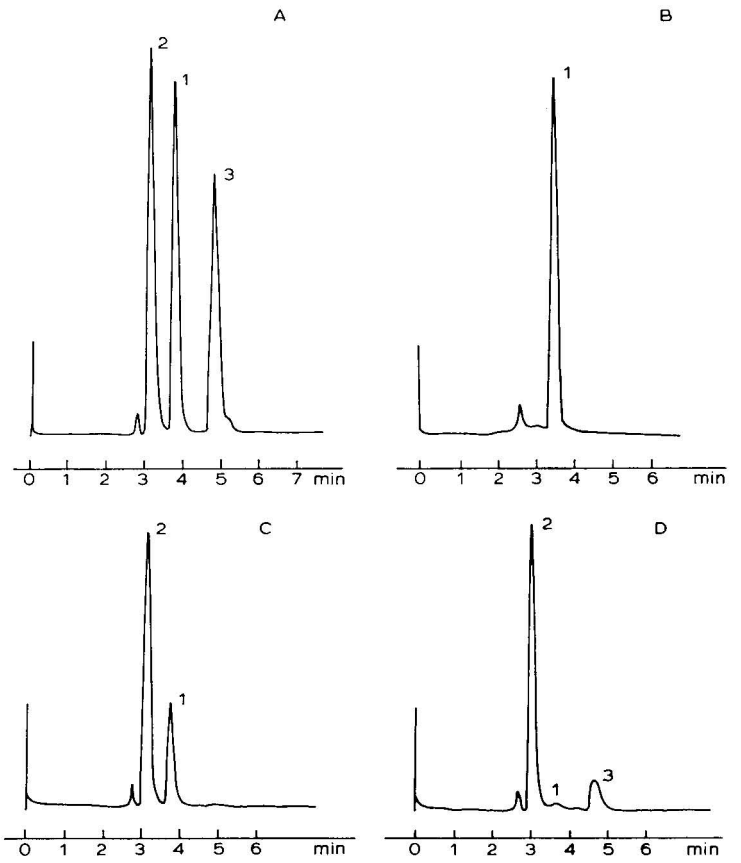

Fig. 2. HPLC of betamethasone 17-valerate and degradation products. Peaks: 1 = betamethasone 17-valerate; 2 = betamethasone 21-valerate; 3 = betamethasone. (A) Standard solutions each 100 ng ml^{-1}; (B) betamethasone 17-valerate in propylene glycol–ethanolamine (100 ng ml^{-1}) at 0 min; (C) after 30 min; (D) after 230 min.

the removal of the hydrocarbon fraction by the hexane, and chromatography of the lower dimethyl sulphoxide phase allowed the ready estimation of the betamethasone 17-valerate level. Recovery studies for these ointments showed levels of 99.80 $\pm$ 1.56% ($P = 0.95$) of the prepared strength (0.1%). The commercial samples also showed satisfactory reproducibility but were found to contain 97.20 $\pm$ 0.52% ($P = 0.95$) of the nominal content (0.1%) of betamethasone 17-valerate. This may reflect the disparate storage conditions for this batch of samples, but formulation differences cannot be excluded.

One further point of interest in the study of the stability of betamethasone and its esters, is the elucidation of the kinetic parameters for the degradation. The method described here also enables the calculation of all rate constants to be achieved during one kinetic run. To illustrate this application, the decomposition of betamethasone 17-valerate in propylene glycol solution catalysed by ethanolamine was undertaken. The reaction sequence in this system is recorded in Fig. 1. It involves rearrangement (I $\rightarrow$ II), hydrolysis (II $\rightarrow$ III) and the loss of betamethasone possibly via condensation

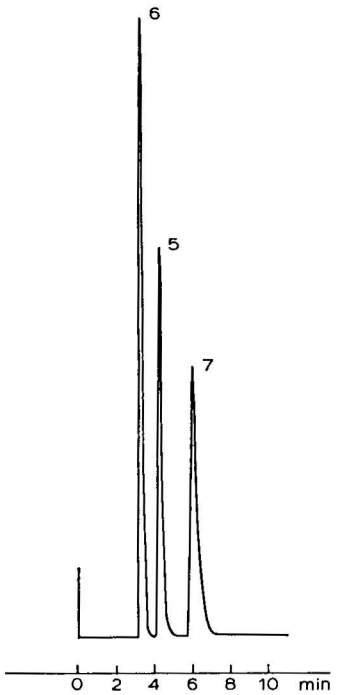

Fig. 3. HPLC of hydrocortisone 17-butyrate and degradation products. Peaks: 5 = hydrocortisone 17-butyrate; 6 = hydrocortisone 21-butyrate; 7 = hydrocortisone; each 100 ng ml^{-1}.

TABLE I

RETENTION DATA FOR BETAMETHASONE AND HYDROCORTISONE AND THEIR ESTERS

Steroid	*Retention time (min)*	*Capacity ratio*	*Resolution*
Betamethasone 21-valerate	3.1	0.11	
			1.20
Betamethasone 17-valerate	3.7	0.32	
			1.69
Betamethasone	4.8	0.71	
Hydrocortisone 21-butyrate	3.2	0.14	
			1.29
Hydrocortisone 17-butyrate	4.1	0.46	
			1.80
Hydrocortisone	5.9	1.11	

(III→IV) and follows the kinetics of an A→B→C→D sequential reaction. The relevant expressions allowing the calculation of the composition of the mixture at time t are shown in Table II. Typical chromatograms showing the progress of the reaction are shown in Fig. 2 (B–D) and illustrate the rapid disappearance of the 17-valerate and the slower appearance of betamethasone. The reaction profile is displayed in Fig. 4 and yields the kinetic parameters of Table III. Although the final product (IV) is not transmitted by the column under the conditions of this analysis, owing to the increased polarity of this compound, the appearance profile may be calculated from eqn. 4 (Table II). The stability of the betamethasone 17-valerate in this system parallels

TABLE II

CALCULATION OF THE COMPOSITION OF BETAMETHASONE 17-VALERATE SOLUTION DURING THE ETHANOLAMINE-CATALYSED DEGRADATION IN PROPYLENE GLYCOL

$$\underset{(I)}{A} \xrightarrow{K_1} \underset{(II)}{B} \xrightarrow{K_2} \underset{(III)}{C} \xrightarrow{K_3} \underset{(IV)}{D}$$

(1) $[A]_t = [A]_0 \, e^{-K_1 t}$

(2) $$[B]_t = \frac{[A]_0 K_1}{(K_2 - K_1)} \{e^{-K_1 t} - e^{-K_2 t}\}$$

(3) $$[C]_t = [A]_0 \left[\left\{ \frac{K_1 K_2}{(K_2 - K_1)(K_3 - K_1)} \right\} e^{-K_1 t} + \left\{ \frac{K_1 K_2}{(K_1 - K_2)(K_3 - K_2)} \right\} e^{-K_2 t} + \left\{ \frac{K_1 K_2}{(K_1 - K_3)(K_2 - K_3)} \right\} e^{-K_3 t} \right]$$

(4) $$[D]_t = [A]_0 \left[1 - \left\{ \frac{K_2 K_3}{(K_2 - K_1)(K_3 - K_1)} \right\} e^{-K_1 t} - \left\{ \frac{K_1 K_3}{(K_1 - K_2)(K_3 - K_2)} \right\} e^{-K_2 t} - \left\{ \frac{K_1 K_2}{(K_1 - K_3)(K_2 - K_3)} \right\} e^{-K_3 t} \right]$$

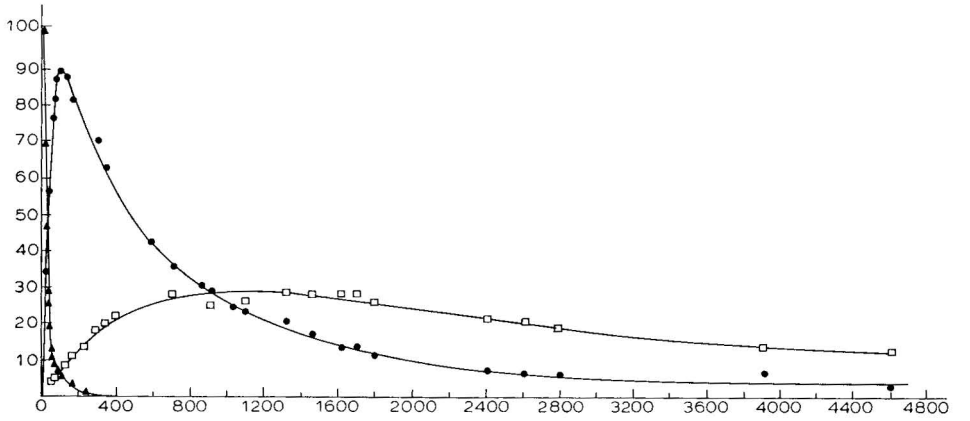

Fig. 4. Reaction profile of the ethanolamine-catalysed degradation of betamethasone 17-valerate in propylene glycol. ▲, Betamethasone 17-valerate; ●, Betamethasone 21-valerate; □, Betamethasone.

TABLE III

KINETIC PARAMETERS FOR THE ETHANOLAMINE-CATALYSED DEGRADATION OF BETAMETHASONE 17-VALERATE IN PROPYLENE GLYCOL

Reaction	*Rate constant (h^{-1})*
Rearrangement (I → II)	$K_1 = 2.4460$
Hydrolysis (II → III)	$K_2 = 0.0859$
Condensation (III → IV)	$K_3 = 0.0502$

that found in ointments[4], with the most rapid degradation being the isomerisation of the 17- to the 21-valerate, followed by a slower hydrolysis to yield betamethasone.

Clearly, the benefits afforded by this HPLC procedure, in particular the speed of assay and the precision of the results, are compelling advantages over current assay methods.

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We are grateful to the West Midland Regional Health Authority for the kind provision of HPLC equipment and for the award of a studentship to Y.W.Y. and to Glaxo Limited for gifts of betamethasone esters.

REFERENCES

1 A. W. McKenzie and R. M. Atkinson, *Arch. Dermatol.*, 89 (1964) 741.
2 R. Gardi, *Hormonal Steroids —Biochemistry, Pharmacology and Therapeutics (Proc. 1st Int. Congr. Hormonal Steroids)*, Vol. 2, Academic Press, New York, 1965, p. 99.
3 M. J. Busse, *Pharm. J.*, 220 (1978) 25.
4 Y. W. Yip and A. Li Wan Po, *J. Pharm. Pharmacol.*, 31 (1979) 400.
5 R. E. Graham, E. R. Biehl and C. T. Kenner, *J. Pharm. Sci.*, 67 (1978) 360.
6 J. C. Touchstone and W. Wortmann, *J. Chromatogr.*, 76 (1973) 244.
7 M. C. Olsen, *J. Pharm. Sci.*, 62 (1973) 2001.
8 J. A. Mollica and R. F. Strusz, *J. Pharm. Sci.*, 61 (1972) 44.
9 L. M. Upton, E. R. Townley and F. D. Sancilio, *J. Pharm. Sci.*, 67 (1978) 913.
10 J. E. Fairbrother and A. Siam, *Proc. Anal. Div. Chem. Soc.*, 15 (1978) 253.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PROSTACYCLIN

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SUMMARY

A high-performance liquid chromatographic method for the analysis of prostacyclin using a laboratory prepared reversed-phase column packing is described. A relative standard deviation of less than 1% was obtained for ten replicate injections. The system resolves prostacyclin from its hydrolysis product, 6-oxo-prostaglandin $F_{1\alpha}$ and from other prostaglandins present as impurities. These can be estimated to levels of approximately 0.5%. The separation of other unrelated prostaglandins by this method is briefly reported.

INTRODUCTION

Prostacyclin (PGI_2) has been shown[1] to be a potent inhibitor of platelet aggregation. In biological fluids PGI_2 was found to be labile being rapidly converted to 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$)[2]. Fitzpatrick[3] has applied a high-performance liquid chromatographic (HPLC) method to the separation and analysis of prostaglandins as their *p*-nitrophenacyl esters. The preparation of the derivative provided a solution to the problem of the lack of sensitivity for these compounds using detection at a fixed wavelength of 254 nm but with the penalty of a loss of time and precision due to the derivatisation steps in the analysis. Cho and Allen[4] reported that this procedure was not suitable for PGI_2 as it spontaneously decomposed to 6-oxo-$PGF_{1\alpha}$ during resolution on a normal-phase silica gel column as well as on a reversed-phase column. This was presumed to be due to the catalytic power of the acidic adsorption sites on the silica. More recently Fitzpatrick[5] has described a separation of prostaglandins and thromboxanes by gas chromatography using glass capillary columns. This again required a derivatisation step, in this case esterification using diazomethane, in which PGI_2 was determined as the derivative of 6-oxo-$PGF_{1\alpha}$.

HPLC offers advantages of specificity, speed and accuracy for the determination of the acid labile PGI_2 especially if the inherent loss of precision associated with the derivatisation step can be avoided. A simple, precise method has been developed for the analysis of PGI_2 using a variable wavelength detector at about 205 nm, a suitably inert reversed-phase column packing and an inert, UV-transparent mobile phase. The analysis of a typical batch of synthetic PGI_2 sodium salt[6,7] is reported. This

shows the presence of minor impurities whose identities can be postulated by comparison of their retention times with those of the primary hydrolysis product, 6-oxo-$PGF_{1\alpha}$[6,7], and the two Δ^4-isomers[8,9] of prostacyclin. The use of the method for monitoring the stability of PGI_2 is shown. The separation of some other prostaglandins on this system is also reported.

EXPERIMENTAL

Apparatus

A Spectra-Physics Model 740B (Spectra-Physics, Santa Clara, Calif., U.S.A.) motor-driven, reciprocating piston pump employing flow feedback control was used. The detector was a Pye Unicam LC3 (Pye Unicam, Cambridge, Great Britain) variable-wavelength UV detector. Chromatographic columns were operated at ambient temperatures and injections were made using a 50-μl split-flow loop injector as described by Webber and McKerrell[10]. Chromatograms were recorded on a Bryans 28000 (Bryans Southern Instruments, Cheshire, Great Britain) single pen recorder 0–10 mV full scale.

Chromatographic column

The column packing material was prepared by the method given below which is a modification of that supplied by Benezra[11]. 10 μm Partisil (Whatman, Maidstone, Great Britain) (10 g) was dried at 80° *in vacuo* for about 3 h in a 250-ml round-bottomed flask. Octadecyltrichlorosilane (10 ml) and dry toluene (100 ml) were added and the solution refluxed for 3 h with paddle stirring, using a reflux condenser fitted with a calcium chloride guard tube. The mixture was allowed to cool and then filtered through a 0.5-μm Millipore filter (Type FHL PO4700). The silica in the filter was washed with 250 ml methanol slurrying the solid continuously, then with 250 ml hot acetone and dried at 80° *in vacuo* for about 2 h. This gave a carbon loading of about 12%.

The sample (11 g) was refluxed with trimethylchlorosilane (10 ml) and dry toluene (100 ml) for 45 min, washed with methanol and then with acetone and dried *in vacuo* for 2 h to yield the product. This gave an increase in carbon load of about 1%.

A 25 cm by 4 mm I.D. seamless stainless-steel column was packed using carbon tetrachloride as the slurry medium[10].

Reagents

HPLC grade methanol (Rathburn Chemicals, Walkerburn, Great Britain), AnalaR boric acid, AnalaR disodium tetraborate, AnalaR sodium chloride, AnalaR sodium carbonate and reagent grade tetramethylammonium hydroxide (TMAH) (BDH, Poole, Great Britain) were used as received. All prostaglandins were supplied by Dr. N. Whittaker, Wellcome Research Laboratories, Beckenham, Great Britain.

Procedure

Samples for analysis were dissolved in 0.025% (w/v) aqueous TMAH. The stability study was carried out in a carbonate–bicarbonate buffer adapted from that

described by Perrin[12] to be 0.05 *M* in buffer and to have an ionic strength of 0.5 using sodium chloride.

RESULTS AND DISCUSSION

Chromatography

PGI_2 has been reported[4] to undergo specific hydrogen ion catalysed hydrolysis to 6-oxo-$PGF_{1\alpha}$. It was also suggested that the reaction was so facile as to occur on the acidic sites still present on a reversed-phase silica column packing. The method used to prepare the column was therefore designed to give a minimum number of these sites. In order to minimise solute hydrolysis the mobile phase should be as alkaline as possible without stripping off the bonded phase. The mobile phase also had to transmit low-wavelength UV light. Methanol–water mixtures with various buffering systems were tried. Initially ammonium carbonate was employed but the system proved difficult to reproduce whilst a carbonate–bicarbonate buffer system stripped the bonded phase from the column. Finally a boric acid–sodium tetraborate buffer was used and found to be satisfactory.

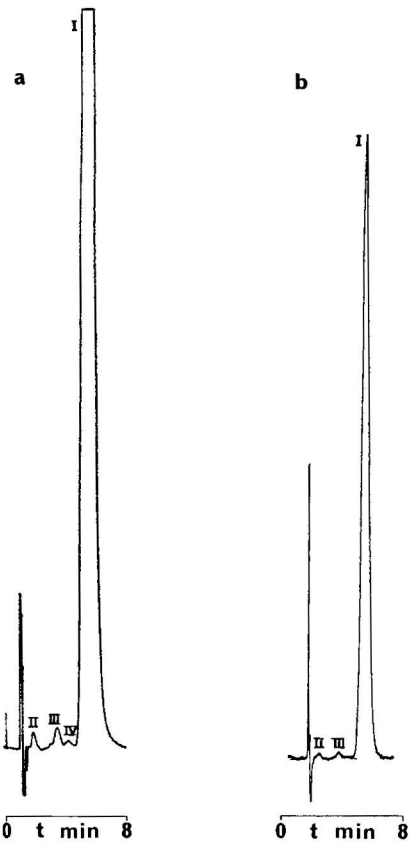

Fig. 1. HPLC of a typical batch of prostacyclin sodium salt. Mobile phase water–methanol (3:2) with 2.5 g l^{-1} boric acid and 3.8 g l^{-1} sodium tetraborate eluent. Flow-rate, 3.6 ml min^{-1}; UV, 205 nm; (a) 0.16 a.u.f.s., 10 μg injected, (b) 0.32 a.u.f.s., 6 μg injected. Structures as in Fig. 2.

The wavelength chosen to monitor the separation, 205 nm, provided the best compromise between maximum sensitivity for PGI_2, which increased as the wavelength was decreased, the efficiency of the detector and interference from the solvent.

Typical chromatograms are shown in Fig. 1. The impurities II, III and IV were found to have the same retention times as the compounds illustrated and named in Fig. 2. The capacity factors (flow-rate 3.6 ml min^{-1}; UV, 205 nm, 0.16 a.u.f.s.; 1 μg injected) of II, III, IV and PGI_2 were 1.4, 2.7, 3.3 and 4.6, respectively; and those at a lower flow-rate (1.5 ml min^{-1}) for PGA_2, PGB_2, PGE_1, PGE_2 and PGI_2 were 4.0, 4.9, 2.7, 2.3 and 4.6, respectively. These data indicate the scope of this approach which requires less sample preparation and only slightly more sophisticated detection equipment than the derivatisation method. Minor modifications to the system should provide analytical methods for use with many prostaglandins.

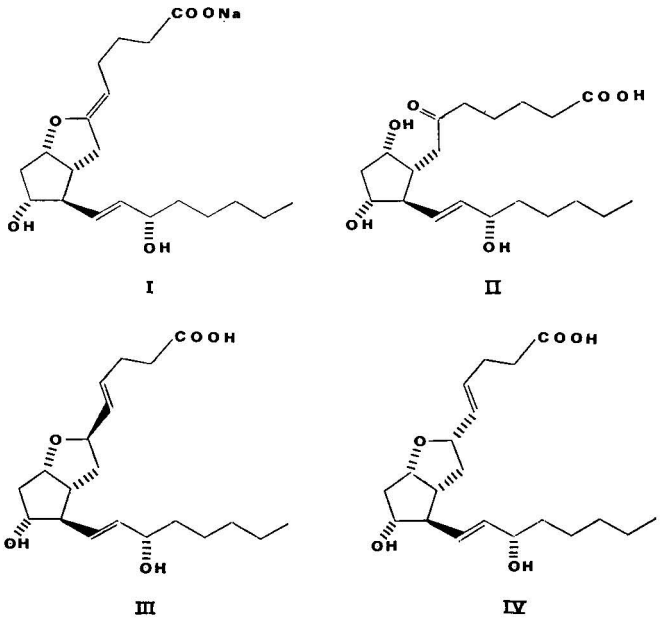

Fig. 2. Structures of prostacyclin and impurities. I = PGI_2 (5*Z*,9α,11α,13*E*,15*S*)-6,9-epoxy-11,15-dihydroxyprosta-5,13-dienoic acid, sodium salt; II = 6-oxo-$PGF_{1\alpha}$ (9α,11α,13*E*,15*S*)-6-oxo-9,11,15-trihydroxyprosta-13-enoic acid; III = (4*E*,6*R*,9α,11α,13*E*,15*S*)-6,9-epoxy-11,15-dihydroxyprosta-4,13-dienoic acid; IV = (4*E*,6*S*,9α,11α,13*E*,15*S*)-6,9-epoxy-11,15-dihydroxyprosta-4,13-dienoic acid.

Calibration

Using peak height measurements the response of PGI_2 was linear in the range 0–50 μg. The relative standard deviation of the peak height for ten successive injections was 0.75% and this method of measurement was found satisfactory for most determinations. The peak height did not decrease significantly over several hours provided the standard was kept at a suitable pH such as that provided by 0.025% TMAH in water.

The small peak height variation indicated that PGI_2 could be analysed by comparing the peak height found in a given sample with that obtained from a

standard solution of an arbitrarily chosen batch of PGI_2 sodium salt and this method has been used to examine the purity and stability of the compound and its formulations.

The purity of a typical batch of PGI_2 was determined by assessing the levels of the observed impurity peaks, using an increased loading and sensitivity to that applied in the analysis of the parent compound as indicated in Fig. 1a. These levels were estimated by peak height against suitable standard solutions as 1% of compounds II and III and 0.5% of compound IV.

Stability

The hydrolytic stability of PGI_2 has been previously examined using a UV spectroscopic method[4]. This HPLC method is ideal for monitoring the decomposition of PGI_2 to 6-oxo-$PGF_{1\alpha}$ and has been carried out, as demonstrated in Fig. 3.

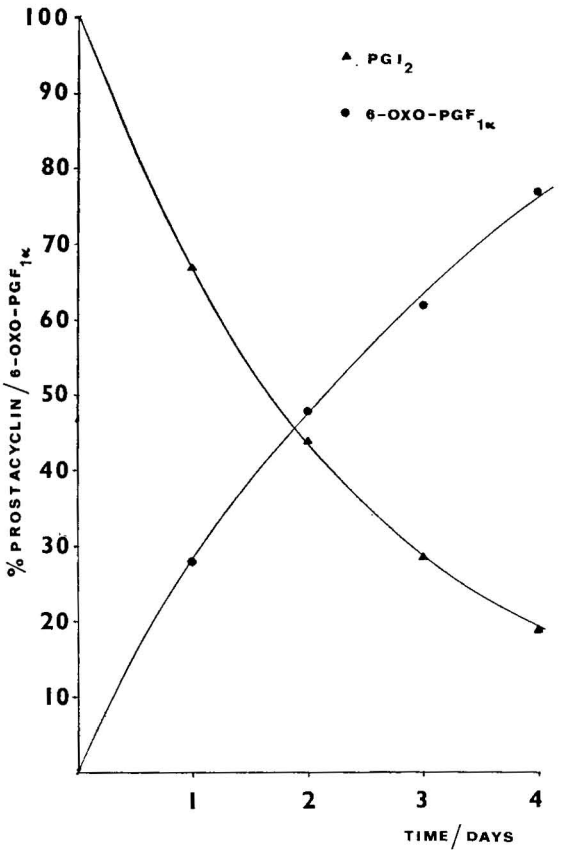

Fig. 3. Stability of prostacyclin at pH 10.5 stored as a 100 μg ml^{-1} solution at 28° in carbonate–bicarbonate buffer. HPLC conditions as in Fig. 1, 2.5 μg injected.

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The author wishes to express his gratitude to Dr. N. Whittaker of the Wellcome Research Laboratories, Beckenham, for supplying the various prostaglandins used, to S. Benezra for the contribution of his unpublished method, and to T. J. Coomber for his assistance in this study and the preparation of the manuscript.

REFERENCES

1 S. Moncada, R. J. Gryglewski, S. Bunting and J. R. Vane, *Nature (London)*, (1976) 263, 663.
2 J. E. Tateson, S. Moncada and J. R. Vane, *Prostaglandins*, 13 (1977) 389.
3 J. A. Fitzpatrick, *Anal. Chem.*, 48 (1976) 499.
4 M. J. Cho and M. A. Allen, *Prostaglandins*, 15 (1978) 943.
5 J. A. Fitzpatrick, *Anal. Chem.*, 50 (1978) 47.
6 R. A. Johnson, F. H. Lincoln, J. L. Thompson, E. G. Nidy, S. A. Mizsak and U. Axen, *J. Amer. Chem. Soc.*, 99 (1977) 4182.
7 N. Whittaker, *Tetrahedron Lett.*, (1977) 2805.
8 K. C. Nicolaou and W. E. Barnette, *J. Chem. Soc., Chem. Commun.*, (1977) 331.
9 E. J. Corey, H. L. Pearce, I. Szekely and M. Ishiguro, *Tetrahedron Lett.*, (1978) 1023.
10 T. J. N. Webber and E. H. McKerrell, *J. Chromatogr.*, 122 (1976) 243.
11 S. Benezra, Burroughs Wellcome & Company, Research Triangle Park, N.C., personal communication, 1977.
12 D. D. Perrin, *Aust. J. Chem.*, 16 (1963) 527.

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Note

High-performance liquid chromatographic assay for prostacyclin

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(First received April 3rd, 1979; revised manuscript received May 7th, 1979)

Prostacyclin, (5-*Z*)-9-deoxy-6,9α-epoxy-Δ5-prostaglandin $F_{1\alpha}$, a recently discovered metabolite of arachidonic acid, is produced by the blood vessels, heart, and lungs[1–3]. Prostacyclin is a potent vasodilator and it inhibits platelet aggregation; consequently, it is considered a potentially important regulator of blood pressure and endothelial integrity[4]. These properties make it an interesting candidate for therapeutic use in cardiovascular, hematological or thrombotic disorders[4]. The investigation of the chemical purity and stability of prostacyclin sodium salt (PGI_2·Na) prepared by total organic synthesis[5] is necessary to complement other analytical methods which are less suitable for measuring impurities and decomposition products[6]. This report describes a reversed-phase high-performance liquid chromatographic (HPLC) method for determining the purity and stability of PGI_2·Na preparations possibly containing four other structurally related compounds. Included are data on PGI_2·Na stability under various condition, and results on the accuracy and precision of the method. The rationale for the use of certain chromatographic conditions and limitations imposed by the chemical nature of the vinyl ether structure of PGI_2·Na are discussed, in terms of the HPLC method of Hill and Coomber[7].

EXPERIMENTAL

Apparatus

Chromatography was performed with a Model 396 pulse damped piston pump (Laboratory Data Control, Riviera Beach, Fla., U.S.A.); a syringe-loaded Model 7120 loop injection valve, with an internal volume of 20 μl (Rheodyne, Berkeley, Calif., U.S.A.); a μBondapak® C_{18} column (Waters Assoc., Milford, Mass., U.S.A.); and a Model LC55 variable-wavelength spectrophotometric detector (Perkin-Elmer, Norwalk, Conn., U.S.A.) operated at 205 nm. Chromatograms were recorded on a Varian A-25 recorder, 1 mV, operated at 0.1 in. min^{-1}.

Reagents

Sterile, distilled water; boric acid and sodium borate (J. T. Baker, Phillipsburg, N.J., U.S.A.); and methanol and acetonitrile (Burdick & Jackson, Muskegon, Mich., U.S.A.) were used without purification. Two mobile phase systems are discussed in this report. Mobile phase 1 consisted of methanol–water (55:45, v/v), buffered at pH 8.9 with 0.04 *M* boric acid and 0.019 *M* sodium borate. Mobile phase 2 consisted of

acetonitrile–water (20:80, v/v), buffered at pH 9.3 with 0.009 *M* boric acid and 0.004 *M* sodium borate. Using mobile phase 1 the column was operated at 1500 p.s.i.g. at a flow-rate of 1.0 ml min^{-1}. Using mobile phase 2, the column was operated at 2000 p.s.i.g. at a flow-rate of 1.2 ml min^{-1}.

Reference standards of possible decomposition products or trace contaminants of the organic synthesis procedure were supplied by the Experimental Chemistry Laboratories of The Upjohn Company. A reference standard of $PGI_2 \cdot Na$ was verified by several complementary analytical techniques to be at least 99.8% (w/w) pure. For chromatographic analysis, accurately weighed samples were dissolved in mobile phase and a 20-μl full-loop injection was made at a recorder setting giving approximately 50% full scale deflection. Peak area measurements were used for all calculations. The injection reproducibility was verified at $\pm 1\%$.

RESULTS

$PGI_2 \cdot Na$, an exocyclic vinyl ether, hydrolyzes rapidly, in a pH dependent fashion, via a hemi-ketal intermediate into a stable end product: 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$)[5,6]. 6β-Δ4-Prostaglandin I_1 (6β-Δ4-PGI_1) and 6α-Δ4-PGI_1 are possible impurities from the synthetic procedure. The structures of these compounds are shown in Fig. 1. Their chemical nature, especially the potential lability of the parent compound, restricts the choice of chromatographic parameters. The vinyl ether moiety of $PGI_2 \cdot Na$ is best stabilized in solution by buffering under basic conditions (>pH 8.8). Thus, a reversed-phase HPLC system was indicated in spite of the fact that the separation of the 6α- and 6β-epimers of Δ4-PGI_1 might be difficult. Also, one would predict that PGI_2 or its principal decomposition product, 6-keto-$PGF_{1\alpha}$, should inter-

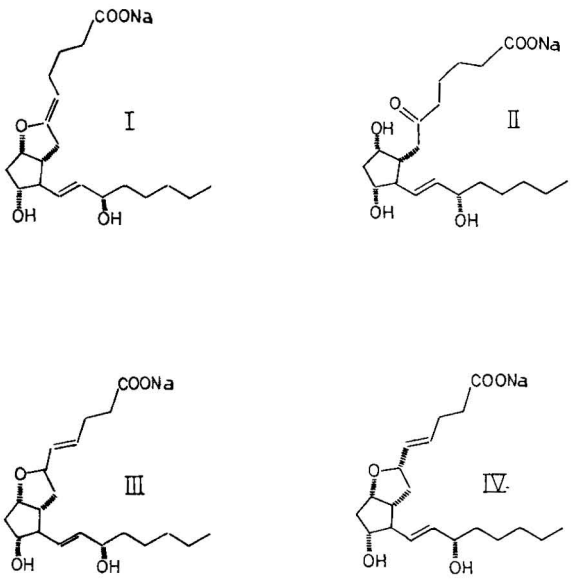
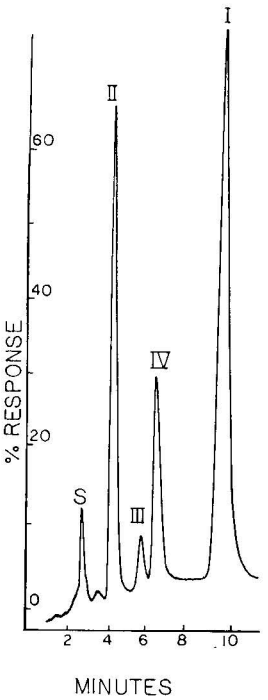


Fig. 1. Structures of prostacyclin (I) and its known decomposition products of contaminants: 6-keto-PS $F_{1\alpha}$ (II), 6β-Δ4-PS I_1 (III) and 6α-Δ4-PS I_1 (IV).

act, as their hemi-ketals, with alcohols (methanol or ethanol) to form methyl or ethyl ketals. These products might, under certain conditions, appear as distinct peaks and complicate the assay. Superficially, the methanol modified, mobile phase 1 adequately separated the compounds of interest (Fig. 2); however, initial quantitative experiments confirmed the predicted problem. Fig. 3 shows that pre-incubation with different proportions of methanol, immediately prior to injection, can distort the chromatographic profile of a pure reference standard of 6-keto-$PGF_{1\alpha}$. When exposed to methanol at concentrations equivalent to those of mobile phase 1, for intervals equivalent to the transit time through the column, the profile for a highly purified sample of 6-keto-$PGF_{1\alpha}$ still contains minor peaks which coincide in retention with 6α- or 6β-Δ4-PGI_1. As noted, the 6α- and 6β-epimers are not decomposition products, but are residual trace impurities from the synthetic procedure. Their concentration in any given sample ought to remain invariant. Thus, apparent increases in 6α- and 6β-Δ4-PGI_1 in several experiments using mobile phase 1 were attributable to the interaction between the hemi-ketal form of 6-keto-$PGF_{1\alpha}$ and methanol. Subsequent chro-



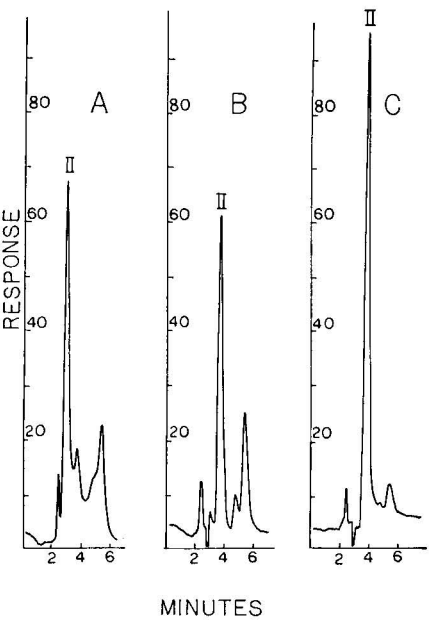
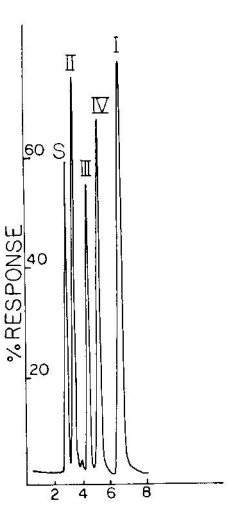


Fig. 2. Typical chromatogram of prostacyclin and its known decomposition products or contaminants. Chromatography was performed on a μBondapak C_{18} column using a mobile phase of methanol–water (55:45, v/v), buffered at pH 8.9 with 0.04 *M* boric acid and 0.019 sodium borate. The flow-rate was 1.0 ml min^{-1} at 1500 p.s.i.g. Compounds were detected with an LC55 variable-wavelength detector fixed at 205 nm and calibrated to register 0.10 a.u.f.s. S = solvent.

Fig. 3. Influence of methanol on 6-keto-PG $F_{1\alpha}$ chromatographic profile. A fixed concentration (1.5 mg/10.0 ml) of 6-keto-$PGF_{1\alpha}$ was incubated in different methanol–water compositions: A = 90:10, B = 50:50, C = 10:90. After 5 min incubation a sample was injected and chromatographed using the identical conditions of Fig. 2.



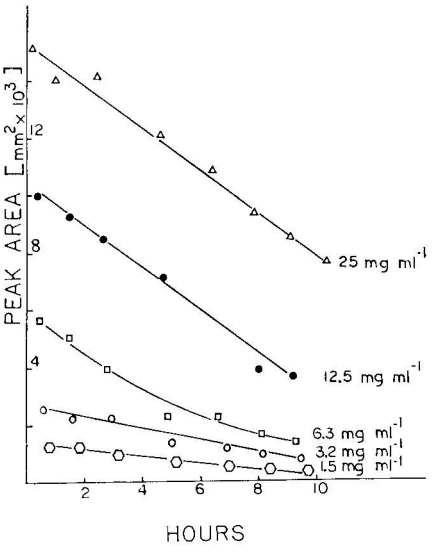


Fig. 4. Typical chromatogram of prostacyclin and its known decomposition products or contaminants. Chromatography was performed on a μBondapak C_{18} column using a mobile phase of acetonitrile–water (20:80, v/v), buffered at pH 9.3 with 0.009 *M* boric acid and 0.004 *M* sodium borate. The flow-rate was 1.2 ml min^{-1} at 2000 p.s.i.g. Compounds were detected with an LC55 variable-wavelength detector fixed at 205 nm and calibrated to register 0.10 a.u.f s.

Fig. 5. The concentration dependence of the stability of prostacyclin at pH 8.9, 23°.

matography using the aprotic organic modifier, acetonitrile, in mobile phase 2, also separated the compounds of interest (Fig. 4). Quantitative experiments with this system confirmed that the increases in 6α- and 6β-Δ4-PGI_1 were artifacts due to the methanol in mobile phase 1. Moreover, a previously unrecognized decomposition product, 6(9α),6(11α)-dioxido-15α-hydroxy-(*E*)-prosta-13-enoic acid[8,9] was resolved

TABLE I

HPLC MEASUREMENT OF PGI_2 STABILITY

Temperature (°C)	*pH*	*Half-life (h)*
0	8.9	21.0
23	8.9	4.4
23	9.3	10.3
23	7.4	0.033

TABLE II

QUANTITATIVE ACCURACY OF HPLC ASSAY

Mass found (mg/5.0 ml)	*Mass expected (mg/5.0 ml)*	*Error (%)*
0.301	0.307	2.0
0.544	0.565	3.8
2.074	2.095	1.1
3.572	3.959	9.8

from the other compounds using mobile phase 2. This impurity co-chromatographed with PGI_2 itself in mobile phase 1. Replicate analyses on several samples showed a precision of $\pm 3\%$. Analysis of several "blind" samples prepared for internal quality control purposes showed an accuracy of 4.2% (Table I). The pH and temperature dependence of the PGI_2 half-life (Table II) determined by HPLC agrees well with values obtained by a spectrophotometric method[6]. The half-life of PGI_2 also shows a concentration dependence (Fig. 5) which has practical implications for formulation purposes.

CONCLUSIONS

The measurement of $PGI_2 \cdot Na$ purity and stability by HPLC is not straightforward. The number of suspected impurities and decomposition products; the lability of the parent compound; and the generic interaction of hemi-ketals with alcoholic modifiers, all restrict the range of chromatographic parameters which are compatible with the assay requirements. HPLC in the reversed phase mode with microparticulate columns was sufficiently versatile to counter these restrictions. Karger and Giese[10] have reviewed this aspect of HPLC.

Our results confirm the need to integrate one's knowledge of the chemical nature of compounds being chromatographed with apparent chromatographic results. In the case of predictable chemical interactions one cannot rely exclusively on results from only one mobile phase. The HPLC method reported here is based on the original method of Hill *et al.*[7]. Our results show that acetonitrile should be used to eliminate systematic errors related to the use of methanol as the organic modifier in the chromatographic eluent. The method described reflects $PGI_2 \cdot Na$ purity with greater accuracy.

REFERENCES

1 S. Moncada, R. Gryglewski, S. Bunting and J. R. Vane, *Nature (London)*, 263 (1976) 663.
2 S. Moncada, E. A. Higgs and J. R. Vane, *Lancet*, 1 (1977) 18.
3 E. A. M. De Deckere, D. Nugteren and F. Ten Hoor, *Nature (London)*, 268 (1977) 160.
4 S. Moncada and J. R. Vane, *Brit. Med. Bull.*, 34 (1978) 129.
5 R. Johnson, F. Lincoln, J. Thompson, E. Nidy, S. Mizsak and U. F. Axen, *J. Amer. Chem. Soc.*, 99 (1977) 12.
6 M. J. Cho and M. Allen, *Prostaglandins*, 15 (1978) 943.
7 G. Hill and T. Coomber, personal communication.
8 K. Shimoji, Y. Konishi, Y. Arai, N. Hayashi and H. Yamamoto, *J. Amer. Chem. Soc.*, 100 (1978) 2457.
9 J. Ueda, T. Yanagisawa, M. Shibasaki and S. Ikegami, *Tetrahedron Lett.*, (1978) 2511.
10 B. Karger and R. Giese, *Anal. Chem.*, 50 (1978) 1073A.

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Note

Electronic signal differentiation as an aid for the comparison of size-exclusion chromatograms

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The use of size-exclusion chromatography on microparticulate silica for the rapid comparison of a wide variety of samples of forensic interest has recently been reported[1]. This form of chromatography, which produces a molecular size profile, often generates chromatograms which consist of a single broad peak with various shoulders and inflections and the comparison of such data can be difficult. To afford a greater level of confidence when judging the significance of such profiles it is the usual practice here to sequentially monitor the eluate with two detectors mounted in series, but a supplementary method is to enhance the visual difference between poorly structured chromatograms generated with a single detector by electronically differentiating the signal. Although derivative spectrophotometry is not new, recent developments in electronics now make it a simple technique to apply[2], and its use for enhancing the discriminatory capability of size exclusion chromatography is an obvious area of application.

EXPERIMENTAL

The column used in these experiments was a stainless-steel tube, 25 cm × 3/8 in. O.D. × 0.8 cm I.D., terminated with Zero Dead Volume (ZDV) reducing unions (3/8–1/16 in.) and packed with an irregular microparticulate silica of *ca.* 5 μm diameter, average pore size 13 nm, and surface area 320 m^2/g. The solvent used was tetrahydrofuran containing 1% water and it was pumped at 4 ml/min. The eluate was monitored with a UV detector (Cecil 212, Cecil Instruments, Cambridge, Great Britain) operated at 254 nm. Samples were dissolved in tetrahydrofuran and injected using a stop-flow technique. The detector signal was split to provide the fundamental chromatogram and its 1st derivative form simultaneously, and both were recorded on flat-bed pen recorders.

The electronic differentiation was carried out using the circuit shown in Fig. 1 which was designed after consideration of various reference sources[3–5]. IC 1 is a non-inverting, unity gain buffer amplifier used to match the output impedance of the detector to the input impedance of IC 2. IC 2 is the differentiator circuit which gives an output voltage proportional to the rate of change of input voltage *i.e.*

$$E_0 = - C_1 R_1 \frac{dE_{in}}{dt}$$

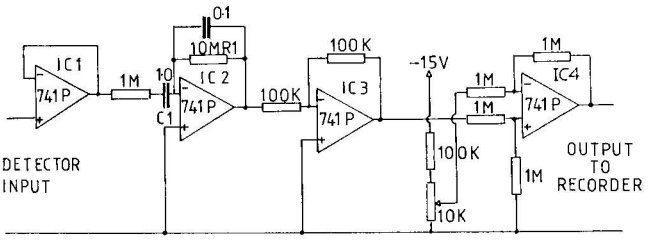

Fig. 1. The circuit used for the first-order differentiation of the detector sinal produced with size-exclusion chromatograms.

As this circuit also inverts the signal a unity gain inverter IC 3 is utilised to correct the signal; the output after IC 3 then becoming $-E_0$. A d.c. level is present at the output of IC 3 due to the leakage of components in and around IC 2 and this standing current is backed off using the subtractor circuit of IC 4 to present a signal suitable for a pen recorder input.

RESULTS AND DISCUSSION

Typical chromatograms produced using the described technique are shown in Fig. 2. It can be readily seen that the first-derivative form of the chromatograms is sufficiently structured to make the visual comparison of two or more profiles a simple task; the derivatised form of the chromatogram is also highly reproducible. The speed and versatility of size exclusion chromatography already makes this a powerful method for evaluating high polymers and complex mixtures of lower molecular weight and the use of electronic differentiation can only add to its versatility.

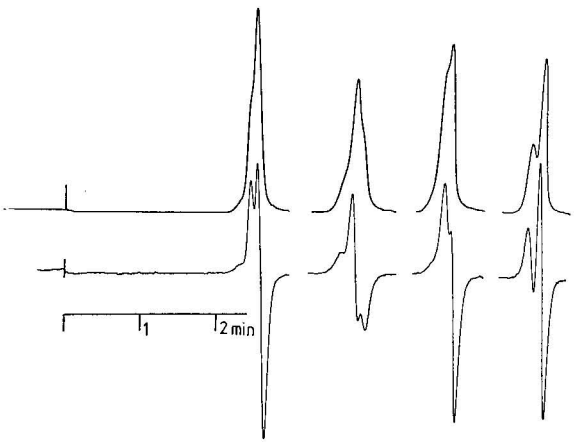

Fig. 2. Size-exclusion chromatograms of engine oils: comparison of the fundamental and first-derivative form of the UV detector response. Column: 25 × 0.8 cm I.D.; packing: 5-μm silica of 13 nm pore size; solvent: tetrahydrofuran–water (99:1); flow-rate: 4 ml/min; pressure: 1400 p.s.i.; detector: UV at 254 nm. The upper traces are the fundamental form of the chromatogram, the lower traces the first-derivative form.

The circuit described costs only about £ 5 to construct and despite the use of low-quality components worked very satisfactorily. One of the main problems in constructing low-frequency differentiator circuits is that of the long time constants involved and ideally low-leakage, non-electrolytic capacitors should be used in conjunction with high input impedance operational amplifiers. Although the circuit shown does not use capacitors of this type it was adequate for first-order differentiation but is unlikely to perform well if higher orders of differentiation are required. Nevertheless there is every indication that differentiation to higher orders can be advantageous[2] and we propose to study this area of application in due course.

ACKNOWLEDGEMENTS

We would like to express our appreciation to Dr. R. Williams, the Director of this laboratory, for bringing ref. 2 to our attention, and thus initiating this study.

REFERENCES

1 B. B. Wheals, *J. Liquid Chromatogr.*, 2 (1979) 91.
2 G. Talsky, L. Mayring and H. Kreuzer, *Angew. Chem.*, 17 (1978) 785.
3 G. B. Clayton (Editor), *Operational Amplifiers*, Butterworths, London 1971.
4 R. Kalvoda (Editor), *Operational Amplifiers in Chemical Instrumentation*, Wiley, Chichester, New York, 1975.
5 W. G. Jung (Editor), *I.C. Operational Amplifier Cookbook*, H. W. Sams (Prentice-Hall), 1974.

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Note

In situ modification of silica with amines and its use in separating sugars by high-performance liquid chromatography

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One of the most convenient methods of sugar analysis at the present time involves a high-performance liquid chromatographic (HPLC) separation with refractive index (RI) monitoring of the eluate. This procedure has advantages over earlier gas chromatographic methods in that it is more rapid, requires no derivatisation, and the interpretation of results is simpler because the anomeric forms of sugars are not resolved by HPLC as they are by gas–liquid chromatography.

Sugar separations are best achieved on silicas that have been chemically changed to display a basic surface chemistry. A chemically bonded material marketed in the form of a "carbohydrate column" by Waters Assoc. (Milford, Mass., U.S.A.), was first introduced in 1974 and has found much subsequent use[1,2]. Comparable separations were achieved using silica modified by reaction with 3-aminopropyltriethoxysilane[3] and the reaction conditions have subsequently been studied in some detail[4]. More recently it has been shown that equally effective chromatography of sugars can be attained by "*in situ*" coating of silica using a poly-functional amine in the eluent[5].

Sugar analysis is of importance in forensic science mainly in the so-called "drugs intelligence" field. Illicit preparations are often diluted with sugars and it can be of interest to have information about their nature and relative proportions in order to compare various seizures. In studying this topic we have found that chemically bonded amino-phase packing materials tend to become "poisoned" when tablet and powder extracts are injected, a phenomenon characterised by a progressive deterioration in resolution. In our experience use of the *in situ* coating technique described by Aitzetmüller[5] produces columns displaying greater long-term stability and, in the work reported here, we have studied the separating capability of silica modified with a variety of amines.

EXPERIMENTAL

The stainless-steel columns used in this study were 12.5 cm × 0.49 cm I.D. × 1/4 in. O.D. terminated with zero-dead-volume reducing unions (1/4–1/16 in.) and were packed with an irregular silica of *ca.* 5 μm diameter, average pore diameter 13 nm, and surface area 320 m^2/g. Chemically bonded stationary phases prepared from the same base silica by reaction with 3-aminopropyltriethoxysilane, or 3-(2-aminoethylamino)-

propyltrimethoxysilane, were also studied (the organic loadings of the two packing materials were 7.5% and 14%, respectively)[4]. The columns packed with silica were treated by pumping an eluent consisting of acetonitrile–water (75:25) containing 0.1% of the amine for 0.5 h at 3 ml/min. The eluent was then changed to contain 0.01% of the amine and was pumped at 2 ml/min until equilibrium conditions were attained (about 30 min). Samples were dissolved in water to give an approximately 5–10% solution and 5 μl aliquots were injected using a stop-flow technique. The eluate was monitored with an Model 750/13 RI detector (Applied Chromatography Systems, Luton, Great Britain).

The amines studied were primary, secondary and tertiary butylamine, an homologous series of *n*-alkylamines, a diamino *n*-alkyl series with terminal amino groups in the 1,2 (*i.e.* ethylenediamine, EDA), 1,3, 1,5, 1,6 and 1,8 positions, and a polyamine series, diethylenetriamine, (DETA), triethylenetetramine (TETA), tetraethylenepentamine (TEPA) and pentaethylenehexamine (PEHA).

Pure sugar standards were used for much of the study but illicit drug preparations were examined by mixing with water, centrifuging and injecting a suitable aliquot of the supernatant.

RESULTS AND DISCUSSION

The findings of this study confirm the work of Aitzetmüller[5] in that they show that silica modified *in situ* with eluents containing polyfunctional amines can provide useful separations of sugars. The efficiency of the *in situ* coated columns and those

TABLE I

THE RETENTION DATA FOR SUGARS ON CHEMICALLY BONDED AND *IN SITU* MODIFIED PACKING MATERIALS

Chromatographic conditions: acetonitrile–water (75:25) + 0.01% amine at 2 ml/min, pressure drop *ca.* 900 p.s.i., column 12.5 cm × 0.49 cm I.D. k' = phase capacity ratio, k_o = 1.5 min.

Packing material or the amine used in the eluent	*k' Values for various sugars*					
	Fructose	*Mannitol*	*Glucose*	*Sucrose*	*Maltose*	*Lactose*
3-Aminopropyl*	1.7	2.2	2.3	3.7	5.0	5.5
3-(2-Aminoethylamino)propyl*	1.5	1.9	2.2	3.6	4.8	4.8
n-Butylamine	0.8	1.2	0.8	0.8	1.6	2.0
Dibutylamine	0.5	0.9	0.5	1.2	1.3	1.4
Tributylamine	0.6	1.0	0.6	1.2	1.3	1.4
n-Octylamine	0.8	1.2	0.8	1.3	1.5	1.9
n-Dodecylamine	0.8	1.2	0.8	1.3	1.6	2.0
1,3-Diamino	1.5	1.9	1.9	2.9	3.8	5.0
1,5-Diamino	1.5	1.9	1.9	2.9	3.8	4.6
1,6-Diamino	1.2	1.5	1.6	2.2	3.0	3.6
1,8-Diamino	0.9	1.2	0.9	1.4	1.8	2.1
EDA	1.3	1.7	1.5	2.3	3.0	3.7
DETA	1.4	1.9	1.9	2.6	3.5	4.2
TETA	1.5	1.9	1.9	2.9	3.8	4.6
TEPA	1.6	2.0	2.0	3.2	4.3	5.0
PEHA	1.3	1.7	1.7	2.7	3.5	4.1

* Bonded phase materials (no amine used in the eluent).

produced with a chemically bonded amino phase were rather similar (*e.g.* number of theoretical plates 1400–2000, *i.e.* 0.09–0.06 mm plate heights). Although not high by HPLC standards, this type of column performance is typical of that previously reported for sugars. At flow-rates higher than 2 ml/min the detector response decreased, hence this flow-rate was used throughout in order to provide a fast analysis with an acceptable response. The retention data for a variety of sugars are shown in Table I and characteristic chromatograms obtained with the series of diamines and polyamines are shown in Figs. 1 and 2. Fig. 3 compares a sugar separation on an aminopropyl bonded phase with that obtainable on silica before and after *in situ* modification with TEPA.

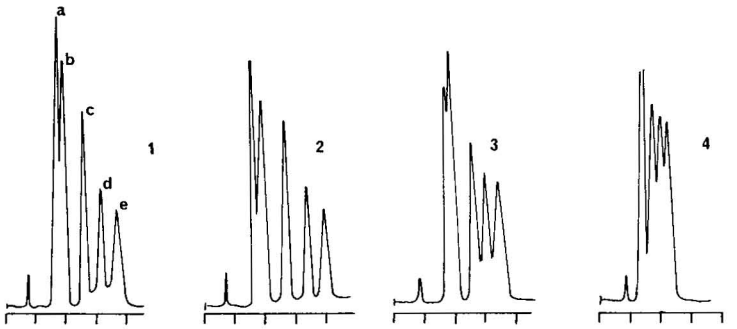

Fig. 1. The separation of (a) fructose, (b) glucose, (c) sucrose, (d) maltose, and (e) lactose on silica modified with diamines. Column: 12.5 cm × 0.49 cm I.D. Solvent: acetonitrile–water (75:25) + 0.01% amine at 2 ml/min. Amines studied: (1) 1,3-diaminopropane, (2) 1,5-diaminopentane, (3) 1,6-diaminohexane, (4) 1,8-diaminooctane. Detector: RI, time intervals 2 min.

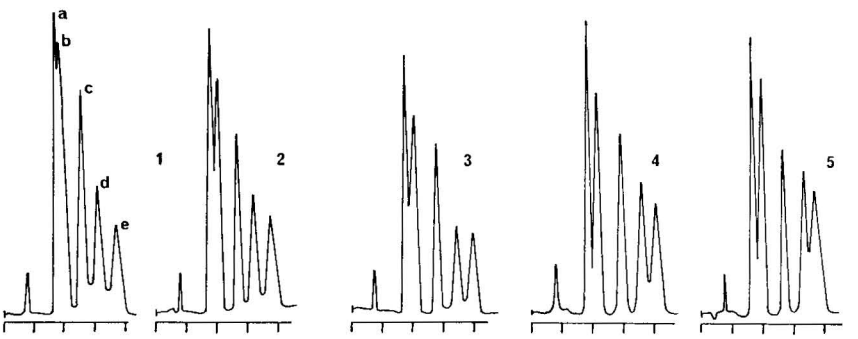

Fig. 2. The separation of sugars on silica modified with polyamines. The sugars and column conditions as indicated in Fig. 1. Amines studied: (1) EDA, (2) DETA, (3) TETA, (4) TEPA, (5) PEHA.

Coating silica with amines using the described procedure leads to greater retention of sugars than is the case when using the uncoated silica. However, the retention of sugars on such columns is somewhat lower than that obtained with chemically bonded amino phases. In general, the retention decreases in the sequence 3-aminopropyl bonded phase > 3-(2-aminoethylamino)propyl bonded phase > polyamines > diamines > amines > silica.

Primary aliphatic amines are of relatively little value for separating sugars

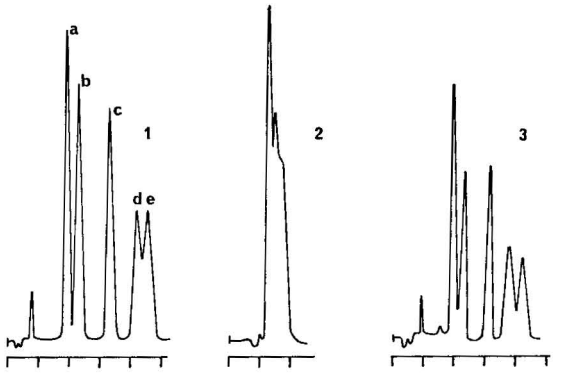

Fig. 3. The separation of sugars on (1) 3-aminopropyl bonded phase and (2) silica using an acetonitrile–water (75:25) eluent. Separation (3) was obtained on silica with 0.01% TEPA added to the eluent. Other conditions as in Fig. 1.

by this method. They do, however, partially resolve mannitol from glucose, and this is a mixture we have encountered in some illicit preparations. In the case of the diamines, those with chain-lengths up to C_5 were able to modify the silica so that it could be used to resolve the disaccharides, sucrose, maltose and lactose, but it was not possible to obtain baseline separations of the monosaccharides, glucose and fructose, on 12.5 cm columns with the eluent used. At chain-lengths greater than C_5, the overall retention of the sugars decreased. In the case of the polyamines the resolution obtained with the monosaccharides was somewhat improved, whereas that between maltose and lactose deteriorated. For the five common sugars, TEPA seems to provide an optimum separation. It is possible to obtain baseline resolution of all five common sugars on a 12.5 cm column by using a TEPA-containing eluent of lower water content, but this can only be attained at the expense of increased analysis time.

The most useful feature of the *in situ* coating method is that the columns prepared by this process are far more tolerant of non-sugar coextractives than are

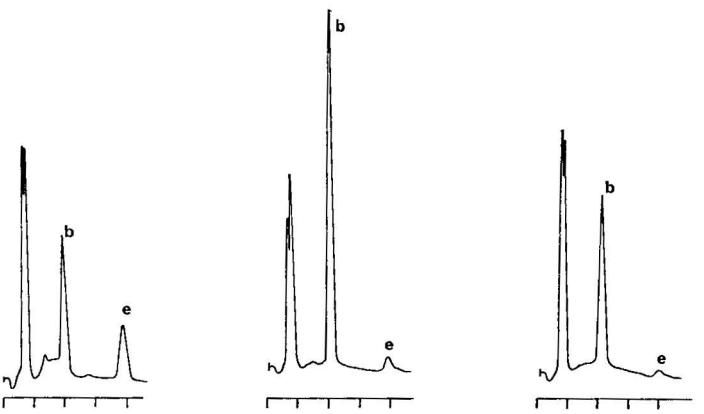

Fig. 4. Typical chromatograms of illicit powder extracts separated on silica using acetonitrile–water (75:25) containing 0.01% TEPA. All other conditions as in Fig. 1.

bonded-phase amino packings. This can be attributed to the fact that the adsorbed amine layer bound to the silica is continually being regenerated by the amine in the eluent. This type of dynamic equilibrium provides an inexpensive basis for preparing columns for sugar analysis having long-term stability; typical analyses are shown in Fig. 4. It has also been found that bonded amino-phase packing materials could be given an extended useful life by using an eluent containing TEPA.

REFERENCES

1 J. C. Linden and C. L. Lawhead, *J. Chromatogr.*, 105 (1975) 125.
2 R. B. Meagher and A. Furst, *J. Chromatogr.*, 117 (1976) 211.
3 R. Schwarzenbach, *J. Chromatogr.*, 117 (1976) 206.
4 A. D. Jones, I. W. Burns, S. G. Sellings and J. A. Cox, *J. Chromatogr.*, 144 (1977) 169.
5 K. Aitzetmüller, *J. Chromatogr.*, 156 (1978) 354.

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Note

Octanol–aqueous partition, distribution and ionization coefficients of lipophilic acids and their anions by reversed-phase high-performance liquid chromatography

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We have described[1] a method for the simultaneous determination of 1-octanol–aqueous partition, distribution and ionization coefficients (P, D and K_a, respectively; $P = D[(K_a/H) + 1]$ where H is the hydrogen ion concentration) by reversed-phase high-performance liquid chromatography (HPLC). 1-Octanol–aqueous partition coefficients for neutral species are of considerable interest in the area of quantitative structure–activity relationships (QSAR)[2], but the cumbersomeness of the classical shake-flask method is a deterrant to the experimental measurement of large numbers of these parameters. Reversed-phase HPLC allows rapid determination of P, D and K_a on small amounts of (possibly) impure material and is therefore of considerable practical interest to the pharmaceutical, pesticide and environmental industries.

In this note we wish to present further evidence that our method is applicable, in particular, to lipophilic acids and ion-pair partitioning, since Wahlund and Beijersten[3] have reported that lipophilic acids can partition to the hydrophobic support under certain conditions used in his studies (1-pentanol on C_2, C_8 and C_{18} bonded supports). Such partitioning to the hydrophobic support would diminish the parallelism between bulk phase and reversed-phase HPLC partitioning if it were to occur. However, this does not appear to be the case under conditions used in our procedure[1]. For example, log P for the lipophilic acid naproxen ((+)-6-methoxy-α-methyl-2-naphthaleneacetic acid) was reported[1] to agree excellently with literature shake-flask values (log $P = 3.20$ compared to 3.18 for literature). These results were obtained at pH $<$ pK_a so that it was not possible to estimate partitioning of the anion (P'). We report here log P, log P' and pK_a for two other lipophilic acids, xanthone-2-carboxylic acid[4] and tiopinac (6,11-dihydro-11-oxodibenzo[*b*,*e*]thiepin-2-acetic acid), a new non-hormonal anti-inflammatory agent[5]. These results confirm that our procedure correctly models bulk phase partitioning for lipophilic acids, including ion-pair partitioning.

EXPERIMENTAL

The procedure of Unger *et al.*[1] was used except that a Waters Model 204 constant-flow high-performance liquid chromatograph was attached to a Scientific Products Model 4000 data system in order to improve accuracy in determining log

k' ($k' = (t_x - t_0)/t_0$, where t_0 is the dead time (volume) and t_x is the sample time). Electronics were slightly modified such that the data system was activated as the injection lever was thrown. Ambient temperature was 22 ± 1°. Stainless-steel columns of 2 mm I.D. and 2.9, 10 and 50 cm length were packed with persilated Corasil C_{18}. Buffers were of 0.15 ionic strength (μ) (with added sodium chloride as necessary; columns were stripped after each workday by purging with water and then methanol; recoating took about 75–100 ml at 5 ml/min) and were saturated by shaking with excess 1-octanol (99.97% certified) and allowing to clarify for about two days. Mobile phases were then filtered through Millipore filters to degas and purify immediately before use. Analytical runs were performed at 2 ml/min as soon as the baseline had stabilized for 5 min. Dimethylformamide was used to dissolve samples (with buffer) and to determine t_0. Triplicate injections of each of the standards was made (2.9-cm column: anisole, chlorobenzene, benzophenone; 10-cm column: benzaldehyde, acetophenone, anisole; 50-cm column: catechol, acetanilide and benzaldehyde). The correlation coefficient for log k' *vs.* log D for standards was always $r \approx 0.999$ with a slope of essentially 1.0. An appropriate amount of dissolved sample was placed into the Waters injector with a 25-μl Hamilton chromatography syringe such that peak areas remained approximately constant.

Shake-flask values were determined[2] at 3–4 dilutions and log D obtained by averaging (xanthone-2-carboxylic acid) or by linear extrapolation (tiopinac). Solutions of tiopinac were protected from light.

Computer work was on an APL language system (Proprietary Computer Computer Systems, Van Nuys, Calif., U.S.A.) or on the Syntex IBM 370/158 for non-linear least squares[1].

RESULTS AND DISCUSSION

Horváth *et al.*[6] have derived equations for the case of both ionized and neutral species partitioning into a lipoid phase (also for multiple ionizations or zwitterionic partitioning) during reversed-phase HPLC. If log D is determined from the standard regression line, then the appropriate equations can be written as eqn. 1a for acid and eqn. 1b for acid and anion partitioning:

$$\log D = \log P - \log (1 + (K_a/H)) \quad (1a)$$

$$\log D = \log (P + P'K_a/H) - \log (1 + (K_a/H)) \quad (1b)$$

An example of ion-pair partitioning is provided by the lipophilic xanthone-2-carboxylic acid (I), which is the parent of a series of 7-substituted xanthone-2-carboxylic acids studied as mast cell inhibitors[4]. The partitioning behavior of I at 7 pH values was well fit by the standard model eqn. 1a with $r = 0.99$ and, in fact, gave insignificant P' when fitted to eqn. 1b. However, points at the most basic pH values deviated systematically, being much more "lipophilic" than expected. Additional points were then taken at still more basic pH values (pH 7.32 and 9.43) by classical shake-flask methods, above the operating range of the column. The combined data (collected in Table I) then fit eqn. 1b[7]. The derived constants from the non-linear fit of eqn. 1b are: $\log P = 3.12 \pm 0.07$; $\log P' = -0.21 \pm 0.07$; and $pK_a = 3.73 \pm 0.10$; with $n = 9$, standard deviation of fit = 0.086 and correlation coefficient between observed and calculated =

0.998. As can be seen in Table I, at pH 2.42–2.47 and pH 5.78–5.82, the agreement between the two methods is excellent. Log D determined at either about 6 or 99% ionization agrees excellently between shake-flask and reversed-phase HPLC methods. The data is internally consistent, being fit by eqn. 1b to a very high precision, irrespective of source of the data. The fourth column of Table I gives calculated log D (eqn. 1b) at all pH values, including those not used in the statistical analysis. Complete log P, log P' and pK_a were obtained for an additional 17 analogs of I in less than one month, demonstrating the productivity of this method[8]. This procedure for log P is more direct than that suggested by Wahlund and Beijersten[3].

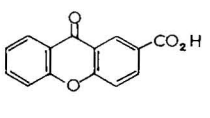
TABLE I

PARTITION DATA ON XANTHONE-2-CARBOXYLIC ACID

pH	*log D*			*Difference (observed − calculated, eqn. 1b)*
	Observed (±S.D.)	*Eqn. 1a***	*Eqn. 1b*	
2.42*,***	3.10 ± 0.14	3.099	3.099	0.001
2.47	3.053 ± 0.007	3.097	3.097	−0.044
3.35	2.977 ± 0.003	2.969	2.969	0.008
3.84	2.865 ± 0.005	2.760	2.761	0.104
4.06	2.617 ± 0.002	2.623	2.624	−0.007
4.35	2.348 ± 0.002	2.407	2.407	−0.059
4.83	1.855 ± 0.003	1.987	1.989	−0.134
5.78	1.190 ± 0.002	1.066	1.088	0.102
5.82*,***	1.10 ± 0.06	1.026	1.051	0.049
7.32*	−0.035 ± 0.03	−0.470	−0.020	−0.015
9.42*	−0.21 ± 0.03	−2.570	−0.208	−0.002

* Shake-flask, all other by reversed-phase HPLC.
** Not used in fitting eqn. 1b.
*** Calculated using log P and pK_a fit to eqn. 1b.

A second example is illustrated by the new non-hormonal antiinflammatory drug, tiopinac (II):



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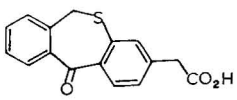

II

Partition data are presented in Table II. These data may be fitted to eqn. 1b, giving log $P = 2.97 \pm 0.06$; log $P' = 0.82 \pm 0.06$; and p$K_a = 3.71 \pm 0.10$; with $n = 7$, standard deviation = 0.069 and $r = 0.998$. These values compare quite favorably with log $P = 3.13 \pm 0.01$ by shake-flask and p$K_a = 3.82 \pm 0.28$ by solubility determination, both at 25° and $\mu = 0.10$. Log k' was found to decrease by 0.02 units at pH = 2.41 on going from $\mu = 0.10$ to $\mu = 0.15$; therefore, log P corrected = 3.11 ± 0.01; there is an insignificant effect on pK_a. Furthermore, pK_a as determined by reversed-phase HPLC is an apparent pK_a since it is determined in the presence of 1-octanol.

TABLE II

PARTITION DATA ON TIOPINAC

pH	*log D*			*Difference (observed – calculated, eqn. 1b)*
	Observed (±S.D.)	*Eqn. 1a**	*Eqn. 1b*	
2.40	2.932 ± 0.001	2.949	2.949	−0.017
3.34	2.813 ± 0.004	2.816	2.817	−0.004
3.92	2.654 ± 0.002	2.551	2.556	0.098
4.00	2.484 ± 0.006	2.500	2.506	−0.022
4.88	1.747 ± 0.001	1.772	1.815	−0.068
6.03	1.100 ± 0.000	0.648	1.042	0.058
7.36	0.802 ± 0.005	−0.680	0.833	−0.031

* Calculated using log P and pK_a fit to eqn. 1b.

This communication helps to establish that our procedure is applicable to lipophilic acids and ion-pair partitioning by confirming that, under these conditions, reversed-phase HPLC partitioning compares excellently with bulk phase distribution coefficients. Furthermore, anions of lipophilic acids can be considerably more lipophilic than previously thought[2]. This observation may have significant impact on various schemes to calculate partition coefficients used in QSAR studies[2,9].

ACKNOWLEDGEMENTS

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REFERENCES

1 S. H. Unger, J. R. Cook and J. S. Hollenberg, *J. Pharm. Sci.*, 67 (1978) 1364.
2 A. Leo, C. Hansch and D. Elkins, *Chem. Rev.*, 71 (1971) 525.
3 K.-G. Wahlund and I. Beijersten, *J. Chromatogr.*, 149 (1978) 313.
4 J. R. Pfister, R. W. Ferraresi, I. T. Harrison, W. H. Rooks, A. P. Roszkowski and A. Van Horn, *J. Med. Chem.*, 15 (1972) 1032.
5 J. Ackrell, Y. Antonio, F. Franco, R. Landeros, A. Leon, J. M. Muchowski, M. L. Maddox, P. H. Nelson, W. H. Rooks, A. P. Roszkowski and M. B. Wallach, *J. Med. Chem.*, 21 (1978) 1035.
6 Cs. Horváth, W. Melánder and I. Molnár, *Anal. Chem.*, 49 (1977) 142.
7 S. H. Unger, *Presented at Environmental Protection Agency Seminar on Structure–Activity Relations, Annapolis, Md., November 8–9, 1978.*
8 S. H. Unger, *Drug Design*, Vol. 9, Ch. 1, in press.
9 A. Leo, P. Y. C. Jow, C. Silipo and C. Hansch, *J. Med. Chem.*, 18 (1975) 865.

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Note

Use of 3-nitro-L-tyrosine as an internal standard for the lithium buffer, single-column amino acid analysis of complex amino acid mixtures

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Internal standards are used during amino acid analysis to monitor losses due to sample preparation and handling and to monitor variations due to chromatography column differences and changes[1]. The infrequent analysis of protein hydrolysates on a system dedicated to the analysis of physiological fluids requires an internal standard which is stable to acid hydrolysis. Although norleucine is most frequently used as an acid-hydrolysis-stable internal standard on both two-column and single-column sodium buffer systems[1–3] and on two-column lithium buffer systems[4], it cannot be used on some single-column lithium buffer systems in that it coelutes with tyrosine[5]. α-Amino-β-guanidinopropionic acid has been recommended as an internal standard for a single-column lithium buffer system[5], but it is not stable to acid hydrolysis[6].

3-Nitrotyrosine has been reported to be suitable as an acid-hydrolysis-stable internal standard on two-column, sodium buffer systems[7]. This communication deals with the suitability of 3-nitro-L-tyrosine as an internal standard for the infrequent analysis of protein hydrolysates on a single-column lithium buffer system.

EXPERIMENTAL

Analyses were conducted on a Beckman (Fullerton, Calif., U.S.A.) 119 CL amino acid analyzer utilizing a 22 × 0.6 cm bed of AA-10 resin and the lithium buffer system specified by the manufacturer[5].

3-Nitro-L-tyrosine was from Sigma (St. Louis, Mo., U.S.A.), ε-aminocaproic acid was from Aldrich (Milwaukee, Wisc., U.S.A.) and S-β-(4-pyridylethyl)-L-cysteine was from Pierce (Rockford, Ill., U.S.A.). A standard mixture containing nitrotyrosine was obtained by mixing 0.50 ml of the Beckman protein hydrolysate amino acid calibration standard (No. 312220), 2.50 ml of the Pierce physiological basic amino acid standard (No. 20076), 1.25 ml of 1.0 m*M* nitrotyrosine in 10 m*M* HCl, and 0.75ml of the Beckman lithium citrate dilution buffer (No. 339594) to give a final concentration of 0.25 μ*M* for each amino acid. Aliquots of 100 μl volume applied to the column gave peaks for 25.0 nmoles of each amino acid.

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RESULTS AND DISCUSSION

ε-Aminocaproic acid has been reported to be suitable as an acid-hydrolysis-stable internal standard[8]. It eluted just before lysine on the basic column of a Beckman 120C two-column amino acid analyzer[8]. However, we have found here that ε-aminocaproic acid coelutes with histidine on the Beckman 119 CL single-column lithium buffer system.

S-β-(4-Pyridylethyl)-L-cysteine has also been reported to be suitable as an acid-hydrolysis-stable internal standard[9]. It eluted just before arginine on the basic column of a Beckman Spinco 120 two-column amino acid analyzer[9]. However, we have found here that S-β-(4-pyridylethyl)-L-cysteine elutes after arginine on the Beckman 119 CL single-column lithium buffer system. This has also been found to be the situation on the long basic (physiological) column of a two-column, sodium buffer amino acid analysis system[10]. Extension of the time of analysis, therefore, makes S-β-(4-pyridylethyl)-L-cysteine undesirable as an internal standard.

S-β-(4-Pyridylethyl)-D,L-penicillamine has been reported as an acid-hydrolysis-stable internal standard but also suffers from the disadvantage of late elution causing an extension of the time of analysis on both the short basic and the long basic (physiological) columns of a two-column sodium buffer amino acid analysis system[10].

In view of the problems involving the elution positions of these internal standards, the suitability of nitrotyrosine[7] was investigated. Whereas it eluted after phenylalanine on a Spinco 120 two-column sodium buffer amino acid analyzer system[7], we found that it elutes just before phenylalanine on the Beckman 119 CL single-column lithium buffer system as shown in Fig. 1. 3-Nitrotyrosine eluted sharply with a peak height-to-valley relationship to phenylalanine which indicated a resolution greater than that of the isoleucine–leucine doublet. The use of 3-nitro-

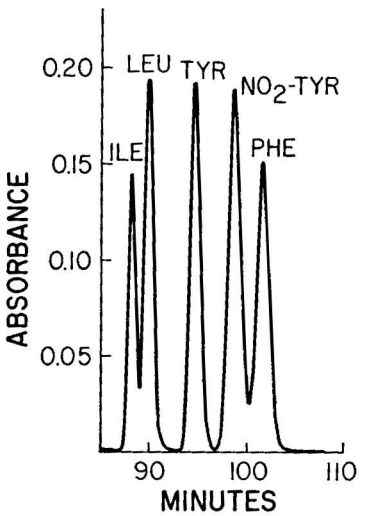

Fig. 1. A typical elution pattern of an amino acid standard mixture containing 3-nitrotyrosine. The portion of the chromatogram prior to the elution of isoleucine and after the elution of phenylalanine has been omitted to facilitate presentation. The buffers for development of the column and the timing sequence were as specified[5]. Each peak represents 25 nmoles.

tyrosine as an internal standard on the Beckman 119 CL single-column lithium buffer system enables the convenient analysis of infrequent protein acid hydrolysates on a system dedicated to the analysis of physiological fluids.

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REFERENCES

1 J. F. Riordan and R. W. Giese, *Methods Enzymol.*, 47 (1977) 31.
2 K. A. Walsh and J. R. Brown, *Biochim. Biophys. Acta*, 58 (1962) 596.
3 *118/119 CL Application Note 001*, Beckman, Fullerton, Calif., April 1977.
4 J. V. Benson, M. J. Gordon and J. A. Patterson, *Anal. Biochem.*, 18 (1967) 228.
5 *118/119 CL Application Note 002*, Beckman, Fullerton, Calif., April 1977.
6 M. J. Crumpton and J. M. Wilkinson, *Biochem. J.*, 88 (1963) 228.
7 R. W. Giese and J. F. Riordan, *Anal. Biochem.*, 64 (1975) 588.
8 L. S. Bates, *Anal. Biochem.*, 41 (1971) 158.
9 J. F. Cavins and M. Friedman, *Anal. Biochem.*, 35 (1970) 489.
10 M. Friedman, A. T. Noma and M. S. Masri, *Anal. Biochem.*, 51 (1973) 280.

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Note

Use of malonamide as a general spray reagent for the fluorimetric detection of reducing sugars on filter papers and thin-layer plates

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There are a number of reagents for fluorimetric determination of carbohydrates. However, most of them fluoresce only under strongly acidic conditions, so they are not suitable for paper chromatographic detection of carbohydrates. Recently we noticed that some aliphatic amines, especially ethylenediamine, were intensely fluorescent under weakly alkaline conditions, and devised a simple procedure for the detection of reducing sugars with this reagent[1]. This procedure allows the detection of as little as 0.5 nmole of reducing sugars, being more sensitive than ordinary colorimetric detection[2–5]. In continuation of the study of fluorimetric analysis of carbohydrates, we found that the malonamide is also usable as a fluorogenic reagent for reducing sugars.

The fluorescence produced by malonamide with reducing sugars shows both excitation and emission maxima at 328–382 and 383–425 nm, respectively. Fluorescence at the longer wavelength is several times as intense as that at the shorter wavelength, and is visible in the greenish-blue region.

Table I gives the lower limits of detection for individual carbohydrates. In this detection, carbohydrate samples were spotted in small circles (diameter, 5 mm) on a sheet of filter paper or a thin-layer plate, which was then sprayed with a 1% solution of malonamide in 1 *M* carbonate buffer (pH 9.2), heated for 5 min (filter paper) or 20 min (thin-layer plate) in an oven at 120°, and irradiated by a mercury lamp which emitted the 365 nm light most abundantly. It is indicated that all the aldoses, ketoses, amino sugars, uronic acids, and reducing disaccharides could be detected at levels as low as 0.25 nmole. The lower limit of detection for 2-deoxy-D-glucose was slightly higher (0.5 nmole). Aldonic acids, alditols, glycosides, non-reducing oligosaccharides, and polysaccharides were far more insensitive. When the spots of carbohydrate samples on a sheet of filter paper were detected after chromatographic development with an appropriate solvent system, such as *n*-butanol–acetic acid–water (4:1:5) (upper layer) or *n*-butanol–pyridine–water (6:4:3), the lower limit of detection rose to the nmole level due to diffusion of samples. Thin-layer chromatographic development did not cause blurring of the spots.

The fluorescence reaction of malonamide is highly selective; ordinary alcohols,

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TABLE I

LIMITS OF DETECTION OF CARBOHYDRATES

Carbohydrate	*Limit of detection (nmole)*	*Carbohydrate*	*Limit of detection (nmole)*
DL-Glyceraldehyde	0.25	Glycolic acid	1000
L-Arabinose	0.25	D-Gluconic acid	1000
D-Lyxose	0.25	Erythritol	10
D-Ribose	0.25	D-Glucitol	10
D-Xylose	0.25	Methyl α-D-glucoside	100
D-Galactose	0.25	Adenosine	1000
D-Glucose	0.25	Trehalose	200
D-Mannose	0.25	Sucrose	200
L-Fucose	0.25	Raffinose	200
L-Rhamnose	0.25	Maltose	0.25
D-Fructose	0.25	Cellobiose	0.25
L-Sorbose	0.25	Lactose	0.25
D-Galactosamine · HCl	0.25	Melibiose	0.25
D-Glucosamine · HCl	0.25	Starch	20
N-Acetyl-D-glucosamine	0.25	Glycogen	1000
D-Galacturonic acid	0.25	Dextran	1000
D-Glucuronic acid	0.25	Pullulan	20
2-Deoxy-D-glucose	0.5		

phenols, aldehydes, ketones, carboxylic acids, including amino acids, esters, amides, nitro compounds, sulfonic acids, thiols, and sulfoxides, did not show fluorescence at these wavelengths.

REFERENCES

1 S. Honda, K. Kakimoto, K. Kakehi and K. Takiura, *Anal. Chim. Acta*, 64 (1973) 310.
2 W. E. Trevelyan, D. P. Procter and J. S. Harrison, *Nature (London)*, 166 (1950) 520.
3 E. Pascu, T. P. Mora and P. W. Kent, *Science*, 110 (1949) 446.
4 L. Hough, J. K. N. Jones and W. H. Wadman, *J. Chem. Soc., London*, (1950) 1702.
5 S. M. Partridge, *Nature (London)*, 164 (1949) 443.

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CHROM. 11,963

Note

Optical brighteners as thin-layer chromatography detection reagents for glycoalkaloids and steroid alkaloids in *Solanum* species

I. Calcofluor® M2R New*

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In the development of a thin-layer chromatographic screening method for the glycoalkaloids and their aglycones, the steroid alkaloids, in *Solanum* species, a sensitive and specific detection reagent is required. As most of the detection reagents for these substances, reported elsewhere[1–4], did not fulfill our requirements regarding sensitivity and specificity, we investigated the use of an optical brightener, Calcofluor® M2R New.

Optical brighteners are used in mycology as microscopic reagents for visualizing cell walls[5]. To test the specificity of Calcofluor, we applied the reagent to substances related to the steroid (glyco)alkaloids, such as alkaloids, glycosides, and saponins. Also, the effect of the optical brightener on glycoalkaloids and steroid alkaloids was compared with the effect of some other reagents. Additionally, we determined the minimal detectable quantity (MDQ) of a few steroid (glyco)alkaloids.

EXPERIMENTAL

All thin-layer chromatography (TLC) was carried out on pre-coated silica gel plates, 250 μm layer thickness (Merck, Darmstadt, G.F.R.). The detection reagents are listed in Table I.

For the determination of the response of Calcofluor M2R New, 5.7 nmol (equivalent to 5 μg solanine) of each of the substances listed in Table II, dissolved in the appropriate solvent, was spotted on a plate. The plate was dipped in reagent 4 and immediately observed under long-wave (365 nm) UV light.

Because only a few steroid (glyco)alkaloids were at out disposal, several *Solanum* species (Table III) were extracted. Part of the glycoside mixtures were hydrolyzed. Of the extracted glycoalkaloids, 5 μl of a 0.1% methanolic solution and 5 μl of a 0.1% chloroform solution of the steroid alkaloids, obtained by hydrolysis, were spotted.

For the glycoalkaloids the plates were developed in *n*-butanol–formic acid–

* Trademark of the American Cyanamid Company.

TABLE I

DETECTION REAGENTS APPLIED TO STEROID (GLYCO)ALKALOIDS, SAPONINS AND RELATED SUBSTANCES

No.	*Reagent*	*Application*	*Heating*	*Substances*	*Colours*			
					Daylight		*Long-wave UV (365 nm)*	
					Spots	*Background*	*Spots*	*Background*
1	5% Sulphuric acid in diethyl ether[6]	Dip	5 min, 100°	Steroid alkaloids, glycoalkaloids	Rose-grey	White	Light blue	Dark pink
2	Antimony trichloride–glacial acetic acid (1:1)[7]	Spray	5 min, 100°	Steroid alkaloids, glycoalkaloids	Rose	White	Orange-yellow	Pink
3	Dragendorff reagent[8]	Dip	–	Steroid alkaloids, glycoalkaloids	Orange-red	Orange-yellow	–	–
4	Calcofluor® M2R New, 0.02% – methanol	Dip	–	See Table II	–	–	Light blue	Blue
5	Anisaldehyde*	Spray	5 min, 100°	Saponins	Various	White	–	–
6	Blood gelatine[9]	Pour	–	Saponins	White	Red	–	–

* Anisaldehyde reagent: Dissolve 0.5 ml anisaldehyde in 80 ml of methanol and 10 ml of glacial acetic acid, then add 5 ml of conc. sulphuric acid.

TABLE II

RESPONSE OF GLYCOALKALOIDS, STEROID ALKALOIDS AND RELATED SUBSTANCES TO CALCOFLUOR® M2R NEW

All the responses are compared with the very weak fluorescence of 5-μl spots of water and of chloroform–methanol (1:1). − = No response; ± = weak; + = positive; ++ = strong.

Compound	*Response*	*Compound*	*Response*	*Compound*	*Response*
Alkaloids		*Saccharides*		*Glycosides*	
Aconitine	−	Galactose	−	Aesculin	−
Atropine	−	Glucose	−	Aloin**	−
Lobeline	−	Rhamnose	−	Apigenin	
Morphine	−	Lactose	−	Monoglucoside	−
Nicotine	−	Mannose	−	Arbutin	−
Pilocarpine	−	Saccharose	−	Digitoxin	±
Quinine*	−	*Steroid alkaloids*		Frangulin**	−
Reserpine*	−	Solanidine	+	Gitoxin	±
Steroidsapogenins		Solasodine	+	Hesperidin	−
Diosgenin	−	Demissidine	+	Rutin	−
Tigogenin	−	Tomatidine	+	Salicin	−
Saponins		*Glycoalkaloids*		Sennoside B**	−
Aescin	+	Solanine	++		
Saponinum purum	+	Tomatine	++		

* These substances already fluoresce before applying the optical brightener.
** The fluorescence of these substances disappears with the optical brightener.

water (4:1:5, upper layer) in a saturated tank over 15 cm. In the case of steroid alkaloids, the plates were also developed with *n*-hexane–acetone (1:1) in an unsaturated tank over 15 cm. After drying the plates, we applied reagents 1, 2, 3, or 4. We also extracted a few saponin-containing drugs (Table IV)[10]. Of these extracts 20 μl was spotted on a plate as a band, together with 10 μl bands of 0.1% solution of aescin and of saponinum purum in 70% alcohol. The plate was developed in an unsaturated chamber with *n*-butanol–acetic acid–water (5:1:4, upper layer) over 15 cm. After drying the plates, the spots were visualized with reagents 4, 5, or 6.

For the determination of the MDQ, we applied decreasing quantities, in steps of 0.01 μg, of solanine, tomatine, solanidine, and solasodine, on a plate. After development, the plate was dried and dipped in reagents 3 or 4.

TABLE III

SURVEYED *SOLANUM* SPECIES AND THEIR CONSTITUENTS AS CITED BY SCHREIBER[11]

Solanum species	*Glycoalkaloids*	*Steroid alkaloids*
S. acaule *S. demissum* *S. polyadenium*	Tomatine, demissine	Tomatidine, demissidine
S. chacoense *S. tuberosum* *S. vernei* *S. verrucosum*	Solanine, chaconine	Solanidine
S. dulcamara *S. triflorum*	Solasonine, solamargine	Solasodine

TABLE IV

SURVEYED SAPONIN-CONTAINING DRUGS

Saponin-containing drugs	*Parts used*	*Saponin type*
Phytolacca americana	Berries	Triterpene
Polygala senega	Roots	Triterpene
Smilax species	Roots	Steroid
Trigonella foenumgraecum	Seeds	Steroid

RESULTS AND DISCUSSION

The responses of the substances to Calcofluor are listed in Table II. The two glycoalkaloids gave a distinct fluorescence, the steroid alkaloids and a few saponins had a fluorescence of lower intensity. The cardiac glycosides, digitoxin and gitoxin, gave a weak fluorescence. All the other compounds listed in Table II gave spots having weak or no fluorescence. The fluorescence of the background changed in time from blue to yellow-green and back to blue.

The chromatograms of the saponin-containing drugs treated with reagents 4, 5, or 6 showed some similarity. When observed immediately after dipping in Calcofluor reagent, the chromatograms of aescin, saponinum purum and *Trigonella foenumgraecum* each showed one band. After 5–10 min more bands became visible, but had a much lower intensity and were of different colours. The bands in the chromatogram of *Smilax* root that fluoresced with Calcofluor gave hemolysis with blood gelatine, as did the bands of aescin, saponinum purum, and *Trigonella foenumgraecum*.

Chromatograms of the glycoalkaloids and the steroid alkaloids from the *Solanum* species gave the same spots with Calcofluor reagent as with Dragendorff reagent. Sulphuric acid and antimony trichloride gave more spots, but these reagents are less specific for steroid (glyco)alkaloids and do not have the same sensitivity for all steroid (glyco)alkaloids.

The MDQ of the steroid (glyco)alkaloids (see Table V) with Calcofluor is at least 10 times less than with Dragendorff reagent.

The results show that Calcofluor is more specific for steroid (glyco)alkaloids than Dragendorff reagent, sulphuric acid, or antimony trichloride. Calcofluor is also more sensitive than Dragendorff reagent.

TABLE V

MDQ OF GLYCOALKALOIDS AND STEROID ALKALOIDS WITH CALCOFLUOR® M2R NEW AND DRAGENDORFF REAGENT

	Calcofluor M2R New		*Dragendorff*	
	$\mu g \cdot 10^{-2}$	$\mu mol \cdot 10^{-5}$	$\mu g \cdot 10^{-2}$	$\mu mol \cdot 10^{-5}$
Solanine	2	2.3	20	23
Tomatine	4	4.0	50	50
Solanidine	5	12.8	230	580
Solasodine	7	16.9	>230	>600

ACKNOWLEDGEMENT

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REFERENCES

1 I. R. Hunter, M. K. Walden, J. R. Wagner and E. Heftmann, *J. Chromatogr.*, 118 (1976) 259.
2 E. G. C. Clarke, *Nature (London)*, 181 (1958) 1152.
3 R. Neher, in E. Stahl (Editor), *Dünnschichtchromatographie*, Springer, Berlin, Heidelberg, New York, 2nd ed., 1967, pp. 313 and 337.
4 F. Šantavý, in E. Stahl (Editor), *Dünnschichtchromatographie*, Springer, Berlin, Heidelberg, New York, 2nd ed., 1967, p. 349.
5 I. F. Preece, in C. Booth (Editor), *Methods in Microbiology*, Academic Press, New York, London, 1971, Vol. 4, p. 509.
6 K. Röder, E. Eich and E. Mutschler, *Pharm. Ztg.*, 40 (1970) 1430.
7 *Anfarbereagenzien für Dünnschicht- und Papierchromatographie*, E. Merck A.G., Darmstadt, p. 4, No. 15.
8 K. G. Krebs, D. Heusser and H. Winner, in E. Stahl (Editor), *Dünnschichtchromatographie*, Springer, Berlin, Heidelberg, New York, 2nd ed., 1967, p. 829, No. 90.
9 E. Stahl and H. Jork, in E. Stahl (Editor), *Dünnschichtchromatographie*, Springer, Berlin, Heidelberg, New York, 2nd ed., 1967, p. 242.
10 E. Stahl, *Chromatographische und Mikroskopische Analyse von Drogen*, Gustav Fischer, Stuttgart, 1970, p. 100.
11 K. Schreiber, in R. Manske (Editor), *The Alkaloids*, Vol. 10, Academic Press, New York, London, 1968, p. 1.

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Note

Formation of diastereoisomeric derivatives from the enantiomers of the antitumour agent cyclophosphamide by reaction with 1-phenethyl alcohol, and their separation by thin-layer chromatography

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During an investigation into the metabolism of the enantiomers[1] of the antitumour agent cyclophosphamide {2-bis(2-chloroethyl)amino-2*H*-1,3,2-oxazaphosphorine-2-oxide, I} a method was required for monitoring the enantiomeric composition of cyclophosphamide recovered from the urine of patients given either the racemate or an enantiomer. Separable diastereoisomeric derivatives of cyclophosphamide have been prepared previously from the racemate by derivatisation at N-3 of the oxazaphosphorine ring using (−)-(*S*)-α-naphthylphenylmethylsilyl chloride[2]. The present procedure depends on the stereospecific cleavage of one of the P–N linkages by a chiral alcohol, 1-phenethyl alcohol, to form diastereoisomeric derivatives in which the chiral centres are separated likewise by only one heteroatom.

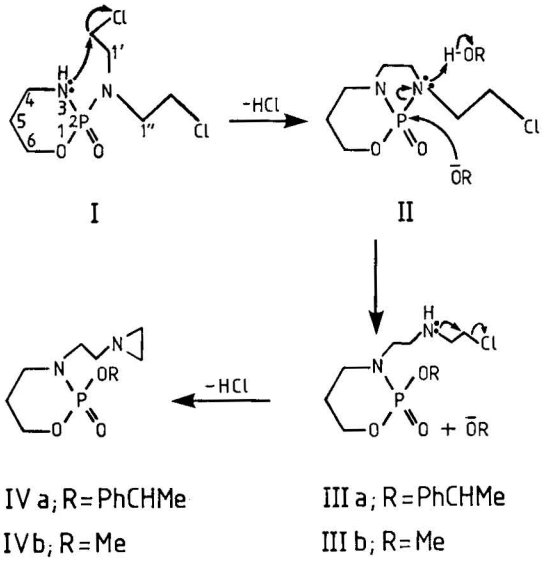
Treatment of cyclophosphamide with sodium hydride under anhydrous conditions affords a bicyclic product (II) containing a strained P–N bridge bond[3]. In the presence of a suitable nucleophile, release of strain by cleavage of one of the P–N linkages would be anticipated. Accordingly, when cyclophosphamide (racemate or enantiomers) was allowed to react with the sodium salts of (+)- or (−)-1-phenethyl alcohol evidence for the reaction sequence I–IVa (Fig. 1) was obtained.

EXPERIMENTAL

Racemic cyclophosphamide was purchased from Koch-Light Labs. (Colnbrook, Great Britain) and (+)- and (−)-1-phenethyl alcohol ($[\alpha]^{20}$ +39.57° and −42.95°, respectively) from Norse Labs., Los Angeles, Calif., U.S.A. (+)-(*R*)- and (−)-(*S*)-cyclophosphamide were prepared by a published procedure[1].

Chromatography

Thin-layer chromatography was carried out on glass plates (5 × 20 cm) coated with a 0.25-mm layer of Kieselgel G. Separated components were visualized by exposure to iodine vapour or by spraying with 1% ethanolic 4-(4-nitrobenzyl)-



IV a; R = PhCHMe III a; R = PhCHMe

IV b; R = Me III b; R = Me

Fig. 1. Scheme for the reaction of cyclophosphamide with (a) 1-phenethyl alcohol and (b) methanol.

pyridine[4]; heating at 150° for 30 min then spraying with 1% ethanolic potassium hydroxide solution (blue spots on white ground).

Reaction of cyclophosphamide in (a) phenethyl alcohol and (b) methanol

(a) To a solution (0.1 ml) prepared by dissolving sodium (46 mg) during 2 h in a mixture of benzene and (—)-1-phenethylalcohol (0.5 ml each) was added cyclophosphamide (racemate or either enantiomer; 1 mg). The progress of the reaction was monitored by thin-layer chromatography in chloroform–ethanol (9:1).

(b) A solution of cyclophosphamide (racemate, 10 mg) in methanolic sodium methoxide (0.5 *M*, 1 ml) was heated under reflux. The reaction was followed by thin-layer chromatography in chloroform–methanol (9:1).

Mass spectrometry

The products were eluted from thin-layer chromatograms using methanol. The concentrated eluates were applied to the direct insertion probe. Mass spectra were determined using an AEI-MS 12 spectrometer at an ionizing voltage of 70 or 12 eV, a trap current of 100 μA and an ion-source temperature of 90–110°.

RESULTS AND DISCUSSION

The reaction of cyclophosphamide in phenethyl alcohol was complete within 15 min. The racemate afforded two products, R_F 0.28 and 0.33 which gave very similar mass spectra consistent with diastereoisomeric structures IVa. Evidence that the principal ion *m/e* 150 (Fig. 2) was formed by loss both of the phenethyl and aziridinomethyl moieties was obtained by carrying out the same reactions with two (racemic) tetradeuterated analogues of cyclophosphamide. The products given by a

ring (4,6-d_4) deuterated analogue[5] gave a base peak at *m/e* 154 (molecular ion $M^{+\cdot}$ at *m/e* 314) whereas the base peak from the side-chain (1′, 1″)-labelled congener[6] was again at *m/e* 150, showing that this ion retains the oxazaphosphorine ring, but not the methylene groups originally adjacent to the exocyclic N-atom in the cyclophosphamide molecule.

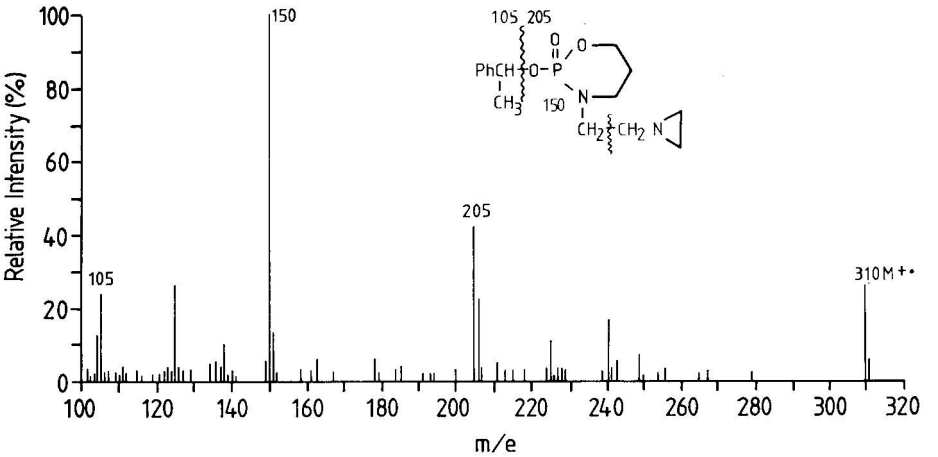


Fig. 2. Mass spectrum of the product of R_F 0.33 in chloroform–ethanol (9:1) formed from (−)-phenethyl alcohol and racemic cyclophosphamide. This spectrum was recorded at an ionizing voltage of 12 eV to maximize the relative intensity of the molecular ion: at 70 eV this fell to 7.3%.

Each enantiomer of cyclophosphamide, when treated as above with (−)-phenethyl alcohol afforded one product only; the (+)-(*R*)-form gave the diastereoisomer of IVa having the higher R_F value. The situation was reversed when the reaction was conducted using (+)-phenethyl alcohol. Hence the step (II → IIIa) in which a bond to the chiral phosphorus atom is cleaved, occurs without detectable racemization. The question of whether complete inversion, or retention of configuration is responsible for the optical purity of the products from the cyclophosphamide enantiomers remain open, although inversion seems more likely, on the basis of mechanisms which have been proposed for the alkaline hydrolysis of phosphoramidates[7].

The putative intermediates (II and IIIa) were not detected, even on chromatograms developed after reaction for only 30 sec, at which time cyclophosphamide was still the major component. However an analogue (IIIb) of one such intermediate was isolated during the slower reaction between cyclophosphamide and methanolic sodium methoxide, which failed to react during 24 h at room temperature, but which afforded after 15 min heating under reflux two principal products of R_F 0.18 and 0.26, having mass spectra (70 eV) corresponding to structures IIIb and IVb respectively. Thus, the former gave a molecular ion at *m/e* 256 (6.7% relative intensity) with the accompanying ^{37}Cl-containing satellite at *m/e* 258, and a base peak at *m/e* 164 that can be ascribed to the ion $[M - CH_2NHCH_2CH_2Cl]^+$. The latter product also gave the base peak ion at *m/e* 164, and the molecular ion at *m/e* 220 (6.9%

relative intensity) appropriate to IVb. The intermediate IIIb was completely converted into IVb after 2 h reaction.

Neither the products (IVa) from 1-phenethyl alcohol nor the product IVb from methanol was isolable in bulk. Attempts at column chromatography led to decomposition, presumably owing to the lability of the aziridino moiety.

The reaction of cyclophosphamide with the optical isomers of 1-phenethyl alcohol affords a simple method of monitoring enantiomeric homogeneity. Thus when the above-described reaction was carried out using either enantiomer of cyclophosphamide containing 5% (w/w) of its antipode (total 1 mg) the minor component was easily detected by exposure to iodine vapour or by the Epstein test following thin-layer chromatography of one-tenth of the reaction mixture. The same fraction of a reaction mixture from 100 μg of racemic cyclophosphamide also afforded readily detectable bands, showing that the method could give at least a qualitative indication of enantiomeric composition (and hence of any stereoselectivity in metabolism) using a quantity of cyclophosphamide which would be easily recoverable[6] from a few millilitres of urine from patients given a conventional therapeutic dose of the racemate.

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REFERENCES

1 R. Kinas, K. Pankiewicz and W. J. Stec, *Bull. Acad. Pol. Sci. Ser. Sci. Chim.*, 12 (1975) 981.
2 T. Kawashima, R. D. Kroshefsky, R. A. Kok and J. G. Verkade, *J. Org. Chem.*, 43 (1978) 1111.
3 G. Zon, S. M. Ludeman and W. Egan, *J. Amer. Chem. Soc.*, 99 (1977) 5785.
4 J. Epstein, R. W. Rosenthal and R. J. Ess, *Anal. Chem.*, 27 (1955) 1435.
5 M. Jarman, E. D. Gilby, A. B. Foster and M. Jarman, *Clin. Chim. Acta*, 58 (1975) 61.
6 L. J. Griggs and M. Jarman, *J. Med. Chem.*, 18 (1975) 1102.
7 C. Brown, J. A. Boudreau, B. Hewitson and R. F. Hudson, *J. Chem. Soc., Chem. Commun.*, (1975) 504.

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Note

Simple and sensitive technique for detecting trace flavour components in beer

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Many flavour components in foodstuffs are sensorily detectable at the sub-ppb* level. However, at present, no simple mild technique exists for detecting these trace components.

A method has been developed which is particularly applicable to the gas chromatographic detection of trace volatile flavour compounds in beer. Beer was vacuum-steam distilled at 25° and 0.02 mmHg pressure, and the distillate was passed through Amberlite XAD-2 porous polymer beads. The resin was eluted with diethyl ether and the extract analysed by gas–liquid chromatography (GLC). Using this technique, sulphur-containing compounds are detectable with a flame photometric detector at the sub-ppb level, and with a flame ionisation detector compounds which do not contain sulphur, are detectable at the ppm level.

EXPERIMENTAL

Instruments

A Pye GCV gas chromatograph, equipped with a linear temperature programmer and synchronous flame ionisation and flame photometric detection (394 nm filter), was used. The GLC column was 2.8 m × 4 mm I.D. glass packed with 10% Carbowax 20M on Chromosorb W AW DMCS (80–100 mesh).

The column was operated with a nitrogen carrier gas flow-rate of 52 ml/min and a temperature programme of 50–200° at 3°/min.

GLC–mass spectrometry (MS) was carried out with the Carbowax 20M column linked to an MS-12 mass spectrometer (Associated Electrical Industries, Manchester, Great Britain) using a membrane separator. Helium was used as a carrier gas and mass spectra were measured with an ionisation energy of 70 eV.

Reagents

Amberlite XAD-2 resin[1–5] (BDH, Poole, Great Britain) was purified by washing sequentially with water, methanol, and diethyl ether just before use, using a procedure similar to that described by Van Rossum and Webb[1].

* Throughout this article, the American billion (10^9) is meant.

Diethyl ether was AnalaR grade (BDH) and was used without further purification.

Sulphur-containing flavour compounds were prepared by known methods[6–8] and were homogeneous according to GLC analysis.

Non-sulphur-containing flavour compounds were commercial samples and were used without further purification.

Nitrogen was OFN grade (oxygen free), (British Oxygen, Wembley, Great Britain) and was dried by passage through calcium chloride.

Antifoam was a solution of 10% silicone DC antifoam RD emulsion (Hopkin & Williams, Chadwick Heath, Great Britain) in water.

No interfering sulphur compounds were detected in any of the reagents described above.

Experimental procedure

Beer (2 l) containing antifoam solution (5 ml) is vacuum-steam distilled at 25° and 0.02 mmHg pressure, using the method of Pickett *et al.*[9]. For a period of 5 h, the vacuum distillate is passed through a column of Amberlite XAD-2 beads (60 mm × 7 mm, 2 g dry weight), which is then washed with deionised water (100 ml). To extract flavour components from the resin beads, they are then eluted with diethyl ether by successive equilibration with five portions of ether (total volume 10 ml) for a period of 10 min each. The combined extract is separated from excess water, dried over sodium sulphate (BDH) and reduced to a volume of 10 μl with a slow stream of nitrogen. The extract (5 μl) is analysed by GLC.

Compounds were identified by comparison of the MS and GLC retention times with those of standard samples.

RESULTS AND DISCUSSION

Beer is a complex mixture of flavour compounds[10], many of which are sensitive to both heat and traces of oxygen. Therefore any technique for extracting the volatile flavour components should be carried out at a temperature below 25° with minimal contact with air. This note describes an extraction method fulfilling these requirements.

Vacuum distillation at 25° effects a separation of volatile flavour components from involatile material which interferes with GLC analysis. Flavour components are then separated from water, ethanol, and most of the lower fusel alcohols present in the vacuum distillate by passage through a column of Amberlite XAD-2 beads. The colourless extract, prepared by extracting the beads with diethyl ether, is suitable for direct analysis by GLC.

In work concerned with flavour chemistry it is important to be able to detect compounds in complex mixtures at concentrations at or below their flavour threshold levels, so as to determine whether the compounds contribute individually to the overall flavour. Several sulphur-containing compounds are easily detected in beer at their flavour threshold levels, in the ppb range, by analysing the extracts prepared as described above using a flame photometric detector. These compounds and their flavour thresholds are shown in Table I. In contrast to the present technique, a headspace sampling procedure[14] does not detect dimethyl trisulphide below 100 ppb, *i.e.* 1000× its flavour threshold concentration.

TABLE I

SULPHUR COMPOUNDS DETECTED IN BEER

Compound	*Flavour threshold (ppb)*
Dimethyl trisulphide[11]	0.1
S-Methyl hexanethioate[12]	1
S-Methyl 4-methylpentanethioate[13]	15
4-(4-Methylpent-3-enyl)-3,6-dihydro-1,2-dithiine[13]	10

A typical gas chromatogram (obtained using a flame photometric detector), showing a profile of the sulphur compounds present in an extract of beer previously treated with dry hops, is given in Fig. 1a.

The method is also capable of detecting many non-sulphur-containing compounds present in beer at the ppm level. A typical gas chromatogram (obtained using a flame ionisation detector) of an extract of a commercial beer is shown in Fig. 1b, together with the identities of a number of the principal components. Decanoic acid, shown to be present in a commercial beer at a level of 1.7 ppm by the method of Taylor and Kirsop[15], was easily detected using the XAD-2 technique. Table II shows the extents to which several beer flavour components were removed, by XAD-2 resin, from a vacuum-steam distillate. It can be seen that whilst the two fatty acids were efficiently adsorbed by the resin, β-phenylethanol was only very poorly adsorbed. Therefore, in accord with the literature, the method appears to be most suited for

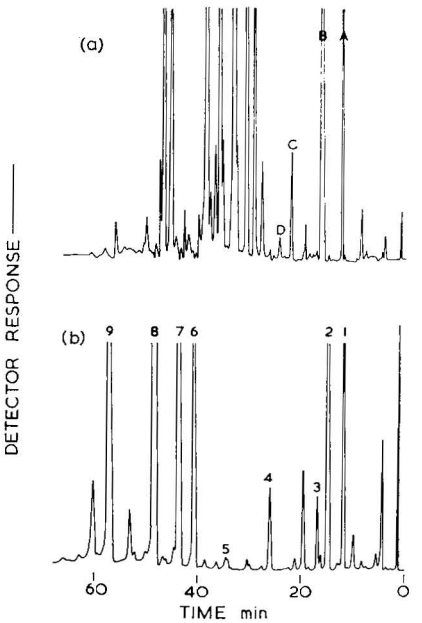

Fig. 1. Gas chromatography of beer extracts. Detection: (a) flame photometric; (b) flame ionisation. Peaks: A = S-methyl 2-methylpropanethioate; B = S-methyl 2-methylbutanethioate; C = S-methyl 4-methylpentanethioate; D = S-methyl hexanethioate: 1 = isoamyl acetate; 2 = isoamyl alcohol; 3 = ethyl hexanoate; 4 = ethyl octanoate; 5 = ethyl decanoate; 6 = β-phenylethyl acetate + hexanoic acid; 7 = β-phenylethanol; 8 = octanoic acid; 9 = decanoic acid.

detecting compounds of a relatively hydrophobic nature. Further work is in progress to establish whether this method provides the basis of a quantitative procedure for estimating beer flavour components which do not contain sulphur.

TABLE II

REMOVAL OF BEER FLAVOUR COMPONENTS FROM A VACUUM STEAM DISTILLATE BY XAD-2 RESIN

Compound	*Concentration in beer vacuum steam distillate (ppm)*	*Removed by XAD-2 resin (%)*
Decanoic acid	1.2	97
Octanoic acid	3.6	89
β-Phenylethanol	19.4	9

CONCLUSION

The technique provides a simple and sensitive method for detecting and quantifying traces of flavour compounds, particularly those containing sulphur, in beer.

Further work is being carried out to improve the sensitivity of the technique. It is believed that this method could be used to detect and quantify sulphur compounds in a wide range of foodstuffs by emulsifying samples in water prior to the initial vacuum steam distillation step.

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REFERENCES

1 P. van Rossum and R. G. Webb, *J. Chromatogr.*, 150 (1978) 381.
2 A. Tateda and J. S. Fritz, *J. Chromatogr.*, 152 (1978) 329.
3 G. A. Junk, J. J. Richard, M. D. Grieser, D. Witiak, J. L. Witiak, M. D. Arguello, R. Vick, H. J. Svec, J. S. Fritz and G. V. Calder, *J. Chromatogr.*, 99 (1974) 745.
4 P. R. Musty and G. Nickless, *J. Chromatogr.*, 89 (1974) 185.
5 F. T. Delbeke and M. Debackere, *J. Chromatogr.*, 133 (1977) 214.
6 T. L. Peppard and J. A. Elvidge, *Chem. Ind. (London)*, in press.
7 F. W. Wenzel and E. E. Reid, *J. Amer. Chem. Soc.*, 59 (1937) 1089.
8 T. L. Peppard and F. R. Sharpe, *Phytochemistry*, 16 (12) (1977) 2020.
9 J. A. Pickett, J. Coates and F. R. Sharpe, *J. Inst. Brew., London*, 82 (1976) 228.
10 G. A. F. Harrison, *J. Inst. Brew., London*, 76 (1970) 486.
11 T. L. Peppard, *J. Inst. Brew., London*, 84 (1978) 337.
12 J. A. Pickett, J. Coates and F. R. Sharpe, *Eur. Brew. Conv., Proc. Congr.*, (1975) 123.
13 T. L. Peppard and D. R. J. Laws, *Eur. Brew. Conv., Proc. Congr.*, (1979) in press.
14 F. H. White and R. Parsons, *Eur. Brew. Conv., Proc. Congr.*, (1975) 721.
15 G. T. Taylor and B. H. Kirsop, *J. Inst. Brew., London*, 83 (1977) 97.

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Note

Determination of L-glutamine in preparations using high-performance liquid chromatography

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L-Glutamine is an important medicine which has been used for the treatment of peptic ulcers, especially in Japan[1]. The determination of L-glutamine has been carried out by means of, *e.g.*, ion-exchange chromatography[2–9], gas chromatography[10–13], fluorimetric methods[14,15], methods for measuring the amount of ammonia liberated by the actions of enzymes[16–21] or acids[22,23] on L-glutamine, and various other methods[24–27]. In this paper, the determination of L-glutamine in preparations using high-performance liquid chromatography is described.

EXPERIMENTAL

Reagents

0.02 *M* Potassium dihydrogen phosphate was prepared by weighing 2.72 g of potassium dihydrogen phosphate in sufficient water to make 1000 ml. 0.2 *M* Sodium hydroxide was prepared by weighing 0.8 g of sodium hydroxide in sufficient water to make 100 ml. Phosphate buffer solution was adjusted to pH 5.0 by mixing 0.02 *M* potassium dihydrogen phosphate and 0.2 *M* sodium hydroxide, filtered through a membrane filter (Toyo, TM-2, 0.45 μm; Toyo Roshi, Tokyo, Japan) and used as a mobile phase after degassing. L-Glutamine (Ajinomoto, Tokyo, Japan) was dried at 80° for 3 h, *ca.* 50 mg accurately weighed and dissolved in sufficient water to make exactly 50 ml, and used as a standard solution. An amount of sample, equivalent to 30 mg of L-glutamine, was weighed accurately in a centrifuge tube with a stopper, 30 ml of water was added, it was shaken for 10 min, filtered through Toyo No. 5 filter paper 10 ml of first filtrate was removed, 5 ml of following clear filtrate was collected and used as a sample solution. All other reagents were commercially available products of analytical reagent grade.

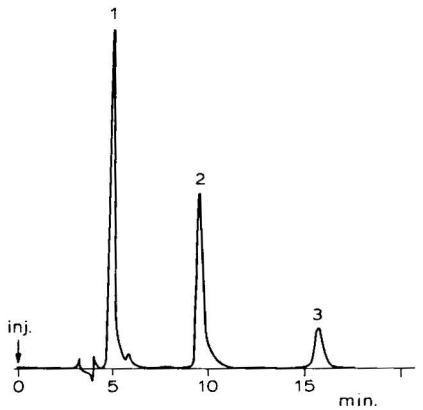
Apparatus and procedure

The chromatograph consisted of an Altex pump (Model 110-A; Berkeley, Calif., U.S.A.) operated at a flow-rate of 0.8 ml/min. The column effluent was monitored by a Shimazu UV variable-wavelength detector (Model 202; Kyoto, Japan) with a 20-μl flow-through cell. The detector was operated at 210 nm ($32 \cdot 10^{-2}$ a.u.f.s.). The column consisted of a stainless-steel tube (25 cm $\times$ 4.6 mm I.D.) packed

with Nucleosil 10 SB (Macherey, Nagel & Co., Düren, G.F.R.) by the balanced viscosity and dispersion slurry-packing technique. All experiments were carried out at room temperature. A 20-μl volume of standard or sample solution was injected accurately by using a syringe loading sample injector (Model 7120; Rheodyne, Berkeley, Calif., U.S.A.). The chromatogram was recorded at a chart speed of 5 mm/min. The L-glutamine content in the sample was calculated by peak height measurements relative to standard solutions.

RESULTS

Under these chromatographic conditions, L-glutamine was separated from nicotinic amide and calcium pantothenate; these have been prescribed frequently together (Fig. 1). L-Glutamine is not very stable in water, and decomposes forming pyrrolidonecarboxylic acid[28] or glutamic acid[29] by the action of moisture, and basic compounds, such as sodium bicarbonate, calcium carbonate, magnesium carbonate, magnesium oxide etc., in preparations stored for long periods of time. L-Glutamine was easily separated from these by-products (Fig. 2). The recovery of L-glutamine was 98.5% and the coefficient of variation was 0.98% for 5 analyses. These authors believe that this chromatographic method is very useful for the determination of L-glutamine in routine preparations, because the procedure is performed easily and rapidly.



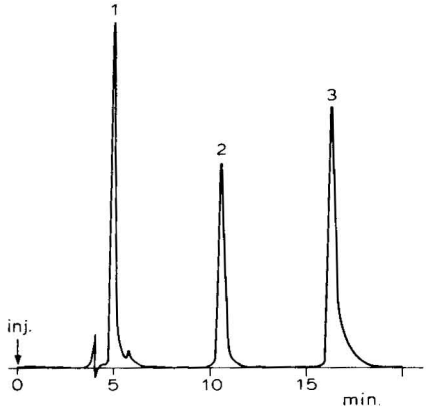


Fig. 1. Chromatogram of L-glutamine (1), nicotinic amide (2) and pantothenate (3).

Fig. 2. Chromatogram of L-glutamine (1), pyrrolidonecarboxylic acid (3) and glutamic acid (2).

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REFERENCES

1 Japan Pharmaceutical Information Center, in M. Horioka, T. Saito, K. Takagi, M. Takasugi and N. Motohashi (Supervisors), *Drugs in Japan, Ethical Drug Edition*, Yakugyo Jiho, Tokyo, 1975, p. 192.

2 I. Oreskes, F. Cantor and S. Kupfer, *Anal. Chem.*, 37 (1965) 1720.
3 H. Lorenz, *J. Chromatogr.*, 27 (1967) 267.
4 T. L. Perry and S. Hansen, *Clin. Chim. Acta*, 25 (1969) 53.
5 A. Vega and P. B. Nunn, *Anal. Biochem.*, 32 (1969) 446.
6 S. Tartarini, *J. Pharm. Sci.*, 61 (1972) 960.
7 J. L. Young and M. Yamamoto, *J. Chromatogr.*, 78 (1973) 349.
8 E. H. F. McGale and I. F. Pye, *J. Chromatogr.*, 89 (1974) 350.
9 P. T. Ozand, R. L. Hawkins, R. M. jun. Collins, J. T. Tildon and M. Cornblath, *Biochem. Med.*, 14 (1975) 170.
10 H. Hediger, R. L. Stevens, H. Brandenberger and K. Schmid, *Biochem. J.*, 133 (1973) 551.
11 J. M. L. Mee and B. Halpern, *Anal. Lett.*, 9 (1976) 605.
12 F. S. Collins and G. K. Summer, *J. Chromatogr.*, 145 (1978) 456.
13 P. Hušek and V. Felt, *J. Chromatogr.*, 152 (1978) 546.
14 S. R. Nahorski, *Anal. Biochem.*, 42 (1971) 136.
15 R. E. Miller, *Anal. Biochem.*, 75 (1976) 91.
16 F. L. Iber and J. Bruton, *Clin. Chem.*, 2 (1956) 413.
17 M. E. A. Ramadan and D. M. Greenberg, *Anal. Biochem.*, 6 (1963) 144.
18 H. G. Preuss, B. B. Bise and G. E. Schreiner, *Clin. Chem.*, 12 (1966) 329.
19 M. Kikuchi, *Anal. Biochem.*, 59 (1974) 83.
20 P. J. Buttery and E. V. Rowsell, *Anal. Biochem.*, 39 (1971) 297.
21 D. Coony and R. Davis, *Anal. Biochem.*, 40 (1971) 312.
22 T. P. Whitehead and S. R. F. Whittaker, *J. Clin. Pathol.*, 8 (1955) 81.
23 P. R. Henderlong and R. R. Schmidt, *Plant Physiol.*, 41 (1966) 1102.
24 E. M. Olsen, D. C. Hill and H. D. Branion, *Can. J. Biochem. Physiol.*, 40 (1962) 381.
25 E. B. Robertson, B. D. Sykes and H. B. Dunford, *Anal. Biochem.*, 9 (1964) 158.
26 H. Kluge, H. Hermann and V. Wieczorek, *Z. Klin. Chem.*, 5 (1967) 86.
27 P. van der Harst, J. D. Baars and A. J. P. F. Lombarts, *Clin. Chim. Acta.*, 50 (1974) 439.
28 H. B. Vickery, G. W. Pucher and H. E. Clark, *Biochem. J.*, 29 (1935) 2710.
29 E. Schulze and G. Trier, *Chem. Ber.*, 45 (1912) 257.

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Note

Identification of amino acid phenylthiohydantoins by gradient, high-performance liquid chromatography on Spherisorb S5-ODS

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A problem in the sequence determination of peptides and proteins by the Edman method is that of the identification of the liberated amino acids which are present as their phenylthiohydantoin derivatives (PTH-amino acids). This task has usually been solved by applying techniques such as gas and/or thin-layer chromatography, hydrolysis to the free amino acids, etc. Over the past few years an increasing number of publications have described the introduction and application of high-performance liquid chromatography (HPLC) in this field. The advantage of HPLC lies in the direct identification of the PTH-amino acids without further derivatization and in their quantitative estimation which is possible with modern equipment.

Current methods employ one- or two-column procedures with either gradient elution or isocratic development of the chromatogram[1–12]. We now report a method which allows the identification of the PTH-amino acids on a commercially available reversed-phase system in a single analysis with a gradient elution program.

MATERIALS AND METHODS

The gradient system was formed with the following solutions: A = 0.02 *M* lithiumacetate buffer, pH 5.2 (checked and adjusted at 25°), obtained by appropriate dilution of a 4 *M*, pH 5.2, stock solution (for amino acid analysis; Pierce, Rockford, Ill., U.S.A.); B = 80% acetonitrile (p.a.; E. Merck, Darmstadt, G.F.R.) and 20% 0.1 *M* lithium acetate buffer, pH 5.2. PTH-Amino acids were obtained from Fluka (Buchs, Switzerland), except for PTH-hydroxyproline which was from Sigma (St. Louis, Mo., U.S.A.). PTH-Carboxamidomethylcysteine was synthesized from carboxamidomethylcysteine by coupling with phenyl isothiocyanate according to conditions used in the manual Edman degradation[13].

Standard mixtures of PTH-amino acids were obtained by preparing 4 m*M* stock solutions of the individual derivatives in acetonitrile–water (50:50). Aliquots of the stock solution, containing 200 nmoles per PTH-amino acid, were mixed together in small vials, lyophilized and stored at 4°. Before use the samples were dissolved in 2 ml of the starting eluent (solution A containing 25% solution B). Usually 20 μl of this mixture (with 2 nmoles of each PTH-amino acid) were injected per analysis.

The column (stainless steel, 250 × 4.6 mm) was packed with Spherisorb S5-ODS (5-μm particles, bonded with octadecylsilane; Phase Separations, Queensferry, Great Britain) by the balanced-density slurry-packing method.

The liquid chromatograph was a Hewlett-Packard Model 1084 A apparatus equipped with two heatable solvent reservoirs, two independently operated pumps for gradient formation which allowed flow-rates from 0.1 to 10 ml min^{-1}, a heatable column compartment, a detector operating at a fixed wavelength of 254 nm and an automated injection mechanism. A built-in microcomputer connected to a Hewlett-Packard 79850 A Terminal allowed the programming of the liquid chromatograph as well as the registration of the chromatograms and their quantitative evaluation.

RESULTS AND DISCUSSION

A chromatogram of a typical separation of the individual PTH-amino acids of a standard mixture (see Table I) is shown in Fig. 1a (PTH-hydroxyproline was used as an internal standard especially when a quantitative evaluation was attempted). The gradient program is shown in Fig. 1b. The time axis of the two graphs is on the same scale and thus permits the elution pattern to be related to the actual chromatographic conditions. A delay of *ca.* 2 min (at a flow-rate of 1.5 ml min^{-1}) has to be taken into account which is due to the time taken by the solvent void volume of the chromatographic system to pass the detector cell.

The chromatogram is started with solvent A containing 25% solvent B. An

TABLE I

COMPOSITION OF A 23-COMPONENT STANDARD MIXTURE OF PTH-AMINO ACID DERIVATIVES WITH INDIVIDUAL RETENTION TIMES (ACCORDING TO A REPRESENTATIVE ANALYTICAL RUN)

PTH-cysteic acid appears exclusively in the aqueous phase and therefore does not interfere with the identification of PTH-Asp.

No.	*Compound*	*Retention time (min)*
1	PTH-Aspartic acid	1.98
2	PTH-Cysteic acid	(~1.95)
3	PTH-(S-carboxymethyl)cysteine (CM-C)	2.46
4	PTH-Glutamic acid	2.73
5	PTH-Asparagine	4.06
6	PTH-Serine	4.43
7	PTH-Threonine	4.80
8	PTH-Glutamine	5.11
9	PTH-Glycine	5.61
10	PTH-(S-carboxamidomethyl)cysteine (CAM-C)	5.95
11	PTH-Histidine monohydrochloride	6.73
12	PTH-Alanine	7.25
13	PTH-Hydroxyproline (HYP)	7.65
14	PTH-Tyrosine	9.11
15	PTH-Arginine	10.21
16	PTH-Valine	11.60
17	PTH-Methionine	11.75
18	PTH-Proline	12.14
19	PTH-Isoleucine	14.99
20	PTH-Tryptophan	15.28
21	PTH-Phenylalanine	15.44
22	PTH-Leucine	15.68
23	PTH-N^{ε}-Phenylthiocarbamoyllysine	16.64

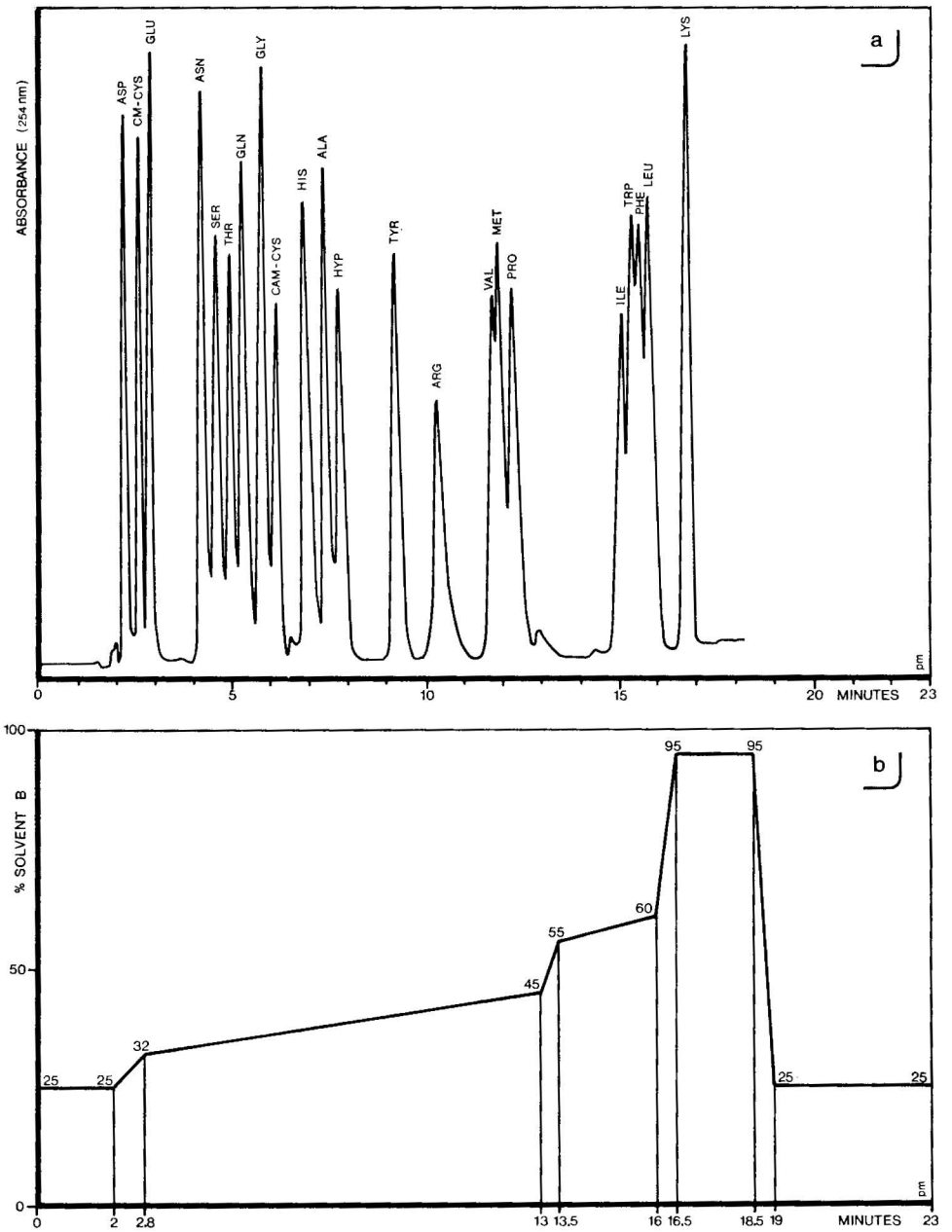

Fig. 1. (a) Typical standard chromatogram of 22 PTH-amino acids. Sample volume: 20 μl (containing *ca.* 2 nmoles per amino acid). Column: 250 × 4.6 mm, Spherisorb S5-ODS (5 μm particles). (b) Gradient program (including regeneration and equilibration). Solvents: A = 0.02 *M* lithium acetate, pH 5.2; B = 80% acetonitrile, 20% 0.1 *M* lithium acetate, pH 5.2. Flow-rate: 1.55 ml min^{-1}. Column pressure: *ca.* 130 bar; temperature, 37°.

initial isocratic phase (0–2 min) is followed by a sharp increase from 25 to 32% solvent B in order to reach the starting conditions for gradient I which leads to 45% solvent B. Under such conditions, most of the PTH-amino acids (PTH-Asp to PTH-Pro) are eluted. The relatively uniform spacing of the peaks is primarily governed by the slope of gradient I. The elution of the last group of derivatives (PTH-Ile to PTH-Lys) within acceptably short retention times requires relatively large amounts of solvent B. Its percentage is therefore raised sharply to a value of 55%. This is the starting point of gradient II (leading to 60% solvent B) the slope of which has to be kept moderate to guarantee a distinction between PTH-Ile, -Trp, -Phe and -Leu, whereas the separation of PTH-Lys is less critical. The regeneration of the column by washing with 95% solvent B is started before the last PTH-amino acid is eluted. The system is finally equilibrated with 25% solvent B.

In this way a near or even complete separation of most of the compounds tested was accomplished, except for PTH-Val and -Met, and for PTH-Ile, -Trp, -Phe and -Leu. As is evident from a number of publications, the separation of these derivatives is difficult and, depending on the analytical system used, is realized with different degrees of success. In our system the elution characteristics of PTH-Val and -Met and also of PTH-Ile, -Trp, -Phe and -Leu appear similar, yet the differences in the individual retention times, of the order of 0.2 min, are sufficient for unequivocal identification of any of these derivatives. The same elution program is also used for the identification of PTH-cysteic acid, -His and -Arg which, after conversion, are constituents of the aqueous phase.

Besides the dependence on the solvent composition, the elution pattern is further influenced by changes in pH and temperature; variations in the ionic strength of the buffer and in the flow-rate, however, have almost no effect.

Influence of pH. The peak positions of the acidic and basic amino acids are primarily affected. With decreasing pH, the retention times of PTH-cysteic acid, -Asp and -Glu are increased, as, but to a lesser extent, is also that of PTH-His. On the other hand, PTH-Arg as well as PTH-Asn and -Gln are eluted earlier and also PTH-Lys shows a drift, although less pronounced, towards shorter retention times.

Influence of temperature. With increasing temperature, the retention times are generally shortened. In addition there is a contrary tendency in terms of the separation power within two groups of PTH-amino acids. The resolution of PTH-Val and -Met decreases at elevated temperature, and PTH-Gln is shifted towards the position of PTH-Thr resulting finally in a fusion of the peaks at *ca.* 50°. On the other hand, the separation within the group of PTH-Ile, -Trp, -Phe and -Leu is generally improved by increasing temperature, except for PTH-Trp which, due to a pronounced drift towards shorter retention times, may interfere with the identification of PTH-Ile at temperatures greater than 45°. As a compromise, due to these limitations, a relatively narrow temperature interval between 35 and 39° gives optimal conditions for the entire chromatogram.

Influence of the amount of solvent B. The amount of solvent B in the eluent mixture is particularly critical for the resolution of the components within two groups of PTH-amino acids. In order to separate PTH-Val and -Met a threshold value of the percentage of solvent B should not be exceeded. On the other side, considerably higher amounts of solvent B are needed as early as possible for the elution of the group comprising PTH-Ile, -Trp, -Phe and -Leu. These requirements are considered

in the elution program by choosing a low percentage of solvent B in the starting eluent and by selecting a gentle slope of gradient I. Only after the elution of the PTH-Val/-Met group is it possible to reach the desired level of solvent B for eluting the remaining PTH-amino acids.

Fine adjustment of the elution program. The possibilities and limits are outlined in principle in the above sections. A fine adjustment is preferably achieved by one of the following measures or by a combination thereof: variation of the column temperature by *ca.* $\pm 1°$, changing the concentration of solvent B by *ca.* $\pm 5\%$ (of the concentration present), alterations in the position and height of the individual steps or gradients or a decrease in the pH in solvents A and B. The last mentioned possibility may become necessary when the elution of PTH-Arg is retarded with increasing age of the column. A slight shift to more acidic pH will push the PTH-Arg peak away from the PTH-Val/-Met/-Pro group.

The method described above has been successfully used in our laboratory for the identification of PTH-amino acids arising mainly from automated Edman degradation of fragments of model proteins with known primary structure as well as of plasminogen fragments of unknown sequence. It has also been applied with success to the identification of PTH-amino acids produced by manual Edman degradation according to Peterson *et al.*[13]. UV-absorbing impurities which may occur with this technique are also characterized in our system by defined retention times, and they normally appear in positions which do not interfere with the established elution pattern of the PTH-amino acids. In all these applications the method has proved its high resolving power and reproducibility even after close to 1000 analytical runs on the same column. Operating costs are low and the time required for one analysis (23 min including regeneration and equilibration) easily allows one to keep pace with the rhythm of the automated Edman degradation.

ACKNOWLEDGEMENTS

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REFERENCES

1 G. Frank and W. Strubert, *Chromatographia*, 6 (1973) 522.
2 J. X. de Vries, R. Frank and C. Birr, *FEBS Lett.*, 55 (1975) 65.
3 E. W. Matthews, P. G. H. Byfield and I. MacIntyre, *J. Chromatogr.*, 110 (1975) 369.
4 M. R. Downing and K. G. Mann, *Anal. Biochem.*, 74 (1976) 298.
5 G. D. Lominac and H. S. Kingdon, *Arch. Biochem. Biophys.*, 173 (1976) 320.
6 J. A. Rodkey and C. D. Bennet, *Biochem. Biophys. Res. Commun.*, 72 (1976) 1407.
7 C. L. Zimmerman, E. Appella and J. J. Pisano, *Anal. Biochem.*, 77 (1977) 569.
8 M. Abrahamsson, K. Gröningsson and S. Castensson, *J. Chromatogr.*, 154 (1978) 313.
9 J. van Beeumen, J. van Damme and J. de Ley, *FEBS Lett.*, 93 (1978) 373.
10 A. S. Bhown, J. E. Mole, A. Weissinger and J. C. Bennett, *J. Chromatogr.*, 148 (1978) 532.
11 J. Elion, M. Downing and K. Mann, *J. Chromatogr.*, 155 (1978) 436.
12 R. Zeeuws and A. D. Strosberg, *FEBS Lett*, 85 (1978) 68,
13 J. D. Peterson, S. Nehrlich, P. E. Oyer and D. F. Steiner, *J. Biol. Chem.*, 250 (1975) 557.

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CHROM. 11,996

Note

High-speed aqueous gel permeation chromatography of proteins

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Since the introduction of gel filtration by Porath and Flodin[1], the method using a cross-linked dextran or a cross-linked polyacrylamide gel column, has been utilized in the field of biological science as one of the essential techniques for separation and molecular-weight estimation of water-soluble macromolecules.

The estimation of the molecular weight of macromolecules is also made by gel filtration together with the other techniques such as analytical ultracentrifuge, light scattering, or electrophoresis. For this purpose, the classical gel filtration method using a soft-gel column does not need sophisticated and expensive instruments nor special techniques; however, the matrix of these gels are quite soft and weak so that the gels do not withstand the high pressure required for high-speed analysis. Therefore, very low flow-rates should be applied to the column and it takes a few days to completely analyse proteins. Moreover, this tedious method also needs a considerable amount of sample for the detection of the peak; usually milligram levels of proteins are required.

Several attempts have been made for the high-speed liquid chromatography of proteins by gel permeation chromatography (GPC) in aqueous media. However, most of the results do not seem to be completely satisfactory due to the low column efficiency and the adsorption of the solute on the packings. To prevent the adsorption of protein molecules, controlled-pore glass beads substituted with glycerol were developed and their applications have been reported[2–5]. The developments of aqueous GPC are reviewed by Cooper and Van Derveer[6]. Recently, Hashimoto *et al.* described[7] the separations of proteins on TSK-GEL PW-type columns. In the present study, we report on the chromatographic properties of another type of TSK-GEL column which uses different gel materials than the PW-type gel.

MATERIALS AND METHODS

Chromatography was carried out on a chromatograph consisting of a Trirotar pump, loop type sample injector, and UV detector of UVIDEC 100-II (JASCO, Tokyo, Japan). TSK-GELs (8–12 μm) were packed in a stainless-steel tube (600 $\times$ 7.5 mm) (Tokyo Soda, Tokyo, Japan).

Proteins were dissolved in water to a concentration of 2 mg/ml. Blue dextran 2000 and alanine were used as the marker compounds for totally excluding and totally permeating molecules, respectively.

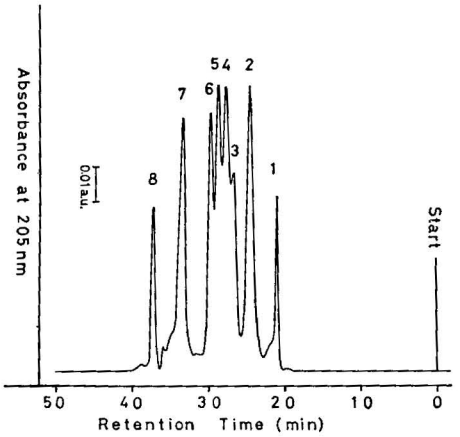

Fig. 1. Chromatogram of proteins on a TSK-GEL SW 2000 column. Elution buffer, pH 6.5 0.01 *M* phosphate + 0.2 *M* sodium sulfate. 1 = Aldolase; 2 = ovalbumin; 3 = lactoglobulin; 4 = trypsin inhibitor; 5 = chymotrypsinogen; 6 = ribonuclease A; 7 = insulin; 8 = alanine.

RESULTS AND DISCUSSION

A typical chromatogram of proteins is shown in Fig. 1. Samples of 2–4 μg of seven proteins with different molecular weights from aldolase to insulin and 40 μg of alanine, were mixed and chromatographed on a TSK-GEL 2000 SW column. The eluent used was 0.01 *M* phosphate buffer containing 0.2 *M* sodium sulfate (pH 6.5) which was pumped at a flow-rate of 0.5 ml/min. Absorbance of the effluent was monitored at 205 nm. Eight compounds were eluted in order of the molecular weights. Fig. 2 shows the chromatograms of six compounds separated on a TSK-GEL 3000 SW column, using different flow-rates.

The relationship between eluent flow-rate and height equivalent of a theoretical

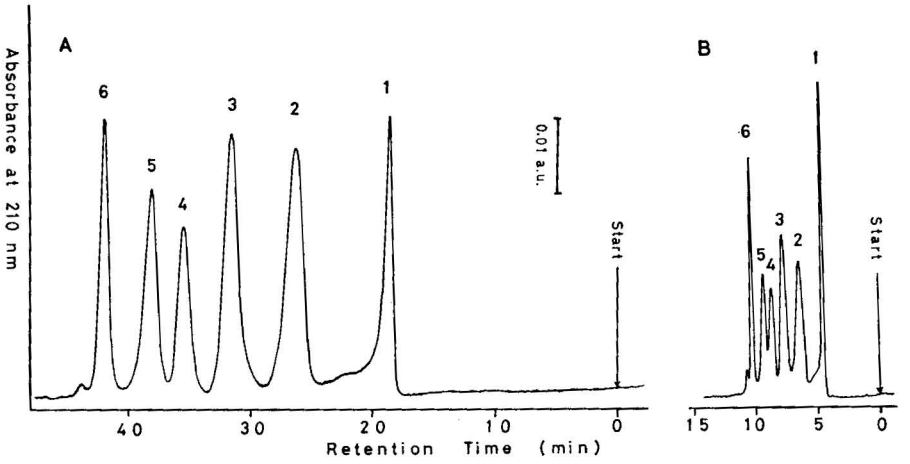

Fig. 2. Chromatograms of proteins on a TSK-GEL SW 3000 column. Elution buffer, pH 6.5 0.01 *M* phosphate + 0.2 *M* sodium sulfate. Flow-rate, (A) 0.5 ml/min, (B) 2.0 ml/min.

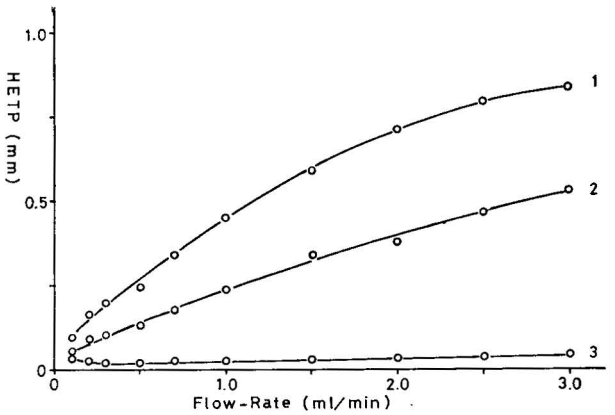

Fig. 3. Effect of flow-rate on the plate height for a TSK-GEL 2000 SW column. 1 = Ovalbumin; 2 = chymotrypsinogen; 3 = alanine.

plate (HETP) is shown in Fig. 3 for a TSK-GEL 2000 SW column at flow-rates ranging from 0.1 ml/min (0.04 mm/sec) to 3.0 ml/min (1.14 mm/sec). HETP for macromolecules is greatly increased with flow-rate due to the small diffusion coefficients as usually observed in the GPC results. On the other hand, HETP of small molecules, such as alanine, depend little on flow-rate and showed a minimum value at about 0.3 ml/min. The value increased at both lower and higher flow-rates. The maximum plate number 48,500/m for this column was obtained for the peak of totally permeating alanine eluted at the flow-rate of 0.3 ml/min. Fig. 4 shows the results from the TSK-GEL 3000 SW column. For macromolecules, a plate number of 8700/m for aldolase (mol. wt. 154,000), and 20,000/m for chymotrypsinogen (mol. wt. 24,500), were obtained at the flow-rate of 0.1 ml/min. The maximum of 30,000 plates/m for this column was obtained with the alanine peak eluted at 0.3 ml/min.

To examine the effects of the flow-rate on the retention volume, aldolase, chymotrypsinogen and alanine were chromatographed at various flow-rates. It was

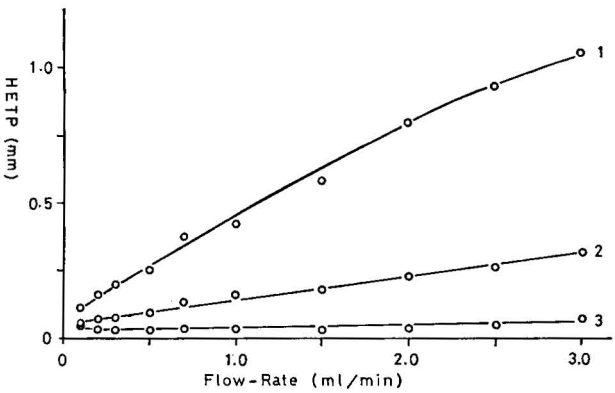

Fig. 4. Effect of flow-rate on the plate height for a TSK-GEL 3000 SW column. 1 = Aldolase; 2 = chymotrypsinogen; 3 = alanine.

found that the retention volume of the compounds was not dependent on the flow-rate (Fig. 5). Therefore, a high flow-rate can be applied to determine the retention volume of the compounds at the sacrifice of resolution. For example, by eluting at 2 ml/min, the analysis of proteins was completed within 11 min as shown in Fig. 2B.

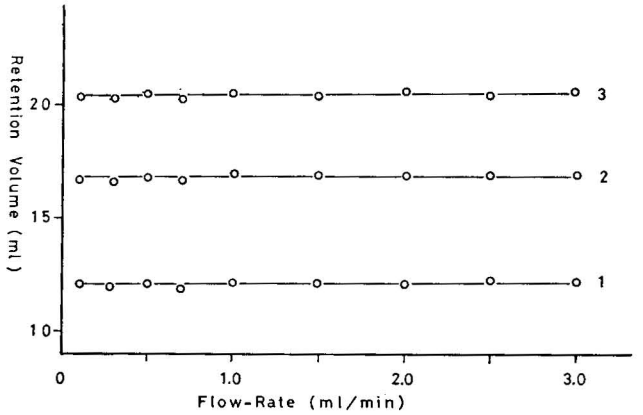

Fig. 5. Effect of flow-rate on the retention volume for a TSK-GEL 3000 SW column. 1 = Aldolase; 2 = chymotrypsinogen; 3 = alanine.

On the TSK-GEL 3000 SW column, a linear relationship between logarithm of molecular weight of proteins and retention volume was obtained for 16 proteins, their molecular weights ranging from 480,000 (ferritin) to 6000 (insulin). As shown in Fig. 6, ferritin eluted from the column after blue dextran 2000. This means that the exclusion limit of this column is somewhat larger than 480,000 daltons. Fig. 6 also gives the protein calibration curve for the TSK-GEL 2000 SW column with the same set of proteins as authentic samples. Ferritin, catalase, aldolase, and bovine serum albumin eluted at the void volume, and ovalbumin and the rest of the smaller compounds were retained on the column, so that the exclusion limit of this column for protein molecules was about 60,000 daltons. Good linearity between the logarithm of molecular weight and retention volume was also obtained on this column for various proteins from ovalbumin to insulin. Lysozyme, however, eluted slower than the expected retention volume on the basis of its molecular weight; when no salt was added to the buffer solution, this compound did not elute from the column. The adsorption of solutes on the column was reduced by the addition of sodium sulfate or sodium chloride. With higher salt concentrations than 0.2 M, the adsorption of lysozyme and smaller aromatic compounds were still observed. Table I shows the distribution coefficient, K, for several amino acids and nucleic acid-related compounds. The K value was obtained from retention volume of the solute, V_r, retention volume of totally excluded blue dextran 2000, V_0, and retention volume of totally permeating alanine, V_t, as $K = (V_r - V_0)/(V_t - V_0)$. These results show that basic amino acids are not adsorbed on the gel, but aromatic amino acids are adsorbed, and nucleic bases are also adsorbed strongly on the gel. The extent of adsorption of nucleic acid-related compounds is in the order of base, nucleoside, and nucleotide. Purine derivatives are adsorbed stronger than pyrimidine derivatives.

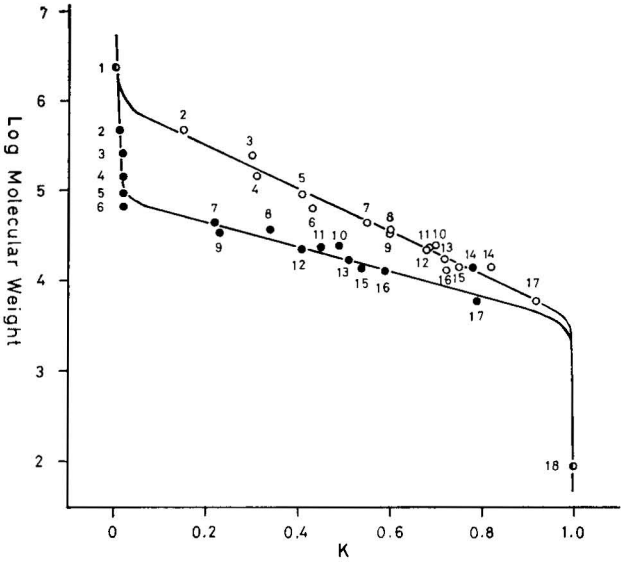

Fig. 6. Molecular weight calibration curves for TSK-GEL 2000 SW (●) and 3000 SW (○) columns. 1 = Blue dextran 2000 (MW 2000,000); 2 = ferritin (480,000); 3 = catalase (244,000); 4 = aldolase (145,000); 5 = transferrin (90,000); 6 = bovine serum albumin (65,000); 7 = ovalbumin (45,000); 8 = lactoglobulin (38 000); 9 = pepsin (33,500); 10 = chymotrypsinogen (24,500); 11 = trypsinogen (24,000); 12 = trypsin inhibitor (22,000); 13 = myoglobin (17,000); 14 = lysozyme (14,400); 15 = ribonuclease A (13,800); 16 = cytochrome *c* (13,000); 17 = insulin (6,000); 18 = alanine (89).

The high performance of these columns permits detection of very small amounts of protein. Even nanogram amounts can be detected satisfactorily by monitoring at a 200–220 nm range. Fig. 7 shows the chromatogram of the mixed solution of aldolase, chymotrypsinogen, and alanine. Between 5–10 ml of elution

TABLE I

ADSORPTION OF SMALL COMPOUNDS ON A TSK-GEL SW 3000 COLUMN

Elution buffer, same as for Fig. 2.

Compound	*K*	*Compound*	*K*
Blue dextran	0	Adenosine	1.37
Alanine	1.00	Cytidine	1.09
Phenylalanine	1.16	Guanosine	1.20
Tyrosine	1.09	Uridine	1.03
Tryptophan	1.54	Thymidine	1.11
Histidine	1.01	5′-AMP	1.11
Lysine	0.97	5′-CMP	0.99
Arginine	0.98	5′-GMP	1.01
Adenine	1.95	5′-UMP	1.00
Cytosine	1.21		
Guanine	1.36		
Uracil	1.21		
Thymine	1.24		

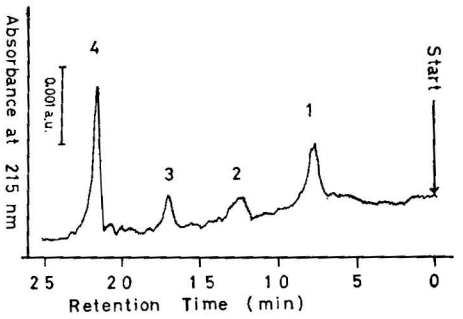

Fig. 7. Chromatogram of nanogram level proteins on a TSK-GEL 3000 SW column. 1 = Ghost peak; 2 = aldolase (20 ng); 3 = chymotrypsinogen (20 ng); 4 = alanine (120 ng).

volume usually a few ghost peaks appeared when detector sensitivity was high. One of these peaks has the same elution volume as the void volume; the other is less.

This work shows the excellent performance of the newly developed TSK-GEL SW-type columns on the hydrophilic GPC of proteins under high pressure. High-speed GPC in aqueous eluent with high resolution and high sensitivity should greatly aid in easing the increasing demands made in the fields of biological and hydrophilic polymer sciences.

REFERENCES

1 J. Porath and P. Flodin, *Nature (London)*, 183 (1959) 1657.
2 F. E. Regnier and R. Noel, *J. Chromatogr. Sci.*, 14 (1976) 316.
3 C. Persiani, P. Cubor and K. French, *J. Chromatogr. Sci.*, 14 (1976) 417.
4 H. D. Crone and R. M. Dowson, *J. Chromatogr.*, 129 (1976) 91.
5 R. J. Blagrove and M. J. Frenkel, *J. Chromatogr.*, 132 (1977) 399.
6 A. R. Cooper and D. S. Van Derveer, *J. Liquid Chromatogr.*, 1 (1978) 693.
7 T. Hashimoto, H. Sasaki, M. Aiura and Y. Kato, *J. Chromatogr.*, 160 (1978) 301.

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CHROM. 11,960

Note

Preparative high-performance gel chromatography for acidic and neutral saccharides

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Isolation of oligosaccharide intermediates liberated during the hydrolysis of a polysaccharide is fundamental for understanding both the structure and mode of hydrolysis of the polysaccharide.

This note describes the large-scale fractionation of acidic and neutral oligo- and monosaccharides by high-performance gel chromatography on H^+ form cation-exchange resins, which provides a rapid and quantitative method for separation of hydrolysates of a polysaccharide.

A volatile eluent is desired for preparative purposes, and one of optimal performance was determined on an analytical column (500 × 8 mm) packed with Hitachi-gel 3011-s (12 μm, H^+)[1]. When 0.5% aqueous acetic acid was used as the eluent, uronic acids gave shorter retention times than the corresponding neutral saccharides. Whereas, 0.5% formic acid provided good separation, uronic acids were eluted at the same retention times as with 0.1% phosphoric acid, which were close to those of the corresponding neutral saccharides. We, therefore, decided to perform the elution with 0.5% formic acid for preparative chromatography.

After equilibration in 1% aqueous NaCl solution about 150 g of Hitachi-gel 3019-s (30–40 μm, Na^+) were treated with 500 ml of 0.5% formic acid to be converted into the H^+ form. A slurry of the gel in 0.5% formic acid (500 ml) was ultrasonized for 1 h and slowly poured into a stainless-steel column (600 × 22 mm) (Umetani Seiki, Osaka, Japan) equipped with a 500-ml packer (Umetani Seiki). The column was packed by pumping the eluent (0.5% formic acid) at a flow-rate of 100 ml/min and at a pressure of *ca.* 80 kg/cm^2 using a double-plunger pump (Umetani Seiki)[2]. After the column pressure was equilibrated, the column was conditioned by recycling the eluent at a pressure of 135 kg/cm^2 for 20 h. The column, thus made, was equipped with an injection valve with a 1-ml loop and a refractive index monitor (type R-2, Japan Analytical Industry). The elutions were executed at room temperature.

The flow-rate dependency of the height equivalent to a theoretical plate (HETP) and resolution (R_s) determined with raffinose and D-galactose (10 mg each) is illustrated in Fig. 1; HETP is almost constant in the range of flow-rate of 1–6 ml/min. At a flow-rate of 5.5 ml/min, which is the optimum condition in efficiency and rapidness

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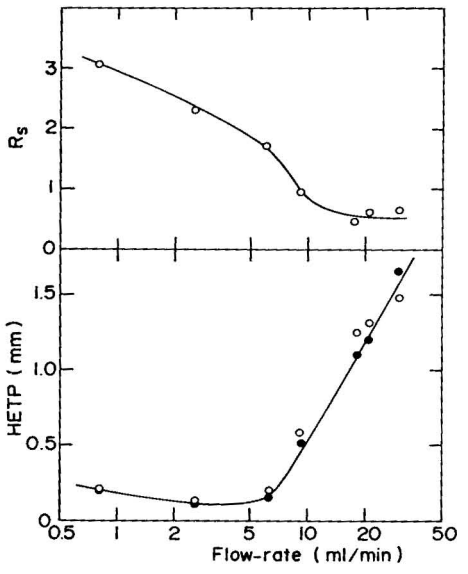
of separation, HETP and R_s were estimated as a function of the amount of charged samples (Fig. 2). HETP is gradually increasing up to the amount of sample of 20 mg. The number of theoretical plates was evaluated to be 7000 at this flow-rate with a sample of 1 mg. Neutral saccharides and uronic acids (10 mg each) could readily be separated in order of their molecular sizes (Fig. 3).



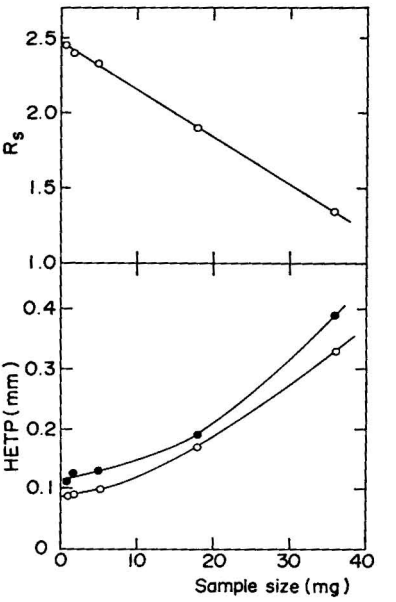


Fig. 1. Flow-rate dependency of R_s and HETP determined with raffinose (●) and D-galactose (○) (10 mg each); a cation-exchange gel column (Hitachi-gel 3019-s, H^+, 30–40 μm, 600 × 22 mm); eluent 0.5% formic acid.

Fig. 2. R_s and HETP determined for raffinose (●) and D-galactose (○) as a function of the amounof at charged sample; flow-rate, 5.5 ml/min; pressure, 7.5 kg/cm^2; other chromatographic conditions as in Fig. 1.

The plant gum[3] (100 mg) isolated from the sap of a lacquer tree (*Rhus succedanea*, Vietnam) was hydrolysed with 0.5 *N* trifluoroacetic acid (TFA) (2 ml) at 100° for 12 h. After removing water and TFA by evaporation at 40° *in vacuo*, the obtained hydrolysate was dissolved in 1 ml of water and the solution was applied to the column and the elution with 0.5% formic acid was performed with the result illustrated in Fig. 4. Each fraction was evaporated at 40° *in vacuo* to yield quantitatively isolated saccharides (peak I, 11.2 mg; II, 23.4 mg; III, 16.1 mg; IV, 10.1 mg; V, 58.2 mg). Peaks I, II and V showed single peaks in an analytical column (TSK-gel LS-212, H^+, 600 × 7.6 mm, 0.1% phosphoric acid)[1], however, peaks III and IV were contaminated with other peaks with close retention times. Rechromatography of these peaks could bring about complete separation of each fraction.

Peak V was identified with D-galactose by analysis on several analytical columns (LiChrosorb-NH_2 (250 × 4 mm), acetonitrile–water (4:1), TSK-gel LS-212 (H^+), 0.1% phosphoric acid[1]; TSK-gel LS-170 (500 × 5 mm), tetrahydrofuran–water (4:1) and by measuring its reducing power[4,5]. Peaks III and IV were due to

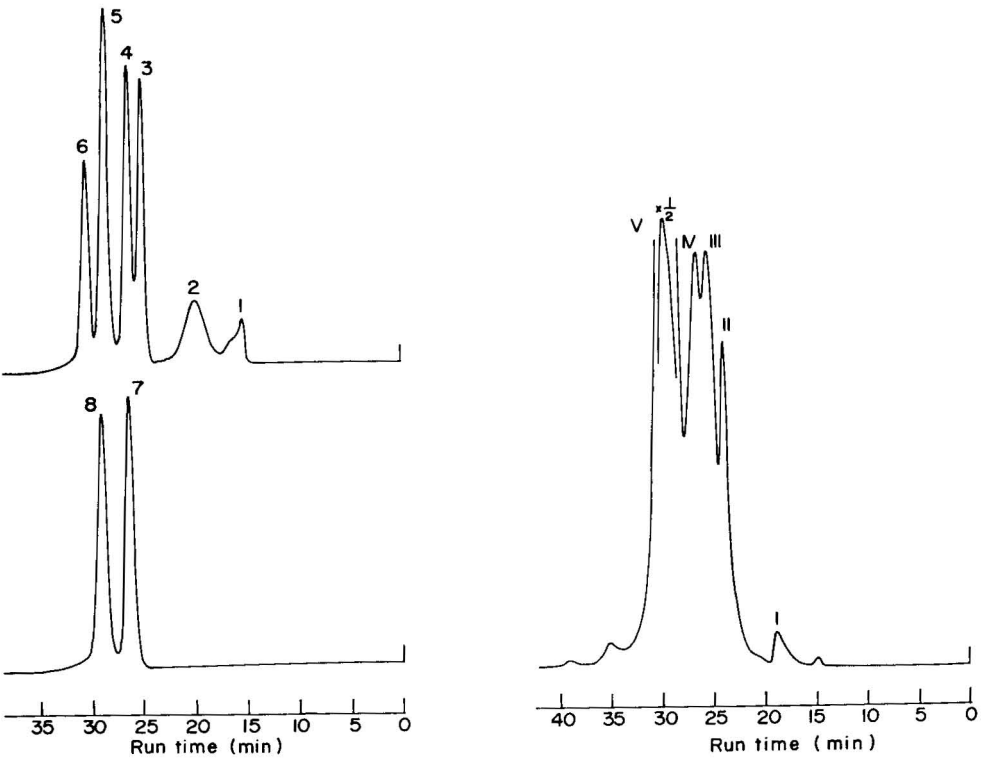


Fig. 3. Chromatogram of standard saccharides (10 mg each); 1 = FITC-dextran 20 (mol.wt. 19,000, Seikagaku Kogyo, Tokyo, Japan); 2 = FITC-dextran 3 (mol.wt. 2900, Seikagaku Kogyo); 3 = raffinose; 4 = lactose; 5 = D-galactose; 6 = 2-deoxy-D-ribose; 7 = D-glucuronic acid; 8 = D-galacturonic acid. Chromatographic conditions as in Fig. 2.

Fig. 4. Chromatogram of acid hydrolysates (100 mg) of the plant gum from the sap of *Rhus succedanea*; chromatographic conditions as in Fig. 2.

dissaccharides according to their retention times, which were confirmed by determining reducing power. Peak III was found to contain a carboxyl group by infrared and ultraviolet measurements and presumed to be an aldobiouronic acid, while Peak IV was thought to be a neutral disachharide. Peak II was also found to contain carboxyl groups and conjectured to be an acidic tri- or tetrasaccharide as judged from its retention time.

Further structural investigation of the obtained oligosaccharides is now in progress.

REFERENCES

1 R. Oshima, Y. Kurosu and J. Kumanotani, *J. Chromatogr.*, in press.
2 H. Mitsui, N. Takai, J. Kumanotani and T. Yamabe, *Seisan-Kenkyu*, 28 (1976) 375.
3 J. Kumanotani and R. Oshima, submitted for publication.
4 N. Nelson, *J. Biol. Chem.*, 153 (1944) 375.
5 M. Somogyi, *J. Biol. Chem.*, 160 (1945) pp. 61 and 74.

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Note

High-performance liquid chromatographic separation of glucose esters and quinic acid esters of hydroxycinnamic acids

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Glucose esters and quinic acid esters of hydroxycinnamic acids, which are widespread in higher plants, are difficult to separate by present methods[1]. Polyamide column chromatography represents an approach to this problem and enables group analyses of these two ester types[2]; however, separation of the individual derivatives requires re-chromatography on thin layers. Thus, the resolution of complex mixtures of these esters is time-consuming and quantitation is difficult.

It has been shown that reversed-phase high-performance liquid chromatography (HPLC) is applicable for the analysis of free hydroxycinnamic acids[3,4]. There have been only a few studies on the separation of simple mixtures of their conjugates by HPLC. Court[5] has reported the HPLC separation of isomers of chlorogenic acid, and Strack and Klug[6] have similarly resolved sinapoylglucose in a mixture of sinapic acid esters. Molderez *et al.*[7] separated glucose and quinic acid esters of *p*-coumaric and caffeic acid on LiChrosorb RP-8 and reported that prepurification and a group separation step were needed prior to the application of HPLC. Ong and Nagel[8] developed an HPLC technique to separate and quantify hydroxycinnamic acid–tartaric acid esters.

This paper describes the resolution of a complex mixture of 10 hydroxycinnamic acid conjugates from tissues of *Spirodela polyrrhiza* (duckweed) without any pretreatment of the crude extracts.

EXPERIMENTAL

Alcoholic extraction of fresh material of *Spirodela polyrrhiza* was done as described previously[9]. Without any pretreatment the crude extract was applied to HPLC. Besides the naturally occurring hydroxycinnamic acid conjugates in *Spirodela*[2], glucose esters of *p*-coumaric acid and caffeic acid were obtained by administration of free *p*-coumaric acid to the intact plant[1] (10 mg/100 ml culture medium for 24 h). Isomers of *p*-coumaroyl- and caffeoylquinic esters were produced by a method described by Sondheimer[10].

The liquid chromatograph used and the chromatographic columns were described in a previous communication[9]. Peak identification was achieved by applying isolated known compounds. HPLC separation was accomplished by gradient elution: in 25 min linear from solvent A (water–methanol–acetic acid, 92.5:5:2.5) to

10% B (water–methanol, 5:95) in A + B. Alternatively acetic acid was replaced by a buffer system (citric acid, 0.1 *M* at defined pH values) in A and B. The flow-rate was 2 ml/min, detection was at 312 nm and the sample size was 25 μl.

Calculations were done with an Autolab System I computing integrator (Spectra-Physics, Santa Clara, Calif., U.S.A.).

RESULTS AND DISCUSSION

The hydroxycinnamic acid conjugates, which were separated with HPLC, are listed in Table I. Fig. 1 shows (a) the resolution of a crude extract of *Spirodela* plants which were incubated with free *p*-coumaric acid (see Experimental) and isomers of *p*-coumaroyl- and caffeoylquinic esters which were added, and (b) the resolution of the quinic acid isomers. As can be seen, the peak pattern allows quantitation of each individual compound.

TABLE I

RELATIVE RETENTIONS (α) OF HYDROXYCINNAMIC ACID CONJUGATES ON LICHROSORB RP-8 USING A WATER–METHANOL GRADIENT

Peak No.	*Compound*	α
1	5-Caffeolquinic acid	1.37
2	1-Caffeoylglucose	1.34
3	5-*p*-Coumaroylquinic acid	1.47
4	4-Caffeoylquinic acid	1.10
5	1-*p*-Coumaroylglucose	1.05
6	3-Caffeoylquinic acid	1.39
7	1-Feruloylglucose	1.11
8	4-*p*-Coumaroylquinic acid	1.06
9	1-Sinapoylglucose	1.22
10	3-*p*-Coumaroylquinic acid	

To determine whether free hydroxycinnamic acids would elute in the retention range of their conjugates, thus interfering with the resolution, we added each of the hydroxycinnamic acids to the *Spirodela* extract. It was observed that these compounds elute after the *Spirodela* constituents. Wulf and Nagel[3] and Ong and Nagel[8] observed a shorter retention time of caffeic acid relative to its 3-quinic acid derivative (chlorogenic acid).

Our results are in agreement with those of Murphy and Stutte[4]. The isocratic system used by Wulf and Nagel[3] for the separation of free hydroxycinnamic acids could not be duplicated in our laboratory. On RP-18, we achieved baseline separation of the four hydroxycinnamic acids using a linear gradient from water to 25% methanol (5% acetic acid in each) in 25 min (flow-rate 2.0 ml/min).

Comparing our results with those of Court[5], we find a difference in the elution sequence of the caffeoylquinic acid esters. In Court's system, the 4-isomer elutes after the 3-isomer, whereas in our profile the 3-isomer is the last of the caffeoylquinic ester isomers to be eluted (see peaks 1, 4 and 6 in Fig. 1).

We found that the content of acetic acid in the elution mixture is very important in the separation of hydroxycinnamic acid conjugates. Slight changes in

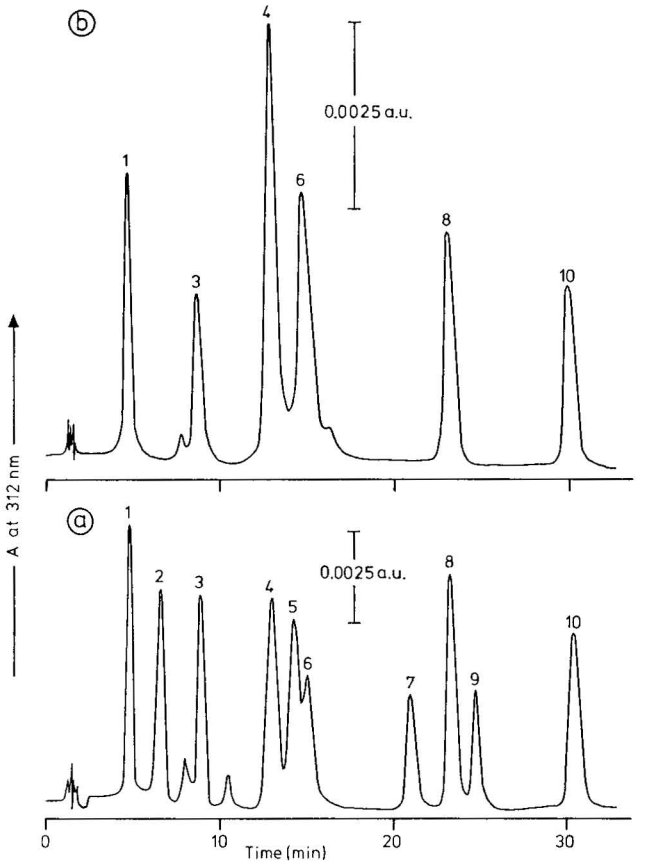

Fig. 1. The separation of hydroxycinnamic acid conjugates on LiChrosorb RP-8 using a water–methanol gradient. (a) The resolution of a crude extract of *Spirodela* plants which were incubated with free *p*-coumaric acid. Isomers of *p*-coumaroyl- and caffeoylquinic acid esters were added to the extract. (b) The resolution of isomers of *p*-coumaroyl- and caffeoylquinic acid esters. For peak identification, see Table I.

concentration (±1%) resulted in marked changes in the retention times and the selectivity. This effect can be used to optimize the separation. We obtained poor resolution using 5% acetic acid, and acetic acid concentrations between 1.5 and 2.5% gave the best resolution pattern.

Ong and Nagel[8] reported a pH sensitivity on the elution of hydroxycinnamic acid–tartaric acid esters and proposed to use this effect to optimize HPLC separation. We examined this pH effect on the isomers of caffeoylquinic acid (1, 4 and 6 in Table I) with a citric acid buffer to find if better separation can be obtained and if the reproducibility is equal to that obtained with 2.5% acetic acid in the solvent mixture.

The influence of different pH values on the capacity factors (k') of the isomers of caffeoylquinic acid is shown in Fig. 2. Whereas little effect was observed on the elution of 5-caffeoylquinic acid, there was a marked influence on 3- and 4-caffeoyl-

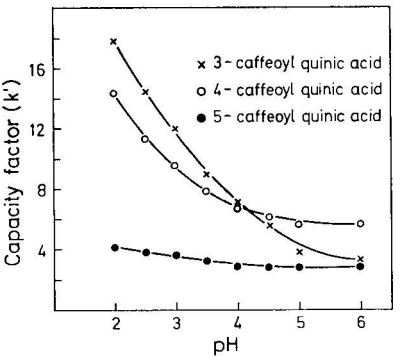

Fig. 2. The effect of pH (0.1 *M* citric acid buffer) on the capacity factors (k') of isomers of caffeoylquinic acid. Each point represents the mean of three determinations.

quinic acid. By shifting the k' values of these two compounds by changing the pH of the solvent mixture, the relative retention (α) for these isomers can be optimized; at pH 5.0 the α-value is 1.48 whereas with 2.5% acetic acid α is 1.16. In addition, the pH of the applied buffer affects the elution sequence (see Fig. 2), which could explain the difference from the results of other authors[3,5,8] (see above).

The reproducibility attained in quantitative analyses with the two elution systems is shown in Table II. The coefficients of variation ($n = 6$) demonstrate that better reproducibility can be obtained for both k' values and peak areas (integrator units) when 2.5% acetic acid is used in the solvent mixture for HPLC of caffeoylquinic acid esters.

TABLE II

CAPACITY FACTORS (k'), INTEGRATED PEAK AREAS AND COEFFICIENTS OF VARIATION (C.V.) OF HPLC-SEPARATED ISOMERS OF CAFFEOYLQUINIC ACID OBTAINED WITH 2.5% ACETIC ACID OR CITRIC ACID BUFFER

Compound	*2.5% Acetic acid*				*Buffer (pH 5.0)*			
	*k'**	*C.V. (%)*	*Area**	*C.V. (%)*	*k'**	*C.V. (%)*	*Area**	*C.V. (%)*
5-Caffeoylquinic acid	2.56	0.66	76,909	1.15	2.75	2.43	75,281	4.41
4-Caffeoylquinic acid	6.03	0.66	71,130	0.27	5.56	2.32	72,501	1.21
3-Caffeoylquinic acid	7.03	0.56	126.514	0.17	3.75	2.32	121,376	0.81

* Average of six runs.

In conclusion, this HPLC system appears to be applicable to physiological studies on the metabolism of hydroxycinnamic acid conjugates in *Spirodela* plants. This plant contains a mixture of quinic acid and glucose derivatives, whose separation with classical methods is elaborate and time-consuming. HPLC offers an efficient and dependable method for investigations on phenylpropanoid metabolism.

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REFERENCES

1 J. B. Harborne and J. J. Corner, *Biochem. J.*, 81 (1961) 242.
2 J. Krause, *Z. Pflanzenphysiol.*, 88 (1978) 465.
3 L. W. Wulf and C. W. Nagel, *J. Chromatogr.*, 116 (1976) 271.
4 J. B. Murphy and C. A. Stutte, *Anal. Biochem.*, 86 (1978) 220.
5 W. A. Court, *J. Chromatogr.*, 130 (1977) 287.
6 D. Strack and M. Klug, *Z. Pflanzenphysiol.*, 88 (1978) 279.
7 M. Molderez, L. Nagels and F. Parmentiers, *Phytochemistry*, 17 (1978) 1747.
8 B. Y. Ong and C. W. Nagel, *J. Chromatogr.*, 157 (1978) 345.
9 D. Strack and J. Krause, *J. Chromatogr.*, 156 (1978) 359.
10 E. Sondheimer, *Bot. Rev.*, 30 (1964) 667.

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CHROM. 11,951

Note

Effect of zinc ions on the reversed-phase separation and quantification of trace isomeric aminobenzoic acids in aqueous solution by high-performance liquid chromatography

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During the course of our investigation on the reactivity of amino radical with benzoic acid, it was necessary to develop a sensitive method for the detection and quantification of isomeric aminobenzoic acids from benzoic acid in aqueous solution. Separations were attempted on μBondapak C_{18}, anion-exchange and cation-exchange columns. The best separation was achieved on μBondapak C_{18} column under reversed-phase conditions. The addition of metal ions either in the mobile phase or chemically bonded to the stationary phase, has been applied for selective separation recently[1]. The effect of the addition of Zn^{2+} in the mobile phase has resulted in increasing the resolution in the separation of isomeric aminobenzoic acids.

EXPERIMENTAL

The main features of the liquid chromatograph used have previously been described[2,3]. It consists of the following components: Water Assoc. Milford, Mass., U.S.A. Model 6000A pump, a 100-μl six-port injection value. The chromatographic columns used were μBondapak C_{18} obtained from Waters Assoc., cation- and anion-exchange columns obtained from Vydac, Los Angeles, Calif., U.S.A. The detector was a Vari-Chrom UV–Vis variable-wavelength detector obtained from Varian. In all of our studies reported here, the photometric range of the detector was set at 0.02. The flow-rate was 1.8 ml/min at an operating pressure of 1000 p.s.i. The detection was carried out at 230 nm, where aminobenzoic acids have maximum absorbance. The UV absorption spectra were taken using a Cary 219 UV–visible spectrophotometer.

RESULTS AND DISCUSSION

Using a Zipax anion-exchange column, Klein *et al.*[4] have separated isomeric hydroxybenzoic acids with considerable difficulty. The *ortho* component was retained in the column for a longer time. Initially the separation of isomeric aminobenzoic acids was attempted using a Vydac anion-exchange column with 0.03 *M* K_2HPO_4 at pH 6.5. Both *ortho*-aminobenzoic acid and benzoic acid eluted together with long retention times (60 min). Varying the pH of the mboile phase to 7.5 did not improve the separation. Using 0.02 *M* borate buffer at pH 9.0 as the mobile phase, the separa-

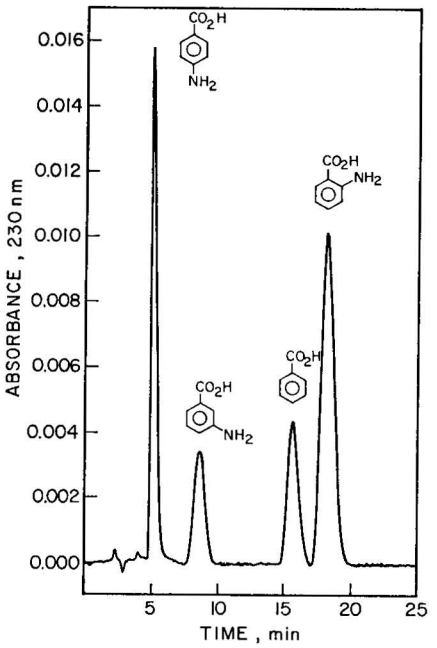

Fig. 1. Separation of isomeric aminobenzoic acids (10 μM each) from benzoic acid (10 μM) on μBondapak C_{18} column with 0.01 M NaH_2PO_4 and water–methanol (96:4) as the mobile phase. The detection was carried out at 230 nm.

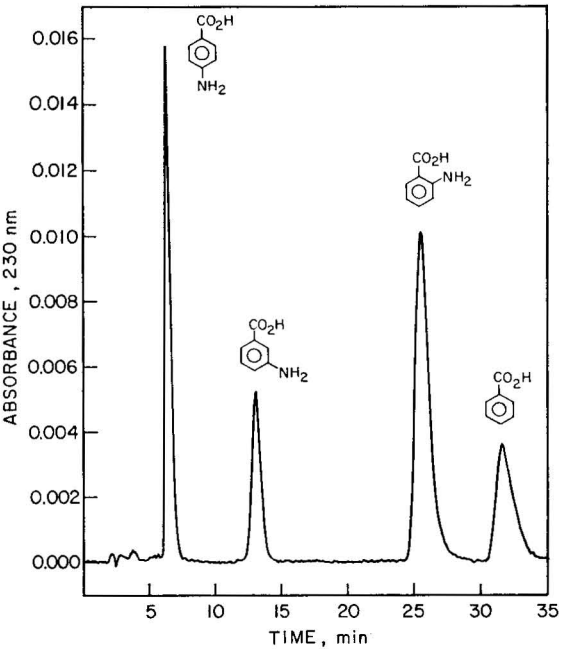

Fig. 2. Separation of isomeric aminobenzoic acids from benzoic acid. Experimental conditions are the same as in Fig. 1, except 0.002 M $ZnCl_2$ was added to the mobile phase.

tion of *ortho* component from benzoic acid was not achieved. Separation was also attempted on Vydac cation-exchange resin using sodium acetate at pH 4.0 as the mobile phase. All the components were not very well resolved.

The separation was achieved on μBondapak C_{18} column using water–methanol (96:4) and 0.01 *M* NaH_2PO_4 and the chromatogram obtained at $\lambda = 230$ nm is shown in Fig. 1. The separation of *ortho*-aminobenzoic acid from benzoic acid is not very well resolved. Since the formation constant of Zn^{2+} with *ortho*-aminobenzoate is much larger than the other components[5], it was hoped that the addition of Zn^{2+} in the mobile phase would show some effect on the chromatogram. The chromatogram obtained on adding 0.002 *M* of Zn^{2+} to the mobile phase is shown in Fig. 2. The pH of the mobile phase was decreased by 0.2 upon the addition of Zn^{2+}. As can be seen from Fig. 2, the *ortho*-aminobenzoic acid and benzoic acid are well separated. Moreover the *ortho* isomer has a shorter retention time which may be due to the formation of labile either mono- or bidentate Zn(II) complex. Very low concentration of Zn^{2+}, $1.0 \cdot 10^{-4}$ *M*, did not show any appreciable effect on the separation. It was possible to detect 2 μM of each of the components with an accuracy of $\pm$ 5%.

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REFERENCES

1 N. H. C. Cooke, R. L. Viavattene, R. Eksteen, W. S. Wong, G. Davies and B. L. Karger, *J. Chromatogr.*, 149 (1978) 391.
2 K. Bhatia and R. H. Schuler, *J. Phys. Chem.*, 77 (1973) 1356.
3 N. V. Raghavan, *J. Chromatogr.*, 168 (1979) 523.
4 G. W. Klein, K. Bhatia, V. Madhavan and R. H. Schuler, *J. Phys. Chem.*, 79 (1975) 1767.
5 L. G. Sillen and A. E. Martell, *Spec. Publ. Chem. Soc.*, No. 17 (1964) 533, and 25 (1971) 547.

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CHROM. 11,930

Note

Separation of insecticides by reversed-phase high-performance liquid chromatography

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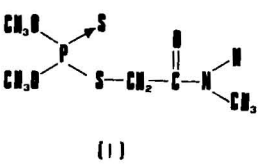
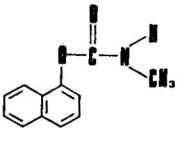
Dimethoate (I) [phosphorodithioic acid S-(2-methylamino-2-oxoethyl) O,O-dimethyl ester], carbaryl (II) (1-naphthalenol methylcarbamate) and tetrachlorvinphos (III) [phosphoric acid 2-chloro-1-(2,4,5-trichlorophenyl)ethenyl dimethyl ester (*Z*)] are pesticides currently employed against *Polychrosis botrana*, and many other insects and acari[1]. Etrimphos (IV) [phosphorothioic acid O-(6-ethoxy-2-ethyl-4-pyrimidinyl) O,O-dimethyl ester] is under experimental trial in our country against *Polychrosis botrana*.



(I)



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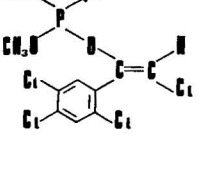
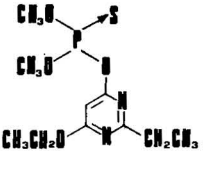


(III)



(IV)

Several analytical techniques are available for the separation and quantitative determination of these pesticides[3–6]. High-performance liquid chromatography (HPLC) has been recently introduced as a means of separating pesticide mixtures[7],

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and determinations of carbaryl[7–10], dimethoate[11] and tetrachlorvinphos[11,12] have been reported. However, few HPLC methods are available for the quantitative determination of these pesticides and have been provided only for carbaryl[8,10]. Moreover, to our knowledge no simultaneous determination of compounds I–III has been performed, and no chromatographic procedure has been applied to etrimphos.

Recently, we have successfully applied HPLC to the simultaneous quantitative determination of a mixture of chlorinated, carbamate and organophosphorus pesticides[12]. Here we describe the conditions which allow simultaneous quantitative determination of I–IV by reversed-phase high-performance liquid chromatography (RPLC).

EXPERIMENTAL

Apparatus

We used a Perkin-Elmer Model 601 liquid chromatograph equipped with a syringe-loading sample injector (Rheodyne 7105) and a variable-wavelength UV detector, Coleman Model LC 55. The column was a Perkin-Elmer reversed-phase, ODS-HC-Sil-X-1 (25 × 0.26 cm I.D.); analyses were performed at room temperature.

Chromatography

Elution was performed with a mixture (50:50) of distilled water and acetonitrile (Merck Analytical Grade, twice distilled) plus 10% of a buffer (0.067 *M* KH_2PO_4–1 *M* NaOH, pH 7.00; Carlo Erba, Milan, Italy). Mixtures of different proportions of these solvents, but without the buffer, at flows varying between 0.40 and 0.75 ml/min were also employed. The best wavelength for simultaneous determination was found to be at 221.0 nm. A standard curve was constructed for each pesticide. Plots of the peak area ratios (A_{prd}/A_{std}) *vs.* concentration showed good linearity in the range 0–100 ppm.

Chemicals

Standard solutions of pesticides were prepared by dissolving analytical-grade samples (purity $\geqslant$ 99.5%; BDH, Milan, Italy) of I–III in acetonitrile containing 30.0 ppm of benzene (purity $\geqslant$ 99.8% RP ACS, Carlo Erba) as internal standard. Etrimphos (IV) (purity $\geqslant$ 97.7%) was kindly donated by Sandoz (Milan, Italy) and prepared by the same procedure.

RESULTS AND DISCUSSION

Table I shows the results obtained with various water–acetonitrile mixtures as mobile phase. It is evident that the best separation was obtained at a 50:50 ratio plus 10% of the buffer, pH 7.00. A representative example of such a separation is shown in Fig. 1.

At a 63:37 ratio of water to acetonitrile the compounds can still be separated, but tetrachlorvinphos and etrimphos have too high retention times and the peaks were less sharp. Initially, we applied this method to the quantitative determination of pesticides I–IV in concentrated (200-fold) extracts of field-sprayed grapes prepared by the Office International du Vin technique[13]. Extracts were analyzed without any

TABLE I

RETENTION TIMES OF PESTICIDES AT DIFFERENT COMPOSITIONS OF MOBILE PHASE

Water–acetonitrile (%)	*Flow (ml/min)*	t_R*				
		I	*II*	*III*	*IV*	*STD*
63:37	0.75	0'42"	2'39"	17'54"	—	—
45:55	0.50	0'27"	1'00"	3'07"	5'10"	3'10"
35:65	0.50	0'20"	0'20"	1'50"	2'52"	—
50:50	0.40	0'40"	1'40"	5'50"	9'38"	2'40"
50:50 + 10% buffer	0.60	0'45"	1'27"	6'45"	12'50"	2'25"

* $t_R = t_{Rprd} - t_{RCH_3CN}$.

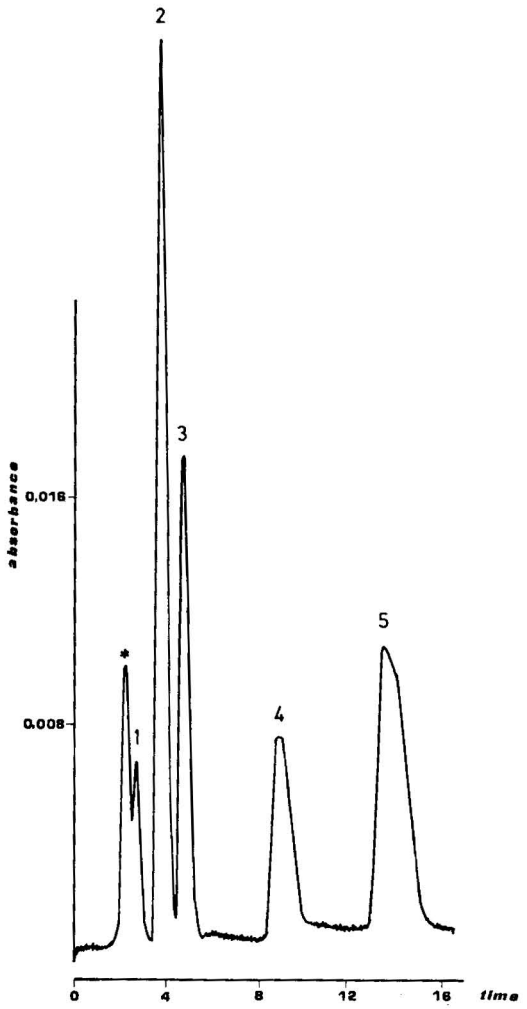

Fig. 1. Chromatogram of 3.0 μl of a mixture of pesticides. Peaks: * = acetonitrile; 1 = dimethoate (60 ppm); 2 = carbaryl (14 ppm); 3 = internal standard; 4 = tetrachlorvinphos (50 ppm); 5 = etrimphos (100 ppm).

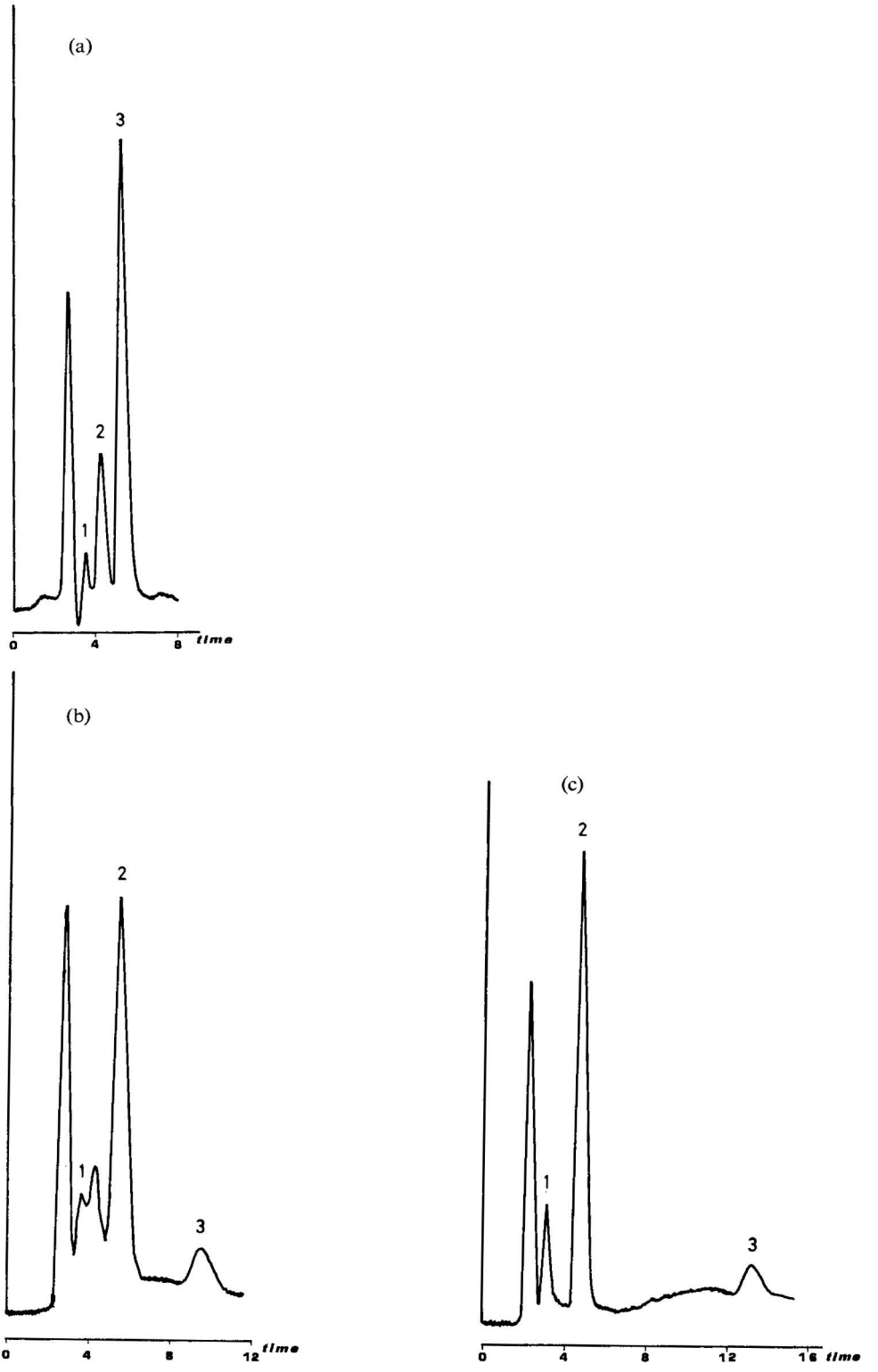

Fig. 2. Chromatograms of concentrated extracts from wine grapes sprayed with: (a) 1 = dimethoate, 2 = carbaryl, 3 = standard; (b) 1 = dimethoate, 2 = standard, 3 = tetrachlorovinphos; (c) 1 = carbaryl, 2 = standard, 3 = etrimphos.

further purification. Fig. 2 shows typical chromatograms of extracts from grapes sprayed with insecticides.

Table II reports the lowest concentration of these compounds that can be determined with reliable accuracy. The minimal acceptable values are considered as those giving a peak area ratio (A_{prd}/A_{std} for a 4.0-μl injection of concentrated extract) of *ca.* 0.04. This value is about three times the standard error of the instrument. The sensitivity of the determination of carbaryl compares favourably with that of other methods reported in the literature[8,10].

TABLE II

LOWEST CONCENTRATION OF PESTICIDES THAT COULD BE DETERMINED

Compound	*Concentration determined at 221.0 nm (ppm)*
I	~0.3
II	~0.002
III	~0.2
IV	~0.2

Although the sensitivity for dimethoate, tetrachlorvinphos and etrimphos is *ca.* 100 times lower than for carbaryl, the procedure enables the determination of these pesticides at concentrations below those permitted in Italy in foodstuffs (1.5, 1.5 and 0.5 ppm, respectively[14,15]).

In conclusion our technique provides a sensitive and rapid means for the simultaneous quantitative determination of chemically different pesticides in foodstuffs.

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REFERENCES

1 E. Egger, *Inform. Agrario*, 17 (1978) 1483.
2 Phytopatologic Station of Sardinia, personal communication.
3 R. T. Wang and S. S. Chou, *J. Chromatogr.*, 42 (1969) 416.
4 J. A. Burke and W. Holswade, *J. Ass. Offic. Agr. Chem.*, 49 (1966) 377.
5 W. Krijgsman and C. G. van de Kamp, *J. Chromatogr.*, 117 (1976) 201.
6 C. R. Turner, *Analyst (London)*, 99 (1974) 431.
7 H. A. Moye, *J. Chromatogr. Sci.*, 13, 268 (1975) and references cited therein.
8 H. M. Passano, *Waters Assoc. publication*, Milford, Mass., No. 76, May, 1977.
9 M. Kojima, N. Shiga, O. Matano and S. Goto, *Nippon Nojaku Gakkishi*, 2 (1977) 311; *C. A.*, 88 (1978) 17121e.
10 J. N. Seiber, *J. Chromatogr.*, 94 (1974) 151.
11 C. Gonnet and J. L. Rocca, *J. Chromatogr.*, 109 (1975) 297.
12 M. Meloni, F. M. Pirisi and P. Cabras, *Rend. Seminario Fac. Sci. Univ. Cagliari*, 48 (1978) 293.
13 P. Garoglio, *Enciclopedia Vitivinicola Mondiale*, Vol. II. Edit. Scientifiche Ufficio Italiano del Vino, Milan, 1973, p. 186, 1282.
14 *Italian Health Department Act Jan. 6, 1979*, Gazz. Uff. of Italian Republic, Feb. 8, 1979, p. 39.
15 Sandoz, S.p.A., Milan, personal communication.

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Note

Analysis of 3′-phosphoadenylylsulphate and related compounds by paired-ion high-performance liquid chromatography

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3′-Phosphoadenylylsulphate (PAPS, active sulphate) is considered as the universal sulphate donor for enzymic sulphation processes in nature. For the *in vitro* studies of the rat-brain phenol sulphotransferase (EC 2.8.2.1) reaction we prepared PAPS enzymically from freeze-dried rat liver and purified it by the method of Tsang *et al.*[1], with some minor modifications as described in a recent paper[2].

Adenosine 3′,5′-bisphosphate (PAP) may be formed by hydrolysis of PAPS during storage. As PAP is a strong inhibitor of sulphotransferases, preparations of PAPS to be used must be free of any contaminating PAP. Therefore, a routine analysis of PAPS is needed, which is capable of monitoring the hydrolysis products of PAPS. The technique should also provide a method for monitoring ATP, as this compound is eluted close to PAPS during the purification of PAPS on DEAE-Sephadex A-25 (ref. 2). Thin-layer chromatography (TLC) and electrophoresis have been used to assay the amount of PAP in preparations of PAPS[1,2]. However, with the advent of high-performance liquid chromatography (HPLC) an excellent technique is at hand, which is rapid and reliable. As a routine analysis technique we have selected reversed-phase paired-ion HPLC on a LiChrosorb 5 RP-18 column, with tetrabutylammonium perchlorate as the pairing agent.

EXPERIMENTAL

All analyses were carried out on a Waters Assoc. (Milford, Mass., U.S.A.) liquid chromatograph consisting of a M-6000A pump, a Model U6K injector and a Model 440 absorbance detector, capable of monitoring 254 nm. The solvent system consisted of 3 m*M* tetrabutylammonium perchlorate (Fluka, Buchs, Switzerland) and 30 m*M* KH_2PO_4 in methanol–water (1:3, v/v), pH 7.0. It was prepared by dissolving the pairing agent in methanol and KH_2PO_4 in water. These solutions were filtered through 0.45-μm Millipore (Molsheim, France) filters before mixing. The resulting solution was adjusted to pH 7.0 with 2 *M* KOH. The analyses were carried out at room temperature on a 15 cm × 4.6 mm I.D. stainless-steel column packed with LiChrosorb 5 RP-18 (Chrompack, Middelburg, The Netherlands). The flow-rate was 1.3 ml/min.

PAPS was prepared and purified as described earlier[2]. Adenosine 5′-monophosphate (AMP), adenosine 5′-sulphatophosphate (APS), PAP and ATP were ob-

tained from Boehringer (Mannheim, G.F.R.). All other chemicals were of analytical grade.

RESULTS AND DISCUSSION

Fig. 1 shows the optimal separation of AMP, APS, PAP, ATP and PAPS, respectively. The retention times for these compounds are 3.1, 5.4, 7.2, 8.5 and 13.5 min, respectively. AMP is co-chromatographed as it appeared to be present as a contaminant in the batch of APS. Assuming these adenosine nucleotides to have approximately equal molar absorption coefficients at 260 nm[3,4] and equal detector responses, the amounts of contaminating APS, PAP and ATP have been determined in the preparation of purified PAPS. The chromatogram is shown in Fig. 2. PAPS contains a maximum of 0.5% of APS, PAP and ATP, together. A purified preparation of PAPS is stable for at least 1 month when stored dry at −20°.

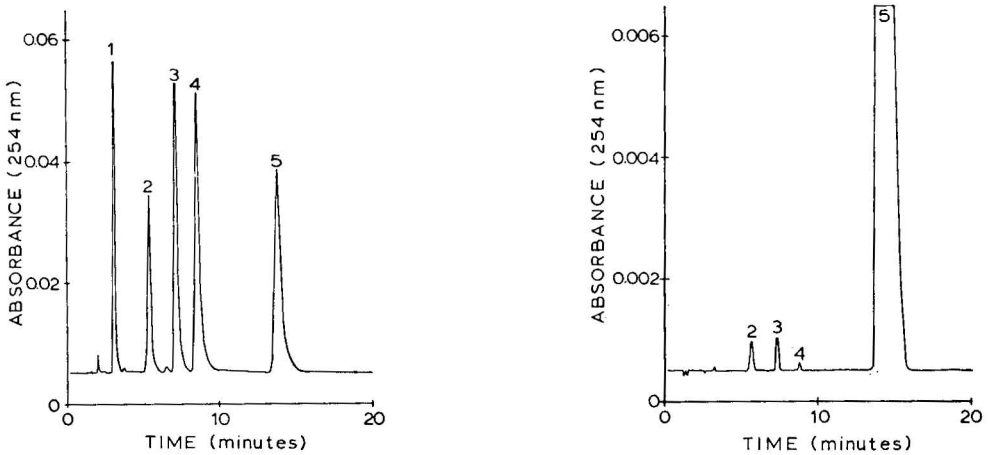

Fig. 1. Optimal separation of a test mixture of AMP (1), APS (2), PAP (3), ATP (4) and PAPS (5). Column: 150 × 4.6 mm I.D., LiChrosorb 5 RP-18. Eluent: 3 m*M* tetrabutylammonium perchlorate, 30 m*M* KH_2PO_4 in methanol-water (1:3, v/v), pH 7.0. Flow-rate: 1.3 ml/min. Detection: UV, 254 nm. Temperature: ambient.

Fig. 2. Analysis of a purified preparation of PAPS. Same conditions as in Fig. 1.

It should be mentioned that ADP and APS have identical retention times under the HPLC conditions used, but, if needed, they can be separated by increasing the concentration of tetrabutylammonium perchlorate in the solvent system. In fact, the amount of pairing agent and the amount of methanol in the solvent system are elegant tools for adapting separation factors and retention times of the compounds studied.

REFERENCES

1 M. L. Tsang, J. Lemieux, J. A. Schiff and T. B. Bojarski, *Anal. Biochem.*, 74 (1976) 623.
2 E. J. M. Pennings, R. Vrielink and G. M. J. van Kempen, *Biochem. J.*, 173 (1978) 299.
3 R. H. de Meio, in D. M. Greenberg (Editor), *Metabolic Pathways, Vol. VII, Metabolism of Sulfur Compounds*, Academic Press, New York, San Francisco, London, 3rd ed,. 1975, Ch. 8, p. 291.
4 In M. Windholz, S. Budavari, L. Y. Stroumtsos and M. N. Fertig (Editors), *The Merck Index*, Merck & Co., Rahway, N.J., 9th ed., 1976, p. 21.

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CHROM. 11,946

Note

Liquid chromatographic analysis of hydralazine and metabolites in plasma

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Hydralazine is an economical effective drug which may be used to treat hypertension and chronic, resistant congestive heart failure. While this drug has been in use for quite some time, the complete metabolism and the pharmacokinetics of hydralazine remain undefined. The complexity of the problem may be in part due to the fact that hydralazine is chemically very reactive; it readily forms hydrazones with biogenic aldehydes and ketones. High-pressure liquid chromatographic (HPLC) studies of the reaction of hydralazine with several aldehydes and ketones at physiological conditions have been effected in this laboratory[1,2]. It was found that the reactions of hydralazine with formaldehyde and acetaldehyde are of special interest, because these reactions lead to the formation of *s*-triazolo[3,4*a*]phthalazine (I) and 3-methyl-*s*-triazolo[3,4*a*]phthalazine (II), respectively, with the hydrazones as intermediates. Both I and II have been reported to be the metabolites of hydralazine[3,4]. In fact, II is the major metabolite accounting for 80% of the total drug in urinary excretion, whereas I is present only in trace amounts in the urine[5].

In order to obtain reliable pharmacokinetic data, the assay procedure used must be highly sensitive and selective. Most of the early studies on hydralazine were based on a spectrophotometric assay[6] which, unfortunately, does not allow one to distinguish between unchanged drug and metabolites[7]. Improved methods have since been published using gas–liquid chromatographic techniques[8,9]. While these methods have been shown to be successful in the analysis of hydralazine, the present study introduces an alternative assay procedure using HPLC. This HPLC method is based on the conversion of hydralazine into compound I with formaldehyde under acid condition. Although I has been shown to be a metabolite of the drug, its concentration in plasma or serum samples is generally non-detectable. The advantages of this method will be discussed.

EXPERIMENTAL

Materials

Hydralazine (kindly supplied by Ciba-Geigy, Summit, N.J., U.S.A.), formaldehyde and 8-chlorotheophylline (both from Aldrich, Milwaukee, Wisc., U.S.A.) were used as obtained. High-purity samples of I and II were prepared by preivous

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literature procedures[10,11] and used as standards. Human plasma was obtained from a local blood bank. All solvents used were HPLC grade (Fisher Scientific, Pittsburgh, Pa., U.S.A.).

Equipment

A HPLC system, including a Model 6000 A pump and a U6K injector (Waters Assoc., Milford, Mass., U.S.A.) equipped with Model SF-770 UV and Model SF-970 fluorescence detectors (both from Schoeffel, Westwood, N.J., U.S.A.), was used. The output of the detectors were displaced on a recorder (Omniscribe, Houston Instruments, Austin, Texas, U.S.A.) having a full-scale range of 10 mV. The HPLC analysis was made with a reversed-phase μBondapak C_{18} column (30 cm × 4 mm I.D.) (Waters Assoc.).

Chromatographic conditions

The mobile phase was acetonitrile–sodium acetate buffer (0.01 *M*, pH 4) (13:87). The column temperature was ambient. Chromatographic analysis was made with a flow-rate of 1.0 ml/min. The sample injection size was 8 μl. The column eluate was monitored by UV detection at 240 nm and by fluorescence detection with excitation at 240 nm and emission above 389 nm. The UV detector was operated at 0.01 a.u.f.s., the fluorescence detector was operated at 0.2, 0.5 or 1 μA.

Sample preparation

Stock solutions of 1 mg/ml of hydralazine and compounds I and II were prepared in methanol. The solutions of authentic I and II were used to identify plasma hydralazine and metabolite in the chromatographic analysis. Synthetic plasma samples were prepared by transferring desired amounts of hydralazine stock into 1 ml plasma with a microsyringe. To convert hydralazine to I, 10 μl of formaldehyde and 10 μl of glacial acetic acid were added to 1 ml plasma containing hydralazine. The resulting plasma sample has a pH of about 5 and contains excess amount of formaldehyde. The sample was then shaken and allowed to stand for 20 min for the completion of the reaction. For blood sample analysis, the plasma obtained by rapid centrifugation of the blood sample were used with and without the formaldehyde treatment. Prior to the analysis; 10 μg/ml of 8-chlorotheophylline was added to all the samples and used as an internal standard for the UV detection.

RESULTS AND DISCUSSION

The problem associated with the analysis of hydralazine is that the drug is notoriously unstable in aqueous solutions. Studies[5] have shown that hydralazine undergoes rapid degradation in aqueous solution depending on the pH conditions and buffer systems. In solutions containing aldehydes or ketones, such as in biological fluids, hydralazine forms hydrazones in an equilibrium process. These phenomena suggest that direct measurement of hydralazine will only give results with poor accuracy. A reasonable approach in the developement of a hydralazine assay would be to derivatize the drug into a stable and easily measured derivative. An example of this approach has been shown by Jack *et al.*[8], in which hydralazine was converted to a tetrazolo phthalazine derivative with sodium nitrite. The reaction of hydralazine

with formaldehyde, which occurs rapidly in aqueous solutions under acid condition, provides an excellent means for the determination of hydralazine in plasma samples. Hydralazine is non-fluorescent and only shows moderate UV absorption. Compound I, the derivative of hydralazine with formaldehyde, however, shows marked enhancement of UV absorption as well as a strong fluorescence at 425 nm, when excited at 240 nm.

The chromatographic analysis of a plasma sample containing 200 ng/ml hydralazine is shown in Fig. 1, where the top chromatograms were obtained with a fluorescence detector, and the bottom chromatograms were obtained with a UV detector. Fig. 1A shows the untreated sample, where peak a represents 8-chlorotheophylline which was used as an internal standard for the UV detection. The added hydralazine was not detectable under the chromatographic conditions. Fig. 1B represents the formaldehyde-treated sample, where a new peak b (or b*) appears with a retention time of 13 min. This new peak was found to be identical in retention time and peak response with a sample containing the authentic I. Fig. 1C represents the same sample of 1B, but the fluorescence detection is monitored at a higher sensitivity setting. Using the fluorescence detector, this method allows one to measure hydralazine levels as low as 3 ng/ml. The minimum detection using the UV detector was found to be about 0.1 μg/ml. The standard curve for the hydral-

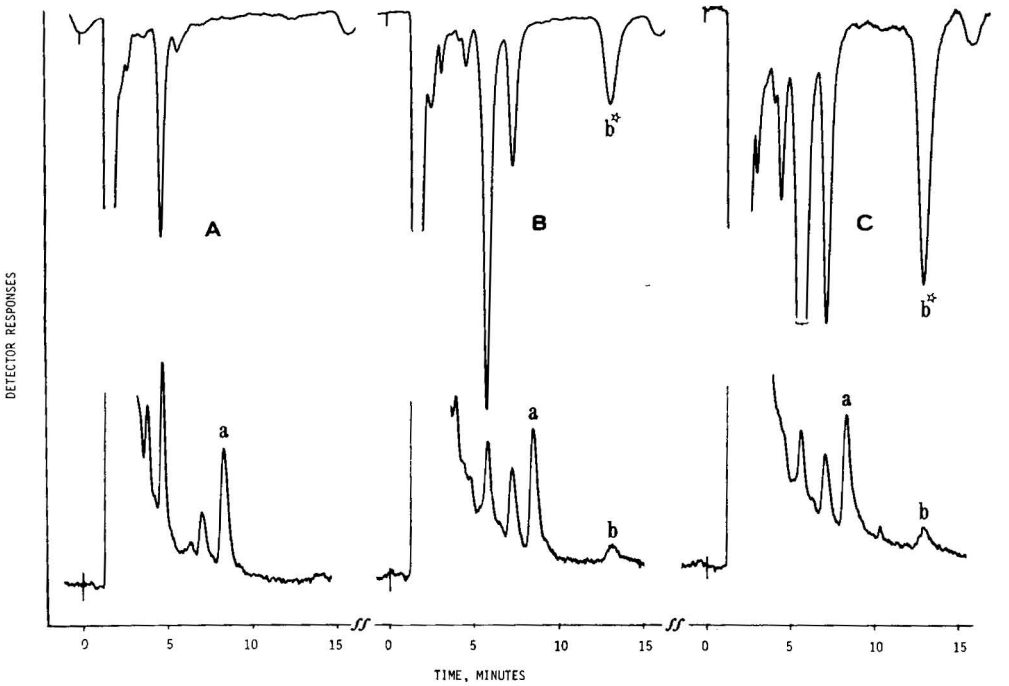

Fig. 1. The UV (bottom) and fluorescence (top) chromatograms of a synthetic plasma sample containing 200 ng/ml hydralazine and 8-chlorotheophylline. (A), Untreated sample, fluorescence range is 0.5 μA; (B), formaldehyde-treated sample, fluorescence range is 0.2 μA; (C), as (B), except for a higher sensitivity setting for the fluorescence detection. The UV detection is set at 0.01 a.u.f.s. Peak a represents 8-chlorotheophylline (internal standard), peak b (or b*) represents compound I.

azine analysis of plasma samples using a fluorescence detector is shown in Fig. 2. It can be seen that this method is applicable to the entire range of hydralazine plasma levels. A standard curve for the determination of II, the major metabolite of hydralazine, was also obtained with the same sensitivity. Under the same chromatographic condition, compound II exhibits a retention time of 24 min.

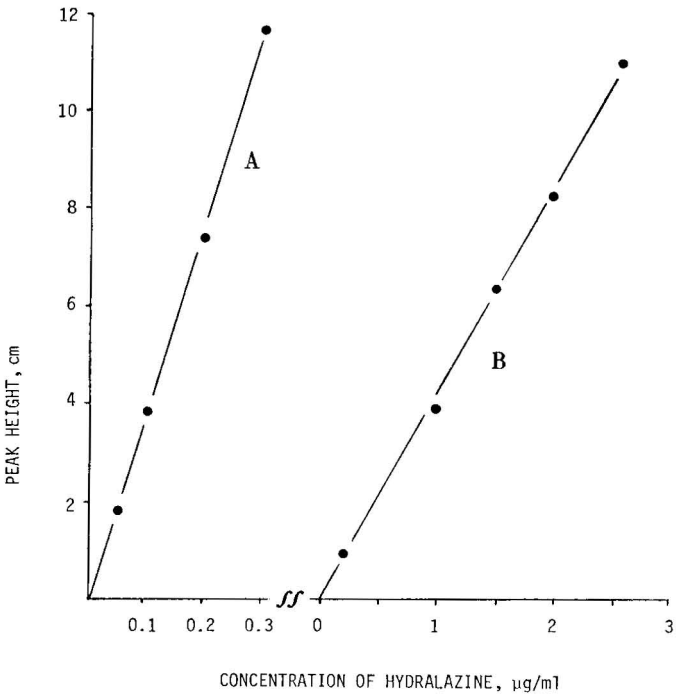

Fig. 2. A calibration curve for hydralazine in synthetic plasma samples with the fluorescence detection. (A), Fluorescence range of 0.2 μA; (B), fluorescence range of 1.0 μA.

Fig. 3 shows the chromatographic analysis of a plasma sample obtained from a patient. The formaldehyde-treated sample is shown by the dashlined chromatograms a patient. The formaldehyde-treated sample is shown by the dashlined chromatograms. It can be seen that the presence of II is indicated by peak c (or c*). When the sample is treated with formaldehyde, the hydralazine level can be easily measured by peak b (or b*).

The method described in this study allows one to use directly plasma samples without protein precipitation or extractions. If one prefers to extract the components after the formation of I with organic solvents, the water soluble constituents in the plasma shown in Fig. 1 and 3 may be removed. In this case, it is more advantageous to use a higher concentration of acetonitrile (up to 20%) in the mobile phase, because at a higher acetonitrile concentration, the peaks of I and II appear at reduced retention times with higher resolution.

Although compound I has been shown to be a minor metabolite of hydralazine its concentration in plasma samples is usually low and non-detectable. There-

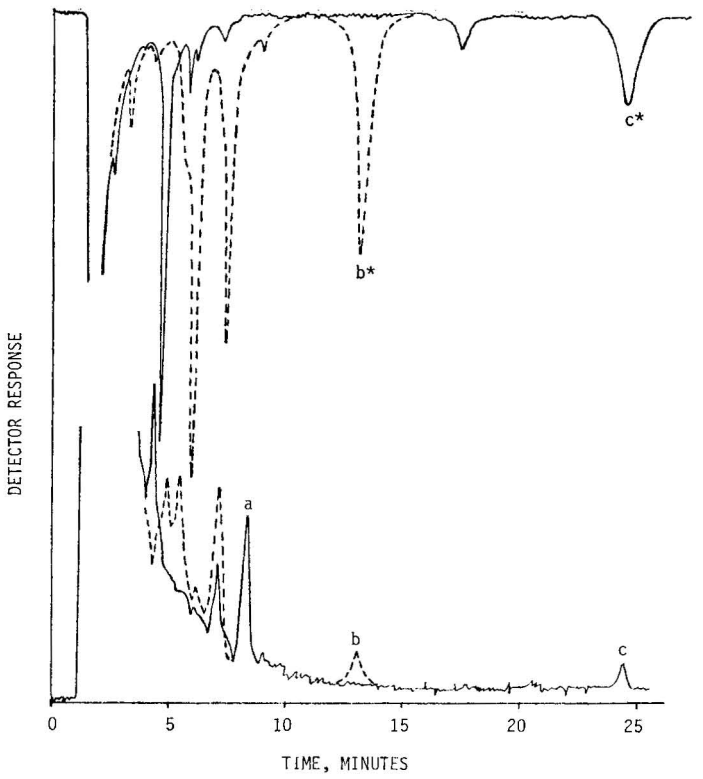

Fig. 3. The UV (bottom) and fluorescence (top) chromatograms of a human plasma sample. ——, Untreated; – – – –, treated with formaldehyde. The fluorescence range was set at 0.5 μA. The UV detection was operated with 0.01 a.u.f.s. Peak a represents 8-chlorotheophylline, b (or b*) represents compound I, c (or c*) represents compound II.

fore, no interference is expected. At high doses of hydralaine, compound I may be present in the plasma in measurable quantities. In this case, the concentration of I may be first quantitated prior to the treatment with formaldehyde.

REFERENCES

1 J. P. O'Donnell, W. J. Proveaux and J. K. H. Ma, *J. Pharm. Sci.*, (1979) submitted for publication.
2 J. P. O'Donnell, W. J. Proveaux and J. K. H. Ma, *J. Pharm. Sci.*, July (1979) in press.
3 M. M. Reidenberg, D. Drayer, A. L. DeMarco and C. T. Bello, *Clin. Pharmacol., Ther.* 14 (1973) 970.
4 J. Wagner, J. W. Faile, P. Imhof and G. Licher, *Arzneim.-Forsch. Drug Res.*, 27 (1977) 2388.
5 T. Talseth, *Clin. Pharmacokinetics*, 2 (1977) 317.
6 A. R. Schulert, *Arch. Int. Pharmacodyn.*, 132 (1961) 1.
7 P. A. Reece, P. E. Stanley and R. Zacest, *J. Pharm. Sci.*, 67 (1978) 1150.
8 D. B. Jack, S. Brechbühler, P. H. Degen, P. Zbinden and W. Riess, *J. Chromatogr.*, 115 (1975) 87.
9 T. Talseth, *Clin. Pharmacol. Ther.*, 21 (1977) 715.
10 J. Druey and B. H. Ringier, *Helv. Chim. Acta*, 34 (1951) 195.
11 K. D. Haegele, H. B. Skrdlant, N. W. Robie, D. Lalka and J. L. McNay, Jr., *J. Chromatogr.*, 126 (1976) 517.

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Note

High-pressure liquid chromatography of androgens

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In connection with our work on the conversion of androstenedione to testosterone by growing pea plants[1], we perceived the need for an efficient method of separating adequate amounts of various metabolites of the androgen series for radiochemical assays. Although much research has gone into chromatographic methods for accomplishing this[2], very few liquid systems capable of resolving the numerous stereoisomers in this class of steroids have been described[3]. High-pressure liquid chromatography (HPLC)[4], particularly on the basis of adsorption, is eminently suitable for this application. For the detection of the androgens, which have a carbonyl group at C-3 and/or C-17, we have used a dual detector, registering the UV absorption at 254 and 280 nm[5].

EXPERIMENTAL*

The HPLC apparatus was assembled from commercially available components. A solvent reservoir, containing a mixture of 89.5 ml dichloromethane, 10 ml acetonitrile, and 0.5 ml 2-propanol (all "Distilled-in-Glass" quality; Burdick & Jackson, Muskegon, Mich., U.S.A.) was connected to the inlet of a dual-piston reciprocating pump (Tracor Model 990; Tracor, Austin, Texas, U.S.A.). The pump outlet was connected to a length of stainless-steel (SS) tubing, 0.02 in. I.D., having a capacity of *ca.* 30 μl, through a SS union. For sample application, this union was disconnected, the solvent in the tubing was removed by aspiration, and the sample was injected directly into the tubing, which formed the column inlet.

The column consisted of two SS chromatographic tubes (Alltech, Arlington Heights, Ill., U.S.A.), each 30 cm × 4.6 mm I.D., packed with Partisil 5 (Whatman, Clifton, N.J., U.S.A.), and connected in series. The column was prepared in our laboratory from a balanced-density slurry of the silica gel in a mixture of tetrabromoethane and tetrachloroethane. The slurry was packed into the tubes with a Haskel HPLC slurry-packing unit (Model 29426; Haskel, Burbank, Calif., U.S.A.). The efficiency of the two sections was determined by using nitrobenzene as the test material and hexane as the eluent. Plate counts were 3200 for each section.

* Reference to a company and/or product named by the Department is only for purpose of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

The outlet of the column was connected to the inlet of a Tracor Model 970 variable-wavelength detector, set at 254 nm. The latter had its outlet connected to the inlet of an Altex–Hitachi Model 100-30 detector (Altex, Berkeley, Calif., U.S.A.), set at 280 nm. The effluent from the latter was returned to the solvent reservoir. A dual-channel recorder (Model 385; Linear, Irvine, Calif., U.S.A.) was attached to the output of both detectors.

RESULTS AND DISCUSSION

Table I lists the eleven androgens separated in a single chromatogram. The chromatogram itself, developed at a flow-rate of 0.4 ml/min, is shown in Fig. 1. The Δ^4-3-ketones (III, VIII, IX) show a strong absorption at 254 nm. As little as 30 ng of testosterone (IX) can be detected. The 17-ketosteroids are detectable by their absorption at 280 nm, but the sensitivity is only about one-fiftieth of that for the Δ^4-3-ketones. The load capacity of our column, tested with etiocholanolone (X), is 12 mg. The amounts of each steroid in the mixture and other details of chromatographic conditions are given in the legend of Fig. 1.

TABLE I

ANDROGENS SEPARATED BY HPLC

Sample No.	*Systematic name*	*Trivial name*
I	5α-Androstane-3,17-dione	Androstanedione
II	5β-Androstane-3,17-dione	Etiocholanedione
III	4-Androstene-3,17-dione	Androstenedione
IV	3β-Hydroxy-5β-androstan-17-one	Epietiocholanolone
V	3β-Hydroxy-5-androsten-17-one	Dehydroepiandrosterone
VI	3β-Hydroxy-5α-androstan-17-one	Epiandrosterone
VII	3α-Hydroxy-5α-androstan-17-one	Androsterone
VIII	1,4-Androstadiene-3,17-dione	Androstadienedione
IX	17β-Hydroxy-4-androsten-3-one	Testosterone
X	3α-Hydroxy-5β-androstan-17-one	Etiocholanolone
XI	17α-Hydroxy-4-androsten-3-one	Epitestosterone

As a rule, the resolution of epimeric steroids by adsorption chromatography is better than that afforded by reversed-phase partition chromatography, except in the case of 17-epimers[3,4]. The C_{19} steroids in our chromatogram fall into three groups: (1) diketones, (2) 3-hydroxy-17-ketones, and (3) 17-hydroxy-3-ketones. As expected, group I (I–III) is first to emerge from the column, followed generally by group 2 (IV–VII) and then group 3 (IX and XI). However, there is some overlap between the three groups: the dienedione VIII, which is much more polar than the monoenedione III, falls between groups 2 and 3, and etiocholanolone (X), a member of group 2, falls between the two 17-hydroxy-3-ketones.

The elution of the A/B *trans*-steroid I before the A/B *cis*-steroid II is contrary to our experience with the 5α- and 5β-cholestan-3-ones[5]. Although the A/B *cis*-steroid with the axial hydroxyl group (IV) is the first compound in group 2 to be eluted, as one would predict[3], the steroids with equatorial hydroxyl groups (V and VI) are less strongly adsorbed than the axial epimer (VII), contrary to expectation. How-

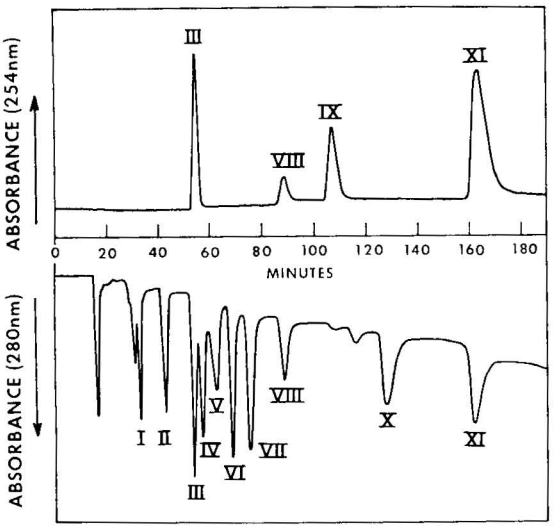

Fig. 1. Elution pattern of androgens. A mixture of 37 μg I, 39 μg II, 34 μg III, 196 μg IV, 188 μg V, 210 μg VI, 214 μg VII, 6 μg VIII, 42 μg IX, 297 μg X, and 99 μg XI was applied by stop-flow to a Partisil 5 column, consisting of two sections (300 × 4.6 mm I.D.). For the identity of I–XI, see Table I. Eluent; dichloromethane–acetonitrile–2-propanol (179:20:1); flow-rate, 0.4 ml/min; pressure, 800 p.s.i. The effluent passed through two detectors in series, the first one set at 280 nm, range 0.1, and the second one at 254 nm, range 1.28. The dual-pen recorder was set at 20 mV for the 254-nm range (top) and at 5 mV for the 280-nm range (bottom). Chart speed, 10 cm/min.

ever, this sequence agrees with the one obtained by triple development of a thin-layer chromatogram on alumina with benzene–diethyl ether (1:1)[6].

The separation of 17-epimers by thin-layer chromatography is difficult[7], but it can be accomplished by partition chromatography on paper[8]. In contrast to paper chromatography, where the 17β-epimer is invariably more polar than the 17α-epimer[3], we find in adsorption HPLC that the 17β-hydroxysteroid testosterone (IX) is eluted before epitestosterone (XI).

REFERENCES

1 J.-T. Lin, W. M. Proebsting and E. Heftmann, *Phytochemistry*, in press.
2 E. Heftmann, *Chromatography of Steroids*, Elsevier, Amsterdam, 1976, p. 87.
3 E. Heftmann, *J. Liquid Chromatogr.*, in press.
4 E. Heftmann and I. R. Hunter, *J. Chromatogr.*, 165 (1979) in press.
5 I. R. Hunter, M. K. Walden, G. F. Bailey and E. Heftmann, *Lipids*, in press.
6 F. Galletti, in C. Cassano (Editor), *Research on Steroids, Trans. Int. Study Group Steroid Hormones, 2nd Meeting, Rome, 1965*. Pensiero Scientifico, Rome, 1966, p. 189.
7 E. Heftmann, *Chromatogr. Rev.*, 7 (1965) 179.
8 E. Heftmann, *Chem. Rev.*, 55 (1955) 679.

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CHROM. 11,925

Note

Determination of α-tocopherol in animal feedstuffs using high-performance liquid chromatography with spectrofluorescence detection

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The importance of vitamin E in animal nutrition has been well established. Of its various molecular forms, α-tocopherol has been recognised as the most biologically important. A comprehensive review has been published by Moustgaard and Hyldgaard-Jensen[1].

Many methods for the determination of the tocopherols in feedstuffs have been introduced, most of them based on separation of the tocopherols by column, paper, or thin-layer chromatography, followed by a colorimetric reaction.

The separation steps, however, are usually laborious and time consuming, and the colorimetric reactions such as that of Emmerie and Engel[2], are often subject to interference from other compounds[3].

Recently, Vatassery and Hagen[4] published a study on the determination of α-tocopherol in brain samples using high-performance liquid chromatography (HPLC) and Vatassery *et al.* have also described the separation of other tocopherols[5] using this technique. Furthermore De Leenheer *et al.*[6] has described the determination of serum α-tocopherol using HPLC.

In the present paper we describe the application of HPLC to the determination of α-tocopherol in animal feedstuffs, such as hay, meal, barley, silage, etc. The method described consists of three main steps *viz.* extraction, saponification, and chromatography.

EXPERIMENTAL

Apparatus

The high-performance liquid chromatograph pump (Model 6000), injector (Model UK6) and column (μBondapak C_{18}, 300 × 3.9 mm) were purchased from Waters Assoc., Hartford, Great Britain. The fluorescence spectrophotometer (Model 204) and flow cell were purchased from Perkin-Elmer, Beaconsfield, Great Britain. The chart recorder (type CR160) was purchased from J. J. Lloyd Instruments, Southampton, Great Britain.

Reagents

AnalaR grade ascorbic acid, potassium hydroxide, methanol, ethanol, and

diethyl ether were used untreated, and were obtained from Hopkin and Williams, Ramford, Great Britain. *n*-Hexane obtained from BDH, Poole, Great Britain, was re-distilled before use to remove fluorescent impurities; D-α-tocopherol was obtained from Eastman-Kodak, Rochester, N.Y., U.S.A. and used without further treatment. Gelatin-coated beadlets of tocopherol acetate feed supplement (Rovimix E25) were obtained from Roche, Dunstable, Great Britain. Barley, hay, grass silage, pig meal etc. were samples submitted for routine α-tocopherol analyses.

Procedure

Extraction. Samples, *e.g.* barley, hay, and meal, were milled before use and wet samples such as silage were finely chopped and mixed. Samples of 5 g were weighed into 125-ml PTFE, wide-mouth bottles and 10 ml of 5% ascorbic acid in 0.1 *N* hydrochloric acid was added, followed by 10 ml ethanol. The bottles were shaken for 15 min in a reciprocating shaker. A 40-ml volume of water and 40 ml of diethyl ether were added, and the samples were again shaken for 1 h, followed by centrifugation at 2000 *g* for 10 min. The upper ether layer was transferred to a 150-ml round-bottomed flask and an additional 40 ml of ether was added to the bottles, which were then shaken for a further 30 min. The bottles were again centrifuged, and the ether layers were combined with those from the first extraction. The ether extracts were reduced in volume to about 5 ml using a rotary film evaporator, and transferred to 100 × 16 mm test tubes, fitted with ground-glass stoppers.

Saponification. The ether was evaporated from the extracts under the stream of nitrogen using a hot water bath (50°) and 3 ml 2% ascorbic acid in ethanol was added. The tubes were placed in a 70° water bath and allowed to equilibrate for a few minutes. A 0.7-ml volume of 60% potassium hydroxide in water was added and the tubes were heated for 15 min at 70°. They were then cooled and 4 ml *n*-hexane and 3 ml distilled water was added and the tubes were shaken vigorously for 1 min, followed by low-speed centrifugation (1000 *g*) for 5 min. The hexane layer was removed for chromatography.

Chromatography. The mobile phase used in the HPLC was methanol–water (95:5). The flow-rate was 3 ml/min. Fluorescence detection was used with an excitation wavelength of 296 nm and emission wavelength of 330 nm.

The standard consisted of 20 μg/ml α-tocopherol in *n*-hexane. 50 μl of this solution was injected, as was 20–100 μl of the unknown sample extracts, depending on the concentration of α-tocopherol. Concentration was related to the height of the fluorescent peaks. A typical trace using different samples is shown in Fig. 1.

RESULTS

Recovery experiments were carried out to check the extraction and saponification steps in the assay. Free α-tocopherol, α-tocopherol acetate, and gelatin-coated beadlets of α-tocopherol acetate were added to a meal sample and carried through the whole procedure. Results are shown in Table I. Recoveries range from 80–92%.

Table II shows a comparison between the wet extraction procedure described, and a standard Soxhlet extraction procedure, where the samples were extracted for 2 h in a Soxhlet apparatus, using diethyl ether. It can be seen that the Soxhlet extrac-

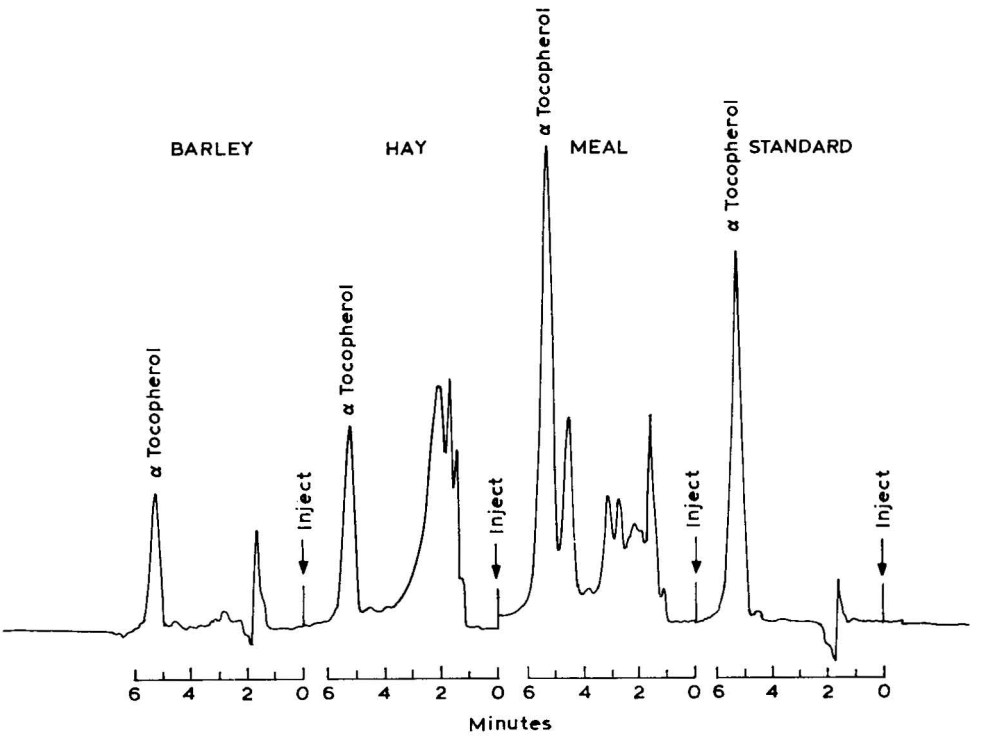

Fig. 1. Typical chromatograms of α-tocopherol standard (1 μg) and three feed extracts.

tion gave generally lower results, particularly for the gelatin beadlet supplemented sample, and also the silage sample.

Table III shows the results of replicate analyses of a meal sample containing natural tocopherol, and also with 20 ppm tocopherol acetate added in the form of gelatin coated beadlets.

TABLE I

RESULTS FROM A MEAL SAMPLE SUPPLEMENTED WITH VARIOUS FORMS OF α-TOCOPHEROL

Sample in duplicate	*α-Tocopherol found (ppm)*	*Recovery (%)*
Meal	5.0	—
	4.8	—
Meal + 100 ppm free α-tocopherol	85.0	80.1
	86.6	81.7
Meal + 100 ppm α-tocopherol acetate	90.2	85.3
	87.1	82.2
Meal + 100 ppm α-tocopherol acetate as gelatin-coated beadlets	88.2	83.3
	96.7	91.8

TABLE II

COMPARISON OF THE WET EXTRACTION PROCEDURE USED IN THE METHOD, WITH SOXHLET EXTRACTION

Sample	*Wet extraction α-tocopherol (ppm)*	*Soxhlet extraction α-tocopherol (ppm)*
Hay	3.5	3.5
	3.7	2.5
Meal	5.4	4.5
	5.4	4.3
Meal supplemented with 100 ppm tocopherol acetate as gelatin beadlets	88	18
	91	10
Silage	28.5	4.2
	24.2	8.7

TABLE III

RESULTS FROM REPLICATE ANALYSIS ($n = 5$) OF A MEAL SAMPLE WITH AND WITHOUT ADDED TOCOPHEROL

Sample	*Mean α-tocopherol (ppm)*	*Range*	*S.D.*	*Coefficient of variation (%)*
Meal	5.36	5.2–5.5	±0.11	2.0
Meal + 20 ppm tocopherol acetate as gelatin coated beadlets	23.3	22.5–24.3	±0.72	3.1

DISCUSSION

The method described has been developed to analyse a wide variety of animal feedstuffs which may contain only naturally occurring tocopherols, or may be supplemented with additional tocopherol in the form of free tocopherol, tocopherol acetate, or gelatin beadlets containing tocopherol acetate. The initial shaking with ethanol–hydrochloric acid prior to extraction was used to dissolve gelatin off any supplement beadlets present. It would also appear that this method of extraction improves the release of tocopherol from plant material, compared with the standard Soxhlet extraction. Ascorbic acid was added to prevent oxidation of the tocopherols. The wet extraction procedure was particularly suitable for supplemented samples and also for wet samples such as silage or grass. Further drying may destroy some of the tocopherols present and is therefore not used in the present procedure. Dry matter determinations were carried out separately. The standard Soxhlet extraction procedure was shown to be unsatisfactory for gelatin beadlet supplemented or wet samples (Table II).

The saponification step was necessary to convert any α-tocopherol acetate to the free form, and also to remove fats and other materials which may cause interference in the chromatographic stage.

Fluorescence rather than ultraviolet detection was used since we found that other compounds with similar retention values to the α-tocopherol caused considerable interference when absorbance was measured at 280 nm. The fluorescence trace

on the other hand, showed clean peaks free from interference of other compounds. The peak preceeding the α-tocopherol has been shown to consist of γ- and β-tocopherols[6], and these two forms cannot be separated using the μBondapak C_{18} column. If required it may be possible to resolve the tocopherols using the column system described by Vatassery *et al.*[5].

The method described has been used in this laboratory for several months and has shown itself to produce fast and accurate results with a minimum of steps. Emulsification of the extract was not found to be a problem compared to some other wet extraction procedures *e.g.* diethyl ether. Recoveries from a variety of samples has been in the region of 80–90%. We have only used the method for animal feedstuffs to date, but it should similarly enable the analysis of a wide range of food in the human nutritional field to be carried out.

REFERENCES

1 J. Moustgaard and J. Hyldgaard-Jensen (Editors), *Acta Agr. Scand., Suppl.*, 19 (1973) 1.
2 A. Emmerie and C. Engel, *Nature (London)*, 142 (1938) 873.
3 W. Hjarde, E. Leerbeck and T. Leth, *Acta Agr. Scand., Suppl.*, 19 (1973) 87.
4 G. T. Vatassery and D. F. Hagen, *Anal. Biochem.*, 79 (1977) 129.
5 G. T. Vatassery, V. R. Maynard and D. F. Hagen, *J. Chromatogr.*, 161 (1978) 299.
6 A. P. de Leenheer, V. O. de Bevere, A. A. Cruyl and A. E. Claeys, *Clin. Chem.*, 24 (1978) 585.

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CHROM. 11,988

Note

Thin-layer chromatographic separation of uracil and dihydrouracil

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The first reaction of uracil degradation is catalysed by uracil reductase and dihydrouracil dehydrogenase, respectively (E.C. 1.3.1.1, E.C. 1.3.1.2)*. Generally its activity is measured spectrophotometrically by oxidation of NADPH or NADH[1] and is visualized with nitroblue tetrazolium[2]. However, in organisms with low activity of uracil reductase, high sensitivity of radiometric methods is required. Quantitative separation of uracil and dihydrouracil is a prerequisite to this determination. Because of restricted resolution of pyrimidines on Sephadex G-10 in gel chromatography[3], on Dowex-1 in ion-exchange chromatography[4] and in paper chromatography[5], separation of uracil and dihydrouracil or thymine and dihydrothymine by thin-layer chromatography (TLC) seems to be the most valuable method[6]. Unfortunately, use of solvent systems described previously[6,7] results in incomplete separation of uracil, dihydrouracil and their degradation product β-ureidopropionic acid, and thymine and dihydrothymine, respectively.

Therefore, adsorption chromatography on Kieselgel D (VEB Chemiewerk, Greiz-Dölau, G.D.R.) was used and modified to give high resolution of uracil and dihydrouracil. The solvent system chloroform–methanol–acetic acid (100:5:1)[6] was used in our studies. TLC plates (20 × 20 cm, 0.5 mm thick) were prepared with Kieselgel D (VEB Chemiewerk, Greiz-Dölau, G.D.R.) activated (10 min at 110°). Separation was performed continuously in an open sandwich chamber (3 mm distance of the plates) at a low temperature (4°) for 12 h. For evaporation of the solvents, chromatographic paper 4 × 20 cm, Filtrak FN 7 (VEB Spezialpapier, Niederschlag, G.D.R.) was placed on the upper edge of adhering adsorbent. [2-^{14}C]Uracil (UVVVR, Prague, Czechoslovakia), [2-^{14}C]dihydrouracil (Rotop, Dresden, G.D.R.), [ureido-^{14}C]propionic acid (prepared from [2-^{14}C]dihydrouracil by the method of Fink *et al.*[8]) and an unlabelled tracer were applied on TLC plates with glass capillaries giving extremely small starting points.

In Fig. 1 complete separation of uracil, dihydrouracil and β-ureidopropionic acid is demonstrated by their scanner profile. Contrary to earlier TLC of pyrimidines and dihydropyrimidines, the use of continuous conditions with the sandwich tech-

* In accordance with Hallock and Yamada[2] the name uracil reductase will be used for the enzyme catalyzing uracil/thymine → dihydrouracil/dihydrothymine reactions, and the name dihydrouracil dehydrogenase for the enzyme catalyzing the reactions dihydrouracil/dihydrothymine → uracil/thymine.

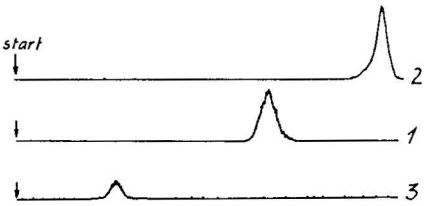

Fig. 1. Separation of uracil (1), dihydrouracil (2) and β-ureidopropionic acid (3) by TLC with Kieselgel D plates in the solvent system chloroform–methanol–acetic acid (100:5:1) at 4° (continuous sandwich conditions, 12 h).

nique and low temperature is of great value. Running time and temperature, determine the separation distance of uracil and dihydrouracil.

REFERENCES

1 P. Fritzson, *J. Biol. Chem.*, 235 (1960) 719.
2 R. O. Hallock and E. W. Yamada, *Anal. Biochem.*, 56 (1973) 84.
3 C. Wasternack, *J. Chromatogr.*, 71 (1972) 67.
4 D. P. Agarwal, K. Eickhoff and H. W. Goedde, *Anal. Biochem.*, 24 (1968) 348.
5 K. Fink and W. S. Adams, *J. Chromatogr.*, 22 (1966) 118.
6 H. W. Goedde, D. P. Agarwal and K. Eickhoff, *Hoppe-Seyler's Z. Physiol. Chem.*, 349 (1968) 1137.
7 G. Knutsen, *Biochim. Biophys. Acta*, 269 (1972) 333.
8 R. M. Fink, C. McGaughey, R. E. Cline and K. Fink, *J. Biol. Chem.*, 218 (1956) 1.

Journal of Chromatography, 176 (1979) 495–496

CHROM. 11,982

Book Review

Contemporary topics in analytical and clinical chemistry, Vol. 2, edited by D. M. Hercules, G. M. Hieftje, L. R. Snyder and M. A. Evenson, Plenum, New York, London, 1978, X + 286 pp., price U.S.$ 33.00, £ 17.33, ISBN 0-306-33522-0.

The five chapters in the second volume of the series deal with a broad spectrum of contemporary analytical chemistry. The first by O'Haver on wavelength modulation spectroscopy gives us a brief introduction to the theoretical and practical aspects of derivative techniques employed in spectroscopy. On the other end of the spectrum a brief, well-written essay is presented on the radioimmunoassay of enzymes by Landon, Carney and Langsley. To the readers of this Journal the remaining chapters are likely to be most interesting. In the field of electrophoresis the employment of lasers and the exploitation of the Doppler effect make it possible to measure directly electrophoretic velocity. Smith and Ware, the pioneers of this approach, which is still in its infancy, give an account of the apparatus and methods involved. The strong interest in everything that is associated with the four letters HPLC may be responsible for the fact that more than two-thirds of the book is directed toward topics associated with this fast growing analytical technique. By far the largest chapter is written by a team of Kissinger on "Detectors for trace organic analysis by liquid chromatography: Principles and applications". In fact it turns out to be an excellent review covering almost all detectors which have found application in modern liquid chromatography. Therefore not only those readers who are interested in trace analysis but all others who want to read about detectors in liquid chromatography will find the chapter interesting.

In many respects the use of sensitive detectors is the key feature of HPLC. The concomitantly low sample concentrations in the eluent (sample load) are mainly responsible that the chromatographic system is quasi-linear and yields high efficiency and reproducibility. The departure from traditional liquid column chromatography with overloaded column has made possible the use of the theory of linear chromatography which was already successfully applied to gas chromatography, and the development of high-performance columns with plate heights much smaller than those of commonly used gas chromatographic columns.

Although well-constructed variable-wavelength photometric detectors are likely to remain the workhorses in HPLC, novel specific and catholic detectors would be desirable. Alternatively, detectors with much smaller sensing volumes than those found in the present instruments would be needed. It is hoped that this review will stimulate further progress in this highly important field.

Whereas gas chromatography has not really made it as a widely used analytical technique in clinical laboratories, the star of HPLC is much brighter in those places and there is a great expectation that HPLC will contribute to the revolution we have witnesses over the recent years. The chapter entitled "Clinical liquid chromatography",

written by Snyder, Karger and Giese, offers an overview of the present, though, still embryonic, state of art. One can argue that right now there is only one liquid chromatography and it is applied to clinical chemistry. The title of the chapter would be germane to a clinical liquid chromatograph, which would be built to meet the needs of clinical analysis and therefore would be different from liquid chromatographs in the general laboratory. Yet such a device is still not available to the chagrin of the reviewer and probably many clinical chemists. We hope that the team of Snyder, which appears to be strongly committed to the pioneering work to put an HPLC instrument in clinical laboratories, will succeed soon. Their chapter deals with the problems of clinical analysis and with their relation to HPLC. It is written with high competence and can serve as an excellent reading material for clinical chemists who want to explore the potential of HPLC in their field, or by chromatographers who are interested in the specific problems of clinical analysis which could be solved by an appropriately designed HPLC system. A caveat, however, is necessary. The chapter, although written in a practice oriented way, is not a cook book to give detailed descriptions of liquid chromatographic procedures for clinical analyses.

The book is well produced and fortunately does not live up to the promise given in the blurb which claims that "each article offers authoritative predictions concerning future improvements in techniques and further developments in their applications." The authors were competent and wise enough to ignore such a foolish pledge and fortunately kept away from casting a horoscope. Finally, no expert would try to embark on such a foretelling exercise without some specific reason. In any case, hardly a reader will cavil at the book not living up to the blurb. She or he will be pleased to get a balanced and well-written account of methods emerging at the frontiers of analytical chemistry.

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Author Index

Errata

J. Chromatogr., 170 (1979) 343–353

The composition of solvents A and B should be: A, water–acetic acid–PIC A (98.35:1.50:0.15); B, methanol–acetic acid–PIC A (98.35:1.50:0.15).

J. Chromatogr., 174 (1979) 97–107

Pages 104 and 105: the structural formulas for 1,2-di-*n*-propylbenzene and 2-alkyl-3-propyloxirane should be interchanged.

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1 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
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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