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USE OF CONVENTIONAL INSTRUMENTATION WITH MICROBORE COLUMNS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method to determine extra-column band broadening in microbore high-performance liquid chromatography (HPLC) (column diameter, *ca.* 1 mm) is developed. The extra-column band broadening of several commercially available UV detectors is compared to that of a home-made electrochemical detector. A trace-enrichment method for microbore HPLC is presented and applied to the determination of a series of aromatic nitro compounds. The effect of miniaturization of HPLC on detection limits is discussed.

INTRODUCTION

In the work on miniaturization of high-performance liquid chromatography (HPLC) several approaches can be discerned according to the type of columns used: open tubular capillary columns, with an internal diameter of 30–120 μm (refs. 1 and 2); packed columns with a diameter of *ca.* 0.1 mm, which are called packed capillary[3–5] or ultra-micro[6] columns, depending on their length; and microbore columns. which have an internal diameter in the range of 0.5–2.0 mm (refs. 7–11).

Various advantages of miniaturized HPLC have been mentioned, *e.g.* the reduction of the cost of packing material and of solvents used as mobile phase[9], the possibility of a direct coupling of HPLC and mass spectrometry (MS)[2,8], the generation of very high plate numbers[3,4,9], high-speed separation[9,11], and a reduction of detection limits[10]. Lower detection limits can be reached because a reduction of column dimensions leads to smaller peak volumes. However, since the maximum allowable sample volume is proportional to the column cross-section, a reduction of the column diameter is only significant in this respect when the amount of sample available is limited.

In all studies on miniaturized HPLC much attention is paid to the construction

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of suitable equipment in order to reduce the extra-column band broadening, which can be considerable when conventional instrumentation is used. We have examined the potential and limitations of the use of conventional instrumentation for the separation of aromatic nitro compounds with miniaturized HPLC. Since it is evident that, on further reducing column dimensions, the instrumental requirements will increase, we have opted for the use of microbore columns with an internal diameter of 1.1 mm. Special attention has been given to the comparison, for microbore HPLC, of a home-made dropping mercury electrode (d.m.e.) detector with that of a number of commercially available UV detectors.

A simple trace-enrichment method has been elaborated which allows the use of relatively large sample volumes. The method has been applied to the determination of nitrobenzene in river water and of 4-nitrophenol in urine.

EXPERIMENTAL

Apparatus

Mobile phase flow-rates between 50 and 250 μl/min were delivered by an Altex (Berkeley, CA, U.S.A.) Model 100 A pump or a one-stroke (300 ml) syringe pump made in our workshop. The samples were injected with a Rheodyne (Berkeley, CA, U.S.A.) Model 089-0932 valve with a 175-μl loop and an appropriate restrictor, through which 12% of the mobile phase flows in the "inject" position of the valve. All connections were made of stainless steel capillary of 1/16 in. O.D. and 0.25 mm I.D.

In Table I the UV detectors used are listed, together with their cell volumes. In order to facilitate comparison, all detectors were used at 254 nm. Though this is not the most suitable wavelength for all the aromatic nitro compounds studied, nitrobenzene has an absorption maximum at 251 nm and all other compounds have molar absorptivities of over 10^3 at 254 nm (ref. 12).

TABLE I

UV DETECTORS EXAMINED

Manufacturer	*Model*	*Cell volume (μl)*
Perkin-Elmer (Norwalk, CO, U.S.A.)	LC 55	8
Pye Unicam (Cambridge, Great Britain)	LC 3	8
Waters (Milford, MA, U.S.A.)	440	12.5
Zeiss (Oberkochen, G.F.R.)	PM 2 DLC	8

For electrochemical detection use was made of a flow-through polarographic cell with a horizontally placed mercury capillary, which has been described earlier[13]. Drop times were between 0.1 and 0.2 sec. The electrical currents were measured with a home-made potentiostat/amplifier. Electronic dampening with a time constant of 1 sec was applied. Potentials were measured against a Ag/AgCl/1 *M* LiCl, methanol–water (50:50) reference electrode.

Reagents

Distilled demineralized water and analytical-grade methanol (Baker, Deventer,

The Netherlands) were used as solvents. Concentrated stock solutions of aromatic nitro compounds, obtained from various sources, were prepared in methanol, and samples of the desired composition were made by diluting aliquots of these stock solutions. Sample solutions were degassed in an ultrasonic bath.

Mobile phases, containing 10^{-2} *M* potassium nitrate and 10^{-3} *M* nitric aid, were filtered over a 0.8-μm Millipore (Bedford, MA, U.S.A.) filter and deaerated by purging with nitrogen at 35°C.

Packing of the microbore columns

Microbore columns were made of stainless steel tubing of 1/16 in. O.D. and 1.1 mm I.D. The inside of the tubing was polished with a wet cotton thread and polishing powder. The ends of the column were sealed with 1/16 in. × 0.13 mm frits, made of 2-μm stainless steel gauze, which fitted in Swagelok zero dead volume unions. In some cases it was more convenient to use male unions; then, a Swagelok 1/16–1/16 in. union was made "low dead volume" by inserting into the central hole of the union a piece of 1/16 in. O.D. and 0.3 mm I.D. PTFE capillary, which had been stretched until it just fitted.

The columns were packed with LiChrosorb RP-18 (E. Merck, Darmstadt, G.F.R.) with a mean particle diameter of 7.7 μm. For a 250 mm × 1.1 mm I.D. column 0.3 g of packing material was wetted with 0.3 ml of methanol, and a slurry was made with 3 ml of tetrachloromethane. The slurry was stirred in an ultrasonic bath for 5 min and injected into a 250 mm × 4.6 mm I.D. slurry reservoir. This reservoir, which was connected to the column by a short 1.1 mm I.D. precolumn, was filled with methanol. The column was packed downwards by pumping through methanol for 20 min at 500 bar.

RESULTS AND DISCUSSION

Measurement of the extra-column band broadening

Several methods have been used to calculate extra-column band broadening, *e.g.*, (1) measuring the band width in a system without column[14], (2) measurements on the peak in an actual chromatogram[15], and (3) measuring the band width as a linear function of the retention volume[16]. The first method does not take into account the extra-column band broadening in frits, column ends and connectors. For the second method purely gaussian on-column band broadening is a prerequisite. The third method lacks a theoretical basis. We therefore developed another approach.

The width of a peak due to solute *i* in a chromatogram is determined by on-column and extra-column band broadening according to

$$\sigma_i^2 = \sigma_{c,i}^2 + \sigma_{ec}^2 \tag{1}$$

where σ_i is half the band width at 0.607 of the peak height, and $\sigma_{c,i}$ and σ_{ec} are the on-column and extra-column contributions, respectively, in volume units.

The on-column band broadening is given by

$$\sigma_{c,i}^2 = \frac{H_i}{L} \cdot V_{R,i}^2 \tag{2}$$

where L is the column length, $V_{R,i}$ and H_i are the retention volume, corrected for the extra-column volume, and the plate height for compound i, respectively. The plate height H_i can be evaluated from a Van Deemter equation such as[17]

$$H_i = \frac{2\,D_{m,i}}{T_m u} + \frac{2\,\lambda_2 d_p}{1 + \lambda_1\,(D_{m,i}/u d_p)^{1/2}} + \frac{1}{5.7}\cdot\frac{k_i'^2}{(1 + k_i')^2}\cdot\frac{\varepsilon_m^{1/2} d_p^{3/2} V^{1/6} u^{1/2}}{(1 - \varepsilon_m)\,D_{m,i}^{2/3}} + \frac{1}{30}\cdot\frac{k_i'}{(1 + k_i')^2}\cdot\frac{(1 - \varepsilon_m)\,T_s\,d_p^2\,u}{\varepsilon_s\,D_{s,i}} \quad (3)$$

Assuming equal diffusion coefficients for the compounds studied, for a particular chromatographic system and a given flow-rate, eqn. 3 can be written as

$$H_i = H_a + \frac{k_i'^2}{(1 + k_i')^2}\cdot H_b + \frac{k_i'}{(1 + k_i')^2}\cdot H_c \quad (4)$$

where k_i' is the capacity factor of solute i and H_a, H_b and H_c are constants. Calculation shows that in eqn. 4 (under normal chromatographic conditions) H_b and H_c are of the same order of magnitude, while H_a is the predominant term. Moreover, the second term on the right-hand side of eqn. 4 increases and the third term decreases with k_i' for $k_i' > 1$. The dependence of H_i on k_i' in this range can therefore be expected to be small.

In order to verify the above conclusion, chromatograms were run for mixtures of aromatic nitro compounds at flow-rates of 0.5–2.5 ml/min on a 120 mm × 4.6 mm I.D. column. An adapted Waters Model 440 UV detector was used; the extra-column band broadening of this detector can be neglected in comparison to the on-column band broadening of 4.6 mm I.D. columns (*cf.* below).

Plate heights of the compounds were calculated according to eqn. 2. It appeared that, although the plate heights depended on the column and flow-rate used, the ratios of the plate heights of different compounds did not. This is illustrated in Table II where the plate heights for a number of aromatic nitro compounds are expressed relative to that of the last eluting compound, 3-chloronitrobenzene. For most compounds the differences in plate height are well within experimental error. That is, the tentative conclusion that H_i does not depend strongly on k_i' is fully confirmed. Only the phenolic compounds show significantly different plate heights. Since, for these solutes, relatively large band broadening is accompanied by strong tailing of the peaks, the deviating result is possibly caused by a specific physico-chemical interaction between solute and stationary phase.

Since the plate heights of different compounds are equal or have a constant ratio, we can write for H_i, for all compounds studied

$$H_i = \beta_i\,H_0 \quad (5)$$

where H_0 is a constant for a particular column and flow-rate, and the plate height ratio β_i is unity for the majority of the compounds tested. The values of β_i used for further calculation are listed in Table II.

TABLE II

CHROMATOGRAPHIC CHARACTERISTICS OF MODEL COMPOUNDS

Plate heights expressed relative to that of 3-chloronitrobenzene. Column: 120 × 4.6 mm I.D., packed with 7.7-μm LiChrosorb RP-18. Mobile phase: methanol–water (50:50) + 10^{-2} *M* potassium nitrate + 10^{-3} *M* nitric acid. Flow-rate: between 0.5 and 2.5 ml/min.

Compound	k'_i	*Relative plate height ± S.D.*	*No. of expts.*	β_i
4-Nitroaniline	1.10	0.97 ± 0.14	16	1.0
4-Nitrophenol	2.25	1.17 ± 0.07	11	1.2
1,2-Dinitrobenzene	3.00	1.03 ± 0.12	9	1.0
2,4-Dinitrophenol	3.05	3.1 ± 0.4	4	−*
1,3-Dinitrobenzene	3.80	1.04 ± 0.13	12	1.0
2-Nitrophenol	4.20	1.30 ± 0.11	11	1.3
Nitrobenzene	4.45	0.88 ± 0.10	14	0.9
2-Nitrotoluene	8.25	1.03 ± 0.07	11	1.0
4-Nitrotoluene	9.10	1.02 ± 0.14	9	1.0
3-Nitrotoluene	9.80	1.02 ± 0.09	10	1.0
3-Chloronitrobenzene	11.9	1		1.0

* Not used for calculations.

The extra-column band broadening can now be easily evaluated from a chromatogram. Combining eqns. 1, 2 and 5 yields

$$\sigma_i^2 = \frac{H_0}{L} \cdot \beta_i V_{R,i}^2 + \sigma_{ec}^2 \qquad (6)$$

In other words, σ_{ec}^2 is the intercept obtained from a plot of σ_i^2 *vs.* $\beta_i V_{R,i}^2$ constructed from relevant data of a number of peaks in a chromatogram.

The validity of the method is illustrated in Fig. 1 where several graphs of σ_i^2 *vs.* $\beta_i V_{R,i}^2$ are seen to yield straight lines ($R \geqslant 0.995$). Moreover, the value of the intercept apparently does not depend on the length of the column used. This proves that on-column band broadening cannot have contributed significantly to this intercept or, in other words, that the said intercept can safely be assumed to represent σ_{ec}^2.

The standard deviation of the σ_{ec} values calculated from different chromatograms run under the same conditions was *ca.* 1 μl (n = 6–8).

Detector performance

The extra-column band broadening of four commercially available UV detectors was examined. The detectors were used without modification and the microbore columns were connected to the cell as prescribed by the manufacturer. Chromatograms were run of 1-μl samples of 5–8 nitro compounds and σ_{ec} was calculated as described above. The calculated σ_{ec} includes the contribution to band broadening of the injection side of the system. The contribution of the sample volume itself can, however, be assumed to be negligible, since no significant changes in σ_{ec} were found on varying the sample volume from 0.5 to 2 μl.

Fig. 2 shows that large differences in σ_{ec} occur from one detector to another

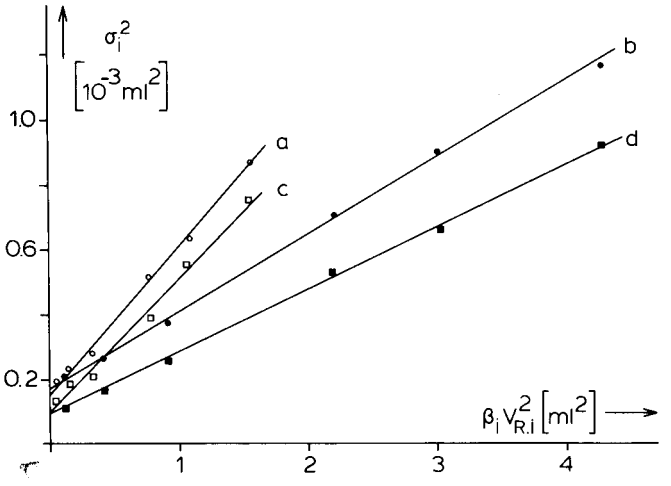

Fig. 1. Plots of σ_i^2 *vs.* $\beta_i V_{R,i}^2$ used for determination of extra-column band broadening. Columns: 1.1 mm I.D. packed with 7.7-μm LiChrosorb RP-18. Mobile phase: methanol–water (50:50) + 10^{-2} *M* potassium nitrate + 10^{-3} *M* nitric acid. Detector, Waters 440. Column length (*L*) and flow-rate (*F*) were as follows: (a) $L = 150$ mm; $F = 190$ μl/min; (b) $L = 250$ mm, $F = 190$ μl/min; (c) $L = 150$ mm, $F = 90$ μl/min; (d) $L = 250$ mm, $F = 90$ μl/min.

which can not be explained by differences in cell volume. For all detectors σ_{ec} increases with flow-rate, which indicates that the laminar flow profile in the flow-through cell and the connecting capillaries is an important source of band broadening[18]. The large σ_{ec} of the Perkin-Elmer LC55 detector is probably caused by the long thermostatting capillary inside the cell housing of this detector.

Since the Waters Model 440 detector combines the highest sensitivity with the smallest extra-column band broadening, this detector was adapted for use with microbore columns in the way described by Hermansson[19]. The optical cell volume was

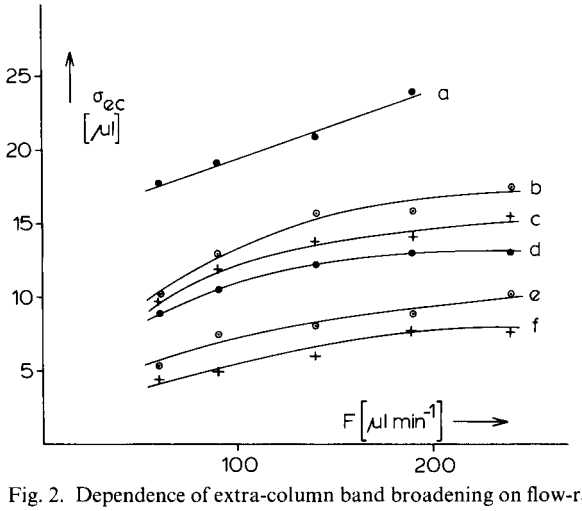

Fig. 2. Dependence of extra-column band broadening on flow-rate for various detectors. (a) Perkin-Elmer LC 55; (b) Zeiss PM 2 DLC; (c) Pye Unicam LC 3; (d) Waters 440; (e) adapted Waters 440; (f) d.m.e.

decreased to 0.7 μl by inserting conically ground pieces of PTFE capillary (0.3 mm I.D.) into the sample and reference cell, and the column was connected as close as possible to the cell. Fig. 2 shows that these adaptations considerably reduce the extra-column band broadening. The consequent gain in resolution was, however, accompanied by a deterioration of the detection limits: although modifying the cell did not influence the detector sensitivity much, the noise increased from $0.05 \cdot 10^{-3}$ to $0.15 \cdot 10^{-3}$ a.u. (see Table III). Therefore the improvement of detection performance which can be obtained by changing from conventional (4.6 mm I.D.) to microbore columns is partially lost when, for resolution's sake, a detector adaptation as described above is required.

TABLE III

DETECTION LIMITS OF NITROBENZENE

Column: 250 × 1.1 mm I.D. packed with 7.7-μm LiChrosorb RP-18. Mobile phase: methanol–water (50:50) + 10^{-2} *M* potassium nitrate + 10^{-2} *M* nitric acid. Sample volume: 1 μl.

Detector	*Signal/ng*	*Noise*	*Detection limit* (ng)*
Waters 440	$1.28 \cdot 10^{-3}$ a.u.	$0.05 \cdot 10^{-3}$ a.u.	0.08
Adapted Waters 440	$1.08 \cdot 10^{-3}$ a.u.	$0.15 \cdot 10^{-3}$ a.u.	0.28
D.m.e.**	7.88 nA	0.5 nA	0.13

* At signal-to-noise ratio of 2:1.

** The signal per ng nitrobenzene and the noise, but not the detection limit, depend on the mercury capillary used.

Relative to UV detection, electrochemical detection has a greater potential in miniaturization of HPLC since there is no need for a "cell path volume" as with spectrometric detection. With, for instance, the d.m.e. detector used in the present work, the column effluent flows via a short capillary directly onto the mercury drops. As indicated in Fig. 2, the extra-column band broadening of this detector is smaller than that of any of the UV detectors studied. As for sensitivity towards the aromatic nitro compounds the d.m.e. detector is comparable to the Waters Model 440 UV detector. As a typical example, detection limits for nitrobenzene with these detectors are given in Table III.

Trace enrichment

The maximum sample volume that can be used without significantly increasing band broadening is proportional to the cross-sectional area of the chromatographic column. With microbore columns this maximum is only one or a few microlitres[20]. Since in practice the amount of sample available is generally much larger the introduction of a trace-enrichment step will often be advantageous.

Scott and Kucera[10] have developed a trace-enrichment method that is based on the use of two (one open and one packed) sample loops; here, an additional (sampling) pump and an extra six-port valve are required. Ishii *et al.*[21] used an off-line method: samples are concentrated on a pre-column which is subsequently inserted prior to the analytical column.

In the present work we have modified our previously published[22] on-line trace-enrichment procedure, which combines a minimum need for extra apparatus with easy handling.

Set up. The design of the pre-column used for trace enrichment is shown in Fig. 3. The pre-column, which has the same internal diameter as the analytical column, was hand-packed with 7.7 μm LiChrosorb RP-18. The length of the plug of packing material is easily varied by changing the length of the PTFE capillary inside the pre-column. The pre-column and analytical column are connected by a Swagelok T-piece (1/16 in.) which is made "low dead volume" by inserting a piece of 0.3 mm I.D. PTFE capillary with a V-shaped cut in the middle. The third branch of the T-piece is connected to a valve.

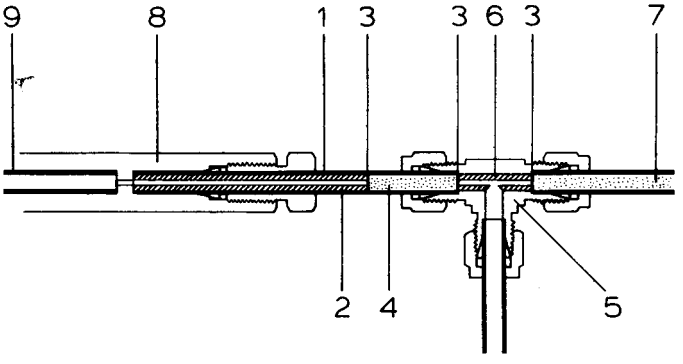

Fig. 3. Construction of pre-column for trace-enrichment studies. 1 = Pre-column, 35 mm × 1/16 in. O.D. (1.1. mm I.D.); 2 = PTFE capillary, 0.3 mm I.D.; 3 = frits; 4 = packing material; 5 = Swagelok T-piece; 6 = PTFE capillary with V-shaped cut; 7 = analytical column; 8 = Swagelok zero dead volume union (1/16–1/16 in.); 9 = to injection valve; 10 = to valve V.

Procedure. A scheme of the trace-enrichment procedure is presented in Fig. 4. The 175-μl loop on the injection valve is rinsed with deaerated water containing 10^{-2} *M* potassium nitrate and 10^{-3} *M* nitric acid. Next, a 100-μl sample is injected (Fig. 4a), the valve V connected to the T-piece is opened, the pump switched on, and the sample loop connected on-line with the mobile-phase flow. Since 12% of this flow is passing through the restrictor of the injection valve, the sample is eluted through the pre-column as a 6% solution in methanol if the mobile phase contains 50%-methanol (Fig. 4b). The presence of 75 μl of water in the last (*i.e.*, the left-hand) part of the sample loop causes non-retarded components present in the sample to be rinsed automatically from the pre-column. The volume of effluent from the pre-column is measured in a syringe connected to valve V. After the passage of 200 μl of effluent, valve V is closed, and the solutes concentrated on the pre-column are eluted and separated on the analytical column (Fig. 4c).

At regular intervals, the pre-column is rinsed with 175 μl methanol to remove strongly retained compounds.

Results. Recoveries of the trace-enrichment method were determined by comparing peak areas with those obtained in the normal injection mode. The results are given in Table IV. As can be seen from this table, if a 10 mm long pre-column is used, compounds with $k' \geq 2$ in methanol–water (50:50) are recovered for over 90%.

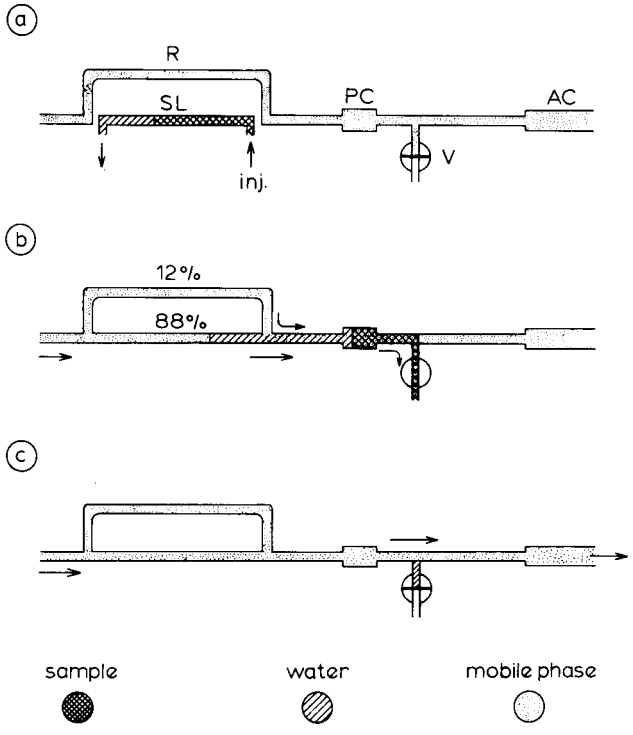

Fig. 4. Scheme of trace-enrichment procedure. SL = sample loop; R = restrictor; PC = pre-column; AC = analytical column; V = valve.

Application of the trace-enrichment method affects the performance of the analytical column negatively. After even a few injections made in the trace-enrichment mode, the plate number of the analytical column was found to drop from,

TABLE IV

RECOVERIES OF MODEL COMPOUNDS IN TRACE-ENRICHMENT STUDIES

For experimental details, see text. Each result is the average of 2–3 experiments.

Compound	k_i' *in methanol–water (50:50)*	*% Recovery on*	
		5-mm pre-column	*10-mm pre-column*
4-Nitroaniline	1.10	9	60
4-Nitrophenol	2.25	70	94
1,2-Dinitrobenzene	3.00	96	103
1,3-Dinitrobenzene	3.80	94	–
2-Nitrophenol	4.20	96	96
Nitrobenzene	4.45	95	108
2-Nitrotoluene	8.25	103	99
4-Nitrotoluene	9.10	97	104
3-Nitrotoluene	9.80	107	100
3-Chloronitrobenzene	11.9	99	99

typically, 5000 to 3500 (flow-rate, 140 μl/min). Fortunately, the plate number then remained stable for a period of a few weeks.

Extra-column band broadening does not materially increase when using a pre-column for trace enrichment. Although the total extra-column volume now is larger, band broadening at the injection side becomes less important, since the solutes are concentrated on top of the pre-column. Strictly speaking, eqn. 6 cannot be applied to calculate σ_{ec}, since the retention volumes, $V_{R,i}$, are not defined exactly in the trace-enrichment mode. If, however, for $V_{R,i}$ the values of the normal injection mode are substituted, straight lines ($R \geqslant 0.994$) are obtained in a σ_i^2 *vs.* $\beta_i V_{R,i}^2$ plot. With a flow-rate of 140 μl/min a value for σ_{ec} of 8.4 μl was found for the adapted Waters Model 440 detector (as against a value of 8.0 μl in the normal injection mode) with a σ_{ec} value of 6.1 μl for the d.m.e. detector (as against a value of 6.0 μl in the normal injection mode).

Applications

Nitrobenzene in river water. Nitrobenzene is a pollutant frequently occurring in river water. In the river Rhine, concentrations of up to 30 ppb have been found[23].

Water samples from the river Amstel were filtered over a Millipore filter and potassium nitrate (10^{-2} *M*) and nitric acid (10^{-3} *M*) were added. Samples of volume 100 μl were concentrated as described above and, next, eluted with methanol–water (50:50). The adapted Waters Model 440 UV detector and the d.m.e. detector were used.

The chromatograms of river water samples spiked with nitrobenzene show that the detection limit both the UV and the electrochemical detector is *ca.* 2 ppb. The nitrobenzene level of the Amstel water obviously is below this detection limit (Fig. 5).

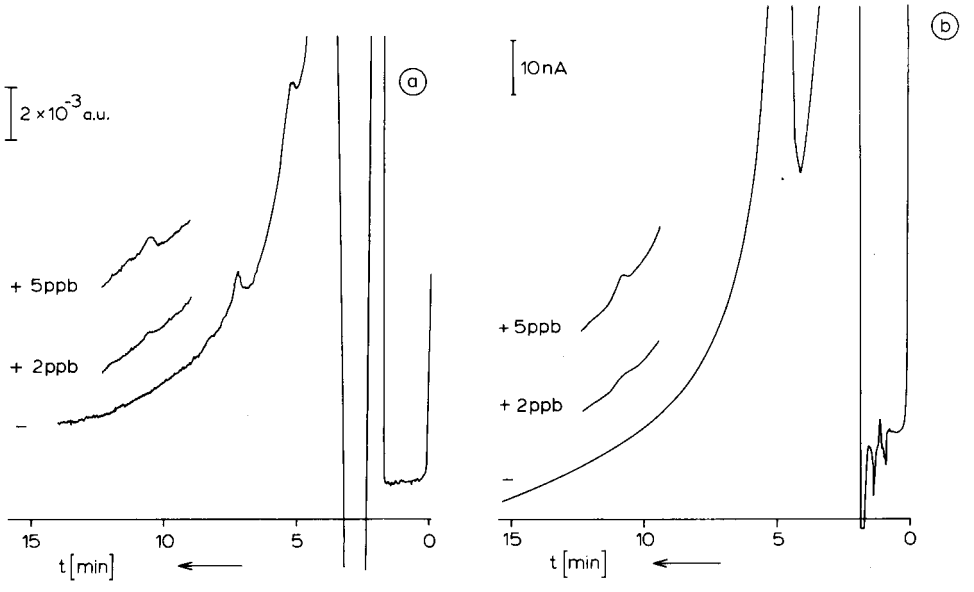

Fig. 5. Determination of nitrobenzene in river water. Column: 250 × 1.1 mm I.D. packed with 7.7-μm LiChrosorb RP-18. Mobile phase, methanol–water (50:50) + 10^{-2} *M* potassium nitrate + 10^{-3} *M* nitric acid; flow-rate, 80 μl/min; 100-μl sample without (–) and with (2 or 5 ppb) added nitrobenzene. (a) Adapted Waters 440 detector (254 nm); (b) d.m.e. detector; $E = -800$ mV.

Interferences due to the tailing solvent or oxygen peak can be eliminated by changing the mobile phase composition to methanol–water (40:60). Nitrobenzene now has a k' value of 8.0; however, since the peak height is considerably reduced, the detection limit does not improve.

4-Nitrophenol in urine. Exposure to the pesticide parathion can be detected by the occurrence of its degradation product 4-nitrophenol in urine. The average concentration of 4-nitrophenol in human urine (U.S.A.), as measured with a derivatization–gas chromatographic technique[24], is 12–26 ppb. After exposure to parathion, concentrations of 18–67 ppb were found. With another derivatization technique even larger differences between exposed and non-exposed subjects have been recorded[25].

In the present study urine samples were filtered over a Millipore filter, 10^{-2} *M* potassium nitrate was added and nitric acid to pH = 3. After ultrasonic degassing, 100-μl samples were pre-concentrated and, next, eluted with methanol–water (40:60). Detection by means of UV absorption at 254 nm was not possible because of very strong interferences. Fig. 6 shows the chromatogram of a urine sample with d.m.e. detection. The concentration of 4-nitrophenol in this sample is apparently less than the detection limit of 25 ppb.

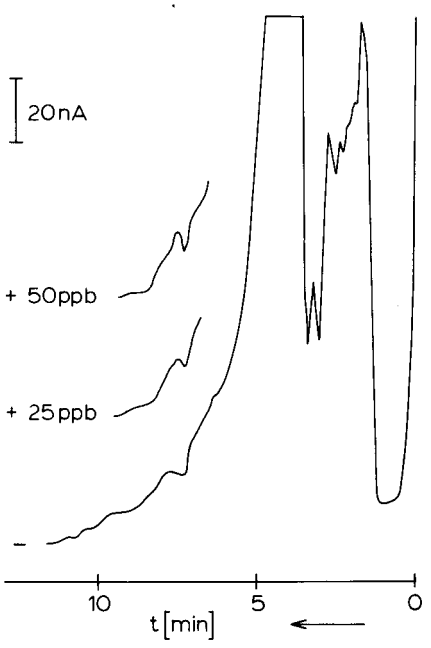

Fig. 6. Determination of 4-nitrophenol in urine. Column, 250 × 1.1 mm I.D. packed with 7.7-μm Li-Chrosorb RP-18. Mobile phase, methanol–water (40:60) + 10^{-2} *M* potassium nitrate + 10^{-3} *M* nitric acid; flow-rate, 100 μl/min; 100-μl sample without (−) and with (25 or 50 ppb) added 4-nitrophenol. D.m.e. detector; $E = -800$ mV.

For the present determination, the peak-height precision in the range 50–200 ppb of added 4-nitrophenol was $\pm 10\%$ (n = 9).

CONCLUSIONS

The use of conventional UV detectors for HPLC on microbore (*ca.* 1 mm I.D.) columns causes considerable extra-column band broadening. Fortunately, this detrimental effect can largely be suppressed by a simple modification of the detector cell, and by reducing the length of connective tubing as much as possible. The electrochemical detector studied is well suited for use in microbore HPLC, as is evident from the σ_{ec} and calculated α_{min} (minimum required relative retention) data summarized in Table V.

TABLE V

BAND BROADENING (σ_{ec}) AND MINIMUM REQUIRED RELATIVE RETENTION (α_{min}) DATA FOR VARIOUS DETECTORS IN NORMAL-INJECTION AND TRACE-ENRICHMENT EXPERIMENTS

Column: 250 × 1.1 mm I.D.; column dead volume, 160 μl; flow-rate, 140 μl/min.

Detector	σ_{ec} (μl)	α_{min} (R_s = 1.0) for peaks with k'_i =		
		1	*3*	*10*
Normal-injection mode (N = 5000)				
Theoretical minimum	–	1.11	1.075	1.062
Perkin-Elmer LC 55	20.9	1.53	1.19	1.081
Pye Unicam LC 3	13.8	1.36	1.14	1.071
Waters 440	12.1	1.32	1.13	1.069
Zeiss PM 2 DLC	15.7	1.41	1.15	1.074
Adapted Waters 440	8.0	1.23	1.10	1.065
D.m.e.	6.0	1.19	1.09	1.064
Trace enrichment (N = 3500)				
Theoretical minimum	–	1.14	1.09	1.074
Adapted Waters 440	8.4	1.25	1.11	1.077
D.m.e.	6.1	1.20	1.10	1.076

A decrease of detection limits is often put forward as one of the advantages of miniaturized HPLC. One should realise, however, that modifying the flow cell of a conventional UV detector (*cf.* above), which may be necessary to improve resolution, increases the baseline noise and, thus, effects a decrease of signal-to-noise ratios. Improved detection performance can therefore only be expected if the amount of sample volume available is small. In other cases, the use of conventional (*i.e.*, 4.6 mm I.D.) columns will lead to lower limits of detection.

The simple trace-enrichment procedure presented in this work allows the injection of samples of up to *ca.* 100 μl. Consequently, for compounds displaying sufficient retention on the stationary-phase material selected, detection limits (in concentration units) can be lowered by two orders of magnitude compared with a direct 1-μl injection. With larger (*e.g.*, 1-ml) sample loops, and for more strongly retained solutes, a three-order-of-magnitude effect can probably be realized. One should keep in mind, however, that with flow-rates of around 100 μl/min, trace enrichment in such a case already takes some 10 min. Handling of significantly larger volumes will, therefore, not be feasible. In other words, leaving apart aspects such as material savings and the

use of special techniques such as on-line HPLC–MS, one can state that HPLC on microbore columns is used to its fullest advantage if the available sample volume is of the order of 0.5–1 ml or less.

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SORPTION OF UNSATURATED MONOCARBOXYLIC ACIDS ON ANION-EXCHANGE RESINS AND NON-IONIC RESINS FROM AQUEOUS SOLUTIONS

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SUMMARY

An increasing number of carbon atoms in unsaturated carboxylic acids gave rise to increased distribution coefficients in sulphate and acetate solutions, both on styrene–divinylbenzene resins and on anion-exchange resins. On the non-ionic resins the double bond led to a large decrease in the retention volume, which was less for 2-alkenoic acids than for isomers with the double bond more distant from the carboxylic acid group. The results indicate that hydrophobic interactions had a larger effect than π-bond interactions with the aromatic resin. For the anion exchangers, hydrogen bonding explains the higher retention of 2-alkenoic than alkanoic acids.

The carboxylate ions derived from 2-alkenoic acids exhibited a higher ion-exchange affinity than those from alkanoic acids. This can be related to the greater acid strength of the parent acid.

INTRODUCTION

Only a few comparisons between the sorption of unsaturated and saturated monocarboxylic acids in aqueous solutions onto anion-exchange resins and non-ionic resins have been published[1–4]. In the present paper the behaviour of unsaturated aliphatic acids with three to six carbon atoms is compared with that of the corresponding saturated acids in solutions containing sodium sulphate, sulphuric acid, sodium acetate or acetic acid.

EXPERIMENTAL

The total retention volumes during the elution of the acids from anion-exchange resins of the quaternary ammonium type and from non-ionic styrene–divinylbenzene resins were determined in jacketed columns. The peak positions were determined with a refractive index detector and UV absorption at 254 nm. The columns were filled by slurry-packing in water and conditioned by pumping the eluent through the column at a high rate (12 cm min^{-1}). The bed volume, X, was determined after the conditioning. No correction was applied for small changes due to compression or

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swelling. To suppress the effect of irreversible shrinkage[5] in aqueous media, the non-ionic resins were kept in 0.001 *M* sulphuric acid for at least 20 days before being used for the determination of retention volumes.

The peak elution volume $\bar{V}$, determined as the total retention volume minus the dead volumes before and after the resin bed, was calculated in bed volumes, $\bar{V}/X$. The interstitial volume is a well-defined quantity for the anion exchangers of the gel type, and the retention is reported as the volume distribution coefficient $D_v = \bar{V}/X - \varepsilon_I$, where ε_I is the relative interstitial volume (0.39). For the non-ionic styrene–divinylbenzene resins the interstitial volume depends on test substance[6]. No attempt was made therefore to calculate the distribution coefficients for these resins.

The anion-exchange column packed with Dowex 1-X10 (7–10 μm) was 200 × 2.5 mm I.D. The column packed with Aminex A-28 (8–12 μm) was 148 × 2.5 mm I.D. The non-ionic resin Amberlite XAD-2 was crushed and fractionated carefully to obtain a particle size of 0.10–0.12 mm before packing the column (160 × 2.5 mm I.D.). The non-ionic Hitachi gel 3011 was supplied as beads with a particle size of 7–17 μm, and the column dimensions were 337 × 2.55 mm I.D. Propenoic acid was purified by crystallization and distillation under vacuum at 53–56°C. Vacuum distillation was used for 3-butenoic acid to remove small amounts of 2-butenoic acid. No interfering impurities were found during chromatography of the other acids, which were applied as received from commercial sources. 2-Butenoic, 2-pentenoic and 2-hexenoic acids were in *trans* form.

The carboxylic acids were dissolved in the eluent and introduced through a 100-μl sample loop. In all experiments referred to in the tables, the applied amounts of carboxylic acids were 2.4 μmol. If not otherwise stated, the experiments were performed at 50°C. An increase in the applied amount led to a lower peak elution volume for all systems in which tailing elution peaks were obtained. Hence, the reported retention data are dependent on the concentration of the solute. In experiments on Dowex 1-X10 in 0.01 *M* sodium sulphate, the adjusted retention volume increased by 6% when the loaded amounts of propanoic and butanoic acids were decreased to 0.6 μmol. For pentanoic acid the decrease was 10%. Similar effects were obtained in 0.05 *M* sodium sulphate.

In the experiments reported in the tables and figures, the nominal linear (empty tube) flow-rate was 5 cm min^{-1}. A change by a factor of two had no significant effect on the experiments with the anion exchangers and with the non-ionic resins in acid media.

Non-ionic styrene–divinylbenzene resins

Most experiments with the non-ionic resins were performed in 0.01 *M* sulphuric acid, which means that the carboxylic acids were virtually non-dissociated. Table I shows that on both non-ionic styrene–divinylbenzene resins the sorption of the saturated as well as of the unsaturated acids increased within each homologous series. For all compounds studied the retention volume on the Hitachi gel was much higher than that recorded on Amberlite XAD-2. The difference between ln $\bar{V}/X$ for one acid and that of its next lower homologue, denoted Δ, exhibited systematic variations. The values were much larger for the Hitachi gel than for XAD-2. An increase in Δ with an increasing number of carbon atoms in the acids was obtained on both resins. The Δ values were higher for saturated than for unsaturated acids.

TABLE I

RETENTION DATA IN 0.01 *M* SULPHURIC ACID ON NON-IONIC STYRENE–DIVINYLBENZENE RESINS

Acids	*Amberlite XAD-2*			*Hitachi gel, 3011*		
	$\bar{V}/X$	Δ	π	$\bar{V}/X$	Δ	π
Propanoic	1.77			3.29		
Propenoic	1.58		−0.11	2.78		−0.17
Butanoic	5.14	1.07		11.8	1.28	
2-Butenoic	4.02	0.93	−0.25	8.83	1.16	−0.29
3-Butenoic	2.85		−0.59	6.22		−0.64
Pentanoic	20	1.35		52	1.48	
2-Pentenoic	15.0	1.31	−0.29	35	1.37	−0.40
4-Pentenoic	8.98		−0.81	21		−0.91
Hexanoic	91	1.52				
2-Hexenoic	62	1.43	−0.38			

On both resins the unsaturated acids were held less strongly than the corresponding saturated acid. The difference in ln $\bar{V}/X$ denoted π was less negative for the 2-alkenoic acids than for those with the double bond more distant from the carboxylic acid group. It has previously been shown that cyclohexylacetic acid is held much more strongly on the Hitachi gel than phenylacetic acid, which is less hydrophobic, and that the same is true for 3-cyclohexylpropanoic acid compared with the corresponding aromatic acid[4]. These and other results led to the conclusion that, in aqueous solution, hydrophobic interactions have a greater influence on the sorption than π-bond interactions between the aromatic compounds and the resins. The results presented in Table I are consistent with these observations. Evidently, the interactions between the double bonds in the solutes and water suppress the hydrophobic contributions to the sorption so effectively that the net result is a significantly decreased retention volume. For molecules with the same number of carbon atoms containing two hydrophilic groups, one of which is terminal (at C-1), the largest hydrophobic interactions will be obtained if the second hydrophilic group is linked to C-2[7]. This explains the larger retention volume of 2-butenoic acid compared with 3-butenoic acid, and the elution order 4-pentenoic < 2-pentenoic acid.

A slight tailing was observed for the acids with low distribution coefficients, whereas acids which appeared late on the chromatograms exhibited a severe tailing. Mixtures of an organic solvent, *e.g.* methanol or ethanol, in water can be used as eluents to suppress the tailing. As a complement, chromatograms were recorded in acetic acid. The example presented in Fig. 1A shows that the elution order in 0.5 *M* acetic acid was the same as that observed in 0.01 *M* sulphuric acid. Although the retention volumes were lower than in the sulphuric acid, the more hydrophobic acids exhibited a severe tailing.

Experiments with butanoic, 3-butenoic and 2-butenoic acids on the Hitachi gel in 0.5 *M* acetic acid showed that the retention volume decreased with increasing temperature. The change in enthalpy was estimated from plots of ln $\bar{V}/X$ *versus* $1/T$ (T = thermodynamic temperature). The most negative value was obtained for butanoic

TABLE II

RETENTION DATA IN SULPHURIC ACID AND SODIUM SULPHATE ON DOWEX 1-X10

Acids	*pK*	*0.01 M Sulphuric acid*			*0.01 M Sodium sulphate*			*0.05 M Sodium sulphate*			*0.25 M Sodium sulphate*		
		D_v	Δ	π	D_v	Δ	π	D_v	Δ	π	D_v	Δ	π
Propanoic	4.87	1.32			2.16			2.06			1.94		
Propenoic	4.25	1.98		0.40	4.0		0.62	3.22		0.45	2.70		0.33
Butanoic	4.80	2.42	0.60		3.50	0.48		3.13	0.42		2.80	0.37	
2-Butenoic	4.70	2.89	0.38	0.18	4.9	0.20	0.34	4.2	0.25	0.28	3.80	0.35	0.31
3-Butenoic	4.35	2.33		−0.03	4.6		0.27	3.7		0.16	3.12		0.11
Pentanoic	4.80	5.3	0.79		7.5	0.76		6.9	0.78		5.4	0.66	
2-Pentenoic	4.74	5.9	0.71	0.10	9.6	0.67	0.25	8.5	0.72	0.22	7.3	0.65	0.29
4-Pentenoic	4.67	3.9		−0.31	6.3		−0.17	5.4		−0.24	4.9		−0.10
Hexanoic	4.80	12	0.82		18	0.90		16	0.86		17	1.15	
2-Hexenoic	4.75	12	0.71	0.0	18	0.66	0.0	18	0.74	0.12	17	0.85	0

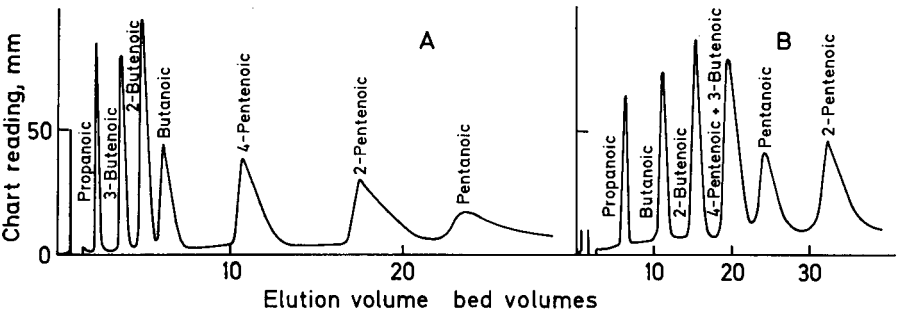

Fig. 1. Chromatography of aliphatic carboxylic acids in 0.5 *M* acetic acid. A, Non-ionic resin (Hitachi gel). B, Anion-exchange resin (Aminex A-28).

acid ($\Delta H = -9.5$), while the values obtained for 2-butenoic and 3-butenoic acids were -8.9 and -6.7 kJ mole^{-1}, respectively.

As expected, much lower retention volumes were observed when the acids were chromatographed as carboxylate ions. Hence, the peak elution volume of propanate on XAD-2 in 0.05 *M* sodium acetate correspond to *ca.* one bed volume, whereas pentanate and hexanate appeared after three and six bed volumes, respectively, of the eluent had passed through the column. The elution curves exhibited a severe fronting, and the peak positions depended on the amount of solute applied to the column. From a practical point of view, it is important that the adsorption of long-chain fatty acids is suppressed when the acids are converted into anions, but it should be noted that they are still held so strongly that large amounts of aqueous eluents are required to displace higher homologues from the resin. For this reason, organic solvents containing a neutralizing agent such as sodium hydroxide or ammonia are applied when strongly hydrophobic acids are to be displaced from non-ionic styrene–divinylbenzene resins.

Anion-exchange resins in sulphuric acid

Table II shows that the retention volumes on Dowex 1-X10 in 0.01 *M* sulphuric acid increased with an increasing number of carbon atoms, both for the saturated and for the unsaturated acids. Again, the Δ-values, here calculated from the D_v values, increased within each homologous series and were lower for the unsaturated than for the saturated species. The Δ-values were much lower than those observed for the non-ionic resins in the same medium. A comparison with the retention volumes given in Table I shows that propenoic acid, which is less hydrophobic and has a much higher dissociation constant than any of the other acids, was retained more strongly by Dowex 1-X10 than by Amberlite XAD-2. All other acids were held much more strongly by the non-ionic resin. The largest differences between ln D_v found on the two resins were observed for the most hydrophobic acids.

These results support the conclusions that hydrophobic interactions, although less important than for the non-ionic resin, contribute markedly to the sorption of the carboxylic acids onto the sulphate form of the anion-exchange resin and that this contribution is most important for the higher homologues. The high swelling pressure in the resin phase of the anion exchanger will suppress the sorption of molecules

with large partial molar volumes more effectively than that of smaller molecules[8]. Evidently, this factor has a comparatively small influence on the net adsorption.

Since the acids were virtually non-dissociated, ion exchange cannot be of great importance. On the other hand, the opposite effect of the double bond in the α-position (positive π-values) compared with the non-ionic resins, indicates that interactions between the carboxylic acid group in the acids and the sulphate form of the anion exchanger contribute to the sorption of the carboxylic acids. One known mechanism for the sorption of free carboxylic acids onto anion-exchange resins is hydrogen bonding[9]. Sulphate ions are known to enter into hydrogen bonding, serving as proton acceptors[10]. Hence, the results suggest that besides non-polar interactions, including hydrophobic interactions, hydrogen bonding with the resin contributes to the sorption of carboxylic acids on the sulphate form of anion exchangers. The effect of the hydrogen bonding with the resin increases with an increasing strength of the acid[11].

The acids with the double bond in the α-position are stronger than the corresponding saturated acids. The largest difference between the pK values is that between propanoic and propenoic acids. As shown in the table, the π-value was larger for propenoic acid than for the other 2-alkenoic acids. A negative π-value was observed for 3-butenoic acid, although this acid has a higher acid strength than butanoic acid. Lowered hydrophobic interactions explain this result. As expected, the most negative π-value was observed for 4-pentenoic acid. These results support the conclusion that hydrogen bonding between the carboxylic proton and the resin contributes markedly to the sorption of the carboxylic acids onto the sulphate form of the anion exchanger in acid solution.

Anion-exchange resins in sodium sulphate solution

The experiments in sodium sulphate solutions were made without any addition of sulphuric acid or alkali. The pH of the solutions was *ca.* 6.5. Some tailing was obtained with the lower homologues, and the tailing increased with an increasing number of carbon atoms. As shown in Table II, the distribution coefficients of the carboxylic acids (carboxylate anions) decreased with an increasing concentration of sodium sulphate. In the idealized case, where the dissociation is complete and the activity coefficients and invasion of cations into the resin phase can be disregarded, an increase in the concentration of sodium sulphate by a factor of 5 should, according to mass-action law, lead to a D_v equal to that at the lower concentration multiplied by $5^{-0.5}$. The results given in the table show that the observed decrease was much smaller. The decrease in the activity coefficients of sodium sulphate with an increasing concentration contributes markedly to the observed effect. The very small influence of the sulphate concentration and the difference in concentration dependency observed for different carboxylate anions show that the deviations from the idealized conditions are very large. It is noteworthy that for hexanoic acid a slight effect on D_v in the opposite direction (salting out) was observed when the eluent concentration was changed from 0.05 to 0.25 M. Similar salting-out effects have been observed for aromatic carboxylate anions, in sodium salt solutions[12].

As previously shown[13], the distribution coefficients for hydrophilic carboxylate anions decrease with an increasing partial molar volume. This is in agreement with the Gibbs–Donnan theory. For the ions studied in the present work, this effect

was offset by the non-polar interactions. Accordingly, positive Δ-values were obtained, both for the saturated and unsaturated carboxylate anions on the anion exchanger in the sulphate form.

Several authors have observed that for ions of similar size and structure, those corresponding to a parent acid of high acid strength are held more strongly than those derived from weaker parent acids[7,14]. In agreement with this rule, propenoic acid exhibited the largest π-values. Again 4-pentenoic acid gave negative values. The small difference in pK_a for hexanoic and 2-hexenoic acids, together with lower hydrophobic interactions for unsaturated species, explains the observation that these acids exhibited very similar distribution coefficients.

Anion-exchange resins in sodium acetate

The acids can in rough calculations be considered as completely ionized in the sodium acetate solution. The results given in Table III show that the D_v of propanoic acid in 1 *M* sodium acetate was in agreement with that calculated by dividing the D_v in 0.25 *M* solution by 4. For propenoic acid the calculated D_v in 1 *M* sodium acetate was lower than that calculated by this method, whereas for the anions with more than three carbon atoms the opposite was found. Salting-out effects on ion-exchange equilibria in sodium acetate have been observed for other hydrophobic anions[12].

Both for the alkanate and the alkenate ions the distribution coefficients increased with an increasing number of carbon atoms. Evidently, non-polar interactions have a decisive influence. The Δ values increased within each homologous series.

Despite the lower hydrophobic interactions, the 2-alkenate ions exhibited higher distribution coefficients than the corresponding alkanate ions. This observation, and the fact that only 4-pentenoic acid exhibited negative π-values, strongly support the theory that when other factors are virtually constant, the anion exchange affinities increase with an increasing strength of the parent acid.

Some other anions held very strongly at a low acetate concentration were studied in 1 *M* sodium acetate. As could be predicted with regard to the increased hydrophobic interactions, heptanate ions (D_v = 62) exhibited a higher Δ-value than

TABLE III

RETENTION DATA IN SODIUM ACETATE AND ACETIC ACID ON AMINEX A-28

Acids	*0.25 M Sodium acetate*			*1 M Sodium acetate*			*0.20 M Acetic acid*		
	D_v	Δ	π	D_v	Δ	π	D_v	Δ	π
Propanoic	6.3			1.6			9.2		
Propenoic	10.4		0.50	2.3		0.35	39		1.44
Butanoic	10.8	0.54		3.4	0.75		17.4	0.66	
2-Butenoic	16.2	0.44	0.40	4.3	0.63	0.23	30	−0.26	0.54
3-Butenoic	13.2		0.20	3.7		0.08	40		0.83
Pentanoic	25	0.84		8.1	0.87		41	0.85	
2-Pentenoic	35	0.77	0.33	10	0.85		62	0.73	0.42
4-Pentenoic	19.3		−0.27	5.8		−0.34	37		−0.10
Hexanoic	66	0.97		19.9	0.90		106	0.95	
2-Hexenoic				26	0.95	0.26	141	0.82	0.29

the carboxylate ions listed in Table III. A larger decrease in D_v resulting from a double bond than for the other unsaturated ions could be predicted for 6-heptanate ions ($D_v = 30$). Accordingly, the π-value was much more negative than that observed for 4-pentenate ions.

A comparison between phenylacetate and cyclohexylacetate ions was also made in this medium. Phenylacetate ions exhibited a D_v of 44, whereas cyclohexylacetate had a D_v of 47, *i.e.* a slightly higher value. The hydrophobic interactions are much larger for cyclohexane derivatives of this type than for the corresponding aromatic compounds[4]. The small difference in D_v is ascribed to the fact that the parent acid is much stronger in the case of phenylacetate.

The effect of a double bond in a cyclohexane ring was also studied. The D_v of cyclohexanecarboxylate was 21, and a lower value ($D_v = 17$) was obtained for 3-cyclohexenate anions. Decreased hydrophobic interactions as a result of π-bond interactions with the water seem to have a greater influence than other factors.

The temperature dependency of the ion-exchange behaviour in sodium acetate solution was very small (Fig. 2). In contrast to results obtained with strongly hydrophilic carboxylate ions[15], straight-line relationships were obtained between ln D_v and $1/T$. No influence of the temperature on D_v was traced for propanate ions. For the other ions the change in enthalpy was negative. It is noteworthy that for the saturated acids the enthalpy change was more negative for higher than for lower homologues. This suggests that hydrophobic interactions (dependent on the water structure) cannot be solely responsible for the increase in D_v with an increasing number of carbon atoms. As expected, the change in enthalpy was more negative for the alkenate ions than for the alkanate ions. Among the studied ions, 2-pentenate exhibited the most negative value (-5.5 kJ mol^{-1}).

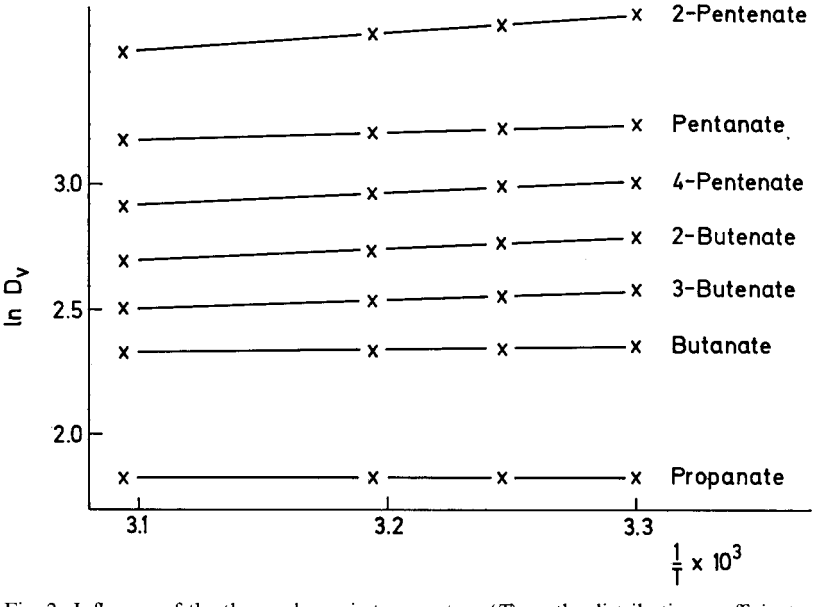

Fig. 2. Influence of the thermodynamic temperature (T) on the distribution coefficients of aliphatic carboxylic acids in 0.25 M sodium acetate on Aminex A-28.

Anion-exchange resins in acetic acid

In agreement with previous[2] determinations of the D_v values on an anion exchange resin in 3 *M* acetic acid, propenoic acid exhibited a much higher D_v in 0.2 *M* acetic acid than propanoic acid (Table III). Similarly, 2-butenoic acid was in both media held more strongly than butanoic acid. The results are mainly explained by a strengthened hydrogen bonding for the stronger acids. Accordingly, 3-butenoic acid, which is stronger than 2-butenoic acid, exhibited the highest D_v among the acids with four carbon atoms. Again, all other unsaturated acids, except 4-pentenoic acid, were held more strongly than the alkanoic acids with the same number of carbon atoms. The same elution order was found in 0.5 *M* acetic acid. Some results are shown in Fig. 1B. A complete separation was obtained of most species. 4-Pentenoic and 3-butenoic acids appeared, however, in the same elution band as shown in Table III, the separation factor for these acids was unfavourable also in 0.2 *M* solution. In this medium pentanoic was also eluted very close to these acids, whereas in 0.5 *M* acetic acid pentanoic appeared later.

A change in the acetic acid concentration affects both the proportion of carboxylate anions and free acids and the formation of associated species by hydrogen bonding, both in the resin and in the eluent. At a high concentration the hydrophobic interactions and the dielectric constant are altered to an appreciable extent. The net result is a decrease in the distribution coefficients with increasing acetic acid concentration.

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PARTIAL EXPLANATION OF THE ANOMALY IN THE RELATIONSHIP BETWEEN THE LOGARITHM OF RETENTION AND THE CARBON NUMBER OF MONOHYDRIC PHENOLS

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SUMMARY

An attempt was made to elucidate the anomaly in the $R_{t(rel)} = f(C_n)$ function. It was found, that this anomaly results from complex and simultaneous actions of several factors. Hydrogen bond formation between the free phenolic hydroxyl group and the TXP polar stationary phase or between the functional groups themselves is certainly not the most important influencing factor. The conditions for using the $R_{t(rel)} = f(C_n)$ function in identification of *n*-alkyl-substituted monohydric phenols were determined.

INTRODUCTION

In our previous papers[1,2] retention data were published for a series of monohydric phenols. These data were obtained on a polar stationary phase of trixylenylphosphate (TXP)–phosphoric acid (95:5). Evaluating the interdependence of log $R_{t(rel)}$ (R_t = retention time) and the number of C atoms (C_n) in the molecule of monohydric phenol examined we proposed an anomalous course for this function.

TABLE I

RELATIVE RETENTION OF *n*-ALKYLPHENOLS ON APIEZON K

Phenol = 1; column temperature, 130°C (Fig. 3).

Substituent	*Position of substituent*			*Number of substituents in molecule*
	ortho	*meta*	*para*	
Methyl	1.25	1.69	1.73	7
Ethyl	1.9	2.72	2.83	8
n-Propyl	2.97	4.46	4.55	9
n-Butyl	5.17	7.79	8.08	10
n-Pentyl	9.28	–	14.91	11
n-Hexyl	15.63	–	25.46	12

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TABLE II

COLUMN PARAMETERS AND CONDITIONS OF GC SEPARATION

	Stationary phase	
	TXP-phosphoric acid (95:5)	*Apiezon K*
Type of column	Open metal capillary	Open metal capillary
Length (m)	50	50
Inner diameter (mm)	0.25	0.25
Column temperature (°C)	125, 130	125, 130
Carrier gas	nitrogen	nitrogen
Nitrogen inlet pressure (kPa)	160	160
Split ratio	1:100	1:100
Evaporator temperature (°C)	250	250
Injected volume (sample size) (μl)	0.5	0.5

The anomaly lies in an abrupt change in the slope of the plot at the point corresponding to *n*-propylphenol.

Owing to the impossibility of explaining this anomalous behaviour of C_9 isomers on the basis of literature data, we have now measured the retention data of some higher members of the homologous series of *n*-alkyl substituted monohydric phenols or similar substances on Apiezon K stationary phase (Table I).

Table II shows the column parameters and GC conditions for the compounds studied. The stationary phase (Apiezon K) was, unlike the TXP phase, non-polar.

In order to elucidate the possible influence of hydrogen bonds we carried out analogous measurements on the methyl ethers of 2- and 4-substituted *n*-alkylphenols, C_7–C_{12}, prepared by the procedure of Sandermann and Weismann[3] (Table III). The experiments were completed by determining the $R_{t(\mathrm{rel})} = \mathrm{f}(C_n)$ function of *n*-alkylbenzenes having a carbon skeleton identical with that of the monohydric phenols. The

TABLE III

RELATIVE RETENTION OF METHYL ETHERS OF *n*-ALKYLPHENOLS DETERMINED AT 125°C

Alkyl phenyl ether of	*Position of alkyl group*				*Number of C atoms in molecule*
	ortho		*para*		
	*Apiezon K**	*TXP***	*Apiezon K**	*TXP***	
Methylphenol	1.58	1.61	1.85	1.87	7
Ethylphenol	2.33	2.45	3.16	3.23	8
n-Propylphenol	3.63	3.85	5.31	5.31	9
n-Butylphenol	6.36	6.89	9.60	10.16	10
n-Pentylphenol	11.05	12.08	17.07	18.29	11
n-Hexylphenol	19.25	21.65	30.40	33.26	12

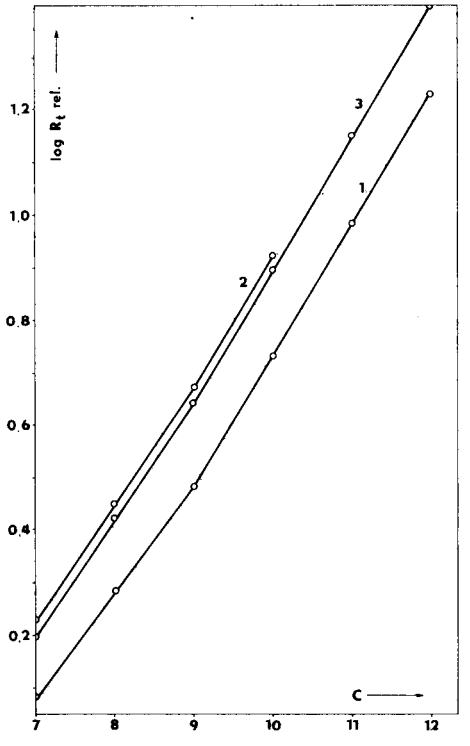
* Fig. 4.
** Fig. 5.

TABLE IV

RELATIVE RETENTION OF *n*-ALKYLBENZENES

Column temperature, 130°C (Fig. 6).

Compound	*Stationary phase*		*Number of C-atoms in molecule*
	Apiezon K	*TXP*	
Benzene	1.00	1.00	6
Methyl benzene	2.35	1.50	7
Ethyl benzene	4.35	2.12	8
n-Propyl benzene	7.65	3.09	9
n-Butyl benzene	14.58	5.15	10
n-Hexyl benzene	48.88	15.20	12
n-Nonyl benzene	296.88	84.47	15
n-Decyl benzene	–	147.20	16



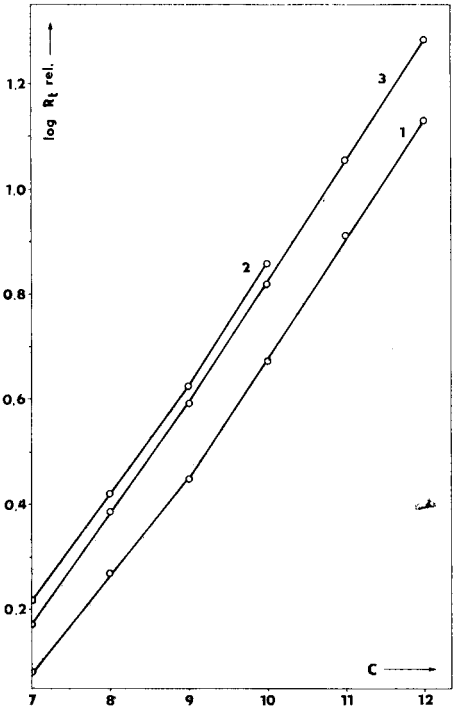


Fig. 1. Relationship between the logarithm of retention time and the carbon number for monohydric *n*-alkylphenols on a open capillary column coated with TXP–phosphoric acid (95:5) as stationary phase. Column temperature, 125°C. Lines: 1, 2-*n*-alkylphenols; 2, 3-*n*-alkylphenols; 3, 4-*n*-alkylphenols.

Fig. 2. Relationship between the logarithm of retention time and the carbon number for monohydric *n*-alkylphenols on a open capillary column coated with TXP–phosphoric acid (95:5) as stationary phase. Column temperature, 140°C. Lines: 1, 2-*n*-alkylphenols; 2, 3-*n*-alkylphenols; 3, 4-*n*-alkylphenols.

results are summarized in Table IV. To show the temperature independence of the data we repeated some measurements, which had previously been published[1], at a column temperature of 125° C. To achieve constant elution conditions an open-capillary column was used for all measurements.

RESULTS AND DISCUSSION

A critical interpretation of the results summarized in Tables I, III and IV enables us to make the following conclusions.

(1) The plot of the log $R_{i(\mathrm{rel})} = \mathrm{f}(C_n)$ function for the homologous *n*-alkyl-monosubstituted monohydric phenols has an anomalous course characterized by a sudden change in slope for all phenols substituted with alkyl substituent longer than *n*-propyl.

(2) This effect is temperature independent over the temperature range examined (125–140° C; Figs. 1 and 2).

(3) For the polar stationary phase the position of the change in slope rises as the *n*-alkyl group is moved nearer to the phenolic hydroxyl group. Very little change in the slope was observed for the 4-*n*-alkyl-substituted phenol series, while the change for the 3-*n*-alkyl-substituted series was more important, with that for the 2-*n*-alkyl-substituted phenols being the most significant.

(4) For the non-polar stationary phase (Apiezon K) there is also a sudden change of course in the plot of the $R_{i(\mathrm{rel})} = \mathrm{f}(C_n)$ function. Large differences in elution of 2-*n*- and 4-*n*-alkyl-substituted phenols up to C_9 are seen, but from this point the slopes of the plots for both series are identical (Fig. 3).

(5) Comparison of the plots of the $R_{i(\mathrm{rel})} = \mathrm{f}(C_n)$ functions for the non-polar and the polar stationary phases (TXP) showed an unambiguous relationship; in all cases the changes in slopes were more pronounced for the 2-*n*- and 4-*n*-alkyl series when a non-polar stationary phase was used. However for the 3-*n*-alkyl series, the changes were approximately the same on both the polar and non-polar stationary phases.

(6) Comparison of the plots of the log $R_{i(\mathrm{rel})} = \mathrm{f}(C_n)$ functions for the polar stationary phase (TXP) with 2-*n*-alkylphenols and with their methyl ethers showed that etherification of the phenolic hydroxyl group leads to an increase in the differences in slope. Etherification of 4-*n*-alkylphenols caused only a small change in the shape of the graph; however there was also in this case a noticeable increase in the difference slope (Fig. 4).

(7) For the 4-*n*-alkylphenols series, using the non-polar stationary phase, the reverse influence of etherification to that observed for the TXP polar stationary phase is seen. The methyl ethers of 4-*n*-alkylphenols show a smaller change in the slope of the $R_{i(\mathrm{rel})} = \mathrm{f}(C_n)$ plot than the original 4-*n*-alkylphenol with a free phenolic function. The 2-*n*-alkylphenols do not change their behaviour and the difference in slope on Apiezon K remains approximately the same as that of the original 2-*n*-alkylphenols (Fig. 5).

(8) On the non-polar stationary phase the course of the $R_{i(\mathrm{rel})} = \mathrm{f}(C_n)$ function for the alkylbenzene series was linear with no change in slope. On the other hand a "break" in the linear plot was observed for the TXP polar stationary phase (Fig. 6).

The occurrence of "breaks" in the $R_{i(\mathrm{rel})} = \mathrm{f}(C_n)$ plot is in accordance with the

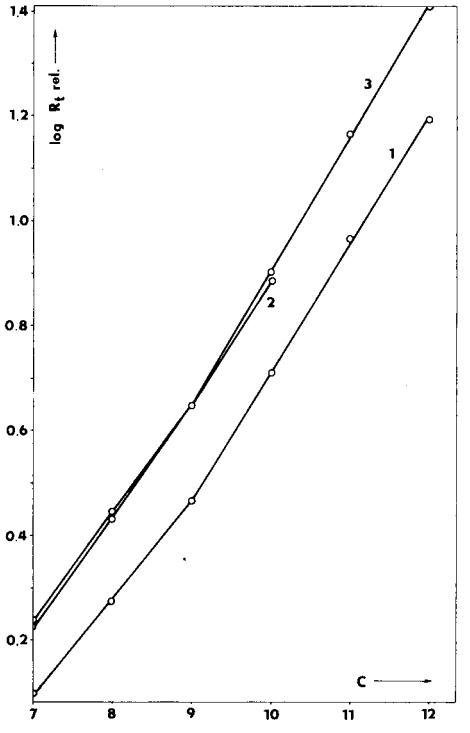


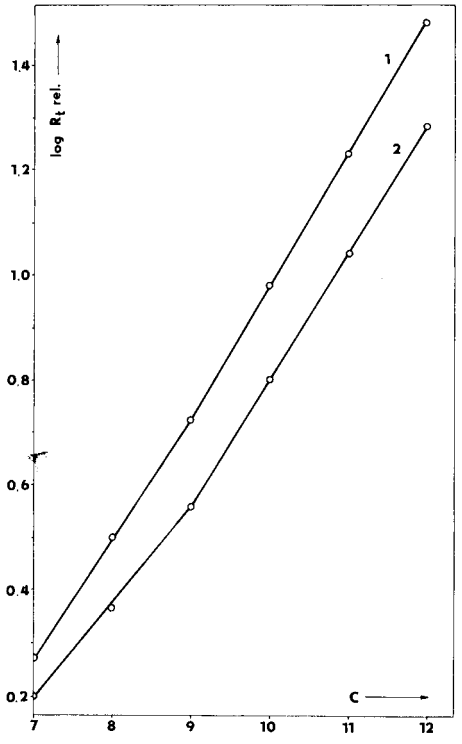
Fig. 3. Relationship between the logarithm of retention time and the carbon number for monohydric *n*-alkylphenols on a open capillary column coated with Apiezon K as stationary phase. Column temperature, 130 C. Lines: 1, 2-*n*-alkylphenols; 2, 3-*n*-alkylphenols; 3, 4-*n*-alkylphenols.

experimental results obtained on polar and non-polar stationary phases, motivated in these two different cases generally by two non-identical sets of influencing factors. This demonstrates for example the change of slope on the plot of log $R_{t\,(\mathrm{rel})} = \mathrm{f}(C_n)$ determined by elution measurements of *n*-alkylbenzenes on the TXP polar stationary phase. On the basis of this plot of $R_{t\,(\mathrm{rel})} = \mathrm{f}(C_n)$ we presume a linkage between the conjugated double-bond system of the benzene aromatic ring and the polar stationary phase with the π-electrons of the aromatic ring playing a predominant role, strongly influenced by the degree of polarity of the stationary phase.

Because of the large amount of data necessary for the quantification and precise description of the bonds formed, it is so far impossible to describe and categorize the "complexes" formed with respect to the types defined in the literature.

In summary, these facts indicate that the anomaly in the gas-chromatographic behaviour is not caused by only one factor but by the complex and simultaneous action of several; the resulting effect can be comprehended only as a complex and mutually penetrating combination of them all.

However, in our work we can with certainty exclude hydrogen-bond formation between the free phenolic hydroxyl group and the stationary phase, or between the functional groups themselves, as being the most important factor causing the anomalous appearance of the $R_{t(\mathrm{rel})} = \mathrm{f}(C_n)$ graph. The "breaks" on the $R_{t(\mathrm{rel})} = \mathrm{f}(C_n)$ plot were found both for the *n*-alkylphenols and for the series of their methyl ethers, and,



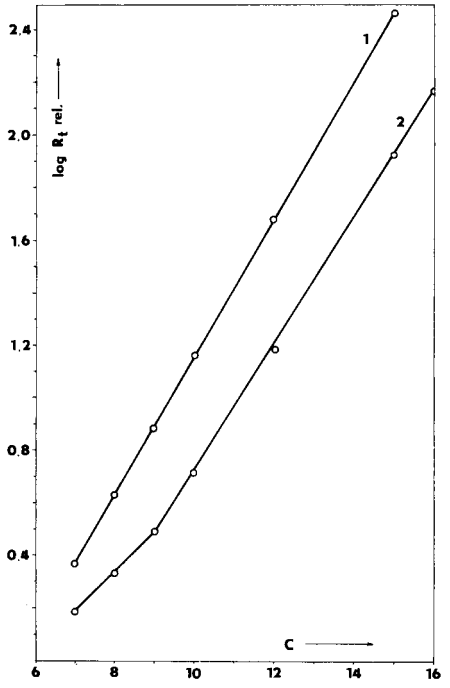


Fig. 4. Relationship between the logarithm of retention time and the carbon number for *n*-alkylphenyl methyl ethers (anisoles) on an open capillary column coated with TXP–phosphoric acid (95:5) as stationary phase. Column temperature, 125°C. Lines: 1, 2-*n*-alkylphenyl methyl ethers; 2, 4-*n*-alkylphenyl methyl ethers.

Fig. 5. Relationship between the logarithm of retention time and the carbon number for *n*-alkylphenyl methyl ethers (anisoles) on a open capillary column coated with Apiezon K as stationary phase. Column temperature, 125°C. Lines: 1, 2-*n*-alkylphenyl methyl ethers; 2, 4-*n*-alkylphenyl methyl ethers.

using the TXP polar stationary phase, also for the corresponding *n*-alkylbenzenes.

On the basis of the measurements it is possible to interpret the data as follows: the necessary condition for the existence of an anomaly in the $R_{t(\mathrm{rel})} = \mathrm{f}(C_n)$ graph for the non-polar stationary phase ("breaks") is the presence of a C_{ar}–O bond in the molecules of the set of compounds studied.

With the polar stationary phase we find a somewhat different situation with regard to the presence of a C_{ar}–O bond, because the anomaly in the $R_{t(\mathrm{rel})} = \mathrm{f}(C_n)$ plot also occurred for elution of *n*-alkylbenzenes. The inductive effect of the C_{ar}–O bond on the aromatic nucleus of phenol and the ability to form hydrogen bonds increases here in importance especially with respect to the total number of influencing variables.

For the *n*-alkyl substituents $< C_3$ the difference in elution characteristics between two subsequent members of a homologous series, *i.e.* when increasing the molecule by a CH_2 group, is equal to the resultant of the altered induction forces between the aromatic nucleus and the alkyl substituent.

For the *n*-alkyl groups $> C_3$ the influence of this substituent on the retention

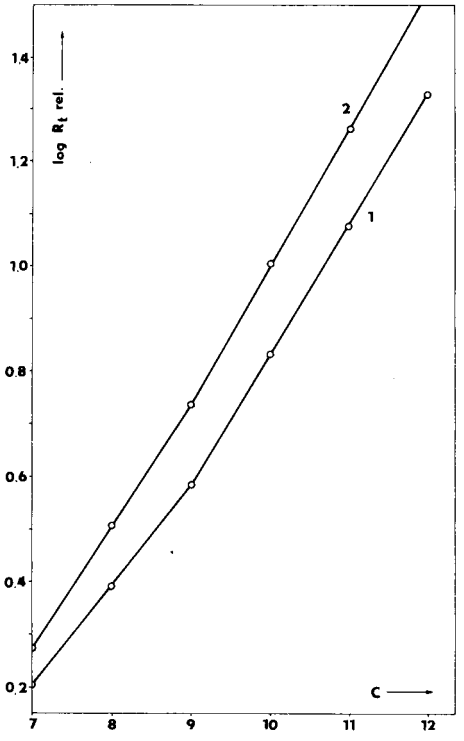


Fig. 6. Relationship between the logarithm of retention time and the carbon number for *n*-alkylbenzenes on a open capillary column coated with Apiezon K (line 1) or TXP–phosphoric acid (95:5) (line 2) as stationary phase. Column temperature, 130°C.

time (or the increase of this characteristic) relative to the CH_2 increment is substantially greater than in the case of a *n*-alkyl substituent < C_3. Here increasing the *n*-alkyl substituent by one CH_2 group does not significantly influence the π-electrons of the aromatic ring; the most important factor here will be solely the subsequent enlargement of the aromatic part of the molecule in question. When a hydroxyl group is introduced into a molecule of an *n*-alkylbenzene, the oxygen atom produces by its positive inductive effect a change in the distribution of electrons in the aromatic nucleus, causing a reduction in the bond-length between the π-electrons of the aromatic nucleus and the polar stationary phase. Nevertheless, these bonds play a part in the gas chromatography of *n*-alkylphenols.

Etherification of the phenolic hydroxyl group in the *n*-alkylphenols leads to a decrease in its inductive power enabling the aromatic nucleus to be better able to form, as described previously, a stronger bond with the polar stationary phase. However, the strength of this linkage is also proportional to the position of the phenolic hydroxyl group (or the methoxy group) in respect to the *n*-alkyl group in the original alkylbenzene molecule.

This interpretation is supported also by the fact that a shift of the alkyl substituent within the neighbourhood of the hydroxyl group produces an enhancement of "break" in the $R_{t(rel)} = f(C_n)$ plot. The smallest differences in slope are charac-

teristic of the 4-*n*-alkylphenol series, larger differences being observed for the 3-*n*-alkylphenols: the largest differences occur for the 2-*n*-alkylphenols. On the basis of these results we conclude that the steric hindrance of hydrogen-bond formation (between the phenolic hydroxyl group and the stationary phase) enhances the other factors affecting the elution characteristics and which arise from the nature of the π-bonding between the stationary phase and the aromatic portion of the molecule.

CONCLUSIONS

In the identification of unknown phenols by gas chromatography on the polar TXP–phosphoric acid (95:5) phase we consider it indispensable when applying the $R_{t(\mathrm{rel})} = \mathrm{f}(C_n)$ relationship to determine by a reliable method the nature of the alkyl substituents present, before the chromatogram is interpreted. In the C_7–C_9 region, where the necessary standards are usually available, we can use the $R_{t(\mathrm{rel})} = \mathrm{f}(C_n)$ relationship directly by extrapolation or by interpolation. In all other cases this method provides wrong results. This statement is valid also for the elution of monohydric *n*-alkylphenols on the non-polar stationary phase Apiezon K and for the separation of *n*-alkylphenol ethers. In the latter case we confirmed this result for both the stationary phases studied (Apiezon K and TXP–phosphoric acid).

It is possible to presume an identical gas-chromatographic behaviour also for other homologous series (*e.g.* for nitrophenols, halogenated phenols, aminophenols, esters, silyl ethers, etc.). The characteristics of the stationary phases used (the elution was performed on both polar and non-polar stationary phases) provide evidence for the general validity of our conclusions for all types of stationary phases. The conclusion what a degree of polarity of the stationary phase causes the "break" in the $R_{t(\mathrm{rel})} = \mathrm{f}(C_n)$ plot for *n*-alkylbenzenes is not borne out by our results.

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GAS CHROMATOGRAPHY OF CANNABIS CONSTITUENTS, INCLUDING CANNABINOID ACIDS, WITH ON-COLUMN ALKYLATION AND ESTERIFICATION

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SUMMARY

Neutral cannabis constituents and cannabinoid acids were gas chromatographed with on-column methylation of phenolic hydroxyl groups and esterification of carboxylic acid functions. Injection of cannabis extracts as solutions in 2 *M* dimethylformamide dimethylacetal in pyridine gave quantitative or near-quantitative conversion of cannabinol and tetrahydrocannabinol into methyl ethers, while cannabidiol gave a monomethyl and a dimethyl derivative. Also cannabidiolic acid, tetrahydrocannabinolic acid and the tetrahydrocannabinol 7-acid metabolite chromatographed well as methyl esters and ethers. The mass spectra of the methyl derivatives gave more pertinent structural information than those of the corresponding TMS derivatives. This simple derivatization procedure should conceivably prove useful for the analysis of other heat-labile biological samples.

INTRODUCTION

Gas chromatography (GC) of neutral cannabis constituents is a simple procedure[1]. Tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabinol (CBN) with their propyl and methyl homologues elute as symmetric peaks from packed[2] as well as capillary columns[3]. Cannabinoid acids[1,4], on the other hand, cannot be gas chromatographed as such. If, for instance, Δ^1-tetrahydrocannabinol-4′-carboxylic acid is introduced into the GC injector it will decarboxylate and give THC[5,6]. The cannabinoid acids are consequently derivatized prior to analysis and chromatographed as trimethylsilyl (TMS) derivatives, with TMS protection of phenolic as well as carboxylic acid functions[5–9], or as methyl esters with TMS protection of phenolic functions only[9–11]. In the preparation of TMS derivatives, however, the reaction mixture must be heated, which may cause decomposition of some cannabinoid constituents[9]. Also, the mass spectra of the TMS derivatives sometimes yield very little structural information[9,12].

Dimethylformamide (DMF) dialkylacetals are excellent derivatizing agents for

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carboxylic acids[13]. The reaction mixture of, for instance, DMF dimethylacetal and a fatty acid in pyridine is usually injected directly onto the GC column, without prior heating, to give a quantitative yield of fatty acid methyl ester. The DMF dialkylacetals also alkylate phenolic hydroxyl functions[14]. They are consequently potential derivatizing agents for neutral as well as acidic cannabis constituents, and this paper presents results demonstrating their practical usefulness. DMF dimethylacetal was used to alkylate cannabinoids under the mildest possible conditions, and the mass spectra of the derivatives proved readily interpretable.

EXPERIMENTAL

Samples and reagents

Samples (100 mg) of cannabis resin (hashish) or marihuana, submitted for forensic analysis, were triturated with 1 ml of methanol[15]. The suspension was placed in an ultrasonic bath for 20 min and then centrifuged. Aliquots of the clear supernatant were either injected on to the GC column or derivatized as described below. Sample extraction, derivatization and analysis were performed on the same day, and the solutions were kept in the dark when not in use.

Synthetic Δ^1-tetrahydrocannabinol-7-acid (NIH, Bethesda, U.S.A.) was a gift from Drs. Magnus Halldin and Stig Agurell. Dimethylformamide dimethylacetal, 2 *M* in pyridine, Methyl-8®, was purchased from Pierce (Rockford, IL, U.S.A.), as was the GC column packing, OV-1, 3% on Chromosorb W HP, 80–100 mesh.

Derivatization procedure

Aliquots (25 μl) of the methanolic cannabis extracts were transferred to conical, glass-stoppered test-tubes, and the solvent was evaporated under a nitrogen stream. The residues were then dissolved in Methyl-8 (100 μl). Samples (2–4 μl) of these solutions were injected on to the GC column. In addition, a "sandwich" injection was tried; 1 μl of hashish extract followed by 5 μl of reagent in the same syringe.

Δ^1-Tetrahydrocannabinol-7-acid was injected as a freshly prepared 10^{-3} *M* solution in Methyl-8.

Equipment and gas chromatographic conditions

The combined gas chromatograph–mass spectrometer was a Finnegan 4023 EI/CI system interfaced with an INCOS data system (Sunnyvale, CA, U.S.A.).

The gas chromatograms were run on a 196 cm × 2 mm I.D. glass column packed with 3% OV-1. The helium flow-rate was 25 ml/min, the injector block temperature was 300°C and the glass jet separator was kept at 250°C. The derivatized or underivatized cannabis extracts were analysed in temperature-programmed runs: the oven temperature was first maintained for 2 min at 200°C, then raised by 10°C/min to 260°C and finally kept there for 5 min. The derivative of Δ^1-THC-7-acid was also analysed at 270°C oven temperature.

Mass spectra were recorded continuously, at one scan every 3 sec, and stored on the data disc. The ion source temperature was 250°C, the electron energy was 70 or 23 eV, the emission current was 0.28 mA, the EM voltage was −1960 V and the preamplifier sensitivity was 10^{-7} A/V. When chemical ionization (CI) spectra were recorded, isobutane (99.5% pure, AGA, Lidingö, Sweden) was added as make-up gas

to an indicated ion source pressure of 27 pA (0.20 torr). The ionizing current was 0.25 mA at 70 eV, the EM voltage was −1850 V and the preamplifier sensitivity was 10^{-8} A/V.

The data-processing included the plotting of chromatograms as reconstructed ion currents (RICs) and of background-subtracted mass spectra. The data system also allows the construction of mass chromatograms, ion intensity *vs.* time, at any *m/z* value. The mass chromatograms can be used to distinguish the mass spectra of two or more substances eluting as an unresolved GC peak and to check whether a peak in a spectrum belongs to "sample" or to "background". They can also, as in selected ion monitoring, be used for quantitation as well as for the localization of compounds with known mass spectra, that elute in amounts too small to give noticeable peaks in the RIC chromatograms.

RESULTS AND DISCUSSION

Gas chromatograms of underivatized and derivatized cannabis extracts

Figs. 1 and 2 show the gas chromatograms of cannabis extracts with and without on-column derivatization. Tables I and II list the compounds identified in the four GC–MS runs, and their structures are given in Fig. 3. The small peaks of underivatized THC (their origin is discussed below) in the chromatograms B and D serve as reference peaks for the comparison of retention times between the four chromatograms.

The methyl ether derivatives of tetrahydrocannabivarin, THC and CBN elute 42, 51 and 51 sec earlier, respectively, than the underivatized compounds. In the

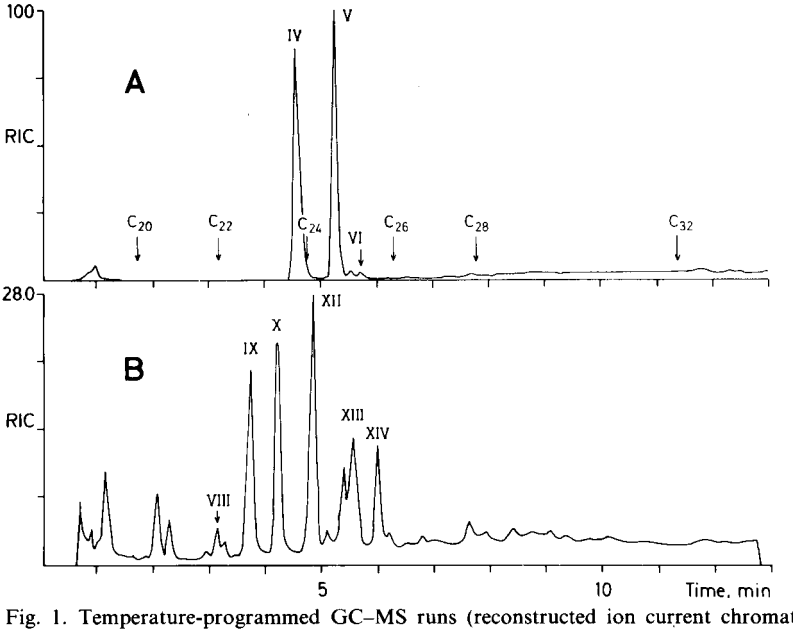

Fig. 1. Temperature-programmed GC–MS runs (reconstructed ion current chromatograms) of (A) a marihuana extract and (B) the same extract with on-column methylation. The arrows, marked C_{20} etc., indicate the positions of the peaks from a reference mixture of straight-chain hydrocarbons run under the same conditions. The roman numerals refer to Tables I and II and to Fig. 3.

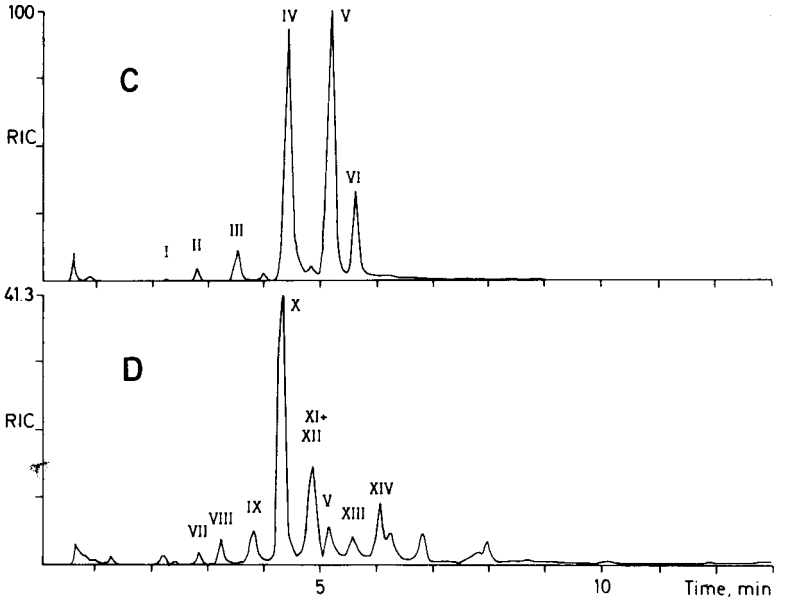

Fig. 2. Temperature-programmed GC–MS runs (reconstructed ion current chromatograms) of (C) a hashish extract and (D) the same extract with on-column methylation. The roman numerals refer to Tables I and II and to Fig. 3.

temperature programme 42 sec corresponds to an elution temperature 7°C lower and 51 sec to an elution temperature 8.5°C lower. Cannabidiol appears as two derivatives, a monomethyl ether (IX) eluting 36 sec (6°C) earlier than the underivatized compound and a dimethyl ether (VIII) eluting another 36 sec (6°C) earlier. The chromatograms B and D contain at least five distinct peaks due to methyl esters of carboxylic

TABLE I

IDENTIFIED CANNABIS CONSTITUENTS IN CHROMATOGRAMS A AND C

Retention time (min) in chromatogram		*Compound*	*Molecular weight**
A	*C*		
–	2.30	Tetrahydrocannabiorcol (I)	258
–	2.80	Cannabidivarin (II)	286
–	3.55	Tetrahydrocannabivarin (III)	286
4.55	4.45	Cannabidiol (IV)	314
5.25	5.20	Tetrahydrocannabinol (V)	314
5.55	–	(Dioctylphthalate)**	
5.70	5.65	Cannabinol (VI)	310

* The sample was re-run under identical GC conditions but with 23 eV electron energy, which afforded clear identification of all molecular ions.

** A ubiquitous contaminant from plasticizers.

TABLE II

IDENTIFIED COMPOUNDS IN CHROMATOGRAMS B AND D

Retention time (min) in		*Compound*	*Molecular weight**
B	*D*		
1.15	1.30	Methyl palmitate	270
2.05	2.20	(Unidentified)	296
2.35	2.40	Methyl stearate	298
–	2.85	3′-O-Methyltetrahydrocannabivarin (VII)	300
3.15	3.25	1′-O, 3′-O-Dimethylcannabidiol (VIII)	342
3.75	3.85	O-Methylcannabidiol (IX)**	328
4.25	4.35	3′-O-Methyltetrahydrocannabinol (X)	328
–	4.80	3′-O-Methylcannabinol (XI)	324
4.85	4.90	1′-O, 3′-O-Dimethylcannabidiolic acid methyl ester (XII)	400
5.10	5.20	Tetrahydrocannabinol (V)	314
5.40	–	(Dioctylphtalate)***	
5.55	5.60	O-Methylcannabidiolic acid methyl ester (XIII) §	386
6.00	6.10	3′-O-Methyltetrahydrocannabinolic acid methyl ester (XIV)	386
6.20	6.30	(Unidentified)	438
–	6.85	(Unidentified)	441
–	8.00	(Unidentified)	381

* The samples were re-run under identical GC conditions but with the mass spectrometer operating in the CI mode. The isobutane CI spectra afforded unambiguous determination of all molecular weights. Indeed, the CI spectra of compounds VII, X, XI and V consisted of an intense $[M+H]^+$ ion peak and no fragment peaks of more than 5% abundance.

** The two phenolic functions of cannabidiol are equivalent.

*** Contaminant from plasticizers.

§ The two phenolic functions of cannabidiolic acid are not equivalent, and the peak is probably due to a mixture of the two isomers.

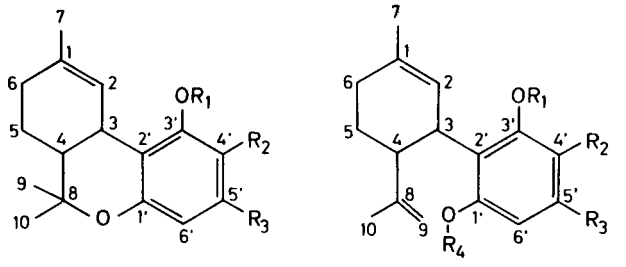

Fig. 3. The structures of the cannabis constituents and their derivatives. The following compounds are represented by formula A: I, $R_1=R_2=H$, $R_3=CH_3$; III, $R_1=R_2=H$, $R_3=C_3H_7$; V, $R_1=R_2=H$, $R_3=C_5H_{11}$; VI, as V, but the C^{1-6} ring aromatic; VII, $R_1=CH_3$, $R_2=H$, $R_3=C_3H_7$; X, $R_1=CH_3$, $R_2=H$, $R_3=C_5H_{11}$; XI, As X, but the C^{1-6} ring aromatic; XIV, $R_1=CH_3$, $R_2=COOCH_3$, $R_3=C_5H_{11}$. Formula B represents: II, $R_1=R_2=R_4=H$, $R_3=C_3H_7$; IV, $R_1=R_2=R_4=H$, $R_3=C_5H_{11}$; VIII, $R_1=CH_3$, $R_2=H$, $R_3=C_5H_{11}$, $R_4=CH_3$; IX, $R_1=R_2=H$, $R_3=C_5H_{11}$, $R_4=CH_3$; XII, $R_1=CH_3$, $R_2=COOCH_3$, $R_3=C_5H_{11}$, $R_4=CH_3$; XIII, $R_1=CH_3$, $R_4=H$ (or vice versa), $R_2=COOCH_3$, $R_3=C_5H_{11}$.

acids. The di- and monomethyl ether derivatives of cannabidiolic acid methyl ester (XII and XIII) and 3′-O-methyltetrahydrocannabinolic acid methyl ester (XIV) elute in the same order and with the same time intervals as the corresponding neutral cannabinoid derivatives. The increase in elution time due to the added methoxycarbonyl functions is 103 ± 2 sec for the three compounds, which corresponds to an elution temperature 17°C higher.

Derivatization yields

The on-column derivatization of cannabinol was more than 99.5% complete, as estimated from the mass chromatograms at $m/z = 309$ (base peak of the derivative, see Table III) and $m/z = 295$ (base peak of CBN). The proportion of underivatized to derivatized THC was estimated by quantitation of the mass chromatogram peaks given by the $m/z = 313$ and $m/z = 299$ ions. This procedure was regarded as permissible, even though the relative molar responses of the two compounds may be slightly different, because the mass spectral behaviour of THC changes very little on methylation (*cf.* Table III). The proportion of THC to methylated THC was 6.1 ± 2.7% (mean ± S.D. of eleven experiments) under the conditions described. When the evaporation residue of 10 µl of cannabis extract was treated with 200 µl of reagent, no underivatized THC could be detected in the gas chromatogram, but the rather high dilution of the sample is a disadvantage in the GC–MS analysis. However, because underivatized tetrahydrocannabinolic acid decarboxylates and also appears as THC in the chromatogram, this peak represents the total amount of underivatized material from two components of the sample, and the methylation yield can stand comparison with that of silylation, which is typically 95–97%[8]. Cannabidiol, as already described, gives two derivatives. Judging by the mass chromatograms at $m/z = 231$ (the base peak of CBD) there is no underivatized cannabidiol left.

The "sandwich" injection of cannabis extract and reagent afforded 76% methylation of cannabinol and 37% methylation of tetrahydrocannabinol (one experiment only).

TABLE III

THE MASS SPECTRA OF METHYLATED NEUTRAL CANNABINOIDS COMPARED WITH THOSE OF THEIR PARENT COMPOUNDS

The major peaks (above $m/z = 100$) of the 70 eV spectra are presented.

Tetrahydrocannabivarin (III)	203(100),	243(75),	–,	271(93),	286(85)		
O-Methyl derivative (VII)	217(60),	257(59),	269(19),	285(100),	300(86)		
Tetrahydrocannabinol (V)	231(100),	243(43),	258(30),	271(57),	–,	299(91),	314(83)
O-Methyl derivative (X)	245(59),	257(37),	272(12),	285(38),	297(21),	313(100),	328(84)
Cannabidiol (IV)	121(17),	–,	–,	174(17),	193(13),	–,	–,
O-Methyl derivative (IX)	–,	–,	174(8),	188(8),	–,	–,	–,
Dimethyl derivative (VIII)	–,	173(35),	–,	–,	221(65),	235(14),	243(44),
	231(100),	246(18),	299(2),	314(7)			
	245(100),	–,	313(3),	328(1)			
	–,	274(100),	–,	342(2)			
Cannabinol (VI)	193(29),	–,	–,	238(19),	295(100),	310(12)	
O-Methyl derivative (XI)	–,	209(12),	238(12),	252(9),	309(100),	324(14)	

The mass spectra of the derivatives

The mass spectra of the derivatives are given in Tables III and IV.

TABLE IV

THE MASS SPECTRA OF THE CANNABINOID ACID DERIVATIVES

The major peaks (above m/z = 100) of the 23 eV spectra are presented.

1′-O, 3′-O-Dimethylcannabidiolic acid methyl ester (XII) 249(14), 263(32), 267(25), 269(18), 279(100), 294(28), 332(99), 353(10), 368(13), 369(13), 400(20)
O-Methylcannabidiolic acid methyl ester (XIII)* 245(1), 303(100), 371(2), 386(8)
3′-O-Methyltetrahydrocannabinolic acid methyl ester (XIV) 270(2), 303(2), 315(3), 330(4), 339(20), 354(8), 355(8), 371(12), 386(100)

* Table II, note §.

The well-explored[16–19] fragmentation paths of tetrahydrocannabinol and tetrahydrocannabivarin are little affected by the introduction of 3′-O-methyl groups. The only major peaks in the spectra of derivatives VII and X that do not have exact counterparts (14 mass units lower) in those of the unmethylated compounds are the ones at M − 31 (loss of CH_3O). Cannabidiol, as well as THC, undergoes fragmentation by two competing paths: loss of carbon atoms 4, 5 and 8–10 in a retro-Diels–Alder reaction gives m/z = 246, and loss of carbon atoms 4–6 and 8–10, with ring-closure between carbon 1 and a phenolic oxygen, gives a chroman-like fragment at m/z = 231[16,18–20]. The latter fragmentation takes place in the monomethyl derivative IX, giving m/z = 245, but it is blocked when both phenolic groups are methylated. Instead, for compound VIII the retro-Diels–Alder fragmentation predominates, giving m/z = 274 and, with further loss of carbon atoms 1, 2, 6 and 7, m/z = 221 (a tropylium ion)[20]. Cannabinol fragments by the loss of a methyl group to give the base peak at m/z = 295 and by further loss of a butyl radical from the side-chain giving m/z = 238[16]. This holds true also for the methyl ether XI, but the spectrum of this compound still has a peak at m/z = 238, possibly indicating a loss of the whole side-chain.

The mass spectrum of cannabidiolic acid methyl ester dimethyl ether (XII) is rather complicated. The expected clevage of CH_3O from the methyl ester function can, however, be observed (m/z = 369, which is the base peak of the isobutane CI spectrum) as well as the loss of carbon atoms 4, 5, 8, 9 and 10 in a retro-Diels–Alder reaction (m/z = 332) and the further loss of carbon atoms 1, 2, 6 and 7, which gives a tropylium type ion at m/z = 279; *cf.* the fragmentation of compound VIII (Table III) and of cannabidiolic acid methyl ester[21]. The fragmentation of the monomethyl ether (XIII) occurs predominantly by loss of carbon atoms 4–6 and 8–10 with ring-closure to a chroman-like fragment (m/z = 303; *cf.* the spectra of CBD and compound IX). There is also a small M − 15 peak. The same fragmentations can be discerned in the spectrum of the tetrahydrocannabinolic acid derivative XIV, which, however, has a much more intense molecular ion peak; *cf.* the difference between the spectra of the analogous neutral cannabinoid derivatives IX and X. This compound also loses

CH_3O and CH_3OH (m/z = 355 and 354) and gives a fragment at m/z = 339, which probably implies a loss of CH_3OH and a methyl group. There is a corresponding peak at m/z = 325 in the mass spectrum of Δ^6-tetrahydrocannabinolic acid methyl ester[22].

Derivatization of tetrahydrocannabinol-7-acid

Δ^1-THC-7-acid, a major metabolite of THC in man[23], gives a derivative with a very simple mass spectrum (Fig. 4). The molecular weight is 372, m/z = 357 equals a loss of a methyl group and m/z = 313 loss of the whole methoxycarbonyl function. This acid is a more polar compound than the naturally occurring cannabinoid acids, and its derivative elutes rather late in the temperature-programmed GC runs, at 7 min. At 270°C column temperature, the retention time of the derivative is 1.2 min.

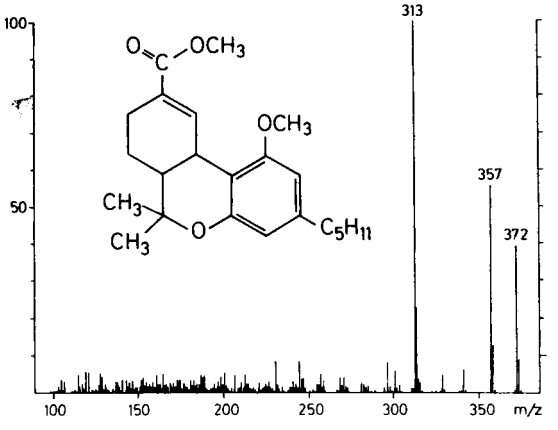

Fig. 4. The structure and 70-eV mass spectrum of 3′-O-methyltetrahydrocannabinol-7-acid methyl ester.

CONCLUSIONS

The usefulness of on-column alkylation and esterification of heat-labile phenols and phenolic acids has been evaluated with cannabis constituents as model compounds. The methylation of the monophenolic compounds CBN and THC was quantitative or near-quantitative, while the diphenolic CBD appeared as two derivatives in the chromatograms. The derivatization yields of the acid cannabinoids could not be estimated owing to lack of reference substances. The carboxylic acid functions can, however, be assumed to react faster than the phenolic ones[14]. The mass spectra of the derivatives are generally readily interpretable and should compare favourably with those of the corresponding TMS derivatives, which are often dominated by an intense M − 15 peak due to cleavage of a methyl group from the TMS function itself[9,12]. This very simple and rapid derivatization method should conceivably prove useful in the qualitative or quantitative analysis of other complex mixtures, such as plant extracts or other biological samples.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF NAPHTHYL-URETHANES WITH FLUORESCENCE DETECTION

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SUMMARY

The method used previously for the fluorodensitometric determination of compounds with an alcoholic hydroxyl group was examined for its applicability in high-performance liquid chromatography. The conversion of alcoholic substances into urethanes was performed with naphthyl isocyanate. Chloroform–benzene–ethanol and *n*-heptane–diethyl ether, were used as eluents for the separation of urethanes of various polarity with silica gel columns. Reversed-phase material is also suitable. The detection limits ascertained by means of fluorescence detection are in the picomole range.

INTRODUCTION

Several workers have reported on the conversion of alcohols into urethanes with phenyl isocyanate and their high-performance liquid chromatographic (HPLC) separation[1–7]. Fluorescence detection of such compounds is not possible, however. In previous papers[8,9] we described the derivatization of substances with an alcoholic hydroxyl group using fluorescent isocyanates, followed by fluorodensitometric measurement. Both naphthyl isocyanate (NI) and anthracene isocyanate (AI) can be used for the derivatization of many alcoholic compounds with different structures. Owing to the greater number of rings in the AI molecule, its reactivity is lower, especially with tertiary alcohols. This may be balanced by increasing the excess of the reagent. However, it was found that this may lead to a complicated separation of the urethanes from the by-products. This paper describes the use of NI, enabling smaller amounts of reagent to be used in the determination of compounds with alcoholic hydroxyl groups by means of HPLC with fluorescence detection.

EXPERIMENTAL

Apparatus

An Altex Model 110A liquid chromatograph equipped with a Perkin-Elmer

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650-10S fluorescence detector and a Perkin-Elmer W + W 1100 recorder were used, together with a Rheodyne variable-volume injector. For the determination of the excitation and emission maxima 10^{-4} *M* solutions of the substances in benzene were prepared and measured with a Perkin-Elmer MPF 44 fluorimeter and recorded with a Perkin-Elmer 023 recorder.

Reagents and materials

A pre-packed 10-μm LiChrosorb Si 100 and a 5-μm LiChrosorb RP-18 column (25 cm × 0.46 mm I.D.) were purchased from Merck (Darmstadt, G.F.R.). Throughout this work doubly distilled water was used. The solvents and chemicals were of analytical-reagent grade from Merck, except for acetonitrile, which was obtained from Rathburn (Walkerburn, Great Britain). They were used without any further pre-treatment.

Procedure

A 1-ml volume of a toluene solution containing not more than 400 ng of the alcoholic compound is pipetted to 0.5 ml of a 1.5-fold molar excess of NI in toluene and to 0.5 ml of a 1 *M* solution of triethylenediamine (TED) in toluene. After heating (primary and secondary alcohols for 60 min at 95°C and tertiary alcohols for 120 min at 140°C, all solutions in xylene), 1 ml of a 3-fold molar excess of diethylamine in toluene is added while still hot. The reaction mixture is shaken briefly and centrifuged at 1000 *g* for 5 min. From the clear, supernatant solution a volume of 200 μl is used for injection on to the chromatographic column.

Determination of the substance 32-468 {4' [2-hydroxy-3-(1,1-dimethylamino)propoxy]-spiro(cyclohexa-2',1-indan)-1'-one} in plasma

To 1 ml plasma, spiked with 100 ng of 32-468, are added 5 ml of 1 *M* sodium hydroxide solution and 10 ml of chloroform and the mixture is shaken for 20 min and centrifuged at 1000 *g* for 5 min. After taking an aliquot of 9 ml of the organic phase and evaporating it under reduced pressure at 35°C, the dry residue is further treated as described above.

RESULTS AND DISCUSSION

Separation on a LiChrosorb Si 100 column

Using silica gel as the stationary phase we used thin-layer chromatography (TLC) as a pilot technique for the selection of suitable mobile phases. We first used benzene–diethyl ether (95:5), which we had previously used in fluorodensitometric analyses. In order to obtain approximately results of the same quality as with TLC separation, less polar eluents have to be applied in HPLC. Instead of benzene, we therefore used toluene, xylene and *n*-heptane. With *n*-heptane–diethyl ether (95:5) a satisfactory separation of the urethanes listed in Table I from their by-products could be achieved.

From Table I it can also be seen how the urethanes are eluted with increasing polarity of their alcoholic component. It takes about 28 min for a run when working isocratically, because the by-products are eluted relatively late. Naphthylamine, which appears as the last peak in the chromatogram, may be eliminated by extraction

TABLE I

CAPACITY FACTORS (k') OF URETHANES AND BY-PRODUCTS USING *n*-HEPTANE–DIETHYL ETHER (95:5) AS THE ELUENT

Flow-rate: 4 ml/min.

Compound	k'	*Compound*	k'
Naphthylamine	31.84	O-Octanol-N-naphthylurethane	4.19
N,N-Diethyl-N′-naphthylcarbamide	18.74	O-Cetyl alcohol-N-naphthylurethane	3.93
N,N′-Dinaphthylcarbamide	10.84	O-Cholecalciferol-N-naphthylurethane	2.67
O-3-Methyl-1-butanol-N-naphthylurethane	4.96	O-Cyclohexanol-N-naphthylurethane	2.62
O-1-Butanol-N-naphthylurethane	4.83	O-Cholesterol-N-naphthylurethane	2.56

with 1 *M* hydrochloric acid and the time for one run is then shortened by about 40% (*cf.*, Fig. 1) and may be further shortened by changing to a more polar eluent after the appearance of the urethane peak.

As the first tests demonstrated, the application of a 30-fold molar excess of the reagent, as chosen for the fluorimetric quantitations on TLC, is unsuitable. The separation of the by-products from the urethanes in this instance involves great difficulties. To overcome this problem, the reagent concentration had to be decreased in order to decrease the peaks of the by-products. This is particularly effective with primary and secondary alcoholic compounds, which react quantitatively within a few

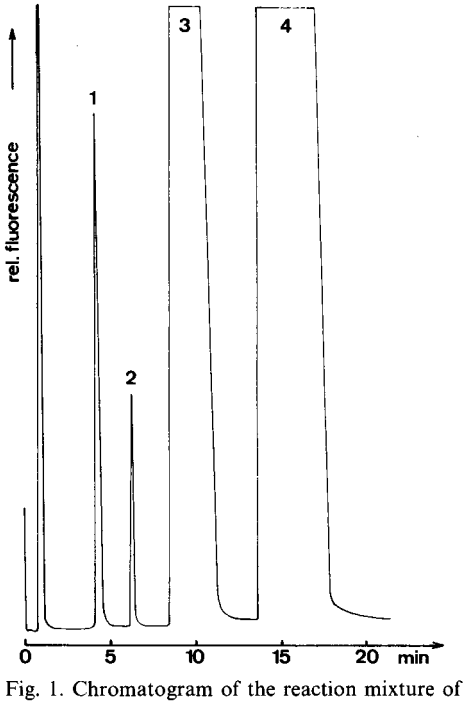


Fig. 1. Chromatogram of the reaction mixture of 200 ng of cetyl alcohol with NI after HCl extraction. Mobile phase, *n*-heptane–diethyl ether (95:5); flow-rate, 4 ml/min. Peaks: 1 = urethane; 2, 3, 4 = by-products.

TABLE II

PEAK AREAS (ARBITRARY UNITS) OF THE REACTION PRODUCTS OF 1-BUTANOL WITH NI

Reaction temperature, 95°C; reaction time, 60 min; injection volume, 10 µl.

Molar excess of reagent	*Amount of alcohol (ng)*		
	200	*75*	*10*
30-fold	896	404	105
3-fold	891	401	102
1.5-fold	891	400	102

minutes with a 30-fold molar excess of reagent. When the reaction time is prolonged to 60 min sufficient derivatization is also achieved after the reagent concentration is decreased to 95% of the initial amount. Table II, which shows the results for the formation of urethane by reaction of 1-butanol with NI, indicates that with a 1.5-fold molar excess of the reagent no reduction in the reaction rate occurs. Steroids with a primary or secondary hydroxyl group, which have low reactivity, need a reaction time of 60 min at 95°C. By increasing the reaction temperature to 140°C compounds with a tertiary hydroxyl group may also be derivatized with a 1.5-fold molar excess of NI within 120 min.

The excess of NI, which is difficult to elute owing to its high polarity, is destroyed by adding diethylamine.

The smallest detectable amounts of urethanes and their excitation and emission maxima are given in Table III. All of the investigated compounds with an alcohol group show a linear relationship between the amount of N-naphthylcarbamic acid ester applied and the peak area. The detection limit is between 1 and 20 ng; the highest tested concentration was 100 ng in an injection volume of 200 µl.

This system, with *n*-heptane–diethyl ether as the mobile phase, can be applied particularly to the separation of reaction mixtures of urethanes with less polar alcohol groups. For urethanes with a polar alcoholic component it is less suitable because

TABLE III

DETECTION LIMITS, MEASURED AT A SIGNAL-TO-NOISE RATIO OF 3:1, AND EXCITATION AND EMISSION MAXIMA

Injection volume, 200 µl; mobile phase, *n*-heptane–diethyl ether (95:5).

Derivatized alcoholic compound	*Limit of detection (ng)*	*Excitation maximum (nm)*	*Emission maximum (nm)*
Isopentyl alcohol	4	310	350
1-Butanol	4	310	355
Cetyl alcohol	1	308	350
Cholecalciferol	20	318	352
Cholesterol	6	319	356
Cyclohexanol	4	318	350
1-Octanol	1	310	350

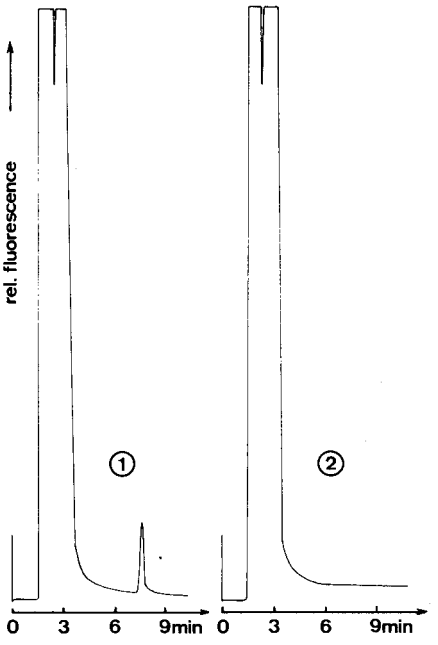


Fig. 2. Chromatogram of the reaction mixture of 200 ng of quinine with NI (1) compared with the blank (2). Mobile phase, chloroform–benzene–ethanol (45:30:0.2); flow-rate, 2 ml/min.

under these conditions separation from the polar decomposition products is difficult. For this purpose, chloroform–benzene–ethanol (45:30:0.2) was found to be a suitable eluent. Whereas the relatively polar by-products of derivatization eluted very late with *n*-heptane–diethyl ether as the eluent, they now appeared within 4 min, followed by the urethane. In contrast to the *n*-heptane–diethyl ether eluent, the excess of reagent could now be increased again. The resulting stronger peaks of the by-prod-

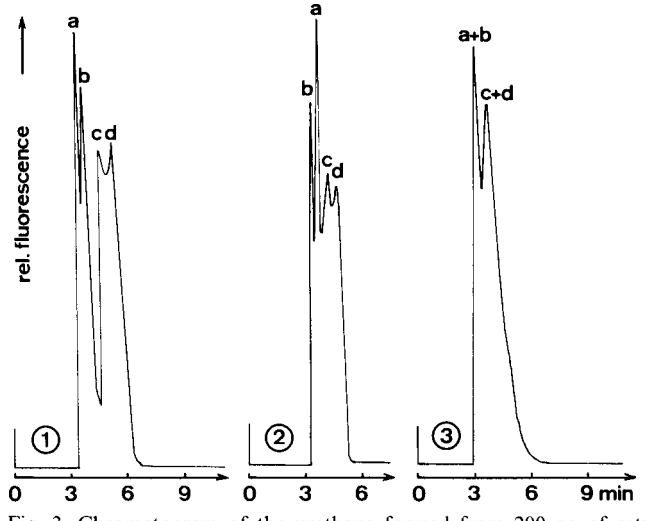


Fig. 3. Chromatogram of the urethane formed from 200 ng of cetyl alcohol and NI. Mobile phase, chloroform–benzene–ethanol: (1) 45:30:0.7; (2) 45:30:5; (3) 15:15:20. Peaks: (a) urethane; (b), (c), (d), by-products.

ucts are eluted very rapidly owing to the higher polarity of the chloroform–benzene–ethanol mixture.

Fig. 2 shows as an example the reaction of quinine with a 1.5-fold molar excess of NI. The run is finished within a few minutes when working isocratically. However, by derivatizing with a 30-fold molar excess of NI separation from the by-products can also be observed.

An attempt was made to use chloroform–benzene–ethanol as the eluent for the analysis of urethanes with a non-polar hydroxyl group, *e.g.*, of butanol or cetyl alcohol. As can be seen in Fig. 3, this solvent system is not appropriate for the separation of urethanes with a lipophilic component. Starting from the mixture chloroform–benzene–diethyl ether (45:30:0.2) the amount of ethanol was gradually increased in an attempt to achieve the separation of by-products. However this was not successful, just as adding of more polar solvents, *e.g.*, acetic acid was also not successful.

The possibility of urethane formation and subsequent HPLC separation with chloroform–benzene–ethanol was also investigated for the determination of 32-468 in plasma.

This substance is a β-blocking agent and possesses a secondary alcoholic hydroxyl group and a secondary amino group in the side-chain, both of which are suitable for derivatization with NI. Three different structures of the derivatives would therefore be conceivable, namely the N-substituted, the O-substituted and the di-substituted products. A closer investigation of the resulting derivative was not performed as this is of no importance for the determination. In comparison with ephedrine[8], the reaction takes place more slowly. As can be seen from Fig. 4, employing a 1.5-fold molar reagent excess, in this instance also within 120 min, a low tendency to react may be observed. When employing a 15-fold molar excess of NI the derivatization is virtually completed within 60 min.

For the determination of 32-468 in plasma the optimal extraction conditions with different organic solvents at different pH values were sought. By shaking once with chloroform at pH 11 an average recovery of 99.8% could be attained. Fig. 5 shows the chromatogram of the reaction product of 300 ng of 32-468 with NI after chloroform extraction from plasma. The detection limit of the urethane was 60 ng for an injection volume of 200 μl.

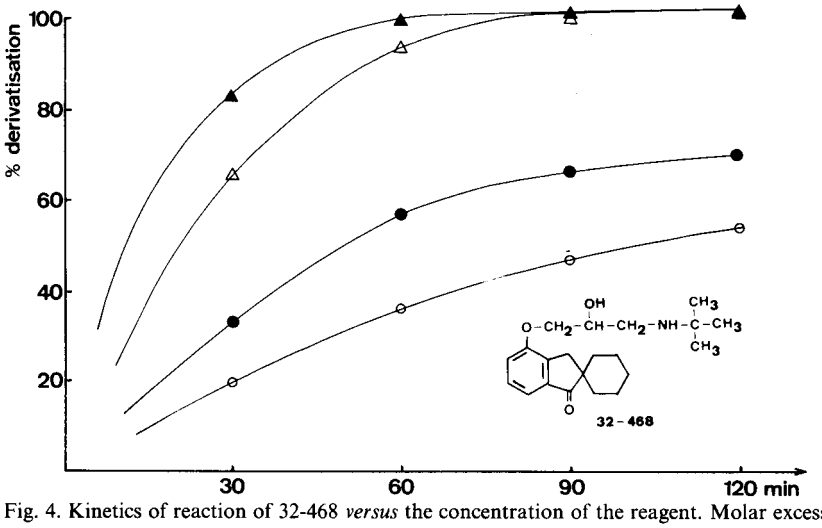


Fig. 4. Kinetics of reaction of 32-468 *versus* the concentration of the reagent. Molar excess: ▲, 150-fold; △, 15-fold; ●, 3-fold; ○, 1.5-fold.

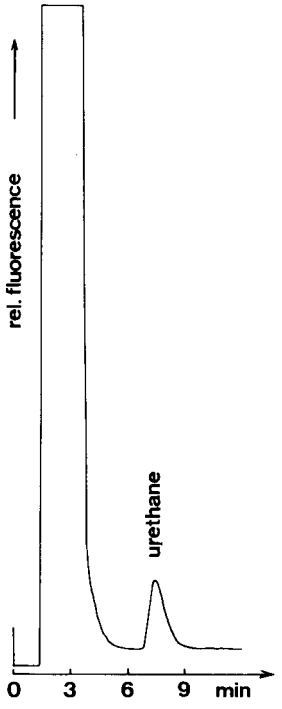


Fig. 5. Chromatogram of the urethane formed from 32-468 and NI. Mobile phase, chloroform–benzene–ethanol (45:30:0.2); flow-rate, 2.5 ml/min.

Separation on a reversed-phase column

As a further possibility, analysis with a reversed-phase support was considered. With such a system, the urethanes must be soluble in acetonitrile–water and methanol–water mixtures. As the solubility tests showed, the N-naphthylcarbamic acid esters with 1-butanol, 2-propanol, 2-phenylethanol or similar alcohols are soluble, whereas the N-naphthylcarbamic acid esters with cetyl alcohol and cholesterol are insufficiently polar and therefore insoluble. In this instance the system with *n*-heptane–diethyl ether in combination with a LiChrosorb Si 100 column is suitable.

By applying acetonitrile-water (60:40) as the eluent the urethanes listed in Table IV may be separated easily from their decomposition products with an RP-8 column. The reactions were carried out with a 1.5-fold molar excess of NI. As Fig. 6 demonstrates, another peak appears after the urethane peak when working isocrati-

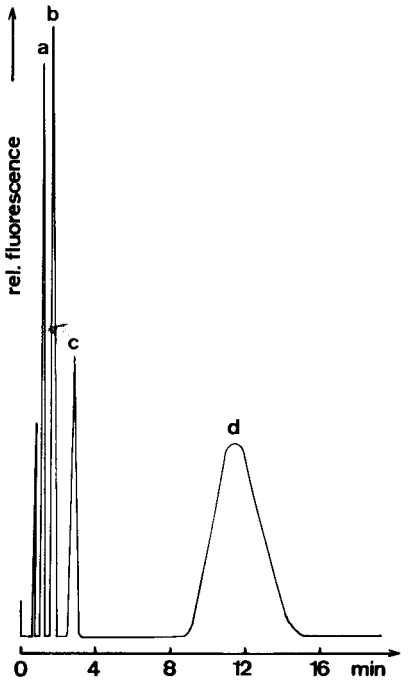
TABLE IV

CAPACITY FACTORS (k') OF NAPHTHYLURETHANES USING ACETONITRILE–WATER (60:40) AS THE ELUENT

Flow-rate: 4 ml/min.

Derivatized alcohol	k'	*Derivatized alcohol*	k'
2-Phenylethanol	2.95	2-Methyl-2-propanol	2.46
Benzyl alcohol	2.83	2-Butanol	2.42
Codeine	2.58	1-Butanol	2.40
2-Propanol	2.49		

cally. This may be eliminated within a short time by changing the eluent to pure acetonitrile after the appearance of the urethane peak.



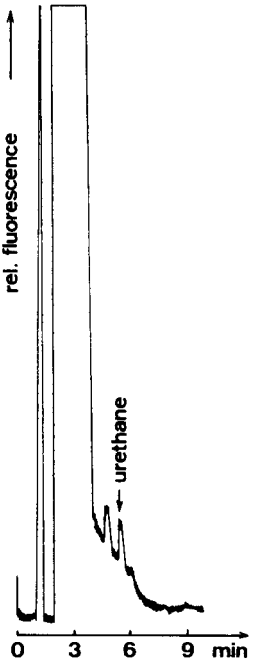


Fig. 6. Chromatogram of the reaction mixture of 200 ng of codeine with NI. Mobile phase, acetonitrile–water (60:40); flow-rate, 4 ml/min. Peaks: (a), (b), (d), by-products; (c), urethane.

Fig. 7. Detection limit of the urethane formed from *n*-butanol and NI; 10 pmol per 100-μl injection volume. Mobile phase, acetonitrile–water (60:40); flow-rate, 2 ml/min.

Fig. 7 gives an example of the urethane with 1-butanol, illustrating that the detection limits for all derivatizations were roughly the same. Linearity was observed from the detection limit to about 400 ng. The correlation coefficients for the function $y = ax + b$ were between 0.997 and 0.999.

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COMPARISON OF THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PEPTIDES AND PROTEINS ON 100- AND 300-Å REVERSED-PHASE SUPPORTS

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SUMMARY

The chromatographic separations of peptides and proteins on commercially available 100- and 300-Å pore size reversed-phase columns have been compared using various buffer systems. The larger-pore-size packing exhibits a slightly more hydrophilic character while maintaining flow and back-pressure characteristics typical of 10-μm supports. In addition to equal or improved resolving capabilities for smaller amino acid derivatives and peptides, this column material is notably superior to the 100-Å supports for the chromatography of proteins with molecular weights exceeding *ca.* 15,000.

INTRODUCTION

Applications of the method referred to as high-performance liquid chromatography (HPLC) are being found in such widely diversified areas as forensic medicine and clinical, as well as analytical, chemistry. Recently, its uses as an analytical or preparative method in the field of biochemistry have become more obvious. Amino acid analysis of either the free acids[1–3] or derivatives[3–5] thereof, as well as the identification of amino acid derivatives from Edman degradations[6,7], have been reported. Peptides from natural sources[8–11] and those from enzymatic digestions[11–15] or from chemical fragmentations[15–18] can be isolated and efficiently desalted[19].

There have been fewer reports describing the chromatography of large peptide fragments[12,16,17–20], proteins[21–23] or enzymes[24–27]. The main reason for this limited use has been the lack of a suitable commercially available reversed-phase packing which does not experience non-specific adsorption, and thereby low recoveries and/or

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reduced resolution. Some researchers have circumvented this problem by synthesizing their own packings, *e.g.* C_8, diphenyl and cyanopropyl groups have been attached to 330- and 500-Å pore size (10 μm) materials[26] or C_8, C_{18} and adamantyl groups on a series of 5- and 10-μm packings ranging in pore sizes from 55 to 1000 Å[20]. Similarly, a commercially available 300-Å pore size C_{18} column has also been utilized[16]. In each of the above-mentioned cases the chromatographic results have suggested that protein recovery increases with increasing pore size.

In this report we compare the applicability of the 100- and 300-Å packings available from Brownlee Labs.; the results indicate that the larger-pore-sized material offers superior resolution and recovery of not only peptides and proteins but also Dns-amino acids[4].

EXPERIMENTAL

Apparatus

An HPLC unit consisting of two Altex Model 100 pumps, a Rheodyne Model 7125 injector (600-μl sample loop), a Kontron Model 200 microprocessor and an Unvikon 725 spectrophotometer equipped with an 8-μl flow-through cell was used for UV detection. Samples were chromatographed on 250 × 4.6 mm 10-μm particle size columns of LiChrosorb RP-18 (100 Å pore size) or Aquapore RP 300 (300 Å pore size) from Brownlee Labs. (Santa Clara, CA, U.S.A.). When necessary the column effluent was collected automatically using an LKB Ultrarac Model 7000 fraction collector. Portions were removed (10–15%), diluted into a scintillator and radioactivity measurements performed using a Packard Model PLD Tri-Carb liquid scintillation counter. No quenching of radioactivity by the buffers was observed under the experimental conditions used.

Chemicals

Acetonitrile and 2-propanol (HPLC quality) were purchased from J. T. Baker (Gross Gerau, G.F.R.), water was quartz bi-distilled. All other chemicals were of reagent-grade quality from Fluka (Buchs, Switzerland) or E. Merck (Darmstadt, G.F.R.) and used without further purification. Obtained from Sigma (St. Louis, MO, U.S.A.) were cytochrome *c* (horse heart), carbonic anhydrase (bovine erythrocytes) serum albumin (chicken) and myoglobin (whale) which was further converted into the apo-form by extraction with methyl ethyl ketone[28]. Alcohol dehydrogenase (yeast), maleic dehydrogenase (pig heart mitochondria), creatine kinase (rabbit muscle) and 3-phosphoglycerate kinase (yeast) were from Boehringer (Mannheim, G.F.R.).

Ovalbumin (egg white) was purchased from Worthington (Freehold, NY, U.S.A.). Parvalbumin (rat muscle) was prepared as described for the isolation of the same protein from chicken muscle[29]. Human serum albumin was isolated and further purified as described[30]. The bovine pancreatic trypsin inhibitor (Trasylol®; Bayer, Leverkusen, G.F.R.) and lysozyme (chicken, Sigma) were used as their carboxymethylated[31] derivatives.

Methods

Prior to reversed-phase chromatography the proteins were labelled by reduc-

tive methylation[32] using sodium cyanoborohydride and [^{14}C]formaldehyde (Radiochemical Centre, Amersham, Great Britain), followed by desalting over Sephadex G-25 and lyophilization. Each sample was then dissolved in water, or in some cases 1.0% phosphoric acid, diluted into the starting buffer for chromatography and injected at concentrations between 5 and 600 μg/μl. Unless otherwise stated, the amounts of protein applied onto the column ranged between 20 and 150 μg. Following effluent collection the percent recovery was determined by liquid scintillation.

Peptides of rat muscle parvalbumin were prepared as described[33] by tryptic digestion (TPCK–trypsin, Worthington) and recovered by lyophilization. Chromatography was carried out on 30–60-μg amounts of the peptide material.

The buffer systems used for chromatography were (I) buffer A, 0.01 *M* sodium perchlorate in 0.1% phosphoric acid, pH 2.1, and B, as A except 60% (v/v) in acetonitrile; or (II) as in (I) except 60% (v/v) in 2-propanol. All chromatography was carried out using a linear gradient of B buffer (2.22% per min) at room temperature (*ca.* 22°C) with a flow rate of 1.0 ml/min. For both the 100- and 300-Å packings the column back pressures increased during gradient development from 500 to 800 p.s.i. with buffer system I and from 725 to 2200 p.s.i. with system II. For a higher pH phosphate system, A buffer of system I (see above) was titrated with 2 *M* sodium hydroxide to pH 7.0. Similarly, the aqueous part of B buffer (containing the appropriate amounts of phosphoric acid and sodium perchlorate to yield the correct concentrations upon final dilution with the organic phase) was adjusted to pH 7.0 prior to addition of 60% (v/v) acetonitrile. The Tris buffer system used consisted of 50 m*M* Tris-HCl, pH 7.5, as A buffer and the same containing 60% (v/v) acetonitrile as B buffer.

RESULTS AND DISCUSSION

The chromatography of peptides and smaller protein fragments by reversed-phase HPLC has been carried out predominantly on either C_8 or C_{18} hydrocarbon ligands bonded to 5- or 10-μm particle size packings with nominal pore sizes of < 100 Å. Fig. 1 illustrates that the more hydrophobic character of a C_{18} hydrocarbon ligand on such packings (Fig. 1B) results in a higher resolution of the chromatographed peptides, ranging in length between 3 and 15 residues[33], than the cyanopropylmoeity (Fig. 1A). The yields from the peptide separation on the C_{18} support (Fig. 1B) averaged 66.9%. That the amino acid compositions of the peptides are in excellent agreement with their known sequences[33,34] supports minimal cross-contamination between closely eluting peaks.

Chromatography of the same peptide mixture on 100- and 300-Å supports at low pH, conditions identical to those in Fig. 1, are illustrated in Fig. 2. The more hydrophilic nature of the 300-Å support is indicated by the quicker elution of the peptides. Peak resolution is not identical on both supports since the two peptides indicated in Fig. 2A (by arrows) were not found to separate on the 300-Å column (Fig. 2B). The similar peak areas from both columns indicate that the 300-Å support can be conveniently used for peptide mapping with yields approximating those from the 100-Å packing (see above).

When the pH of the chromatographic buffers were increased, the peptides generally became more hydrophilic owing to deprotonation and were thus eluted

TABLE I

EFFECT OF MOBILE PHASE pH ON PEPTIDE RETENTION

*Peak number**	*Peptide sequences***	*Retention times (min)*		*Peptide charge****		*Peptide hydrophobicities*[§]		
		pH 2.2 (Fig. 1B)	*pH 7.0 (Fig. 3A)*	*pH 1.9*	*pH 6.5*	*Wilson et al.*[§§]	*Meek and Rossetti*[§§§], *pH 2.1*	*Meek*[†], *pH 7.4*
1	ETK	5.1	2.0	+2	0	− 5.8	− 2.9	−14.1
2	SADDVKK	12.2	2.5	+3	0	− 5.0	− 3.2	−11.8
3	DLSAK	13.8	5.8	+2	0	+ 0.7	+ 5.4	+ 2.4
4	GFSSDAR	17.1	8.3	+2	0	+ 4.7	+ 5.9	+ 8.7
5	TLMAAGDKDGDGK	18.7	9.2	+3	−1	− 1.5	+ 7.7	− 7.1
6	VFHILDKDK	26.0	20.8	+4	+0.5	+13.0	+31.1	+18.9
7	AIGAFTAADSFDHKK	27.3	17.9	+4	+0.5	+ 5.2	+26.9	+26.5
8	Ac-SMTDLLSAEDIKK	29.0	16.2	+2	−2	−	+29.2	+14.4
9	IGVEEFSTLVAES	29.7	17.2	+1	−3	+27.7	+39.6	− 3.8
10	FFQMVGLK	30.5	22.9	+2	+1	+27.4	+44.1	+38.0
11	SGFIEEDELGSILK	31.1	16.2	+2	−3	+25.5	+44.2	+ 2.2

* Refers to those numbered peaks in Figs. 1B and 3A which were identified by amino acid analysis.

** From ref. 33.

*** Calculated according to Offord[35] and assuming histidine to have a charge of +0.5 at pH 6.5.

[§] Calculated by summation of the hydrophobic values for each amino acid side chain within the peptide; lists of amino acid hydrophobicities are given in the indicated refs.

[§§] Ref. 36.

[§§§] Ref. 37.

[†] Ref. 38.

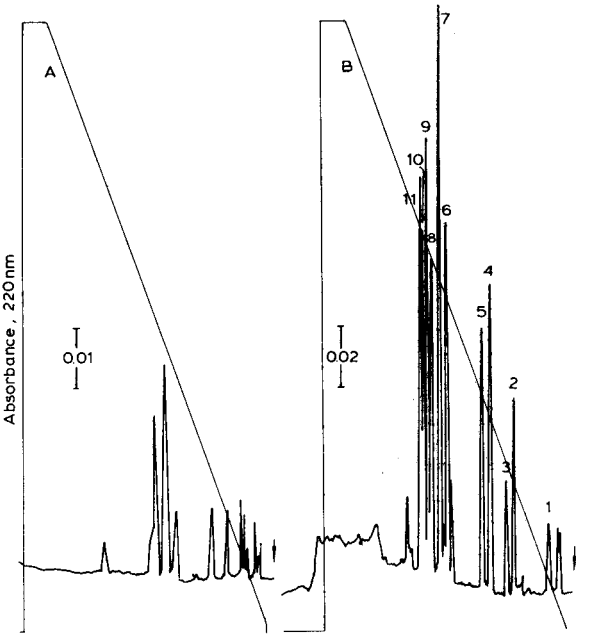

Fig. 1. Chromatography of the tryptic peptides from rat muscle parvalbumin on (A) 70-Å (6 μm) cyanopropyl- and (B) 100-Å (10 μm) C_{18} packings. The peptides were dissolved in 100 μl of A buffer [2.7 nmol and 5.0 nmol for (A) and (B), respectively] and chromatographed with an acetonitrile gradient (buffer system I at pH 2.1, see Experimental). The arrows indicate the points of injection. The peaks in (B) are numbered so that comparisons can be made with Fig. 3A; see Table I.

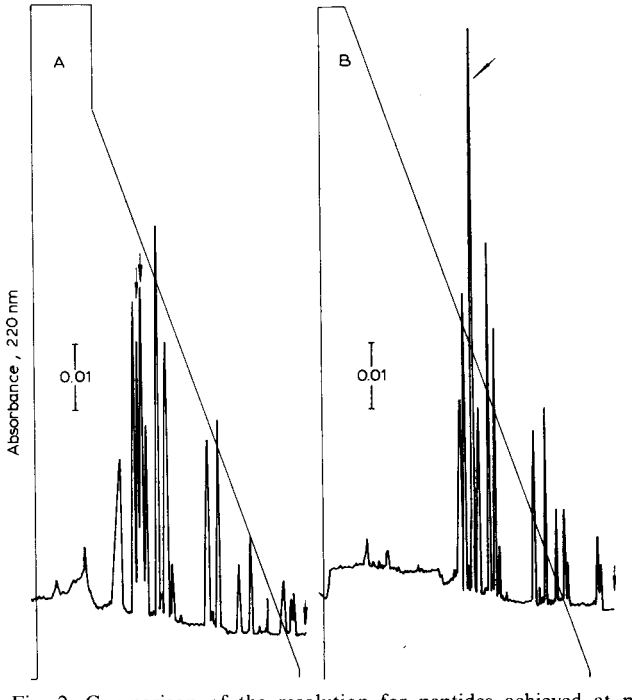

Fig. 2. Comparison of the resolution for peptides achieved at pH 2.1 by 100- (A) and 300-Å (B) supports. The peptide mixtures (2.5 nmol) were chromatographed as above using buffer system I.

earlier, *i.e.* at lower buffer *B* concentrations (*cf.* Figs. 2B and 3A). Such pH changes do not effect all peptides equally, *e.g.* resulting simply in an elution at lower acetonitrile concentrations while maintaining the same elution order. Some peptides were found to be greatly effected while others not at all. Table I gives the sequences of the peptides and their chromatographic as well as charge characteristics at both pH 2.2 and 7.0. At the lower pH the peptides do elute as a function of their increasing apolarity when the newer values from Meek and Rossetti[37] for amino acid hydrophobicities are used to calculate peptide hydrophobicity. This would be expected since the buffer systems used in this study and that of Meek and Rossetti[37] are essentially the same. However, when the amino acid hydrophobicity values determined by us in a different buffer system[36] (*e.g.* pyridine–formate–acetate using 1-propanol as the organic eluant) are used the estimation of peptide elution order is less satisfactory. Using the values from a previous investigation by Meek[38], calculated peptide hydrophobicities for pH 7.4 digress considerably from the observed elution order seen in Fig. 3A. Conceivably this might be due to one (or more) of the amino acid hydrophobicity values being incorrectly assessed. The differences in peptide chromatographic behaviour are sufficient enough, however, to utilize simply a change in the pH as a further isolation step or as a check for purity. Utilizing these columns at pH 7.0 to 7.5 for several weeks has not resulted in any noticeable chromatographic changes in either resolution or yield. Similarly, switching from one buffer system to another with a different pH is conveniently performed without effecting column performance.

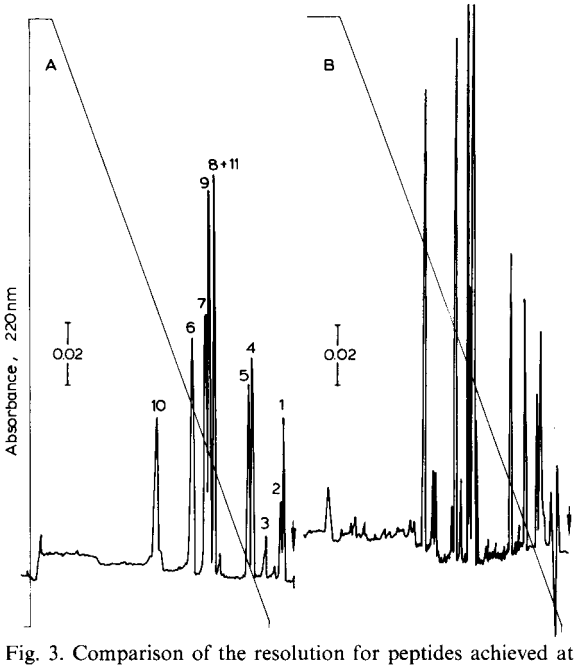

Fig. 3. Comparison of the resolution for peptides achieved at neutral pH using 100-(A) and 300-Å (B) supports. The buffer systems were for (A) as in Figs. 1 and 2 except that the pH of the buffer was titrated to 7.0 and for (B) a 50 m*M* Tris–HCl, pH 7.5, 60% (v/v) acetonitrile system (see Experimental). The peptide amounts injected were for 5.0 nmol for both (A) and (B). The peaks in (A) are numbered so that comparisons can be made with Fig. 1B; see Table I.

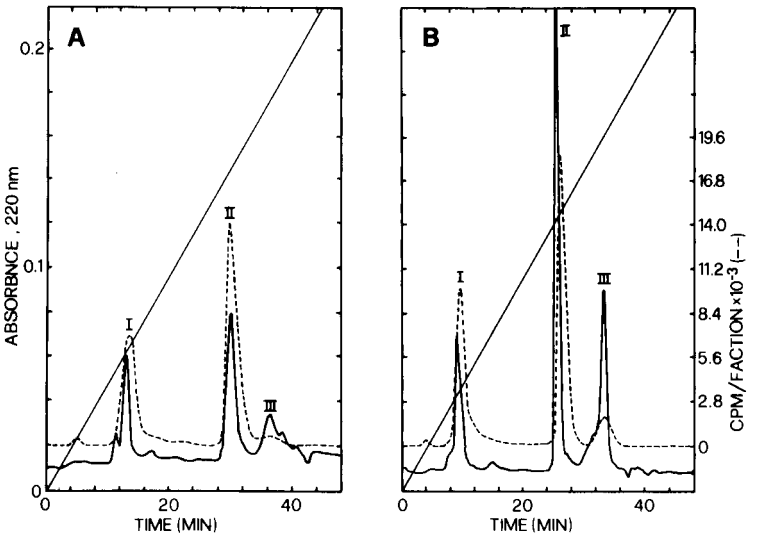

Fig. 4. HPLC of [^{14}C]methylated proteins on 100-(A) and 300-Å (B) packings. The samples injected were: I = bovine pancreatic trypsin inhibitor (57 μg, $1.6 \cdot 10^5$ cpm); II = myoglobin (48 μg, $2.2 \cdot 10^5$ cpm); III = ovalbumin (76 μg, $5.3 \cdot 10^4$ cpm). Elution was carried out with a 2-propanol gradient at low pH (buffer system II, see Experimental) and 1.4-ml fractions were collected. Solid lines represent absorbance at 220 nm and dashed the ^{14}C-label incorporated into the proteins by reductive methylation (see Experimental).

The chromatography of a protein mixture on such supports indicates that resolution as well as yield are a function of both the packing pore size and protein molecular weight. On the 300-Å column (Fig. 4A) peak broadening is significantly

TABLE II

COMPARISON OF PROTEIN REVERSED-PHASE CHROMATOGRAPHY ON 100- AND 300-Å (10 μm) PACKINGS

Elution carried out using buffer system II: A buffer, 10 m*M* $NaClO_4$ in 0.1 % H_3PO_4; B buffer, as A except 60 % (v/v) in 2-propanol.

Protein	*Molecular weight (ref.)*	*A, LiChrosorb RP-18, 100 Å*		*B, Aquapore RP 300, 300 Å*		*Differences (column A minus column B)*	
		B (%)	*Yield (%)*	*B (%)*	*Yield (%)*	*B (%)*	*Yield (%)*
Bovine pancreatic trypsin inhibitor	6500 (39)	29.0	81.1	20.5	59.8	+ 8.5	+21.3
Cytochrome *c*	11,700 (39)	58.5	48.0	56.7	45.6	+ 1.8	+ 2.4
Parvalbumin	11,700 (33)	74.0	102.9	60.5	78.2	+13.5	+24.7
Lysozyme	14,300 (39)	62.5	67.1	63.4	44.1	− 0.9	+23.0
Myoglobin	17,200 (39)	64.0	89.0	59.0	94.9	+ 5.0	− 5.9
Creatine kinase	42,000 (40)	73.5	33.7	69.0	67.2	+ 4.5	−33.5
Ovalbumin	43,000 (41)	82.0	36.5	75.5	57.3	+ 5.5	−20.8
Serum albumin, chicken	65,000 (42)	62.5	76.5	56.0	101.1	+ 6.5	−24.6
Serum albumin, human	69,000 (43)	59.5	48.3	50.6	98.5	+ 8.9	−50.2

decreased in comparison to a 100-Å packing (Fig. 4B) and the yields of the larger proteins, here ovalbumin, are improved. As shown in Table II the protein elution times also decrease by 5–10 % owing to the more hydrophilic character of the support. The differences in per cent yields thus appear to be a function of both protein molecular weight and pore size, *i.e.* under *ca.* 15,000 the yields are higher with the 100-Å material; conversely, the 300-Å support is superior for higher-molecular-weight proteins.

Overall yields remain constant or slightly increase when more protein is progressively chromatographed (Fig. 5). That portion not recovered in the effluent appears to remain irreversibly bound to the column since neither radioactivity nor absorbance can be detected when changing to either higher concentrations of 2-propanol or other buffer solutions (*e.g.* 0.1 % trifluoroacetic acid). This observation is supported by the results from the isolation of various proteins from both muscle and brain extracts by reversed-phase HPLC using above-mentioned chromatographic conditions[44,45]. In such cases the eluted proteins were collected, desalted and radioactively labelled by reductive methylation. They exhibited only single spots by either protein staining or autoradiography following two-dimensional sodium dodecyl sulphate isoelectric focusing, thereby indirectly supporting the idea that once protein is lost (presumably by either precipitation or hydrophobic association with the packing) it is slowly, if at all, eluted during subsequent separations.

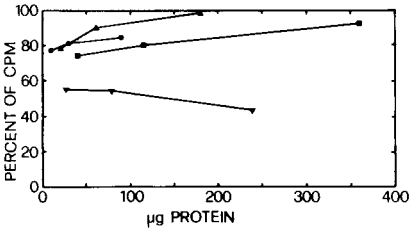

Fig. 5. Influence of protein quantity on per cent [^{14}C] recovery. The samples injected on the 300-Å C_{18} column were: ▲, myoglobin; ●, bovine pancreatic trypsin inhibitor; ■, carbonic anhydrase; ▼, ovalbumin. Elution was carried out with the 2-propanol gradient (buffer system II).

Table III summarizes the results from the chromatography of a number of proteins, molecular weights ranging between 6500 and 69,000, on the RP 300 support with buffer systems I and II. With the exception of myoglobin, all the proteins chromatographed were eluted in higher yields when 2-propanol, rather than acetonitrile, was used as the organic eluent. The protein elution points were decreased by approximately 25 % owing to the higher solvent strength while maintaining comparable peak heights and widths (results not shown). Other proteins which have been chromatographed with similar yields are α- and β-chains of haemoglobin, calmodulin, insulin-like growth factors, S-100 protein and troponin c[44,45].

In summary, the C_{18} reverse-phase support of 300-Å pore size offers a number of improvements: (a) proteins of molecular weights exceeding *ca.* 15,000 can be chromatographed with higher yields; (b) the resolution on such supports for proteins is better; (c) peptide mapping can be performed under conditions which give results approximating those of the smaller-pore-size packings; and (d) smaller molecules

TABLE III

ELUTION POSITIONS AND YIELDS FROM PROTEIN REVERSED-PHASE CHROMATOGRAPHY ON AQUAPORE RP-300 (300 Å, 10 μm) PACKINGS UTILIZING ACETONITRILE AND 2-PROPANOL GRADIENTS

nd = Not determined.

Protein	*Molecular weight*	*A, buffer system I (acetonitrile)*		*B, buffer system II (2-propanol)*		*Differences (column A minus column B)*	
		B (%)	*Yield (%)*	*B (%)*	*Yield (%)*	*B (%)*	*Yield (%)*
Bovine pancreatic trypsin inhibitor	6500 (39)	42.5	36.1	20.5	59.8	22.0	−23.7
Cytochrome *c*	11,700 (39)	nd	nd	56.7	45.6	–	–
Parvalbumin	11,700 (39)	85.6	65.0	60.5	78.2	25.1	−13.2
Lysozyme	14,300 (39)	nd	nd	63.4	44.1	–	–
Myoglobin	17,200 (39)	88.6	98.3	59.0	94.9	29.6	+ 3.4
Carbonic anhydrase	31,000 (46)	nd	nd	68.5	81.3	–	–
Maleic dehydrogenase	35,000 (47)	nd	nd	?	0	–	–
Alcohol dehydrogenase	36,700 (46)	nd	nd	78.4	18.9	–	–
Creatine kinase	42,000 (40)	88.7	60.3	64.0	67.2	24.7	− 6.9
Ovalbumin	43,000 (41)	>100	36.9	75.5	57.3	?	−20.4
3-Phosphoglycerate kinase	47,000 (48)	nd	nd	78.4	37.9	–	–
Serum albumin, chicken	65,000 (42)	93.7	97.7	56.0	101.1	37.7	− 3.4
Serum albumin, human	69,000 (43)	85.0	68.0	50.6	98.5	34.4	−30.5

such as Dns-amino acids chromatograph extremely well on the larger-pore-size material[3].

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SEPARATION OF PEPTIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON A WEAK ANION-EXCHANGE BONDED PHASE

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(Received November 11th, 1981)

SUMMARY

Multicomponent peptide mixtures were separated by high-performance liquid chromatography on a MicroPak AX-10 column, a silica-based bonded-phase weak anion exchanger. A gradient of increasing 0.01 *M* triethylammonium acetate buffer (pH 6.0) into acetonitrile was usually used for elution. For peptides containing a number of acidic amino acids without compensating basic residues, such as delta sleep-inducing peptide, a dilute 0.04 *M* formic acid solution (pH 2.6) was employed as the eluent. Peptides of up to about 30 residues were successfully tested, including peptides such as somatostatin, neurotensin, ribonuclease s-peptide, α-endorphin, glucagon, and various angiotensins and bradykinins. Tryptic digests of horse heart cytochrome *c*, calmodulin and reduced and alkylated hen egg-white lysozyme were also successfully examined. Because of the volatility of the eluents used, peptides can be readily isolated for further investigation. Recoveries of over 80% were observed in those cases tested by comparative amino acid analysis.

INTRODUCTION

In the past several years, high-performance liquid chromatography (HPLC) has become one of the most important techniques for peptide separation. Reversed-phase HPLC, utilizing a variety of solvent systems[1–14], has been the type most widely used for this purpose. More recently, HPLC employing a macroreticular anion-exchange column was used to separate tryptic digestion mixtures of peptides[15].

We recently reported a method for the separation of dipeptides, including resolution of sequence isomeric and diastereoisomeric dipeptides, by HPLC on a weak anion-exchange bonded phase using mixtures of triethylammonium acetate (TEAA) buffer and acetonitrile for elution[16]. In this paper, we describe the separation

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of a large variety of peptides containing up to approximately 30 amino acid residues using a similar system. These peptides include somatostatin, α-endorphin, ribonuclease s-peptide, glucagon, various angiotensins and bradykinins as well as tryptic digestion mixtures of horse heart cytochrome *c*, calmodulin and reduced and alkylated hen egg-white lysozyme. This methodology has been developed for the study of the effects of ionizing radiation on peptides.

EXPERIMENTAL*

Apparatus

Separations were performed on a Hewlett-Packard Model 1084B liquid chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) equipped with a microprocessor, an automatic injector and a variable wavelength detector. The buffer solutions were filtered (Millipore, Bedford, MA, U.S.A.) prior to use. All separations were carried out on a 30 × 0.4 cm MicroPak AX-10 column (Varian, Walnut Creek, CA, U.S.A.), a difunctional weak anion-exchange bonded phase prepared on LiChrosorb Si-60 silica (10 μm)[17]. Prior to chromatography of most peptide mixtures, the column was first equilibrated with at least 50 ml of 0.01 *M* TEAA pH 6.0 buffer, to insure that column pH was also 6.0. Then, after column equilibration with the starting solution, usually acetonitrile–buffer (75:25), elution was begun with a gradient terminating at 100% buffer. Specific conditions are described in the Figures. For elution of acidic peptides, an isocratic flow of 0.04 *M* formic acid pH 2.6 buffer was employed. Again, the column was first pre-equilibrated with at least 20 ml of this eluent to insure that column pH was also 2.6.

Materials

Peptides were obtained from Sigma (St. Louis, MO, U.S.A.), Research Plus (Bayonne, NJ, U.S.A.) and Beckman (Palo Alto, CA, U.S.A.). Triethylamine was purchased from Eastman Kodak (Rochester, NY, U.S.A.) and purified by distillation. Buffer solutions were prepared by titrating 0.01 *M* acetic acid solutions with triethylamine to pH 6.0. Glass-distilled water and acetonitrile were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Trypsin was obtained from Boehringer (Indianapolis, IN, U.S.A.).

Tryptic digestion

Tryptic digestions were carried out according to the procedure described by Margoliash[18]. 0.3 μmol of peptide were dissolved in 0.1 ml of 0.03 *M* Tris–HCl buffer (pH 8.0). 0.1 mg of trypsin was added and the mixture was incubated at 40°C for 24 h.

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* Certain commercial equipment, instruments, or materials are identified in this paper in order to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Amino acid analysis

For recovery studies, the eluted peptides were dried *in vacuo* and hydrolyzed with constant boiling HCl in evacuated and sealed tubes at 110°C for 24 h. The hydrolyzates were analyzed on a Durrum D-500 amino acid analyzer.

RESULTS AND DISCUSSION*

The separation of a multicomponent peptide mixture is shown in Fig. 1, and peak identifications are given in Table I. Excellent peak symmetry for all peptides in this mixture was obtained with the exception of somatostatin (peak 1), which gave a tailing peak. Glucagon (peak 9) gave two peaks; amino acid analysis of collected material showed the same amino acid composition for both components. All of the other peptides in this chromatogram gave a single peak when freshly prepared solutions were individually injected. The 0.01 *M* TEAA pH 6.0 buffer used with acetonitrile in the above separations is the usual gradient system employed in peptide separation. It buffers well at the pH employed and is compatible with the pumps and column. It also allows sensitive detection of peptides at wavelengths in the range of 210–225 nm. Furthermore, this buffer is readily removed from eluted compounds by freeze-drying[19], facilitating the easy recovery of the separated peptides. Some pep-

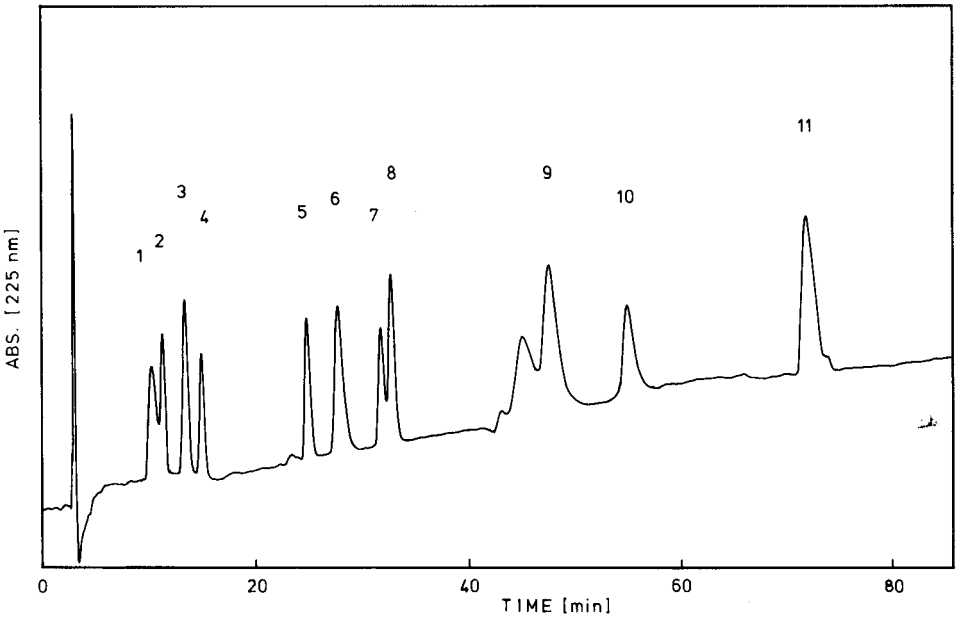


Fig. 1. Separation of various peptides. Column, MicroPak AX-10 (10 μm), 30 × 0.4 cm. Temperature, 30°C. Eluent: A, acetonitrile; B, 0.01 *M* triethylammonium acetate (pH 6.0), gradient program: linear starting from 25% B with a rate of 1% B per min. Flow-rate, 1 ml/min. Peak identification and sequences are given in Table I. Amount of injection, 0.5–5 μg per peptide.

* Abbreviations for amino acids follow IUPAC-IUB recommendations (see *Biochem. J.*, 126 (1972) 773).

TABLE I

PEAK IDENTIFICATION AND SEQUENCES IN FIGURES

Peak	*Peptide*	*Sequence*
Fig. 1		
1	Somatostatin	Ala–Gly–Cys–Lys–Asn–Phe–Phe–Trp–Lys–Thr–Phe–Thr–Ser–Cys
2	Proctolin	Arg–Tyr–Leu–Pro–Thr
3	Neurotensin	pGlu–Leu–Tyr–Glu–Asn–Lys–Pro–Arg–Arg–Pro–Tyr–Ile–Leu
4	Met–Enkephalin	Tyr–Gly–Gly–Phe–Met
5	Bradykinin potentiator C	pGlu–Gly–Leu–Pro–Pro–Gly–Pro–Pro–Ile–Pro–Pro
6	—	Lys–Glu–Thr–Tyr–Ser–Lys
7	α-Endorphin	Tyr–Gly–Gly–Phe–Met–Thr–Ser–Glu–Lys–Ser–Gln–Thr–Pro–Leu–Val–Thr
8	EAE-peptide	Phe–Ser–Trp–Gly–Ala–Glu–Gly–Gln–Arg
9	Glucagon	His–Ser–Gln–Gly–Thr–Phe–Thr–Ser–Asp–Tyr–Ser–Lys–Tyr–Leu–Asp–Ser–Arg–Arg–Ala–Gln–Asp–Phe–Val–Gln–Trp–Leu–Met–Asn–Thr
10	Ribonuclease s-peptide (RSP)	Lys–Glu–Thr–Ala–Ala–Ala–Lys–Phe–Glu–Arg–Gln–His–Met–Asp–Ser–Ser–Thr–Ser–Ala–Ala
11	IgE-peptide	Asp–Ser–Asp–Pro–Arg
Fig. 2		
1	Ribonuclease s-peptide	see above
2	IgE-peptide	see above
3	Glutathione oxidized form	γ-Glu-Cys-Gly | γ-Glu-Cys-Gly
4	–	Phe–Leu–Glu–Glu–Ile
5	DSIP	Trp–Ala–Gly–Gly–Asp–Ala–Ser–Gly–Glu
6	–	γ-Glu–Leu
7	–	γ-Glu–Glu
Fig. 3		
1	Angiotensin III (A III)	Arg–Val–Tyr–Ile–His–Pro–Phe
2	(Sar^1-Ala^8)-A II	Sar–Arg–Val–Tyr–Ile–His–Pro–Ala
3	A II	Asp–Arg–Val–Tyr–Ile–His–Pro–Phe
4	A I	Asp–Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu
5	(Val^5)-A II	Asp–Arg–Val–Tyr–Val–His–Pro–Phe
Fig. 4		
1	Bradykinin	Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg
2	Met, Lys–Bradykinin	Met–Lys–Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg
3	Lys-Bradykinin	Lys–Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg
Fig. 5		
1	–	Leu–Trp–Met–Arg–Phe–Ala
2	–	Leu–Trp–Met–Arg–Phe
3	–	Leu–Trp–Met–Arg
4	–	Met–Arg–Phe–Ala
5	–	Met–Arg–Phe
6	–	Arg–Phe–Ala
7	–	Leu–Trp–Met

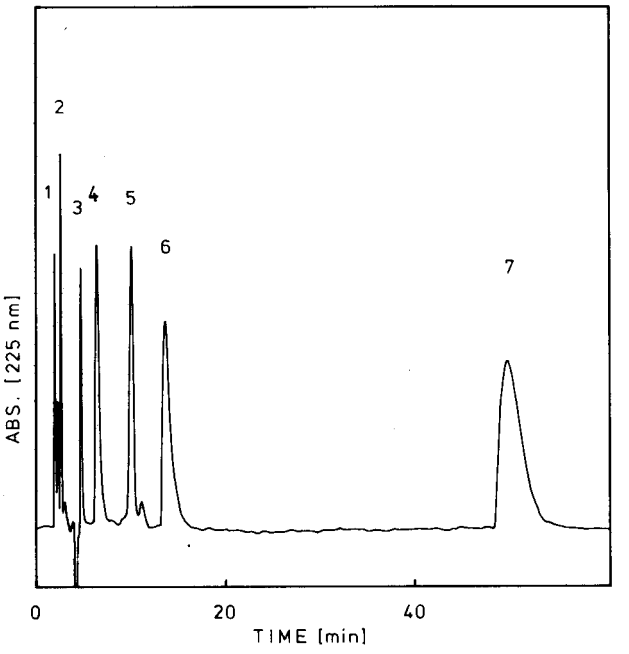
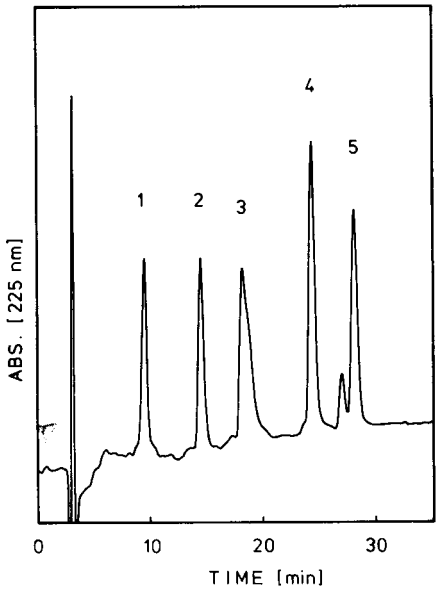


Fig. 2. Separation of some acidic peptides. Column as in Fig. 1. Temperature, 60°C. Eluent, 0.04 *M* formic acid (pH 2.6). Flow-rate, 1 ml/min. Peak identification and sequences are given in Table I. Amount of injection as in Fig. 1.

tides which contain a number of acidic amino acids with no compensating basic residues, such as delta sleep-inducing peptides (DSIP) (see Table I) or the dipeptide γ-Glu–Glu, have unacceptably long retention times when the elution conditions described in Fig. 1 are used. These peptides were chromatographed using an isocratic flow of dilute formic acid (pH 2.6) instead as the eluent, as is shown in Fig. 2. Both ribonuclease s-peptide (RSP) and IgE-peptide (peaks 1 and 2, respectively) could also be eluted with the solvent system described in Fig. 1. Although RSP contains three acidic amino acids (see Table I), it elutes much earlier than the other peptides in this mixture. This is probably due to the previously mentioned compensation of the acidic amino acids by the three basic amino acids or possibly steric hindrance to interaction of the carboxyl groups with the stationary phase. With increasing chain length, conformation of the peptide may also play a role. Thus, the pentapeptide with two Glu residues (peak 4) and DSIP (peak 5) elute later than RSP and IgE-peptide. As migth be expected from these other results, γ-Glu–Glu has the longest retention time. Dilute formic acid solutions are also compatible with the column and pumps used. In addition, formic acid is also volatile, allowing recovery of peptides for further use. As with the TEAA buffer, peptide recoveries are 80 % or greater. The figures also demonstrate that sensitive detection of eluted peptides is also obtained with this eluent.

Separation of closely related peptides

The weak anion-exchange column is also capable of separating closely related peptides such as angiotensins or bradykinins. For example, Fig. 3 shows the separa-



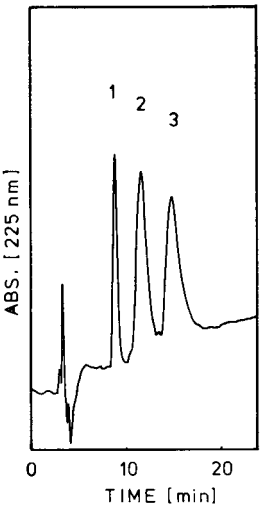


Fig. 3. Separation of some angiotensins. Column details as in Fig. 1. Peak identification and sequences are given in Table I. Amount of injection *ca.* 1 μg per peptide.

Fig. 4. Separation of several bradykinins. Column details as in Fig. 1 except gradient program: linear starting from 25% B with a rate of 1.7% B per min. Peak identification and sequences are given in Table I. Amount of injection, *ca.* 2 μg per peptide.

tion of a mixture of five angiotensins with excellent resolution, and all angiotensins gave symmetrical peaks. The peak representing angiotensin II showed a shoulder (peak 3) which corresponds to an impurity in this peptide. The small peak in front of (Val^5)–angiotensin II peak also represents an impurity.

The separation of three bradykinins is given in Fig. 4. Bradykinin gave a symmetrical peak whereas Met,Lys–bradykinin and Lys–bradykinin (peaks 2 and 3, respectively) yielded somewhat broad peaks.

Fig. 5 shows the separation of the hexapeptide Leu–Trp–Met–Arg–Phe–Ala (Research Plus Labs.) from the synthetic fragments that would be obtained by digestion with proteolytic enzymes or chemical cleavage. Excellent separation of these peptides was achieved by isocratic elution at 50°C.

Separation of tryptic digestion mixtures

Enzymatic digestion is frequently employed in sequence analysis of large peptides and proteins. For this reason, the weak anion-exchange column methodology was also employed to separate the peptides resulting from tryptic digestion of horse heart cytochrome *c*, calmodulin or reduced and alkylated egg-white lysozyme.

Fig. 6 shows the separation by gradient elution of the peptides resulting from tryptic digestion of horse heart cytochrome *c*. The elution became isocratic at 100% buffer after 60 min, and another four peaks were observed with continued flow of buffer. According to the sequence of horse heart cytochrome *c* given by Margoliash *et al.*[20], sixteen fragments and free lysine are expected upon digestion with trypsin.

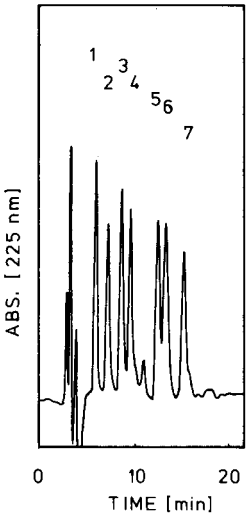


Fig. 5. Separation. of Leu-Trp-Met-Arg-Phe-Ala and its simulated digestion fragments. Column and eluent as in Fig. 1. Isocratic elution with 21% B. Flow-rate, 1 ml/min. Temperature, 50°C. Peak identification and sequences are given in Table I. Amount of injection, *ca.* 1 μg per peptide.

Fourteen major peaks and some other small peaks were observed. Further work would be needed to characterize the separated fragments. The large peaks probably represent the fragments containing Tyr and Trp residues[20]. Since some digestion products contain a number of acidic amino acids, the separation of the above digestion mixture employing conditions described in Fig. 2 was also undertaken (Fig. 7). As expected, the majority of the fragments have no or little retention using these

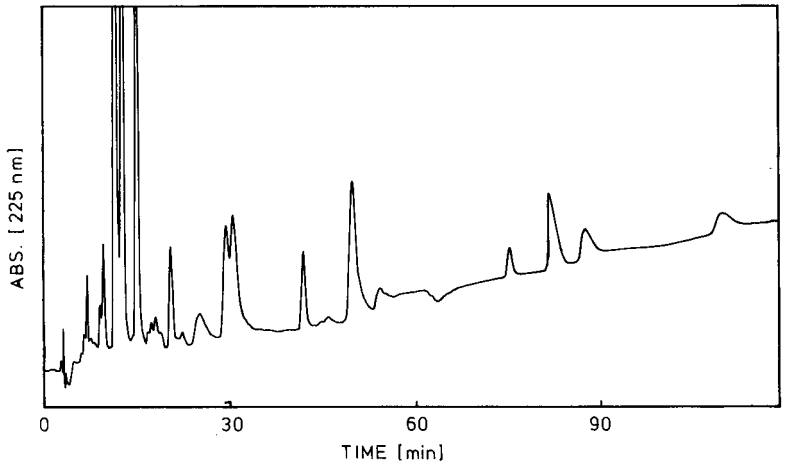


Fig. 6. Separation of a tryptic digest of horse heart cytochrome *c*. Column details as in Fig. 1 except gradient program: linear starting from 25% B with a rate of 0.6% B per min to 50% B then 4.5% B per min to 100% B. Amount of injection, *ca.* 10 nmol of cytochrome *c*.

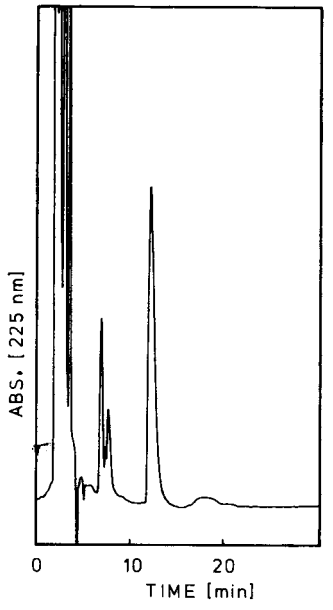


Fig. 7. Separation of a tryptic digest of horse heart cytochrome *c*. Column details as in Fig. 2. Amount of injection, *ca.* 10 nmol of cytochrome *c*.

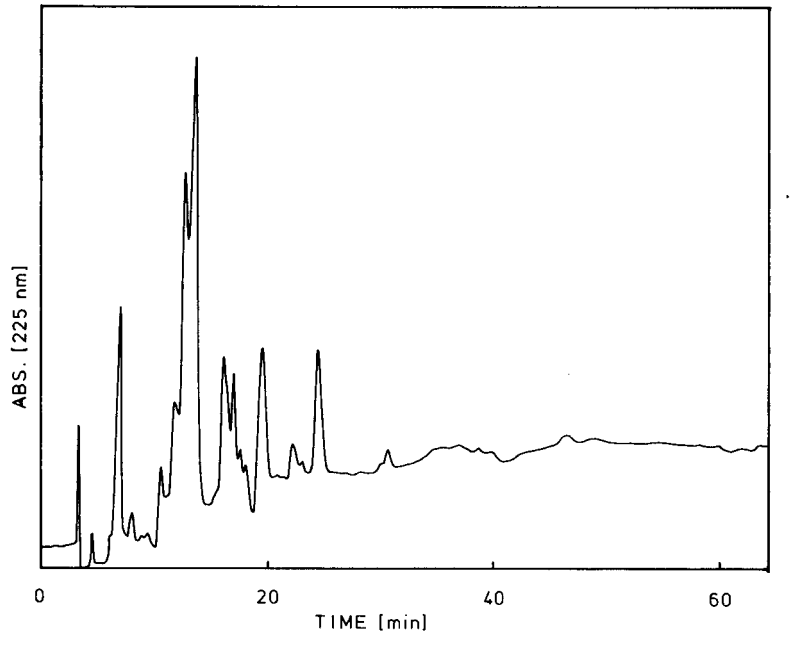


Fig. 8. Separation of a tryptic digest of calmodulin. Column details as in Fig. 1 except gradient program: linear starting from 25% B with a rate of 2% B per min. Amount of injection, *ca.* 15 nmol of calmodulin.

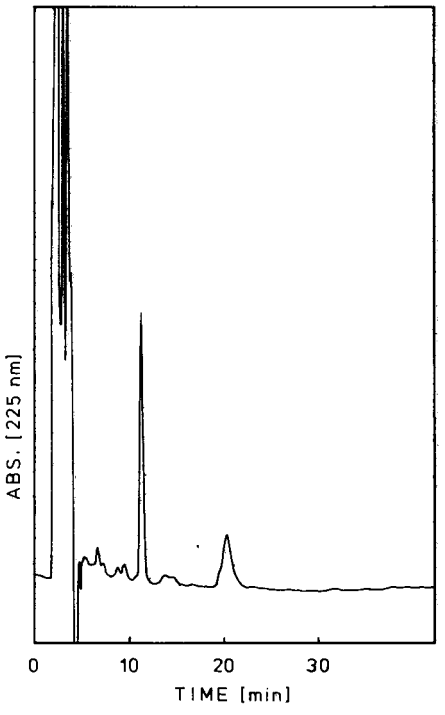


Fig. 9. Separation of a tryptic digest of calmodulin. Column details as in Fig. 2. Amount of injection as in Fig. 8.

conditions. However, in addition to these peaks, four other peaks were observed. The last peak in Fig. 7 may represent the fragment that contains four Glu residues and one Lys residue[20]. No other peaks were observed, even after an elution time of 90 min had elapsed. This data indicated that full separation of all peptides resulting from tryptic digestion of this protein was achieved.

The separation of the peptides from tryptic digestion of calmodulin is given in Fig. 8. Twelve fragments and free lysine are expected by digestion of calmodulin with trypsin[21]. Nine large peaks were observed besides material represented by small peaks. Fig. 9 shows the separation of this sample under the dilute formic acid conditions given in Fig. 2. In addition to the majority of the fragments with little retention, two more peaks were observed. Since tryptic fragments of calmodulin contain a large number of acidic amino acids, and the retention of such peptides shows a strong dependence on pH (see below *Effect of pH* and Fig. 11), a 0.07 *M* formic acid solution (pH 2.5) was also employed in an attempt to elute other possible fragments. However, no more peaks were observed after an elution time of 60 min.

Fig. 10 shows the separation of the tryptic digest of reduced and alkylated lysozyme. Again, the number of major peaks detected corresponds closely to the number of the fragments expected from tryptic digestion of lysozyme[22]. The two large peaks probably represent fragments with Trp and Tyr residues. Unlike the digests of cytochrome *c* and calmodulin, no other peaks in addition to those with no or litte retention, were observed when the dilute formic acid buffer was employed for elution.

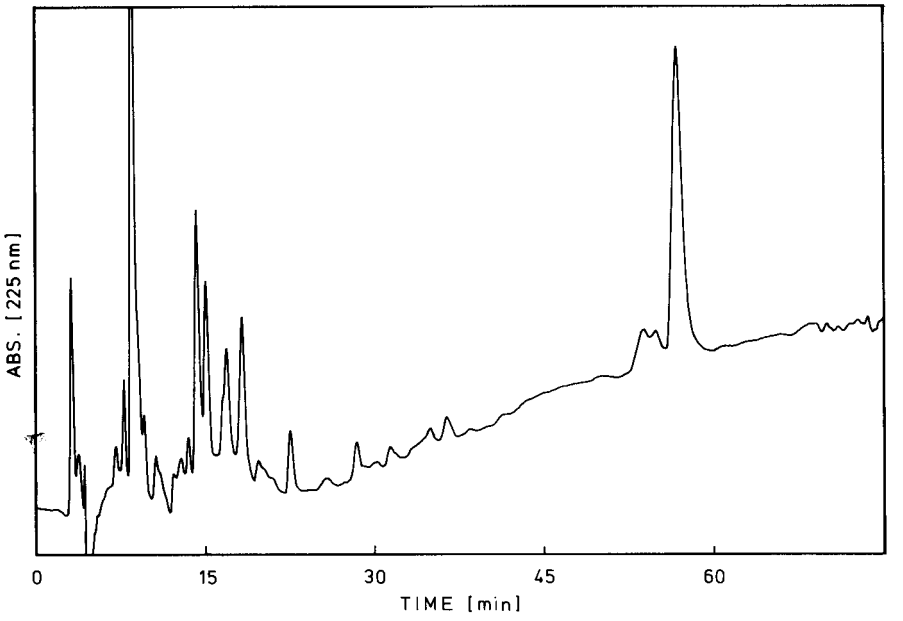


Fig. 10. Separation of a tryptic digest of reduced and alkylated lysozyme. Column details as in Fig. 1 except temperature: 40°C. Amount of injection, *ca.* 10 nmol of lysozyme.

Effect of temperature on retention and resolution

An increase in column temperature generally causes an increase in retention time. In some cases, resolution could be improved by increasing temperature to a certain limit starting from room temperature. For instance, the mixtures in Figs. 2 and 5 could be optimally separated at 60°C and 50°C, respectively. However, when gradient elution was used, an up-scale drift of the baseline was observed at temperatures above 40°C, due to higher UV absorption of the buffer. Nevertheless, baseline drift is still acceptable, and temperatures up to 60°C can be used to improve resolution of a given mixture if necessary.

Effect of pH

In a recent paper, we described separation of dipeptides using the TEAA buffer–acetonitrile solvent system, except the pH of the TEAA buffer was 4.3 (ref. 16). In the present work with peptides, a pH value of 6.0 was more suitable in terms of retention, peak symmetry and resolution. The separation selectivity of the stationary phase thus shows a quite strong dependence on the pH value of the eluent. This means that any pH value between 4 and 6 with this buffer system can be tried in order to improve resolution in a given separation using the chromatographic system described here.

The retention of acidic peptides even more strongly depends on the pH value of the eluent, as can be seen by the conditions described in Fig. 2 for their separation. This was further demonstrated in Fig. 11, where capacity factors (k') of DSIP were plotted *versus* pH of the eluent. In order to obtain a constant solvent strength, a 0.04 *M* formic acid solution was used and pH was changed by adding triethylamine to the

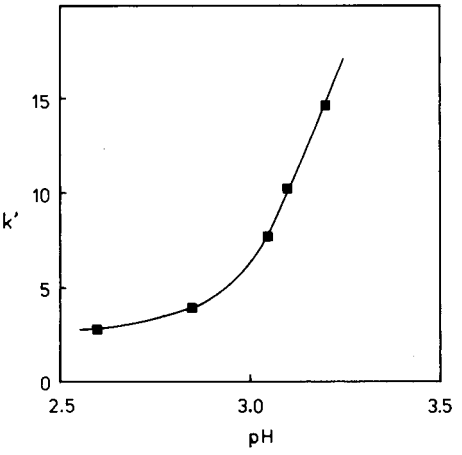


Fig. 11. Dependence of the k' value of DSIP (peptide No. 5 in Fig. 2) on the pH value of the eluent. Column as in Fig. 1. Eluent, 0.04 *M* formic acid, the pH value was adjusted by adding triethylamine. Flow-rate, 1.5 ml/min. Temperature, 60°C.

eluent. As can be seen from this plot, a very strong dependence of k' values on pH above ca. 2.9 was observed.

CONCLUSIONS

The results obtained in this paper clearly show that multicomponent mixtures of peptides or closely related peptides can be successfully resolved by HPLC on a weak anion-exchange bonded phase. The suggested method offers great advantages in terms of sensitivity, peak symmetry, reproducibility and high recoveries. The composition and pH of the mobile phase and the column temperature all contribute to resolution. The optimal conditions for resolution of a given separation problem can be determined by varying these parameters. Because the eluents used for elution are volatile, isolation of separated peptides for further use, such as structure determination, is facilitated. In addition, the experimental conditions appear to allow long column life (up to one year with several daily injections).

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PRÄPARATIVE ISOLIERUNG VON OLIGOTHYMIDINPHOSPHATEN AUS PARTIALHYDROLYSATEN CHEMISCH ABGEBAUTER DNA MIT HILFE DER TEMPLATE-CHROMATOGRAPHIE*

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(Eingegangen am 20. November 1981)

SUMMARY

Preparative isolation of oligothymidine phosphates from partial hydrolysates of chemically degraded DNA using template-chromatography

Herring sperm DNA (500 g) is chemically degraded to oligothymidine phosphates which are subsequently fractionated on a QAE-Sephadex column according to the increasing charges of their phosphate groups. The final fraction contains a mixture (1.5 g) of long-chain oligothymidine phosphates. Chromatographically pure, single substances (1–4 mg) of the $(dT)_{3-12}$ series are separated from the mixture using template chromatography and rechromatography on QAE-Sephadex. Sequence and purity of the isolated oligothymidine phosphates are checked using paper chromatography, high-pressure liquid chromatography and homochromatography as well as the two-dimensional fingerprint method.

EINLEITUNG

Desoxyribonucleinsäure (DNA) lässt sich unter bestimmten Bedingungen chemisch zu Gemischen von Oligothymidinphosphaten abbauen. Mit bekannten chromatographischen Trennmethoden sind aus dem erhaltenen Gemisch bisher nur Oligothymidinphosphate mit bis zu 7 Monomereinheiten in präparativen Mengen zugänglich[1]. Mit Hilfe der Template-Chromatographie[2], in der die Spezifität des Basenpaarungsmechanismuses nach Watson und Crick zur Trennung genutzt wird, ist es inzwischen möglich, chromatographisch reine Oligothymidinphosphate mit bis zu 12 Monomereinheiten in präparativen Mengen aus dem Partialhydrolysat zu isolieren, wie im folgenden gezeigt wird.

* Bei Abkürzungen werden prinzipiell IUPAC–IUB Regeln befolgt [*Eur. J. Biochem.*, 15 (1970) 203]. A_{260}-Einheit = Nucleotid-Menge in 1 cm^3 Solvens, die bei 260 nm die Absorption 1 ergibt (Schichtdicke 1 cm). PVp$(dA)_n$-DEAE-Cellulose = Desoxyoligoadenosin-5′-phosphat-DEAE-Cellulose. Tris = a,a,a-Tris(hydroxymethyl)methylamin.

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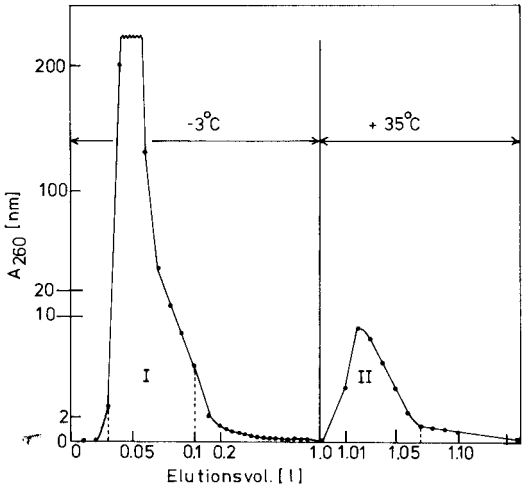

Fig. 1. Elutionsprofil der Template-Chromatographie von 650 mg (10320 A_{260}-Einheiten) einer Oligothymidinphosphatmischung ("1 *M* Fraktion") an PVp(dA)$_n$-DEAE-Cellulose. Säulenmasse: 20 × 2 cm; Fliessgeschwindigkeit: 350 ml/h. Die Elution erfolgt mit 1.0 *M* NaCl–0.01 *M* Na_2HPO_4 (pH 6.5) in der 1. Stufe (−3°C) mit 1 l, in der 2. Stufe (+35°C) mit 150 ml. Fraktionen zu 10 ml werden gesammelt und innerhalb der senkrechten Strichelung vereinigt. Ergebnisse siehe Tabelle I.

EXPERIMENTELLES

Reagenzien

QAE-Sephadex A-25 (Pharmacia, Uppsala, Schweden); Enzyme (Boehringer, Mannheim, B.R.D.); Membranen (Amicon, Lexington, MA, U.S.A.); [^{32}P]ATP (Amersham Buchler, Braunschweig, B.R.D.); Nucleosil 5 μm C_{18} (Macherey-Nagel, Düren, B.R.D.); $(dT)_6$ sowie PVp(dA)$_n$-DEAE-Cellulose[5] wurden in unserem Labor synthetisiert. Die höhermolekularen Oligothymidinphosphate ("1 *M* Fraktion") werden auf dem früher beschriebenen Weg[1,3] aus dem Partialhydrolysat der depurinierten Heringsspermen-DNA gewonnen.

Template-Chromatographie der Oligothymidinphosphate "1 M Fraktion" an PVp(dA)$_n$-DEAE-Cellulose (vgl. Fig. 1)

Die in der folgenden Trennung verwendete PVp(dA)$_n$-DEAE-Cellulose Säule (20 × 2 cm) ist mit einem Kühlmantel versehen, der mit einem Thermostaten verbunden ist. Die Elution der Säule erfolgt mit 1 *M* NaCl, 0.01 *M* Na_2HPO_4 (pH 6.5) bei einem Durchfluss von *ca.* 350 ml/h.

Die unterschiedlichen Proben (vgl. Tabelle I) werden jeweils in 2 ml Elutionspuffer gelöst und bei +35°C auf die Säule aufgetragen. Nachdem die Probenlösung in das Gelbett eingezogen ist, wird die Säule auf −3°C gekühlt und anschliessend so lange bei dieser Temperatur eluiert, bis die Absorption des Eluats nach einem steilen Anstieg (Peak I der Fig. 1) unter 0.04 A_{260}-Einheiten fällt. Dieser Wert wird spätestens nach 1 l erreicht. Dann stoppt man die Elution und erwärmt die Säule auf +35°C. Anschliessend wird die Elution fortgesetzt, bis die Absorption nach einem zweiten Anstieg (Peak II der Fig. 1) wieder auf 0.04 A_{260}-Einheiten gesunken ist.

TABELLE I

ERGEBNISSE DER TEMPLATE-CHROMATOGRAPHIE VON OLIGOTHYMIDINPHOSPHATEN ("1 *M* FRAKTION") AN $PVp(dA)_n$-DEAE-CELLULOSE (VGL. FIG. 1)

Aufgetragene Probenmengen		*Eluierte Mengen in A_{260}-Einheiten (%)*	
mg	*A_{260}-Einheiten*	*Peak I bei −3°C*	*Peak II bei +35°C*
(a) "1 M Fraktion"			
100	1480	1360 (91.89)	95 (6.42)
300	4360	4020 (92.20)	240 (5.50)
500	7520	6990 (92.95)	390 (5.19)
650	10320	9610 (93.12)	510 (4.94)
(b) Rechromatographie von Peak I			
500	10650	10480 (98.40)	50 (0.47)
(c) Rechromatographie von Peak II			
–	310	80 (25.81)	225 (72.58)

Hierzu werden *ca.* 150 ml Elutionspuffer benötigt. Fraktionen zu 10 ml werden gesammelt und photometrisch bei 250, 260, 280 nm gemessen. Die Auftragung der bei A_{260} erhaltenen Messwerte gegen das Elutionsvolumen führt zu den in Fig. 1 und 2 abgebildeten Elutionsprofilen. Die Fraktionen von Peak I und II werden innerhalb der senkrechten Strichelung vereinigt, an einer UM 2 Membran entsaltzt und anschliessend lyophilisiert. Die Lyophilisate von Peak II werden aus allen Läufen vereinigt und zur Entfernung terminaler Phosphatgruppen mit alkalischer Phosphatase behandelt[1]. Anschliessend wird der Ansatz mit 30 ml 7 *M* Harnstoff versetzt und wie folgt an einer QAE-Sephadex Säule fraktioniert.

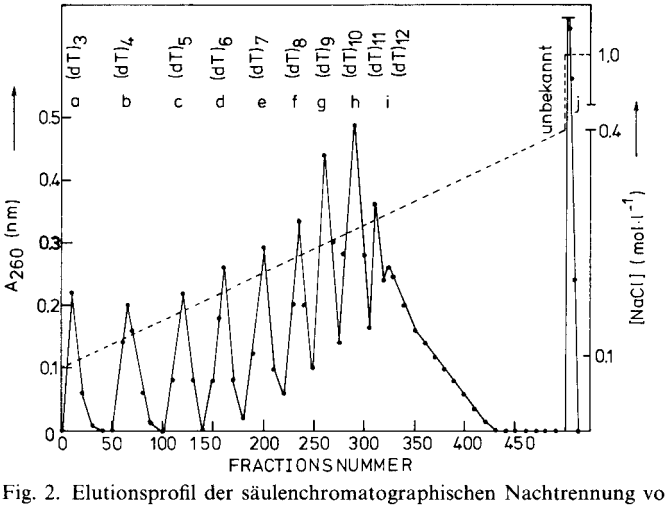

Fig. 2. Elutionsprofil der säulenchromatographischen Nachtrennung von *ca.* 1300 A_{260}-Einheiten einer dephosphorylierten Oligothymidinphosphatmischung (Peak II, Fig. 1) an QAE-Sephadex. Säulenfüllung: 50 × 2 cm. Die Säule wird bei Raumtemperatur im steigenden NaCl Gradienten eluiert, der mit 0.05 *M* Tris–HCl auf pH 7.5 gepuffert und mit 7 *M* Harnstoff versetzt ist. Fliessgeschwindigkeit *cq.* 300 ml/h. Ergebnisse siehe Tabelle II.

TABELLE II

ERGEBNISSE DER SÄULENCHROMATOGRAPHISCHEN NACHTRENNUNG AN QAE-SEPHADEX (VGL. FIG. 2) VON OLIGOTHYMIDINPHOSPHATEN (*CA.* 1300 A_{260}-EINHEITEN), DIE VON DER PVp(dA)$_n$-DEAE-CELLULOSE SÄULE IN PEAK II BEI +35°C (VGL. FIG. 1) ELUIERT WERDEN

Vereinigte Fraktionen		*Isolierte Oligothymidinphosphate*		
Peak	*Nr.*	*Bezeichnung*	A_{260}-*Einheiten*	%
a	1–25	$(dT)_3$	41	3.2
b	50–80	$(dT)_4$	56	4.4
c	100–140	$(dT)_5$	74	5.9
d	145–170	$(dT)_6$	58	4.6
e	185–210	$(dT)_7$	65	5.1
f	220–245	$(dT)_8$	72	5.7
g	255–270	$(dT)_9$	82	6.5
h	280–300	$(dT)_{10}$	102	8.1
i	310–370	$(dT)_{11,12}$	166	13.1
j	1 *M* Fraktion	Unbekannt	181	14.3
	Zwischenfraktionen		367	29.0

Säulenchromatographische Fraktionierung der dephosphorylierten Produkte aus Peak II der Fig. 1 an QAE-Sephadex A-25 (vgl. Fig. 2)

Die Reaktionslösung (*ca.* 1300 A_{260}-Einheiten) wird auf eine mit 0.1 *M* NaCl, 0.05 *M* Tris–HCl (pH 7.5), 7 *M* Harnstoff äquilibrierte QAE-Sephadex A-25 Säule (50 × 2 cm) aufgetragen. Die Säule wird bei Raumtemperatur zunächst mit 200 ml 7 *M* Harnstoff, dann mit 600 ml 0.1 *M* NaCl, 0.05 *M* Tris–HCl (pH 7.5), 7 *M* Harnstoff eluiert. Die Elutionsgeschwindigkeit wird mit einer Schlauchpumpe auf *ca.* 300 ml/h eingestellt. Anschliessend wird die Elution in einem steigenden NaCl-Gradienten fortgesetzt, 5 l 0.1 *M* NaCl, 0.05 *M* Tris–HCl, 7 *M* Harnstoff im Mischgefäss; 5 l 0.4 *M*

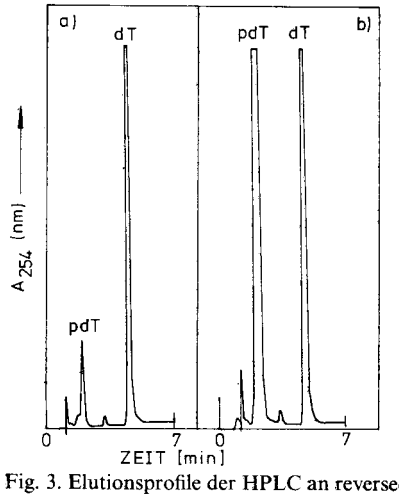

Fig. 3. Elutionsprofile der HPLC an reversed-phase nach Totalhydrolyse der Lyophilisate von (a) Peak h, (b) Peak i mit Phosphodiesterase aus Schlangengift. Einzelheiten siehe Experimentelles.

NaCl, 0.05 *M* Tris–HCl, 7 *M* Harnstoff im Vorratsgefäss. Nach dem Gradienten wird die Säule mit *ca.* 400 ml 1 *M* NaCl eluiert. Fraktionen zu 20 ml werden gesammelt. Fraktionen von Peak a–i der Fig. 2 werden nach den Angaben in Tabelle II vereinigt, durch Ultrafiltration an einer UM 2 Membran entsalzt und anschliessend lyophilisiert. Die Ergebnisse sind in Tabelle II zusammengefasst.

Hochdruckflüssigkeitschromatographie (HPLC) der enzymatisch hydrolysierten Produkte aus Peak e–i der Fig. 2

1–5 A_{260} Einheiten des jeweiligen Oligothymidinphosphats werden in 20 µl Wasser, 5 µl 1 *M* Tris–HCl (pH 8.1), 5 µl 0.1 *M* $MgCl_2$ gelöst und mit *ca.* 5–10 µl der käuflichen Phosphodiesterase aus Schlangengift 6 h bei 37°C inkubiert. Anschliessend wird der Reaktionsansatz an einer Nucleosil 5 µm C_{18}-Säule (0.46 × 11.25 cm) mittels eines Waters Assoc. Hochdruckflüssigkeitschromatographen mit folgendem linear steigenden Puffergradienten bei Raumtemperatur fraktioniert. Puffer A = 0.1 *M* Ammoniumacetat (pH 7.5); Puffer B = 60% CH_3OH, 40% H_2O. Innerhalb von 10 min steigt Puffer B von 0 auf 45%, bei einem Durchfluss von 1 ml/min. Der Elutionsvorgang wird automatisch bei 254 nm registriert, wobei die Trennungen von Peak h und i zu den in Fig. 3 wiedergegebenen Elutionsprofilen führen, die stellvertretend für die übrigen Trennungen abgebildet sind.

ERGEBNISSE

DNA aus Heringsspermen wird nach dem in Schema 1 skizzierten und in früheren Arbeiten[1,3] ausgeführten Weg in zwei Schritten zu Oligothymidinphosphaten abgebaut.

DNA (≈500 g)
1 ↓ (a) Ameisensäure–Diphenylamin/12 h/37°C
(b) Neutralisation (NaOH)
(c) Ultrafiltration
(d) DEAE-Cellulose/pH 7.5

Längerkettige Pyrimidinoligonucleotide (≈80 g)
2 ↓ (a) 7 *M* KOH/15 h/100°C
(b) Neutralisation ($HClO_4$)
(c) Ultrafiltration
(d) QAE-Sephadex/pH 3.5

"1 *M* Fraktion" (≈1.5 g)

Schema 1. Chemischer Abbau einer DNA zu Oligothymidinphosphaten

Im 1. Hydrolyseschritt[3] werden nach der Methode von Burton[4], die wir in den präparativen Massstab übertragen haben, mit Ameisensäure in Anwesenheit von Diphenylamin die Purinbausteine der DNA zerstört. Das anfallende Partialhydrolysat wird mit NaOH neutralisiert, von Salz und niedermolekularen Bruchstücken durch Ultrafiltration befreit und schliesslich säulenchromatographisch an DEAE-Cellulose bei pH 7.5 fraktioniert. Auf diesem Weg werden die kurzen Fragmente, die den Hauptanteil des Partialhydrolysats bilden, von den längerkettigen Pyrimidinoligonucleotiden getrennt, die nur *ca.* 16% der zur Spaltung eingesetzten DNA ausmachen.

Im 2. Hydrolyseschritt[1] werden die längerkettigen Fragmente mit 7 *M* KOH zu Oligothymidinphosphaten abgebaut. Durch die alkalische Hydrolyse werden bevorzugt die Cytidylateinheiten der Pyrimidinoligonucleotide zerstört, während die intakten Thymidylatsegmente aus dem Molekülverband freigesetzt werden. Das neutralisierte und ultrafiltrierte Partialhydrolysat wird anschliessend säulenchromatographisch an QAE-Sephadex bei pH 3.5 im steigenden Salzgradienten fraktioniert, wobei zum Schluss mit 1 *M* NaCl ein Gemisch von Oligothymidinphosphaten mit mehr als 7 Monomereinheiten eluiert wird. Von dieser Mischung, die im folgenden als "1 *M* Fraktion" bezeichnet wird, erhält man beim Abbau von *ca.* 500 g Heringsspermen-DNA *ca.* 1.5 g. Mit herkömmlichen Trennmethoden ist uns bisher keine weitere Auftrennung der "1 *M* Fraktion" gelungen. Erst mit Hilfe der Template-Chromatographie[2] und nachfolgender Fraktionierung an QAE-Sephadex ist es möglich, definierte Oligothymidinphosphate aus der "1 *M* Fraktion" zu isolieren, wie im folgenden ausgeführt wird.

Zunächst wird die "1 *M* Fraktion" an PVp$(dA)_n$-DEAE-Cellulose[5] in zweistufigen Temperaturgradienten in zwei Fraktionen getrennt (vgl. Fig. 1). Die 1. Fraktion (Peak I) enthält Verbindungen, die mit den immobilisierten Oligodesoxyadenylsäuren keine Wechselwirkungen eingehen, während die Komponenten der 2. Fraktion (Peak II) an der stationären Phase adsorbiert werden. Bei der nachfolgenden Auftrennung der 2. Fraktion (Peak II), die an QAE-Sephadex durchgeführt wird (vgl. Fig. 2), werden Oligothymidinphosphate mit 3–12 Monomereinheiten erhalten.

Bei der PVp$(dA)_n$-DEAE-Cellulose, deren Struktur in Fig. 4 schematisch dargestellt ist, handelt es sich um DEAE-Cellulose, an der kovalent an Polyvinylalkohol (PV) gebundene Oligomere der Desoxyriboadenylsäure p$(dA)_n$, über Nebenwirkungskräfte immobilisiert sind. Dieses Trennmaterial haben wir früher zur präparativen Trennung synthetischer Oligothymidylsäuren verwendet[5].

Die Auftrennung der "1 *M* Fraktion" an PVp$(dA)_n$-DEAE-Cellulose wird wie folgt durchgeführt. Unterschiedliche Mengen (100, 350, 500, 650 mg) werden in vier Läufen chromatographiert. Das Lyophilisat wird jeweils im Elutionspuffer gelöst und auf die auf +35°C erwärmte PVp$(dA)_n$-DEAE-Cellulose aufgetragen. Anschliessend wird die Säule auf −3°C gekühlt und zunächst bei dieser Temperatur eluiert. Unter diesen Bedingungen bilden die im Lyophilisat enthaltenen Oligothymidinphosphate mit den stationär gebundenen Oligodesoxyadenylsäuren Basenpaare im Sinne von Watson und Crick aus. Die Folge ist, dass die hybridisierten DNA-Fragmente retardiert bleiben, während alle Substanzen, die bei −3°C keine Basenpaare eingehen, eluiert werden. Der Elutionsvorgang wird photometrisch verfolgt. Alle vier Trennungen zeigen praktisch die gleichen Elutionsprofile. Stellvertretend für die übrigen Trennungen ist daher in Fig. 1 das Elutionsprofil der Trennung abgebildet, in der 650 mg der "1 *M* Fraktion" chromatographiert werden. Nachdem die bei −3°C nicht adsorbierten Produkte in Peak I die Säule verlassen haben, werden anschliessend die hybridisierten DNA-Fragmente bei +35°C gemeinsam in Peak II eluiert. Aus zwei Gründen wählen wir als Elutionstemperatur +35°C. Aus anderen Arbeiten[2,6] ist bekannt, dass Oligothymidinphosphate mit bis zu 12 Monomereinheiten ab *ca.* +30°C mit stationär gebundenen Oligoadenylsäuren keine stabilen Basenpaare mehr ausbilden. Andererseits sind im Partialhydrolysat einer natürlichen DNA präparative Mengen an Oligothymidinphosphaten mit über 12 Monomereinheiten sicherlich nicht zu erwarten. Eine Elutionstemperatur über +35°C erscheint daher nicht zweck-

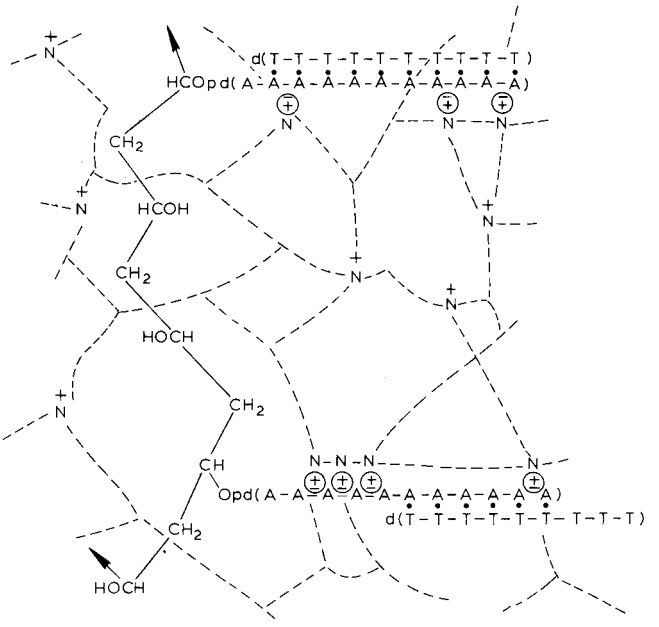

Fig. 4. Schematischer Aufbau der PVp(dA)$_n$-DEAE-Cellulose.

mässig, zumal DNA-Fragmente mit steigender Temperatur instabiler werden. Die Trennergebnisse sind in Tabelle I zusammengefasst.

Von der aufgetragen "1 *M* Fraktion" verlassen bei −3°C bereits *ca.* 93% die Säule, während *ca.* 5–6% hybridisieren und dann bei +35°C eluiert werden. Die Menge an adsorbiertem Material nimmt bei Erhöhung der aufgetragenen Probenmenge zu und strebt gegen einen Sättigungswert, der schätzungsweise bei *ca.* 80 A_{260}-Einheiten/ml PVp(dA)$_n$-DEAE-Cellulose liegt. Berücksichtigt man, dass die verwendete Säule seit fast 6 Jahren in Gebrauch ist, so beweist die noch vorhandene Kapazität eine beachtliche Stabilität des Materials. Aus allen Läufen werden die Fraktionen von Peak I und II vereinigt, durch Ultrafiltration entsalzt und anschliessend lyophilisiert. Bei der Entsalzung tritt ein Verlust von *ca.* 20% auf, den wir vorallem darauf zurückführen, dass bei der Ultrafiltration Oligothymidinphosphate teilweise abgebaut werden und dann die Membran passieren.

Die Ergebnisse der folgenden zwei Versuche legen nahe, dass die PVp(dA)$_n$-DEAE-Cellulose nach dem Basenpaarungsmechanismus trennt. Zunächst werden 500 mg (10650 A_{260}-Einheiten) des Lyophilisats von Peak I, das keine hybridisierfähigen Verbindungen enthalten soll, unter identischen Bedingungen an der PVp(dA)$_n$-DEAE-Cellulose rechromatographiert. Hierbei werden nur 0.47% bei +35°C eluiert (vgl. Tabelle I). Bei der Rechromatographie des Lyophilisats aus Peak II, in dem Oligothymidinphosphate zu vermuten sind, die mit der stationären Phase Basenpaare ausbilden, werden dagegen nur 25.81% bei −3°C aber 72.58% bei +35°C eluiert (vgl. Tabelle I). Ausserdem zeigen die Absorptionsverhältnisse von A_{250}/A_{260} bzw. A_{280}/A_{260}, die bei pH 7 für Peak I 0.90 bzw. 0.76 für Peak II 0.73 bzw. 0.65 ergeben, bereits deutlich, dass beide Fraktionen unterschiedlich zusammengesetzt sind.

Zur Isolierung definierter Einzelsubstanzen werden die in Peak II eluierten

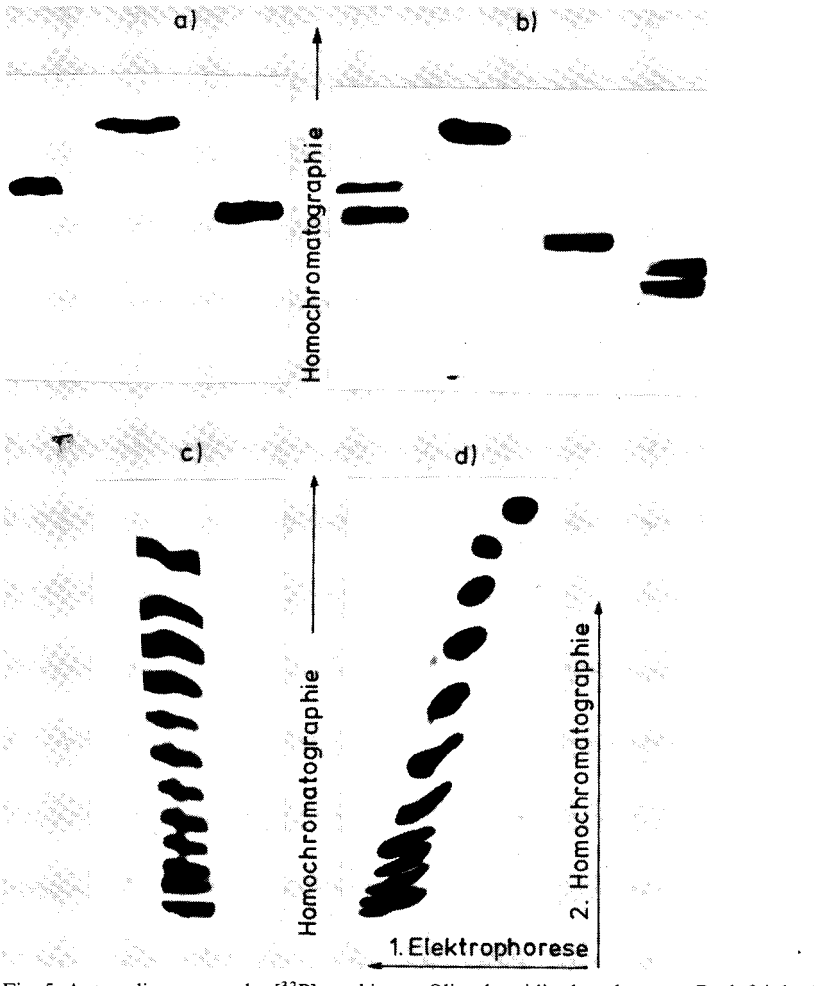

Fig. 5. Autoradiogramme der [^{32}P] markierten Oligothymidinphosphate aus Peak f–i der Fig. 2. (a) Nach der Homochromatographie von Peak f, g; (b) von Peak h, i; (c) Auftrennung des Partialhydrolysats von Peak i in der Homochromatographie; (d) in der zweidimensionalen Chromatographie. In (a) und (b) entsprechen die Flecken der Autoradiogramme von links nach rechts folgenden Oligothymidinphosphaten: Peak f, $(dT)_8$; Referenz $(dT)_6$; Peak g, $(dT)_9$; Referenz $(dT)_{8,9}$; Referenz $(dT)_6$; Peak h, $(dT)_{10}$; Peak i, $(dT)_{11,12}$.

Produkte aller Läufe anschliessend gemeinsam an QAE-Sephadex fraktioniert. Um die Trennung zu erleichtern, werden zunächst die terminalen Phosphatgruppen der Oligothymidinphosphate enzymatisch mit alkalischer Phosphatase entfernt[1]. Nach der enzymatischen Dephosphorylierung resultiert eine Mischung homologer Oligothymidinphosphate $(dT)_n$ (*ca.* 1300 A_{260}-Einheiten), die sich problemlos an QAE-Sephadex wie folgt auftrennen lässt (vgl. Fig. 2 und Tabelle II).

Die Lösung der dephosphorylierten Oligothymidinphosphate wird auf eine QAE-Sephadex Säule aufgetragen und bei Raumtemperatur im linear steigenden NaCl-Gradienten eluiert, der mit Tris-HCl auf pH 7.5 gepuffert und mit 7 *M*

Harnstoff versetzt ist. Unter diesen Bedingungen verlassen Peak a–i der Fig. 2 die Säule. Nach dem Gradienten werden mit 1 *M* NaCl nicht näher untersuchte Produkte gemeinsam in Peak j eluiert. Die Peakfraktionen werden wie in Tabelle II aufgeführt vereinigt, die Menge der darin enthaltenen Oligonucleotide photometrisch bestimmt, durch Ultrafiltration entsalzt und lyophilisiert. Bei dieser Aufarbeitung gehen wiederum bis zu 30% der in den einzelnen Peaks enthaltenen Produkte verloren.

Die Identifizierung der in Peak a–d enthaltenen Oligothymidinphosphate (vgl. Tabelle II) nach dem bereits früher beschriebenen Weg[1] zeigt, dass $(dT)_3$, $(dT)_4$, $(dT)_5$, $(dT)_6$ nacheinander von der Säule eluiert werden.

Die Produkte von Peak e–i werden nach den in der Oligonucleotidsynthese üblichen Methoden identifiziert. Zunächst werden die Lyophilisate einzelner Peaks auf Papier gemeinsam mit $(dT)_6$ als Referenzsubstanz chromatographiert. Im Papierchromatogramm bilden alle Lyophilisate einheitliche Flecken, die zwar unterschiedlich schnell aber langsamer als $(dT)_6$ wandern.

Die Absorptionsverhältnisse der vom Papier eluierten Produkte entsprechen den für Thymidinphosphate charakteristischen Werten von $A_{250}/A_{260} = 0.65 \pm 0.02$ (pH 7), 0.63 ± 0.02 (pH 2) bzw. $A_{280}/A_{260} = 0.71 \pm 0.02$ (pH 7), 0.71 ± 0.02 (pH 2). Aliquote der vom Papier eluierten Produkte werden nach der Totalhydrolyse mit Phosphodiesterase aus Schlangengift an einer Nucleosil 5 μm C_{18}-Säule chromatographiert (vgl. Fig. 3). Hierbei zeigt sich, dass über 99% des Hydrolysats aus pdT und dT besteht. In den Hydrolysaten von Peak h und i werden vernachlässigbare Spuren von pdC gefunden. Aus dem Verhältnis von dT zu pdT, das sich aus den Peakflächen der HPLC-Trennung errechnet, lässt sich die Kettenlänge des hydrolysierten Oligothymidinphosphates in unserem Fall aus folgendem Grund nicht ermitteln. Wir verwenden einen grossen Überschuss an Enzym und inkubieren bis zu 6 h, um eine vollständige Hydrolyse mit Phosphodiesterase sicherzustellen. Unter diesen Bedingungen kann die in der Enzympräparation als Verunreinigung geringfügig enthaltene Phosphatase pdT weiter zu dT abbauen. Der resultierende erhöhte dT-Gehalt täuscht eine verkürzte Oligonucleotidkette vor.

Die exakte Bestimmung der Kettenlänge wird daher wie folgt durchgeführt. Zunächst werden die Produkte aus Peak e–i mit T_4-Polynucleotid-Kinase in der 5′-Position mit [γ^{32}P]ATP phosphoryliert und anschliessend der Homochromatographie[7,8] unterzogen wobei $[^{32}P](dT)_6$ bzw. $[^{32}P](dT)_{7,8}$ als Marker verwendet werden (vgl. Fig. 5). Aufgrund der R_F-Werte werden den Oligothymidinphosphaten, die in Peak e–i der Fig. 2 enthalten sind, die dort aufgeführten Kettenlängen zugewiesen. Die Homochromatogramme der Produkte von Peak f–i (vgl. Fig. 5) zeigen ausserdem, dass oberhalb der jeweiligen Hauptprodukte Oligothymidinphosphate wandern, die jeweils um eine Monomereinheit verkürzt sind. Es liegt nahe, dass diese Nebenprodukte, deren Anteil unter 5% liegt, bei der Aufarbeitung bzw. Lagerung der vereinigten Peakfraktionen gebildet werden, da Oligonucleotide bekanntlich leicht depolymerisieren. Für unsere Annahme spricht, dass als Nebenprodukte immer nur kürzere aber keine längeren Fragmente gefunden werden. Bei Peak i liegt dagegen eine Mischung von $(dT)_{11,12}$ vor, was aufgrund der ungenügenden Peakauftrennung an QAE-Sephadex (vgl. Fig. 2) nicht anders zu erwarten ist.

Kettenlänge und Sequenz des in Peak i der Fig. 2 enthaltenen längsten Oligothymidinphosphats werden zusätzlich nach der Fingerprintmethode[7,8] exakt ermittelt und bestätigt. Die Homochromatographie (vgl. Fig. 5c) beweist das Vorliegen eines

zwölfer DNA-Fragmentes, das aufgrund des Fingerprints (Fig. 5d) als $(dT)_{12}$ identifiziert wird.

Die Oligothymidinphosphate sind auf dem hier beschriebenen Weg in Mengen zugänglich, die auch bei der chemischen Synthese von Genfragmenten angestrebt werden. Den Vorteil dieser Methode sehen wir darin, dass die Isolierung weitaus einfacher zu praktizieren ist, als die komplizierte Synthese, die Spezialisten vorbehalten ist. Allerdings ist die chromatographische Isolierung auf DNA-Sequenzen begrenzt, die einerseits in natürlicher DNA gehäuft vorkommen und anderseits mit chemischen Abbaumethoden aus dem Molekülverband unversehrt freigesetzt werden können. Trennleistung und Effektivität der chromatographischen Verfahren in der hier praktizierten Kombination sind bemerkenswert, wenn man bedenkt, dass bspw. die erhaltenen *ca.* 4 mg an chromatographisch reinem $(dT)_{11,12}$ aus dem äusserst komplex zusammengesetzten Partialhydrolysat isoliert werden müssen, das bei chemischem Abbau von 500 g DNA anfällt.

DANK

Die Arbeit wurde von der Deutschen Forschungsgemeinschaft unterstützt.

ZUSAMMENFASSUNG

DNA aus Heringsspermen (500 g) wird chemisch zu Oligothymidinphosphaten abgebaut, die anschliessend an QAE-Sephadex in Verbindungen steigender Phosphatladungen fraktioniert werden. In der letzten Fraktion wird ein Gemisch (1.5 g) längerkettiger Oligothymidinphosphate erhalten. Aus dem Gemisch werden mit Hilfe der Template-Chromatographie und anschliessender Nachtrennung an QAE-Sephadex homologe Oligothymidinphosphate (1–4 mg) der Reihe $(dT)_{3-12}$ chromatographisch rein isoliert. Sequenz und Reinheit der isolierten Oligothymidinphosphate werden mittels Papier-, Hochdruckflüssigkeits- und Homochromatographie sowie der zweidimensionalen Fingerprintmethode bestimmt.

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GAS CHROMATOGRAPHIC DETERMINATION OF TRACE AMOUNTS OF β-METHYLMERCAPTOPROPIONALDEHYDE (METHIONAL) IN THE FREE FORM USING FLAME PHOTOMETRIC DETECTION

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SUMMARY

The gas chromatographic (GC) determination of trace amounts of β-methylmercaptopropionaldehyde (methional) in the free form using a flame photometric detector (FPD) was investigated. The GC conditions were as follows: stationary phase, 6% DEGS; support, Chromosorb W HP (80–100 mesh); glass column, 2 m × 3 mm I.D., column temperature (programming), holding for 1 min at 100°C, heating the column oven at a rate of 10°C/min from 100 to 190°C, maintaining this temperature for 20 min and then cooling to the initial temperature; carrier gas, nitrogen, flow-rate, 60 ml/min; flow-rates of hydrogen and air for the FPD, each 40 ml/min. The minimum detectable amounts for the methional were *ca.* 0.3 ng; the repeatabilities of retention times and peak areas (measured as integrator counts) at 1-ng level of the compound were less than *ca.* 1.5% and *ca.* 15%. The percentage recovery in the concentration method by bubbling the nitrogen carrier gas (0.2 l/min, at 28°C) through the standard solution of methional (at 1 ng per μl ethanol) from 10 to 0.5 ml was *ca.* 72%; however, the percentage recovery of the standard solution (at 10 ng/μl ethanol) after 10 l of the laboratory air had been passed through the impinger containing the standard solution at a rate of 0.5 l/min was 97.5%.

The method was applied to the determination of methional in air over waste water from a corn starch factory.

INTRODUCTION

It is well known that β-methylmercaptopropionaldehyde (methional) is very important as an intermediate in the synthesis of methionine and as a product during the thermal degradation of methionine. Patton[1] has reported that the compound has a broth-like flavour, which is responsible for sunlight flavour defect of milk, and that the flavour and aroma of cooked meats, soup and boiled vegetables frequently suggest the presence of the compound. He also reported that the odour of methional is detectable in milk at a level of 0.05 ppm. However, by sniffing the odour of methional in air using a 14 l Tedlar bag containing a known concentration of the compound, a concentration of 0.7 ppb* was recognisable by trained odour panellists[2]. The com-

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

0021-9673/82/0000–0000/$02.75

pound is thus very important as specific odourant, being a sulphur-containing lower aliphatic carbonyl compound responsible for the sulphur-like pollution odour in the food industries, because the threshold recognition value of methional in air corresponds to that of methanethiol and diethyl trisulphide[3,4].

The determination of methional has been performed by paper chromatography as its 2,4-dinitrophenylhydrazone[1] and by gas chromatography (GC)–mass spectrometry as its *n*-butyl ester[5]. However, there are few reports on the direct determination of methional in its free form.

In this paper, the direct determination of methional in its free form by GC with a flame photometric detector (FPD) is described. The methional-in-air sample was collected in ethanol, through which was bubbled nitrogen carrier gas. The concentrated sample solution was subsequently introduced into the gas chromatograph with an FPD and 6% diethylene glycol succinate polyester (DEGS) on Chromosorb W HP (80–100 mesh) as the column packing. The GC conditions produced peaks with no tailing.

EXPERIMENTAL

Reagents

Acrolein (90–95% purity) was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Methanethiol (99% purity) was obtained from Seitetsu Kagaku Kogyo (Osaka, Japan). Copper(II) acetate was obtained from Katayama (Osaka, Japan). Ethanol was obtained from Wako (Osaka, Japan). All reagents were of guaranteed or analytical-reagent grade. The column packings were purchased from Wako.

Preparation of methional

Methional was prepared by the method of Pierson[6]. The reaction was carried out by the addition of methanethiol to acrolein, and was catalysed by the addition of a small amount of copper(II) acetate to the reaction mixture, *i.e.*, gaseous methanethiol was bubbled for 30 min with a constant flow of *ca.* 0.7 l/min into a cooled stirred mixture of 56 g of acrolein and 0.5 g of copper(II) acetate, while the temperature of the reaction mixture was maintained at 35–40°C with an ice–water bath. The mixture obtained was stirred for 1 h and then distilled under reduced pressure [b.p. 76–78°C at 34 mmHg, (lit.[1], 71–73°C at 23 mmHg; lit.[6], 52–57°C at 11 mmHg)], yield 80%.

Standard solutions of the methional were prepared by dissolving the synthesized methional in ethanol to give concentrations of 0.01, 0.1, 1 and 10 mg per 10 ml of ethanol. A calibration graph was obtained using these standard solutions. The volume of the standard solution or the diluted solutions injected into the gas chromatograph was usually 1–5 μl. A 10-μl Hamilton 701-N microsyringe was used.

Apparatus

The gas chromatograph used was a Shimadzu Model GC5AP$_5$Fp (on-column and on-detector system) equipped with an FPD; the FPD had a flame-ionization detector (FID) for monitoring. The FPD and its FID accessory were operated with a separate electrometer (Shimadzu Model EM-5S). The chromatograph was also equipped with a digital integrator (Shimadzu Model ITG-2A) for the determination

TABLE I

EVALUATION OF COLUMN PACKINGS FOR THE DETERMINATION OF TRACE AMOUNTS OF METHIONAL IN THE FREE FORM (FPD DETECTOR, 10 ng)

Carrier gas, nitrogen; flow-rate, 60 ml/min.

Column packing and GC conditions	*Evaluations*
A 5% TCEP on Carbopack B (60–80 mesh), 1.5 m × 3 mm I.D., glass; 130°C	No peak until 40 min
B 5% SE-30 on Chromosorb W AW DMCS (60–80 mesh), 3 m × 3 mm I.D., glass; 100°C, 1 min holding, 10°C/min programming	Peak at 3.27 min with tailing
C 3% OV-17 on Chromosorb W AW DMCS (60–80 mesh), 3 m × 3 mm I.D., glass; 100°C, 1 min holding, 10°C/min programming	Peak at 5.93 min with tailing
D 6% DEGS on Chromosorb W HP (80–100 mesh), 2 m × 3 mm I.D., glass; 100°C 1 min holding, 10°C/min programming	Peak at 5.40 min without tailing

of retention time and for quantitative analysis. The detector signals were simultaneously recorded at 10 mV b.s.d. on a Shimadzu Model R-201 double-pen recorder.

Operating conditions for gas chromatography

The glass analytical column (2 m × 3 mm I.D.) was packed with 6% DEGS on Chromosorb W HP (80–100 mesh) (Wako). The chromatographic conditions for the FPD were as follows: column temperature (programming), holding for 1 min at 100°C, heating column oven at a rate of 10°C/min from 100 to 190°C, maintaining at this temperature for 20 min and then cooling to the starting temperature; injection port and detector temperature, 200°C; carrier gas (nitrogen) flow-rate, 60 ml/min; flow-rates of hydrogen and air for the FPD, each 40 ml/min. The column was preconditioned with the column oven temperature at 200°C for 6 h with a constant flow of nitrogen carrier gas (60 ml/min) before being connected to the FPD.

Collection method for the methional-in-air sample and concentration procedure for the ethanol solution of trace amounts of methional

The collection method for the methional-in-air sample was carried out by using a mini-impinger containing 20 ml of ethanol. The sample gas (10 l) was directly collected in the impinger at 30°C using a Handy Sampler (Model HS-6, Kimoto Electric Industries, Osaka, Japan; maximum sampling rate, 2.5 l/min). The sampling rate was *ca.* 0.5 l/min. The concentration procedure for the ethanol solution containing trace amounts of methional was performed by the nitrogen-gas-bubbling method (0.2 l/min at 28°C).

RESULTS AND DISCUSSION

Evaluation of column packings

Five kinds of column packing were investigated for the GC determination of trace amounts (*ca.* 10 ng) of methional in its free form. As listed in Table I, on the

TABLE II

RELATIVE RETENTION TIMES OF SEVEN SULPHUR-CONTAINING COMPOUNDS ON THE DEGS COLUMN

Retention time of methional = 1.00. Operating conditions for the DEGS column are the same as for condition D in Table I.

Sulphur compound	*Relative retention time*
Dimethyl disulphide	0.21
Diethyl disulphide	0.32
n-Heptanethiol	0.31
Di-*n*-butyl disulphide	0.89
Methional	1.00 (true value, 5.40)
Di-*n*-amyl disulphide	1.37
Diphenyl disulphide	3.00

stationary phases SE-30 (condition B) and OV-17 (condition C) on Chromosorb W AW DMCS (60–80 mesh), the methional peak tailed.

In the TCEP on Carbopack B[7,8] stationary phase, which was used for the determination of the lower aliphatic carbonyl compounds in their free forms, the peak was not detected until 40 min. Under these conditions, the retention time of *n*-valeraldehyde (C_5) was *ca.* 2.5 min. Use of DEGS on Chromosorb W HP as stationary phase, however, gave a well resolved peak for methional with no tailing.

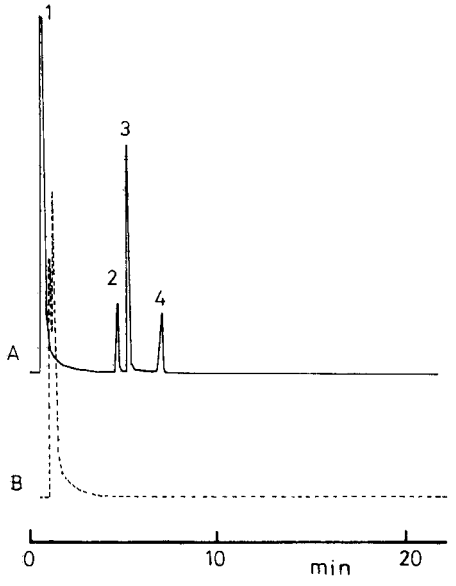

Fig. 1. Typical gas chromatograms of methional, di-*n*-butyl disulphide and di-*n*-amyl disulphide. Chromatogram A: FPD sensitivity, 16 × 10. Peaks: 1 = ethanol; 2 = di-*n*-butyl disulphide (1 ng); 3 = methional (2 ng); 4 = di-*n*-amyl disulphide. Chromatogram B: FID (monitor), sensitivity, 16 × 10. 6% DEGS on Chromosorb W-HP (80–100 mesh), 2 m × 3 mm I.D., glass; temperature programming, holding for 1 min at 100°C, heating the column oven at 10°C per min from 100 to 190°C, maintaining at this temperature for 20 min and then cooling to the starting temperature; nitrogen carrier gas, flow-rate 60 ml/min.

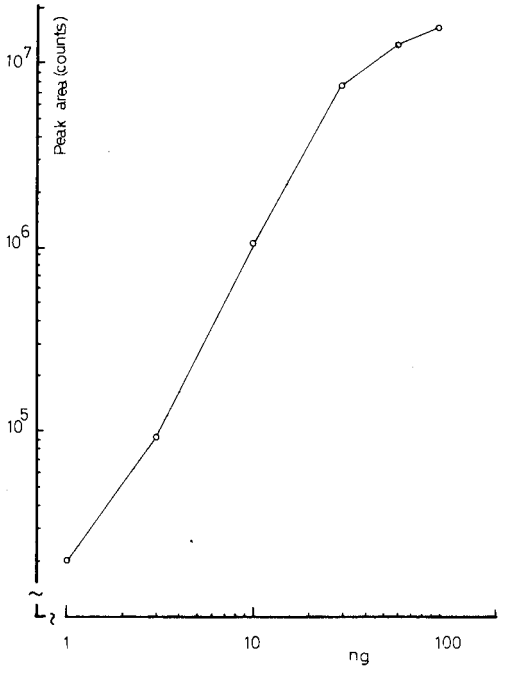

Fig. 2. Calibration graph for methional in its free form with FPD.

The retention times of seven sulphur containing compounds under the GC conditions labelled as D in Table I, are listed in Table II. The amount of each sulphur-containing compound used was 5 ng.

Fig. 1 shows a typical gas chromatogram of methional, di-*n*-butyl disulphide and di-*n*-amyl disulphide. The peak of the methional in its free form occurred between the peaks of di-*n*-butyl disulphide and di-*n*-amyl disulphide with no tailing. Fig. 1 shows that under the GC conditions D of Table I, these conditions are the test for a rapid, specific and sensitive detection of methional in its free form.

Calibration graph

The FPD response produced a straight line in the approximate range 1–30 ng of methional in its free form (Fig. 2), and the detection limit (at twice the noise level) was *ca.* 0.3 ng.

Repeatability of retention times and peak areas for methional in its free form

The repeatability and uniformity of the retention times and the peak area (as counts of the digital integrator) for the methional in its free form were evaluated by using 1- and 10-ng amounts. Table III shows that there was good repeatability.

Percentage recovery in the concentration procedure of the methional–ethanol standard solution

Table IV lists the percentage recovery in the concentration procedure of the standard solution (1 ng/μl) by the bubbling method with a constant flow of nitrogen

TABLE III

REPEATABILITY OF RETENTION TIMES AND PEAK AREAS FOR METHIONAL IN ITS FREE FORM ON THE DEGS COLUMN (n = 7)

Operating conditions for the DEGS column are the same as for conditions D in Table I.

	1 ng		*10 ng*	
	Retention time (min)	*Peak area (counts × 10^3)*	*Retention time (min)*	*Peak area (counts × 10^3)*
Average	5.39	19	5.44	1091
S.D.	0.02	3	0.08	82
Coefficient of variation (%)	0.4	15.8	1.5	7.5

carrier gas (0.2 l/min) at 28°C for 6 h, and after 10 l of laboratory air had passed through the standard solution (10 ng/μl). These tests were required for preliminary information on the oxidative degradation and photolysis of methional. Table IV shows that no interference by the presence of air was observed; however, loss of methional during the bubbling procedure occurred.

Typical gas chromatogram of methional-in-air sample collected from over waste water from a corn starch factory

Fig. 3 shows a typical gas chromatogram for a sample of methional and other sulphur-containing compounds in air collected from over waste water from a corn starch factory. The procedure for preparation of concentrated solution was as described in the Experimental section. The volume of the concentrated sample solution injected was 50 μl. The FPD, which is selective for analysis of sulphur compounds, permits the use of large sample volumes. Thus the merit of this method is that up to 50 μl of the sample solution can be used. The concentration of the methional in free form in the air sample was *ca.* 0.5 ppb. The odour characteristics (quality and intensity) of the air sample were sulphur-like and moderate.

CONCLUSION

The direct gas-chromatographic determination of the trace concentration of a methional-in-air sample, using a DEGS on Chromosorb W HP column with detec-

TABLE IV

PERCENTAGE RECOVERIES OF THE METHIONAL FROM ETHANOL STANDARD SOLUTIONS

Conditions	*Recovery (%)*
(a) After passage of 10 l of laboratory air through the standard solution (10 ng/μl) in 10-ml impinger	97.5
(b) After concentration of the standard solution (1 ng/μl) from 10 to 0.5 ml with a nitrogen carrier gas flow of 0.2 l/min at 28°C for 6 h	72.6

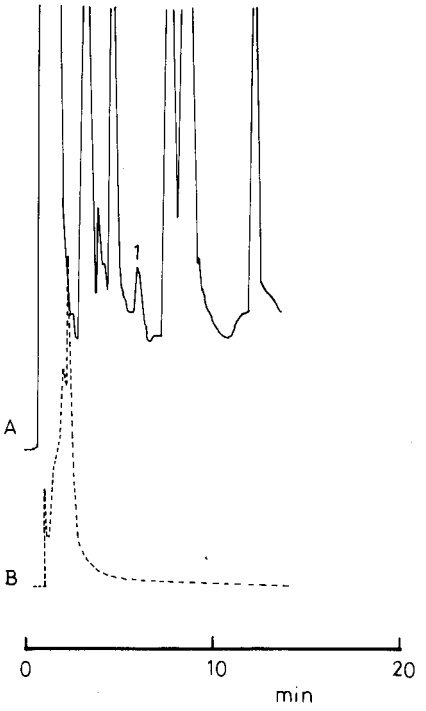

Fig. 3. Typical gas chromatogram of methional in its free form from air sample collected from over waste water from a corn starch factory. Chromatogram A: FPD sensitivity, 16 × 10. Peak: 1 = methional (*ca.* 0.5 ppb). Chromatogram B: FID (monitor), sensitivity, 16 × 10. Sample gas, 10 l collected into 20 ml ethanol impinger at 0.5 l/min, then 10 ml of the ethanol solution concentrated to 0.5 ml, 50 μl injected.

tion by FPD, has been demonstrated. The proposed method may be capable of giving rapid, sensitive and specific detection of both methional in its free form in air and of odour pollution related to food industries, etc.

ACKNOWLEDGEMENT

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SYSTEMS FOR THE SEPARATION OF METABOLITES OF THE CARCINOGEN, N-2-FLUORENYLACETAMIDE, BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Separation procedures for N-2-fluorenylacetamide (2-FAA) and its metabolites have been hampered by the unusually strong adsorption of N-hydroxy-N-2-fluorenylacetamide (N-OH-2-FAA), the proximate carcinogenic metabolite, to chromatographic packings. Results of investigations with C_2, C_3, C_8, and C_{18} column packings are presented showing that N-OH-2-FAA could be eluted and the separation of 2-FAA and eight other metabolites achieved with acidic mobile phases on C_2 and C_8 columns after a period of column conditioning. Elution of C_8 columns with mobile phases containing acetohydroxamic acid was less destructive to the columns while permitting the separation of 2-FAA and its metabolites with recovery yields of N-OH-2-FAA of 91.5%. Quantitation of these compounds was by integration of peaks detected by the spectroscopic method. Areas were linear for peaks representing from 5 to at least 50 ng of N-OH-2-FAA and of the eight other metabolites.

INTRODUCTION

The development of a separation procedure for a carcinogen, N-2-fluorenylacetamide (2-FAA), and its metabolites (structures and abbreviations of the compounds are listed in Table I) is warranted by the importance of 2-FAA in studies of N-arylamide carcinogenesis, the mechanism of xenobiotic biotransformations as well as the role of mixed-function oxidases. The primary metabolites of 2-FAA generated by the hepatic enzymes include phenols, N-OH-2-FAA, an alcohol, a ketone and an amine[1–4]. The development of an efficient separation procedure for these metabolites has been hampered by the unusually strong adsorption of N-OH-2-FAA to chromatographic column packings. Since N-OH-2-FAA is a proximate carcinogenic metabolite of 2-FAA[5], estimates of the amounts of the N-hydroxy compound formed in hepatic and extra-hepatic tumor targets are of particular importance in carcinogenesis. An additional difficulty with the separation procedures arises when conditions under which quantitative elution of N-OH-2-FAA is possible must be balanced with conditions allowing separation of the remaining metabolites.

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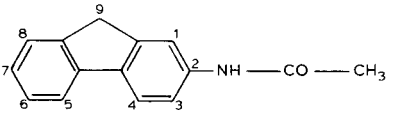
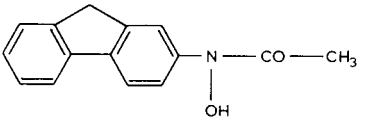
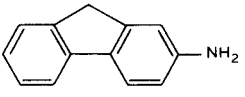
TABLE I

STRUCTURES OF 2-FAA AND ITS METABOLITES CORRESPONDING TO THE PEAK NUMBER IN THE HPLC ELUTION PROFILES

Structure	*Compound*	*Peak No.*
	N-2-Fluorenylacetamide [2-FAA]	10
Ring-oxidized derivatives:		
1-OH	N-(1-Hydroxy)-2-FAA [1-OH-2-FAA]	8
3-OH	N-(3-Hydroxy)-2-FAA [3-OH-2-FAA]	7
5-OH	N-(5-Hydroxy)-2-FAA [5-OH-2-FAA]	3
7-OH	N-(7-Hydroxy)-2-FAA [7-OH-2-FAA]	1
9-OH	N-(9-Hydroxy)-2-FAA [9-OH-2-FAA]	2
9=O	N-(9-Oxo)-2-FAA	6
	N-Hydroxy-2-FAA [N-OH-2-FAA]	9
	N-2-Fluorenamine [2-FA]	4
Derivative:		
9=O	N-(9-Oxo)-2-FA*	5

* Potential metabolite of 2-FA.

Initially, applications of high-performance liquid chromatography (HPLC) to the separation of the metabolites of 2-FAA involved a combination of normal-phase and reversed-phase HPLC[6,7]. A separation procedure employing only reversed-phase HPLC was developed by Thorgeirsson and Nelson[8]. Procedures based on reversed-phase HPLC developed subsequently[9–11] were modifications of the original procedure[8] and employed a mobile phase buffered to a pH of 8.5 or 9.0, at which dissolution of the silica matrix occurs. Moreover, elution of N-OH-2-FAA was slow, its peak broad and its quantitation unreliable. The nature of the secondary interactions contributing to the adsorption of N-OH-2-FAA has not been elucidated. It is suspected that the interactions may be due to metal impurities found in silica[12]. It has been shown, that N-OH-2-FAA and certain other hydroxamic acids can be selectively eluted from reversed-phase columns after complexation with ferric ions during chromatography[13]. However, this method was not applicable to the separation of other metabolites of 2-FAA. More recently, 2-FAA and eight of its metabolites were separated by reversed-phase HPLC with a mobile phase containing desferal mesylate[14]; this agent presumably chelates the sites of adsorption for the hydroxamic acids[12]. However, the addition of desferal mesylate by itself was not sufficient to block the chemisorptive sites, since the procedure required pre-saturation of the column with a solution of N-OH-2-FAA for several hours[14].

We present a procedure for separation of 2-FAA and eight of its metabolites,

which involves isocratic elution from a reversed-phase column with a mobile phase containing acetohydroxamic acid. Employing the labelled compound, we show that N-OH-2-FAA is recovered quantitatively, peak detection is by the spectroscopic method and quantitation by peak integration.

MATERIALS AND METHODS

Preparation of compounds

2-FAA (Aldrich, Milwaukee, WI, U.S.A.) was recrystallized from ethanol–water (7:3) and had a m.p. of 196–198°C. N-OH-2-FAA, m.p. 150–151°C (ref. 15); 1-OH-2-FAA, m.p. 210–212°C (ref. 16); 3-OH-2-FAA, m.p. 247–249°C (ref. 17); 5-OH-2-FAA, m.p. 214–216°C (ref. 18); 7-OH-2-FAA, m.p. 230–232°C (ref. 19); 9-OH-2-FAA, m.p. 249–250°C and N-(9-oxo)-2-FAA, m.p. 233–236°C (ref. 20); 2-FA, m.p. 127–129°C (ref. 21) and N-(9-oxo)-2-FA, m.p. 154–156°C (ref. 22) were prepared by the published procedures. The IR and UV spectra of the compounds matched those of the authentic samples. The compounds were found to be pure by thin-layer chromatography on silica gel GF_{254} with chloroform–methanol (95:5) or ethyl acetate–benzene (7:3) as mobile phase. The compounds for HPLC analyses were dissolved in methanol, and a stock solution containing 0.5 or 1.0 mg/ml of each compound was prepared.

Solvents and chemicals for HPLC

Sodium heptanesulfonate, oxalic acid and all solvents used for chromatography were glass-distilled/HPLC grade from Matheson, Coleman and Bell (Cincinnati, OH, U.S.A.). The methanesulfonic acid and trimethylamine hydrochloride were from Aldrich. The chloroacetic acid was from Mallinckrodt (Paris, KE, U.S.A.). Acetic acid was spectral grade, called Photorex reagent grade, from J. T. Baker (Phillipsburg, NJ, U.S.A.). Acetohydroxamic acid was purchased from Sigma (St. Louis, MO, U.S.A.). All solvents were pre-mixed to minimize exothermic mixing effects.

Columns

The C_{18} (MCH-10) and the end-capped C_{18} (N-MCH-10) columns were from Varian Instruments (Sunnyvale, CA, U.S.A.). The C_8 columns included Zorbax C_8 from DuPont (Wilmington, DE, U.S.A.), Ultrasphere Octyl from Altex (Berkeley, CA, U.S.A.), IBM Octyl from IBM Instruments (Danbury, CT, U.S.A.), LiChrosorb RP-8 from E. Merck (Darmstadt, G.F.R.). The 5- and 10-μm LiChrosorb RP-2 columns were from E. Merck, and the Zorbax ODS and Zorbax TMS were from DuPont.

Chromatographic equipment

All chromatographic analyses were carried out on the Varian Instruments Model 5060 liquid chromatograph. Spectroscopic peak detection was done with a Varian Instruments UV50 variable-wavelength detector whose flow cell was maintained at 31°C with a circulating water bath (type K2/R, MGW Lauda, G.F.R.). Peak areas and peak heights were measured by an integrating recorder from Hewlett-Packard (Model 3390A). Samples were injected by means of a pneumatically actuated injector from Valco Instruments (Houston, TX, U.S.A.) equipped with a 10-μl loop.

Preparation and determination of the recovery of N-OH-2-[9-^{14}C]FAA

N-OH-2-[9-^{14}C]FAA was prepared from 2-nitro[9-^{14}C]fluorene (California Bionuclear Corp. (Sun Valley, CA, U.S.A.), Lot No. 2529, specific radioactivity 14.7 mCi/mmole) that had been diluted with the unlabelled compound to a specific radioactivity of 5.8 mCi/mmole. 2-Nitro-[9-^{14}C]fluorene was reduced and subsequently acetylated as described previously[21]. N-OH-2-[9-^{14}C]FAA was recrystallized from ethanol–water (8:2) and had a m.p. of 148–150°C. The labelled compound was purified by HPLC employing an Altex Ultrasphere Octyl column (150 × 4.6 mm, 5 μm particle diameter) and 10% (v/v) dimethyl sulfoxide (DMSO), 16% (v/v) isopropanol, 10% (v/v) 0.5 *M* sodium chloroacetate, pH 3.0, as mobile phase. The specific radioactivity of the pure compound was 5.4 mCi/mmole. From 15,000 to 40,000 dpm of radioactivity in 10 μl of methanol was used per injection. The amount of radioactivity collected under the N-OH-2-FAA peak was compared to the amount of radioactivity collected directly from the 10-μl injector loop. All collected samples were diluted with mobile phase to the same final volume (15 ml), frozen in a dry ice–ethanol bath, and lyophilized overnight in 40-ml conical tubes. A 10-ml volume of scintillation liquid[21] was then added to each tube, which contained a transparent yellowish residue, and the tubes were shaken to insure that the residue became suspended in the liquid. The precipitate which formed was removed by centrifugation in an International Equipment Company Centrifuge (Model PR-2) at 600 *g* for 20 min. The amount of radioactivity in the supernatant was determined using a Packard Liquid Scintillation Spectrometer (Model 3255). The counts were corrected for quenching by means of an external standard. The counting efficiency was 70%.

RESULTS AND DISCUSSION

Adsorption of N-OH-2-FAA to reversed-phase columns

N-OH-2-FAA was eluted with methanol at a flow-rate of 2 ml/min as a broad peak for 30 to 50 min from C_{18} (MicroPak MCH-10, MicroPak N-MCH-10, Zorbax ODS), C_8 (Zorbax C_8), and C_3 (Zorbax TMS) columns. The use of acetonitrile, isopropanol or tetrahydrofuran (THF) and the addition of sodium heptanesulfonate or tetramethylammonium chloride to these solvents did not improve the elution of this hydroxamic acid.

Elution of N-OH-2-FAA as a sharp, unretained peak from C_{18} columns was achieved by the use of DMSO, dimethylformamide (DMF) or dimethylacetamide (DMA). Dilution of these solvents to increase the retention time of N-OH-2-FAA resulted in large decreases in the recovery of this compound and in excessive peak broadening. Acidification of the mobile phase with acetic acid, formic acid or methanesulfonic acid improved recovery of the compound and peak shape, while addition of ion-pairing agents such as sodium heptanesulfonate or tetramethylammonium chloride did not. However, even acidified mobile phases containing DMSO, DMF or DMA could not be diluted to the extent needed to simultaneously increase the retention time of N-OH-2-FAA, to permit its quantitative recovery and to separate the other 2-FAA metabolites on C_{18} columns.

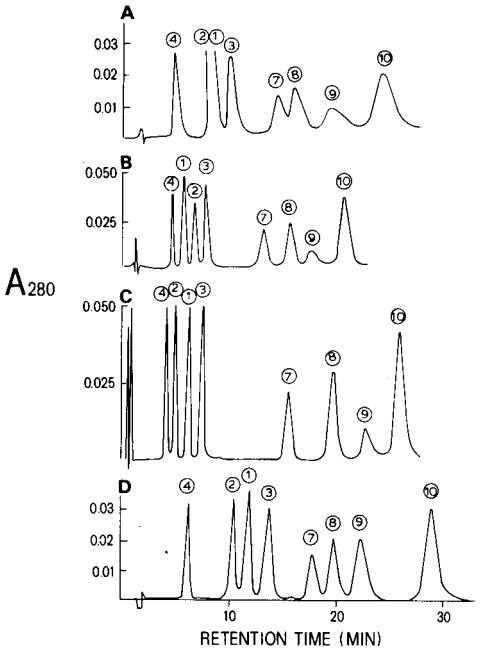


Fig. 1. Separation of 2-FAA and its metabolites on C_2 and C_8 columns. A, LiChrosorb RP-2, 10-μm particles, 250 × 4.6 mm (I.D.); flow-rate 2 ml/min; mobile phase, DMSO (10%, v/v)–formic acid (18%, w/v). B, Zorbax C_8, 250 × 4.6 mm I.D.; flow-rate 2 ml/min; mobile phase, DMSO (30%, v/v)–formic acid (17.6%, w/v)–phosphoric acid (0.85%, w/v). C, Ultrasphere Octyl, 5-μm particles, 150 × 4.6 mm I.D.; flow-rate 2 ml/min; mobile phase, DMSO (20%, v/v)–isopropanol (10%, v/v)–oxalic acid (1%, w/v). D, LiChrosorb RP-2, 5-μm particles, 250 × 4.6 mm I.D.; flow-rate 1.8 ml/min; mobile phase, methanol (25%, v/v–)acetic acid (5%, v/v)–oxalic acid (1%, w/v)–Na_2HPO_4 (50 m*M* final concentration). Compounds are identified in Table I and were injected each at a concentration of 50 μg/ml in 10 μl.

Separation of 2-FAA and its metabolites on C_2 and C_8 columns

The C_2 and C_8 columns provided more suitable stationary phases for separation of 2-FAA and its metabolites. Four separation procedures were developed for these compounds on such columns by adjusting the relative proportions of DMSO, isopropanol, methanol, formic acid, acetic acid and oxalic acid in the mobile phase (Fig. 1). A feature common to all four procedures is that mobile phases are strongly acidic. Although it appeared that DMSO or DMF was necessary for elution of N-OH-2-FAA as a result of work with C_{18} columns, given the proper conditions (Fig. ID), such solvents could be eliminated from the mobile phaxe. DMSO was used in preference to DMF because 1-OH-2-FAA and N-OH-2-FAA tend to co-elute when DMF is added as the organic modifier.

A common drawback to the four procedures (Fig. 1) is that separation can be achieved only after the columns are conditioned by washing with the acidic mobile phase for an extended period of time. Hence, these mobile phases will not work immediately when applied to new columns. Column packings were found to be unstable when such mobile phases were used. A gradual decline in retention capacity of columns occurred initially and became rapid after 70 to 80 sample injections. The loss

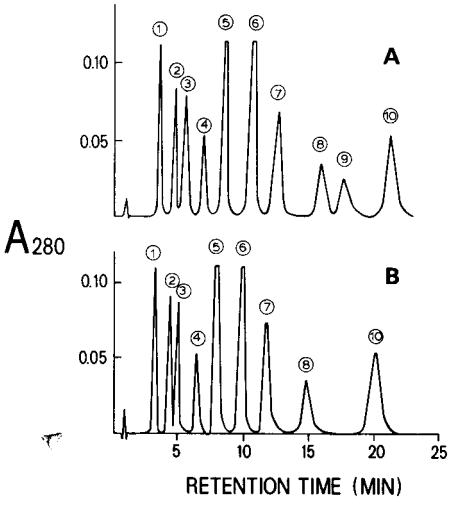


Fig. 2. Effect of the presence in (A) or absence from (B) the mobile phase of acetohydroxamic acid on the separation of 2-FAA and its metabolites on an Ultrasphere Octyl column, 150 × 4.6 mm I.D. operated at 30°C and a flow-rate of 2 ml/min with an operating pressure of 4750 p.s.i. The mobile phase contained DMSO (10%, v/v), isopropanol (16%, v/v), THF (1%, v/v), 0.5 *M* sodium chloroacetate, pH 3.0 (10%, v/v) and acetohydroxamic acid (1%, w/v), when added. Compounds are identified in Table I and were injected each at a concentration of 50 μg/ml in 10 μl.

of retention capacity was due presumably to the hydrolysis of the organic matrix from silica. Column voiding became evident after 80 to 100 sample injections.

The difficulties associated with the use of such separation procedures might be due to a limitation of derivatized silica gel packing material. Nevertheless, the procedures were used to separate 2-FAA and its metabolites for 25 to 50 sample injections. Should developments in the technology of packing materials result in more stable column packings, these solvent systems would be of greater value.

Elution of N-OH-2-FAA with a mobile phase containing acetohydroxamic acid

Elution of N-OH-2-FAA and separation of the other metabolites of 2-FAA with less damage to columns became possible with mobile phases containing acetohydroxamic acid. The addition of acetohydroxamic acid to the mobile phase is required for elution of N-OH-2-FAA, but has little effect on the elution of the other compounds (Fig. 2). With the use of acetohydroxamic acid, the strongly acidic conditions used previously to achieve the separation (Fig. 1) were found unnecessary. The pH of the aqueous portion of the mobile phase was 5.65 if the chloroacetate buffer was not added. To achieve the separation, the mobile phase was buffered to pH 3.0 to control the retention time of 2-FA, which increased with increasing pH and at pH 4.68 became identical with that for 1-OH-2-FAA.

Extensive washing of the column with mobile phase containing acetohydroxamic acid for several weeks did not condition the column to permit elution of N-OH-2-FAA with a mobile phase devoid of this modifier (Fig. 2B). Elution of N-OH-2-FAA with acetohydroxamic acid, therefore, was achieved by reversible competition for adsorption sites rather than by an irreversible conditioning process which elim-

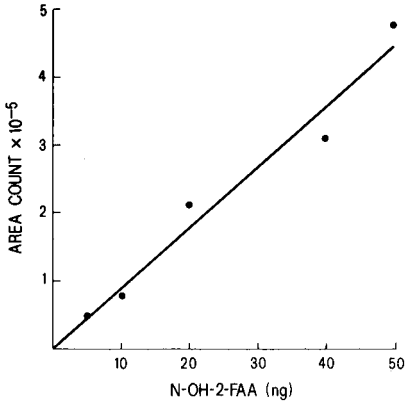


Fig. 3. Linearity of integrator response with the amount of N-OH-2-FAA injected in 10 μl of methanol and eluted as described in Fig. 2A.

inates such sites. In three experiments, an average of 91.5% of injected radioactivity of N-OH-2-[9-^{14}C]FAA was recovered under its peak.

Acetohydroxamic acid of high purity has no appreciable absorbance at 280 nm, which permits elution profiles for the 2-FAA metabolites to be monitored by spectroscopic methods. Minimum detectable quantitites of N-OH-2-FAA are limited by detector noise levels. When the detector noise level was stabilized to 10^{-4} absorbance unit, peak areas could be accurately determined for quantities of N-OH-2-FAA as low as 5 ng (Fig. 3). Integrator response was found to be linear for peaks representing 5 to 50 ng of N-OH-2-FAA and of the other metabolites. The upper limit for linear integrator response was not determined, but appeared to be much greater than 50 ng. Peaks representing <5 ng of N-OH-2-FAA were visible in recorder tracings, but could not be integrated at this noise level. The use of fixed-wavelength detectors, with noise specifications nearly an order of magnitude lower than those of variable-wavelength detectors, should permit quantitative detection of N-OH-2-FAA at <5 ng.

The separation of 9-OH-2-FAA from 5-OH-2-FAA (peaks 2 and 3, respectively), and the separation of 1-OH-2-FAA, N-OH-2-FAA and 2-FAA (peaks 8, 9 and 10, respectively), required fine adjustments in mobile-phase composition. Applications in which the quantities of 2-FAA are large compared to those of the metabolites, conditions encountered during *in vitro* metabolic studies, necessitated that the peak for 2-FAA be well separated from the other peaks. The position of the peak for N-OH-2-FAA was shifted away from the 2-FAA peak toward the 1-OH-2-FAA peak by the addition of THF. Simultaneously, the separation between 9-OH-2-FAA and 5-OH-2-FAA was improved by the presence of THF in the mobile phase. The amount of THF which could be added to the mobile phase was limited by the tendency of the 1-OH-2-FAA and N-OH-2-FAA peaks to superimpose with increasing THF content.

Acetohydroxamic acid and stainless steel

With mobile phases containing acetohydroxamic acid, the liquid which sometimes leaked around high-pressure fittings was orange to red in color. When nuts made of 316 stainless steel were placed in solutions containing 1% (w/v) of ac-

TABLE II

RETENTION TIMES FOR 2-FAA AND ITS METABOLITES AND MOBILE PHASE COMPOSITIONS IN SEPARATIONS ON SEVERAL C_8 COLUMNS

Column dimensions, 150 mm × 4.6 mm I.D.; flow-rate, 2 ml/min, except for III, for which it was 3 ml/min; column temperature, 30°C; a guard column of Perisorb RP-2, 35 × 4 mm I.D. was used for all separations.

Columns		*Retention time (min)*					
		7-OH-2-FAA	*9-OH-2-FAA*	*5-OH-2-FAA*	*2-FA*	*9-Oxo-2-FA*	*9-Oxo-2-FAA*
I	Ultrasphere Octyl (No. 1)***	3.42	4.60	5.34	6.75	8.27	10.40
II	Ultrasphere Octyl (No. 2)	3.39	4.91	5.68	7.28	8.79	10.93
III	Zorbax C_8	2.95	3.90	4.78	5.51	7.81	10.17
IIIa	Zorbax C_8 §	3.07	4.09	4.85	6.07	7.80	9.02
IV	IBM C_8	3.57	4.54	5.22	8.72	–	–

* Added as percentage w/v

** 0.5 *M* Sodium chloroacetate, pH 3.0.

*** See Fig. 2A for chromatogram.

§ See Fig. 4 for chromatogram.

§§ Methanesulfonic acid added to a final concentration of 20 m*M*.

etohydroxamic acid, the solution became yellow within 1 h at room temperature. If, after 2 days of contact with the solution of acetohydroxamic acid, the nuts were washed with isopropanol and transferred to a fresh solution of acetohydroxamic acid, the solutions became weakly discolored only after more than 1 week. A surface impurity in the steel which becomes complexed by acetohydroxamic acid could account for the initial discoloration.

New Ultrasphere Octyl columns

When the mobile phase used to achieve the separation in Fig. 2A was applied to new Ultrasphere Octyl columns, resolution between the 9-OH-2-FAA and the 5-OH-2-FAA peaks was diminished and the N-OH-2-FAA peak was broader, tailing into the 2-FAA peak. Increasing the concentration of acetohydroxamic acid from 1 to 2% (w/v) restored the separation between the N-OH-2-FAA and the 2-FAA peaks. Minor adjustments in isopropanol, DMSO and THF (Table II) restored the remainder of the separation to that shown in Fig. 2A.

Although losses in retention capacity were not observed with buffered mobile phases containing acetohydroxamic acid, column voiding appeared to be a persistent problem with the Ultrasphere Octyl columns. Voiding was evident as a broadening of the peaks near their bases, obscuring the separation. Column function could be restored by running the column in the direction opposite to that prescribed by the manufacturer. With the three Ultrasphere Octyl columns tested, column voiding was apparent within 1 week of daily use. However, after being turned upside down, the columns could be used daily for at least 2 months.

Pre-equilibration of the mobile phase with silica by interposing a silica precolumn between the pump and the injector did not alleviate the voiding problem, nor did the use of a guard column between the injector and the analytical column. The

				Percent of mobile phase (v/w)				
3-OH-2-FAA	*1-OH-2-FAA*	*N-OH-2-FAA*	*2-FAA*	*Isopropanol*	*DMSO*	*THF*	*Acetohydroxamic acid**	*Chloroacetate***
12.37	15.68	17.37	20.97	16	10	1	1	10
14.11	18.17	20.03	23.78	15.5	9	1.5	2	10
11.94	15.25	17.59	21.37	16	10	1	1	10
11.54	14.57	16.25	19.54	18	9.5	1.5	1.25	10
12.92	16.21	18.54	22.77	16	10	1	1	0§§

reason for the voiding is not understood. However, the operating pressure of 4750 p.s.i. needed to maintain a flow-rate of 2 ml/min in a 150 × 4.6 mm column packed with 5-μm Ultrasphere Octyl may be a factor.

Zorbax C_8 column

With the mobile phase used to generate the chromatogram in Fig. 2A, 2-FAA and its metabolites were separated on a Zorbax C_8 column (150 × 4.6 mm). The Zorbax C_8 column was found to have a greater retention capacity than the Ultrasphere Octyl column, hence a flow-rate of 3 ml/min was required to complete the separation in less than 25 min (Table II). Increasing the strength of the mobile phase and making adjustments in its composition allowed the separation to be achieved in the same amount of time at a flow-rate of 2 ml/min (Fig. 4). The use of the Zorbax column had two important advantages over the use of Ultrasphere Octyl columns. Less acetohydroxamic acid (1.25%, w/v) was required to elute N-OH-2-FAA with the Zorbax column as compared to the new Ultrasphere columns (2.0%, w/v). The operating pressure required to maintain a flow-rate of 2 ml/min with the Zorbax C_8 columns (Fig. 4) was 2700 p.s.i. or 2000 p.s.i. less than that required with the Ultrasphere Octyl columns.

Coverage of the silica matrix

As a final note, during the work to achieve elution of N-OH-2-FAA and a separation of the 2-FAA metabolites, it was apparent that column packings with more extensive coverage of the silica matrix by the organic matrix were better suited to this purpose. Both the Zorbax C_8 and the Ultrasphere Octyl packings were end-capped for maximum coverage of surface silanols. Packings which are not end-capped, such as LiChrosorb RP-8 (data not shown) and the IBM C_8 (Table II) pose a

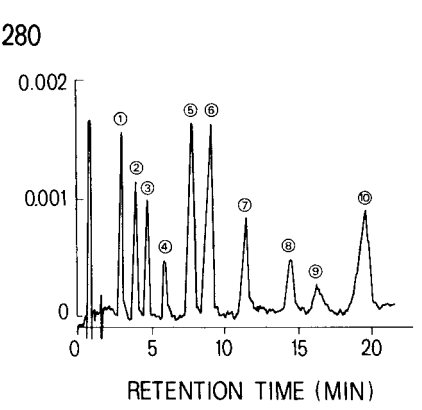


Fig. 4. Separation of 2-FAA and its metabolites on a Zorbax C_8 column 150 × 4.6 mm I.D. operated at 30°C and a flow-rate of 2 ml/min with an operating pressure of 2700 p.s.i. The mobile phase contained DMSO (9.5%, v/v), isopropanol (18%, v/v), 0.5 *M* sodium chloroacetate, pH 3.0 (10%, v/v), and acetohydroxamic acid (1.25%, w/v). Compounds are identified in Table I and were injected each at a concentration of 0.5 μg/ml in 10 μl.

problem to the elution of 2-FA in addition to the other problems associated with separations of the 2-FAA metabolites. Perhaps it is the more extensive coverage of the silica found with C_2 packings that permitted the elution of N-OH-2-FAA from such packings more readily.

ACKNOWLEDGEMENTS

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF CHLORHEXIDINE

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SUMMARY

Chlorhexidine, a common antiseptic, has been extracted from blood and urine by shaking with diethyl ether, chromatographed in methanol–water using an ion-pair agent to control k', and detected by ultraviolet absorption. A variety of ion-pair agents have been evaluated. Quantitation was achieved via a calibration curve and a detection limit of 0.2 $\mu g\ ml^{-1}$ proposed. Studies on blood and urine reveal no interference problems. The method has been applied to a preliminary study of post-cystoscopy urine specimens where chlorhexidine had been used as the irrigant, and to the quality control of pharmaceutical preparations.

INTRODUCTION

The bis-biguanide compound chlorhexidine (1,1′-hexamethylene-bis[5-(4-chlorophenyl)biguanide] (Fig. 1) is an antiseptic developed for use in hospital practice (trademark "Hibitane", I.C.I., Alderley Park, Great Britain). It is a strong base readily forming salts from which a range of pharmaceutical preparations have been formulated. For example the skin cleanser "Hibiscrub" (I.C.I.) contains the digluconate salt in aqueous solution. Chlorhexidine is reported to carry four positive charges in strong acid[1] delocalised over all ten nitrogen atoms[2]. However, only two pK_a values of 2.2 and 10.3 have been reported[3]. Thus in neutral or mild alkaline solution the compound is dicationic. At bacteriostatic concentrations the bacterial cell membrane is damaged with irrevocable loss of cell contents and inhibition of enzyme proteins. At higher concentrations, precipitation of cellular proteins and nucleic acids occurs resulting in effective bactericidal action[4–6]. Animal studies indicate that, at

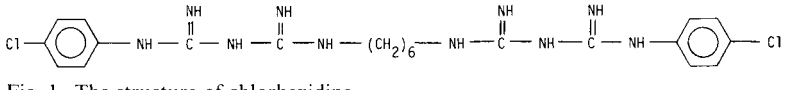

Fig. 1. The structure of chlorhexidine.

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high concentrations, chlorhexidine binds to rat liver microsomes interfering with enzyme metabolism and causing protein precipitation[1]. Such effects have been attributed to the cationic nature of chlorhexidine.

Chlorhexidine-containing preparations are commonly used in hospital practice for disinfection of hands and of the operation site. Further applications involve contact with microsal surfaces other than the intact skin, for example irrigation of the bladder to prevent urinary tract infection, cleansing of post-operative wounds and in the treatment of burns. Little is known of the possible absorption of chlorhexidine administered by such routes. Absorption through intact skin is known to be negligible, even after prolonged use[7], and there is no evidence of systematic toxicity. Low levels of chlorhexidine have been reported in the blood of neonates following topical application of 4% chlorhexidine gluconate[8] or 1% chlorhexidine powder[9]. Further studies of possible systematic absorption of chlorhexidine administered by other routes in urological and obstetric practice are indicated.

The colorimetric method used to estimate chlorhexidine in pharmaceutical preparations[10] lacks the sensitivity required for analysis of body fluids. To date, the only published method fulfilling this requirement for blood samples is gas–liquid chromatography (GLC)[11], involving solvent extraction and hydrolysis to 4-chloroaniline followed by diazotisation and iodination to 4-chloroiodobenzene. This method fails to distinguish 4-chloroaniline resulting from the *in vitro* or *in vivo* decomposition of chlorhexidine prior to analysis. Thermal decomposition occurs slowly at room temperature although increased levels of 4-chloroaniline have been detected in aqueous chlorhexidine solutions autoclaved at 130°C for 30 min[12]. The amount of 4-chloroaniline in pharmaceutical preparations is monitored by a colorimetric method and is unlikely to be sufficiently high to cause toxic side effects or an appreciable loss in antibacterial potency. However for pharmacological and absorption studies, a sensitive method for the independent detection of chlorhexidine and 4-chloroaniline is preferable. Such considerations prompted the investigation of high-performance liquid chromatography (HPLC) as an alternative to the GLC and colorimetric methods. We now report an HPLC assay of chlorhexidine which may be applied to urine and blood with a detection limit of 0.2 μg ml^{-1}. The method may also be used for the rapid determination of chlorhexidine in pharmaceutical preparations.

EXPERIMENTAL

An LC-XPD Model 100 pump (Pye Unicam, Cambridge, Great Britain), loop injection system (Rheodyne 7120 with 50 μl capacity), reversed-phase column (10 μm, ODS Waters Assoc., Cheshire, Great Britain) and ultraviolet detector (Model LC3, Pye Unicam) were used.

Water was double distilled before use. Methanol (Willot Industrial, Bristol, Great Britain), acetonitrile (BDH, Poole, Great Britain), and acetone (Chas. Tennants, London, Great Britain) were used as received, as were sodium lauryl sulphate, sodium cyclamate, toluene-4-sulphonic acid, sodium acetate, Pic B-7 (Waters Assoc.), pentadecafluorooctanoic acid and acetic acid (glacial). The mobile phase was degassed by ultrasonic vibration under vacuum (17 mmHg) for 15 min prior to use and stirred continuously during use.

Optimised chromatographic parameters were: mobile phase 1000 μg ml^{-1} toluene-4-sulphonic acid in methanol–water (65:35); flow-rate, 1.5 ml min^{-1}; injection volume 50 μl; detector wavelength 238 nm; sensitivity 0.04 a.u.f.s. into 10 mV f.s.d. (unless otherwise stated).

To extract 1 ml blood, serum or urine 1 ml of 1 *N* NaOH was added followed by 10 ml diethyl ether. Mechanical shaking (30 min) and centrifugation (15 min, 1110 *g*) was followed by decantation of the organic layer (*ca.* 9.5 ml). This was then blown to dryness (under nitrogen) and redissolved in mobile phase (300 μl) to give a suitable sample for injection.

Chlorhexidine base powder, Hibitane (20% chlorhexidine gluconate solution), Hibiscrub (4% chlorhexidine gluconate) and "Corsodyl" mouthwash (0.2% chlorhexidine gluconate) were obtained from I.C.I. 0.02% chlorhexidine acetate (for irrigation) was obtained from Travenol, (Thetford, Great Britain) and solutions of 0.2% aqueous chlorhexidine gluconate and 0.5% chlorhexidine gluconate in 70% methylated spirit were obtained as dispensed from the Pharmacy Department, Bristol Royal Infirmary, Great Britain.

RESULTS AND DISCUSSION

Optimisation of chromatography

Chlorhexidine itself, usually referred to as chlorhexidine base, is exceedingly insoluble in water (0.008%) and is only slightly soluble in organic solvents such as hexane and acetone. Solubility of the salts is variable with the gluconate salt being the most soluble. Thus in aqueous medium (pH $\approx$ 7) the chlorhexidine moiety exists as the di-cation and thus readily forms ion pairs. These considerations dictated the selection of a reversed-phase column (ODS) and the use of an ion-pair agent possessing an organic anionic moiety.

The mobile phase originally investigated consisted of acetonitrile–water mixtures but impurity problems prompted a change to methanol–water. A relative proportion of 75:25 was selected after studying the chromatography of standard compounds at various relative proportions. In view of the ionic nature of chlorhexidine the effect of pH of the mobile phase was investigated. The mobile phase consisting of methanol–20 m*M* aqueous sodium acetate (75:25) was adjusted to the required pH with acetic acid. The resultant capacity factors (k') suggested that, for the pH range 3.8–5.5, 4-chloroaniline was poorly retained ($k' = 0.2$). For chlorhexidine k' varied from 0.2 (pH 3.8), to 3.0 (pH 5.5) but the peak shape was unacceptable. For these experiments the acetate ion may be considered as an ion-pairing agent albeit a very poor one. A change of mobile phase proportion to methanol–water (pH 4.0) (50:50) raised k' (chlorhexidine) from 0.4 to 10.3. Thus control of k' (chlorhexidine) may be achieved by control of the methanol–water ratio and pH but peak shape (and thus column efficiency) are poor.

The addition of ion-pairing agents to the mobile phase for reversed-phase chromatography of ionic species increases their retention on the column. Various possible ion pair agents were studied, using a constant methanol–water ratio (75:25) at pH 4. In a search for a large organic counter-ion sodium lauryl sulphate and sodium cyclamate were used at 10, 100 and 1000 μg ml^{-1} concentrations. Whilst variation of k' for chlorhexidine was achieved peak shape remained rather broad. In

contrast 0.005 *M* heptanesulphonic acid (Pic B-7, Waters Assoc.) provided excellent peak shape with a k' (chlorhexidine) of 1.2 (Fig. 2a). The argument against the use of this reagent is financial and hence heptanoic acid was evaluated. This proved unsuccessful.

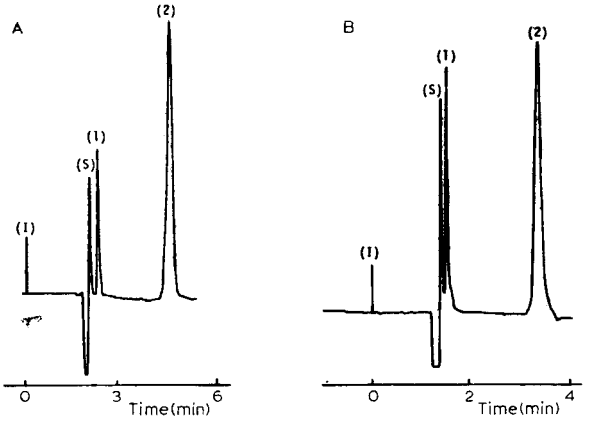

Fig. 2. (A) Chromatogram of chlorhexidine (2) and 4-chloroaniline (1) with Pic B-7 as ion-pair agent. (Detector: 254 nm, sensitivity 0.04 a.u.f.s.). (B) Chromatogram of chlorhexidine (2) and 4-chloroaniline (1) with toluene-4-sulphonic acid as ion-pair agent. (Detector 238 nm; sensitivity 0.04 a.u.f.s. I = injection point; S = solvent front).

Attention was thus turned to aromatic sulphonic acids. At first sight the use of an aromatic constituent of the mobile phase when an ultraviolet detector is employed appears self-defeating but examination of the ultraviolet spectrum of toluene-4-sulphonic acid revealed a significant *minimum* in the main absorption band. This minimum was centred at about 242 nm. Chlorhexidine absorbs strongly between 215 nm and 260 nm and thus monitoring the column eluate at approximately 242 nm to coincide with the absorbance minimum for toluene-4-sulphonic acid was apparently possible. Mobile phase was thus prepared containing 1000 μg ml^{-1} of this ion-pair agent. The background absorption of the solvent was increased by the ion-pair agent but was still acceptable. Fine adjustment of the detector-wavelength setting located the point of minimum background absorbance (238 nm). Under these conditions chlorhexidine was eluted very shortly after the solvent front with a capacity factor of 0.2. Changing the mobile phase to methanol–water (65:35) eluted chlorhexidine as a single sharp symmetrical peak (k' = 1.0; Fig. 2B). Using this solvent system a "ghost" peak associated, possibly, with the use of toluene-4-sulphonic acid was observed. It is suggested that this peak is caused by the different solubilities of the sulphonic acid between methanol and water. Injection of a mixture of methanol–water other than in the ratio of the mobile phase causes a discontinuity in the background absorbance due to the change in the concentration of the acid in the eluent (caused by re-quilibration). This "ghost" peak interferes with the chlorhexidine peak when a methanol–water (60:40) mobile phase ratio is employed but is of no significance provided a mixture such as methanol–water (65:35) is maintained. Alternatively use of the mobile phase for the preparation of samples (rather than water) eliminates the discontinuity. Column efficiency using these conditions is com-

parable with that achieved with heptanesulphonic acid. Efficiency decreases with increasing flow-rate. The compromise selected was 1.5 ml min^{-1} to give reasonable chromatographic analysis times.

Thus, although toluene-4-sulphonic acid functioned successfully as an ion-pair agent for chlorhexidine its use provided a high background absorbance.

From these experiments it was apparent that a suitable ion-pair agent should be difunctional, that is it should possess a non-polar part with which to interact with the stationary phase and a polar ionic part with which to interact with the solute. Secondary considerations were solubility in the mobile phase, a low UV absorbance at the detection wavelength, low cost and easy availability. These criteria are satisfied by perfluorocarboxylic acids of which pentadecafluorooctanoic acid is probably the most common.

Used as an ion-pair agent at a concentration of 1000 $\mu g\ ml^{-1}$ pentadecafluorooctanoic acid permitted the elution of chlorhexidine as a sharp symmetrical peak ($k' = 1.2$).

The ion-pair agents studied divide into two groups. The successful ones were heptane sulphonic acid, toluene-4-sulphonic acid and pentadecafluorooctanoic acid. The less successful ones were sodium acetate, sodium cyclamate and sodium lauryl sulphate even when used at pH 4 or lower. It would thus appear that the counter ion has either a beneficial (H^+) or deleterious (Na^+) effect. A possible explanation is that the mobility of solvated sodium ions is significantly less than that of solvated hydrogen ions. The presence of sodium ions thus obstructs the association–dissociation mechanism of the chlorhexidine molecules with the ionic sites of the ion pair agent.

Quantitation

An internal standard is desirable in the trace analysis of biological samples because it compensates for possible errors in the extraction step. Two possible internal standards were considered of which one, an undefined white powder, available on special request from the manufacturer of chlorhexidine, was rejected because of commercial non-availability in spite of being chromatographically acceptable. The other, benzyl-hibitane, although chromatographically suitable *i.e.* being well resolved from chlorhexidine was rejected because it was only poorly recovered from biological fluids (see below).

Quantitation was thus achieved via use of a calibration curve. A stock solution of chlorhexidine diacetate (1000 $\mu g\ ml^{-1}$) and a set of standards (1–10 $\mu g\ ml^{-1}$) produced daily by serial dilution was used. [Studies of the adsorption of chlorhexidine onto glass suggests that it is of little significance[13] hence daily serial dilution is a precautionary measure only]. For this concentration range and injection of a 50 μl sample response was linear (regression equation $y = 13.9x - 1.27$; correlation coefficient = 0.9997). Linear response extended to at least 20 $\mu g\ ml^{-1}$. Below 1 $\mu g\ ml^{-1}$ results became increasingly irreproducible. At high detector sensitivity (0.01 a.u.f.s.; 10 mV f.s.d.) a 1-$\mu g\ ml^{-1}$ sample gave almost full scale response on the recorder. However the relative standard deviation (10 injections) was only 5%. We therefore tentatively suggest a detection limit of 0.2 $\mu g\ ml^{-1}$ but a sensitivity for the method of only 1 $\mu g\ ml^{-1}$.

Recovery from spiked samples

Blank samples of urine, horse blood and horse serum were spiked with chlorhexidine to give a concentration of 2 μg ml^{-1}. Samples were made alkaline with 1 *N* NaOH and extracted with either dichloromethane, ether containing 3% amyl acetate or ether. Initially benzyl-hibitane was added to all samples as internal standard but it was soon apparent that it was not recovered by any of these three solvents from blood, urine or serum. Hence quantitation via calibration curve was used. A comparison between ether and ether containing 3% amyl acetate for the extraction of chlorhexidine from urine showed that interferents were co-extracted if amyl acetate was used. A similar result was obtained for the recovery from blood. Thus ether was the selected solvent for extraction. Its volatility reinforces its selection permitting easy removal prior to dissolution of the sample in the mobile phase. A typical chromatogram (Fig. 3) of an extract from blood spiked with chlorhexidine indicates that no serious interferences are present. A similar statement is applicable to extracts obtained from spiked urine (Fig. 4).

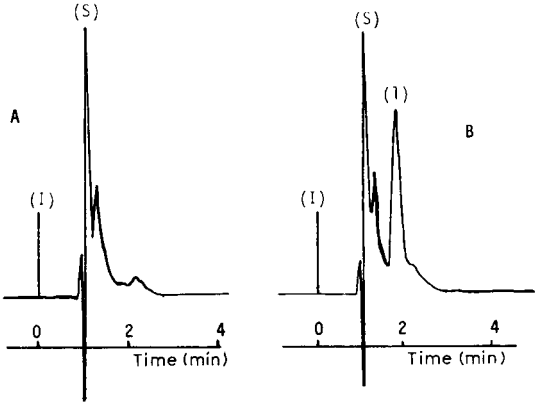

Fig. 3. (A) Chromatogram of blank blood extracted with ether. (B) Chromatogram of spiked blood [chlorhexidine concentration = 2 μg ml^{-1}], (I = injection point; S = solvent front; I = chlorhexidine; flow-rate 2.5 ml min^{-1}; detector 238 nm; sensitivity 0.08 a.u.f.s.; methanol–water (65:35), 1000 μg ml^{-1} toluene-4-sulphonic acid as ion-pair reagent.

Recovery efficiency from urine was assessed from a standard pool sample spiked to give a concentration of 2 μg ml^{-1}. Ten 1-ml aliquots were removed and extracted. Two methods of agitation were assessed. Use of an ultrasonic bath was found to be less effective than mechanical agitation. The latter procedure is thus recommended. Recovery of chlorhexidine from urine was determined to be 89% with a coefficient of variation for the ten samples of 1.9%. A similar experiment using blood gave a recovery efficiency of 68% (10 aliquots, coefficient of variation 6.3%) at the 2-μg ml^{-1} level. This recovery reduced to 48% (coefficient of variation 7.8%) if ultrasonic agitation was used.

Chlorhexidine in urine

The developed assay has been successfully used in a preliminary study of chlorhexidine in urine. Specimen samples of urine were collected from patients (8) immediately prior to cystoscopy. An instillation of chlorhexidine (200 μg ml^{-1}) was

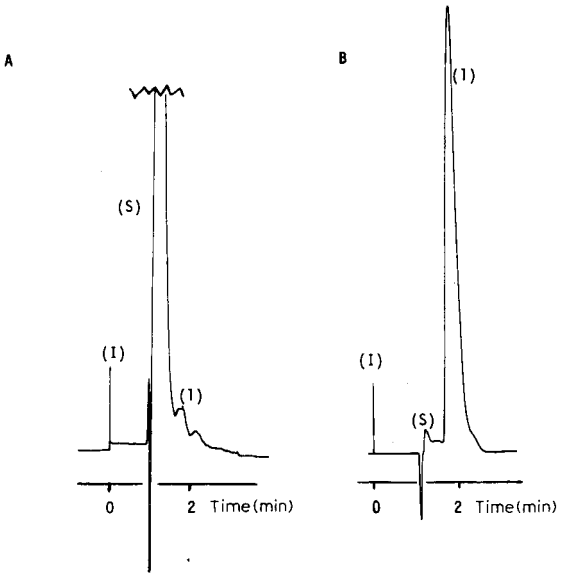

Fig. 4. (A) Chromatogram of blank urine extracted with ether. (B) Chromatogram of spiked urine extracted with ether (conditions as for Fig. 3).

made and the examination carried out. Urine samples were then collected about 5 min after completion of the examination. No trace of chlorhexidine was found in any patient prior to instillation of the chlorhexidine solution. All patients showed relatively high levels of chlorhexidine after treatment with values in the range 112–170 μg ml^{-1} with a mean value of 153 μg ml^{-1}. After 25 min the level of chlorhexidine in one patient had reduced from 170 to 159 μg ml^{-1} probably due to dilution by accumu-

TABLE I

THE CHLORHEXIDINE CONTENT OF SOME COMMON PHARMACEUTICAL PREPARATIONS

Pharmaceutical preparation	*Nominal concentration ≡ 100% (w/v)*	*Specific function*	*Chlorhexidine relative to standard (= 100%) (%)*
"Hibitane" (gluconate concentrate)	20	Stock solution for pharmacy use	101
"Hibiscrub" (gluconate in detergent)	4	Skin disinfection	92
Chlorhexidine gluconate in 70% industrial methylated spirit	0.5	Skin disinfection	102
"Hibiscrub" (gluconate in water)	0.2	Skin cleansing	98
"Corsodyl" (gluconate in water)	0.2	Mouthwash	96
Chlorhexidine acetate	0.02	Bladder irrigation	109

lated urine. No interference from other co-extracted compounds was observed for any of the samples.

Chlorhexidine in pharmaceutical preparations

To facilitate accurate comparison between the nominal value of a pharmaceutical preparation containing chlorhexidine and the determined value all preparations investigated were diluted to a nominal 10 μg ml^{-1}. Triplicate injections were made, the average response measured, corrected for the conjugate base if necessary and compared with a freshly prepared 10 μg ml^{-1} solution of chlorhexidine diacetate. The results are presented in Table I. Hospital preparations diluted in the pharmacy are normally acceptable if the composition is within 10% of the nominal value. In a related study[13] of the shelf life of chlorhexidine we observed no obvious deterioration of concentration for solutions in the range 50–2500 μg ml^{-1} when stored either in acid-washed or deactivated (silanised) glass over a period of 49 days.

CONCLUSIONS

The method described provides a sensitive and quantitative assay for chlorhexidine in blood, urine and pharmaceutical preparations. The extraction step is short and simple. Recoveries from urine (89%) and blood (68%) are acceptable and reproducible. Heptane sulphonic acid, toluene-4-sulphonic acid and pentadecafluorooctanoic acid may be used as ion-pair agents. The method is sufficiently sensitive to permit the detection of chlorhexidine in body fluids and studies on clinical specimens obtained following urological applications of chlorhexidine are in progress.

Concern has been expressed about the possible toxic consequences of chlorhexidine degradation to 4-chloroaniline. Although 4-chloroaniline may not be determined simultaneously with chlorhexidine by this method, the use of HPLC prevents interference between the two species. A significant advance over the GLC method of determining chlorhexidine is thus achieved.

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Note

Preparation of capillary columns coated with phenylsilicone gum

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Phenylsilicones have been used extensively as stationary phases in packed column gas chromatography. As such phases have a wide field of application, there is considerable interest in the development of capillary columns with the same type of stationary phase. At present, the performance of phenylsilicone columns is by far surpassed by that of equivalent non-polar columns. Therefore, the use of phenyl columns instead of non-polar columns will mostly lead only to marginal improvements in separation. The advantages of phenylsilicones will be evident, however,when the efficiency and thermostability of such columns are of the same magnitude as those of a corresponding non-polar column. To obtain this, a glass surface is required that is readily wetted by phenylsilicones, and a phenylsilicone stationary phase that forms a stable film. Further, the support must be well deactivated. One possibility of achieving wettability and deactivation is to silanize the support surface with reagents having phenyl groups. Grob and Grob presented a method for silinization with some phenyldisilazanes[1], but this modification method facilitates wetting only with silicones that have a low phenyl content. Further, Schomburg *et al.*[2] attempted to obtain capillary deactivation and wettability by treatment with OV-17 at high temperatures.

High-temperature silanization with cyclic siloxanes is a powerful method for the modification of glass surfaces. Silanization with octamethylcyclotetrasiloxane was described by Stark *et al.*[3], and this method gives excellently deactivated non-polar columns. We have developed methods for silanization with cyclic siloxanes having special side-groups, 3,3,3-trifluoropropyl[4,5] and cyanopropyl[5]. By applying these methods, we obtained deactivation and also wettability for silicone stationary phases having corresponding functional groups.

Modification of glass capillaries by silanization with hexaphenylcyclotrisiloxane is described in this paper. Capillaries treated in this manner are well deactivated and they are readily wetted by phenylsilicone stationary phases.

A stationary phase film of silicone gum is generally much more stable than a film of silicone fluid[6]. Phenylsilicone gums were not commercially available, however, when this work was started: we therefore synthezised such a gum to be used as a stationary phase.

Stable columns are also obtained when the stationary phase is bonded to the glass surface and/or cross-linked[7,8]. The highest column efficiencies could not be attained, however, when using these methods. More promising results in this respect

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TABLE I

PROPERTIES OF TYPICAL 20-m AR-GLASS CAPILLARY COLUMNS, DEACTIVATED WITH HEXAPHENYLCYCLOTRISILOXANE AND COATED WITH METHYLPHENYLSILICONES

Efficiency measured for naphthalene at 100°C.

Stationary phase	*Column diameter (mm)*	*Film thickness (μm)*	*Capacity ratio, k (naphthalene)*	*HETP (mm)*	*UTE* (%)*	*Kováts retention index*	
						Octanol	*Naphthalene*
Phenyl gum	0.22	0.28	15.5	0.24	84	1181	1390
OV-25	0.23	0.29	12.3	0.33	66	1197	1468
OV-17	0.24	0.30	13.7	0.46	47	1155	1373
OV-1701	0.23	0.29	9.9	0.21	98	1180	1288

* UTE = Utilization of the theoretical best efficiency.

were achieved by peroxide-initiated stationary phase vulcanization[9,10]. This method, however, cannot be directly applied to phenylsilicones, as such vulcanization is sterically hindered by the phenyl groups[9].

EXPERIMENTAL

An OV-17 type siloxane was synthezised from methylphenyldichlorosilane[11] (Silar Labs., Scotia, NY, U.S.A.). This siloxane was further polymerized to a gum using sodium trimethylsilanolate (Ventron, Beverly, MA, U.S.A.) as a catalyst according to Johannson[12]. In order to remove the catalyst after reaction, the gum was carefully rinsed with dilute hydrochloric acid (pH = 1) and then with distilled water to neutrality. Methylene chloride was added and, after separation, the gum was dried over calcium sulphate. Finally, capping with trimethylchlorosilane (Merck, Darmstadt, G.F.R.) was performed under refluxing toluene. The product was a thick, clear gum.

AR-glass capillaries were leached according to Grob *et al.*[13], then the capillaries were coated dynamically with a saturated solution of hexaphenylcyclotrisiloxane (Petrarch Systems, Levittown, PA, U.S.A.) in ethyl acetate, at a speed of 4 cm/sec. After solvent evaporation, the capillaries were filled with dry ammonia gas (Matheson Gas Products, East Rutherford, NJ, U.S.A.), sealed with caution in a microflame and heated at a rate of 5°C/min to 400°C, then isothermal at 400°C for 15 h and finally slowly cooled. Excess of reagent was rinsed out with 5 ml of methylene chloride.

The silanized capillaries were statically coated with a 0.5% (w/v) solution of the methylphenylsilicone gum, OV-25, OV-17 or OV-1701 in methylene chloride.

RESULTS

Silanization with diphenylsilanes leads mostly to low coverages (*e.g.*, see ref. 14). Reaction at high temperatures in the presence of a catalyst (ammonia), however gave a surface modification suitable for our purposes.

Three types of methylphenylsilicone stationary phases were applied to phenyl-modified capillaries (Table I). Capillaries coated with the methylphenylsilicone gum, prepared in this Laboratory, show high efficiencies. The gum has 50% phenyl substitution and it was of interest to test a phase having 75% phenyl substitution. For this

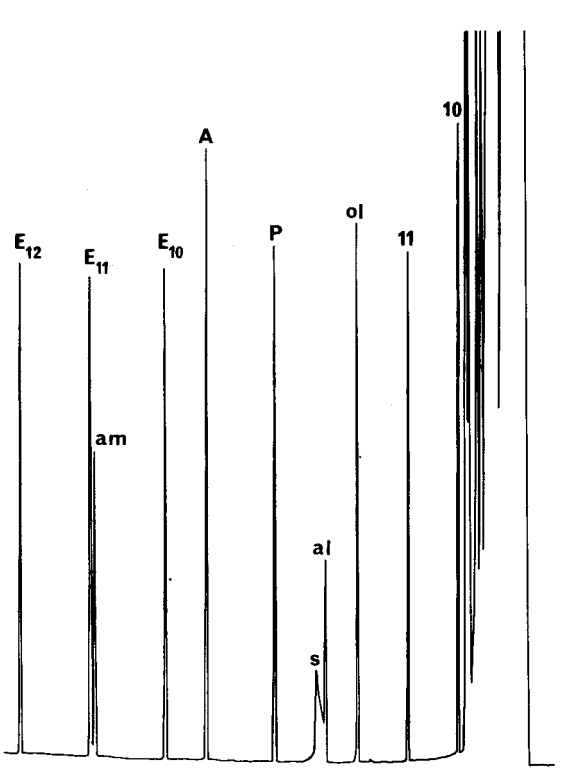

Fig. 1. Gas chromatogram (flame-ionization detector) of a Grob test mixture on a 20-m AR-glass capillary column, modified with hexaphenylcyclotrisiloxane and coated with methylphenylsilicone gum. Initial temperature 60°C, programmed to 150°C at 5°C/min. Peaks: 10 = decane; 11 = undecane; ol = octanol; al = nonanal; s = 2-ethylhexanoic acid; P = 2,6-dimethylphenol; A = 2,6-dimethylaniline; am = dicyclohexylamine; E_{10}, E_{11}, E_{12} = C_{10}, C_{11}, C_{12}-acid methyl ester, respectively. Each peak corresponds to *ca.* 1 ng of substance.

purpose OV-25, which is a silicone oil but still relatively viscous, was chosen[15]. As might be expected, an increased phenyl content led to decreased column efficiencies. Finally, the methylphenylsilicone oil OV-17 (50% phenyl) was tested and was found to give poor results.

All three phases gave columns of low adsorptive activity. A typical test chromatogram is shown in Fig. 1.

The gum showed the best temperature stability of the three phases tested. Columns coated with this phase can be used isothermally at 320°C without difficulty. OV-25 can be used up to 300°C but bleeding is much higher than with the gum phase. OV-17 could only be used up to 240°C.

An example of the possibilities of efficient, deactivated and temperature-stable phenylsilicone columns is demonstrated in Fig. 2, where the separation of a mixture of underivatized tricyclic antidepressants is shown.

A viscous substitute for OV-17, OV-1701, will soon be commercially available (Alltech Europe, Eke, Belgium), and a sample was obtained and tested. The phase was slightly opaque. Preliminary results indicated that highly efficient columns can be prepared and that the polarity is lower than that for our home-made phenylsilicone gum (Table I). The bleeding properties seems to be in the same range as for OV-25.

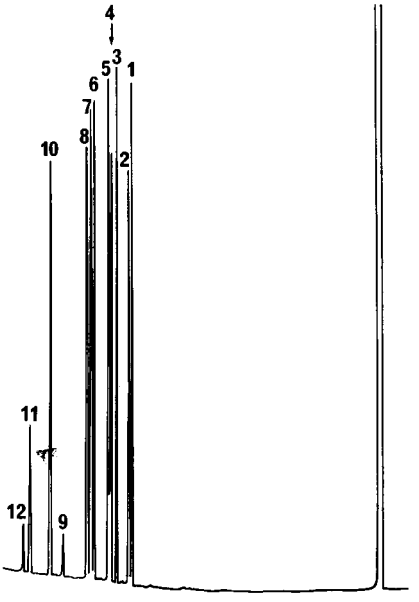

Fig. 2. Gas chromatogram (flame-ionization detector) of a test mixture of underivatized tricyclic antidepressants. Column as in Fig. 1. Initial temperature 185°C, programmed at 5°C/min. Peaks: 1 = amitriptyline; 2 = trimipramine; 3 = imipramine; 4 = nortriptyline; 5 = desmethylnortriptyline; 6 = desipramine; 7 = protriptyline; 8 = desmethyldoxepine; 9 = desmethylmaproteline; 10 = chlomipramine; 11 = maprotiline; 12 = desmethylchlomipramine. Each peak corresponds to *ca.* 1 ng of substance.

ACKNOWLEDGEMENTS

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Note

Gas chromatographic analyses of diastereomeric lactamides

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The enantiomers of lactic acid derivatives have been separated[1–3] by gas chromatography (GC) using chiral stationary phases. The diastereomers of lactic acid esters have also been separated by GC using achiral stationary phases (SE-30, SP-1000), by protecting the hydroxyl groups using trimethylsilyl (TMS)[4], trifluoroacetyl (TFA)[4] or acetyl groups[5]. However, no examples were reported of the separation of diastereomeric lactic acid derivatives (lactamides or lactic acid esters) whose hydroxyl groups are free by using a packed column with an achiral stationary phase.

In this paper, the GC separation of diastereomeric lactamides by the use of a packed column filled with a silica support coated with an achiral stationary phase (SE-52) is reported. The diastereomeric lactamides in which the hydroxyl groups were not protected have not been separated by the use of a packed column with SE-52 as a stationary phase. However, it was found that the diastereomeric lactamides were clearly separated when the samples were injected immediately after injection of a pyridine solution of N-trimethylsilylimidazole (TMS-Im).

EXPERIMENTAL

Gas chromatography

A Hitachi 163 gas chromatograph equipped with a flame-ionization detector was used. A stainless-steel column (4 m × 3 mm I.D.) was filled with Chromosorb W AW DMCS, which was coated with SE-52 silicone gum (5%, 80–100 mesh). The temperature of the injection port was 300°C. The oven temperature was increased at the rate of 2°C/min from 100 to 250°C. The flow-rate of the carrier gas (nitrogen) was 39 ml/min. A chloroform solution of the sample was applied 10 sec after the injection of 3 μl of a pyridine solution of TMS-Im (TMS-Im–pyridine = 2:1).

Materials

N-[(S)-Lactoyl]-(S)-α-methylbenzylamine (Ia). To a cooled solution of (*S*)-lactic acid (0.011 mol), (*S*)-α-methylbenzylamine (0.011 mol) and N-hydroxysuccinimide (0.013 mol) in ethyl acetate (20 ml), dicyclohexylcarbodiimide (0.012 mol) was added. After the usual work-up, an oily product was obtained. This crude oil was purified by the use of preparative silica gel thin-layer chromatography [developing solvent: chloroform–ethanol (19:1)]. Yield: 34% (oil). $[\alpha]_D^{25} = -116°$ ($c = 1.4$, chloro-

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form). Analysis: Calculated for $C_{11}N_{15}NO_2$, C68.36, H7.82, N7.24%; found, C67.47, H7.82, N7.53%.

The following five lactamides were prepared in a similar manner.

N-[(S)-Lactoyl]-(R)-α-methylbenzylamine (Ib). Yield: 18% (oil). $[\alpha]_D^{25} = +82°$ ($c = 1.4$, chloroform). Analysis: calculated for $C_{11}H_{15}NO_2$, C 68.36, H 7.82, N 7.24%; found, C 67.64, H 7.88, N 7.30%.

N-[(S)-Lactoyl]-(S)-α-ethylbenzylamine (IIa). Yield: 27% (oil). $[\alpha]_D^{16} = -121°$ ($c = 1.4$, chloroform). Analysis: calculated for $C_{12}H_{17}NO_2$, C 69.53, H 8.26, N 6.75%; found, C 68.81, H 8.24, N 6.90%.

N-[(S)-Lactoyl]-(R)-α-ethylbenzylamine (IIb). Yield: 43% (oil). $[\alpha]_D^{24} = +109°$ ($c = 1.4$, chloroform). Analysis: calculated for $C_{12}H_{17}NO_2$: C 69.53, H 8.26, N 6.75%; found, C 68.82, H 8.33, N 6.76%.

N-[(S)-Lactoyl]-(S)-α-naphthylethylamine (IIIa). Yield: 10%. M.p.: 104–105°C. $[\alpha]_D^{24} = -76°$ ($c = 1.4$, chloroform). Analysis: calculated for $C_{15}H_{15}NO_2$, C 74.04, H 7.04, N 5.75%; found, C 73.52, H 7.00, N 5.68%.

N-[(S)-Lactoyl]-(S)-alanine isobutyl ester (IVa). To a cooled mixture of (*S*)- $+32°$ ($c = 1.1$, chloroform). Analysis: calculated for $C_{15}H_{15}NO_2$, C 74.04, H 7.04, N 5.75%; found, C 73.13, H 7.02, N 5.63%.

N-[(S)-Lactoyl]-(S)-alanine isobutyl ester (IVa). To a cooled mixture of (*S*)-lactic acid (0.011 mol), (*S*)-alanine isobutyl ester (0.011 mol) and N-hydroxysuccinimide (0.013 mol) in ethyl acetate (20 ml), dicyclohexylcarbodiimide (0.012 mol) was added. After the usual work-up, an oily product was obtained. This crude oil was purified by silica gel column chromatography [developing solvent: ethyl acetate–benzene (1:1)]. Yield: 19% (oil). $[\alpha]_D^{26} = -21°$ ($c = 1.4$, chloroform). Analysis: calculated for $C_{10}H_{19}NO_4$, C 55.28, H 8.81, N 6.44%; found, C 55.18, H 8.82, N 6.71%.

The following five lactamides were prepared in a similar method.

N-[(S)-Lactoyl]-(R)-alanine isobutyl ester (IVb). Yield: 13%. M.p.: 63–64°C. $[\alpha]_D^{26} = -0.75°$ ($c = 1.4$, chloroform). Analysis: calculated for $C_{10}H_{19}NO_4$: C 55.28, H 8.81, N 6.44%; found, C 55.72, H 8.80, N 6.36%.

N-[(S)-Lactoyl]-(S)-valine isobutyl ester (Va). Yield: 64% (oil). $[\alpha]_D^{26} = -1.8°$ ($c = 1.4$, chloroform). Analysis: calculated for $C_{12}H_{23}NO_4$, C 58.75, H 9.45, N 5.70%; found, C 58.50, H 9.40, N 5.77%.

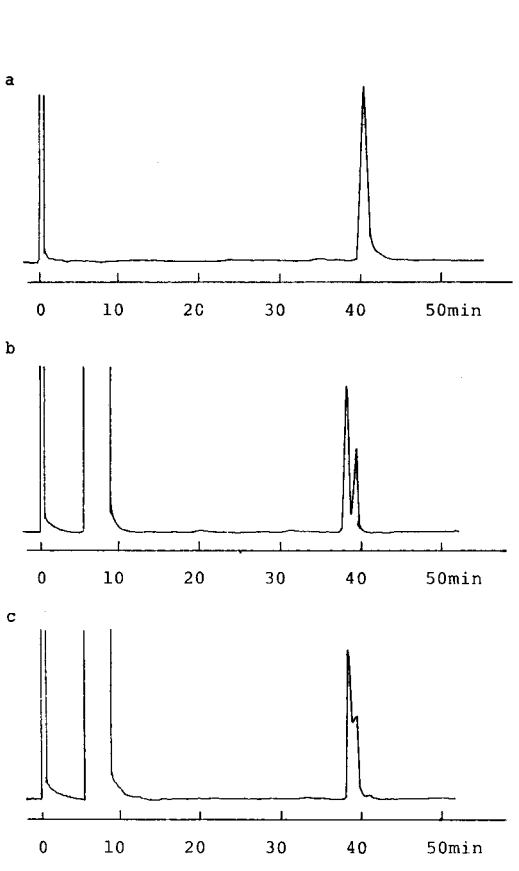
N-[(S)-Lactoyl]-(R)-valine isobutyl ester (Vb). Yield: 49% (oil). $[\alpha]_D^{26} = -10°$ ($c = 1.6$, chloroform). Analysis: calculated for $C_{12}H_{23}NO_4$, C 58.75, H 9.45, N 5.70%; found, C 58.50, H 9.46, N 5.87%.

N-[(S)-Lactoyl]-(S)-leucine isobutyl ester (VIa). Yield: 61% (oil). $[\alpha]_D^{26} = -14°$ ($c = 1.4$, chloroform). Analysis: calculated for $C_{13}H_{25}NO_4$, C 60.20, H 9.70, N 5.40; found, C 59.82, H 9.51, N 5.69%.

N-[(S)-Lactoyl]-(R)-leucine isobutyl ester (VIb). Yield: 65% (oil). $[\alpha]_D^{26} = +4.5°$ ($c = 1.4$, chloroform). Analysis: calculated for $C_{13}H_{25}NO_4$, C 60.20, H 9.70, N 5.40%; found, C 60.04, H 9.69, N 5.56%.

RESULTS AND DISCUSSION

The usual GC analysis of all diastereomeric pairs of the above prepared lactamides by using an SE-52 packed column gave a single peak on the chromatogram (Fig. 1a). However, when the application of the samples was performed immediately



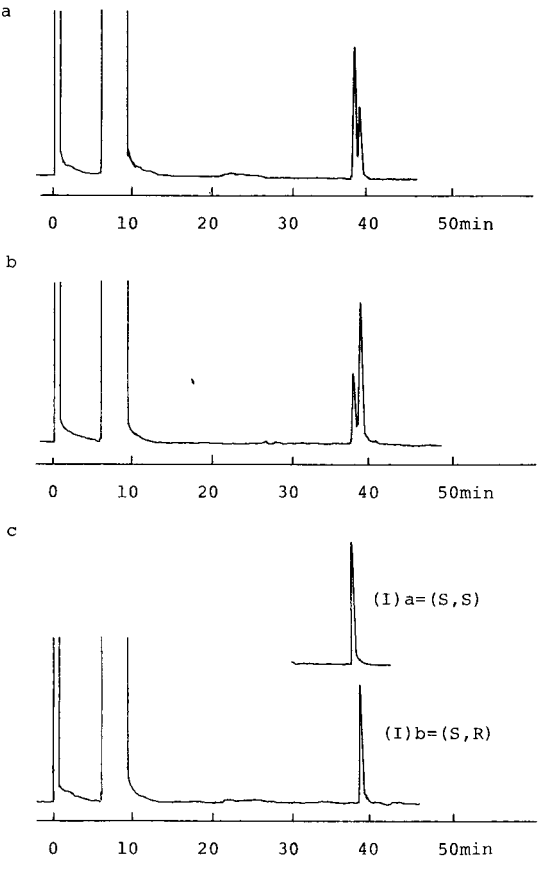


Fig. 1. Chromatograms of a mixture of diastereomeric lactamides Ia and Ib. (a) Without TMS-Im; (b) sample applied 10 sec after injection of TMS-Im; (c) sample applied 30 sec after injection of TMS-Im.

Fig. 2. Chromatograms of mixtures of diastereomeric lactamides Ia and Ib, and authentic diastereomers Ia and Ib. (a) Ia and Ib [a-rich, a = (*S*,*S*)]; (b) Ia and Ib [b-rich, b = (*S*,*R*)]; (c) authentic diastereomers.

after the injection of the pyridine solution of TMS-Im, the two diastereomers of each lactamide were well separated on the chromatogram and the tailing of the peaks was minimized. However, when the sample was injected more than 30 sec after injection of the TMS-Im solution, the two diastereomers were not separated (Fig. 1c). In this study all samples were injected 10 sec after the injection of TMS-Im solution (Fig. 1b).

In the GC analyses with TMS-Im, the identification of the peaks was carried out by using an authentic diastereomeric mixture in which the amount of one diastereomer was greater than the other. Fig. 2a shows a chromatogram of an (*S*,*S*)-lactamide-rich mixture and Fig. 2b shows a chromatogram of an (*S*,*R*)-lactamide-rich mixture. The chromatograms of the two authentic diastereomers in the analyses with TMS-Im are shown in Fig. 2c. Each of the two diastereomers emerged as a single peak. The retention times of the chromatographic peaks and the analytical conditions are given in Table I. It was found that the first peak was due to (*S*,*S*)-lactamide and

TABLE I

SEPARATION OF DIASTEREOMERIC LACTAMIDES BY GC WITH TMS-Im

Conditions: stainless-steel column (4 m × 3 mm I.D.) with 80–100 mesh Chromosorb W AW DMCS coated with 5% SE-52; carrier gas, nitrogen at a flow-rate of 39 ml/min; oven temperature, programmed from 100 to 250°C at 2°C/min.

Lactamide	*Retention time (min)*			*Separation factor**
	Without TMS-Im	*With TMS-Im*		
		First peak	*Second peak*	
Ia, Ib	40.5	39.0(*S,S*)**	39.9(*S,R*)***	1.023
IIa, IIb	43.6	42.0(*S,S*)	42.9(*S,R*)	1.021
IIIa, IIIb	68.0	64.4(*S,S*)	66.0(*S,R*)	1.024
IVa, IVb	38.9	37.5(*S,S*)	38.3(*S,R*)	1.021
Va, Vb	46.0	43.7(*S,S*)	45.2(*S,R*)	1.034
VIa, VIb	48.4	46.7(*S,S*)	47.5(*S,R*)	1.017

* Separation factor $= \frac{\text{retention time of second peak}}{\text{retention time of first peak}}$.

** a = (*S,S*): N-[(*S*)-lactoyl]-(*S*)-amine or -amino acid ester.

*** b = (*S,R*): N-[(*S*)-lactoyl]-(*R*)-amine or -amino acid ester.

the second peak to (*S,R*)-lactamide for all of the lactamides studied. The retention time of the peak analysed with TMS-Im was slightly higher than that of the peak without TMS-Im for all of the lactamides.

It seems that TMS-Im makes the stationary phase temporarily inert, and the adsorption of the hydroxyl and amide groups on the stationary phase weak. Thus it could be considered that the TMS-Im works as a temporary column conditioner, and the individual lactamides were separated clearly without tailing on the chromatogram. This method is simple and should be applicable to many other diastereomeric samples that are difficult to separate by the usual GC techniques.

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Note

Direct resolution of enantiomers by liquid affinity chromatography on albumin-agarose under isocratic conditions

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The ability of proteins, as highly chiral species, to exhibit enantioselectivity in their binding of certain ligands is well documented in the case of enzyme–substrate interactions[1], but much less information in this area is available for other proteins. It has been shown, however, by isotopic labelling experiments, that plasma albumin has a higher affinity for L-tryptophan of the D,L-pair[2] and that a corticosteroid-binding globulin selects (+)-aldosterone for similar reasons[3]. In 1973 it was demonstrated for the first time that D,L-tryptophan could be resolved by chromatography on a bovine serum albumin (BSA)-agarose column due to this difference in antipodal affinity[4]. As judged from two recently published reviews[5,6] on direct chromatographic resolution, this result seems to have been overlooked and never developed further, despite the potential value of this new technique.

In this paper, we demonstrate its use under optimized isocratic conditions with fluorimetric and continuous UV or electrochemical detection for the direct resolution of D,L-tryptophan, D,L-5-hydroxytryptophan, D,L-kynurenine and D,L-3-hydroxykynurenine.

EXPERIMENTAL

Racemic and optically active compounds investigated

D,L-, L- and D-tryptophan (TRP), D,L-, L- and D-5-hydroxytryptophan (5-HTP), D,L- and L-kynurenine (KYN), D,L-3-hydroxykynurenine (3-HKN), D,L-, L- and D-3,4-dihydroxyphenylalanine (DOPA) and D,L- and L-norepinephrine (NE) were obtained from Sigma (St. Louis, MO, U.S.A.).

Synthesis of BSA-agarose

Method 1. Activated CH-Sepharose-4B (Pharmacia, Uppsala, Sweden) (5 g of dry material) was swollen in 1 m*M* hydrochloric acid and washed on a glass filter with 1 m*M* hydrochloric acid followed by 0.1 *M* sodium hydrogen carbonate + 0.5 *M* sodium chloride (pH 8.0) used as the buffer for coupling. This amount of gel has a maximum binding capacity of 90 μmol for low-molecular-weight compounds.

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Defatted BSA (Sigma) (670 mg, 10 μmol) was dissolved in 30 ml of the coupling buffer, mixed immediately with the washed gel, and the suspension was ultrasonicated for 10 min and then placed in a mechanical shaker for 2 h at 25°C. The gel was filtered off, washed with coupling buffer and finally treated with 1 *M* ethanolamine at pH 8.5 for 30 min. Then the gel was washed thoroughly with 1 m*M* hydrochloric acid followed by 0.1 *M* borate buffer (pH 8.8).

Method 2. Epoxy-activated Sepharose 6B (Pharmacia) (7.5 g of dry material) was swollen in water and washed on a glass filter. BSA (1.5 g, 22 μmol) was dissolved in 50 ml of 0.1 *M* sodium hydrogen carbonate–sodium carbonate buffer/0.2 *M* sodium chloride (pH 9.75), and mixed immediately with the washed gel. After ultrasonication the suspension was placed in a shaker for 24 h at 25°C. The gel was then treated as described under method 1.

Chromatography

A 250 × 9 mm column (Pharmacia) was packed with the gel and equilibration of the system was carried out with the borate buffer of pH 8.8 for 2 h. No leakage of BSA could be detected. The bed height after equilibration was 185 mm unless stated otherwise. The column was connected to a peristaltic pump and the flow-rate determined. Flow-rates up to 50 ml/h could be used without unfavourable compression of the gel. Application of the sample was performed by means of a micropipette (for volumes >10 μl) and, more easily, by direct injection into the column bed with a Hamilton syringe (for volumes <10 μl).

In a second series of experiments a 500 × 3.0 mm glass column (Altex, Berkeley, CA, U.S.A.) was used. This was coupled to an HPLC pump (Altex Model 110A) and injections of the sample were made directly on to the column.

Detection of the species eluted from the column was carried out either by fluorescence measurements on collected fractions (used during the initial studies on D,L-TRP) or by continuous monitoring by means of a UV or an amperometric detector. In all instances aqueous buffers were used as the mobile phase.

Instrumentation

Fluorescence was measured with a Perkin-Elmer Model 204 spectrofluorimeter. The UV detector used was a variable-wavelength LDC Spectromonitor III. The amperometric detector connected to the column outlet consisted of a thin-layer flow cell equipped with a carbon paste anode and connected to a reference electrode compartment containing a silver–silver chloride electrode. The cell was provided with a 50-μm spacer and an anode potential of +0.75 V was used. The potentiostat/amplifier (Model LC-2A) and the other detector equipment were obtained from Bioanalytical Systems (West Lafayette, IN, U.S.A.). A Linear Model 264 potentiometric recorder was used to register the output signal as a function of time.

RESULTS AND DISCUSSION

Resolution of the enantiomers is highly dependent on the pH of the mobile phase. Thus, under the conditions used by Stewart and Doherty[4], L-TRP is strongly retained at pH 9.2 and 0.1 *M* acetic acid was used for its displacement. We were able to confirm their results completely and we also found that on decreasing the pH to 6.5

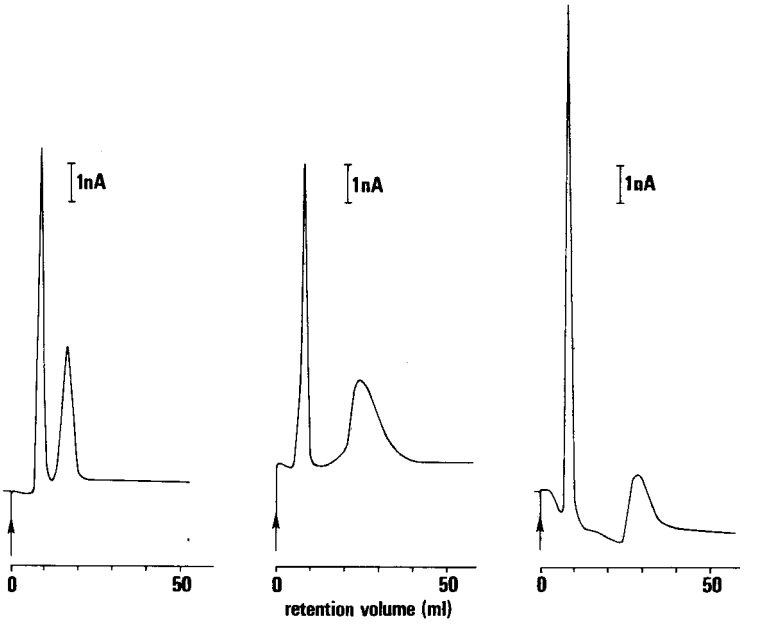

Fig. 1. Chromatograms showing the effect of the pH of the mobile phase. A 5-μl volume of a 4.46 mM solution of D,L-3-hydroxykynurenine was injected. Column, 140 × 9 mm; flow-rate, 44.5 ml/h; electrochemical detection at +0.75 V. Mobile phases (from left to right): 0.20 M phosphate buffer (pH 7.05); 0.05 M borate buffer (pH 8.00); 0.20 M Tris–HCl buffer (pH 9.00).

there was still a sufficient difference in retention between the antipodes to give a complete baseline separation under the isocratic conditions of elution used. Under the latter conditions, however, D,L-5-HTP was not resolved, but on increasing the pH to 7.9 complete resolution was achieved. Some results from a study of the effect of buffer pH on the chromatographic resolution of D,L-3-HKN are shown in Fig. 1. The

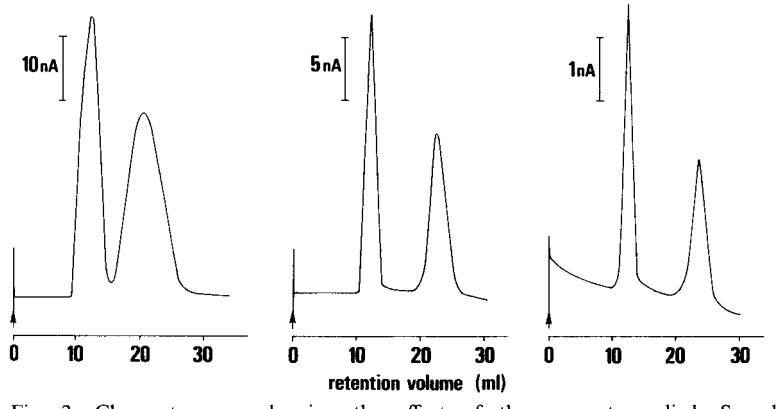

Fig. 2. Chromatograms showing the effect of the amount applied. Sample: 3.7 mM D,L-5-hydroxytryptophan. Volumes applied to the column (from left to right): 50, 5.0 and 0.50 μl. Column, 185 × 9 mm; flow-rate, 15 ml/h; electrochemical detection at +0.75 V. Mobile phase: 0.05 M borate buffer (pH 7.95).

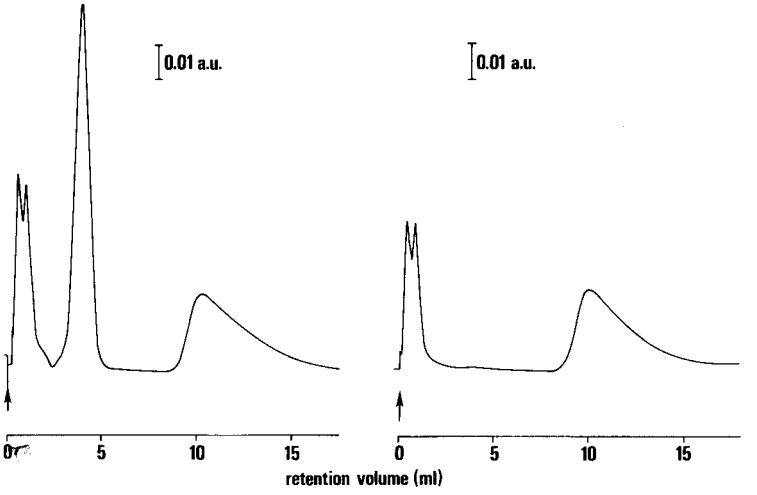

Fig. 3. Enantiomer identification. Chromatograms of D,L-kynurenine (30.6 nmol; left) and L-kynurenine (15.3 nmol; right) obtained under identical conditions. Volume injected: 4 μl. Column, 500 × 3.0 mm; flow-rate, 6.0 ml/h; UV detection at 264 nm. Mobile phase: 0.05 *M* borate buffer (pH 7.95).

progressive increase in k' for the L-enantiomer with increasing pH is readily observed.

Owing to the relatively low capacity of these gels, the amount of sample applied to the column is of great importance, as shown in Fig. 2. Two effects of a decrease in the sample load are obvious: (a) an increase in the column efficiency and (b) an increase in the separation factor, α, due almost exclusively to an increased k' value of the second component (the L-form).

Of the compounds resolved so far, the naturally occurring L-enantiomer was always found to be that most retained on these columns. Identification was performed by chromatography of one of the optically active forms, as shown in Fig. 3 for the case of kynurenine. Preliminary experiments with two catechol derivatives with different types of chirality, *viz.*, D,L-DOPA and D,L-NE, were unsuccessful with respect to resolution under the conditions used. Some of the chromatographic data obtained are summarized in Table I.

TABLE I

SEPARATION FACTORS (α) OBTAINED BY CHROMATOGRAPHY OF VARIOUS D,L-PAIRS ON ALBUMIN-AGAROSE UNDER DIFFERENT CONDITIONS

Compound	*Buffer*	*pH*	α
TRP	Borate, 0.05 *M*	7.95	2.0
5-HTP	Phosphate, 0.20 *M*	7.05	1.3
	Borate, 0.05 *M*	9.25	1.7
3-HOK	Phosphate, 0.20 *M*	7.05	4.5
	Borate, 0.05 *M*	8.00	11.5
	Tris–HCl, 0.20 *M*	9.00	14.4

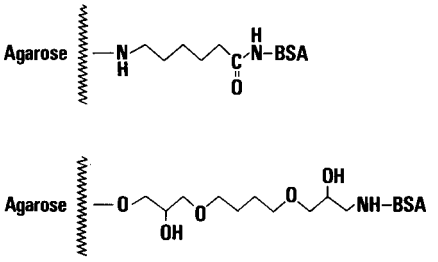

Fig. 4. Assumed structures of BSA-agarose synthesized by method 1 (above) and method 2 (below).

The gels prepared according to method 1 were found to give a better resolution than those prepared by method 2. Fig. 4 shows the expected chemical structures of the gels. However, because the degree of albumin substitution on these gels has not yet been determined, the reason for this effect is still unknown. Further work is in progress.

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Note

Potential sweetening agents of plant origin

I. Purification of *Stevia rebaudiana* sweet constituents by droplet counter-current chromatography*

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Stevia rebaudiana Bertoni (Compositae) is a sweet herb indigenous to elevated terrain on the borders of Brazil and Paraguay[1]. Eight sweet constituents have so far been isolated from this plant, namely, stevioside, steviolbioside, rebaudiosides A–E and dulcoside A[2–7]. In the last decade *S. rebaudiana* extracts and isolates have been used commercially in Japan to sweeten a variety of food products[8]. Currently, there is a great deal of interest in the potential use of stevioside and other *S. rebaudiana* constituents as non-nutritive sweetening agents for other markets around the world[9–13].

Our group recently became involved in a multi-disciplinary investigation of the physico-chemical and biological properties of certain plant-derived sweeteners. Prior to any biological testing, we have studied the applicability of a number of phytochemical isolation methods to purify individual sweet constituents of the plants under investigation. In this communication we wish to present details of a droplet counter-current chromatographic (DCCC) method for the rapid purification of stevioside, the most abundant sweet *ent*-kaurene glycoside from *S. rebaudiana*[2–4]. In addition, the suitability of this technique for the separation of other known *n*-butanol-soluble sweet principles of this plant (Fig. 1) will be discussed. DCCC has been used successfully for the separation of a variety of polar plant principles[14–17].

EXPERIMENTAL

Plant material

Stevia rebaudiana Bertoni (Compositae) was collected in its native habitat at

* This work was presented in part at the *Joint Meeting of the American Society of Pharmacognosy and the Society for Economic Botany, Boston, MA, July 13–17, 1981.*

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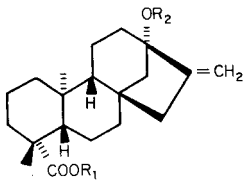

COMPOUND	R_1	R_2
Stevioside	-G	$-G^2-{}^1G$
Steviolbioside	-H	$-G^2-{}^1G$
Rebaudioside A	-G	$-G^2_3(-{}^1G,\ -{}^1G)$
Rebaudioside B	-H	$-G^2_3(-{}^1G,\ -{}^1G)$
Rebaudioside C (Dulcoside B)	-G	$-G^2_3(-{}^1Rh,\ -{}^1G)$
Dulcoside A	-G	$-G^2-{}^1Rh$

G = glucose
Rh = rhamnose

Fig. 1. Structures of *n*-butanol-soluble sweet *ent*-kaurene glycosides from *Stevia rebaudiana*.

Cerro Kuatiá, near P. J. Caballero, Paraguay in April, 1981, by one of us (D.D.S.). Voucher specimens representing this collection have been deposited at the John G. Searle Herbarium of the Field Museum of Natural History, Chicago. An additional quantity of *S. rebaudiana* leaves, cultivated in California, was provided by Stevia Inc. (Arlington Heights, IL, U.S.A.).

Apparatus

DCCC separations were carried out on a Model-A instrument (Tokyo Rikakikai, Tokyo, Japan). Preliminary separations of certain *S. rebaudiana* sweet principles were achieved using preparative high-performance liquid chromatography (HPLC) on a Jobin-Yvon Chromatospac Prep 100 liquid chromatograph (Instruments SA, Metuchen, NJ, U.S.A.). Melting points were determined on a Kofler hot-stage instrument and are uncorrected, and optical rotations were obtained on a Perkin-Elmer 241 polarimeter. Ultraviolet (UV) and infrared (IR) spectra were recorded, respectively, using a Beckman Model DB-G grating spectrophotometer and a Beckman IR-18A spectrophotometer (polystyrene calibration at 1601 cm^{-1}).

Chemicals

Authentic samples of stevioside, rebaudioside A and rebaudioside C were generously provided by Professor O. Tanaka, Hiroshima University, Hiroshima, Japan. A reference sample of dulcoside A was donated by Professor H. Mitsuhashi, Hokkaido University, Sapporo, Japan.

Larger quantities of these *n*-butanol-soluble *S. rebaudiana ent*-kaurene glycosides were generated in the following manner. Dried *S. rebaudiana* leaves were macerated in methanol–water (4:1) and defatted with ethyl acetate. The diterpene glycosides listed in Fig. 1 were then partitioned into *n*-butanol from water, and a portion of the *n*-butanol fraction was subjected to preparative HPLC over silica gel 60 (Merck, Darmstadt, G.F.R.) eluted with mixtures of chloroform–methanol–water[4], using an elution pressure of 6 atm. Elution with chloroform–methanol–water (45:9:1) yielded separate fractions containing dulcoside A and stevioside, while elution with chloroform–methanol–water (45:12:1.5) yielded separate fractions containing rebaudioside A and rebaudioside C. Stevioside and rebaudioside A were isolated in a pure form without further chromatographic frationation by recrystallization from methanol. Additional small-scale gravity column chromatography over silica gel 60 of appropriate HPLC column cuts, using a similar elution profile to that listed above, was necessary to obtain dulcoside A and rebaudioside C in a form free from contamination by diterpene glycoside congeners. Dulcoside A was then obtained pure by recrystallization from methanol, while rebaudioside C was finally purified using the DCCC system described herein.

Isolated stevioside, rebaudioside A, rebaudioside C and dulcoside A exhibited closely comparable data [m.p., $[\alpha]_D$, IR, UV, thin-layer chromatography (TLC)] to authentic samples of these compounds.

Steviolbioside and rebaudioside B were obtained from stevioside and rebaudioside A, respectively, by alkaline hydrolysis according to published conditions[18]. These reaction products, when purified by recrystallization from methanol provided m.p., optical rotation and TLC data consistent with literature values[3,4,19].

Extraction and fractionation of S. rebaudiana leaves

Milled *S. rebaudiana* leaves (10 g), collected in Paraguay from a native habitat, were macerated in 4 × 200 ml methanol–water (4:1). Macerates were combined and solvent was removed under reduced pressure to yield 3.5 g of dried residue. This residue was dissolved in water (200 ml) and defatted with 7 × 100 ml ethyl acetate. The aqueous layer was then partitoned with *n*-butanol (6 × 50 ml), and the combined organic layer washed with water and evaporated to dryness to produce a residue. This extractive was dissolved in methanol (30 ml), stood at 5°C for 12 h, and crude stevioside (0.8 g) was precipitated, filtered, washed with methanol and dried in a vacuum oven. The remaining filtrate, constituting the *S. rebaudiana n*-butanol fraction minus the majority of the stevioside originally present, was evaporated to dryness to produce 1.3 g of a solid.

Droplet counter-current chromatography

The solvent system chloroform–methanol–isopropanol–water (11:9:4:8) was used for all DCCC separations reported in this communication. Ascending development was employed, with the upper layer of the partitioned solvent acting as the mobile phase. Solutes were dissolved in the stationary phase (10 ml) and injected into a 10-ml sample chamber. Fractions (5 ml) were collected at a rate of 10 ml/h in a Buchler Fractomette Alpha-200 (Buchler, Fort Lee, NJ, U.S.A.) automatic fraction collector, and were weighed on removal of solvent. All separations were carried out at ambient temperature.

RESULTS AND DISCUSSION

In Fig. 2 a DCCC elution chromatogram of crude stevioside (200 mg), obtained by methanol precipitation from an *n*-butanol fraction of *S. rebaudiana* collected in the field in Paraguay, is presented. Pure stevioside (120 mg; 4.8 %, w/w; m.p. 202–204°C) (lit.[3] 198–202°C), uncontaminated by any other *S. rebaudiana* constituents, including rebaudioside C (see below), was collected as crystals in fractions 31–42 on removal of solvent. A total solvent consumption of only 750 ml was needed for the DCCC purification of stevioside and the isolation was achieved within 36 h after the commencement of the solvent partitioning of *S. rebaudiana* leaves. This process was highly reproducible and required very little technical manipulation during the DCCC operation.

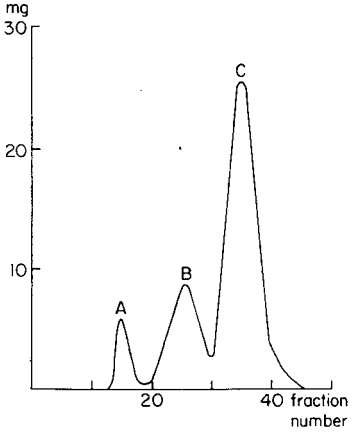

Fig. 2. DCCC elution chromatogram of crude stevioside (200 mg), crystallized by methanol from an *n*-butanol fraction of *S rebaudiana*. Solvent system, chloroform–methanol–isopropanol–water (11:9:4:8), upper layer used as mobile phase. A = Solvent front; B = rebaudioside A; C = stevioside.

Fig. 3 shows the separation obtained when equal weights (30 mg) of the diterpene glycosides listed in Fig. 1 were submitted to DCCC. Compounds were eluted in a series representative of the number of sugar units attached to the *ent*-kaurene nucleus, with the more polar compounds being eluted before less polar compounds. While there are considerable differences in polarity in the six compounds shown in the DCCC chromatogram in Fig. 3, the efficiency of the method was such that almost complete resolution of every component in the mixture was obtained. Attempts to resolve rebaudioside C and stevioside better by varying the solvent composition were unsuccessful. Rebaudiosides D and E, two sweet *S. rebaudiana* constituents omitted from Fig. 1 because they are more soluble in water than in *n*-butanol, were not included in the present investigation.

Rebaudioside C and residual stevioside were also found to elute together when 160 mg of the Paraguayan *S. rebaudiana n*-butanol fraction, after crude stevioside removal, was submitted to DCCC (Fig. 4). Therefore, a preliminary separation procedure is necessary to resolve rebaudioside C from stevioside before DCCC, using this solvent system, may be applied to the purification of rebaudioside C from *S.*

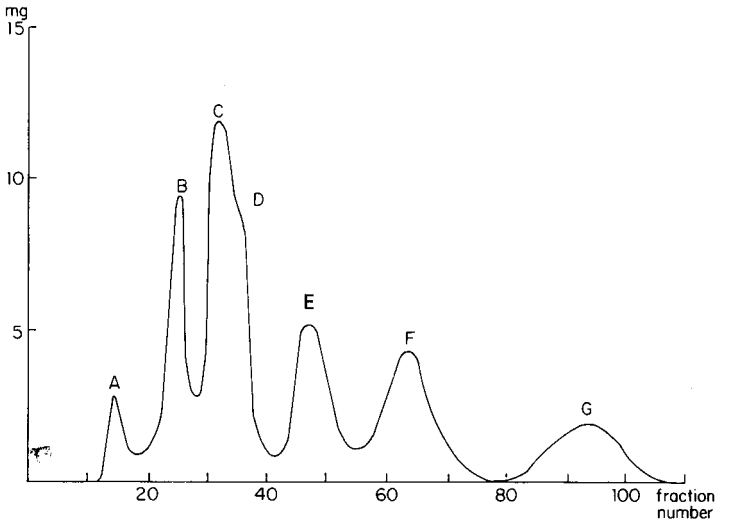

Fig. 3. DCCC elution chromatogram of a mixture (30 mg per compound) of *S. rebaudiana* sweet principles. A = Solvent front; B = rebaudioside A; C = rebaudioside C; D = stevioside; E = dulcoside A; F = rebaudioside B; G = steviolbioside.

rebaudiana. However, pure rebaudioside A (40 mg; m.p., 236–238°C) (lit.[4] 242–244°C) and pure dulcoside A (15 mg; 0.9%, w/w; m.p. 188–190°C) (lit.[7] 193–195°C) were obtained after recrystallization from methanol of the solutes in fractions 21–29 and 42–52, respectively (Fig. 4). The combined yield of rebaudioside A from this *S. rebaudiana* sample, as obtained in the DCCC runs represented in Figs. 2 and 4, was 4.25% (w/w) (total weight obtained pure 65 mg). Rebaudioside B and steviolbioside were not detected in this Paraguayan source of *S. rebaudiana* leaves, although these compounds have been found as natural products in *S. rebaudiana* cultivated in Japan[4].

Thus the DCCC methodology described in this paper constitutes a rapid, direct preparative procedure for the small-scale isolation of stevioside, rebaudioside A and

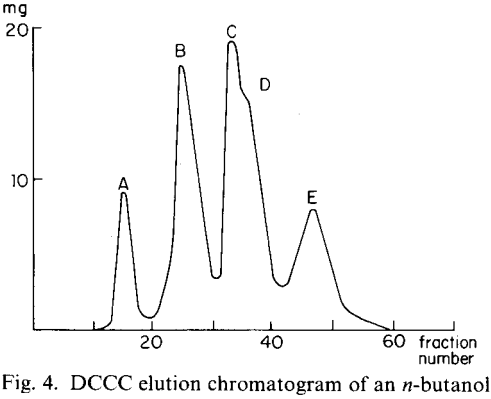

Fig. 4. DCCC elution chromatogram of an *n*-butanol fraction (160 mg), after methanol precipitation, of leaves of *S. rebaudiana* collected in Paraguay. A = Solvent front; B = rebaudioside A; C = rebaudioside C; D = stevioside; E = dulcoside A.

dulcoside A in high purity from a crude extract of *S. rebaudiana*. The method is also applicable to the final purification of the other main *n*-butanol-soluble *S. rebaudiana* sweet principle, rebaudioside C, after preliminary column chromatography has been performed to resolve this compound from stevioside.

ACKNOWLEDGEMENTS

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Note

Analysis of unquenched reaction mixtures of chlorine dioxide and phenols by reversed-phase high-performance liquid chromatography

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In the course of a study of the mechanisms of the reaction of chlorine dioxide (ClO_2) with phenols[1], we sought a method whereby dilute aqueous reaction mixtures, sometimes containing excess oxidant, could be separated and the products analyzed without prior quenching and extraction. Mixtures of chlorophenols and chlorinated benzoquinones and hydroquinones resulting from treatment of dihydric phenols with aqueous hypochlorite have been analyzed by gas chromatography (GC) without derivatization[2], but the procedure required quenching and extraction and was not suitable for large numbers of product studies of fast reactions in very dilute solutions. However, in recent years high-performance liquid chromatography (HPLC)[3–5], with the use of both normal[3] and reversed[4,5] phases, has been the method of choice for determination of phenols and chlorophenols in dilute aqueous solutions. We here describe a reversed-phase HPLC method by which standard samples of the chlorophenols, chlorobenzoquinones and chlorohydroquinones anticipated as possible products of the reaction of ClO_2 with phenol and with hydroquinone were separated and quantified, and by which unquenched oxidation mixtures were analyzed directly.

EXPERIMENTAL

Chemicals

All solutions were made with deionized doubly distilled water. Acetonitrile was Mallinkrodt chromatographic grade. Phenol (MCB reagent) was purified by distillation under nitrogen; *p*-benzoquinone (Baker reagent) and 2-chlorohydroquinone (Pfaltz and Bauer) were steam distilled. Hydroquinone (Aldrich), 4-chlorophenol (Chemical Service Co.) and 2,6-dichlorobenzoquinone (Pfaltz and Bauer) were purified by recrystallization. 2-Chlorophenol (Chemical Service Co.) and 2,4-dichlorophenol (Eastman) were used as received. 2,6-Dichlorobenzoquinone was prepared from 2,4,6-trichlorophenol[6], and 2,3-dichlorobenzoquinone from 2,3-dichlorophenol[7] by CrO_3–acetic acid oxidation. 2,6- and 2,3-Dichlorohydroquinones were prepared by treatment of the respective dichlorobenzoquinones with $NaBH_4$ in ethanol.

* National Research Council Postdoctoral Associate, 1980–1981.

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TABLE I

STANDARDS FOR HPLC ANALYSIS OF UNQUENCHED MIXTURES

Compound	*Minimum concentration (M)*
Hydroquinone	$8.18 \cdot 10^{-6}$
Benzoquinone	$8.38 \cdot 10^{-6}$
2,6-Dichlorobenzoquinone	$6.00 \cdot 10^{-6}$
Phenol	$6.11 \cdot 10^{-6}$
2,5-Dichlorohydroquinone	$6.63 \cdot 10^{-6}$
2,6-Dichlorohydroquinone	$6.77 \cdot 10^{-6}$
2,3-Dichlorohydroquinone	$6.77 \cdot 10^{-6}$
2,5-Dichlorobenzoquinone	$6.25 \cdot 10^{-6}$
2-Chlorophenol	$5.31 \cdot 10^{-6}$
4-Chlorophenol	$6.22 \cdot 10^{-6}$
2,6-Dichlorophenol	$7.82 \cdot 10^{-6}$
2,4-Dichlorophenol	$8.05 \cdot 10^{-6}$

HPLC analyses

The liquid chromatographic system consisted of the following: (1) two Model 6000A pumps and Model M660 solvent programmer (Waters Assoc.), (2) Model 7120 syringe loading sample injector with 200-μl sample loop (Rheodyne), (3) 300 × 3.9 mm I.D. 10-μm μBondapak C_{18} reversed-phase column (Waters Assoc.), (4) SF-770 variable-wavelength detector (Schoeffel) set at 220 nm (0.04 a.u.f.s.), (5) Sigma 10 chromatographic data station (Perkin-Elmer). A linear gradient elution program was used in which the eluent was changed from 100% 0.01 *M* KH_2PO_4 (pH 2.8) to 50% acetonitrile–water (80:20) in 30 min at 1.5 ml/min and 1200 p.s.i. Solutions of standards at $6.25 \cdot 10^{-6}$ *M*, $1.25 \cdot 10^{-5}$ *M*, $2.5 \cdot 10^{-5}$ *M*, $5 \cdot 10^{-5}$ *M*, and $1 \cdot 10^{-4}$ *M* were used to determine a standard concentration curve for each compound. Analyses of ClO_2 oxidation mixtures were performed under the same conditions. Solutions of phenol and of hydroquinone (10^{-6}–10^{-4} *M*) were each mixed with equal volumes of ClO_2 solutions (10^{-5}–10^{-4} *M*) and 200-μl aliquots were removed at times varying from 45 sec to 2.5 h after mixing and analyzed immediately. Concentrations of products at time *t* were determined from the standard curves. Detection limits are listed in Table I.

RESULTS AND DISCUSSION

Fig. 1 displays a typical chromatogram of twelve of the thirteen standards. It can be seen that the separations, with the exception of 2,5- and 2,6-dichlorohydroquinone, which eluted within < 0.5 min of one another, were very clean. Even under these optimum conditions, however, 2,3-dichlorobenzoquinone co-eluted with 2-chlorophenol, and since only the latter was expected to be an important reaction product, the quinone was not further utilized. For reaction mixtures in which this peak (retention time 24.5 min) was found, the presence of 2-chlorophenol and the absence of 2,3-dichlorobenzoquinone after quenching and extraction were verified by GC–mass spectrometry (MS).

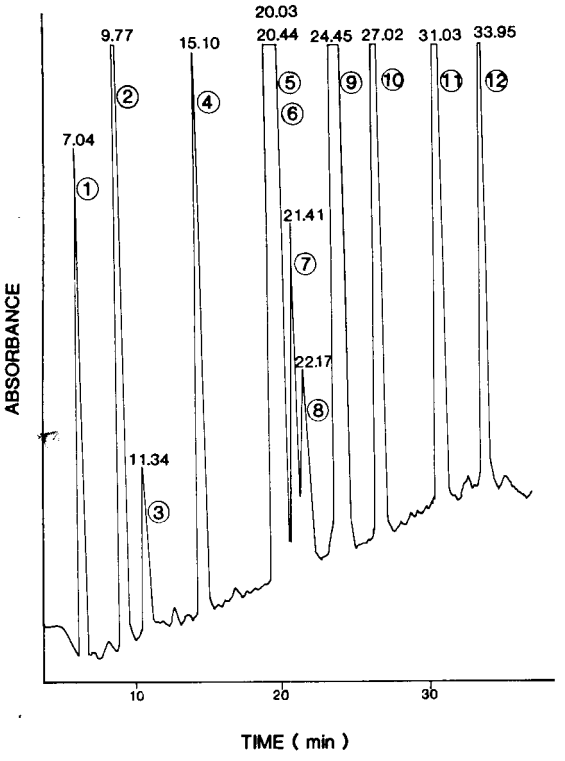

Fig. 1. HPLC separation of chlorinated phenol, hydroquinone, and benzoquinone standards, on μBondapak C_{18} in buffered acetonitrile–water (pH 2.8). Peaks: 1 = Hydroquinone ($2.7 \cdot 10^{-5}$ *M*); 2 = benzoquinone ($3.9 \cdot 10^{-5}$ *M*); 3 = 2,6-dichlorobenzoquinone ($1.8 \cdot 10^{-5}$ *M*); 4 = phenol ($2.3 \cdot 10^{-5}$ *M*); 5 = 2,5-dichlorohydroquinone ($3.1 \cdot 10^{-5}$ *M*); 6 = 2,6-dichlorohydroquinone ($2.1 \cdot 10^{-5}$ *M*); 7 = 2,3-dichlorohydroquinone ($2.0 \cdot 10^{-5}$ *M*); 8 = 2,5-dichlorobenzoquinone ($1.7 \cdot 10^{-5}$ *M*); 9 = 2-chlorophenol ($1.5 \cdot 10^{-5}$ *M*); 10 = 4-chlorophenol ($3.9 \cdot 10^{-5}$ *M*); 11 = 2,6-dichlorophenol ($2.9 \cdot 10^{-5}$ *M*); 12 = 2,4-dichlorophenol ($2.1 \cdot 10^{-5}$ *M*).

With the isomeric dichlorobenzoquinones and dichlorohydroquinones, it should be noted that HPLC and GC are complementary methods. 2,5- and 2,6-Dichlorohydroquinones, poorly separated under the above HPLC conditions, were well separated by GC (4.8 and 6.7 min, respectively, on 3% OV-1), whereas 2,5- and 2,6-dichlorobenzoquinone, with HPLC retention times of 22.2 and 11.3 min, respectively, did not separate by GC on OV-1.

Preliminary HPLC studies with a Partisil-10-ODS column (Whatman) with unbuffered methanol–water as eluent had shown peak broadening of the standards after analysis of one oxidation mixture and, after two injections of oxidation mixtures, splitting of the slower moving standards into double peaks. Restoration of column efficiency according to the manufacturer's instructions (successive washes with water, dilute phosphoric acid, water, and methanol) after each analysis was prohibitively time-consuming. Use of the μBondapak C_{18} column with buffered (pH 2.8) acetonitrile–water eliminated this problem, and successive analyses of oxidation mixtures were performed without loss of column efficiency. It was not determined,

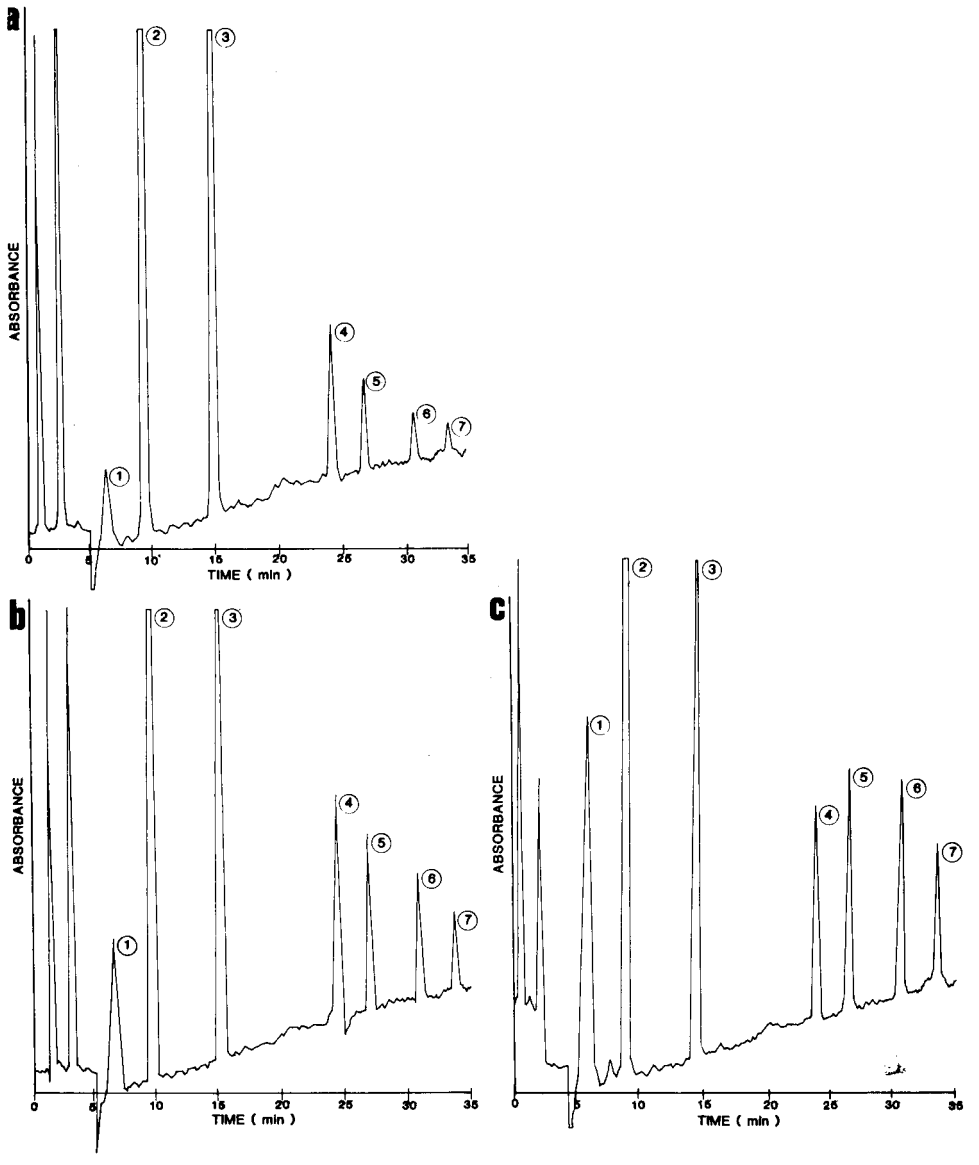

Fig. 2. Products of the reaction of $5 \cdot 10^{-4}$ *M* ClO_2 with $5 \cdot 10^{-4}$ *M* phenol after (a) 1.25 min, (b) 4.0 min, (c) 150 min, analyzed under the conditions of Fig. 1. Peaks: 1 = hydroquinone; 2 = benzoquinone; 3 = phenol; 4 = 2-chlorophenol; 5 = 4-chlorophenol; 6 = 2,6-dichlorophenol; 7 = 2,4-dichlorophenol.

however, whether the Partisil column would also retain its efficiency with the buffered solvent system.

A typical product analysis of an unquenched buffered reaction mixture of ClO_2 with phenol in excess, with aliquots taken at 1.25, 4.0 and 150 min after mixing, is displayed in Fig. 2. The slow increase in concentrations of hydroquinone and the chlorinated products, 2- and 4-chlorophenol and 2,4- and 2,6-dichlorophenol, and a

concomitant decrease in concentration of the excess phenol, with increasing reaction times, can be readily seen. Similar analyses of reaction mixtures of a stoichiometric amount of phenol with ClO_2 or with ClO_2 in excess showed no chlorinated products, and in all cases *p*-benzoquinone was the major product and was formed extremely rapidly ($\ll$ 45 sec)[1]. Chlorinated benzoquinones or hydroquinones were not detected at any time.

Similar analyses of oxidation mixtures of ClO_2 and hydroquinone showed *p*-benzoquinone as the only product, and even with hydroquinone in excess and at times longer than 2 h nó evidence was found for chlorinated products.

In summary, these reversed-phase HPLC conditions permitted repeated analyses of unquenched dilute aqueous buffered reaction mixtures of ClO_2 and phenols with high resolution and unimpaired column efficiency. In the one case of coelution, quenching followed by GC–MS was a useful complement.

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Note

High-performance liquid chromatographic analysis of the *in vivo* metabolites of [^{14}C]pyridine

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Nitrogen heteroaromatic ring systems may undergo metabolic reactions at the heteroatom (*e.g.* N-oxidation or N-methylation) and at ring carbons (C-oxidation)[1]. The simple heterocycle, pyridine, has often been used as a model compound for studying the occurrence and enzymology of each of these metabolic pathways[2–9]. N-Oxidation of pyridine has previously been studied using gas–liquid chromatography (GLC)[10] and recently by high-performance liquid chromatography (HPLC)[9]. The N-methylation product, N-methylpyridinium hydroxide, has been isolated and identified by traditional chemical methods[2] (m.p., CHN analysis, colour reactions) and quantitatively determined by paper chromatography (PC)[4], by ultraviolet (UV) spectroscopy[4] and by radiochemical techniques (reverse isotope dilution)[8]. Data are available in the literature[11] on thin-layer chromatography (TLC) of 2-pyridone, 4-pyridone and 3-hydroxypyridine, but reports on their metabolic formation and quantitative determination are lacking.

In order to understand the relative importance of oxidative and methylation reactions in the metabolism of drugs and xenobiotics containing nitrogen heteroaromatic ring systems, we have chosen to carry out detailed *in vivo* metabolic studies with [^{14}C]pyridine. To this end we have developed an analytical procedure which allows the simultaneous determination of six potential *in vivo* metabolites of [^{14}C]pyridine by utilising a combination of cation-exchange and reversed-phase HPLC. The details of this assay procedure are described in this paper.

MATERIALS AND METHODS

[2,6-^{14}C]Pyridine (250 μCi; specific activity 27.1 mCi/mmole) was obtained from The Radiochemical Centre, Amersham, Great Britain and administered to various laboratory animal species. Urine samples were collected over a 24-h period as previously described[8]. Pyridine, phosphoric acid, glacial acetic acid, and HPLC-grade methanol and acetonitrile were purchased from Fisons, Loughborough, Great Britain. 3-Hydroxypyridine was from Fluka, Glossop, Great Britain. Potassium dihydrogen orthophosphate and disodium hydrogen orthophosphate (anhydrous)

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were obtained from BDH, Poole, Great Britain, and a batch selected which gave minimal absorption at 260 nm. Luma gel (liquid scintillation cocktail) was purchased from Lumac Systems, Basel, Switzerland.

Pyridine N-oxide[12], 2-pyridone[13], 4-pyridone[14], N-methyl-2-pyridone[15] and N-methylpyridinium iodide[16] were all synthesised by literature methods. All chromatographic standards were prepared as 10 m*M* solutions in methanol, where solubility permitted. Analyses were carried out on a modular unit constructed in our laboratory consisting of a HPLC Technology pump (Model RR/015) and a Pye Unicam LC3 variable-wavelength UV detector operating at 254 or 260 nm. Samples were introduced via a Rheodyne® loop injector. All eluent buffers used were filtered and degassed *in vacuo* before use. A Partisil-10 SCX microparticle column (Whatman), 25 cm × 4.6 mm I.D., was used for cation-exchange chromatography, whereas a Partisil-10 ODS microparticle column (Whatman), 25 × 0.50 cm I.D., was used for reversed-phase chromatography. Essential chromatographic operating parameters are to be found in the legends to the tables and figures.

Urine samples (20–100 μl) from animals dosed with [^{14}C]pyridine were injected, either directly or after protein precipitation using an equal volume of acetonitrile, on the HPLC columns. Radioactive effluent was monitored by collecting fractions of known volume on a LKB 7000 Ultrorac® fraction collector. Sufficient scintillation cocktail (Luma Gel, 4 ml) was added to each fraction to form a clear, homogenous gel or solution. A Packard Tri-Carb liquid scintillation spectrometer was used to measure the radioactivity in the samples. Radiochromatograms were constructed by plotting the radioactivity of the fractions against their retention times. To determine the recovery of radioisotope from analytical columns, an identical volume of radioisotopic urine applied to the column was added directly to a scintillation vial containing an appropriate volume of column effluent and treated as described above.

RESULTS AND DISCUSSION

Initial investigations were carried out on a Partisil-10 SCX cation-exchange

TABLE I

THE INFLUENCE OF ELUENT pH ON THE RETENTION OF POTENTIAL PYRIDINE METABOLITES BY PARTISIL-10 SCX CATION-EXCHANGE CHROMATOGRAPHY

Column, Partisil-10 SCX 25 cm × 4.6 mm I.D.; eluent, methanol–0.3 *M* ammonium acetate buffer (30:70, v/v): the ammonium acetate solution was adjusted to the pH shown before dilution with methanol; flow-rate, 1.5 ml/min at 1800 p.s.i.g.; detection, 254 nm.

Compound	*Retention time (min)**				
	pH 3.7	*pH 3.9*	*pH 4.9*	*pH 6.0*	*pH 7.0*
N-Methylpyridinium ion	10.3	12.6	14.9	17.5	21.0
Pyridine	3.6	4.5	4.4	3.0	2.3
Pyridine N-oxide	1.5	2.0	2.3	2.1	1.9
3-Hydroxypyridine	1.3	1.3	1.3	1.2	1.1
4-Pyridone	1.3	1.3	1.2	1.1	1.1
2-Pyridone	0.8	0.8	1.1	0.9	0.8

* Corrected retention time ($t_R - t_0$); t_0 = 1.8 min.

TABLE II

THE INFLUENCE OF ELUENT IONIC STRENGTH ON THE RETENTION OF POTENTIAL PYRIDINE METABOLITES BY PARTISIL-10 SCX CATION-EXCHANGE CHROMATOGRAPHY

Column, Partisil-10 SCX 25 cm × 4.6 mm I.D.; eluent, methanol–ammonium acetate, pH 3.7 (30:70, v/v); flow-rate, 1.5 ml/min at 1800 p.s.i.g.; detection, 254 nm.

Compound	*Retention time (min)**				
	0.1 M	*0.3 M*	*0.5 M*	*0.8 M*	*1.0 M*
N-Methylpyridinium ion	20.4	10.3	7.9	6.4	5.9
Pyridine	7.8	3.6	2.8	2.1	1.7
Pyridine N-oxide	2.1	1.5	1.4	1.2	1.2
3-Hydroxypyridine	1.1	1.0	0.9	0.9	0.8
4-Pyridone	1.0	0.9	0.9	0.8	0.8
2-Pyridone	0.7	0.6	0.6	0.7	0.7

* Corrected retention time ($t_R - t_0$); t_0 = 1.8 min.

column using a mixture of methanol and ammonium acetate buffer as the eluent (see Table I). Variation of hydrogen ion concentration has a marked effect on the retention times of the more basic solutes over the pH range 3.7–7.0, whereas the neutral pyridone derivatives, as expected, elute early and are relatively unaffected by change in pH. Optimum pH for separation of pyridine, pyridine N-oxide and N-methylpyridinium ion is in the range 3.7–4.9. As would be expected of an ion-exchange process, a decrease in the ionic strength of the buffer leads to longer retention times of all the three basic solutes (see Table II). The effect of varying the methanol content of the eluent (Table III) is a complex phenomenon, leading to a considerable change in the retention time of N-methylpyridinium ion accompanied by extensive peak broadening, while not affecting to any great degree the retention behaviour of pyridine, pyridine N-oxide or the neutral pyridones. These results in part, may reflect the

TABLE III

THE INFLUENCE OF METHANOL CONTENT OF THE ELUENT ON THE RETENTION OF POTENTIAL PYRIDINE METABOLITES BY PARTISIL-10 SCX CATION-EXCHANGE CHROMATOGRAPHY

Column, Partisil-10 SCX 25 cm × 4.6 mm I.D.; eluent, various proportions of methanol with 0.3 *M* ammonium acetate, pH 3.7; flow-rate: 1.5 ml/min at 1800 p.s.i.g.; detection: 254 nm.

Compound	*Retention time (min)**			
	20%	*30%*	*40%*	*60%*
N-Methylpyridinium ion	9.0	9.0	9.4	14.4
Pyridine	3.4	3.2	3.1	2.3
Pyridine N-oxide	1.6	1.6	1.6	1.6
3-Hydroxypyridine	0.8	1.0	1.1	1.2
4-Pyridone	0.9	0.9	1.0	1.1
2-Pyridone	0.5	0.6	0.7	0.8

* Corrected retention time ($t_R - t_0$); t_0 = 1.8 min.

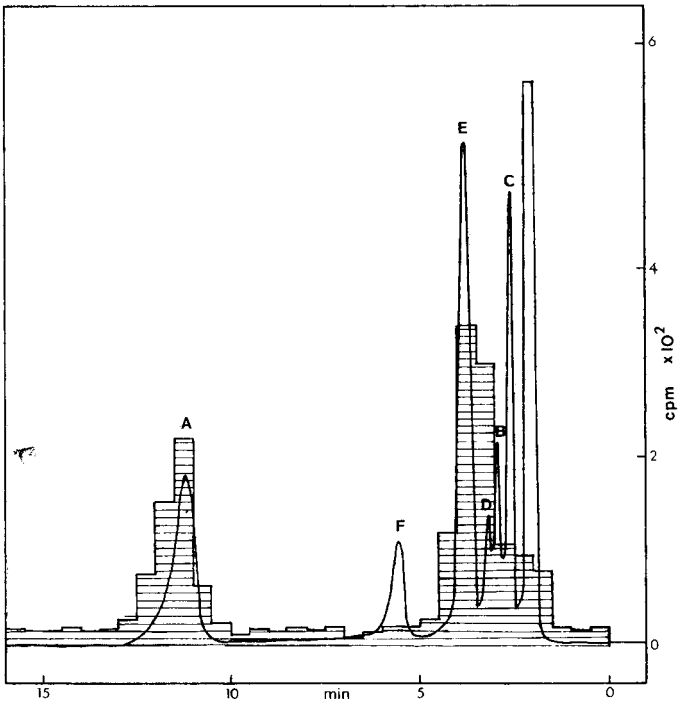

Fig. 1. Radiochromatogram of 24-h hamster urine, after intraperitoneal administration of [^{14}C]pyridine, using cation-exchange HPLC. Column, Partisil-10 SCX (25 cm × 4.6 mm I.D.); eluent, methanol–0.3 *M* ammonium acetate buffer (30:70, v/v); flow-rate, 1.5 ml/min at 1800 p.s.i.g.; detection, 254 nm; fractions collected every 30 sec for determination of ^{14}C (see text for details). Peaks: A = N-methylpyridinium ion; B = 4-pyridone; C = 2-pyridone; D = 3-hydroxypyridine; E = pyridine N-oxide; F = pyridine. R_t = Retention time.

relative solubilities of N-methylpyridinium ion, pyridine and pyridine N-oxide in water and methanol. Because of the many variables investigated, choosing optimum conditions was almost impossible. For practical purposes, a methanol–ammonium acetate buffer (0.3 *M*, pH 3.7) (30:70, v/v) was utilised for the analysis of radioactive urine samples (see Fig. 1). Under these conditions, the N-methylpyridinium ion, pyridine (protonated at pH 3.7), and pyridine N-oxide are well separated over a short elution period. However, 3-hydroxypyridine and the neutral pyridone derivatives co-elute early, almost in the void volume.

Reversed-phase chromatography was investigated as a method for analysis of the C-oxidation metabolites of [^{14}C]pyridine which co-elute early by cation-exchange chromatography. Table IV shows results obtained using a Partisil-10 ODS column and a 0.067 *M* phosphate buffer (pH 7.4) as eluent with acetonitrile concentrations varying from 0 to 10% (v/v). As expected, the very water-soluble N-methylpyridinium ion elutes in the void volume in this system, whereas pyridine, because of its lipophilic character, has a high affinity and a long retention time. Baseline separation of 3-hydroxypyridine, 2- and 4-pyridones and pyridine N-oxide was achieved using this method of analysis with no organic modifier. Incorporation of even small amounts of acetonitrile or methanol produce a marked improvement in the chroma-

TABLE IV

THE INFLUENCE OF ACETONITRILE CONTENT OF THE ELUENT ON THE RETENTION OF POTENTIAL PYRIDINE METABOLITES BY PARTISIL-10 ODS REVERSED-PHASE CHROMATOGRAPHY

Column, Partisil-10 ODS 25 × 0.5 cm I.D.; eluent, various proportions of acetonitrile with 0.067 *M* phosphate buffer, pH 7.4; flow-rate, 2 ml/min at 1700 p.s.i.g.; detection, 260 nm.

Compound	*Retention time (min)**				
	0.0%	*2.0%*	*2.5%*	*5.0%*	*10.0%*
Pyridine	36.8	31.6	26.5	16.2	11.5
Pyridine N-oxide	10.1	6.7	6.2	5.7	4.7
3-Hydroxypyridine	6.0	5.6	5.5	5.3	4.3
2-Pyridone	5.0	4.3	4.2	4.1	3.5
4-Pyridone	3.4	3.3	3.1	3.1	2.9
N-Methyl-2-pyridone	10.6	10.8	10.7	7.9	5.9
N-Methylpyridinium ion	0.0	0.0	0.0	0.0	0.0

* Corrected retention time ($t_R - t_0$); $t_0 = 0.5$ min.

tography of pyridine (*i.e.* retention time and peak shape), but lead to unstatisfactory separation of the C- and N-oxidation metabolites. Varying the ionic strength of the buffer appears to have little effect on the retention of any of the solutes examined (Table V), whereas varying the pH of the eluent, as expected, had a marked effect on the retention for pyridine, but little effect on that of the other solutes (Table VI). The eluent composition chosen for the reversed-phase analysis of the urinary metabolites of [^{14}C]pyridine was 0.067 *M* phosphate buffer, pH 7.4 without any organic modifier at a fast flow-rate of 4.2 ml/min at 2750 p.s.i.g. to achieve a relatively short analysis time (see Fig. 2). This analytical system, in combination with the Partisil-10 SCX chromatographic system described above, allows the analysis of six potential metabolites of [^{14}C]pyridine.

TABLE V

THE INFLUENCE OF ELUENT IONIC STRENGTH ON THE RETENTION OF POTENTAL PYRIDINE METABOLITES BY PARTISIL-10 ODS REVERSED-PHASE CHROMATOGRAPHY

Column, Partisil-10 ODS 25 × 0.5 cm I.D.; eluent, Acetonitrile–phosphate buffer, pH 7.4 (2.5:97.5 v/v); flow-rate, 2 ml/min at 1700 p.s.i.g.; detection, 260 nm.

Compound	*Retention time (min)**			
	0.034 M	*0.067 M*	*0.134 M*	*0.200 M*
Pyridine	24.4	26.5	26.0	25.7
Pyridine N-oxide	6.0	6.2	7.6	7.1
3-Hydroxypyridine	5.5	5.5	5.8	5.3
2-Pyridone	4.1	4.2	4.6	4.1
4-Pyridone	3.1	3.1	3.3	2.6
N-Methyl-2-pyridone	10.5	10.7	12.2	11.5
N-Methylpyridinium ion	0.0	0.0	0.0	0.0

* Corrected retention time ($t_R - t_0$); $t_0 = 0.5$ min.

TABLE VI

THE INFLUENCE OF ELUENT pH ON THE RETENTION OF POTENTIAL PYRIDINE METABOLITES BY PARTISIL-10 ODS REVERSED-PHASE CHROMATOGRAPHY

Column, Partisil-10 ODS 25 cm × 5.0 mm I.D.; eluent, acetonitrile–0.067 *M* phosphate buffer (2.5:97.5, v/v): the phosphate buffer was adjusted to the pH shown before dilution with acetonitrile; flow-rate, 2 ml/min at 1700 p.s.i.g.; detection, 260 nm.

Compound	*Retention time (min)**				
	pH 6.0	*pH 6.8*	*pH 7.0*	*pH 7.4*	*pH 8.0*
Pyridine	34.0	32.5	31.5	26.5	27.0
Pyridine N-oxide	7.5	8.2	7.9	6.2	7.7
3-Hydroxypyridine	5.7	6.3	6.2	5.5	5.9
2-Pyridone	4.7	4.9	4.8	4.2	4.7
4-Pyridone	3.2	3.4	3.4	3.1	3.3
N-Methyl-2-pyridone	12.5	13.5	12.1	10.7	12.3
N-Methylpyridinium ion	0.0	0.0	0.0	0.0	0.0

* Corrected retention time ($t_R - t_0$); t_0 = 0.5 min.

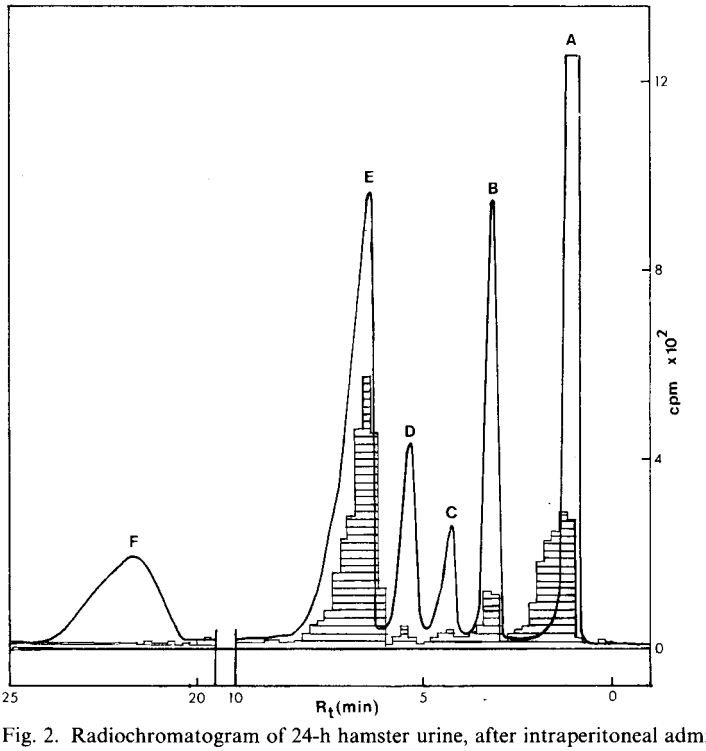

Fig. 2. Radiochromatogram of 24-h hamster urine, after intraperitoneal administration of [^{14}C]pyridine, using reversed-phase HPLC. Column, Partisil-10 ODS (25 cm × 5.0 mm I.D.); eluent, 0.067 *M* phosphate buffer; flow-rate, 4.2 ml/min at 2750 p.s.i.g.; detections, 260 nm; fractions collected every 12 sec for determination of ^{14}C (see text for details). Peaks: A = N-methylpyridinium ion; B = 4-pyridone, C = 2-pyridone; D = 3-hydroxypyridine; E = pyridine N-oxide; F = pyridine.

The very low dose of [^{14}C]pyridine utilised in this study (7 mg/kg body weight, *ca.* 10 μCi), and the consequent low activity of the urine samples collected, necessitated both the injection of the intact urine samples (20–100 μl, *ca.* 1000–2000 cpm) on to the column, and the monitoring of column effluent for ^{14}C by liquid scintillation counting for detecting and quantitating the small amount of metabolites present. The radiochromatograms obtained from analysis of a 24-h urine sample from a female Golden Syrian hamster dosed with [^{14}C]pyridine are illustrated in Fig. 1 and 2, and are typical of results obtained from analyses of urine samples from several other animal species. With each system, the amount of radioactivity applied to the column was almost totally recovered in the effluent. The cation-exchange column allows the estimation of the percentage urinary radioactivity present as N-methylpyridinium ion (*ca.* 25%) and as pyridine (*ca.* 0%). Analysis of an identical sample by reversed-phase chromatography shows that *ca.* 25% of the applied radioactivity elutes in the void volume (N-methylpyridinium ion), *ca.* 60% of ^{14}C co-elutes with the pyridine N-oxide peak and *ca.* 6% with the 4-pyridone peak, whereas 2-pyridone and 3-hydroxypyridine appear to be only minor urinary metabolites. The results from both analyses complement each other well and allow the quantitative determination of the five *in vivo* metabolites of pyridine present in hamster urine.

The above analytical procedure is being used for the analysis of urine samples obtained from several other animal species dosed with [^{14}C]pyridine, and these results will be the subject of future communications.

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Note

Improved separation of biologically relevant C_{14}–C_{20} fatty acids by reversed-phase high-performance liquid chromatography

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UV-absorbing derivatives of many fatty acids may be separated by high-performance liquid chromatography (HPLC)[1–10]. However, the presently available methods are not satisfactory for the investigation of naturally occuring C_{14}–C_{20} fatty acids, as some of these derivatives are not separated. This subgroup of fatty acids is of primary interest when dealing with the analysis of lipids in mammalian cells and tissues[11].

We have devised a method for the simultaneous chromatographic separation of nine of the biologically most important C_{14}–C_{20} fatty acids as their *p*-bromophenacyl esters. The method is based on the use of a high-performance 5-μm reversed-phase column and the isocratic three-component eluent methanol–acetonitrile–water (82:9:9).

EXPERIMENTAL

HPLC-grade methanol and acetonitrile were purchased from Rathburn Chemicals (Walkerburn, Great Britain). Ultrapure water was prepared by filtration of distilled water through a Gelman Water I Filtration Unit (Gelman Sciences, Ann Arbor, MI, U.S.A.). *p*-Bromophenacyl bromide, N,N-diisopropylethylamine and dimethylformamide were obtained from Fluka (Buchs, Switzerland). Traces of water were eliminated from the reagents by drying the dimethylformamide over molecular sieve 4A (Pearlform; E. Merck, Darmstadt, G.F.R.) and by adding sodium hydroxide pellets to the N,N-diisopropylethylamine. Margaric acid was purchased from Sigma (St. Louis, MO, U.S.A.) and the other fatty acid standards were obtained from Supelco (Bellefonte, PA, U.S.A.).

p-Bromophenacyl esters of fatty acids were synthesized as described by Jordi[5]. HPLC analyses were carried out on a modular system consisting of a Consta Metric-III HPLC pump [Laboratory Data Control, Riviera Beach, FL, U.S.A. (LDC)]

working at a flow-rate of 1.00 ml/min, and a Consta Metric Spectromonitor-III (LDC) variable-wavelength UV detector set to monitor absorbance at 254 nm. The detector was connected to a Rec-2 recorder (Pharmacia, Uppsala, Sweden). The column system consisted of a 49 × 4.6 mm I.D. guard column, dry packed with 40-μm pellicular reversed-phase material (Pelliguard LC-18) and a 250 × 4.6 mm I.D. Supelcosil LC-18 analytical column, all purchased from Supelco. Reversed-phase columns were also purchased from Gene Tec, Sweden (Nucleosil ODS) and Supelco (Chromosorb LC-7). The Supelcosil LC-18 column was packed with 5-μm spherical packing and the other columns with 10-μm spherical packing. Injection was performed through a Rheodyne fixed-loop (20 μl) injector.

RESULTS AND DISCUSSION

When mixtures of acetonitrile and water were used as eluents, the *p*-bromophenacyl esters of palmitic and oleic acids eluted together as a single peak, and there was a poor separation of myristic and palmitoleic acid derivatives (Fig. 1A). An eluent system based on mixtures of methanol and water gave an incomplete separation of the derivatives of myristic and linoleic acids (Fig. 1B).

The two principal parameters determining the polarity and thereby the elution volume of the fatty acid *p*-bromophenacyl esters are the number of carbon atoms and the number of unsaturated bonds in the fatty acid chain[5,7]. However, as can be seen from the chromatograms (Fig. 1A and B, Table I), the relative importances of these two parameters are different in acetonitrile and methanol. When acetonitrile was used as the eluent the number of unsaturated bonds seemed to be of greater importance in determining the elution volume, whereas with methanol the elution volume was relatively more influenced by the chain length. By mixing acetonitrile and methanol in varying proportions, it was possible to vary the relative retention of the different fatty acid derivatives in a systematic and predictable manner. Thus it was possible to find the proportions of acetonitrile and methanol that resulted in the optimal overall resolution. An eluent consisting of methanol–acetonitrile–water (82:9:9) gave a good separation of all of the fatty acid derivatives investigated, with $R \geqslant 1.5$ for any two neighbouring peaks (Fig. 1C).

Margaric acid (17:0) is suitable as an internal standard for the analysis of the fatty acid composition of samples of mammalian origin. The *p*-bromophenacyl ester of margaric acid was completely separated from the other fatty acid esters used in the study (Fig. 1C, Table I).

The results described were obtained using a high-performance 5-μm reversed-phase column (Supelcosil LC-18). The column-to-column reproducibility was found to be excellent. The two 10-μm columns (Nucleosil ODS-18 and Chromosorb LC-7) did not give satisfactory separations of the fatty acid derivatives in any solvent system tested (data not shown). Our study has demonstrated that for some reversed-phase chromatographic separations, changing from a two-component to a three-component eluent system may result in a substantial increase in chromatographic selectivity[12,13]. Using *p*-bromophenacyl esters of myristic (14:0) and arachidonic (20:4) acids as standards the detection limit was determined to be about 0.1 pmol injected. This corresponds to 20–50 pg of fatty acid, which is of the same order of magnitude as that reported elsewhere[5,9].

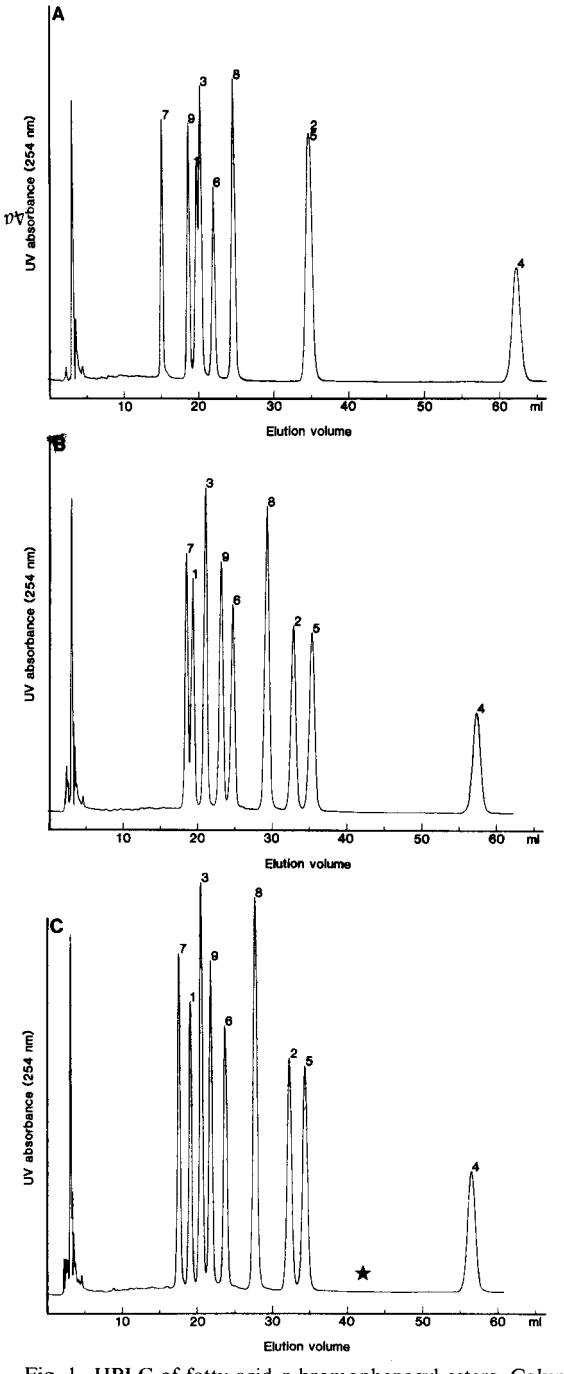

Fig. 1. HPLC of fatty acid *p*-bromophenacyl esters. Columns: 49 × 4.6 mm guard column dry packed with 40-μm pellicular reversed-phase material (Pelliguard LC-18) and a 250 × 4.6 mm Supelcosil LC-18 analytical column. Flow-rate: 1.0 ml/min with pressure *ca.* 1000 p.s.i. Eluents: A, acetonitrile–water (91:9); B, methanol–water (91:9); C, Methanol–acetonitrile–water (82:9:9). ★, Elution volume of margaric acid (17:0). See Table I for peak identification.

TABLE I

RETENTION VOLUMES OF FATTY ACID STANDARDS

p-Bromophenacyl esters of different fatty acids were subjected to HPLC on a 250 × 4.6 mm Supelcosil LC-18 reversed-phase column. Flow-rate, 1.0 ml/min with pressure *ca.* 1000 psi. Eluents: A: acetonitrile–water (91:9); B: methanol–water (91:9); C: methanol–acetonitrile–water (82:9:9).

*No.**	*Trivial name*	*Structure***	*Retention volume (ml)*		
			A	*B*	*C*
1	Myristic	14:0	19.7	19.3	19.3
2	Palmitic	16:0	34.8	32.8	32.8
3	Palmitoleic	16:1	20.2	21.0	20.7
4	Stearic	18:0	62.8	57.4	57.4
5	Oleic	18:1	34.8	35.3	35.0
6	Linoleic	18:2	22.0	24.7	24.0
7	Linolenic	18:3	15.1	18.4	17.8
8	Eicosatrienoic	20:3	24.6	29.3	28.1
9	Arachidonic	20:4	18.6	23.1	22.0
	Margaric	17:0			44.0

* These numbers correspond to the peaks in Fig. 1.

** The number to the left of the colon is the number of carbon atoms; the number to the right represents the number of double bonds present in the molecule.

The method described here has several advantages compared with previously published methods[1,2,4,5,7,9,10]. Good resolution of all the individual fatty acids of interest is obtained. The chromatographic procedure is fast and the elution volumes are very reproducible. The procedure is based on an isocratic constant-flow system, and can easily be adapted to automatic injection. It is thus a simple, sensitive and reliable method for the separation of naturally occuring C_{14}–C_{20} fatty acids, which has been used successfully in our laboratory for the quantification of free fatty acids in human serum and in the analysis of the fatty acid composition of lipids in human monocytes cultured *in vitro*.

ACKNOWLEDGEMENTS

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Note

Preparative purification and group separation of mono- and dinucleotides by combining charge-transfer and affinity chromatography

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(Received November 17th, 1981)

The study of the binding sub-sites of the well known enzyme bovine pancreatic ribonuclease (RNAase, E.C. 3.1.27.5) has been attempted in several ways (X-ray diffraction, NMR spectroscopy, affinity labelling)[1–8]. All of these techniques require non-substrate ligands and, although most studies have been carried out with mononucleotides, it is obvious that a dinucleotide can provide a better picture of the interaction between the enzyme sub-sites and a high-molecular-weight substrate. It is for this reason that the chromatographic separation on a preparative scale of 2′-O-methylated dinucleotides which are alkali-stable was undertaken. The interaction of these compounds with the enzyme should give a closer idea of the enzyme–substrate complex than that obtained with either the phosphonate analogues of the dinucleoside monophosphates in which the critical oxygen of the 5′-phosphoester bond is replaced by a CH_2 group[1] or a 2′,5′-dinucleotide[2] with distorted geometry in the phosphodiester bond.

On the other hand, alkali-stable dinucleotides are found in a low proportion (about 3%) in RNA. The separation of the sixteen different dinucleotides has only been accomplished on a small scale by means of very complicated and tedious procedures[9,10]. In this work partial purification on the milligram scale was achieved by means of a combination of charge-transfer chromatography on acriflavin-Sephadex G-25 and affinity chromatography on Sepharose 4B-RNAase.

The existence and characteristics of charge-transfer complexes between biomolecules has been known for over 10 years[11,12]. The pioneering work of Porath and his collaborators laid the theoretical basis[13,14] and showed the applicability of this type of interaction to the purification of many substances[15,16]. The best characterized gel of the electron-acceptor type is acriflavin-Sephadex G-25. Its behaviour and response to a set of different conditions (temperature, ionic strengh, pH, buffer composition and presence of different ions) has been thoroughly studied[16,17]. These are some of the reasons that justified the choice of this gel for the partial resolution of complex dinucleotide mixtures. The charge-transfer chromatography on acriflavin-Sephadex G-25 was also used for the final purification of some brominated nucleotides from the unreacted starting materials. Halogenated nucleotides are interesting compounds for the affinity labelling of RNAase[7] and other enzymes[18].

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EXPERIMENTAL

Acriflavin-Sephadex G-25 was synthesized as described by Egly and Porath[16]. Commercial bovine pancreatic ribonuclease (crystallized twice) was obtained from Cambrian Chemicals (Croydon, Great Britain) and was immobilized on Sepharose-4B according to the method of Axén *et al.*[19]. 8-bromoadenosine 5′-monophosphate (5′-Br^{8}AMP) and 8-bromoadenosine 2′,3′ cyclic monophosphate (2′,3′-Br^{8}AMP) were synthesized from 5′-AMP (Merck, Darmstadt, G.F.R.) and 2′,3′-AMP (Cambrian Chemicals) respectively, by direct bromination according to the method of Ikehara *et al.*[20]. After the reaction, the unreacted bromine was extracted with carbon tetrachloride and the nucleotides were freeze-dried. They were stored in a desiccator at −20°C.

The fraction corresponding to the sixteen 2′-O-methylated alkali-stable dinucleotides was obtained by the method of Trim and Parker[10] from wheat germ ribosomal RNA prepared as described by Glitz and Decker[21]. The dinucleotide fraction was stored as a freeze-dried powder at −20°C.

Some of the dinucleotide peaks were identified by means of thin-layer chromatography (TLC) and UV absorbance ratios[10].

RESULTS AND DISCUSSION

Separation of halogenated from non-halogenated mononucleotides

A 3-ml volume of an equimolar mixture of 5′-AMP and 5′-Br^{8}AMP (3 mg/ml each) in 0.2 *M* ammonium acetate–acetic acid buffer (pH 6.0) were loaded on to a column (46 × 2.6 cm I.D.) of acriflavin-Sephadex G-25 at a flow-rate of 40 ml/h. Elution was carried out at 4°C with the same buffer and the corresponding V_e/V_t values are given in Table I. The temperature (4°C) and the pH (6.0) were chosen so as to obtain maximal retardation and resolution among the different nucleotides as reported by Egly and Porath[16]. In the preparative separation of the brominated nucleotides from the unreacted starting materials (5′-AMP and 2′,3′-AMP) the same chromatographic conditions were used except that the sample volume was 1.5 ml at a concentration of 150 mg/ml. The V_e/V_t values are also listed in Table I.

The fact that the cyclic nucleotides are more retarded than the non-cyclic nucleotides is in agreement with published results on the analytical separation of 5′-AMP and 3′,5′-AMP[17]. Nevertheless, the high retardation of the brominated nucleotides with respect to the non-brominated ones would, in principle, be in disagreement

TABLE I

REDUCED ELUTION VOLUMES OF SOME MONONUCLEOTIDES ON ACRIFLAVIN-SEPHADEXG-25

Nucleotide	V_e/V_t*
5′-AMP	3.0
5′-Br^{8}AMP	7.5
2′,3′-AMP	5.4
2′,3′-Br^{8}AMP	12.6

* V_e is the elution volume, V_t the bed volume and V_e/V_t the reduced elution volume. The values were obtained in 0.2 *M* ammonium acetate buffer (pH 6.0).

with the behaviour of acriflavin-Sephadex G-25 as an acceptor gel[16,17]. The bromine, an electron-withdrawing substituent, should diminish the interaction of the halogenated nucleotide with the gel compared with the non-halogenated nucleotide. The results are, however, in agreement with the strong retardation of iodinated compared with non-iodinated tyrosines in dinitrophenyl-Sephadex G-25 (another π-electron acceptor gel)[13]. This effect was explained by the participation of the halogen itself in an electron-transfer interaction with the gel[13]. This could also be the case in the present experiments. An alternative explanation for the unexpected result could be the fact that acriflavin is situated in an intermediate position in the scale of donor and acceptor molecules[14]. Acriflavin could then act as an electron acceptor with 5′-AMP and as an electron donor with 5′-Br^{8}AMP, although other explanations are also possible.

If this behaviour of the halogenated nucleotides in acriflavin-Sephadex G-25 columns were general it would provide a powerful tool for separating halogenated from non-halogenated nucleotides in complex mixtures. This procedure has the advantage of being quick and suitable for scaling-up.

Fractionation of complex alkali-stable dinucleotide mixtures

The chromatographic fractionation of the 2′-O-methylated alkali-stable dinucleotides was attempted by combining charge-transfer chromatography on acriflavin-Sephadex G-25 and affinity chromatography on Sepharose 4B-RNAase. A mixture of the sixteen dinucleotides (3 ml at a total concentration of 7 mg/ml) in 0.2 *M* am-

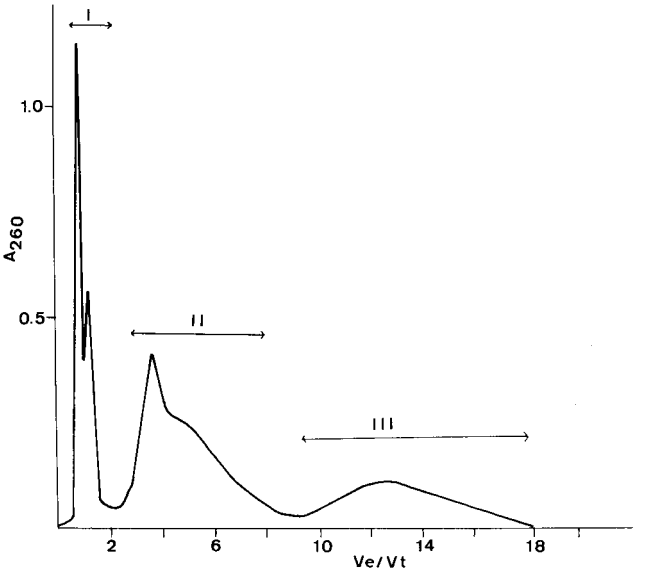

Fig. 1. Chromatography of a mixture of alkali-stable dinucleotides on acriflavin-Sephadex G-25. A 3-ml volume of 2′-O-methylated alkali-stable dinucleotides (7 mg/ml) was loaded on an acriflavin-Sephadex G-25 column (46 × 2.6 cm I.D.) in 0.2 *M* ammonium acetate buffer (pH 6.0). Elution was carried out with the same buffer at a flow-rate of 40 ml/h. V_e is the elution volume, V_t is the total volume and V_e/V_t is the reduced elution volume. Peak I represents dinucleotides of the PyrpPyrp type, peak II contains PyrpPurp and PurpPyrp dinucleotides and peak III dinucleotides of the PurpPurp type.

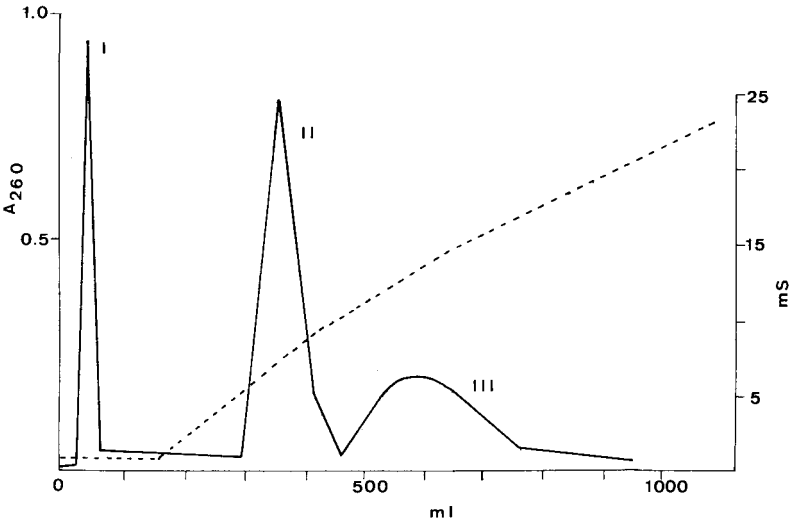

Fig. 2. Chromatography of a mixture of alkali-stable dinucleotides on Sepharose 4B-RNAase. A 1-ml volume of 2′-O-methylated alkali-stable dinucleotides (10 mg/ml) in 10 m*M* ammonium acetate buffer (pH 7.0) was loaded on a Sepharose 4B-RNAase column (35 × 1.2 cm I.D.). The flow-rate was 30 ml/h and the temperature was 4°C. Elution was carried out with a linear ionic strength gradient from 10 m*M* to 0.3 *M* (400 ml in each vessel) of ammonium acetate buffer (pH 7.0). Peak I represents PurpPurp dinucleotides, peak II a mixture of PyrpPyrp and PurpPyrp dinucleotides and peak III dinucleotides of the PyrpPurp type.

monium acetate buffer (pH 6.0) was loaded on to a column (46 × 2.6 cm I.D.) of acriflavin-Sephadex G-25. The flow-rate was 40 ml/h and the elution was carried out with the same buffer at 4°C. Three well separated fractions were obtained and identified as shown in Fig. 1. The order of elution was in agreement with the published data for some natural dinucleoside monophosphates[16].

The elution profile of a chromatogram of a similar sample of total dinucleotides applied to a Sepharose 4B-RNAase column is shown in Fig. 2. Fraction I, not retained in the conditions of equilibration of the column, was shown to be constituted by dinucleotides of the Pur–Pur type whereas the other two, eluted by means of an ionic strength gradient, followed the order expected from the known affinities for the binding sub-sites of the enzyme[1]. It should be noted that affinity chromatography has been widely used to purify proteins by immobilizing a suitable low-molecular-weight ligand[22]. In the case of nucleic acid fragments or analogues, purification has been mainly attempted by immobilizing not proteins but complementary strands of nucleic acids[22]. In this work a different approach was used, namely immobilization of the enzyme for the fractionation of a complex mixture of low-molecular-weight ligands based on the different affinity of the individual ligands towards the enzyme.

In order to fractionate further peak II of the Sepharose 4B-RNAase column it was chromatographed, after freeze-drying, through a long acriflavin-Sephadex G-25 column (110 × 1.2 cm I.D.). The experimental conditions and results are shown in Fig. 3. Experiments are in progress to fractionate further the unresolved dinucleotides by means of other chromatographic procedures.

These examples show the great potential of the combination of charge-transfer

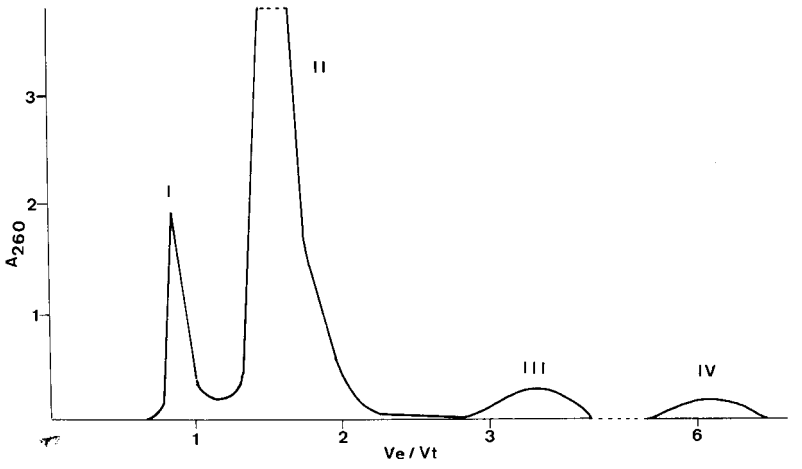

Fig. 3. Chromatography of a partially purified mixture of alkali-stable dinucleotides on acriflavin-Sephadex G-25. A 0.3-ml volume of peak II from the chromatogram in Fig. 2 at a concentration of 11 mg/ml in 0.2 *M* ammonium acetate buffer (pH 6.0) was chromatographed on an acriflavin-Sephadex G-25 column (110 × 1.2 cm I.D.) at a flow-rate of 15 ml/h at 4°C. The assignment of the peaks was as follows: I, CmpCp (V_e/V_t = 1); II, CmpUp, UmpCp, UmpUp (V_e/V_t = 1.5); III, AmpCp, GmpCp (V_e/V_t = 3.1); IV, AmpUp, GmpUp (V_e/V_t = 6). m in the above abbreviations represents a methyl group in the 2′-position of the ribose ring of the corresponding nucleoside.

and affinity chromatography for the preparative purification of complex mixtures of mono- and dinucleotides. The method is rapid and simple and avoids the tedious procedures that use ion-exchange chromatography with[23] or without[9,10] urea. Obviously, the step that uses the immobilized enzyme can be used only when the nucleotides are not substrates for the enzyme such as, in the case of RNAase, the 2′-O-methylated di- and oligonucleotides, 2′,5′-oligonucleotides or 2′-deoxyribonucleotides (single-stranded DNA).

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Note

Capillary tube isotachophoretic separation of nucleotides using complex-forming equilibria

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Many papers have described the separation of nucleotides using various methods, *e.g.*, liquid chromatography[1–7], electrophoresis[8–12] and isotachophoresis[13–16].

Beckers and Everaerts[13] studied the separation of nucleotides by isotachophoresis and showed that the separation of complex mixtures was difficult and the use of some other techniques, *e.g.*, a counter-flow of electrolyte, might be necessary. We found that the differences among the effective mobilities of nucleoside triphosphates were small in comparison with those of di- or monophosphates.

The use of complex-forming equilibria in capillary tube isotachophoresis is a powerful technique for improving the separability. Inorganic anions[17], organic acids[18], some EDTA complexes[19], alkaline earth metal cations[20] and lanthanide cations[21] have been separated using this technique. In this paper we show that nucleotides can be effectively separated by capillary tube isotachophoresis using complex-forming equilibria between magnesium(II) ion and the nucleotides.

EXPERIMENTAL

Isotachopherograms were recorded with a Model IP-1B capillary tube isotachophoretic analyser with a PGD-1 potential gradient detector (Shimadzu, Kyoto,

TABLE I

LEADING AND TERMINATING ELECTROLYTE SYSTEMS

Electrolyte	*Parameter*	*Value*
Leading	Leading ion	10.4 mmol dm^{-3} NO_3^- [added as nitric acid and magnesium(II) nitrate]
	Complexing agent	2.86 mmol dm^{-3} Mg^{2+} (added as nitrate)
	Additive	5% ethanol 0.005% poly(vinyl alcohol)
	Buffering counter ion	Adenosine
	pH	3.00
Terminating	Terminating ion	10 mmol dm^{-3} 2,2-dimethylpropanoic acid
	pH	3.5

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Japan). The length of the capillary tube (PTFE, I.D. 0.5 mm) was 20 cm. Measurements of pH were made with a Model F-7ss expanded-scale pH meter (Horiba, Japan).

Adenosine and 5′-nucleotides were obtained from Boehringer (Mannheim, G.F.R.). The other chemicals used were of the highest grade commercially available and deionized water was used. The leading and terminating electrolyte systems are shown in Table I. The nitric acid solution and the magnesium(II) ion solution were standardized by acid–base and chelatometric titration, respectively. In all experiments metal ions were added as nitrate and the leading ion (*i.e.*, nitrate) concentration was kept constant.

RESULTS AND DISCUSSION

Fifteen 5′-nucleotides (AMP, CMP, GMP, IMP, UMP, ADP, CDP, GDP, IDP, UDP, ATP, CTP, GTP, ITP and UTP)* were used as sample constituents. All experiments were carried out in a low pH region of the leading electrolyte because the differences among the effective mobilities of the nucleotides were larger than those in

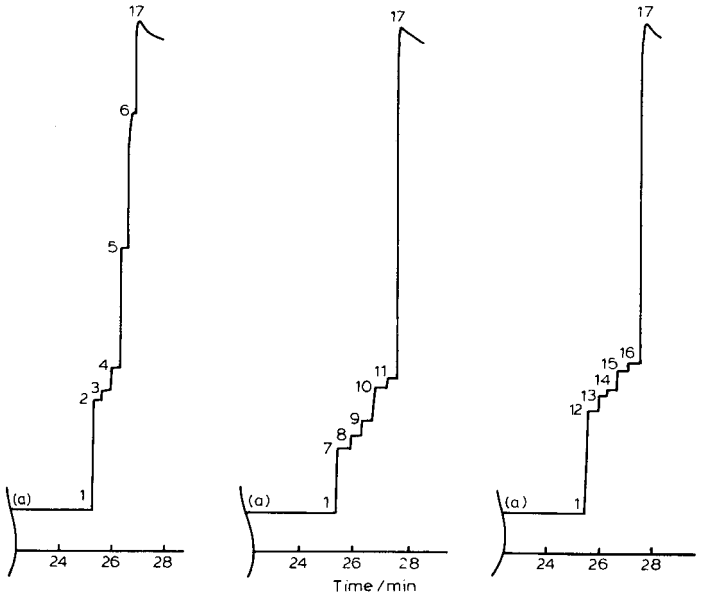

Fig. 1. Isotachopherograms of nucleoside mono-, di- and triphosphates. Conditions as in Tables I and II except chart speed, 10 mm min^{-1}. Differential potential gradient curves are not shown, to simplify the figures. (a) Potential gradient. 1 = Nitrate; 2 = UMP; 3 = IMP; 4 = GMP; 5 = AMP; 6 = CMP; 7 = UDP; 8 = IDP; 9 = GDP; 10 = ADP; 11 = CDP; 12 = UTP; 13 = ITP; 14 = GTP; 15 = CTP; 16 = ATP; 17 = 2,2-dimethylpropanoic acid.

* Abbreviations: AMP = adenosine monophosphate; CMP = cytidine monophosphate; GMP = guanosine monophosphate; IMP = inosine monophosphate; UMP = uridine monophosphate; ADP = adenosine diphosphate; CDP = cytidine diphosphate; GDP = guanosine diphosphate; IDP = inosine diphosphate; UDP = uridine diphosphate; ATP = adenosine triphosphate; CTP = cytidine triphosphate; GTP = guanosine triphosphate; ITP = ionsine triphosphate; UTP = uridine triphosphate.

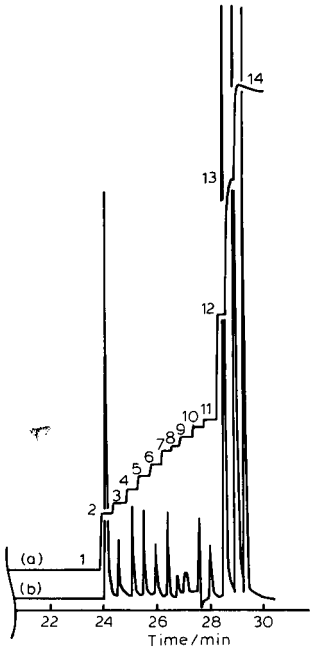

Fig. 2. Isotachopherogram of eleven nucleotides and phosphate. Conditions as in Fig. 1 except sample volume, 6.0 μl. (a) Potential gradient; (b) differential potential gradient. 1 = Nitrate; 2 = phosphate; 3 = UDP; 4 = IDP; 5 = GDP; 6 = UTP; 7 = ITP; 8 = GTP; 9 = CDP; 10 = CTP; 11 = ATP; 12 = AMP; 13 = CMP; 14 = 2,2-dimethylpropanoic acid.

a high pH region. In the initial experiments several metal cations were tested as complex-forming reagents with the nucleotides. It was found that magnesium(II) was one of the most effective ions and calcium(II) had almost the same effect as magnesium(II).

The effective mobilities of the nucleotides decreased with increasing magnesium(II) ion concentration in the leading electrolyte and the differences among the mobilities of some nucleotides were increased. To obtain a sufficient separation of nucleotides it was preferable to make the magnesium(II) ion concentration high. Such conditions, however, make the buffering ability of the leading electrolyte low because the amounts of the buffering agent, *i.e.*, adenosine, decrease with increase in the magnesium(II) ion concentration to keep the pH of the leading electrolytes constant.

As the stability constants of metal complexes are higher in organic than in aqueous media[22], ethanol was added to the leading electrolyte to enhance the interaction between magnesium(II) ion and the nucleotides. This effect appeared when several percent of ethanol were added. Not only were the differences among the mobilities of some nucleotides increased and the separabilities improved by adding ethanol to the leading electrolyte, but also the isotachopherograms obtained were better than those obtained in experiments with no ethanol: the sloping steps obtained with some sample constituents were improved to a flat shape and the drift of the potential gradient of the leading ion became very small.

As shown in Fig. 1, five kinds of nucleoside mono-, di- and triphosphates could

TABLE II

PR VALUES AND ZONE LENGTHS

The PR value (potential gradient ratio) is the ratio of the potential gradient of the leading ion to that of the sample ion (PG_L/PG_S), which corresponds to the ratio of the mobility of sample ion to that of the leading ion (m_S/m_L)[16]. Conditions as in Table I; other conditions, migration current, 75 μA; chart speed, 40 mm min^{-1}; sample, about 4 nmol; sample volume 2.0 μl.

Compound	*PR value**	*Relative standard deviation (%)*	*Zone length (mm)**	*Relative standard deviation (%)*
Phosphate	0.427	1.9	14.9	2.7
AMP	0.135	2.0	14.4	2.0
CMP	0.0293	2.0	12.2	1.2
GMP	0.225	2.3	14.7	1.2
IMP	0.257	1.9	12.6	1.7
UMP	0.272	2.0	14.3	2.0
ADP	0.246	2.7	22.2	2.7
CDP	0.233	2.9	17.7	2.1
GDP	0.308	2.4	17.3	2.3
IDP	0.344	2.3	16.6	2.1
UDP	0.387	2.5	21.0	1.7
ATP	0.216	1.4	20.1	2.1
CTP	0.226	1.5	16.8	2.2
GTP	0.251	1.6	16.2	1.7
ITP	0.261	1.7	12.9	1.7
UTP	0.287	1.3	16.8	1.8

* Average of six determinations.

be separated into individual constituents. Furthermore, twelve sample constituents, including five nucleoside triphosphates, four nucleoside diphosphates, two nucleoside monophosphate and phosphate, could be separated simultaneously, as shown in Fig. 2. The complexing agent was 2.86 mmol dm^{-3} magnesium(II) ion and 5% of ethanol was added to the leading electrolyte. Table II shows that the relative standard deviations of the PR values of the nucleotides and phosphate are 1.3–2.9% and those of the zone lengths are 1.2–2.7% with about 4 nmol of sample constituents.

The effective mobilities of the nucleotides were very sensitive to the ethanol and magnesium(II) ion concentrations in the leading electrolyte. In other words, suitable conditions for given samples can be chosen by adjusting the ethanol or magnesium(II) ion concentration in the leading electrolyte. The mobilities of the nucleotides were also sensitive to the pH of the leading electrolyte. Satisfactory separations of the nucleotides could not be obtained at pH 5.3 and 8.5.

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Note

Separation of human insulin and some structural isomers by high-performance liquid chromatography

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Insulin is a polypeptide hormone of molecular weight *ca.* 6000 composed of two chains (A and B) joined by two disulphide bonds. The A chain contains a further internal disulphide bond. The cystine residues involved in these bonds are apparently invariant in natural insulins[1], but the sequence of amino acids in each chain may change. At present the only insulins used therapeutically are of porcine or bovine origin. However, the synthesis of human insulin by chemical[2], semisynthetic[3] or DNA recombinant[4] methods has been described. If the A and B chains are synthesized separately and then combined, theoretically a number of disulphide bond isomers could be formed.

Reversed-phase high-performance liquid chromatography (HPLC) has proved to be a powerful technique for the analysis of insulins, capable of separating human, bovine and porcine insulins[5,6] although human and porcine insulin differ in only a single amino acid residue in the B chain and bovine and porcine insulin differ in only two amino acid residues in the A chain.

We have now examined the behaviour of synthetic human insulin, a disulphide bond isomer and several D-Cys analogues[7,8] in two reversed-phase HPLC systems[6,9] and suggest that such systems may be suitable for detection of by-products in the synthesis of insulin.

EXPERIMENTAL

Materials

Chemicals and solvents were obtained from BDH (Poole, Great Britain) and were of "AnalaR" grade where possible. Other materials were: L(+)-tartaric acid from Sigma (Poole, Great Britain), acetonitrile (HPLC grade) from Fisons (Loughborough, Great Britain) and 2-methoxyethanol (autoanalyser grade) from Koch Light (Colnbrook, Great Britain).

Synthetic human insulin, three analogues synthesized with D-cysteine at positions A7, A11, and B7, respectively, and the disulphide bond isomer A7-A11, A6-B7 were obtained from Ciba-Geigy (Basle, Switzerland). Because of their limited availability all except the first of these insulins had been recovered by Ciba-Geigy from previous spectroscopic experiments. Each sample was dissolved in 0.01 *M* hydrochloric acid to give a nominal concentration of 2 mg/ml.

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HPLC

An Altex Model 110A pump and a Cecil CE 2012 variable-wavelength UV monitor were used. Injections were made with a Rheodyne 7125 sample-injection valve fitted with a 20-μl loop. A precolumn (50 × 5.0 mm I.D.) dry packed with LiChroprep Si 60 (15–25 μm, Merck, Darmstadt, G.F.R.) was fitted to the system before the injection valve. Separations were performed at ambient temperature on either a prepacked 250 × 4.6 mm I.D. column of Zorbax-TMS (6 μm) (Dupont, Hitchin, Great Britain) or a 150 × 5.0 mm I.D. column of ODS Hypersil (5 μm, Shandon Southern, Runcorn, Great Britain) slurry packed in propan-2-ol. Solvent mixtures were degassed in an ultrasonic bath and filtered before use. Two systems were examined:

System I (Zorbax TMS column). The mobile phase was prepared as follows: solution A: 0.1 *M* Sodium dihydrogen orthophosphate in water–2-methoxyethanol (95:5) adjusted to pH 2.0 with orthophosphoric acid; solution B: acetonitrile–2-methoxyethanol (95:5)[10]. These solutions were mixed in the proportions 74% A–26% B. The column eluate was monitored at 210 nm.

System II (ODS Hypersil column). The mobile phase consisted of acetonitrile–0.1 *M* ammonium sulphate (25:75) + 0.005 *M* L(+)-tartaric acid in water pH 3.0. Cetrimide was added to give a final concentration of $14 \cdot 10^{-6}$ *M*. The eluate was monitored at 225 nm.

Thin-layer chromatography (TLC)

TLC was performed on plates precoated with microcrystalline cellulose (Schleicher and Schüll G1440) in two systems[2]: *System 155:* pentan-1-ol–pyridine–water–butan-2-one–formic acid (40:28:15:11:5) and *system 112E:* butan-1-ol–pyridine–water–formic acid (44:24:20:2). The spots were located with ninhydrin.

RESULTS AND DISCUSSION

Separations of human insulin and its analogues in the two HPLC systems are shown in Fig. 1, and the capacity factors (k') obtained from each compound are listed in Table I. The k' quoted is for the major peak in each sample as some heterogeneity (not detected by TLC) was observed. All analogues were well separated from human insulin in both HPLC systems and almost complete resolution was achieved using system I. The order of elution was similar in both cases although the disulphide bond isomer eluted before the D-Cys B7 analogue in system I (Fig. 1A) but after, and only partially resolved from it, in system II (Fig. 1B). The D-Cys A11 analogue showed the greatest difference in retention behaviour relative to human insulin. This was most marked in system II.

The TLC results are presented in Table I. The chromatographic mobilities of the analogues relative to those of insulin are in good agreement with published results[7]. However the discrimination of the TLC systems is not as great as that of the HPLC systems. Although TLC possesses advantages as a rapid screening procedure, analyses by HPLC provide more detailed information.

The behaviour of these synthetic insulins on HPLC confirms previous work showing that the analogues appear to possess more hydrophobic character than native insulin[11]. For the diastereoisomers, the effect of substitution on the retention

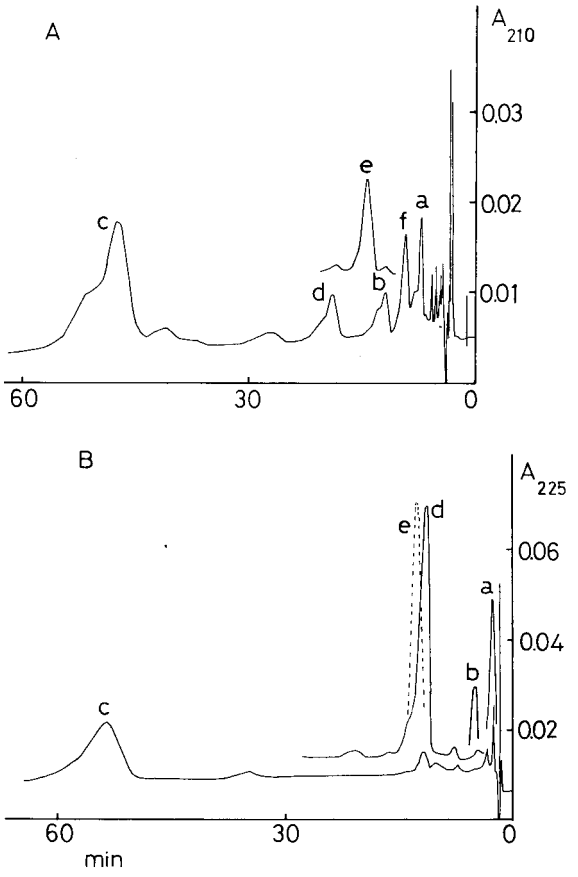

Fig. 1. Composite chromatograms showing the behaviour of synthetic human insulin and some analogues. A, Column: Zorbax TMS; solvent: see text; monitored by UV at 210 nm. B, Column: ODS-Hypersil; Solvent: see text; Monitored by UV at 225 nm; Peaks a and b 1/10 actual size. Analyses conducted at ambient temperature, flow-rate 1.0 ml/min. a = Synthetic human insulin; b = analogue with D-Cys A7; c = analogue with D-Cys A11; d = analogue with D-Cys B7; e = disulphide isomer A7-A11, A6-B7.

time reflects the extent of conformational change necessary to accommodate the appropriate D-Cys residue as predicted from examination of the crystal structure of native insulin[12]. All three substitutions appear to increase the accessibility of residues in the hydrophobic "core" of the molecule, thus explaining the observed increase in hydrophobic behaviour. On the same basis[12], the interchange of the disulphide bonds at residues A6 and A7 might also be expected to produce a considerable conformational change in the molecule with comparable effects. The chromatographic behaviour of the disulphide bond isomer suggests that this is indeed the case.

Insulin readily degrades to monodesamidoinsulin in solution, especially at acid pH, by deamidation of the terminal asparagine residue at A21. Analysis of mixtures of porcine, bovine, and their respective monodesamidoinsulins is not practicable under simple isocratic conditions because native porcine insulin and beef monodesamidoinsulin coelute. However, such mixtures may be completely resolved by gradient elution[13] or by use of a hydrophobic ion-pair system[9]. The latter, employed as system

TABLE I

CHROMATOGRAPHIC BEHAVIOUR OF SYNTHETIC HUMAN INSULIN AND SOME ANALOGUES

For details of chromatographic systems see text.

	HPLC k'		*TLC**	
	System I	*System II*	*System 155*	*System 112E*
Human insulin	1.9	1.2	1.0 (R_F 0.08)	1.0 (R_F 0.31)
D-Cys A7	4.1	3.1	1.52	1.14
D-Cys A11	21.9	43.7	2.10	1.23
D-Cys B7	7.2	8.9	1.48	1.12
Disulphide bond isomer A-7-A11, A6-B7	5.1	9.7	1.90	1.15

* Migration distances on microcrystalline cellulose relative to human insulin.

II here, is also able to separate the isomeric analogues of human insulin that we have studied. It will not, however, resolve human and porcine insulins.

The availability of insulin analogues of defined structure has enabled us to assess the value of HPLC for their separation. Our results demonstrate the potential of such a technique for analysis of insulin obtained by synthesis, in particular in separating disulphide bond isomers. The differences in behaviour of the analogues in the two HPLC systems employed, however, and the inability of one system to separate human and porcine insulins having different amino acid sequences, suggest that undue reliance should not be placed on homogeneity of such complex molecules in a single HPLC system.

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Note

Determination of cefsulodin sodium [D(—)-SCE-129] by high-performance liquid chromatography

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Cefsulodin sodium or D(−)-SCE-129 (I, Fig. 1) is a unique semisynthetic cephalosporin antibiotic having pronounced antipseudomonal activity[1–4]. The drug substance is chemically [6R-[6α,7β(R*)]]-4-(aminocarbonyl)-1-[[2-carboxy-8-oxo-7-[(phenylsulfoacetyl)amino]-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl[methyl]-pyridinium hydroxide inner salt, monosodium salt. This paper describes the analysis of cefsulodin sodium in 500 mg (potency) vials using reversed-phase high-performance liquid chromatography (HPLC). Assay results obtained by HPLC are compared with those obtained by the currently accepted hydroxylamine and microbiological agar diffusion methods.

Procedures reported in the literature for the quantitation of β-lactam antibiotics use various microbiological[5], polarographic[6–8], gas–liquid chromatographic[9] and chemical techniques. Included in the chemical assays for these compounds are iodometric titration[10,11], fluorometry[12,13] and colorimetric procedures after reaction with either hydroxylamine[14–16], ninhydrin[17] or nicotinamide[18]. The two official procedures specified most often for cephalosporins are the microbiological agar diffusion[19] and the hydroxylamine[20] methods.

In this work HPLC is used because it offers the desired combination of speed, accuracy and sensitivity. The HPLC procedure is specific and stability indicating, since cefsulodin sodium is resolved from its various manufacturing impurities and/or degradation products (II–V, Fig. 1). Procedures using both ion-exchange and reversed-phase HPLC are reported in the literature for the quantitation of β-lactam antibiotics in bulk drug[21,22], dosage forms[23–25], fermentation broths[26,27] and biological matrices[28–32].

EXPERIMENTAL

Apparatus

The HPLC system consisted of a model M-6000A pump and U6K injector (Waters Assoc., Milford, MA, U.S.A.), a SF 770 variable-wavelength UV detector (Schoeffel, Westwood, NJ, U.S.A.) and a SP-4100 data handling system (Spectra-Physics, Santa Clara, CA, U.S.A.). The chromatographic separations were achieved using a Zorbax® C_8 (4.6 mm I.D. × 25 cm) column (DuPont, Wilmington, DE, U.S.A.) fitted with a RP-8 (4.6 mm I.D. × 3 cm) pre-column (Brownlee, Santa Clara,

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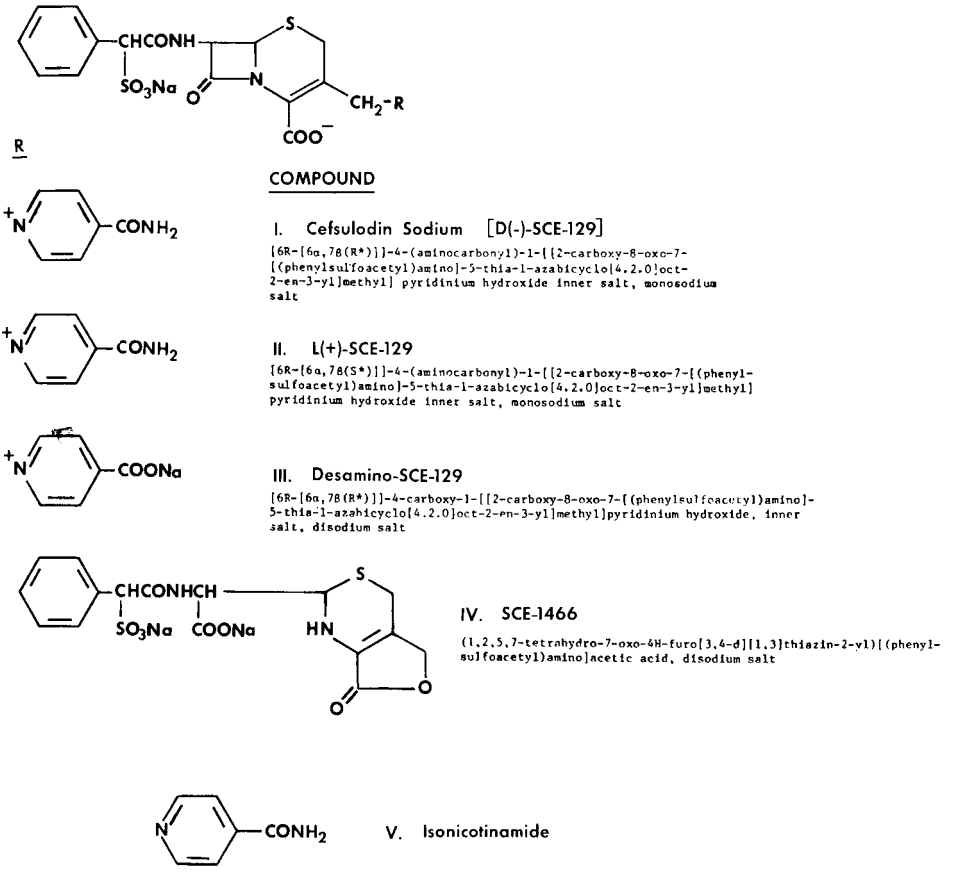

Fig. 1. Chemical structures of cefsulodin sodium (I) and manufacturing impurities and/or degradation products (II–V).

CA, U.S.A.). The HPLC eluent and solutions chromatographed were filtered through 0.45-μm polycarbonate membranes (Nuclepore, Pleasanton, CA, U.S.A.).

Reagents

Acetonitrile and methanol used were distilled-in-glass UV grade from Burdick and Jackson (Muskegon, MI, U.S.A.). Ammonium acetate was ACS grade from J. T. Baker (Phillipsburg, NJ, U.S.A.). Cefsulodin sodium reference standard and 500 mg (potency) vials were from Takeda (Osaka, Japan). Phenoxy acetic acid was 98% minimum purity from Aldrich (Milwaukee, WI, U.S.A.).

Chromatographic conditions

HPLC Eluent. 0.02 *M* ammonium acetate in water–methanol–acetonitrile (950:35:15). A 1.54-g portion of ammonium acetate was dissolved in 1 liter of aqueous solution containing 35 ml of methanol, 15 ml of acetonitrile and 4 ml of glacial acetic acid. If necessary, the eluent was adjusted to pH 4.1 by addition of more acetic acid.

Flow-rate. 1.5 ml/min.

Pressure. 1700 p.s.i.

Detector. 254 nm at 0.04 a.u.f.s., attenuation at 16 (with 1 mV integrator output).

Injection volume. 10 μl.

Analytical procedure

Cefsulodin sodium 500 mg (potency) vials were reconstituted and diluted with distilled water to obtain drug concentrations of approximately 2 mg/ml. A 5-ml portion of each sample was mixed with 5 ml of internal standard solution [16 mg/ml of phenoxy acetic acid in water–acetonitrile (9:1)], diluted to 100 ml with distilled water and chromatographed. The peak area response for each sample preparation was compared to that of a 100 μg/ml solution of cefsulodin reference standard prepared in a similar manner.

RESULTS AND DISCUSSION

The HPLC conditions described in this procedure give optimum separation of the drug substance and various impurities while maintaining a reasonable assay time. In preliminary work, cefsulodin sodium was quantitated using a reversed-phase ODS

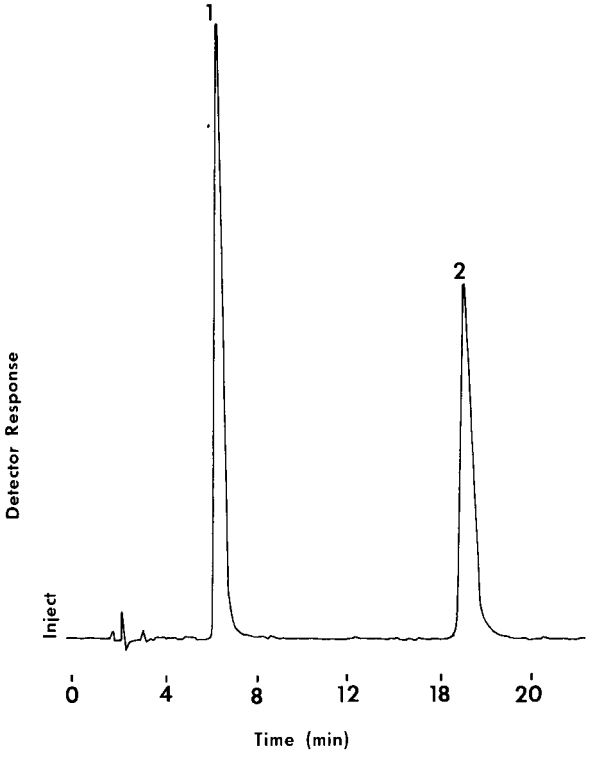

Fig. 2. Chromatogram of a typical cefsulodin sodium vial preparation. Conditions stated in text. Peaks: 1 = cefsulodin sodium, 2 = internal standard.

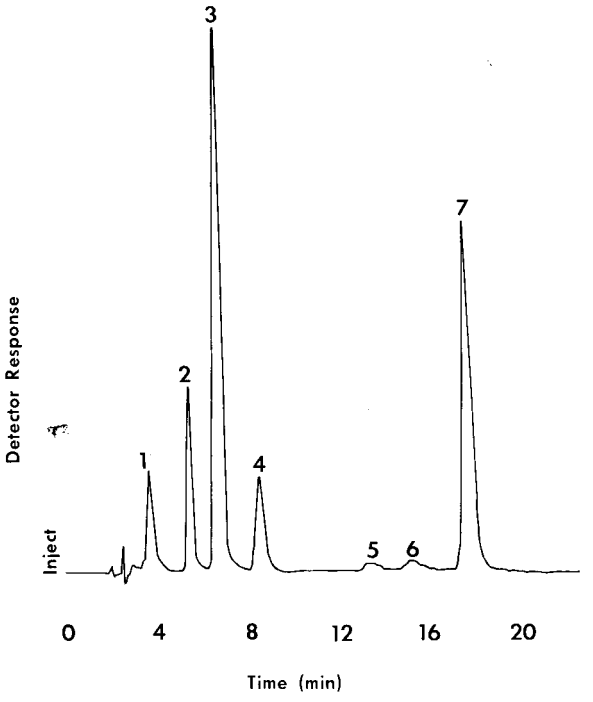

Fig. 3. Chromatogram of cefsulodin sodium standard preparation spiked with impurities (approx. 10% level), conditions stated in text. Peaks: 1 = desamino-SCE-129; 2 = L(+)-SCE-129; 3 = cefsulodin sodium; 4 = isonicotinamide; 5, 6 = SCE-1466 [possibly D(−) and L(+) isomers]; 7 = internal standard.

column with a 0.02 *M* ammonium acetate buffer–acetonitrile (98:2) eluent. This system, while equivalent to that described here for potency determination, failed to completely resolve the minor impurities from each other.

Presented in Fig. 2 is a typical chromatogram of a cefsulodin sodium vial preparation described in the text, while Fig. 3 is a chromatogram of the cefsulodin sodium reference standard preparation which was spiked (at about the 10% level) with possible degradation products and manufacturing impurities. Linearity of the detector response was demonstrated for cefsulodin sodium free acid concentrations

TABLE I

POTENCY DETERMINATION (PERCENT LABEL CLAIM) FOR CEFSULODIN SODIUM 500-mg VIALS BY VARIOUS METHODS

Sample	*HPLC*	*Hydroxylamine*	*Microbiological*
1	104.0	103.4	104.8
2	106.2	103.8	103.4
3	101.8	105.4	103.0
4	107.2	106.8	103.6
5	104.6	104.8	101.4
6	104.2	102.4	101.0

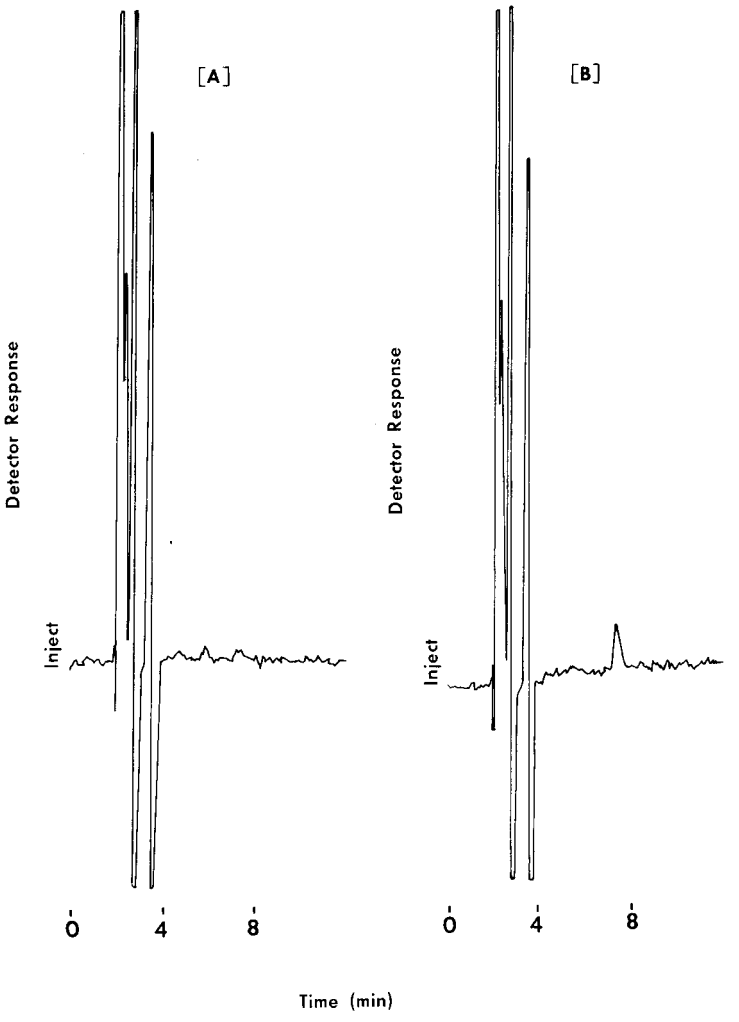

Fig. 4. Chromatograms of [A] solution blank and [B] 0.10 μg/ml cefsulodin standard. Conditions stated in text; using 75 microliter injection volume, UV detector at 261 nm, sensitivity 0.04 AUFS at attenuation 2.

of 29 to 145 μg/ml. The plot of free acid concentration *vs.* peak area ratios of drug to internal standard essentially intersected the origin and had a correlation coefficient of 0.9999.

In this work, the HPLC detector was operated at 254 nm since this allows the use of fixed-wavelength instruments. Cefsulodin sodium exhibits a wavelength maxima at 261 nm in aqueous solution ($\varepsilon = 1.52 \times 10^4$ l mole^{-1} cm^{-1}). Using a UV detector operated at the maxima and larger injection volumes cefsulodin sodium is detectable and may be quantitated to 0.10 μg/ml. In Fig. 4 a 0.10 μg/ml cefsulodin sodium solution and solution blank are chromatographed under conditions described in the text.

To compare the HPLC procedure for cefsulodin sodium with official chemical

TABLE II

POTENCY DETERMINATION (PERCENT LABEL CLAIM) FOR DEGRADED CEFSULODIN SODIUM 500-mg VIALS BY VARIOUS METHODS

Sample	*HPLC**	*Hydroxylamine*	*Microbiological*
Heat	103.4	102.6	104.6
UV	103.8	103.0	104.4
Water–heat	84.2	91.0	87.8
pH 8 Buffer–heat	43.0**	76.2	50.4

* Solutions were chromatographed on an ODS column using a 0.02 *M* ammonium acetate–acetonitrile (98:2) eluent.

** The HPLC assay showed 22% label claim of the L(+) isomer of cefsulodin sodium.

and microbiological assays, six lots of vials were assayed by HPLC, by the hydroxylamine[20] procedure and by the agar diffusion[19] method. As shown in Table I, potencies (as percent label claim) obtained by the three methods agreed well. As an additional comparison of the potency methods, individual cefsulodin sodium vials (Sample 6, Table I) were stressed under the following conditions: (a) dry heat at 110°C for 1.25 h, (b) intensive UV radiation for 1.25 h, (c) diluted to 50 ml with water and heated at 70°C for 30 min and (d) diluted to 50 ml with pH 8.0 phosphate buffer and heated at 70°C for 30 min. Each sample was assayed by the three methods described above and the results are summarized in Table II. For normal stability situations (heat, light and simple solution degradation), the methods are comparable. When cefsulodin is degraded in base, the chemical method shows a high bias, probably resulting from the conversion of the drug to its L(+) isomer.

Precision data for the HPLC procedure are summarized in Table III. Assays were performed by three analysts over several days and as shown the relative standard deviation was ±1.4%.

TABLE III

PRECISION DATA FOR THE HPLC ASSAY OF CEFSULODIN SODIUM 500-mg VIALS

Day	*Analyst*	*Percent Label Claim*
1	1	104.6
1	1	106.0
2	2	102.8
2	2	108.2
3	3	105.2
3	3	104.8
3	3	104.2
4	1	105.0
4	1	106.4
Mean		105.2
Standard deviation		±1.5
Relative standard deviation		±1.4%

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Note

Use of 6-*p*-toluidino-2-naphthalenesulfonic acid to quantitate lipids after thin-layer chromatography

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One of the most useful and generally applicable of all of the methods for the detection of lipids on thin-layer chromatography (TLC) is the use of compounds that fluoresce in hydrophobic environments. Several reports[1–3] have described the results obtained using 8-anilinonaphthalene-1-sulfonate (ANS). In this report, we present data which demonstrate that 6-*p*-toluidino-2-naphthalenesulfonic acid (TNS) is considerably more sensitive in the detection of lipids on thin-layer chromatograms than ANS because of its greater quantum yield. We have shown that very small amounts of lipid compounds such as cholesterol can be detected and quantitated using TNS. In addition, several other advantages in using a detection method such as the TNS procedure are presented.

EXPERIMENTAL

The TNS was obtained from Eastman (Rochester, NY, U.S.A.). The working solution was 1 m*M* TNS in 50 m*M* Tris-HCl, pH 7.4, and this solution was stable for several weeks stored in the dark. The reagent required gentle heating to bring the TNS into solution before use. TLC was conducted as usual using HETLC uniplates from Analtech (Newark, DE, U.S.A.) and a solvent of chloroform–methanol–water (65:25:4, v/v/v). After developing the chromatogram, the solvent was evaporated and the plate sprayed with a fine mist of the TNS solution. The lipid spots were observed under a UV lamp (short wave) or scanned using a Helena Quick Scanner equipped with a fluorescent lamp monitoring system. The lipids appear as bright spots on a dark background. For quantitative work, the amount of the lipid was determined by measuring the area of the peak after scanning. Standard cholesterol was obtained from Sigma (St. Louis, MO, U.S.A.). Myelin from rat spinal cord was isolated by the method of Norton and Poduslo[4], and lipids were extracted by the method of Bligh and Dyer[5]. The lipids were stored at −20°C in chloroform. Dolichol was isolated from pig liver as described by Richards and Hemming[6].

RESULTS AND DISCUSSION

The detection limits differed for each lipid, indicating that TNS has a different

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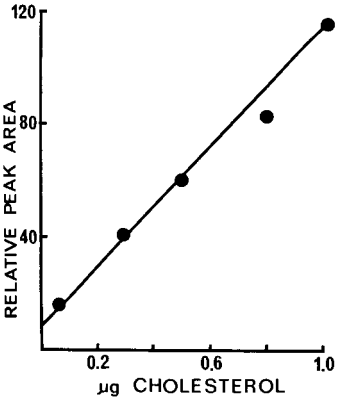
fluorescence quantum yield with different lipids; therefore, for quantitative work, standards must be used. For cholesterol, the linear range was 0.06–1.0 μg (Fig. 1). This is also the range detectable by UV light using ANS[1–3]. However, as little as 0.03 μg of cholesterol may be detected with the TNS reagent, using a UV lamp and direct visualization, making the TNS qualitatively more sensitive than the ANS reagent.

TNS was also a non-destructive reagent since it could be removed from the cholesterol after TLC. The UV-visible spots were scraped from the plate, and extracted with chloroform–methanol–water (1:1:0.9) by the procedure of Bligh and Dyer[5]. After re-applying the chloroform extract on a new plate, the extracted cholesterol co-chromatographed with authentic cholesterol and exhibited no fluorescence while the solvent front was fluorescent. Respraying the plate with TNS again showed the position of the cholesterol. The TNS did not significantly change the cholesterol R_F position. The ability to remove TNS from the lipid would allow for the further processing of a valuable material. The lipid could also be removed from the TLC



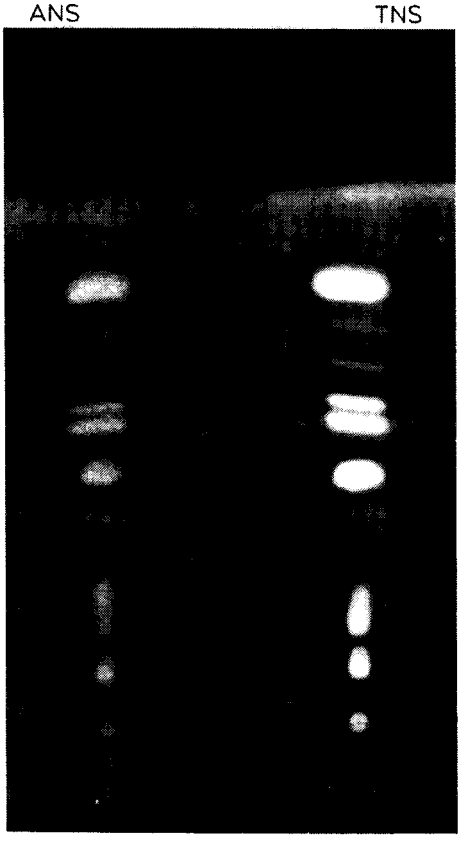


Fig. 1. The relative peak area measured after TNS treatment of cholesterol on TLC. The concentration of cholesterol was varied as indicated.

Fig. 2. Comparison of TNS and ANS with myelin lipids chromatographed on HETLC uniplates. The same amount of lipid extract was applied to each lane. ANS spray is on the left and TNS spray is on the right of the photograph.

plate and radioactivity evaluated, after drying the plate, since the TNS did not interfere with scintillation counting (data not shown).

To compare directly the sensitivity of TNS and ANS, both reagents were made up to the same molar concentration and used, on the same day, to evaluate myelin lipids. The same amount of extract was applied to each lane. The results are shown in Fig. 2. The left side of the plate was sprayed with ANS and the right side with TNS. The photograph (taken using Kodak Tri-X Pan film and UV light) clearly shows that the TNS fluorescence was more intense than that of ANS for each of the myelin lipid spots.

In another another experiment, authentic cholesterol standards were used to quantitate the cholesterol content of rat spinal cord myelin. The myelin lipid extract (10 μl) was applied to a HETLC uniplate and run as before. After evaporation of the solvent, spots of cholesterol standards were applied to another lane of the plate, and the plate was sprayed with TNS and scanned. Comparisons of the areas of the standard peaks indicated that the myelin cholesterol content was 0.81 μg per 10 μl of chloroform extract. The myelin chloroform extract was also evaluated for the cholesterol content by the enzymatic method of Allain *et al.*[7] which required 200 μl of chloroform to be read on the standard curve for this assay. This analysis indicated that 10 μl of chloroform contained 0.65 μg of cholesterol, which is in good agreement with the TNS data. The TNS value, however, was obtained more quickly and required considerably less sample.

In our laboratory, we have also used the TNS spray to detect dolichol in the range 0.04–0.70 nmoles on thin-layer chromatograms. This enabled us to monitor dolichol during the isolation process. TNS is a stable reagent which can be used in a rapid, sensitive, and non-destructive procedure for evaluating lipids on TLC. This procedure should have wide applicability in isolation and quantitation of lipids.

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Note

Thin-layer chromatography of neutral and acidic sugars from plant cell wall polysaccharides

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Thin-layer chromatography (TLC) has been used to separate carbohydrates[1-4]. However, these methods have been of limited use for the separation of mixtures of a large number of sugars, and so far no appropriate TLC technique exists which allows for a complete separation of all monomers obtained after hydrolysis of plant cell walls. The present report describes a two-dimensional thin-layer technique on unimpregnated cellulose for the separation of seven neutral and two acidic sugars such as those found in hydrolysates of plant cell wall polysaccharides.

EXPERIMENTAL

The thin-layer plates were prepared as follows: 15 g of cellulose MN 300 (Brinkman, Westbury, NY, U.S.A.) were homogenized with 100 ml of distilled water in a Wareing blender for 30 sec. The mixture was then spread onto glass plates (20 × 20 cm) at a thickness of 0.25 mm using an adjustable applicator (Desaga, Heidelberg, G.F.R.). After drying, the plates were heated for 15 min at 100°C. The sugars were applied to the lower corner of the plate at 2 cm from the bottom and 2 cm from the right edge. The following solvents were used: first direction, *n*-butanol–2-butenone–formic acid–water (8:6:3:3); second direction, phenol–water–formic acid (100:98:2, w/v/v organic phase only).

The sugar mixture was developed twice in the first direction. The plates were then chromatographed once in the second direction. Chromatography was carried out in glass tanks under saturated atmospheres. Visualization of the spots was achieved by spraying the plates with either anisidine phthalate[5] or *p*-aminobenzoic acid spray[6] and heating at 100°C for 10 min.

RESULTS AND DISCUSSION

Nitella axillaris cell walls were hydrolyzed in 2 *N* trifluoracetic acid for 1 h at 120°C. The hydrolysate was neutralized and chromatographed by the two-dimensional TLC method described above, and yielded the following sugars: glucose, galac-

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tose, mannose, xylose, arabinose, rhamnose, fucose, glucuronic acid and galacturonic acid (Fig. 1). The present method allowed for a clean separation of galactose and glucose, as well as arabinose and mannose which are difficult to separate in one-directional systems[1–3]. Quantitative evaluations of the separated sugars can be achieved by autoradiography[7] or fluorography[7]. Alternatively the sugars can be visualized directly with spray reagents. The colored spots are then decolorized with sodium borohydride to avoid quenching during the liquid scintillation radioassay[8].

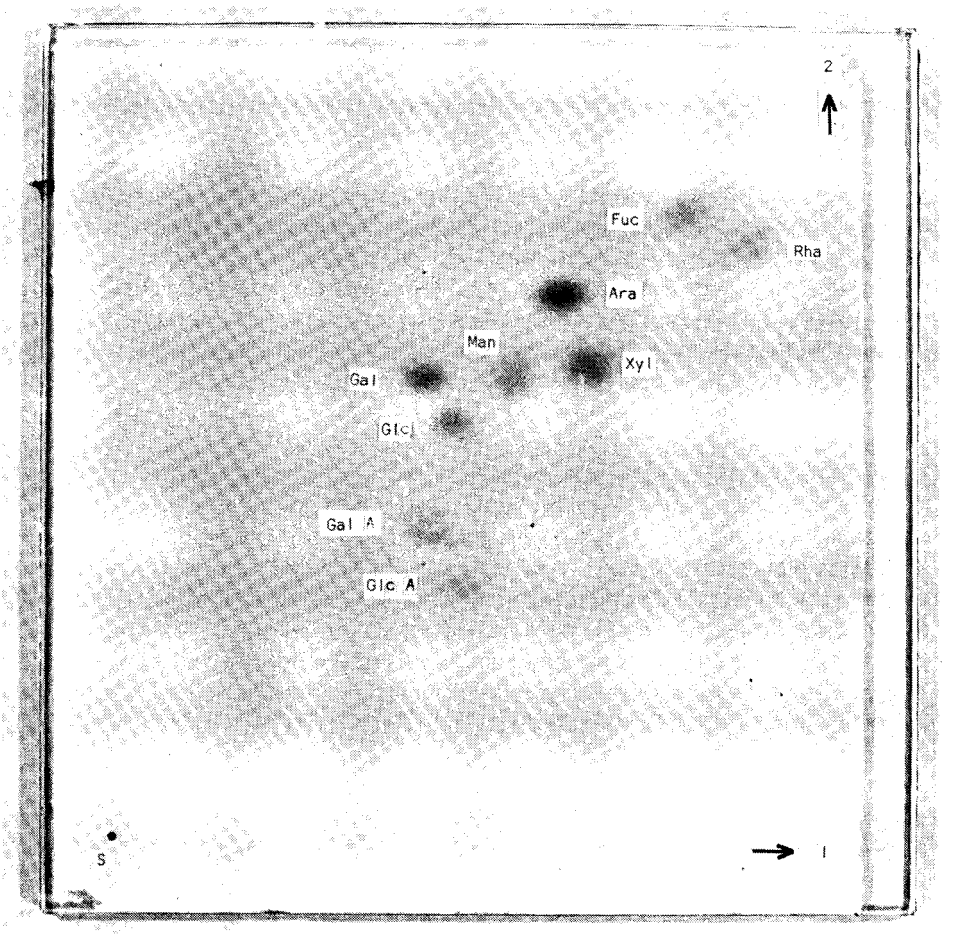

Fig. 1. Two-dimensional TLC on cellulose of a hydrolysate of *Nitella* cell wall polysaccharides. The solvents used are those described under Experimental. Spots: Glc = glucose; Gal = galactose; Man = mannose; Xyl = xylose; Ara = arabinose; Fuc = fucose; Rha = rhamnose; Glc A = glucuronic acid; Gal A = galacturonic acid. S = start; 1 = first direction; 2 = second direction. The plate was sprayed with anisidine phthalate. Development times: first solvent, 3 h; second solvent, 3.5 h.

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Note

Semi-quantitative thin-layer mass-screening detection of 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid in human urine

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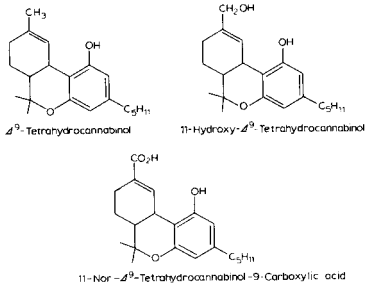
Marijuana is the term used to present the various preparations derived from the plant *Cannabis sativa* (family Cannabinaceae). Marijuana is not a simple drug; it is a complex mixture of over 400 individual chemicals[1]. Marijuana contains four constituents of similar structure, *i.e.*, Δ^9-tetrahydrocannabinol (Δ^9-THC); cannabidiol (CBD); cannabinol (CBN); and cannabichromene. The widespread abuse of marijuana throughout the world is primarily via smoking. Δ^9-THC is the most active of the principal constituents of marijuana and is contained in various parts of the plants in amounts varying from only trace to as high as 4% (w/w)[2]. Initial metabolism of cannabinoids in marijuana smoke takes place in the lungs, whereas initial cannabinoid metabolism of orally ingested marijuana takes place in liver. Major lung metabolites are usually side-chain hydroxylated metabolites, whereas major liver metabolites are usually hydroxylated derivatives of the cyclohexene ring system. There are over 35 metabolites of Δ^9-THC, 22 metabolites of CBD, and 22 metabolites of CBN known. Metabolism of Δ^9-THC in humans has been reported by several groups, with findings that 11-hydroxy-Δ^9-tetrahydrocannabinol and 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid are the principal metabolites. The identification of either of the above metabolites in body fluids will be the evidence that Δ^9-THC had been present. Because of the very small amounts of THC and 11-hydroxy-THC which appear in human plasma and urine, the major urinary metabolite, THC-9-carboxylic acid (THC-9-acid), can be used as an indicator of recent cannabis use. The concentration of this metabolite in plasma exceeds that of THC within 50 min



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after smoking and then remains higher for more than 24 h. This acid seems to be the result of further metabolism of the 11-methyl group, which is oxidized to a carboxyl group via the aldehyde[3,4]. Wall *et al.*[5] suggested that identification of this acid may be a practical method to identify cannabis users. Green *et al.*[6] also pointed out that nearly half of the total THC-9-acid present in urine can be extracted without hydrolyzing the specimen. In fact they postulated that unconjugated free THC-9-acid may be a practical indicator of the degree of physical impairment caused by the smoking of marijuana. Qualitative and/or quantitative procedures for the determination of carboxy-THC in body fluids have been reported using gas chromatography–mass spectrometry by Foltz *et al.*[7], Green *et al.*[6], Green[8], and Nordquist *et al.*[4]; high-performance liquid chromatography (HPLC) alone[9,10] or combined with radioimmunoassay (RIA)[11], RIA alone[12,13], and homogeneous enzyme immunoassay[14] techniques, have also been reported.

The purpose of this communication is to report a very sensitive thin-layer chromatographic TLC procedure which can be used as an indicator of a recent marijuana use in the urine of marijuana smokers. The procedure can detect the presence of marijuana in the urine of smokers who either smoke a few puffs through a tobacco pipe by burning small amounts of street marijuana (*ca.* 200 mg) or smoke *ca.* 1/4th of a marijuana cigarette for recreational purposes. The procedure is not only simple but also very specific and reproducible. It permits mass testing at very low cost since it neither needs sophisticated and costly equipment nor imposes special requirements such as (i) prior silylation of glassware, (ii) evaporation of extraction solvent under vacuum or at low temperatures under stream of nitrogen, or use of expensive reagents within a stipulated period of time. The total laboratory cost per specimen is less than US $ 2.00 including the labor of one skilled technician who performs 40 complete tests per day.

The test involves total extraction of free and conjugated 11-nor-tetrahydrocannabinolic acid (THCA). The urine specimen is subjected to a very mild alkaline hydrolysis and total acid is extracted at a pH of *ca.* 4.0 (3.0–4.0) with cyclohexane–ethyl acetate. The evaporation of the extraction solvent is accomplished either by employing a boiling water bath or in the air-circulated oven maintained at 80–85°C. A sample of standard 11-nor-Δ^8-THC-9-carboxylic acid in control urine is carried concomitantly through the assay procedure to be used as a standard for TLC along with unknown specimens. TLC separation is achieved on a 20 × 20 cm Gelman precoated silica gel glass microfiber sheet. The sensitivity of this proposed procedure is 50–100 ng/ml of urine and the volume of urine needed is 20 ml. An immunochemical test called the enzyme multiplied immunoassay technique (EMIT) is already on the market for the measurement of cannabinoid metabolites in human urine[14,15]. It is the most sensitive to 11-nor-Δ^9-THC-carboxylic and 11-hydroxy-Δ^9-THC, the predominant metabolites of Δ^9-THC. The detection limit of the assay is 50 ng/ml of urine[16]. The procedure is rapid and semi-quantitative, but any positive should be confirmed by an alternate non-immunological technique of comparable sensitivity. This laboratory analyzed more than 100 urine specimens using the EMIT system and all the positives obtained by the EMIT system were analyzed using the proposed TLC procedure. The results were 100% in agreement for the positives shown by the EMIT system. The authors are of the opinion that the proposed TLC procedure is suitable for mass testing, and only the positive urines need to be confirmed by the EMIT System in situations where a punitive action on a urine specimen is contemplated.

EXPERIMENTAL

Materials

11-nor-Δ^8-tetrahydrocannabinol-9-carboxylic acid and 11-nor-Δ^9-THC-9-carboxylic acid, Δ^9-THC, CBN, and 11-hydroxy-Δ^9-THC were obtained from the National Institute of Drug Abuse (Rockville, MD, U.S.A.).

Solvents

All solvents used for the extraction and TLC separation were of reagent grade as used in a standard analytical laboratory. The extraction solvent was cylohexane–ethyl acetate (96:4), and the TLC development solvent was chloroform–methanol–concentrated ammonium hydroxide (28–30%) (85:15:2, v/v/v).

Reagents

Potassium hydroxide (10%) solution in methanol, and glacial acetic acid. The detection reagent was Fast Blue RR, 0.5% (w/v) solution in an equal volume of methanol and water (Fast Blue RR must be highly pure, the one specially prepared for histochemical phosphatase determination by Calbiochem-Behring Corporation, Cat. No. 34134, mol.wt. 272, should be used. The reagent of lower purity could give false positives.)

Thin-layer plates

Gelman precoated silica gel glass microfiber sheets (ITLC) with a layer thickness of 250 μm were used. The use of these plates results in high sensitivity since a colored spot could form either on one side or both sides of the plate, and even a spot of a very minute intensity can be seen visually.

Extraction procedure

A 20-ml aliquot of urine (10–25 ml depending on the volume of urine available) is transferred to a 4-oz wide-mouth glass jar, and 2 ml of a methanolic solution of potassium hydroxide are added (10%, w/v). The contents are swirled, the mouth of the jar is covered with tinfoil, and the jar is placed in the air-circulating oven previously maintained at 100°C. After 12 min heating (10–15 min range), the rack containing jar (S) is removed and the contents are allowed to cool. The pH is then adjusted to a range of 3–4 by the addition of 3 ml of glacial acetic acid and 15 ml of cyclohexane–ethyl acetate (96:4) solution are added. The rack containing jar (S) is placed on a reciprocating shaker (Eberbach table model shaker) and after shaking for 10 min at low speed, the upper layer is pipetted out into a 40–50 ml plain conical glass centrifuge tube in a steel rack. The extraction is repeated twice more (total 3 × 15 ml extractions) and all the extracts are combined into the same test-tube. If the results are needed on the same day, the steel rack is placed in the boiling water bath and then evaporation of first extract is continued until the second extract is ready to be pipetted out. It is recommended that the second extract is pipetted out into the same tube when the solvent of the first extract has evaporated to *ca.* 5 ml and the third extract is pipetted out similarly. The steel rack containing the tube (S) is taken out when the solvent has just evaporated to dryness. Alternatively, all three extracts are combined and the rack containing tube (S) is placed in the air-circulating oven maintained at

80–85°C (the rack is placed a little farther from the air-circulating fan). The evaporation is carried out just to dryness, and the sides of the test-tube are washed with 0.5 ml of methanol, vortexed, and rinsed again with a few drops of methanol. The methanol is removed as above.

It is imperative that the standard of 11-nor-Δ^8-THC-9-carboxylic acid (THCA) must be carried through the assay procedure concomitantly with the unknown specimens(s). This standard should be used for the TLC identification of unknown specimens. A 20-ml aliquot of controlled urine is spiked with 2–4 μg of the tetrahydrocannabinolic acid* (100 ng/ml of urine), 3 ml of methanolic potassium hydroxide are added and the assay is carried out as above. Another 20-ml aliquot of the same controlled urine without spiking with the acid is also recommended to be carried through the assay procedure (blank controlled urine).

Thin-layer chromatography

One specimen of standard THCA (THCA which has been carried through the assay procedure in controlled urine) and 8–10 unknown specimens are spotted on a 20 × 20 cm ITLC Gelman precoated silica gel glass microfiber sheet. The residue obtained from the extraction procedure is spotted dexterously as follows. Methanol (10 μl) is added into conical tip of the tube, the tube is vortexed with swirling for 20–30 sec, and methanol is spotted on the plate using a 5-μl capillary tube. The process is repeated twice more. The sides of the tube are then washed with 0.5 ml of methanol, vortexed, sides rinsed with a few drops of methanol, the methanol is evaporated to dryness on a water bath or in the oven as described under *Extraction procedure.* Methanol (10 μl) is added and transferred to the plate on to the same spot as above. The process is repeated again with 10 μl of methanol (in all five 10-μl spottings are done). The spotting may be done under nitrogen or without nitrogen. The plate is dried in the oven at 90°C for 5 min after the standard and unknown specimens are all spotted. It is then placed in the freshly prepared development solvent (chloroform–methanol–concentrated ammonium hydroxide (85:15:2, v/v/v)**, shaken well until it forms a clear solution) and taken out after the solvent has travelled a distance of 15 cm or more (time taken to travel 15 cm is *ca.* 55–60 min).

Detection of tetrahydrocannabinolic acid

The plate is air-dried for 10 min and then dried at 100°C for 5 min and sprayed on both sides with a freshly prepared 0.5% Fast Blue RR solution (0.5%, w/v, in equal volumes of methanol and water). One pink spot of the acid compound is immediately seen at an R_F of *ca.* 0.25–0.38(3.8–5.8 cm). This spot becomes of greater intensity after the plate is allowed to stay in the air for 10–25 min. Respraying after 10–25 min also increases the intensity of the spot. In some specimens this pink spot is touching the greyish streak, but it is very distinct even when it is of the lowest intensity. A second pink spot simultaneously seen with the upper spot in some standards of tetrahydrocannabinolic acid carried through the assay procedure and in

* Prepare a solution of the standard 11-nor-Δ^8-THC-9-carboxylic acid by dissolving 10 mg of the acid in 10 ml of methanol (1 mg/ml) and use this solution for spiking. Store this in refrigerator.

** Since the quality of TLC plates differs from batch to batch, the volume of the solvent to be used for development must be weighed out in each batch, it varied from 45 to 100 ml.

unknown specimens, at the lower R_F value of *ca.* 0.093–0.23 (1.4–3.5 cm) should be ignored as it is sometimes seen in controlled urines as well. The positive unknown specimen can be semi-quantitated by scanning the thin-layer plate using Helna Quick Scan R and D TLC Densitometer. The areas under the peaks can be quantitated by attaching Quick Scanner out-put to the Hewlett-Packard Computer GC Terminal 5880A series.

RESULTS AND DISCUSSION

The proposed procedure is simple, specific, reproducible and cost effective. It is imperative that a controlled urine spiked with Δ^8-tetrahydrocannabinolic acid be carried through the assay procedure concomitantly with unknown urine specimen(s) since the Δ^8-tetrahydrocannabinolic acid not carried through the assay procedure gives a spot lower than the one carried through the assay procedure. The possibility that the acid might have decarboxylated to form tetrahydrocannabinol was eliminated since no tetrahydrocannabinol was seen on the thin layer plate. The tetrahydrocannabinolic acid (11-nor-Δ^8-THC-9-carboxylic acid) after it was carried through the assay procedure did not react with EMIT enzyme substrate, malate dehydrogenase, though it gave a positive reading prior to carrying through the assay procedure.

The reaction product needs rigorous purification since gas chromatography gave multiple peaks. The possibility of methyl ester formation* was also eliminated by forming the methyl ester and then performing gas chromatography on the ester, which gave a single peak as compared to the multiple peaks given by the reaction product. The acid product formed is highly specific and very sensitive to Fast Blue RR spray. No body metabolite or other known marijuana components gave this pink spot at the reported R_F value other than the THCA gone through the assay procedure. The efficacy and the sensitivity of the proposed TLC procedure were tested on the urine specimens which were collected in a controlled study at time intervals of 20, 24 and 35 h. In this study *ca.* 200 mg of street marijuana (equivalent to 1/4th a cigarette) was puffed four times using a tobacco pipe. Urine specimens collected at 20-, 24- and 35-h intervals gave strong positive test using the TLC procedure for the presence of 11-nor-Δ^9-THC-9-carboxylic acid; urine specimens collected at 4.0 and 10.0 h could not be tested owing to insufficient volumes. All of these specimens (including the 4.0-h specimen) gave positive readings on the EMIT system. The proficiency of the proposed TLC procedure was evaluated by applying this procedure to the urine specimens shipped by the Center for Disease Control** in its Fourth CDC Proficiency Testing Survey, 1980 and the first survey of 1981. Two of the ten specimens in each survey were spiked with 100 ng and 150 ng of 11-nor-Δ^8-THC-9-carboxylic acid per millilitre of urine, respectively. The spiked specimens in both

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* The gas chromatographic separation of the ester and of the reaction product was performed by Drs. Michael Schaffer and Reng-Lang Lin at the Cook County Medical Examiner's Laboratory, Chicago, IL, U.S.A.

** Center for Disease Control (CDC) Atlanta, GA, U.S.A., Department of Health and Human Service conducts a quarterly Proficiency Testing Program in Toxicology Drugs of Abuse. Each quarter, the center submits ten urine specimens each spiked with three or four commonly abused drugs.

surveys gave strongly positive tests for the acid on a 10-ml aliquot of each urine for the first survey and 14 ml of each urine for the second survey. A total of ten specimens, each spiked with a mixture of three or four commonly abused drugs, were submitted for each survey. This laboratory identified both of the spiked specimens in each survey with 100% accuracy.

ACKNOWLEDGEMENT

The authors thank Mr. Thomas B. Kirkpatrick, Jr., Executive Director of the Illinois Dangerous Drugs Commission, for his keen interest in the operation of this laboratory.

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Errata

J. Chromatogr., 215 (1981) 99–108

Page 100, section Vanillate hydroxylase assay, 7th line, "Syringyl" should read "Syringol".

Page 105, 6th line from top, "2,3-dihydroxybenzoate" should read "2,4-dihydroxybenzoate".

Page 106, section Effect of inhibitors of purified vanillate hydroxylase (Table IV), 1st, 4th, 5th, and 6th lines, "nM" should read "mM".

Page 107, 23rd line from top, "nM" should read "mM".

J. Chromatogr., 217 (1981) 369–386

Page 370, 5th line, "135 msec" should read "290 msec".

Page 382, 9th and 10th lines, "The total baseline response time is 135 msec." should read "The W_{95} response time is 290 msec."
17th line, "30, 300, and 3000 msec" should read "70, 200, and 2000 msec".

Page 382, Fig. 12, abcissa scale should be labelled 0 to 1.5 seconds.

Page 382, legend to Fig. 12, 2nd and 3rd lines, delete "The time required to return to the original light level is taken as the instrumental response time".

Page 383, legend to Fig. 13, 2nd line, "1.35-sec" should read "0.85-sec" and "0.135-sec" should read "0.29-sec".

Page 383, legend to Fig. 14, 2nd line, "1.35-sec" should read "0.85-sec" and "0.135-sec" should read "0.29-sec".

Page 385, line 8, "instrumental bandwidth" should read "band width containing 95% of peak area".

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