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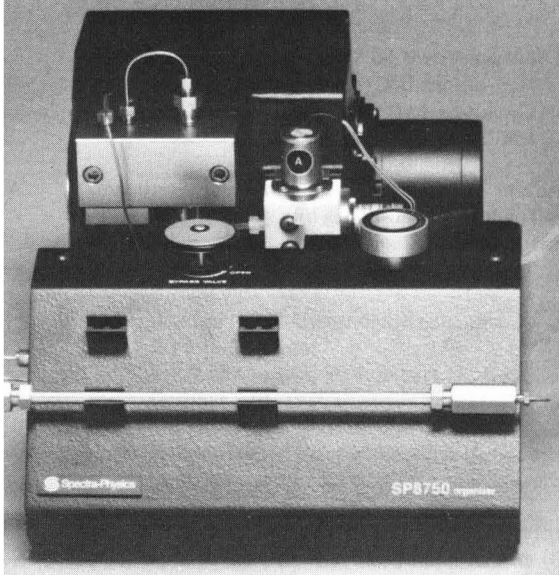

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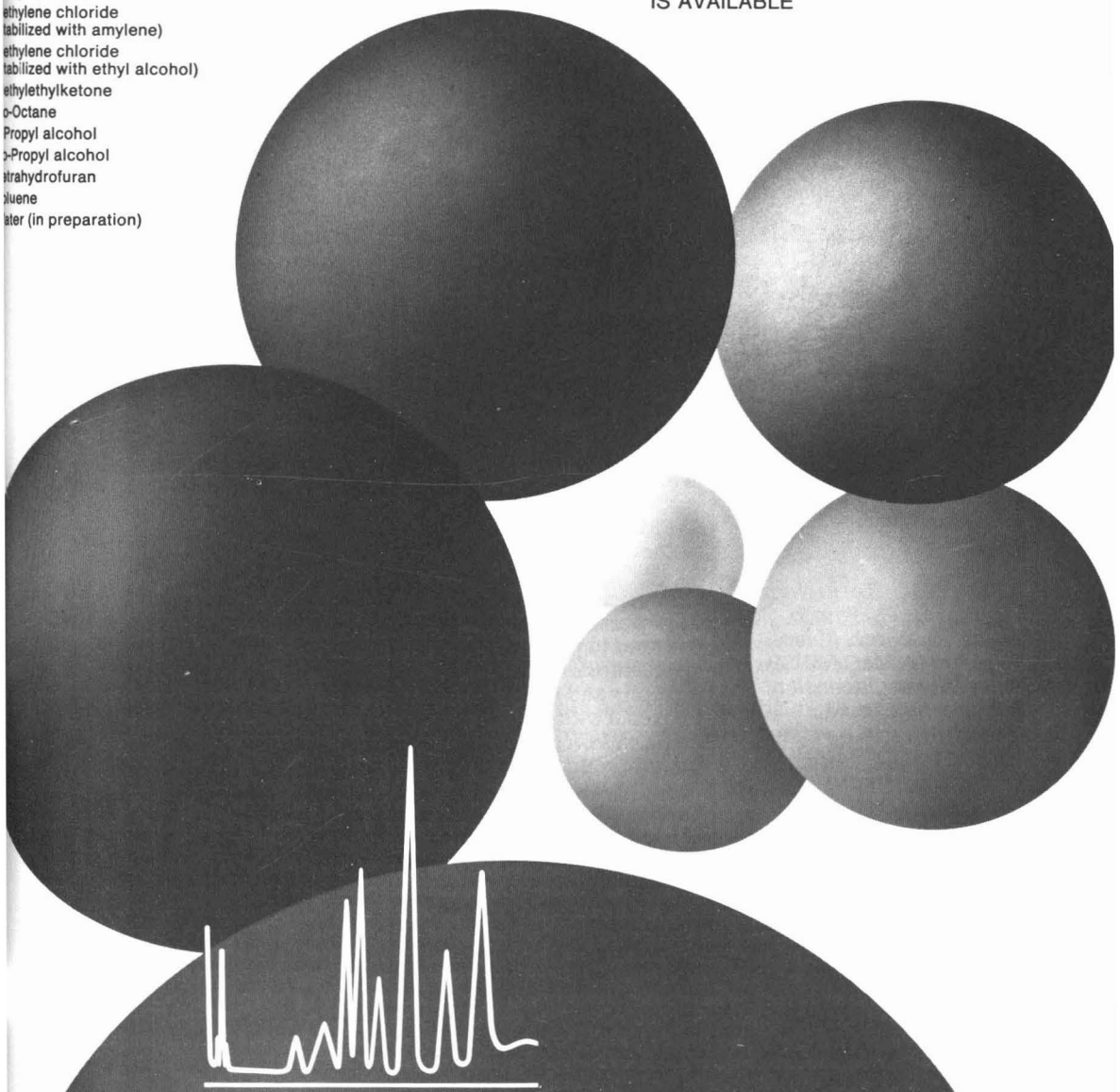

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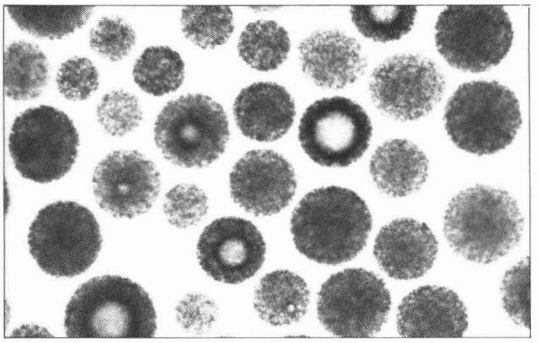

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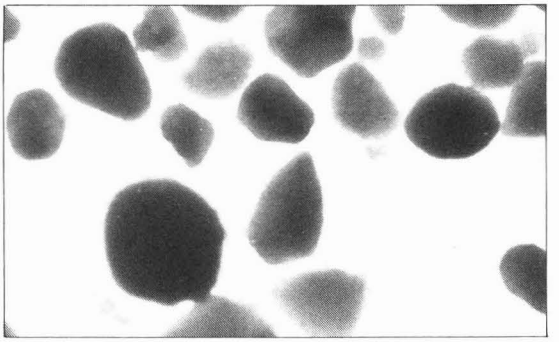

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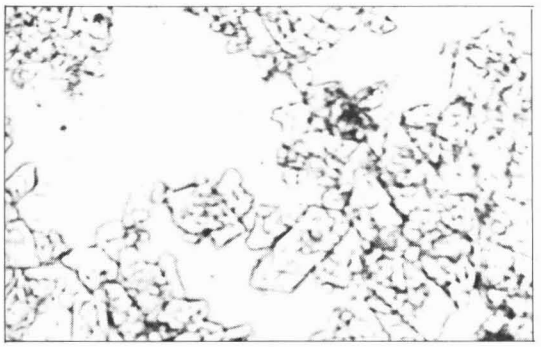

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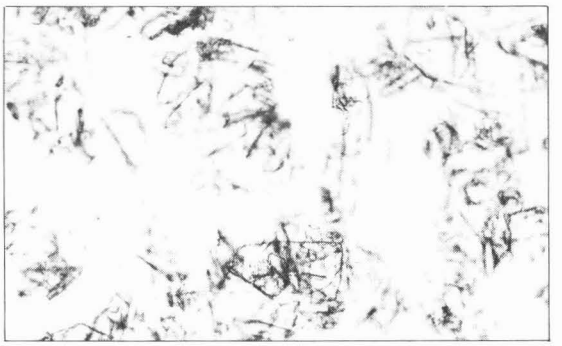

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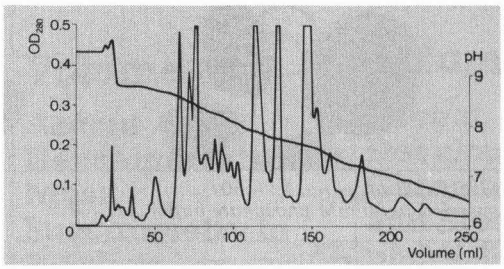

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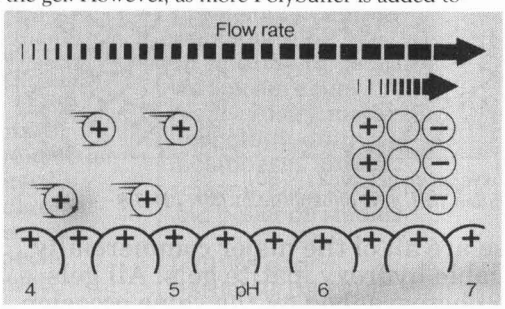

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MIXED RETENTION MECHANISMS IN GAS–LIQUID CHROMATOGRAPHY

III. DETERMINATION OF THE GAS–LIQUID ADSORPTION COEFFICIENT FOR DIISOPROPYL ETHER ON *n*-OCTADECANE

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(Received August 27th, 1980)

SUMMARY

The contribution of adsorption to the retention volume for diisopropyl ether was measured on columns with different loadings of *n*-octadecane coated on glass beads. A theoretical calculation of the magnitude of the adsorption on the glass surface, through the liquid, enables the adsorption coefficient at the gas–liquid interface, K_I, to be calculated.

INTRODUCTION

In a recent study[1] of adsorption effects on gas–liquid chromatographic (GLC) columns with *n*-octadecane as the stationary phase, coated on a silanized diatomaceous support, it was found that the adsorption on the surface of the liquid phase plays an important role in the retention of some polar compounds. To interpret the results that study, a knowledge of the area of the gas–liquid interface is required. To calculate such areas, values of gas–liquid adsorption coefficients (K_I) can be used, but they are not available in the literature.

Adsorption effects at gas–liquid interfaces have been reviewed by Martire[2] and more recently by Conder and Young[3]. There has been a debate about the existence os such adsorption, probably settled by Conder[4].

In this work the GLC determination of K_I for diisopropyl ether on the surface on *n*-octadecane has been attempted. This system is one of those studied earlier[1].

THEORETICAL

Retention in GLC is generally due to a combination of partition and adsorption, causing two separable[5] contributions to the total retention volume. At an infinitely small sample concentration, the adsorption contribution can be written[6] (see the list of symbols at the end of this paper):

$$V_N^A = K_I A_I + K_S A_S + K_A A_A \quad (1)$$

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If we can assume that the liquid phase completely wets the support surface, A_A is zero. Further, if the liquid film is thin enough and if the surface is smooth, the areas A_I and A_S will be identical, so

$$V_N^A = (K_I + K_S)A \tag{2}$$

To approach the conditions that make eqn. 2 valid, we used unsilanized glass beads as the solid support. The surface area, A_I, of the liquid coated on the glass beads will be lower than the geometrical area of the beads, as the liquid will tend to accumulate at the points where the beads are in close contact with each other. This necessitates retention volume measurements at different liquid loadings and extrapolation to zero liquid loading.

According to eqn. 2, in order to determine K_I from experimental results for V_N^A and A we need an estimation of K_S. This constant is small and difficult to measure; therefore, a theoretical calculation is attempted.

By definition, K_S is given by

$$K_S = \frac{\Gamma_S}{C_g} \tag{3}$$

at infinite dilution where both Γ_S and C_g approach zero. Assuming that the gas phase is ideal, and that the solution of sample in the liquid phase follows Raoult's law, we can express K_S in terms of molar fractions of sample in the bulk liquid and in the surface layer at the support surface:

$$K_S = \frac{X_1^S}{X_1} \cdot \frac{RT}{a_1 p_1^0} \tag{4}$$

From Everett[6], who systematized the thermodynamics of adsorption from solutions, we obtain an expression for X_1^S/X_1 (when those molar fractions approach zero) giving

$$K_S = \frac{RT}{a_1 p_1^0} \cdot \exp\left[\frac{a_1(\sigma_2^0 - \sigma_1^0)}{RT}\right] \tag{5}$$

The quantities σ_1^0 and σ_2^0 are liquid–solid interface tensions between the pure liquids and the solid. Such interfacial tensions are difficult to measure, but Eon[7] has pointed out an interesting connection between relative interfacial tensions and the solvent strength parameters, ε^0, introduced into liquid chromatography theory by Snyder[8]. The following relationship was given[7]:

$$\sigma_{\text{pentane}}^0 - \sigma_i^0 = 0.125\,\varepsilon_i^0 \tag{6}$$

Eqn. 6 is written for 60°C and for interfacial tensions expressed in SI units, so the numerical factor is different from that in ref. 7.

Values of ε_i^0 have been tabulated for different pairs of liquids and solvents[8], which makes it possible to calculate an approximative value of K_S for diisopropyl ether adsorbed on glass from a solution in *n*-octadecane.

The molar area, a_1, of diisopropyl ether is estimated from data for critical temperature and pressure[9] by means of Hill's equation[10]; the result is $2.6 \cdot 10^5$ m^2 mole^{-1}. The vapour pressure, p_1^0, at 60°C of the same substance[9] is $8.5 \cdot 10^4$ Pa. The solvent strength parameter, ε_1^0, is taken from ref. 8 (Table 8-1 and eqn. 8-6a), giving $\varepsilon_1^0 = 0.22$ on silica, which has a surface structure similar to that of glass. For *n*-octadecane, $\varepsilon_2^0 = 0.05$ was used in the calculation. This value should be a low estimate, as $\varepsilon^0_{\text{pentane}}$ is 0.00 by definition and $\varepsilon^0_{\text{decane}}$ is 0.03 (ref. 8). If the real value of ε_2^0 is higher, it will result in a smaller value of K_S.

Inserting these values into eqns. 5 and 6 gives $K_S = 0.9 \cdot 10^{-6}$ m. Taking into account the uncertainties in the values given above, we estimate the true value of K_S to be between 0 and $2 \cdot 10^{-6}$ m.

It should be pointed out that we have assumed ideal solution behaviour of diisopropyl ether in *n*-octadecane. This was tested, considering the deviation of the infinite dilution activity coefficient, γ_1^∞, from unity. The following equation[11] was used:

$$\gamma_1^\infty = \frac{RT\delta_2}{K_L p_1^0 M_2} \tag{7}$$

K_L for the system under study was determined[5,12] to be 99.4. With the density for *n*-octadecane of $\delta_2 = 0.7558$ g cm^{-3} (ref. 13), we find $\gamma_1^\infty = 0.96$, indicating a fairly ideal solution.

It should also be mentioned that we ignored any temperature dependence of the ε^0 values.

As will be seen later, the calculated value of K_S is small enough to be neglected in comparison with K_1, so slight errors in the calculation of K_S are of minor importance.

EXPERIMENTAL

Glass beads (80–100 mesh; Alltech, Arlington Heights, IL, U.S.A.) were covered with 99% *n*-octadecane (Riedel de Haën, Hannover, G.F.R.) at different loadings between 0.05 and 1% (w/w). The coating was performed by dissolving the *n*-octadecane in *n*-pentane (p.a. grade; Merck, Darmstadt, G.F.R.), adding the glass beads and evaporating the solvent with gentle stirring. At normal room temperature the *n*-octadecane is solid, making the coated glass beads dry and easy to handle.

The surface areas of the glass beads were measured by the nitrogen thermal desorption method, first described by Nelsen and Eggertsen[14]. The value found was 0.0134 m^2 g^{-1}, corresponding to a mean diameter of the beads of 163 μm (the density was assumed to be 2.5 g cm^{-3}), in good agreement with the sieve gradings (149–177 μm).

Glass columns (800 × 4 mm I.D.) were filled with the prepared packings.

All measurements were made with the apparatus described previously[15]. Diisopropyl ether (p.a. grade; Merck) was used as the sample. Dead volumes were measured with methane. Hydrogen was used as the carrier gas and the temperature was 60.0°C. Net retention volumes were measured at widely different sample concentrations and values of V_N^A were calculated using the procedure in ref. 5.

RESULTS AND DISCUSSION

The experimentally measured values of V_N^A are presented in Fig. 1. A decrease in the V_N^A values with increasing liquid loading (λ') is seen. This should be extrapolated to $\lambda' = 0$. The fitting of a straight line to the data gives an intercept of 0.73 ± 0.17 ml g^{-1} (95% confidence interval). If an exponential equation is fitted instead we obtain an intercept of 0.76 ± 0.16 ml g^{-1}. Although the difference between these values is not significant, the value of 0.76 ml g^{-1} is considered to be more reliable. From eqn. 2 and the surface area of the glass beads (0.0134 m^2 g^{-1}) we obtain $K_I + K_S = (57 \pm 12) \cdot 10^{-6}$ m. From the estimation of K_S above we can calculate the rounded-off final result for K_I to be $(55 \pm 15) \cdot 10^{-6}$ m. This value of K_I can be used to estimate the area of the gas–liquid interface for the columns in ref. 1.

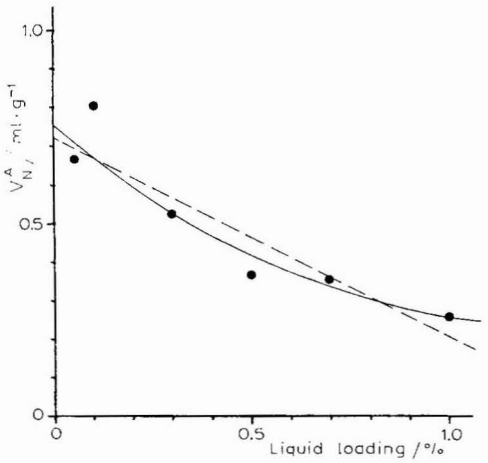

Fig. 1. Retention volume contribution from adsorption V_N^A, measured for diisopropyl ether on *n*-octadecane on glass beads at 60.0°C for various liquid loadings λ'.

The maximum value of V_N^A for diisopropyl ether on *n*-octadecane, which can be attributed to liquid surface adsorption, is *ca.* 15.5 ml per gram of packing. With our value of K_I, the area of the liquid surface at this maximum is calculated to be 0.28 m^2 g^{-1}. This can be compared with the specific surface area of the support, which was measured as 0.55 m^2 g^{-1} by the Nelsen and Eggertsen method[14].

It is of interest to compare the K_I value with others obtained in similar systems of polar compounds on surfaces of non-polar liquids. Such literature data are rare. Pecsok and Gump[16], however, have reported values evaluated from surface tension measurements. The general magnitude of these K_I values is much lower than our value of K_I for diisopropyl ether on *n*-octadecane. The discrepancy is due to the lack of measurements at low concentration. Inspecting Fig. 2 in ref. 16, one finds that the lines giving the K_I values can equally well be drawn with a considerably higher slope, giving values corresponding to the magnitude of our result. This supports the view expressed by Conder[4] that the gas–liquid interface adsorption is strongly dependent on concentration; it might be considerable with very small samples, but negligible with larger samples.

ACKNOWLEDGEMENT

We thank Dr. Z. Suprynowicz, Lublin, Poland, for surface area measurements.

LIST OF SYMBOLS

A Specific surface area of the support ($m^2\ g^{-1}$)
A_A Surface area of uncovered support (gas–solid interface area) ($m^2\ g^{-1}$)
A_I Surface area of the stationary liquid (gas–liquid interface area) ($m^2\ g^{-1}$)
A_S Surface area of support, covered with liquid (liquid–solid interface area) ($m^2\ g^{-1}$)
a_i Molar area of component i ($m^2\ mole^{-1}$)
C_g Concentration in gas phase ($mol\ m^{-3}$)
K_A Adsorption coefficient, gas–solid interface (m)
K_I Adsorption coefficient, gas–liquid interface (m)
K_L Partition coefficient, gas–bulk liquid
K_S Adsorption coefficient liquid–solid interface (m)
M_i Molecular weight of component i ($g\ mole^{-1}$)
p_1^0 Saturated vapour pressure of component i (Pa)
R Gas constant ($8.314\ J°K^{-1}\ mole^{-1}$)
T Temperature (°K)
V_N^A Contribution of adsorption to the net retention volume (ml)
X_i Molar fraction of component i, bulk liquid
X_i^S Molar fraction of component i, surface layer
Γ_S Surface concentration ($mole\ m^{-2}$)
γ_1^∞ Infinite dilution activity coefficient
δ_i Density of component i ($g\ m^{-3}$)
ε_i^0 Solvent strength parameter, component i
λ' Liquid loading (weight of liquid phase/weight of packing)
σ_i^0 Interfacial surface tension between component i and solid support ($N\ m^{-1}$)

REFERENCES

1 L. Mathiasson and J. Å. Jönsson, *J. Chromatogr.*, 179 (1979) 7.
2 D. E. Martire, in J. H. Purnell (Editor), *Progress in Gas Chromatography*, Wiley-Interscience, New York, 1968, p. 93.
3 J. R. Conder and C. I. Young, *Physicochemical Measurement by Gas Chromatography*, Wiley, Chichester, 1979, Ch. 11.
4 J. R. Conder, *Anal. Chem.*, 48 (1976) 917.
5 J. Å. Jönsson and L. Mathiasson, *J. Chromatogr.*, 179 (1979) 1.
6 D. H. Everett, *Trans. Faraday Soc.*, 61 (1965) 2478.
7 C. H. Eon, *Anal. Chem.*, 47 (1975) 1871.
8 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968.
9 G. G. Schlessinger, in R. C. West (Editor), *Handbook of Chemistry and Physics*, CRC, Cleveland, OH, 33rd ed., 1972, p. D151.
10 T. L. Hill, *J. Chem. Phys.*, 16 (1948) 181.
11 J. R. Conder and C. I. Young, *Physicochemical Measurement by Gas Chromatography*, Wiley, Chichester, 1979, Ch. 5.
12 A. M. Olsson, *Acta Chem. Scand., Ser. A*, 34 (1980) 699.
13 W. G. Cutler, R. H. Mickle, W. Webb and R. W. Schiesser, *J. Chem. Phys.*, 29 (1958) 727.
14 F. M. Nelsen and F. T. Eggertsen, *Anal. Chem.*, 30 (1958) 1387.
15 J. Å. Jönsson, R. Jönsson and K. Malm, *J. Chromatogr.*, 115 (1975) 57.
16 R. L. Pecsok and B. H. Gump, *J. Phys. Chem.*, 71 (1967) 2202.

Journal of Chromatography, 206 (1981) 7–15
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DETERMINATION OF HYDROPHOBIC PARAMETERS FOR PYRIDAZINONE HERBICIDES BY LIQUID–LIQUID PARTITION AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The retention behaviour of eight herbicidal pyridazinones in a reversed-phase high-performance liquid chromatographic (RP-HPLC) system has been examined. Using methanol–water as the mobile phase, a linear relationship between the volume fraction of the organic modifier and the logarithm of the capacity factor (log k') over a limited range was established for every solute. A comparison of the resulting curves showed that the separation system is selective with respect to the lipophilic trifluoromethyl substituent with otherwise the same structure. The influence of such a selective effect on the correlation between log k' and the partition coefficient, P, obtained using the standard *n*-octanol–water system, is demonstrated. This effect can be eliminated if log P is related to the extrapolated k' value with pure water as eluent. The resulting curve accommodates the two sets of data ($r = 0.992$). It is concluded that the organic modifier in RP-HPLC exerts subtle effects on the retention behaviour of pyridazinones, a discriminative feature which may also be important in biological membranes.

INTRODUCTION

Since the development of the quantitative structure–activity relationship (QSAR) by Hansch and co-workers[1,2], it has become apparent that the biological activity of a given class of chemicals is in many instances predominantly a function of their lipophilic behaviour. The use of partition coefficients P obtained from an *n*-octanol–water partitioning system has become a standard method[2] for modelling biological membranes and thereby quantifying the hydrophobicity of a given compound as $\log P = \log C_{OCT} - \log C_{WATER}$. Log P is either determined experimentally or calculated[2,3]. The calculations have limitations, however, and there are innumerable compounds for which log P values have to be determined. The conventional shaking flask method has limited application range up to $\log P = 4$ (ref. 3) and is a laborious and time-consuming procedure, often complicated by instability in aqueous media, impurities and the tendency for the compound to dissociate.

In recent years attempts have been made to introduce chromatographic

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techniques for the determination of the lipophilicity of different chemicals, especially thin-layer chromatography[4–6] and reversed-phase high-performance liquid chromatography (RP-HPLC). The latter technique has attracted much interest because it produces very efficiently high-precision data with respect to retention, which is believed to be a measure of the partition behaviour between the non-polar bonded stationary phase and the more polar eluent.

The few reports published so far[7–14] deal with the correlation between log P and the logarithm of the capacity factor, k', mainly obtained with octadecylsilica as the bonded stationary phase. k' is given by

$$k' = (t_R - t_0)/t_0 \tag{1}$$

where t_R and t_0 are the retention times of a retained and an unretained solute, respectively, in a given system. The most direct approach was made by Mirrlees *et al.*[7], who covered trimethylchlorosilane-treated silica *in situ* with a thin layer of *n*-octanol and used *n*-octanol-saturated water as the eluent. This seems to be a true analogy to liquid–liquid partitioning in octanol–water, and therefore the correlation between log k' and log P is excellent with a slope of the regression curve very close to 1.0. The same is perhaps true for buffers[8] or a very small amount of organic modifier in water[9] when used together with octadecylsilica. Here the slope also has a value near 1.0, although the correlation in the case of buffer is much poorer.

Several workers have used methanol–water and acetone–water as eluents to extend the applicability of the method to more hydrophobic compounds. They also found good correlations between log k' and log P[10–14] but varying slopes on regression analysis.

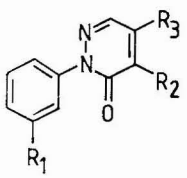
It has been emphasized[9] that free silanol groups at the accessible surface of the stationary phase can influence to some extent the retention behaviour of the solutes, so that vigorous silylation of the silica is recommended. However, several commercially available packings already have a very high surface coverage and can be used without further treatment[14].

On the basis of the above experience, RP-HPLC seems to be a convenient technique with respect to accuracy, sensitivity and application range for the determination of hydrophobicity data for chemical groups beyond the few tested so far. As we are interested in the QSAR for pyridazinone herbicides[15], the partition data for which have not yet been published and which have a complicated structure with respect to hydrophilic and hydrophobic substituents (Fig. 1), a study of the retention and partition behaviour should give a good basis for testing the validity of RP-HPLC for the determination of these pharmacologically important physico-chemical parameters.

EXPERIMENTAL

Materials

SAN 6706 [4-chloro-5-(dimethylamino)-2-(α,α,α-trifluoro-*m*-tolyl)-3(2H)-pyridazinone], SAN 9789 [4-chloro-5-(methylamino)-2-(α,α,α-trifluoro-*m*-tolyl)-3(2H)-pyridazinone; Norflurazon], SAN 9774 [5-amino-4-chloro-2-(α,α,α-trifluoro-*m*-tolyl)-3(2H)-pyridazinone], SAN 9785 [4-chloro-5-(dimethylamino)-2-phenyl-3(2H)-pyrida-



No.	Compound	Substituents
1	SAN 6706	$R_1 = CF_3, R_2 = Cl, R_3 = N(CH_3)_2$
2	BAS 44521	$R_1 = CF_3, R_2 = Cl, R_3 = OCH_3$
3	SAN 9789	$R_1 = CF_3, R_2 = Cl, R_3 = NHCH_3$
4	SAN 9774	$R_1 = CF_3, R_2 = Cl, R_3 = NH_2$
5	SAN 9785	$R_1 = H, R_2 = Cl, R_3 = N(CH_3)_2$
6	BAS 33650	$R_1 = H, R_2 = Br, R_3 = OCH_3$
7	SAN133-410	$R_1 = H, R_2 = Cl, R_3 = NHCH_3$
8	BAS13033	$R_1 = H, R_2 = Cl, R_3 = NH_2$

Fig. 1. Chemical structures of pyridazinone derivatives.

zinone] and SAN 133-410 H [4-chloro-5-(methylamino-2-phenyl-3(2H)-pyridazinone] were gifts from Sandoz (Basle, Switzerland). BAS 44521 [4-chloro-5-methoxy-2-(α,α,α-trifluoro-*m*-tolyl)-3(2H)-pyridazinone], BAS 33650 (4-bromo-5-methoxy-2-phenyl-3(2H)-pyridazinone], and BAS 13033 [5-amino-4-chloro-2-phenyl-3(2H)-pyridazinone; Pyrazon) were kindly supplied by BASF (Ludwigshafen, G.F.R.). Some of these compounds were technical products and recrystallized twice prior to use. Distilled water was prepared with an all-glass double distilling unit (Heraeus-Schott, Mainz, G.F.R.). All other reagents were of analytical-reagent grade (Merck, Darmstadt, G.F.R.). A 10-μm LiChrosorb RP-18 column (25 cm $\times$ 4.6 mm I.D.) (Merck) was used without further treatment in all experiments.

Partition coefficient measurements

Previously water-saturated *n*-octanol and *n*-octanol-saturated water were used as the liquid phases. Two different amounts of the herbicides (10 and 30 mg) were introduced into 25-ml glass flasks equipped with glass stoppers and dissolved in 5 ml of *n*-octanol. A few drops of methanol improve dissolution without a measurable effect on the partition coefficients. Then 5 ml of water were added and the flasks were shaken mechanically with a Model TR 1 shaker (Infors, Basle, Switzerland) at a frequency of 300 min^{-1} for 1 h. All experiments were performed at room temperature (20–23°C). The contents of the flaks were decanted into centrifuge tubes and the two phases were allowed to separate. The upper *n*-octanol phase was carefully removed and the remaining aqueous phase was centrifuged for 1 h at 5000 rpm in a Labofuge III (Heraus-Christ, Osterode, G.F.R.) to remove *n*-octanol droplets. Subsequently the absorbance at the wavelength maximum was measured in a Gilford Model 250 spectrophotometer (Gilford, Oberlin, OH., U.S.A.).

Quantitative evaluation was performed with molar absorption coefficients which were determined by dissolving two different amounts of the herbicides in *n*-octanol-saturated water and measuring the absorbance as described above. The samples were diluted, if necessary, to yield an absorbance of 0.2–0.5.

The partition coefficients represent the means of six independent measurements and the standard error of log *P* was better than ± 0.05.

Chromatography

The liquid chromatograph consisted of a Series 2/2 reciprocating pump

(Perkin-Elmer, Norwalk, CT, U.S.A.), a Model LC-55 variable-wavelength UV–visible detector (Perking-Elmer) set at 280 nm, and a Servogor Model S pen recorder (Metrawatt, Nürnberg, G.F.R.).

A volume of 5 μl of a 10^{-4} M sample solution was injected with a 10-μl precision syringe and the retention times were measured with a stop-watch. The mobile phase consisted of different volume fractions of methanol in water, prepared with the gradient former of the chromatograph. The flow-rate was 1.7 ml/min at room temperature. The reproducibility of the retention times was checked by making a series of six injections under fixed conditions and was found to be better than 1%, so that in all other experiments two independent runs were carried out. The column dead time was determined by the injection of a small amount of acetone dissolved in water. Acetone has a log P of -0.29 (ref. 3) and was expected not to be retained in our system. The retention times were indeed the same at volume fractions of 0.55 and 0.80, so that retention due to interactions with the stationary phase could be excluded. The standard error of log k' determinations was better than ± 0.005.

RESULTS AND DISCUSSION

Several studies have been reported on the variation of sample k' values with the volume fraction, Φ, of organic solvent in water–organic solvent mixtures[16–18]. Snyder *et al.*[16] give this relationship as

$$\log k' = \log k_W - S\,\Phi_B \tag{2}$$

where k_W represents the k' value of a compound with pure water as the mobile phase (usually an extrapolation where the intercept on the ordinate is taken as log k_W) and S is related to the solvent strength of pure solvent B. For methanol–water and for other polar organic solvents S should therefore be constant for a given column and different types of solutes. Snyder *et al.* gave average values of S for methanol (3.0), acetonitrile (3.1), tetrahydrofuran (4.4) and other organic solvents. If this suggestion is true, the retention mechanism for different solutes in a particular system should be the same, which is a prerequisite for a strong relationship between retention data in RP-HPLC and partition coefficients. Table I shows the k' values for pyridazinones over a range of 0.55–0.80 for Φ_M, the volume fraction of methanol in the mobile phase.

As can be seen from Table II, eqn. 2 describes appropriately the behaviour of the different solutes with a small standard error of fit and a high linear correlation coefficient. From these data we conclude, in accordance with Snyder *et al.*[16], that the relationship between Φ_M and log k' over a limited range of Φ_M is perfectly linear and that there is not need to introduce a parabolic function, as has been previously suggested[19]. However, with respect to S the solutes have to be divided into two different groups as the values of S are significantly different. The pyridazinones with a trifluoromethyl substituent and different polar groups at the C-5 position have high values of 4.45–4.68 for S, whereas the same pyridazinones without this lipophilic substituent have values of only 3.42–3.72. The RP-HPLC system can clearly discriminate between solutes with and without a trifluoromethyl substituent due to different retention mechanisms, which probably depend on electronic or steric contributions in addition to the lipophilicity of this group. As a result, S depends not only on the solvent strength

TABLE I

ISOCRATIC k' VALUES FOR DIFFERENT VOLUME FRACTIONS OF METHANOL IN WATER, Φ_M, AND PARTITION COEFFICIENTS IN *n*-OCTANOL–WATER (LOG *P*)

*Compound No.**	*Volume fraction, Φ_M*					*log P*
	0.55	*0.60*	*0.65*	*0.70*	*0.80*	
1	6.21	3.57	2.12	1.23	0.40	2.67
2	3.89	2.32	1.53	0.81	0.25	2.39
3	3.77	2.21	1.43	0.79	0.25	2.30
4	2.55	1.52	1.00	0.55	0.17	2.41
5	1.50	0.98	0.67	0.41	—	1.55
6	1.07	0.71	0.47	0.29	—	1.42
7	0.78	0.51	0.37	0.22	—	1.19
8	0.47	0.30	0.21	—	—	1.14

* See Fig. 1.

TABLE II

REGRESSION ANALYSIS OF THE RELATIONSHIP BETWEEN THE VOLUME FRACTION OF METHANOL Φ_M AND LOG k': LOG k' = LOG $k_W - S\Phi_M$

*Compound No.**	*Log k_W*	*S*	*r***	*s****
1	3.340	4.642	0.9999	0.003
2	3.042	4.452	0.9959	0.007
3	3.134	4.636	0.9992	0.005
4	3.003	4.684	0.9972	0.016
5	2.164	3.619	0.9976	0.006
6	2.081	3.721	0.9987	0.005
7	1.768	3.422	0.9925	0.012
8	1.614	3.552	0.9954	0.002

* See Fig. 1.
** Linear correlation coefficient.
*** Standard error of fit ($\alpha = 0.001$).

of the mobile phase but also to a considerable extent on specific interactions between solutes, stationary phase and mobile phase. Tanaka *et al.*[18] have shown that the composition of the mobile phase can have such an effect on hydrophobic group selectivity and especially polar group selectivity. Pyridazinones offer several possibilities for selective interactions as both hydrophilic and hydrophobic substituents are available, whereas only the trifluoromethyl substituent can realize this property in our particular separation system.

The relationship between log *P* and log k' is shown in Fig. 2, from which the following linear equation is obtained:

$$\log P = 1.537 \log k' + 1.473 \tag{3}$$

with $n = 8$, $r = 0.964$ and $s = 0.664$ ($\alpha = 0.001$). The correlation is not as good as reported earlier for other types of solutes (see Table IV), as expected from the results

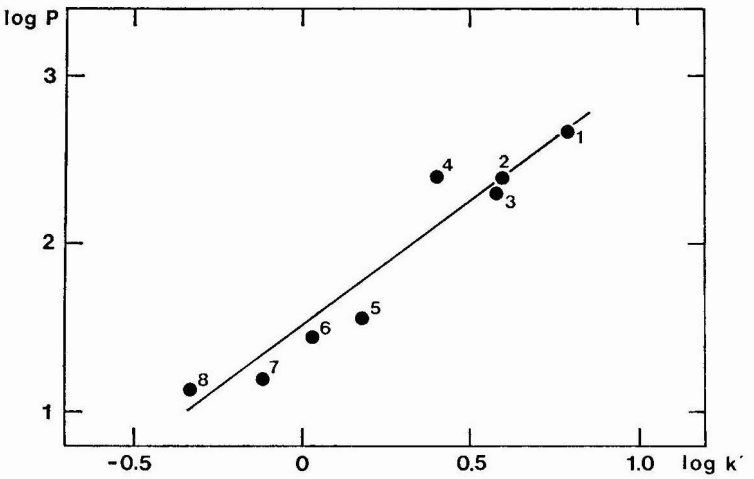


Fig. 2. Log k' *versus* log P for eight pyridazinones (numbered as in Fig. 1). k' was measured at a volume fraction of methanol in water of $\Phi = 0.55$.

described above. However, the selective effects in Table II do not distort the relationship. For comparison we performed the same experiments with a few benzene derivatives for which literature data for partition coefficients were related to the capacity factor at $\Phi_M = 0.80$, and obtained the equation

$$\log P = 2.746 \log k' + 3.021 \tag{4}$$

with $n = 4$ and $r = 0.994$. Table III shows the relevant data for the benzenes.

TABLE III

CAPACITY FACTOR, k', AND PARTITION COEFFICIENTS FOR SOME BENZENE DERIVATIVES

Mobile phase, 80% methanol–water; flow-rate, 1.7 ml/min; column, 10-μm LiChrosorb RP-18.

Compound	k'	*Log* k'	*Log* P*
Benzene	0.47	−0.33	2.14
Toluene	0.81	−0.09	2.69
1,3-Dichlorobenzene	1.48	0.17	3.60
1,3,4-Trichlorobenzene	2.24	0.35	3.93

* Data taken from ref. 3.

A similar regression curve was reported earlier[14] for over 30 benzenes, toluenes and anilines. The slope was nearly twice that for the pyridazinones, which indicates different retention mechanisms for the two types of solutes. What has been shown before within the class of heterocyclic pyridazinones now finds its counterpart in the different log P *versus* log k' relationships for different chemical classes. Together with some previously published data the following picture emerges (Table IV). When water and buffers consisting of water with a small amount of an organic modifier (*e.g.*, 1%

TABLE IV

COMPARISON OF LITERATURE REGRESSION DATA FOR THE RELATIONSHIP LOG $P = a$ LOG $k' + b$

In some instances not all of the relevant data were included in the work reported. In these instances we have carried out the regression analysis from the published k' and log P, not knowing the accuracy of the data, so that minor errors have to be taken into account.

Class of compounds	*a*	*b*	*r*	*Reference*	*Separation system**
Sulphonamides	0.98	−8.03	0.937	7	ODS/buffer
Miscellaneous	1.086	1.14	0.982	8	ODS/1% TEA in water
Miscellaneous	1.006	−0.622	0.999	6	*n*-Octanol-coated silica/water
Phenols	1.907	1.922	0.961	9	ODS/acetone–water
Anilines	2.240	1.438	0.968	9	ODS/acetone–water
Miscellaneous	1.701	1.293	0.988	19	ODS/methanol–water
Miscellaneous	1.451	1.016	0.983	19	ODS/acetonitrile–water
Benzenes, toluenes, anilines	2.50	2.12	0.989	13	ODS/methanol–water

* ODS = octadecylsilica; TEA = triethylamine.

of triethylamine[9]) are used as eluents in RP-HPLC, the resulting k' values of a variety of substances are based on comparable mechanisms, indicated by the same slope of nearly 1.0 in all instances. Organic–water mixtures, on the other hand, produce selective effects that depend on both solute type and mobile phase composition.

The view that the solutes are partitioning between the hydrocarbonaceous surface layer of the non-polar stationary phase and the mobile phase is too simple an analogy in this instance and does not account for the differences between RP-HPLC with organic–water eluents and a true liquid partitioning system such as *n*-octanol–water. The bonded phase may be only a monolayer thick and the bonded molecules have fewer translational and rotational degrees of freedom than those comprising a true liquid. The behaviour of the bonded phase further depends on its type and surface coverage. A theoretical treatment of the binding process has shown[20] that the capacity factor of a given solute depends on the volume ratio of the stationary and mobile phases, the free energy change for binding of the solute to the bonded phase, the free energy change for cavity formation in the mobile phase and Van der Waals and electrostatic contributions that result from the interaction between the solvent and solute. Every term, except the volume ratio, is a function of the molecular structures of the solutes and eluents, so that it is expected that k' will be sensitive to small variations in the physical properties of the structures concerned. Within a group of compounds of comparable size, shape and polarity, good correlations between log k' and log P can always be obtained, but a relationship derived for a particular groups cannot be generalized to other solutes and separation systems. This conclusion restricts the value of such relationships and offers little help for the case of unknown samples.

An alternative is provided by the measurement of k' values at different Φ_M as described above. The regression analysis results in an extrapolated log k_w, the capacity factor with pure water as eluent. If these theoretical k' values are correlated with the partition coefficients of the solutes, a much improved relationship is obtained:

$$\log P = 0.901 \log k_w - 0.384 \quad (5)$$

with $n = 8$, $r = 0.992$ and $s = 0.207$ ($\alpha = 0.001$). The selective effects of the mobile phase are eliminated as the slope is close to 1.0 and the linear correlation coefficient is much better than before. Schoenmaker *et al.*[19] have shown that the relationship between log k' and Φ is not really linear over the whole range of Φ, especially at the upper and lower end of the range. They suggested a function of the following type:

$$\log P = A\,\Phi^2 + B\,\Phi + C \tag{6}$$

The simple linear extrapolation to $\Phi_M = 0$ nevertheless yields lipophilicity data that are strongly related to the partition coefficients of the *n*-octanol–water system. The validity of this approach for other chemical classes however, has not yet been demonstrated but eqn. 2 certainly implies such a relationship.

The determination of log k_W requires detailed studies of the elution of the solute in question under different isocratic conditions and is necessary only if one has to correlate partition and retention data of more complex molecules. This is generally of theoretical interest for gaining an insight into retention mechanisms in RP-HPLC. In QSAR the question is whether the model for the behaviour of chemicals during the passage through biological membranes is adequate. It is now well established that these membrane structures are highly compartmentalized with respect to their lipid and protein moieties. Therefore, membranes are expected to be far more discriminative than is indicated by the gross lipophilic behaviour in *n*-octanol–water partitioning. Secondly, biomembranes will not behave like bulky liquids because their components are asymmetrically arranged with fewer translational and rotational degrees of freedom. Most of the membranes should therefore be able to distinguish between minor steric and electronic variations in molecular structure, a feature that is also common in RP-HPLC. We have shown that in our system pyridazinones with and without a trifluoromethyl substituent possess different retention mechanisms. Preliminary results, obtained in this laboratory, indicate that biomembranes of green plants can also discriminate between these two types of structures so that the question arises of whether the capacity factor in RP-HPLC could be a better alternative for modelling the hydrophobicity of reagents in QSAR.

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REFERENCES

1 A. Leo, C. Hansch and D. Elkins, *Chem. Rev.*, 71 (1971) 525.
2 C. Hansch and A. Leo, *Substituent Constants for Correlation Analysis in Chemistry and Biology*, Wiley, New York, 1979.
3 R. F. Rekker, *The Hydrophobic Fragmental Constant*, Elsevier, Amsterdam, 1977.
4 C. B. C. Boyce and B. V. Milborrow, *Nature (London)*, 208 (1967) 2275.
5 E. Tomlinson, *J. Chromatogr.*, 113 (1975) 1.
6 W. Steurbaut, W. Dejonckheere and R. H. Kips, *J. Chromatogr.*, 160 (1978) 37.

7 M. S. Mirrlees, S. J. Moulton, C. T. Murphy and P. J. Taylor, *J. Med. Chem.*, 19 (1976) 615.
8 D. Henry, J. H. Block, J. L. Anderson and G. R. Carlson, *J. Med. Chem.*, 19 (1976) 619.
9 J. M. McCall, *J. Med. Chem.*, 18 (1975) 549.
10 R. M. Carlson, R. E. Carlson and H. L. Kopperman, *J. Chromatogr.*, 107 (1975) 219.
11. I. Molnár and Cs. Horváth, *J. Chromatogr.*, 142 (1977) 623.
12 T. Hanai, *Chromatographia*, 12 (1979) 77.
13 S. H. Unger and T. F. Feuerman, *J. Chromatogr.*, 176 (1979) 426.
14 H. Könemann, R. Zelle, F. Busser and W. E. Hammers, *J. Chromatogr.*, 178 (1979) 559.
15 H. W. Kümmel and L. H. Grimme, *Z. Naturforsch., C*, 30 (1975) 333.
16 L. R. Snyder, J. W. Dolan and J. R. Gant, *J. Chromatogr.*, 165 (1979) 3.
17 J. W. Dolan, J. R. Gant, and L. R. Snyder, *J. Chromatogr.*, 165 (1979) 31.
18 N. Tanaka, H. Goodell and B. L. Karger, *J. Chromatogr.*, 158 (1978) 233.
19 P. J. Schoenmakers, H. A. H. Billiet and L. de Galan, *J. Chromatogr.*, 185 (1979) 179.
20 Cs. Horváth and W. Melander, *J. Chromatogr. Sci.*, 15 (1977) 393.

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SURFACE AFFINITY CHROMATOGRAPHIC SEPARATION OF BLOOD CELLS

II. INFLUENCE OF MOBILE PHASE COMPOSITION ON THE CHROMATOGRAPHIC BEHAVIOUR OF HUMAN PERIPHERAL BLOOD CELLS ON POLYETHYLENE GLYCOL-BONDED SEPHAROSE

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SUMMARY

The influence of mobile phase composition on the chromatographic behaviour of human platelets, granulocytes, lymphocytes and erythrocytes has been studied by using a bisoxirane-coupled polyethylene glycol 20M–Sepharose 6B column at pH 7.5.

Lowering of the concentration of dextran T40 from 8 to 2% (w/w) produced the highest separation factor between platelets and granulocytes. Addition of 0.5% (w/w) of DEAE-dextran to a mobile phase containing 2% dextran T40 or T500 increased the retention of platelets, and discriminated the cells from erythrocytes. Addition of sodium chloride increased the retention volumes of lymphocytes, granulocytes and platelets. These blood cells were adsorbed to the column in isotonic phosphate-buffered eluent, whereas in imidazole-buffered eluent about 0.15–0.154 *M* sodium chloride improved their resolution.

INTRODUCTION

In the previous paper[1] we described the partition of blood cell particles between the bonded stationary phase and the mobile phase in a manner similar to aqueous polymeric two-phase (APTP) systems established by Albertsson[2] by means of binding of polyethylene glycol (PEG) to the support material in liquid chromatography. The chromatographic separation of an artificial mixture of erythrocytes, granulocytes and lymphocytes from human and rabbit peripheral blood was achieved by the use of bisoxirane-coupled PEG 20M–Sepharose 6B (PEG 20M–Sepharose) as the column packings and phosphate-buffered solution of 4.5 or 8.0% (w/w) dextran as the mobile phase. Bonded PEG stationary phases and mobile phases containing dextran systems offer an approach to the chromatographic separation of blood cell populations.

It is, therefore, necessary to determine the effects of variables on the behaviour of the blood cells in such chromatographic systems. The present paper describes the

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influence of the mobile phase composition on the elution behaviour of platelets, granulocytes and lymphocytes from human peripheral blood on a PEG 20M–Sepharose column. The variables studied include: concentrations of dextran T40 and T500; several anionic or cationic buffers; addition of a neutral salt, such as sodium chloride, and of a positively charged dextran, *e.g.*, DEAE-dextran.

EXPERIMENTAL

Materials

Epoxy-activated Sepharose 6B, dextran T40 (weight-average molecular weight $M_w = 40{,}000$), dextran T500 ($M_w = 500{,}000$) and diethylaminoethyldextran (DEAE-dextran, $M_w = 500{,}000$) were obtained from Pharmacia (Uppsala, Sweden). Polyethylene glycol, number-average molecular weight $M_n = 6000$–7500 and 15,000–20,000 was purchased as PEG 6000 and 20M, respectively (extra pure grade; Wako, Osaka, Japan). Other reagents were of analytical reagent grade.

Instruments

A Hitachi Model 034 liquid chromatograph, equipped with a Model 0037 multi-wavelength effluent monitor (Hitachi, Tokyo, Japan), was used. An LKB 2112 RediRac fraction collector (LKB, Bromma, Sweden) was employed for fractionation of eluates. A Coulter Model D counter (Coulter Electronics, Harpenden, Great Britain) was used for counting the number of blood cells.

Preparation of column packing

Bisoxirane-coupled PEG 20M–Sepharose 6B was prepared by coupling of epoxy-activated Sepharose 6B with PEG 20M for 16 h at 40°C in a solution of pH 12.0, as described previously[1]. The product was treated with 1 *M* 2-aminoethanol for the purpose of blocking residual free epoxy groups. The amount of PEG 20M coupled under the optimal conditions as the bonded stationary phase was determined by spectrophotometry[1] as 16 μmol per gram of dry powder.

Collection and isolation of blood cells

Human blood was drawn from normal male adult donors by venous puncture and heparin was added, 0.05 ml of a 1000 U/ml solution per 10 ml of the blood. Siliconized glassware was used in all procedures.

Erythrocytes. Blood was centrifuged at 500 *g* for 10 min, and the supernatant and buffy coat layer were removed. The cells were washed three times with saline and packed by centrifugation.

Granulocytes. The sodium metrizoate–dextran T500 sedimentation technique[3] was used. The granulocyte preparation contained a variable amount of contaminating erythrocytes.

Lymphocytes. The sodium metrizoate–Ficoll sedimentation technique[4] was used. The erythrocyte contamination of the lymphocyte preparation was usually between 1 and 5% of the total number of cells. The isolation procedures for both the above kinds of blood cells have been described in detail[1].

Platelets. A centrifugal isolation technique based on that of Leeksma and Cohen[5] was employed. A 10-ml volume of heparinized whole blood was mixed with

1 ml of 1 % (w/v) disodium ethylenediaminetetraacetate in 0.9 % (w/v) saline, and was centrifuged at 65 *g* for 20 min after filtration through an absorbent gauze. The supernatant (platelet-rich plasma, PRP) was removed with a siliconized pipette. Generally, not all of the PRP could be removed without disturbing the buffy coat. The PRP was centrifuged at 250 *g* for 20 min. Most of the leukocytes and erythrocytes (if present) were then collected on the bottom of the tube. On the top of these the platelets were already partly sedimented. These were resuspended, precaution being taken to prevent stirring of the bottom layer. The PRP was pipetted into another tube, centrifuged at 250 *g* for 20 min and the sedimented platelets were then washed thrice with 0.9 % (w/v) saline to remove other contaminating cells.

Chromatography

Nineteen kinds of eluents were used, the compositions of which are shown in Tables I and II.

A jacketed glass column (25 × 0.9 cm I.D.) filled with bisoxirane-coupled PEG 20M–Sepharose 6B was used. The packing material was suspended in each of the eluents, and the column was filled with the slurried packing. The column was thoroughly washed with the eluent to equilibrate the chemically bonded phase, using a reciprocating or peristaltic pump.

The total amount of platelets, granulocytes, lymphocytes and erythrocytes prepared as above was suspended in 0.6 ml of the eluent used. A 0.5-ml volume of the cell suspension containing $6.6 \cdot 10^5$–$24.4 \cdot 10^5$ platelets, $13.7 \cdot 10^4$–$20 \cdot 10^4$ granulocytes, $9.5 \cdot 10^4$–$14.5 \cdot 10^4$ lymphocytes or $2.5 \cdot 10^4$–$3.4 \cdot 10^4$ erythrocytes was loaded in the column, and the column was eluted with each of the eluents. These operations were performed at 4°C, the temperature being maintained by circulation of cold water through the column jacket. A flow-rate of 3–12 ml/h was maintained by the use of a pump. The absorbance of the eluate at 230, 260 and 570 nm was monitored continuously with a multi-wavelength effluent monitor. The fractions were collected in glass vials every 10 min or 15 min, the volume of each fraction being about 0.75–2.0 ml. An aliquot of each fraction was diluted with 5 ml of Isoton (aqueous electrolyte diluent for blood cell counting; Coulter Diagnostics, Hialeah, FL, U.S.A.) and the number of blood cells was counted with a Coulter counter. The recovery of the eluted cells was calculated from the combined number in each fraction compared with the cells loaded on the column.

RESULTS

Influence of concentration and molecular weight of dextran in the mobile phase

Each suspension of erythrocytes, platelets, granulocytes and lymphocytes from human peripheral blood was chromatographed by the use of a chemically bonded PEG 20M–Sepharose column. Seven kinds of mobile phases (I–VII) containing either dextran T40 or T500 (except for eluent V) were used. These mobile phases were kept essentially isotonic with 0.09 *M* sodium phosphate buffer, pH 7.5.

Table I shows retention volumes and separation factors for erythrocytes, platelets, granulocytes and lymphocytes eluted independently from the PEG 20M–Sepharose column. All reported retention volumes of the blood cells are the means of triplicate or further determinations. It can readily be seen that erythrocytes, plate-

TABLE I

RETENTION VOLUMES AND SEPARATION FACTORS OF HUMAN ERYTHROCYTES (e), PLATELETS (p), GRANULOCYTES (g) AND LYMPHOCYTES (l)

Column: bisoxirane-coupled PEG 20M–Sepharose 6B (25 × 0.9 cm I.D.). Mobile phases I–X contained 0.045 *M* NaH_2PO_4 and 0.045 *M* Na_2HPO_4 (pH 7.5). All retention volumes are the means of triplicate or further determinations.

Mobile phase	*Concentration (% w/w)*			*Retention volume (ml)*				*Separation factor*		
	Dextran T40	*Dextran T500*	*DEAE-dextran*	*e*	*p*	*g*	*l*	*p/e*	*g/p*	*l/g*
I	8.0	—	—	3.8*	7.6	8.3*	22.0*	2.00	1.09	2.65*
II	4.5	—	—	5.4*	8.9	9.0*	21.0*	1.64	1.01	2.33*
III	2.0	—	—	5.8	7.4	13.2	16.2	1.28	1.78	1.23
IV	1.0	—	—	5.6	5.7	6.1	14.6	1.02	1.07	2.39
V	—	—	—	5.1	5.7	6.2	4.9	1.12	1.15	1.04(e/l)
VI	—	4.5	—	ads.*	11.6	12.1*	18.0*	—	1.04	1.49*
VII	—	2.0	—	ads.	8.3	10.6	12.5	—	1.28	1.18
VIII	4.5	—	0.5	8.7**	8.6	14.0	15.4	0.99	1.63	1.10
IX	2.0	—	0.5	5.9**	11.0	15.4	18.8	1.86	1.40	1.22
X	—	2.0	0.5	10.3**	10.3	12.6	14.8	1.00	1.22	1.17

* Data from ref. 1. ads.: adsorbed on the column.

** A proportion of the erythrocytes was adsorbed at the top end of the column.

lets, granulocytes and lymphocytes were eluted from the column in the order of their increasing retention volumes with every mobile phase used, except for eluent V in which dextran was absent. Of the various factors investigated, the concentration and the molecular weight of dextran in the eluent had a significant effect on the retention volume of these blood cells. As shown previously[1], the retention volumes of erythrocytes and granulocytes increased with decreasing concentration of dextran T40 [from 8.0 to 4.5% (w/w)]. In the present work, however, when the concentration of dextran T40 and T500 was decreased from 4.5 to 2.0% (w/w), the retention volumes of platelets, lymphocytes and granulocytes were decreased, the only exception being those of granulocytes with eluent III. Retention volumes of erythrocytes, platelets and granulocytes became very similar because of the reduced retention of the last two groups of blood cells with eluent IV containing 1.0% (w/w) of dextran T40, whereas lymphocytes were still appreciably retained with this eluent. Evidently, in the absence of dextran in the mobile phase (V), all these kinds of blood cells were only weakly retained on the stationary phase.

Of the seven kinds of mobile phases used, eluent III, containing 2.0% (w/w) of dextran T40, produced the best separation between platelets and granulocytes, whereas that of erythrocytes and platelets was not good because of the reduced retention of the latter on the stationary phase. On the other hand, the best resolution of granulocytes and lymphocytes was achieved by the use of eluent I. Increasing the concentration of dextran T40 or T500 from 2.0 to 4.5% (w/w) retarded the elution of these blood cells, and the separation factor between platelets and granulocytes was reduced to 1.01–1.04 because of the delayed elution of the former cells.

An increase in the molecular weight of dextran from T40 to T500 appreciably increased the retention of platelets and decreased those of granulocytes and lympho-

cytes. With eluents VI and VII erythrocytes aggregated and were adsorbed on the column. In general, of the seven mobile phases used, eluent II, containing 4.5% (w/w) of dextran T40, produced the best resolution for all these kinds of blood cells.

Effect of addition of DEAE-dextran to the mobile phase

In order to examine the influence of a polycationic polymer in the eluents, three kinds of mobile phases containing 0.5% (w/w) of DEAE-dextran in addition to either dextran T40 or T500 were also used. It has been pointed out in APTP systems that dextran–PEG systems can be modified by incorporation of DEAE-dextran to enhance separations based on the surface charge of mammalian erythrocytes of a number of species[6,7].

Table I shows that addition of 0.5% (w/w) of DEAE-dextran to a mobile phase containing 4.5% (w/w) dextran T40 (eluent VIII) decreased the retention volumes of all these kinds of blood cells and the separation factors were reduced as compared with those obtained with eluent II. On the other hand, the cationic polymer increased the retention of these cells in mobile phases containing 2.0% (w/w) of either dextran T40 (eluent IX) or T500 (eluent X) in comparison with those values obtained with eluents III or VII. In every case the separation factor between platelets and granulocytes was appreciably reduced because of the increased retention volume of the platelets. The usefulness of mobile phase systems containing DEAE-dextran was demonstrated by the fact that one can discriminate between erythrocytes and platelets due to the retarded elution of the latter when using the eluent IX.

Effect of addition of neutral salt to the mobile phase

The effect of neutral salts, such as sodium chloride, on the retention of blood cells in mobile phases buffered with either phosphate or organic amines was investigated. Table II summarizes the results of increasing the concentration of sodium chloride on the retention volumes of platelets, granulocytes and lymphocytes. An

TABLE II

RETENTION VOLUMES AND SEPARATION FACTORS OF HUMAN PLATELETS (p), GRANULOCYTES (g) AND LYMPHOCYTES (l) USING MOBILE PHASES CONTAINING PHOSPHATE OR CATIONIC BUFFER (pH 7.5) IN THE PRESENCE OF SODIUM CHLORIDE

Column as in Table I. Mobile phases XI–XIX contained 2.0% (w/w) of dextran T40.

Mobile phase	*Concentration (M)*					*Retention volume (ml)*			*Separation factor*	
	NaCl	*NaH_2PO_4*	*Na_2HPO_4*	*Imidazole*	*Tris*	*p*	*g*	*l*	*g/p*	*l/g*
XI	0.07	0.03	0.03	—	—	5.8	8.6	12.6(ads.)	1.48	1.47
XII	0.11	0.02	0.02	—	—	5.5	10.4(31.4)	12.1(ads.)	1.89	1.16
XIII	0.13	0.01	0.01	—	—	5.3	17.2(ads.)	21.9(ads.)	3.25	1.27
XIV	0.12	—	—	0.465	—	3.6	3.6	3.6	—	—
XV	0.13	—	—	0.465	—	4.2	5.3	5.3	1.26	—
XVI	0.14	—	—	0.465	—	4.2	5.3	12.0	1.26	2.26
XVII	0.15	—	—	0.465	—	4.2	8.7	12.4	2.07	1.43
XVIII	0.154	—	—	0.465	—	5.3	9.8	13.1	1.85	1.34
XIX	0.03	—	—	—	0.07	4.5	9.5	15.8	2.11	1.66

increase in sodium chloride concentration with a concomitant decrease in phosphate concentration to keep the overall salt concentration essentially isotonic caused a slight decrease in the retention volume of platelets. On the other hand, the retention volumes of granulocytes and lymphocytes decreased and a proportion of these cells were adsorbed on the column when eluent XI containing 0.07 *M* of sodium chloride was used. In particular, the affinity of granulocytes for the stationary phase was significantly increased by the use of eluents XII and XIII, and lymphocytes were also adsorbed with eluent XIII. Erythrocytes were entirely adsorbed on the column with eluents XII and XIII.

From the effect of DEAE-dextran on retention of blood cells described above, it is considered that positively charged organic amines are distributed in the bonded PEG stationary phase rather than in the mobile phase, in contrast to the anionic phosphate. In order to examine the influence of buffer ions and of the concentration of sodium chloride, three kinds of blood cells were chromatographed by using mobile phases containing 2.0% (w/w) of dextran T40 and several organic amines instead of phosphate. Table II shows the retention volumes of these blood cells with eluents XIV–XVIII which comprised 0.465 *M* imidazole–hydrochloric acid buffer solution, pH 7.5, and 0.12–0.145 *M* sodium chloride. In these eluents, from hypotonic to hypertonic concentrations there was very little lysis of human erythrocytes. As shown in Table II, a marked change of the retention was observed for granulocytes and lymphocytes. Retention volumes of the three kinds of cells became the same with eluent XIV containing 0.12 *M* of sodium chloride. These values were still approximately the same for platelets and granulocytes in 0.13 and 0.14 *M* sodium chloride. The retention of lymphocytes was enhanced in 0.15 *M* sodium chloride, and the most remarkable distinction of these cells was attained with 0.154 *M* sodium chloride. Lysis of erythrocytes due to the hypertonicity of the eluent was observed above this concentration.

By use of isotonic 0.07 *M* Tris–hydrochloric acid–0.03 *M* sodium chloride buffer, pH 7.5, containing 2% (w/w) dextran T40 (eluent XIX), the retention volumes of these cells were differentiated; however, their recoveries from the column were very poor. Four kinds of Good's buffer materials, N,N-bis(2-hydroxyethyl)glycine (Bicine), N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES), N-tris-(hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES) and 2-(N-morpholino)-ethanesulphonic acid (MES), in concentrations of 0.01–0.1 *M* resulted in irreversible adsorption on the column and/or poor recoveries of these cells in the presence of isotonic 0.03–0.09 *M* sodium chloride.

DISCUSSION

In the previous paper[1], mobile phases containing either 8.0 or 4.5% (w/w) of dextran T40 or T500 and 0.09 *M* sodium phosphate buffer, pH 7.5, were used for the sub-fractionation of human and rabbit peripheral blood cells, except platelets. These concentrations of dextran were chosen according to the concentrations used in the APTP systems. It is convenient to imagine that charge-associated properties of the membrane surface are a major determinant of the partition of blood cells between the bonded PEG stationary phase and these mobile phases, since there is an electrostatic potential difference between the two phases.

It was shown in the present work that erythrocytes were eluted first, followed by platelets, granulocytes and lymphocytes, with every eluent containing dextran. It was also found the retention of lymphocytes and platelets to the stationary phase requires the presence of dextran in the mobile phase. As is seen from Table I, it was very difficult to resolve platelets and erythrocytes by means of this chromatographic system using eluents containing $<2.0\%$ (w/w) dextran T40. In APTP systems, Walter[8,9] partitioned platelets obtained from a number of different species in a system containing 5.5% (w/w) dextran–4% (w/w) PEG and 0.11 *M* sodium phosphate buffer, pH 6.8, and found that the partitions of platelets and erythrocytes from the same species are remarkably similar. This indicates that platelets and erythrocytes in a given species have similar surface properties, at least with respect to those characteristics which determine partition.

Lowering of the concentration of dextran T40 in the eluents from the 8% (w/w) used in the previous work to 2% (w/w) results in several advantages such as an improvement of the separation factor between platelets and granulocytes and an increase in flow-rate based on the decrease in the viscosity of the eluent.

In APTP systems, it has long been known[10], but not further explored, that the partition coefficient of cells increases when the polymer concentration is reduced in the presence of a constant salt composition and concentration. Walter *et al.*[11] pointed out that since the electrostatic potential difference between the phases diminishes with reduction of polymer concentration, the species-specific increase in partition coefficients of mammalian erythrocytes must be due to membrane surface properties other than charge. Furthermore, since the potential difference between the two aqueous polymeric phases caused by unequal partition of salts, notably phosphate, diminishes as the critical point is approached, it follows that the increase in partition of sells in phases close to the critical point must be related to something other than membrane surface charge. It is most likely that the reduction in interfacial tension that accompanies reduction in polymer concentration[12] results in less cell adsorption at the interface. The extent of the interaction of the cell surface with polymer determines the cell partition.

It has been also pointed out[6,7] in APTP systems that the partition of rabbit erythrocytes of low surface charge in a dextran–PEG phase system which also contains DEAE-dextran is dependent on the partition of DEAE-dextran itself. DEAE-dextran is distributed unevenly between the two aqueous polymeric phases[13]. In 5% (w/w) dextran T500 and 4% (w/w) PEG 6000 containing 0.11 *M* sodium phosphate, pH 6.8, *ca.* 90% of DEAE-dextran was found in the dextran-rich lower phase, which is negatively charged relative to the the upper phase[13]. The binding of DEAE-dextran to the anionic surface of blood cells causes the latter to behave as more positively charged particles.

Based on these results, in the phosphate-buffered mobile phase, it might be expected that the binding of DEAE-dextran would lead to a shift of the blood cells from the bonded PEG stationary phase to the mobile phase. By the use of a phosphate-buffered dextran mobile phase containing 0.5% (w/w) of DEAE-dextran (eluents VIII, IX and X), erythrocytes, and in particular platelets, were retained to the stationary phase with eluents VIII and X (Table I). On the other hand, erythrocytes were pulled into the mobile phase with eluent IX. The retention volumes of platelets, granulocytes and lymphocytes were slightly increased, however, with eluent IX compared to the

values obtained with eluent III. It is considered that this increase in retention may be due to an affinity of discharged cells binding with the cationic polymer for the stationary phase. The affinity is enhanced by increasing the concentration of dextran T40 from 2.0 to 4.5% (w/w). It must be noted that mobile phases containing DEAE-dextran are not ideal. As has been reported by Marikovsky *et al.*[14], the presence of charged polyelectrolytes has a tendency to cause agglutination of erythrocytes. In some of the mobile phases containing DEAE-dextran, after standing for a long time, the erythrocytes were aggregated.

It is known in APTP systems that some salts, such as phosphate, sulphate and citrate, are distributed unevenly, giving rise to an electrical and a zeta potential between the two aqueous polymeric phases[13,15,16]. This phase charge interacts with membrane surface-charge associated properties of cells added to the phases[17,18]. Thus the partition of suspended materials is dependent, although not exclusively, on charge. In addition, it has been pointed out that if sodium chloride is substituted for the phase close to the critical point, there is no potential difference between the phases[13] since this salt partitions almost equally[15]. Erythrocytes from most sources collect at the interface in such a system. Cells that do partition in such a phase system must do so through surface properties not related to their membrane charge.

In the present work, the interaction of blood cells with chemically bonded PEG stationary phase is apparently selective, as lymphocytes and granulocytes are more strongly retained on the column than platelets. The extent of retention of these cells is significantly affected by incorporation of sodium chloride in the eluent buffer. As shown in Table II, the retention behaviour of the blood cells gives an insight into the rôle played by the surface properties of the cells in this chromatographic system. Elution with mobile phases which are assumed to have zero potential difference with the bonded PEG stationary phase results in increased retention volume and adsorption of granulocytes. The retention of lymphocytes is increased in the presence of 0.13 *M* sodium chloride and these cells are adsorbed on the column with eluents XI–XIII. The chromatographic behaviour of platelets is little affected by the addition of sodium chloride. It is therefore considered that the retention of these blood cells when using a mobile phase containing sodium chloride and phosphate must depend on properties other than the membrane surface charge, since the mobile phase and the stationary phase provide no remarkable electrostatic potential difference with which the membrane charge could interact. The retention volumes of lymphocytes and granulocytes are also increased in the presence of 0.14–0.154 *M* sodium chloride in the mobile phase buffered with imidazole (eluents XVI–XVIII). It is assumed that these cells may be retained on the stationary phase by an interaction which is enhanced in sodium chloride concentrations above 0.14 *M*.

The effect of addition of sodium chloride to the mobile phase shows that the charge on the cell surface is one determinant of the retention of blood cells on the column. Ionic interactions may play a rôle in the retention of erythrocytes on the bonded PEG stationary phase; however, it is possible that the affinity of lymphocytes and granulocytes is considerably dependent on the interaction of the cell surface with the stationary phase. The contribution of hydrophobic interactions should also be taken into consideration. A study of the surface interaction of blood cells will be presented in a subsequent paper.

REFERENCES

1 U. Matsumoto and Y. Shibusawa, *J. Chromatogr.*, 187 (1980) 351.
2 P.-Å. Albertsson, *Partition of Cell Particles and Macromolecules*, Almqvist & Wiksell, Stockholm, and Wiley-Interscience, New York, 2nd ed., 1971.
3 A. Bøyum, *Nature (London)*, 204 (1964) 793.
4 E. Thorsby and A. Bratlie, in P. I. Terasaki (Editor), *Histocompatibility Testing*, Munksgaad, Copenhagen, 1970, p. 655.
5 C. H. W. Leeksma and J. A. Cohen, *J. Clin. Invest.*, 35 (1956) 964.
6 H. Walter and F. W. Selby, *Biochim. Biophys. Acta*, 148 (1967) 517.
7 H. Walter, R. Garza and R. P. Coyle, *Biochim. Biophys. Acta*, 156 (1968) 409.
8 H. Walter, *Proc. Protides Biol. Fluids*, 15 (1968) 367.
9 H. Walter, in T. Gerritsen (Editor), *Progress in Separation and Purification, Vol. 2, Modern Separation Methods of Macromolecules and Particles*, Wiley-Interscience, New York, 1969, p. 138.
10 P.-Å. Albertsson and G. D. Baird, *Exp. Cell Res.*, 28 (1962) 296.
11 H. Walter, E. J. Krob and D. E. Brooks, *Biochemistry*, 15 (1976) 2959.
12 J. Ryden and P.-Å. Albertsson, *J. Colloid Interface Sci.*, 37 (1971) 219.
13 R. Reitherman, S. D. Flanagan and S. H. Barondes, *Biochim. Biophys. Acta*, 297 (1973) 193.
14 Y. Marikovsky, D. Danon and A. Katchalsky, *Biochim. Biophys. Acta*, 124 (1966) 154.
15 G. Johansson, *Biochim. Biophys. Acta*, 221 (1970) 387.
16 G. V. F. Seaman and H. Walter, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 30 (1971) 1182a.
17 H. Walter, F. W. Selby and R. Garza, *Biochim. Biophys. Acta*, 136 (1967) 148.
18 H. Walter, R. Tung, L. J. Jackson and G. V. F. Seaman, *Biochem. Biophys. Res. Commun.*, 48 (1972) 565.

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DISSOLUTION OF SILACEOUS CHROMATOGRAPHIC PACKINGS IN VARIOUS AQUEOUS ELUENTS

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SUMMARY

Two pairs of DuPont Zorbax PSM Bimodal gel permention chromatography columns, packed with porous silica particles have been used for the fractionation of dextran. High initial efficiencies decreased with use and shrinkage of the bed suggested silica was dissolving. The eluent required a certain ionic strength to prevent some of the dextran being excluded.

The dissolution of two types of porous silica packings, LiChroprep SI-60 and Zorbax BP-SIL, has been investigated in a batch system using several aqueous solutions of different ionic strengths at ambient temperature. It was found that where a low ionic strength was required to supress ionic exclusion of the dextran, the best eluents were 0.001 *M* potassium phthalate and 0.001 *M* potassium dihydrogen phosphate. However, silica dissolution with these eluents or even with high purity water will give columns packed with porous silica a finite life.

INTRODUCTION

We have recently reported our experiences with various chromatographic packings used in the aqueous gel permeation chromatography (GPC) of dextran[1]. We remarked in that paper on the doubtful long term stability of microparticular silaceous chromatographic packings in aqueous eluents.

Silica packings are used to a considerable extent with aqueous eleuents in reversed-phase chromatography and there has been little adverse comment on the long term stability of these packings. However, comparatively little high-performance GPC is carried out with aqueous eluents.

That silaceous chromatographic packings would dissolve in aqueous solvents with a pH greater than about 8 has long been recognised[2]. Only recently[3] has dissolution of silaceous reverse phase chromatographic packings been accepted as a problem at lower pH values and the rate of silica loss reported is extremely high.

It is not surpising that problems of silica dissolution in aqueous eluents should exist. The normally accepted[4] equilibrium solubility is about 100 ppm at pH values between 1 and 8 although this can be much greater for microparticles[5]. The complicated

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interaction between silica and water is summarised succintly by Unger[5]. It would appear that chromatography on silaceous packings in aqueous eluents is only possible because of the low rate of dissolution.

We have tested one of the new generation of high-performance silaceous packings and subjected some of this packing to stability tests in various aqueous eluents.

EXPERIMENTAL

Chromatography

Two pairs of DuPont Zorbax PSM Bimodal columns were tested. A simple chromatographic system was used, consisting of a pump (Series II; Metering Pumps Ltd., London, Great Britain), a sample injection valve (Type 30.501; Spectroscopic Accessory Co., Sideup, Great Britain) fitted with a 20-μl sample loop and a differential refractometer detector (Model 1107LJ; Laboratory Data Control, Stone, Great Britain).

Any deterioration in the chromatographic columns could be easily monitored by measurement of their efficiency. The efficiency was calculated, with glucose as the solute, by

$$N = 8\left(\frac{t_R}{W_{h/e}}\right)^2$$

where N is the number of theoretical plates, t_R is the peak retention time and $W_{h/e}$ is the peak width at the peak height, h, divided by e, the base of the natural logarithm.

The pairs of columns were calibrated using Dextran "T" fractions (Pharmacia, Uppsala, Sweden) and the elution of the solutes was compared in terms of the Wheaton and Bauman distribution coefficient, K_d[7]

$$K_d = (V_e - V_0)/V_i$$

where V_e is the elution volume of the solute and V_0 is the void volume, measured by the elution of a totally excluded solute, Dextran 2000 (Pharmacia). V_i is the internal pore volume which is assumed to be the difference between the elution volume of glucose, as a small solute, and the void volume.

A number of eluents were used in this work, all were made up from distilled water and analytical grade reagents.

For the second pair of Zorbax columns a pre-column (25 $\times$ 0.4 cm) filled with LiChroprep Si-60, 15 to 20 μm (E. Merck, Darmstadt, G.F.R.) was introduced. Such a pre-column acts as an eluent filter and should contribute towards saturating the eluent with silica.

Silica dissolution

Using static experiments, the dissolution of loose silaceous chromatographic packings in various possible eluents was measured. The silica dissolution was measured as the silicon content of the solutions as assayed by atomic absorbtion spectroscopy.

The atomic absorption spectrophotometer was a Model 151 from Instrumentation Laboratories Inc. (Lexington, MA, U.S.A.), fitted with a silicon hollow cathode lamp from S & J Juiper and Co. (Harlow, Great Britain). The instrument set-

tings were as recommended by the manufacturer: lamp current 12 mA; slit width 80 μm; wavelength 251.6 nm; a rich acetylene–nitrous oxide flame with a burner height of 7 mm.

Two silaceous chromatographic packings were used in this work: (i) Li-Chroprep Si-60, a 15–25 μm packing normally used for preparative chromatography (E. Merck); (ii) Zorbax BP-SIL, a microparticular packing very closely resembling the Zorbax PSM 60 used in chromatography.

The various solutions were made up from analytical grade reagents in either deionised water or "Water for Liquid Chromatography" (BDH, Poole, Great Birtain).

The experimental procedure involved preparing 50 cm^3 of the solutions, monitoring the pH and then placing 0.2 g of the packing in the solution. The solutions were vigorously agitated occasionally, though they were allowed to settle for at least 1 h before the dissolved silicon concentration was determined directly by aspirating solutions from above the solid packing. Measurements were taken as the average of five 4-sec integrated signals. It was assumed that all the silicon was dissolved silica, the concentrations being expressed as parts per million (ppm) SiO_2.

All the work was carried out using polythene containers although the "Water for Liquid Chromatography" was supplied in glass bottles.

RESULTS

Chromatography

The initial characteristics of the first pair of Zorbax PSM Bimodal columns were very impressive (Table I). A flow-rate of 1 cm^3/min was maintained throughout the life of this pair of columns.

TABLE I

INITIAL CHARACTERISTICS OF THE FIRST PAIR OF ZORBAX COLUMNS

Column	*Efficiency (plates)*	*Pressure drop (bar)*
PSM 60	7000	55
PSM 1000	10,000	55
Combined	18,400	120

A slow deterioriation in efficiency was observed over the first 80 h use, but then there was a dramatic drop in efficiency which coincided with the application of a number of samples of Dextran 2000, used to determine the void volume. After this sudden drop in efficiency the columns maintained a steady performance for a further 250 h use. During this latter period of operation a variety of dextran samples were successfully analysed. It was not the reduced efficiency which caused problems but the non-Gaussian peak shape. When the inlet ends of the columns were opened up it was found that a definite void of about 2 mm existed on the PSM 60 column and a slight settlement of the PSM 1000 also appeared to have taken place. The final characteristics of this first pair of columns are shown in Table II.

A second pair of Zorbax PSM Bimodal columns were brought into use. The initial characteristics of this pair of columns were even more impressive (Table III).

TABLE II

FINAL CHARACTERISTICS OF THE FIRST PAIR OF ZORBAX COLUMNS

Column	*Efficiency (plates)*	*Pressure drop (bar)*
PSM 60	3600*	60
PSM 1000	8000	60
Combined	5000*	100

* Poor peak shape.

TABLE III

INITIAL CHARACTERISTICS OF THE SECOND PAIR OF ZORBAX COLUMNS

Column	*Efficiency (plates)*	*Pressure drop (bar)*
PSM 60	9900	88
PSM 1000	17,200	72
Combined	21,200	145

The fractionating range of both pairs of columns was ideal for the analysis of clinical dextran fractions with no noticeable lower limit and an exclusion limit of about 10^6 daltons. However, the second pair of columns did show a consistent excluded peak with dextran samples. This was presumed to be due to ionic exclusion and previous experience had shown us that this could usually be suppressed by increasing the ionic strength of the eluent. Our experiences with a number of eluents is summarised in Table IV.

TABLE IV

EXPERIENCES WITH VARIOUS ELUENTS

Eluent	*Hours used*	*Samples analysed*	*Comments*
Distilled water	60	160	Small excluded peaks
0.5% NaCl	1	3	*
0.02% NaN_3	3	3	*,**
0.02% Sodium pentachlorophenol	2	3	**
0.02% KH_2PO_4	80	140	***
0.02% Potassium hydrogen phthalate	9	4	***, also a dramatic drop in efficiency

* Suppressed excluded peaks, but it produced large negative peaks due to the absence of salt in the sample.

** With pH values of less than 7 these eluents were expected to enhance silica dissolution.

*** Suppressed excluded peaks with only a small negative peak introduced.

The efficiency dropped slowly during the period of time that phosphate was used in the eluent. After 50 h use of the phosphate eluent the combined column efficiency was 16,200 plates. After the sudden drop in efficiency it was no longer possible to maintain the 1 cm^3/min flow-rate. The column efficiencies were measured after being used with the phthalate eluent (Table V).

TABLE V

CHARACTERISTICS OF THE SECOND PAIR OF ZORBAX COLUMNS AFTER 150 h USE

Column	*Efficiency (plates)*	*Pressure drop (bar)*	*Flow-rate (cm^3/min)*
PSM 60	1190	115	1
PSM 1000	8040	115	1
Combined	6650	130	0.7

* Poor peak shape.

When the inlet ends of the second pair of columns were opened up it was found that there had been a considerable amount of settling with 2-mm and 1-mm voids on the PSM 60 and PSM 1000 columns respectively. The columns were "topped-up" using the Zorbax BP-SIL material and a combined efficiency of 15,000 plates was obtained. A further 250 h use was obtained from these columns with occasional "topping-up". Although the combined efficiency was always kept well above 5000 plates, there was a steady increase in pressure drop and decrease in flow-rate. The phthalate eluent was used for this later work.

The elution volumes of Dextran 2000 and glucose were recorded regularly to allow the use of the Wheaton and Bauman distribution coefficient. No change in the calibration of these columns was observed.

Silica dissolution

The initial silica dissolution work was carried out with the LiChroprep Si-60 packing. The silica dissolution with time is recorded in Table VI.

TABLE VI

DISSOLUTION OF LICHROPREP Si-60

Solution	*Approx. molarity*	*Silica concentration (ppm)*				*Initial pH*
		16 h	*98 h*	*122 h*	*27 days*	
Deionised Water	—	41	73	92	118	5.5
0.5% NaCl	0.1	51	81	105	113	5.6
0.05% NaCl	0.01	41	75	101	113	5.2
0.05% KH_2PO_4	0.004	21	43	60	88	5.0
0.05% $KO_2C(C_6H_4)CO_2H$	0.003	9	15	21	49	4.2
0.05% KSCN	0.001	32	65	94	88	5.7
0.05% NaN_3	0.01	51	79	103	113	7.3

Zorbax BP-SIL was used in two separate dissolution experiments. In the first experiment the solutions were made up in deionised water and the results are summarised in Table VII. In the second dissolution experiment with Zorbax, the solutions were made up in "Water for Liquid Chromatography" (Table VIII).

TABLE VII

DISSOLUTION OF ZORBAX IN SOLUTIONS FROM DEIONISED WATER

Solution	*Silica concentration (ppm)*			*Initial pH*
	1 h	*24 h*	*48 h*	
Deionised water	2.1	2.4	9.0	5.7
0.1 *M* NaCl	3.4	24.2	54.6	5.7
0.05% NaN_3	5.7	$\gg$40	$\gg$70	7.3
0.1 *M* KH_2PO_4	4.3	25.3	65.4	4.5
0.01 *M* KH_2PO_4	3.2	18.9	44.6	4.5
0.001 *M* KH_2PO_4	2.1	9.0	19.1	5.2
0.1 *M* $KO_2C(C_6H_4)CO_2H$	7.1	24.4	50.1	3.8
0.01 *M* $KO_2C(C_6H_4)CO_2H$	1.5	16.7	31.9	4.0
0.001 *M* $KO_2C(C_6H_4)CO_2H$	2.4	7.9	15.0	4.2
0.1 *M* KSCN	2.1	20.6	48.4	5.5
0.1 *M* HCl	1.7	7.9	12.0	0.6

TABLE VIII

DISSOLUTION OF ZORBAX IN SOLUTIONS FROM "WATER FOR LIQUID CHROMATOGRAPHY"

Solution	*Silica concentration (ppm)*		*Initial pH*
	48 h	*114 h*	
Deionised water	2.1	8.8	5.7
"Water for Liquid Chromatography"	3.2	9.2	4.8
0.001 *M* $KO_2C(C_6H_4)CO_2H$	8.9	19.3	4.2
0.1 *M* Na_2SO_4	34.4	50.6	6.2
0.01 *M* Na_2SO_4	22.5	35.4	6.8
0.001 *M* Na_2SO_4	11.5	23.4	6.8

DISCUSSION

It can be seen from the results reported here on chromatography with the DuPont Zorbax PSM Bimodal columns that a useful practical life can be obtained from microparticular silaceous GPC packings when used with aqueous eluents. The first pair of columns exhibited an early dramatic drop in efficiency which was then followed by a long period of stability. With the second pair of columns a significant period of high efficiency was achieved before a sudden drop in efficiency again followed by a steady lower efficiency. The later silica dissolution work indicates that there is probably no connection between the sudden drop in efficiency of the second pair of columns and the change to the phthalate eluent solution.

The difference in the initial pressure drops across the two pairs of columns is to some extent explained by the introduction of the pre-column when using the second pair. However, the higher efficiency and the higher presure drop of the second pair of columns probably reflect an improvement in the column packing technique of the manufacturer.

The very high efficiency found with the silaceous microparticular columns is not generally required in GPC, though the short analysis time is a great asset. When a

void is created at the top of the column, a shoulder will usually be seen on the glucose peak. It should be stressed that it was this lack of quality in the peak shape, rather than the drop in efficiency, that caused problems. However these voids at the top of the column can be "topped-up", even with a different packing, to improve the peak shape and increase the effective life of the column.

The silica dissolution work with LiChroprep Si-60 was originally carried out to test the experimental method on a less valuable chromatographic packing. Since this packing is likely to have different surface properties to the Zorbax, the results have been included to allow comparisons to be made.

The two experiments with the Zorbax BP-SIL packing used two test solutions common to each experiment [deionised water and 0.001 *M* $KO_2C(C_6H_4)CO_2H$]. That the measured silica dissolution is so different in each case indicates that there must be other important parameters (*e.g.*, the degree and frequency of agitation) and only the relative solubilities should be considered.

From the first experiment with Zorbax BP-SIL it can be clearly seen that the concentration of salts in solution have a dramatic effect on the amount of silica dissolution. The concentration of salts is obviously more important than small changes in pH around this mildly acidic area. The more extreme pH values have a large effect on silica dissolution. The effect of sodium azide solution is of particular interest; its pH of 7.3 would normally be considered acceptable and in fact sodium azide is frequently used to inhibit bacterial growth either in aqueous samples or aqueous eluents. However, these results suggest that sodium azide enhances dissolution of silica quite significantly.

The second experiment with Zorbax BP-SIL was principally intended to compare the silica dissolution properties of deionised water and "Water for Liquid Chromatography", though an additional salt, recommended to suppress "ghost" peaks[8], sodium sulphate was also tested. There appeared to be no significant difference between the silica dissolution properties of deionised water and "Water for Liquid Chromatography". It was also surprising that this "Water for Liquid Chromatography" had no recordable silica concentration as it was supplied in glass bottles; this may well be because the ultra pure water does not contain the salts necessary to promote silica dissolution.

Comparison of the phthalate and sodium sulphate solutions suggests that sodium sulphate will cause similar silica dissolution to the other salts used at the same concentration.

Subsequent to the work reported here it was found[9] that if the columns are flushed with and stored in acetone after use, their lifetime is considerably increased. It is also reported[8] that aqueous methanol (10% methanol–90%water, to which 2% glycerol is added) has been successfully used as eluent elsewhere.

CONCLUSIONS

There would appear to be always a significant dissolution of microparticular silaceous chromatographic packings in aqueous eluents. In the slightly acidic region of pH (3–6), frequently used for chromatography, the concentration of salt in solution is the important parameter. Usually only a low concentration of salts is required to suppress any adverse ionic effects of a chromatographic column. We have found that

0.001 *M* potassium phthalate or potassium dihydrogen phosphate suppresses the small excluded "ghost" peaks, without introducing large negative peaks and these solutions do not significantly enhance silica dissolution.

The life of the new generation of microparticular silaceous chromatographic columns must be limited when used in aqueous eluents because of silica dissolution. Whether or not the life of microparticular silaceous chromatography columns will justify the cost of manufacturers pre-packed columns will depend on the application. Where bulk chromatography packings are available this is probably a more sensible approach as the columns can be readily re-packed.

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REFERENCES

1 P. E. Barker, B. W. Hatt and S. R. Holding, *J. Chromatogr.*, 174 (1979) 143.
2 A. Wehrli, J. C. Hildenbrand, H. P. Keller and R. Stamfli, *J. Chromatogr.*, 149 (1978) 199.
3 F. M. Rabel, *Int. Lab.*, March (1980) 53,
4 R. K. Iler, in E. Matijevic (Editor), *Surface and Colloid Science*, Vol. 6, Wiley-Interscience, London, 1973, p. 1.
5 G. B. Alexander, *J. Phys. Chem.*, 61 (1957) 1563.
6 K. K. Unger, *Porous Silica—Its Properties and Use as Support in Column Liquid Chromatography*, Elsevier, Amsterdam, Oxford, New York, 1979.
7 R. M. Wheaton and W. C. Bauman, *Ann. N.Y. Acad. Sci.*, 57 (1953) 159.
8 E. I. Du Pont de Nemours and Co., private communication, 1978.
9 G. J. Vlachogiannis, Department of Chemical Engineering, University of Aston, Birmingham, personal communication.

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GRAPHITIZED CARBON BLACK COLUMNS FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Commercially available graphitized carbon black, after a fractionation process to obtain particles of various sizes can be used for the preparation of columns for high-performance liquid chromatography where it behaves as a natural reversed phase. The chromatographic and packing performances of these columns have been determined. Reduced plate heights of less than four have been obtained for retained compounds ($k' = 7$), coupled with a satisfactory permeability. A critical evaluation of the advantages and limitations of these columns is made. Some practical applications are reported to allow a direct comparison between graphitized carbon black and other reversed-phase chromatographic supports.

INTRODUCTION

Since the pioneering work of Kiselev and co-workers[1,2], graphitized carbon black (GCB) has increasingly been applied in the gas chromatography of complex organic mixtures[3]. The versatility of this material is mainly due to the characteristics of its surface which is almost free of unsaturated bonds and polar active sites so that adsorption of various molecules occurs non-specifically. These features have been exploited[2–4] in gas–solid chromatography (GSC) as well as in gas–liquid–solid chromatography (GLSC).

Although GCB is particularly suitable as a reversed phase in liquid chromatography and in high-performance liquid chromatography (HPLC), no direct use of GCB has been reported mainly because, as pointed out by Colin *et al.*[5], practical application is prevented owing to its poor mechanical properties. Those authors tried to overcome this limitation by hardening the GCB surface by benzene pyrolysis in an inert gas stream. This treatment, which yields a deposit of pyrolytic carbon, renders the particles hard enough to stand the high pressures commonly required

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when the so-called balanced-density slurry packing method[6] is employed. However, drastic changes are produced in the properties of the original particles: the surface area is decreased and the material is not as inert and homogeneous[5,7]. Tailing is frequently observed in peaks with a rather high capacity ratio, and a further graphitization process has been proposed[5].

Since both the hardening and the graphitization processes require careful control of some parameters (such as temperature, time and benzene flow-rate) in order to obtain a sufficiently reproducible support, an investigation has been carried out with commercially available GCB in an attempt to obtain HPLC columns. This paper describes the preparation procedure and an evaluation of the performance of the columns in terms of plate height as a function of mean particle diameter and capacity ratio, k'. Some practical applications are presented, together with a comparison between GCB and the most widely used materials in reversed-phase chromatography. The limitations and advantages of GCB in HPLC are also discussed.

EXPERIMENTAL

Column preparation

Carbopack B (80–100 mesh) supplied by Supelco (Supelco, Bellefonte, PA, U.S.A.) surface area *ca.* 80 m^2/g, was selected because of its mechanical stability compared to other GCB. The material was ground on a mechanical sieving machine with rubber balls. The resulting particles were then placed on the top of a set of metal screens (200, 150, 88, 75, 54, 45, 33, 25, 15 μm) having a diameter of 22 cm and acetone was continuously added to the top screen. This preliminary treatment breaks down those particles formed by simple agglomeration of microparticles present in the original material. The frictional forces due to the flow of acetone dislocate the particles as their cohesion is reduced by the decrease of the interparticular electrostatic interactions; consequently microcrystallites from the core of the particles are washed out. After this treatmenent the various GCB sizes were dried and the dried material was sieved again. Examination under the microscope showed that particles having mean diameters greater than 20 μm are well defined and almost spherical, whereas those having mean diameters less than 20 μm appear to be mainly comprised of irregularly shaped microparticles. Only particles in the former category were selected for our investigation. Because we wished to check the packing arrangement during the packing process as well as during the chromatographic analysis, glass columns, 25–35 cm in length, instead of stainless steel were employed. Glass tubes (6 mm O.D., 1.6 mm I.D.) can be used even above 200 kg/cm^2, although the column capacity is reduced.

Columns were prepared by a dry packing technique similar to that employed for packing GCB micropacked columns commonly used in gas chromatography[8]. GCB was continuously placed on the top of the column with constant vibration of the walls as well as of end of the column. As the use of fritted metal ends was found to damage the GCB particles, the column ends were separated from the packing by means of 180–200 μm metal particles. These were kept in place by a 10-μm metal screen supported on a PTFE O-ring placed in contact with the internal wall of the metal fitting. Polyamide ferrules were used to ensure a tight metal–glass connection and were found to be stable above 200 kg/cm^2.

Apparatus

To obtain a low dead volume, a home-made injector was employed. This device permits observation of the sample injection and prevents column plugging caused by the syringe needle; it is quite useful especially when soft particles are used.

The columns were set into a Varian Model 4100 liquid chromatograph (Varian Aerograph, Walnut Creek, CA, U.S.A.) equipped with a variable-wavelength Variscan UV detector and fed with a liquid of low viscosity (pentane) by slowly increasing (2 h) the flow-rate from 0.1 to 2 ml/min. As this procedure generally causes a slight rearrangement and a small decrease (*ca.* 2 cm) of the height of the packed material, additional particles are required to fill the extra volume. For column safety, it is suggested that GCB of larger particle size (120–180 μm) be added. A schematic diagram of the injection device and of the connections of the glass columns with the liquid chromatograph is shown in Fig. 1.

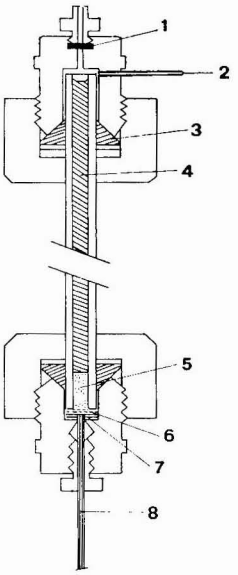

Fig. 1. Scheme of the injector and its connections to the column. 1 = Septum; 2 = liquid inlet; 3 = polyamide ferrules; 4 = glass column filled with Carbopack B particles; 5 = 180–200 μm metal particles; 6 = 10-μm metal screen; 7 = 1 mm thick PTFE O-ring; 8 = stainless-steel capillary tube (0.2 mm I.D.).

RESULTS AND DISCUSSION

GCB column performance

Three sets of columns filled with particles with mean diameters corresponding to 75–88, 33–45 and 25–33 μm respectively were prepared. Each column was then connected to the chromatographic apparatus and its efficiency was measured for compounds having a capacity ratio ranging from about 0.01 to 7. The test mixture injected contained benzene, 1,3,5-trimethylbenzene, naphthalene, methylnaphthalene and 3,6-dimethylnaphthalene and the elvent was pentane. Fig. 2 shows typical plots of HETP *vs.* linear velocity for two of the most retained compounds ($k' = 3.07$ and

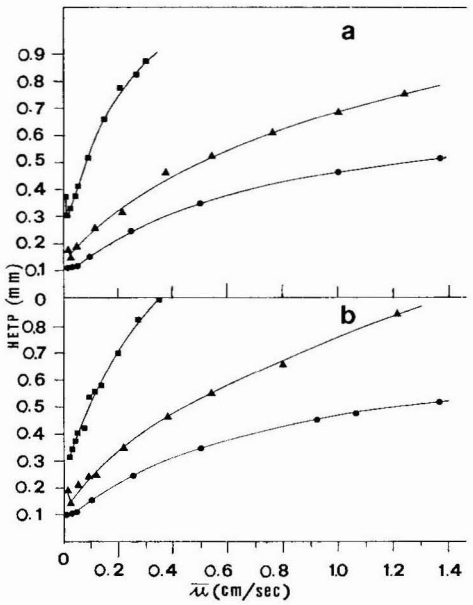

Fig. 2. HETP *vs.* linear velocity for glass columns (25 cm × 1.6 mm I.D.) packed with Carbopack B particles of different sizes. Eluent: pentane. Particle size: ■, 75–88 μm; ▲, 33–45 μm; ●, 25–33 μm. Compounds: a = methylnaphthalene ($k' = 3.07$); b = 3,6-dimethylnaphthalene ($k' = 7.01$).

7.01 respectively). The variations of HETP values *vs.* increasing values of the capacity ratio for various compounds are shown in Fig. 3a. All HETP values correspond to the of minimum values observed in the HETP *vs.* linear velocity curves. The dashed line refers to the HETP values measured at a linear velocity of 1 cm/sec with a column filled with 25–33 μm particles.

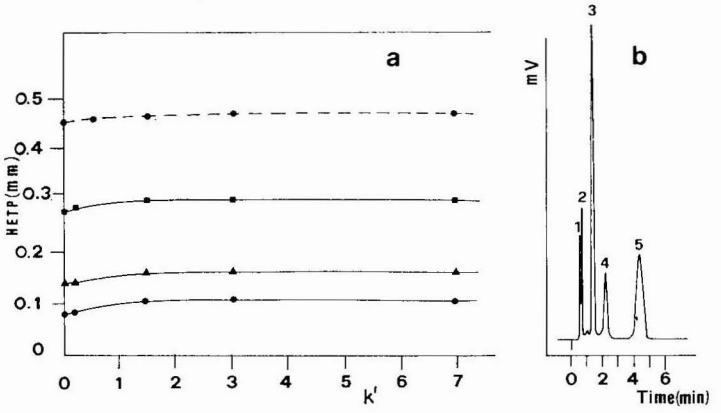

Fig. 3. a, HETP *vs.* capacity ratio calculated at a linear velocity corresponding to the minimum HETP value for Carbopack B particles of different sizes (see Fig. 2). The dashed line corresponds to the HETP value measured at a linear velocity of 1 cm/sec. b, Typical chromatogram obtained with a glass column (25 cm × 1.6 mm I.D.) packed with Carbopack B (particle size, 25–33 μm). Peaks: 1 = benzene; 2 = 1,3,5-trimethylbenzene; 3 = naphthalene; 4 = methylnaphthalene; 5 = 3,6-dimethylnaphthalene.

When the above fractionating procedure described was carefully followed, the differences in HETP between the various columns in each set never exceeded the values in Figs. 2 and 3 by more than 10%. Fig. 3b shows a typical chromatogram obtained with a 25-cm column filled with 25–33 μm particles and working at a linear velocity of 1 cm/sec.

From the results reported in Figs. 2 and 3 the following conclusions can be made:

(1) The average minimum HETP values obtained for retained compounds (k' from 3 to 7) are almost equal to four times the particle diameter (reduced plate height ranging from 3.7 to 4.0). This indicates that quite efficient columns can be obtained with GCB. If the results obtained at low k' with GCB do not differ appreciably from those obtained with hardened carbon black particles[5], the slope of the HETP *vs.* $\bar{u}$ plots obtained at high linear velocity appears to be less with GCB than with pyrolytic carbon.

(2) Plots of HETP *vs.* k' are almost flat for different values of the mean particle diameter and for the range of linear velocities employed. This means that efficiency losses arising from non-linearity of the adsorption isotherm are negligible and there is only slight tailing of the peaks due to the dead volume. This explains why the values obtained at $k' = 0$ are slightly higher than expected on the basis of plate height theory. It should be added, however, that whereas the value of the minimum HETP at $k' = 0$ is of theoretical interest, in practice of greater importance is the HETP value measured for a fairly strongly retained compound ($k' = 3$) when the linear velocity approaches the values commonly used during analytical separations (0.5–1 cm/sec). In this content, GCB columns show a comparable or better performance than those filled with the commonly used reversed-phase packings, having the same particle diameter.

The above considerations demonstrate the advantage in using Carbopack B columns in comparison with pyrolytically hardened carbon black particles. The high efficiency observed with the former at high values of the capacity ratio should undoubtedly be attributed to its greater homogeneity in comparison with the latter. The pyrolytic treatment produces an increase of the number of active sites on the carbon surface having an intermediate structure between amorphous and graphitized carbon, and therefore a lower degree of homogeneity[9].

The packing performance of GCB columns has been evaluated also by measuring the permeability exhibited by each set of columns characterized by different particle size. Column permeabilities, φ, were calculated from the equation[10] $\varphi = \Delta P d_p^2 / L u \eta$, where ΔP is the column pressure drop, L the column length, u the mobile phase velocity and η the eluent viscosity. Values ranging from 650, when $d_p > 33$ μm, to 870 when $d_p < 33$ μm, have been calculated. Although the packing permeability is not as good as that calculated for pyrolysed carbon black, the values obtained indicate that GCB may be suitable for HPLC.

It has to be stressed, however, that Carbopack B columns have a more limited field of application than those packed with modified carbon black. As they require solvents of viscosity less than 0.7 cP, any attempt to use water–methanol (30:70) or even pure ethanol as eluent has been unsuccessful. Although this limitation might in practice be overcome by operating with eluents of low viscosity, which in the case of carbon black cover almost the entire scale of eluotropic strength[7], care has to be raken to prevent columns plugging when viscous eluents are needed in connection with rapid gradients.

Moreover it has so far been impossible to prepare GCB particles smaller than 20 μm, a size available for other packing materials.

Practical application of GCB columns

Some analytical applications which can be performed rapidly without the use of gradients or viscous solvents have been examined to provide a direct comparison with the packings commonly used in reversed-phase liquid chromatography.

Fig. 4b shows the separation of a mixture of chlorinated pesticides and Fig 4a the fingerprint of a commercial PCB mixture (Fenclor 64). As the former sample was analyzed by using a more selective but also weaker eluent (methanol) than the latter, it is suggested that GCB columns might fruitfully be employed for sample fractionation.

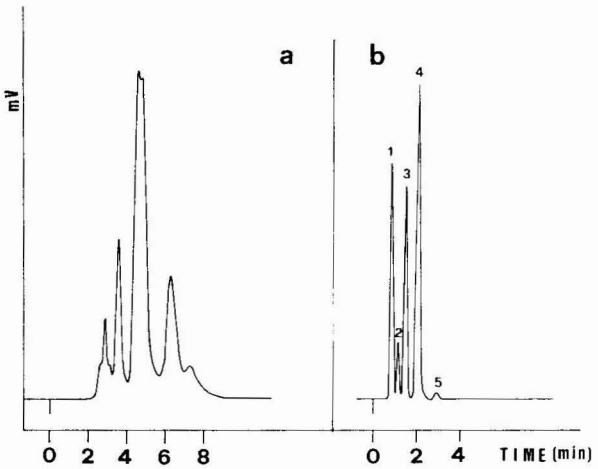

Fig. 4. a, Separation of a PCB mixture (Fenclor 64) on a glass column (25 cm × 1.6 mm I.D.) packed with Carbopack B (25–33 μm). Eluent: pentane. Flow-rate: 1 ml/min. b, Analysis of a mixture of chlorinated pesticides on the same column as in a. Eluent: methanol. Flow-rate: 0.5 ml/min. Peaks: 1 = chlordane; 2 = aldrin; 3 = *pp'*-DDT; 4 = Metoxichlor; 5 = unknown.

Fig. 5a shows the chromatogram of a mixture of alkyl phthalates commonly used as plastifiers, and Fig. 5b illustrates the analysis of an environmental sample consisting of a water condensate collected from the gaseous emission of an urban waste incinerator. Although these analyses can be performed with many other packings, it is worth noting the selectivity exhibited by GCB in the separation of diisobutyl and di-*n*-butyl phthalate isomers.

Fig. 6a shows the separation of atrazine, a known herbicide, from some of its metabolites produced by bacterial degradation[11].

Fig. 6b illustrates the direct analysis of the products formed during an "*in vitro*" bacterial degradation of an atrazine solution.

Fig. 7 shows a typical pharmaceutical analysis, which is generally carried out by reversed-phase chromatography: the separation of active components of an analgesic pharmaceutical product consisting of phenylacetamide, phenacetin and caffeine. Acetylsalicylic acid, if present in such drugs, is eluted after the phenylacetamide peak.

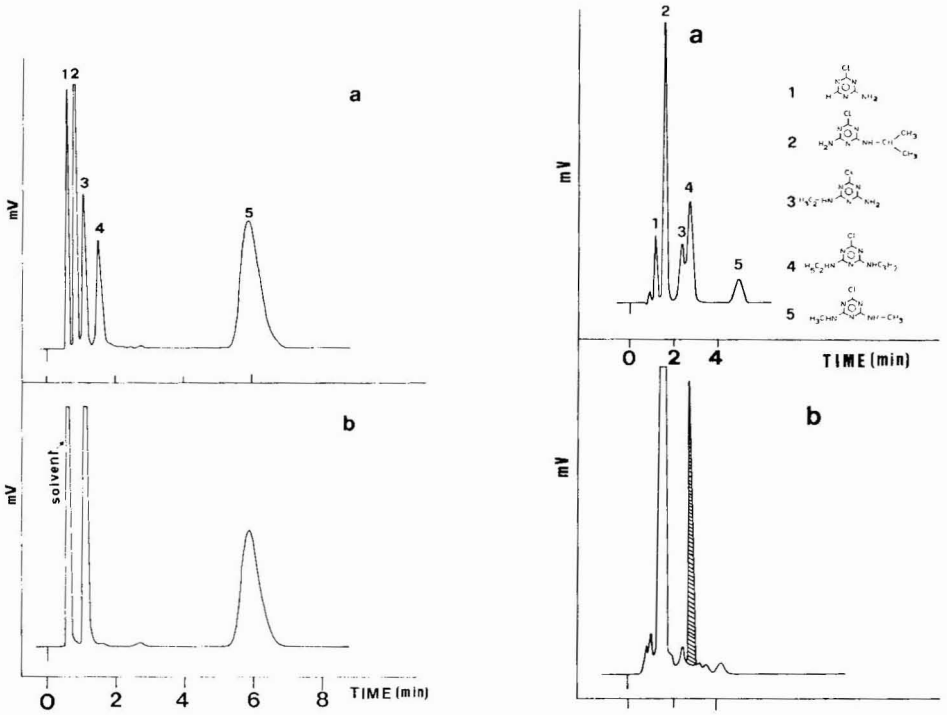

Fig. 5. Separation of a mixture of plastifiers on a glass column (25 cm × 1.6 mm I.D.) packed with Carbopack B (25–33 μm). Eluent: methanol. Flow-rate: 1 ml/min. a, Chromatogram of a standard mixture. Peaks: 1 = dimethyl phthalate; 2 = diethyl phthalate; 3 = Diisobutyl phthalate; 4 = di-*n*-butyl phthalate; 5 = di-*n*-octyl phthalate. b, Water condensate from urban waste incinerator.

Fig. 6. Analysis of some triazine isomers. Column and eluent: same as in Fig. 5. Flow-rate: 0.5 ml/min. a, Pure standards; b, sample collected from an "*in vitro*" bacterial degradation of atrazine.

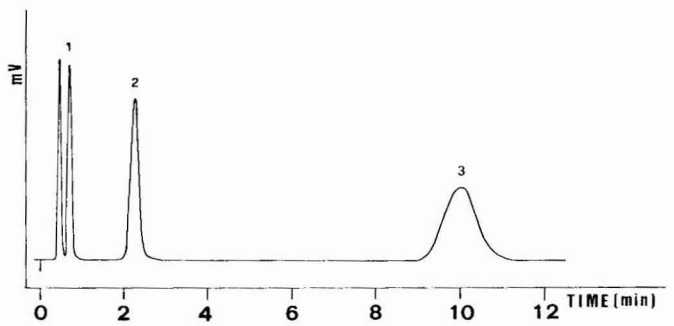

Fig. 7. Separation of some constituents of a pharmaceutical preparation (analgesic). Conditions as in Fig. 5. Peaks: 1 = phenylacetamide; 2 = phenacetin; 3 = caffeine.

Finally Fig. 8 shows the separation of two UV absorbing free amino acids in methanol solution: phenylalanine and tryptophan. This separation should be regarded only as an attempt to demonstrate the versatility of GCB for the analysis of such fractionations.

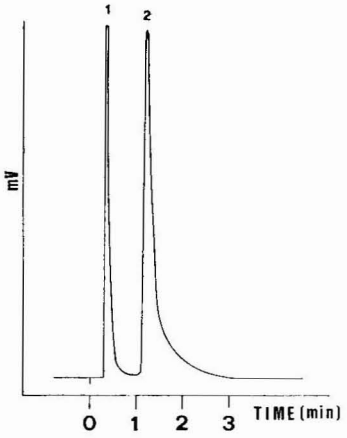

Fig. 8. Separation of two UV absorbing amino acids. Conditions as in Fig. 5. Peaks: 1 = phenylalanine; 2 = tryptophan.

CONCLUSIONS

Commercially available GCB can successfully be used for the preparation of HPLC columns providing the material is fractionated into various sizes. Although there are some limitations related to the viscosity of the eluents, GCB, which is a natural reversed phase, may be specific in the analysis of polar low boiling isomers as in the separation of complex mixtures, especially when particles having small diameters can be employed.

ACKNOWLEDGEMENTS

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REFERENCES

1 A. V. Kiselev and J. P. Yashin, *Gas Adsorption Chromatography*, Plenum, New York, 1969, p. 128.
2 A. V. Kiselev, N. V. Kovaleva and Yu. S. Nikitin, *J. Chromatogr.*, 58 (1971) 19 and citations therein.
3 A. Di Corcia and A. Liberti, *Advan. Chromatogr.*, 14 (1974) 305–363.
4 F. Bruner, P. Ciccioli, G. Crescentini and M. T. Pistolesi, *Anal. Chem.*, 45 (1973) 1851.
5 H. Colin, C. Eon and G. Guiochon, *J. Chromatogr.*, 119 (1976) 41.
6 R. E. Majors, *Anal. Chem.*, 30 (1958) 914.
7 H. Colin, C. Eon and G. Guiochon, *J. Chromatogr.*, 122 (1976) 223.
8 A. Di Corcia, A. Liberti and R. Samperi, *J. Chromatogr.*, 167 (1978) 243.
9 T. V. Barmakova, A. V. Kiselev and N. V. Kovaleva, *Kolloid Zh.*, 36 (1974) 133.
10 J. H. Knox, in C. F. Simpson (Editor), *Practical High Performance Liquid Chromatography*, Heyden & Son, London, 1976, pp. 34–39.
11 M. C. Giardina, M. T. Giardi and G. Filacchioni, *Agr. Biol. Chem.*, 9 (1980) 821–825.

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AUTOMATED ON-LINE MULTI-DIMENSIONAL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC TECHNIQUES FOR THE CLEAN-UP AND ANALYSIS OF WATER-SOLUBLE SAMPLES*

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SUMMARY

The application of an automated on-line multi-dimensional liquid–liquid chromatographic technique for the clean-up and analysis of water-soluble samples was investigated. The use of microparticulate aqueous-compatible steric exclusion columns as the primary separation step coupled to either reversed-phase, normal-phase or ion-exchange columns as the secondary step allowed the direct injection of complex samples without prior clean-up. The entire operation was automatically controlled by a microprocessor-based liquid chromatograph with time-programmable events which allowed precise switching of high-pressure pneumatically operated valves. Both heart-cutting and on-column concentration methods were used. The heart-cutting technique had the advantage of selectivity but lacked sensitivity; more successful was the on-column concentration technique, which, by the concentration of the solute from a larger volume of exclusion column effluent on to the secondary column, gave better sensitivity. The technique was applied to the analysis of theophylline and caffeine in biological fluids, catecholamines in urine, vitamins in a protein food supplement and sugars in molasses and candy bars.

INTRODUCTION

Multi-dimensional chromatography (also known as coupled column chromatography or column switching) refers to the technique in which fractions from one column are selectively transferred to one or more secondary columns for further

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separation. Off-line multi-dimensional liquid chromatographic (LC) techniques have been in use for some time. In particular, the use of gel permeation chromatography (GPC) for the clean-up of complex sample matrices using organic solvents followed by either an LC step or a gas chromatographic (GC) separation of the collected fractions is widespread (for an example, see ref. 1). Such off-line techniques are often time consuming, inconvenient and difficult to quantitate and/or reproduce. Obviously, on-line techniques are to be preferred.

The on-line combination of high-performance GPC and reversed-phase chromatography (RPC) employing two chromatographic pumps has been reported earlier[2]. In the earlier approach, complex organic soluble samples were first fractionated on a microparticulate cross-linked polystyrene exclusion column using tetrahydrofuran (THF) as the mobile phase and, using a heart-cutting approach, these fractions were directed through a six-port sampling valve on to a reversed-phase chromatographic column using water–acetonitrile as the mobile phase. Two difficulties were encountered with this approach: (1) water-soluble samples could not be handled and (2) because THF is a powerful modifier in reversed-phase chromatography, only a small volume could be injected from the GPC column on to the reversed-phase column. Large injection volumes of THF would cause partial migration of injected fractions down the reversed-phase column, thereby limiting resolution. However, too small volumes would limit the sensitivity of the technique.

With the recent developments in aqueous-compatible, rigid microparticulate (10-μm) exclusion (gel filtration) columns[3], it was of interest to explore the feasibility of handling complex aqueous samples by multi-dimensional chromatography. Erni and Frei[4] reported the successful coupling of aqueous-exclusion and reversed-phase columns but used large porous glass particles as the exclusion media. Broad elution profiles from low-efficiency primary columns limit the ultimate resolution and sensitivity of the multi-dimensional technique. In this work we utilized 10-μm high-performance microparticulate aqueous-exclusion columns coupled on-line with reversed-phase, ion-exchange and normal bonded phase chromatographic columns. Such a combination allows the on-line clean-up of a wide range of aqueous samples, ranging from physiological fluids to water-soluble industrial polymers. This combination technique also provides the capability of easily concentrating samples on to a secondary column.

The combination of aqueous-exclusion chromatography (EC) and RPC is ideal from the standpoint that the solvents used in both techniques are compatible. The predominant aqueous mobile phase used in EC is, fortunately, a "weak" mobile phase in RPC. Such solvent compatibility allows, in addition to heart cutting, the technique of on-column concentration to be carried out. In this mode of operation, the flow of column 1 is directed for a finite period of time on to column 2; in effect, it directs a portion of the chromatogram from column 1 on to column 2. By the use of automatic valving, the entire operation can be carried out unattended.

In our earlier report[2], two or three pumps were employed. With the advent of modern single-pump ternary gradient chromatographs, this less expensive alternative appeared to be more attractive. Therefore, one aim of this work was also to investigate the use of a microprocessor-controlled single pump chromatograph in performing all required tasks.

EXPERIMENTAL

Instrumentation and columns

Fig. 1 is a schematic diagram of the multi-dimensional system employed. The chromatograph is a Varian Model 5060, which has a single pump with three-solvent capability. It was equipped with an automatic six-port sampling valve (Valco) for injection of the sample on to the exclusion column. In addition, up to three other automatic six-port, two-position valves (Valco) were employed. All valves could be controlled by time-programmable external events (powered contact closures) from the chromatograph. They could be automatically switched either off or on at predetermined times by single keyboard programming.

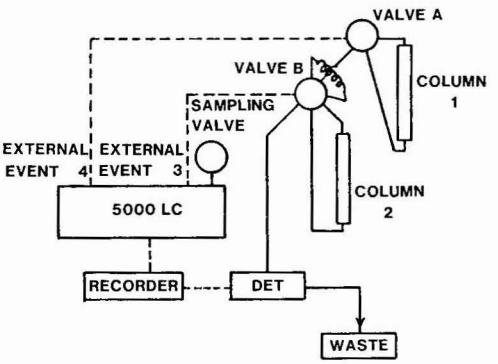

Fig. 1. System configuration for automated on-line column-switching techniques using Varian Model 5000 LC external events. Dashed lines represent electrical connections.

The aqueous compatible microparticulate (10-μm) exclusion columns were 30 cm $\times$ 7.5 mm I.D. MicroPak TSK Type SW or TSK Type PW columns. The Type SW columns are recommended for biopolymers and are packed with rigid particles of silica gel especially chemically deactivated to avoid adsorption of proteins. The Type PW columns are packed with microparticulate polyether gels which contain hydrophilic hydroxyl surface groups. They are recommended for simple saccharides, polysaccharides and water-soluble industrial polymers, oligomers and additives. Column 2 (30 cm $\times$ 4 mm I.D.) was one of three: (1) a MicroPak-MCH-10 reversed-phase column which contains 10-μm particles with a monolayer of bonded octadecylsilane; (2) an Aminex HPX-87 (polystyrene–divinylbenzene) cation-exchange resin in the Ca^{2+} form, recommended for carbohydrates; or (3) a MicroPak-NH_2-10 column, which possesses surface aminopropyl groups. All of these columns are available from Varian (Palo Alto, CA, U.S.A.).

A Varian UV-50 variable-wavelength detector, a Varian Fluorichrom filter fluorimeter or a Bioanalytical Systems (W. Lafayette, IN, U.S.A.) LC-2 electrochemical detector with a glassy carbon electrode were employed.

Valving configurations

By appropriate configurations of the switching valves, a number of flow options were available. Fig. 2 depicts the normal configuration when two switching valves

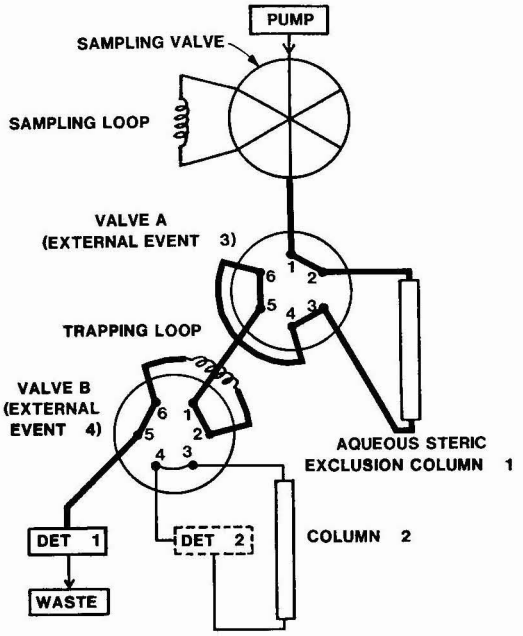

Fig. 2. HPLC solvent flow diagram for automated on-line multi-dimensional chromatography. Heavy lines represent solvent flow path for the normal configuration.

were employed. Such a configuration allows both heart cutting or on-column concentration to be carried out with no plumbing change. By using external events 3 and 4 from the Model 5060, valve A or valve B can be switched at different times in order to obtain different flow paths. The heavy lines represent the flow paths for the normal configuration at time zero. For heart cutting, when the solute of interest enters the trapping loop, having been eluted from column 1, both valves are switched and the trapped solute (the volume of which is determined by the loop volume) is directed on to column 2. In this switched configuration, using a single-pump chromatograph, some mobile phase used for the exclusion separation that resides in the chromatograph hydraulics and connecting lines is routed through column 2. In the usual case, the passage of this volume of mobile phase, which at most represents 4 ml in the Model 5060, is not detrimental since water or buffered water is a weak solvent for RPC. By switching valve B back to its normal state after all of the sample has been directed on to column 2, most of this solvent goes to waste. Then chromatography on column 2 can be carried out by again switching valve B. Note that column 1 is isolated and other fractions could, in turn, be chromatographed on column 2 when the first separation has been completed.

In the on-column sample concentration configuration, valve B is switched from the normal configuration in Fig. 2 when one desires to concentrate a portion of the chromatogram. The total time selected is dependent on the volume desired to be injected. When the volume is diverted from column 1 to column 2, then valve A is placed in its switched position and now chromatography proceeds on column 2.

In cases where the solvent used in column 1 remains in the lines and is,

therefore, partially pumped through column 2 during its chromatography step is detrimental to the column or the separation, a second valving configuration employing three valves can be used. Although not shown, an optional valve is placed between valves A and B. Its purpose is to act as a holding loop during heart cutting so that the solvent in the lines can be directed to waste. Once all lines have been flushed, then the sample is directed to column 2 and chromatography begins.

Solvents and chemicals

Acetonitrile and methanol were distilled-in-glass materials obtained from Burdick and Jackson (Muskegon, MI, U.S.A.). Water was purified by reverse osmosis and deionization. Buffer salts were of reagent grade.

Samples of molasses, candy bar and protein supplement were obtained locally. Sugars and vitamins used as standards were of reagent grade. Samples of reagent-grade DOPA (3,4-dihydroxyphenylalanine) standard were obtained from Aldrich (Milwaukee, WI, U.S.A.). Urine samples for DOPA analysis were obtained from the University of California at San Francisco, Department of Dermatology.

Preparation of samples

Samples of molasses and candy bar were either suspended or shaken with water for several minutes, then centrifuged, and the supernatant was filtered through an 0.45-μm membrane filter prior to injection. Urine and plasma were filtered through a membrane filter. Standard samples were dissolved in water or buffer at the appropriate concentration and then chromatographed.

Chromatography

The basic chromatographic procedures were carried out in a similar manner. The filtered sample was injected, either manually or with an AutoSampler, on to the exclusion column. The Model 5060 pump allows use of one, two or three solvents and either isocratic or gradient operation. Blending is accomplished by high-speed solvent proportioning valves on the low-pressure side of the pump. These solvent compositions can be time programmed. Thus, the EC solvent used in column 1 can be different from that used in column 2. For example, solvent A for EC can be water, and while EC is proceeding solvent A only can be pumping. When the second separation step commences on column 2, solvents B (aqueous buffer) and C (acetonitrile or methanol) can be programmed to begin flow and solvent A stopped. Thus a single pump can serve the purpose of a two- or three-pump system.

At the time heart cutting or on-column concentration is to occur, the chromatograph is simultaneously programmed to actuate the correct valves, thereby directing the desired trapped volume or portion of chromatogram as well as the desired solvent or solvent combination.

RESULTS

Caffeine and theophylline in biological fluids

Theophylline (1,3-dimethylxanthine) is used in the treatment of bronchial asthma and has been applied as a CNS stimulant and diuretic. High-performance liquid chromatographic (HPLC) analyses of theophylline and a common interferent,

caffeine, in plasma and urine have been given considerable attention recently. These analyses, however, rely on sample pre-treatment, such as extractions or pre-concentrations[5–7]. The following application allows the direct injection of biological fluids without pre-treatment.

The analysis of caffeine and theophylline in biological fluids was carried out using both the heart-cutting and on-column concentration methods. In both instances, the first column was a MicroPak TSK 2000SW exclusion column (50 cm × 7.5 mm I.D.) and the second column was a MicroPak MCH-10 reversed-phase column. The effluent from both columns was monitored at 272 nm. Exclusion chromatography was performed using water as the mobile phase at 1 ml/min. Caffeine and theophylline were co-eluted from the aqueous exclusion column at 18.8 ml under these conditions.

In the heart-cutting method used for the analysis of caffeine, 50 μl of raw urine were injected on to the exclusion column. At the elution volume of the caffeine peak from the exclusion column (18.8 ml corresponding to the peak apex), the solute was trapped by means of a 50-μl trapping loop. Solvent flow was switched to the reversed-phase column by appropriate valve actuation and, after an equilibration period of 4 min at a mobile phase flow-rate of 4 ml/min, the trapped solute was injected on to the reversed-phase column. RPC analysis of caffeine was performed isocratically using a mobile phase containing 20 m*M* tetramethylammonium chloride, 10 m*M* potassium dihydrogen orthophosphate and 24% acetonitrile at a flow-rate of 1 ml/min. Fig. 3A depicts the analysis of a caffeine standard using this technique. The elution volume of caffeine is slightly above 4 ml under these conditions. The heart-cutting method yielded a minimum detectable concentration of $5 \cdot 10^{-3}$ mg/ml of caffeine with 4 decades of linearity. Fig. 3B shows the analysis of a urine sample containing approximately $6 \cdot 10^{-3}$ mg/ml of caffeine. Although the heart-cutting method allowed high selectivity owing to the ability to trap small solute volumes, in this instance it was insufficiently sensitive to detect theophylline at therapeutic levels.

In the on-column concentration method for blood plasma and urine analysis, a 2.8-ml volume, corresponding to the elution of caffeine and theophylline from the exclusion column, was concentrated on to the head of the reversed-phase column. Caffeine and theophylline were then analyzed using gradient elution, as shown in Fig. 4A and B. Using this approach, a minimum detectable concentration of approximately $5 \cdot 10^{-4}$ mg/ml was obtained for both caffeine and theophylline, with linearity to 1 mg/ml. This can be compared with values in the literature[7] of $2 \cdot 10^{-5}$ mg/ml. It should be noted that although the method lacks the absolute sensitivity of previously reported analyses, it accomplishes automatic sample handling of complex matrices and yields detection limits within therapeutic ranges. Although not as selective as the heart-cutting method (necessitating gradient elution), the on-column method yielded substantially better sensitivity.

Analysis of vitamins in protein food supplement

Increasingly, many diverse food products are being fortified with vitamins to enhance their nutritional value. One such food product, a protein supplement, was selected for analysis of several B vitamins at levels ranging from 0.001% to 0.04% by weight in the protein supplement.

Direct injection of the protein supplement on to a reversed-phase column for vitamin analysis would result in irreversible adsorption of the protein content of the

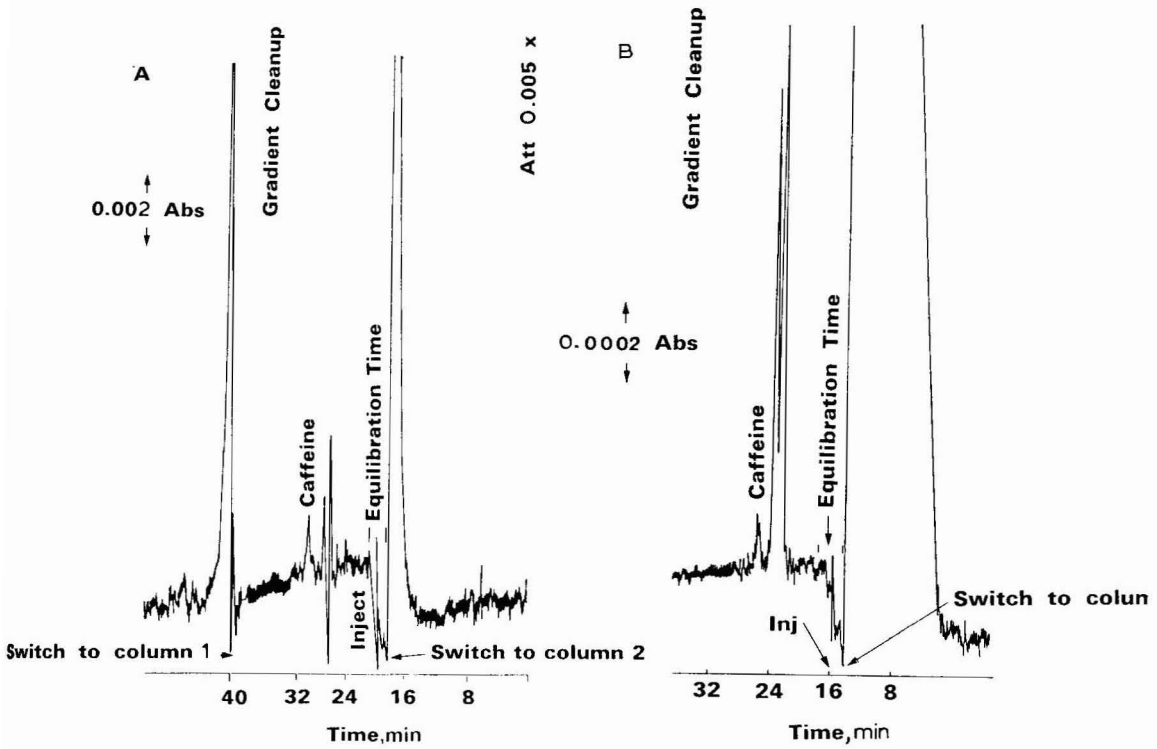

Fig. 3. Heart-cutting technique for separation of caffeine in raw urine. (A) Caffeine standard; (B) raw urine sample. Conditions: EC column, MicroPak TSK 2000SW (50 cm × 7.5 mm I.D.); flow-rate, water at 1.0 ml/min; detection at 272 nm; 50 μl injected. RPC column: MicroPak MCH-10 (30 cm × 4 mm I.D.); flow-rate, 1 ml/min 20 m*M* tetramethylammonium chloride, 10 m*M* KH_2PO_4 and 24% acetonitrile; 50 μl injected from trapping loop; detection at 272 nm; 0.002 a.u.f.s.

supplement to the column packing as well as possible matrix interferences. Therefore, an on-column concentration technique was used for sample clean-up in addition to concentration of low levels of the vitamins, accomplished by utilizing the high sample capacity of the exclusion columns.

An on-column concentration technique employing a MicroPak TSK 2000SW aqueous-exclusion column and a MicroPak MCH-10 reversed-phase column was used for the analysis of vitamins in the protein supplement. The chromatogram of the aqueous-exclusion analysis of the protein supplement is shown in Fig. 5A. A 1.3-ml volume of the eluent, corresponding to the elution interval of the vitamins from the exclusion chromatogram, was concentrated on to the head of the reversed-phase column by appropriate switching valve actuation. Fig. 5B depicts the analysis of niacin, pyridoxine, thiamine and riboflavin vitamin standards by reversed-phase gradient elution using 1-heptanesulfonic acid (HSA) as an ion-pairing reagent.

The vitamin analysis of the protein supplement with on-column concentration is shown in Fig. 5C. A 10–15-mg sample load was injected on to the aqueous-exclusion column and the solute eluting from 8.2 to 9.5 ml (1.3 ml volume) was concentrated on to the reversedphase column. Gradient elution of the vitamins was then initiated.

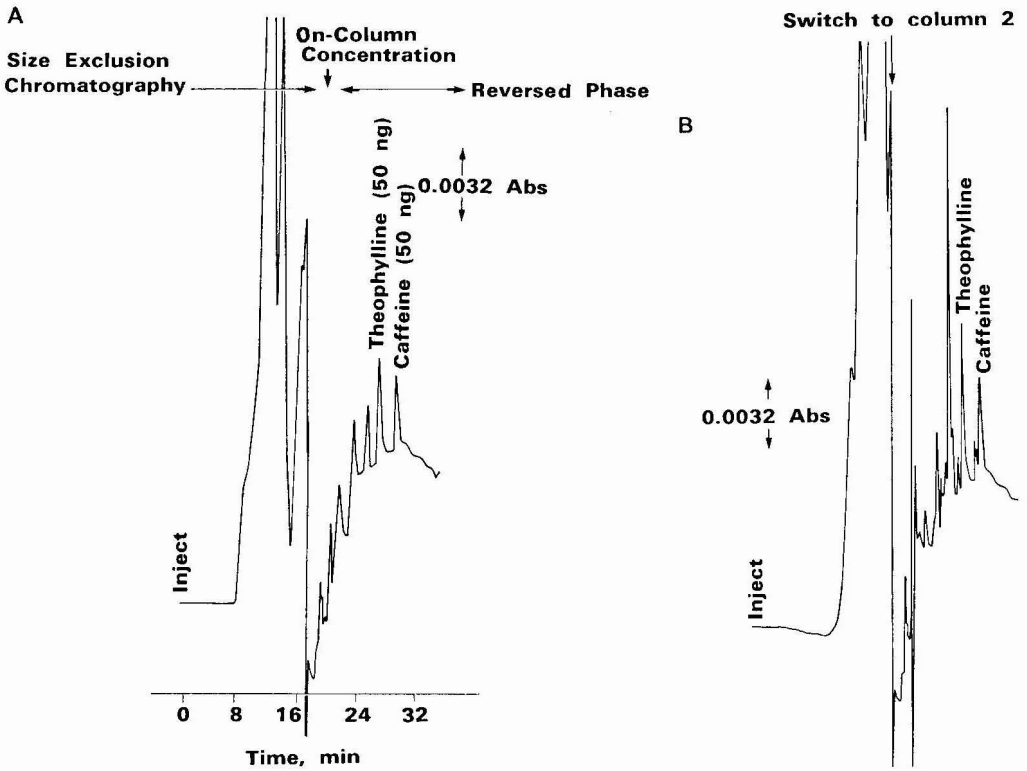

Fig. 4. On-column concentration technique for separation of caffeine and theophylline in biological fluids. (A) Blood plasma; (B) raw urine. EC conditions as in Fig. 3 except injection volume (10 μl). RPC conditions: column, MicroPak MCH-10 (30 cm × 4 mm I.D.); flow-rate, 1 ml/min; volume concentrated from EC column, 2.8 ml; gradient, water with 0.01 *M* KH_2PO_4 (pH 4)–acetonitrile, 0 to 12% acetonitrile at 6%/min, then held for 10 min; detection at 272 nm; 0.032 a.u.f.s.

The chromatographic conditions used were the same as those in Fig. 5A for exclusion analysis and Fig. 5B for reversed-phase analysis. Approximately 0.02% of niacin, 0.003% of pyridoxine, 0.009% of riboflavin and 0.003% of thiamine were found in the food supplement. The analysis of these B vitamins by HPLC has been favorably compared to chemical and microbiological analyses of these vitamins in foodstuff matrices[8,9]. On-column concentration for the analysis of vitamins in food matrices not only offers the advantage of speed by reducing the sample preparation time, but also extends the technique to trace levels by effectively increasing the detection sensitivity through the use of higher sample loadings on the EC column.

Analysis of DOPA in urine

The analysis of DOPA and other catecholamines by HPLC has received considerable attention owing to the advent of the highly sensitive and selective electrochemical detector. One of the major limitations of the currently available electrochemical detectors is their susceptibility to electrode poisoning, which is particularly pronounced with complex biological samples. This factor has required that usual

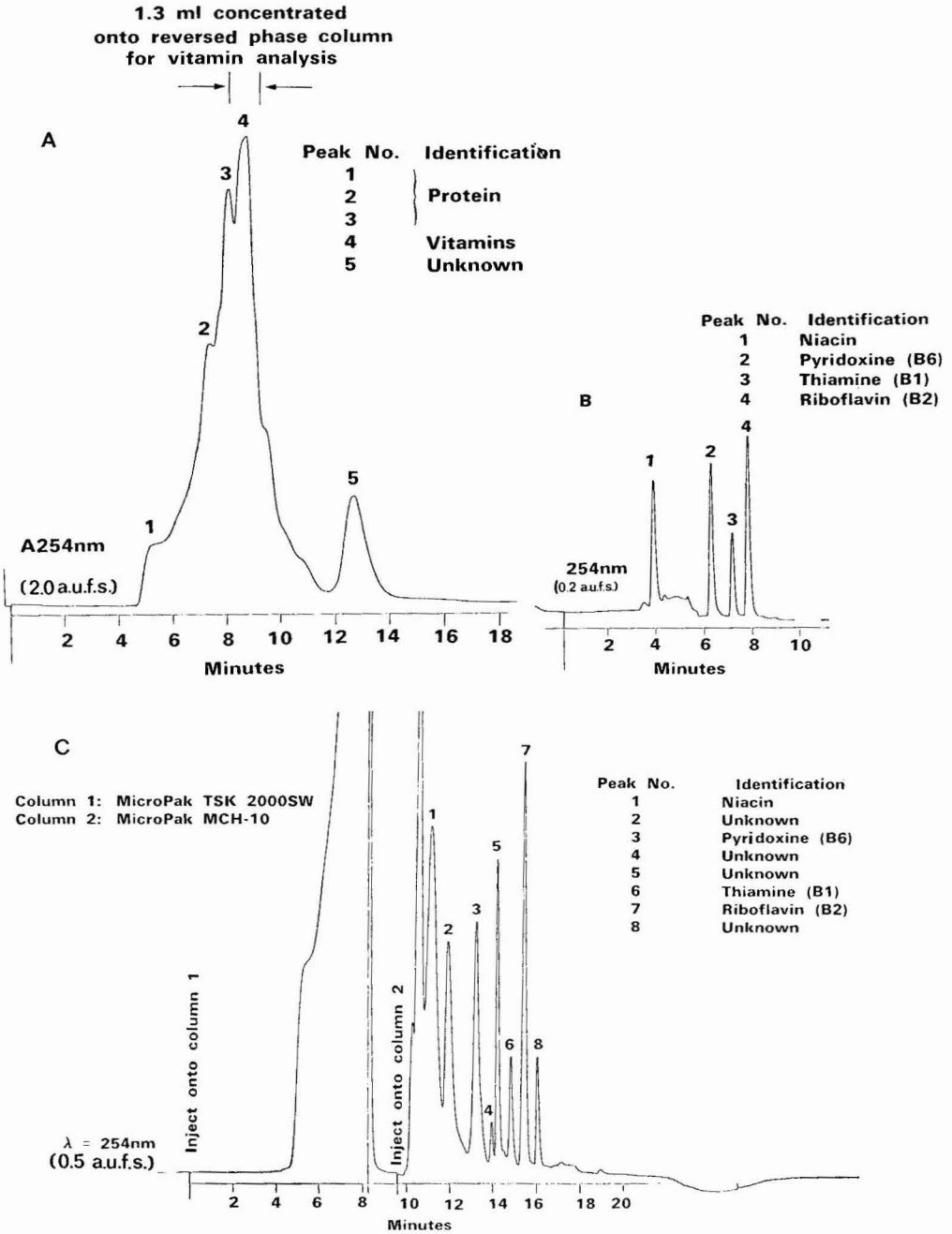

Fig. 5. On-column concentration technique for separation of B vitamins in food protein supplement: (A) EC analysis of supplement. Column, MicroPak TSK 2000SW (30 cm × 7.5 mm I.D.); methanol–water (1:9) with 0.1 *M* KH_2PO_4 and 0.01 *M* 1-heptanesulfonic acid (HSA) at 1.2 ml/min; detection at 254 nm; 2.0 a.u.f.s.; injection volume, 100 μl. (B) RPC analysis of vitamin standards. Column, MicroPak MCH-10 (30 cm × 4 mm I.D.); flow-rate, 2 ml/min; injection volume, 10 μl; gradient, methanol–water (10:90), to 80% methanol with 0.01 *M* KH_2PO_4 and 0.01 *M* HSA at 15% methanol/min; detection at 254 nm; 0.1 a.u.f.s. (C) On-column concentration techniques for vitamin analysis in supplement; conditions as in (A) and (B) except volume concentrated from exclusion chromatogram for injection on to RPC column (1.3 ml).

HPLC techniques include extensive sample pre-treatment procedures[10,11]. The use of two-dimensional on-line HPLC, as described below, eliminates sample pre-treatment by allowing only the portion of the sample that includes the compound of interest to pass through the electrochemical detector.

The analysis of DOPA in urine was carried out by a heart-cutting method. The exclusion column used was a MicroPak TSK 2000SW with water as the mobile phase. The second column was a MicroPak MCH-10 reversed-phase column using 20 m*M* camphorsulfonic acid, 100 m*M* sodium dihydrogen orthophosphate and 0.1 m*M* NaEDTA for the isocratic elution of DOPA. DOPA eluted at 8.9 ml on the exclusion column and a 100-μl volume was trapped at this point. The trapped solute was then injected on to the reversed-phase column and eluted at 15.2 ml. The exclusion chroma-

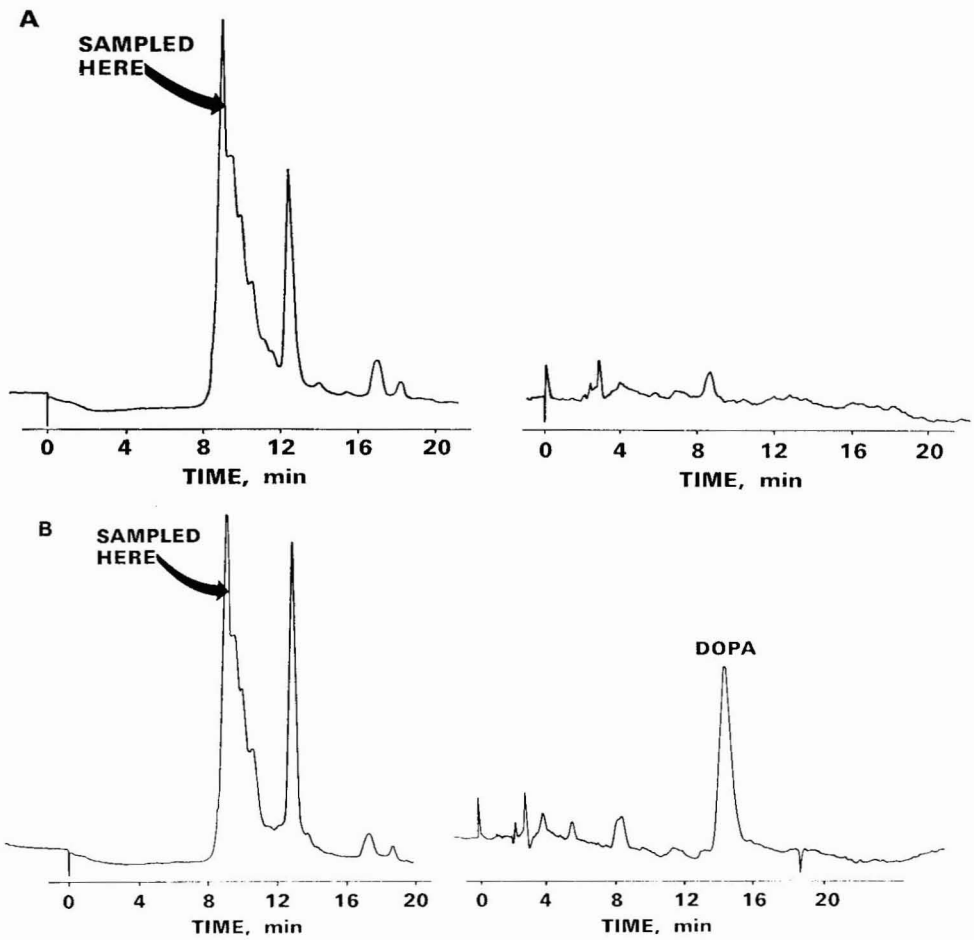

Fig. 6. Heart-cutting technique for the analysis of DOPA in urine. (A) Normal urine; (B) abnormal urine. EC analysis (left): column, MicroPak TSK 2000SW (30 cm × 7.5 mm I.D.); water at 1.0 ml/min; injection volume, 10 μl raw urine; detection at 230 nm; 2.0 a.u.f.s. RPC analysis (right): column, MicroPak MCH-10 (30 cm × 4 mm I.D.); water with 20 m*M* camphorsulfonic acid, 100 m*M* NaH_2PO_4 and 0.1 m*M* NaEDTA at 1 ml/min; detection, electrochemical detector with glassy carbon electrode operated at 0.720 mV *vs.* Ag–AgCl; attenuation, 2 nA/V; sample, 100 μl.

tography was monitored by UV at 230 nm and the reversed-phase effluent was monitored using an electrochemical detector operated at 720 mV *vs.* a silver–silver chloride electrode. Fig. 6A shows the analysis of normal urine by this method and Fig. 6B the analysis of urine from a melanoma patient with elevated DOPA levels. Note that by using this approach not only is the reversed-phase separation simplified considerably, but also the electrochemical detector is protected from poisoning by the raw urine sample.

The purpose of this application was to illustrate the possibility of on-line sample clean-up. Quantitative aspects are under investigation.

Analysis of sugars in molasses and candy formulations

The amounts and types of sugars present in food formulations has become a subject of great concern to both food processors and consumers. LC techniques employing both aminopropyl bonded phase columns for normal-phase chromatography and ion-exchange resins in the Ca^{2+} form have been used successfully for the analysis of sugars in various foodstuffs.

An on-column concentration technique was employed for the analysis of sugars in a candy bar formulation using a TSK Type PW aqueous-exclusion column and an Aminex HPX-87 cation-exchange column. The results for sucrose, glucose and fructose standards on these columns are shown in Fig. 7A.

The chromatogram of the EC analysis of the candy bar formulation is shown in Fig. 7B. A 1.1-ml portion of the chromatogram, encompassing the elution interval of the sugar standards, was concentrated on to the ion-exchange column for subsequent separation. The higher molecular weight components seen as peak 1 in this chromatogram are passed to waste to prevent contamination of the analytical ion-exchange column.

The results of the on-column concentration technique are shown in Fig. 7C. A sample of the candy formulation dissolved in water was injected on to the EC column and the solute eluting from 5.4 to 6.5 ml was concentrated on to the ion-exchange column by activation of switching valves. Analysis of the sugars was then begun on the HPX-87 column. The chromatographic conditions for analysis on both columns were the same as those for the sugar standards in Fig. 7A. Approximately 975 μg of sucrose and 75 μg of glucose were detected in the sample, representing 0.008% and 0.1% by weight, respectively, in the formulation.

The heart-cutting technique used for the analysis of sugars in molasses using normal-phase chromatography represents a special, non-ideal case in that the problem of non-compatible solvents for columns 1 and 2 greatly restricts the volume of solvent from the EC column (1) that can be passed over the normal-phase column (2). For this analysis, an EC column was employed with water as a mobile phase. A MicroPak-NH_2 column was used for sugar analysis. For such a normal-phase column water is a strong solvent. To circumvent this complication, a third switching valve was installed between the two valves shown in Fig. 2 such that, by appropriate actuation, the analytical columns and trapping loop (50-μl volume) can be isolated from the solvent flow path while the dead volume of the chromatographic pumping system is flushed directly to waste.

Fig. 8A depicts the EC analysis of molasses using a TSK 2000PW column. Note that the higher molecular weight polysaccharides eluting in peak 1 are flushed to waste

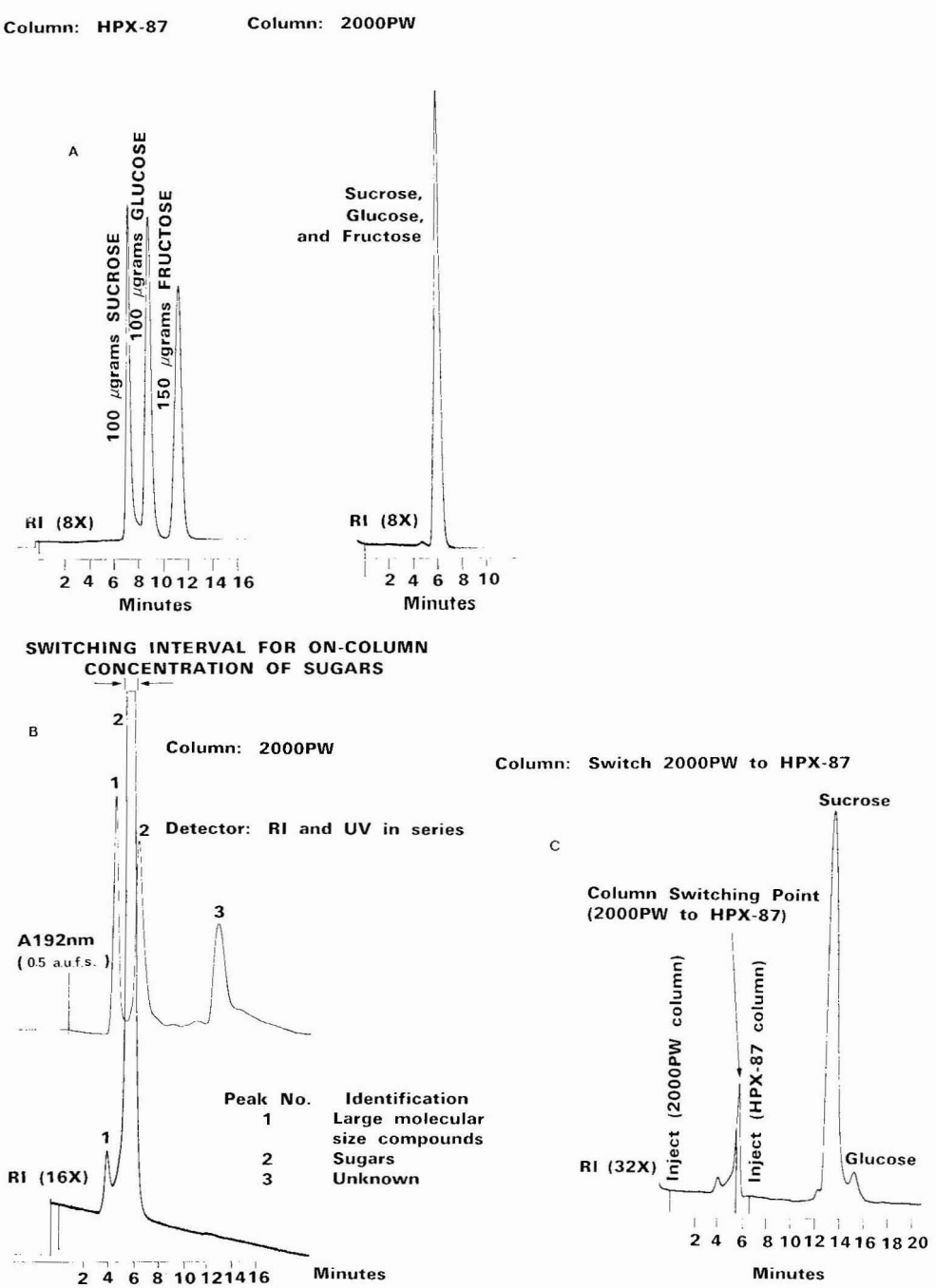

Fig. 7. On-column concentration method for sugar analysis in a candy bar formulation. (A) Sugar standards; (B) exclusion chromatography of candy bar formulation; (C) on-column concentration method for sugars in candy bar formulation. EC analysis: column, MicroPak TSK 2000PW (30 cm × 7.5 mm I.D.); water at 1.5 ml/min; injection volume, 10 μl; concentration volume, 1.1 ml; detection, RI; attenuation, 8×; UV, 192 nm, 0.5 a.u.f.s. Cation-exchange chromatography: column HPX-87; temperature, 85°C; water at 0.7 ml/min; detection as above.

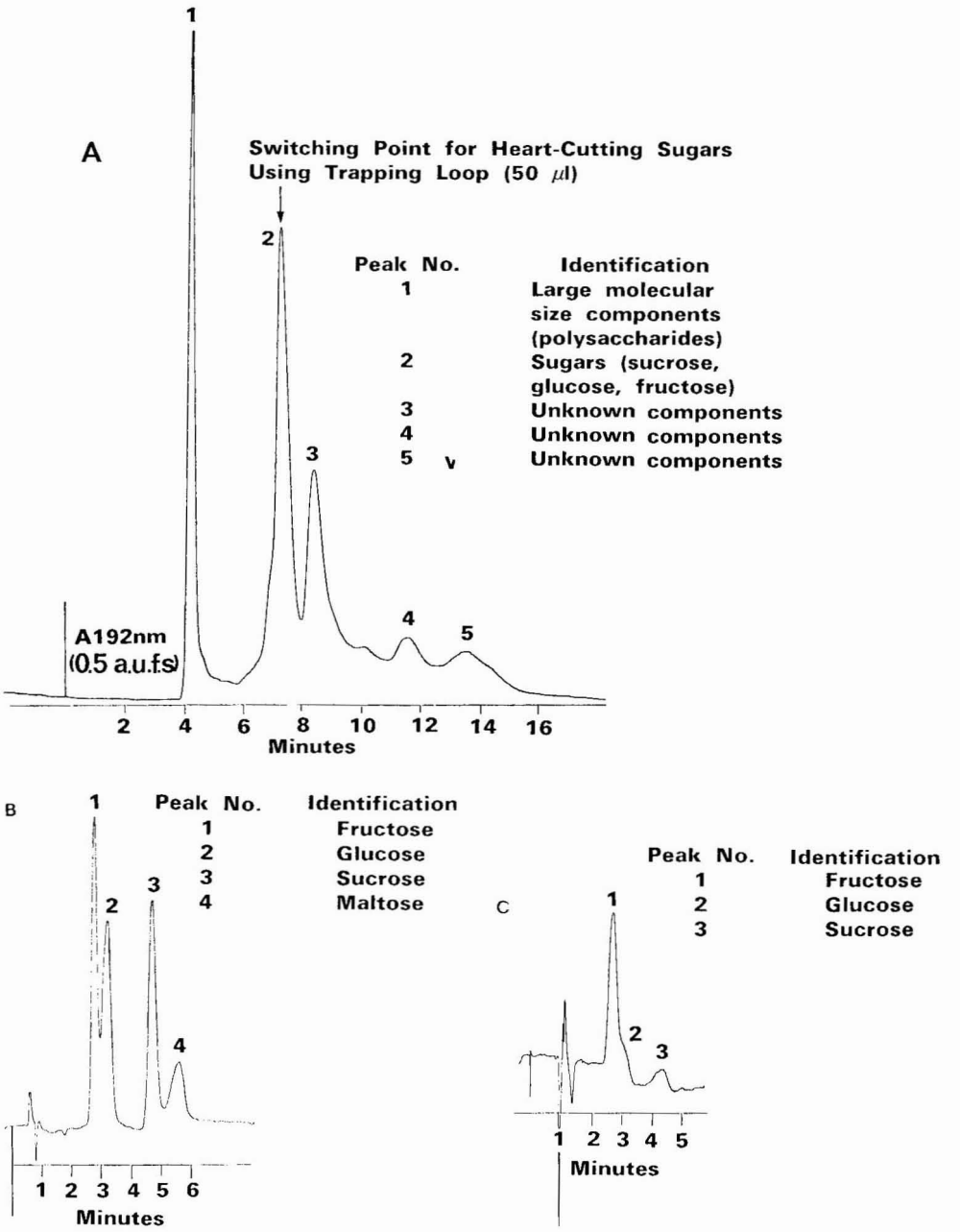

Fig. 8. Heart-cutting method for sugars in molasses. (A) Exclusion chromatography of molasses; (B) normal-phase chromatography of sugar standards; (C) normal-phase chromatography of molasses heart cut. Exclusion chromatography: column, MicroPak TSK 2000PW (30 cm × 7.5 mm I.D.); 0.1% acetic acid in water at 1.2 ml/min; detection, UV, 192 nm, 0.5 a.u.f.s.; injection volume, 100 μl. Normal-phase chromatography: column, MicroPak NH_2-10 (30 cm × 4 mm I.D.), acetonitrile–water (65:35) at 2.8 ml/min; detection, UV, 192 nm, 0.2 a.u.f.s. (B), 0.1 a.u.f.s. (C), trapping loop volume, 50 μl.

to prevent contamination of the analytical column for sugar analysis. At an elution volume of 7.8 ml, the sugars were trapped by means of a 50-μl trapping loop. The trapping loop was then isolated and solvent flow directed to waste by appropriate valve actuation. After flushing the chromatographic pumping system, switching valves were again actuated to direct the trapped sugar solutes onto a MicroPak-NH_2-10 normal-phase column for isocratic analysis of the sugars. Fig. 8B shows the analysis of several sugar standards on the normal-phase column. A chromatogram of the trapped sugar solutes from a molasses sample is shown in Fig. 8C. In this manner, sucrose, fructose and glucose can be analyzed in the molasses sample. Levels of 15% of fructose and 5% of sucrose were found in the molasses.

Methods for the HPLC analysis of sugars in molasses have been well documented using aminopropyl bonded phase columns[12], but lengthy sample preparation and column clean-up procedures are often required. It should also be noted that, although an additional valve is utilized in the heart-cutting scheme involving non-compatible solvents, through judicious choice of solvent flow schemes a third powered contact closure for actuation of this additional valve is not required and the third valve can be actuated from one of the contact closures already employed.

DISCUSSION

For aqueous samples, the technique of multidimensional LC has proved to be useful when aqueous-exclusion chromatography is the primary mode chosen. The LC technique separates on the basis of size so that the high-molecular-weight components in complex mixtures that frequently contaminate regular HPLC columns by being irreversibly adsorbed can be effectively eliminated by directing them to waste while the lower molecular weight components can be further separated on the second column, as was illustrated for the molasses sample.

A further advantage of EC is that, provided the appropriate pore size is selected, low-molecular-weight components frequently elute as a single peak that can be totally or partially directed to column 2 by on-column concentration or by heart cutting. EC also has a higher sample capacity than the other LC modes and thus a large sample can be fractionated and the individual fractions still contain an appreciable amount of material for detection during the chromatographic step. This was amply illustrated in the protein supplement example, where 10–15 mg of sample were injected and trace amounts of vitamins were clearly observed in the reversed-phase ion-pair separation.

For the secondary aqueous-compatible chromatographic step, the modes of RPC or ion-exchange chromatography appear to have the most promise. Reversed-phase chromatography is particularly attractive as it can be used for a wide variety of ionic, ionizable and non-ionic compounds. If water or buffer is used for the EC step, use of RPC is attractive in that water is a weak solvent in this mode. If for some reason, such as for elimination of hydrophobic interactions in the EC column, an organic solvent must be added to the mobile phase, care should be exercised when directing large sample volumes on to the RPC column. A large sample volume of a moderately strong solvent may cause a partial movement (*i.e.*, spreading out) of some sample components. The technique of using column 2 as a holding column or by use of a third valve as a holding loop will prove useful in such instances. After the sample is in holding, the

solvent lines can be flushed of the strong solvent to waste. After flushing, the chromatography on column 2 can be carried out under more typical elution conditions.

Analysis of molasses using an aqueous EC column as the primary column and a normal-phase NH_2 column for sugar analysis illustrates an example of a non-compatible solvent situation, water being the strong solvent for normal-phase columns. Through the use of a third switching valve to isolate the two columns and the trapping valve from the solvent flow, systems involving otherwise incompatible chromatographic modes can be effectively coupled provided that trapping loop volumes are kept small ($<$100–200 μl). Further work to evaluate such column-switching schemes more fully is currently under way.

As demonstrated with the urine and plasma samples, an exciting possibility is the direct injection of an untreated biological fluid on to an EC column. The columns show little interaction with the sample matrix and under normal circumstances all sample components elute in one column volume. Samples merely have to be filtered to remove particulate matter. With the use of sensitive electrochemical and fluorescence detectors, trace levels of endogenous compounds and drugs and drug metabolites can be determined.

CONCLUSION

The technique of multi-dimensional LC combining aqueous-exclusion with reversed-phase or ion-exclusion chromatography provides a powerful combination for the approach of a "universal" LC separation system for aqueous samples. Such a system can be used to analyze components in complex sample matrices with minimal sample pre-treatment. For maximal selectivity, a heart-cutting technique shows advantages over an on-column concentration technique, while the on-column concentration approach yields greater sensitivity for LC analyses.

REFERENCES

1 D. E. Mundy and A. F. Machin, *J. Chromatogr.*, 139 (1977) 321.
2 E. L. Johnson, R. Gloor and R. E. Majors, *J. Chromatogr.*, 149 (1978) 571.
3 Y. Kato, K. Komiya, H. Sasaki and T. Hashimoto, *J. Chromatogr.*, 190 (1980) 297.
4 F. Erni and R. W. Frei, *J. Chromatogr.*, 149 (1978) 561.
5 R. Verbesselt, T. B. Tjandaranga and P. J. DeSheppert, *Curr. Med. Res. Opin.*, 6 (Suppl. 16) (1979) 151.
6 G. Menillet, M. C. Santais and F. Ruff, *Toxicol. Eur. Res.*, 2 (2) (1979) 111.
7 L. Larson, *LC At Work*, No. 74, Varian Associates, Walnut Creek, CA, 1978.
8 J. F. Gregory, *J. Agr. Food Chem.*, 28 (1980) 486.
9 G. C. Show and W. R. Day, *J. Chromatogr. Sci.*, 15 (1977) 262.
10 J. E. Heady and K. D. Agee, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 2 (1979) 617.
11 H. Hallman, L. O. Farnebo, B. Hamberger and G. Jonsson, *Life Sci.*, 23 (1978) 1049.
12 C. E. Damon and B. C. Pettitt, Jr., *J. Ass. Offic. Anal. Chem.*, 63 (1980) 476.

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THE USE OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH RADIAL COMPRESSION FOR THE ANALYSIS OF PEPTIDE AND PROTEIN MIXTURES

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SUMMARY

This report describes the separation of peptide and protein mixtures on a C_{18}-microparticulate support which was packed in a polyethylene cartridge and subjected to radial compression of *ca.* 2600 p.s.i. The purpose of the radial compression was to minimise inhomogeneities in the column packing, in particular in the region of the column wall and end fittings. The effectiveness of this new chromatographic system was demonstrated by the efficient separation of the following mixtures: the C-apolipoproteins isolated from human very low density lipoproteins; the polymorphs of apolipoprotein A-I; the tryptic fragments of apolipoprotein C-II; the complex mixture generated by partial proteolysis of apolipoprotein B; the tryptic fragments of ^{3}H- and ^{14}C-labelled β-chain of murine IA alloantigen and the tryptic fragments of carboxymethylated lambda chain isolated from human immunoglobulin G. The separated peaks were identified by amino acid analysis, radioactivity counting and in the last example by amino acid sequence determination. The mobile phase consisted of 1% aqueous solution of triethylammonium phosphate, pH 3.2 with acetonitrile or isopropanol as the organic modifier.

INTRODUCTION

Recently reversed-phase high-performance liquid chromatography (HPLC) has allowed the separation of a variety of peptide and protein samples[1–11]. An important factor in the resolution of such a diverse range of samples, by a single chromatographic system, was the introduction of a number of ionic materials which can be added to

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the mobile phase[1,4,11–14]. Such materials probably influence the chromatographic separation by a mixture of ion pairing effects[12–15], solvophobic interactions[16] and column modification[11,17]. Despite these important applications, the separations achieved by reversed-phase HPLC still require additional improvements, particularly for complex mixtures present in partial protein hydrolysates and in mixtures of closely related proteins.

Recent reports have described the purification of peptides on a support which consisted of 70-μm C_{18}-silica particles, packed in large polyethylene cartridges which can be radially compressed[18,19]. These reports have demonstrated that radial compression does greatly improve the efficiency of the column, so that rapid preparative separations can be achieved. The purpose of this publication is to demonstrate that the corresponding analytical system, namely 10-μm C_{18}-silica particles packed in a 10×0.8 cm polyethylene cartridge and subjected to a radial compression, can be used to generate high efficiency separations of peptide and protein mixtures.

EXPERIMENTAL

Apparatus

A Waters Assoc. (Milford, MA, U.S.A.) HPLC system was used for the analytical separations, which consisted of two M6000A solvent delivery units, an M660 solvent programmer and a U6K universal liquid chromatograph injector, coupled to an M450 variable-wavelength UV spectrophotometer and an Omniscribe two-channel chart recorder (Houston Instruments, Austin, TX, U.S.A.). Sample injections were made using a Microliter 802 syringe (Hamilton, Reno, NV, U.S.A.). The Radial Compression Module and the Radial-PAK A (C_{18}) cartridge were purchased from Waters Assoc.

Chemicals

Orthophosphoric acid (analytical-reagent grade) was obtained from May & Baker (Dagenham, Great Britain). Water and glass distilled, acetonitrile (UV grade) was purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Triethylamine was obtained from Aldrich (Milwaukee, WI, U.S.A.) and used without further purification. The A-I and C-apolipoprotein mixtures were obtained using established procedures[20,21]. Typical conditions for the preparation of a tryptic digest of a protein sample are described in the publication on the determination of the amino acid sequence of apolipoprotein C-II[22].

Methods

The triethylammonium phosphate (TEAP) was prepared from a 1% aqueous solution of phosphoric acid, to which triethylamine was added until a pH of 3.2 was reached. All pH values quoted refer to the aqueous phase before addition of the organic component in the gradient analysis.

All chromatography was carried out at room temperature (*ca.* 22°C). The samples were dissolved in a solvent which corresponded to the initial component of the mobile phase. Other chromatographic conditions were the same as has been described previously[9].

RESULTS

The separation of a mixture of C-apolipoproteins isolated from human very low density lipoproteins (VLDL) is shown in Fig. 1. This mixture represents a partially purified serum fraction which was isolated by ultracentrifugal flotation, delipidation and Sephacryl S-200 fractionation[9]. The order of elution of the apolipoproteins on the radially compressed C_{18} (RC-C_{18}) column is apolipoprotein C-I, C-$III_{1,2}$ and C-II respectively. Apolipoprotein C-III is a glycoprotein and exists in three major isomeric forms which contain two, one or no sialic acid residues (C-III_2, C-III_1 and C-III_0 respectively). The RC-C_{18} column does not separate the major isomers C-III_2 and C-III_1, while the small amount of C-III_0 present in this sample (as shown by polyacrylamide gel electrophoresis) probably elutes at the trailing edge of the C-III peak. As is shown in Table I amino acid analysis data allowed identification of these peaks. The additional minor peaks in the chromatogram probably represent other apolipoproteins which are coisolated with the C-apolipoproteins. The amounts present in these minor peaks were insufficient for identification by amino acid analysis.

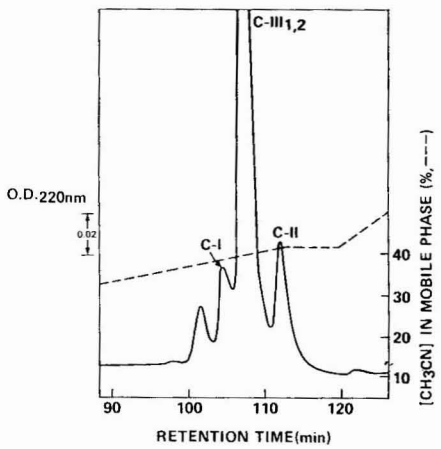

Fig. 1. The elution profile of a mixture of C-apolipoproteins (0.2 mg) on a radially compressed C_{18} cartridge (RC-C_{18}). A flow-rate of 1.5 ml/min was used with a mobile phase consisting of 1 % TEAP. After elution from the column, the fractions containing the apolipoproteins were pooled separately, freeze dried, hydrolysed with acid and identified by amino acid compositional data. The identity of the protein is indicated by lettering.

The analysis of a highly purified sample of apolipoprotein A-I on the RC-C_{18} column is shown in Fig. 2. The three protein containing peaks (3, 4 and 5 respectively) were examined by amino acid analysis. Peaks 4 and 5 gave compositional data which agreed closely with the expected values for apolipoprotein A-I, while the amino acid content of peak 3, although present in amounts too small for quantitation, was also suggestive of this apolipoprotein.

Fig. 3 shows the elution profile for the tryptic digest of apolipoprotein C-II on a RC-C_{18} column. Each peak was collected and identified by amino acid analysis (see Table III). Some large optical density peaks contained no significant amounts of amino acids, such as peak 11, while a few small peaks accounted for some of the

TABLE I

AMINO ACID COMPOSITIONAL DATA OBTAINED IN THE SEPARATION OF A C-APOLIPOPROTEIN MIXTURE ON A RC-C_{18} COLUMN

Other peaks did not give significant amino acid analyses. Trp and Cys were not measured. Values expressed as moles per 100,000 g protein; expected values from A. M. Scanu, *Biochemistry of Atherosclerosis*, Academic Press, New York, 1979, p. 3.

Amino acid	*C-I*		*C-II*		*C-III*$_{1,2}$	
	Obtained	*Expected*	*Obtained*	*Expected*	*Obtained*	*Expected*
Asp	79	81	72	64	91	80
Ser*	52	45	82	101	57	57
Thr*	96	106	81	99	120	125
Pro	—	15	29	26	24	23
Glu	140	136	125	104	118	114
Gly	26	15	36	28	28	34
Ala	42	45	75	75	120	114
Val	21	30	49	45	74	68
Met	9	15	20	21	11	23
Ile	47	45	13	10	0	0
Leu	102	91	91	92	98	57
Tyr	8	0	55	53	19	23
Phe	47	45	25	24	48	46
His	0	0	0	0	12	11
Lys	131	136	83	70	75	68
Arg	—	45	10	13	24	23

* Uncorrected for losses during acid hydrolysis.

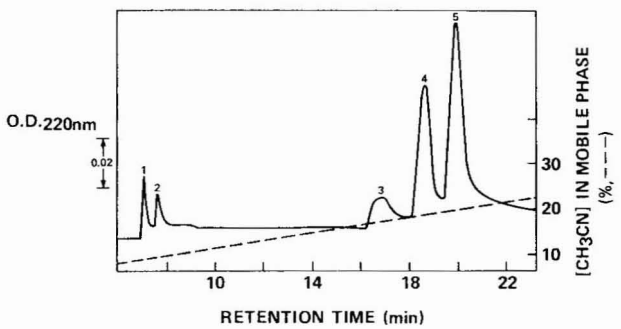

Fig. 2. The elution profile obtained for 100 μg purified apolipoprotein A-I when analysed on the RC-C_{18} column. A flow-rate of 2 ml/min was used with a mobile phase of 1% TEAP. Peaks: 1 and 2 = artifacts caused by the solvent and salts present in the sample; 3, 4 and 5 = collected, freeze-dried, hydrolysed and subjected to amino acid analysis.

tryptic fragments (peaks 6 and 7). These fragments were small peptides which did not contain any significant UV chromophores.

In Fig. 4 the gradient map for a partial digest of apolipoprotein B is described. The apolipoprotein was solubilised using the copper(II) procedure of Huang and Lee[23]. Due to the presence of multiple basic residues in this protein, a partial digestion yielded an extremely complex mixture of peptides. The presence of overlap fragments

TABLE II

AMINO ACID COMPOSITIONAL DATA OBTAINED IN THE ANALYSIS OF APOLIPOPROTEIN A-I ON A RC-C_{18} COLUMN

Amino acid	*Peak number**			
	3	*4*	*5*	*apo A-I***
Asp	+ + ***	25	26	21
Ser	+	15	11	10
Thr	+ +	15	17	15
Pro	+	7.0	7.9	10
Glu	+ + +	44	46	46
Gly	+	13	24	10
Ala	+ +	16	13	19
Val	+	15	11	13
Met	—	3	2.5	3
Ile	—	2.5	—	0
Leu	+ + +	28	32	37
Tyr	+	8.0	7.1	7
Phe	+	6.5	6.2	6
His	+	3.8	4.1	5
Lys	+ +	24	19	21
Arg	+ +	14	14	16

* Other data same as for Table I.

** Theoretical values obtained from ref. 21.

*** Not sufficient material to allow quantitation of the amounts of each amino acid, but the relative amounts are indicated by the notation +, ++ and +++.

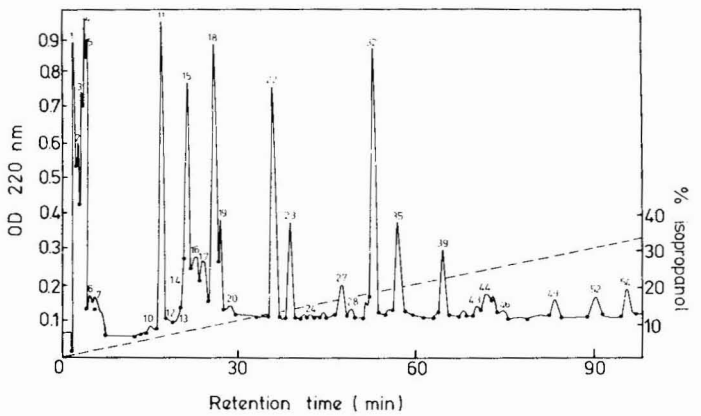

Fig. 3. The elution profile obtained by chromatography of the peptide mixture obtained from the digestion of apolipoprotein C-II with trypsin. The amount of peptides loaded on to the column was 0.7 mg. The mobile phase consisted of 1% TEAP and a flow-rate of 1.5 ml/min was used; solvent B was isopropanol. A 0.5-ml sample was taken from the reaction mixture of the tryptic digestion which contained 0.2 *M* ammonium bicarbonate and 0.5 g of guanidine hydrochloride. To this aliquot, 0.5 ml of 1% TEAP, which contained 6 *M* guanidine hydrochloride, was added and the total sample was injected. A 2-h linear gradient from 0 to 40% isopropanol was run. No significant peaks were observed after 100 min of the gradient run and thus this section of the elution profile is not shown. Each peak was collected and subjected to amino acid analysis after acid hydrolysis.

TABLE III

PEAK ASSIGNMENT FOR THE TRYPTIC PEPTIDES OF APOLIPOPROTEIN C-II SEPARATED ON A RC-C_{18} COLUMN

Mobile phase: 1% TEAP; flow-rate, 1.5 ml/min with a gradient of 0–40% isopropanol. Assignments based on amino acid analytical data and the amino acid sequence data of Jackson *et al.*[22].

Peak number	*Assignment*
6	T-8
7	T-5
15	T-6
18	T-3
19	T-5,6
22	T-4
27	T-1 (deamidated?)
32	T-2
35	T-1
43–52	Fragments of partial tryptic digests
54	T-7,8

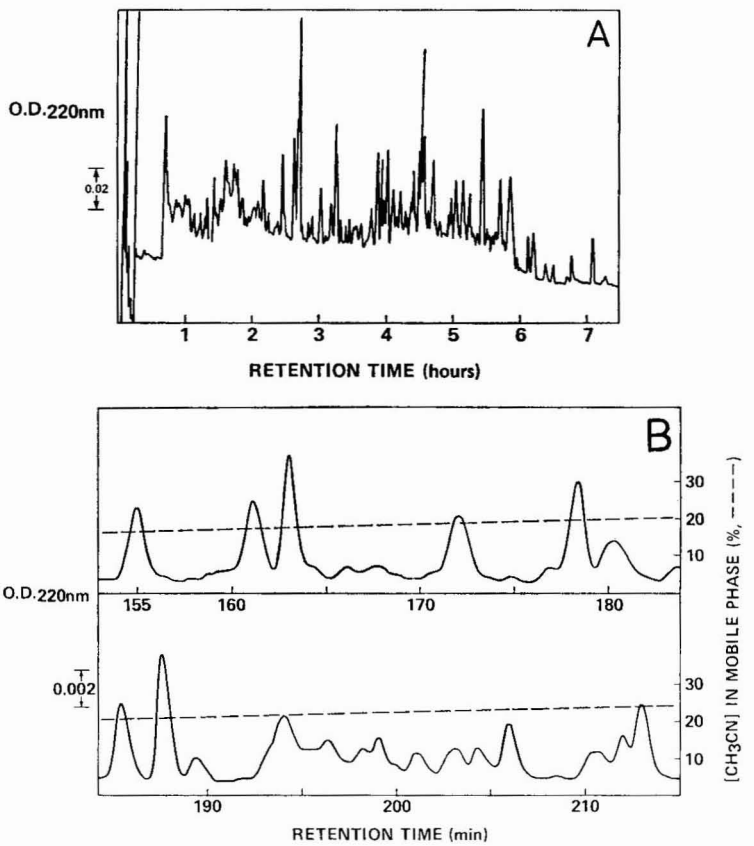

Fig. 4. The elution profile of the tryptic digest of apolipoprotein B on the RC-C_{18} column: A, the full gradient; B, an expansion of a 1-h segment of the full analysis. A 10-mg sample of the tryptic digest was dissolved in 0.5 ml of 1% TEAP, 6 *M* guanidine hydrochloride, pH 3.2 and applied to the column. The mobile phase was 1% TEAP, pH 3.2 at a flow-rate of 1 ml/min. A linear gradient of 0–35% acetonitrile over 10 h was run, 1-ml fractions were collected and the major peaks were pooled, hydrolysed and examined by amino acid analysis. The apolipoprotein B was solubilised using the 6 *M* guanidine–20 m*M* NH_4Cl–NH_4OH procedure of Huang and Lee[23].

in this mixture was confirmed by amino acid analysis studies which demonstrated that many of the peptides still contained more than one arginine or lysine residue. Conditions which give complete digestion of the protein, yielded a much less complex mixture. Over 200 peptides were separated in a single gradient run and Fig. 4B shows an expansion of a 1-h segment of the gradient to illustrate more clearly the complex nature of the elution profile.

The elution profile from a RC-C_{18} analysis of the murine IA alloantigen beta chain tryptic digest is shown in Fig. 5. This membrane glycoprotein was labelled *in vitro* with [^{14}C]- and [^{3}H]-Leu, -Phe, -Arg and -Lys. The eluted peaks were monitored by a flow through radioactive cell. Fig. 6 shows the elution profile for a tryptic digest

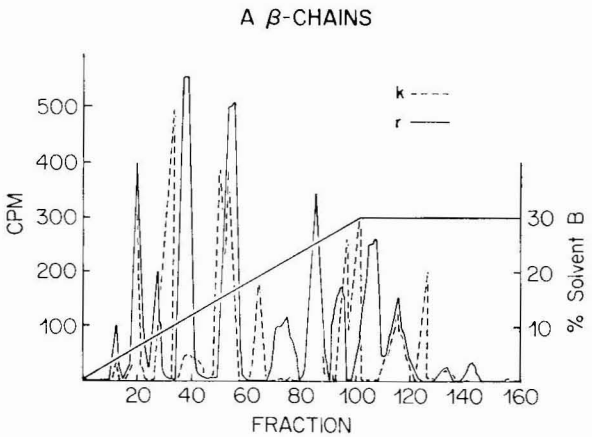

Fig. 5. The elution profile for the separation of the tryptic fragments of the murine IA alloantigen beta chains. The tryptic digestion was carried out for 4 h. The mobile phase was 1% ammonium bicarbonate and the acetonitrile gradient used is shown by a solid line. The flow-rate was 1 ml/min.

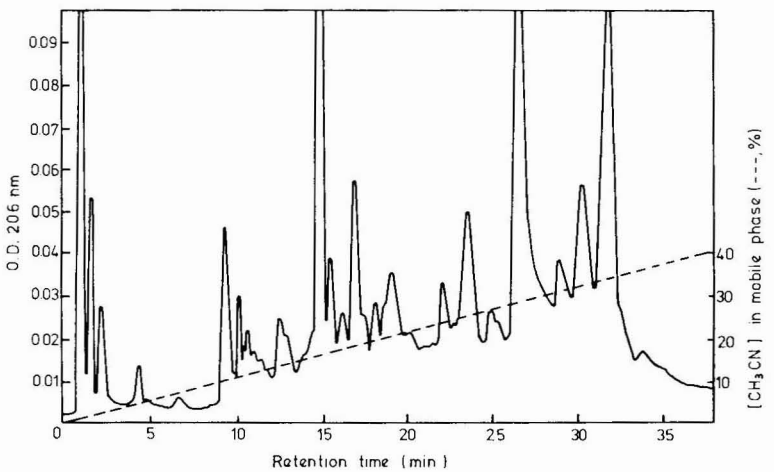

Fig. 6. The separation of the tryptic fragments from the digestion of a carboxymethylated derivative of a human lambda chain. A 10-mg sample was dissolved in 0.17 ml of 1% ammonium bicarbonate and injected onto the column. The mobile phase was 1% ammonium bicarbonate and the flow-rate was 2 ml/min. A linear gradient of 0–40% acetonitrile over 40 min was used.

of a carboxymethylated sample of a human immunoglobulin lambda chain. The major peptides isolated from this separation were subjected to an automated Edman sequence analysis[24]. The results of the sequence analysis are shown in Table IV.

TABLE IV

SEQUENCE DATA FOR THE MAJOR ELUTED PEAKS FROM THE SEPARATION OF THE TRYPTIC FRAGMENTS OF A CARBOXYMETHYLATED LAMBDA CHAIN OF HUMAN IMMUNOGLOBULIN G

The one letter code for amino acids was used[25]: C = Cys; H = His; I = Ile; M = Met; S = Ser; V = Val; A = Ala; G = Gly; L = Leu; P = Pro; T = Thr; F = Phe; R = Arg; Y = Tyr; W = Trp; D = Asp; N = Asn; E = Glu; Q = Gln; K = Lys.

Peak number	*Sequence*
1	AEK
2	QSNNK
3	SHK
4	AAPSVTLFPPSSEELQANK
5	SYAGR
6	TVAPTECS
7	LIIYGVWK
8	FIILLK
9	ATLVCLISDFYPGAVTVAWK

DISCUSSION

Even in well packed columns, a "wall region" is present where the dispersion of solutes is much greater in the packed region close to the walls than in the core of the column. In certain cases the "wall region" can be very significant, for example it is stated that, for a column of 5 mm bore containing 20-μm particles, the fraction of the column cross-section taken up by the annular wall layer is about 40%[27]. In an effort to minimise this region, Waters Assoc. have introduced a Radial Compression Separation System, in which flexibly walled cartridges are compressed radially into high efficiency columns[26]. The use of a 30 $\times$ 5.7 cm polyethylene cartridge with radial compression has allowed the preparative separation of multigram amounts of crude materials[28].

In recent studies[18,19] this chromatographic system was used successfully to purify a variety of synthetic peptides. It was observed[18] that despite the relatively large size particles (70 μm) packed in the polyethylene cartridge, high efficiency separations were observed. Such results suggested that radial compression did indeed improve the separation efficiencies obtainable for a given silica-based support.

Recently, Waters Associates introduced the corresponding analytical system. The flexibly walled columns consisted of a 10 $\times$ 0.8 cm polyethylene cartridge which was packed with 10-μm octadecyl-silica particles (RC-C_{18}). Fig. 7 shows scanning electron micrographs of the interior of one of these columns before and after radial compression. These photographs indicate that such a column should have a smaller dispersion region near the column wall than for columns with inflexible walls. Because of the successful use of this technology for preparative isolations, it was decided to

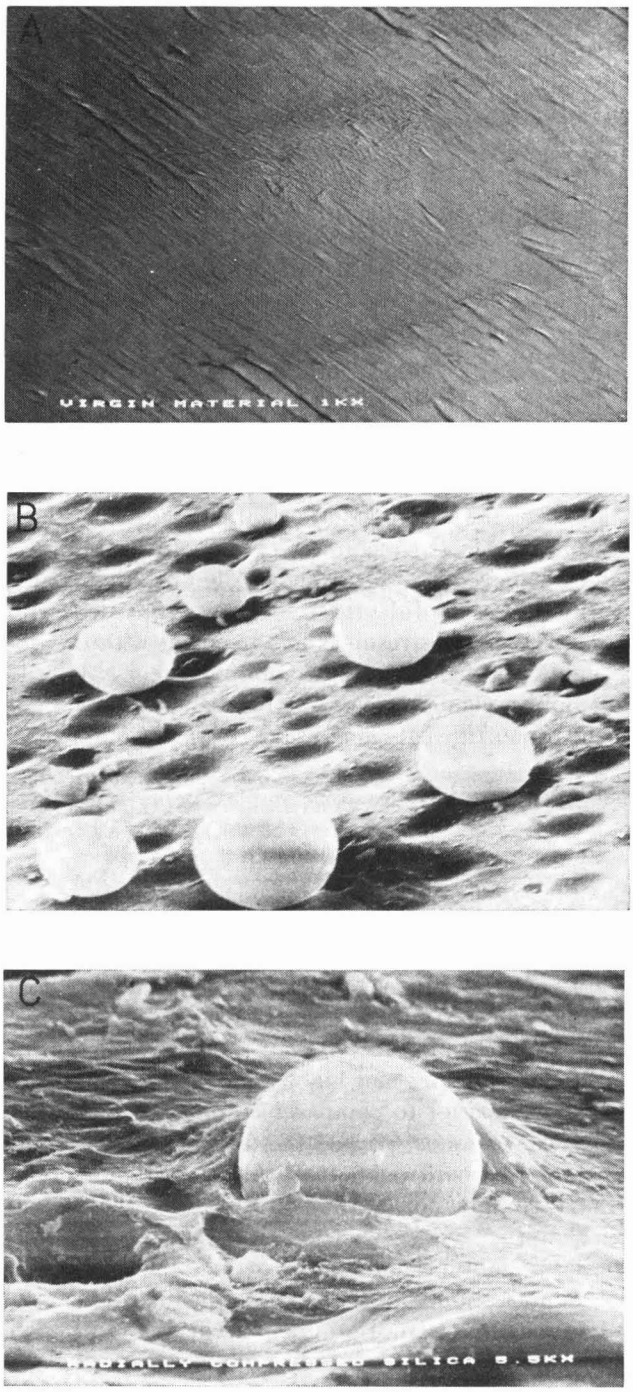

Fig. 7. Scanning electron micrographs of the interior of a polyethylene cartridge before compression (A) and after compression (B and C). In the latter two examples some of the radially compressed silica particles can be seen adhering to the column walls after the column had been emptied.

examine the potential of the analytical system for the separation of complex peptide and protein mixtures.

Fig. 1 shows that the major C-apolipoproteins isolated from human very low density lipoproteins (VLDL) can be separated on a RC-C_{18} column. The elution order and separation is similar to that obtained on a μBondapak-alkylphenyl column[9]. Both columns give much better separation of the C-apolipoproteins than the μBondapak C_{18} column. The lower surface loadings of the organosilane present in the former column packings (5 and 10% *versus* 15% respectively) is suggested as a possible reason for the differences in selectivity between the columns[29]. Although the RC-C_{18} can be used at high flow-rates (up to 10 ml/min) the separation of proteins was more efficient at lower flow-rates (0.5–1.5 ml/min), because of the lower diffusion rates of the high-molecular-weight solutes.

The molecular dimensions of the C-apolipoproteins (which range from 57 to 79 residues) is probably comparable to the pore size of the RC-C_{18} packing material (*ca.* 90 Å). Apolipoprotein A-I, which contains 245 residues, is unlikely to be able to penetrate most pores in this packing material and thus interaction of this protein with the support probably occurs mainly on the exterior surface of the silica particles. Despite this limitation, the separation of at least 1-mg amounts of apolipoprotein A-I into three components was readily achieved on the RC-C_{18} column. Amino acid analysis indicated that the composition of each of the three peaks was consistent with the values expected for the apolipoprotein. This result is in agreement with the observation of Herbert *et al.*[32] that highly purified samples of apolipoprotein A-I could be resolved into three species on polyacrylamide electrophoretic analysis. It is not known if these multiple forms of the protein are caused by aggregation, deamidination or some other process.

Figs. 3–6 show a number of elution profiles for complex peptide mixtures produced by tryptic digestions of high-molecular-weight proteins. A common difficulty with apolipoprotein proteolytic digestions is the partial aggregation of the protein during the cleavage reaction, which results in incomplete digestion of the protein. In Figs. 3 and 4 a number of additional fragments could be detected in the complex elution profile for the digestion products from apolipoprotein C-II and B respectively. In addition the yield of purified peptides based on the amount of protein digested was lower for the apolipoproteins (20–60%) than for other proteins (often quantitative). In each example the RC-C_{18} column gave high efficiency separations which were characterised by excellent selectivities and high sample capacities (up to 20 mg). These separations were clearly superior to comparable analyses carried out on the corresponding 10-μm analytical columns, packed in stainless steel columns without radial compression. The improved performance of the RC-C_{18} column can probably be attributed both to radial compression of the polyethylene cartridge and to a mixed mode separation mechanism. Evidence is presented in a companion publication that the packing material present in the RC-C_{18} cartridge can give rise to both a silanol and a reversed phase interaction with the solute[33]. Such a mechanism can be attributed to significant levels of free silanol groups present in the packing material. For this reason, the mobile phase used in this study contained an amine salt at relatively high ionic strength, which minimises the interaction of basic peptides with silanol groups.

Other chromatographic parameters were chosen to be suitable for the analysis

of peptides and proteins. For example, organic modifiers were chosen which exhibited good solvent properties for the solutes, and slow, shallow solvent gradients were used to avoid the precipitation and/or denaturation of the samples[31].

In conclusion, this publication has demonstrated that the RC-C_{18} system is capable of high efficiency separations of complex peptide and protein mixtures. The application of this new separation system to the analysis and purification of a number of protein samples is currently under investigation.

ACKNOWLEDGEMENTS

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REFERENCES

1 W. S. Hancock, C. A. Bishop, R. L. Prestidge, D. R. K. Harding and M. T. W. Hearn, *Science*, 200 (1978) 1168.
2 E. Lundanes and T. Greibrokk, *J. Chromatogr.*, 149 (1978) 241.
3 S. Terabe, R. Konaka and K. Inouye, *J. Chromatogr.*, 172 (1979) 163.
4 J. E. Rivier, *J. Liquid Chromatogr.*, 1 (1978) 343.
5 M. J. O'Hare and E. C. Nice, *J. Chromatogr.*, 171 (1979) 209.
6 J. Rivier, R. Kaiser and R. Galyean, *Biopolymers*, 17 (1978) 1927.
7 E. C. Nice and M. J. O'Hare, *J. Chromatogr.*, 162 (1979) 401.
8 M. T. W. Hearn, C. A. Bishop, W. S. Hancock, D. R. K. Harding and G. D. Reynolds, *J. Liquid Chromatogr.*, 2 (1979) 1.
9 W. S. Hancock, C. A. Bishop, A. M. Gotto, D. R. K. Harding, S. M. Lamplugh and J. T. Sparrow, *Lipids*, submitted for publication.
10 W. Mönch and W. Dehnen, *J. Chromatogr.*, 140 (1970) 260.
11 I. Molnár and Cs. Horváth, *J. Chromatogr.*, 143 (1977) 391.
12 W. S. Hancock, C. A. Bishop, R. L. Prestidge, D. R. K. Harding and M. T. W. Hearn, *J. Chromatogr.*, 153 (1978) 391.
13 W. S. Hancock, C. A. Bishop, L. J. Meyer, D. R. K. Harding and M. T. W. Hearn, *J. Chromatogr.*, 161 (1978) 291.
14 W. S. Hancock, C. A. Bishop, J. E. Battersby, D. R. K. Harding and M. T. W. Hearn, *J. Chromatogr.*, 168 (1979) 377.
15 R. P. W. Scott and P. Kucera, *J. Chromatogr.*, 142 (1977) 213.
16 Cs. Horváth, W. Melander and I. Molnár, *J. Chromatogr.*, 125 (1976) 129.
17 B. A. Bildlingmeyer, S. N. Deming, W. P. Price, Jr., B. Sachok and M. Petrusek, *J. Chromatogr.*, 186 (1979) 419.
18 C. A. Bishop, D. R. K. Harding, L. J. Meyer, W. S. Hancock and M. T. W. Hearn, *J. Chromatogr.*, 192 (1980) 222.
19 J. Rivier, in J. Hawk (Editor), *Biological/Biomedical Applications of Liquid Chromatography II*, Marcel Dekker, New York, 1979.
20 A. L. Catapano, R. L. Jackson, E. B. Guilliam, A. M. Gotto and L. C. Smith, *J. Lipid Res.*, 19 (1978) 1047.

21 H. N. Baker, A. M. Gotto and R. L. Jackson, *J. Biol. Chem.*, 250 (1975) 2725.
22 R. L. Jackson, H. N. Baker, E. B. Guillam and A. M. Gotto, *Proc. Nat. Acad. Sci. U.S.*, 74 (1977) 1942.
23 S. S. Huang and D. M. Lee, *Biochim. Biophys. Acta*, 577 (1979) 424.
24 C. H. Hirs and C. N. Timasheff (Editors), *Methods Enzymol.*, 47 (1977) Section VIII.
25 M. O. Dayhoff, *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, MD, 1972.
26 *Prep PAK-500/C_{18} Cartridges for Reversed Phase Preparative Liquid Chromatography*, Bulletin No. D83, Waters Assoc., Milford, MA.
27 J. H. Knox (Editor), *High Performance Liquid Chromatography*, Edinburgh University Press, Edinburgh, 1978, p. 82.
28 J. N. Little, R. L. Cotter, J. A. Prendergast and P. D. McDonald, *J. Chromatogr.*, 126 (1976) 439.
29 W. S. Hancock and J. T. Sparrow, in preparation.
30 H. B. Brewer, R. Shulman, P. Herbert, R. Ronan and K. Wehrly, *Advan. Exp. Med. Biol.*, 26 (1972) 280.
31 W. S. Hancock and J. T. Sparrow, *A Laboratory Manual for the Separation of Biological Materials by HPLC*, Marcel Dekker, New York, in press.
32 P. N. Herbert, R. S. Shulman, R. I. Levy and D. S. Fredrickson, *J. Biol. Chem.*, 248 (1973) 4941.
33 W. S. Hancock and J. T. Sparrow, *J. Chromatogr.*, 206 (1981) 71.

Journal of Chromatography, 206 (1981) 71–82
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USE OF MIXED-MODE, HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE SEPARATION OF PEPTIDE AND PROTEIN MIXTURES

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SUMMARY

The packing material recently introduced for use in a radial compression chamber, with a partially flexible cartridge, has a low C_{18}-coating (5% w/w) combined with an absence of secondary capping. This report demonstrates that such a support, which contains significant concentrations of both free silanol and hydrocarbon groups, can allow a mixed-mode separation to occur via adsorption and reversed-phase separation mechanisms. In any given separation, the predominant mechanism depends both on the nature of the sample and the mobile phase. For efficient peptide and protein separations, it was necessary to suppress most silanol group interactions by the use of a mobile phase which contained a high concentration of an amine phosphate, *e.g.*, 0.17 *M* triethylammonium phosphate, pH 3.2. In addition, it was necessary to deactivate further the silanol groups by an initial column wash of at least 20 column volumes of methanol. Samples which contained strongly basic groups, for example the guanidino group of arginine, can still exhibit poor separation efficiencies on such a support. These problems were largely overcome, however, with the use of isopropanol as an organic modifier. If these precautions were followed, the packing material gave excellent selectivities in the separation of closely related materials, as well as allowing increased sample capacities.

These observations will be supported by an examination of the chromatographic properties of a range of small peptides, the C-apolipoprotein mixture present in human very-low-density lipoproteins and the purification of an 8-mg sample of a synthetic pentadecapeptide in a single chromatographic run.

INTRODUCTION

We described earlier[1] the use of the Radial Compression Separation System[2] for the high-efficiency separation of complex peptide and protein mixtures. This

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system uses flexibly walled cartridges packed with spherical 10-μm silica particles which were derivatised with a relatively light loading (*ca.* 5% w/w) of a C_{18}-silane[3]. Since the unreacted silanol groups are not blocked by "end-capping" with trimethylchlorosilane, this packing material contains a significant concentration of free silanol groups which can interact with the solute molecules as well as with the mobile phase. Several authors[4–7] have suggested that such a packing material would give mixed retention mechanisms. Also Rabel and co-workers[7,8] found that the use of a support with low organic loadings gave greater selectivities in the separation of polar, low-molecular-weight solutes. The relatively high column efficiencies obtained with non-polar stationary phases with low surface coverages can be attributed to a higher concentration of accessible silanol groups. Such groups, which are likely to be hydrated, may reduce mass transfer resistance for a polar solute from the aqueous eluent to the surface of the stationary phase. In addition silanol groups, in the presence of low pH mobile phases, may introduce specific hydrogen bonding interactions with polar solutes.

This report, therefore, examines the chromatography of small basic peptides on this column, and demonstrates that a strong interaction between basic solutes and silanol groups present in the packing material gives irreproducible results. However, the use of hydroxylic organic solvents and amine phosphate buffers minimises the effect of silanol groups, so that satisfactory peak shapes and sample recoveries can be obtained. The resulting mixed-mode chromatographic system was shown to allow the efficient separation of a range of peptide and protein samples.

EXPERIMENTAL

Apparatus

A Waters Assoc. (Milford, MA, U.S.A.) high-performance liquid chromatographic (HPLC) system was used for the analytical separations. This consisted of two M6000A solvent delivery units, an M660 solvent programmer and a U6K universal liquid chromatograph injector, coupled to an M450 variable wavelength UV spectrophotometer (Waters Assoc.) and an Omniscribe two-channel chart recorder (Houston Instruments, Austin, TX, U.S.A.). Sample injections were made using a Microliter 802 syringe (Hamilton, Reno, NV, U.S.A.). The Radial Compression Module and the Radial-PAK A(C_{18}) cartridge were also purchased from Waters Assoc. For convenience the radially compressed cartridges will be referred to as RC-C_{18}.

Chemicals

The solvents and chemicals used in this study are identical to those described in a previous paper[1]. The synthetic peptides were prepared by the solid phase method[8] and the details of the synthesis will be described elsewhere. The C-apolipoprotein mixture from very-low-density lipoproteins (VLDL), and the partial tryptic digest of apolipoprotein B were prepared as described[1].

The triethylammonium phosphate (TEAP) buffer (1%, pH 3.2) was prepared by the addition of phosphoric acid (1%, v/v) to purified water and adjusting the pH with triethylamine (unpurified; Aldrich, Milwaukee, WI, U.S.A.). The buffer was purified by passage through a C_{18}-radially compressed cylinder (30 × 5.7 cm) in a Waters Prep-500 preparative liquid chromatograph. Before use the cartridge was

washed successively with 10 l each of isopropanol, isopropanol–water (1:1) and water, to remove any unbonded organosilane polymer. The amine phosphate buffer (20 l) was then pumped through the column at a flow-rate of 100 ml/min and collected in dark glass bottles. The use of plastic tubing was avoided whenever possible, because the strong solvent properties of this buffer made further contamination inevitable. The first 5 l of buffer were discarded. Each bottle was stored under nitrogen at 4°C in the dark. The buffer had satisfactory stability under these conditions, for at least 1 month.

All chromatography was carried out at room temperature (*ca.* 22°C). The samples were dissolved in a solvent which corresponded to the initial component of the mobile phase.

RESULTS

Fig. 1 shows the elution profile obtained for the tripeptide Gly-Gly-Tyr on a new radially compressed (RC-C_{18}) column, with a mobile phase of acetonitrile–1% TEAP (20:80). Each successive injection (B to F respectively) represents 50 μg of the peptide chromatographed under identical conditions. Although a reproducible elution

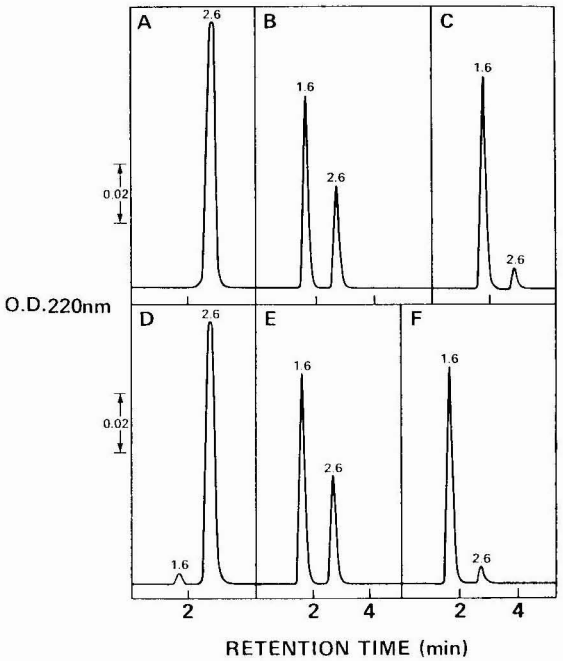

Fig. 1. The elution profile for the peptide Gly-Gly-Tyr on a RC-C_{18} column. The mobile phase consisted of 1% TEAP–acetonitrile (80:20) at a flow-rate of 2 ml/min. The elution profiles shown in parts B to F were obtained with a RC-C_{18} column which had not been subjected to a pre-wash with 200 ml of methanol at a flow-rate of 2 ml/min. The elution profile shown in A was obtained with a column which had a methanol pre-wash before equilibration with the mobile phase. All injections consisted of 50 μg of the peptide dissolved in 50 μl of the mobile phase. All chromatographic parameters were identical for elution profiles A–F.

profile could not be obtained, a pattern of two optical density peaks with constant retention times was always observed. A different result was obtained if the RC-C_{18} column was washed with at least 200 ml of methanol at a flow-rate of 2 ml/min. After equilibration of the column with the same mobile phase as before, chromatography of the tripeptide now only gave one peak with a retention time of 2.6 min. This effect was quite reproducible and could be demonstrated with a fresh column.

Other peptides, when chromatographed on the column without a methanol pre-wash, exhibited either two peaks, an extremely broad peak, or were retained indefinitely on the column even in the presence of high levels of acetonitrile. In most cases, the use of a methanol pre-wash improved the elution profile of the peptide so that a reasonably sharp peak was observed.

For small peptides, which contained the very basic guanidino group of arginine, the methanol pre-wash was not effective, however, and Fig. 2B and D show broad peak shapes for the peptides Leu-Trp-Met-Arg and Met-Arg-Phe respectively with a mobile phase of 1% TEAP–acetonitrile (80:20). The replacement of acetonitrile with the hydroxylic solvent isopropanol caused a dramatic improvement in the peak shapes as is shown in Fig. 2A and C, respectively. A similar observation was made for other peptides which exhibited poor peak shapes with acetonitrile as the organic modifier.

Fig. 3 shows the successful chromatography of a mixture of C-apolipoproteins from human VLDL using two different gradient programmes. As was described[1],

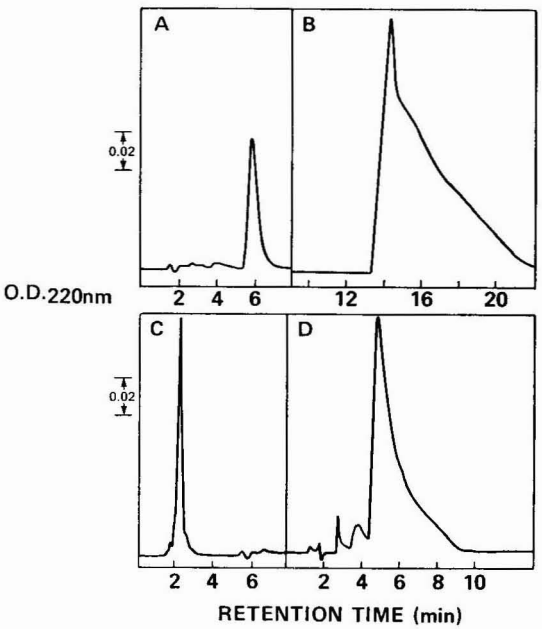

Fig. 2. The chromatography of the peptides Leu-Trp-Met-Arg (A and B) and Met-Arg-Phe (C and D) on a RC-C_{18} column. The column had been subjected to the methanol pre-wash. Each analysis was carried out on 50 μg of the peptide dissolved in 50 μl of the mobile phase. The O.D. sensitivity was increased two-fold in B and D to allow for the broader peak shape. The flow-rate was 2 ml/min. In A and C the mobile phase was 1% TEAP–isopropanol (80:20) while B and D used a mobile phase of 1% TEAP–acetonitrile (80:20).

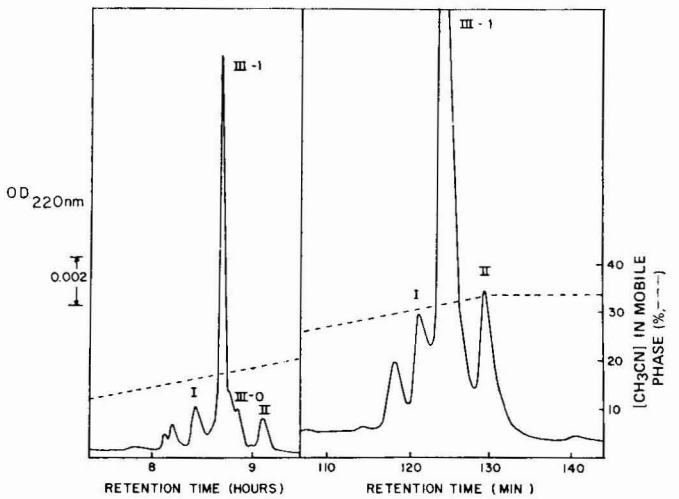

Fig. 3. The elution profiles of a mixture of C-apolipoproteins obtained from human VLDL when chromatographed on a RC-C_{18} column and with different acetonitrile gradients (see dashed lines). A 0.1-mg sample of the protein mixture was dissolved in 0.1 ml of 1% TEAP, 6 *M* guanidine hydrochloride and then chromatographed on the RC-C_{18} column with an initial mobile phase of 1% TEAP, pH 3.2. The TEAP used in the mobile phase was unpurified.

the RC-C_{18} column clearly separated apolipoprotein C-I from C-$III_{1,2}$ and C-II. While a 2-h gradient of 0 to 40% acetonitrile gave adequate resolution of the protein mixture, a much longer gradient of 0 to 40% over 10 h did further improve the resolution (see Fig. 3). A distinct advantage of the longer gradients was that the C-apolipoproteins were eluted at a significantly lower concentration of organic modifier, for example C-$III_{1,2}$ was eluted at 33% acetonitrile with the 10-h gradient, while the 2-h gradient required 38% acetonitrile for elution of the protein. In a research situation where repetitive assays are not required, the long separation time is not necessarily a disadvantage, particularly as the separation can be run overnight, and the separated proteins collected with a fraction collector. A key feature was the use of a high concentration of TEAP (0.17 *M*) in the mobile phase. If a ten-fold lower concentration of the salt was used, a much lower recovery of the C-apoproteins was observed (41% compared to 84% with the higher concentration of the ion-pairing reagent). In addition poor peak shapes were observed with significant tailing.

For preparative studies, it was found necessary to purify the TEAP as it contained a non-polar impurity which co-eluted with apolipoprotein C-II. The salt was purified as a 1% (v/v) solution by preparative HPLC on a C_{18}-cartridge using the Waters Prep-500 liquid chromatograph. The purification procedure resulted in the removal of impurities with a range of polarities. Fig. 4 shows the considerable improvement in the low wavelength UV transparency of the 1% TEAP solution after passage through the preparative C_{18}-column. A blank gradient analysis (see Fig. 5) also showed considerable improvement in the number of optical density peaks eluting during an acetonitrile gradient. In the analysis of both crude and purified TEAP mobile phases a 30-min column equilibration with just the aqueous mobile phase was used to allow any impurities to accumulate on the column, and thus increase the

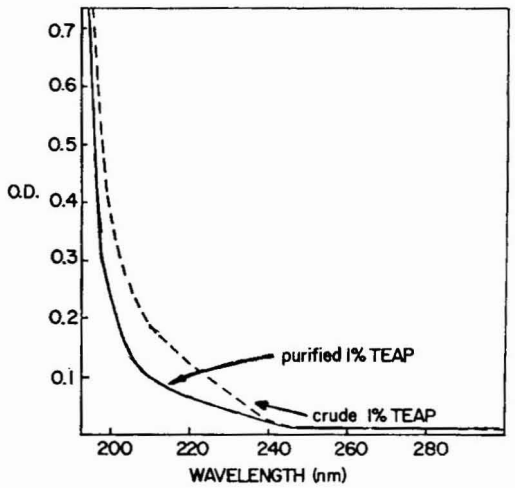

Fig. 4. The UV absorption spectra of a sample of crude 1% TEAP (dashed line) and purified 1% TEAP (solid line). The purification procedure was described in Experimental.

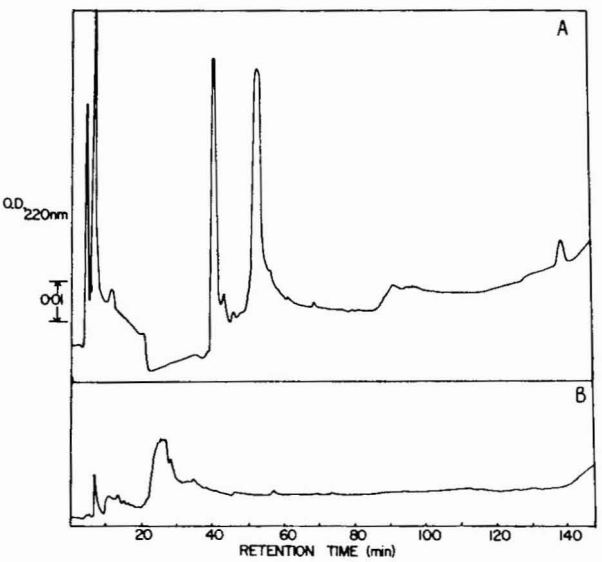

Fig. 5. A blank gradient run for the analysis of the purity of the 1% TEAP aqueous component of the mobile phase. A and B show the elution profile for crude and purified 1% TEAP respectively. In both analyses the column was equilibrated for 30 min at a flow-rate of 1.5 ml/min with the 1% TEAP solution. A 250-μl sample of each mobile phase was injected and then gradient analysis of 0 to 80% acetonitrile was carried out over 2 h.

sensitivity of the analysis. A very different elution profile was observed with the purified buffer as the C-apolipoproteins exhibited a much stronger interaction with the column. With the purified buffer, approximately twice as much acetonitrile was required to elute the protein sample and poor peak shapes with tailing were observed. As was found with small basic peptides, the use of isopropanol instead of acetonitrile

overcame these problems, and allowed the satisfactory analysis of the C-apolipoproteins in the presence of purified TEAP in the mobile phase. Fig. 6 shows the elution profile of the C-apolipoprotein mixture with a mobile phase containing the purified buffer and a isopropanol gradient. Again two different gradient programmes were used to illustrate that a very shallow organic solvent gradient gave a better separation of the protein mixture. The similarity in the elution profile obtained for the protein mixture with the two different mobile phases was confirmed by amino acid analysis of an 24-h, 6 *M* HCl hydrolysate of the pooled peaks (see ref. 1 for representative values).

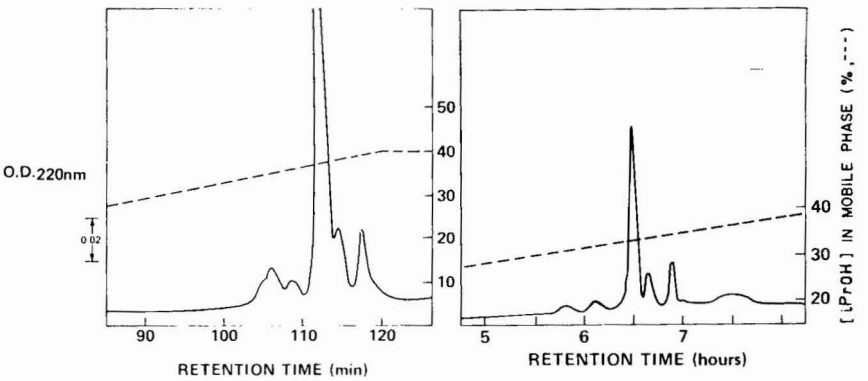

Fig. 6. The elution profiles of a mixture of C-apolipoproteins with different isopropanol gradients (see dashed lines). The TEAP used in the mobile phase was purified as described in Experimental. All other chromatographic parameters were as described in Fig. 3. The identity of the eluted peaks was the same as shown in Fig. 3.

The successful purification by the column of a synthetic pentadecapeptide is shown in Fig. 7. This peptide represents an analogue of the amino terminal region of apolipoprotein C-I (1–15), in which phenylalanine at residue 14 is replaced with *p*-iodophenylalanine. The peptide was purified by successively larger injections of material (0.5, 2 and 8 mg shown in Parts A to C respectively) without any diminution of the separation efficiency. A shallower gradient was used, however, at the higher loadings as a slight decrease in retention times was observed with these concentrations. In this example injections of up to 8 mg of crude synthetic product could be separated without any decrease in resolution (Fig. 7). The material from peak 3 in the different runs was pooled, and analysed by analytical HPLC (Fig. 7D). The isolated material was shown to be homogeneous by the single peak in the elution profile and by amino acid analysis of a hydrolysate. Peaks 1 and 2 consisted of small amount of deletion products (less than 10%) formed by incomplete coupling reactions near the end of the synthesis. The early eluting large O.D. peaks were either due to the guanidine hydrochloride added to the injection mixture or to non-peptidic contaminants.

DISCUSSION

The development of a packing with a significant concentration of free silanol groups does run counter to recent progress in liquid chromatography for the produc-

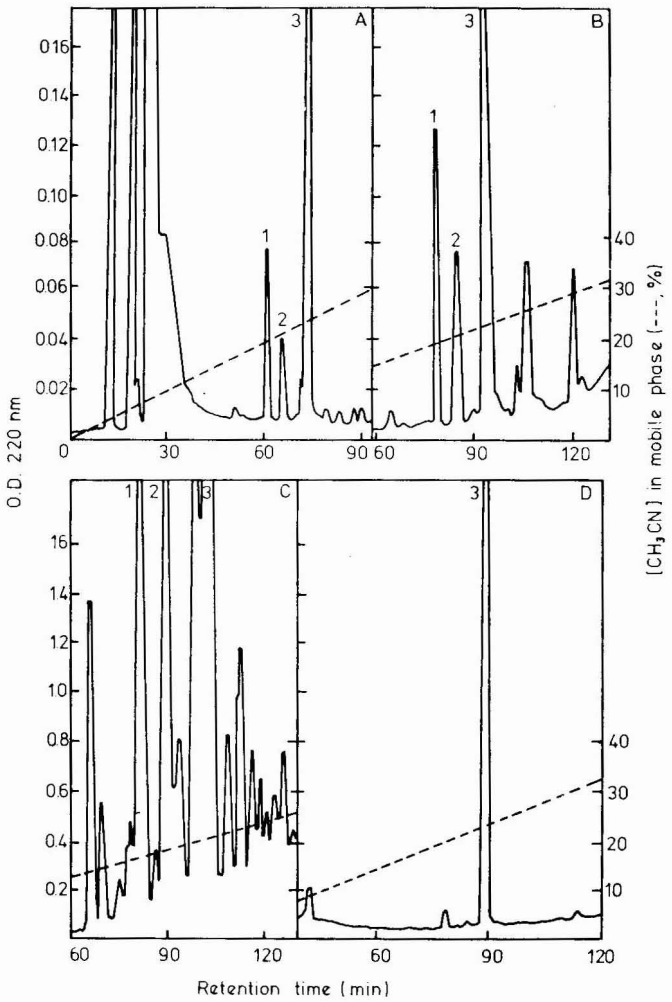

Fig. 7. The purification of a synthetic analog of the 1–15 segment of human apolipoprotein C-I, in which Phe-14 is replaced with *p*-iodophenylalanine in the chemical synthesis. The mobile phase was purified 1% TEAP with a linear gradient of acetonitrile (see the dashed lines). The flow-rate was 1.5 ml/min. The loadings in A to C were 0.5, 2 and 8 mg of crude peptide dissolved in 1% TEAP, 3 *M* guanidine hydrochloride at a concentration of 10 mg/ml. Peak 3 corresponded to the desired peptide, and this fraction from each run was pooled. The analysis of an aliquot of this pool is shown in D.

tion of hydrocarbonaceous supports with very high carbon bondings and minimal silanol content[9,10]. While such supports are undoubtedly successful for the separation of low-molecular-weight solutes such as phenylthiohydantoin (PTH)-amino acids or aromatic compounds, the high carbon bondings can cause difficulties in the chromatography of high-molecular-weight samples such as proteins. For example both C- and A-apolipoproteins are retained indefinitely on C_{18}-reversed-phase packings which have a surface loadings of greater than 20% w/w. This strong interaction between the non-polar stationary phase and the apolipoproteins cannot be overcome by high

levels of organic solvents, as the proteins precipitate and denature irreversibly under these conditions.

For these reasons, the development of a reversed-phase column packing which had a higher polarity due to the presence of free silanol groups, could provide a useful medium for the chromatography of relatively hydrophobic proteins. In addition the silanol groups would be expected to increase the hydration of the pores in the microparticulate silica, thereby facilitating diffusion of the protein molecules. A serious problem with the use of such packings, however, is the propensity of proteins to bind irreversibly to silanol groups[11]. It was decided, therefore, to investigate the use of the RC-C_{18} column with small basic peptides and with protein samples known to be sensitive to silanol groups.

Microparticulate silica presents a heterogeneous surface with silanol groups of varying accessibility and acidity, with a pK_a range of 5–6 (refs. 10, 12). The presence of intramolecular hydrogen bonding is probably the cause of this variation in pK_a values. In adsorption chromatography the more reactive silanol groups are deactivated by treatment of the silica with polar solvents such as water and methanol[10]. Fig. 1 shows the importance of a methanol pre-wash of the RC-C_{18} column before chromatography of peptide samples. In the absence of this pre-wash, the tripeptide Gly-Gly-Tyr exhibited irreproducible elution profiles. The peptide had been previously shown to be homogeneous by a variety of chromatographic studies[13], so that the observation of multiple peaks could be attributed to the packing material and not the sample. After deactivation of silanol groups by the methanol wash, a single elution peak was observed for all subsequent injections. All other studies were performed on a RC-C_{18} column which had been subjected to the methanol wash.

The addition of salts to the mobile phase is also an important procedure for minimising interactions between the silanol groups of the column and ammonium groups present in the solute. The salt can either act as a general electrolyte thereby suppressing ionic interactions, or in certain cases a more specific interaction may occur. It was shown[13,14] that phosphate is capable of forming an ion pair with suitable solutes, as described in the following equation:

$$R\text{–}NH_3^+ + H_2PO_4^- \rightleftharpoons [RNH_3^+\ H_2PO_4^-]$$

Such a reaction would be expected to also decrease interactions between ammonium groups of the sample and silanol groups in the column packing.

Recently amine phosphate buffers have become popular in the HPLC of peptides and proteins[15–18]. Part of the success of these buffers can be attributed to the deactivation of silanol groups by ionic and/or hydrogen bonding interactions with amines added to the mobile phase. Although a mobile phase of 0.1% H_3PO_4 was adequate for the successful chromatography of Gly-Gly-Tyr, other peptides such as Gly-Leu-Tyr required a mobile phase which contained an amine phosphate such as triethylammonium phosphate (TEAP).

The presence of arginine in a peptide presents an additional strongly basic site due to the guanidino side chain. It is not surprising, therefore, that the peptides Met-Arg-Phe, and Leu-Trp-Met-Arg exhibit poor peak shapes on the RC-C_{18} column even in the presence of 1% TEAP in the mobile phase (see Fig. 2B and D). The replacement of acetonitrile with isopropanol as the organic modifier, however,

dramatically improves the elution profile for these basic peptides (see Fig. 2A and C). Methanol does not have this effect when it is used instead of acetonitrile. A possible explanation is that isopropanol, which has a significant non-polar region, is particularly suitable for penetrating the reversed-phase support and further deactivating the silanol groups by hydrogen bonding.

In addition, the following peptides were also examined on the RC-C_{18} column with the different mobile phases described above: Leu-Trp-Met-Arg-Phe, Gly-Phe, Phe-Ser-Lys-Gly-Asp-Gly, Gly-Leu-Tyr, Ala-Lys and Ser-Lys. The elution profiles observed with these peptides demonstrated again that the methanol pre-wash of the column was essential. The only peptides which could be satisfactorily chromatographed in the absence of an amine salt in the mobile phase, were simple peptides which did not contain strongly basic and/or hydrophobic groups, *e.g.*, Gly-Gly-Tyr or Gly-Phe. The other peptides required an amine salt in the mobile phase to suppress the major proportion of silanol group effects, while possibly retaining sufficient polar interactions to allow useful selectivities to still be achieved. These studies suggested that a mobile phase which contained 1% TEAP, pH 3.2, with isopropanol should allow the chromatography of most peptides and proteins on the RC-C_{18} column.

The chromatography of the C-apolipoprotein mixture has been shown to be extremely sensitive to silanol group effects[16]. For example, it was recently shown that the separation of this protein mixture on a μBondapak–alkylphenyl column with a mobile phase of 0.1% H_3PO_4–acetonitrile (20:80) was predominantely a normal rather than reversed-phase separation[16]. In this system the most polar apolipoprotein C-I was eluted last, rather than first as in the reversed-phase separation shown in Fig. 3. Also an increase in concentration of organic modifier resulted in an increased retention time, an observation which is consistent with the proposed normal phase separation mechanism. Not surprisingly this separation was characterised by low recoveries of the C-apoliproteins[16]. If the mobile phase was modified from 0.1% H_3PO_4 to 1% TEAP then a reversed-phase separation was observed similar to the elution profiles shown in Fig. 3.

In a similar manner the separation of the C-apolipoproteins on the RC-C_{18} column could be shown to be extremely sensitive to the nature and concentration of the amine phosphate used in the mobile phase. Triethylamine was shown to be most effective of a number of amines, *e.g.*, ammonia, triethylamine, hexylamine and ethanolamine. In the comparative study each amine was converted to the phosphate salt as described for TEAP in Experimental. Also a high ionic strength was required, as a decrease from 1% to 0.1% in the concentration of TEAP caused a two-fold reduction in recoveries of the apoproteins, as well as resulting in poor peak shapes with excessive tailing.

When purified TEAP was used for the aqueous mobile phase, a distinct difference was observed in the analysis of the C-apolipoprotein mixture. As was described in Results, the use of the purified amine phosphate buffer apparently allowed a stronger interaction to occur between the apolipoproteins and the column. If acetonitrile was used as the organic modifier, the proteins could not be eluted in a satisfactory manner from the column. In fact the results obtained parallel those obtained with the small basic peptides, in that a change in organic modifier from acetonitrile to isopropanol allowed the convenient elution of the C-apolipoproteins. At this stage it is not clear what impurities are present in the crude solution of TEAP

that can account for the differences in chromatographic behaviour of the apolipoproteins between the crude and purified mobile phases. Since the apolipoproteins are known to be extremely sensitive to silanol group effects, it is possible that a basic impurity present in the crude triethylamine was able to complex with the silanol groups still accessible in the RC-C_{18} packing after methanol deactivation.

The important feature of these studies, however, is that operating conditions have been described, which allow the separation of a range of peptide and protein samples by a mixed-mode separation mechanism. Apparently the mixed retention mechanism, which involves both reversed-phase and adsorption interactions, allows for a greater discrimination to be made in a mixture of closely related polar materials. The separation of the extremely complex mixture of tryptic fragments from a partial digestion of apolipoprotein B on a RC-C_{18} column was described previously[1]. An extra-ordinary degree of selectivity was obtained in this separation with over 200 distinct peptides separated in a single gradient run. The maximum number of peptides separated on a μBondapak C_{18} or μBondapak–alkylphenyl column was 50–60 in a gradient run. The major reason for the poorer performance of the fully silanized analytical columns relative to the RC-C_{18} system was not in terms of column efficiencies, but in the lack of selectivity of the former system where a large number of peptides coeluted at *ca.* 20% acetonitrile in the gradient analysis. In the separation shown on the RC-C_{18} column[1] these peptides were spread throughout the gradient, suggesting that greater selectivity is an important factor in the success of the Radial Compression Separation System in the analysis of complex peptides mixture.

Another important feature of this system is that higher sample capacities are available, despite the amount of packing material which is comparable to conventional analytical columns. Often 10–20-mg loadings of complex peptide mixtures can be chromatographed on the RC-C_{18} column without any decrease in separation efficiency, for example Fig. 7 shows the purification of 8 mg of a crude synthetic peptide (the 1–15 amino terminal fragment of apolipoprotein C-I). The corresponding maximum loading on a μBondapak C_{18} column is usually up to 0.2 mg.

REFERENCES

1 W. S. Hancock, J. D. Capra, W. A. Bradley and J. T. Sparrow, *J. Chromatogr.*, 206 (1981) 59.
2 *Radial Compression Separation System*, Bulletin No. F99, Waters Assoc., 1979.
3 C. Pidacks, personal communication.
4 E. J. Kikta and E. Grushka, *Anal. Chem.*, 48 (1976) 1098.
5 J. J. Kirkland, *J. Chromatogr. Sci.*, 9 (1971) 206.
6 R. P. W. Scott and P. Kucera, *J. Chromatogr.*, 142 (1977) 213.
7 R. M. Rabel, E. T. Butts, M. L. Hellman and D. J. Popovich, *J. Liquid Chromatogr.*, 1 (1978) 631.
8 F. M. Rabel, A. G. Caputo and E. T. Butts, *J. Chromatogr.*, 126 (1976) 731.
9 B. L. Karger and R. W. Geise, *Anal. Chem.*, 50 (1978) 1048A.
10 J. H. Knox (Editor), *High Performance Liquid Chromatography*, Edinburgh University Press, Edinburgh, 1978, pp. 43, 48.
11 F. E. Regnier and R. Noel, *J. Chromatogr. Sci.*, 14 (1976) 316.
12 R. K. Iler, *The Colloid Chemistry of Silica and Silicates*, Cornell University Press, New York, 1955.
13 W. S. Hancock, C. A. Bishop, R. L. Prestidge, D. R. K. Harding and M. T. W. Hearn, *J. Chromatogr.*, 153 (1978) 391.
14 W. S. Hancock, C. A. Bishop, R. L. Prestidge, D. R. K. Harding and M. T. W. Hearn, *Science*, 200 (1978) 1168.

15 W. S. Hancock, C. A. Bishop, J. E. Battersby, D. R. K. Harding and M. T. W. Hearn, *J. Chromatogr.*, 168 (1979) 377.
16 W. S. Hancock, C. A. Bishop, A. M. Gotto, D. R. K. Harding, S. M. Lamplugh and J. T. Sparrow, *J. Lipid Res.*, in press.
17 M. T. W. Hearn and W. S. Hancock, *Trends Biochem. Sci.*, 4, No. 3 (1979) 58.
18 J. E. Rivier, *J. Liquid Chromatogr.*, 1 (1978) 343.

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DERIVATISATION AND GAS–LIQUID CHROMATOGRAPHY OF 3-AMINOMETHYL-2-PHENYLBICYCLO[2.2.2]OCTANES

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SUMMARY

Gas–liquid chromatographic methods are described for measurement of the isomer content of 3-methylaminomethyl-2-phenylbicyclo[2.2.2]octane following chemical derivatisation. The stereospecificity of its synthesis is confirmed and the resolved enantiomers are shown to be essentially pure.

INTRODUCTION

Members of a series of *cis*- and *trans*-fused 3-aminomethyl-2-phenylbicyclo-[2.2.2]octanes have been shown to have potential antidepressant properties[1] in animal models. The *cis*-series is the more potent. The most active compound (I) in the *cis*-series has been resolved into its two enantiomers, the laevorotatory isomer showing the greater activity. In support of the synthesis of I for use in toxicological and clinical trials it was necessary to measure both optical and geometric isomer purities of itself and selected synthetic precursors.

The latter stages of the synthesis[1] are shown in Fig. 1. Catalytic reduction of the Diels–Alder adduct formed between *cis*-3,4-dichlorocinnamonitrile and 1,3-cyclohexadiene gives the *cis*-fused 3-cyano-2-phenylbicyclo[2.2.2]octane (II). Compound I is formed in two successive reactions, either by formation of the primary amine (III) or the secondary amine (IV). Chromatographic separation and purity measurements of I were complicated by the presence of numerous homologue and dechlorinated impurities. As high-performance liquid chromatography (HPLC) was not successful in handling these molecules, gas–liquid chromatography (GLC) was utilised to analyse the principal components, even though extensive derivatisation procedures were required. GLC–mass spectrometry (MS) was also the primary technique utilised to characterise impurities at the various synthetic stages.

EXPERIMENTAL

Instruments

GLC analyses were carried out using either a Pye-Unicam Model 104 or a

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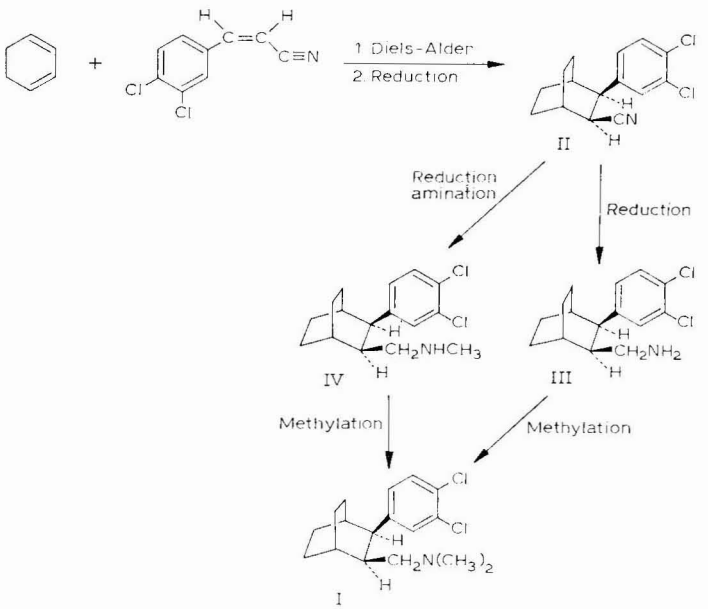

Fig. 1. Synthesis of 3-dimethylaminomethyl-2-(3′,4′-dichlorophenyl)-bicyclo[2.2.2]octane.

Hewlett-Packard Model 5700 gas chromatograph equipped with a flame ionisation detector. An LKB Model 9000S mass spectrometer (MS) was used for the GLC–MS measurements.

Determination of trans-fused molecule (Ia) in cis-fused molecule (I) by formation of the carbamate (Ib)

The hydrochloride of I (3 mg) was dissolved in chloroform (1 ml) and shaken with 0.1 *M* sodium hydroxide solution. An aliquot (100 μl) of the chloroform layer was transferred to a 1-ml Reacti-vial (Pierce, Rockford, IL, U.S.A.). Aliquots (1, 3 and 5 μl) of isomer Ia (0.3 mg/ml) were added to further 100-μl volumes of I. 100 μl of ethyl chloroformate (Koch-Light, Colnbrook, Great Britain) and sodium carbonate (10 mg) were added to each solution. The solutions were heated at 100°C for 45 min and then blown to dryness under nitrogen. The residue was redissolved in chloroform (50 μl) and a sample analysed on a 2% XE60 column at 210°C.

Conversion of compound I to IV

Compound I (3 mg) was converted to Ib as previously described. Hydrobromic–glacial acetic acid (1:1) (0.5 ml) was added to the carbamate residue. The solution was heated at 100°C for 90 min, basified with sodium hydroxide solution to pH 10, and diluted with water. Compound IV was extracted into heptane ($\times$6) and blown to dryness.

Derivatisation of compound IV with optically acid chlorides

The residue of compound IV was reacted with 50 μl of either (+)-α-methoxy-α-trifluoromethylphenylacetyl chloride or N-heptafluorobutyryl-α-prolyl chloride

(Regis, Morton Grove, IL, U.S.A. and triethylamine (10 μl). After 5 min the solution was blown to dryness and the residue dissolved in methylene chloride. (+)-α-Methoxy-α-trifluoromethylphenylacetic acid (Aldrich, Milwaukee, WI, U.S.A.) was converted to the corresponding acid chloride by reaction with oxalyl chloride in triethylamine. The diastereomers V were analysed by GLC using an Apiezon M column whilst VI were analysed using an N,N-bis(*p*-methoxybenzylidene-α,α'-bi-*p* toluidine) (Eastman-Kodak, Rochester, NY, U.S.A.) liquid crystal column at 225°C.

Formation of diastereomeramide VI via formation of N-oxide VII

Compound I (0.05 mg) was dissolved in methylene chloride (50 μl) and treated with *m*-chloroperoxybenzoic acid (50 μg). The solution was blown to dryness after 5 min, again after the addition of triethylamine, and finally after the addition of ethereal diazomethane. (+)-α-Methoxy-α-trifluoromethylphenylacetyl chloride (30 μl), chloroform (50 μl) and triethylamine were added and the diastereomers analysed as previously indicated.

Analysis of compounds III and IV

Aliquots (20 μl) of chloroform solution (1 mg/ml) of compound III or IV were reacted with 10 μl of pentafluorobenzoyl chloride (Fluorochem, Dinting Vale, Great Britain) and triethylamine (2 μl) at 60°C for 15 min. After cooling the resultant solution was shaken with distilled water (350 μl) and 0.880 ammonia (50 μl). An aliquot of the chloroform layer was analysed on an Apiezon J column at 270°C.

RESULTS AND DISCUSSION

Determination of isomeric trans-fused molecule (Ia) in cis-fused 3-dimethylaminomethyl-2-(3',4'-dichlorophenyl)bicyclo[2.2.2]octane (I)

Compound I not only exhibits poor GLC characteristics but it is difficult to separate from the isomeric *trans*-fused molecule (Ia). An optimum relative retention of only 1.05 was achieved for these two molecules on an Apiezon L column. The separation was not suitable for measuring low levels of isomer Ia in I. Several attempts have been made to improve the gas chromatographic properties of tertiary amines by derivatisation. The Hofmann degradation reaction[2] can be utilised to form olefins characteristic of the parent amine. Reaction of a tertiary amine with an alkyl chloroformate[3,4] has been shown to be a satisfactory method for determining low concentrations of drugs.

The levels of Ia in I were determined by this approach using ethyl chloroformate (Fig. 4). The resultant derivatives were then readily resolved by GLC. The formation of the carbamate (Ib) was monitored at 10 min intervals using GLC. On an XE-60 (cyanoethyl silicone) column, the two derivatives show a relative retention time of 1.2. The peak area ratios of *cis*-carbamate to *n*-triacontane internal standard are shown (Fig. 2). Optimum yield (90%) was observed after 60 min, GLC–MS confirming the identity of both carbamate peaks, *viz*: *m/e* 369/371/373 [$M^{+}\cdot$]; 266/268/270 [$M - N(CH_3)COOC_2H_5]^+$ and 116 [$CH_2{=}\overset{+}{N}(CH_3)COOC_2H_5$]. As the synthesis route of I shows a high degree of stereospecificity[1], levels of the *trans*-isomer in I were expected to be below one percent although it was not possible to obtain a standard sample free of *trans*-isomer Ia. Consequently the levels of Ia in I were determined by a spiking

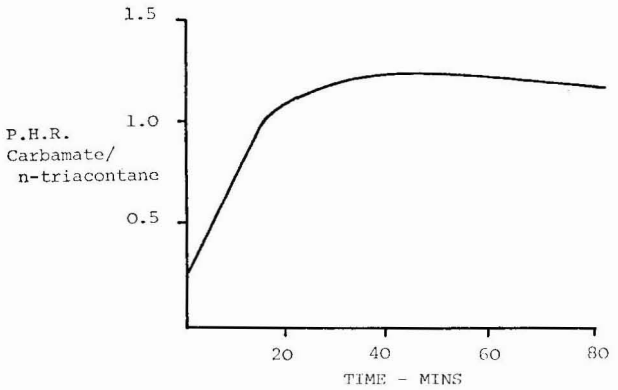

Fig. 2. Graph showing rate of formation of carbamate derivative (Ib).

technique and ratioing the areas of the respective carbamate peaks, assuming an equality of response from each molecule. Standard aliquots of unknown I were spiked with known amounts of Ia. From the calibration graph (Fig. 3) the intercept value representing the required impurity level could be measured. Typical levels of 0.1–0.2% were observed. A precision measurement on six samples gave a coefficient of variation of 3.0% for a mean value of 0.145% *trans*-isomer in I.

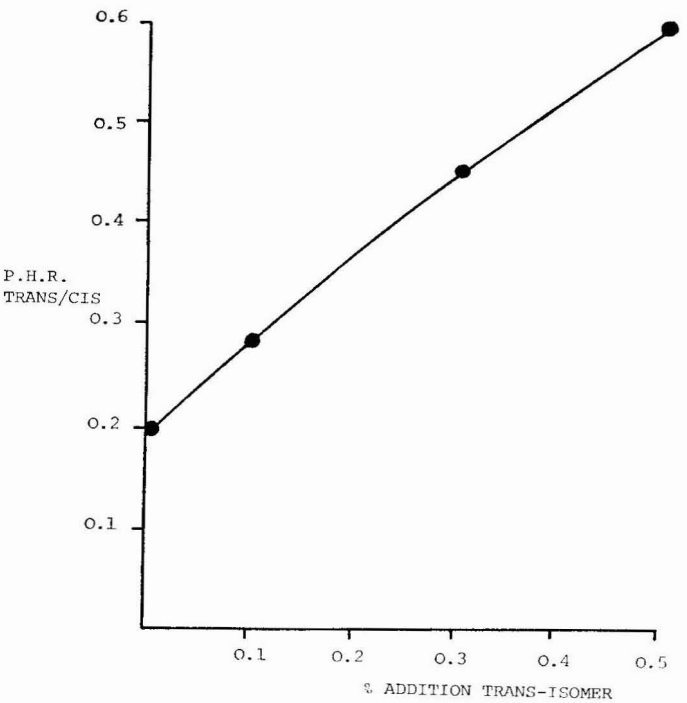

Fig. 3. Calibration graph for determining the level of the *trans*-fused isomer in *cis*-fused 3-dimethylaminomethyl-2-(3′,4′-dichlorophenyl)bicyclo[2.2.2]octane (I).

Analysis of 3-aminomethyl-2-(3',4'-dichlorophenyl)bicyclo[2.2.2]octane (III) and 3-methylaminomethyl-2-(3',4'-dichlorophenyl)bicyclo[2.2.2]octane (IV)

It was not necessary to regularly measure the levels of the *trans*-fused isomer in III or IV once it was established that no inversion of *cis*-fused material took place at either of these synthetic stages. In contrast to the cyano-functional molecule (II), neither III nor IV gave satisfactory chromatographic peaks and were best analysed after benzoylation or acetylation when the respective isomeric derivatives were readily resolved. The pentafluorobenzoate derivatives of IV (*m/e* 491/493/495 [$M^{+\cdot}$], 266/268/270 [$M - N(CH_3)COC_6F_5]^+$ and 238[$CH_2{=}\overset{+}{N}(CH_3)COC_6F_5$] were quantitatively formed and easily resolved by GLC. They indicated that in both cases minimal levels of the *trans*-isomer were present and no inversion took place. Similar results were obtained from the derivatives of III.

Determination of optical purity of cis-fused 3-dimethylamino-2-(3',4'-dichlorophenyl)-bicyclo[2.2.2]octane (I)

Using GLC amine enantiomers can either be directly determined on optically active stationary phases or as diastereoisomers on achiral phases[5,6]. Although the former approach is more direct, it was not possible to implement it for I due to upper temperature limits imposed by the volatility of the optically active phase.

To form diastereoisomers from I it was first necessary to introduce a functional group which could be derivatised. In one approach compound I was demethylated after initial conversion to the carbamate (Ib). Compound Ib was then hydrolysed to give compound IV, a secondary amine. The overall yield of this reaction was 70%. Reaction of IV with N-heptafluorobutyryl-L-prolyl chloride[7] (Fig. 4) gave a diastereoisomer pair (V) which could be resolved on an Apiezon M column. However, this

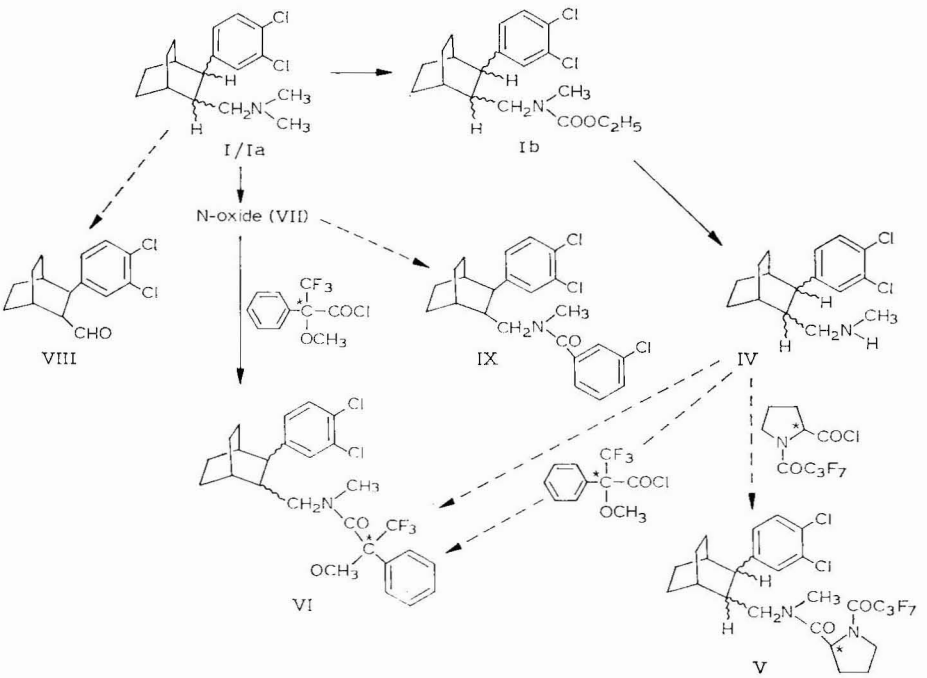

Fig. 4. Derivatisation routes for the analysis of *cis*-fused 3-dimethylamino-2-(3',4'-dichlorophenyl)-bicyclo[2.2.2]octane (I).

latter reagent proved unsuitable for quantitative work due to its variable purity and inversion during derivatisation. When IV was reacted with (+)-α-methoxy-α-trifluoromethylphenylacetyl chloride[8] (Fig. 4) no inversion took place. In contrast to the prolyl reagent the latter acid chloride lacks an α-hydrogen and hence is resistant to racemisation. The resultant diastereoisomers (VI) were resolved into two equal intensity peaks using the liquid crystal phase N,N′-bis(*p*-methoxybenzylidene-α,α′-bi-*p*-toluidine). Super-cooling of the liquid crystal improved the separation[9].

In a preferred alternative route to VI, compound I was converted to the corresponding N-oxide (VII) by reaction with *m*-chloroperoxybenzoic acid. Reaction of VII with (+)-α-methoxy-α-trifluoromethylphenylacetyl chloride[10] (Fig. 4) formed the diastereoamides (VI) ([$M^{+}\cdot$] *m/e* 513/515/517). The formation of VI can be followed by GLC on a dimethyl silicone phase. The average overall conversion yield for both diastereoisomers was 43% (relative standard deviation = 4.9%). The aldehyde (VIII) ([$M^{+}\cdot$] *m/e* 182/184/186) was a significant by-product whilst the formation of the unwanted amide (IX) ([$M^{+}\cdot$] *m/e* 435/437/439/441) could be minimised by reaction of excess *m*-chloroperoxybenzoic acid, *m*-chlorobenzoic acid or its acid chloride with diazomethane.

TABLE I

PERCENTAGE OPTICAL PURITY OF *CIS*-FUSED 3-DIMETHYLAMINO-2-[3′,4′-DICHLOROPHENYL] BICYCLO-[2.2.2]OCTANE(I)

(+)-Isomer		*(−)-Isomer*	
Experimental	*Composition Synthetic mixture*	*Experimental*	*Composition Synthetic mixture*
100.0	100.0		
		100.0	100.0
88.8	90.6	11.2	9.4
93.2	95.3	6.8	4.7
10.7	10.7	89.3	89.3
6.2	5.3	93.8	94.7

As the method required the determination of relative isomer concentration, the respective peak area ratios gave a satisfactory measurement. Analysis of resolved samples of the two enantiomers by this method suggest them to be essentially pure. Synthetic mixtures of the enantiomers were prepared from these pure components. The experimental composition of the synthetic mixtures was in close agreement with expected levels (See Table I).

REFERENCES

1 C. H. Cashin, J. Fairhurst, D. C. Horwell, I. A. Pullar, S. Sutton, G. H. Timms, E. Wildsmith and F. Wright, *Eur. J. Med. Chem.*, 13 (1978) 495.
2 H. V. Street, *J. Chromatogr.*, 73 (1972) 73.
3 P. Hartvig and J. Vessman, *J. Chromatogr. Sci.*, 12 (1974) 722.
4 P. H. Degen and W. J. Reiss, *J. Chromatogr.*, 85 (1973) 53.
5 E. Gil-Av, *J. Mol. Evol.*, 6 (1975) 131.
6 C. H. Lochmuller and R. W. Souter, *J. Chromatogr.*, 113 (1975) 283.
7 R. W. Souter, *J. Chromatogr.*, 108 (1975) 265.
8 L. R. Pohl and W. F. Trager, *J. Med. Chem.*, 16 (1973) 475.
9 S. Wasik and S. Chester, *J. Chromatogr.*, 122 (1976) 451.
10 A. Cave, C. Kan-Fan, P. Potier and J. Le Men, *Tetrahedron*, 23 (1967) 4681.

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QUANTITATIVE GAS CHROMATOGRAPHIC DETERMINATION OF LOW-MOLECULAR-WEIGHT STRAIGHT-CHAIN CARBOXYLIC ACIDS AS THEIR *p*-BROMOPHENACYL ESTERS AFTER EXTRACTIVE ALKYLATION IN ACIDIC MEDIUM

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SUMMARY

The measurement of acetate by gas chromatography at low concentrations in biological fluids is a difficult task. A significant improvement can be brought about by derivatization of acids as *p*-bromophenacyl esters.

By such a procedure, the determination of low-molecular-weight straight-chain carboxylic acids (acetic, propionic and butyric acids) was enhanced with respect to sensitivity and specificity. The volatile fatty acids were esterified by means of an extractive alkylation mechanism in acidic conditions from aqueous solutions. Tetrahexylammonium was used as counter-ion and the alkylating agent was α,*p*-dibromoacetophenone.

The phenacylation reaction was studied with respect to pH, concentration of counter-ion and kinetics. The yield of derivatization was *ca.* 70% after 2 h at 42°C.

A mass spectrometry study was performed in order to ascertain the structure of derivatives using electron impact and chemical ionization.

The gas–liquid chromatographic behaviour of the *p*-bromophenacyl esters of acetic, propionic and butyric acids was studied on an OV-225 packed column and an OV-1 capillary column. Flame ionization detection was shown to be linear between 0.2 and 4 nmol injected, with a quantitative limit of detection of 31 pmol (*i.e.* 1.8 ng of acetic acid).

INTRODUCTION

Many gas chromatographic (GC) methods for the measurement of acetate at low concentrations (10–100 μM) have been reported[1–5]. An extensive review of the techniques was compiled by Cochrane[6]. In most of these procedures, columns packed with liquid phases suitable for the chromatography of the free acid were used. Many pitfalls could be found in GC of volatile fatty acids (VFA). Among these, adsorption of the acid into the chromatographic systems (inlet and columns) resulted in tailing, irregular shape of peaks and ghosting[1–4]. Addition of formic acid to the carrier gas[1,7,11]

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and/or addition of phosphoric acid to the stationary phase[1–5,8] was proposed to improve this situation, but the lifetime of the columns then decreased. In addition, nonreproducible recoveries of VFA in aqueous biological fluids can occur during pre-chromatographic manipulation, such as extraction with organic solvents, concentration by evaporation or vacuum microdistillation[5,9,10,12].

Another problem occurs in the detection level of acetate. Owing to its structure (one methyl only), acetate has a very low response factor to the flame ionization detector (FID). Since the FID has a sensitivity of 20 millicoulombs per gram of carbon, the detection limit can be estimated at *ca.* 700 ng of injected acetate at the usual sensitivity of 10^{-10} A (attenuation $\times 100$) consistent with the noise level in biological analysis. In such conditions, acetate concentrations as low as 60 μM can be measured without pre-concentration. Such a value is close to the normal acetate concentration in human plasma. Accordingly, it is necessary to enhance the detectability of acetate by FID. This can be achieved by derivatization of the molecule.

Our objective was to select a suitable derivative for acetic acid that could be prepared quantitatively, chromatographed and detected in good analytical conditions.

MATERIALS AND METHODS

Reagents

α,*p*-Dibromoacetophenone (Merck, Darmstadt, G.F.R.) was dissolved in methylene chloride at 36 mM concentration. All organic acids (Merck) were dissolved in water, and freshly prepared solutions were used. Different counter-ions were tried: tetrahexylammonium hydrogen sulphate, tetrabutylammonium hydrogen sulphate or chloride or bromide, triethylbenzylammonium chloride, trimethylhexadecylammonium bromide, tributylhexadecylphosphonium bromide. They were all of analytical grade and were purchased from Merck or Interchim (Montluçon, France) or INC (Irvine, CA, U.S.A.) or Eastman (Rochester, NY, U.S.A.). They were dissolved at concentrations of 0.3–1 mM in phosphate buffer solution of variable pH.

Gas–liquid chromatography

The reaction conditions were evaluated with a Pye Unicam 104 Model 84 gas chromatograph equipped with a FID. The glass column, 2.50 m $\times$ 0.4 cm I.D., was packed with 2.1% OV-225 on Gas-Chrom Q (Applied Science Labs., State College, PA, U.S.A.). Nitrogen was used as carrier gas at a flow-rate of 40 ml/min. The column oven and detector temperatures were, respectively, 185 and 250°C. Studies in the nanogram range were carried out with a Carlo-Erba 2150 gas chromatograph equipped with a FID and a splitless injector. The carrier gas was hydrogen at a flow-rate of 1 ml/min. The glass capillary column, 30 m $\times$ 0.3 mm I.D., was a soft persilylated glass as described by Grob[13] and coated with OV-1, film thickness 0.15 μm.

Mass spectrometry

Mass spectrometry (MS) was carried out on a Ribermag R-10-10B (Rueil Malmaison, France) apparatus. Sample introduction was via the GC inlet, OV-225 3% on Chromosorb W HP 100–120 mesh support at 180°C. Helium was used as carrier gas at 1.8 bar. The solvent peak was diverted from the GC–MS interface held at 250°C. The electron impact mass spectra were recorded at 70 eV; the ionization current was 210 μA. Chemical ionization mass spectra were recorded with ammonia as reagent gas.

Evaluation of reaction conditions

The derivatization was carried out as follows. For analytical purposes, 2 ml of diluted solution of acetic, propionic and butyric acids in the range 0.1125–1.125 m*M* were added to 8 ml of buffer solution containing the counter-ion. A 1 ml volume of methylene chloride containing the alkylating reagent (36 m*M*) and *n*-hexacosane (1.22 m*M*) was added. The reaction mixture was shaken in screw-cap PTFE vials for various times at 42°C. Then 2 μl of the organic phase were injected into the gas chromatograph. Reaction yields were calculated from peak heights ratios with *n*-hexacosane as internal standard.

For preparative purposes, 2 mmol of acetic acid in 150 ml of buffer solution (pH 5) were derivatized with 2.2 mol of α,*p*-dibromoacetophenone in 20 ml of methylene chloride. The organic phase was removed under vacuum. The residue, redissolved in benzene, was filtered through 20 g of dry silica gel, 35–70 mesh (Merck). The alkylated derivative was eluted with benzene and recrystallized from heptane.

RESULTS AND DISCUSSION

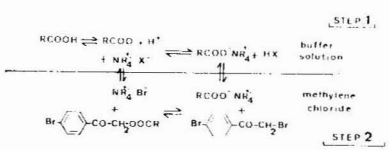
Critical examination of current techniques of synthesis of carboxylic acids esters prior to chromatographic analysis[14–17] indicates that a need exists for methods of derivatizing VFA, such as acetic acid, in very dilute aqueous biological samples. Indeed, many of these procedures were found to be tedious because they entailed successive steps of extraction, concentration and derivatization. Furthermore, when using solid–liquid phase-transfer catalysis[18,19] aqueous media were excluded, and a preliminary ion-exchange step was found necessary to resolve problems due to the interference of Na^+, Mg^{2+} and Ca^{2+} ions[20].

One interesting breakthrough was the application of extractive alkylation, which is well known in organic chemistry[21–25]. The present study applied this derivatization procedure while using a very reactive alkylating reagent, α,*p*-dibromoacetophenone[17].

The phenacyl esters so prepared exhibit good chromatographic properties. In GC, the separation and detectability are noticeably enhanced whereas in liquid chromatography such derivatives are nowadays largely used[26–28] because of their UV properties[19].

Derivatization reaction

Extractive alkylation, also called liquid–liquid phase-transfer catalysis[15], is a very convenient technique, coupling extraction and derivatization. (i) Extraction of the anion of the acid as an ion-pair. This ion-pair, with a bulky lipophilic radical R, is extracted into an organic phase where the alkylation takes place. As the anion present is highly reactive in poorly solvating solvents, the nucleophilic properties of the carboxylate anion are enhanced. (ii) Alkylation with a highly reactive reagent: α,*p*-dibromoacetophenone.



Study of the phenacylation reaction

The extractive alkylation of VFA was the subject of our investigation. Many parameters, such as pH and the nature and concentration of the counter-ion were studied.

pH influence. The influence of pH on the reactivity is shown in Fig. 1. The optimal pH for acetate and propionate is between 5 and 6 and for butyrate it is in the range 4–8.

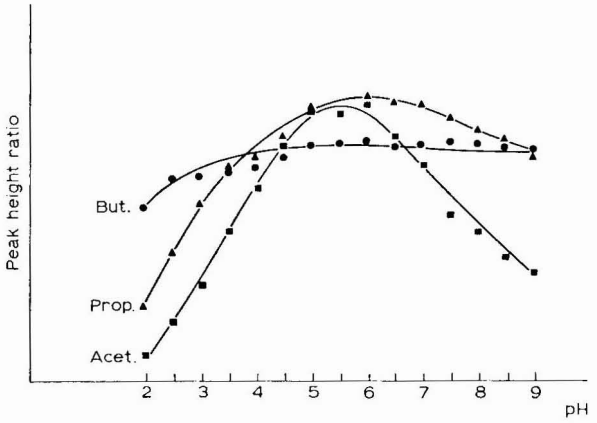


Fig. 1. Influence of pH on the reaction rate: 8 h at 42°C. Reactants: tetrahexylammonium hydrogen sulphate 0.3 m*M* in buffer solution 1 *M*, organic acids 1.5 μmol. Results are expressed in arbitrary units as peak height ratio of organic acids to internal standard (*n*-hexacosane). The peak height ratio was, respectively, 1.13, 1.11 and 1.04 for equal amounts of acetic, propionic and butyric acids with respect to internal standard.

The partition ratio of the carboxylate anion between the organic and aqueous phases is a function of the concentrations of the quaternary ammonium ion and the anion in the aqueous phase and of the extraction coefficient for the ion pair. Accordingly, it is to be expected that the dissociation coefficients of the organic acids will influence their reactivities. At a pH lower than the pK_a, VFA are not dissociated enough to be extracted as ion-pairs in the organic phase. In other respects, all fatty acids are totally dissociated at pH 9–10, which is the pH usually used for extractive alkylation[14,15]. Fig. 1 shows that, above the pK_a value of acetic acid, the reactivity decreases significantly. This abnormal behaviour can be explained by the well-known observation that the $CH_3COO^-Na^+$ salt is more stable than the $CH_3COO^-\overset{+}{N}R'_4$ salt. Consequently, at pH 9–10 the acetate ion cannot be extracted quantitatively as the $CH_3COO^-\overset{+}{N}R'_4$ ion pair.

When the length of the alkyl chain of the organic acid increases, so does the extraction coefficient of the ion-pair because of greater lipophilicity. Consequently, for the propionate and butyrate anions, the equilibrium of step 1 is displaced to the the right, *i.e.* the $RCOO^-\overset{+}{N}R'_4$ ion-pair.

Nature and concentration of counter-ion. The results are listed in Table I for acetic, propionic and butyric acids at pH 5 and 9.

The reactivity seems independent of the nature of the tetra-alkylammonium

TABLE I

INFLUENCE OF THE NATURE OF THE COUNTER-ION ON REACTION RATE

Reactants: counter-ion $0.3 \cdot 10^{-3}$ *M* in buffer solution, organic acid 1.5 μmol, internal standard: *n*-hexacosane. 8 h at 42°C. Results are expressed in arbitrary units relative to internal standard.

Counter-ion	*Buffer solution, pH 5*			*Buffer solution, pH 9*		
	Acetic acid	*Propionic acid*	*Butyric acid*	*Acetic acid*	*Propionic acid*	*Butyric acid*
Tetrabutylammonium hydrogen sulphate	0.03	0.06	0.10	0.05	0.12	0.31
Tetrabutylammonium bromide	0.04	0.05	0.16	0.04	0.06	0.18
Tetrabutylammonium chloride	0.05	0.11	0.24	0.05	0.08	0.23
Tetrahexylammonium hydrogen sulphate	1.45	1.40	1.23	0.45	1.23	1.21
Triethylbenzylammonium chloride	0	0	0	0.05	0.08	0.23
Trimethylhexadecyl-ammonium bromide	0.04	0.14	0.41	0.08	0.22	0.53
Tributylhexadecyl-phosphonium bromide	0.66	0.73	0.74	0.35	0.98	0.96

anion. On the other hand, as the extraction coefficient is a function of the lipophilic character of the counter-ion, the reaction rate is influenced by the length of the alkyl chain of the quaternary ammonium ion. Table I shows that the tetrabutylammonium salts are too hydrophilic. The best results were obtained with the tetrahexylammonium ion. This result is in good agreement with those published by Dehmlow[23], which showed that the counter-ion must contain not less than 15 carbon atoms. When the phosphonium salt was used, the reactivity did not increase. For the following experiments, tetrahexylammonium salts were used.

According to the scheme described above, the reactivity can be increased by a higher concentration of the quaternary ammonium ion. However, only the rate of derivatization of acetic acid increased with concentration of the tetrahexylammonium ion (Fig. 2). For propionic and butyric acids, the derivatization was practically independent of this parameter. This observation can be explained by the more lipophilic character of these acids.

Kinetics of reaction. Fig. 3 shows the results for the three acids at two concentrations of the quaternary ammonium ion. The yield of phenacyl acetate was constant at reaction times over 2 h for a 10^{-3} *M* concentration of tetrahexylammonium ion and over 18 h at a $0.3 \cdot 10^{-3}$ *M* concentration. It is clear that a fast reaction for hydrophilic acids will require a high concentration of the lipophilic counter-ion. The yield of the phenacyl ester ion remains constant at reaction times between 3 and 24 h, indicating that no hydrolysis takes place. The phenacyl esters are highly lipophilic and mainly present in the organic phase, which decreases the possibility of decomposition in buffer phase solution.

Choice of solvent. The distribution of the ammonium salt between the aqueous and organic phases depends to a large extent on the nature of the organic phase.

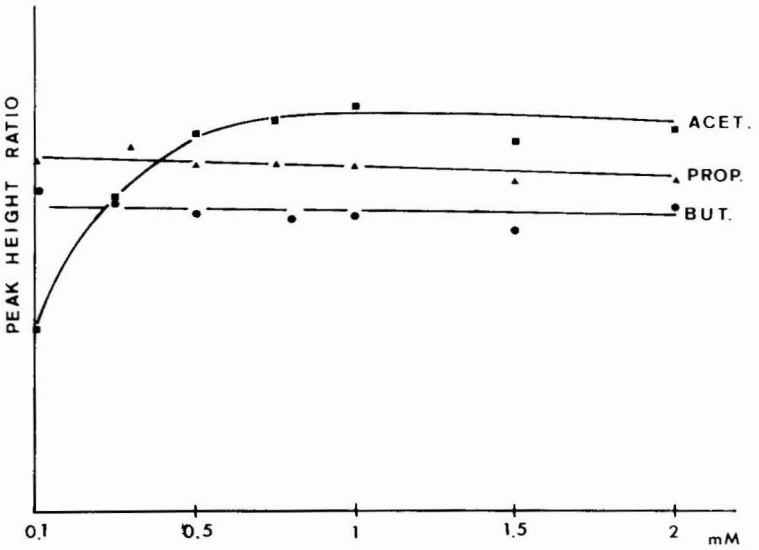


Fig. 2. Influence of tetrahexylammonium ion concentration on the reaction rate. Reactants: organic acids 1.5 μmol. For units see Fig. 1.

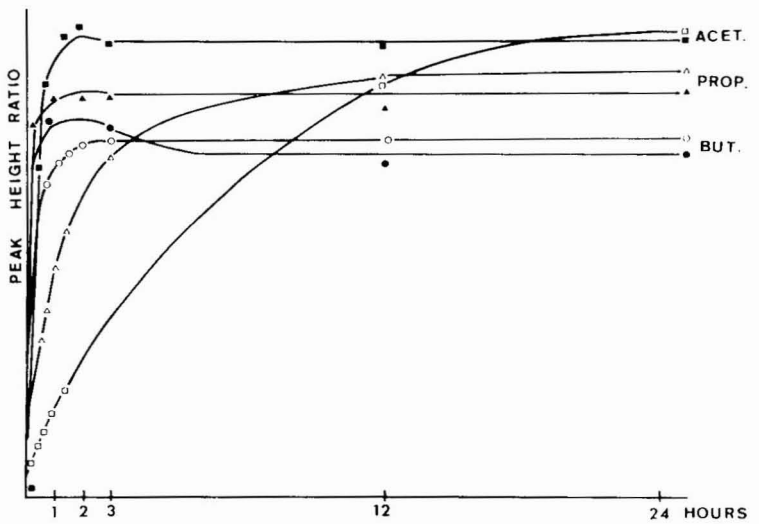


Fig. 3. Phenacyl esterification of acetic, propionic and butyric acids (1.5 μmol) at pH 5. □, Acetic acid; △, propionic acid; ○, butyric acid; all with tetrahexylammonium ion 10^{-3} *M*. ■, Acetic acid; ▲, propionic acid; ●, butyric acid; all with tetrahexylammonium ion $0.3 \cdot 10^{-3}$ *M*.

Methylene chloride, which is often recommended, showed by far the most favorable extraction coefficient. At 42°C, in optimal conditions, the yield of phenacyl acetate was *ca.* 70% (determined with a known amount of pure phenacyl acetate as reference). Benzene and dichloroethane were also tested as solvents, principally to increase the reaction temperature; however, interfering side reactions occurred.

Chromatographic analysis

Fig. 4 shows separation of acetic, propionic and butyric acids as phenacyl esters on an OV-225 packed column. All these peaks were quantitatively resolved when using this polar liquid phase. The retention indices (RI) of the *p*-bromophenacyl esters of acetic, propionic and butyric were, respectively, 2334, 2409 and 2491 at 185°C on OV-225, and the α,*p*-dibromoacetophenone reagent (RI = 2237) gave a pronounced peak with marked tailing. This tailing, if too great, made quantitative measurement of acetic acid difficult. Thus, the use of high-performance gas chromatography (HPGC) on glass capillary columns was attempted.

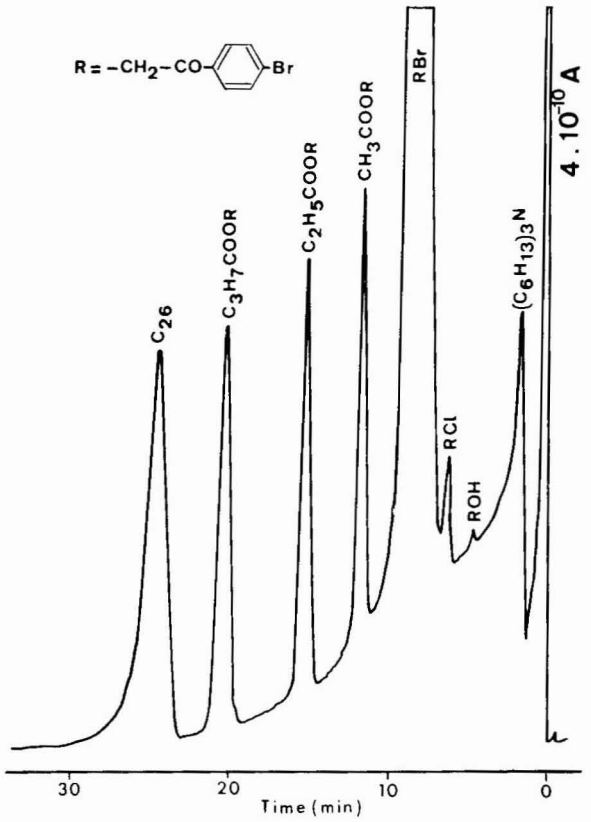


Fig. 4. Gas chromatogram of acetic, propionic and butyric acids as phenacyl esters on 2.1% OV-225 at 185°C.

Fig. 5 illustrates the effect of the efficiency of GC on the separation of the reagent, RBr, and the *p*-bromophenacyl esters of acids. This separation was carried out on a non-polar liquid phase OV-1. It should be noted that when such an analysis was carried on an OV-1 packed column, the acetic acid peak was obscured by tailing of the reagent peak. Retention indices of α,*p*-dibromoacetophenone and the *p*-bromophenacyl esters of acetic, propionic and butyric acids were, respectively, 1582, 1642, 1721 and 1778 on OV-1.

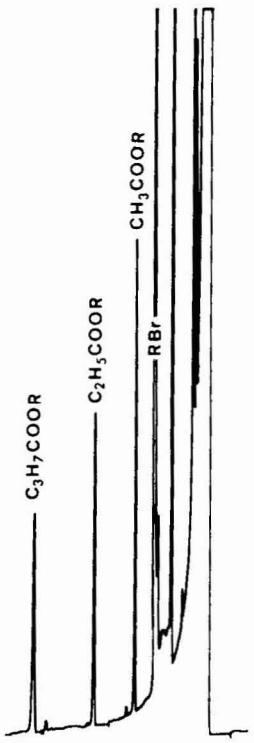


Fig. 5. Gas chromatogram of acetic, propionic and butyric acids as phenacyl esters on glass capillary column OV-1 30 m × 0.3 mm I.D., film thickness, 0.15 μm; oven temperature, 150°C; carrier gas, hydrogen at 0.5 bar. Split injection.

Mass spectrometry of p-bromophenacyl esters

In order to identify *p*-bromophenacyl ester derivatives, mass spectrometry was performed with a GC–MS coupled system using two ionization modes.

The electron impact (EI) ionization gave a base peak at *m/e* 183, 185, corresponding to the fragment ion $BrC_6H_4CO^+$. Such a fragment was present in the mass spectra of all the phenacyl esters studied (Fig. 6). It resulted from the elimination of the $RCOOCH_2$ residue from the molecular ion by a β-cleavage without hydrogen transfer. The presence of one bromine atom in the molecule provided a characteristic isotopic pattern. This doublet was present in all the fragments containing an bromine atom (^{79}Br and ^{81}Br) with a relative abundance of about one. The ions at *m/e* 155 and 76 corresponded, respectively, to the fragments $BrC_6H_4^+$ and $C_6H_4^+$. Finally, while using EI, the identity of the derivatives was confirmed only by the molecular ions at *m/e* 256–258, 270–272 and 282–284. These molecular ions were *ca.* 3% of the intensity of the base peak. However, fragments characteristic of derivatized acids were observed at low *m/e* 43, 57 and 71 for acetic, propionic and butyric acids, respectively. These ions corresponded to the fragments CH_3CO^+, $C_2H_5CO^+$ and $C_3H_7CO^+$.

Chemical ionization (CI) with ammonia as reagent gas gave quasi-molecular ions (QM^+) at $(M + 1)^+$: *m/e* = 257–259, 271–273 and 285–287 for the three VFA studied. A base peak (Fig. 7) was obtained at $(M + 18)^+$, corresponding to ions

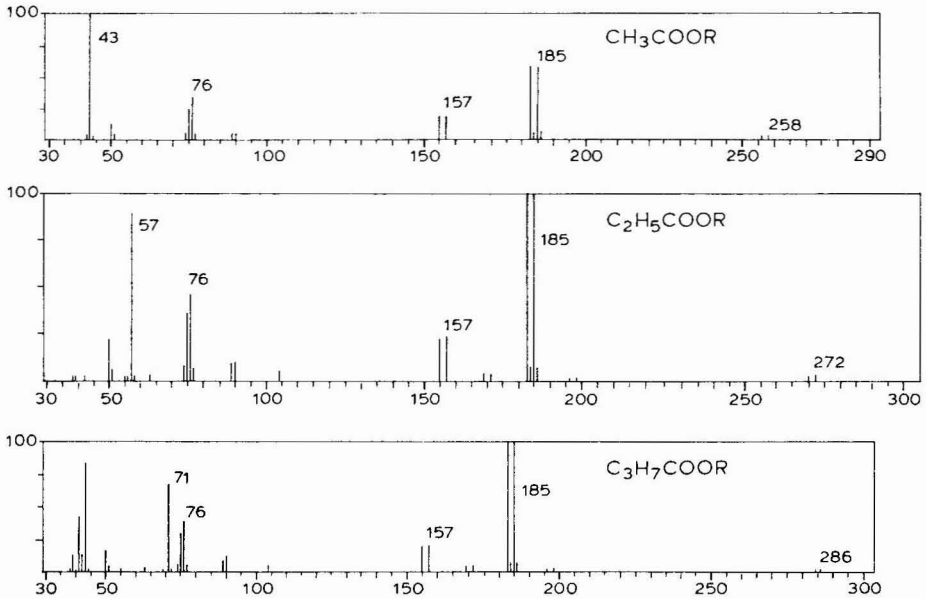


Fig. 6. EI mass spectra of phenacyl esters of acetic, propionic and butyric acids. R = Br–C_6H_4–CO–CH_2. For experimental conditions see text.

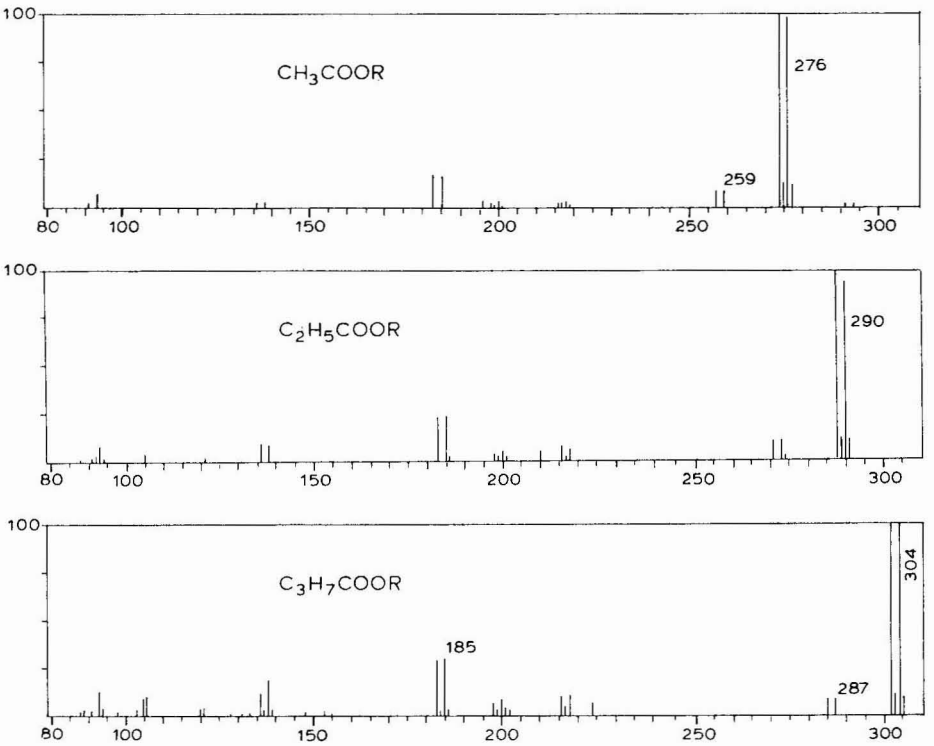


Fig. 7. CI mass spectra of phenacyl esters of acetic, propionic and butyric acids. Ammonia was used as reagent gas.

($Q - NH_4^+$). Furthermore, the fragment ions at $m/e = 183, 185$ were still present in all the mass spectra. This observation can be explained by the stabilization of the fragment ion, $p\text{-}BrC_6H_4CO^+$, obtained by a resonance mechanism.

Measurement of VFA

In order to establish the advantages of the derivatization of VFA for quantitative purposes, the linearity of response and the limit of detection were studied. A linear response from 200 pmol to 4 nmol injected of each derivatized acid was obtained, as shown in Fig. 8.

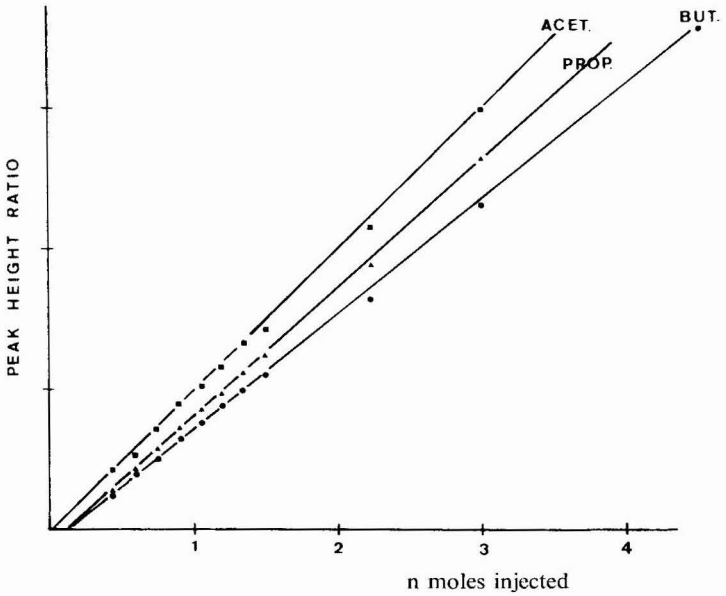


Fig. 8. Standard curves for acetic, propionic and butyric acids as *p*-bromophenacyl esters. Chromatographic conditions: 2.5 m × 0.4 cm I.D. column packed with 2.1% OV-225 on Gas-Chrom Q; oven temperature, 185°C; flame ionization detection; sensitivity, 2×10^{-10} A (full-scale deviation); internal standard, *n*-hexacosane.

By measuring the response of pure crystallized *p*-bromophenacyl ester of acetic acid, it was shown that the yield of derivatization was only *ca.* 70% in optimal conditions. However, this lack of total derivatization did not prevent quantitative analysis (Fig. 8). Nevertheless for quantitative analysis of biological samples, it will be necessary to check that the reaction yield is identical with that of the standards.

The limit of detection is an important criterion. According to Curie[29], a quantitative detection limit, *Lq*, can be defined as the level at which the precision of the measurement will be satisfactory for quantitative determination. This term *Lq* is calculated as $Lq = 10\,\sigma_0$, where σ_0 is the standard deviation of measurement at very low levels; for acetic acid it is 31 pmol or 1.8 ng injected in the gas chromatograph at 10^{-10} A sensitivity. Under these conditions the determination of the plasma acetate level in normal human subjects, 51 μM[5], could be performed satisfactory. This detection limit could be noticeably improved by using a capillary column.

CONCLUSIONS

Although several GC methods for the quantitative determination of acetate in biological fluids have been reported, almost all were concerned with pathological cases, such as hemodialysis or metabolic diseases. The main problem is due to the relatively low concentration of acetate in the plasma of healthy subjects. When such small amount have to be measured, the GC adsorption gives rise to tailing, irregular peaks and ghosting, making quantitative analysis difficult. Furthermore, owing to its structure, acetic acid gives a low signal in a FID (formic acid does not respond to a FID). Accordingly, in order to enhance detectability, volatile fatty acids have often been converted into their esters. However, owing to the non-quantitative recoveries of the esters, especially for the lower acids, these procedures are not suitable for quantitative analysis.

The authors attempted to improve the esterification procedure by using phenacyl esters via an extractive alkylation of acetic, propionic and butyric acids. Phenacyl esters can be prepared easily and quantitatively, allowing the detection of as little as 1.8 ng of C_2 acid. The method of preparation is inexpensive and rapid, coupling the extraction and derivatization steps. The reaction conditions do not require the exclusion of water. GC detectability and separation can be improved by using a glass capillary column. Furthermore, phenacyl esters are recognized as suitable tags for UV detection in HPLC analysis[19].

The formation of phenacyl esters of volatile fatty acids must thus be considered as a useful method for the quantitative analysis of acetate. Indeed, such a change in the polarity of the molecule cannot but enhance the specificity of the method.

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REFERENCES

1 B. Pileire, *Clin. Chim. Acta*, 88 (1978) 321.
2 L. G. Nielsen, K. O. Ash and E. Thor, *Clin. Chem.*, 24 (1978) 348.
3 G. Desch and B. Descomps, *Clin. Chim. Acta*, 92 (1977) 193.
4 M. Kveim and J. E. Bredesen, *Clin. Chim. Acta*, 92 (1979) 27.
5 C. D. Tollinger, H. J. Vreman and M. W. Weiner, *Clin. Chem.*, 25 (1979) 1787.
6 G. C. Cochrane, *J. Chromatogr. Sci.*, 13 (1975) 440.
7 R. G. Ackman and R. D. Burgher, *Anal. Chem.*, 35 (1963) 647.
8 R. E. Hillman, *Clin. Chem.*, 24 (1978) 800.
9 H. J. Vreman, J. A. Dowling, R. A. Raubach and M. W. Weiner, *Anal. Chem.*, 50 (1978) 1138.
10 J. E. Tyler and G. H. Dibdin, *J. Chromatogr.*, 105 (1975) 71.
11 D. A. M. Ceddes and M. N. Gilmour, *J. Chromatogr. Sci.*, 8 (1970) 394.
12 R. G. Richards, C. L. Mendenhall and J. MacGee, *J. Lipid. Res.*, 16 (1975) 395.
13 K. Grob, G. Grob and K. Grob, Jr., *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 1 (1979) 31.
14 H. Ehrsson, *Acta Pharm. Suecica*, 8 (1971) 113.
15 A. M. Tivert and K. Gustavii, *Acta Pharm. Suecica*, 16 (1979) 1.
16 A. M. Tivert and K. Gustavii, *Acta Pharm. Suecica*, 16 (1979) 233.

17 E. O. Umeh, *J. Chromatogr.*, 56 (1971) 29.
18 A. Arbin, H. Brink and J. Vessman, *J. Chromatogr.*, 170 (1979) 25.
19 H. D. Durst, M. Milano, E. J. Kikta, S. A. Connelly and E. Grushka, *Anal. Chem.*, 47 (1975) 1797.
20 M. J. Barcelona, H. M. Liljestrand and J. J. Morgan, *Anal. Chem.*, 52 (1980) 321.
21 A. Brandstrom and U. Junggren, *Acta Chem. Scand.*, 23 (1969) 2204.
22 M. Makosza and B. Serafin, *Rocz. Chem.*, 39 (1965) 1799.
23 E. V. Dehmlow, *Angew. Chem., Int. Ed. Engl.*, 16 (1977) 493.
24 J. Dockx, *Synthesis*, (1973) 441.
25 J. Jonkmann, *Pharm. Weekbl.*, 110 (1975) 649.
26 R. F. Borch, *Anal. Chem.*, 47 (1975) 2437.
27 E. Grushka, H. D. Durst and E. J. Kikta, Jr., *J. Chromatogr.*, 112 (1975) 673.
28 H. C. Jordi, *J. Liquid Chromatogr.*, 1 (1978) 215.
29 L. A. Curie, *Anal. Chem.*, 40 (1968) 586.

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DEVELOPMENT OF A SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR DETECTION OF AFLATOXINS IN PISTACHIO NUTS

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SUMMARY

A very sensitive method is described for the detection of aflatoxins in pistachio nuts. The method employs the extraction procedure used in the BF method (described in the Association of Official Analytical Chemists' Book of Methods) followed by an acid alumina column clean-up to remove sample components which would otherwise interfere in the final determinative step. The quantitation of the aflatoxins is carried out by high-performance liquid chromatography on a C_{18} reversed-phase column after conversion of aflatoxins B_1 and G_1 to their more fluorescent derivatives, B_{2a} and G_{2a}. The limit of detection was found to be 0.5 μg/kg. The chromatograms were found to be free of interfering peaks and multiple injections showed excellent reproducibility.

INTRODUCTION

Most methods commonly used for analysis of pistachio nuts for aflatoxins are based on a clean-up procedure originally developed for grains and employ silica gel thin-layer chromatography (TLC) in the final determination step. The dependence of these methods upon quantitation can lead to variations in the results, presumably because the resolution and fluorescent intensity of aflatoxins on thin-layer plates varies with the type of silica gel and with the environmental conditions prevailing in a given laboratory[1–4]. Intralaboratory collaborative studies where TLC was used to quantitate the aflatoxin content have reported coefficients of variation ranging from 20 to 60% (refs. 5–7).

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In recent years instrumentation for high-performance liquid chromatography (HPLC) and high-resolution columns has become available. These advances have resulted in several applications of this technique to the analysis of foodstuffs for the presence of aflatoxins and other mycotoxins with the advantages of speed, good resolution and high degree of accuracy and precision. HPLC has been applied to the detection of aflatoxins in ground *Aspergillus*-infested grain samples[8], mold extracts[9], cottonseed[10], wine[11], corn[12] and some other mycotoxins including patulin in apple juice[13]. Recently we have also applied this technique successfully to the determination of ochratoxin A in corn[14].

Application of the HPLC technique to semipurified extracts of naturally contaminated pistachio nuts obtained from Official Methods, AOAC[15] showed that residual non-aflatoxin artifacts in the extract interfere with the resolution of aflatoxins. In this article a very sensitive HPLC method for aflatoxin determination in pistachio nuts is proposed in which the sample extract from Official Methods (BF method) is further purified on a small acid alumina clean-up column designed to completely remove the non-aflatoxin impurities followed by conversion of aflatoxins B_1 and G_1 to their hydroxylated derivatives B_{2a} and G_{2a}. The fluorescence characteristics of the aflatoxins B_{2a} and G_{2a} have been reported, and they were found to be more fluorescent in polar solvents than B_1 and G_1 (refs. 12 and 16).

EXPERIMENTAL

Equipment

A DuPont Model 840 liquid chromatograph was used with a Valco septumless injector and a Model 836 fluorescnece detector with a Corning CS-7-54 excitation filter (UV range 328–385 nm) and a CS-3-72 emission filter (UV cut-off 451 nm) connected to a 1-mV recorder. The stainless-steel HPLC column employed was 30 cm × 4 mm I.D., and contained 10-μm ODS-bonded silica gel (μBondapak; Waters Assoc., Milford, MA, U.S.A.). A Schoeffel Model SD 3000 fluorodensitometer was employed in TLC mode. A 300 × 15 mm I.D. glass column with a PTFE stopcock and a 125-ml reservoir was used for acidic alumina column chromatography.

Reagents

Acidic alumina (Fisher A-948, 80–200 mesh) was prepared by adding 3% water (w/w), shaking for several minutes and allowing to stand overnight to equilibrate. Crystalline aflatoxins B_1, B_2, G_1 and G_2 were obtained from the Food and Drug Administration (Washington, DC, U.S.A.) and standard solutions were prepared containing 0.030, 0.045, 0.060, 0.075 and 0.090 μg/ml of each aflatoxin. The HPLC elution solvent employed was distilled water–methanol–acetonitrile (67:20:13) degassed before use.

Sample preparation and extraction

Pistachio nut samples were prepared and 50 g of each sample was extracted according to Official Methods, AOAC, 26.020–26.023[15] and evaporated to dryness.

Column chromatography

A ball of glass wool was placed in the bottom of the column and 2 g anhydrous

sodium sulfate was added as a base for the acidic alumina. Benzene was added until the column was about half full, then 5 g of acidic alumina was added. The sides of the column were washed with about 10 ml of benzene and stirred to disperse the alumina. The benzene was drained to about 3–5 cm above the alumina, then 5 g of anhydrous sodium sulfate was slowly added and the benzene was drained to the top of the packing. The sample extract was dissolved in 2 ml of chloroform–benzene (1:4) and quantitatively transferred onto the column. The sample beaker was rinsed with an additional 5 ml of chloroform–benzene (1:4) and the contents added to the column. To the column, 50 ml of benzene was added, drained at maximum flow-rate and discarded. Finally, the aflatoxins were eluted with 50 ml of chloroform–methanol (97:3) into a 100-ml beaker. This solution was evaporated to about 2 ml on a steam bath, transferred to a 5-ml vial and evaporated to dryness under a stream of nitrogen.

Derivative formation

The conversion of aflatoxins B_1 and G_1 to their water adducts B_{2a} and G_{2a} is catalyzed by acid. A 1-ml volume of each of the standard solutions was pipetted into separate 5-ml vials and evaporated to dryness under a stream of nitrogen. To each vial of standard and sample 100 μl of trifluoroacetic acid and 100 μl of water were added. The vials were heated on a steam bath (at about 50°C) for 30 min. Then the solutions were evaporated to dryness under nitrogen. Each sample or standard was dissolved in 1 ml of mobile phase. If there were any particles in the sample solution, these were filtered through a 5-μm filter prior to injection into the HPLC column.

HPLC determination

Pressure was applied to the HPLC column to give a flow-rate of 1 ml/min. A 20-μl volume of the most dilute standard solution was injected and the detector was set to give about "½" pen deflection for the first two peaks (B_{2a} and G_{2a}).

A standard curve was prepared for each aflatoxin by injecting 20 μl of each standard solution into the HPLC column and plotting peak height *vs.* standard concentration (in ng/ml). The four aflatoxins were eluted in the order G_1, B_1 (as G_{2a} and B_{2a}), G_2, and B_2.

A 20-μl volume of the sample extract was injected into the HPLC column and the aflatoxin peaks were identified by comparing the retention times with those of the stanards. The peak heights were measured and the concentrations were determined in sample from the standard curves. The amount of each aflatoxin in the original sample was calculated using the equation $\mu g/kg = C_s \times V_s/5$, where V_s is the final sample volume (ml) and C_s is the aflatoxin concentration as read from the standard curve (ng/ml).

If the aflatoxin peaks in the sample are larger than the most concentrated aflatoxin standard solution, the samples should be diluted and re-injected.

RESULTS AND DISCUSSION

Several HPLC mobile-phase solvent mixtures were tried before deciding on the mixture of water–methanol–acetonitrile (67:20:13). This solvent elutes the four aflatoxins in from 6.8 to 15.8 min at a flow-rate of 1.0 ml/min. Fig. 1 is a chromatogram of 1.7 ng of each of the four aflatoxins. It was found that the solvent mixture

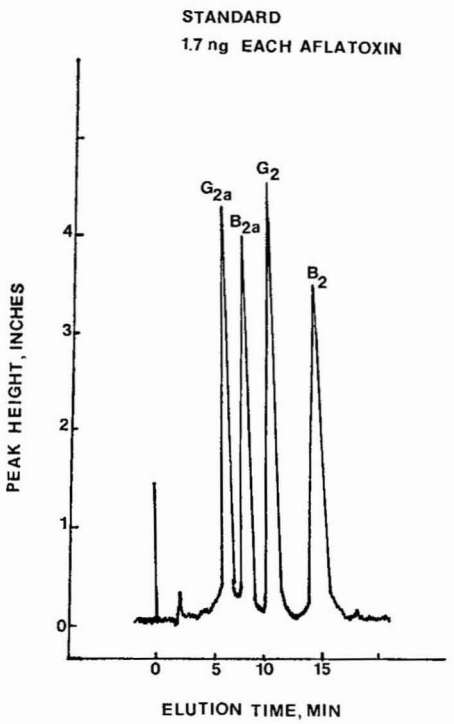

Fig. 1. Resolution of aflatoxins in reference standards, 1.7 ng each B_1, B_2, G_1 and G_2. Chromatographic conditions: column, C_{18} (octadecyl reversed phase), detector, fluorescence at 8 NAFS; pressure, 1200 p.s.i. to give a flow-rate of 1 ml/min; chart speed: 0.1 in./min; mobile phase, water–methanol–acetonitrile (67:20:13).

had to be varied slightly in order to achieve the same separation of the aflatoxins on a series of C_{18} reversed-phase columns from the same manufacturer. With a new column the solvent mixture described above was used as a starting point and a comparable separation of the aflatoxins was usually achieved by altering the methanol:water ratio by 1 or 2%. Once a satisfactory solvent mixture was found the separation was found to be reproducible over several months.

TABLE I

REPRODUCIBILITY OF RETENTION TIMES OF AFLATOXINS IN STANDARD SOLUTIONS AND SAMPLE EXTRACTS

For standard solutions 20-μl injections of solutions containing 0.3–1.8 ng of each aflatoxin were made. For sample extracts 20-μl injections of pistachio nut sample extracts which contained 10 μg/kg of each of the aflatoxins were made. Number of injections was 6 in each case.

	Aflatoxin standards				*Sample extracts*			
	G_{2a}	B_{2a}	G_2	B_2	G_{2a}	B_{2a}	G_2	B_2
Mean retention time (min)	6.81	8.79	11.39	15.8	6.82	8.79	11.21	15.88
Standard deviation	0.036	0.026	0.026	0.017	0.02	0.02	0.028	0.028
Coefficient of variation (%)	0.4	0.3	0.24	0.10	0.3	0.2	0.25	0.2

To demonstrate the reproducibility of the retention times 20 μl of six standard solutions ranging in concentration from 0.015–0.090 ng/μl were injected into the HPLC column and six 20-μl injections of a pistachio nut extract to which each of the four aflatoxins had been added at levels of 10 μg/kg were also made. The mean retention times, standard deviations and coefficients of variation for these injections are given in Table I. The low values for the standard deviations and coefficients of variation (0.1–0.4% for standards and 0.2–0.3% for samples) demonstrate that the retention times

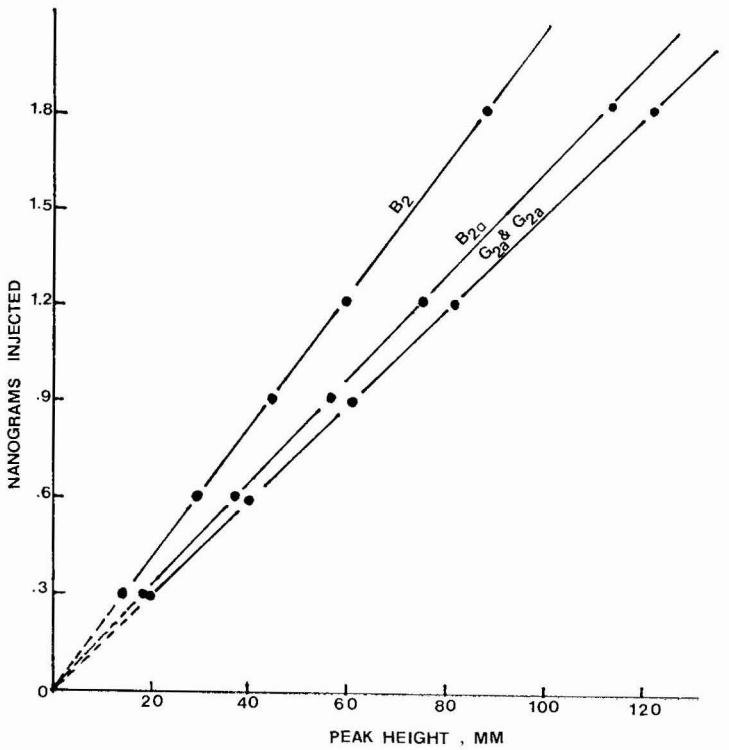

Fig. 2. Typical standard curves of aflatoxins B_{2a}, B_2, G_{2a} and G_2. Chromatographic conditions are the same as those in Fig. 1.

are reproducible within the concentration range studied and are not affected by other components in the sample extract.

The linearity of the HPLC fluorescence detector response for the four aflatoxins was investigated over the range of 0.3–1.8 ng. The response was found to be linear for all four aflatoxins. Typical standard curves of peak height *vs.* amount injected (in ng) are reproduced for B_{2a}, B_2, G_{2a} and G_2 (Fig. 2).

Before development of a new clean-up procedure, both BF (26.020–26.024) and CB (26.014–26.019) methods of the AOAC methods[15] for pistachio nuts were modified by substituting HPLC for TLC in the determinative steps. In Fig. 3 are reproduced the HPLC chromatograms which were obtained following clean-up of a pistachio nut sample by the BF and CB methods. Although sample clean-up by the CB method was

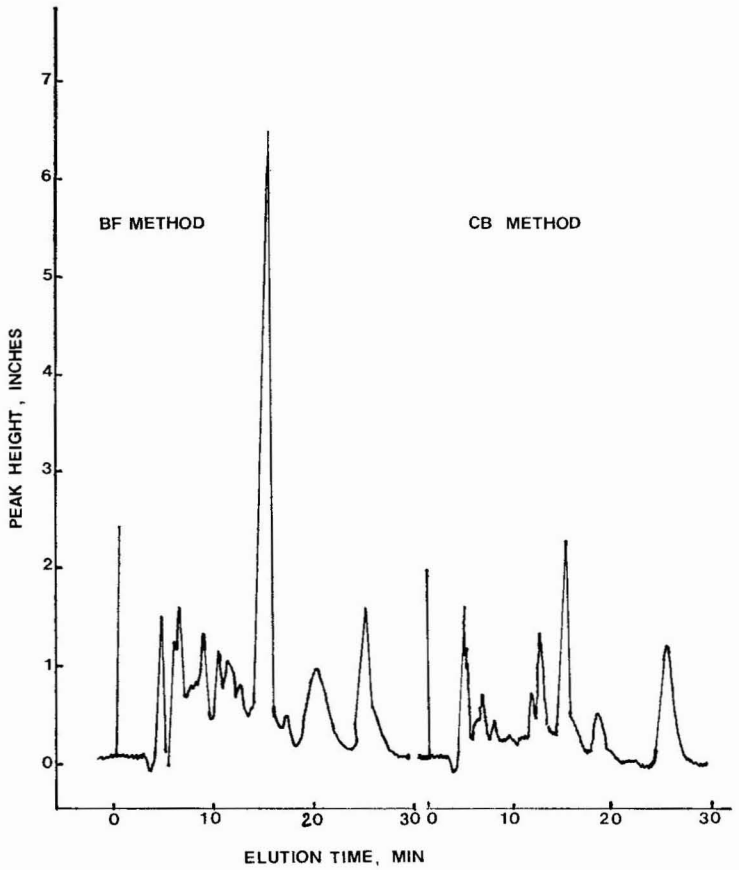

Fig. 3. High-performance liquid chromatograms of pistachio nut extracts prepared by the BF and CB methods. Chromatographic conditions are the same as those in Fig. 1.

more satisfactory, it was concluded that other components in the sample would interfere with the analysis for aflatoxins.

In Fig. 4 three chromatograms are reproduced of the HPLC analysis of pistachio nut extracts after sample clean-up by the procedure described earlier. The first chromatogram is of a sample to which each of the four aflatoxins has been added at the 10 μg/kg level. The second chromatogram is of a naturally contaminated pistachio nut sample which contained a high level of B_1 and smaller amounts of G_1, G_2 and B_2. The third chromatogram is from a sample in which no aflatoxins were detected. These chromatograms illustrate the effectiveness o the clean-up achieved; in no case was an appreciable background due to the sample matrix observed.

A total of nine pistachio nut samples, one sample to which no aflatoxins were added, six samples to which each of the four aflatoxins was added at levels ranging from 0.5–18.0 μg/kg, and two naturally contaminated samples, were cleaned up by the procedure described earlier. The aflatoxins were determined first by TLC using a fluorodensitometer, then by HPLC; in each case the same extracts were used. The results of these analyses are tabulated in Table II. The total aflatoxins recovered ranged

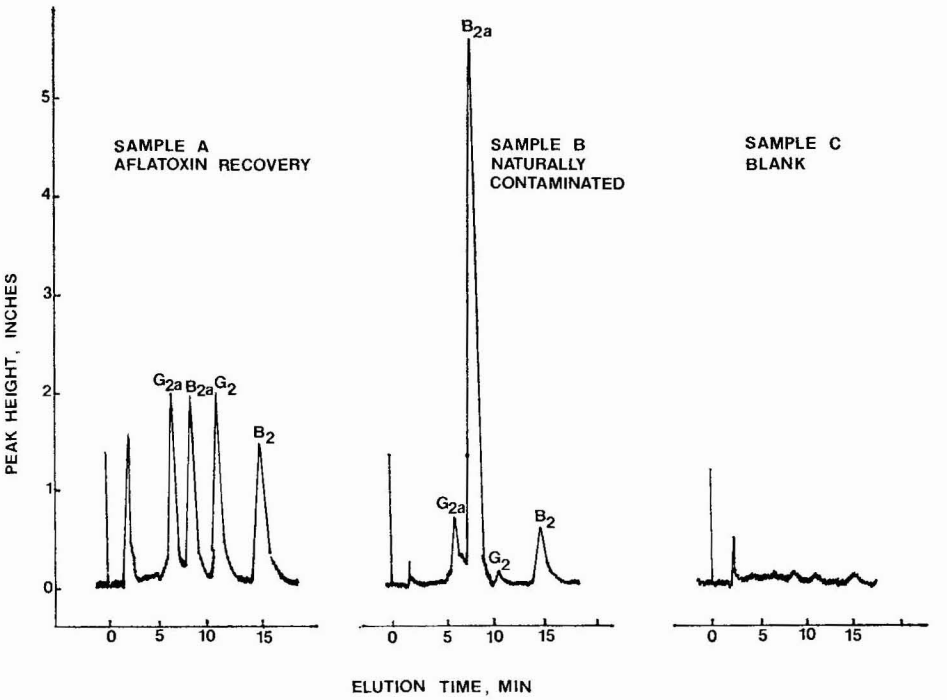

Fig. 4. High-performance liquid chromatograms of pistachio nut extracts prepared by the procedure described in the Experimental section. Chromatographic conditions are the same as those in Fig. 1.

TABLE II

COMPARISON OF THE DETERMINATION OF AFLATOXINS IN PISTACHIO NUTS BY TLC AND HPLC

NC = Naturally contaminated.

Aflatoxins added (μg/kg)	B_1		B_2		G_1		G_2		*Total*	
	Found		*Found*		*Found*		*Found*		*Found*	
	μg/kg	*%*	*μg/kg*	*%*	*μg/kg*	*%*	*μg/kg*	*%*	*μg/kg*	*%*
HPLC analysis										
0	0		0		0		0		0	
0.5	0.46	92	0.22	44	0.47	94	0.27	54	1.42	71
2.0	1.43	72	1.2	60	1.4	70	1.2	60	5.23	65
6.1	5.0	82	3.09	51	4.83	79	2.8	46	15.72	64
10.0*	8.9	89	6.77	68	8.82	88	6.75	67	31.24	78
10.0**	6.04	60	7.0	70	6.8	68	6.6	66	26.44	66
18.0	13.07	73	8.15	45	12.51	70	7.29	41	41.02	57
NC	2.06		0.3		0.83		0		3.19	
NC	100.4		13.8		11.4		1.91		127.5	
TLC analysis										
0	0		0		0		0		0	
0.5	0		0		0		0		0	
2.0	1.46	73	1.26	63	1.31	66	1.12	56	5.15	64
6.1	4.78	78	3.66	60	3.23	53	3.26	54	14.93	61
10.0**	5.4	54	7.1	71	7.32	73	6.9	69	26.7	67
18.0	12.6	70	9.54	53	12.8	71	8.46	47	43.4	60
NC	1.88		0		0		0		1.88	
NC	101.5		14.0		11.0		1.4		122.9	

* First sample.

** Second sample. Tgis sample was used for TLC analysis in this table and in Table III.

TABLE III

DETERMINATION OF AFLATOXINS IN PISTACHIO NUTS BY TLC PRIOR TO ALUMINA COLUMN CLEAN-UP STEP

NC = naturally contaminated.

Aflatoxins Added (μg/kg)	B_1		B_2		G_1		G_2		*Total*	
	Found		*Found*		*Found*		*Found*		*Found*	
	μg/kg	*%*	*μg/kg*	*%*	*μg/kg*	*%*	*μg/kg*	*%*	*μg/kg*	*%*
0	0		0		0		0		0	
6.1	4.8	79	3.54	58	3.42	56	2.9	48	14.7	61
10.0*	5.7	57	7.4	74	8.3	83	8.3	83	29.7	74
18.0	12.06	67	9.72	54	10.26	57	12.6	70	44.6	62
NC	1.42		0		0		0		1.42	
NC	97.4		14.4		13.0		1.32		126.1	

* See footnote ** to Table II.

from 57–78% for the HPLC analyses and from 60–67% for the TLC analyses although recoveries of individual aflatoxins showed greater variations. The agreement between the HPLC and TLC analyses was excellent. To determine if there was any loss of aflatoxins during the acid alumina column clean-up step, the same samples were analyzed by TLC after the initial extraction. The results of these determinations are given in Table III. A comparison of these results with those in Table II indicates that there is no loss of aflatoxins in the alumina column clean-up step.

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REFERENCES

1 W. A. Pons, Jr., J. A. Robertson and L. A. Goldblatt, *J. Amer. Oil Chem. Soc.*, 43 (1966) 665.
2 A. C. Beckwith and L. Stoloff, *J. Ass. Offic. Anal. Chem.*, 49 (1968) 740.
3 D. R. Beljaars and F. H. M. Fabry, *J. Ass. Offic. Anal. Chem.*, 55 (1972) 775.
4 O. L. Shotwell and R. D. Stufflefield, *J. Ass. Offic. Anal. Chem.*, 55 (1972) 781.
5 W. A. Pons and L. A. Goldblatt, *Aflatoxin*, Academic Press, New York, 1969, p. 77.
6 A. E. Waltking, *J. Ass. Offic. Anal. Chem.*, 53 (1970) 104.
7 M. E. Stack, *J. Ass. Offic. Anal. Chem.*, 57 (1974) 871.
8 G. H. R. Rao and M. W. Anders, *J. Chromatogr.*, 84 (1973) 402.
9 R. C. Garner, *J. Chromatogr.*, 103 (1975) 186.
10 W. A. Pons, Jr., *J. Ass. Offic. Anal. Chem.*, 60 (1977) 89.
11 D. M. Takahashi, *J. Chromatogr.*, 131 (1977) 147.
12 C. W. Thorpe and L. Stoloff, *J. Ass. Offic. Anal. Chem.*, 58 (*1975*) 15.
13 G. M. Ware, *J. Ass. Offic. Anal. Chem.*, 58 (1975) 754.
14 B. Haghighi, B. Iravani, M. Suzanger and F. Farin, in preparation.
15 *Official Methods of Analysis*, 12th ed., Ass. Offic. Anal. Chem., Washington, DC, 1975, 26.014–26.023.
16 K. K. Maggon, S. Gopal, L. Viswanathan, T. A. Venkitasubramanian and S. S. Rathi, *Indian J. Biochem. Biophys.*, 9 (1972) 195.

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DETERMINATION OF OPIATES IN BIOLOGICAL SAMPLES BY GLASS CAPILLARY GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

A gas chromatographic method for the simultaneous determination of morphine, 6-acetylmorphine and codeine in human plasma or blood has been developed. The samples are buffered to pH 9 and extracted on silica columns, cleaned by extraction and finally acylated with pentafluoropropionic anhydride. The derivatives formed are separated on a glass capillary column with falling glass needle injection and electron-capture detection. The choice of the extraction conditions and the preparation of suitable capillary columns are discussed.

INTRODUCTION

There is a great need for sensitive and specific assays of drugs in tissues. A large number of analyses are often necessary, so that long-term reproducibility and time of analysis are important. It is also of interest to be able to detect metabolites simultaneously. Codeine is metabolized to morphine, which is of interest in pharmacokinetic investigations. The determination of 6-acetylmorphine and morphine in *post mortem* blood specimens after acute heroin poisoning is another example.

Liquid chromatographic assays for morphine often make use of the reactivity of the phenol group. Oxidation is used in amperometric detection[1] and formation of dimeric products by pre-column derivatization and fluorescence detection[2].

Derivatization of morphine and related compounds is necessary in order to reduce the polarity and to increase the sensitivity (for electron-capture detection) prior to gas chromatography (GC). Acylation with fluorinated anhydrides and N-heptafluorobutyrylimidazole has been used in combination with electron-capture detection[3–7] or mass fragmentographic detection[8]. Silylation has been used prior to flame-ionization detection[9] and mass fragmentography[10]. Only mass fragmentography and electron-capture detection give detection limits low enough for pharmacokinetic investigations where only a few nanograms per millilitre are to be detected. We have been using a GC method with electron-capture detection for several years[6]. However,

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this method is laborious and we report here the results of our efforts to reduce the time of analysis and to improve the reproducibility at low concentrations with the aid of capillary chromatography.

EXPERIMENTAL

Morphine, codeine and nalorphine were obtained from the WHO Centre for Chemical Reference Substances (Solna, Sweden). Diacetylmorphine was prepared by heating 0.4 g of morphine hydrochloride with 0.2 g of sodium acetate and 2 ml of acetic anhydride for 20 min at 100°C, followed by addition of 30 ml of 1 *M* sodium carbonate and extraction three times with 10 ml of dichloromethane–cyclohexane (1:5). The solvent was distilled off in a rotary evaporator and the product was recrystallized from ethanol–water. 6-Acetylmorphine was synthesized according to Wright[11] and its purity was checked by thin-layer, liquid and gas–liquid chromatography. The product contained 0.5% of morphine but no other impurities were detected by these methods. Pentafluoropropionic anhydride (PFPA) was purchased from Massanalys (Stockholm, Sweden).

Dichloromethane, 1-butanol and ethyl acetate were of LiChrosolv quality (Merck, Darmstadt, G.F.R.). Extractions of samples were performed on columns packed with Extrelut silica (Merck). The silica was packed in a large column and purified with methanol and ethanol until the eluent was free from impurities, followed by drying in an oven at 100°C.

A 1.2-g amount of the pure silica was packed in a glass ion-exchange column (190 × 6 mm I.D.) with silanized glass-wool as outlet filter. Columns and test-tubes were silanized with 2% Drifilm (Pierce, Rockford, IL, U.S.A.) in cyclohexane, followed by three washes with methanol and drying at ambient temperature.

Preparation of buffers

Buffer A. Ammonia solution (1 *M*) was titrated with 2 *M* hydrochloric acid using a pH meter with a glass electrode until pH 9.0 was obtained.

Buffer B. Ammonia solution (5 *M*) was added to buffer A until it gave pH 9.0 when mixed with an equal volume of 0.05 *M* sulphuric acid. This calibration should be checked whenever a new dilution of sulphuric acid is prepared.

Preparation of capillary columns

Borosilicate columns (25 m × 0.36 mm I.D.) were filled with 20% hydrochloric acid and sealed under vacuum. The columns were heated at 160°C for 16 h, followed by washing with ten column volumes of distilled water and drying at 120°C with a flow of nitrogen.

Deposition of sodium chloride according to de Nijs *et al.*[12] was used for the preparation of columns with polar stationary phases. Deactivation of the glass surface was performed with N-cyclohexyl-3-azetidenol (CHAZ) according to Sandra and Verzele[13] or gas-phase deactivation with Carbowax 20M[14,15]. Polar stationary phases were applied by dynamic coating using the mercury plug method. Non-polar stationary phases were applied with static coating by distillation of the solvent under vacuum. The columns were conditioned by temperature programming, followed by further conditioning at 220°C overnight.

Instruments

A Hewlett-Packard gas chromatograph equipped with a ^{63}Ni electron-capture detector was used. The injection port was enlarged to take a Packard glass solid injector with a falling needle. Argon containing 5% of methane as make-up gas for the detector was connected with a tee-piece and glass-lined tubing.

The column (25 m × 0.36 mm I.D.) was packed with OV-1. The injection temperature was 250°C, the column temperature 220°C, and the detector temperature 300°C. The flow-rate of the carrier gas (helium) was 35 cm/sec and that of the make-up gas was 40 ml/min.

Extraction procedure

Plasma (1 ml) and 0.1 ml of internal standard solution containing nalorphine and 1 ml of buffer A (pH 9.0) are mixed and poured on to an extraction column. After 10 min, elution is carried out with a 5% solution of 1-butanol in dichloromethane until 8 ml have been collected.

The organic phase is extracted with 0.5 ml of 0.05 *M* sulphuric acid and the aqueous phase is separated by centrifugation and transferred into a new tube with a pasteur pipette. The aqueous phase is made alkaline with 0.5 ml of buffer B and extracted with the same solvent as above. The aqueous phase is aspirated off and the organic phase is poured into a 3-ml tube. The organic phase is evaporated with a stream of nitrogen at 50°C in a heating bath and 100 μl of PFPA are added. The tubes are sealed with glass stoppers and heated in a heating block at 65°C for 30 min, then the PFPA is evaporated with a stream of nitrogen at ambient temperature. A 0.25-ml volume of ethyl acetate is added and the contents of the tube are thoroughly mixed on a Whirlmixer. Then 1–3 μl of this mixture is injected to the gas chromatograph. The concentrations of morphine, codeine and 6-acetylmorphine are evaluated by using a calibration graph established by use of six standard samples.

RESULTS AND DISCUSSION

Extraction

Morphine is an ampholytic compound and the optimal pH for extraction has been determined to be 8.96 (ref. 16). We earlier used carbonate buffers for adjustment of pH prior to extraction, but have now changed to ammonium chloride buffers owing to their better pH stability on storage. The extraction method described above is not suitable for the quantitative determination of 6-acetylmorphine in samples containing diacetylmorphine owing to partial hydrolysis of the latter during extraction. For analysis of samples containing diacetylmorphine, addition of enzyme inhibitors and extraction at lower pH must be used[17]. 6-Acetylmorphine is more stable and is not hydrolysed to morphine in the extraction method described above. This was checked by extraction of spiked plasma samples containing 6-acetylmorphine alone. Less than 1% of 6-acetylmorphine was hydrolysed to morphine during extraction.

The hydrolysis of diacetylmorphine in blood or plasma is very rapid. Nakamura and Thornton[18] determined the half-life of diacetylmorphine in blood to be 9 min and concluded that *post mortem* blood specimens should be analysed for the presence of 6-acetylmorphine. Halogenated hydrocarbons mixed with alcohols have been

widely used or the extraction of morphine from biological samples[2,3,8,10,19]. High extraction recoveries are obtained with these polar solvents, but polar solvents also extract more of the sample matrix. Less polar solvents such as toluene–butanol[5,6] and benzene–butanol[20] have been used to achieve clean extracts. If the more polar solvents are used the pH adjustments becomes less critical, and adsorption to glass surfaces will be reduced.

Emulsions are formed when chlorinated hydrocarbons are shaken with buffered plasma samples, which makes phase separation difficult. Column extraction has proved to be useful for solving this problem. Different materials have been used to absorb the aqueous phase, such as cellulose powder[21] silica[22] and gauze sponges[23]. A good absorption matrix to hold the aqueous phase should be inert with respect to adsorption of the compounds of interest and it should not contain extractable impurities. Extrelut silica was found to be suitable after purification with methanol as described above. Extracts with a low background (Fig. 1) and a high recovery were obtained (Table I).

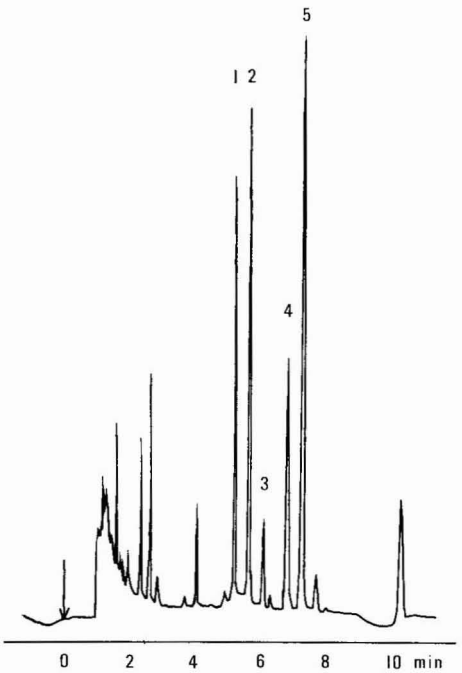

Fig. 1. Chromatogram obtained from a 1-ml serum standard containing 62 ng of morphine (1), 546 ng of codeine (2), 76 ng of normorphine (3), 189 ng of 6-acetylmorphine (4) and 100 ng of nalorphine (5). Injection: 1 μl from 250 μl by falling needle on to a 25 m $\times$ 0.36 mm I.D. OV-1 column (column 1, Table II). Carrier gas, helium (35 cm/sec; make-up gas, argon–5% methane (40 ml/min). Injection temperature, 250°C; column temperature, 220°C; detector temperature, 300°C. Hewlett-Packard electron-capture detector (^{63}Ni source).

Injection and chromatography

A solid injector with a falling glass needle was used due to its inertness and the possibility of injecting large fractions of the total sample. It is possible to evaporate

TABLE I

RECOVERY AND PRECISION

Compound	*Elution time (sec)*	*Amount added (ng)*	*Recovery ± S.D. (%)*	*Coefficient of variation* (%)*	*No. of determinations*	*Detection limit (ng/ml)*
Morphine	314	62	98 ± 6	4	7	1
		0.8		10	8	
Codeine	341	546	98 ± 4	3	7	5
		4		7	8	
Normorphine	368	76	24 ± 2	7	7	
6-Acetylmorphine	389	409	98 ± 2	2	7	2

* Relative peak height *versus* nalorphine.

the PFPA directly on the falling needle, but this is not recommended for routine work. The density of PFPA is high, which makes it difficult to inject without releasing a drop down to the column.

Our first attempts to use capillary columns for morphine and codeine analysis gave contradictory results. No peaks appeared after injection of nanogram amounts of the derivatives on some columns, while others appeared to be inert. Surface pretreatment and deactivation are usually not carried out with commercially available columns. Therefore, we decided to test some different methods described in the literature to find one suitable for opiate analysis. After some preliminary tests it became clear that some kind of standard chromatographic test for opiates was needed in order to be able to compare different columns and to control a column in use. A methanolic solution of morphine, codeine and pentazocine was evaporated and treated with PFPA. The PFPA was evaporated and the test sample was dissolved in ethyl acetate containing aldrin. The test sample was chromatographed and the peak area per mole relative to aldrin was calculated. The results are presented in Table II.

TABLE II

RESULTS FOR DETERMINATION OF OPIATES USING DIFFERENT COLUMNS

Columns 3 and 6 were supplied by Hewlett-Packard; the others were prepared as described under Experimental. Carbowax 20M deactivation, columns 1, 2 and 3; CHAZ deactivation columns 4 and 5.

Column	*No.*	*Column temperature (°C)*	*Relative peak area/mole versus aldrin*		
			Pentazocine	*Morphine*	*Codeine*
OV-1	1	220	0.53	1.34	0.25
OV-1	2	220	0.41	1.13	0.15
Methylsilicone fused silica	3	220	0.38	0.63	0.24
OV-17	4	220	0.31	0.74	0.09
OV-225	5	180	0.59	1.82	0.27
		200	0.47	1.56	0.19
		220	0.52	1.62	0.103
		240	0.48	0.58	0.013
Carbowax 20M fused silica	6	220	0.19	0.13	0.074

Table II indicates that degradation of morphine and codeine occurs on all columns to some extent, and the sensitivity to degradation decreases in the order codeine $\approx$ morphine $\gg$ pentazocine.

It is difficult to estimate how much of the derivatives have survived chromatography from chromatographic data alone, as no absolutely inert column is available, and the degradation might proceed without catalysis. It seems probable that the degradation follows a first-order reaction with catalysis (pyrolysis of esters on glass beads has been used for the synthesis of alkenes; Tschugaev elimination). De Nijs *et al.*[15] measured the first-order rate constant for the degradation of endrin in order to compare the activity of different columns. It should be possible to make similar measurements for opiate derivatives, but it is very time consuming. To be able to obtain a figure for the peak area ratio with small or negligible degradation and for kinetic investigations, a 10 m $\times$ 0.5 mm I.D. capillary coated with OV-225 was prepared. Test mixtures of aldrin, morphine and codeine derivatives were chromatographed at almost constant elution time at different temperatures using extreme flow-rates (Fig. 2). One experiment with variation of the flow-rate at constant temperature was also carried out (Fig. 3).

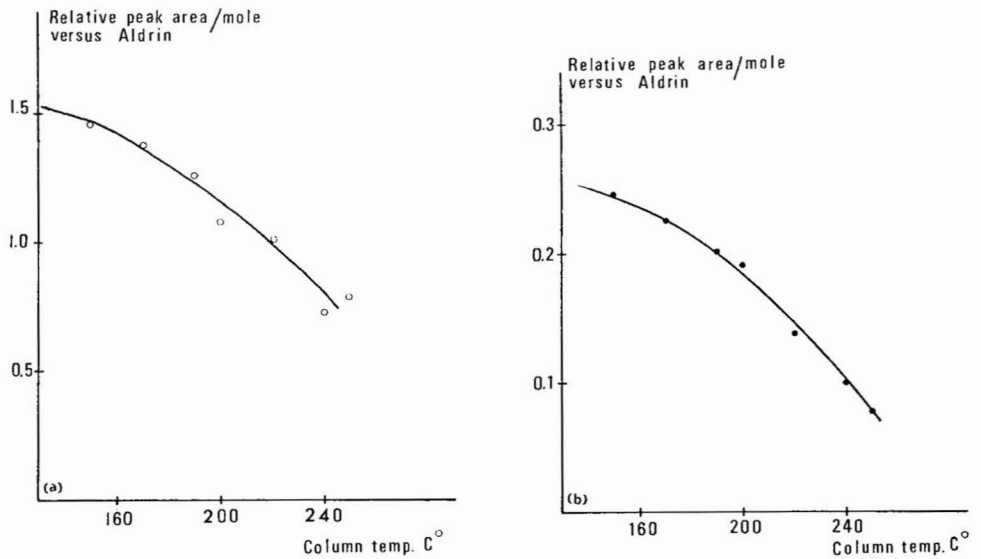

Fig. 2. Variation with column temperature of relative peak area for PFPA-acylated morphine (a) and codeine (b). The elution time was kept constant by variation of the flow-rates. Column: 10 m $\times$ 0.5 mm I.D. with sodium chloride deposition and CHAZ deactivation followed by coating with OV-225.

From Figs. 2 and 3 it is clear that degradation will always occur on columns used for analysis as excessive flow-rates cannot be used and a column temperature of at least 200°C is needed in order to achieve a reasonable time of analysis. However, the degradation is very reproducible so that analyses can be carried out in spite of degradation, but regular column control and calibration are advisable. If there is interest in analysing codeine alone, 3-O-ethylmorphine might be used rather than nalorphine as a more suitable internal standard.

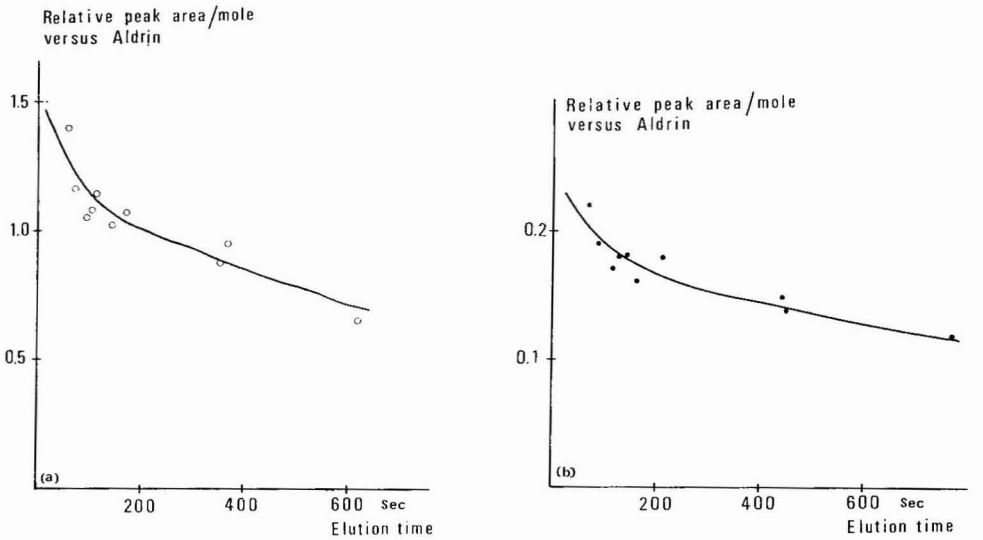

Fig. 3. Variation of relative peak area peak area for PFPA-acylated morphine (a) and codeine (b) at different elution times by variation of flow-rate. Column as in Fig. 2, operated at 200°C.

Both the CHAZ and Carbowax 20M deactivation methods gave useful columns. The advantage of using gas-phase deactivation with Carbowax 20M is that it can be used to deactivate old columns. Good results were most easily obtained with Carbowax 20M deactivation and coating with OV-1. One of these OV-1 columns has been used for the analysis of at least 500 biological samples so far without any sign of deterioration.

The method described is suitable for the simultaneous determination of morphine, codeine and 6-acetylmorphine in biological samples. Normorphine can also be determined, but the extraction recovery is only 24%. The method has been used for the investigation of the specificity of radioimmunoassay of morphine in plasma, the determination of morphine and codeine after codeine administration and the determination of 6-acetylmorphine and morphine in different tissues after acute heroin poisoning to determine the cause of death.

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REFERENCES

1 M. W. Wite, *J. Chromatogr.*, 178 (1979) 229–240.
2 I. Jane and J. F. Taylor, *J. Chromatogr.*, 109 (1975) 37–42.
3 J. E. Wallace and H. E. Hamilton, *Anal. Chem.*, 46 (1974) 2107–2110.
4 R. A. Sams and L. Malspeis, *J. Chromatogr.*, 125 (1976) 409–420.
5 B. Dahlström and L. Paalzow, *J. Pharm. Pharmacol.*, 27 (1975) 172–176.
6 B. Dahlström, L. Paalzow and P. O. Edlund, *Acta Pharmacol. Toxicol.*, 41 (1977) 273–279.
7 A. S. Christophersen and K. E. Rasmussen, *J. Chromatogr.*, 168 (1979) 216–220.

8 W. O. R. Ebbighausen, J. H. Mowat, H. Stearns and P. Vestergaard, *Biomed. Mass Spectrom.*, 1 (1974) 305–311.
9 F. Medzihradsky and P. J. Dahlstrom, *Pharmacol. Res. Commun.*, 7 (1975) 55–69.
10 P. A. Clarke and R. L. Foltz, *Clin. Chem.*, 20 (1974) 465–469.
11 C. I. Wright, *J. Pharmacol. Exp. Ther.*, 71 (1941) 164.
12 R. C. M. de Nijs, G. F. M. Rutten, J. F. Franken, R. P. M. Dooper and J. A. Rijks, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 2 (1979) 447–455.
13 P. Sandra and M. Verzele, *Chromatographia*, 10 (1977) 419–425.
14 J. J. Franken, R. C. M. de Nijs and F. L. Schulting, *J. Chromatogr.*, 144 (1977) 253–256.
15 R. C. M. de Nijs, J. J. Franken, R. P. M. Dooper, J. A. Rijks, H. J. J. M. de Ruwe and F. L. Schulting, *J. Chromatogr.*, 167 (1978) 231–241.
16 G. Shill and K. Gustavii, *Acta Pharm. Suecica*, 1 (1964) 24–35.
17 E. R. Garret and T. Gürkan, *J. Pharm. Sci.*, 68 (1979) 26–32.
18 G. R. Nakamura, J. I. Thornton and T. T. Noguchi, *J. Chromatogr.*, 110 (1975) 81–89.
19 W. J. Serfontein, D. Botha and L. De Villiers, *J. Pharm. Pharmacol.*, 27 (1975) 937–939.
20 E. R. Garret and T. Gürkan, *J. Pharm. Sci.*, 67 (1978) 1512–1517.
21 A. Arbin and P. O. Edlund, *Acta Pharm. Suecica*, 12 (1975) 119–126.
22 P. O. Edlund, *J. Chromatogr.*, 187 (1980) 161–169.
23 G. L. Spraque and A. E. Takemori, *J. Pharm. Sci.*, 68 (1979) 660–662.

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DETERMINATION OF Δ^9-TETRAHYDROCANNABINOL IN PHARMACEUTICAL VEHICLES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A procedure for the determination of Δ^9-tetrahydrocannabinol (Δ^9-THC) in the presence of its degradation products in pharmaceutical vehicles by high-performance liquid chromatography (HPLC) is described. The method compares favorably with a standard gas–liquid chromatographic procedure used for the analysis of Δ^9-THC in sesame oil USP. The HPLC method is suitable for quantitating Δ^9-THC in the presence of several pharmaceutical vehicles and excipients including: sesame oil USP, polyvinylpyrrolidone, Emulphor EL620 and Cremophor EL. Extractions are not required and samples require little preparation. Only the addition of an internal standard in an appropriate solvent is necessary before injection. The procedure has been applied to stability studies of Δ^9-THC in various pharmaceutical vehicles.

INTRODUCTION

The preparation of dosage forms of Δ^9-tetrahydrocannabinol (Δ^9-THC) suitable for human use presents interesting and challenging problems. The water solubility of Δ^9-THC has been determined by Garrett and Hunt[1] to be 2.8 μg/ml. This poor solubility coupled with its viscous tar-like nature make routine handling and solubilization difficult. Additionally, Δ^9-THC decomposes more readily in the presence of light or oxygen[2,3]. Much work has been directed toward the formulation of products of Δ^9-THC that are useful in evaluating the pharmacological properties of the drug. However, more attention needs to be directed to the optimization of formulations suitable for routine therapeutic use in humans. Specifically of interest are products suitable for use in antiemetic therapy in combination with antitumor agents. The usefulness of Δ^9-THC as an antiemetic was first reported by Sallan *et al.*[4]

in 1975. Since then the activity of Δ^9-THC has been confirmed in several clinical trials *versus* chemotherapy induced emesis[5–8]. These trials were conducted with Δ^9-THC products distributed by the National Institute on Drug Abuse, specifically a standardized marihuana cigarette and a soft gelatin capsule containing Δ^9-THC in sesame oil. Some problems were encountered with the use of these dosage forms. There is reluctance in some older patients towards the use of marihuana cigarettes due to the associated social and legal implications. Also, the effectiveness of the cigarette as a dosage form depends heavily on the smoking process itself. Patients must be carefully trained in proper smoking techniques for administration to be successful. At best, only a fraction of the actual amount of Δ^9-THC available is introduced to the lungs for absorption and the amounts of drug absorbed may be less predictable than desired[9]. Also, the use of the cigarette as a dosage form inherently involves the administration of various other marihuana components and combustion products which may present problems of their own. Likewise, the absorption of Δ^9-THC from the gastrointestinal tract has been unpredictable[5–8]. Chang *et al.*[5] reported blood levels of Δ^9-THC which suggested variable absorption of the drug after oral administration. Likewise, Frytak *et al.*[6] observed similar variations in plasma levels of Δ^9-THC. These problems might be avoided by the use of alternate dosage forms.

Due to some of these drug delivery problems, formulation studies were begun to develop new dosage forms of Δ^9-THC suitable for intravenous, intramuscular or oral administration to humans. Numerous methods for the quantitation of Δ^9-THC in a variety of biological media and botanical products have been reported. Many have been summarized in monographs or reviews[10–14]. A gas–liquid chromatographic (GLC) method employing multiple extractions has been used for analysis of delta-9-THC in capsules containing the drug in sesame oil[15]. Our goal was to develop a reversed-phase high-performance liquid chromatographic (HPLC) method that would be generally applicable to the analysis of Δ^9-THC in the presence of its decomposition products in a variety of pharmaceutical vehicles. A HPLC method was desirable to avoid laborious extractive work-up of samples. This non-destructive technique would also permit the collection and subsequent identification of degradation products. This report describes the development of such a method and its utility for the analysis of Δ^9-THC in several potential pharmaceutical vehicles.

EXPERIMENTAL

HPLC apparatus and conditions

Method A. A modular high-performance liquid chromatograph (Model 3500B, Spectra-Physics, Santa Clara, CA, U.S.A.), including a reciprocating piston pump with flow feedback control, delivered mobile phase at a constant rate (1 ml/min) to a stainless-steel column (250 × 4.6 mm I.D.) packed with 10-μm particles of silica gel chemically bonded to a C_8 hydrocarbon phase (Altex LiChrosorb RP-8, 10 μm, Altex Scientific, Berkeley, CA, U.S.A.). A fixed-wavelength (280 nm) ultraviolet detector (Model 8200, Spectra-Physics) with a sensitivity setting of 0.08 a.u.f.s. detected the eluted compounds. The detector output signal was recorded with a strip chart recorder equipped with variable chart speed controls (Model A5211-1, Omniscribe, Houston Instruments, Austin, TX, U.S.A.). Samples were introduced to the column

with a manual injection valve equipped with a 10-μl sample loop (Model CV-6-UHP-a-N60, Valco Instrument Company, Houston, TX, U.S.A.).

The mobile phase consisted of methanol–distilled water (78:22). The column pressure was about 630 p.s.i. at a flow-rate of 1 ml/min. All separations were affected isocratically at ambient temperature.

Method B. This procedure is identical to Method A with the following exceptions: mobile phase consisted of methanol–distilled water (73:27). The column packing material consisted of a dimethylsilane phase chemically bonded to silica gel (Altex LiChrosorb RP-2, 10 μm).

Quantitation was performed using an internal standard method. Standard curves constructed from the ratio of peak heights of Δ^9-THC to the internal standard tetraphenylethylene (I.S.) *versus* concentration were linear ($r > 0.99$).

Reagents

Δ^9-THC was supplied by the National Institute on Drug Abuse (Rockville, MD, U.S.A.). Tetraphenylethylene (Aldrich, Milwaukee, WI, U.S.A.) was used as received. Methanol, HPLC grade (Fisher Scientific, Fair Lawn, NJ, U.S.A.) and distilled water were filtered through 0.5-μm and 0.8-μm solvent-resistant filters, respectively (Millipore, Bedford, MA, U.S.A.). Reference samples of Δ^8-THC, cannabinol and cannabidiol were used as received (Supelco, Bellefonte, PA, U.S.A.). Solutions of ethanol–Emulphor EL620 (1:1) and of ethanol–Cremophor EL (1:1) were supplied by the Division of Cancer Treatment, National Cancer Institute (National Institutes of Health, Bethesda, MD, U.S.A.). All other chemicals were reagent grade and were used as received.

Most Δ^9-THC-containing samples were diluted or reconstituted with absolute ethanol to give a drug concentration of 5 or 10 mg/ml. An aliquot of this solution (100 μl) was diluted with 600 μl of a solution of tetraphenylethylene in ethanol (150 μg/ml). This solution (10 μl) was injected directly for HPLC analysis. Δ^9-THC in seame oil USP was quantitated by weighing individual samples (25–40 mg) of the drug in sesame oil (3% Δ^9-THC, w/w) and then dissolving the sample in 600 μl of *n*-butanol containing tetraphenylethylene (128.5 μg/ml). The resulting solution was injected directly for HPLC analysis. Samples for GLC analysis were prepared in ethanol to contain about 0.3 mg/ml of Δ^9-THC and 0.45 mg/ml androst-4-ene-3,17-dione (internal standard) according to a modification of a standard method[13,15].

Preparation of experimental formulations

Several formulations were investigated. The following vehicle and packaging variations were prepared and the contents subjected to HPLC analysis:

(A) An evacuated vial containing 10 mg Δ^9-THC prepared by a low-temperature vacuum drying procedure[16].

(B) A solution containing 10 mg/ml Δ^9-THC in ethanol–Emulphor EL620–sodium chloride injection USP (5:5:90) was prepared as described previously[17]. Aliquots (0.5 ml) of this solution were dispensed and sealed under room air in clear glass ampules.

(C) A solution containing 10 mg/ml Δ^9-THC in ethanol–Cremophor EL–sodium chloride injection USP (5:5:90) was prepared and dispensed in a manner similar to formulation B.

(D) A solution containing 100 mg/ml Δ^9-THC in ethanol–Emulphor EL620 (1:1) sealed under nitrogen in a glass ampule.

(E) A solution containing 100 mg/ml Δ^9-THC in ethanol–Cremophor EL (1:1) sealed under nitrogen in a glass ampule.

(F) Adsorbates with polyvinylpyrrolidone containing 3.5% (w/w) Δ^9-THC.

(G) A solution of 3% (w/w) of Δ^9-THC in sesame oil USP.

RESULTS AND DISCUSSION

Chromatography

Δ^9-THC and several related cannabinoids: Δ^8-THC, cannabinol and cannabidiol could be resolved by either HPLC method (Fig. 1). Preliminary studies of Δ^9-THC decomposition in formulation B under accelerated conditions of heat and light in the presence of air indicated cannabinol, cannabidiol and several relatively polar products were formed. Little or no Δ^8-THC was detected. The elution of the various cannabinoids was more prolonged from the C_8 column as compared to the C_2 column with equivalent mobile phases. Increasing the organic component in either system resulted in faster elution of all components; however, Δ^9-THC and Δ^8-THC could no longer be resolved. Mobile phases containing less organic component required more time for elution of the components with subsequent peak broadening and tailing. Ultraviolet detection at 280 nm was satisfactory as both Δ^9-THC and Δ^8-THC have absorbance maxima in that region (Δ^9-THC, $UV_{max.}$ in ethanol: 282 and 278 nm (ε, 2075 and 2040 $l \cdot mol^{-1} \cdot cm^{-1}$; Δ^8-THC, $UV_{max.}$ in ethanol: 283, 276

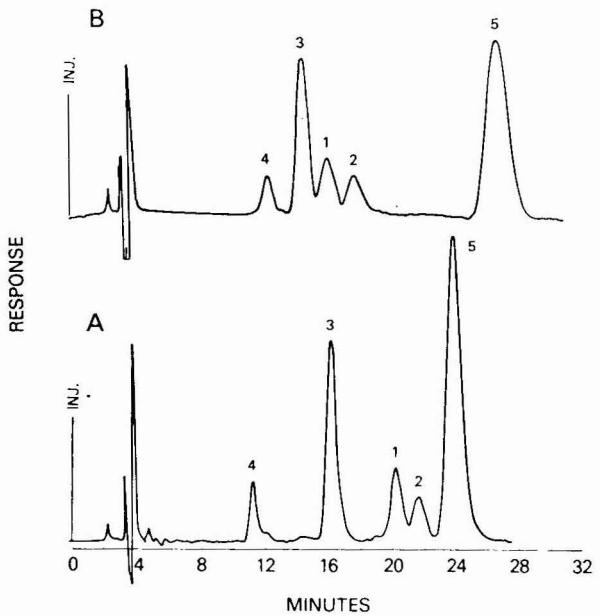

Fig. 1. HPLC chromatograms of a mixture of Δ^9-THC (1), Δ^8-THC (2), cannabinol (3), cannabidiol (4) and the internal standard tetraphenylethylene (5) using Method B and Method A.

and 209 nm (ε, 1390, 1330 and 41,000 $l \cdot mol^{-1} \cdot cm^{-1}$; ref. 18). Cannabinol, the aromatic degradation product of $\triangle^9$–THC, has a much greater molar absorptivity than $\triangle^9$–THC or $\triangle^8$–THC ($UV_{max.}$ in ethanol: 286 nm (ε, 18,300 $l \cdot mol^{-1} \cdot cm^{-1}$; ref. 19) and consequently displays peaks which are disproportionately large for the amount present.

Method A was chosen for stability studies because of the somewhat better resolution of the cannabinol and Δ^9-THC peaks. This was important because cannabinol is produced from the decomposition of Δ^9-THC and possesses a strong chromophore. The resolution of Δ^9-THC and Δ^8-THC was of lesser concern because Δ^8-THC was not produced in significant amounts in preliminary studies and contains a weaker chromophore than that of Δ^9-THC.

The HPLC system (Method A) was compared to a standard GLC method which has been used for the analytical evaluation of the Δ^9-THC sesame oil capsules produced for the National Institute on Drug Abuse[13,15]. Duplicate shelf life samples of formulation A were subjected to HPLC and GLC analysis. Results of these comparative analyses are presented in Table I. These data show reasonable agreement for the two methods for samples stored at 25° or 50°C.

TABLE I

COMPARISON OF GLC AND HPLC METHODS ON ANALYSIS OF LOW-TEMPERATURE VACUUM DRIED Δ^9-THC (FORMULATION A)

Time (weeks)	*Percent of initial assay*			
	50°C		*25°C*	
	HPLC	*GLC*	*HPLC*	*GLC*
4	100.0	101.4	101.1	100.7
8	92.0	93.5	100.0	102.2
12	82.1	80.4	98.0	97.8
20	76.2	74.6	—	—

Δ^9-THC in sesame oil (formulation G) was also determined by HPLC and GLC. The GLC procedure required multiple extraction (six times) and an evaporation step[15]. Six individual samples of formulation G were weighed (250–350 mg for GLC analysis and 25–40 mg for HPLC analysis) for analysis by each method. The results were in agreement. The precision, as measured by percent relative standard deviation, of the HPLC method was somewhat better (2.25% as compared to 3.05% for the GLC method.)

The analysis of other formulations some of which contain various surfactants and oils can also be accomplished directly with this HPLC method. Direct injection offers distinct advantages over the GLC method which would require multiple extractions in most instances. Emulphor EL620 and Cremophor EL do not exhibit UV absorbance at 280 nm and are thus not detected during HPLC analysis. However, detection at 220 nm indicated two large peaks eluted after the injection of samples containing either Emulphor EL620 (retention volume, $V_R \approx$ 27–37 ml) or Cremophor EL ($V_R \approx$ 28–35 ml). Several peaks in sesame oil USP are eluted and detected

at 280 nm with retention volumes ranging from 3.8 to 8.1 ml. Polyvinylpyrrolidone produced no peaks that were detectable at 280 nm. At 220 nm, a large tailing peak was observed between V_R 1.8 and 12.0 ml. Fortunately none of the peaks eluted from the various vehicles interfered with the Δ^9-THC peak or the internal standard peak at 280 nm. Repeated injections of any of the formulations described did not affect resolution, retention volumes or overall column performance. As a precaution, however, the column was routinely flushed with methanol for 1–2 h after each 2–3 days of analysis.

This HPLC method was suitable for stability studies of Δ^9-THC in any of the vehicles described. Typical stability data in several vehicles are seen in Table II. Formulations B and C demonstrate the more rapid decomposition of Δ^9-THC in the presence of air and water. Other vehicles with the product stored an inert nitrogen atmosphere (formulations D and E) are considerably more stable. Day-to-day variation based on the analysis of a standard solution of Δ^9-THC in ethanol over a period of 6 months resulted in a relative standard deviation of 4.40%.

TABLE II

STABILITY OF Δ^9-THC IN VARIOUS VEHICLES AT 25°C

Time	*Percent of initial assay*			
	Formulation B	*Formulation C*	*Formulation D*	*Formulation E*
0	100.0	100.0	100.0	100.0
2 Days	86.0	91.6	—	—
7 Days	82.9	86.5	—	—
10 Days	81.1	84.2	—	—
6 Weeks	—	—	96.2	98.1
14 Weeks	—	—	97.0	96.0
20 Weeks	—	—	96.2	97.5

HPLC has proven to be a useful method for formulation and stability studies with Δ^9-THC. The procedure compares favorably to a standard GLC method used in other formulation related analysis of Δ^9-THC. The method is simple and requires no extraction prior to analysis in the vehicles investigated.

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REFERENCES

1 E. R. Garrett and C. A. Hunt, *J. Pharm. Sci.*, 63 (1974) 1056.
2 J. W. Faubairn, J. A. Liebmann and M. G. Rowan, *J. Pharm. Pharmacol.*, 28 (1976) 1.
3 R. K. Razdan, A. J. Puttick, B. A. Zitko and G. R. Handrick, *Experientia*, 28 (1972) 121.
4 S. E. Sallan, N. E. Zinberg and E. Frei, *N. Engl. J. Med.*, 293 (1975) 795.
5 A. E. Chang, D. J. Shiling, R. C. Stillman, N. H. Goldberg, C. I. Seipp, I. Barofsky, R. M. Simon and S. A. Rosenberg, *Ann. Intern. Med.*, 91 (1979) 819.

6 S. Frytak, C. G. Moertel, J. R. O'Fallon, J. Rubin, E. T. Creagan, M. J. O'Connell, A. J. Schutt and N. W. Schwartau, *Ann. Intern. Med.*, 91 (1979) 825.
7 S. E. Sallan, C. C. Cronin, M. Zelen and N. Zinberg, *N. Engl. J. Med.*, 302 (1980) 135.
8 V. S. Lucas and J. Laszlo, *J. Amer. Med. Ass.*, 243 (1980) 1241.
9 E. B. Truitt, *Pharmacol. Rev.*, 23 (1971) 273.
10 R. E. Willette (Editor), *Cannabinoid Assays in Humans*, National Institute on Drug Abuse Research Monograph Series No. 7, National Institute on Drug Abuse, Rockville, MD, 1976, pp. 1–120.
11 J. A. Vinson (Editor), *Cannabinoid Analysis in Physiological Fluids*, American Chemical Society Symposium Series No. 98, American Chemical Society, Washington, DC, 1979, pp. 1–242.
12 J. M. Parker and B. L. Stembal, *J. Ass. Offic. Anal. Chem.*, 57 (1974) 888.
13 K. H. Davis, N. H. Martin, C. G. Pitt, J. W. Wildes and M. E. Wall, *Lloydia*, 33 (1970) 453.
14 E. E. Knaus, R. T. Contts and C. W. Kazakoff, *J. Chromatogr. Sci.*, 14 (1976) 525.
15 K. H. Davis, Research Triangle Institute, Research Triangle Park, NC, personal communication, 1980.
16 D. W. Flamberg, D. L. Francis, S. L. Morgan and G. F. Wickes, *Bull. Parenteral Drug Assoc.*, 24 (1970) 209.
17 J. C. Cradock, J. P. Davignon, C. L. Litterst and A. M. Guarino, *J. Pharm. Pharmacol.*, 25 (1972) 345.
18 *Merck Index*, 8th ed., Merck and Co., Inc., Rahway, NJ, 1973, p.1206.
19 K. H. Davis and H. Setzman, Research Triangle Institute, Research Triangle Park, NC, personal communication, 1980.

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MÉTHODE DE DOSAGE PAR CHROMATOGRAPHIE LIQUIDE HAUTE PERFORMANCE DE L'ÉTHYLÈNETHIOURÉE DANS LES FORMULATIONS À BASE D'ÉTHYLÈNE-BIS-DITHIOCARBAMATES

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(Reçu le 11 septembre 1980)

SUMMARY

Reversed-phase high-performance liquid chromatography determination of ethylenethiourea in ethylenebisdithiocarbamate formulations

The ethylenethiourea (ETU) contents of ethylenebisdithiocarbamate formulations have been determined by reversed-phase chromatography. The method is simple, rapid (25 min), and allows to control easily the analysis time in order to minimize the ETU formation from the intermediary degradation products in solutions. The analyzed products contain less than 0.2% ETU except for the more than three years old formulations. Samples stored under normal conditions have shown an ETU increase of 30 to 80% after two years.

INTRODUCTION

Les fongicides du groupe des éthylène-bis-dithiocarbamates (EBDC) sont probablement les fongicides les plus utilisés à l'échelle mondiale. Les recherches sur la toxicité des EBDC ont montré que cette toxicité était principalement due à l'éthylènethiourée ou imidazolidine-2-thione (ETU). Ce composé est connu depuis longtemps comme agent cancérigène, tératogène et comme responsable de modifications de la fonction thyroïdienne. Les résidus d'ETU sur les végétaux peuvent provenir de la dégradation ou de la métabolisation des EBDC après les traitements et de l'ETU initialement présent dans les formulations commerciales. Afin de pouvoir évaluer les teneurs en ETU dans les formulations, il est indispensable de disposer d'une méthode de dosage spécifique et précise. De nombreuses méthodes ont été décrites dans la littérature. Elles sont basées sur différentes techniques: couche mince, électrophorèse, polarographie, chromatographie en phase gazeuse etc. Un relevé de ces méthodes a été publié dans le rapport sur l'éthylènethiourée présenté par les experts de la "Commission on Terminal Pesticide Residues" du IUPAC[1].

En 1962, Johnson et Tyler[2] ont prouvé, en chromatographiant sur papier des extraits aqueux des divers dérivés dithiocarbamiques, que les fongicides éthylène-bis-dithiocarbamiques contenaient une impureté, identifiée comme étant de l'ETU. En 1972, Bontoyan *et al.*[3], en appliquant une méthode de chromatographie en phase

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gazeuse (GLC), ont décelé dans les formulations commerciales des teneurs en ETU variant de 0.1 % à plus de 2 %. Et plus tard, Bontoyan et Looker[4] ont prouvé que de l'ETU se formait au cours du stockage des EBDC, en fonction de la teneur en humidité, de la température et de la durée de stockage.

La méthode de chromatographie en phase liquide à haute performance (HPLC), décrite dans le présent article, a été présentée au Symposium CIPAC en 1978 (bibl. 5) et comparée à une méthode de chromatographie en phase gazeuse après dérivatisation de l'ETU en S-benzyl ETU. La méthode GLC donnait des résultats systématiquement supérieurs. Cette différence avait été attribuée aux produits intermédiaires de décomposition qui peuvent former de l'ETU[1,5–8].

La méthode par chromatographie liquide a l'avantage d'opérer à température ambiante, d'être rapide et reproductible si le temps entre l'extraction et l'injection est strictement respecté car, dans ces conditions, la formation d'ETU en cours d'analyse est fortement limitée.

MATÉRIEL ET MÉTHODE

Matériel et réactifs

(1) Filtre Millipore de 0.45 μm de porosité (réf. HAWP 02500) ou équivalent; (2) tube à centrifuger de 100 ml, fond rond et bouchon à visser Sovirel no. 22 ou équivalent; (3) centrifugeuse (2900 *g*); (4) évaporateur rotatif sous vide; (5) bain thermostatique de précision; (6) ETU pur; (7) méthanol p.a.; (8) tétrahydrofuranne Uvasol (Merck, Darmstadt, R.F.A., 8110 ou équivalent); (9) solution de stock: préparer une solution contenant 20 mg d'ETU dans 200 ml d'eau distillée; (10) solutions étalons: diluer la solution de stock de manière à obtenir des solutions étalons contenant 4, 10, 15, 20, 30 et 40 μg d'ETU par ml.

Appareillage et conditions de la chromatographie liquide

(1) Chromatographe liquide Tracor 990 équipé d'un détecteur à longueur d'onde variable Tracor 970, d'une vanne Rheodyne 7120 munie d'une boucle d'injection de 20 μl, d'un enregistreur et d'un intégrateur Infotronics CRS 304; (2) colonne de 200 × 4.6 mm contenant du Nucléosil 50 C_{18} de 5 μm ou colonne de 125 × 4 mm contenant du LiChrosorb RP-18 de 5 μm; (3) éluant: eau distillée contenant 0.05 % de tétrahydrofuranne (agiter magnétiquement et chauffer à environ 40°C pour dégazer); (4) débit: 1.0 ml/min, pression: 1500 p.s.i. pour la colonne de Nucléosil et 1000 p.s.i. pour la colonne de LiChrosorb; (5) température ambiante; (6) longueur d'onde: 233 nm.

Mode opératoire

Dans le tube à centrifuger, peser à 0.1 mg près une prise d'essai (Pg) contenant environ 0.8 g d'EBDC. Ajouter, à la pipette, 50 ml de méthanol. Boucher hermétiquement et agiter pendant exactement 5 min. Centrifuger à 2900 *g* pendant 5 min. Prélever immédiatement 10 ml de surnageant dans un erlenmeyer de 50 ml à rodage normalisé. Évaporer à sec, sous vide au moyen d'un évaporateur rotatif ($t = 35$°C). Reprendre le résidu sec par addition, à la pipette, de 20 ml d'eau. Agiter 2 à 3 min pour dissoudre l'ETU. Filtrer sur filtre Millipore. Suivant la teneur en ETU, la solution filtrée peut être diluée (facteur de dilution = d). Injecter la solution finale exacte-

ment 15 min après le prélèvement dans les conditions décrites ci-dessous. Ne traiter qu'une prise d'essai à la fois. La température d'évaporation et les temps entre la mise en solution, le prélèvement et l'injection doivent être strictement respectés.

Détermination en HPLC

Injecter 20 μl d'une ou deux solutions étalons, injecter ensuite la solution à analyser, laisser le chromatogramme se développer pendant 15 min, puis injecter une ou plusieurs solutions étalons de concentrations voisines. Calculer la quantité d'ETU en μg/ml (Q) dans la solution analysée en rapportant la hauteur du pic à celle du pic de la solution étalon de concentration la plus voisine. Teneur en ETU (% m/m) = $Q \cdot d/P \cdot 100$ où P est poids de la prise d'essai en g et d le facteur de dilution éventuelle après filtration.

RÉSULTATS ET DISCUSSION

L'extraction de l'ETU soit par l'eau, soit par le méthanol donne des extraits contenant parfois un grand nombre de substances pouvant interférer avec l'ETU dans les conditions de la méthode. De plus, l'extraction par l'eau ou par un mélange eau–méthanol donne, même après centrifugation, un surnageant relativement trouble.

Les essais ont montré que la meilleure manière de procéder était d'extraire l'ETU par agitation de la prise d'essai dans du méthanol, de centrifuger, de prélever une partie aliquote de l'extrait méthanolique, d'évaporer le méthanol et de reprendre le résidu par de l'eau. La récupération dans l'eau possède l'avantage d'éliminer un certain nombre de substances extraites par le méthanol mais insolubles dans l'eau et d'éviter ainsi l'accumulation de ces substances sur la colonne. La filtration de l'extrait aqueux sur papier filtre ne permet pas d'obtenir des solutions limpides; par contre, après une filtration sur filtre Millipore, le filtrat est parfaitement clair. Au cours d'essais de reproductibilité, les résultats obtenus indiquaient une augmentation de la teneur en ETU en fonction du temps lors d'injections successives d'une même prise d'essai.

Le Tableau I montre qu'il est nécessaire de contrôler le temps d'analyse, de maintenir la prise d'essai aux environs de 0.8 g d'EBDC et de ne procéder qu'à une seule injection de l'échantillon car la teneur en ETU passe de: 0.096 à 0.123, soit une augmentation d'environ 30%, lorsque l'extrait méthanolique est conservé pendant deux heures avant de poursuivre l'analyse, et de 0.096 à 0.115, soit une augmentation de 20%, lorsque la solution aqueuse, après filtration, est conservée pendant trois heures avant d'être injectée.

TABLEAU I

TENEUR EN ETU EN %, MESURÉE EN FONCTION DU TEMPS DANS L'EXTRAIT MÉTHANOLIQUE ET EN SOLUTION AQUEUSE POUR L'ÉCHANTILLON NO. 1

Prélèvement de l'extrait méthanolique	*Temps entre la mise en solution dans l'eau et l'injection (min)*		
	20	*100*	*180*
Immédiatement	0.096	0.105	0.115
Après 1 h	0.114	0.125	0.135
Après 2 h	0.123	0.134	0.145

Des essais préliminaires sur des colonnes de gel de silice normal ou modifié chimiquement par des groupements –CN ou $-N(CH_3)_2$ n'ont pas permis de séparer parfaitement l'ETU de nombreuses substances se trouvant dans l'extrait méthanolique sans utiliser un gradient d'élution. Cependant, Farrington et Hopkins[8] ont depuis publié une méthode utilisant une colonne de Sphérisorb CN de 5 μm avec comme éluant 35% (v/v) d'éthanol absolu dans l'hexane. L'ETU étant polaire et soluble dans l'eau n'est pas ou peu retenu sur une colonne de "reversed-phase" si on utilise pour l'élution un mélange tel que eau–méthanol. C'est pourquoi, l'élution a été effectuée avec de l'eau contenant 0.05% de tétrahydrofuranne. Dans ces conditions, le temps de rétention de l'ETU est de 4 min 35 sec pour la colonne de Nucléosil C_{18}, mais n'est

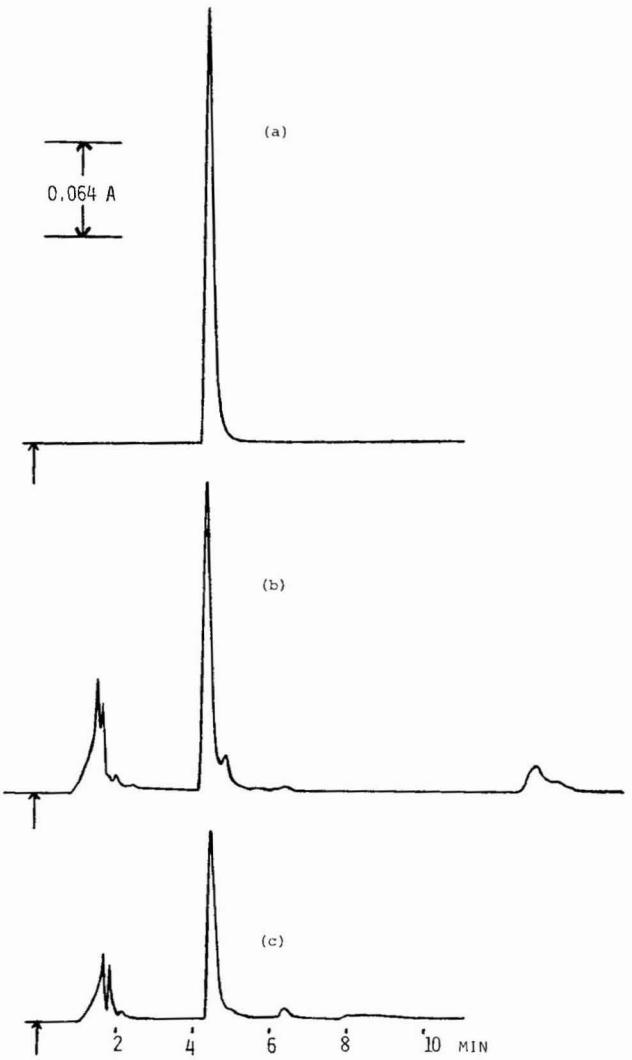

Fig. 1. (a) Chromatogramme d'une solution étalon contenant 20 μg ETU par ml. (b) Chromatogramme de l'échantillon no. 5 (voir Tableau IV). (c) Chromatogramme de l'échantillon no. 9 (voir Tableau IV).

séparé que d'environ 30 sec d'une substance présente en faible quantité dans la plupart des formulations (Fig. 1). Pour cette raison, il est préférable de mesurer la hauteur des pics et non leur surface. Certaines des analyses de 1980 ont été effectuées sur une colonne LiChrosorb RP-18. Le temps de rétention est alors de 2 min 40 sec; le pic et la séparation obtenus sont également satisfaisants.

La reproductibilité obtenue pour neuf injections de solutions étalons de différentes concentrations est illustrée au Tableau II. Le coefficient de variation est de 0.17% et le rapport hauteur/concentration indique une bonne linéarité dans la gamme des concentrations utilisées.

TABLEAU II

REPRODUCTIBILITÉ ET LINÉARITÉ OBTENUES POUR NEUF INJECTIONS DE SOLUTIONS ÉTALONS

Concentration en ETU (µg/ml)	*Hauteur/Concentration**
10	111.6
10	112.0
15	111.6
15	111.7
20	112.2
40	111.8
15	111.9
10	111.9
10	111.8
Moyenne	111.8
Ecart-type	0.19
Coefficient de variation	0.17%

* Unité arbitraire.

Les coefficients de variation de l'ordre de 2%, obtenus pour des prises d'essai croissantes d'une formulation de zirame dopée à 0.1 et 0.5% d'ETU, montrent que l'extraction est complète et la reproductibilité de la méthode est satisfaisante car sept répétitions sur l'échantillon no. 10 ont donné un coefficient de variation de 2.5% (Tableau III).

TABLEAU III

TAUX DE RÉCUPÉRATION ET REPRODUCTIBILITÉ DE LA MÉTHODE

Zirame dopé à 0.1% d'ETU		*Zirame dopé à 0.5% d'ETU*		*Répétitions sur l'échantillon no. 10*	
Prise d'essai (g)	*ETU (%)*	*Prise d'essai (g)*	*ETU (%)*	*Prise d'essai (g)*	*ETU (%)*
0.4982	0.104	0.3024	0.482	1.0358	0.104
0.6952	0.102	0.5031	0.493	1.0043	0.103
0.9849	0.105	0.7491	0.493	1.0043	0.101
1.5489	0.106	1.0010	0.505	1.0073	0.106
2.1174	0.101	1.5203	0.483	1.0330	0.100
2.6402	0.101	2.1738	0.501	1.0155	0.099
				1.0067	0.100
Moyenne	0.103		0.493		0.102
Coefficient de variation (%)	2.1		1.9		2.5

Les teneurs en ETU des formulations commerciales analysées en 1978 et en 1980 sont présentées au Tableau IV. Ces teneurs sont de l'ordre de 0.1% pour les échantillons de manèbe de fabrication récente (1977–1978); par contre, pour des produits plus anciens tels que l'échantillon no. 7 (1975) et l'échantillon no. 8 (1971), elles sont respectivement de 0.32 et de 0.63% et augmentent de 40 à 60% au cours de deux années supplémentaires de stockage dans des conditions normales. Les deux échantillons de mancozèbe ont des teneurs inférieures à 0.1% et, après deux années de stockage, ces teneurs sont de l'ordre de 0.1%. Les échantillons contenant du mancozèbe et du manèbe en mélange avec d'autres fongicides contiennent très peu d'ETU bien que ces échantillons aient été conservés pendant dix-huit mois au laboratoire avant d'être analysés.

TABLEAU IV

TENEURS EN ETU DANS LES FORMULATIONS*

No. Échantillon	*Type de produit*	*Année de fabrication*	*ETU (%)*	
			Résultats de 1978	*Résultats de 1980*
1	Zirame dopé à 3% ETU	—	2.95	—
2	Manèbe 85%	—	0.07	0.09
3	Manèbe 80%	1978	0.10	0.17
4	Manèbe 80%	1977	0.10	—
5	Manèbe 80%	1977	0.11	—
6	Manèbe 80%	—	0.12	—
7	Manèbe 80%	1975	0.32	0.43
8	Manèbe 80%	1971	0.63	0.89
9	Mancozèbe 83%	—	0.06	0.11
10	Mancozèbe 83%	1978	0.07	0.10
11	Mancozèbe 67% + carbendazime 8%	—	—	0.04
12	Mancozèbe 35.5% + carbendazime 3.3% + soufre 40%	—	—	0.01
13	Manèbe 60% + thiophanateméthyl 14%	—	—	0.03
14	Manèbe 25% + thiophanate-méthyl 7% + soufre 50%	—	—	0.02

* Moyenne d'au moins 3 déterminations.

CONCLUSION

La méthode décrite permet de doser avec une reproductibilité satisfaisante l'ETU dans les formulations d'EBDC; la durée d'analyse est courte et les conditions opératoires doivent être strictement contrôlées afin de limiter au maximum la formation d'ETU pendant les manipulations.

REMERCIEMENTS

Les auteurs ont grandement apprécié l'aide technique de Monsieur R. Luxen et de Madame J. Huybreck-Potvin. Ils remercient les firmes phytopharmaceutiques qui ont eu l'obligeance de leur fournir les formulations commerciales.

RÉSUMÉ

Les teneurs en éthylènethiourée (ETU) de formulations à base d'éthylène-bis-dithiocarbamates ont été déterminées par chromatographie liquide au moyen d'une colonne de Nucléosil C_{18} de 5 μm ou de LiChrosorb RP-18 de 5 μm. La méthode est simple et rapide (25 min) et permet de contrôler aisément le temps d'analyse afin de limiter au maximum la formation d'ETU au cours des manipulations. Les teneurs trouvées sont inférieures à 0.2% sauf pour les formulations de plus de trois ans. Dans des conditions normales, les teneurs en ETU ont augmenté, en deux ans, de 30 à 80% dans les formulations stockées.

BIBLIOGRAPHIE

1 *Pure and Appl. Chem.*, 49 (1977) 675–689.
2 E. I. Johnson et J. F. C. Tyler, *Chem. Ind. (London)*, (1962) 305–306.
3 W. R. Bontoyan, J. B. Looker, T. E. Kaiser, P. Giang et B. M. Olive, *J. Ass. Offic. Anal. Chem.*, 55 (1972) 923–925.
4 W. R. Bontoyan et J. B. Looker, *J. Agr. Food Chem.*, 21 (1973) 338–341.
5 J.-C. van Damme et J. Verdier, *Symposium CIPAC, Versailles, 1978*, résultats non-publiés.
6 W. R. Benson, R. D. Ross, J.-Y. Chen, R. P. Barron et D. Mastbrook, *J. Ass. Offic. Anal. Chem.*, 55 (1972) 44–46.
7 W. D. Marshall, *J. Agr. Food Chem.*, 25 (1977) 357.
8 D. S. Farrington et R. G. Hopkins, *Analyst (London)*, 104 (1979) 111–116.

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Note

Sample applicator for granular gel slabs: effect of sample orientation on the sensitivity of detection in isoelectric focusing

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Numerous methods have been described for sample application on gel slabs of the continuous matrix type (*e.g.* polyacrylamide)[1]. These methods are generally not suitable on slabs of the granular type (*e.g.* Sephadex), because the gels are comparatively fragile. In analytical focusing on granular layers, the sample is usually applied to the slab on the edge of a cover-glass. This is done either freehand or with the hand steadied on a platform over the gel plate[2]; either way, the procedure is tedious because the gel is easily smeared. The requirement for a steady hand becomes increasingly important when contact with the slab must be prolonged while the sample soaks in.

The applicator described in Figs. 1–3 reduces much of the tedium of this procedure and provides a steady sample application. The applicator will accomodate standard 20 × 20 cm, and 10 × 20 cm plates. In practice, the gel plate is placed under the applicator, and then the latter is shifted along the *y*-axis of the plate to the desired position. The sample rod is detached from the magnetic surface of the slide bar, and the cover-glass is loaded with sample. The sample rod is returned to the slide bar and the latter shifted along the bridge to the desired position on the *x*-axis of the gel plate.

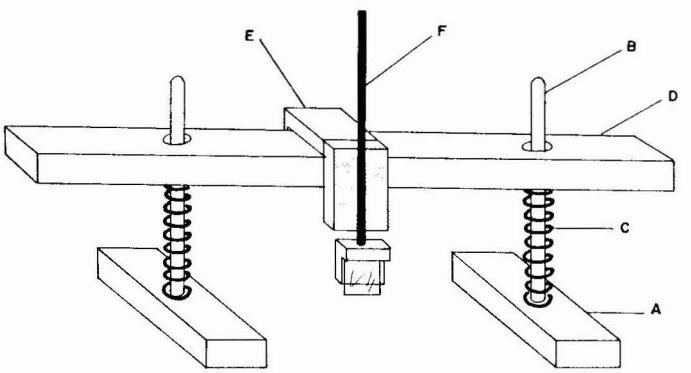

Fig. 1. Diagram of sample applicator. A, Base: lucite, or wood, 11.5 × 4.5 × 1.9 cm. A hole, ¼ in. in diameter and 1.5 cm deep, is drilled in the center of each base to receive the spindles (B): ¼ in. aluminum rods, 14 cm long. A little Pliobond cement (GC Electronics, Rockford, IL, U.S.A.) is introduced into each hole, and the spindles are tapped into position. C, Compression springs: medium strength, 2 3/4 in. long, 5/16 in. I.D., 3/8 in. O.D. D, Bridge: lucite, 36.5 × 2.0 × 2.0 cm. A hole, 5/16 in. in diameter, is drilled 5 cm from each end. E, Slide-bar (see Fig. 2). F, Sample rod (see Fig. 3).

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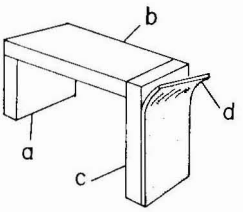
The sample rod is lowered to about 1 cm above the gel plate and then adjusted from side to side until the cover-glass is coplanar with the gel surface. As the bridge is depressed, the cover-glass is observed at eye level to insure that coplanarity is maintained. There is sufficient clearance between the moving parts of the applicator to permit small adjustments in each plane as the bridge is depressed.



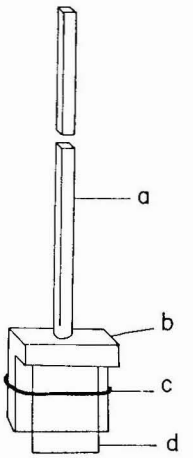


Fig. 2. Diagram of slide-bar. The following components are cut from $\frac{1}{4}$ in. lucite sheet, and glued together with lucite cement. a, 2.0 × 2.0 cm; b, 3.0 × 2.0 cm; c, 5.8 × 2.0 cm. After the components are assembled, a piece of adhesive magnetic strip d (Walbead, New York, NY, U.S.A.) is cut to size and fixed to component c, as shown in the figure.

Fig. 3. Diagram of sample rod. a, Adjusting rod: 1/8-in. steel rod, square cross-section, 14 cm long. b, Cover-glass holder: lucite, $\frac{1}{4}$-in. sheet, 2.2 × 1.9 cm. The lower 1.0 cm of one face is recessed to a depth of 2 mm, as shown in the figure. A hole, 1.8 in. in diameter and 6 mm deep, is drilled in the top. Six mm of one end of the ajusting rod is filed round and inserted into the hole with a little Pliobond cement; one face of (b) should be parallel with one face of the rod. A rubber band (c) holds a cover-glass (d) to the cover-glass holder. Cover-glasses (18 × 18 mm, or 20 × 20 mm) are easily slipped under the rubber band, as required. The sample rod is held firmly to the slide-bar by the magnetic strip, and can be raised and lowered, rotated around its axis, and pivoted from side to side, without danger of falling.

The sample rod is capable of rotation around its long axis, which permits sample application in either of two directions perpendicular to each other. This is of some advantage in the electrofocusing of dilute samples, owing to the concentrating effect achieved when the sample is applied in a line parallel with the electric field (y-axis).

Fig. 4 shows the effect of sample orientation on protein detectability. The protein used in this study was β-lactoglobulin, 1% stock solution in water. Decreasing concentrations of protein were electrofocused on 10 × 20 cm plates containing 16 g of gel slurry of the following composition: Sephadex G-75 (superfine) 5%, and carrier ampholyte (Servalyt, pH 2 to 11) 3% (w/v). Samples were applied with 18 × 18 mm cover-glasses oriented either perpendicular or parallel to the electric field. Plates were run at constant voltage at 4°C for 16 h at 200 V, followed by 4 h at 850 V. Paper prints of focused plates were made as described by Radola[3]; they were stained with Coomassie brilliant blue G-250. The photograph shows three paper prints, each with four sample tracks. With perpendicular application the smallest

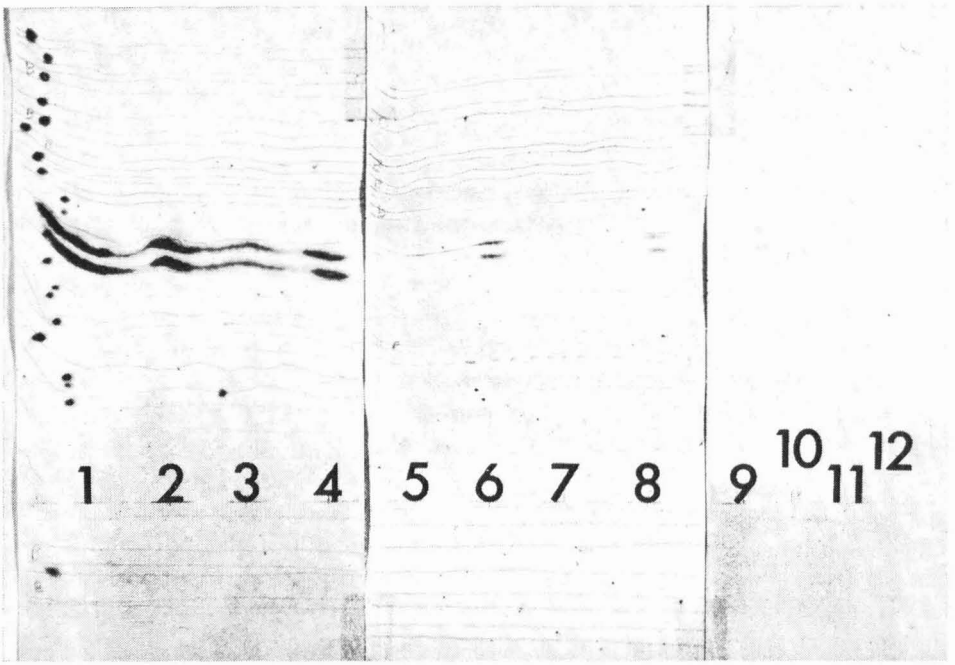


Fig. 4. Effect of sample orientation on protein detection ability. See text for details. Numbers refer to sample track. After each number is listed, in order: the sample concentration (g%), the sample volume, and the direction of application. 1 = 1%, 5 μl, perpendicular (perp); 2 = 1%, 5 μl, parallel (para); 3 = 0.5%, 5 μl, perp; 4 = 0.5%, 5 μl, para; 5 = 0.1%, 10 μl, perp; 6 = 0.1%, 10 μl, para; 7 = 0.1%, 5 μl, perp; 8 = 0.1%, 5 μl, para; 9 = 0.1%, 5 μl, para; 10 = 0.05%, 10 μl, para; 11 = 0.05%, 5 μl, para; 12 = 0.01%, 10 μl, para.

amount of protein detectable is 10 μg, on track 5. With parallel application the smallest amount is 2.5 μg, in track 11. Horizontal pencil lines on the prints, and dark spots on the first print (left), are location guides which were utilized in unrelated experiments and should be disregarded.

As seen in Fig. 4, a four-fold increase in sensitivity of sample detection is achieved with parallel compared with perpendicular application. In the former arrangement the sample is concentrated into a narrower area than in the latter arrangement.

A further advantage of parallel sample application is that a larger number of samples can be run on a given plate: twice as many samples may be accomodated with parallel compared with perpendicular application.

In parallel application, samples should be placed as far away as possible from the expected focusing site, because the application slit sometimes distorts the focused sample bands.

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REFERENCES

1 P. G. Righetti and J. W. Drysdale, *Isoelectric Focusing*, North-Holland, Amsterdam, 1976, pp. 459–460.
2 *Operating Manual No. 171 E for Isoelectric Focusing*, Brinkman Instruments, Westbury, NY, 1977.
3 B. J. Radola, *Biochim. Biophys. Acta*, 295 (1973) 412.

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Note

Packing of Toyopearl columns for gel filtration

II. Dependence of optimal packing velocity on column size

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It is well known in high-performance liquid chromatography that the column packing technique greatly influences the performance of the columns obtained. However, the importance of the packing technique has not been recognized for soft gels such as Sephadex, as pointed out by Sosa[1]. Only a few studies have been reported on packings of soft gels[2,3] and efficient and reproducible packing procedures still seem to be lacking. We have been investigating the influence of various packing conditions on the column performance for semi-soft gels by using Toyopearl (Toyo Soda, Tokyo, Japan), which is a hydrophilic porous polymer packing material for gel filtration, resistant to pressures up to several atmospheres. The dependence of column performance on packing velocity for constant-velocity packings has already been reported[4]. The influence of column size on the dependence of column performance on packing velocity is described in this note.

Toyopearl HW55S (Lot No. 55009-16M) of particle size 20–40 μm was used. This is the same material as Fractogel TSK HW55 (0.025–0.037 mm) available from E. Merck (Darmstadt, G.F.R.). This gel was packed into commercial glass columns (Amicon, Lexington, MA, U.S.A.) of several different sizes (Table I) by the constant-velocity method and the performance of the packed columns was tested with a mixture of bovine serum albumin and myoglobin as described previously[4], with two exceptions. A slurry with a 40% gel concentration was employed in the packing of

TABLE I

SIZES OF CHROMATOGRAPHIC COLUMNS AND SLURRY RESERVOIRS

Chromatographic column (cm)	*Slurry reservoir (cm)*
30 × 2.2 I.D.	45 × 2.2 I.D.
45 × 2.2 I.D.	60 × 2.2 I.D.
60 × 2.2 I.D.	90 × 2.2 I.D.
90 × 2.2 I.D.	120 × 2.2 I.D.
60 × 1.0 I.D.	120 × 1.0 I.D.
60 × 1.6 I.D.	90 × 1.6 I.D.
60 × 3.2 I.D.	90 × 3.2 I.D.
60 × 4.4 I.D.	90 × 4.4 I.D.

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60 × 1.0 cm I.D. columns because of inferior packing reproducibility with a slurry concentration of 45% for this size of column. The protein concentrations of the solutions injected were varied in proportion to the column capacities.

The resolution factor for bovine serum albumin and myoglobin, *R*(BSA, myoglobin), was calculated by using eqn. 1 in ref. 4 as a measure of column performance. The dependences of *R*(BSA, myoglobin) and the final packing pressure on packing velocity are shown in Figs. 1 and 2. Very similar tendencies were observed for all columns (except the 90 × 2.2 cm I.D. column), *viz.*, *R*(BSA, myoglobin) was constant above some critical packing velocity and decreased with packing velocity below the critical point. On the other hand, the final packing pressures increased approximately linearly with increasing packing velocity at low packing velocities but began to rise more rapidly at higher packing velocities. Although these two kinds of critical packing velocities differed according to column size, the final packing pressures corresponding to them were independent of column size and were approximately 0.6 and 1.0 atm, respectively. Accordingly, we defined the packing velocities between the final packing pressures of 0.6 and 1.0 atm as optimum, and these optimal packing velocities are summarized in Table II and are plotted against column length and

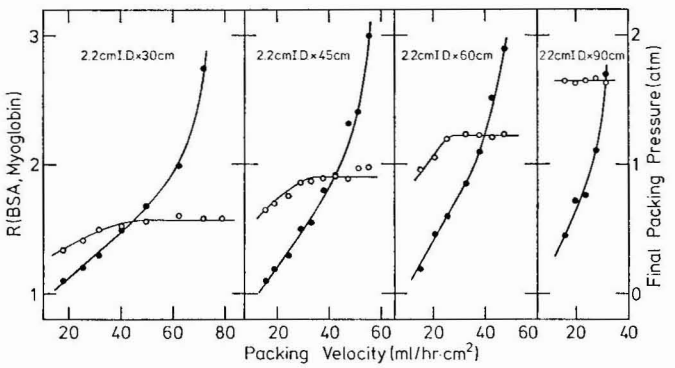


Fig. 1. Dependences of *R* (BSA, myoglobin) (○) and final packing pressure (●) on packing velocity in constant-velocity packings of Toyopearl HW55S in columns of 2.2 cm I.D. and various lengths.

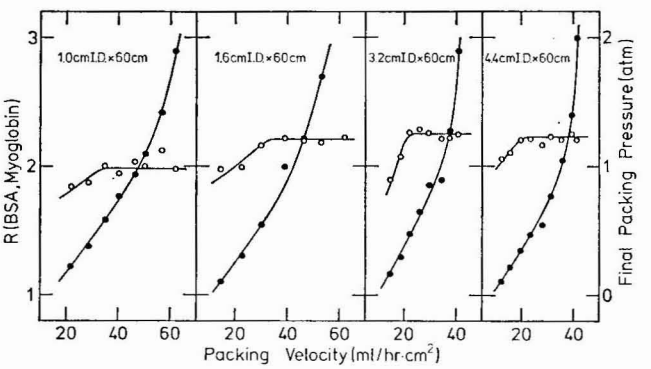


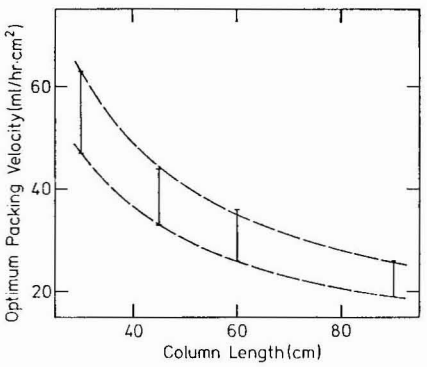
Fig. 2. Dependences of *R*(BSA, myoglobin) (○) and final packing pressure (●) on packing velocity in constant-velocity packings of Toyopearl HW55S in columns of various inner diameters and 60 cm length.

TABLE II

OPTIMAL PACKING VELOCITIES AND *R*(BSA, MYOGLOBIN) FOR COLUMNS PACKED AT OPTIMAL VELOCITIES FOR TOYOPEARL HW55S

Column dimensions (cm)	*Optimal packing velocity (ml/h·cm²)*	*R(BSA, myoglobin)*
30 × 2.2 I.D.	47–63	1.57
45 × 2.2 I.D.	33–44	1.90
60 × 2.2 I.D.	26–36	2.22
90 × 2.2 I.D.	19–26	2.65
60 × 1.0 I.D.	36–47	1.98
60 × 1.6 I.D.	31–42	2.21
60 × 3.2 I.D.	23–32	2.25
60 × 4.4 I.D.	22–32	2.23

column inner diameter in Figs. 3 and 4, respectively. These results indicate that the optimal packing velocities vary with both column length and column inner diameter. Therefore, the optimal packing velocity must be known for each column to be packed. However, it cannot be established exactly from the above results, but an approximate estimate may be possible by assuming that the optimal packing velocities are inversely proportional to column length, as shown in Fig. 5 for columns of 2.2 cm I.D. First, the optimal packing velocity for the 60 cm long column of the same inner diameter as the column to be packed is known from Fig. 4. Then, the optimal packing velocity is corrected for the column length by utilizing the assumption of the above inversely proportional relationship.



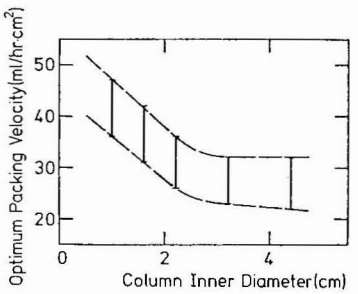
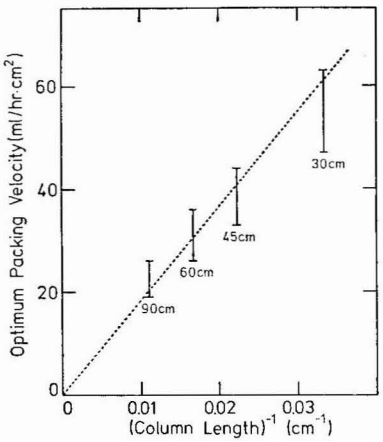


Fig. 3. Dependence of optimal packing velocity on column length in constant-velocity packings of Toyopearl HW55S in 2.2 cm I.D. columns.

Fig. 4. Dependence of optimal packing velocity on column inner diameter in constant-velocity packings of Toyopearl HW55S in 60 cm long columns.

Table II or Fig. 3 shows that the optimal packing velocity decreases with increasing the column length. This means that the time required to pack longer column increases more than proportionately to the column length. Moreover, as the maximal operating velocities of packed columns are lower than the packing velocities



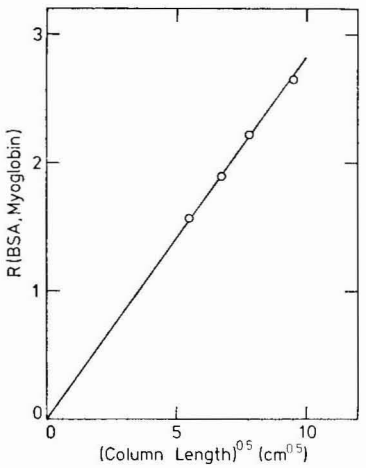


Fig. 5. Plots of optimal packing velocity against reciprocal of column length in constant-velocity packings of Toyopearl HW55S in 2.2 cm I.D. columns.

Fig. 6. Dependence of R(BSA, myoglobin) on column length for 2.2 cm I.D. columns packed with Toyopearl HW55S by the constant-velocity method at optimal velocities.

used initially, it is more convenient to use sever alshort columns in series when a longer column is required. Table II or Fig. 4 also shows that the optimal packing velocity increases with decreasing column inner diameter, especially with columns of small inner diameter. However, although almost the same resolutions were obtained with columns with inner diameters larger than or equal to 1.6 cm, a considerable decrease in resolution was observed with columns of 1.0 cm I.D., as shown in Table II. Therefore, very narrow columns are not desirable when there is no limitation to the column size. Columns of about 2 cm I.D. seem to be best in such instances.

Fig. 6 shows the dependence of R(BSA, myoglobin) on column length for 2.2 cm I.D. columns. R(BSA, myoglobin) was proportional to the square root of column length, as theoretically expected, indicating that longer columns were packed as well as shorter columns.

REFERENCES

1 J. M. Sosa, *Anal. Chem.*, 52 (1980) 910.
2 P. Flodin, *J. Chromatogr.*, 5 (1961) 103.
3 R. Widén and K.-E. Eriksson, *J. Chromatogr.*, 15 (1964) 429.
4 Y. Kato, K. Komiya, T. Iwaeda, H. Sasaki and T. Hashimoto, *J. Chromatogr.*, 205 (1981) 185.

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Note

Integral high-performance liquid chromatographic–electron spin resonance spectrometer for *in situ* studies of thermal and photochemical free radical reactions

Separation of phenoxy, hydrazyl, nitroxide and silyl-substituted semiquinone radicals

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The potential and wide applications of high-performance liquid chromatography (HPLC) in chemical and biochemical studies are beyond debate. In the past ten years we have extensively used electron spin resonance (ESR) spectroscopy to characterize transient free radicals and paramagnetic intermediates involved in organic and organometallic reactions. Many of these radical intermediates are very persistent and it is often desirable to separate them for a full spectroscopic characterization, including electronic, vibrational and nuclear magnetic spectroscopy. Prompted by the recent reports of a Japanese group[1–4] on the separation of the stable nitroxide radicals formed in radiolysis by HPLC with ESR detection, we wish to present here a more extensive use of a simple integral HPLC–ESR system assembled in our laboratory for *in situ* studies of thermal and photochemical free radical reactions. Three separate chemical systems will be used to illustrate the general applications of our integral HPLC–ESR spectrometer. They include the thermal reactions of diphenylpicrylhydrazyl radical (DPPH) and di-*tert.*-butyl peroxide (BOOB) with substituted phenols[5], the photochemical reaction of 2-methyl-2-nitrosopropane (TBNO) and the photochemical reaction of 2,6-di-*tert.*-butyl-*p*-benzoquinone (DTBQ) and triphenylsilane in the presence of BOOB[6].

EXPERIMENTAL

The principal components of the integral HPLC–ESR system are shown in Fig. 1. It consists of a positive-displacement pump (Laboratory Data Control, Model 396) coupled to a home-made pulse damper, a Valco sample-injection 6-port valve (universal HPLC injection system) with a sample loop modified for *in situ* thermal and photochemical reactions. For thermal reactions, the sample coil was thermostatted in an oil bath at a pre-selected temperature for a pre-determined period of time, usually 20 sec. For photochemical studies, a short length of a small quartz tubing was included in the modified sample loop for irradiation by a mercury arc. All columns were packed in our laboratory with Whatman Partisil-10 (silica gel); the short column is 30 cm × 2 mm and the long one measures 60 cm × 3 mm.

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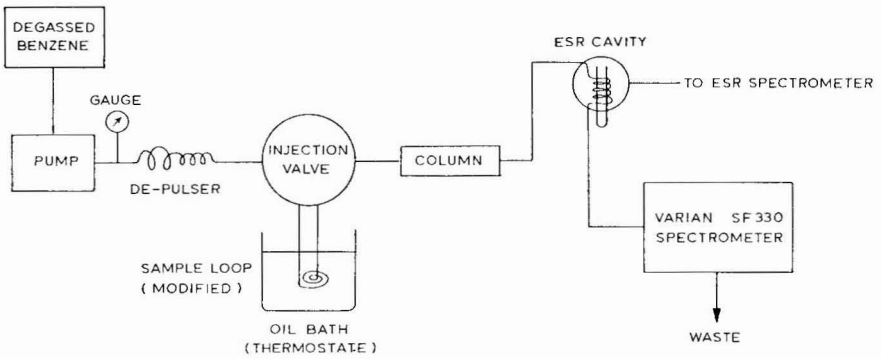


Fig. 1. Flow diagram of an integral HPLC–ESR system.

All HPLC components were mounted on a platformed next to the 10-in. magnet of an old Varian V4500 X-band ESR spectrometer with 100 kHz field modulation. The connection between the HPLC column and the ESR cavity is made by PTFE tubings (0.03 in. I.D.). The PTFE tubings are coiled around a quartz tube inserted into the cavity and the coil is approximately 2 cm in length and in double layers. A standard radical sample placed in a capillary tube can be inserted into the quartz tube in the cavity without distrubing the PTFE sample coil. The magnetic field is manually set by the standard sample and locked in by a Varian F-8A fluxmeter. The standard is then removed before the HPLC operation begins. In order to enhance the sensitivity of the old ESR spectrometer, the state-of-the-art low noise GaAs microwave preamplifier (Narda, Model N6244S-43) was installed at the signal front ends. This provides an approximately 3-fold improvement in sensitivity and a detection limit of about 10^{-12} mole of free radicals.

In the dual-detector configuration, the sample solution was routed from the ESR cavity directly to a Varian SF330 double beam spectrofluorometer for either emission or absorption studies.

All chemicals used were supplied by Aldrich; quinones and phenols were vacuum sublimed before use. Benzene was used as the solvent in all chemical systems and degassed benzene was also used as eluting solvent. In a typical operation, a benzene solution of the chemical system was injected into the sample loop (modified as the *in situ* reactor, 30–50 μl) and after a pre-determined reaction time the system was eluted at a flow-rate of 0.6 ml/min.

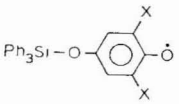
RESULTS AND DISCUSSION

Table I summarizes the retention times of various free radicals in benzene under identical operational conditions as detected by ESR. It is remarkable that the nitroxide radicals have a relatively very long retention time. This is not too surprising as the nitroxide is the most polar radical among those studiec here. The potential usefulness of the HPLC–ESR systems in studies of free radical reactions is illustrated in the following three chemical systems.

TABLE I

RETENTION TIMES OF SOME PHENOXY, HYDRAZYL, NITROXIDE AND SILYL-SUBSTITUTED SEMIQUINONE RADICALS IN BENZENE

Flow-rate: 0.6 ml/min.

Radical	*Retention time (min)*
2,4,6-tri-*tert.*-Butylphenoxy	0.70
1,1-Diphenylpicrylhydrazyl	1.06
$(t\text{-Bu})_2N^{\cdot}\text{–O}$	8.03
	0.72
For comparison, the parent quinone, 2,5-di-*tert.*-butyl-*p*-benzoquinone	2.22

Thermal reaction of DPPH and BOOB with phenol

The thermal reactions of BOOB and DPPH with substituted phenols are well established and accounted for by the following mechanisms:

$$\text{BOOB} \xrightarrow{\triangle} 2\,\text{B}\dot{\text{O}} \quad (1)$$

$$\text{B}\dot{\text{O}} + \text{PhOH} \longrightarrow \text{BOH} + \text{Ph}\dot{\text{O}} \quad (2)$$

$$\text{DP}\dot{\text{P}}\text{H} + \text{PhOH} \xrightarrow{\triangle} \text{DPPH}_2 + \text{Ph}\dot{\text{O}} \quad (3)$$

Here PhOH represents the 2,4,6-tri-*tert.*-butylphenol. In the first BOOB–PhOH system, the increase in $\text{Ph}\dot{\text{O}}$ radical concentration with temperature as seen by the HPLC–ESR spectrometeer reflects the increases of rates for both reactions 1 and 2.In the second system, both the $\text{DPP}\dot{\text{H}}$ and the $\text{Ph}\dot{\text{O}}$ were detected and separated by the HPLC–ESR spectrometer but the ratios of the concentrations of the two radicals varied with temperature. For example, $[\text{DPP}\dot{\text{H}}]/[\text{Ph}\dot{\text{O}}] = 0.82$ at 30°C and the ratio decreased to 0.72 at 49°C. This is consistent with the fact that although reaction 3 is "reversible"[5], the forward reaction proceeds much faster with increasing temperature.

Photolysis of 2-methyl-2-nitrosopropane in benzene

TBNO has been used widely as a spintrap to convert short-lived intermediate radicals into the more stable nitroxides. However, TBNO itself is relatively photosensitive and in a benzene solution a self-trapping reaction will proceed as follows:

$$t\text{-BuNO} + h\nu \rightarrow (\text{CH}_3)_3\dot{\text{C}} + \text{N}\dot{\text{O}} \quad (4)$$

$$(\text{CH}_3)_3\dot{\text{C}} + t\text{-BuNO} \rightarrow [(\text{CH}_3)_3\text{C}]_2\text{N}^{\cdot}\text{–O} \quad (5)$$

The concentration of the nitroxide radicals increases with the irradiation time. Because of the extraordinarily long retention times, Makino and co-workers[1–4] have demonstrated that in the radiolysis of an aqueous solution of TBNO, at least five kinds of self-trapped nitroxids radicals were separated by HPLC and characterized by ESR. The photodecomposition of TBNO in a benzene solution appears to be much less extensive and only one major nitroxide radical was produced via reaction 5.

Photolysis of DTBQ–triphenylsilane–BOOB in a benzene solution

The photolysis of a typical benzene solution containing 10^{-3} *M* of DTBQ and triphenylsilane with an excess of BOOB led to a strong blue fluorescence and the observation of a persistent ESR spectrum. The ESR spectrum can be assigned to the triphenylsilyl radical adduct of DTBQ formed by the following mechanism[6]:

$$\text{BOOB} + h\nu \rightarrow 2\ \text{BO} \tag{6}$$

$$\text{BO} + \text{Ph}_3\text{SiH} \rightarrow \text{BOH} + \text{Ph}_3\dot{\text{S}}\text{i} \tag{7}$$

$$\text{Ph}_3\dot{\text{S}}\text{i} + \text{DTBQ} \rightarrow \text{Ph}_3\text{Si}-\text{O}-\text{C}_6\text{H}_2(\text{X})_2(\dot{\text{O}}) \tag{8}$$

It was not immediately obvious whether the strong blue fluorescence is due to the persistent triphenylsilyl radical adduct of DTBQ, since both the fluorescence and the ESR spectrum appear simultaneously. When this chemical system was studied in the HPLC–ESR spectrometer with the sample irradiation time of about 15 min, the persistent radical adduct formed in reaction 8 was separated and identified by ESR. However, the eluent containing this radical did not give any fluorescence. Subsequently, the fraction of eluent containing the fluorescent material was run through another commercial high-performance liquid chromatograph (Waters Assoc., refractive index detector) and the fluorescent material was tentatively identified as the di-silyl substituted hydroquinone, probably formed by the combination of the DTBQ radical adduct with another triphenylsilane radical:

$$\text{Ph}_3\text{Si}-\text{O}-\text{C}_6\text{H}_2(\text{X})_2(\dot{\text{O}}) + \text{Ph}_3\dot{\text{S}}\text{i} \longrightarrow \text{Ph}_3\text{Si}-\text{O}-\text{C}_6\text{H}_2(\text{X})_2-\text{O}-\text{SiPh}_3$$

Finally, while the present note demonstrates adequately the potential and the wider general applications of an integral HPLC–ESR spectrometer in free radical studies, other novel metalorganic radicals involved in organometallic reactions are now being successfully separated and characterized by this technique.

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REFERENCES

1 K. Makino, N. Suzuki, F. Moriya, S. Rokushika and H. Hatano, *Anal. Lett.*, Part A, 13 (1980) 301.
2 K. Makino, N. Suzuki, F. Moriya, S. Rokushika and H. Hatano, *Anal. Lett.*, Part A, 13 (1980) 311.
3 K. Makino, *J. Phys. Chem.*, 84 (1980) 1012, 1016.
4 K. Makino and H. Hatano, *Chem. Lett.*, (1979) 119.
5 P. B. Ayscough and K. E. Russell, *Can. J. Chem.*, 43 (1965) 3039.
6 K. S. Chen, T. Foster and J. K. S. Wan, *J. Chem. Soc. Perkin II*, (1979) 1288.

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Note

Gas chromatographic separation of α-hydroxycarboxylic acid ester enantiomers using amino acid derivatives as chiral stationary phase

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It is well known that amino acid enantiomers in the form of their N-acyl esters[1,2] or N-acyl amides[3] can be resolved by gas chromatography (GC) with amino acid derivatives as optically active stationary phase. This separation has been attributed to diastereomeric interaction including hydrogen bonding between CONH groups.

In contrast, α-hydroxycarboxylic acid enantiomers have never been separated in the form of their O-acyl esters and it was necessary to use the corresponding O-acyl amides to resolve the enantiomers[4]. This has been considered to be due to the absence of a nitrogen-attached hydrogen in O-acyl α-hydroxycarboxylic acid esters.

In this paper we describe the direct separation of enantiomers of α-hydroxycarboxylic acid esters, which have no NH groups, using amino acid derivatives as chiral stationary phase.

EXPERIMENTAL

A Shimadzu GC-7A gas chromatograph equipped with a flame ionization detector was employed. Glass capillary columns (40 m × 0.25 mm I.D.) were coated with the optically active stationary phases N,N′-[2,4-(6-ethoxy-1,3,5-triazine)diyl]bis (L-valyl-L-valine isopropyl ester) (OA-200)[5], N,N′-[2,4-(6-ethoxy-1,3,5-triazine)diyl] bis(L-valyl-L-valyl-L-valine isopropyl ester) (OA-300)[6] and N,N′,N″-[2,4,6-(1,3,5-triazine)triyl]tris(N^{α}-lauroyl-L-lysine *tert.*-butylamide) (OA-400)[7] which were prepared as described previously.

L- and DL-lactic acid and DL-α-hydroxybutyric acid were purchased from Wako (Osaka, Japan). The various esters or amines for GC were prepared from these acids by treatment with corresponding alcohols or amides. Some esters and amides were O-acylated with acetic anhydride or trifluoroacetic anhydride.

RESULTS AND DISCUSSION

The results are summarized in Tables I and II. A typical chromatogram is shown in Fig. 1.

Enantiomers of several α-hydroxycarboxylic acid esters were resolved into their antipodes when the α-hydroxy group was not acylated, as shown in Table I. The

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TABLE I

GC SEPARATION OF α-HYDROXYCARBOXYLIC ACID ESTER ENANTIOMERS

Chromatographed on 40 m × 0.25 mm I.D. glass capillary columns. Carrier gas: helium at 0.7 ml/min.

H R–C*–COOR′ OH			*OA-400*				*OA-300 + OA-200 (1:1)*			
			Column temp. (°C)	*Retention time* (min)*		*Separation factor, α (2nd/1st)*	*Column temp. (°C)*	*Retention time* (min)*		*Separation factor, α (2nd/1st)*
X	*R*	*R′*		*1st peak*	*2nd peak*			*1st peak*	*2nd peak*	
H	CH_3	C_2H_5	90	3.48	3.60	1.034	130	1.4	1.4	1.000
H	CH_3	$CH(CH_3)_2$	90	3.84	3.95	1.029				
H	CH_3	$CH_2CH(CH_3)_2$	90	9.85	10.20	1.036	130	3.36	3.43	1.021
$COCH_3$	CH_3	$CH_2CH(CH_3)_2$	90	10.8	10.8	1.000	130	4.5	4.5	1.000
H	CH_3	cyclo-C_6H_{11}	100	32.0 (L)	33.1 (D)	1.034	130	16.9 (L)	17.4 (D)	1.030
H	CH_3	n-C_6H_{13}	100	24.5 (L)	25.4 (D)	1.037	130	12.5 (L)	12.8 (D)	1.024
$COCH_3$	CH_3	n-C_6H_{13}	100	24.4	24.4	1.000	130	16.1	16.1	1.000
H	C_2H_5	n-C_6H_{13}					130	18.1	18.6	1.028

* Measured from solvent peak.

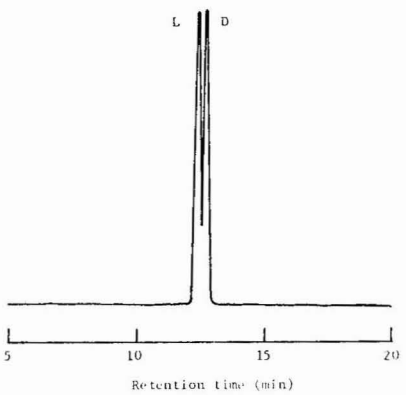


Fig. 1. Gas chromatogram of DL-*n*-hexyl lactate. Column: glass capillary (40 m × 0.25 mm I.D.) coated with OA-300 + OA-200 (1:1). Temperature: 130°C.

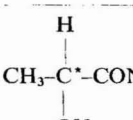
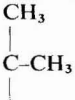
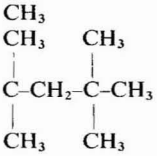
fact that enantiomers could not be resolved under the same chromatographic conditions when the α-hydroxy group was acylated indicates the free α-hydroxy group makes a large contribution to the separation of α-hydroxycarboxylic acid ester enantiomers.

The enantiomers of lactic acid amide were resolved even when the α-hydroxy group was acylated, as shown in Table II. This result suggests the CONH group exerts the main influence on the enantiomeric separation of these amides.

TABLE II

GC SEPARATION OF LACTIC ACID AMIDE ENANTIOMERS

Chromatographed on 40 m × 0.25 mm I.D. glass capillary columns coated with OA-300 + OA-200 (1:1). Carrier gas: helium at 0.6 ml/min. Column temperature: 150°C.

		*Retention time* (min)*		*Separation factor, α*
		1st peak	*2nd peak*	*(2nd/1st)*
X	*R*			
H	*n*-C_6H_{13}	161.1 (D)	163.8 (L)	1.017
$COCF_3$	*n*-C_6H_{13}	20.5 (D)	20.7 (L)	1.010
H	cyclo-C_6H_{11}	181.3 (D)	182.7 (L)	1.008
$COCF_3$	cyclo-C_6H_{11}	14.7 (D)	15.0 (L)	1.020
H		14.4	14.6	1.014
H		60.8	62.3	1.025

* Measured from solvent peak.

Peak identifications were made by chromatographing successively racemic and 1:3 mixtures of enantiomers of cyclohexyl-, *n*-hexyl esters and cyclohexyl-, *n*-hexyl amides of lactic acid. It is very interesting that L-isomers were eluted prior to D-isomers in the ester form, but D-isomers were eluted prior to L-isomers in the amide form. This reversal of the order of emergence on the same chiral stationary phase apparently indicates that the solute–solvent interaction in the ester form is different from that in the amide form. Hitherto it had been believed that the existence of a nitrogen-attached hydrogen was required for the formation of a strong diastereomeric association complex in the separation of enantiomers on amino acid derivatives.

It is noticeable that a free OH group linked to the asymmetric carbon atom is effective in the separation of enantiomers. We consider this finding throws new light on the mechanism of the separation of enantiomers.

REFERENCES*

1 E. Gil-Av, B. Feibush and R. Charles-Sigler, *Tetrahedron Lett.*, (1966) 1009.
2 C. H. Lochmüller and R. W. Souter, *J. Chromatogr.*, 113 (1975) 283.
3 N. Ôi, M. Horiba and H. Kitahara, *J. Chromatogr.*, 202 (1980) 299.
4 H. Frank, G. J. Nicholson and E. Bayer, *J. Chromatogr.*, 146 (1978) 197.
5 N. Ôi, H. Takeda, H. Shimada and O. Hiroaki, *Bunseki Kagaku (Jap. Anal.)*, 28 (1979) 69.
6 N. Ôi, O. Hiroaki and H. Shimada, *Bunseki Kagaku (Jap. Anal.)*, 28 (1979) 125.
7 N. Ôi, O. Hiroaki, H. Shimada, M. Horiba and H. Kitahara, *Bunseki Kagaku (Jap. Anal.)*, 29 (1980) 270.

* *Editor's Note:* The paper by W. A. König and S. Sievers (*J. Chromatogr.*, 200 (1980) 189) was accepted by this journal on July 16th, 1980, and had not yet appeared when the present article was submitted.

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Note

Gas–liquid chromatography of the isomers of dichlorobenzophenone, di-(chlorophenyl)methane and 1,1-di(chlorophenyl)ethane

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(Received September 11th, 1980)

The identification and quantitative estimation of the six dichloro isomers of dichlorobenzophenone, the six dichloro isomers of di(chlorophenyl)methane and the six dichloro isomers of 1,1-di(chlorophenyl)ethane were required, because the dichloro isomers in the three series* were expected reaction products in a number of Friedel–Crafts syntheses. All six isomers of dichlorobenzophenone are known[1], but no attempts to identify and to estimate the six isomers in mixtures have been reported. Only four of the di(chlorophenyl)methane isomers and two of the 1,1-di(chlorophenyl)-ethane isomers have been mentioned in the literature, but all six dichloro isomers in each of the three series have now been prepared by unambiguous synthetic routes[2]. Although several groups have used infra-red and ^{1}H nuclear magnetic resonance (NMR) spectroscopy to determine isomer ratios in compounds related to the present series[3–6], we found that these techniques, as well as that of mass spectroscopy, were of limited use. We did find, however, that ^{1}H NMR could be used to determine ratios between various groups of isomers in the di(chlorophenyl)methane and the 1,1-di-(chlorophenyl)ethane series, *viz.* the groups (2,2′) to (2,3′ plus 2,4′) to (3,3′ plus 3,4′ plus 4,4′).

Since ^{1}H NMR could not be used to identify or to determine all the six isomers in any of the three series, we therefore studied the gas–liquid chromatographic (GLC) characteristics of the isomers in all three series.

EXPERIMENTAL

The apparatus used for the GLC analyses was a Perkin-Elmer F-11 instrument with a flame-ionisation detector. All analyses were carried out isothermally. The columns and operating conditions that were finally used for the analyses were as follows. For the dichlorobenzophenones a 2 m × 2 mm column of 2½% Carbowax 20M on 80–100 mesh Chromosorb W was used; the column temperature was 180°C, the injection temperature 200°C, nitrogen pressure was 24 p.s.i., air pressure 19 p.s.i.,

* In each series, the aromatic nuclei are monosubstituted, making six possible dichloro isomers.

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hydrogen pressure 22 p.s.i., and the chart run at 5 mm per min. The column and operating conditions for the di(chlorophenyl)methanes were the same except that the column temperature was 160°C and the chart speed 15 mm per min. For analyses of the 1,1-di(chlorophenyl)ethanes a 2 m × 2 mm column of 6% diethyleneglycol succinate on 85–100 mesh Celite AW DMCS was used; the column temperature was 150°C, the injection temperature 200°C, nitrogen pressure 20 p.s.i., air pressure 24 p.s.i., hydrogen pressure 24 p.s.i., and the chart speed 5 mm per min.

For both qualitative and quantitative analyses, it was found convenient to use internal standards, making sure that no peaks were masked. The standards used were benzophenone for the dichlorobenzophenone and di(chlorophenyl)methane series and methyl stearate in the case of the 1,1-di(chlorophenyl)ethane series. Qualitative analyses were carried out by the addition of small amounts of the known dichloro isomers to an unknown mixture, in order to identify the various components present. The retention times of the six dichloro isomers in each series of compounds are given in Table I. In quantitative analyses, peak areas were calculated from the product of peak height and peak width at half peak height. It was found that in each series of isomers the areas were proportioned to the weights of isomers in the mixture, so that within each series the molar response of the six dichloro isomers was the same. The compounds (singly or as mixtures) were injected as 5% solutions in acetone (0.1 μl).

TABLE I

RETENTION TIMES OF THE DICHLORO ISOMERS IN THE DICHLOROBENZOPHENONE, DI(CHLOROPHENYL)METHANE AND 1,1-DI(CHLOROPHENYL)ETHANE SERIES

Isomer	*Retention time (min)*		
	*Dichlorobenzophenone**	*Di(chlorophenyl)methane***	*1,1-Di(chlorophenyl)ethane****
2,2′	31.3	9.9	16.0
2,3′	33.0	11.4	17.8
2,4′	34.8	12.0	20.4
3,3′	36.8	12.7	23.0
3,4′	38.0	13.3	26.2
4,4′	41.6	13.9	28.8
Internal standard	20.0§	24.1§	14.1§§

* On $2^1/_2$% Carbowax at 180°C.
** On $2^1/_2$% Carbowax at 160°C.
*** On 6% diethyleneglycol succinate at 150°C.
§ Benzophenone.
§§ Methyl stearate.

RESULTS AND DISCUSSION

The retention times given in Table I show that in all three series of compounds, the order of increasing retention time follows the sequence:

$$2,2' < 2,3' < 2,4' < 3,3' < 3,4' < 4,4' \quad (1)$$

This agrees with previous work on related series. For example the retention times of dichlorobiphenyls on a large number of stationary phases always follow the above

sequence[7,8]. In compounds of type YC_6H_4X, where X is F, Cl, Br or I and Y is $PhCH_2$ or CH_3, etc., the order of retention times quite generally is 1,2 < 1,3 < 1,4, which again follows the pattern of sequence 1 (ref. 9). It seems possible, therefore, that the general sequence 1 could be assumed to hold in cases where authentic samples of all the isomers are not available. The only major exception to this general sequence, known to us, is that for the difluorobenzophenones retention times, on the same Carbowax column as used for the dichlorobenzophenones, are in the unusual order of 3,4′ < 2,4′ < 4,4′ (ref. 2).

Quantitative analyses of the isomers within each series was helped by our observation that the molar responses of the six isomers were identical. In other related series this has not been found to be the case; Albro and Fishbein[7] observed that for the corresponding six isomers in the dichlorobiphenyl series, molar responses varied by up to about 20% (*i.e.* to within ±10%) between the various isomers. To some extent we were able to check our results against ^{1}H NMR analyses for the di(chlorophenyl)methanes and 1,1-di(chlorophenyl)ethanes. Details are in Tables II and III, for representative analyses. Agreement between the two methods is reasonably good and confirms our quantitative procedure. Since the ^{1}H NMR analyses are carried out with respect to the aliphatic proton signals of the Ar–CH_2–Ar and Ar–CH–Ar groups, the method is not applicable to the dichlorobenzophenones, and we were unable to use the NMR method as a check in this series.

TABLE II

ANALYSIS OF THE DI(CHLOROPHENYL)METHANE ISOMERS BY GLC AND ^{1}H NMR

Isomer	*Composition (%)*		
	GLC		*^{1}H NMR*
2,2′	9.3	9.3	9.2
2,3′	27.2	43.2	42.4
2,4′	16.0		
3,3′	20.1	47.5	48.4
3,4′	18.7		
4,4′	8.7		

TABLE III

ANALYSIS OF THE 1,1-DI(CHLOROPHENYL)ETHANE ISOMERS BY GLC AND ^{1}H NMR

Isomer	*Composition (%)*		
	GLC		*^{1}H NMR*
2,2′	5.2	5.2	4.6
2,3′	6.2	47.5	48.5
2,4′	41.3		
3,3′	0.2	47.3	46.9
3,4′	16.1		
4,4′	31.0		

We also tested the reproducibility of the quantitative analyses by repeated injections of a mixture of isomers. Some representative analyses of the 1,1-di(chlorophenyl)ethane isomers are given in Table IV, with different mixtures of the isomers obtained as reaction products. The reproducibility of the analysis varies somewhat from mixture to mixture, but in general the percentage composition of a mixture of all six isomers can be ascertained to within about one "absolute" percent.

TABLE IV

REPRODUCIBILITY OF ANALYSES OF THE 1,1-DI(CHLOROPHENYL)ETHANE ISOMERS BY GLC

Isomer	*Composition of mixture (%)* *		
	A	*B*	*C*
2,2′	4.5 ± 0.3	4.2 ± 0.4	3.3 ± 0.4
2,3′	6.8 ± 0.9	5.9 ± 0.7	4.8 ± 0.6
2,4′	32.7 ± 0.7	35.4 ± 4.0	37.4 ± 1.8
3,3′	2.0 ± 0.5	1.3 ± 1.4	<1
3,4′	18.5 ± 0.4	17.1 ± 1.7	17.3 ± 1.1
4,4′	35.6 ± 1.4	35.8 ± 1.4	37.2 ± 1.4
Number of analyses	8	12	8

* Standard deviations are given in all cases. Note that mixtures A, B and C are not the same.

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REFERENCES

1 H. L. Haller, P. D. Bartlett, N. L. Drake, M. S. Newman, S. J. Cristol, C. M. Eaker, R. A. Hayes, G. W. Kilmer, B. Magerlein, G. P. Mueller, A. Schneider and W. Wheatley, *J. Amer. Chem. Soc.*, 67 (1945) 1591.
2 D. Huq, *Ph.D. Thesis*, University of Surrey, Guildford, 1978.
3 D. S. Hoffenberg, E. M. Smolin and K. Matsud, *J. Chem. Eng. Data*, 9 (1964) 104.
4 J. Blackwell and W. J. Hickinbottom, *J. Chem. Soc.*, (1963) 366.
5 G. S. Mironov, K. A. Chemyakovskaya, M. I. Farberov, I. M. Tyuleneva and M. S. Rusakova *Zh. Prikl. Khim.*, 43 (1970) 620.
6 L. H. Keith, A. L. Alford and A. W. Carson, *J. Ass. Offic. Anal. Chem.*, 52 (1969) 1074.
7 P. W. Albro and L. Fishbein, *J. Chromatogr.*, 69 (1972) 273.
8 P. W. Albro, J. K. Haseman, T. A. Clemmer and B. J. Corbett, *J. Chromatogr.*, 136 (1977) 147.
9 G. A. Olah, S. J. Kuhn and S. H. Flood, *J. Amer. Chem. Soc.*, 84 (1962) 1695.

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Note

Quantitative determination of naproxen in formulated rat feed by gas chromatography–mass spectrometry and gas chromatography–flame-ionization detection

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Good laboratory practices require quality control of the drug–feed mixtures fed to animals on long term toxicity studies. In the past few years an increasing number of papers have been published which described methods for the analysis of a wide variety of compounds in feed[1,2]. These procedures employed a range of techniques including UV spectrophotometry, gas chromatography (GC) and high-performance liquid chromatography (HPLC).

Drug dosage is dependent upon body weight so drug concentrations in feed are low for the young rats and increase in proportion as the rat matures, typical concentrations being in the range 0.002–0.05% of naproxen in the feed. It is essential with this type of feeding approach to toxicity studies that the homogeneous mixing of the drug in feed and its stability therein be constantly monitored.

Once the drug has been mixed with feed and subjected to the repelleting process it is necessary to use polar solvents for complete extraction. None of the currently available methods for the determination of naproxen in plasma or urine[3–6] could be applied to feed either for efficiency of extraction or their ability to clean up the tremendous amount of background interference.

This paper describes the analysis of naproxen, (+)-6-methoxy-α-methyl-2-naphthaleneacetic acid, from feed using either GC–mass spectrometry (MS) or GC–flame-ionization detector (FID) techniques. The two techniques between them provide the necessary specificity, sensitivity and a method for routine analysis of large numbers of feed samples. Application of the GC–MS method to demonstrate the homogeneity and stability of naproxen in repelleted formulated rat feed is described.

EXPERIMENTAL

Reagents

Diazomethane was prepared from N-methyl N-nitroso-*p*-toluene sulfonamide as per the instructions on the manufacturers label (Diazald; Aldrich, Milwaukee, WI, U.S.A.). All solvents used were either glass distilled or analytical reagent grade.

Instrumentation

The gas chromatograph–mass spectrometer (Finnigan Model 3200) was equipped

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with a chemical-ionization source and a Model 6000 data system. Methane was used as the carrier gas (20 ml/min) and also as chemical ionization reagent gas, the source pressure being maintained at approximately 1 torr. The column was 150 cm × 2 mm I.D. glass packed with 3% OV-1 in 100–120 mesh Chromosorb W.

Column, injection port, transfer oven and source temperatures were maintained at 150, 260, 260 and 120°C, respectively. The mass spectrometer was operated in the selected ion recording mode, monitoring ions at 231 and 245 a.m.u.

The gas chromatograph (Model 2100, Varian, Walnut Creek, CA, U.S.A.) was equipped with an FID. The column was 180 cm × 2 mm I.D. glass packed with 3% SP-2401 on 100–120 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.). Injector, column and detector were maintained at 275, 150 and 300°C, respectively.

Procedure

Method A: GC–MS. To 25 grams of well ground and mixed sample in a 500-ml erlenmeyer flask was added the appropriate amount of internal standard, 6-methoxynaphthylacetic acid in methanol.

Extraction was achieved by stirring in 240 ml of acetonitrile–water (1:1) for 20 min at 35°C. After centrifugation, a portion of the supernatant, approximately 3 ml, was transferred to a separatory funnel. A 9-ml volume of acetate buffer (pH 3.4) was added and the solution extracted with 10 ml of ether. The organic layer was filtered through a plug of anhydrous sodium sulfate and evaporated to dryness at 40°C under nitrogen. Diazomethane in ether (0.8 ml) was added and the methylation reaction was allowed to proceed for 5 min at room temperature. After evaporation of excess reagent under nitrogen, the residue was dissolved in 200 μl of methanol and 2–5 μl were injected for analysis in the GC–MS system.

Method B: GC–FID. Initial extraction from the feed was similar to method A with 6-ethoxy naphthyl acetic acid being used as the internal standard. A 2-ml volume of the supernatant was acidified with 2 ml of 2 *N* HCl, extracted with 10 ml of dichloromethane–hexane (3:7) and centrifuged. The organic layer was evaporated to dryness at 40°C under nitrogen. The residue was dissolved in 3 ml of 1 *N* sodium hydroxide, washed with 7 ml of dichloromethane, acidified with 1.7 ml of 2 *N* HCl and extracted with 5 ml of ether. The residue after evaporation of the ether was methylated in the same manner as in method A. The final residue was dissolved in 100 μl of carbon disulfide and 1–2 μl injected for analysis on the gas chromatograph.

RESULTS AND DISCUSSION

Naproxen is a polar compound containing a carboxylic acid function which necessitates the use of very polar solvents to achieve complete extraction. This, of course, tends to elute more background interference from the very complex and variable feed matrix. The GC–MS technique, due to its greater specificity, did not detect much of the background interference thereby allowing the development of a simple procedure in a short time. The specificity afforded by the GC–MS stems from the use of chemical ionization and selected ion monitoring techniques. Chemical ionization spectra are usually simpler than electron impact spectra and therefore give rise to less interference from endogenous compounds. The selected ion monitoring technique provides sensitivity and specificity since only the quasi-molecular ions of

naproxen and the internal standard are actually monitored so lessening the chance of background interference. In fact GC analysis of the extracts did show background interference that was not visible on the GC–MS ion chromatograms. The chemical ionization mass spectra obtained for the methyl esters of naproxen and the internal standard are shown in Fig. 1. Typical ion chromatograms obtained by monitoring the quasi-molecular ions of $MH^+ = 245$ for naproxen and $MH^+ = 231$ for the internal standard are shown in Fig. 2. The sensitivity necessary to measure the lowest required level of 0.002% could easily be obtained and calibration curves prepared in the range of 0.001 to 0.03% were linear.

The data presented in Table I show that the drug is accurately formulated and homogenously distributed in the feed. Assay results were usually within 5% of the label strength. In order to determine homogeneous mixing of the drug in feed, samples

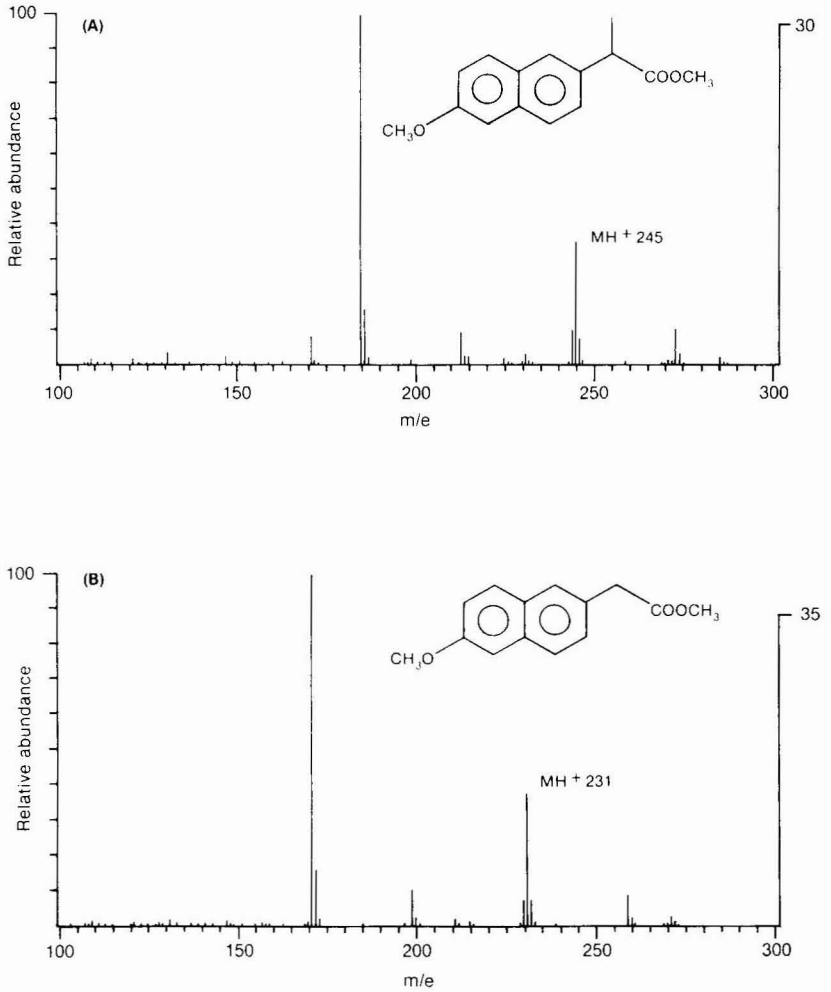

Fig. 1. Methane chemical ionization mass spectra of the methyl esters of naproxen (A) and internal standard (B).

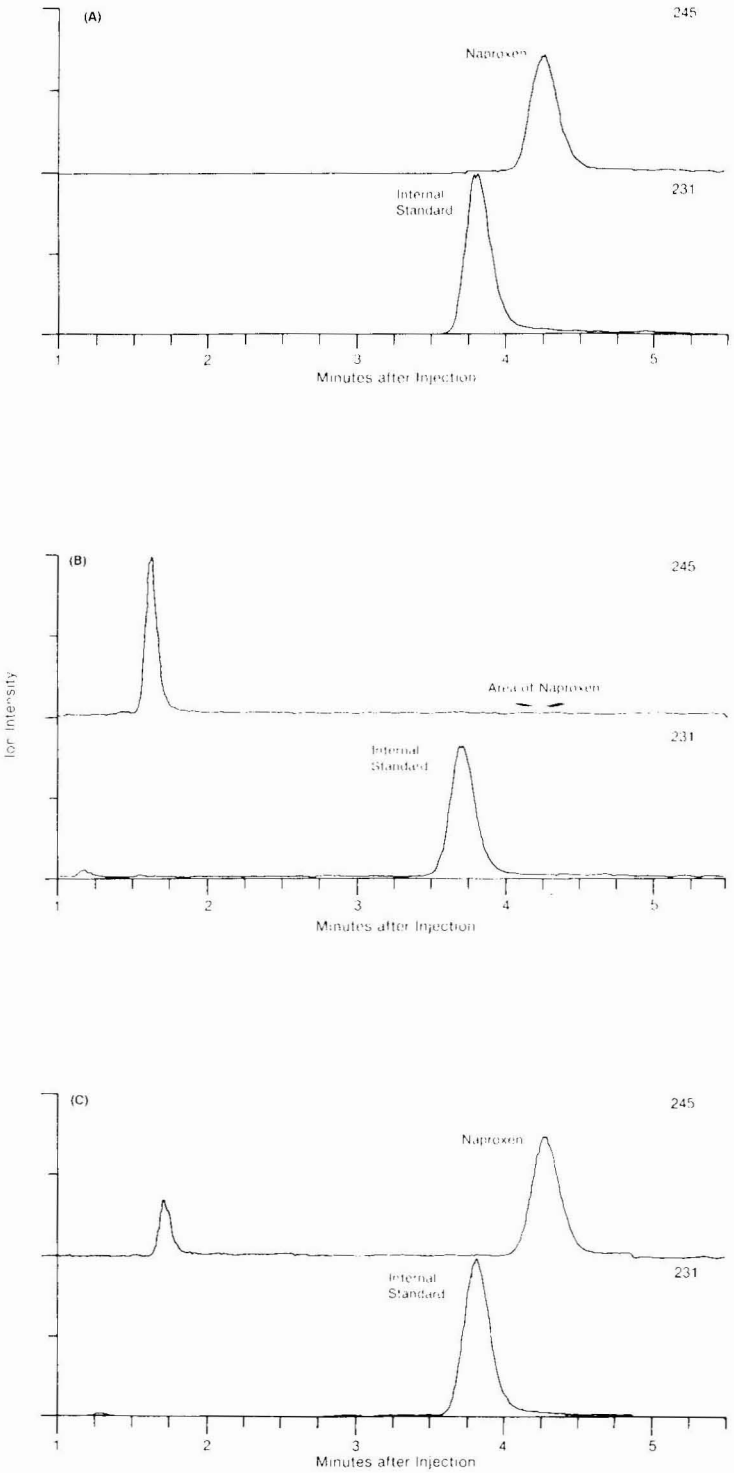

Fig. 2. Selected ion chromatograms obtained for method A. (A), reference standards of the methyl esters of naproxen and internal standard; (B), blank feed sample spiked with internal standard; (C), formulated feed sample spiked with internal standard.

TABLE I

ASSAY RESULTS INDICATING ACCURACY OF FORMULATION AND HOMOGENEITY OF NAPROXEN IN RAT FEED

The feed was repelletized after formulation and samples taken for analysis during the beginning, middle and end of the pelleting run to check homogeneity.

Sample	*Label strength (%)*	*Assay value (%)*
Beginning	0.0040	0.0037
Middle	0.0040	0.0038
End	0.0040	0.0039
Beginning	0.0080	0.0078
Middle	0.0080	0.0077
End	0.0080	0.0078
Beginning	0.0120	0.0117
Middle	0.0120	0.0116
End	0.0120	0.0118
Beginning	0.0053	0.0051
Middle	0.0053	0.0051
End	0.0053	0.0050
Beginning	0.0159	0.0154
Middle	0.0159	0.0153
End	0.0159	0.0158

TABLE II

ASSAY RESULTS INDICATING THE STABILITY OF NAPROXEN IN FORMULATED FEED

Sample	*Label strength (%)*	*Initial assay (% of l.s.)**	*1 Week room temp. (% of l.s.)*	*3 Week room temp. (% of l.s.)*	*4 Week room temp. (% of l.s.)*	*1 Week 37°C (% of l.s.)*
"Stability"	0.0100	100	101			101
No. 850	0.0053	96		98		
No. 851	0.0106	95		95		
No. 852	0.0159	96		93		
No. 864	0.0075	87			89	
No. 865	0.0150	96			95	
No. 866	0.0225	93			96	

* l.s. = Label strength.

of the feed were taken for analysis at the beginning, middle and end of the repelleting process.

It is an essential requirement that the drug be stable in the feed for the period elapsing between formulation and utilization. The data presented in Table II show that naproxen levels were unchanged when analyzed one month later.

As the rat toxicity study progressed we adapted the GC–MS method to the more routine and economically attractive GC–FID technique. To achieve this end, it was necessary to increase the number of clean-up steps, as outlined in method B. Typical gas chromatograms are shown in Fig. 3. A slightly modified procedure was eventually used to monitor the remainder of the two-year rat toxicology study.

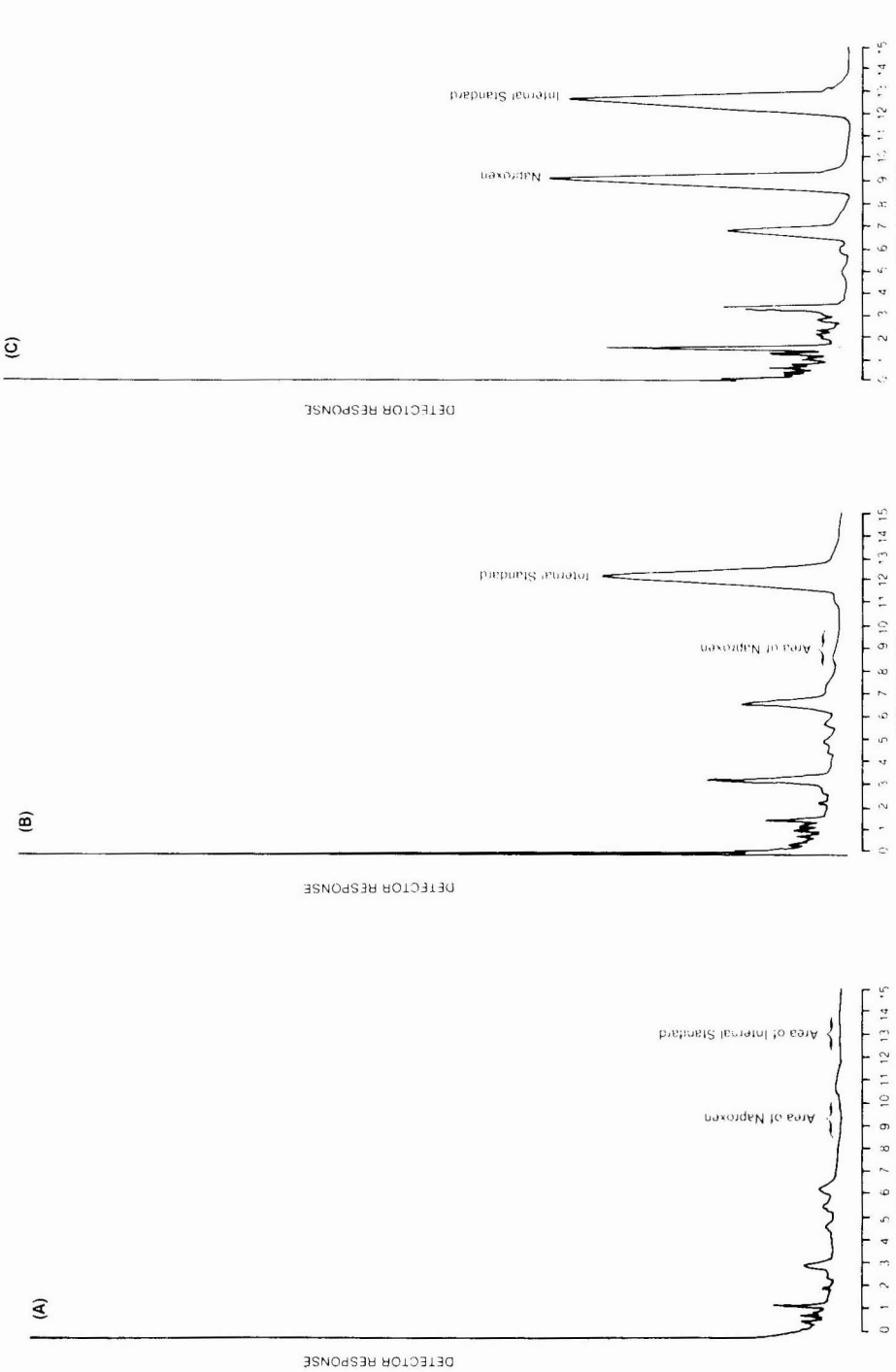

Fig. 3. Gas chromatograms obtained for method B. (A), blank feed sample; (B), blank feed sample spiked with internal standard; (C), formulated feed sample spiked with internal standard.

GC–MS is an expensive technique when compared with GC and HPLC and would not normally be the method of choice for economic reasons. However, it should be remembered that when the instrument is available it can be used to considerable advantage as a highly specific detector as well as in its more traditional role as a highly sensitive technique.

In conclusion, analytical procedures have been presented which covered all the feed assay requirements for a 2-year chronic toxicity study for naproxen in rats.

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REFERENCES

1 J. R. Markus, *J. Ass. Offic. Anal. Chem.*, 61 (1978) 1289–1298.
2 A. Somogyi, *J. Ass. Offic. Anal. Chem.*, 61 (1978) 1198–1200.
3 *Syntex Analytical Method No. 10,123C*, Syntex, Palo Alto, CA, 1973.
4 S. H. Wan and S. B. Matin, *J. Chromatogr.*, 170 (1979) 473–478.
5 J. T. Slattery and G. Levy, *Clin. Biochem.*, 12, (1979) 100.
6 D. Weskerlund, A. Theordorsen and Y. Jaksch, *J. Liquid Chromatogr.*, 2 (1979) 969.

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Note

Determination of N-nitrosodiethanolamine in cosmetics by gas chromatography with electron capture detection

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N-Nitrosodiethanolamine (NDELA) has been shown to be weakly carcinogenic in rats after oral administration[1] and in hamsters after subcutaneous injections[2]. Hepatocellular carcinomas and renal adenomas are induced in rats, and adenocarcinomas of the nasal cavity, papillary tumours of the trachea, hepatocellular adenomas and local fibrosarcomas are produced in hamsters. NDELA has also been reported to be mutagenic on *Actinomyces* either *olivaceus* or *griseoflavus*[3], as well as on *Salmonella typhimurium* strains TA 100 and TA 1535 in the absence of a metabolic activation system[4].

NDELA has been detected in the urine of a human wearing a NDELA-contaminated facial cosmetic, thereby demonstrating its ready absorption in the skin[5]. Very high levels (up to 3%) of NDELA have been found in nitrite–triethanolamine based cutting and grinding fluids used in metal working[6,7]. Levels up to 4.9×10^4 ppb have also been shown to be present in facial cosmetics, hand and body lotions and hair shampoos[8]. Several methods have been described for the analysis of NDELA, including high-performance liquid chromatography combined with a thermal energy analyser (TEA), and gas chromatography (GC) coupled either with a TEA or with a high resolution mass spetrometer (MS).

As those sophisticated instruments are not available in most laboratories, we have developed a method for the determination of NDELA using conventional GC with electron capture detection. This sensitive and selective method has been applied to the detection of NDELA present at the ppb level in several cosmetics and dermopharmaceuticals.

EXPERIMENTAL

Reagents

All reagents were of analytical grade. Diethanolamine, 2-chloroethanol, 3-hydroxy-*n*-propylamine, thionyl chloride and trifluoroacetic anhydride were obtained from Aldrich Europe (Beerse, Belgium) and hydrogen peroxide from Solvay (Brussels, Belgium). The reagents were used without further purification, except for thionyl chloride and trifluoroacetic anhydride which were purified by distillation.

NDELA and N-nitrosopropanolethanolamine (NPELA), used as the internal

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standard, were synthesized by reaction of the corresponding amines with sodium nitrite according to Jones and Wilson[9] with minor modifications: the pH of the medium was maintained at the optimal value of 3 and the N-nitrosamines were purified by extraction with *p*-dioxan after elimination of water and solvents under vacuum. Their purity was checked by thin-layer chromatography (TLC) and GC–MS. *n*-Propanolethanolamine was obtained by direct reaction between 2-chloroethanol and 3-hydroxy-*n*-propanolamine in water at 120°C during 24 h, and subsequently purified.

Peroxotrifluoroacetic acid (PTFA) reagent was prepared as follows: 0.5 ml 98% hydrogen peroxide was added to 7 ml ethyl acetate kept at 0°C. Five 0.5-ml aliquots of trifluoroacetic anhydride were successively added under constant shaking to the solution cooled at 0°C. The reagent was kept in the dark and in ice; a new amount was prepared every week.

Apparatus and conditions

A Perkin-Elmer Model 3920 gas chromatograph, equipped with a ^{63}Ni electron capture detector (ECD) was used. A spiral borosilicate glass column (2 m × 2 mm I.D.) packed with 3% OV-225 on Supelcoport (100–120 mesh) was employed. The operating conditions were as follows: column temperature, 210°C; injector and detector temperature, 250°C; carrier gas (argon–methane, 95:5) flow-rate, 100 ml/min; standing current for the ECD, 2 nA.

A LKB 9000 S gas chromatograph–mass spectrometer was used for confirmation of the identity of the N-nitrosamine derivatives. The operating conditions were as follows: spiral glass column (2 m × 3 mm I.D.) packed with 1% OV-1 on chromosorb W (60–80 mesh); carrier gas (helium) flow-rate, 30 ml/min; oven temperature, 200°C; injector temperature, 230°C; separator temperature, 240°C; ion-source temperature, 270°C; trap current ,60 nA; energy, 70 eV; accelerating voltage, 3.5 kV.

Clean-up procedure

In order to prevent nitrosation artifacts during the analytical procedure, ammonium sulphamate (1 g) was added to 1 g of the cosmetic to be analysed; NPELA (100 μl of an aqueous solution of 1 μg NPELA/ml) was added as the internal standard. The sample was stirred at 30°C for 20 min in a mixture of 10 ml of 2,2-dimethoxypropane, 25 ml of methyl *tert.*-butyl ether and 100 μl of 10% aqueous oxalic acid solution. The mixture was then neutralized with an excess of calcium carbonate and the solvent removed under vacuum in a rotary evaporator operated at 30°C.

The residue was extracted four times with 50 ml light petroleum (b.p. 45–60°C) and four times with 50 ml ethyl acetate. The extracts were successively loaded on a silica gel column (20 × 2 cm, Merck silica gel 60A, 70–230 mesh). Ethyl acetate (150 ml) was used to wash out the impurities and 250 ml acetone subsequently eluted the N-nitrosamines. The acetone fraction was evaporated to dryness under vacuum at 50°C and the residue dissolved in 5 ml acetone.

Derivatization

A 2-ml aliquot of the final acetone solution was evaporated under a stream of nitrogen in a reaction vial; the residue was dissolved in 2 ml ethyl acetate, 10 μl of thionyl chloride were added and the vial was then heated at 55°C for 1 h. The organic phase was washed by shaking with 1 ml of 1 *M* sodium hydroxide and the two phases

were separated after freezing the aqueous phase at −50°C. A 200-μl volume of the PTFA reagent was added to the ethyl acetate solution, followed by standing at 55°C for 1 h. The organic solution was washed with 1 ml of 1 *M* NaOH and an aliquot (1 μl) of the reaction mixture used for GC analysis.

Photolysis

Two aliquots (1 ml) of the acetone solution were evaporated to dryness under nitrogen. The residues were dissolved in 500 μl ethyl acetate and the solution placed in photolysis tubes of soft glass (16 × 0.25 I.D. × 0.35 cm O.D.) which were then flame sealed. One tube was irradiated with UV light for 3 h; the other was kept in the dark for the same period and served as a control. The UV light was emitted from a 8-W germicide lamp situated 4 cm from the samples and submitted to a oscillatory movement so as to make the irradiation uniform. The solutions were then diluted in 1.5 ml of ethyl acetate and 10 μl of thionyl chloride, heated at 55°C for 1 h and finally derivatized as above.

RESULTS AND DISCUSSION

Denitrosation of N-nitrosamines with thionyl chloride in methylene chloride, leading to the formation of nitrosyl chloride, has been used by others[10] as a method for the analysis of non-volatile nitrosamines. It was therefore essential to carefully select the reaction conditions (nature of the solvent, temperature and duration of the treatment, thionyl chloride concentration) to enable the specific transformation of NDELA into volatile bis(2-chloroethyl)-N-nitrosamine with minimal denitrosation.

The reaction has been carried out in different solvents and the products examined by TLC. Bis(2-chloroethyl)-N-nitrosamine was found to be the only derivative formed when ethyl acetate was used as the solvent; in other solvents, such as dichloromethane or diethyl ether, two or more other products were present. Under our conditions, 5 μg thionyl chloride/ml has been found to be the optimal concentration. The results presented in Fig. 1 indicate that the reaction is complete after standing for 1 h at 55°C. At higher temperatures the derivative formed starts to break down.

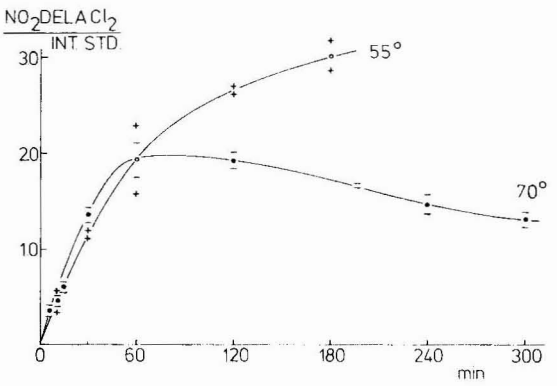

Fig. 1. Effect of time on the reaction of $SOCl_2$ (8 mg/ml) with NDELA (5 μg/ml) at 55 and 70°C.

Fig. 2 indicates that similar reaction conditions were also the most appropriate for the subsequent transformation of bis(2-chloroethyl)-N-nitrosamine into the corresponding nitramine with the PTFA reagent. Moreover, the utilization of ethyl acetate as solvent permits aliquot samples to be taken of the organic phase, previously washed with a sodium hydroxide solution, for direct injection into the gas chromatograph–ECD; the hydroxide solution is used to destroy the excess of PTFA reagent.

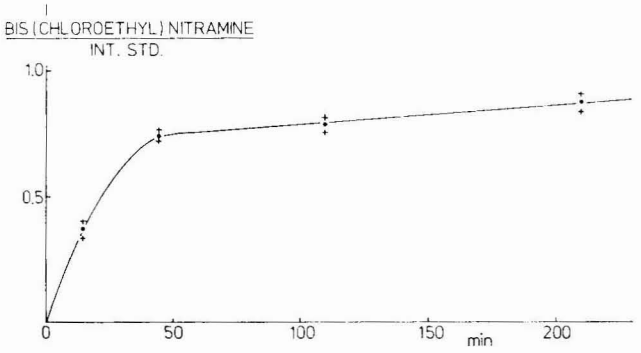

Fig. 2. Effect of time on the oxidation of bis(2-chloroethyl)-N-nitrosamine to nitramine with peroxotrifluoroacetic acid (100 μl PTFA reagent per ml mixture) in ethyl acetate at 55°C.

When pure NDELA was treated, a linear relationship was found between ECD response and NDELA concentration, from 0 to 200 ng NDELA/ml. The sensitivity of the method allows the determination of less than 5 ng NDELA/ml.

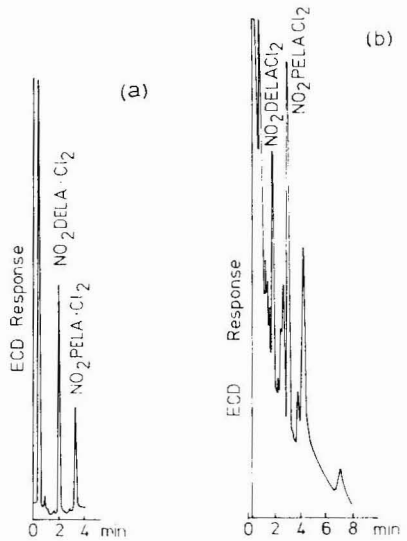

Fig. 3. Gas chromatograms: a, pure NDELA and NPELA (500 ng/ml of each) after derivatization; b, NDELA (1 ppm) after extraction from a cosmetic preparation and derivatization (sample 1, Table I).

With cosmetic samples, the chromatograms are more complex and it is not possible to determine less than 50 ng NDELA/ml (Fig. 3). The concentrations of NDELA present in the analysed samples were computed from a standard graph (Fig. 4) constructed from chromatograms of cosmetic preparations containing known added amounts (0–400 μg/kg) of NDELA and a fixed amount (600 μg/kg) of NPELA used as internal standard. The results obtained are presented in Table I, and were

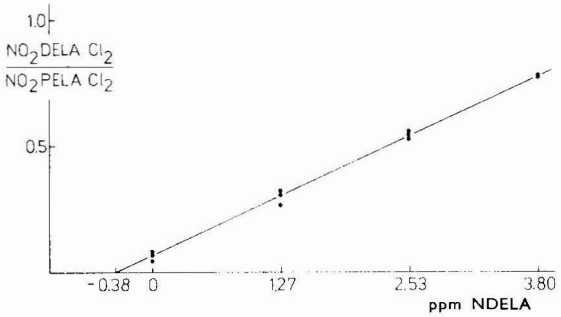

Fig. 4. Calibration curve for NDELA in cosmetic preparation, obtained by addition of increasing amounts of NDELA to sample (1 in Table I).

TABLE I

LEVELS OF NDELA (μg/kg) IN COSMETIC AND DERMOPHARMACEUTICAL PREPARATIONS COMMERCIALLY AVAILABLE IN BELGIUM

For each preparation, two different samples purchased in separate shops were submitted to analysis. n.d. = Not detectable.

Preparation	*Utilization*	*NDELA (μg/kg)*
	Cosmetics	
1	Cream	350
		350
2	Cream	n.d.
		n.d.
3	"For night" cream	100
		380*
4	Cream	100
		100
	Dermopharmaceutical preparations	
5	Slimming gel	n.d.
		n.d.
6	Cleaning gel for eyes	
	Lot J810	300
	Lot T826	300
7	Cream	n.d.
		200
8	Cream (1.34% triethanolamine)	170
		250
9	Cream (1.3% triethanolamine)	110
		220
10	Cream (1.35% triethanolamine)	100
		100

* Confirmed by photolysis.

confirmed for samples with the highest levels of NDELA by photolysis according to Doerr and Fiddler[11].

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REFERENCES

1 H. Druckrey, R. Preussmann, S. Ivankovic and D. Schmahl, *Z. Krebsforsch.*, 69 (1967) 103.
2 J. Hilfrich, I. Schmeltz and D. Hoffmann, *Cancer Lett.*, 4 (1977) 55.
3 L. L. Gumonov, *Spetzifichnost Khim. Mutageneza, Mater. Vses Simp.*, (1968) 65.
4 A. Mesbert, M. Lemonnier and C. Cavelier, *Mutat. Res.*, 68 (1979) 207.
5 G. S. Edwards, M. Peng, D. Hfine, B. Spiegelhalder and J. Kann, *Toxicol. Lett.*, 4 (1979) 217.
6 T. Y. Fan, J. Morrison, D. P. Rounbehler, R. Ross, D. H. Fine, W. Miles and N. P. Sen, *Science*, 136 (1977) 70.
7 P. A. Zingmark and C. Roppe, *Ambio*, 6 (1977) 237.
8 T. Y. Fan, Y. Goff, L. Song, D. H. Fine, G. P. Arsenault and K. Biemann, *Food Cosmet. Toxicol.*, 15 (1977) 423.
9 E. R. M. Jones and W. Wilson; *J. Chem. Soc., London*, (1949) 547.
10 T. G. Lunt, D. G. Fueggle and C. L. Walters, *Anal. Lett.*, 6 (1973) 369.
11 R. C. Doerr and W. Fiddler, *J. Chromatogr.*, 140 (1977) 284.

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Note

Metal chelate affinity chromatography

II. Group separation of mono- and dinucleotides

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In a recent communication[1] we investigated the influence of various parameters such as pH, buffer composition and ionic strength on the retention of various nucleotides and related compounds in copper chelate affinity chromatography. In the present paper, we report an extension of our studies to illuminate further the potential of our method to separate nucleotides according to their metal affinity.

EXPERIMENTAL

The gel used was a bis-carboxymethylamino Sepharose 6B. It was prepared according to the published procedure[1,2] by activation of Sepharose 6B with epichlorohydrin, followed by treatment with sodium iminodiacetate. The adsorbent was packed into columns (diameter 1 cm, V_t = 2–5 ml), loaded with the desired metal ion (20 mM aqueous solution of $CuSO_4 \cdot 5\ H_2O$ or $NiSO_4 \cdot 7\ H_2O$), washed with distilled water and equilibrated with the desired buffer until loosely bound metal was completely eluted.

Artificial mixtures to be tested were prepared from aqueous solutions (2.5 mg/ml) of different nucleotides. The elution profile was recorded at 280 nm. Identification of the peaks was made from the reduced elution volumes, V_e/V_t, of the compounds chromatographed as pure samples.

Specific conditions used in each separation experiment (composition of the nucleotide mixture, V_t of the column, elution buffer) are indicated in the legends to the figures.

RESULTS AND DISCUSSION

Group fractionation of pyrimidine and purine mononucleotides can easily be achieved on a copper chelate adsorbent, as shown in Fig. 1. Since all the pyrimidines show little interaction with the bound metal[1] they are eluted together in a single peak at the void volume. The stronger complexation of purine mononucleotides allows their separation from pyrimidine mononucleotides and also provides sufficient selectivity to differentiate AMP from GMP.

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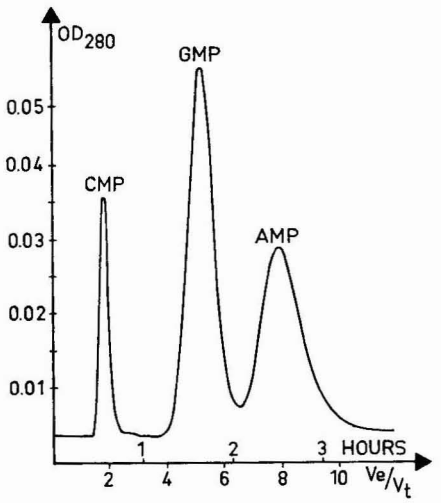


Fig. 1. Fractionation of CMP (62.5 μg per 25 μl) + GMP (62.5 μg per 25 μl) + AMP (250 μg per 100 μl). Copper column: V_t = 4.8 ml. Flow-rate: 15 ml/h. Buffer: 0.1 *M* ethylmorpholine–acetic acid (pH 6.5)–0.2 *M* $MgSO_4$.

The same behaviour was observed with the deoxy derivatives (Fig. 2). However, the fractionation of AMP or GMP from their respective deoxy homologues could not be achieved (results not shown), indicating a minor contribution of the 2′-OH group in the retention process.

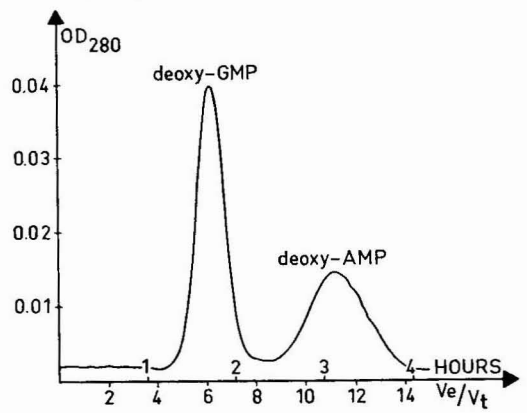


Fig. 2. Fractionation of deoxy-GMP (100 μg per 40 μl) + deoxy-AMP (300 μg per 120 μl). Copper column: V_t = 4.5 ml. Other details as in Fig. 1.

Copper chelate affinity chromatography affords excellent separation of dinucleotides. However, with this metal the dipurine compounds are generally strongly adsorbed (ApA: $V_e/V_t \approx 45$ in 0.05 *M* Tris–HCl (pH 7.0)–1 *M* NaCl) and cannot be eluted within a reasonably short time. To overcome this difficulty, the ligand exchange capacity of the gel can be decreased by raising the pH, the molarity of the elution buffer or /and its nucleophilic character[1]. Alternatively, we performed the group experiment on a gel loaded with Ni^{2+}, a less potent chelating ion under our conditions. Fig. 3 shows that isocratic elution of a mixture of dinucleotides allows total fractiona-

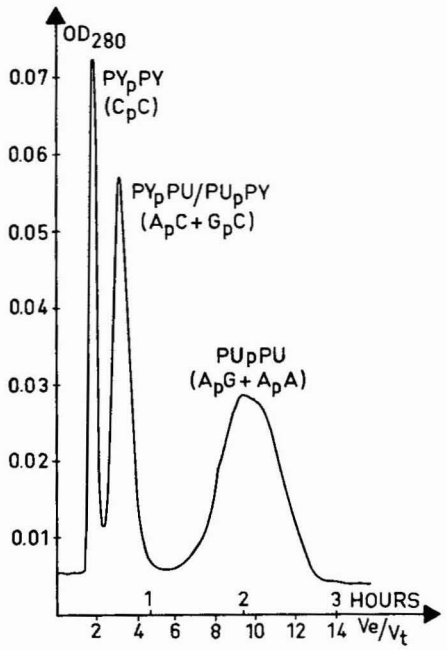


Fig. 3. Group fractionation of CpC (17.5 μg per 7 μl) + GpC (25 μg per 10 μl) + ApC (37.5 μg per 15 μl) + ApC (75 μg per 30 μl) + ApA (125 μg per 50 μl). Nickel column: V_t = 3 ml. Flow-rate: 14 ml/h. Buffer as in Fig. 1.

tion into three peaks on the basis of the purine content of the dinucleotide molecules: PUpPU > PUpPY, PYpPU≫ PYpPY. Fractionation within the PUpPY/PYpPU group can be obtained when the experiment is performed on the copper chelate gel (Fig. 4).

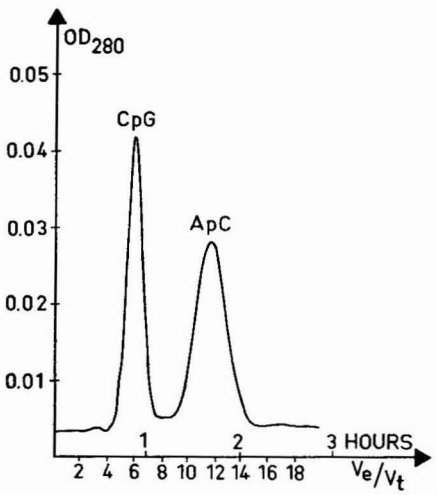
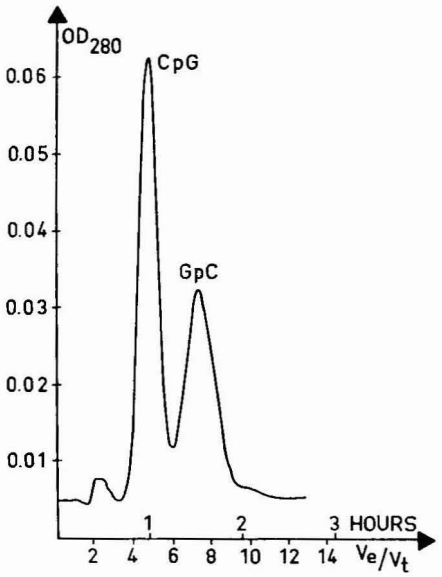


Fig. 4. Fractionation of CpG (62.5 μg per 25 μl) + ApC (250 μg per 100 μl). Copper column: V_t = 2.45 ml. Flow-rate: 17 ml/h. Buffer as in Fig. 1.

The high selectivity of metal chelate affinity chromatography is further demonstrated by the total separation afforded of a mixture of dinucleotides belonging to the PUpPY/PYpPU group, on the basis of the position of the purine moiety in the molecule. Figs. 5 and 6 show, respectively, the separation of CpG from GpC and CpA from ApC in an isocratic elution process.



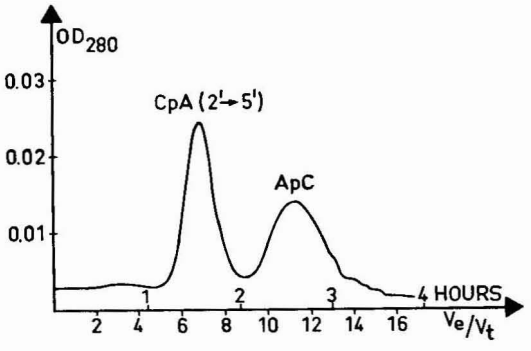


Fig. 5. Fractionation of CpG (87.5 μg per 35 μl) + GpC (87.5 μg per 35 μl). Copper column: V_t = 2.75 ml. Flow-rate: 13.2 ml/h. Buffer as in Fig. 1.

Fig. 6. Fractionation of ApC (87.5 μg per 35 μl) + $CpA_{(2'\rightarrow 5')}$ (87.5 μg per 35 μl). Copper column: V_t = 4.5 ml. Flow-rate: 19.2 ml/h. Buffer as in Fig. 1.

The retention of nucleotides on metal chelate affinity chromatography is due to complex phenomena. The abundance of potential binding sites (nitrogen and oxygen atoms on the bases, hydroxyl groups on the ribose and negatively charged oxygen atoms in the phosphate residue) account for this complexity, although our data seem to rule out any major contribution of either the phosphate or the ribose moieties to the binding.

The dominant role played by the bases in the coordination mechanism, as suggested by our results, has already been demonstrated by crystallographic studies as well as experiments in solution[3]. The nature of the bases present in an oligonucleotide is obviously very important (Figs. 1–4), but the conformation of the molecule should also be taken into account. In the case of ApC and CpA for instance, the examination of models as well as theoretical calculations[4] indicates the existence of very small differences, concerning mainly the orientation of the heterocyclic rings. These differences presumably affect the overlapping of the orbital systems of the two bases and modify also the environment of the most strongly interacting centre, *i.e.*, purine N_7 (ref. 3). This different environment of the N_7 purine binding site, along with the fact that the immobilization of the metal on a rather rigid matrix may favour the approach

of molecules having a certain type of conformation, may explain the surprisingly good separation obtained. Alternatively, the occurrence of a fifth or even sixth copper coordination bond[5] and the participation of the pyrimidine ring in a weak binding could also be responsible for the effect observed, due to a slight difference (between the two derivatives) in the base–metal–base spatial arrangement. These differences and their reflection in the chromatographic behaviour should be further explored in order to develop particularly efficient methods for fractionation of nucleotides on a preparative scale.

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REFERENCES

1 P. Hubert and J. Porath, *J. Chromatogr.*, 198 (1980) 247.
2 J. Porath and L. Sundberg, *Protides Biol. Fluids*, 18 (1970) 401.
3 H. Sigel (Editor), *Metal Ions in Biological Systems*, Vol. 8, Marcel Dekker, New York, 1979.
4 C. H. Lee, E. Charney and I. Tinoco, *Biochemistry*, 18 (25) (1979) 5636.
5 A. F. Wells, *Structural Inorganic Chemistry*, 3rd ed., Oxford University Press, London, 1967.

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Note

High-performance liquid chromatographic method for the determination of diosgenin in plants

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(First received August 26th, 1980; revised manuscript received October 7th, 1980)

Diosgenin is one of the most important and versatile starting materials for the manufacture of steroid drugs, including oral contraceptives. The steroid drug industry in India relies almost entirely on diosgenin as the base material, the chief sources being the yams of the different Dioscorea spp. New sources of diosgenin, such as *Costus speciosus*[1,2] and *Kallstroemia pubescens*[3–5], are being developed for commercial exploitation. Whether it is a Dioscorea tuber or a plant sample to be screened for the presence of diosgenin, or whether it is a cell mass from tissue culture or a plant part from a breeding programme, the diosgenin content must be determined by an accurate and reproducible method. Several procedures are available for the determination of diosgenin, such as gravimetric[6–11], spectrophotometric[12,13], gas–liquid chromatographic (GLC)[14], densitometric thin-layer chromatographic (TLC)[15,16] and IR spectrometric[17] methods. The gravimetric method is widely used on account of its simplicity and inexpensive apparatus. However, it has some disadvantages, *e.g.*, it gives the total sapogenin content and not only diosgenin. The other methods mentioned above also have disadvantages[18]. The GLC method developed by Cooke[14], although it works well with certain Dioscorea spp. where there is no interference from other substances, is unsatisfactory when such interfering compounds are present.

High-performance liquid chromatographic (HPLC) techniques have been reported for determination of various materials, including glycyrrhizin[19,20], cholesterol autoxidation products[21], potato glycoalkaloids[22] and monoterpenes[23]. The separation of diosgenin by HPLC has also been reported[24]. This paper reports an HPLC method for the determination of diosgenin in some plant species.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of a Waters Assoc. Model 6000A pump, U6K injector, Model 440 absorbance detector with a fixed wavelength of 254 nm and an R-401 differential refractometer connected to an Omniscribe recorder. The chromatograph contained a Waters Assoc. 30 cm × 3.9 mm I.D. stainless-steel column packed with a μPorasil microparticulate (10 μm) silica gel column. The mobile phase was light petroleum (b.p. 60–80°C)–isopropanol (12:1), the flow-rate 0.8 ml/min, the pressure 125 p.s.i., the temperature 20°C, the attenuation 8 × and the recorder chart speed 1 cm/min.

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Chemicals and reagents

All solvents used were glass-distilled. The diosgenin used for the preparation of the calibration graph was isolated from *Kallstroemia pubescens* and purified by chromatography [m.p. 206–207°C; $[\alpha]_D$ −119° ($CHCl_3$)].

Preparation of standards

A set of ten standard solutions were prepared containing 2.26–0.226 mg/ml of diosgenin and were stored at ambient temperature.

Extraction procedure

Plant samples were dried, either in air or at 110°C in an air oven, and then powdered. Each of the samples (500 mg) was placed in a 25-ml conical flask and hydrolysed with 2.5 *N* hydrochloric acid (7 ml) for 4 h by keeping the flask partially immersed in boiling water, keeping the mouth loosely closed with a stopper. The contents of the flask were cooled, filtered quantitatively using a Whatman No. 41 filter-paper and washed until the residue was free from acid. The washed residue was dried at 105°C and then extracted with light petroleum (b.p. 60–80°C) in a Soxhlet extractor for 4 h. The solvent was evaporated and the residue was dissolved in HPLC-grade light petroleum–isopropanol (12:1) and filtered into a measuring flask using a sample clarification kit (Millipore, Bedford, MA, U.S.A.) consisting of a 10-ml syringe, Swinney filter holder and Millipore filters (0.5 μm). The solution was then made up to a suitable concentration by dilution with the same solvent in a volumetric flask. A 20-μl volume of the solution was injected into the chromatograph with a 25-μl Hamilton syringe.

RESULTS AND DISCUSSION

As diosgenin does not exhibit any UV absorption that would be helpful for detection with the 254-nm UV detector, a differential refractometer was used. With a 30 cm × 3.9 mm I.D. column and a mobile phase flow-rate of 0.8 ml/min the retention time was 347 ± 5 sec for diosgenin based on 20 measurements. The retention time was very sensitive to the light petroleum–isopropanol mobile phase composition. Differences in retention time between batches existed, indicating a slight variation in the composition of the mobile phase from time to time. An internal standard was not incorporated in the assay procedure, because the recoveries were consistent and almost identical with the theoretical recovery.

Of all column and mobile phase combinations tried, the μPorasil column with light petroleum–isopropanol (12:1) proved to be the best because there were no interfering peaks. The line obtained by plotting peak height against concentration was always straight. Calibration graphs were constructed every time a new series of plant material was extracted. A calibration graph covering the range from 2.26 to 0.226 mg/ml of diosgenin is shown in Fig. 1.

For each analysis 500 mg of dried plant sample was used and the method was found to be quantitative and reproducible. The same results were obtained when more than 500 mg of plant material was used. However, less than 500 mg of plant sample gave a slightly low value. Ten samples of *Kallstroemia pubescens*, two samples of *Dioscorea prazeri* and one sample each of *Dioscorea floribunda*, *Dioscorea deltoidea*

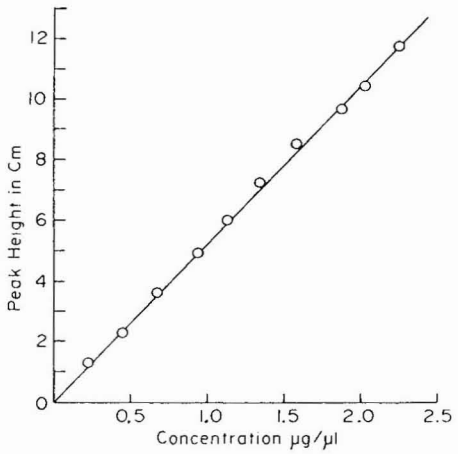

Fig. 1. Calibration graph for diosgenin.

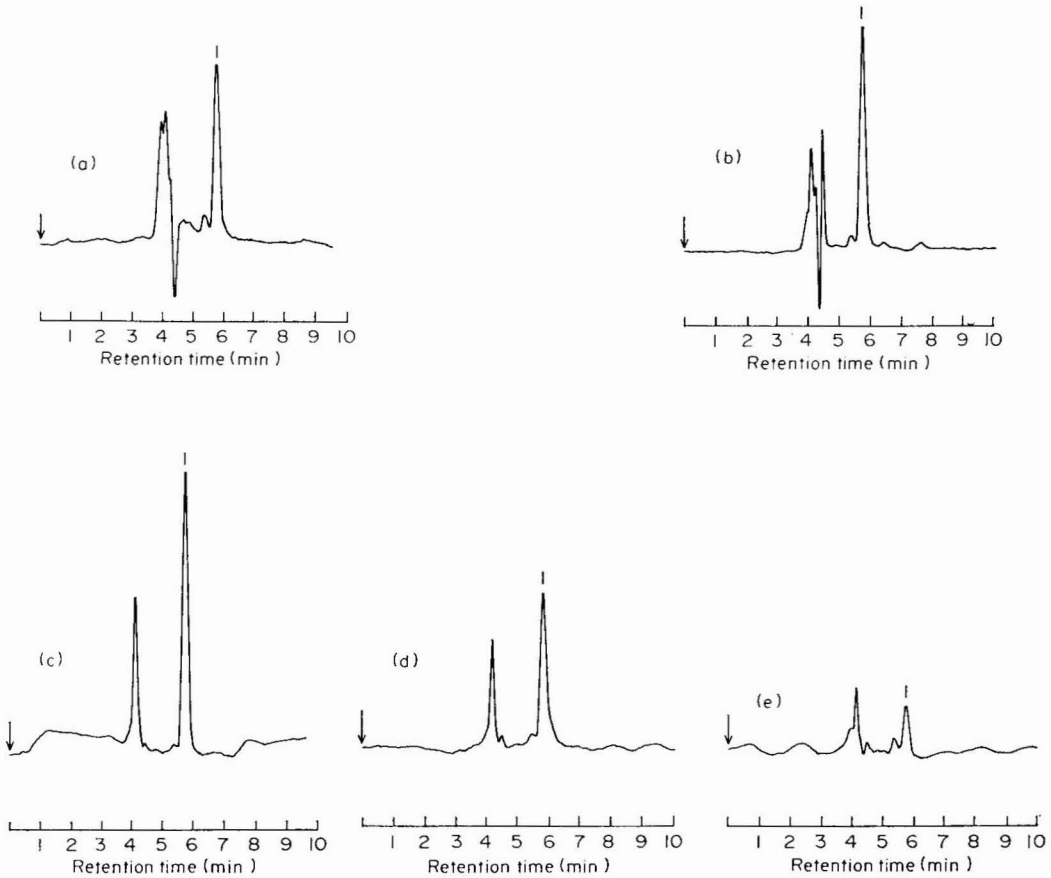

Fig. 2. Representative chromatograms of (a) *Kallstroemia pubescens*; (b) *Dioscorea floribunda*; (c) *Dioscorea deltoidea*; (d) *Dioscorea prazeri*; (e) *Costus speciosus*. Peak 1 = diosgenin.

and *Costus speciosus* were assayed by the proposed HPLC method. Chromatograms of one sample of each species are shown in Fig. 2.

Attempts were made to combine the hydrolysis and extraction stages by heating the plant sample with 2.5 *N* hydrochloric acid and *n*-hexane and working up in the usual way, but much too low values were obtained even after heating for 6 h so these attempts were abandoned.

The results obtained by HPLC were checked against the usual gravimetric determination by using aliquots from a larger sample. A comparison of the values obtained is shown in Table I. As would be expected, the results obtained by HPLC were found lower as diosgenin alone is determined, whereas in the gravimetric method other constituents present in the diosgenin extract are also determined.

TABLE I

COMPARISON OF RESULTS OBTAINED FOR SAMPLES OF *KALLSTROEMIA PUBESCENS* (KP), *DIOSCOREA FLORIBUNDA* (DF), *DIOSCOREA DELTOIDEA* (DD), *DIOSCOREA PRAZERI* (DP) AND *COSTUS SPECIOSUS* (CS) BY HPLC AND GRAVIMETRIC METHODS

Sample	*Diosgenin (%)*	
	HPLC method	*Gravimetric method*
KP-1	0.93	1.04
KP-2	1.14	1.27
KP-3	1.05	1.19
KP-4	0.88	1.01
KP-5	0.88	1.02
KP-6	0.93	1.12
KP-7	1.04	1.08
KP-8	1.06	1.11
KP-9	1.05	1.09
KP-10	1.04	1.21
DF-1	1.42	1.63
DD-1	3.48	3.70
DP-1	0.62	0.65
DP-2	1.92	2.08
CS-1	0.34	0.39

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REFERENCES

1 B. Das Gupta and V. B. Pandey, *Experientia*, 26 (1970) 475.
2 R. N. Chakravarti, S. B. Mahato, N. P. Sahu, B. C. Pal and S. C. Dutta, *J. Inst. Chem., Calcutta*, 48 (1976) 148.
3 R. N. Chakravarti, S. B. Mahato, N. P. Sahu, B. C. Pal and D. Chakravarti, *J. Inst. Chem., Calcutta*, 48 (1976) 170.
4 R. N. Chakravarti, S. B. Mahato, N. P. Sahu and B. C. Pal, *Indian Pat.*, 139136, 1975.
5 S. B. Mahato, N. P. Sahu, B. C. Pal, R. N. Chakravarti, D. Chakravarti and A. Ghosh, *J. Inst. Chem., Calcutta*, 50 (1978) 46.

6 J. W. Rothrock, P. A. Hammes and W. J. McAleer, *Ind. Eng. Chem.*, 49 (1957) 186.
7 M. P. Morris, B. A. Roark and B. Cancel, *J. Agr. Food Chem.*, 6 (1958) 856.
8 R. N. Chakravarti, D. Chakravarti and M. N. Mitra, *J. Proc. Inst. Chem., Calcutta*, 30 (1958) 106.
9 W. H. Preston (Jr), J. R. Haun, J. W. Garvin and R. J. Daum, *Econ. Bot.*, 18 (1964) 323.
10 R. N. Chakravarti, S. N. Dash and D. Chakravarti, *J. Inst. Chem., Calcutta*, 42 (1970) 165.
11 Y. Selvaraj, *Indian J. Hort.*, 28 (1971) 135.
12 E. A. Safowora and R. Hardman, *Planta Med.*, 26 (1974) 385.
13 A. K. Rishi, R. Kapoor and V. K. Vakhlu, *Curr. Sci.*, 45 (1976) 327.
14 B. K. Cooke, *Analyst (London)*, 95 (1970) 95.
15 G. Blunden, R. Hardman and J. C. Morrison, *J. Pharm. Sci.*, 56 (1967) 948.
16 K. R. Brain and R. Hardman, *J. Chromatogr.*, 38 (1968) 355.
17 K. R. Brain, F. R. Y. Fazli, R. Hardman and A. B. Wood, *Phytochemistry*, 7 (1968) 1815.
18 L. V. Asolkar and Y. R. Chadha, *Diosgenin and Other Steroid Drug Precursors*, Publication and Information Directorate, CSIR, New Delhi, 1979, p. 89.
19 S. Ogawa, A. Yoshida and Y. Mitani, *Yakugaku Zasshi*, 96 (1976) 122 and 1488.
20 Y. Akada and Y. Tanase, *Yakugaku Zasshi*, 96 (1976) 1035.
21 G. A. S. Ansari and L. L. Smith, *J. Chromatogr.*, 175 (1979) 307.
22 R. J. Bushway, E. S. Barden, A. W. Bushway and A. A. Bushway, *J. Chromatogr.*, 178 (1979) 533.
23 B. B. Jones, B. C. Clark, Jr., and G. A. Iacobucci, *J. Chromatogr.*, 178 (1979) 575.
24 J. W. Higgins, *J. Chromatogr.*, 121 (1976) 329.

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Note

Analysis of testolactone and its formulations by high-performance liquid chromatography

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(Received October 1st, 1980)

Testolactone (D-homo-17a-oxaandrosta-1,4-diene-3,17-dione) is used in the adjunctive and palliative treatment of inoperable breast cancer in women[1]. Although structurally related to the androgens, it is essentially devoid of androgenic activity in therapeutic doses. It is obtained from a number of steroidal compounds (testosterone, progesterone, 11-deoxycortisol) by microbial transformation using, for example, *Cylindrocarpon radicicola* ATCC 11011 (ref. 2). Such starting materials are therefore potential impurities.

The compendial method[3] for testolactone raw material, suspension and tablets is colorimetric, based on condensation with isoniazid. It is, therefore, non-specific, since it would be interfered with by the above mentioned starting materials if they were present as impurities. To overcome this problem partially, The National Formulary[3] (NF) requires in the raw material monograph a test for foreign steroids and other impurities, which is based on thin-layer chromatography (TLC). There is no such requirement for the suspension or the tablets, and consequently a laboratory performing the NF analysis of testolactone in formulations would be unable to detect any impurities which might be present. To alleviate this problem, a high-performance liquid chromatographic (HPLC) procedure has been developed which allows the assay of the drug and the detection of starting materials.

EXPERIMENTAL

Apparatus

A modular HPLC system was used, consisting of a pump (Constametric II, Laboratory Data Control, Riviera Beach, FL, U.S.A.) operated at 1.75 ml/min, a variable-wavelength UV detector (Schoeffel Model SF 700, Westwood, NJ, U.S.A.) set at 240 nm and a loop injector (Rheodyne septumless valve injector Model 7120, Berkeley, CA, U.S.A.) equipped with a 10-μl loop. The column was a 250 $\times$ 4.6 mm I.D. commercially available octyl silane, chemically bonded to totally porous irregularly shaped 10 μm micro-silica particles (RP-8, Brownlee Labs., Santa Clara, CA U.S.A.).

Peak retention times and areas were obtained by the use of a reporting integrator (Automation System 3385A, Hewlett-Packard, Avondale, PA, U.S.A.).

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Reagents

Testolactone was NF reference standard. Propylparaben (Aldrich, Milwaukee, WI, U.S.A.) was recrystallized commercial grade. Methanol was HPLC grade (Fisher, Fair Lawn, NJ, U.S.A.). Water was double-distilled in glass.

Mobile phase

Methanol in water (55:45); previously filtered through a membrane (HA 0.45 μm, Millipore, Bedford, MA, U.S.A.) and degassed was used.

Internal standard solution

A solution of propylparaben in aqueous methanol (60% v/v) was prepared at a concentration of 1 mg/ml.

Standard preparation

About 20.0 mg of testolactone reference standard, accurately weighed, was transferred to a 100-ml volumetric flask and dissolved in 50 ml of methanol. Internal standard solution (20.0 ml) was added. While mixing, water was added to volume.

Sample preparation

Tablets. A portion, accurately weighed, of finely powdered tablets, equivalent to about 50 mg of testolactone was transferred to a 50-ml volumetric flask, to which was added 30 ml of methanol. The flask was stoppered and was vigorously shaken for 30 min. Water was added to volume and the contents were well mixed and filtered. A 20.0-ml portion of the filtrate was pipetted and transferred to a 100-ml volumetric flask. Internal standard solution (20.0 ml) was added and mobile phase was added to volume and the contents were mixed.

Suspension. An accurately measured volume of well mixed testolactone suspension, equivalent to 100 mg, was transferred to a 100-ml volumetric flask, to which was added 60 ml of methanol. The flask was made up to volume with water, and the contents were well mixed and filtered (if necessary). A 20.0-ml portion of the clear solution was treated as above (*Tablets*).

Procedure

A 10-μl portion of the standard preparation and the sample preparation was successively injected into the column via the 10-μl loop injector.

The ratio (R) of the area of testolactone to the area of internal standard was recorded for the standard preparation and sample preparation. The concentration of testolactone in sample preparation was obtained by the following formula:

$$C_u = C_s \cdot R_u / R_s$$

where C_u is concentration of testolactone in sample preparation, C_s is concentration of testolactone in standard preparation (mg/ml), R_u is ratio of areas for sample preparation and R_s is ratio of areas for standard preparation.

RESULTS AND DISCUSSION

Baseline resolution was achieved between solvent front, benzyl alcohol, testolactone internal standard and with the three potential synthesis residues, namely testosterone, progesterone and 11-deoxycortisol[2] (Table I). The limit of detectability of each impurity was found to be 0.1 % w/w of testolactone. No impurity was detected from either the tablets or the suspension. Sample preparations were relatively stable, and no detectable degradation products appeared within 48 h.

TABLE I

CAPACITY FACTORS OF TESTOLACTONE AND RELATED COMPOUNDS

Substance	$k'(t\text{-}t_0/t_0)$
Testolactone	1.89
Testosterone	14.35
Progesterone	32.27
11-Deoxycortisol	6.12
Propylparaben	4.78
Benzylalcohol*	1.05
Degradation product	3.69
Androsta-1,4-diene-3,17-dione**	5.35
Androsta-1,4-diene-17-B-ol-3-one**	8.51

* Benzylalcohol was included because it is present in the suspensions.
** These two compounds are included because they are potential impurities.

Linear response *versus* concentration was found up to a concentration of 3 μg injected. Within this range, the standard curve passed close to the origin and its correlation coefficient was nearly ideal (0.99996).

Quantitative analysis of two commercial formulations are compared in Table II to the NF colorimetric procedure. All results are within compendial limits (90–110 % for tablets and 90–120 % for suspensions). Coefficients of variation are excellent. The difference of about 2.0 % between results is most likely explained by the inherent differences between the two techniques.

TABLE II

COMPARISON OF HPLC TO NF ASSAY PROCEDURE

Values are the average of five determinations.

Formulation	*HPLC (%)*	*NF (%)*
Testolactone tablet	99.0 $\pm$ 0.6	100.4 $\pm$ 0.6
Testolactone suspension	108.1 $\pm$ 1.1	109.9 $\pm$ 1.9

CONCLUSION

This HPLC procedure for the analysis of testolactone and its formulations is fast, specific and accurate.

REFERENCES

1 J. E. Hoover (Editor) *Remington's Pharmaceutical Sciences*, Mack Publishing, Easton, PA, 15th ed., 1975, p. 932.
2 J. Fried, R. W. Thoma and A. Klingsberg, *J. Amer. Chem. Soc.*, 75, 5764 (1953).
3 *The National Formulary*, Mack Publishing, Easton, PA, XIVth ed., 1975, p. 683.

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Note

Shortened purification procedure of a spleen-derived immunosuppressive peptide

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Several studies have shown that activated T cell supernatants[1–3], sera fractions[4,5] and lymphoid organs[6–11] contain immunosuppressive agents which depress the humoral response in mice.

Working with bovine spleen extracts, we demonstrated[12] that in this case the immunosuppressive activity could be attributed to the presence of a low-molecular-weight spleen-derived immunosuppressive peptide (SDIP) which was non-covalently bound to high-molecular-weight carriers. Taking advantage of this observation, we isolated the active fraction through ultrafiltration (Amicon PM 10) of an acetic extract prepared from a bovine spleen acetonic powder[13]. Then we developed a reproducible but tedious purification procedure (Fig. 1) which led to 10^7-fold purified SDIP[14].

As a preliminary high-performance liquid chromatographic (HPLC) analysis of SDIP showed that the biologically active agents could be separated from some contaminants which were still present in the purified fraction, this technique has been developed in order to curtail the actual purification procedure. The results presented here show that, from the ultrafiltrate, a three-step purification procedure (one molecular sieving plus two HPLC steps) yields an homogeneous substance.

EXPERIMENTAL

As evaluation of the isolated substance by weighing was inpractical in routine work, the concentration and quantities were estimated by measuring the optical density of aqueous solutions at 220 nm. One $A_{220\ nm}$ unit represents the quantity of substance dissolved in 1 ml of water which has an optical density of 1 at 220 nm in a 1-ml cuvette.

Preparation of the ultrafiltrate, U

The extraction procedure and the ultrafiltration were performed in a pilot plant, using 10–100 kg spleen. An acetone extract was obtained from spleen, the solvent removed and the powder was extracted with water. The extract was concen-

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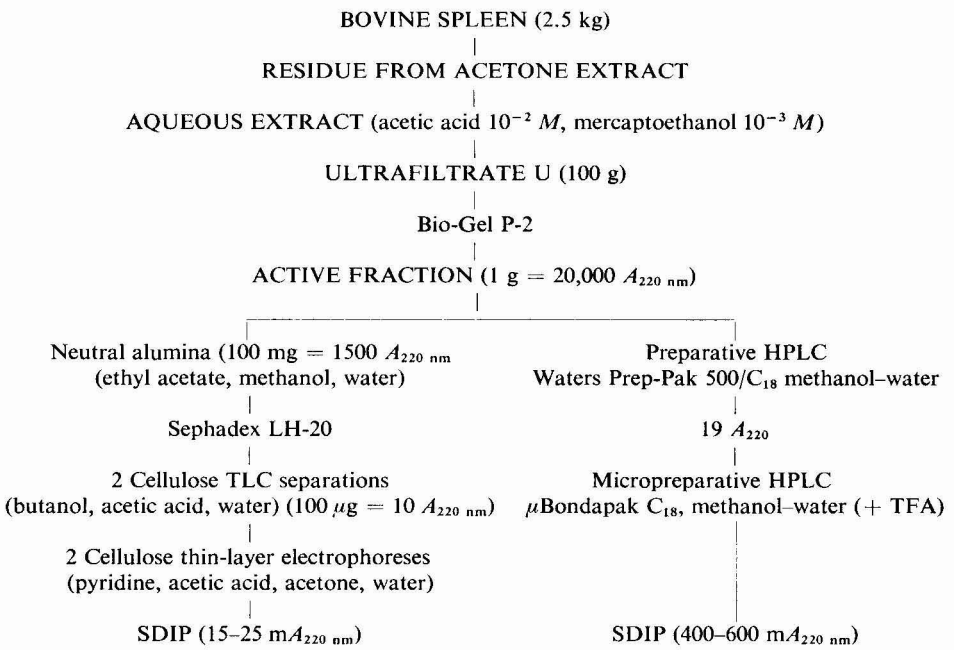

Fig. 1. SDIP purification procedures.

trated ten-fold by ultrafiltration on Sartorius membranes. After 1:50 dilution with water, the concentrate was again subjected to ultrafiltration. The successive ultrafiltrates thus obtained contained the active factor.

Chromatography of fraction U

The ultrafiltrates were chromatographed on a Bio-Gel P-2 column (750 × 95 mm) as previously described[13], using 10^{-2} *M* acetic acid as solvent (flow-rate 3 ml/min). The active fraction was eluted between 2.6 and 3.4 V_0, where V_0 is the void volume of the column.

Preparative HPLC

A Waters Assoc. Prep LC/system 500 and a Gilson Spectochrom UV detector were used. Two cartridges of Prep-Pak 500/C_{18} (30 × 5.7 cm, particle size 37 μm) (Waters) were used and equilibrated in the eluent prior to sample injection. A 1.6-g amount of the active fraction was dissolved in 40 ml methanol–water (80:20) and injected. The column was eluted with methanol–water (80:20) at a rate of 200 ml/min.

Microscale HPLC purification of SDIP

A Waters HPLC system equipped with a Pye Unicam LC_3 UV detector was used. Separation was carried out on a μBondapak C_{18} stainless-steel column (30 cm × 4 mm) (Waters) using methanol–water (+ 0.1% trifluoroacetic acid, TFA) (38:62) as solvent. Flow-rate was 1 ml/min. Peak heights at 215 nm were recorded. The sample

to be injected was dissolved in water (1 ml) and passed through a Millipore filter. All experiments were carried out at room temperature.

The bulk of biological activity was eluted between 34 and 40 min (Fig. 2). The active peak was collected and analysed under the same conditions and on the same column using acetonitrile–isopropanol–water (+ 0.1 % TFA) (225:75:700) as eluent, as shown in Fig. 3. Electrophoresis confirmed that the isolated substance is homogeneous.

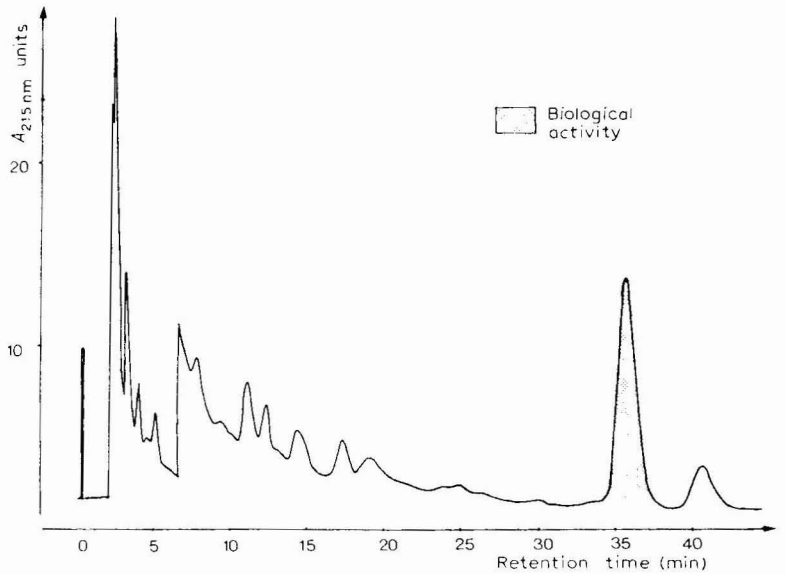

Fig. 2. HPLC chromatogram of SDIP on a μBondapak C_{18} column, with methanol–water (+ 0.1 % TFA) (38:62) as solvent. $A_{215\,nm}$ units are $\times 10^3$.

Compared to previously established purification schemes, application of HPLC permits very rapid recovery of a highly pure substance, with a considerably improved yield. Furthermore, these analytical conditions should allow the detection of SDIP in different organ extracts, and the comparison of SDIP to the different lymphokines described in the literature which exhibit analogous biological activities.

HPLC analysis of purified SDIP

The previously described apparatus and conditions were used. The solvent was acetonitrile–isopropanol–water (+ 0.1 % TFA) (225:75:700) or methanol–water (+ 0.1 % TFA) (38:62).

RESULTS AND DISCUSSION

Bovine spleen was extracted in a pilot plant as previously described[13]. In brief, lipids and water were removed from bovine tissue by acetone extraction. The powder was extracted in acetic acid (10^{-2} *M*) in presence of 2-mercaptoethanol (10^{-3} *M*) and the aqueous extract was submitted to ultrafiltration. The ultrafiltrates were chromatographed on a Bio-Gel P-2 column, using dilute acetic acid solution as eluent.

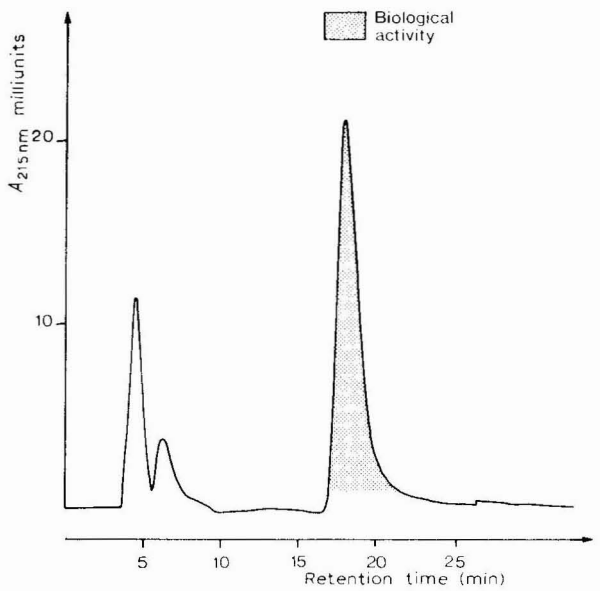

Fig. 3. HPLC analysis of purified SDIP on a μBondapak C_{18} column, with acetonitrile–isopropanol–water (+ 0.1% TFA) (225:75:700) as solvent.

The active fractions were pooled and submitted to preparative reversed-phase liquid chromatography on cartridges of Prep-Pak 500/C_{18} (Waters), using methanol–water (80:20) as solvent. This technique allowed the separation of roughly 99% of the inactive material eluted before 16 min from the active material which is retained on the column and eluted between 42 and 60 min.

These fractions were then submitted to a micropreparative separation on a μBondapak C_{18} column using as elution system the mixture methanol–water (+ 0.1% TFA) (38:62), which has recently been recommended for peptide separations[15].

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REFERENCES

1 R. H. Gisler and W. H. Fridman, *J. Exp. Med.*, 142 (1975) 507.
2 A. S. Rubin, *Immunology*, 36 (1979) 629.
3 D. W. Thomas, W. K. Roberts and D. W. Talmage, *J. Immunol.*, 114 (1975) 1616.
4 M. Voorting Hawkin and J. G. Michael, *J. Immunol.*, 118 (1977) 505.
5 B. F. Wang, A. M. Badger, R. R. Nimberg, S. R. Cooperband, K. Schmid and J. A. Mannick, *Cancer Res.*, 37 (1977) 3022.
6 I. Blazsek and D. Gaal, *Cell Tissue Kinet.*, 11 (1978) 265.
7 J. Grundboeck-Jusko, *Acta Biochim. Pol.*, 23 (1976) 165.
8 J. C. Houck, *Reticuloendothel. Soc.*, 24 (1978) 571.
9 N. Kiger, I. Florentin, G. Lorans and G. Mathe, *Immunology*, 33 (1977) 439.
10 U. Ernstrom and K. Nordlind, *Acta Pathol. Microbiol. Scand., Sect. A*, 85 (1977) 105.
11 K. Ward and A. J. Munro, *Immunology*, 37 (1979) 61.
12 M. Lenfant, M. Privat de Garilhe, E. Garcia-Giralt and C. Tempete, *Biochim. Biophys. Acta*, 451 (1976) 106.
13 M. Lenfant, E. Garcia-Giralt, M. Thomas and L. Di Giusto, *Cell Tissue Kinet.*, 11 (1978) 455.
14 M. Lenfant, E. Garcia-Giralt, L. Di Giusto and M. Thomas, *Mol. Immunology*, 17 (1980) 119.
15 D. Voskamp, C. Olieman and H. C. Beyerman, *Rec. Trav. Chim. Pays-Bas*, 99 (1980) 105.

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Note

High-performance liquid chromatographic analysis of technical Ruelene insecticide

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Ruelene insecticide which contains the active ingredient, 4-*tert.*-butyl-2-chlorophenyl methyl methylphosphoramidate (common name, crufomate) has found use as an anthelmintic and insecticide[1,2]. Besides the active ingredient, this product contains two significant impurities, 4-*tert.*-butyl-2-chlorophenyl dimethyl phosphate (triester) and 4-*tert.*-butyl-2-chlorophenyl N,N^1-dimethylphosphorodiamidate (diamidate).

Technical Ruelene insecticide has been analyzed internally by an infrared (IR) method using the $POCH_3$ vibration. Although giving good results, it requires two scans, a differential scan for impurities and a scan for assay. Based on the concentrations for those impurities having $POCH_3$ absorption, a correction of the assay value is made.

Gas chromatography[3–5] has been used for trace crufomate determination. It has also been used internally for assay; however, extensive column conditioning and frequent standardization are necessary to obtain satisfactory results.

This product has been analyzed gravimetrically[6] based on phosphorus content but this approach is non-specific.

High-performance liquid chromatography (HPLC) seemed like a viable approach for analysis of the technical product.

EXPERIMENTAL

Reagents

(a) 4-Chlorophenol, 99%+, Cat. No. 18578-7 (Aldrich, Milwaukee, WI, U.S.A.).

(b) Crufomate, 99% (Dow Chem.). Purity and identification were made by appropriate tests.

(c) Triester (see b).

(d) Diamidate (see b).

(e) 4-*tert.*-Butyl-2-chlorophenol (see b).

(f) Acetonitrile, distilled-in-glass grade (Burdick & Jackson Labs., Muskegon, MI, U.S.A.).

(g) Eluent, 50% v/v acetonitrile–water.

(h) Diamidate–triester stock solution. Weigh 25 mg of the diamidate and triester standard to the nearest 0.01 mg into the same 25-ml volumetric flask. Dissolve and dilute to volume with acetonitrile.

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(i) Calibration solution. Weight 20–30 mg 4-chlorophenol to the nearest 0.01 mg into a 4-dram vial. Into the same vial weigh 40–60 mg of crufomate standard. Pipet in 2 ml of diamidate–triester standard (h) and follow with 8 ml of acetonitrile.

Apparatus

Liquid chromatograph-modular: a unit composed of a model M-6000 pump (Waters Assoc., Milford, MA, U.S A.), a Model 70-10 injection valve with 20-μl loop (Rheodyne, Berkeley, CA, U.S.A.), a variable wavelength detector, Model LC 55 (Perkin-Elmer, Norwalk, CT, U.S.A.), a computing integrator, Model 3380A, Hewlett-Packard, Avondale, PA, U.S.A.), a μBondapak C_{18} reversed-phase column (30 cm $\times$ 3.9 mm), Cat. No. 27324 (Waters Assoc.) protected with a guard column (50 $\times$ 2.1 mm) packed with pellicular Corasil C_{18} (Waters Assoc.).

Preparation of sample

Weigh 20–30 mg 4-chlorophenol internal standard to the nearest 0.01 mg into a 4-dram vial. Into the same vial weigh 40–60 mg of Ruelene insecticide sample. Add 10 ml of acetonitrile$_1$

Determination

Inject 20-μl aliquots of the calibration solution (i) until the response factor varies $<1\%$ for consecutive injections. Inject 20-μl duplicate aliquots of the sample solution. Record the peak areas on a computing integrator and average the results. Calculation

$$R_F = P_{ir} W_r / W_{ir} P_r$$

where R_F = response factor, P_{ir} = internal standard peak area in reference standard solution, P_r = crufomate standard peak area in reference standard solution, W_{ir} = weight in grams of internal standard and W_r = weight in grams of crufomate standard.

$$\% \text{ crufomate} = P_c W_{is} R_F P / P_{is} W_s$$

where R_F = response factor, P_c = peak area of crufomate peak in sample solution, P_{is} = peak area of internal standard peak in sample solution, W_s = weight in grams of sample; W_{is} = weight in grams of internal standard and P = purity of standard.

RESULTS AND DISCUSSION

High-performance liquid chromatograms for a Ruelene insecticide sample and standard are presented in Fig. 1.

Coefficient of variation data for the analysis of technical Ruelene insecticide was obtained by analyzing one sample five times on two successive days. Results are given in Table I. The values for the diamidate and triester were obtained in the same run as the assay value therefore their coefficients of variation would be expected to greater than crufomate.

Recovery data was obtained by preparing synthetic samples and analyzing these by the method. Results are presented in Table II.

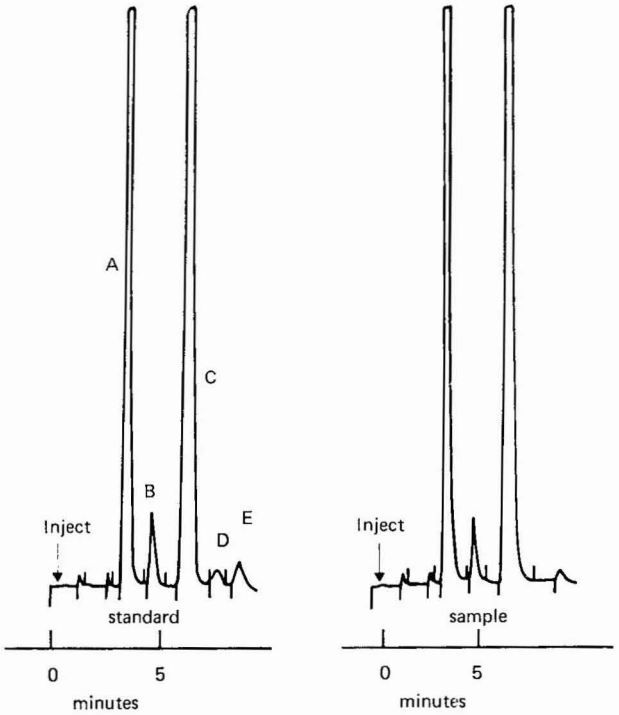

Fig. 1. Liquid chromatogram for 4-chlorophenol (internal standard) (A), diamidate (B), crufomate (C), 4-*tert*.-butyl-2-chlorophenol (D) and triester (E).

TABLE I

PRECISION STUDY FOR THE ANALYSIS OF RUELENE INSECTICIDE

	Diamidate (%)	*Crufomate* (%)	*Triester* (%)
	2.64	94.08	1.75
	2.61	93.91	1.75
	2.67	94.17	1.86
	2.61	93.69	1.79
	2.63	93.21	1.69
	2.43	93.78	1.73
	2.42	93.72	1.84
	2.44	93.85	1.89
	2.45	93.63	1.81
	2.44	93.79	1.83
Mean (%)	2.53	93.78	1.79
S. D. (%)	0.10	0.26	0.063
Coeff. of Var. (%)	4.0	0.28	3.5

The retention times for a number of potential impurities were determined. None of these was found to interfere with the internal standard or assay peak. They are shown in Table III.

TABLE II

ANALYSIS OF KNOWN MIXTURES OF RUELENE INSECTICIDE

Mixture	*Diamidate (%)*			*Crufomate (%)*			*4-tert.-Butyl-2-chlorophenol (%)*		*Triester (%)*		
	By wt.	*By LC*	*Recovery*	*By wt.*	*By LC*	*Recovery*	*By wt.*	*By LC*	*By wt.*	*By LC*	*Recovery*
1	3.86	3.48	90	92.40	92.49	100.1	0.60	0.62	3.09	3.13	101
2	6.12	5.35	87	88.92	88.56	99.6			5.31	4.96	93
3	2.51	2.42	96	95.38	95.72	100.4	0.59	0.61	1.36	1.52	112
4	1.00	1.17	117	94.15	94.25	100.1			5.00	4.85	97
5	1.06	1.21	114	97.15	97.13	100.0	0.50	0.50	1.18	1.29	109
6	3.54	3.25	92	93.04	93.25	100.2	0.55	0.59	2.64	2.87	109
7	5.14	4.55	89	92.10	92.12	100.0	1.20	1.38	1.48	1.56	105
8	2.52	2.35	93	92.88	93.03	100.2			4.96	4.60	92
9	1.04	1.14	110	92.15	92.50	100.4	0.49	0.55	6.56	6.32	96
10	3.14	2.88	92	95.59	95.56	100.0			1.17	1.27	109
Mean recovery (%)			98			100.1					102
Coeff. of var. (%)			11			0.23					7

TABLE III

HPLC RETENTION TIMES FOR RUELENE INSECTICIDE AND POTENTIAL IMPURITIES

Component	*Time (min)*
Solvent front	1.04
4-*tert.*-Butyl-2-chlorophenyl methyl phosphate dimethylamine salt	1.18
Methyl phenyl N-methylphosphoramidate	2.13
2-Chlorophenyl methyl N-methylphosphoramidate	2.56
4-Chlorophenyl methyl N-methylphosphoramidate	2.71
4-Chlorophenol (internal standard)	3.26
2,4-Dichlorophenyl methyl N-methylphosphoramidate	3.56
4-*tert.*-Butyl-2-chlorophenyl N,N'-dimethylphosphorodiamidate (diamidate)	4.50
4-*tert.*-Butyl methyl N-methylphosphoramidate	4.58
4-*tert.*-Butyl-2-chlorophenyl methyl N-methylphosphoramidate (crufomate)	6.32
4-*tert.*-Butyl-2,6-dichlorophenyl methyl N-methylphosphoramidate	7.49
4-*tert.*-Butyl-2-chlorophenyl dimethyl phosphate (triester)	8.97
Bis(4-*tert.*-butyl-2-chlorophenyl) methyl phosphate	~30
Bis(4-*tert.*-butyl-2-chlorophenyl) methylphosphoramidate	~30
Tris(4-*tert.*-butyl-2-chlorophenyl) phosphate	~30

A wavelength of 270 nm was chosen for monitoring the separation because it is very near the wavelength of maximum absorption for crufomate and related impurities.

A methanol–water eluent was also tried for this separation; however, 4-*tert.*-butyl-2-chlorophenol was not separated from the other components.

REFERENCES

1 E. C. Turner, D. F. Watson and F. S. McClaugherty, *J. Am. Vet. Med. Ass.*, 141 (1962) 360–361.
2 C. O. Knowles and J. F. Casida, *J. Agr. Food Chem.*, 14 (1966) 566–572.
3 M. C. Bowman and M. Beroza, *J. Ass. Offic. Anal. Chem.*, 53 (1970) 499–508.
4 V. V. Ermakov, *Zh. Anal. Khim.*, 24 (1969) 1264–1270.
5 R. R. Watts and R. W. Storherr, *J. Ass. Offic. Anal. Chem.*, 52 (1969) 513–521.
6 K. I. Korol'kova and I. M. Shabanov, *Tr. Inst. Kraev. Patol. Min. Zdravookhr. Kaz. SSR*, 16 (1969) 65–67.

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Note

Isolation and purification of Amadori compounds by semi-preparative reversed-phase high-performance liquid chromatography

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During heat processing or storage of foods and beverages the non-enzymatic Maillard browning reaction occurs between the carbonyl group of a reducing sugar and the amino group of an amine or amino acid. In the first steps of the reaction, Amadori compounds (1-amino-1-deoxy-2-ketoses) are formed. These compounds have been separated from several biological materials and are considered as precursors of the colour, aroma and flavour of processed foods[1–5]. The bound amino acid in an Amadori compound is not available as a source of amino acid which results in a decrease in the nutritional value of the foods[6–8]. Furthermore, the Maillard reaction has been shown to be responsible for some physiological and toxicological effects[9–11]. There is thus a growing interest in these compounds.

For their analysis in natural products, high-performance liquid chromatography (HPLC) on a cation-exchange resin[12] or in the reversed-phase mode[13] has been proved to be very suitable. However, the Amadori compounds have been investigated to date in relatively few biological products. This fact is due to the tedious procedures for their isolation and purification. The methods generally described[14–18] comprise multiple ion-exchange chromatographic separations which are time-consuming and result in poor yields.

In the first step of our work on the separation of Amadori compounds from biological materials we tried to isolate and purify them in a short time and in a sufficient amount for further systematic studies. We first confirmed the results described in the literature[13] on the separation of Amadori compounds from D-glucose and the aromatic amino acids by ionic suppression and ion-pairing reversed-phase HPLC. These procedures were suitable for the investigation of a number of Maillard reactions starting from aliphatic amino acids. In some cases, the separation of the Amadori compound on a C_{18} reversed-phase analytical column, using water as eluent, has proved to be efficient. An application of these results to the analysis on a C_{18} reversed-phase semi-preparative column of crude Maillard reactions between valine and glucose and between proline and maltose allowed the direct isolation and purification of the Amadori compounds as free products. In our preliminary work, we found it more convenient to use as a model system a mixture of proline (PRO) and its Amadori compound proline–fructose (PRO–FRU) previously obtained by crystallization[1,19].

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EXPERIMENTAL

Reagents

Amino acids and sugars were from Fluka (Buchs, Switzerland). Water as eluent for the HPLC analysis was very high purity grade. Samples and water for HPLC were passed through a 0.45-μm Sartorius filter.

Thin-layer plates were coated with Polygram SIL G silica gel (Machery, Nagel & Co, Düren, G.F.R.).

Preparation of model Maillard browning systems

L-Valine (0.01 mole) and (+)-D-glucose monohydrate (0.03 mole) were dissolved in methanol (200 ml) and refluxed for 1 h. After cooling, the solution was evaporated to dryness under reduced pressure at 15°C. The residue was extracted with 150 ml anhydrous methanol and refluxed for another 3 h, removing water as its benzene azeotrope. After cooling, the mixture was evaporated to dryness and dissolved in a small amount of water for the HPLC analyses. The crude samples were injected directly into the liquid chromatograph.

L-Proline (0.010 mole) and (+)-D-maltose monohydrate (0.011 mole) were refluxed for 1 h in 200 ml methanol. After cooling, the solution was evaporated to dryness and the residue dissolved in 150 ml anhydrous methanol. Refluxing was continued, removing water as its benzene azeotrope, for another 8 h. The solution was then evaporated to dryness and the residue dissolved in a small amount of water for the HPLC analyses. The reactions were followed by thin-layer chromatography (TLC) on silica gel (eluent: methanol).

HPLC apparatus and procedures

The chromatographic system (Waters Assoc., Milford, MA, U.S.A.) included a Model 6000 A pump, a Model U6K injector and a Model R-401 differential refractometer. The analytical column (300 × 3.9 mm I.D.) was packed with Nucleosil 10 C_{18} (Machery, Nagel & Co.). The semi-preparative column (600 × 8 mm I.D.) was packed with 30-μm RSIL C_{18} HL (R.S.L., Eke, Belgium). The precolumn (25 × 3.9 mm I.D.) was packed with 30–38 μm CO:PELL ODS (Chrompack, Middelburg, The Netherlands).

On the analytical column, aliquots (25–30 μl) of the Maillard reactions were separated at a water flow-rate of 0.3–1 ml/min. On the semi-preparative column, aliquots (500–700 μl) were separated at a water flow-rate of 3–7 ml/min. In both these separations, the mobile phase flow-rate was increased after the elution of the Amadori compounds.

Spectrometric apparatus and procedures

The IR spectrophotometer was a Model PE 580 E (Perkin-Elmer). The Cameca NMR spectrometer was operated at 250 MHz. The ^{13}C NMR spectra were conducted in ^2H_2O as solvent with DSS as internal standard (δ TMS = δ DSS*). The Ribermag R 10/10 quadrupole mass spectrometer coupled to the Sidar 111 A data processing system was purchased from Nermag. The analyses were carried out according to the chemical ionization–desorption (CI/D) technique[20]. The reactant gas

* DSS = Sodium trimethylsilyl propionate-d_4.

used was NH_3. In this case the CI/D analyses generally give three ions corresponding to M, M + H^+ (M + 1) and M + NH_4^+ (M + 18). Conditions: desorption, 40–500 mA; speed 0.7 mA/sec; desorption point 378 mA; source temperature 70°C. For the desorption process, 1 μl of a methanolic solution of the sample (5 mg in 5 ml) was deposited on the filament.

RESULTS AND DISCUSSION

The analytical HPLC profiles of the crude Maillard reactions between valine and glucose and between proline and maltose are shown in Figs. 1A and 2A respectively. In the latter case, the presence on the chromatogram of glucose and PRO–FRU is proof of some thermal decomposition of PRO–maltulose during the reflux process.

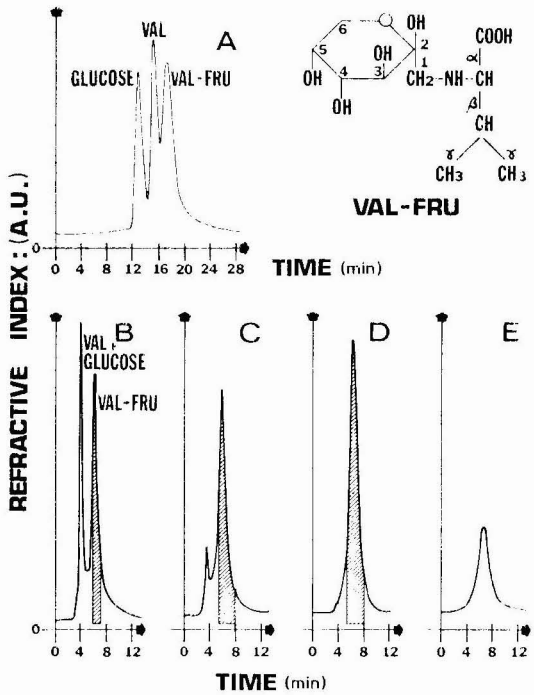

Fig. 1. HPLC profiles of the crude Maillard reaction between valine and glucose on the analytical column (A) and the semi-preparative column (B). Two successive purifications (C and D) of the collected peak of VAL–FRU in B give a pure compound (E). Chromatographic conditions: A, Nucleosil 10 C_{18}, mobile phase (water) flow-rate 0.3–1 ml/min; B, 30-μm RSIL C_{18} HL, mobile phase (water) flow-rate 3–7 ml/min. A.U. = Arbitrary units.

Figs. 1B and 2B show the analysis of the two samples on the semi-preparative column. The peaks are not as well resolved as in A, but the fraction containing the Amadori compound can be recovered (shaded portions on the chromatograms).

After evaporation of the fractions to a small volume a second chromatographic separation was carried out. Three purifications were generally sufficient to obtain

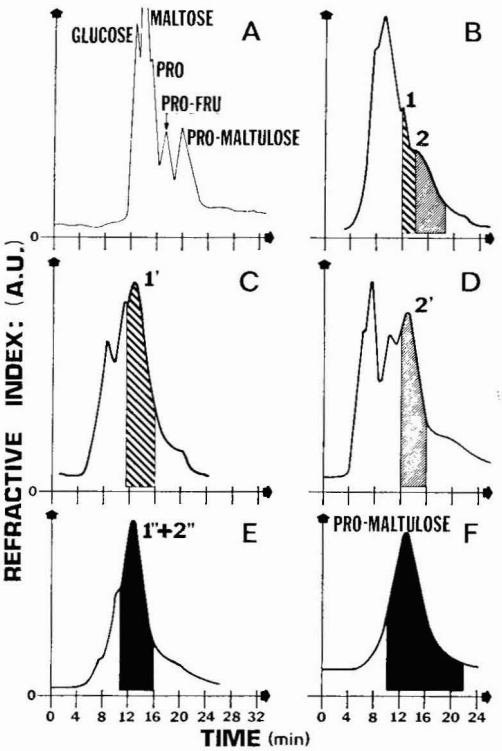

Fig. 2. HPLC profiles of the crude Maillard reaction between protine and maltose on the analytica column (A) and the semi-preparative column (B). Fraction 1 containing PRO–FRU and significant traces of PRO–maltulose is purified in C. Fraction 2 containing PRO–maltoluse is purified in D. Both collected fractions 1′ and 2′ contain PRO–maltulose and are purified together in E and F. Chromatographic conditions as in Fig. 1.

the pure Amadori compound. The residue, obtained after evaporation to dryness of the last purified fractions, was crystallized three times from a small amount of anhydrous methanol. We obtained 900 mg VAL–FRU (yield: 30% from valine) and 500 mg PRO-maltulose (yield: 10% from proline). Both yields can be optimized. The purity of the Amadori compounds was examined by TLC and HPLC on the analytical column. Their structures were confirmed as follows.

VAL–FRU

IR (KBr): ν (C = 0) at 1620 cm^{-1}. Mass spectrum: parent VAL–FRU, 280 (M + 1); fragments: fructose, 180 (M) and valine, 118 (M + 1). ^{13}C NMR: the chemical shifts with respect to tetramethylsilane (TMS) (Table I) were compared with literature data[21–23]. In ^2H_2O as solvent, VAL–FRU can occur as an equilibrium mixture of the furanose and pyranose structures α and β via the open-chain structure. Based on C-2 signals, the percentages of the different configurations are: β-pyranose, 71%; β-furanose, 23% and α-furanose, 6%. The predominance of the β-pyranose configuration at neutral pH confirms the literature[12] results.

TABLE I

^{13}C NMR ASSIGNMENTS FOR VAL–FRU (FIG. 1)

p = Pyranose; f = furanose.

δ (ppm)/TMS	*Fructose*		*Valine*
	Carbon atom	*Configuration*	*carbon atom*
175.21 175.03			COOH
101.59 98.06 98.00	2	α-p, β-f, β-p	
85.38 85.29	3 4	α-f, α-f, β-f	
80.88 78.73	3 5	β-f α-f, β-f	
72.91 72.11 71.85 71.73 71.67 71.52	3, 4, 5	α-p, β-p	
66.64 63.55	1, 6	α-p, α-f, β-f	
60.22 56.13			C-α
31.80 31.68			C-β
21.24 21.09 19.89 19.74			C-γ

PRO–maltulose

IR (KBr): ν (C = O) at 1627 cm^{-1}. Mass spectrum: no parent peak of PRO–maltulose was found, probably due to the thermal instability of this compound. Fragmentation peaks: proline 116 (M + 1), 133 (M + 18); glucose, fructose 180 (M), 198 (M + 18), 216 (M + 2 × 18); PRO–FRU 278 (M + 1); maltulose 342 (M − H_2O), 378 (M + 2 × 18). ^{13}C NMR: the assignments to the sugar moiety could not be made with precision on account of the complexity of the spectrum due to the equilibrium in solution between the different configurations (Table II). The assignments were compared with the literature data[21–23].

Based on the above results, we consider that the semi-preparative HPLC separations of Amadori compounds from crude Maillard reactions can be conducted rapidly, with good recoveries of the desired product and low solvent cost. The availability of sufficient amounts of the Amadori compounds in a shorter time would permit more systematic studies of them and of their biological roles.

TABLE II

^{13}C NMR ASSIGNMENTS FOR PRO-MALTULOSE (FIG. 3)

δ (ppm)/TMS	*Maltulose*		*Proline*
	Carbon atom	*Configuration*	*carbon atom*
176.59			COOH
176.45			
103.36			
100.65			
98.50	2	α-p, α-f, β-p, β-f	
98.45			
85.20			
82.97	3, 4	α-f, β-f	
80.53			
75.61			
75.50			
75.29			
75.17	2′, 3′, 4′, 5′		
74.52			
74.23			
73.91			
72.41			
72.23	3, 4, 5	α-p, β-p	
72.11			
71.58			
66.58			
63.37	1, 6	α-p, α-f, β-f	
63.23			
60.02			C-α
51.72			C-δ
31.48			C-β
31.04			
26.15			
26.06			C-γ
26.01			

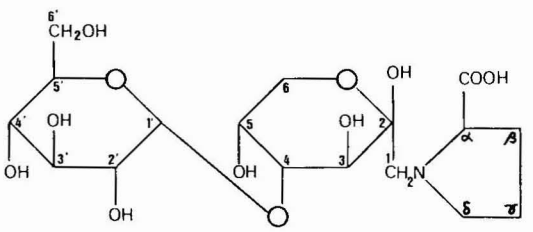

Fig. 3. Structure of PRO-maltulose.

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REFERENCES

1 F. D. Mills and J. E. Hodge, *Carbohyd. Res.*, 51 (1976) 9.
2 F. D. Mills, *J. Agr. Food Chem.*, 27 (5) (1979) 1136.
3 R. Tressl, *Monatsschr. Brau.*, 32 (1979) 240.
4 T. Kossa, D. Bahri and R. Tressl, *Monatsschr. Brau.*, 32 (1979) 249.
5 L. Friese, F. Fendesack and R. Tressl, *Monatsschr. Brau.*, 32 (1979) 255.
6 J. Adrian and J. C. Favier, *Ann. Nutr. Aliment.*, 15 (1961) 181.
7 J. Adrian, *Ann. Nutr. Aliment.*, 21 (1967) 129.
8 M. Tanaka, T. C. Lee and C. O. Chichester, *J. Nutr.*, 105 (8) (1975) 989.
9 M. Tanaka, M. Kimiagar, T. C. Lee and C. O. Chichester, *Advan. Exp. Med. Biol.*, 86 B (1977) 321
10 E. Spingarn and C. T. Garvie, *J. Agr. Food. Chem.*, 27 (6) (1979) 1319.
11 K. Heyns, S. Röper, H. Röper and B. Meyer, *Angew. Chem., Int. Ed. Engl.*, 18 (11) (1979) 878.
12 G. A. M. Van den Ouweland, H. G. Peer and S. B. Tjan, in G. Charalambous (Editor), *Flavor of Foods and Beverages, Chemistry and Technology*, Academic Press, New York, San Francisco, London, 1978, p. 131.
13 G. R. Takeoka, J. R. Coughlin and G. F. Russell, in G. Charalambous (Editor), *Liquid chromatographic Analysis of Food and Beverages*, Vol. 1, Academic Press, New York, San Francisco, London, 1979, p. 179.
14 A. Abrams, P. H. Lowy and H. Borsook, *J. Amer. Chem. Soc.*, 77 (1955) 4794.
15 E. F. L. J. Anet, *Aust. J. Chem.*, 10 (1957) 193.
16 F. D. Mills, B. G. Baker and J. E. Hodge, *J. Agr. Food Chem.*, 17 (1969) 723.
17 J. E. Hodge and B. E. Fischer, *Methods Carbohyd. Chem.*, 2 (1963) 99.
18 H. Hashiba, *J. Agr. Food Chem.*, 24 (1976) 70.
19 G. A. M. Van den Ouweland, personal communication.
20 P. J. Arpino and G. Devant, *Analusis*, 7–8 (1979) 348.
21 E. Breitmaier and V. Voelter (Editors), *^{13}C-NMR Spectroscopy, Methods and Applications in Organic Chemistry*, Verlag Chemie, Weinheim, New York, 2nd ed., 1978.
22 D. Doddrell and A. Allerhand, *J. Amer. Chem. Soc.*, 93 (11) (1971) 2779.
23 W. Funcke and A. Klemer, *Carbohyd. Res.*, 50 (1976) 9.

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Note

Potential errors in benzoylecgonine and cocaine analysis

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Benzoylecgonine is considered to be the major metabolite of cocaine in man. Many methods have been reported for its detection and measurement in urine[1–13] and some workers have reported its detection in blood. Others, however, suggest that benzoylecgonine may not be a true metabolite of cocaine, in the sense that it is produced by non-enzymic hydrolysis[14–16]. These workers have shown that conversion of cocaine to benzoylecgonine occurs at a substantial rate in plasma and urine at alkaline pH.

Since most of the methods reported for benzoylecgonine analysis have involved an alkaline extraction stage, it may be that some proportion of the amount detected could be due to conversion of cocaine during such extractions. In order to check this possibility it was considered essential to determine the rate of conversion of cocaine to benzoylecgonine at different pH values.

EXPERIMENTAL

Solutions of cocaine hydrochloride were made in distilled water at a concentration of 50 μg/ml. These were adjusted to pH values ranging from 2.0 to 9.4 with 0.1 *M* HCl or 0.1 *M* NaOH. The solutions were analysed immediately, and after standing for various times at room temperature, by high-performance liquid chromatography (HPLC).

HPLC was carried out using a Waters Assoc. (Northwich, Great Britain) M6000A pump with a reversed-phase support, Hypersil-5-ODS (5 μm; Shandon Southern Products, Runcorn, Great Britain) column (10 cm $\times$ 4.6 mm I.D.) and an eluent composition of methanol–water (55:45) (adjusted to pH 3.8 with syrupy phosphoric acid). The eluate was monitored by determining UV absorbance at 232 nm with a CE212 UV monitor (Cecil Instruments, Cambridge, Great Birtain). Flow-rate was 2 ml/min. Under these conditions benzoylecgonine eluted at a retention time of 1.4 min and cocaine at 3.4 min. Conversion of cocaine to benzoylecgonine was calculated from the peak heigths for the two compounds.

The precision of the method was estimated by replicate (10) determinations of a solution containing 10 μg/ml cocaine and 10 μg/ml benzoylecgonine at pH 4.0. The means and standard deviations were, for cocaine, 9.7 $\pm$ 0.4; and for benzoylecgonine, 9.8 $\pm$ 0.5.

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RESULTS AND DISCUSSION

The degrees of conversion of cocaine to benzoylecgonine taking place at different pH values over the period of one hour are shown in Table I. The conversion taking place at pH 8 over a longer period is given in Table II.

TABLE I

THE EFFECT OF pH ON THE CONVERSION OF COCAINE TO BENZOYLECGONINE

pH of solution	*Conversion (%)**	
	Initial	*After 1 h*
2	0	0
4	0	0
6	0	0.6
7	0	1.4
8	1.7	16.7
9	3.2	27.5
9.4	3.9	40.7

* Mean of 3 determinations.

TABLE II

THE CONVERSION OF COCAINE TO BENZOYLECGONINE AT pH 8

Age of solution (h)	*Conversion (%)**
0	0.6
1.0	15.6
2.0	21.3
3.0	26.2
4.5	34.7
6.75	45.1

* Mean of 3 determinations.

These results clearly indicate that cocaine is susceptible to hydrolysis in slightly alkaline conditions. Since most published methods for cocaine and benzoylecgonine analysis involve an alkaline extraction step the results obtained with these methods must be reconsidered. It is likely that cocaine levels will have been underestimated and benzoylecgonine levels overestimated. Relevant details of the extraction procedures of most of the methods are given in Table III.

Significant cocaine hydrolysis at neutral and slightly alkaline pH has important implications in toxicological analysis since the delay in analysing samples after they have been taken can be considerable. Blood samples generally become alkaline (to pH 8–9) on standing and alkaline urine is often encountered. Unless such samples are stored frozen, or adjusted to acid pH upon receipt, any cocaine present may have disappeared by the time analysis is performed.

TABLE III

DETAILS OF ALKALINE CONDITIONS USED FOR THE EXTRACTION OF COCAINE OR BENZOYLECGONINE FROM BLOOD AND URINE BY VARIOUS WORKERS

Ref.	*pH of extraction stage*	*Contact time with alkaline phase (min)*
1	8.0	?
2	8.5	?
3	8.9	10
4	9.5	4
5	sat. Na_2CO_3	10
6	*ca.* 9.6	5
7	*ca.* 10.0	15
8	sat. $NaHCO_3$	20
9	9.5	?
10	sat. $NaHCO_3$	10
11	8.9	5
12	9.0	?
13	8.5	11

The evidence presented here does not exclude the possibility of enzymic hydrolysis of cocaine in the body, but it could explain the widely divergent estimates of how much cocaine is excreted unchanged.

REFERENCES

1 F. Fish and W. D. C. Wilson, *J. Chromatogr.*, 40 (1969) 164.
2 N. N. Valanju, M. M. Baden, S. N. Valanju, D. Mulligan and S. K. Verma, *J. Chromatogr.*, 81 (1973) 170.
3 M. M. Bastos, D. Jukofsky and S. J. Mulé, *J. Chromatogr.*, 89 (1974) 335.
4 J. W. Blake, R. S. Ray, J. S. Noonan and P. W. Murdick, *Anal. Chem.*, 46 (1974) 288.
5 N. C. Jain, D. M. Chinn, R. D. Budd, T. S. Sneath and W. J. Leung, *J. Forensic Sci.*, 22 (1975) 7.
6 P. I. Jatlow and D. N. Bailey, *Clin. Chem.*, 21 (1975) 1918.
7 B. H. Dvorchik, S. H. Miller and W. P. Graham, *J. Chromatogr.*, 135 (1977) 141.
8 K. K. Kaistha and R. Tadrus, *J. Chromatogr.*, 135 (1977) 385.
9 M. J. Kogan, K. G. Verebey, A. C. Depace, R. B. Resnick and S. J. Mule, *Anal. Chem.*, 49 (1977) 1965.
10 S. J. Mule, D. Jukofsky, N. Kogan, A. Depace and K. Verebey, *Clin. Chem.*, 23 (1977) 796.
11 J. I. Javaid, H. Dekirmenjian, J. N. Davis and C. R. Schuster *J. Chromatogr.*, 152 (1978) 105.
12 S. P. Jindal and P. Vestergaard, *J. Pharm. Sci.*, 67 (1978) 811.
13 B. Holmstedt, J. E. Lindgren and L. Rivier, *J. Ethnopharmacol.*, 1 (1979) 69.
14 D. Taylor, V. S. Estevez, L. F. Englert and B. T. Ho, *Res. Commun. Chem. Pathol. Pharmacol.*, 14 (1976) 249.
15 D. J. Stewart, T. Inaba, B. K. Tang and W. Kalow, *Life Sci.*, 20 (1977) 1557.
16 D. J. Stewart, T. Inaba, M. Lucassen and W. Kalow, *Clin. Pharmacol. Ther.*, 25 (1979) 464.

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CHROM. 13,410

Note

Preadsorbent thin-layer chromatography

III. Direct detection of quinine in urine as a presumptive test for heroin

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Many methods are currently available for detecting morphine in the urine of heroin users. Those which have proven useful in screening large numbers of samples are thin-layer chromatography (TLC)[1,2], gas–liquid chromatography[3,4], fluorometry[5–7], radioimmunoassay[8], enzyme multiplied immunoassay[9] and hemagglutination inhibition[10,11]. These methods have recently been critically reviewed[12,13].

The immunoassays are highly sensitive, detecting from 30–400 ng/ml of total morphine for a preliminary screen[13]. They are easy to use, rapid and do not require sample treatment. However, these assays suffer from cross-reactivity with a number of drugs and thus give rise to false positives as compared with TLC of the hydrolyzed extract. In addition, the cost is quite high for immunoassays.

Fluorometry is also quite sensitive and reliably detects 0.22 μg/ml of free morphine[6]. A new manual method is sensitive to 0.04 μg/ml[7]. However, fluorometry requires considerable sample pre-treatment, and is subject to non-specific endogenous background interference. In addition, the extract for morphine analysis by fluorometry cannot then be used to screen for other drugs.

Gas chromatography is also very sensitive but the expense and relatively long retention times for morphine with many liquid stationary phases limits gas chromatography to confirmation rather than initial screening.

TLC suffers from two faults, a lack of sensitivity and the need for sample treatment, although the extract can be utilized for screening other drugs. The sensitivity of TLC for free morphine is 1 μg/ml at the 100% detection level[12]. Since the amount of free morphine excreted varies from 5 to 20% of the total, only an acid-hydrolyzed urine is suitable for efficient screening using TLC. The big advantages of TLC are its low cost and selectivity. It is the least expensive and thus the most widely used of all the screening methods.

Quinine is a major diluent of heroin, especially in the eastern part of the United States. It is used chiefly as a camouflage to prevent user determinations of purity by taste. It is readily detectable by the same fluorometric methods used to detect morphine in urine. The limit of detection of quinine by fluorometry after extraction is 0.1 μg/ml[5]. A New York City study in 1973[12] showed that in 42 urine samples from methadone patients, of those positive for quinine, 78.5% were positive for morphine by TLC after acid hydrolysis. Mule and Hushin[5] found only 1.3% of urines were

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positive for morphine and negative for quinine by fluorometry. The false negatives were 6.6%, *i.e.*, quinine positive and morphine negative. However, some of these samples were probably positive for morphine but below the detection limit of the assay. Thus, it appears quinine is an excellent marker for prediction of heroin use.

This work describes a simple, rapid procedure for the detection of quinine in urine by TLC without extraction.

MATERIALS

Microcaps, Drummond, 10 μl disposable; developing tank, Kontes Glass, 22 × 10 × 23 cm; developing solvent: chloroform–methanol–acetic acid (80:20:3); ultraviolet visualization chamber, Ultraviolet Products, Model CC-20; silica gel plates (Whatman, Clifton, NJ, U.S.A.), precoated LKD-5, 20 × 20 cm glass plates with nineteen 1-cm channels containing a 250-μm layer of silica gel and a 3-cm preadsorbent area.

Urine samples from a methadone program were obtained from the Allegheny County Coroner Drug Urine Screening Program.

PROCEDURE

Urine samples were stored frozen until use. After thawing, they were filtered or centrifuged to remove solids. A 50-μl volume of each urine is spotted with a 100-μl syringe along with 10 μl of a solution of quinine sulfate standard (1 mg/ml) in methanol. These were spotted in the middle of the preadsorbent area of each channel. A maximum of eighteen samples and one standard can be spotted on each plate. The samples are applied with the TLC plates on a hot plate at a maximum temperature of 65°C or with the aid of a hair dryer. No attempt is made to keep the spots small but the preadsorbent area should not be completely flooded vertically with sample for optimum chromatography. After the plate is dried, it is placed in a developing tank which has been equilibrated with solvent for 30 min using a sheet of filter paper wet with solvent. The plate was developed at 20°C for 10 cm from the top of the preadsorbent area. After removal, the plate was air-dried for 5 min. The plate was then observed for the blue fluorescence of quinine and its metabolites in long (366 nm) or short wave (254 nm) UV light. Those samples positive for quinine and/or its metabolites should then be confirmed for morphine by other methods such as TLC, gas chromatography or immunoassay. The samples negative for quinine should then be screened for drugs and morphine by the usual TLC method.

RESULTS AND DISCUSSION

In a study of quinine metabolism, 325 mg of quinine sulfate (Lilly, Indianapolis, IN, U.S.A.) was ingested and urine samples obtained daily for twelve days. The R_F values (measured from the top of the preadsorbent area) for the bands due to quinine and its two metabolites are shown in Table I. M_1 and M_2 are probably hydroxy metabolites of quinine (Q) and are more polar thus having lower R_F values than quinine. Q and M_1 are detected the day after ingestion and decrease rapidly thereafter. M_2 is seen the day after ingestion and then increases to a maximum five days after

TABLE I

R_F VALUES OF QUININE AND ITS METABOLITES

Compound	*R_F range*
Quinine Q (standard)	0.77–0.79
Quinine Q (urine)	0.74–0.77
Metabolite M_1 (urine)	0.73–0.75
Metabolite M_2 (urine)	0.46–0.53

ingestion. It can still be seen ten days later. No spots were visible eleven days after ingestion. A similar detection limit was seen by Mule and Hushin[5] using fluorometry. A sensitivity study using a blank urine spiked with quinine gave a limit of detection of 0.4 ng in 50 μl which is equivalent to 8 ng/ml of urine. This is much lower than the 100 ng/ml found for the solution fluorometric method. The limitation of solution fluorometry is due to the endogenous background fluorescence in all urines which must be "blanked out" using drug-free urine. The acidic nature of the developing solvent in the TLC method appears to increase the fluorescence. Complete removal of the developing solvent by heating in an oven greatly diminishes the fluorescence.

The specially manufactured preadsorbent area retains the highly polar compounds in urine and allows the less polar materials to move with the developing solvent until the top of the preadsorbent area is reached. Then the developing solvent in contact with silica gel separates these compounds as bands. Bands are preferable to spots, which are the norm for conventional TLC, because improved resolution and sensitivity result. Endogenous fluorescent spots are often seen below an R_F of 0.3 and appear as blue, yellow and purple bands.

A study was made of thirteen drugs which were fluorescent in a published TLC street drug procedure[14] and thus, possible interferences. The results are shown in Table II. Due to the excellent TLC separation, no drug constitutes an interference to detecting quinine or its metabolites by this method. No fluorescence was seen for morphine, phenmetrazine, diphenhydramine, chloroquine, adrenaline, thiopropazate and perphazine in this TLC procedure.

Fifty urines were obtained from a Pennsylvania methadone clinic and subjected to the direct TLC procedure. They were also analyzed by conventional extraction and TLC for quinine, morphine, amphetamines, barbiturates and methadone. Of the 50 samples, 11 were found positive for morphine by conventional methodology and 9 of these 11 or 82% were found to be positive for quinine by direct TLC (Table III). Ten of these 11 samples were found to contain quinine by conventional TLC. The

TABLE II

$R_{Quinine}$ VALUES OF QUININE METABOLITES AND DRUGS RELATIVE TO QUININE

Drug	*Color*	*$R_{Quinine}$*
Trifluoperazine	Blue–green	0.43
Morphine	Non-fluorescent	0.45
Mephentermine	Blue	0.51
Metabolite M_2	Blue	0.68
Metabolite M_1	Blue	0.96
Quinine	Blue	1.00
Thioridiazine	Blue	1.10
Pentazocine	Blue	1.28
Quinidine	Blue	1.31

false negative rate was 1/11 or 9%. Interestingly, unchanged quinine rather than its metabolites appears to be the most prevalent form in these urines.

TABLE III

A COMPARISON OF THE STANDARD EXTRACTION/TLC METHOD AND THE DIRECT TLC METHOD FOR THE DETECTION OF QUININE (Q), ITS METABOLITES M_1 AND M_2, AND MORPHINE

Sample number	*Results*	
	Conventional TLC	*Direct TLC*
1	+Q, +Morphine	+Q, $+M_1$, $+M_2$
2	+Q, +Morphine	+Q, $+M_1$, $+M_2$
3	+Q, +Morphine	+Q, $+M_1$, $+M_2$
4	+Q, +Morphine	+Q, $+M_1$, $+M_2$
5	+Q, +Morphine	+Q, $+M_1$, $-M_2$
6	+Q, +Morphine	+Q
7	+Q, +Morphine	+Q
8	+Q, +Morphine	+Q
9	+Q, +Morphine	+Q
10	+Q, +Morphine	+Q
11	−Q, +Morphine	−Q

CONCLUSION

This paper describes a simple, rapid TLC procedure for the detection of quinine in urine. Quinine is a common diluent of heroin and its presence in the urine is an indicator of heroin abuse. Urine is directly spotted on a commercial TLC plate equipped with a preadsorbent area and detected by native fluorescence.

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REFERENCES

1 B. Davidow, N. L. Petri and B. Quame, *Amer. J. Clin. Pathol.*, 50 (1968) 714.
2 V. P. Dole, W. K. Kim and I. Eglitis, *J. Amer. Med. Ass.*, 198 (1966) 349.
3 B. S. Finkle, F. J. Cherry and D. M. Taylor, *J. Chromatogr. Sci.*, 9 (1971) 393.
4 R. F. Adams, J. E. Purcell and L. S. Ettre, *Amer. Lab.*, (1973) 51.
5 S. J. Mule and P. L. Hushin, *Anal. Chem.*, 43 (1971) 708.
6 M. Sansur, A. Buccofuri and S. Morgenstern, *J. Ass. Offic. Anal. Chem.*, 55 (1972) 880.
7 V. F. Cordova and T. A. Banford, *J. Forensic Sci.*, 20 (1975) 58.
8 S. Spector and C. W. Parker, *Science*, 168 (1970) 1347.
9 K. E. Rubenstein, R. S. Schneider and E. F. Ullman, *Biochem. Biophys. Res. Commun.*, 47 (1972) 846.
10 F. Adler and C.-T. Liu, *J. Immunol.*, 106 (1971) 1683.
11 F. Adler, C.-T. Liu and D. H. Catlin, *Clin. Immunol. Immunopathol.*, 1 (1972) 53.
12 D. Sohn, J. Simon, M. A. Hanna, G. V. Ghali, R. A. Tolba and V. Melkonian, *Anal. Chem.*, 45 (1973) 1498.
13 S. J. Mule, M. L. Bastos and D. Jukofsky, *Clin. Chem.*, 20 (1974) 243.
14 J. A. Vinson, J. E. Hooyman and C. E. Ward, *J. Forensic Sci.*, 20 (1975) 552.

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Note

Thin-layer chromatography of phospholipids

Separation of major phospholipid classes of milk without previous isolation from total lipid extracts

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(Received October 8th, 1980)

The triglyceride content of milk lipids, amounting up to 99 %, causes problems in quantifying and characterizing other lipid subclasses. For instance, the direct enzymatic determination of cholesterol in milk samples is impossible without previous alkaline hydrolysis of triglycerides and subsequent extraction of the non-saponifiable matter[1,2]. With regard to phospholipids, we have been looking for a method for separating and determining single phospholipid classes by thin-layer chromatography (TLC). However, the amounts of lipid extract applied to the plates required for the determination or further analysis of the major phospholipid classes of milk caused unsatisfactory separation owing to overloading by neutral lipids. We therefore investigated several solvent systems, known to move only neutral lipids from the starting region, in order to ensure that they do not influence the recovery in addition to the composition of phospholipids.

Further, the one-dimensional TLC separation of sphingomyelin, lecithin, phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine could not be achieved using commercially available pre-coated plates. Phosphatidylserine and phosphatidylinositol either chromatographed together or they could not be resolved properly by any of the solvent systems under investigation[3–6]. We know of only one system that permits the separation of all phospholipid classes we are interested in; almost equidistant spots are obtained by development with chloroform–methanol–acetic acid–water (25:15:4:2) if silica gel plates prepared with 1 m*M* sodium carbonate solution instead of water are used[7].

Owing to the time- and space-consuming technique of preparing thin-layer plates by hand, we first looked for a method for post-impregnating ready-for-use plates with sodium carbonate. Early results were encouraging but eventually the method proved not to be really satisfactory. We therefore tried to find a new solvent system or a combination of solvent systems that could meet our demands for a reliable TLC separation of all of the major phospholipid classes of milk.

EXPERIMENTAL

Materials

Phospholipid standards were obtained from Sigma (St. Louis, MO, U.S.A.)

0021-9673/81/0000–0000/$02.50

and Applied Science Europe (Oud-Beijerland, The Netherlands). TLC plates were purchased from E. Merck (Darmstadt, G.F.R.) and Macherey, Nagel & Co. (Düren, G.F.R.). All chemicals were of analytical-reagent grade (Merck).

Methods

A total lipid extract was prepared by a slightly modified procedure of Bligh and Dyer[8]. Extraction and centrifugation were performed in 140 × 24 mm O.D. sampling tubes (Sarstedt, Nümbrecht, G.F.R.). A 4-ml volume of milk was thoroughly mixed with 10 ml of methanol. After addition of 5 ml of chloroform and thorough shaking, the samples were incubated for 15 min at 37°C, cooled to room temperature and mixed with a further 5 ml of chloroform and 5 ml of distilled water. Before phase separation by centrifugation (15 min at 2500 *g*) the samples were kept in an ice-bath for at least 10 min with intermittent shaking. The organic solvent layer, containing the lipid material, was removed by syringe and cannula. The solvent was evaporated under reduced pressure and the lipids were redissolved in 4 ml of chloroform–methanol (19:1).

TLC was performed using pre-coated silica gel 60 plates. Phospholipid standards (up to 200 μg) or aliquots of the lipid extract (up to 10 mg) were applied as a narrow band (2.5 cm in length) 1.5 cm above the lower edge of the thin layer plate.

The first and second developments were performed using light petroleum (boiling range 40–60°C)–diethyl ether–acetic acid (90:10:1)[9] and acetone, respectively, each to a distance about 15 cm above the starting line. Separation of phospholipid classes was achieved by subsequent development with ethyl acetate–2-propanol–water (50:35:15) to a distance 12 cm above the starting line (third development). Finally, chloroform–methanol–water (75:25:4) was allowed to rise 8.5 cm above the starting line (fourth development).

All developments were carried out in well equilibrated chromatographic tanks with intermediate drying of the plates under an electric fan. Staining was performed either by exposure to iodine vapour or by spraying with a 20% (w/v) solution of molybdophosphoric acid in ethanol and heating the plates for 10 min at 105°C. Phospholipid classes were identified using authentic markers.

Total phospholipids and single phospholipid classes were determined by phosphorus assay as described previously[10].

RESULTS

If run singly, none of the solvent mixtures or organic solvents known to separate neutral lipids and phospholipids by TLC, the latter remaining at the starting line, is able to move all of the neutral lipids to the upper part of the TLC plate. However, development with light petroleum–diethyl ether–acetic acid (90:10:1) followed by a second development with acetone is successful (Fig. 1). Thus it is possible to eliminate all interferences to the TLC separation of phospholipid classes caused by neutral lipids.

Using phospholipid standards we were able to show that no lytic products are generated, that none of the phospholipid classes is lost in considerable amounts, recovery ranging between 95% and 97.5%, and that the composition of the phospholipid fraction is not influenced by this procedure (Table I).

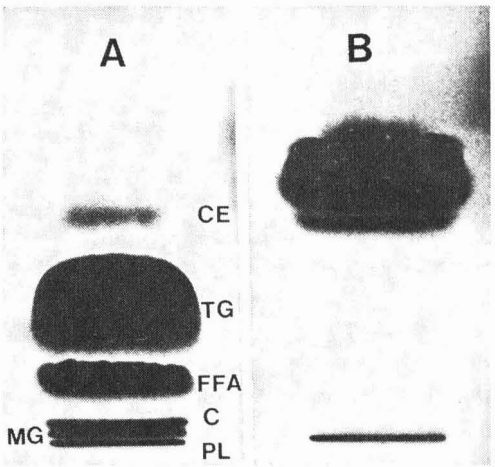

Fig. 1. Elution of neutral lipids from the lower part of the TLC plates. (A) First development with light petroleum–diethyl ether–acetic acid (90:10:1). (B) First development as for (A) and second development with acetone. PL = phospholipids; MG = monoglycerides; C = cholesterol; FFA = free fatty acids; TG = triglycerides; CE = cholesterol esters.

TABLE I

RECOVERY AND RELATIVE AMOUNTS OF SINGLE PHOSPHOLIPID CLASSES AFTER TLC SEPARATION ACCORDING TO THE PROPOSED PROCEDURE

Aliquots of a phospholipid standard mixture were applied to the plates and subjected to TLC separation including all four consecutive developments. Recoveries and relative amounts were calculated from data obtained from quantitative analysis of the single phospholipid classes by phosphorus assay.

Phospholipid	*Recovery after TLC separation (%)*		*Relative amounts (mole-% of total PL)**	
	*Mean**	*Range*	*Standard mixture*	*After TLC separation*
Sphingomyelin	95.5	95.2–95.9	19.7	19.6
Lecithin	96.7	96.4–97.2	20.5	20.6
Phosphatidyl-serine	97.1	96.5–97.5	19.5	19.8
Phosphatidyl-inositol	95.4	95.1–95.8	18.1	18.0
Phosphatidyl-ethanolamine	95.4	95.0–95.7	22.2	22.1

* Mean of five determinations.

Satisfactory TLC separation of the phospholipid classes so far remaining at the starting line can be achieved by consecutive developments with ethyl acetate–propanol–water (50:35:15) and chloroform–methanol–water (75:25:4) to 12 and 8.5 cm, respectively, above the starting line. The former solvent system is able to separate phosphatidylserine and phosphatidylinositol and the latter system accomplishes the separation of sphingomyelin and lecithin and also phosphatidylinositol and phosphatidylethanolamine (Fig. 2). Lyso-lecithin chromatographs beneath sphingomyelin, and lyso-phosphatidylethanolamine between phosphatidylserine and phosphatidylinositol.

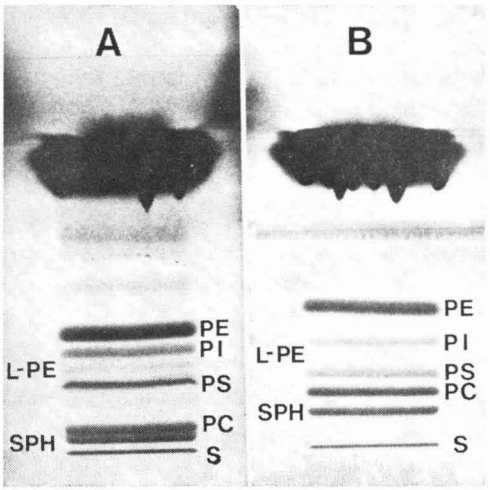

Fig. 2. TLC separation of phospholipid classes from milk. Pre-developments as in Fig. 1. (A) Third development with ethyl acetate–propanol-2–water (50:35:15). (B) Pre-developments and third development as for (A); fourth development with chloroform–methanol–water (75:25:4). SPH = sphingomyelin; PC = lecithin; PS = phosphatidylserine; PI = phosphatidylinositol; PE = phosphatidylethanolamine; L-PE = lyso-phosphatidylethanolamine; S = starting line.

DISCUSSION

The TLC separation of phospholipid classes from lipid extracts containing large amounts of neutral lipids is impossible without previous isolation of total phospholipids, for instance by column chromatography or acetone precipitation[4,5,11–15]. However, the time required, possible reduction of yield, and influences on relative amounts of single phospholipid classes (*e.g.*, refs. 12, 16 and 17) involved in these procedures are inconvenient if large sample series, containing only small amounts of phospholipids, have to be analysed. In addition, we have not been able to find any solvent system described in the literature that was really able to separate the five major phospholipid classes of milk by one-dimensional TLC using pre-coated silica gel plates, the separation of phosphatidylinositol and phosphatidylserine being the main problem.

Problems due to overloading the plates with neutral lipids, by application of lipid extract in sufficient amounts to analyse phospholipid classes quantitatively, could easily be avoided by pre-development with light petroleum–diethyl ether–acetic acid (90:10:1) and acetone successively. However, we had to test many different solvent systems before we achieved a satisfactory one-dimensional TLC separation of all the phospholipid classes under investigation. The proposed combination of four consecutive developments not only makes it possible to apply sufficient amounts of phospholipids to TLC plates without previous isolation from total lipid extracts, even if neutral lipids greatly predominate, but also allows the separation of phospholipids, *e.g.*, from milk, using commercially available pre-coated silica gel plates. If neutral lipids are present in small amounts the first two developments can be omitted without changing the chromatographic behaviour of the single phospholipids.

Moreover, using non-destructive detection methods, the present procedure can also be applied to the direct small-scale preparative isolation of single phospholipid classes from total lipid extracts and therefore might be of interest to many laboratories engaged in analytical work on phospholipids.

REFERENCES

1 A. Grossmann, H. Timmen and H. Klostermeyer, *Milchwissenschaft*, 31 (1976) 721.
2 P. R. Gentner and A. Haasemann, *Milchwissenschaft*, 34 (1979) 344.
3 O. Renkonen and A. Luukonen, in G. V. Marinetti (Editor), *Lipid Chromatographic Analysis*, Vol. 1, Marcel Dekker, New York, Basle, 2nd ed., 1976, Ch. 1, p. 1.
4 A. Kuksis, *J. Chromatogr.*, 143 (1977) 3.
5 G. J. Nelson, in E. G. Perkins (Editor), *Analysis of Lipids and Lipoproteins*, American Oil Chemists' Society, Champaign, IL, 1975, Ch. 5, p. 70.
6 H. P. Kaufmann, *Fette, Seifen, Anstrichm.*, 72 (1970) 1091.
7 V. P. Skipski, R. F. Peterson and M. Barclay, *Biochem. J.*, 90 (1964) 374.
8 E. G. Bligh and W. J. Dyer, *Can. J. Biochem. Physiol.*, 37 (1959) 911.
9 D. C. Malins and H. K. Mangold, *J. Am. Oil Chem. Soc.*, 37 (1960) 576.
10 P. R. Gentner and A. Haasemann, *Fette, Seifen, Anstrichm.*, 81 (1979) 357.
11 C. H. Lea, D. N. Rhodes and R. D. Stoll, *Biochem. J.*, 60 (1955) 353.
12 D. N. Rhodes and C. H. Lea, *J. Dairy Res.*, 25 (1958) 60.
13 R. Olegard and L. Svennerholm, *Acta Paediat. Scand.*, 59 (1970) 637.
14 R. C. MacDonald and S. P. Rempas, *J. Chromatogr.*, 131 (1977) 157.
15 G. Rouser, G. Kritchevsky and A. Yamamoto, in G. V. Marinetti (Editor), *Lipid Chromatograhic Analysis*, Vol. 3, Marcel Dekker, New York, Basle, 2nd ed., 1976, Ch. 12, p. 713.
16 O. Renkonen, *J. Lipid Res.*, 3 (1962) 181.
17 L. Gosselin and J. M. Foidart, *Clin. Chim. Acta*, 81 (1977) 209.

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Chemistry of heterocyclic compounds, Vol. 38, Part 1: Isoquinolines, edited by G. Grethe, Wiley, Chichester, New York, 1980, ca. 700 pp., price ca. US$ 100.00, £ 44.00, ISBN 0-471-37481-4.

Polymers in nature, by E.A. MacGregor and C.T. Greenwood, Wiley, Chichester, New York, 1980, ca. 328 pp., price ca. US$ 53.20, £ 19.00, ISBN 0-471-27762-2.

General chemistry: Principles and structure, by J.E. Brady and G.E. Humiston, Wiley, Chichester, New York, **2nd ed. SI version**, 1980, ca. 800 pp., price ca. US$ 25.00, £ 10.80, ISBN 0-471-06315-0.

Thin-layer chromatography: Quantitative environmental and clinical applications, edited by J.C. Touchstone and D. Rogers, Wiley, Chichester, New York, 1980, ca. 475 pp., price ca. US$ 36.60, £ 16.80, ISBN 0-471-07958-8.

Wilson and Wilson's Comprehensive analytical chemistry, Vol. XI, The application of mathematical statistics in analytical chemistry; mass spectrometry; ion selective electrodes, edited by G. Svehla, Elsevier, Amsterdam, Oxford, New York, 1980, XIV + 408 pp., price Dfl. 245.00, US$ 119.50, ISBN 0-444-41886-5.

Methods of plasma protein fractionation, edited by J.M. Curling, Academic Press, London, New York, Toronto, Sydney, San Francisco, 1980, XIV + 326 pp., price £ 23.20 (U.K.), US$ 53.50, ISBN 0-12-199550-X.

Developments in food analysis techniques – 2, edited by R.D. King, Applied Science, Barking, 1980, IX + 268 pp., price £ 23.00, ISBN 0-85334-921-5.

Handbook of protein sequence analysis, by L.R. Croft, Wiley, Chichester, New York, **2nd ed.**, 1980, XIV + 628 pp., price US$ 105.00, £ 38.00, ISBN 0-471-27703-7.

Spectral and chemical characterization of organic compounds: A laboratory handbook, by W.J. Criddle and G.P. Ellis, Wiley, Chichester, New York, **2nd ed.**, 1980, ca. 123 pp.; price ca. US$ 33.60, £ 12.00, ISBN 0-471-27813-0 (cloth); ca. US$ 12.60, £ 4.50, ISBN 0-471-27812-2 (paper).

MEETINGS

12th ANNUAL SYMPOSIUM ON THE ANALYTICAL CHEMISTRY OF POLLUTANTS

The twelfth Annual Symposium on the Analytical Chemistry of Pollutants will be held April 14–16, 1982, in the Congress Centre of the Free University, De Boelelaan 1105, 1081 HV Amsterdam, The Netherlands. The scientific programme will consist of invited plenary lectures, invited and submitted research lectures and poster presentations covering the whole field of environ-

mental analytical chemistry. One day will be devoted to interdisciplinary topics: Analytical Chemistry and Water Chemistry; and Analytical Chemistry and Mutagenicity with special emphasis on water pollutants.

Those interested in giving a research lecture (20 min incl. discussion) or a poster presentation should send an abstract of no more than 200 words to the Congress Office by March 15, 1981. The congress language will be English: there will be no simultaneous translation. Further information can be obtained from Prof. Dr. R.W. Frei, Congress Office, 12th Annual Symposium on the Analytical Chemistry of Pollutants, Congress Bureau, Vrije Universiteit, P.O. Box 7161, 1007 MC Amsterdam, The Netherlands.

9th INTERNATIONAL MASS SPECTROMETRY CONFERENCE

The ninth International Mass Spectrometry Conference will be held from August 30 until September 3, 1982, in the historical castle "Hofburg" in the center of Vienna, Austria. The conference is organized by the Austrian Mass Spectrometry Group, the Austrian Society for Microchemistry and Analytical Chemistry, the Austrian Chemical Society and the Institute of Analytical Chemistry of the University of Vienna in cooperation with an International Scientific Committee. The scientific program will cover all aspects of mass spectrometry. The program will consist of invited lectures, submitted papers and posters. Also an extensive commercial exhibition will be included. Further information is available from the secretariat of the conference: Interconvention, P.O. Box 105, A-1014 Vienna, Austria.

CALENDAR OF FORTHCOMING MEETINGS

March 9–13, 1981
Atlantic City, N.J., U.S.A.

1981 Pittsburgh Conference

Contact:
John A. Queiser, Programme Chairman, 1981 Pittsburgh Conference, 2523 Greenboro Lane, Pittsburgh, PA 15220, U.S.A.
Tel. (412) 795-7110.

March 23–June 5, 1981

Course: Biochemical Separation Methods

Contact:
Secretary, Eva Linder, Institute of Biochemistry, University of Uppsala, Box 576, S-751 23 Uppsala, Sweden.

Apr. 13–16, 1981
Cardiff, Wales,
United Kingdom

International Symposium on Electroanalysis in Clinical, Environmental and Pharmaceutical Chemistry

Contact:
Short Courses Section (Electroanalysis Symposium), UWIST, Cardiff CF1 3NU, Wales, United Kingdom.

May 3–7, 1981
Hindelang (Bavarian Alps),
F.R.G.

4th International Symposium on Capillary Chromatography

Contact:
Dr. J. Rijks, Laboratory of Instrumental Analysis, Eindhoven University of Technology, P.O. Box 513, NL-5600 MB Eindhoven, The Netherlands.

May 4–7, 1981
Brussels, Belgium

XXIXth Annual Colloquium Protides of the Biological Fluids

Contact:
Dr. Hubert Peeters, Colloquium secretariat, c/o Lipid and Protein Dept., Institute for Medical Biology, Alsembergsesteenweg 196, B-1180 Brussels, Belgium. Tel. (32-2) 344.19.50.

May 5–8, 1981
Gatlinburg, TN, U.S.A.

Separation Science and Technology for Energy Applications

Contact:
A.P. Malinauskas, Oak Ridge National Laboratory, P.O. Box X, Oak Ridge, TN 37830, U.S.A.

May 11–15, 1981
Avignon, France

5th International Symposium on Column Liquid Chromatography

Contact:
Professor G. Guiochon, Ecole Polytechnique, Laboratoire de Chimie Analytique Physique, Route de Saclay, 91128 Palaiseau, France. (Further details published, Vol. 194, No. 3).

May 18–20, 1981
Jekyll Island, GA, U.S.A.

11th Annual Symposium on the Analytical Chemistry of Pollutants

Contact:
Prof. Dr. R.W. Frei, The Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

May 19–22, 1981
Munich, F.R.G.

11th German Hospital Conference

Contact:
Joint Working Committee of German Hospitals, Tersteegenstr. 9, D-4000 Düsseldorf 30, F.R.G. Tel. (02 11) 43 46 83.

May 20–22, 1981
Eger, Hungary

Symposium on the Analysis of Steroids

Contact:
Prof. S. Görög, c/o Hungarian Chemical Society, 1061 Budapest VI, Anker köz 1, Hungary.

June 16–17, 1981
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1st International Symposium on Chromatography in Biochemistry, Medicine and Environmental Research

Contact:
Dr. A. Frigerio, Italian Group for Mass Spectrometry in Biochemistry and Medicine, c/o Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan, Italy. Tel. 35.54.546.

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Venice, Italy

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June 22–26, 1981
Veldhoven, The Netherlands

4th International Symposium on Affinity Chromatography and Related Techniques

Contact:
Secretariat, 4th Int. Symp. on Affinity Chromatography and Related Techniques, Department of Organic Chemistry, Faculty of Sciences, Katholieke Universiteit, Toernooiveld, 6525 ED Nijmegen, The Netherlands. (Further details published in Vol. 205, No. 2).

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Congress Secretariat, Interconvention, P.O. Box 35, A-1095 Vienna, Austria. Tel. (0222) 421352.

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Contact:
Gesellschaft Deutscher Chemiker, P.O. Box 90 04 40, D-6000 Frankfurt/Main 90, F.R.G.

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Siofok, Hungary

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Contact:
Hungarian Chemical Society, H-1368 Budapest, P.O.B. 240, Hungary. Tel. Budapest 427–343. (Further details published in Vol. 189, No. 2).

Sept. 29–Oct. 2, 1981
Basle, Switzerland

ILMAC 81; 8th International Exhibition of Laboratory, Chemical Engineering, Measurement and Automation Techniques in Chemistry

Contact:
D. Gammeter, Secretariat ILMAC 81, Postfach, CH-4021 Basle, Switzerland. Tel. 061 20 20 20.

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Resource Recovery and Environmental Issues of Industrial Solid Wastes

Contact:
J.S. Watson, Oak Ridge National Laboratory, P.O. Box X, Oak Ridge, TN 37830, U.S.A.

Nov. 23–25, 1981
Barcelona, Spain

2nd International Congress on Analytical Techniques in Environmental Chemistry

Contact:
Dr. Joan Albaigés, General Secretary, Plaza de Espana, Barcelona-4, Spain. Tel. 223 31 01.

Apr. 14–16, 1982
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PUBLICATION SCHEDULE FOR 1981

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INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 193, No. 3, pp. 529–532. A free reprint can be obtained by application to the publisher)

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