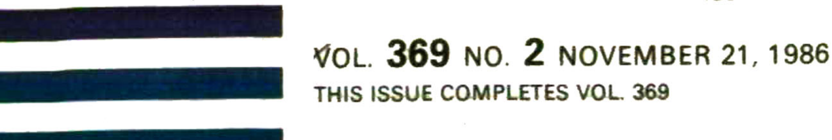

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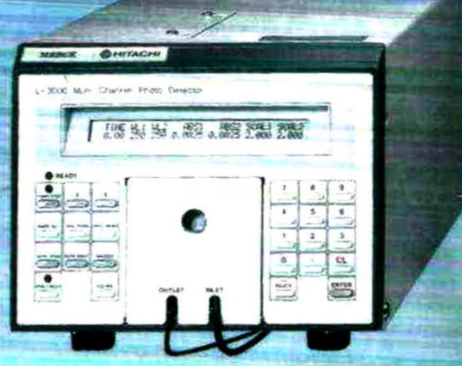

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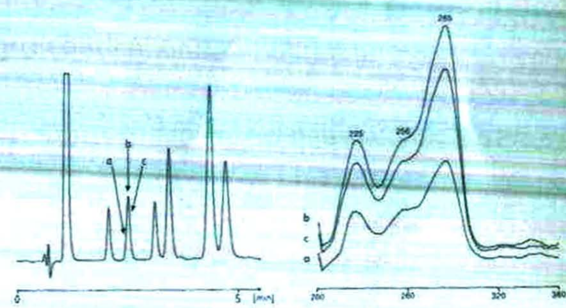

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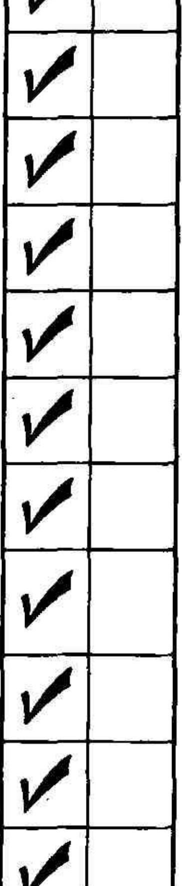

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MEASUREMENT OF SOLUBILITY PARAMETERS BY GAS–LIQUID CHROMATOGRAPHY

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(Received June 19th, 1986)

SUMMARY

Gas–liquid chromatography has been used to calculate thermodynamic data for a variety of probe molecules in three polymers at 25°C. The method of Guillet and co-workers has been applied to calculate solubility parameters, δ, and good agreement was found with literatue values. The results avoid extrapolation from higher temperatures and add further validity to the method. Discussion of the role of δ in polymer solution thermodynamics is also given.

INTRODUCTION

Although of limited theoretical significance in solution thermodynamics, the concept of a solubility parameter, δ, or a cohesive energy density (c.e.d.), δ^2, as a measure of intermolecular forces remains useful for many practical applications[1]. The definition of δ^2 as an internal energy of vapourization per unit volume allows straightforward measurement of δ for small molecule liquids but for polymers, this definition is of little or no use so that the physical meaning of the polymer solubility parameter, δ_2, is doubtful.

Guillet and co-workers[2–4] have developed a technique for the estimation of δ_2 at infinite dilution from thermodynamic measurements made using gas–liquid chromatography (GLC) and have obtained consistent results for a number of polymers. However, GLC measurements have to be made well above the glass transition temperature, T_g, of the polymer[5] so that data have to be extrapolated to near ambient temperature for comparison with δ_2 values from classical techniques such as swelling equilibrium.

This paper presents results on polymers with low T_g values, polydimethylsiloxane (PDMS), polyisobutylene (PIB) and ethylene-propylene rubber (EPR), so that measurements could be made at room temperature allowing a direct comparison with literature values of δ_2. The thermodynamic significance of the polymer solubility parameter is also examined further.

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EXPERIMENTAL

Materials

The probes used were obtained from a number of commercial sources and were of reagent grade or better.

The PDMS was an OV-101 stationary phase from Chromatographic Specialites, the EPR was a low-molecular-weight polymer from Aldrich and the PIB was a Kalene 800 sample of butyl rubber from Hardman (Toronto, Canada). The molecular weights and other physical properties are shown in Table I. The column packings were prepared in the usual way by coating Chromosorb G (80–100 mesh) from a suitable solvent. Column loadings in the region of 10% were used and were calculated by calcination for EPR and PIB, and by exhaustive solvent extraction to constant weight for PDMS.

Apparatus

The chromatograph used was designed for the application of finite concentration GLC techniques and has been described in detail previously[6]. For the present work, a carrier gas flow of pure helium was used for performing the usual infinite dilution measurements.

The column temperature was controlled and measured to ±0.01°C using a water bath and high-precision thermometers. Probe samples in the region of 0.005–0.02 μl or 5 μl of air as a non-absorbing marker were injected using Hamilton syringes and the results reported here are the average of at least three values within experimental error. The necessary conditions for obtaining thermodynamic data by GLC, peak symmetry and absence of flow-rate and sample size effects, were satisfied directly with PDMS and EPR. This was as expected since the measurements were made well above T_g (T_g = 150 K for PDMS and 150–180 K for EPR)[7]. However, as noted by several workers[5,8] some non-equilibrium effects were noticed with PIB (T_g = 200–210 K)[7]. In this case a range of sample sizes and flow-rates was used and the values extrapolated to zero in each case.

RESULTS AND DISCUSSION

The primary GLC datum, the specific retention volume, V_g^0, was calculated from the usual expression[9]

TABLE I

PHYSICAL PROPERTIES OF THE POLYMERS AT 25°C

Polymer	*Molecular weight*	*Density (g cm⁻³)*	*Column load (wt. %)*
PDMS	30000	0.965	10.2
PIB	40000	0.917	8.9
EPR	24500	0.910	9.8

TABLE II

PHYSICAL PROPERTIES OF THE PROBES AT 25°C

	Vapour pressure (Torr)	*Density (g cm⁻³)*	*2nd Virial coefficient (cm³ mol⁻¹)*	*Molar volume (cm³ mol⁻¹)*
Pentane	512.48	0.6214	1260	116.10
Hexane	150.42	0.6549	1935	131.59
Heptane	45.72	0.6795	2861	147.46
Octane	13.98	0.6985	4188	179.91
Cyclohexane	97.29	0.7738	1717	108.76
Benzene	94.90	0.8738	1478	89.40
Toluene	28.44	0.8623	2375	106.85
Carbon tetrachloride	115.25	1.5843	1520	97.10
Chloroform	194.18	1.4799	1207	80.67
Methylene chloride	435.86	1.3163	857	64.53

$$V_g^0 = [F(t_R - t_M)]/w \tag{1}$$

where w is the weight of polymer used, t_R and t_M are the retention times of the probe and marker, respectively, and F is the flow-rate of carrier gas, fully corrected to standard temperature and pressure in the usual manner[9].

Infinite dilution activity coefficients rationalized on a weight fraction basis, Ω_1^∞, and Flory–Huggins interaction parameters, χ^∞, were calculated using[10]

$$\ln \Omega_1^\infty = \ln\left(\frac{273.15\, R}{P_1^0 M_1 V_g^0}\right) - \frac{P_1^0}{RT}(B_{11} - V_1^0) \tag{2}$$

$$\chi^\infty = \ln \Omega_1^\infty - \ln(\rho_1/\rho_2) - [1 - (V_1^0/V_2^0)] \tag{3}$$

In eqns. 2 and 3 P_1^0, B_{11} and M_1 are the saturated vapour pressure, second virial coefficient and molecular weight of the probe at column temperature, T, respectively, while ρ and V^0 represent the density and molar volume. In calculating the results, values for these physical properties were taken from a number of sources[11–15] and are shown in Tables I and II. The results calculated from eqns. 1–3 are given in Table III.

Bearing in mind the small temperature difference, the results for EPR are in good agreement with those of Ito and Guillet[3] measured at 30°C. Reasonable agreement is also seen for the PIB results compared to literature values[16–18]. The small divergence may be explained by the non-equilibrium effects noted above. Several workers have measured thermodynamic data for PDMS by GLC and obtained conflicting results although a more recent comparison of GLC values with those extrapolated from static equilibrium measurements showed good agreement[19]. The results from this study agree well with those of the latter work.

Although the interaction parameter was originally introduced to account for enthalpic mixing effects, later work recast it as a free energy parameter allowing

TABLE III

SPECIFIC RETENTION VOLUMES, V_g^0 ($cm^3\ g^{-1}$), INFINITE DILUTION ACTIVITY COEFFICIENTS, Ω_1^∞, AND INTERACTION PARAMETERS, χ^∞, FOR PROBES AT 25°C

	PDMS			*PIB*			*EPR*		
	V_g^0	$\ln \Omega_1^\infty$	χ^∞	V_g^0	$\ln \Omega_1^\infty$	χ^∞	V_g^0	$\ln \Omega_1^\infty$	χ^∞
Pentane	77.4	1.822	0.382	54.8	2.091	0.702	72.5	1.887	0.505
Hexane	219.5	1.803	0.415	184.0	1.983	0.646	217.4	1.816	0.487
Heptane	604.7	1.824	0.473	575.3	1.873	0.573	632.2	1.779	0.468
Octane	1633.5	1.833	0.511	1781.1	1.793	0.521	1973.7	1.691	0.426
Cyclohexane	390.4	1.683	0.461	338.8	1.922	0.476	473.8	1.489	0.327
Benzene	359.1	1.864	0.764	405.1	1.646	0.874	452.7	1.633	0.592
Toluene	1053.4	1.824	0.710	1062.1	1.816	0.754	1364.7	1.565	0.511
Carbon tetrachloride	371.5	0.960	0.455	387.4	0.918	0.465	450.4	0.768	0.322
Chloroform	226.6	1.190	0.616	262.9	1.041	0.520	289.8	0.944	0.430
Methylene chloride	95.0	1.599	0.907	137.2	1.232	0.593	145.9	1.170	0.539

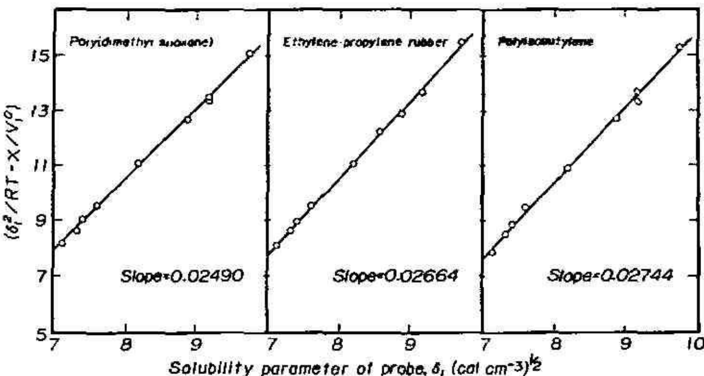

Fig. 1. Calculation of polymer solubility parameters at 25°C: (a) poly(dimethyl siloxane), (b) ethylene-propylene rubber, (c) polyisobutylene.

separation into an entropic contribution χ_S^∞ in addition to the enthalpy contribution, χ_H^∞.

$$\chi^\infty = \chi_H^\infty + \chi_S^\infty \tag{4}$$

Combining regular solution theory[20], which uses the solubility parameter to estimate enthalpy effects, with eqn. 4 leads to

$$\chi^\infty = \frac{V_1^0 (\delta_1 - \delta_2)^2}{RT} + \chi_S^\infty \tag{5}$$

where δ_1 and δ_2 are the solubility parameters of the probe and polymer, respectively. Expanding the term in parentheses and rearranging yields[2],

$$\frac{\delta_1^2}{RT} - \frac{\chi^\infty}{V_1^0} = \left(\frac{2\delta_2}{RT}\right)\delta_1 - \left(\frac{\delta_2^2}{RT} - \frac{\chi_S^\infty}{V_1^0}\right) \tag{6}$$

Hence, a plot of the term on the left hand side of eqn. 6 *versus* δ_1 should yield a linear relationship with slope $(2\delta_2/RT)$.

Fig. 1 shows the results for the three polymers plotted in the form suggested by eqn. 6. As predicted the plots show excellent linear correlation (regression coefficients > 0.99) in each case. The values of the derived polymer solubility parameters are given in Table IV along with literature data for comparison[7]. The PDMS result

TABLE IV

SOLUBILITY PARAMETERS (cal cm^{-3})$^{1/2}$ AT 25°C

	Current work	*Literature*[7]
PDMS	7.38	7.3–7.6
PIB	7.90	7.7–8.1
EPR	8.13	7.9–8.4

agrees very well with the value of 7.33 (cal cm^{-3})$^{\frac{1}{2}}$ measured by the same method as used here with static equilibrium data[21]. Table IV shows that, as has been found previously, results obtained by GLC and by more classical methods are in excellent agreement. However, in this work, GLC measurements were made in the temperature range where the classical methods are useful rather than being extrapolated from, in some cases, considerably higher temperatures. Thus the method used here is again shown to give useful results for δ and gives further credence to results obtained previously. However, it should be noted that the solubility parameter measured here is at infinite dilution of solvent (probe) whereas conventional techniques are usually useful for dilute polymer solutions. Since Regular Solution and basic Flory–Huggins theories do not allow for concentration dependent interaction parameters although there is ample experimental evidence that χ does vary with concentration, the relationship, if any, between δ_2 in the different regions of concentration is unclear.

The data analysis used in this work has yielded consistent results for a large range of probes and a number of polymers, a correlation which is worthy of further comment and speculation.

The Flory–Huggins relation for the thermodynamic activity of a solvent in a solution, a_1 (*i.e.*, the ratio of the solution fugacity to that of a standard state, usually taken as pure solvent), is

$$\ln a_1 = \ln \varphi_1 + [1 - (1/r)]\varphi_2 + \chi\varphi_2^2 \quad (7)$$

where r is the ratio of the molar volumes of polymer and solvent. Useful activity coefficients for polymer solutions may be defined in terms of volume fraction, φ, or of weight fraction, w, as earlier in the text.

$$a_1 = \varphi_1 {}^V\gamma_1 = w_1 \Omega_1 \quad (8)$$

where ${}^V\gamma_1$ is the activity coefficient on a volume fraction basis. Mole fraction based activity coefficients are of little use for polymer solutions since they require precise knowledge of molecular weights[10]. It is readily shown that, at infinite dilution,

$$\Omega_1^\infty = {}^V\gamma_1^\infty (\rho_2/\rho_1) \quad (9)$$

Hence, from eqn. 3 with the assumption that $r \gg 1$ as is the case for most polymers.

$$\begin{aligned} \chi^\infty &= \ln \Omega_1^\infty + \ln (\rho_2/\rho_1) - 1 \\ &= \ln {}^V\gamma_1^\infty - 1 \end{aligned} \quad (10)$$

By analogy with an excess free energy in small molecule solutions, which accounts for all mixing interactions other than the ideal entropy, and following Flory's definition[22], a residual partial molar free energy of mixing, ${}^\phi\Delta G_1^{R,\infty}$, can be used to account for all interactions not ncluded in the combinatorial entropy of mixing given by the Flory–Huggins expression.

$$\begin{aligned} {}^\phi\Delta G_1^{R,\infty} &= RT \ln {}^V\gamma_1^\infty \\ &= RT(\chi^\infty + 1) \end{aligned} \quad (11)$$

Hence

$$\chi^{\infty} = \frac{{}^{\phi}\Delta G_1^{R,\infty} - RT}{RT} \tag{12}$$

$$\approx \frac{{}^{\phi}\Delta G_1^{R,\infty} - PV}{RT} \tag{13}$$

$$\approx \frac{{}^{\phi}\Delta A_1^{R,\infty}}{RT} \tag{14}$$

assuming ideal behaviour of the probe vapour where A represents a Helmholtz free energy. It should be noted that eqns. 11–14 will be valid only for polymer solutions.

Thus, the infinite dilution interaction parameter as defined here has the form of a Helmholtz free energy or work function corresponding to the work required to remove a probe molecule from infinite dilution in the polymer compared to its removal from the pure liquid.

Since

$$\Delta A = \Delta U - T\Delta S \equiv RT\chi^{\infty} \tag{15}$$

then a more appropriate way of splitting χ^{∞} may be into an entropic contribution and one due to internal energy rather than enthalpy.

$$\chi^{\infty} = \chi_U^{\infty} + \chi_S'^{\infty} \tag{16}$$

where the prime on $\chi_S'^{\infty}$ differentiates it from the same quantity in eqn. 4.

Since the probe solubility parameter δ may be found from

$$\delta^2 = \Delta U^{vap}/V_1^0 \tag{17}$$

it is also an internal energy parameter. Thus, inspection of eqn. 6 shows that δ_2^{∞} is calculated from a combination of parameters representing changes in internal energies. This may provide at least a partial explanation of the consistency of results calculated according to eqn. 6 since δ_2^{∞} is obtained from the slope while χ_S^{∞} (or $\chi_S'^{\infty}$) appears only in the intercept.

Lipson and Guillet[4] have commented on the physical significance of χ_S values which were calculated from the intercepts of eqn. 6 but no general agreement of results has been found. It is possible that eqn. 5 needs to be recast in the form

$$\chi^{\infty} = \frac{V_1^0(\delta_1 - \delta_2)^2}{RT} + \chi_S^{\infty} + \chi_{PV}^{\infty}$$

where χ_{PV} accounts for pressure–volume effects. These effects would be included in the χ_S calculated as above and so may go some way toward explaining some inconsistencies found in previous discussions. It is also pertinent to note that more recent rigorous theories of the thermodynamics of polymer solutions[22–24] treat χ as a residual chemical potential with pressure–volume effects included by considering equation of state effects.

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REFERENCES

1 F. W. Harris and R. B. Seymour (Editors), *Structure–Solubility Relationships in Polymers*, Academic Press, New York, 1977.
2 G. DiPaola-Baranyi and J. E. Guillet, *Macromolecules*, 11 (1978) 228.
3 K. Ito and J. E. Guillet, *Macromolecules*, 12 (1979) 1163.
4 J. E. G. Lipson and J. E. Guillet, in R. B. Seymour and G. Stahl (Editors), *Solvent–Property Relations in Polymers*, Pergamon, New York, 1982.
5 J. M. Braun and J. E. Guillet, *Macromolecules*, 8 (1975) 557.
6 G. J. Price and J. E. Guillet, *J. Macromol. Sci. Chem.*, A23 (1986) 1487.
7 J. Brandrup and E. H. Immergut (Editors), *Polymer Handbook*, Wiley, New York, 1975
8 R. N. Lichtenthaler, D. D. Liu and J. M. Prausnitz, *Macromolecules*, 7 (1974) 565.
9 J. R. Conder and C. L. Young, *Physicochemical Measurement by Gas Chromatography*, Wiley, Chichester, 1979.
10 D. Patterson, Y. B. Tewari, H. P. Schreiber and J. E. Guillet, *Macromolecules*, 4 (1971) 356.
11 J. H. Dymond and E. B. Smith, *The Virial Coefficients of Pure Gasses and their Mixtures*, Clarendon Press, Oxford, 1969.
12 R. R. Dreisbach, *Physical Properties of Chemical Compounds*, Vol. 3, American Chemical Society, Washington, DC, 1965, Table 23-10-2 (10.011)d.
13 T. Boublik, V. Fried and E. Hala, *The Vapour Pressure of Pure Substances*, Elsevier, New York, 1973.
14 *Selected Values of the Prorerties of Hydrocarbons*, A.P.I. Project 44.
15 *Selected Values of the Properties of Chemical Compounds*, T.R.C. Data Project, Texas A&M University College Station, TX, 1965.
16 Y. K. Leung and B. E. Eichinger, *J. Phys. Chem.*, 78 (1974) 60.
17 R. N. Lichtenthaler, J. M. Prausnitz, C. S. Su, H. P. Schreiber and D. Patterson, *Macromolecules*, 7 (1974) 136.
18 R. N. Lichtenthaler, R. D. Newman and J. M. Prausnitz, *Macromolecules*, 6 (1973) 650.
19 A. J. Ashworth, C. F. Chien, D. L. Furio, D. M. Hooker, M. M. Kopecni, R. J. Laub and G. J. Price, *Macromolecules*, 17 (1984) 1090.
20 J. H. Hildebrand, J. M. Prausnitz and R. L. Scott, *Regular and Related Solutions*, Van Nostrand, New York, 1972.
21 A. J. Ashworth and G. J. Price, *Macromolecules*, 19 (1986) 362.
22 P. J. Flory, *Discuss. Farad. Soc.*, 49 (1970) 7.
23 D. Patterson, *Macromolecules*, 2 (1969) 672.
24 I. Sanchez and R. H. Lacombe, *Macromolecules*, 11 (1978) 1145.

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COMPARATIVE COMPUTERISED GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC ANALYSIS OF PETROPORPHYRINS

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SUMMARY

The ability of computerised gas chromatography–mass spectrometry (C-CG–MS) to afford detailed information on petroporphyrin composition is exemplified through analyses of Boscan crude oil and La Luna shale (Maracaibo Basin, W. Venezuela), an oil-source rock pair. The petroporphyrins of both samples are complex mixtures, comprising at least 224 and 175 compounds, respectively. Five structural classes (A, A-2, A-4, A-6 and A-8) have been characterised and shown to contain at least 5 pseudo-homologous series through linear Kováts' plots and co-injection. The two samples are shown to be qualitatively and quantitatively very similar in composition. These related samples are compared and contrasted to an unrelated bitumen, Gilsonite, examined in an earlier paper. The data show that petroporphyrin, analysis by C-GC–MS can provide classical biological marker information, *e.g.* thermal maturity. This paper provides the first such comparative examination of petroporphyrins by GC–MS analysis.

INTRODUCTION

The potential of endogenous petroporphyrins to yield geochemical information such as depositional palaeoenvironment and thermal history is now well established[1–4]. To obtain such information, the petroporphyrin components must be isolated and characterised. Direct insertion probe mass spectrometry (MS)[5,6] and high-performance liquid chromatography (HPLC)[3,7,8] have both been used extensively as means of petroporphyrin "fingerprinting" and characterisation; both have their shortcomings[9,10]. We have recently developed procedures whereby petroporphyrins can be rigorously analysed by computerised gas chromatography–mass spectrometry (C-GC–MS) as their bis(*tert.*-butyldimethylsiloxy)Si(IV) derivatives. Porphyrin com-

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1

1a R^{1-8} = Et

1b R^{1-8} = Me

1c R^{1-8} = Me_5Et_2H

1d $R^{1-3,5-7}$ = Me, $R^{4,8}$ = Et

1e $R^{1-3,5,6,8}$ = Me, $R^{4,7}$ = Et

1f $R^{2,4,5,7,8}$ = Me, $R^{1,3,6}$ = Et

1g $R^{2,4,6,7,8}$ = Me, $R^{1,3,5}$ = Et

1h $R^{2,3,4,5,8}$ = Me, $R^{1,6,7}$ = Et

1i $R^{1,3,4,5,8}$ = Me, $R^{2,6,7}$ = Et

1j $R^{1,3,5,8}$ = Me, $R^{2,4,6,7}$ = Et

2

2a R^1 = Et, R^2 = Me

2b R^1 = Me, R^2 = Me

2c R^1 = Et, R^2 = H

3

R^{1-6} = alkyl

4

R^{1-6} = alkyl

5

R^{1-5} = alkyl

6

R^{1-5} = alkyl

9

7

$R^{1,2}$ = alkyl

8

R = H or Me

10 11 12

11a R = Me

11b R = Et

position is readily determined[9–13]. Structural type is deduced from the characteristic $(M-131)^+$ ions. Further classification into pseudo-homologous series is achieved via assignment of pseudo-Kováts retention indices and co-chromatography of derivatised standards of known structure. Quantitative information, readily obtainable from ion-intensity data, can be used to plot distribution profiles for use in comparative studies.

The procedural details of the C-GC–MS and data handling techniques are well documented[10,13,14]. To date, however, detailed analysis has been reported for only a single sample, the Gilsonite bitumen (Eocene, Uinta Basin, UT, U.S.A.)[14]. The results demonstrated the power of the technique to yield compositional information inaccessible by other methods. Over 100 individual components were observed, comprising four structural classes.

We now extend C-GC–MS investigations of petroporphyrins to Boscan crude oil and its proposed source rock, the La Luna shale (Cretaceous, Maracaibo Basin, W. Venezuela). Studies of the Maracaibo Basin have firmly demonstrated this source–oil relationship[15,16]. Petroporphyrin analysis has been used to establish this close relationship[17]. The oils and shales of this region are well known for their high vanadyl porphyrin content (typically several thousand ppm of the crude oils and shale extracts) and have been the subject of a number of porphyrin investigations. Ultraviolet–visible spectrophotometry (UV–VIS) initially revealed their high vanadyl porphyrin content[17]. Subsequently[5,6], MS of both metallo- and metal-free (following sequestration in acid media) porphyrins showed them to be a complex mixture of structural types proposed to be aetio (1), deoxophylloerythroaetio-(DPEP; 2), di-DPEP (3), rhodo-aetio- (4) and rhodo-DPEP (5). Recently, the nomenclature benz-aetio- and benz-DPEP- has been used for the latter two structural types[2] on the basis of structures (4 and 5) suggested earlier[6]. Structures of the benz-DPEP type have now been proven unambiguously for two compounds (C_{32} and C_{33})[18]. ^1H NMR evidence has been presented[4] for the structure of the so-called di-DPEP porphyrins; a tetrahydro-benz-DPEP structure (6) was proposed. Extensive carbon number homology has been detected by MS for the various proposed structural types[5,6,19–21]. Chromium trioxide oxidation of Boscan porphyrins has been employed in attempts to determine the nature of β-alkyl substituents of the porphyrin ring[20,22–24]. GC–MS

analyses showed the presence of maleimide (7) and phthalimide (8) oxidation products. Hence, evidence was obtained for the presence of compounds bearing extended β-alkyl substituents[24] and fused benzo-rings[20]; both structural features are unknown in modern-day biogenic porphyrins. The presence of fused benz(o) rings in some compounds has recently been unambiguously proved[18]. Thin-layer chromatography (TLC) and HPLC of Boscan porphyrins have been used to afford carbon number separations[7,25]. Kováts'-type plots of log isocratic retention times *vs.* carbon number provided evidence for the presence of at least three homologous or pseudohomologous series of aetioporphyrins in Boscan oil and La Luna shale[26]. Indications of pseudo-homologous series were also obtained in early GC–MS examinations of Boscan porphyrins on packed GC columns[25]. The poor resolution and high adsorptive properties of the packed GC columns limited further investigation[27] and led us to employ flexible fused silica capillaries and bonded phases in order to obtain the detailed C-GC–MS information required[9,11,12,28].

It was envisaged that analyses of the Boscan and La Luna petroporphyrins would enable us to begin to test the versatility of the C-GC–MS data for use in comparative geochemical investigations. Furthermore, by choosing samples that have experienced very different geological histories to the Gilsonite bitumen (the only other sample so far studied and reported in this way[14]) it may be possible to build up an explicit picture of the way in which petroporphyrin content and distributions vary with differing geological regimes.

EXPERIMENTAL

Samples

The geological histories of the La Luna shales and Boscan oils have been described in detail elsewhere[17,29]. The oil examined was Boscan 8E-4, which had been studied previously by TLC, HPLC and MS[8,17]. The La Luna sample examined herein was obtained from an outcrop in the type-area. Gilsonite data derive from an earlier publication[14].

Extraction of free-base porphyrins

After Soxhlet extraction of the shale (83 h, dichloromethane), the porphyrins were extracted as their free bases by demetallation with methanesulphonic acid, according to established procedures[30]. Typically, crude oil or shale extract (1 g) was heated (100°C, 2 h) with at least a five–ten fold excess of methanesulphonic acid (98%, *ca.* 10 ml, Aldrich). The reaction was quenched by pouring the acid–organic mixture into distilled water (25 ml). After allowing to cool, the coagulated organic material was removed by filtration. The aqueous filtrate, containing the porphyrins as dications, was extracted with dichloromethane (3 × 15 ml), which was then neutralised (sodium bicarbonate) and dried (sodium sulphate). The free-bases obtained were purified by TLC (silica gel–dichloromethane). In neither the Boscan nor La Luna case was separation of nickel and vanadyl porphyrins attempted. In each case the nickel porphyrins are present in much lower abundance (< 100 ppm)[31].

La Luna shale (513 g) produced a total extract (7.7 g) yielding free-base porphyrins (127 mg: 247 ppm of shale, 1500 ppm of extract). Boscan oil (1 g) yielded free-base porphyrins (2.5 mg: 2500 ppm of oil). All UV–VIS spectra were recorded

in dichloromethane solution, quantification being performed on the α absorption maximum of the vanadyl porphyrins (570 nm, ε = 17 600)[32] or absorption band IV of the free-base porphyrins (498 nm, ε = 15 600)[32].

Compounds for co-injection

The compounds employed in the co-injection studies were those used earlier[14]. The compound identities, their structure references, pseudo-Kováts retention indices [KRI, as $(TBDMSO)_2Si$ derivatives], MS $(M-131)^+$ ion m/z, source, and occurrence in La Luna shale and Boscan crude oil are listed in Table I.

Friedel-Crafts acetylation

An aliquot of the free-base porphyrins from Boscan oil was converted to its copper(II) chelates[39] prior to acetylation[40], as described previously for Gilsonite[14]. TLC separation yielded a fully β-alkyl substituted fraction which was derivatised for GC–MS analysis.

Derivatisation

The insertion of silicon into aliquots of the free-bases and formation of $(TBDMSO)_2Si$ derivatives was carried out as described earlier[9,14].

Instrumentation, data acquisition and KRI calculation

The instrumentation and data acquisition parameters have been fully detailed elsewhere[14]. GC–MS analyses were performed on a Finnigan 4000 quadrupole mass spectrometer, linked to a Finnigan 9610 gas chromatograph with modified SGE OCI-2 on-column injector. The column was a flexible fused-silica capillary (Hewlett Packard "Ultra" series; 25 m × 0.31 mm I.D.) coated with cross-bonded polydimethysiloxane (0.17 μm film thickness); temperature programming details as for the Gilsonite analyses[14]. Data acquisition was in multiple ion detection (MID) mode monitoring the ion m/z 113 and then scanning the range m/z 545–850[14]. Data processing utilised a Finnigan INCOS 2300 data system.

Retention indices were calculated by computer program (RRI[41]), using n-alkane standards[42]. Improved reproducibility of KRI calculation was achieved by use of two standard porphyrin derivatives [$(TBDMSO)_2Si$ OMP and $(TBDMSO)_2Si$ OEP (Table I)] as retention standards, their pseudo-Kováts retention indices being defined as 3455 and 3800, respectively. The KRI values of the more numerous n-alkanes were then adjusted in the RRI program by a constant offset (generally <10 KRI units) until these defined values were realised. Thus, problems caused by differences in GC behaviour of the porphyrin derivatives and the n-alkanes are effectively minimised.

Processing of GC–MS data

Detailed data processing for the Boscan and La Luna samples followed the procedures described previously[9,13,14].

Data presentation

The presentation of data in this paper, while developed from our earlier published methods[9,14], has been designed to allow direct comparison of multiple sample analyses in a variety of ways.

TABLE I

CO-INCIDENCE OF STANDARD PORPHYRINS WITH GILSONITE, LA LUNA AND BOSCAN PORPHYRINS AS $(TBDMSO)_2Si$ DERIVATIVES

Abbreviations: OMP = 2,3,7,8,12,13,17,18-octamethylporphyrin; aetio-III = aetiporphyrin III; OEP = 2,3,7,8,12,13,17,18-octaethylporphyrin.

Porphyrin	*Structure*	*KRI*	*m/z* $[M-131]^+$	*Co-chromatographs or co-incident**			*Source of compound*
				Gilsonite	*La Luna*	*Boscan*	
C_{28} A (OMP)	1b	3455	579	Yes	Yes	Yes	P. S. Clezy***
C_{29} A**	1c	3528	593	Yes	Yes	Yes	
		3540	593	Yes	Yes	Yes	Gilsonite bitumen[33,34]
C_{30} A	1d,e§	3541	607	Yes	Yes	Yes	Gilsonite bitumen[34,35]
C_{31} A§§	1f–i§	3583	621	Yes	Yes	Yes	Gilsonite bitumen[34]
C_{32} A (aetio-III)	1j	3624	635	Yes	Yes	Yes	J. G. Erdman***
C_{36} A (OEP)	1a	3800	691	No	No	No	Porphyrin Products Inc.***
C_{31} 13,15-ethano	2b	3715	619	Yes	Yes	Yes	Serpiano shale[32]
C_{32} 13,15-ethano	2a	3754	633	Yes	Yes	Yes	Serpiano shale[32]
C_{32} 13^1-methyl-13,15-ethano	9	3715	633	Yes	Yes	Yes	Gilsonite bitumen[35,36]
C_{32} 15^1-methyl-15,17-propano	10	3795	633	No	No	No	Serpiano shale[37]
C_{31} 15,17-butano	11a	3842	619	Yes (trace)	No	No	Serpiano shale[32,35]
C_{32} 15,17-butano	11b	3894	633	No	No	No	Serpiano shale[32,38]

* Co-chromatography in the cases of Gilsonite and Boscan, KRI comparison (see text) in the case of La Luna.
** At least two components, partially resolved into two peaks.
*** Synthetic compound.
§ A number of possible structural (positional) isomers.
§§ At least two components, not resolved.

Figs. 1 and 2 show simple GC–MS reconstructed ion current (RIC) traces presented on a KRI scale *vs.* ion intensity. The regions above KRI 3775 have been expanded by a factor of 5 in the vertical scale.

Figs. 3 and 4 contain plots of KRI *vs.* carbon number. Rather than showing all the points (≡ porphyrins detected) for one sample (*cf.* Figs. 3–6, 8 of ref. 14), the

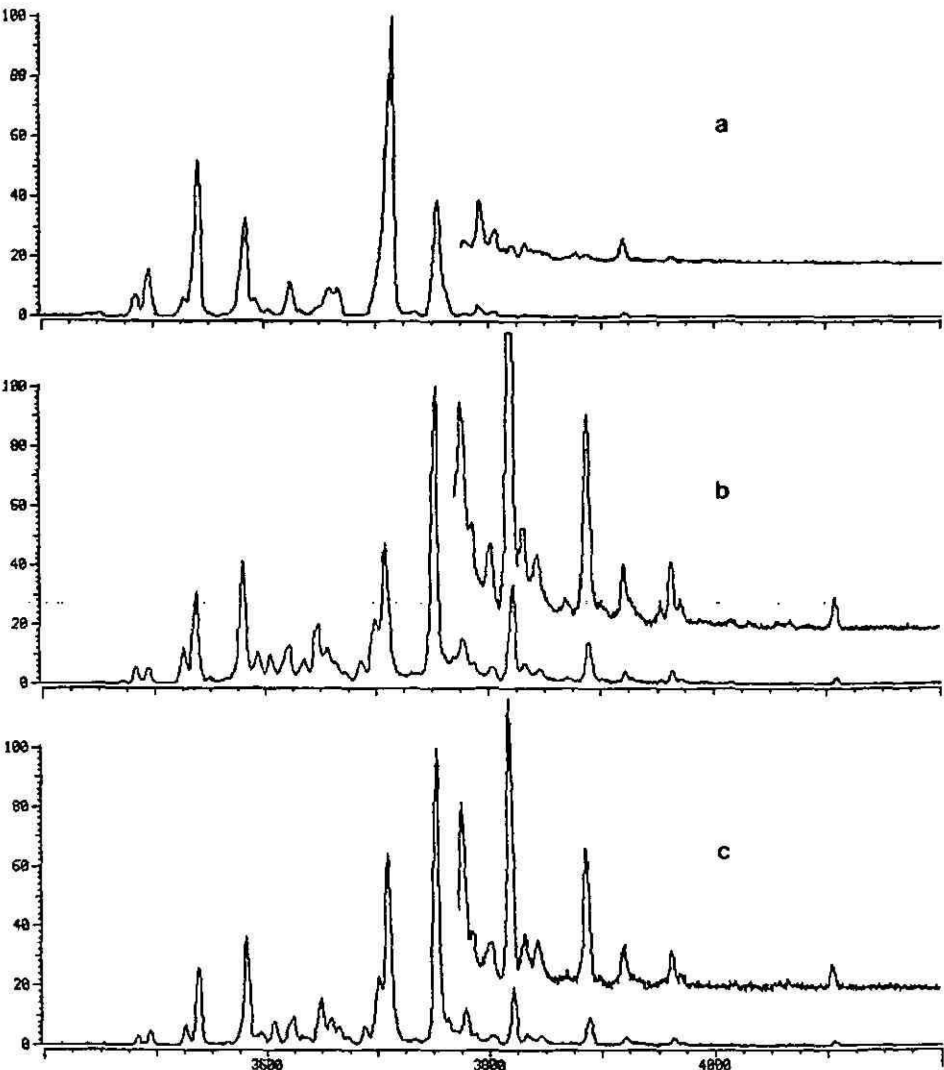

Fig. 1. Reconstructed ion current (RIC; m/z 545–850) GC–MS traces for the total porphyrin mixtures [as their $(TBDMSO)_2Si$ derivatives] of (a) Gilsonite bitumen, (b) La Luna shale, and (c) Boscan oil. The retention time scale has been converted to KRI by computer interpolation, using data for co-chromatographed *n*-alkanes. Considerable co-elution of components is occurring but some peaks are broad and fronting due to high loading of the GC column, required to enable detection of trace components. Injection of *ca.* 1–5 μg of porphyrin derivatives on-column produced ion intensity values for the largest peak of 206 592 (a), 56 896 (b) and 45 312 (c). See Experimental for conditions.

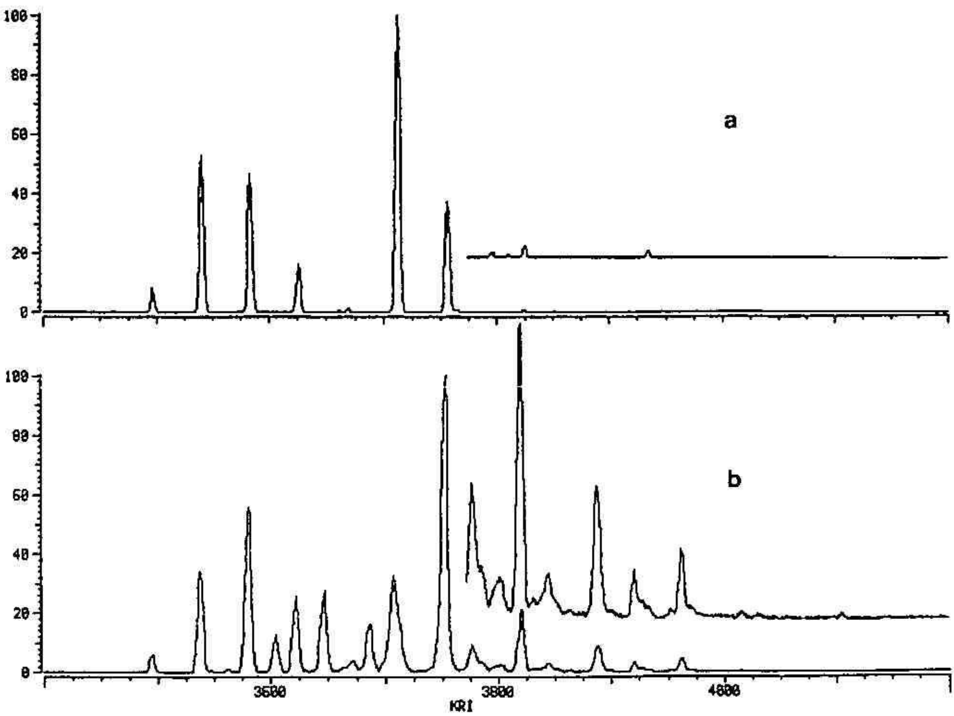

Fig. 2. RIC (m/z 545–850) traces for the fully alkylated petroporphyrin fractions [as their $(TBDMSO)_2Si$ derivatives] of (a) Gilsonite bitumen and (b) Boscan oil. Compared to Fig. 1, the greatly improved peak shapes are the consequence of simplification brought about by the removal of β-unsubstituted components, so eliminating much of the co-elution found in the total mixtures. Column loadings are comparable, with ion intensities of 281 088 (a) and 78 208 (b). See Experimental for details.

plots show comparisons of two samples. Thus, Fig. 3a contrasts the porphyrin content of La Luna shale with the unrelated Gilsonite bitumen; Fig. 3b compares the shale with its sourced oil, Boscan; and Fig. 4 contrasts only the fully alkylated porphyrins of Gilsonite and Boscan. In each plot, a carbon number range of 27–36 has been used. Some samples (especially Boscan, Table II) contain porphyrins outside this range, but these fall below the abundance thresholds used. Only porphyrins having abundances >2% of the largest have been displayed in the A and A-2 class plots, and only those >0.4% of the largest in the A-4, A-6 and A-8 classes. Each plot has a vertical axis range of 500 KRI units, the actual range chosen is varied to suit the class (*e.g.* 3400–3900 for the A class, 3800–4300 for the A-8 class).

Fig. 5 contains histograms of abundance (≡ ion intensity) *vs.* carbon number for the five petroporphyrin series partially identified (by co-chromatography and KRI comparisons) in the three samples studied. The use of carbon number rather than KRI as the horizontal scale allows more direct comparison of samples (*cf.* Fig. 7 of ref. 14). Abundances are normalised to the largest individual component as 100%.

Fig. 6 contains similar histograms to the above, but for the five unsaturation classes found in these samples. The abundances are normalised to the largest total

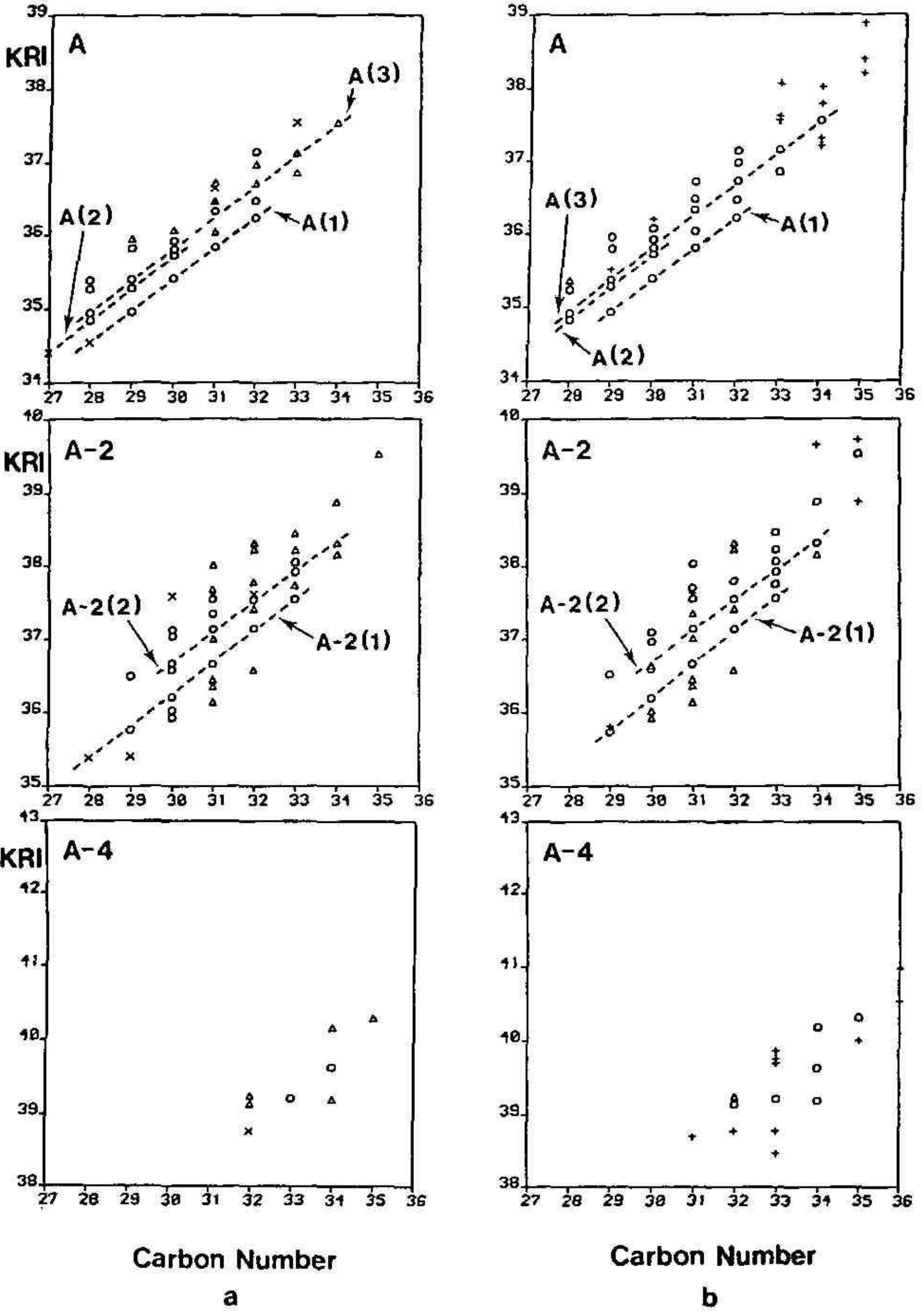

Fig. 3.

(Continued on p. 290)

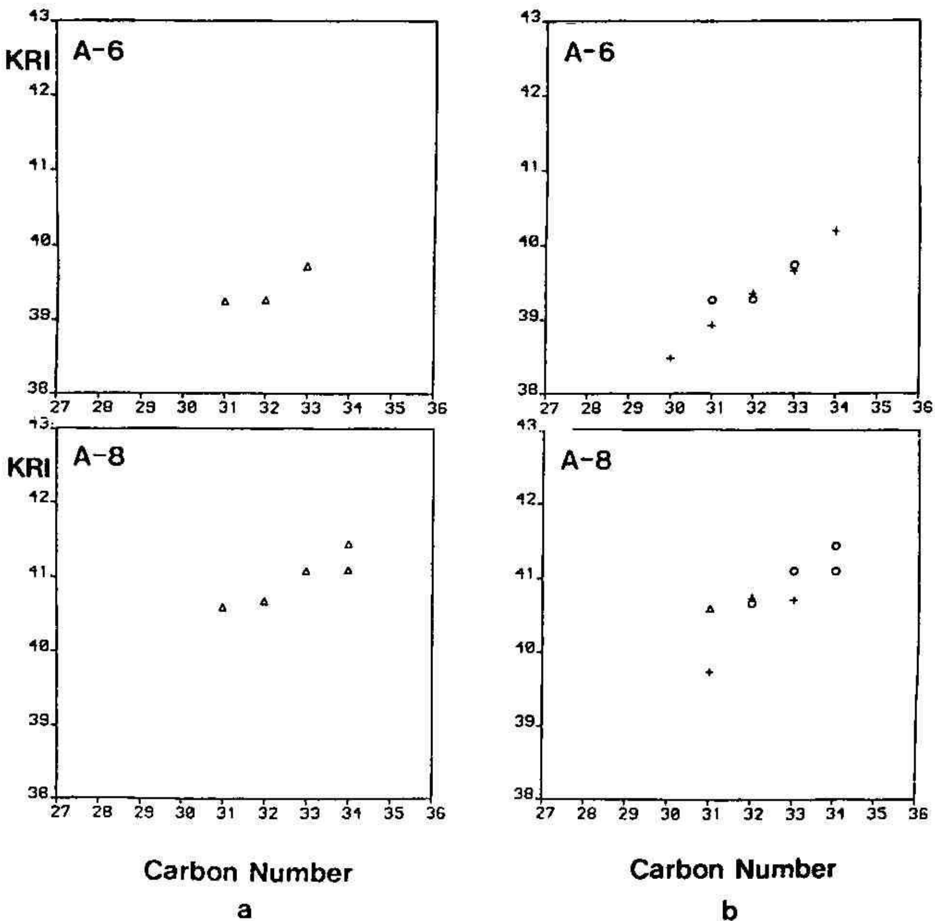

Fig. 3. Plots of KRI *vs.* carbon number for GC–MS analyses of porphyrins as their $(TBDMSO)_2Si$ derivatives, comparing the petroporphyrin compositions, displayed as A, A-2, A-4, A-6 and A-8 classes for (a) La Luna shale and the unrelated Gilsonite bitumen, (b) La Luna shale and its sourced oil, Boscan. Before being plotted, threshold values of intensity were applied to reduce the number of points. The lower limits used were >2% of the largest peak for A and A-2 classes, and >0.4% of the largest peak for A-4, A-6 and A-8 classes. Different symbols illustrate those points (≡ porphyrin components) common to two samples, present only in one sample, or only in the other sample (see key). Dashed lines indicate proposed pseudo-homologous series[14], partially identified by co-chromatography or KRI and MS comparison. Experimental conditions were as for Figs. 1 and 2 (see text). Key: (a) ×, Gilsonite only; △, La Luna only; ○, Gilsonite and La Luna; (b) △, La Luna only; +, Boscan only; ○, La Luna and Boscan.

carbon number as 100% and these plots include all porphyrins detected in the C_{26}–C_{38} range.

Fig. 7 contains histograms for all the pseudo-homologous series co-injected or KRI compared with the samples, even where the series represented by the standards was not present in the samples. All are normalised to the largest individual compound as 100%. Histograms are included for the sum of those compounds remaining after the partially identified series have been subtracted from the total for that class. This

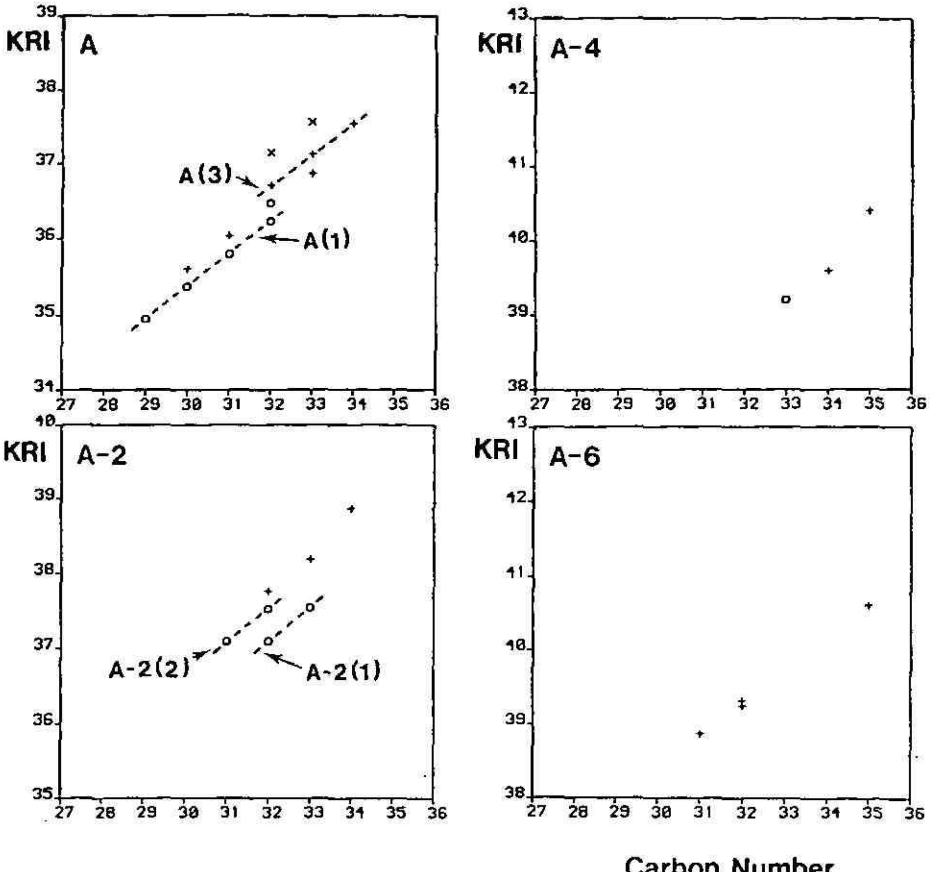

Fig. 4. Plots of KRI *vs.* carbon number comparing the A, A-2, A-4 and A-6 classes of porphyrins in the fully alkylated fractions of Gilsonite bitumen and Boscan oil, as their $(TBDMSO)_2Si$ derivatives. The simplification which has ensued following removal of β-unsubstituted porphyrins is evident by comparison with Fig. 3. However, such removal may not be complete (see text). Thresholds are applied as for Fig. 3; A-8 class porphyrins were below 0.4% of the largest peak. Again, different symbols are used to show common components and those present only in one or other of the samples (see key). Pseudo-homologous series are indicated by dashed lines. Experimental conditions were as for Figs. 1 and 2 (see text). Key: ×, Gilsonite only; +, Boscan only; O, Gilsonite and Boscan.

figure forms the basis for expansion as further series are identified and co-injected (*e.g.* structures 2c[43], 12[44]). In Figs. 3–7, components were assumed to be identical in two samples if possessing identical mass spectral ions and KRI values within ± 5 units.

Tables III and IV are similar to Table III of ref. 14. It should be noted that components are listed below each carbon number in order of elution, so that horizontal rows do not represent pseudo-homologous series.

TABLE II

CARBON NUMBER RANGES AND MAXIMA OF THE FIVE UNSATURATION CLASSES OF PORPHYRINS OBSERVED IN GILSONITE BITUMEN, LA LUNA SHALE AND BOSCAN OIL, AS DETERMINED BY C-GC-MS

n.d. = Not detected.

Class	*Carbon number range (maximum)*		
	Gilsonite	*La Luna*	*Boscan*
A	26–35 (30)	26–36 (30)	27–38 (30)
A-2	27–35 (31)	27–36 (32)	28–38 (32)
A-4	31–34 (33)	31–36 (34)	31–37 (34)
A-6	31–34 (32)	31–34 (33)	30–34 (32)
A-8	n.d.	31–34 (33)	31–36 (33)

RESULTS

Gilsonite bitumen

The extraction, demetallation and derivatisation of the petroporphyrins of Gilsonite have been described in detail elsewhere[14]. Some results are repeated and extended herein, with display formats chosen to allow comparison with La Luna shale and Boscan oil.

La Luna shale

Fig. 1b shows the RIC (m/z 545–850) obtained from the total petroporphyrin derivatives of La Luna shale. Thirty-five fully or partially resolved peaks are discernable. As is generally the case in GC–MS analysis of complicated mixtures, considerable co-elution of components is occurring, as evidenced by subsequent mass chromatography. Single-ion mass chromatograms were examined for the intense $(M-131)^+$ ions of all alkyl porphyrins from C_{20} to C_{42}, and for the classes A, A-2, A-4, A-6 and A-8. The porphyrins present were of the carbon numbers and classes listed in Table II. To enable the checking of mass chromatogram peaks, spectra were obtained, summed in groups of 20 scans, sequentially over the entire region of porphyrin elution. These were processed to allow the detection and rejection of "spurious" peaks, *i.e.* peaks not due to $(M-131)^+$ ions of porphyrin derivatives (discussed in detail elsewhere[9]). The remaining "genuine" mass chromatogram peaks were listed (Table III). Quantification of the detected porphyrin derivatives listed in Table III was performed by published methods[14], and is therefore subject to the same errors arising when two porphyrins, with masses differing by two a.m.u., co-elute (*e.g.* a C_{30} A class porphyrin co-elutes with a C_{30} A-2 class porphyrin at KRI 3620 (see Table III). Such instances of co-elution occurred 28 times in the La Luna analysis, the quantification error introduced from this source varying from being negligible (<1%, where the compound of lower mass is much less abundant than that of higher mass) to a worst case in which a compound, though present, is not detected, because its $(M-131)^+$ ion is "swamped" by the isotope peak of a co-eluting

compound. The approximate quantification adopted was, therefore, simply to use the peak areas of the characteristic $(M-131)^+$ ions in the GC-MS data. These areas were obtained from retention index (RI) lists, output by program RRI[9]. The areas of the peaks may be read directly from the list, and are then expressed as fractions (%) of the area of the major C_{32} A-2 porphyrin (KRI = 3754, 2a), read from the m/z 633 list, and also as fractions of the total porphyrins. Table III contains details of each porphyrin detected by GC-MS analysis of the petroporphyrins of La Luna shale (175 in all), its class, carbon number, KRI and abundance.

To allow further investigation of the tabulated petroporphyrins, Kováts' plots were prepared. For the porphyrins within each class (A, A-2, etc.), KRI was plotted against carbon number after application of threshold values (see *Data presentation*). Two sets of plots show comparative co-plotting of data from Gilsonite and La Luna (Fig. 3a), and La Luna and Boscan (Fig. 3b). The series partially identified in Gilsonite[14] are present also in La Luna and are marked on the plots by dashed lines.

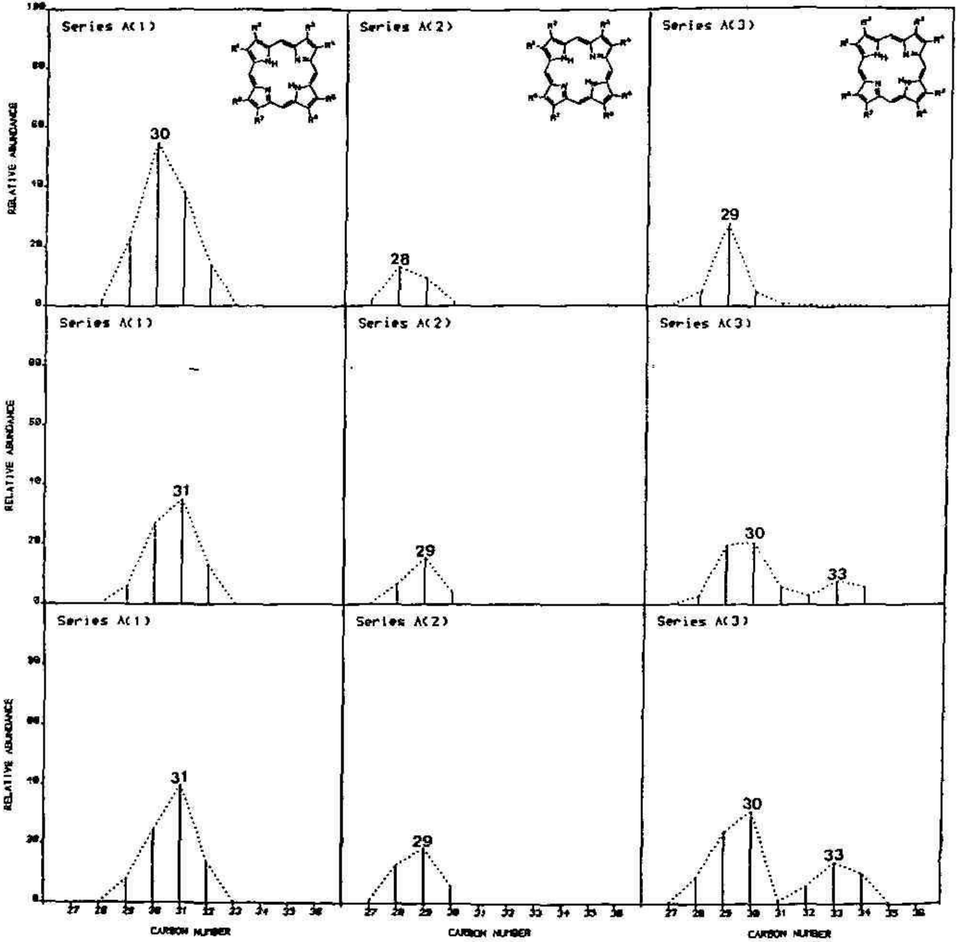

Fig. 5.

(Continued on p. 294)

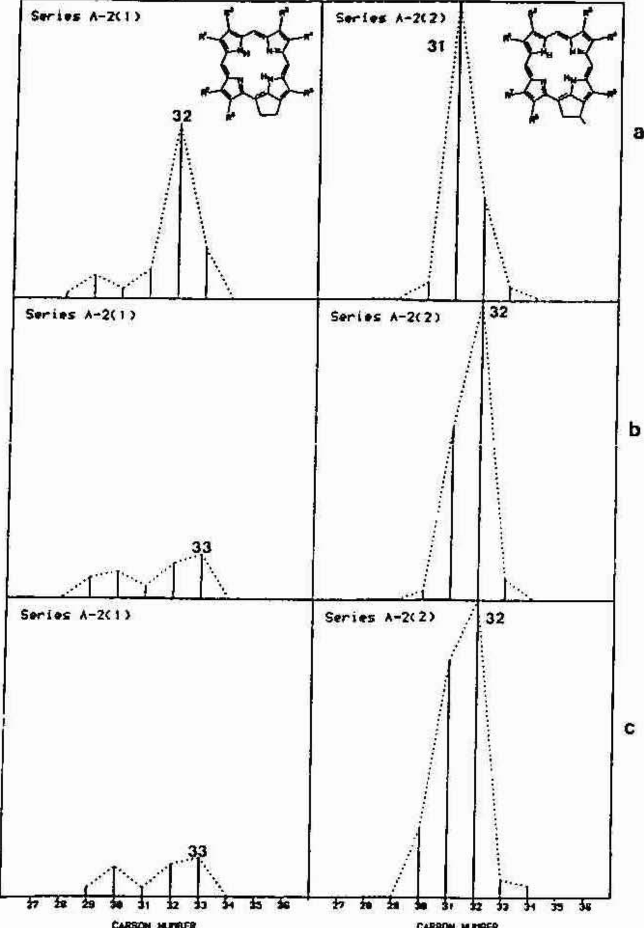

Fig. 5. Relative abundance histograms for the five partially identified series of petroporphyrins occurring in (a) Gilsonite bitumen, (b) La Luna shale, and (c) Boscan oil. Assignment is based on similarity (±5) of KRI values to those of derivatised porphyrins of known structure and by extrapolation from them on Kováts' plots (see Fig. 3a,b). Abundances are derived from ion intensity data[9]. Compounds of elucidated structure which have been co-injected are: A(1) series, C_{30}, C_{31} and C_{32}; A(2) series, C_{29} (partially identified); A(3) series, C_{29} (partially identified); A-2(1) series, C_{32}; A-2(2) series, C_{31} and C_{32}. The structure of A(1) C_{29} is known from comparison of abundance with the work of Quirke *et al.*[33]. As contrasted to ref. 14, the data are plotted here by carbon number rather than KRI, allowing more direct comparison between different samples, in which KRI values differ by up to ±5 units for the same components.

There is reason to suppose, by analogy with the behaviour of standards, that if the points on the Kováts' plots are joined by straight lines with gradients of *ca.* 40–50 KRI units per carbon number, then these lines represent pseudo-homologous series of structurally related petroporphyrins. Further evidence for this hypothesis has come from co-injection experiments. Table I sets out the standards employed, their GC–MS characteristics (KRI values and major MS ions), and indicates occurrences of co-incidence with a component of the La Luna porphyrins. Co-injection

experiments with standard porphyrin derivatives were not performed for La Luna shale. Knowledge of the retention indices of the standards concerned (Table I), and of the coincidence of straight-line relationships of the La Luna Kováts' plots with those of Gilsonite[14] and Boscan, allows reliable identification of those components of the La Luna porphyrin mixture which correspond to the known standards. Thus, for example, it is possible to state that the C_{30} aetioporphyrin (KRI = 3539; Table III) is identical to, or isomeric with, the C_{30} aetioporphyrin isolated from Gilsonite (1d or 1e[34]).

By use of the KRI comparison method, a number of compounds have been tentatively identified in the petroporphyrins of La Luna shale, certain other compounds can be definitely stated to be absent from the mixture (or, at least, to be below the GC–MS detection limit for $(TBDMSO)_2Si$ derivatives). The standard porphyrins are listed in Table I, showing whether or not they are present in La Luna shale.

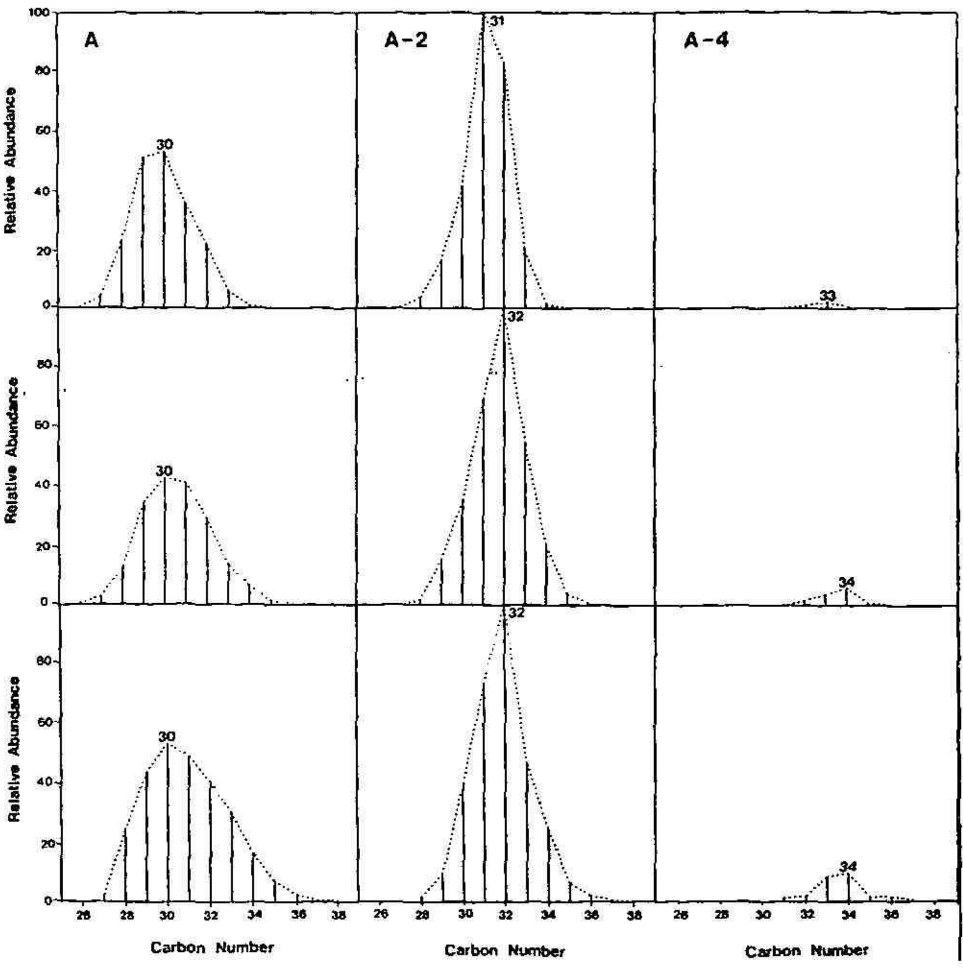

Fig. 6.

(Continued on p. 296)

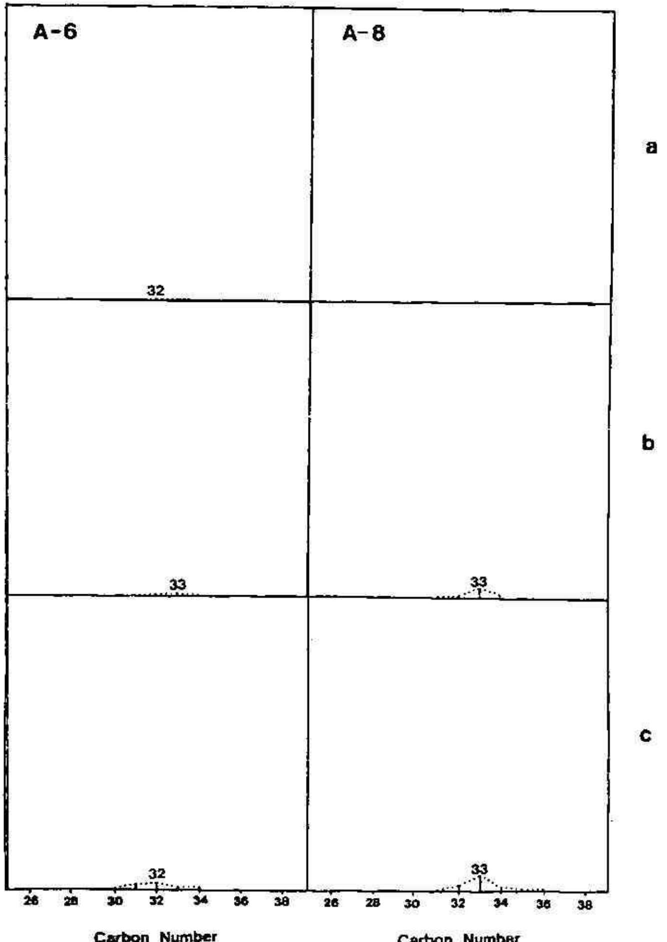

Fig. 6. Relative abundance histograms for the 5 unsaturation cla_ses A, A-2, A-4, A-6, A-8, as detected in (a) Gilsonite, (b) La Luna, and (c) Boscan samples.

Following this procedure, five pseudo-homologous series of petroporphyrins may be partially identified, as for Gilsonite bitumen[14]: A(1), A(2), A(3), A-2(1) and A-2(2).

Histograms (Fig. 5b) were prepared for each of the five series, forming distribution profiles, showing each component of the series, with its KRI value and relative abundance. In each case, the abundances are presented as percent fractions of the abundance of the C_{32} A-2 of KRI = 3754 (structure 2a). Additional histograms of this type are shown in Fig. 6b. These show the abundance and carbon number relationships within the unsaturation classes, A, A-2, A-4, A-6 and A-8. Histograms of different types appear in Fig. 7b, showing all the series for which standards were available, plotting abundance against carbon number for each series A(1), A(2), A(3), A-2(1) and A-2(2). In addition, series A-2(3) and A-2(4) appear. These have structures as shown and are included, though absent from the samples studied in this

Fig. 7.

(Continued on p. 298)

A-2(1)	32	33	33	R^{1-7} = Alk
A-2(2)	31	32	32	R^{1-7} = Alk
A-2(3)				R^{1-6} = Alk
A-2(4)				R^{1-6} = Alk

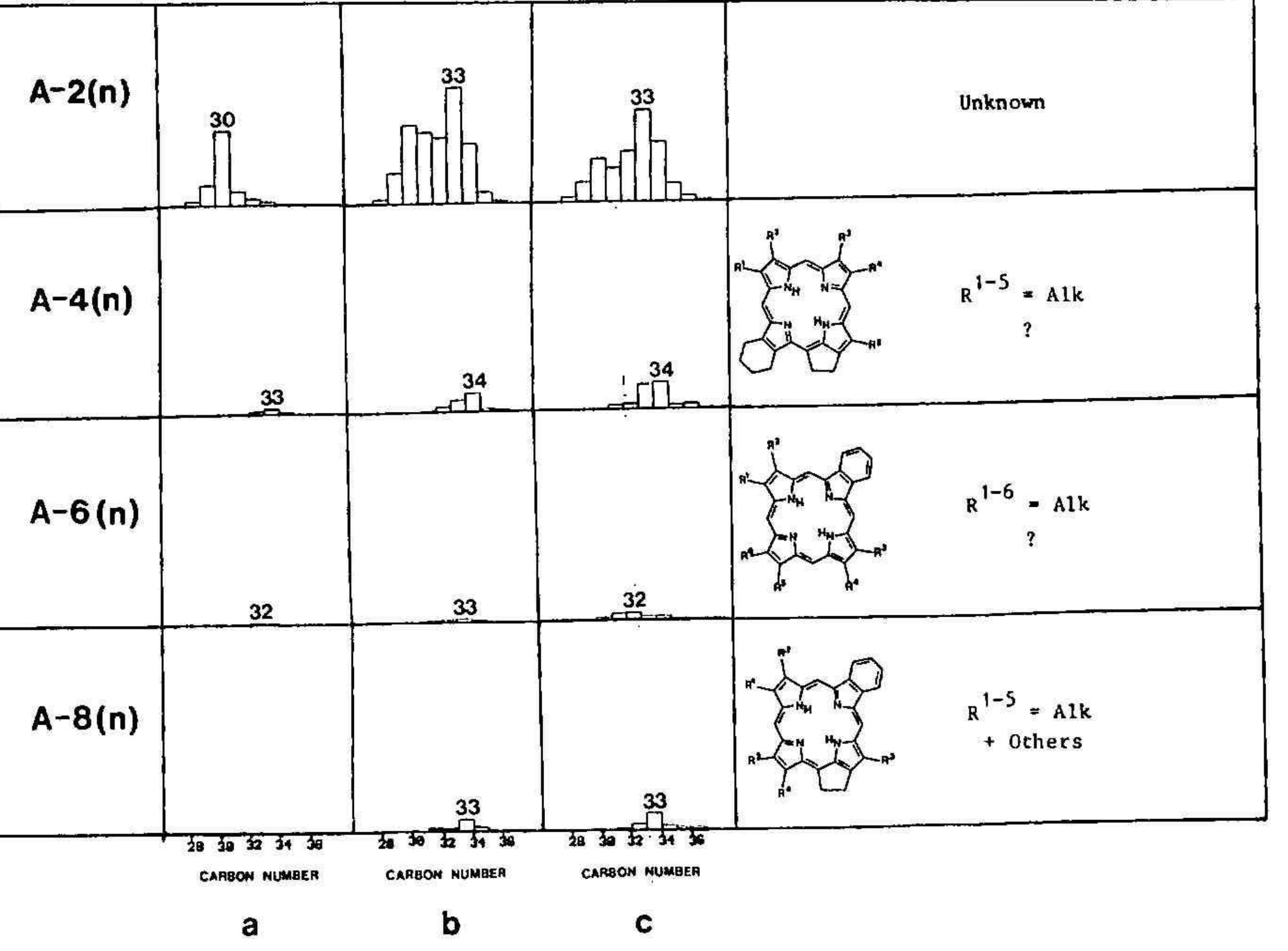

Fig. 7. Relative abundance histograms for the series of porphyrins identified to date in geological materials by C-GC–MS analysis. Results are presented for three samples and 12 series. The samples are labelled across the x-axis and the series in the left hand column, with partial structures to the right of the histograms, where known. Series names are as used previously, with the addition of series A(n), A-2(n), A-4(n), A-6(n) and A-8(n) to include all compounds not assigned to one of the partially identified series. a, Gilsonite; b, La Luna; c, Boscan.

TABLE III

PETROPORPHYRINS DETECTED BY GC–MS ANALYSIS OF LA LUNA SHALE

tr = less than 0.1% of total petroporphyrins.

Class	*Carbon numbwer, retention index (KRI), relative abundance (%)**										
	26	*27*	*28*	*29*	*30*	*31*	*32*	*33*	*34*	*35*	*36*
A	3426 (0.2:tr)	3441 (0.5:tr)	3455 (0.2:tr)	3495 (6:0.8)	3539 (27:4)	3580 (35:5)	3622 (13:1.8)	3666 (0.1:tr)	3755 (6:0.8)	3820 (0.8:0.1)	3884 (0.1:tr)
	3437 (0.2:tr)	3448 (0.4:tr)	3483 (7:1.0)	3527 (16:2)	3572 (4:0.5)	3604 (8:1.1)	3647 (16:2)	3687 (9:1.2)	3778 (1.0:0.1)	3823 (0.4:tr)	3928 (0.3:tr)
	3447 (0.2:tr)	3468 (1.4:0.2)	3493 (3:0.4)	3537 (20:3)	3579 (21:3)	3633 (6:0.8)	3672 (3:0.4)	3714 (8:1.1)	3800 (1.0:0.1)	3837 (0.1:tr)	3960 (0.1:tr)
	3467 (0.1:tr)	3482 (1.2:0.2)	3523 (5:0.7)	3551 (0.9:0.1)	3592 (5:0.7)	3648 (8:1.1)	3698 (5:0.7)	3751 (0.5:tr)	3816 (0.6:tr)	3859 (0.2:tr)	
	3487 (0.3:tr)	3492 (0.3:tr)	3537 (2:0.3)	3579 (4:0.5)	3607 (2:0.3)	3673 (2:0.3)	3714 (5:0.7)	3757 (1.4:0.2)	3849 (0.6:tr)	3890 (0.5:tr)	
		3498 (0.3:tr)	3554 (0.8:0.1)	3595 (3:0.4)	3619 (1.5:0.2)		3731 (0.3:tr)	3772 (0.3:tr)			
					3655 (1.3:0.2)						
A-2		3542 (0.1:tr)	3528 (0.3:tr)	3527 (0.2:tr)	3578 (0.6:tr)	3614 (4:0.5)	3658 (6:0.8)	3755 (15:2)	3799 (0.2:tr)	3886 (0.3:tr)	3968 (0.6:tr)
			3563 (1.7:0.2)	3538 (0.7:0.1)	3593 (8:1.1)	3636 (7:1.0)	3713 (12:1.6)	3774 (5:0.7)	3816 (2:0.3)	3892 (1.1:0.1)	4017 (0.4:tr)
			3577 (0.4:tr)	3548 (0.7:0.1)	3603 (2:0.3)	3645 (4:0.5)	3742 (3:0.4)	3794 (7:1.0)	3820 (1.6:0.2)	3910 (0.4:tr)	4058 (0.2:tr)
			3588 (0.2:tr)	3559 (0.3:tr)	3618 (9:1.2)	3667 (4:0.5)	3754 (100:14)	3805 (4:0.5)	3832 (4:0.5)	3917 (0.2:tr)	
				3574 (7:1.0)	3646 (0.7:0.1)	3701 (6:tr)	3779 (17:2)	3822 (42:6)	3841 (0.3:tr)	3926 (0.5:tr)	
				3591 (1.1:0.1)	3657 (4:0.5)	3712 (58:7.8)	3823 (3:0.4)	3845 (4:0.5)	3873 (0.7:0.1)	3953 (2:0.2)	
				3603 (0.2:tr)	3665 (3:0.4)	3734 (1.9:0.3)	3832 (3:0.4)	3904 (0.8:0.1)	3889 (19:3)	3970 (1.0:0.1)	
				3617	3699	3755		3921	3921		

	(0.2:tr) 3630 (0.9:0.1) 3652 (9:1.2) 3714 (1.9:0.3)	(15:2) 3711 (6:0.8) 3721 (0.9:0.1) 3756 (1.0:0.1)	(5:0.7) 3769 (5:0.7) 3782 (1.8:0.2) 3802 (3:0.4)		(0.5:tr)	(0.2:tr) 3963 (1.4:0.2)		
A-4			3870 (0.3:tr)	3832 (0.2:tr) 3871 (0.1:tr) 3887 (0.1:tr) 3915 (1.3:0.2) 3926 (0.5:tr) 3942 (0.1:tr)	3879 (0.2:tr) 3922 (5:0.7) 3973 (0.3:tr)	3921 (1.1:0.1) 3964 (7:1.0) 3992 (0.2:tr) 4018 (0.4:tr)	4005 (0.2:tr) 4031 (0.6:tr) 4057 (0.3:tr) 4082 (0.1:tr)	4054 (0.2:tr) 4078 (0.1:tr) 4102 (0.3:tr)
A-6			3925 (0.7:0.1)	3927 (0.7:0.1) 3978 (0.3:tr)	3967 (0.2:tr) 3972 (0.6:tr) 3981 (0.2:tr) 3991 (0.3:tr)	4018 (0.3:tr) 4026 (0.2:tr) 4034 (0.2:tr)		
A-8			3972 (0.3:tr) 4017 (0.3:tr) 4060 (0.4:tr)	4026 (0.2:tr) 4057 (0.3:tr) 4068 (0.7:0.1)	4073 (0.2:tr) 4108 (5:0.7)	4110 (0.5:tr) 4145 (0.4:tr) 4157 (0.3:tr) 4174 (0.2:tr)		

* 1st relative to C_{32}A-2 (KRI = 3754) = 100%: 2nd relative to total petroporphyrins = 100%.

work, to allow future expansion of this type of figure to further samples. In addition, A(n), A-2(n), A-4(n), A-6(n) and A-8(n) appear; these "series" contain the unidentified components which can not be assigned to any of the series co-chromatographed to date.

Boscan crude oil

Fig. 1c shows the RIC (m/z 545-850) obtained from the total petroporphyrins of Boscan oil analysed as their $(TBDMSO)_2Si$ derivatives, and displayed over the KRI range 3400–4300; some 30 peaks are fully or partially resolved. Considerable co-elution of components is occurring, as evidenced later by mass chromatography. The m/z range of 545–850 shows the "total porphyrin chromatogram", and does not display the co-injected n-alkane retention index standards. Single-ion mass chromatograms were examined for the intense $(M-131)^+$ ions of all alkyl porphyrins from C_{20} to C_{42}, and for the classes A, A-2, A-4, A-6 and A-8. The porphyrins present were of the carbon numbers and classes listed in Table IV. "Genuine" components were assigned after checking of mass spectra as for La Luna. Quantification of the detected porphyrin derivatives was performed by the usual method and is therefore subject to the same errors arising when two porphyrins, with masses differing by two a.m.u., co-elute. Such instances of co-elution occurred 43 times in the analysis of Boscan oil. As before, therefore, the approximate quantification adopted was simply to use the peak areas of the characteristic $(M-131)^+$ ions in the GC–MS data. These areas were obtained from RI lists, output by program RRI. Table IV contains details of each porphyrin detected in GC–MS analysis of the petroporphyrins of Boscan oil (224 in all), its class, carbon number, KRI, and abundance (relative to the C_{32} A-2 porphyrin of KRI = 3754, structure 2a, and also as a percentage fraction of the total petroporphyrins).

To allow further investigation of the tabulated petroporphyrins, Kováts' plots were prepared as before. The five plots produced appear in Fig. 3b, where comparative co-plotting with La Luna has been performed after applying the same threshold values. As was done for Fig. 3a, proposed pseudo-homologous series are marked by dashed lines such that the components of these series are structurally related to co-injected standards. The same five series were identified as were found in Gilsonite bitumen and La Luna shale.

Histograms were prepared for these series, showing each component with its KRI value and relative abundance. In each case the abundances are presented as percent fractions of the abundance of the C_{32} A-2 of KRI = 3754 (structure 2a). The histograms appear in Fig. 5c, forming distribution profiles for these five pseudo-homologous series. Histograms of abundance *vs.* carbon number for each class (A, A-2, A-4, A-6, A-8) of porphyrins present in Boscan oil are presented in Fig. 6c, for comparison with Gilsonite (Fig. 6a), and La Luna shale (Fig. 6b). Fig. 7c contains similar histograms, plotted for all 7 series for which standards were available for co-injection, though two of these, A-2(3) and A-2(4), were not present in any of the three samples studied to date (see Table I).

Fully alkylated Boscan porphyrins

After acetylation and removal of β-unsubstituted porphyrins, the fully alkylated porphyrins of Boscan oil were derivatised and GC–MS analysis performed as

for the total petroporphyrin mixture. The resultant GC–MS data were processed by the methods set out previously. Co-injections were not performed, since the identities of the standard compounds are readily obtained by comparison of the retention and mass spectral characteristics (especially Fig. 4) with those for the total mixtures (especially Fig. 3b).

Fig. 2b is the RIC, where it is compared with that for the fully alkylated fraction of petroporphyrins from Gilsonite (Fig. 2a). Comparison of Fig. 2a with Fig. 1a and Fig. 2b with Fig. 1c, reveals that the distribution of components is greatly simplified by removal of the β-unsubstituted components. The mass chromatograms obtained for the fully alkylated petroporphyrins of Boscan oil were cross-checked with the mass spectra, quantified and used to prepare a table similar to Table IV. Comparison with the total mixture (Table IV) revealed a reduction in the number of petroporphyrins detected after acetylation and removal of β-unsubstituted compounds from 224 to 87. This simplification is apparent in the Kováts' plots for the fully alkylated fraction, Fig. 4, when compared with the equivalent plots from the total petroporphyrins, Fig. 3b. Fig. 4 compares the fully alkylated porphyrin fractions of Gilsonite bitumen and Boscan oil in the same way as Fig. 3 compared the total porphyrin distributions of Gilsonite and La Luna (Fig. 3a), and La Luna and Boscan (Fig. 3b).

DISCUSSION

Origin of samples

The two samples analysed in this work were obtained from the Maracaibo Basin area of northwestern Venezuela. This basin formed during the Oligocene epoch, and has been described[45].

Sedimentation of the area now occupied by the basin began in the early Cretaceous with the transgression of a marine environment from the north, which by the end of the Early Cretaceous had covered the major part of the basin. In this marine environment were laid down the rocks of the La Luna formation. This is a thinly-bedded to finely-laminated, organically-rich, shaley limestone of Cretaceous age (*ca.* 90 million years[15]). It is widespread throughout northern South America, where similar sediments have been detected in eastern Venezuela, Trinidad, Colombia and Ecuador[15].

The deposition of the La Luna formation is believed to have occurred in an anoxic marine environment, as a result of the existence of a stratified water column. The high sulphur content, up to 10% of the organic extract, of the La Luna formation suggests that active sulphate reduction was occurring at this time[46].

The La Luna formation has been proposed[15,16] to be the source rock of the oils of the various reservoirs of northwestern Venezuela. A study based on the petroporphyrin content of crude oils and sediments of the Basin established a direct genetic relationship between the La Luna formation and a range of crude oils from the Maracaibo Basin, including certain Boscan oils[17].

The La Luna shale sample used in the present study is taken from an exposure of the formation in the La Luna type area on the western border of the Maracaibo Basin. It was supplied by Dr. F. Cassani (INTEVEP, Venezuela), and no precise information of its maximum depth of burial and/or temperature is available.

TABLE IV

PETROPORPHYRINS DETECTED BY GC–MS ANALYSIS OF BOSCAN OIL

tr = less than 0.1% of total petroporphyrins.

Class	*Carbon number, retention index (KRI), relative abundance (%)**											
	27	*28*	*29*	*30*	*31*	*32*	*33*	*34*	*35*	*36*	*37*	*38*
A	3442 (1.1:0.1)	3455 (0.3:tr)	3493 (8:1.0)	3539 (25:3)	3580 (40:5)	3622 (14:1.7)	3664 (0.3:tr)	3721 (2:0.2)	3785 (0.3:tr)	3857 (0.1:tr)	4023 (0.3:tr)	4052 (0.2:tr)
	3447 (0.5:tr)	3482 (13:1.6)	3528 (19:2)	3572 (6:0.7)	3603 (10:1.2)	3647 (20:2)	3684 (13:1.6)	3732 (2:0.2)	3798 (0.4:tr)	3887 (0.6:tr)	4031 (0.1:tr)	4075 (0.1:tr)
	3470 (0.7:tr)	3492 (9:1.1)	3537 (24:3)	3580 (31:4)	3626 (1.0:0.1)	3673 (6:0.7)	3715 (14:1.7)	3756 (10:1.2)	3821 (3:0.4)	3899 (0.7:tr)	4047 (0.2:tr)	4116 (0.2:tr)
	3481 (0.5:tr)	3525 (9:1.1)	3551 (2:0.2)	3593 (3:0.4)	3633 (7:0.8)	3697 (2:0.2)	3756 (6:0.7)	3779 (3:0.4)	3840 (2:0.2)	3927 (0.7:tr)	4072 (0.1:tr)	
		3536 (0.9:0.1)	3580 (3:0.4)	3607 (4:0.5)	3649 (5:0.6)	3717 (12:1.4)	3762 (3:0.4)	3802 (3:0.4)	3855 (0.7:tr)	3945 (0.4:tr)	4076 (0.1:tr)	
		3545 (0.7:tr)	3593 (3:04)	3620 (3:0.4)	3670 (3:0.4)		3778 (0.8:0.1)	3832 (0.9:0.1)	3889 (3:0.4)	3978 (0.6:tr)		
							3790 (1.5:0.2)	3848 (1.0:0.1)				
							3806 (2:0.2)					
A-2		3538 (1.0:0.1)	3534 (1.0:0.1)	3576 (0.8:0.1)	3607 (0.4:tr)	3655 (1.4:0.2)	3756 (13:1.6)	3801 (0.5:tr)	3889 (2:0.2)	3893 (0.3:tr)	4030 (0.3:tr)	4105 (0.2:tr)
		3546 (0.1:tr)	3576 (3:0.4)	3591 (0.9:0.1)	3634 (1.6:0.2)	3714 (11:1.3)	3776 (3:0.4)	3817 (1.6:0.2)	3923 (0.5:tr)	3925 (0.2:tr)	4057 (0.3:tr)	
		3563 (0.5:tr)	3581 (3:0.4)	3620 (10:1.2)	3666 (3:0.4)	3756 (100:12)	3791 (6:0.7)	3833 (4:0.5)	3954 (2:0.2)	3946 (0.2:tr)	4115 (0.2:tr)	
		3583 (0.3:tr)	3622 (0.3:tr)	3661 (23:3)	3715 (80:10)	3782 (21:3)	3807 (4:0.5)	3849 (1.6:0.2)	3972 (3:0.4)	3960 (0.3:tr)	4148 (0.1:tr)	
		3606 (0.2:tr)	3650 (4:0.5)	3696 (6:0.7)	3733 (0.1:tr)	3822 (0.6:tr)	3823 (28:3)	3861 (0.2:tr)	4001 (0.5:tr)	3965 (0.4:tr)		

	3651 (0.4:tr)	3658 (1.0:0.1) 3760 (0.5:tr)	3708 (12:1.4) 3757 (0.9:0.1)	3756 (4:0.5) 3763 (2:0.2) 3770 (5:0.6) 3804 (3:0.4)	3836 (1.1:0.1)	3846 (8:1.0) 3923 (1.0:0.1)	3890 (21:3) 3922 (1.1:0.1) 3966 (3:04)	4009 (0.4:tr)	4019 (0.8:0.1) 4055 (0.5:tr) 4102 (0.1:tr)	
A-4				3803 (0.1:tr) 3818 (0.2:tr) 3826 (0.3:tr) 3870 (0.8:0.1) 3888 (0.3:tr) 3893 (0.2:tr)	3821 (0.2:tr) 3835 (0.2:tr) 3878 (1.2:0.1) 3915 (1.3:0.2) 3927 (0.3:tr) 3935 (0.2:tr)	3847 (0.4:tr) 3878 (0.5:tr) 3890 (0.2:tr) 3923 (9:1.1) 3970 (0.4:tr) 3976 (0.5:tr) 3987 (0.8:0.1)	3920 (2:0.2) 3953 (0.2:tr) 3965 (10:1.2) 3992 (0.3:tr) 4019 (0.5:tr)	4001 (0.5:tr) 4012 (0.2:tr) 4034 (0.7:tr) 4058 (0.3:tr) 4086 (0.2:tr)	3973 (0.3:tr) 4019 (0.3:tr) 4054 (0.4:tr) 4065 (0.3:tr) 4077 (0.1:tr) 4099 (0.9:0.1)	4055 (0.1:tr) 4067 (0.2:tr) 4070 (0.2:tr)
A-6			3848 (0.4:tr) 3882 (0.1:tr) 3891 (0.3:tr) 3898 (0.1:tr)	3893 (1.4:0.2) 3917 (0.2:tr) 3927 (0.9:0.1) 3935 (0.2:tr)	3928 (2:0.2) 3936 (0.7:tr) 3953 (0.1:tr) 3966 (0.1:tr) 3974 (0.2:tr)	3966 (0.5:tr) 3974 (0.5:tr) 3984 (0.2:tr) 3994 (0.1:tr)	4003 (0.1:tr) 4020 (0.6:tr) 4025 (0.2:tr) 4036 (0.3:tr) 4047 (0.2:tr) 4063 (0.1:tr)			

(Continued on p. 306)

TABLE IV (*continued*)

Class	Carbon number, retention index (KRI), relative abundance (%)*											
	27	28	29	30	31	32	33	34	35	36	37	38
A-8					3974 (0.7:tr) 3987 (0.1:tr)	3988 (0.3:tr) 4030 (0.3:tr) 4066 (0.4:tr) 4074 (1.5:0.2) 4106 (0.2:tr)	4071 (0.5:tr) 4110 (7:0.8) 4119 (0.2:tr)	4104 (0.1:tr) 4110 (0.7:tr) 4144 (0.4:tr) 4158 (0.2:tr) 4172 (0.3:tr) 4179 (0.1:tr)	4171 (0.1:tr) 4186 (0.3:tr) 4203 (0.2:tr) 4216 (0.2:tr) 4228 (0.2:tr)	4232 (0.2:tr) 4244 (0.2:tr) 4250 (0.1:tr) 4262 (0.2:tr) 4277 (0.2:tr)		

* 1st relative to C_{32}A-2 (KRI = 3756) = 100%; 2nd relative to total petroporphyrins = 100%.

The Boscan oil field is situated in the northwestern part of the Maracaibo Basin, west of the Maracaibo Lake and east of the La Luna shale outcrop areas. This field has been described in detail[29]. The oil-bearing sandstones (Upper Eocene, *ca.* 40 m years) can be detected over a large depth range, from *ca.* 1500 m in the north to over 3000 m in the south. As a result of these differences in depth, the temperatures of the oils in the reservoir also vary, between *ca.* 65°C and 85°C[19]. The Boscan oil sample examined in the present paper was obtained from well 8E-4 at a depth of *ca.* 2230–2310 m, with a formation temperature estimated from present-day geothermal gradient[47] as 77°C. These two oil-source samples are compared and contrasted herein, by GC–MS examination of their petroporphyrins, with the unrelated bitumen Gilsonite.

Gilsonite bitumen is found in veins in the sandstone Uinta formation of Utah (U.S.A.), and is believed to be sourced from the Parachute Creek member of the underlying Green River formation[48,49]. The shales of the Green River formation were deposited by Lake Uinta during the Eocene. During most of its existence, this lake was stratified, with an aerated upper layer containing abundant life, and a saline, anoxic, lower layer. The salinity (sulphates, carbonates, bicarbonates, only small amounts of chlorides) increased through the life-span of the lake so that the Gilsonite source beds were laid down in a strongly saline anoxic non-marine environment[50–53]. It is estimated from the present-day geothermal gradient, that the Gilsonite source beds, 1400 m deep, have experienced temperatures no higher than 50°C.

Thus, Gilsonite, studied in our earlier publication[14], is of immature, non-marine origin, contrasting with the more mature, marine La Luna shale and the further matured Boscan oil, sourced from the La Luna shale.

Petroporphyrin analyses

As earlier reported for Gilsonite[14], in Boscan oil and La Luna shale five pseudo-homologous series of petroporphyrins have been partially identified. Certain members of these series co-chromatograph with (or have near-identical KRI values to) known porphyrin derivatives and so are presumed to have structures identical to, or isomeric with, those standards. The other members of the series may therefore be assumed to have closely related structures of different carbon number. From arguments presented previously[14], these series, containing 32 compounds in both Boscan oil and in La Luna shale, and representing over 80% of the total porphyrin content of each, are suggested to possess *n*-alkyl substituents only.

Two of the series, A(2) and A(3), are each known to contain a C_{29} member with two ethyl and five methyl substituents, and therefore one unsubstituted β-position. The two series are therefore each proposed to consist of monounsubstituted aetioporphyrins. A(2) and A(3) are separated by *ca.* 10 KRI units, with the members of A(2) eluting earlier. This KRI difference is presumably an effect of the relationship of the free β-H to the other groups, this relation being somehow different in the two series. Clearly, it would be very useful to possess complete and unambiguous structural assignments of these two C_{29} isomers.

Full structural determination has not been carried out for most of these compounds. Hence, the assumption that points lying on straight lines on the plots represent porphyrins of related structures is not fully tested. However, all those instances in which known structures have been co-injected support this proposition. Com-

pounds of related type (*e.g.* the fully-alkylated aetios) fall on one such line, compounds of different types (*e.g.* 13^1-methyl-13,15-ethanoporphyrins and 13,15-ethanoporphyrins) fall on different lines. Two other A-2 types, a 15^1-methyl-15,17-propanoporphyrin and two 15,17-butanoporphyrins, which are not present in these samples, fall at much larger KRI values and, presumably, would lie on other straight lines in samples known to contain series of these types (*e.g.* Serpiano oil shale[32,37]).

Petroporphyrin distributions

Histograms were prepared for these five series of petroporphyrins. As revealed by GC–MS analysis of La Luna shale, they have carbon number ranges and maxima as set out in Fig. 5b. In general, the abundances of the compounds show an increase from low carbon numbers, up to a maximum, and then decrease again at high carbon numbers. A slight deviation occurs in series A-2(1), a series of 13^1-methyl-13,15-ethanoporphyrins, where the C_{31} member is lower in abundance than its C_{30} and C_{32} counterparts, producing an apparently "bimodal," distribution. This "bimodality" is more evident in series A(3), which has maxima at C_{30} and C_{33}. In Boscan oil, the distribution profiles of the five series are very similar to the corresponding La Luna series. Their carbon number ranges and maxima are presented in Fig. 5c. Minor differences from La Luna are apparent:

(i) In series A(3), mono β-unsubstituted aetioporphyrins, a C_{35} component was detected in Boscan oil, but not in La Luna shale.

(ii) In series A-2(1), a fully alkylated series of 13^1-methyl-13,15-ethanoporphyrins, a C_{28} member was found in the shale, but was absent from Boscan oil.

(iii) In series A-2(2), the major series of fully alkylated 13,15-ethanoporphyrins, the C_{30} and C_{31} components are relatively much more abundant in Boscan oil than in La Luna shale.

Fig. 5 (the distribution histogram for Gilsonite, Boscan and La Luna) allows simple visual comparison of the major components of each sample. The porphyrin distribution of Boscan crude oil is similar to that of its source rock, emphasising the potential utility of GC–MS analysis in the correlation of geochemical materials by their petroporphyrin distributions. In contrast the petroporphyrins of Gilsonite[14] show a different distribution to those of the two Maracaibo Basin samples:

(i) Though their carbon number ranges are similar, the five partially identified series, A(1), A(2), A(3), A-2(1) and A-2(2), all maximise one carbon number lower in Gilsonite.

(ii) The Boscan and La Luna samples each contain more porphyrins, particularly the later-eluting components and the components with higher degrees of unsaturation. Thus, in the Maracaibo Basin samples, there are more aetioporphyrins with KRI values higher than those of series A(3), more A-2 porphyrins with KRI values above those of series A-2(2) and more porphyrins of classes A-4 and A-6, while the A-8 class is absent from Gilsonite. One possibility is that these high carbon number homologues represent the remains of bacteriochlorophyll precursors[54].

Acetylation of petroporphyrins

The Friedel-Crafts acetylation procedure, as applied to petroporphyrins[40], is expected to replace any aromatic hydrogen atom by an acetyl function. The acetylated species may then be separated from the remaining porphyrins by TLC. How-

ever, it should be noted that in the supposed fully alkylated fraction of Boscan petroporphyrins, compounds of classes A-6 and A-8 remain, though at much reduced relative abundance. Since porphyrins of these classes are known[6,18] to possess a fused benzo-ring structure (*e.g.* 4 and 5), they should be acetylated and removed; it may be that the conditions employed for the reaction were not sufficiently forcing to acetylate fully compounds of this type. In particular, longer reaction times may be required. Similar observations have been made in the case of Gilsonite[14], where certain A-6 class porphyrins remain after acetylation, and where the higher carbon number members of series A(3) were not removed, despite the fact that the C_{29} member (1c), at least, possesses an unsubstituted β-position.

Comparative C-GC–MS analyses of petroporphyrin distributions

The use of C-GC–MS analysis with comparison of graphical displays shows differences and similarities among and between the porphyrin distributions of different samples. Thus, Figs. 5 and 6 show strong similarities between the related La Luna shale and Boscan oil, with significant differences from the Gilsonite bitumen.

By comparison of Fig. 5 (which shows abundances for all identified components and their pseudo-homologues) with Fig. 6 (containing abundance data for the sum of all porphyrins present), it is possible to determine easily the presence of significant components of unknown structure. Tables III and IV then give the KRI values of the relevant compounds. These data are useful to the planning of future structural elucidation studies. As more structures are completely assigned to the porphyrins, it will become possible to unravel the geochemical processes operating to produce, and alter, the distributions observed in the GC–MS analyses. Knowledge of the chemical processes may, in turn, enable specific features of the porphyrin distributions to be employed as indicators of palaeoenvironment of the original site of deposition of the sedimentary organic matter and/or the thermal maturity reached by such organic matter.

Detailed numerical correlation will require the use of statistical software packages and some means of entering GC–MS data, either "raw" or partially processed. One approach is the use of a "reverse search" procedure[13]. Statistical treatment of C-GC–MS data will provide opportunities for the numerical investigation of maturity and palaeoenvironmental parameters.

CONCLUSIONS

GC–MS analysis of the petroporphyrins of Boscan oil and its source rock, La Luna shale, has revealed the presence of large numbers of individual porphyrins. Plotting the KRI values of the $(TBDMSO)_2Si$ derivatives against the carbon numbers of the parent porphyrins produces a number of straight-line relationships between the points. These are proposed to be pseudo-homologous series of structurally related porphyrins. Co-injection experiments with compounds of known, or partially known, structures provide some information regarding the nature of these series. Comparison of the distributions of porphyrins within these series, their carbon numbers and relative abundances, demonstrates the similarity of these two related samples, at least in regard to their major components. These two Maracaibo Basin samples are demonstrably different in their porphyrin distributions from the unrelated Gilsonite bitumen.

The following conclusions may be drawn:

(i) GC–MS analysis of petroporphyrin fractions, as $(TBDMSO)_2Si$ derivatives, yields useful and detailed information on their alkyl porphyrin content. The class, carbon number and abundance are obtained directly, and co-injection and acetylation experiments provide some information on the structures of these porphyrins.

(ii) Visual comparisons of relative abundance profiles produced for five major pseudo-homologous series demonstrate the high degree of similarity between a crude oil (Boscan) and its source rock (La Luna).

(iii) The ratio of A-2 class to A class porphyrins (generally termed the DPEP–aetio ratio), which has been suggested, and employed, as a maturity measure of geological materials[3], is readily obtained from GC–MS data.

(iv) The procedure described reveals petroporphyrin distributions of geological or similar samples and, hence, provides information complementary to that gained from other biomarkers, which may include oil to source correlation, assessment of maturity and palaeoenvironment of deposition, evidence for migration and effects (if any) of biodegradation.

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REFERENCES

1 A. S. MacKenzie, J. M. E. Quirke and J. R. Maxwell, in A. G. Douglas and J. R. Maxwell (Editors), *Advances in Organic Geochemistry 1979*, Pergamon Press, Oxford, 1980, pp. 239–248.

2 E. W. Baker and J. W. Louda, in M. Bjorøy, P. Albrecht, C. Cornford, K. de Groot, G. Eglinton, E. Galimov, D. Leythauser, R. Pelet, J. Rullkötter and G. Speers (Editors), *Advances in Organic Geochemistry 1981*, Heyden and Sons, London, 1983, pp. 401–421.

3 A. J. G. Barwise and P. J. D. Park, in M. Bjorøy, P. Albrecht, C. Cornford, K. de Groot, G. Eglinton, E. Galimov, D. Leythauser, R. Pelet, J. Rullkötter and G. Speers (Editors), *Advances in Organic Geochemistry 1981*, Heyden and Sons, London, 1983, pp. 668–674.

4 A. J. G. Barwise and I. Roberts, *Org. Geochem.*, 6 (1984) 167–176.

5 E. W. Baker, *J. Am. Chem. Soc.*, 88 (1966) 2311–2315.

6 E. W. Baker, T. F. Yen, J. P. Dickie, R. E. Rhodes and L. F. Clarke, *J. Am. Chem. Soc.*, 89 (1967) 3631–3639.

7 S. K. Hajlbrahim, P. J. C. Tibbetts, C. D. Watts, J. R. Maxwell, G. Eglinton, H. Colin and G. Guiochon, *Anal. Chem.*, 50 (1978) 549–553.

8 G. Eglinton, S. K. HajIbrahim, J. R. Maxwell and J. M. E. Quirke, in A. G. Douglas and J. R. Maxwell (Editors), *Advances in Organic Geochemistry 1979*, Pergamon Press, Oxford, 1980, pp. 193–203.
9 P. J. Marriott, J. P. Gill, R. P. Evershed, C. S. Hein and G. Eglinton, *J. Chromatogr.*, 301 (1984) 107–128.
10 G. Eglinton, R. P. Evershed and J. P. Gill, *Org. Geochem.*, 6 (1984) 157–165.
11 P. J. Marriott, J. P. Gill and G. Eglinton, *J. Chromatogr.*, 249 (1982) 291–310.
12 P. J. Marriott, J. P. Gill, R. P. Evershed, G. Eglinton and J. R. Maxwell, *Chromatographia*, 16 (1982) 304–308.
13 C. S. Hein, J. P. Gill, R. P. Evershed and G. Eglinton, *Anal. Chem.*, 57 (1985) 1872–1879.
14 J. P. Gill, R. P. Evershed, M. I. Chicarelli, G. A. Wolff, J. R. Maxwell and G. Eglinton, *J. Chromatogr.*, 350 (1985) 37–62.
15 H. D. Hedburg, *Bull. Am. Assoc. Petrol. Geol.*, 48 (1964) 1755–1803.
16 S. Talukdar, O. Gallango and M. Chin-A-Lien, *Org. Geochem.*, in press.
17 J. A. Gransch and E. Eisma, in G. D. Hobson and G. C. Speers (Editors), *Advances in Organic Geochemistry 1966*, Pergamon Press, Oxford, 1970, pp. 193–203.
18 S. Kaur, M. I. Chicarelli and J. R. Maxwell, *J. Am. Chem. Soc.*, 108 (1986) 1347–1348.
19 B. M. Didyk, *Ph.D. Thesis*, University of Bristol, Bristol, 1975.
20 A. J. G. Barwise and E. V. Whitehead, in A. G. Douglas and J. R. Maxwell (Editors), *Advances in Organic Geochemistry 1979*, Pergamon Press, Oxford, 1980, pp. 181–192.
21 T. F. Yen, L. J. Boucher, J. P. Dickie, E. C. Tynan and G. B. Vaughan, *J. Inst. Pet.*, 55 (1969) 87–99.
22 G. W. Hodgson, M. Strosher and D. J. Casagrande, in H. R. von Gaertner and H. Wehner (Editors), *Advances in Organic Geochemistry 1971*, Pergamon Press, Oxford, 1972, pp. 151–161.
23 E. W. Baker and S. E. Palmer, in D. Dolphin (Editor), *The Porphyrins*, Vol. I, Academic Press, New York, 1978, pp. 486–552.
24 J. M. E. Quirke, G. J. Shaw, P. D. Soper and J. R. Maxwell, *Tetrahedron*, 36 (1980) 3261–3267.
25 Y. I. A. Alturki, *Ph.D. Thesis*, University of Bristol, Bristol, 1972.
26 S. K. HajIbrahim, J. M. E. Quirke and G. Eglinton, *Chem. Geol.*, 32 (1981) 173–188.
27 D. B. Boylan, Y. I. A. Alturki and G. Eglinton, in P. A. Schenck and I. Havenaar (Editors), *Advances in Organic Geochemistry 1968*, Pergamon Press, Oxford, 1969, pp. 227–240.
28 R. Alexander, G. Eglinton, J. P. Gill and J. K. Volkman, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 3 (1980) 521.
29 J. A. F. Sutherland, in R. E. King (Editor), *Stratigraphic Oil and Gas Fields: Classification, Exploration Methods and Case Histories*, Am. Assoc. Petrol. Geol. Memoir 16, 1972, pp. 559–567.
30 J. G. Erdman, *U.S. Pat.*, 3,190,829 (1965).
31 S. K. HajIbrahim, *Ph.D. Thesis*, University of Bristol, Bristol, 1978.
32 M. I. Chicarelli, *Ph.D. Thesis*, University of Bristol, Bristol, 1985.
33 J. M. E. Quirke, G. Eglinton and J. R. Maxwell, *J. Am. Chem. Soc.*, 101 (1979) 7693–7697.
34 G. A. Wolff, *Ph.D. Thesis*, University of Bristol, Bristol, 1983.
35 G. A. Wolff, M. I. Chicarelli, G. J. Shaw, R. P. Evershed, J. M. E. Quirke and J. R. Maxwell, *Tetrahedron*, 40 (1984) 3777–3786.
36 M. I. Chicarelli, J. R. Maxwell and R. A. Pitt, in preparation.
37 M. I. Chicarelli, G. A. Wolff, M. Murray and J. R. Maxwell, *Tetrahedron*, 40 (1984) 4033–4039.
38 G. A. Wolff, M. Murray, J. R. Maxwell, B. K. Hunter and J. K. M. Sanders, *J. Chem. Soc., Chem. Commun.*, (1983) 922–924.
39 J.-H. Fuhrhop and K. M. Smith, in K. M. Smith (Editor), *Porphyrins and Metalloporphyrins*, Elsevier, Amsterdam, 1976, p. 798.
40 J. M. E. Quirke, in M. Bjorøy, P. Albrecht, C. Cornford, K. de Groot, G. Eglinton, E. Galimov, D. Leythauser, R. Pelet, J. Rullkötter and G. Speers (Editors), *Advances in Organic Geochemistry 1981*, Heyden and Sons, London, 1983, pp. 733–745.
41 C. S. Hein and G. Eglinton, in preparation.
42 E. Kováts, *Helv. Chim. Acta.*, 41 (1958) 1915–1932.
43 M. I. Chicarelli and J. R. Maxwell, *Tetrahedron Lett.*, 25 (1984) 4701–4704.
44 R. Ocampo, H. J. Callot, P. Albrecht and J. R. Kintzinger, *Tetrahedron Lett.*, 25 (1984) 2589–2592.
45 J. B. Miller, K. L. Edwards, P. P. Wolcott, H. W. Anisgard, R. Martin and H. Anderegg, in *Habitat of Oil Symposium, New York 1955*, Am. Assoc. Petrol. Geol., Tulsa, 1958, pp. 601–640.
46 J. A. Gransch and P. Posthuma, in B. Tissot and F. Bienner (Editors), *Advances in Organic Geochemistry 1973*, Editions Technip, Paris, 1974, pp. 727–739.

47 E. E. Hagan, personal communication (1974).
48 J. M. Hunt, F. Stewart and P. A. Dickey, *Bull. Am. Assoc. Petrol. Geol.*, 38 (1954) 1671–1698.
49 K. G. Bell and J. M. Hunt, in I. Breger (Editor), *Organic Geochemistry*, Pergamon Press, London, 1963, pp. 333–366.
50 W. H. Bradley, *U.S. Geol. Survey Prof. Paper 496-A*, 1964.
51 W. H. Bradley and H. P. Euger, *U.S. Geol. Survey Prof. Paper 496-B*, 1969.
52 W. H. Bradley, *Bull Geol. Soc. Am.*, 81 (1970) 985–1000.
53 W. H. Bradley, *Bull. Geol. Soc. Am.*, 84 (1973) 1121–1124.
54 R. Ocampo, H. J. Callot and P. Albrecht, *J. Chem. Soc., Chem. Commun.*, (1985) 200–201.

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GAS-LIQUID CHROMATOGRAPHY, THIN-LAYER CHROMATOGRAPHY AND CARBON-13 NUCLEAR MAGNETIC RESONANCE SPECTROMETRY IN THE ANALYSIS OF DIASTEREOMERIC MIXTURES OF R–CH(OH)–CH(CH_3)–CH = CH_2 ALCOHOLS

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SUMMARY

Gas–liquid chromatography, thin-layer chromatography and ^{13}C NMR spectrometry have been employed to identify and analyse diastereomeric mixtures of the β-methylalcohols R–CH(OH)–CH(CH_3)–CH = CH_2 where R = CH_3, C_2H_5, *n*-C_3H_7, *n*-C_4H_9, *n*-C_5H_{11}, *iso*-C_3H_7, *sec.*-C_4H_9, *tert.*-C_4H_9. The existence of a relationship between the analytical data and diastereomeric configuration of the alcohols is pointed out for all the three methods adopted.

INTRODUCTION

The formation of stereochemically defined β-methylalkanols of the type R–CH(OH)–CH(CH_3)–CH = CH_2, prepared by addition of metal enolates or allylmetal compounds to aldehydes, is very important in the synthesis of macrolides and polyether antibiotics, as well as of some pheromones[1,2]. So far, mixtures of diastereomeric alcohols have been obtained by addition of many crotylmetal compounds to aldehydes[1,3–5].

In the present work, gas–liquid chromatography (GLC), thin-layer chromatography (TLC) and ^{13}C NMR spectrometry have been used to identify and analyse the diastereomeric mixtures of R–CH(OH)–CH(CH_3)–CH = CH_2, where R = CH_3, C_2H_5, *n*-C_3H_7, *n*-C_4H_9, *n*-C_5H_{11}, *iso*-C_3H_7, *sec.*-C_4H_9, *tert.*-C_4H_9.

EXPERIMENTAL

Materials

The diastereomeric mixtures of R–CH(OH)–CH(CH_3)–CH = CH_2 were prepared by the addition reaction of dibutylcrotyltin chloride with aldehydes RCHO[5].

Pure samples of *threo*- and *erythro*-homoallylic alcohols were obtained by separation of the mixtures with a Hewlett-Packard Scientific 700 gas chromatograph/thermal energy analyser equipped with a 3 m × 6.4 mm O.D. column packed with

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10% (w/w) Carbowax 20M on Chromosorb P (30–60 mesh). The helium carrier gas flow-rate was 20 ml/min, the injector temperature was 240°C, the detector temperature was 250°C and the oven temperature was in the range 100–130°C according to the alcohol to be analysed.

Gas chromatography

Pure *threo-* and *erythro*-alcohols were dissolved in diethyl ether (1%) solution. The samples were analysed with a Perkin-Elmer Model Sigma 3B gas chromatograph equipped with flame ionization detection (FID) and a 2 m × 3.2 mm O.D. column packed with 10% (w/w) LAC 860 on Chromosorb. The nitrogen carrier gas flow-rate was 20 ml/min, and the injector, detector and oven temperatures were 250, 270 and 105°C respectively. The retention times of the alcohols were measured with respect to the diethyl ether. The relative detector response factors for the *threo* and *erythro* isomers have the same values.

Thin-layer chromatography

The pure *threo-* and *erythro*-alcohols were dissolved in diethyl ether and 1–10 μl of the samples were applied with a microsyringe 1.5 cm from the lower edge of the plate (Kieselgel 60 F_{254}, 10 × 5 cm plates, Merck art. 5720) and dried by an air blower. Ascending development at room temperature was effected in a glass chamber equilibrated with the solvent. The following solvent systems were employed: S_1 = 10% solution of diethyl ether in light petroleum (b.p. 40–60°C); S_2 = 20% solution of diethyl ether in light petroleum; S_3 = 10% solution of ethyl acetate in hexane and S_4 = 10% solution of ethyl acetate in light petroleum.

Usually 10–15 min were required for the solvent front to cover a distance of 8 cm. The plates were then dried and the spots were detected in an iodine vapour chamber or by spraying with 50% sulphuric acid and kept for 15 min at 110°C. In both cases the alcohols were revealed as brown spots on a white background.

Multiple development of the plates was also carried out. The diastereomeric mixtures of each alcohol (1% diethyl ether solution) were applied (1–10 μl) and TLC was carried out with all the solvent systems (S_1–S_4). The plates were then removed from the chamber and the solvent was allowed to evaporate; they were then returned to the same solvent and developed a number of times depending on the separation to be achieved[6].

^{13}C NMR spectrometry

The spectra of diastereomeric mixtures of the β-methylalcohols were recorded at a fixed temperature (303K) on a Bruker WH 90 spectrometer operating in Fourier Transform (FT) mode. The off-resonance decoupling technique permitted the assignment of the ^{13}C NMR signals, since the typical multiplet structure of the carbon resonance lines was retained. In order to determine the mixture composition, sufficiently long pulse intervals (at least 25 s) were used to avoid partial saturation of ^{13}C resonances, and the gated decoupling method was employed to eliminate the nuclear Overhauser effect (NOE)[7].

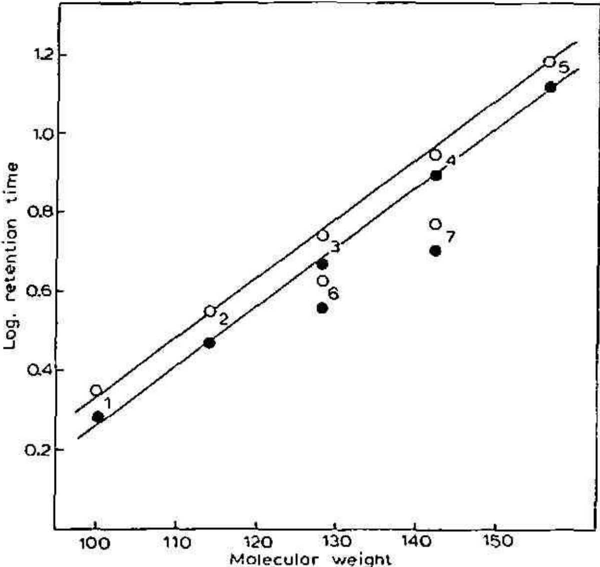

Fig. 1. Plot of log t_R against the molecular weight for β-methylalcohol (1–7 in Table I). ●, *threo*-isomer; ○, *erythro*-isomer.

RESULTS AND DISCUSSION

GC

The retention times of the *threo*- and *erythro*-alcohols are reported in Table I and a plot of log t_R *versus* molecular weight is given in Fig. 1. Linearity is found only for the homologous series of compounds 1–5 (*cf.*, Table I), as expected.

The four diastereomeric alcohols with R = *sec.*-C_4H_9 could not be separated under the conditions employed, only three peaks were detected, having retention times of 5.9, 7.8 and 8.0 min. The corresponding collected fractions were designated as A, B and C respectively and used in the other analytical methods (see below).

The results obtained reveal the existence of a relationship between the configuration of the alcohols and the relative retention times, *i.e.*, t_R (*erythro*) > t_R (*threo*).

TABLE I

GLC RETENTION TIMES, t_R, OF THE EXAMINED ALCOHOLS R–CH(OH)–CH(CH_3)–CH = CH_2

Alcohol No.	*R*	*t_R (min)*	
		Threo-isomer	*Erythro-isomer*
1	CH_3	2.0	2.3
2	C_2H_5	3.0	3.6
3	*n*-C_3H_7	4.8	5.6
4	*n*-C_4H_9	8.1	9.1
5	*n*-C_5H_{11}	13.7	15.7
6	*iso*-C_3H_7	3.7	4.4
7	*tert.*-C_4H_9	5.3	6.2

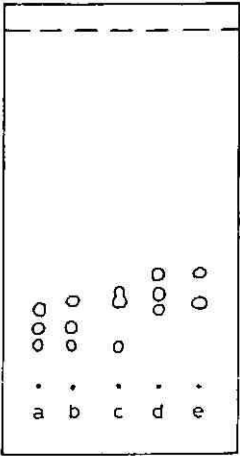

Fig. 2. TLC separation of R–CH(OH)–CH(CH_3)–CH=CH_2 mixtures using solvent system S_2. (a) R = CH_3, C_2H_5, *n*-C_3H_7 (R_F = 0.11, 0.17, 0.21); (b) R = CH_3, C_2H_5, *n*-C_4H_9 (R_F = 0.11, 0.17, 0.23); (c) R = CH_3, *n*-C_4H_9, *n*-C_5H_{11} (R_F = 0.11, 0.23, 0.25); (d) R = *n*-C_3H_7, *n*-C_5H_{11}, *tert.*-C_4H_9 (R_F = 0.21, 0.25, 0.31); (e) R = *iso*-C_3H_7, *tert.*-C_4H_9 (R_F = 0.23, 0.31).

This finding is in agreement with previous reports[8,9] since in the *threo* configuration the vinyl group is more able to form an intramolecular hydrogen bond with the hydroxyl group.

TLC

The R_F differences between the *threo*- and *erythro*-isomers of each alcohol are in the range 0.01–0.02, for all the solvent systems used. As a consequence, this method cannot be adopted for resolving these mixtures.

Nevertheless, it is to be noted that the R_F (*threo*) values are always greater than the R_F (*erythro*) values. This trend was confirmed by the results obtained with multiple development (see below). In addition, in the light of previous reports[10–13],

1 multiple development | 3 multiple developments | 5 multiple developments

Fig. 3. TLC separation of diastereomeric alcohols, using solvent system S_1. (a) R = *n*-C_3H_7, *threo*/*erythro* mixture; (b) R = *iso*-C_3H_7, *threo*/*erythro* mixture; (c) R = *sec.*-C_4H_9, A and B mixture; (d) R = *sec.*-C_4H_9, A and C mixture; (e) R = *sec.*-C_4H_9, B and C mixture; (f) R = *sec.*-C_4H_9, sample of the original alcohol prepared. A, B and C represent the three fractions collected by GC.

1 multiple development	3 multiple developments	5 multiple developments
a b c d e f	a b c d e f	a b c d e f

Fig. 4. TLC separation of diastereomeric alcohols, using solvent system S_3. Details as in Fig. 3.

TLC with ascending development offers a good, rapid resolution of alcohol mixtures having different R groups, as shown in Fig. 2, where the corresponding R_F values are also reported.

The multiple developments performed for alcohols with R = n-C_3H_7, *iso*-C_3H_7 and *sec.*-C_4H_9 using the solvent systems S_1 and S_3 are shown in Figs. 3 and 4 respectively. Analogous results have been obtained with the solvent systems S_2 and S_4. A good separation of the examined diastereomeric mixtures takes place only when R is a branched group, *cf.*, samples b–f in Figs. 3 and 4.

A significant example of the separation and identification is given by the ΔR_F value (0.06) between the *threo*- and *erythro*-alcohols with R = *iso*-C_3H_7 found after the third multiple development with both systems S_1 and S_3, *cf.*, sample b, Figs. 3 and 4. The individual R_F values are: 0.36 (*threo*) and 0.30 (*erythro*) with system S_1; 0.56 (*threo*) and 0.50 (*erythro*) with system S_3.

This method allows the complete separation of the four diastereomeric alcohols with R = *sec.*-C_4H_9, after five multiple developments (sample f, *cf.*, Figs. 3 and 4): four spots are found having R_F 0.56, 0.51, 0.47 and 0.42 with system S_1 and R_F 0.70, 0.66, 0.63 and 0.59 with system S_3.

A comparison of sample f with c, d and e (*cf.*, Figs. 3 and 4) allows one to establish that the fractions A and B are pure diastereoisomers, whereas C is a mixture of the other two.

^{13}C NMR spectrometry

The carbon-13 chemical shifts of the examined alcohols are listed in Table II. The signal of each carbon is split into a doublet whose intensity ratio is dependent on the *threo/erythro* composition.

In order to identify the *threo*- and *erythro*-isomers in a given mixture of alcohols, pure samples of the *threo*- and *erythro*-isomers of alcohol 3 (*cf.*, Table I) (separated by GC) were mixed in the ratio 2:1. The spectrum of this system in the olefinic region shows two inner lines, $\delta(=CH)$ 141.0 ppm and $\delta(=CH_2)$ 115.4 ppm, *cf.*, Table II), corresponding to the *threo*-isomer and other two outer lines, $\delta(=CH)$ 142.0 ppm and $\delta(=CH_2)$ 114.5 ppm, corresponding to the *erythro*-isomer, as shown in Fig. 5. The spectra of all the other alcohols examined show an analogous pattern.

We have observed that along this series the differences, $\Delta\delta$(ppm), between the

TABLE II

^{13}C NMR CHEMICAL SHIFTS* OF THE CARBINOLS

Carbinol	Diastereoisomer	Carbon atom										
		1	*2*	*3*	*3'*	*4*	*5*	*5'*	*6*	*7*	*8*	*9*
1 2 3 3' 4 5 $CH_2=CH{-}CH(CH_3){-}CH(OH){-}CH_3$**	*erythro*	114.4	141.2	45.3	15.9	70.8	19.8					
	threo	114.8	141.4	45.6	15.3	71.0	20.5					
1 2 3 3' 4 5 6 $CH_2=CH{-}CH(CH_3){-}CH(OH){-}CH_2{-}CH_3$**	*erythro*	114.1	142.1	44.3	15.6	76.6	27.6		10.6			
	threo	114.8	141.0	44.0	16.3	76.6	27.4		10.6			
1 2 3 3' 4 5 6 7 $CH_2=CH{-}CH(CH_3){-}CH(OH){-}CH_2{-}CH_2{-}CH_3$	*erythro****	114.5	142.0	44.5	15.0	74.8	36.9		19.6	14.2		
	*threo****	115.4	141.0	44.5	16.4	74.8	36.9		19.4	14.2		
1 2 3 3' 4 5 6 7 8 $CH_2=CH{-}CH(CH_3){-}CH(OH){-}CH_2{-}CH_2{-}CH_2{-}CH_3$												
	*erythro****	113.0	142.0	44.4	15.2	75.1	34.4		28.7	23.1	14.2	
	*threo****	114.4	141.0	44.4	16.4	75.1	34.3		28.6	23.1	14.2	
1 2 3 3' 4 5 6 7 8 9 $CH_2=CH{-}CH(CH_3){-}CH(OH){-}CH_2{-}CH_2{-}CH_2{-}CH_2{-}CH_3$												
	*erythro****	114.5	142.0	44.4	14.9	75.0	34.7		32.4	26.2	23.1	14.2
	*threo****	115.4	141.0	44.4	16.4	75.0	34.7		32.4	26.0	23.1	14.2

Compound	Isomer	Configuration	1	2	3	3'	4	5	5'	6	7
$CH_2=CH\text{-}CH(CH_3)\text{-}CH(OH)\text{-}CH(CH_3)\text{-}CH_3$** (carbons 1, 2, 3, 3', 4, 5, 5', 6)											
	erythro		113.9	142.5	41.8	15.4§	79.7	30.9	16.7§	20.1§	
	threo		115.0	140.8	41.5	15.7§	80.0	31.2	17.8§	19.8§	
$CH_2=CH\text{-}CH(CH_3)\text{-}CH(OH)\text{-}CH(CH_3)\text{-}CH_2\text{-}CH_3$** (carbons 1, 2, 3, 3', 4, 5, 5', 6, 7)											
	erythro§§	*SSS,RRR*	113.9	142.8	41.2	13.6§§§	79.0	37.8§§§	16.0§§§	24.4§§§	11.4
			(113.9)	(142.5)	(41.9)		(78.3)				(11.4)
		RSS,SRR	113.9	142.2	42.7	12.7	77.6	37.5	16.8	26.8	11.4
	threo§§	*SRS,RSR*	115.2	141.7	41.8	13.3	77.4	37.2	17.2	26.8	11.7
			(115.2)	(141.2)	(41.5)		(78.2)				(11.7)
		SSR,RRS	115.2	140.7	41.2	14.5§§§	79.0	38.1§§§	18.0§§§	23.8§§§	11.7
$CH_2=CH\text{-}CH(CH_3)\text{-}CH(OH)\text{-}C\equiv(CH_3)_3$** (carbons 1, 2, 3, 3', 4, 5, 6)											
	erythro		112.5	144.9	40.5	16.1	81.6	36.1		27.1	
	threo		114.2	141.1	40.5	21.6	82.8	36.1		27.1	

* In ppm from internal tetramethylsilane for pure liquids.

** See also ref. 5.

*** Chemical shifts in $C^2H_2Cl_2$.

§ The assignment is only tentative.

§§ This formalism is used in order to compare this system with the others. The calculated barycentres of the resonance lines of the pairs of diastereoisomers are given in parentheses.

§§§ These figures may be interchanged within each column.

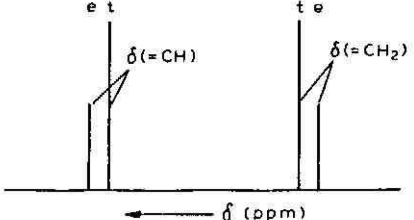

Fig. 5. ^{13}C NMR pattern of the olefinic carbon signals. t = *threo*-isomer; e = *erythro*-isomer.

chemical shifts $\delta(=CH)$ and $\delta(=CH_2)$ of the inner lines lie in a narrower range in comparison with the outer lines: $\Delta\delta$(inner) = 26.2 ± 0.6 ppm, $\Delta\delta$(outer) = 29.6 ± 2.8 ppm.

It is to be noted that the *threo*-form is stabilized in the eclipsed structure owing to intramolecular interactions between –OH and $-CH=CH_2$ groups[8]. Thus, it is likely that the chemical environment around the olefinic carbons in such isomers does not change very much on varying the steric hindrance of the R group. On the contrary, changes are to be expected for the *erythro*-isomer. Hence the pattern depicted in Fig. 5 applies to all the alcohols examined, that is the inner lines belong to the *threo*-isomer and the outer to the *erythro*-isomer.

Integration of these signals, as indicated above, allows a quantitative analysis of the *threo/erythro* mixtures. The results obtained are in good agreement with the GC data. For example, the *threo/erythro* composition of alcohols 3–5 (*cf.*, Table I) detected by GC and ^{13}C NMR are: 53/47 (GC), 53/47 (^{13}C NMR); 50/50, 49/51; 55/45, 54/46 respectively.

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REFERENCES

1 R. W. Hoffmann, *Angew. Chem., Int. Ed. Engl.*, 21 (1982) 555 and references therein.
2 P. A. Bartlett, *Tetrahedron*, 36 (1980) 3 and references therein.
3 G. Courtois and L. Miginiac, *J. Organomet. Chem.*, 69 (1974) 1.
4 V. Rautenstrauch, *Helv. Chim. Acta*, 57 (1974) 496.
5 A. Gambaro, D. Marton, V. Peruzzo and G. Tagliavini, *J. Organomet. Chem.*, 226 (1982) 149.
6 J. G. Kirchner, in E. S. Perry and A. Weissberger (Editors), *Thin-layer Chromatography, Technique of Organic Chemistry*, Wiley-Interscience, New York, 1967, p. 111.
7 C. H. Sotak, C. L. Dumoulln and G. C. Levy, *Anal. Chem.*, 55 (1983) 782.
8 Y. Gault and H. Felkin, *Bull. Soc. Chim. Fr.*, (1965) 742.
9 J. Sicher, M. Cherest, Y. Gault and H. Felkin, *Collect. Czech. Chem. Commun.*, 28 (1963) 72.
10 R. Subbarao, M. W. Roomi, M. R. Subbaram and K. T. Achaya, *J. Chromatogr.*, 9 (1962) 295.
11 P. B. Rao and B. Sreenivasan, *Chem. Ind. (London)*, (1966) 1376.
12 E. J. Sing and L. L. Gershbein, *J. Chromatogr.*, 23 (1966) 180.
13 A. Nobuhara, *J. Chromatogr.*, 30 (1967) 235.

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INTERACTION OF DNA WITH HYDROXYAPATITE

STUDIES ON THE EFFECT OF THE PHOSPHATE CONCENTRATION OF THE COLUMN EQUILIBRATION AND WASHING BUFFER

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SUMMARY

The ability of hydroxyapatite to bind DNA effectively in phosphate solutions used for column equilibration, sample loading and column washing has been examined. It was demonstrated that substantial amounts of DNA (up to 40%) were eluted in the washing buffer when the phosphate concentration in the lysing solution or urea–phosphate used for column equilibration, sample loading and column washing was 0.24 *M*. A reduction in the phosphate concentration from 0.24 to 0.15 *M* in urea–phosphate solution led to almost 100% binding, whereas a similar reduction in the lysing solution did not. A modified method for loading and eluting DNA from hydroxyapatite columns is presented.

INTRODUCTION

Hydroxyapatite (HaP) is widely used for nucleic acid separation[1–4]. It has been used for separating RNA and DNA after *in vitro*[3] and *in vivo*[5] exposure to chemical carcinogens, in order to analyse each for covalent adducts. However, to avoid ambiguity in this type of analysis two factors require close attention: (1) the maximum useful capacity of HaP for DNA and (2) the critical relationship between the phosphate concentration and nucleic acid[1,6] as well as protein[7,8] adsorption.

Previously published[5,9] HaP chromatographic procedures tend to suggest that samples can be applied as solutions in which the phosphate concentration is as high as 0.24 *M*. Attempts to use these procedures for establishing the maximum useful capacity of hydroxyapatite for DNA revealed that almost 20% of the DNA loaded was not effectively bound by the column. Hence it was considered useful to examine the effect of the phosphate concentration of the column equilibration, loading and washing buffer on the DNA–HaP interaction.

MATERIALS AND METHODS

Calf thymus DNA and bovine serum albumin (BSA), fraction V, were obtained

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from Sigma Chemical Company (London, U.K.), whereas yeast RNA and hydroxyapatite were from BDH (Poole, U.K.).

Loading and elution of sample from HaP column

HaP columns (3.0 cm × 1.0 cm) were first equilibrated with either lysing solution (LS) or urea–phosphate (UP). Aliquots of DNA, RNA or BSA solution (1 mg/ml) in lysing solution were then loaded. The columns were washed as described by Shoyab[5] or Markov and Ivanov[9]. In some experiments the phosphate concentration in the washing buffer solutions was changed, as indicated in Results.

Quantitation of nucleic acids and protein

Fractions of 2 ml were collected and the presence of nucleic acid or protein was monitored spectrophotometrically. The absorbance of nucleic acids was measured at 260/320 nm while that of protein was measured at 280 nm. The amounts of nucleic acids or protein recovered from the column could then be estimated.

RESULTS

When purified calf thymus DNA was loaded and eluted from HaP as described by Shoyab[5], 30–40% was eluted in the washing buffer (data not shown). Consequently the phosphate concentration in the lysing solution was reduced from 0.24 to 0.15 *M*. Though this change did not lead to complete binding, it decreased the proportion of DNA that was prematurely eluted, from 30–40% to 9–19% (Table I,

TABLE I

EFFECT OF REDUCING THE PHOSPHATE CONCENTRATION OF THE LYSING SOLUTION AND UREA–PHOSPHATE ON THE DNA–HaP INTERACTION

Known amounts of DNA in lysing solution were loaded on 3.0 cm × 1.0 cm HaP columns pre-equilibrated with lysing solution containing 0.15 *M* (A) or 0.05 *M* (B) sodium phosphate. The columns were washed sequentially with LS (8 *M* urea, 1.0% sodium dodecyl sulphate, 0.01 *M* EDTA, 0.15 *M* sodium phosphate, pH 6.8), UP (8 *M* Urea, 0.15 *M* sodium phosphate, pH 6.8) and 0.1 *M* sodium phosphate, pH 6.8 to remove any unbound DNA (These three solutions are referred to as "washing buffer"; the concentration of phosphate in LS and UP was reduced to 0.05 *M* in condition B.) The bound DNA was eluted by 0.24 and 0.48 *M* sodium phosphate, pH 6.8 (referred to as eluting buffer). RNA and BSA were separately dissolved in lysing solution, loaded and eluted using condition B.

*Expt.**	*% Distribution of recovered macromolecule*			
	A		*B*	
	Washing buffer	*Eluting buffer*	*Washing buffer*	*Eluting buffer*
1	19.0	81.0	–	–
2	9.0	90.0	–	–
3	–	–	3.0	97.0
4	–	–	0.0	100.0
5	–	–	92.0	8.0
6	–	–	63.0	36.0

* Mean load of DNA in expts. 1–4: 106 μg. Load of RNA in expt. 5: 500 μg; of BSA in expt. 6, 1600 μg.

expts. 1 and 2, column A). However, total binding was subsequently achieved when the phosphate concentration in both the lysing solution and urea–phosphate was reduced to 0.05 *M* (Table I, expts. 3 and 4, column B). Evidently, a considerable reduction in phosphate concentration is required for satisfactory DNA binding when using Shoyab's procedure.

Apart from satisfactory binding, another parameter that deserves attention is the purity of the DNA eluted from HaP columns, especially when it is being isolated from tissue lysate. One of the ways to achieve this is by ensuring that the phosphate concentration of the washing buffer solution is just sufficient to elute RNA and protein without desorbing the DNA. This condition is not satisfied by 0.05 *M* sodium phosphate which enhanced the DNA–HaP interaction but did not effect the complete elution of RNA and protein from the column (Table I, expts. 5 and 6, column B). As a result of this, Shoyab's procedure was abandoned.

Markov and Ivanov[9] used a method slightly different from Shoyab's to load and elute DNA from HaP columns. In their procedure, lysing solution was omitted and the columns were equilibrated with urea–phosphate solution containing 0.2 *M* sodium phosphate. When DNA was loaded and eluted using this procedure, a significant amount was also eluted in the washing buffer, UP (8 *M* urea, 0.24 *M* sodium phosphate, pH 6.8) and 0.15 *M* sodium phosphate, pH 6.8 (data not shown). Hence the concentration of phosphate in UP was reduced to 0.15 *M*. As a result, 100% binding was achieved (Table II, expts. 1 and 2, column A). Under the same conditions, BSA was loaded and completely eluted in the washing buffer (Table II, expts. 3 and 4, column A). However, when RNA was applied about 75% of it was eluted

TABLE II

EFFECT OF REDUCING THE PHOSPHATE CONCENTRATION OF UREA–PHOSPHATE SOLUTION ON THE ADSORPTION OF DNA, RNA AND BSA TO HaP

DNA, BSA and RNA were dissolved separately in lysing solution and 0.5 ml of each was loaded on 3.0 cm × 1.0 cm HaP columns pre-equilibrated with urea–phosphate (UP). After loading, the columns were washed with UP (8 *M* urea, 0.15 M sodium phosphate, pH 6.8), 0.15 *M* sodium phosphate (A) and additionally with 0.20 *M* sodium phosphate (B) and then tightly bound DNA, BSA or RNA was eluted with 0.48 *M* sodium phosphate, pH 6.8.

*Expt.**	*% Distribution of recovered macromolecule*						
	A			*B*			
	UP	*0.15 M*	*0.48 M*	*UP*	*0.15 M*	*0.20 M*	*0.48 M*
1	0.0	0.0	100.0	–	–	–	–
2	0.0	0.0	100.0	–	–	–	–
3	2.1	97.9	0.0	–	–	–	–
4	2.5	97.7	0.0	–	–	–	–
5	55.6	19.3	25.6	–	–	–	–
6	57.5	18.3	24.3	–	–	–	–
7	–	–	–	54.6	11.9	33.5	0.0
8	–	–	–	0.0	0.0	50.0	50.0

* Mean loads: 230 μg DNA in expts. 1, 2 and 8; 1640 μg BSA in expts. 3 and 4; 160 μg RNA in expts. 5–7.

in the washing buffer while the remaining 25% was subsequently eluted in 0.48 *M* sodium phosphate, the eluting buffer (Table II, expts. 5 and 6, column A).

Consequently, 0.2 *M* sodium phosphate was introduced as an additional washing solution after 0.15 *M*. This solution caused RNA to be eluted completely in the washing buffer (Table II, expt. 7, column B), but when DNA was chromatographed under identical conditions the 0.20 *M* sodium phosphate desorbed 50% of the DNA (Table II, expt. 8, column B).

The effect of 0.20 *M* sodium phosphate on the adsorption of DNA and RNA to hydroxyapatite, Table II, suggests that the elution of both substances overlaps at this concentration. To resolve this, a gradient elution of DNA and RNA from HaP was performed with the sodium phosphate concentration between 0.15 and 0.20 *M* (see Fig. 1). It was deduced that 0.18 *M* phosphate can desorb RNA completely without any effect on the stability of the DNA–HaP interaction.

The procedure of Markov and Ivanov[9] was, therefore, modified as follows. The column was equilibrated with UP containing 0.15 instead of 0.24 *M* sodium phosphate. After loading, the columns were washed sequentially with UP, 0.15 *M* and additionally with 0.18 *M* sodium phosphate before eluting the DNA with 0.48 *M* sodium phosphate, pH 6.8. This procedure was successfully used to establish the maximum useful capacity of HaP columns and also for separating RNA and DNA in nucleic acid extracts.

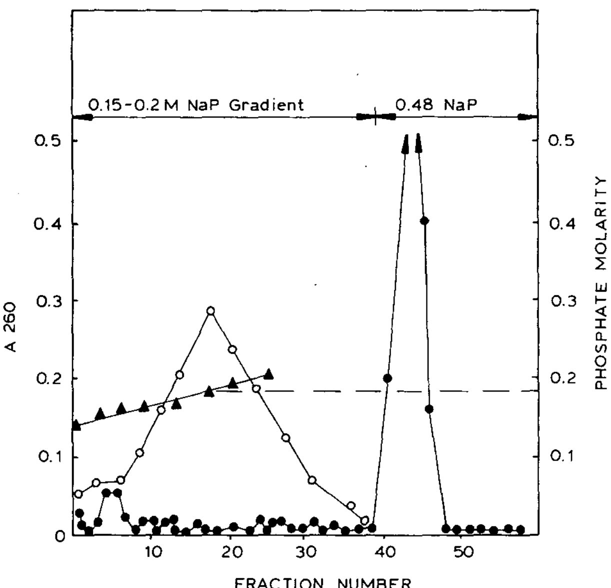

Fig. 1. Continuous gradient elution of 1.0 mg RNA (O—O) and 0.30 mg DNA (●—●) from a 3.0 cm × 1.0 cm HaP column. The column was equilibrated and loaded as described in Table II. After washing with UP, gradient elution between 0.15 *M* (40 ml only) and 0.20 *M* sodium phosphate (40 ml only) was commenced. Two-ml fractions were collected and the absorbance at 260 nm and conductivity of each were measured. Conductivities were converted into phosphate molarities (▲—▲) using an appropriate calibration curve.

DISCUSSION

The chromatographic behaviour of DNA loaded on HaP columns pre-equilibrated and subsequently washed with lysing solution or urea–phosphate containing different amounts of phosphate described in this paper highlights a problem inherent in a number of published procedures for HaP column chromatography. These reports[5,9] suggest that DNA can be loaded on HaP columns pre-equilibrated and later washed with solutions whose phosphate concentration is 0.24 *M*. On the basis of the present observations, this concentration appears to be rather high for these purposes and may be responsible for the premature elution of DNA in the washing buffer, as experienced in this work and by others[10]. This view is confirmed by the observation (see Table II) that DNA can even be eluted by 0.20 *M* phosphate. Furthermore, this single factor, more than any other, explains why all attempts to bind DNA in lysing solution or urea–phosphate containing a higher phosphate concentration (0.24 *M*) were completely unsuccessful. Still, a recent report[11], like the earlier ones[5,9] recommends 0.24 *M* phosphate for column equilibration and DNA loading. In view of the present results, it is probable that some proportion of DNA is eluted prematurely from the column when 0.24 *M* sodium phosphate is used for column equilibration and DNA loading.

Since the concentration of phosphate is a potent factor that determines the strength of nucleic acid–HaP interaction[1,6], the actual concentration of phosphate in the lysing solution used for sample loading, column equilibration and washing should be evaluated and stated. The reason is that (as in this work) when solid urea is dissolved in a solution of 0.24 *M* sodium phosphate, the volume changes lead to a new phosphate concentration of *ca.* 0.15 *M*, that shown to allow complete DNA binding. Indeed, this is the way Johnson and Illan[11] prepared their 8 *M*/0.24 *M* urea–phosphate solution. The concentration (0.24 *M*) stated does not reflect the actual concentration of phosphate in this solution, and may therefore be misleading.

HaP has been successfully used for different nucleic acid separation problems. One of these is the separation of DNA and RNA present in cellular or nuclear lysates after *in vivo*[5] and *in vitro*[3] modification by radiolabelled chemical carcinogen. This enables investigators to establish which of the two is more susceptible to chemical modification by the carcinogen. The use of HaP in this kind of investigation and the fact that DNA can be desorbed from HaP columns prematurely under certain conditions as noted in this paper indicate the importance of accurate monitoring of the purity of RNA or DNA eluted from HaP columns, especially when the sample chromatographed contains both substances and HaP is the sole separation and purification technique used. In such situations, the problem of cross-contamination can be minimized if the appropriate concentration of phosphate for column equilibration, sample loading and column washing is not exceeded.

REFERENCES

1 G. Bernardi, *Methods Enzymol.*, 21 (1971) 95–139.
2 G. Bernardi, *Methods Enzymol.*, 22 (1971) 325–345.
3 P. I. Andriaenssens, J. Bixler and M. W. Anderson, *Anal. Biochem.*, 123 (1982) 162–169.
4 F. A. Beland, K. L. Dooley and D. A. Casciano, *J. Chromatogr.*, 174 (1979) 177–186.

5 M. Shoyab, *Chem. Biol. Interact.*, 25 (1979) 71–85.
6 H. G. Martinson, *Biochemistry*, 12 (1973) 139–145.
7 G. Bernardi and W. H. Cook, *Biochim. Biophys. Acta*, 44 (1960) 96.
8 G. Bernardi and T. Kawasaki, *Biochim. Biophys. Acta*, 160 (1968) 301–310.
9 G. G. Markov and I. G. Ivanov, *Anal. Biochem.*, 59 (1974) 555–563.
10 S. Brown, K. Ocraft, A. H. Alwan and C. O'Callaghan, *Cell Biol. Int. Rep.*, 5 (1981) 6.
11 T. R. Johnson and J. Illan, *Anal. Biochem.*, 132 (1983) 20–25.

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PREPARATIVE REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF IODINATED INSULIN RETAINING FULL BIOLOGICAL ACTIVITY*

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SUMMARY

Insulin monoiodinated in Tyr A14, A19, B16 and B26 can be separated from insulin and diiodoinsulins using reversed-phase high-performance liquid chromatography on LiChrosorb RP-18 columns. Monoiodoinsulins with high and low specific activities were isolated from a number of buffer systems without any reduction in binding affinity and biological activity in isolated rat fat cells. The reason for the previously observed reduction in the binding affinity was probably column bleeding, *i.e.*, chemical degradation of the column support.

INTRODUCTION

Iodination of insulin leads to a heterogeneous mixture of insulin, mono- and diiodinated insulins. Insulin monoiodinated in Tyr A14, A19, B16 or B26 can be isolated after iodination in urea-containing buffer using disc electrophoresis/ion-exchange chromatography[1,2]. It has been shown that A14 monoiodoinsulin exhibits the same binding affinity and biological activity in fat cells as native insulin, making it an ideal tracer for insulin[3].

Several groups have been studying the isolation of monoiodoinsulins, recently mostly using reversed-phase high-performance liquid chromatography (RP-HPLC)[4–12]. It is generally accepted that the A19 isomer shows a decreased binding affinity[1–4,13] and the B26 isomer an increased binding affinity in isolated rat adipocytes[2,3,7,8]. The receptor binding properties of these isomers also differ in other cell types, such as hepatocytes and cultured human IM-9 lymphocytes[1,2,5–8].

We have recently described several RP-HPLC systems for the separation of

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insulin and all four monoiodoinsulins using different C_{18} columns, organic modifiers and triethylammonium buffers at various pH values[9–12,14,15]. We observed a reduced binding affinity of these HPLC-purified monoiodoinsulin tracers to isolated rat adipocytes compared with the affinity of similar tracers isolated by disc electrophoresis/ion-exchange chromatography. Different observations of the deterioration of the biological properties of other peptides and proteins after RP-HPLC fractionation have been published in recent years[16–23], although some reports of retention of biological activity have also been published[24,25].

The present paper describes the RP-HPLC separation of all four monoiodoinsulins with maximum specific activity, as well as the preparative isolation in milligram-amounts of insulin and monoiodoinsulins with low specific activity using RP-HPLC on LiChrosorb RP-18 columns. By employing different isolation procedures it was possible to obtain monoiodoinsulins (and insulin) with retained biological properties in isolated rat adipocytes.

MATERIALS AND METHODS

Insulin

Highly purified porcine insulin was prepared as described previously[10].

Iodinated insulin with high specific activity

Insulin was iodinated in phosphate buffer containing 6 *M* urea using the lactoperoxidase method as described previously[1,26]. The average iodination degree was 0.14 I/mol of insulin. The iodination mixture (40 μl) was adjusted with 15 μl glacial acetic acid to pH about 3 prior to RP-HPLC fractionation as described below.

Iodinated insulin with low specific activity

A 150-mg amount of insulin was iodinated on a preparative scale (25 mg/ml) analogously to the method for preparing iodinated insulin with high specific activity, except that the added iodide was "cold" $Na^{127}I$ plus 1 mCi $^{125}I^-$ as tracer. The average iodination degree was 0.16 I/mol of insulin. To remove lactoperoxidase and iodide, the iodination mixture was applied to a 90 cm × 2.5 cm I.D. Sephadex G-50 column eluted with 0.1 *M* ammonium hydrogencarbonate, pH 8.0. The fractions containing the iodinated insulin were pooled and lyophilized.

RP-HPLC

The HPLC system consisted of a Spectra-Physics SP 8700 chromatograph, a U6K (Waters) injector and a Pye Unicam UV detector. The columns were LiChrosorb RP-18, 5 μm, 250 mm × 4 mm I.D. (Merck) and LiChrosorb RP-18, 7 μm, 250 × 25 mm I.D. (Merck). The buffers were 0.25 *M* triethylammonium phosphate (TEAP), pH 4.0 and 0.25 *M* triethylammonium formate (TEAF), pH 6.0. Acetonitrile and isopropanol were used as organic modifiers. The columns were eluted isocratically, the eluate collected in 1-min fractions (Pharmacia, FRAC 300 fraction collector) and the radioactivity was measured in a 16-channel γ-counter (Hydrogamma 16) or in a manual gamma spectrometer (Mølsgaard Medical ApS, Denmark) equipped with a damping device (1000 times damping) made from stainless steel. All separations were performed at room temperature.

Reagents

Phosphoric acid and formic acid (p.a.) were from Merck, triethylamine (99%) from Janssen Chimica and acetonitrile and isopropanol (HPLC grade S) from Rathburn Chemicals. All other chemicals were of analytical reagent grade.

Distilled water was drawn from a Millipore Milli Q plant and all buffers were filtered (0.45 μm, Millipore) and vacuum/ultrasound degassed before use.

Silicon estimation

The silicon content in the column eluate (0.25 *M* TEAF, pH 6.0–21.5% isopropanol) was measured using inductively coupled plasma atomic emission spectrometry (ICP-AES). The effect of different organic modifiers on the silicon content was analyzed using graphite-furnace atomic absorption spectrometry (GFAAS).

Isolation procedures

(1) A Sep-pak C_{18} cartridge (Waters) was prewashed with 10 ml isopropanol–water (90:10, v/v) followed by 10 ml water. The pooled HPLC fractions were diluted in 1 volume of water and loaded on the Sep-Pak, followed by 10 ml water and 10 ml of 1 *M* acetic acid. The [^{125}I]insulin was eluted with 5 ml isopropanol–1 *M* acetic acid (90:10, v/v).

(2) Gel chromatography on a 70 cm × 1.6 cm I.D. Fractogel TSK HW-40 (s) (Merck) column eluted at 20 ml/h with 0.1 *M* ammonium hydrogencarbonate pH 8.0 containing 40% ethanol. The pooled fractions containing the [^{125}I]insulin were diluted in 2 volumes of water and lyophilized.

(3) Extraction with cyclohexane: 4 volumes of cyclohexane were shaken with the pooled fractions from HPLC or the concentrate from the Sep-Pak. The water phase was isolated, flushed with nitrogen and lyophilized.

The purity of the monoiodoinsulins

The purity was determined using analytical RP-HPLC (as described above).

The iodine distribution was determined using oxidative sulphitolysis and enzymatic cleavage of the separated A- and B-chains as previously described[13].

The diiodoinsulin content was estimated by disc electrophoresis, slicing the gel and measuring the radioactivity in the slices[13]. The content of diiodoinsulin with two iodine atoms in the same tyrosine group was determined by pronase digestion followed by gel chromatographic determination of diiodotyrosine (DIT) as described[27].

Biological determinations

The binding affinity to isolated rat adipocytes was measured as previously described[1–3,13].

The biological activities of mono[^{125}I]iodoinsulin and mono-[^{127,125}I]iodoinsulin were determined using the enhancement of the conversion of [U-^{14}C]glucose into lipid in isolated rat adipocytes as described previously[2,3]. In some of the determinations of mono[^{127,125}I]iodoinsulin the conversion of [3-^{3}H]glucose into lipid was measured without separation of the water and toluene phases.

The samples for biological determinations were dissolved in 0.01 *M* hydrochloric acid to give two different stock concentrations ($\approx$0.3 and $\approx$0.1 mg/ml) and

the optical density was measured at 277 nm. The insulin concentration was calculated based on an optical density of 0.958 for an insulin solution containing 1 mg/ml in 0.01 *M* hydrochloric acid.

The concentration in the most concentrated stock solutions of insulin and the monoiodoinsulins with low specific activity was determined using amino acid analysis (Kontron Liquimat III). The determination was based on eight stable amino acids from the 24-h hydrolysis in 6 *M* hydrochloric acid (lys, his, arg, asp, glu, gly, ala, leu). The concentration in the more diluted solutions was determined using staining with Folin-Ciocalteau's reagent according to Lowry *et al.*[28] and with Coomassie Brilliant Blue in the microassay procedure (Bio-Rad). Linear calibration plots were obtained in the range 1–10 μg/ml. The validity was assessed by the identity of the calibration plots for insulin and iodoinsulin substituted to an average of 1 I/mol of insulin.

RESULTS

Fractionation of iodination mixtures

The fractionation of 20 μg insulin iodinated with 1 mCi $^{125}I^-$ using isocratic elution in TEAF–isopropanol, pH 6.0 is shown in Fig. 1. This iodination mixture contained about 17.5 μg of unsubstituted insulin plus 2.5 μg of monoiodoinsulins. The amount of unreacted $^{125}I^-$ was in agreement with the amount (about 5%) determined by TCA precipitation of the iodination mixture. Using this buffer, baseline separation of the four monoiodoinsulins and unlabelled insulin is obtained. Using TEAP–acetonitrile, pH 4.0 as eluent, the critical separation between A19 monoiodoinsulin and unsubstituted insulin was less satisfactory, as previously reported[11].

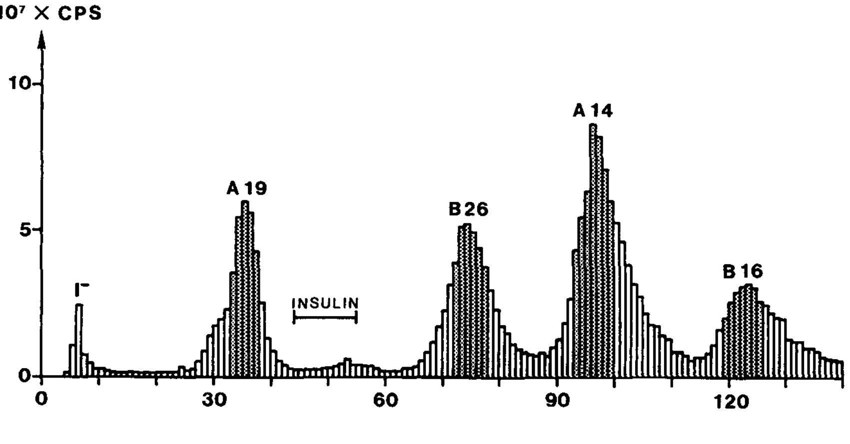

Fig. 1. Isocratic RP-HPLC separation of 50 μl iodination mixture (20 μg insulin iodinated with 1 mCi $^{125}I^-$) using a 250 mm × 4.0 mm I.D. LiChrosorb RP-18 column (5 μm) eluted at 0.5 ml/min with 0.25 *M* TEAF, pH 6.0–21.5% isopropanol. The histogram represents the radioactivity in the collected 1-min fractions. The dotted fractions in each peak were pooled and purified further (see Materials and Methods).

Fractionation of milligram amounts of iodinated insulin

Fractionation of [^{127,125}I]iodoinsulin in the TEAF–isopropanol, pH 6.0 system on a 250 mm × 4 mm column was satisfactory only with an amount up to 1 mg iodoinsulin containing about 170 μg of monoiodoinsulins (data not shown).

Scaling up to a 250 mm × 25 mm column and applying 25 mg of iodinated insulin was satisfactory with respect to the separation of A19 monoiodoinsulin and insulin, whereas A14 and B16 monoiodoinsulin were eluted as a single peak (data not shown). When the same column was eluted with TEAP–acetonitrile, pH 4.0 a satisfactory separation was obtained when 30 mg iodinated insulin were applied, as shown in Fig. 2.

Column bleeding

The contents of silicon in eluates from different batches of the LiChrosorb RP-18 column are given in Table I. The influence of different organic modifiers on the bleeding of silicon from a single LiChrosorb RP-18 column is shown in Table II.

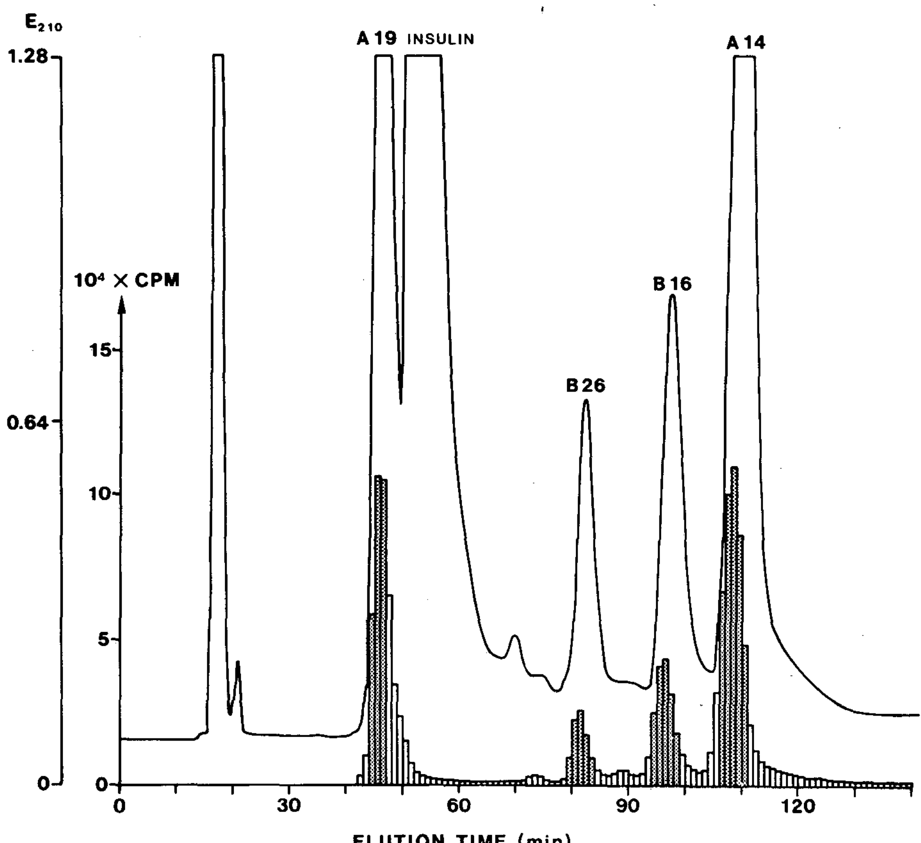

Fig. 2. Isocratic RP-HPLC separation of 300 μl iodinated insulin (30 mg insulin iodinated to 0.16 I/mol containing trace amounts of $^{125}I^{-}$) using a 250 mm × 25 mm I.D. LiChrosorb RP-18 column (7 μm) eluted at 5 ml/min with 0.25 *M* TEAP, pH 4.0–26% acetonitrile. The continuous curve represents the UV absorbance at 210 nm and the histogram represents the radioactivity in the collected 1-min fractions. The dotted fractions and fractions 53–56 containing insulin were pooled and purified further (see Materials and Methods).

TABLE I

THE CONTENTS OF SILICON (ng/ml) IN ELUATES FROM DIFFERENT BATCHES OF LICHROSORB RP-18, 5-μm COLUMNS ELUTED WITH 0.25 *M* TEAF, pH 6.0–21.5% ISOPROPANOL

Column batch No.	*Silicon (ng/ml)**
414999	188
415000	216
415016	30
Eluent	11

* By ICP-AES, see Materials and Methods.

Isolation of monoiodoinsulins

The recovery of monoiodoinsulins concentrated using Sep-Pak is shown in Table III. It was possible to obtain 90% of the monoiodoinsulin in 1 ml (loaded volume up to 50 ml). The recovery in the water phase after extraction with cyclohexane was *ca.* 90%.

Purity of monoiodoinsulins

The purity of monoiodoinsulin tracers with high specific activity was evaluated using the different methods summarized in Table IV. These results are representatives for several batches.

The purity of monoiodoinsulins with low specific activity is shown in Table V. Although the purity was *ca.* 95%, the B-chain labelled monoiodoinsulins were less pure than the A-chain labelled ones, probably because the amount of B-chain incorporation is lower than that of the A-chain incorporation.

Binding affinity of monoiodoinsulin tracers

Full binding affinity relative to that of the same isomer prepared by disc electrophoresis/ion-exchange chromatography was retained following isolation by gel chromatography in 40% ethanol (A), extraction with cyclohexane (B) and dilution in large volumes of albumin-containing buffer (C) (Table VI).

TABLE II

THE CONTENT OF SILICON (ng/ml) IN ELUATES FROM LICHROSORB RP-18, 5-μm COLUMN NO. 208810.

Eluent	*Silicon (ng/ml)**
0.25 *M* TEAF, pH 6.0–21.5% isopropanol	504
0.25 *M* TEAF, pH 6.0–27% acetonitrile	244
0.25 *M* TEAF, pH 6.0	88
Eluent	56

* By GFAAS, see Materials and Methods.

TABLE III

RECOVERY OF MONOIODOINSULIN MEASURED AS THE RADIOACTIVITY AFTER SEP-PAK CONCENTRATION

	% of radioactivity ± S.D. (n = 7)
Loaded Sep-Pak	100
First ml eluted	91 ± 2
Second ml eluted	1 ± 0.5
Empty Sep-Pak	7 ± 2

TABLE IV

THE PURITY OF MONOIODOINSULIN TRACERS PREPARED BY RP-HPLC AS PER CENT OF RADIOACTIVITY IN CONTAMINATING MONOIODOINSULIN

Methods: A = analytical RP-HPLC; B = iodine distribution analysis; C = pronase digestion. The content of DIT (diiodotyrosine) represents the molar (per cent) amount of diiodoinsulin with two iodine substitutions in the same tyrosine group.

Monoiodo-insulin	*Method*	*Iodide*	*A14*	*A19*	*B16*	*B26*	*DIT*
A14	A	2.1	96.8		1.1		
	B		95.6	2.4		2.0	
	C	1.3					0.2
A19	A	2.6		97.4			
	B			99.7		0.3	
	C	1.7					0.3
B16	A	4.8			93.8	1.4	
	B		-	2.7	93.3	4.0	
	C	3.6					1.9
B26	A	2.9				97.1	
	B			4.3		95.7	
	C	2.0					0.2

TABLE V

THE PURITY (%) OF MONOIODOINSULINS (MILLIGRAM AMOUNTS) WITH LOW SPECIFIC ACTIVITY PREPARED BY RP-HPLC AND EVALUATED BY ANALYTICAL RP-HPLC OF 25 μg

	Iodide	*Insulin*	*A14*	*A19*	*B16*	*B26*
A19 monoiodoinsulin*				100		
Insulin*		100				
A19 monoiodoinsulin**				99		
Insulin**		100				
B26 monoiodoinsulin**		4.2		1.6		94.4
B16 monoiodoinsulin**	0.5		1.7	0.8	95.1	1.8
A14 monoiodoinsulin**			98.0		2.0	

* The preparative RP-HPLC was performed with TEAF, pH 6.0–isopropanol.

** The preparative RP-HPLC was performed with TEAP, pH 4.0–acetonitrile.

TABLE VI

BINDING AFFINITY (%)* OF RP-HPLC PURIFIED MONOIODOINSULIN TRACERS RELATIVE TO THE CORRESPONDING REFERENCE TRACER (= 100%)

Methods: A = gel chromatography in 40% ethanol; B = extraction with cyclohexane; C = dilution with albumin-containing buffer.

Isolation procedure	*A14*	*A19*	*B16*	*B26*
A	97 ± 1	85 ± 17	93 ± 24	89 ± 3
B	93 ± 18	105 ± 5	107 ± 6	104 ± 10
C	112 ± 11	106 ± 5	104 ± 11	111 ± 14
Reference** tracer	100	100 (57)	100 (114)	100 (183)

* The binding affinity is mean ± S.D. of 2–6 independent experiments each consisting of four replicates.

** Purified by disc electrophoresis/ion-exchange chromatography. The values in parentheses are the binding affinities relative to A14 monoiodoinsulin.

Biological activity of monoiodoinsulins with low specific activity

The biological activity was calculated relatively based on the assumption that the specific activity of the four monoiodoinsulins is identical when prepared from the same iodination, *i.e.*, the amount of radioactivity is a measurement of the amount of monoiodoinsulin and is shown in Table VII. The absolute biological activity was based on the determination of the insulin/monoiodoinsulin concentrations primarily using amino acid analysis, since these cannot be determined based on weighing (less than 1 mg yields) or spectrophotometry (the molar extinction coefficients are not known). The concentrations found by amino acid analysis of the insulin isolated after RP-HPLC were in agreement with those estimated by spectrophotometry at 277 nm.

The absolute biological activities are shown in Table VIII.

TABLE VII

RELATIVE BIOLOGICAL ACTIVITY OF MONOIODOINSULINS ISOLATED AFTER PREPARATIVE RP-HPLC USING TEAP–ACETONITRILE AS ELUENT

Iodine substitution	*Per cent biological activity* (mean ± S.D.)*
Tyr A19	51 ± 1
Tyr B26	164 ± 9
Tyr B16	104 ± 0
Tyr A14	100

* Determined per 10 000 cpm, calculated relative to A14 monoiodoinsulin (= 100%). The mean biological activity was determined from two different stock solutions, as described in Materials and Methods. The reproducibility varied from 3 to 13% within the same stock solution.

TABLE VIII

ABSOLUTE BIOLOGICAL ACTIVITY OF INSULIN AND MONOIODOINSULINS ISOLATED AFTER PREPARATIVE RP-HPLC AS IN TABLE VI

The absolute biological activity is calculated relative to an insulin standard prepared by low-pressure methods. The biological determinations were performed as described in Table VII.

Insulin/monoiodoinsulin	*Per cent biological activity (mean ± S.D.)*
A19*	61 ± 7
Insulin*	113 ± 4
A19**	49 ± 4
Insulin**	91 ± 4
B26**	157 ± 5
B16**	127 ± 0
A14**	95 ± 1

* The preparative RP-HPLC was performed with TEAF, pH 6.0–isopropanol.
** The preparative RP-HPLC was performed with TEAP, pH 4.0–acetonitrile.

DISCUSSION

Since it is well established that monoiodoinsulins substituted in different tyrosine groups behave differently in biological systems, the need for homogeneous well characterized monoiodoinsulin tracers is evident. The A14 monoiodoinsulin is widely accepted as the ideal tracer for insulin whereas the A19, B16 and B26 monoiodoinsulins are substituted in tyrosyl residues considered to be part of the putative binding site[30,31] and thus valuable in various biological examinations.

Insulin was iodinated in 6 *M* urea buffer by the lactoperoxidase method to increase the iodine substitution in the B-chain tyrosine groups to about 40% of the iodine in the B-chain. Without urea, the iodine substitution in the B-chain was about 10%[13]. These results are representative for carrier-free iodination with ^{125}I to an iodination degree of 0.14 I/mol of insulin.

Iodination of 150 mg insulin with $^{127}I^-$ containing trace amounts of $^{125}I^-$ resulted in a lower iodine incorporation in the B-chain (about 25% calculated from Fig. 2). This is probably a consequence of the higher insulin concentration during the preparative iodination. An increase in the average degree of iodination did not result in a higher incorporation in the B-chain and at the same time the iodine incorporation was less reproducible (data not shown).

Since diiodoinsulins may have different binding affinities and decay to iodide and polymers with high non-specific binding[27], the amount of diiodosubstitution must be minimized. With iodination degrees lower than 0.2 I/mol of insulin, the diiodoinsulin content was maximally 5% on a molar basis[13].

We have recently described a one-step RP-HPLC fractionation of insulin, all four monoiodoinsulin isomers and five different types of diiodoinsulins from diluted iodination mixtures[12]. A LiChrosorb RP-18, 5-μm column is eluted isocratically with 0.25 *M* TEAF, pH 6.0 containing 21.5% isopropanol and the separation between A19 monoiodoinsulin and insulin is superior to that with other RP-HPLC systems[8]. Several silica-C_{18} columns could be used, but batch-to-batch variations in individual

columns might be a problem[32]. In contrast to other reported RP-HPLC systems[8], A19 monoiodoinsulin is obtained free from unlabelled insulin in a single step.

When this system was applied to a 250 mm × 25 mm LiChrosorb RP-18 (7-μm) column the separation was not satisfactory, perhaps due to the greater particle size and a lower linear flow-rate. The linear flow-rate was not increased in accordance with the analytical system due to practical limitations (the elution was performed at 5 instead of 20 ml/min).

Fig. 2 shows that the preparative column performs very well using the TEAP, pH 4.0–acetonitrile system, yielding all four monoiodoinsulins plus insulin in high purity (Table V).

The use of acetonitrile instead of isopropanol as organic modifier resulted in sharper peaks and the individual batch properties tend to disappear. The critical separation between A19 monoiodoinsulin and insulin was even better in the preparative column than in previous separations using analytical columns[11].

Separations on analytical columns in TEAP–acetonitrile, pH 4.0 resulted in an A19 monoiodoinsulin tracer contaminated with insulin, as determined by comparison of the biological activity of the four monoiodoinsulins (data not shown). A reason for the better results on the preparative column could be the four-fold decrease in flow-rate.

Lyophilization of an RP-HPLC column eluate containing the four monoiodoinsulins always resulted in reduced binding affinity[12]. The biological activity in isolated adipocytes was also reduced when applying insulin on the analytical column in amounts increasing from 0.5 to 10 mg (data not shown). The addition of the lyophilization residue from an RP-HPLC column eluate to monoiodoinsulin reference tracers (disc electrophoresis/ion-exchange chromatography) resulted in a decrease in binding affinity, which provides supports to the assumption that column bleeding (chemical degradation of the column support) could be the responsible factors[12]. Other evidence is provided by the direct precipitations in the eluate from a preparative C_{18} cartridge[12].

The quantitative analyses of the products of bleeding obtained by elution of several LiChrosorb RP-18 stationary phases are given in Tables I and II. In previous RP-HPLC separations of diluted iodination mixtures, the amounts of monoiodoinsulins were about 1 ng of each isomer in 2–3 ml of column eluate. From the tables, the amount of silicon is several times higher than the amount of monoiodoinsulin.

If TEAF–isopropanol extracts of LiChrosorb RP-18 stationary phases were hydrolyzed under alkaline conditions, octadecane and octadecanol could be demonstrated using gas chromatography[29].

The variation with respect to different batches of LiChrosorb (Table I) and organic modifiers (Table II) may explain the observed fluctuations in binding affinity found previously[11,12]. Increasing the amount of monoiodoinsulins fractionated (about 2.5 μg in Fig. 1) did not *per se* preserve the binding affinity of the isolated monoiodoinsulins.

The addition of serum albumin to RP-HPLC column eluates containing minute amounts of the monoiodoinsulin isomers has been reported[5–8], but the resulting binding affinity has never been compared to that of the corresponding tracers prepared by low-pressure methods. Addition of serum albumin to the column eluate immediately after the HPLC separation was examined. The tracers were stored frozen

until the measurement of binding affinity (Table VI, C), which showed that the binding activity was retained compared to the reference tracers. The added albumin probably binds to Si-C_{18} derivatives in the eluate (tracers stored frozen without albumin showed decreased binding affinity). The use of isolation procedures under conditions with minimum hydrophobic interaction between the polypeptide and the Si-C_{18} derivatives, such as gel chromatography in ethanol-containing buffer or removal of the organic modifier by extraction with cyclohexane, resulted in retained binding affinity as shown in Table VI, A and B.

The relative biological activity of isolated milligram amounts of monoiodoinsulin with low specific activity (Table VII) showed consistency with the binding affinities of the corresponding mono[^{125}I]iodoinsulin tracers (Table VI, values in parentheses).

The absolute biological activity of insulin, A14 and A19 monoiodoinsulin (Table VIII) was in agreement with our previously determined activities for the corresponding isomers prepared by low-pressure methods[3]. The observation that A14 monoiodoinsulin and insulin have the same biological activity in isolated adipocytes and A19 monoiodoinsulin has about half this activity is widely accepted[1–3,6–13,23], although Jørgensen *et al.*[4] found evidence for about 20% higher potency of A14 monoiodoinsulin relative to insulin. The values for the four isomers agreed well with the relative biological activities and the binding affinities of corresponding monoiodoinsulin tracers prepared by disc electrophoresis/ion-exchange chromatography (shown in parentheses in Table VI). There was a minor discrepancy in the biological activity of B26 monoiodoinsulin determined relatively and absolutely, but the conclusion that the monoiodoinsulins purified by RP-HPLC retain full biological activity is still valid.

The increasing number of reports describing the reduction of the biological activity of peptides and proteins (often enzymes) after HPLC purification demonstrates the need to consider the potential risks. The reasons for the reduced biological activity are frequently the irreversible denaturating effect of the organic modifiers used[20,21] or a direct instability in acidic and/or organic solvents[23,32,33]. In some cases the biological activity can be restored[34]. Böhlen *et al.*[22] reported that the loss of biological activity in the isolation of bovine pituitary fibroblast growth factor can be prevented by using highly polar organic solvents such as ethylene glycol or glycerol. Sharifi *et al.*[19] found that a residue from the acetonitrile used gave 20% inhibition of 3T3 cell protein synthesis, whereas residues from isopropanol and ethanol did not inhibit the synthesis. Vacuum concentration of *n*-propanol-containing fractions from an RP-HPLC fractionation of human fibroblast interferon (Hu IFN) leads to 99% loss of IFN activity[20]. The activity was retained when the HPLC fractions were stored at −20°C or when the fractions were first extracted with cyclohexane to remove *n*-propanol and then vacuum concentrated, in accordance with the present results.

Until now it had not been demonstrated that column bleeding can be responsible for the decrease in biological activity. The effect on the biological activity of other proteins is often ascribed to the denaturation effect of the organic modifiers in the eluent, but since it is known that the insulin molecule is quite stable during, *e.g.*, acid–ethanol extraction from the pancreatic glands, this is unlikely for insulin and monoiodoinsulin.

The detection of silicon and C_{18} molecules in the RP-HPLC column eluate in various amounts depending on the column support and buffer system explains the fluctuating reduction of binding affinity and biological activity of insulin and mono-iodoinsulins.

Using isolation methods with minimum hydrophobic interaction between insulin and silica-C_{18} degradation products, the biological properties were retained. We cannot exclude that other types of RP-HPLC column available now or in the future will have the same tendency for bleeding, but it should be emphasized that the bioactivity of any compound purified by HPLC should always be compared to that of the same compound purified to a similar degree using more "physiological" methods.

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REFERENCES

1 S. Linde, O. Sonne, B. Hansen and J. Gliemann, *Hoppe-Seyler's Z. Physiol. Chem.*, 362 (1981) 573–579.
2 O. Sonne, S. Linde, T. R. Larsen and J. Gliemann, *Hoppe-Seyler's Z. Physiol. Chem.*, 364 (1983) 101–110.
3 J. Gliemann, O. Sonne, S. Linde and B. Hansen, *Biochem. Biophys. Res. Commun.*, 87 (1979) 1183–1190.
4 K. H. Jørgensen, A. J. Moody and M. C. Christensen, *11th Congress of The International Diabetes Federation, Nairobi, 1982.*
5 F. B. Stentz, R. K. Wright and A. E. Kitabchi, *Diabetes*, 31 (1982) 1128–1131.
6 B. H. Frank, M. J. Beckage and K. A. Wiley, *J. Chromatogr.*, 266 (1983) 239–248.
7 D. A. Podlecki, B. H. Frank, M. Kao, H. Horikoshi, G. Freidenberg, S. Marshall, T. Ciaraldi and J. M. Olefsky, *Diabetes*, 32 (1983) 697–704.
8 B. H. Frank, D. E. Peavy, C. S. Hooker and W. C. Duckworth, *Diabetes*, 32 (1983) 705–711.
9 B. S. Welinder, S. Linde and J. S. Brush, *J. Chromatogr.*, 257 (1983) 162–165.
10 B. S. Welinder, S. Linde and B. Hansen, *J. Chromatogr.*, 265 (1983) 301–309.
11 B. S. Welinder, S. Linde, B. Hansen and O. Sonne, *J. Chromatogr.*, 281 (1983) 167–177.
12 B. S. Welinder, S. Linde, B. Hansen and O. Sonne, *J. Chromatogr.*, 298 (1984) 41–57.
13 S. Linde, B. Hansen, O. Sonne, J. J. Holst and J. Gliemann, *Diabetes*, 30 (1981) 1–8.
14 L. Benzi, P. Marchetti, P. Cecchetti, V. Pezzino, D. Gullo, A. Masoni and R. Navalesi, *J. Nucl. Med. Allied Sci.*, 28 (1984) 277–281.
15 M. N. Lioubin, M. D. Meier and B. H. Ginsberg, *Prep. Biochem.*, 14 (1984) 303–311.
16 J. Rivier, C. Rivier, D. Branton, R. Miller, J. Spiess and W. Vale, in D. H. Rich and E. Gross (Editors), *Peptides: Synthesis, Structure, Function*, Pierce, Rockford, IL, 1982, pp. 771–776.
17 A. F. Bristow, C. Wilson and N. Sutcliffe, *J. Chromatogr.*, 270 (1983) 285–292.
18 J. W. Wilks and S. S. Butler, *J. Chromatogr.*, 298 (1984) 123–130.
19 B. G. Sharifi, C. C. Bascom, V. K. Khurana and T. C. Johnson, *J. Chromatogr.*, 324 (1985) 173–180.
20 H. Smith-Johannsen and Y. H. Tan, *J. Interferon Res.*, 3 (1983) 473–477.
21 S. A. Cohen, K. P. Benedek, S. Dong, Y. Tapuhi and B. L. Karger, *Anal. Chem.*, 56 (1984) 217–221.
22 P. Böhlen, A. Baird, F. Esch, N. Ling and D. Gospodarowicz, *Proc. Natl. Acad. Sci. U.S.A.*, 81 (1984) 5364–5368.
23 D. E. Peavy, J. D. Abram, B. H. Frank and W. C. Duckworth, *Endocrinology*, 114 (1984) 1818–1824.
24 H. Tojo, T. Teramoto, T. Yamano and M. Okamoto, *Anal. Biochem.*, 137 (1984) 533–537.
25 J. H. O'Keefe, L. F. Sharry and A. J. Jones, *J. Chromatogr.*, 336 (1984) 73–85.
26 B. S. Welinder, S. Linde and B. Hansen, in J. Larner and S. L. Pohl (Editors), *Methods in Diabetes Research*, Vol. 1, Wiley, New York, Toronto, 1984, Part B, pp. 341–354.

27 B. P. Maceda, S. Linde, O. Sonne and J. Gliemann, *Diabetes*, 31 (1982) 634–640.
28 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265–275.
29 B. S. Welinder, K. R. Hejnæs and B. Hansen, in B. Hancock (Editor), *High Performance Liquid Chromatography in Biotechnology*, Wiley, New York, Toronto, in press.
30 R. A. Pullen, D. G. Lindsay, S. P. Wood, I. J. Tickle, T. L. Blundell, A. Wollmer, G. Kiail, D. Brandenburg, H. Zahn, J. Gliemann and S. Gammeltoft, *Nature (London)*, 259 (1976) 369–373.
31 T. L. Blundell, G. G. Dodson, D. C. Hodgkin and D. Mercola, *Adv. Protein Chem.*, 26 (1972) 279–402.
32 B. S. Welinder, S. Linde and B. Hansen, *J. Chromatogr.*, 348 (1985) 347–361.
33 E. M. Kniep, B. Kniep, W. Grote, H. S. Conradt, D. A. Monner and P. F. Mühlradt, *Eur. J. Biochem.*, 143 (1984) 199–203.
34 J. Luiken, R. Van der Zee and G. W. Welling, *J. Chromatogr.*, 284 (1984) 482–486.

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METHOD FOR COMPLETE SEPARATION OF THE HIGH MOBILITY GROUP (HMG) PROTEINS HMG I AND HMG Y FROM HMG 14 AND HMG 17 AND A PROCEDURE FOR PURIFICATION OF HMG I AND HMG Y

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SUMMARY

A purification procedure which separates the four low-molecular-weight high mobility group (HMG) proteins, HMG 14, 17, I and Y, is described. The procedure includes chromatography on phosphocellulose and Blue Sepharose combined with reversed-phase high-performance liquid chromatography. The blue Sepharose column separates HMG I and Y completely from HMG 14 and 17, and should therefore be an useful tool for the identification of these proteins which in several reports have been confused with HMG 14 and 17. HMG I and Y on the one hand and HMG 14 and 17 on the other exhibited considerable differences in their affinities for Blue Sepharose, probably reflecting fundamental differences in biological function.

INTRODUCTION

The high mobility group (HMG) non-histone chromosomal proteins are a group of relatively abundant chromatin-associated proteins present in most eucaryotic cells[1]. They are characterized by their solubility in 5% perchloric acid (PCA) and 2% trichloroacetic acid (TCA) and their high content of basic and acidic amino acid residues, as well as an high content of proline. This unusual amino acid composition is the main reason for the lack of secondary structure in parts of the molecules[1].

The HMG proteins can be divided into two subgroups: the high-molecular-weight proteins consisting of HMG 1 and 2 with molecular weight (MW) of approximately 28 000, and the low-molecular-weight group consisting of HMG 14 and 17 with MW of about 10 000[2]. In addition to these well characterized HMG proteins, two novel HMG proteins, of the low-molecular-weight type have recently been reported by this laboratory[3–5]. These two proteins, designated HMG I and Y, seem to be characteristic of, or at least are expressed at elevated levels, in proliferating cells[3–6]. They are both phosphorylated in interphase and superphosphorylated in metaphase and exhibit a high affinity for A-T rich DNA sequences[7,8]. The two novel small HMG proteins are of interest due to their apparent correlation with cell proliferation[6] and possible involvement in the mitotic process[3–5].

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When analysing the group of small HMG proteins, one has to consider four polypeptide chains with very similar molecular weights and amino acid compositions, resulting in similar migration velocities on both sodium dodecyl sulphate (SDS) and acetic acid–urea gels and a concomitant risk of misinterpretation of the gel patterns. For instance, in several reports on phosphorylation of HMG proteins *in vivo*, HMG I and Y have been mistaken for phosphorylated HMG 14 and 17[9–11] We therefore considered it important to develop a simple method for separating the closely related HMG I and Y from HMG 14 and 17. This paper describes how this separation can be achieved by chromatography on Blue Sepharose, and in addition the purification to apparent homogeneity of all four small HMG proteins by including chromatography on phosphocellulose and reversed-phase high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Propagation of cells

HeLa S3-cells were propagated in suspension culture containing Eagles minimum essential medium with 10% foetal calf serum, 4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid (HEPES) buffer (pH 7.3) and non-essential amino acids.

Extraction of HMG proteins

The cells (usually 10^9) were extracted twice with 5% PCA, and the acid-soluble proteins were precipitated with TCA (final concn. 20%). TCA-precipitated proteins were washed with acetone–conc. hydrochloric acid (400:1), followed by acetone–0.1 *M* hydrochloric acid (6:1) and finally three times with acetone. The protein pellet was suspended in 5% PCA, 1 volume of acetone was added and the precipitated material was removed by centrifugation. To the supernatant were added 6 volumes of acetone–0.07 *M* hydrochloric acid, and the resulting precipitate was washed with acetone–0.1 *M* hydrochloric acid (6:1) and three times with acetone. This protein fraction, which contains the PCA-soluble HMG proteins, was used for further purifications.

Purification by phosphocellulose chromatography

PCA-soluble proteins were dissolved in 10 ml of 0.4 *M* sodium chloride, 10 m*M* Tris–HCl (pH 8.0) and applied to a Whatman P 11 phosphocellulose column (7 cm × 1.9 cm) equilibrated with the same buffer. Chromatography was carried out with a linear sodium chloride gradient as indicated in the legends, at a flow-rate of 1.3 ml/min. The eluate was monitored with a zinc lamp (Pharmacia) having a 214-nm filter and collected in fractions of 4 ml.

Purification by Blue Sepharose chromatography

The selected fractions from the phosphocellulose column were diluted to 0.2 *M* sodium chloride in 10 m*M* Tris–HCl (pH 8.0) and aplied to a Blue Sepharose CL-6B (Pharmacia) column (5 cm × 1.5 cm), equilibrated with the same buffer. The proteins were eluted with a sodium chloride gradient as indicated in the legends, at a flow-rate of 1.3 ml/min. The eluate was monitored at 214 nm, collected in fractions of 4 ml and the proteins were subsequently precipitated with TCA (final concn. 20%).

Purification by reversed-phase HPLC

Crude PCA-soluble proteins or partially purified HMG proteins were dissolved in 0.01% trifluoroacetic acid (TFA), and applied to an Ultrapore RPSC *n*-propyldimethyl silane column (Beckman) (75 mm × 4.6 mm for analytical use and 250 mm × 10 mm for preparative purposes), which was equilibrated with 0.01% TFA. The elution was a modification of that described by Anzano *et al.*[12], using a linear acetonitrile gradient in 0.01% TFA, with 0% acetonitrile at time 0 and 35% at 50 min, at flow-rates of 1 ml/min for the small column and 4 ml/min for the large column. The eluate was monitored with a Kontron Uvikon 725 spectrophotometer at 220 nm, the eluted protein was collected manually, and pooled fractions were dried under vacuum.

Polyacrylamide gel electrophoresis (PAGE)

Proteins were analysed by electrophoresis in polyacrylamide slab gels (13 cm × 13 cm × 0.1 cm), with a resolving gel (15% acrylamide) as described by Panyim and Chalkley[13], and a stacking gel as described by Spiker[14].

RESULTS

The aim of the present work was to establish a simple and safe procedure for the separation and identification of four related HMG proteins, HMG 14, 17, I and Y. The purified proteins were analysed by acetic acid–urea PAGE, which gives a better separation of these proteins than SDS-PAGE.

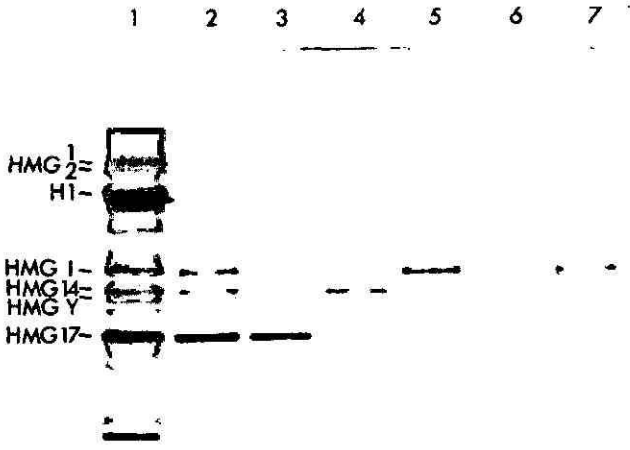

Fig. 1. Acetic acid–urea PAGE of HMG proteins at different levels of purification. Lanes: 1 = total PCA-extracted proteins from HeLa S3-cells; 2 = fractions 23–35 from phosphocellulose chromatography; 3 = fractions 14–17 from Blue Sepharose chromatography; 4 = fractions 21–24 from Blue Sepharose chromatography; 5 = fractions 34–36 from Blue Sepharose chromatography; 6 = HMG Y purified by HPLC, 7 = HMG I purified by HPLC.

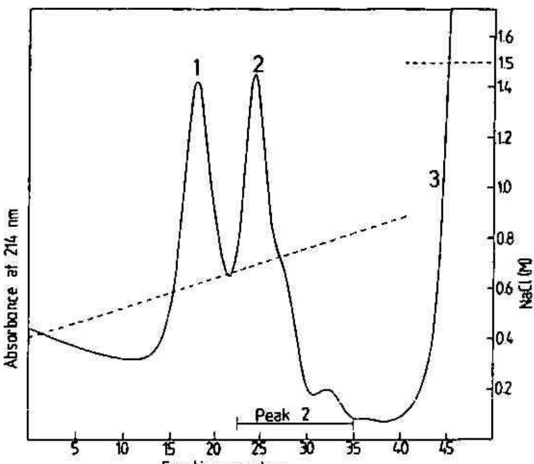

Fig. 2. Chromatography on phosphocellulose of PCA-soluble proteins from HeLa S3-cells. The chromatography was carried out with a sodium chloride gradient. ———, Absorbance at 214 nm; ---------, sodium chloride concentration.

HeLa S3-cells, which contain relatively high levels of low-molecular-weight HMG proteins, were used as the source for the purifications. In the PCA extract from HeLa S3-cells the amounts of HMG I and 14 are approximately one third that of HMG 17, and the amount of HMG Y is about one ninth that of HMG 17, judged from scans of Coomassie Brilliant Blue-stained acetic acid–urea gels (Fig. 1, lane 1, scans not shown).

The low-molecular-weight HMG proteins were separated from most of the other PCA-soluble proteins, including histone H1, by chromatography on phosphocellulose with a linear gradient ranging from 0.4 to 0.9 *M* sodium chloride (Fig. 2). The proteins which remained bound were step-eluted with 1.5 *M* sodium chloride. Analysis of TCA-precipitated proteins by acetic acid–urea PAGE revealed that HMG 14, 17, I and Y were quantitatively eluted in peak 2 (Fig. 1, lane 2), while peak 1 contained HMG 1 and 2 and peak 3 mainly H1. Analysis of each fraction (4 ml) in peak 2 showed that HMG 14 was eluted first followed by HMG 17, I and Y, but none of the proteins was completely separated from the others.

The eluate corresponding to peak 2 was fractionated further by Blue Sepharose chromatography. The eluate was diluted to 0.20 *M* sodium chloride by adding 10 m*M* Tris–HCl (pH 8.0) and applied to the Blue Sepharose column which was equilibrated with the same buffer. The chromatography was carried out with a linear gradient from 0.20 to 0.70 *M* sodium chloride (which elutes HMG 14 and 17), and the remaining proteins step-eluted with 1.5 *M* sodium chloride to avoid the unfavourable dilution of HMG I and Y when these were gradient-eluted (Fig. 3). Acetic acid–urea PAGE of TCA-precipitated proteins revealed that fractions 14–17 contained HMG 17 (Fig. 1, lane 3), fractions 21–24 HMG 14 (Fig. 1, lane 4) and fractions 34–36 a mixture of HMG I and Y (Fig. 1, lane 5). Fractions 18–20 contained a mixture of HMG 14 and 17. These results so far show that HMG I and Y can be completely separated from HMG 14 and 17 by Blue Sepharose chromatography and,

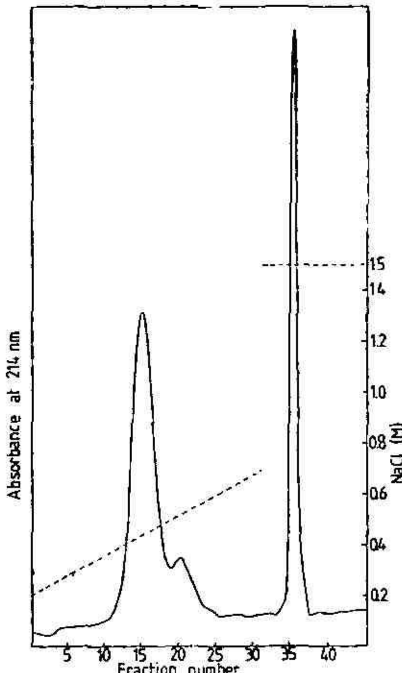

Fig. 3. Chromatography on Blue Sepharose of fractions 23–35 from phosphocellulose chromatography (Fig. 2). The proteins were eluted by increasing the sodium chloride concentration (---------). Absorbance at 214 nm (———).

in addition, pure HMG 17 and 70–90% pure HMG 14 can be obtained. Further purification of HMG I and Y was carried out by HPLC.

Fractionation of HMG proteins by HPLC was carried out with a *n*-propyl-dimethyl silane, trimethyl-encapped reversed-phase column, using a linear acetoni-

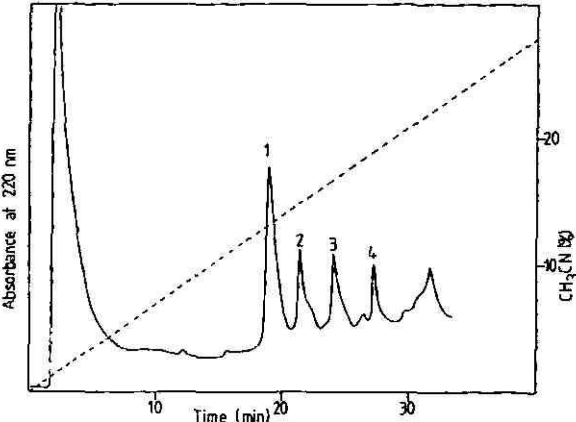

Fig. 4. Reversed-phase HPLC of PCA-soluble proteins from HeLa S3-cells with an Ultrapore RPSC column (75 mm × 4.6 mm). The flow-rate was 1 ml/min. ---------, Acetronitrile concentration; ———, absorbance at 220 nm.

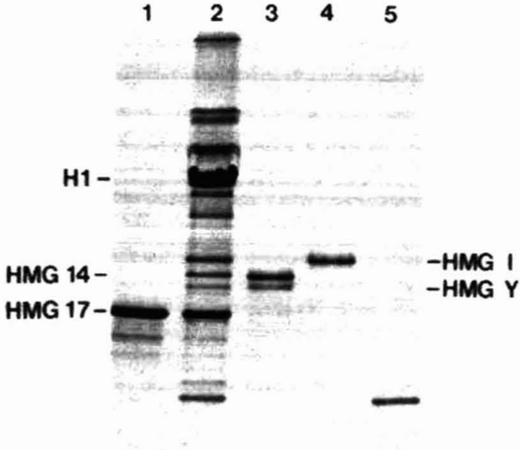

Fig. 5. Acetic acid–urea PAGE of fractions from HPLC (Fig. 4). Lanes: 1 = proteins from peak 1; 2 = PCA-soluble proteins which were applied to the column; 3 = proteins from peak 2; 4 = proteins from peak 3; 5 = proteins from peak 4.

trile gradient. The elution profile obtained with total PCA-soluble proteins from HeLa S3-cells is shown in Fig. 4 (only the first part of the profile is shown). The subsequent gel analysis of some of the peaks (Fig. 5) revealed that HMG I (peak 3, lane 4) was separated from HMG Y (peak 2, lane 3) and was not contaminated by any other proteins. Another protein (peak 4, lane 5) with higher mobility than HMG 17 also seems to be obtained pure from this column. HMG Y and 14 were eluted in the same peak (peak 2, lane 3), which excludes the use of this HPLC column for a single-step purification of these proteins. HMG 17 was slightly contaminated by some unidentified proteins (peak 1, lane 1).

HPLC was also used to fractionate the mixture of HMG I and Y obtained from chromatography on Blue Sepharose. Analysis by acetic acid–urea PAGE revealed that the resulting HMG Y and I preparations were essentially pure (Fig. 1, lanes 6 and 7). From 10^9 HeLa cells, approximately 40 μg HMG I and 10 μg HMG Y were obtained, which is a higher recovery than that obtained by preparative PAGE[3].

There is an alternative procedure for purification of HMG I without using HPLC. When fractions 24–30 from the phosphocellulose column (Fig. 2) were used instead of fractions 24–35, a preparation containing about 80% of the total amount of HMG I and no HMG Y (results not shown) was obtained. HMG I can then be purified to homogeneity by Blue Sepharose chromatography.

DISCUSSION

The low-molecular-weight HMG proteins are relatively abundant chromosomal proteins without enzymatic activity. Their identification has been based on

one- or two-dimensional gel-electrophoretic analysis. HMG 14, 17, I and Y exhibit just minor differences in electrophoretic mobilities, and the interpretation of the gel pattern is further complicated by the fact that there exist species differences in the electrophoretic mobilities, especially with the poorly conserved HMG 14[3]. This means that HMG proteins from different species, exhibiting the same electrophoretic mobility, do not necessarily represent the same or homologous proteins. There has been much confusion concerning the identification of HMG proteins separated by PAGE [9–11]. Accordingly, there is a need for analytical and preparative methods exploiting more subtle properties than molecular mass and charge upon which gel electrophoretic mobilities depend. More subtle differences have already been revealed since these proteins have different affinities for poly(dA · dT) sequences (see Introduction), while the affinity for total DNA is approximately the same.

Blue Sepharose, which contains the dye Cibacron Blue and seems to function as a dinucleotide analogue, has been successfully used for the purification of a variety of chromatin proteins, among them poly(ADP-ribose) polymerase[15–17]. We find that fractionation of the low-molecular-weight HMG proteins on Blue Sepharose completely separates HMG I and Y from HMG 14 and 17 and is a useful step in their identification. Chromatography on Blue Sepharose and subsequent electrophoresis on acetic acid–urea gels enables confirmation of the identity of each individual HMG protein. The considerable difference in the affinity for Blue Sepharose probably reflects fundamental differences in biological function between HMG I and Y on the one hand and HMG 14 and 17 on the other.

In the present work HMG I and Y have been purified by chromatography to homogeneity, as judged by acetic acid-PAGE. The purity is comparable to that obtained by using preparative gel electrophoresis. The advantage of the described chromatographic procedure compared to preparative acetic acid–urea PAGE is the higher recovery; it is also less technically demanding.

HMG I has also been purified in one step by HPLC of PCA-extracted proteins in this work, and previously by Goodwin *et al.*[6] by use of a similar column. In the present work the HMG I isolated seems to be devoid of an additional protein with slightly higher mobility than HMG I, which was present in the preparation of Goodwin *et al.*[6]. However, we found that the high resolution was gradually lost when the HPLC column was repeatedly used to fractionate crude PCA-extracted proteins. If there is a need for highly purified HMG I, preceding purification steps such as chromatography on phosphocellulose and Blue Sepharose should therefore be used in addition to HPLC.

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REFERENCES

1 E. W. Johns, in E. W. Johns (Editor), *The HMG Chromosomal Non-histone Proteins*, Academic Press, New York, 1982, pp. 1–9.
2 R. H. Nicolas and G. H. Goodwin, in E. W. Johns (Editor), *The HMG Chromosomal Non-histone Proteins*, Academic Press, New York, 1982, pp. 41–68.
3 T. Lund, J. Holtlund, M. Fredriksen and S. G. Laland, *FEBS Lett.*, 152 (1983) 163.

4 T. Lund, J. Holtlund and S. G. Laland, *FEBS Lett.*, 180 (1985) 275.
5 S. G. Laland, T. Lund and J. Holtlund, in G. R. Reeck, G. A. Goodwin and P. Puigdomenech (Editors), *Chromosomal Proteins and Gene Expression*, Plenum, New York, 1986, pp. 239–247.
6 G. H. Goodwin, P. N. Cockerill, S. Kellam and C. A. Wright, *Eur. J. Biochem.*, 149 (1985) 47.
7 F. Strauss and A. Varshavsky, *Cell*, 37 (1984) 889.
8 M. J. Solomon and A. Varshavsky, *Proc. Natl. Acad. Sci. U.S.A.*, 82 (1985) 6470.
9 J. S. Bhorjee, *Proc. Natl. Acad. Sci. U.S.A.*, 78 (1981) 6944.
10 B. Levy-Wilson, *Proc. Natl. Acad. Sci., U.S.A.*, 78 (1981) 2189.
11 J. D. Saffer and R. I. Glazer, *J. Biol. Chem.*, 257 (1982) 4655.
12 M. A. Anzano, A. B. Roberts, J. M. Smith, L. C. Lamb and M. B. Sporn, *Anal. Biochem.*, 125 (1982) 217.
13 S. Panyim and R. Chalkley, *Arch. Biochem. Biophys.*, 130 (1969) 337.
14 S. Spiker, *Anal. Biochem.*, 108 (1980) 263.
15 S. T. Thompson, K. H. Cass and E. Stellwagen, *Proc. Natl. Acad. Sci. U.S.A.*, 72 (1975) 669.
16 J. E. Wilson, *Biochem. Biophys. Res. Commun.*, 72 (1976) 816.
17 T. Kristensen and J. Holtlund, *J. Chromatogr.*, 192 (1980) 494.

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SEPARATION AND HEAT STABILITY OF THE CORTICOSTEROID-INDUCED AND HEPATIC ALKALINE PHOSPHATASE ISOENZYMES IN CANINE PLASMA

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SUMMARY

A convenient method has been developed for the separation of alkaline phosphates (AP) isoenzymes from canine plasma. The various forms of AP activity were extracted by ethanol and separated on an anion exchanger by fast protein liquid chromatography. In this way a complete discrimination was achieved between the increase in plasma AP activity due to liver disease and that due to corticosteroid induction. The corticosteroid-induced form of AP could be separated from the other isoenzymes because of its relative heat stability at 65°C. A quantitation of the contribution of liver and corticosteroid-induced AP isoenzymes to the total plasma AP activity could be made from the respective heat inactivation plots. The separation of the isoenzymes may be valuable in the purification of the different isoenzymes for further characterization.

INTRODUCTION

Alkaline phosphatase (AP) (E.C. 3.1.3.1; orthophosphoric monoester phosphohydrolase) consists of a group of isoenzymes which catalyse the hydrolysis of monophosphate esters at alkaline pH. The heterogeneity of these isoenzymes may be partly due to the expression of different gene loci and partly to post-translational modifications[1]. In man several isoenzymes contribute to the total amount of plasma AP, some of which (liver, intestinal and bone AP) can be found in the plasma of normal healthy individuals, and may have elevated levels in diseased ones. Other isoenzymes are present in plasma only during pregnancy (placental AP) or disease (kidney AP) and sometimes in association with cancer, *e.g.*, Regan isoenzyme[1–5].

In the dog separate AP isoenzymes have also been isolated from the kidney, placenta, intestinal mucosa, liver and bone[6–7]. However, renal, placental and intestinal isoenzymes have never been demonstrated in canine plasma, neither in healthy nor in diseased dogs. This may be due to their very short half-lives (3–6 min in plasma)[8,9]. Besides the normal liver and bone isoenzymes, an additional hepatic isoenzyme has been found in canine plasma following corticosteroid excess[10–12]. This isoenzyme has not yet been found in man or in any other species. There is a marked difference in

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heat stability at 65°C between liver AP and corticosteroid-induced AP isolated from liver tissue[13].

Due to the close similarities between the AP isoenzymes, a quantitative analysis of the contribution of the various forms to the total plasma activity is difficult. Several methods have been employed to distinguish between different AP isoenzymes, including selective heat inactivation, inhibition with L-phenylalanine or L-homoarginine, separation by means of electrophoresis, isoelectric focusing and immunochemical techniques[7,9,12,14–17]. We describe here the results of heat inactivation and of separation by fast protein liquid chromatography (FPLC) of canine plasma AP isoenzymes caused by liver disease and hyperadrenocorticism.

EXPERIMENTAL

Animals

Studies were performed on ten clinically healthy mature Beagles and 26 canine patients (in the Small Animal Clinic of Utrecht State University) which had elevated plasma AP levels. In 14 of the patients, hyperadrenocorticism (Cushing's syndrome) was diagnosed by use of the dexamethasone screening test[18] and in the remaining 12 dogs the presence of liver disease was confirmed by biopsy, and they had no history of corticosteroid treatment or signs of Cushing's syndrome.

Samples

Blood samples were collected by jugular venapuncture in tubes containing lithium–heparine. The plasma was separated by centrifugation at 2800 *g* and stored at −20°C until used for further analysis. Bone AP isoenzyme was isolated from the cystic fluid of a dog with histologically diagnosed osteosarcoma. The canine intestinal AP isoenzyme was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Enzyme measurement

Alkaline phosphatase activity was measured on a Multistat III-FLS centrifugal analyser (Allied, Instrumentation Laboratory, Lexington, MA, U.S.A.) at 30°C, using *p*-nitrophenyl phosphate as substrate (Boehringer Mannheim Diagnostica, Mannheim, F.R.G.). The method used was an "optimized standard method" conforming to the recommendations of the German Society of Clinical Chemistry[19].

Heat inactivation

Plasma samples were incubated at 65°C in a Lauda MT-LCE 004 thermostatic water-bath (Messgeräte-Werk Lauda, Lauda-Königshofen, F.R.G.), which had a guaranteed temperature control of ± 0.1°C. Samples of 3 ml at a temperature of 20°C were placed in thin-walled glass tubes (55 mm × 9 mm, wall thickness 0.5 mm) in the water-bath and at intervals of 0, 0.5, 1, 1.5, 2, 2.5 and 4 min, aliquots of 0.25 ml were transferred to identical glass tubes chilled in ice. The activity remaining after each interval of heat inactivation was expressed as a percentage of the activity in the unheated samples.

Extraction and ion-exchange chromatography

Canine plasma AP was isolated using ethanol extraction according to Dorner

et al.[10]: briefly, 1 ml plasma was diluted in an equal volume of 0.9% sodium chloride and placed in an ice-bath. With continuous stirring, 1 ml of 96% ethanol, cooled to −20°C, was added to give a final ethanol concentration of 32% (v/v). After centrifugation of the mixture for 10 min at 6000 *g*, an equal volume of 96% ethanol was added to the supernatant with continuous stirring. The pellet, collected after centrifugation for 10 min at 6000 *g*, was dissolved in 1 ml of 10 m*M* sodium acetate buffer (pH 5) containing 0.5 m*M* magnesium chloride and dialyzed overnight against the same buffer. After dialysis at 4°C the precipitated protein was removed by centrifugation for 2 min in an Eppendorf microcentrifuge (Eppendorf, Hamburg, F.R.G.).

AP isoenzymes were separated by an FPLC system consisting of a Mono Q anion-exchange column, two P-500 pumps, a GP-250 gradient programmer, an UV-1 single path monitor and an FRAC-100 fraction collector (Pharmacia Fine Chemicals, Uppsala, Sweden). The column was equilibrated with 10 m*M* sodium acetate–acetic acid buffer (pH 5.0) and 0.5 m*M* magnesium chloride. Samples of 0.1–0.5 ml were injected at a solvent flow-rate of 1 ml/min. Proteins were eluted with a linear gradient from 0 to 0.2 *M* sodium chloride in the equilibration buffer, followed by a wash with 1 *M* sodium chloride. The gradient was completed within 20 min. The absorbance was measured at 280 nm and fractions of 0.5 min (0.5 ml) were collected for subsequent estimation of the AP activity.

Statistics

The results were analyzed by the distribution-free method of Wilcoxon–Mann–Whitney[20]. Differences were considered to be significant at $p < 0.05$. The results are presented as the median and range.

RESULTS

Incubation of plasma from the control dogs at 65°C produced a rapid linear decline in the logarithm of AP activity within 2 min, after a short initial plateau (Fig. 1). This linearity indicated the inactivation reaction kinetics to be of pseudo-first order. Inactivation was complete after 2 min (median 0%, range 0–2.1%, $n = 10$). Half-inactivation times, $t_{1/2}$, were derived from the straight lines when plotted on semilog paper. The $t_{1/2}$ for control dogs was 17.3 s (14.0–19.0 s).

Heating for 2 min at a lower temperature was also investigated (Fig. 2). A decrease in temperature of less than 1°C had little effect on the results, but between 64 and 60°C a linear decrease in inactivation was observed.

The results of heat inactivation at 65°C of AP in plasma from dogs with hepatic disease ($n = 12$) were similar to those in plasma from control dogs (Fig. 3). The activities remaining after 2 min (median 2.0%, range 0–8.1%) and $t_{1/2}$ values (median 17.5 s, range 10–25 s) were not significantly different ($p > 0.05$) from those of control dogs. There was only slight heat inactivation of plasma AP from twelve dogs with Cushing's syndrome. Both the remaining activity of 83.1% (62.2–99.9%, $n = 12$) and the $t_{1/2}$ of 350.0 s (138–1080 s) were significantly higher than in the other groups ($p < 0.0001$).

For the plasma of two dogs with hyperadrenocorticism, the plot of the logarithm of the remaining AP activity against time could be resolved into two components (Fig. 3). The $t_{1/2}$ (mean for the two dogs) was 18.5 s for the first component and 262.5 s for the second component.

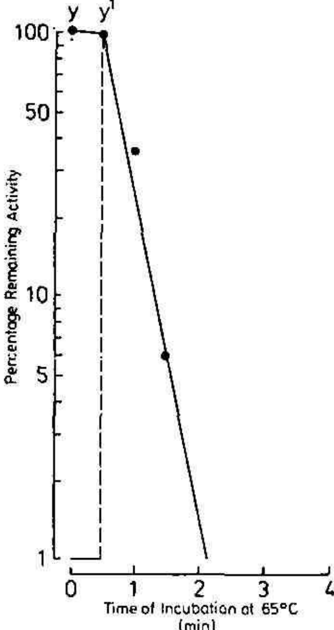

Fig. 1. Heat inactivation at 65°C expressed as the median of the individual activities of alkaline phosphatase in the plasma of ten control dogs. The horizontal part Y–Y[1] is due to the warming up of the glass tubes.

Optimum conditions for the separation of AP isoenzymes were determined on an anion-exchange column using plasma from dogs with either hyperadrenocorticism or liver disease. The efficiency of extraction of the total AP activity from the various plasmas with chilled ethanol was 75%, as determined after dialysis against sodium acetate buffer (pH 5) and separation of the precipitated protein by centrifugation. In a buffer of pH 8, normally used for separation by electrophoresis on agarose or

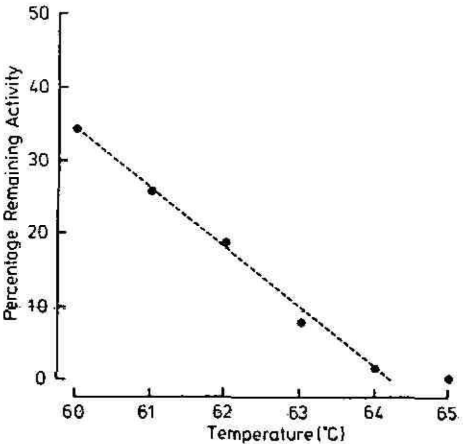

Fig. 2. Effect of different temperatures (2-min exposure) on the mean alkaline phosphatase activity in the plasma of four dogs.

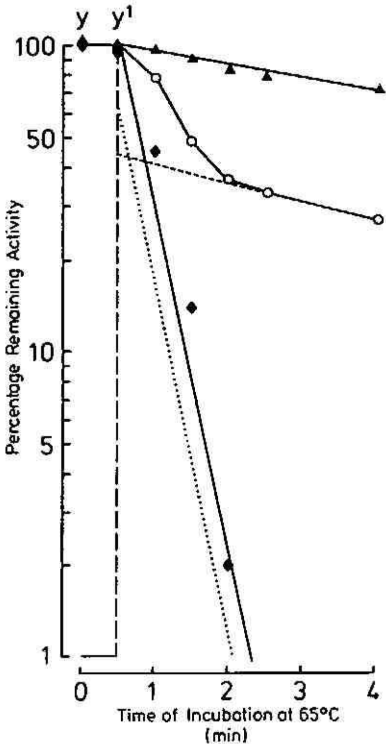

Fig. 3. median percentages of alkaline phosphatase activities in plasma after heating at 65°C for dogs with liver disease (◆—◆; n = 12) and those with hyperadrenocorticism (▲—▲; n = 12). In two other dogs with hyperadrenocorticism (O—O), the biexponential plot could be resolved into two straight lines (..... and -----).

cellulose acetate, only partial separation was obtained between the liver and corticosteroid-induced AP activity. However, at pH 5 a clear separation was obtained between the two isoenzymes (Fig. 4). A general pattern for the protein absorption at 280 nm was seen in all separations, indicating only small differences in the extraction of plasma proteins with ethanol. The AP activity in the fractions obtained after FPLC was determined before and after heating for 2 min at 65°C. About 60% of the activity added to the Mono Q column was recovered. After heat inactivation the AP of dogs with hyperadrenocorticism appeared to be stable to heat, while the dogs with liver disease only had a heat-labile AP, confirming the identity of the two isoenzymes.

The elution positions of intestinal and bone AP were also established (Fig. 5). Both isoenzymes appeared to be heat labile. Hence all four AP isoenzymes could be separated by anion-exchange chromatography, intestinal AP being eluted with the column volume, bone AP at 0.06 *M* sodium chloride, liver AP at 0.11 *M* sodium chloride, and corticosteroid-induced AP at 0.16 *M* sodium chloride. In the plasma samples investigated, no intestinal or bone AP isoenzyme was found. Occasionally the liver AP and corticosteroid-induced AP isoenzymes coexisted in the same plasma of dogs with Cushing's syndrome (Fig. 5), which was confirmed by differences in heat

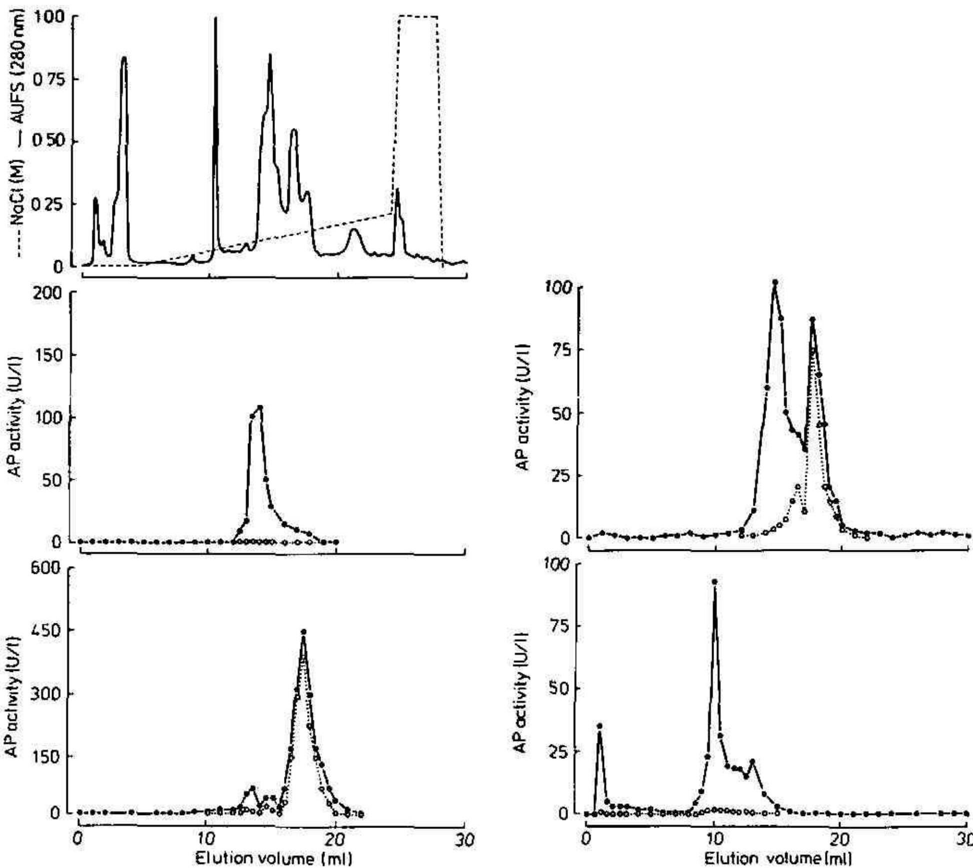

Fig. 4. Anion-exchange chromatography of canine plasma alkaline phosphatases extracted with ethanol on a Mono Q column. The column was equilibrated with 10 m*M* sodium acetate–acetic acid buffer (pH 5) and 0.5 *M* magnesium chloride. The proteins were then eluted with a linear gradient of 0–0.2 *M* sodium chloride in the same buffer at a flow-rate of 1 ml/min. The upper figure shows the general absorption pattern at 280 nm. The middle and bottom figures show the AP enzyme activity in the collected fractions before (●—●) and after (O- -O) heating for 2 min at 65°C of plasma from animals with liver disease and animals with Cushing's disease, respectively. The AP from the plasma of a dog with liver disease was eluted at 0.11 *M* sodium chloride, while that corticosteroid-induced AP was eluted at 0.16 *M* sodium chloride.

Fig. 5. Elution profiles of ethanol-extracted canine plasma AP on a Mono Q anion exchanger (●—●). The chromatographic conditions were as in Fig. 4. The upper figure shows the existence of two AP activity peaks in the plasma of an animal with Cushing's disease, at 0.11 and 0.16 *M* sodium chloride, respectively. After heating at 65°C (O—O) the second peak remained, which proved it to be due to corticosteroid-induced AP. In the bottom figure the elution position of bone AP is shown. Bone AP was eluted mainly at 0.06 *M* sodium chloride. Intestinal AP was eluted completely within the column volume.

stability. A small unidentified peak of AP activity was sometimes present between the liver AP and corticosteroid-induced AP peaks obtained by FPLC.

DISCUSSION

Heat inactivation as a means of separation of different AP isoenzymes is a simple method in comparison with electrophoresis, isoelectric focusing or immunochemical techniques. Because of its simplicity, it can easily be adapted to routine laboratory use. The results of this study demonstrate that AP in plasma from dogs with hyperadrenocorticism is more heat resistant than is that from normal dogs or those with hepatic disease. Heat inactivation at 65°C for 2 min allows the two isoenzymes to be distinguished. Because of individual variations in the half-inactivation times of corticosteroid-induced AP, a quantitative measurement of the different isoenzymes can be obtained only by construction of a multi-point inactivation plot. The possible presence of bone AP in canine plasma does not influence the determination of corticosteroid-induced AP, because the former has an even lower heat stability than liver AP[7,9,12].

Two dogs with hyperadrenocorticism had different denaturation plots than the other twelve dogs of this group. The plots could be resolved into two components with mean half-inactivation times of 18.5 and 262.5 s, indicating the existence of both liver AP and corticosteroid AP in the plasma. The non-linear part is due to the simultaneous inactivation of the two isoenzymes, the heat-labile liver AP being the cause of the rapid decline in the first part of the plot and the heat-stable corticosteroid AP representing the linear part. The contribution of both isoenzymes to the plasma AP could be calculated by extrapolation of each part of the plot.

The rate of heat inactivation of plasma AP isoenzymes appears to be higher than that of AP in liver extracts as reported by Wellman *et al.*[13]. This difference may be due to the effects of precipitation of plasma proteins that would enhance the denaturation of plasma AP[21].

The separation of hepatic and corticosteroid-induced AP isoenzymes by electrophoresis on cellulose acetate may result in overlapping bands, making this technique less useful for quantitative measurement of these AP isoenzymes. In the present study a new technique is described for separation of AP isoenzymes by FPLC. One isoenzyme is predominant in plasma of healthy dogs and dogs with liver disease. Corticosteroid induction leads to the presence of an isoenzyme more stable to heat which is eluted from the anion-exchange column at higher sodium chloride concentrations. Both the heat stability and the difference in elution of the corticosteroid-induced enzyme from the anion-exchange column may be caused by an increase in sialic acid residues as reported by Wellman *et al.* The isoelectric point of the corticosteroid-induced enzyme, p*I* 3.5, indicates an acidic isoenzyme in contrast with p*I* 4.5 for the liver isoenzyme[14]. This lower isoelectric point may be one of the reasons for the stronger retention of this isoenzyme by the Mono Q column at pH 5. The close similarity of liver and corticosteroid-induced isoenzymes after treatment with neuraminidase suggests that both isoenzymes are derived from a single gene and that differences are due to post-translational modification resulting in the addition of sialic acid residues. The small heat-resistant peak between the main liver and corticosteroid-induced peaks in FPLC may be a transient form between different stages of incorporation of sialic acid residues.

The separation of isoenzymes by FPLC, giving isolated isoenzyme fractions, may be valuable for further characterization of AP isoenzymes as well as for the

generation of isoenzyme-specific antiserum, which in turn may have diagnostic value in some diseases and in oncology.

REFERENCES

1 D. W. Moss, *Clin. Chem.*, 28 (1982) 2007–2016.
2 A. L. Latner and A. W. Skillen, *Isoenzymes in Biology and Medicine*, Academic Press, London, New York, 1968, p. 68.
3 W. H. Fishman, *Am. J. Med.*, 56 (1974) 617–645.
4 W. H. Fishman, N. R. Inglis, S. Green, C. L. Anstiss, N. K. Gosh, A. E. Reif, R. Rustigian, M. J. Krant and L. L. Stolbach, *Nature (London)*, 219 (1968) 697–699.
5 P. G. Steinherz, L. J. Steinherz, J. S. Nisselbaum and M. L. Murphy, *J. Am. Med. Assoc.*, 252 (1984) 3289–3292.
6 W. E. Moore and B. F. Feldman, *J. Am. Anim. Hosp. Assoc.*, 10 (1974) 420–429.
7 L. A. Nagode, A. Koestner and C. L. Steinmeyer, *Clin. Chim. Acta*, 26 (1969) 45–54.
8 W. E. Hoffmann and J. L. Dorner, *Am. J. Vet. Res.*, 38 (1977) 1553–1556.
9 P. K. Saini and S. K. Saini, *Am. J. Vet. Res.*, 39 (1978) 1510–1513.
10 J. L. Dorner, W. E. Hoffmann and G. Long, *Am. J. Vet. Res.*, 35 (1974) 1457–1458.
11 A. R. Dillon, J. S. Spano and R. D. Powers, *J. Am. Anim. Hosp. Assoc.*, 16 (1980) 831–837.
12 P. K. Saini, G. M. Peavy, D. E. Hauser and S. K. Saini, *Am. J. Vet. Res.*, 39 (1978) 1514–1518.
13 M. L. Wellman, W. E. Hoffmann, J. L. Dorner and R. E. Mock, *Am. J. Vet. Res.*, 43 (1982) 1204–1207.
14 P. D. Eckersall and A. S. Nash, *Res. Vet. Sci.*, 34 (1983) 310–314.
15 W. E. Hoffmann and J. L. Dorner, *J. Am. Anim. Hosp. Assoc.*, 11 (1975) 283–285.
16 M. L. Wellman, W. E. Hoffmann, J. L. Dorner and R. E. Mock, *Am. J. Vet. Res.*, 39 (1978) 1200–1203.
17 P. K. Saini and S. K. Saini, *Am. J. Vet. Res.*, 39 (1978) 1506–1509.
18 J. C. Meijer, J. J. de Bruijne, A. Rijnberk and R. J. M. Croughs, *J. Endocrinol.*, 77 (1978) 111–118.
19 Empfehlungen der Deutschen Gesellschaft für Klinische Chemie. Standardisierung von Methoden zur Bestimmung von Enzymaktivitäten in biologischen Flüssigkeiten, *Z. Klin. Chem. Klin. Biochem.*, 10 (1972) 182–192.
20 R. R. Sokal and F. J. Rohlf, *Biometry*, Freeman, San Francisco, CA, 1969, p. 391.
21 L. A. Nagode, A. Koestner and C. L. Steinmeyer, *Clin. Chim. Acta*, 26 (1969) 55–65.

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IDENTIFICATION AND ASSAY OF PHOSPHOSERINE AND TYROSINE-O-SULPHATE IN FIBRINOPEPTIDES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A procedure utilizing reversed-phase high-performance liquid chromatography (HPLC) is described for the identification and quantitation of individual phosphorylated and sulphated fibrinopeptides present in fibrin clot supernatants. Fibrinopeptides from human, rabbit and canine fibrinogens, which have different structures and degrees of phosphorylation and sulphation, were used to demonstrate the applicability of these methods. The procedure relies on the increased peptide hydrophobicity following removal of highly charged phosphate or sulphate groups. Dephosphorylated or desulphated peptides are thus more strongly retained on the reversed-phase HPLC column and are eluted later than their corresponding phosphorylated or sulphated peptide counterparts. Dephosphorylation is achieved by treatment of fibrinopeptide-containing clot supernatants with alkaline phosphatase. Phosphorylated peptides are characterized by an increased retention time resulting from loss of phosphate, whereas non-phosphorylated peptides remain unaffected. Similarly, a prolongation of the peptide retention time resulting from desulphation by mild acid hydrolysis serves to verify sulphation of a peptide.

INTRODUCTION

Examples of biological peptides covalently modified by phosphorylation or sulphation include neuropeptides and peptide hormones[1], fibrinopeptides[2,3] and others[4]. The chemical instability of the modified amino acid residues, phosphoserine and tyrosine-O-sulphate, prevents their identification by standard protein-chemical methods such as amino acid composition and sequence analysis. Therefore, specific procedures are required to demonstrate their presence. Enzymatic degradation of the purified peptide has often been used to generate free phosphoserine or tyrosine-O-sulphate which can subsequently be identified by paper electrophoresis or thin-layer

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chromatography[2,5]. More recent methods have achieved higher sensitivities by radioactive labelling with ^{32}P or ^{35}S, but these are generally applicable only to proteins[6,7].

Fibrinopeptides belong to the earliest peptides found to be modified by phosphorylation or sulphation.[8,9] These peptides are cleaved from fibrinogen by the protease thrombin to form insoluble fibrin, which co-polymerizes to form the major component in the structural network of blood clots. The primary structures and sites of phosphorylation and sulphation have been determined for fibrinopeptides of many different species[2,3]. In order to assign the peptide peaks in high-performance liquid chromatography (HPLC) of fibrin clot supernatants from various species which contain different phosphorylated, sulphated and unmodified fibrinopeptides, a simple, non-radioactive HPLC procedure was developed which enables phosphorylated, sulphated and unmodified peptides to be determined without peptide isolation and individual analysis. A brief description of this work has been given[10].

Due to the small size of a peptide, highly ionic phosphate or sulphate groups contribute considerably to its net charge and polarity. An alteration in these properties leading to a change in peptide retention on a reversed-phase HPLC column would be anticipated upon removal of a phosphate or sulphate group. The individual contributions of tyrosine-O-sulphate as well as other amino acids to the overall retention coefficients of peptides have been investigated and quantitated[11].

Dephosphorylation with alkaline phosphatase followed by HPLC analysis has been applied to the identification of the phosphorylated form of human corticotropin[12] or of human fibrinopeptide A, which in the latter case was cleaved from phosphatase-treated fibrinogen by thrombin[13,14]. In the procedures reported here, fibrinopeptides are first cleaved from fibrinogen and subsequently analysed for phosphorylation or sulphation. HPLC chromatograms of the fibrinopeptide-containing clot supernatants are compared with those of phosphatase-treated clot supernatants in order to identify phosphorylated peptides from human and canine fibrinogen. A similar approach is used for the analysis of sulphated peptides present in rabbit and canine fibrin clot supernatants, whereby sulphate is removed from tyrosine-O-sulphate by hydrolysis with mild acid.

MATERIALS AND METHODS

Clotting of fibrinogen

Approximately 20 mg fibrinogen from human (Deutsche Kabi, Munich, F.R.G.), rabbit (Sigma, St. Louis, MO, U.S.A.) or dog (Sigma) were dissolved in 4 ml of 0.15 *M* ammonium acetate, brought to pH 8.5 with ammonia. Thrombin (Behringerwerke, Marburg/Lahn, F.R.G.) was added to a final concentration of 1 NIH (U.S. National Institute of Health trombin reference standard) unit/ml. After digestion for 2 h at 20°C, the clotted sample was placed in a bath of boiling water for 1 min, then centrifuged (4800 *g*) and part of the supernatant was applied directly to HPLC.

Enzymatic removal of phosphate

The dephosphorylation was carried out directly on 500 μl of clot supernatant. Alkaline phosphatase (Boehringer Mannheim, Marburg/Lahn, F.R.G.) was added

to a final concentration of 4 U/ml (U = Boehringer units). After digestion for 2 h at 20°C, an aliquot was applied to HPLC.

Removal of sulphate by acid hydrolysis

A 500-μl volume of clot supernatant was lyopholized to remove ammonium acetate and redissolved in 500 μl of 1 *M* hydrochloric acid. After heating for 1 min in a bath of boiling water, the sample was frozen, lyopholized and redissolved in 1 *M* formic acid. Part of the clot supernatant was applied to HPLC.

HPLC conditions

The conditions for the analysis of fibrinopeptides were essentially the same as described previously[15], using an analytical steel column (200 mm × 5.0 mm) packed with LiChrosorb RP-18 (Merck, Darmstadt, F.R.G.). A linear elution gradient was employed, with the following solvents: (A) 0.025 *M* ammonium acetate brought to pH 6.0 with orthophosphoric acid; (B) 0.05 *M* ammonium acetate brought to pH 6.0, plus an equal volume of acetonitrile (Uvasol grade, Merck).

Amino acid analysis

Fractions to be analysed were hydrolyzed under vacuum in 5.7 *M* hydrochloric acid for 24 h at 110°C. The samples were analysed on a Biotronic LC 600 amino acid analyser[16].

RESULTS

Two types of fibrinopeptides, referred to as A and B, are cleaved by the protease thrombin from the Aα- and Bβ-fibrinogen chains. This proteolytic action intitiates the formation of a fibrin blood clot. The resulting fibrinopeptides released into the clot supernatant were dephosphorylated by treating clot supernatants with alkaline phosphatase or desulphated by subjecting clot supernatants to mild acid hydrolysis. The primary structures and sites of phosphorylation or sulphation of the fibrinopeptides investigated here are shown in Fig. 1. In human fibrinopeptides a proportion of the fibrinopeptide A has been shown to be phosphorylated at the single serine residue[2]. Thus two fibrinopeptide A forms exist, a non-phosphorylated and a phosphorylated form, designated as A and AP respectively.

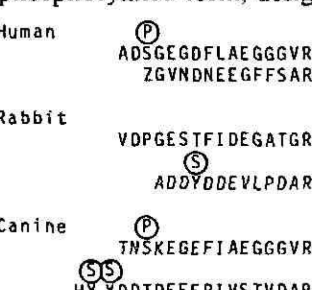

Fig. 1. Primary structures of the fibrinopeptides A and B of human, rabbit and canine. The structures are according to refs. 2–5, using the one-letter code[18]. Encircled P or S denotes phosphate or sulphate groups respectively. Due to incomplete phosphorylation or sulphation, modified fibrinopeptides often exist as mixtures together with unmodified forms.

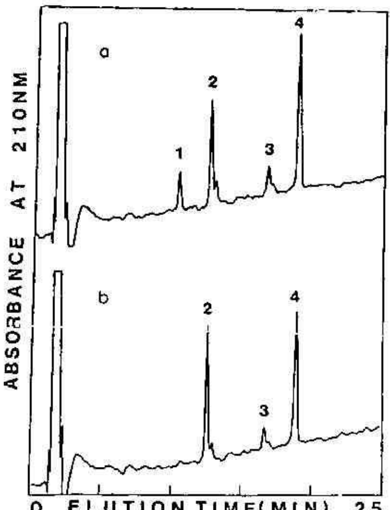

Fig. 2. The HPLC elution pattern of the clot supernatant peptides of human fibrin. The pattern of the untreated clot supernatant (a) is aligned with that of clot supernatant treated with alkaline phosphatase (b) as described under Materials and Methods. Approximately 10 μg were injected in each case onto a column packed with LiChrosorb RP-18 and the peptides separated using a linear gradient from 10 to 30% B over 30 min at a flow-rate of 1.5 ml/min. 0.05 a.u.f.s.

HPLC analysis of the clot supernatant of human fibrinogen gave the elution pattern shown in Fig. 2a. The amino acid compositions were determined (data not shown) from fractions corresponding to each peptide peak and compared to the known human fibrinopeptide structures shown in Fig. 1. Peaks 1 and 2 had identical amino acid compositions corresponding to the known sequence of human A, whereas peaks 3 and 4 corresponded to the structure of B. In order to to identify the peptide peaks in Fig. 2a corresponding to fibrinopeptides A and AP, clot supernatant was treated with alkaline phosphatase and subsequently analysed by HPLC as described under Materials and Methods, giving the chromatogram shown in Fig. 2b. Upon inspection of Fig. 2b, a peak eluting at the same position as peak 1 of the untreated sample (Fig. 2a) is absent and a concomitant increase in peak 2 is observed. This can be interpreted as the result of the removal of phosphate from AP, thereby converting it into A. Thus peak 1 in Fig. 2a represents AP and peak 2, peptide A. On the basis of peak heights, the relative concentrations are approximately 30% AP and 70% A. Since fibrinopeptide B is not phosphorylated, the retention times of peaks 3 and 4 are not affected by the phosphatase treatment.

As previously reported [13,14], a slow degradation of fibrinopeptide B occurs after its release by thrombin from fibrinogen, resulting in minor amounts of desarginine B, which was found in Fig. 2 to be eluted as peak 3 on the basis of its amino acid composition, since it lacked arginine but corresponded otherwise to the human fibrinopeptide B structure (Fig. 1). Peak 4 was identified as the intact peptide B, since it contained arginine and corresponded fully to the structure of B. It has been suggested that trace amounts of the enzyme responsible for this degradation copurify with Kabi fibrinogen and that the enzyme is specific towards fibrinopeptide B but

not fibrinopeptide A, which also contains a carboxy-terminal arginine[13,14]. This example serves to demonstrate the need to examine peptide retention time shifts which might result from cleavage. For example, the formation of desarginine B from intact B was established after amino acid analyses of both peptides. This degradation is apparently caused by carboxypeptidease-like enzyme contaminants present in Kabi fibrinogen and is not the result of the dephosphorylation procedure, since similar amounts of desarginine B occur in the untreated sample (Fig. 2a, peak 3) as well. Here, and in the following experiments, amino acid analyses were consistently carried out on all peptides before and after dephosphorylation or desulphation to confirm the integrity of their primary structures and to exclude the possibility of proteolytic or hydrolytic cleavage.

Sulphated peptides were investigated using a similar approach to that for phosphorylation, whereby sulphate removal was achieved chemically by mild acid hydrolysis. The rabbit fibrinopeptide B, whose structure is shown in Fig. 1, is sulphated at tyrosine[3], the sulphated form being designated here as BS. The chromatogram of the rabbit fibrin clot supernatant, shown in Fig. 3a, contains two major peaks. Peak 1 was identified by amino acid analysis as B and peak 3 as A. Rabbit clot supernatant was subjected to mild acid hydrolysis to remove sulphate as described in Materials and Methods. The hydrolysis products were analysed by HPLC, giving the chromatogram shown in Fig. 3b. Comparison with Fig. 3a shows that peptide peak 2 in Fig. 3b has a similar relative height but is eluted later than peak 1 of the untreated sample (Fig. 3a). Furthermore, peaks 1 and 2 (in Fig. 3a and b, respectively) had an identical amino acid composition which corresponded to the rabbit fibrinopeptide B structure. These results indicate that peak 1 in Fig. 3a corresponds to BS, which upon sulphate removal is converted into the less polar B, eluted later as peak 2. Peak 3, however, is not altered by mild acid since it represents fibrinopeptide A which does not contain any acid-hydrolyzable sulphate groups.

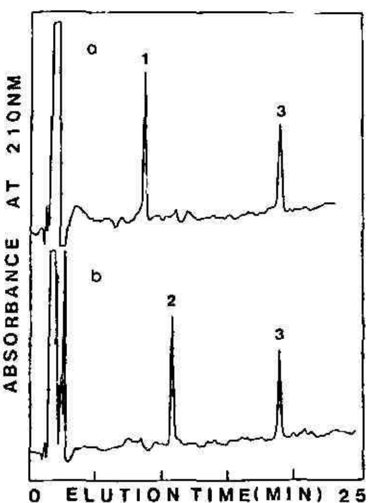

Fig. 3. The HPLC elution pattern of peptides in the clot supernatant of rabbit fibrin. The elution pattern of the untreated control material (a) is aligned with that of clot supernatant subjected to mild acid hydrolysis (b) as described under Materials and Methods. Approximately 10 μg were injected onto the column. Linear gradient: from 5 to 25% B over 30 min at a flow-rate of 1.5 ml/min. 0.05 a.u.f.s.

Both phosphorylation and sulphation have been described for the canine fibrinopeptides[5,17]. Canine fibrinopeptide A is found as a non-phosphorylated and phosphorylated form (A and AP) and the fibrinopeptide B as a mono and disulphated form (BS and BS_2 respectively) resulting from the possible sulphation of one or two of the adjacent tyrosine residues, as seen by their structures shown in Fig. 1. The HPLC elution of the dog fibrinopeptides, presented in Fig. 4a, consists of four major peaks. Peaks 1 and 2 in Fig. 4a were identified as canine A and peaks 3 and 4 as B by their amino acid compositions. Phosphorylation of A was investigated by treatment of the clot supernatant with alkaline phosphatase, as described in Materials and Methods. Subsequent HPLC analysis showed one peak to increase beyond the full scale of detection. Therefore, a smaller amount of phosphatase-treated material was analysed which corresponded to one-half of the untreated material analysed previously in Fig. 4a. The resulting elution pattern is shown in Fig. 4b. From the observed disappearance of peak 1 and the concomitant increase in peak 2, resulting from dephosphorylation of AP to A, it can be concluded that peak 1 represents AP and peak 2, A. Based on peak heights, the relative concentrations are approximately 30% A and 70% AP. Interestingly, these proportions are reversed for the human fibrinopeptides, *i.e.*, 70% A and 30% AP. The mono- and disulphated B peptides were identified as follows; clot supernatants were subjected to mild acid hydrolysis resulting in removal of most of the sulphate groups. The hydrolysis products were then analysed by HPLC and the resulting elution profile (Fig. 4c) aligned with that of the untreated sample (Fig. 4a). It is seen that the disappearance of peak 3 and the decrease in peak 4 is accompanied by the appearance of a more hydrophobic peak 5 in Fig. 4c, which had an amino acid composition identical to that of peaks 3 and 4. Peaks 3 and 4 were thus identified as BS_2 and BS respectively, since upon mild acid hydrolysis apparently all of the BS_2 is degraded to BS and B and most of the

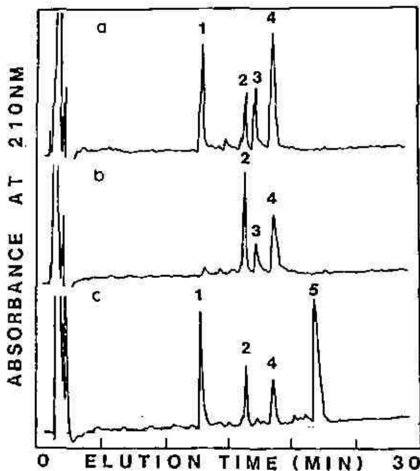

Fig. 4. The HPLC elution pattern of peptides in the clot supernatant of canine fibrin. The elution pattern of the untreated material (a) is aligned with that of phosphatase-treated (b) and of acid-treated (c) clot supernatants as described under Materials and Methods. In (b) half the amount of peptide material was analysed as in (a) and (c) where the amount was approximately 20 μg. Peptides were eluted by a linear gradient from 10 to 30% solvent B over 30 min at a flow-rate of 1.5 ml/min. 0.05 a.u.f.s.

BS is degraded to B. The relative concentrations of the sulphated peptides were 30% BS_2 and 70% BS. Furthermore, all of the canine B peptides were found to be sulphated, since no peak corresponding to desulphated B (peak 5 in Fig. 4c) is observed with the untreated sample (Fig. 4a).

DISCUSSION

The methodology described here enables the identification and assay of individual phosphorylated and sulphated peptides in peptide mixtures by comparison of the peptide elution pattern before and after dephosphorylation or desulphation. This treatment leads to changes in charge and polarity which are sufficient to alter their retention on reversed-phase HPLC columns. This is a property characteristic of peptides containing phosphate or sulphate which enables them to be distinguished from peptides lacking such modifications.

This approach offers an alternative to labelling with ^{32}P or ^{35}S, particularly in cases where radioactive labelling may not be feasible. The analysis is carried out with standard HPLC equipment and the peptides can readily be recovered in a lyophilizable solvent with their primary structures intact. Therefore, these procedures can also be adapted for preparative dephosphorylation or desulphation. Furthermore, the results obtained from the canine fibrinopeptides demonstrate that phosphorylated and sulphated peptides can be individually analysed in sample containing both phosphorylated and sulphated peptides, since the dephosphorylation or desulphation procedures appear to be specific. No side reactions were detected, particularly during the mild acid hydrolysis procedure used for desulphation of rabbit and canine fibrinopeptides. In these cases, retention shifts of sulphated peptides occurred as expected, whereas no alterations of other peptides present were found. The finding that these procedures can be used successfully on fibrinopeptides of differing structures suggests that they may also be useful for other types of peptides.

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REFERENCES

1 Y. P. Loh and H. Gainer, in D. T. Krieger, M. J. Brownstein and J. B. Martin, (Editor), *Brain Peptides*, Wiley, New York, 1980, pp. 108–109.
2 B. Blomback, M. Blomback, P. Edman and B. Hessel, *Biochim. Biophys. Acta*, 115 (1966) 371–396.
3 B. Blomback, M. Blomback and N. J. Grondahl, *Acta Chem. Scand.*, 19 (1965) 1789–1791.
4 W. C. Barker, L. T. Hunt, B. C. Orcutt, D. G. George, L. S. Yeh, H. R. Chen, M. C. Blomquist, G. C. Johnson, E. I. Seibel-Ross, M. K. Hong and R. S. Leydly, *Protein Sequence Data Base Release 4*, National Biomedical Research Foundation, Washington, DC, 1985.
5 T. Krajewsky and B. Blomback, *Acta Chem. Scand.*, 22 (1968) 1339–1346.
6 T. M. Martensen, *Methods Enzymol.*, 107 (1984) 3–23.
7 W. B. Huttner, *Methods Enzymol.*, 107 (1984) 200–223.
8 B. Blomback, M. Blomback and P. Edman, *Nature (London)*, 193 (1962) 883–884.
9 F. R. Bettelheim, *J. Am. Chem. Soc.*, 76 (1954) 2828–2839.
10 A. Lucas and A. Henschen, *Thromb. Haemostasis*, 54 (1985) abstract No. 344.

11 J. L. Meek, *Proc. Natl. Acad. Sci. U.S.A.*, 77 (1980) 1632–1636.
12 H. P. J. Bennet, P. L. Brubaker, M. A. Seger and S. Solomon, *J. Biol. Chem.*, 258 (1983) 8108–8112.
13 J. A. Koehn and R. E. Canfield, *Anal. Biochem.*, 116 (1981) 349–356.
14 F. E. Ebert and W. R. Bell, *Anal. Biochem.*, 148 (1985) 70–78.
15 M. Kehl, F. Lottspeich and A. Henschen, *Hoppe-Seyler's Z. Physiol. Chem.*, 362 (1981) 1661–1664.
16 D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, 30 (1958) 1190–1206.
17 A. J. Osbahr, Jr., R. W. Colman, K. Laki and J. A. Gladner, *Biochem. Biophys. Res. Commun.*, 14 (1964) 555–558.
18 IUPAC-IUB Commission on Biochemical Nomenclature, *Eur. J. Biochem.*, 5 (1968) 151–153.

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PROFILING OF IMPURITIES IN ILLICIT AMPHETAMINE SAMPLES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING COLUMN SWITCHING

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SUMMARY

A simple high-performance liquid chromatographic method, suitable for routine profiling of impurities in illegally produced amphetamine, has been developed. Amphetamine is dissolved in acetonitrile–citrate buffer (pH 3) (2:8) and injected directly without further sample pre-treatment. The impurities are enriched on-line on a C_8 extraction column, while amphetamine and polar diluents are washed out with water. After washing for 1.5 min, a six-port valve is switched and an acetonitrile–0.2 *M* butylamine in water (pH 8) gradient elutes the impurities from the extraction column on to a C_{18} analytical column where they are separated. The compounds are monitored by UV detection at 220 and 254 nm. The total extraction and analysis time is 30 min. The method allows automated extraction and analysis to be performed.

INTRODUCTION

Illegal amphetamine samples often contain traces of by-products and intermediates from the illicit manufacture of the drug. The demand for an intimate knowledge of the composition of these impurities has increased in recent years, as detailed impurity profiles can provide valuable information concerning illegal methods of manufacture[1,2]. In addition, the presence or absence of impurities can aid in identifying drug samples of common origin[3,4]. Both capillary gas chromatographic (GC)[5,6] and high-performance liquid chromatographic (HPLC) methods[7] have proved to be useful in giving detailed impurity profiles suitable for the comparison of illicit amphetamine samples.

When different samples are compared, it is important to obtain an analytical method that causes minimum changes to the sample composition. One means of obtaining this is to use chromatographic systems that make direct injection of the sample possible.

On-line extraction columns and column switching in HPLC have recently been extensively performed for the direct injection of plasma samples[8–10]. The method is

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also a powerful technique for the enrichment of trace impurities in environmental samples[11,12].

In this work, a column switching procedure was used for the isolation and enrichment of impurities in illegally produced amphetamine. A sample solution of amphetamine is injected directly on to an on-line extraction column and the main constituents, amphetamine and water-soluble diluents, are washed out with water. Less polar trace impurities from the synthesis, often present in amounts of less than 1%, are enriched on the column and are later eluted and separated.

An off-line liquid–solid extraction method with Bond Elut columns has earlier been described for the extraction of impurities in amphetamine[13]. However, the present on-line extraction procedure can easily be automated and is suitable for the routine screening of impurities in illegal samples.

In several countries in Europe amphetamine is most frequently synthesized by the Leuckart procedure[5], samples produced by other procedures being only occasionally encountered. A typical Leuckart profile has been compared with impurity profiles of amphetamine synthesized by three other routes. The contents of some of the impurities in illegal seizures have been determined.

EXPERIMENTAL

Chemicals

HPLC-grade water was obtained by purifying distilled water in a Milli-Q (Millipore, Bedford, MD, U.S.A.) filtration system. HPLC-grade acetonitrile was purchased from Rathburn (Walkerburn, U.K.). Analytical-reagent grade butylamine and dichloromethane were obtained from Fluka (Buchs, Switzerland) and Riedel de Häen (Hannover, F.R.G.), respectively. Citrate and phosphate buffer–Titrisol (pH 3 and 7) and orthophosphoric acid were provided by E. Merck (Darmstadt, F.R.G.).

Samples of seized amphetamine were obtained from the Forensic Laboratory Department, National Bureau of Crime Investigation (Oslo, Norway). Standards of N,N-di(β-phenylisopropyl)amine sulphate, 4-methyl-5-phenylpyrimidine, 2-benzyl-2-methyl-5-phenyl-2,3-dihydropyrid-4-one and a mixture of the high-boiling pyridines described by Van der Ark *et al.*[14] mainly consisting of 2,6-dimethyl-3,5-diphenylpyridine and amphetamine synthesized by reductive amination with Raney nickel as catalyst[15,16] were obtained as a gift from the Forensic Science Laboratory of the Ministry of Justice, Rijswijk, The Netherlands. Amphetamine synthesized by the nitrostyrene route[17] were kindly supplied by the National Laboratory of Forensic Science, Linköping, Sweden. Amphetamine were synthesized by the aluminium method[18] for research purposes at the Department of Chemistry, University of Oslo, Norway.

HPLC and column switching

Apparatus. An SP 8700 (Spectra-Physics, San Jose, CA, U.S.A.) gradient pump was used in combination with a Model 709 (LDC/Milton Roy, Riviera Beach, CA, U.S.A.) isocratic pump with a Model 7000 (Rheodyne, Berkely, CA, U.S.A.) six-port switching valve to perform the column switching. A Spectromonitor III variable-wavelength UV detector (LDC) was used for detection at 220 and 254 nm. Chromatograms were recorded on an SP 4270 integrator (Spectra-Physics). The injector was a Model 7120 (Rheodyne) with 100, 250 and 500 μl sample loops.

TABLE I

TIME SCHEDULE, SWITCHING

Time after injection (min)	*Event*
0	Sample is injected on to the extraction column with water as mobile phase (switching valve is in position 1)
1.5	Washing with water is finished. Switching valve: switching from position 1 to 2. Gradient started. Printer started. Desorption of trace impurities from extraction column, separation on analytical column starts
16.5	Reset of switching valve: position 2 to 1. Re-equilibration of extraction column with water before next injection
26.5	Elution and separation from analytical column are finished
30	Analytical column re-equilibrated with initial gradient. New injection.

Mobile phases and columns

MPLC^{TM} cartridge columns (Brownlee Labs., Santa Clara, CA, U.S.A.) were used for both extraction and separation of the impurities. For the extraction and enrichment 7 μm C_2, C_4 and C_8 (15 × 3.2 mm I.D.) cartridges were evaluated. A 5 μm Spheri 5 C_{18} analytical column (100 × 4.6 mm I.D.) with a 5 μm C_{18} (30 × 3.2 mm I.D.) guard column were used for the separation of impurities. Measurements were repeated on two different analytical columns to ensure that no irreversible changes occured during the method development with the first column.

The washing procedure was performed with HPLC-grade water with a flow-rate of 1.0 ml/min. The following gradient was used for the desorption and separation of the impurities: (A) 0.2 *M* butylamine in water, the pH being adjusted to 8.0 with orthophosphoric acid; (B) 20% (v/v) of A in acetonitrile. The gradient was programmed linearly from 30 to 100% B in 20 min, then isocratic 100% B for 5 min. The flow-rate was 1.0 ml/min and the analyses were carried out at ambient temperature.

At the end of the day, the system was washed with 75% acetonitrile in water for about 30 min.

Samples. Samples were prepared at a concentration of 50 mg/ml in acetonitrile–citrate buffer (pH 3) (2:8). If necessary, the solutions were placed on an ultrasonic bath for 15 min.

Scheme of column-switching events. The scheme of switching events is given in Table I and the positions of the switching valve are shown in Fig. 1.

Quantitation. Quantitative estimates of the N-formylamphetamine (N-f-A), 4-methyl-5-phenylpyrimidine (4-me) and N,N-di(β-phenylisopropyl)amine (di-iso-an) contents were based on peak-height measurements. The content was calculated as the weight percentage of the impurity in "uncut" amphetamine sulphate. Contents of less than 0.003, 0.001 and 0.006% of N-f-A, 4-me and di-iso-an, respectively, were not quantified.

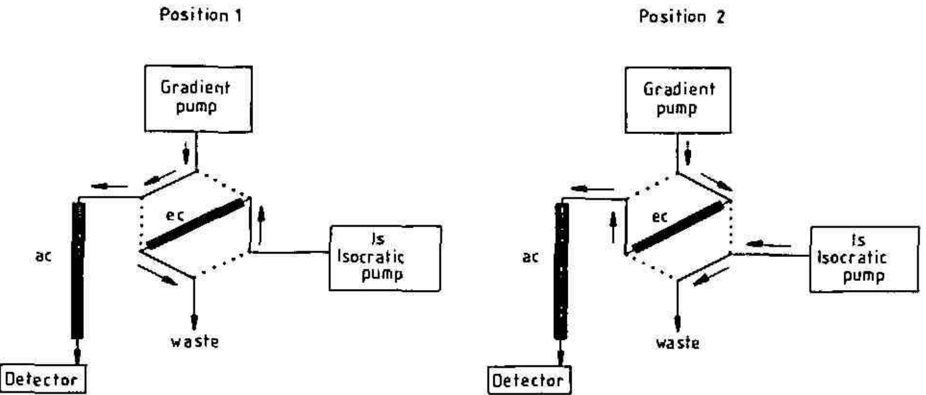


Fig. 1. Switching configuration for on-line pre-concentration and clean-up. ec = Extraction column; ac = analytical column; is = injection system.

Bond Elut extraction

The off-line liquid–solid sample preparation procedure with Bond Elut columns has been described elsewhere[13].

Gas chromatography–mass spectrometry (GC–MS)

A Micromass 7070 F mass spectrometer (VG-Micromass, Altrincham, U.K.) combined with a Fractovap 4200 gas chromatograph (Carlo Erba, Milan, Italy) was used to check the standards and to identify the impurities eluted from the HPLC column. The eluates from ten injections were concentrated under a stream of nitrogen at 35°C, then extracted with dichloromethane. The dichloromethane extract was evaporated to dryness under a stream of nitrogen at 35°C. The residue was dissolved in 50 μl of acetonitrile and 1 μl of the solution was injected into the gas chromatograph. The experimental conditions for the GC–MS analysis have been described earlier[7].

RESULTS AND DISCUSSION

Extraction and pre-concentration of trace impurities

The impurity profile of an illegal amphetamine sample (sample 1) obtained by on-line pre-concentration on a C_8 reversed phase column is given in Fig. 2. The method allows a 100 μl sample solution (50 mg/ml) to be injected directly, as the dominating component, amphetamine, is efficiently removed during the washing procedure. Water-soluble diluents such as glucose and sucrose, which are often added during the drug distribution process in concentrations varying from 10 to 90%, are also washed out of the column. However, the less polar trace impurities originating from the synthesis are retained and enriched on the extraction column.

Structures, names and abbreviations of amphetamine and the impurities from sample 1 that are identified by GC–MS are given in Table II. The sample, which contained 90% amphetamine and was diluted with glucose, was synthesized by the Leuckart procedure. Several impurities associated specifically with this method were detected and identified (N-f-A, 4-me and di-iso-ad)[2]. The characteristic difference in

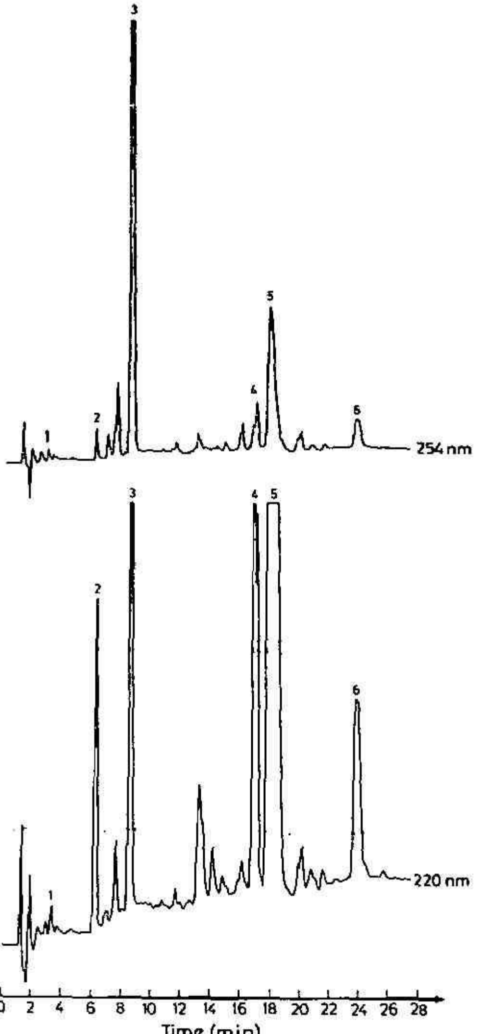


Fig. 2. HPLC impurity profile of a Leuckart-synthesized sample (sample 1) obtained by direct injection of 100 µl of a 50 mg/ml sample solution. Detection by UV absorption at 254 and 220 nm. Peaks: 1 = amphetamine; 2 = N-formylamphetamine; 3 = 4-methyl-5-phenylpyrimidine; 4 = N,N-di(β-phenylisopropyl)formamide; 5 = N,N-di(β-phenylisopropyl)amine; 6 = N,N-di(β-phenylisopropyl)methylamine.

absorbance at 254 and 220 nm of N-f-A, di-iso-ad, di-iso-an and di-iso-mn is demonstrated in Fig. 2.

A more detailed impurity profile is obtained by the on-line extraction method than with the off-line Bond Elut procedure described earlier[13], as a higher sample volume is injected. When only small sample amounts are available, the whole sample can be utilized for one injection to provide the highest possible sensitivity.

Several reversed-phase extraction columns were evaluated for the extraction of impurities. Short columns (2–5 mm) and large particle size (30–50 µm) packing

TABLE II

AMPHETAMINE AND IMPURITIES IN ILLEGALLY SYNTHESIZED AMPHETAMINE

Compounds as discussed in the text.

Formula	*Abbreviation*	*Name*
$C_6H_5-CH_2-CH(CH_3)-NH_2$		Amphetamine
$C_6H_5-CH_2-CH(CH_3)-NH-CHO$	N-f-A	N-Formylamphetamine
4-methyl-5-phenylpyrimidine ring structure (C_6H_5, CH_3, N, N)	4-mc	4-Methyl-5-phenylpyrimidine
$[C_6H_5-CH_2-CH(CH_3)]_2N-CHO$	di-iso-ad	N,N-Di(β-phenylisopropyl)formamide
$[C_6H_5-CH_2-CH(CH_3)]_2NH$	di-iso-an	N,N-Di(β-phenylisopropyl)amine
$[C_6H_5-CH_2-CH(CH_3)]_2N-CH_3$	di-iso-mn	N,N-Di(β-phenylisopropyl)methylamine

materials are usually recommended for plasma samples, but for the trace enrichment of impurities in amphetamine samples, longer columns (1.5 cm) with small particle size (7 μm) packing material were chosen. These "high-capacity" extraction cartridges were preferred because of the large variation in the contents of impurities in different samples. Problems with clogging of the columns or increased back-pressure were not observed.

C_2, C_4 and C_8 extraction columns from Brownlee Labs. were compared by connecting them directly to the detector. With water as the mobile phase the difference in retention of amphetamine and the early eluting impurity in Leuckart-synthesized amphetamine, N-f-A, was measured. The experiment showed that N-f-A was most efficiently retained on the C_8 column, while amphetamine was washed out. A washing period of 1.5 min with water at 1.0 ml/min gave reproducible peak heights of the impurities [the switching valve is switched to position II 1.5 min after injection (Fig. 1)]. The switching valve was reset to position I (Fig. 1) after 15 min of elution

and the extraction column was equilibrated with water for 13.5 min before the next injection (30 min working cycle).

No significant difference was noted in extra peak broadening between back- and forward-flush elution. The forward-flush mode was chosen in order to maintain the protective filter aspect of the pre-column. Extra-column band broadening for the foreward-flush mode was less than 10%.

Efficient clean-up was obtained for concentrations up to 100 mg/ml amphetamine solutions and for the injection of 100–500 μl sample solutions. In general, 100 μl of the sample at a concentration of 50 mg/ml were injected.

Development of mobile phase for efficient desorption and separation of impurities

Several mobile phases were investigated to obtain the best desorption from the extraction column and separation of the impurities on the analytical column. When the acetonitrile–water gradient described previously[13] was used, a washing procedure was required after ten injections because of baseline drift. A decrease in efficiency of

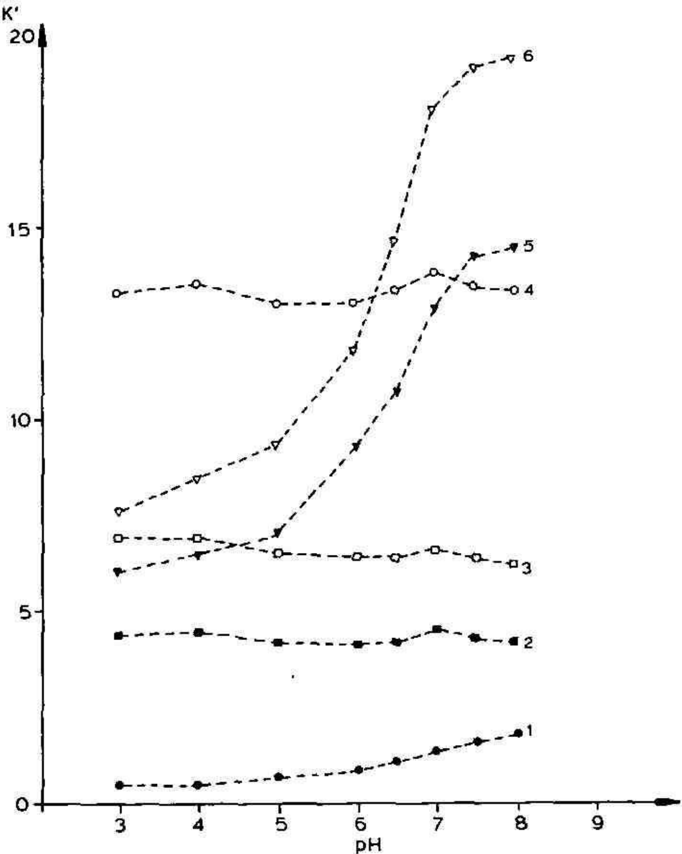


Fig. 3. Influence of the pH of the mobile phase on the capacity ratios (k') of amphetamine and impurities. 1 = Amphetamine; 2 = N-formylamphetamine; 3 = 4-methyl-5-phenylpyrimidine; 4 = N,N-di(β-phenylisopropyl)formamide; 5 = N,N-di(β-phenylisopropyl)amine; 6 = N,N-di(β-phenylisopropyl)methylamine.

the analytical column after a few injections was also observed. As most of the impurities in illicit amphetamine samples are weakly basic[1], this phenomenon was thought to be due to adsorption effects of some of the basic amine impurities to uncovered silanol groups. The addition of an amine[19,20] or a quaternary ammonium compound[21] to the mobile phase has proved to be an effective method of masking the silanol groups on the packing material. With addition of the amine modifier, butylamine, more than 100 samples could be injected on to the same extraction column without an increase in baseline drift or column back-pressure. No memory effects were observed when a sample blind was injected after every ten samples. Extra peak broadening and tailing of some of the components were also reduced.

The mobile phase was optimized to separate most of the impurities commonly encountered in Leuckart-synthesized amphetamine, as this method still seems to be the most popular in Europe. The pH of the mobile phase was varied by the addition of orthophosphoric acid and the capacity ratios (k') of amphetamine, N-f-A, 4-me, di-iso-ad, di-iso-an and di-iso-mn were measured. The k' values of di-iso-an and di-iso-mn showed a drastic increase when the mobile phase pH was raised from 3 to 8, as shown in Fig. 3, whereas the k' values of the other compounds were almost constant in the pH range 3–8. In the pH range 5–6.5 di-iso-an gave broad peaks and extensive tailing.

The UV responses of some compounds were strongly dependent on pH. Measurements with sample 1 showed that the peak areas of di-iso-an and di-iso-mn were reduced by about the half on going from pH 8 to 6. The final mobile phase with the addition of orthophosphoric acid to give pH 8.0 gave the best separation and sensitivity and minimum peak tailing and baseline drift at 220 nm.

TABLE III

REPRODUCIBILITY OF CAPACITY RATIOS (k') OF PEAKS FROM SAMPLE 1

t_0 = 1.17 min.

Compound	*Variation**	*k' (mean, n=8)*	*Standard deviation*	*Relative standard deviation (%)*
Amphetamine (peak 1)	A	1.7	0.01	0.6
	B	1.8	0.05	2.3
N-Formylamphetamine (peak 2)	A	4.2	0.02	0.5
	B	4.2	0.07	1.7
4-Methyl-5-phenylpyrimidine (peak 3)	A	6.2	0.03	0.5
	B	6.1	0.05	0.8
N,N-Di(β-phenylisopropyl)formamide (peak 4)	A	13.3	0.04	0.3
	B	13.3	0.04	0.3
N,N-Di(β-phenylisopropyl)amine (peak 5)	A	14.3	0.07	0.5
	B	14.4	0.08	0.6
N,N-Di(β-phenylisopropyl)methylamine (peak 6)	A	19.3	0.10	0.5
	B	19.3	0.13	0.7

* A = run-to-run variations; B = day-to-day variations with eight different batches of mobile phase.

The reproducibility of the k' values of the identified impurities from sample 1 is shown in Table III. The relative standard deviations are less than 1% on a run-to-run basis (A), which are equivalent to the relative standard deviations when Bond Elut-extracted samples were injected without using the column switching system. The reproducibility based on day-to-day variations was less than 3.0% for eight different batches of mobile phase (B).

The guard column lasts for about 1–2 months of continuous use and the analytical column has been used for about 6 months without a significant decrease in efficiency.

Preparation of sample solutions

Several solvents were evaluated for the dissolution of amphetamine samples. A screening of buffers in the pH range 2–9 showed that amphetamine was most efficiently washed out of the extraction column when injected in an acidic solution. The trace impurities from the synthesis, although some of them were probably protonated at acidic pH, were retained on the column. Variation of pH from 2–7 did not have a significant influence on the recovery, but at alkaline pH the intensities of several peaks decreased.

Some of the impurities were more soluble in a mixture of water and acetonitrile than in pure water. Acetonitrile at concentrations of 10–50% was therefore added to the buffer to improve the solubility of these compounds. The addition of acetonitrile gave higher recoveries of di-iso-ad, di-iso-an and di-iso-mn and lower recoveries of N-f-A and 4-me. At an intermediate concentration of acetonitrile (20%), the loss of N-f-A and 4-me was negligible, whereas higher intensities of di-iso-an, di-iso-ad and di-iso-mn were obtained. Table IV shows the reproducibility of peak-height ratios of impurities from sample 1 (peak height of impurities relative to the 4-me peak) dissolved in citrate buffer (pH 3)–acetonitrile (8:2). The relative standard deviations of the peak-height ratios were less than 4% and are acceptable for the comparison of samples.

To avoid stability problems of the impurities in the solvent, it is advisable to

TABLE IV

REPRODUCIBILITY OF PEAK-HEIGHT RATIOS OF IMPURITIES FROM SAMPLE 1

$h_{N\text{-}f\text{-}A}$ = peak height of N-formylamphetamine (peak 2); $h_{4\text{-}me}$ = peak height of 4-methyl-5-phenylpyrimidine (peak 3); $h_{di\text{-}iso\text{-}ad}$ = peak height of N,N-di(β-phenylisopropyl)formamide (peak 4); $h_{di\text{-}iso\text{-}an}$ = peak height of N,N-di(β-phenylisopropyl)amine (peak 5); $h_{di\text{-}iso\text{-}mn}$ = peak height of N,N-di(β-phenylisopropyl)methylamine (peak 6).

Peak-height ratio	*Value (mean, n = 8)*	*Standard deviation*	*Relative standard deviation (%)*
$h_{N\text{-}f\text{-}A}/h_{4\text{-}me}$	0.435	0.011	2.5
$h_{di\text{-}iso\text{-}ad}/h_{4\text{-}me}$	0.761	0.022	2.9
$h_{di\text{-}iso\text{-}an}/h_{4\text{-}me}$	1.450	0.056	3.9
$h_{di\text{-}iso\text{-}mn}/h_{4\text{-}me}$	0.370	0.003	0.8

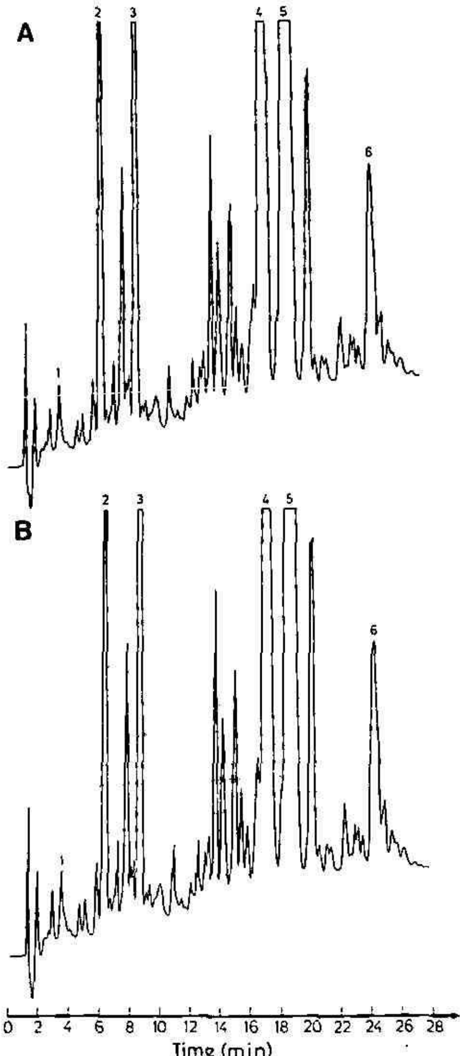


Fig. 4. HPLC impurity profile of two separately analysed samples (A and B) from the same seizure synthesized by the Leuckart method (sample 2). The sample (100 μl sample solution at a concentration of 50 mg/ml) was injected directly. Detection by UV absorption at 220 nm. Peaks as in Fig. 1.

prepare the sample solutions immediately prior to the analysis. If necessary, centrifugation instead of filtration should be performed. An investigation with filtration of sample solutions through Millex HV-4 filters (Millipore) indicated adsorption of some of the components on the filter.

Screening of amphetamine samples

A screening of a series of twenty amphetamine samples seized in Norway in 1985 and 1986 was performed. Wide variations in the purity of the samples and the

TABLE V

RETENTION OF COMMON DILUENTS IN AMPHETAMINE SEIZURES

Diluent	*Capacity ratio (k')*
Caffeine	0.7
Ephedrine chloride	1.5
Phenazone	1.8
Procaine chloride	4.8

addition of diluents were observed. When monitoring at 220 nm, detailed impurity profiles suitable for the comparison of samples were obtained.

The analyses showed that most of the samples had been synthesized by the Leuckart procedure. Fig. 4 shows the impurity profiles of two different samples from a seizure synthesized by this method. The comparison of the two profiles monitored at 220 nm demonstrates the good reproducibility of the method. This sample contained 70% amphetamine and was diluted with glucose (sample 2).

The k' values of commonly used diluents in Norwegian seizures are given in Table V. Most of the diluents elute in or near the front and will not interfere with the trace impurity profile. Glucose and sucrose will not be detected. The k' values of two impurities identified in Leuckart seizures, 2-benzyl-2-methyl-5-phenyl-2,3-dihydropyrid-4-one and 2,6-dimethyl-3,5-diphenylpyridine, were 15.9 and 9.8, respectively. Other high-boiling pyridines described by Van der Ark *et al.*[14] are expected to elute near 2,6-dimethyl-3,5-diphenylpyridine because of structural similarity.

The results of the determination of N-f-A, 4-me and di-iso-an in eight different Leuckart-synthesized samples are shown in Table VI. The contents of N-f-A and 4-me were less than 1%, while di-iso-an showed concentrations up to 2.4%. This is

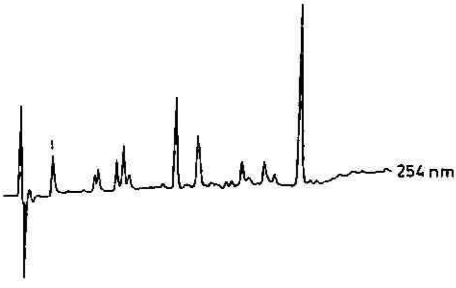
TABLE VI

QUANTITATIVE HPLC DATA FOR N-f-A, 4-me AND di-iso-an IN EIGHT AMPHETAMINE SAMPLES SYNTHESIZED BY THE LEUCKART METHOD

Sample No.	*N-f-A content* (%, w/w)*	*4-me content* (%, w/w)*	*di-iso-an content* (%, w/w)*
1	0.3	0.09	1.9
2	0.7	0.3	2.4
3	—**	0.1	0.9
4	—**	0.02	0.7
5	0.03	0.2	0.6
6	0.2	0.1	0.9
7	0.02	0.1	2.0
8	0.3	0.1	1.3

* These values represent concentrations calculated as % (w/w) of the impurity in "uncut" amphetamine sulphate.

** Quantitation not possible.



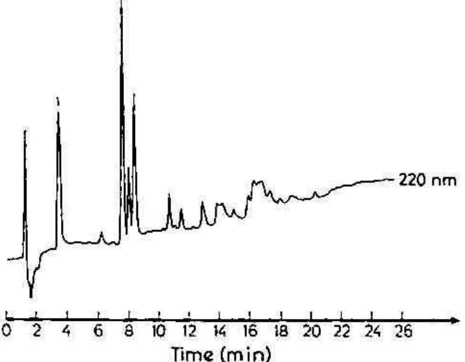


Fig. 5. HPLC impurity profile of amphetamine synthesized by the nitrostyrene route obtained by direct injection of 100 μl of a 100 mg/ml sample solution. Detection by UV absorption at 254 and 220 nm. Attenuation at 254 nm is double that at 220 nm. Peaks: 1 = amphetamine; others not identified.

in agreement with the findings of Huizer *et al.*[22], who showed that di-iso-an was often the main impurity in Leuckart-synthesized samples produced in The Netherlands in recent years.

A typical Leuckart profile was compared with the impurity profiles of amphetamine synthesized by the nitrostyrene route, reductive amination with Raney nickel as catalyst and the aluminium method. A significant difference in impurity profiles between these manufacturing methods was observed, and these samples contained less impurities than the Leuckart-synthesized samples. Fig. 5 shows the chromatogram of a sample synthesized by the nitrostyrene route detected at 254 and 220 nm.

CONCLUSIONS

By using a C_8 on-line extraction column and column switching in HPLC, automated enrichment, isolation and separation of impurities in illegally produced amphetamine can be performed. The method is suitable for routine impurity screening of illegal amphetamine samples.

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The forensic science laboratories in Oslo (Norway), Linköping (Sweden) and Rijswijk (The Netherlands) are gratefully acknowledged for supplying amphetamine samples and standards of the impurities. Mr. H. Dugstad (Department of Chemistry, University of Oslo, Norway) is thanked for performing the amphetamine syntheses.

REFERENCES

1 A. Sinnema and A. M. A. Verweij, *Bull. Nar.*, 33 (1981) 37.
2 M. Lambrechts, T. Klemetsrud, K. E. Rasmussen and H. J. Storesund, *J. Chromatogr.*, 284 (1984) 499.
3 L. Strömberg and A. C. Maehly, *J. Chromatogr.*, 109 (1975) 67.
4 L. Strömberg, *J. Chromatogr.*, 106 (1975) 335.
5 M. Lambrechts and K. E. Rasmussen, *Bull. Nar.*, 36 (1984) 47.
6 L. Strömberg, H. Bergkvist and E. A. M. K. Edirisinghe, *J. Chromatogr.*, 258 (1983) 65.
7 M. Lambrechts and K. E. Rasmussen, *J. Chromatogr.*, 295 (1984) 264.
8 W. Roth, K. Beschke, R. Jauch, A. Zimmer and F. W. Koss, *J. Chromatogr.*, 222 (1981) 13.
9 R. Huber, K. Zech, M. Wörz, T. Kronbach and W. Voelter, *Chromatographia*, 16 (1982) 233.
10 U. Juergens, *J. Chromatogr.*, 310 (1984) 97.
11 R. L. Smith and D. J. Pietrzyk, *J. Chromatogr. Sci.*, 21 (1983) 282.
12 C. E. Goewie, P. Kwakman, R. W. Frei, U. A. Th. Brinkman, W. Maasfeld, T. Seshadri and A. Kettrup, *J. Chromatogr.*, 284 (1984) 73.
13 M. Lambrechts and K. E. Rasmussen, *J. Chromatogr.*, 331 (1985) 339.
14 A. M. van der Ark, A. M. A. Verweij and S. Sinnema, *J. Forensic Sci.*, 23 (1978) 693.
15 A. B. E. Theeuwen and A. M. A. Verweij, *Forensic Sci. Int.*, 15 (1980) 237.
16 H. Huizer, A. B. E. Theeuwen and A. M. A. Verweij, *J. Forensic Sci. Soc.*, 21 (1981) 225.
17 G. A. Alles, *J. Am. Chem. Soc.*, 54 (1932) 271.
18 B. H. Groot Wassink, A. Duijndam and A. C. A. Jansen, *J. Chem. Educ.*, 51 (1974) 671.
19 J. Kraak and P. Bijster, *J. Chromatogr.*, 143 (1977) 499.
20 R. Gill, S. P. Alexander and A. C. Moffat, *J. Chromatogr.*, 247 (1982) 39.
21 K. E. Bij, Cs. Horváth, W. R. Melander and A. Nahum, *J. Chromatogr.*, 203 (1981) 65.
22 H. Huizer, H. Brusse and A. J. Poortman-van der Meer, *J. Forensic Sci.*, 30 (1985) 427.

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RAPID PURIFICATION OF ANTISTEROID ANTIBODIES BY HIGH-PERFORMANCE LIQUID AFFINITY CHROMATOGRAPHY

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SUMMARY

An adsorbent for the high-performance affinity chromatography of antisteroid antibodies was prepared, based on a commercial pre-packed column. The column contained activated microparticulate silica beads bearing epoxide functions, on which the steroid dexamethasone was covalently linked. The column was used successfully for the rapid and complete isolation of several hundred microgram amounts of specific antidexamethasone antibodies from rabbit antisera. The practical aspects of the purification procedure, especially the optimization of the washing and of the elution steps, are detailed. Despite non-biospecific elution with 20% acetonitrile in an acidic buffer, the purification yield was very satisfactory and the biological activity of the purified immunoglobulins appeared excellent.

INTRODUCTION

Since the initial report of Ohlson *et al.*[1], who were the first to use HPLC-grade silica beads for preparing bioaffinity matrixes and thus to initiate high-performance liquid affinity chromatography (HPLAC), increasing attention has been paid to this new technique, which combines the high-speed characteristics of high-performance liquid chromatography (HPLC) with the selectivity of biospecific interactions. Numerous papers have appeared in the last few years on improving the derivatization of silica[2], discussing both the practical[3–7] and theoretical[7–9] aspects of HPLAC and describing many separations[3–27]. However, most of the work involved analytical applications and only a few dealt with the particular problems encountered during preparative work[15,24,25]. Moreover, owing to the lack of ready-to-use activated silica beads, the spread of HPLAC was restricted to laboratories with expertise in silica chemistry. Thus, the relative scarcity of reports on preparative HPLAC applications from biochemical laboratories with the need to solve specific purification problems was not unexpected. However, the very recent appearance of various commercial pre-activated HPLAC supports will probably change the situation. In this paper we report the use of a commercial macroporous silica matrix bearing activated epoxide groups for the covalent immobilization of the steroid dexamethasone and the appli-

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cation of the stationary phase obtained to the complete purification of rabbit polyclonal antidexamethasone antibodies.

EXPERIMENTAL

Materials

[1,2(*n*)-^{3}H]Dexamethasone, 40 Ci mmol^{-1}, was obtained from Amersham International (Amersham, U.K.) and unlabelled dexamethasone from Roussel-Uclaf (Romainville, France). The Ultraffinity-EP column (50 × 4.6 mm I.D.) and the Ultraffinity-EP column capacity kit were obtained from Beckman (Berkeley, CA, U.S.A.). Ultrogel ACA 202 was purchased from Industrie Biologique Francaise (Villeneuve-la-Garenne, France). All others chemicals were of analytical-reagent grade.

Antisera

Antisera to dexamethasone were raised in New Zealand rabbits by immunization with dexamethasone-21 hemisuccinate–bovine serum albumin conjugates using conventional techniques[28,29]. Immunoglobulins were precipitated by ammonium sulphate, redissolved in phosphate-buffered saline (10 m*M* sodium phosphate, 150 m*M* sodium chloride, pH 7.0) and submitted to gel filtration on an *Ultrogel* ACA 202 column equilibrated in the same buffer. The excluded fractions containing the immunoglobulins were pooled and used for further purification by HPLAC.

Derivatization of the column

A preliminary derivatization experiment was performed using the Ultraffinity-EP column capacity kit. Eight micromoles of [^{3}H]dexamethasone (specific activity 2 mCi mmol^{-1}) were dissolved in 1.6 ml of acetonitrile–water (96:4) and added to the vial containing the activated silica (106 mg), which was then placed on a rotating shaker for 40 h at 37°C. After careful washing with acetonitrile and tetrahydrofuran, the amount of silica-bound steroid was assayed by scintillation counting of an aliquot of the derivatized gel. Deactivation of the remaining epoxy functions was obtained by incubation of the gel with 10% glycerol in 1 *M* phosphate buffer (pH 7.0) as recommended by the manufacturer.

The Ultraffinity-EP column was connected to a Beckman Model 110B solvent-delivery system. An 11.5-μmol amount of [^{3}H]dexamethasone (specific activity 1.75 mCi mmol^{-1}) was dissolved in 15 ml of acetonitrile–water (98:2), the solution was degassed by sonication and pumped through the column at a 0.2 ml min^{-1} (closed circuit) as recommended by the manufacturer. The column was immersed in a water-bath thermostated at 45°C and the derivatization was carried on for 40 h. The column was then washed with 70 ml of acetonitrile at room temperature. The amount of steroid bound to the column was obtained from differential assay of the radioactivity in the coupling solution before and after derivatization. The column was then equilibrated in an aqueous solvent after several intermediary washing steps with acetonitrile–water mixtures of increasing water content. The remaining epoxy functions were deactivated by pumping 20 ml of 0.5 *M* mercaptoethanol in 0.1 *M* potassium phosphate (pH 6.5) overnight (closed circuit) at 0.23 ml min^{-1}. The column was then washed with water containing 0.02% sodium azide.

Affinity chromatography procedure

Apparatus. The HPLAC system consisted of a Beckman 1108 solvent-delivery system, a Waters U6K injector equipped with a 10-ml sample loop, an Altex 210A sample injection valve used as a column-switching valve and fitted with the Ultraffinity-EP column in place of the sample loop, a Beckman Model 160 absorbance detector connected to a recorder and a programmable Gilson Model 201 fraction collector. The detection wavelength was 280 nm. The Altex 210A valve was used to direct the eluent either to the column (in the "inject" position) or to a column bypass (in the "load" position).

Adsorption. The γ-globulin samples were filtered on a 0.22 μm Millex GS filter (Millipore) before use and injected at room temperatrure and at a definite flow-rate (0.2–1.0 ml) in the column previously equilibrated in phosphate-buffered saline. The absorbance at 280 nm was recorded and 0.8-ml fractions were collected and assayed for [^{3}H]dexamethasone specific binding activity. As soon as the absorbance began to decrease, the flow-rate was increased to 2 ml min^{-1} and washing with phosphate-buffered saline was continued until a complete return to the baseline was achieved.

Elution. The column was rapidly isolated from the solvent line by switching the Altex valve, and was immersed in a tank filled with ice. The solvent line and the detector were then rapidly equilibrated at 0°C with the eluting buffer, 6.7 m*M* sodium citrate–6.7 m*M* sodium phosphate–11.4 m*M* sodium borate–20% acetonitrile (pH 3.1). Once the new baseline had been obtained the flow-rate was reduced to 0.2 ml min^{-1} and the column was switched on the line. Fractions of 0.4 ml were collected in tubes already containing 0.1 ml of 1 *M* potassium phosphate buffer (pH 8.5). In some instances a stopped-flow elution was performed, which consisted in diverting the flow around the column as soon as the phosphate-buffered saline was replaced in the column by the acidic strong eluting buffer. Then stopped-flow elution was continued in the column and after a few minutes the column was switched on the solvent line again and the eluate was collected.

Antibody detection assay

Rabbit antisera, ammonium sulphate fractionated immunoglobulins and affinity chromatography effluents were submited to the appropriate dilution with phosphate-buffered saline and 0.4-ml aliquots were incubated in duplicate at 0–4°C with 30 n*M* [^{3}H]dexamethasone. After 17 h the bound radioactivity was determined in duplicate by charcoal adsorption assay[30]. The non-specific binding was measured by incubating parallel samples in the presence of a 1000-fold excess of non-radioactive dexamethasone.

Miscellaneous

The Coomassie blue assay of Bradford[31] modified according to Read and Northcote[32] was used for protein determination, using purified rabbit immunoglobulins G as standard. Radioactivity was measured in an Intertechnique SL 4000 liquid spectrometer, using Aqualyte (Baker Chemicals, Deventer, The Netherlands) as scintillation cocktail (35% tritium efficiency).

Fig. 1. Covalent binding of dexamethasone to epoxy-silica beads.

RESULTS AND DISCUSSION

Coupling of dexamethasone to the epoxy silica

The Ultraffinity-EP column and kit contained an already activated silica, pore size 300 Å and particle diameter 10 μm, bearing epoxide functions. Dexamethasone appeared to react fairly easily in an organic solvent with this activated silica to yield a covalent linkage, probably by reaction of the epoxide with the primary 21-OH group of the steroid, the reactivity of which was probably increased by the adjacent 20-keto group (Fig. 1). However, we observed a striking difference in the coupling efficiencies of dexamethasone to the ultraffinity-EP column capacity kit and to the ultraffinity-EP column itself. With the kit the silica-bound dexamethasone was only 0.21 μmol per gram of silica, whereas it reached 4.1 μmole with the column. Both the coupling procedures were performed on the same activated silica, bearing 69.1 μmol of titratable epoxy groups according to the manufacturer, using the same solvent and coupling time at only slightly different temperatures. The much higher derivatization yield obtained with the column procedure probably resulted from the fact that, using the closed circuit process, the coupling solution was repeatedly forced through the compacted silica and penetrated all the pores of the beads, whereas this certainly occurred to a far lesser extent using the batch procedure. To obtain improved results with the latter process would probably require working at higher temperature, with sonication of the beads during the coupling step. On the other hand, the ligand substitution level obtained on the column was in the micromolar range already reported by others[1,12,13] and was considered satisfactory for our purpose.

Purification of antidexamethasone antibodies on the dexamethasone column

The overall results of six successive purification experiments performed on the column starting from various dexamethasone antisera samples obtained from the same rabbit are summarized in Table I. The column performances may be discused with respect to both the adsorption and elution steps.

Adsorption step. Adsorption appeared to be very rapid, as shown in experiment 1 where 89% of the specific immunoglobulins present in the sample were retained on the column injected at a 1 ml min^{-1} flow-rate, *i.e.*, a contact time with the stationary

phase of only 30 s (the 50 × 4.6 mm I.D. column corresponded to a total solvent diffusion volume of about 0.5 ml). This result was in excellent agreement with preliminary experiments performed with the column capacity kit (data not shown). The maximal capacity of the column was tested by injecting large sample amounts in order to saturate the affinity matrix in experiments 2 and 3. This maximal capacity was in the range 0.7–1.0 mg of specific immunoglobulins, *i.e.*, 4–7 nmol, for a column of total volume 0.83 ml, or 4.8–8.4 nmol per ml of gel. This capacity was very similar to that obtained after a longer adsorption time on a conventional "soft" gel (of the agarose type) derivatized with dexamethasone in a similar fashion (data not shown).

The rapidity of the antibody binding on the dexamethasone–silica is illustrated in Fig. 2, which shows the residual specific antidexamethasone activity found in the effluent of the affinity column during the injection in experiment 3. It can be seen that the first fraction was almost totally cleared of specific antidexamethasone antibodies and that the column then became rapidly saturated, as the subsequent effluent displayed the same antidexamethasone activity as the injected sample. Here, the injection could have been stopped from the fourth fraction and a sample load reduced to 3 ml would have yielded the same gel saturation as the 6 ml used. Moreover, the column washing procedure used between two sucessive injections appeared to be of utmost importance in order to obtain reproductible purification. In our first attempts we used only a simple washing with 6 *M* urea, a procedure that appeared almost satisfactory during our routine purification procedure using a conventional agarose

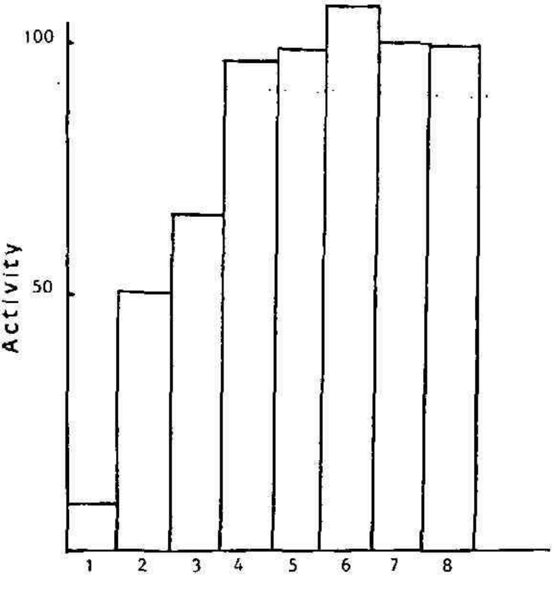

Fig. 2. Residual dexamethasone specific binding activity in the effluent of the dexamethasone derivatized column (50 × 4.6 mm I.D.) during the injection of a 6-ml sample of rabbit antidexamethasone antibodies. Flow-rate, 0.2 ml min^{-1}. Fractions of 0.8 ml were collected and assayed for [^{3}H]dexamethasone binding. Results are expressed as a percentage of the binding activity of the injected sample.

TABLE I

COMPARATIVE DATA OF SIX SUCCESSIVE PURIFICATION EXPERIMENTS PERFORMED ON THE SAME 50 × 4.6 mm I.D. COLUMN CONTAINING DEXAMETHASONE DERIVATIZED ULTRAFFINITY-EP SILICA BEADS

Antidexamethasone antiserum pre-fractionated by ammonium sulphate precipitation was loaded on the column. Between two successive experiments the column was washed with 6 *M* urea, except after experiment 5, where an additional wash with 0.1 *M* acetic acid containing 2 *M* NaCl and then with formic acid and propanol was used.

Step	*Parameter*	*Experiment*					
		1	*2*	*3*	*4*	*5*	*6*
Adsorption	Amount of specific immunoglobulins present in the sample (μg)	275	42 768	2864	1061	1261	4303
	Sample volume (ml)	4	27	6	3	5.8	10
	Injection flow-rate (ml min^{-1})	1	1	0.2	0.5	0.4	0.5
	Amount of specific immunoglobulins adsorbed (μg)	225	1029	686	281	230	673
	Adsorption yield (% of injected specific material)	89	2.4	24	26	18.2	15.6
Elution	Total amount of active specific immunoglobulins eluted (μg)	71	324	266	40	70	202
	Elution yield (% of adsorbed Ig)	32	31.5	39	14	30	30
	% of the total eluted immunoglobulins found in the main 0.5-ml eluate fraction	65.4	49.7	48.3	51	52	37
	Apparent homogeneity of the purified immunoglobulins (%)	51	27	86	—	—	—

TABLE II

QUANTITATIVE DATA CONCERNING THE ELUTION OF EXPERIMENT 3 (SEE TABLE I), DETAILED IN FIG. 2

The concentration of the active specific antidexamethasone immunoglobulins was calculated from the [^{3}H]dexamethasone binding activity assayed in saturating conditions. Total protein content was assayed by the Coomassie blue method.

Fraction	*Volume (ml)*	*Protein ($\mu g\ ml^{-1}$)*	*Binding activity (cpm · 10^{-6} ml^{-1})*	*Active specific immunoglobulins*		*Eluted activity (% of the total)*	*Specific activity (cpm · 10^{-6} per μg of protein)*	*Apparent homogeneity (%)*	*Purification (fold)*
				$\mu g\ ml^{-1}$	*Total μg*				
Starting serum extract	6	5200	229.6	477	2864	–	0.044	9.2	1
Affinity eluate fractions:									
1–3	1.5	0	0	0	0	0	0	0	0
4	0.5	56	21.5	45.4	22.7	8.3	0.384	80	8.7
5	0.5	82	34.8	73.2	36.6	13.6	0.424	88	9.6
6	0.5	92	42.0	88.3	44.2	16.4	0.456	96	10.4
7	0.5	302	123.6	260.0	130.0	48.3	0.409	86	9.3
8	0.5	82	29.3	61.6	30.8	11.4	0.357	75	8.1
9	0.5	29	5.2	10.9	5.5	2.0	0.179	38	4.1
Total					270	100			

gel. However, with the silica matrix this washing procedure was insufficient, especially after the massive loads applied in experiments 2 and 3, and this could explain the relative decrease in performance in terms of column capacity observed during experiments 4 and 5, where, according to the sample load, we expected to retain a larger amount of immunoglobulins on the column. However, an additional wash of the column using 0.1 *M* acetic acid containing 2 *M* sodium chloride and then 0.1 *M* propionic acid–propanol allowed the column capacity to be restored to its previous value (experiment 6). In our opinion, the column was probably contaminated by hydrophobic components adsorbed on the hydrophobic steroid stationary phase. The relatively high derivatization level of the matrix, in comparison with the agarose gel, reinforced this hypothesis.

Elution. Non-biospecific elution was obtained with an almost aceptable yield (30–39%), owing to the drastic physico-chemical conditions used (acidic buffer of pH 3.1, containing 20% acetonitrile). The severity of these conditions was required because of the high affinity for dexamethasone displayed by the γ-globulins to be purified. The same buffer supplemented with acetonitrile has already been used with success by others for the purification by affinity chromatography of anticortisol antibodies[33]. The elution yield obtained appeared to be reproducible, except in experiment 4 where its low value could perhaps be ascribed to the inadequate column washing which had already hindered the adsorption step in this trial. In each exper-

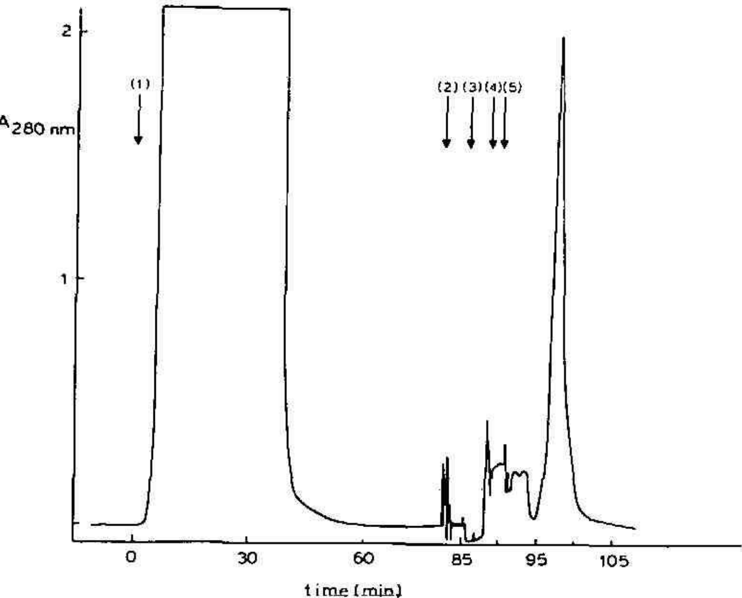

Fig. 3. Absorbance at 280 nm recorded during the purification experiment 3 (see Table 1). A 6-ml sample containing the antidexamethasone immunoglobulins to be purified was injected on to the column at 0.2 ml min^{-1} and 20°C (1). The column was washed with phosphate buffer saline at 1 ml min^{-1}, then switched off the solvent line to a stopped-flow position (2) and immersed in an ice-bath, whereas the solvent line was rapidly equilibrated with the cold acidic elution buffer at 4 ml min^{-1}. The flow-rate was then reduced to 0.2 ml min^{-1} and the column switched to the line (3), 0.4-ml fractions being collected. After 4 min the column was switched to the stopped-flow position (4) for 2 min and then switched to the line again (5).

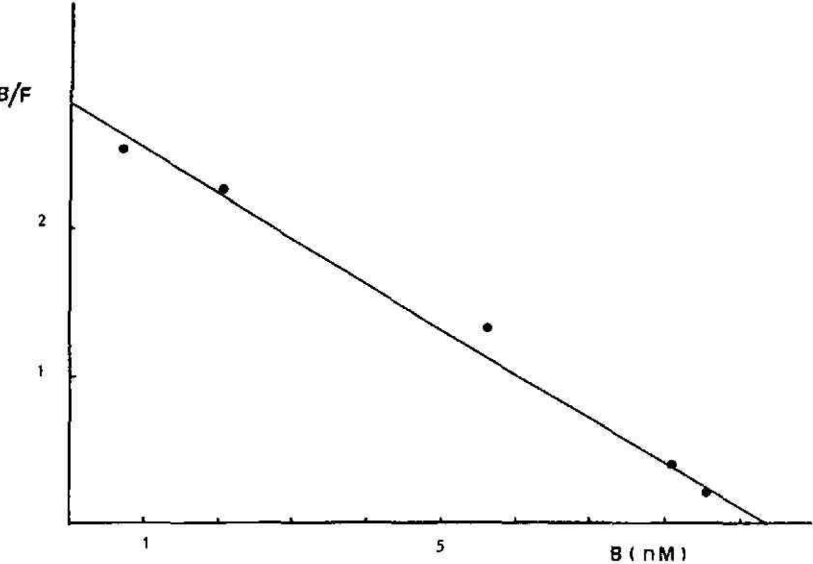

Fig. 4. Scatchard plot obtained by incubating the affinity-purified antidexamethasone immunoglobulins (from experiment 2 in Table I) with various concentrations of [^{3}H]dexamethasone in the nanomolar range for 16 h at 0°C. B, Concentration of antibody specifically bound [^{3}H]dexamethasone; F, concentration of the free [^{3}H]dexamethasone. Separation of bound from free steroid was performed by charcoal assay.

iment it could be assessed that the missing specific antibodies were either still retained on the column or already eluted, but in a denatured form that could not be restored to an active form by the rapid readjustment of the pH of the collected fractions. The assay of the protein content of each fraction and the determination of its specific activity helped to clarify the choice between these two hypotheses.

Indeed, when calculated on the basis of the antidexamethasone specific activity (with the assumption that we were dealing only with immunoglobulin G displaying a molecular weight of 150 kD and able to bind two molecules of dexamethasone per molecule of antibody), the level of homogeneity of the purified immunoglobulins was very satisfactory, as in experiment 3 the eluate was made of almost pure active anti-dexamethasone antibodies (86% apparent homogeneity). The high purity of the antibodies obtained is significant, and appeared impressive with regard to the non-specific mode of elution used here. Table II gives the detailed results of this experiment, the absorbance line at 280 nm of which is depicted in Fig. 3. We used chromatographic conditions similar to those used by Muller and Carr[7], using a configuration that allowed us to switch the column to the solvent line or to isolate it in a stopped-flow position. This configuration appeared very useful in saving time and allowing, if required, stopped-flow elution. The elution peak was obtained rapidly in a concentrated form (260 μg of specific immunoglobulins per ml of eluate in fraction 7), clearly more concentrated (4–6-fold more) than that usually obtained with a conventional agarose gel (data not shown). Generally as high as 50% of all the eluted material was found in a single 0.5-ml fraction (see Table I). The more efficient diffusion of the eluting solution through the pores of a "high-performance" stationary

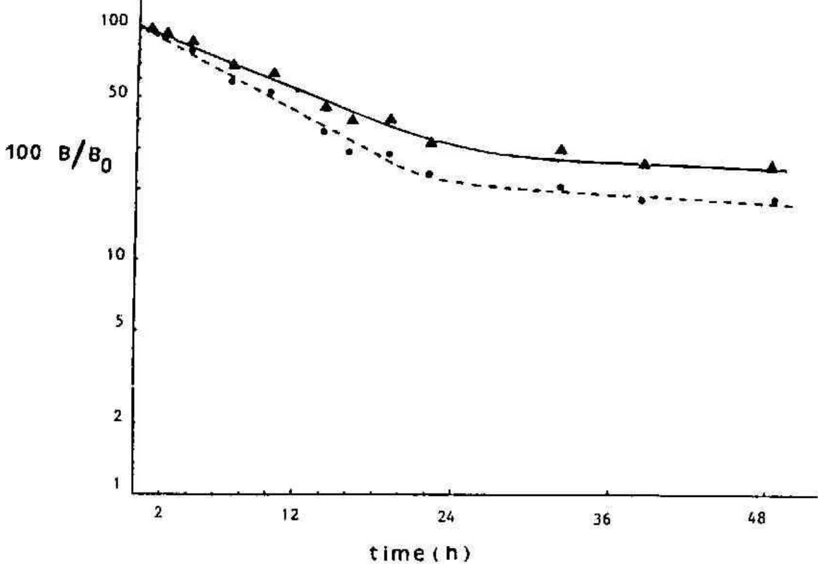

Fig. 5. Dissociation kinetics of both crude antidexamethasone antiserum (●) and of the affinity-purified γ-globulins (▲). Each sample was pre-incubated with 30 n*M* [^{3}H]dexamethasone and then supplemented with a 500-fold excess of unlabelled dexamethasone. The evolution of the [^{3}H]dexamethasone binding activity B was followed by periodic charcoal assay and expressed as a percentage of the initial activity B_0 assayed just before the addition of the unlabelled steroid.

phase made of rigid beads of small diameter probably explains this result. Moreover, the HPLC hardware used in the system precluded any post-column dilution effects, which were more difficult to avoid when handling conventional agarose gels and columns devices.

Characterization of the affinity-purified antidexamethasone antibodies

A crucial point to be checked after a purification by affinity chromatography using a drastic non-specific elution procedure is the biological quality of the eluted material. We therefore measured the binding parameters of the purified antibodies towards tritiated dexamethasone and these parameters were then compared with those of the starting antisera. The dissociation constant assayed at equilibrium using a Scatchard plot (Fig. 4) was roughly the same before and after purification (3.0 n*M* *versus* 3.5 n*M*). Moreover, the dissociation rate constant measured by isotopic dilution was significantly lower for the purified immunoglobulins than for the starting antiserum, corresponding to a half-life 1.4-fold longer in the former instance (Fig. 5). Hence the affinity chromatography step seemed to have allowed isolation from various polyclonal antidexamethasone immunoglobulins present in the plasma of the rabbit, if not the most avid then at least those forming the most stable complexes with the steroid. This constitutes a very interesting result and was one of the aims of the study.

CONCLUSIONS

Dexamethasone has been shown to bind covalently to commercial activated silica beads bearing epoxide functions. The resultant chromatographic adsorbent appeared to be suitable for the rapid purification of antidexamethasone antibodies. The yield and the level of purification were excellent. HPLAC compared very favorably with conventional affinity chromatography on agarose beads and allowed a more rapid purification of small amounts of purified immunoglobulins, obtained in a very small volume. However, the purification factor required for complete purification in the application reported here was relatively modest (11-fold for the affinity step). It would be of great interest to see if HPLAC would be a satisfactory alternative in more difficult purification problems such as that of cellular steroid receptors, for which we recently described an improved purification by affinity chromatography on a dexamethasone-bearing agarose gel[34].

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REFERENCES

1 S. Ohlson, L. Hansson, P. O. Larsson and K. Mosbach, *FEBS Lett.*, 93 (1978) 5.
2 P. O. Larsson, *Methods Enzymol.*, 104 (1984) 212.
3 K. Nilsson and P. O. Larsson, *Anal. Biochem.*, 134 (1983) 60.
4 R. R. Walters, *J. Chromatogr.*, 249 (1982) 19.
5 V. Kasche, K. Buchholz and B. Galunsky, *J. Chromatogr.*, 216 (1981) 169.
6 A. J. Muller and P. W. Carr, *J. Chromatogr.*, 284 (1984) 33.
7 A. J. Muller and P. W. Carr, *J. Chromatogr.*, 294 (1984) 235.
8 J. R. Sportsman and G. S. Wilson, *Anal. Chem.*, 52 (1980) 2013.
9 H. E. Swaisgood and I. M. Chaiken, *J. Chromatogr.*, 327 (1985) 193.
10 M. Glad, S. Ohlson, L. Hansson, M.-O. Månsson and K. Mosbach, *J. Chromatogr.*, 200 (1980) 254.
11 A. Borchert, P.-O. Larsson and K. Mosbach, *J. Chromatogr.*, 244 (1982) 49.
12 C. R. Lowe, M. Glad, P.-O. Larsson, S. Ohlson, D. A. P. Small, T. Atkinson and K. Mosbach, *J. Chromatogr.*, 215 (1981) 303.
13 D. A. P. Small,. T. Atkinson and C. R. Lowe, *J. Chromatogr.*, 216 (1981) 175.
14 D. A. P. Small, T. Atkinson and C. R. Lowe, *J. Chromatogr.*, 248 (1982) 271.
15 D. A. P. Small, T. Atkinson and C. R. Lowe, *J. Chromatogr.*, 266 (1983) 151.
16 E. Hagemeier, K.-S. Boos, E. Schlimme, K. Lechtenbörger and A. Kettrup, *J. Chromatogr.*, 268 (1983) 291.
17 L. Hansson, M. Glad and L. Hansson, *J. Chromatogr.*, 265 (1983) 37.
18 S. C. Crowley and R. R. Walters, *J. Chromatogr.*, 266 (1983) 157.
19 R. Ledger and E. Stellwagen, *J. Chromatogr.*, 299 (1984) 175.
20 T. M. Phillips, N. S. More, W. D. Queen, T. V. Holohan, N. C. Kramer and A. M. Thompson, *J. Chromatogr.*, 317 (1984) 173.
21 T. M. Phillips, N. S. More, W. D. Queen and A. M. Thompson, *J. Chromatogr.*, 327 (1985) 205.
22 T. M. Phillips, W. D. Queen, N. S. More and A. M. Thompson, *J. Chromatogr.*, 327 (1985) 213.
23 J. N. Kinkel, B. Anspach, K. K. Unger, R. Wieser and G. Brunner, *J. Chromatogr.*, 297 (1984) 167.
24 R. F. Taylor and I. G. Marenchic, *J. Chromatogr.*, 317 (1984) 193.

25 S. K. Roy, D. V. Weber and W. C. McGregor, *J. Chromatogr.*, 303 (1984) 225.
26 S. K. Roy, W. C. McGregor and J. T. Orichowskyj, *J. Chromatogr.*, 327 (1985) 189.
27 M. E. Rogers, M. W. Adlard, G. Saunders and G. Holt, *J. Chromatogr.*, 326 (1985) 163.
28 M. Hichens and A. F. Hogans, *Clin. Chem.*, 20 (1974) 266.
29 G. E. Abraham and P. K. Grover, in W. D. Odell and W. Daughaday (Editors), *Principles of Competitive Protein Binding Assay*, J. B. Lippincott, Philadelphia, 1971, pp. 140–157.
30 G. E. Abraham, F. S. Manlimos and R. Garza, in G. E. Abraham (Editor), *Handbook of Radioimmunoassay*, Marcel Dekker, New York, 1977, pp. 591–656.
31 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
32 S. M. Read and D. H. Northcote, *Anal. Biochem.*, 116 (1981) 53.
33 J. G. Lewis and P. A. Elder, *J. Steroid Biochem.*, 22 (1985) 387.
34 T. Idziorek, P. Formstecher, P. M. Danzé, B. Sablonnière, P. Lustenberger, C. Richard, V. Dumur and M. Dautrevaux, *Eur. J. Biochem.*, 153 (1985) 65.

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HIGH-PERFORMANCE CHROMATOGRAPHIC METHOD FOR THE PURIFICATION OF TISSUE-TYPE PLASMINOGEN ACTIVATOR

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SUMMARY

High-performance affinity chromatography was performed on five ligand-bound columns in an attempt to purify tissue-type plasminogen activator (t-PA), which is a glycoprotein with a high affinity for fibrin and also has two Kringle structures and finger-domain in its molecule. The five columns were concanavalin A-5PW, *p*-aminobenzamidine-5PW, imidinodiacetic acid-5PW, boric acid-5PW and lysine-5PW. All five were able to rapidly separate t-PA from contaminating proteins, with high resolution and recovery.

INTRODUCTION

Recently there has been great progress in the study of blood fibrinolysis[1–3]. A new plasminogen activator (tissue-type plasminogen activator, t-PA), which differs from urinary plasminogen activator (urokinase, UK), has been found to induce plasminogen activation on the surface of fibrin. Formerly, t-PA was purified from tissue homogenates of the heart, ovary or uterus by precipitation with ammonium sulphate, acid precipitation or gel filtration[4–6]. However, the development of affinity chromatography has made it easy and convenient to purify t-PA[7]. Further, the introduction of tissue culture medium as a starting material has promoted the use of high-performance liquid chromatography (HPLC) for rapid separation because of the low protein contamination.

In the present study, affinity chromatography with HPLC was employed for the purification of t-PA from melanoma (Bowes) tissue culture medium.

MATERIALS AND METHODS

The following high-performance affinity chromatography columns were prepared with TSK gel G5000PW, which is a hydrophilic resin-based material of large pore size for high-performance gel filtration, with a particle diameter of 10 μg: (1) concanavalin A-5PW; (2) *p*-aminobenzamidine-5PW; (3) iminodiacetic acid-5PW; (4) boric acid-5PW; (5) lysine-5PW. All affinity chromatographic measurements were

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carried out with a computer-controlled multipump (CCPM) at a flow-rate of 1.0 ml/min and with a variable wavelength UV detector (Toyo Soda Kogyo). Proteins were usually detected at 280 nm, unless stated otherwise. The ligands were concanavalin A (Seikagaku Kogyo, Tokyo), *p*-aminobenzamidine dihydrochloride (Sigma, St. Louis, MO, U.S.A.), iminodiacetic acid (Tokto Kasei, Tokyo), *m*-aminophenylboronic acid hemisulphate (Aldrich, Milwaukee, WI, U.S.A.) and L-lysine hydrochloride (Nakarai Kagaku, Kyoto).

The equilibration buffer and elution buffer were (1) 50 m*M* Tris–HCl (pH 7.5) containing 0.01% Tween 80; 0.4 *M* methyl α-D-mannopyranoside and 0.6 *M* potassium thiocyanate dissolved in the equilibration buffer for concanavalin A-5PW; (2) 50 m*M* Tris–HCl (pH 7.5) containing 0.01% Tween 80; 1 *M* potassium thiocyanate dissolved in the equilibration buffer for *p*-aminozamidine-5PW; (3) 50 m*M* Tris–HCl (pH 8.0) containing 0.5 *M* sodium chloride and 0.01% Tween 80; 0.1 *M* glycine dissolved in the equilibration buffer for iminodiacetic acid-5PW; (4) 50 m*M* Tris–HCl (pH 8.5) containing 50 m*M* magnesium chloride and 0.01% Tween 80; 0.4 *M* methyl α-D-mannopyranoside and 0.6 *M* potassium thiocyanate dissolved in the equilibration buffer for boric acid-5PW; (5) 50 m*M* Tris–HCl (pH 7.5 containing 0.01% Tween 80; 0.2 *M* arginine and 0.8 *M* potassium thiocyanate dissolved in the equilibration buffer for lysine-5PW.

The t-PA activity was measured by the fibrin-film method using plasminogen-rich fibrinogen (bovine, Organon Teknika) and thrombin (bovine, Mochida Pharmaceuticals)[8].

The purity of proteins was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Weber and Osborn[9]. The proteins in the gels were visualized by silver staining[10].

The molecular weight and plasminogen activator activity were measured simultaneously using electrophoretic enzymography[11]. A fibrin film was produced in polyacrylamide gel, and the position of the plasminogen activator appearing as a clear zone in the fibrin film yielded the molecular weight.

Tissue culture of melanoma (Bowes) was performed as described elsewhere[12], and the monolayered cells were cultured with essential Eagle medium for harvesting. In the conventional method, t-PA was purified from tissue culture medium according to the methods of Rijken and Collen[13]. Human urokinase was purified according to Holmberg *et al.*[14], and had molecular weights of 53000 and 33000.

The activity of t-PA was expressed in international units using standard t-PA (Lot 83/517)[15].

RESULTS AND DISCUSSION

Elution profile of t-PA by high-performance affinity chromatography

Since t-PA is a glycoprotein, high-performance chromatography on concanavalin A-5PW (75 mm × 7.5 mm I.D.) was carried out as follows. After equilibrating the column with 50 m*M* Tris–HCl (pH 7.5) containing 0.01% Tween 80, 1 ml of melanoma (Bowes) tissue culture medium was injected. First the unbound protein was eluted (Fig. 1), and then bound protein was eluted with a linear gradient (0–0.4 *M* methyl α-D-mannopyranoside and 0–0.6 *M* potassium thiocyanate). The t-PA was eluted as a single peak at 0.4 *M* methyl α-D-mannopyranoside and 0.6 *M* potassium

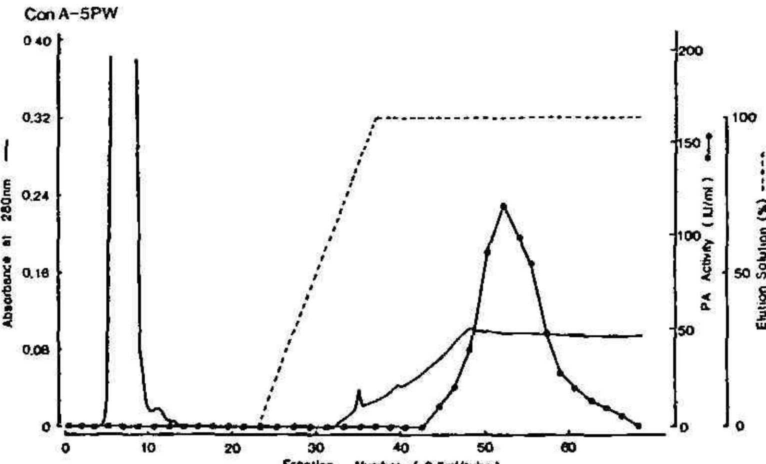


Fig. 1. High-performance affinity chromatography on concanavalin A-5PW. A 1-ml volume of tissue culture medium was applied to concanavalin A-5PW (75 mm × 7.5 mm) and eluted with a linear gradient of methyl α-D-mannopyranoside (0–0.4 *M*) and potassium thiocyanate (0–0.6 *M*).

thiocyanate. All of the t-PA activity was eluted and none was observed in the unbound portion. Thus, the separation of t-PA from other contaminating proteins by high-performance affinity chromatography on concanavalin A-5PW was rapid, with high resolution and recovery.

High-performance affinity chromatography on *p*-aminobenzamidine-5PW (75 mm × 7.5 mm I.D.) was carried out as follows. After equilibrating the column with 50 m*M* Tris–HCl (pH 7.5) containing 0.01% Tween 80, 3 ml of melanoma (Bowes) tissue culture medium were injected. First the unbound protein was eluted, and then

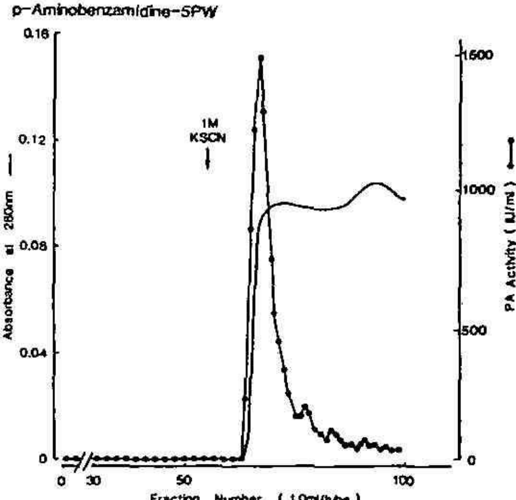


Fig. 2. Elution profile on *p*-aminobenzamidine-5PW. A 3-ml volume of tissue culture medium was applied to *p*-aminobenzamidine-5PW (75 mm × 7.5 mm), washed with 1 *M* sodium chloride buffer solution and eluted with 1 *M* potassium thiocyanate buffer solution.

the column was washed with 1 *M* sodium chloride dissolved in the equilibration buffer. Finally, 1 *M* potassium thiocyanate dissolved in the equilibration buffer was used to elute the bound protein, as a single peak (Fig. 2). All of the t-PA activity was eluted and none was observed in the unbound portion or in the portion eluted with 1 *M* sodium chloride solution. Thus, the separation of t-PA from other contaminating proteins by high-performance affinity chromatography on *p*-aminobenzamidine-5PW was rapid, with high resolution and recovery.

Using two different high-performance metal chelate affinity chromatography columns, an iminodiacetic acid column and a boric acid column, purification of t-PA was carried out as follows. The iminodiacetic acid column (75 mm × 8.0 mm I.D.) was equilibrated with 50 m*M* Tris–HCl (pH 8.0) containing 0.5 *M* sodium chloride and 0.01% Tween 80, and 20 ml of zinc chloride (5 mg/ml) were applied to the column. This amount of zinc chloride was sufficient to saturate the column with zinc ion. After the column had been re-equilibrated with the initial buffer, 30 ml of melanoma (Bowes) tissue culture medium were applied. The unbound protein passed through first, and the bound protein was then eluted with a linear gradient of glycine (0–0.1 *M*). As shown in Fig. 3, the bound protein was eluted at a glycine concentration of 0.067 *M*. The t-PA was eluted as two peaks: the first peak appeared at a glycine concentration of 0.083 *M*, just after the protein peak; the second and major peak appeared at a glycine concentration of 0.1 *M*. All of the t-PA activity was eluted and none was observed in the unbound portion. Thus, the separation of t-PA from other contaminating proteins was effectively achieved by high-performance iminodiacetic acid affinity chromatography. When boric acid-5PW was used, the column (75 mm × 7.5 mm I.D.) was equilibrated with 50 m*M* Tris–HCl (pH 8.5) containing 50 m*M* magnesium chloride and 0.01% Tween 80, and 1 ml of melanoma (Bowes) tissue culture medium was applied. The unbound protein passed through first, and

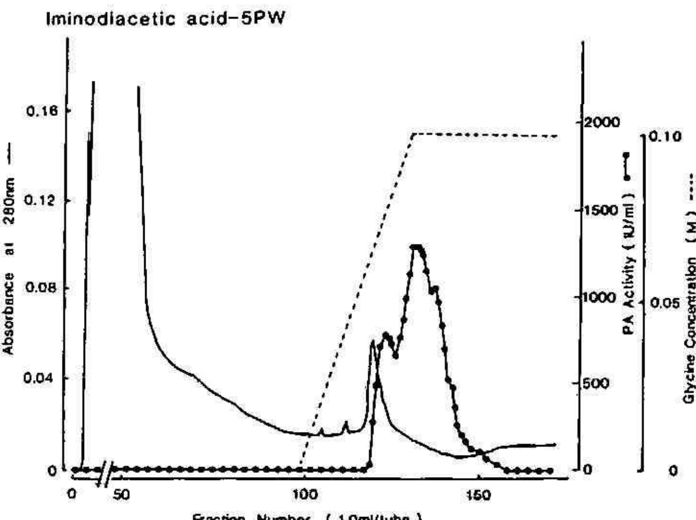


Fig. 3. High-performance affinity chromatography on iminodiacetic acid-5PW. A 30-ml volume of tissue culture medium was applied to iminodiacetic acid-5PW (75 mm × 8.0 mm), which had previously been equilibrated with buffer containing zinc chloride, and eluted with a linear gradient of glycine (0–0.1 *M*).

the bound protein was then eluted with a linear gradient of 0–0.4 *M* methyl α-D-mannopyranoside and 0–0.6 *M* potassium thiocyanate. As shown in Fig. 4, the t-PA was eluted as a single peak and no activity was observed in the unbound portion. Thus, high-performance boric acid affinity chromatography is also useful for the separation of t-PA from other contaminating proteins, with high resolution and recovery.

When the separation of t-PA on lysine-5PW was performed, the column (75 mm × 7.5 mm I.D.) was equilibrated with 50 m*M* Tris–HCl (pH 7.5) containing 0.01% Tween 80, and 3 ml of melanoma (Bowes) tissue culture medium were applied. The unbound protein passed through first, and the column was then washed with 0.3 *M* sodium chloride dissolved in the equilibration buffer. The bound protein was eluted with 0.2 *M* arginine and 0.8 *M* potassium thiocyanate. The t-PA was eluted as a single, sharp peak. All of the activity was observed in the eluted portion and none was observed in the unbound portion. Thus, high-performance lysine-5PW affinity chromatography was effective for the separation of t-PA from other proteins in a rapid and convenient manner.

Purification of t-PA

In each of the above experiments, a single-stage affinity chromatography with HPLC was employed to purify t-PA from tissue culture medium. In order to obtain t-PA as a single band in SDS-PAGE, several variations of affinity chromatography were investigated. Among them, the combination of iminodiacetic acid-5PW, concanavalin A-5PW and *p*-aminobenzamidine-5PW was found to be excellent. A 30-ml volume of tissue culture medium (872.7 I.U.) was first applied to iminodiacetic acid-5PW, and the eluted t-PA was then applied to concanavalin A-5PW. On washing the column, the bound t-PA was eluted with 0.4 *M* methyl α-D-mannopyranoside.

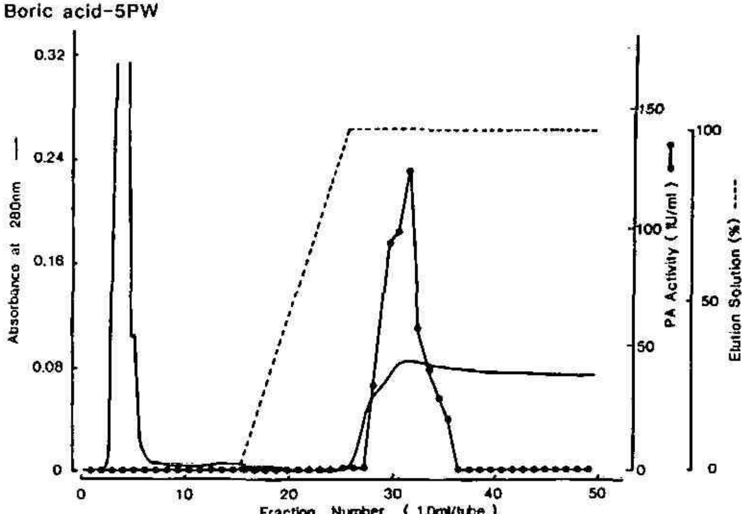
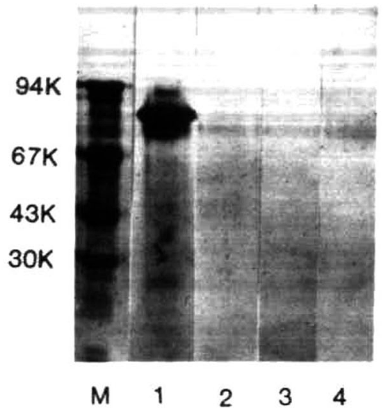


Fig. 4. Elution profile on boric acid-5PW. A 1-ml volume of tissue culture medium was applied to boric acid-5PW (75 mm × 7.5 mm) and eluted with a linear gradient of methyl α-D-mannopyranoside (0–0.4 *M*) and potassium thiocyanate (0–0.6 *M*).

TABLE I

PURIFICATION OF TISSUE-TYPE PLASMINOGEN ACTIVATOR BY HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY

Purification step	*Vol. (ml)*	*Absorbance at 280 nm*		*PA activity*		*Specific activity*	*Purification factor*	*Recovery (%)*
		Per ml	*Total*	*(I.U./ml)*	*(I.U.) Total*			
Medium	30	1.300	39.0	872.7	26179	671.3	1	100
Iminodiacetic acid-5PW	12	0.023	0.276	1280.3	15364	55665	82.6	58.7
Concanavalin A-5PW	8.5	0.017	0.145	1060.2	9011	62149	92.6	34.4
p-Aminobenzamidine-5PW	17.7	0.002	0.035	407.7	7208	205928	306.8	27.5



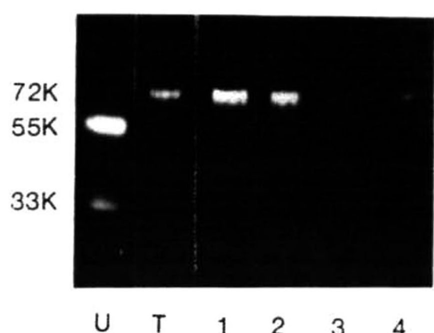


Fig. 5. SDS-PAGE with silver staining of t-PA eluted by high-performance affinity chromatography. 1 = Starting material; 2 = protein eluted from zinc chelate-5PW; 3 = protein eluted from concanavalin A-5PW; 4 = protein eluted from *p*-aminobenzamidine-5PW; M = marker protein.

Fig. 6. Electrophoretic enzymography of the protein eluted by high-performance affinity chromatography. 1–4 as in Fig. 3; U = urokinase; T = t-PA purified from melanoma (Bowes) tissue culture medium by a conventional method[13].

The portion with t-PA activity was applied to *p*-aminobenzamidine-5PW and eluted with 1 *M* potassium thiocyanate buffer solution. The purification factor was about 300, and the recovery rate was 27.5% (Table I). The purity of the final product was confirmed by SDS-PAGE, which upon silver staining revealed a single band with a molecular weight of 72000 (Fig. 5). Electrophoretic enzymography demonstrated a single active band with a molecular weight of 72000 (Fig. 6). The above combination of affinity chromatographies is thus useful for the rapid and convenient purification of t-PA.

REFERENCES

1 O. Matsuo, D. C. Rijken and D. Collen, in P. M. Mannucci and A. D'Angelo (Editors), *Urokinase: Basic and Clinical Aspects*, Academic Press, London, 1982, pp. 55–61.
2 F. Bachmann and E. K. O. Kruithof, *Semin. Throm. Hemostasis*, 10 (1984) 6–17.
3 O. Saksela, *Biochim. Biophys. Acta*, 823 (1985) 35–65.
4 E. R. Cole and F. Bachmann, *J. Biol. Chem.*, 252 (1977) 3729–3737.
5 P. Kok, *Thromb. Haemostasis*, 41 (1979) 718–733.
6 J. G. Corasanti, C. Celik, S. M. Camiolo, A. Mittelman, J. L. Evers, A. Barbasch, G. H. Hobika and G. Markus, *J. Natl. Cancer Inst.*, 65 (1980) 345–351.
7 D. C. Rijken and D. Collen, *J. Biol. Chem.*, 256 (1981) 7035–7041.
8 O. Matsuo, H. Mihara and Y. Rokushima, *Acta Haematol. Jpn.*, 39 (1976) 298–305.
9 K. Weber and M. Osborn, *J. Biol. Chem.*, 244 (1969) 4406–4412.
10 P. Tunón and K. E. Johansson, *J. Biochem. Biophys. Methods*, 9 (1984) 171–179.
11 O. Matsuo, T. Sakai, H. Bando, K. Okada, S. Nakajima, O. Takagi and S. Izaki, *Haemostasis*, 16 (1986) 43–50.
12 O. Matsuo, T. Sakai, C. Matsuo, Y. Nishida, K. Akazawa and H. Mihara, *Acta Physiol. Latinoam.*, 33 (1983) 305–313.
13 D. C. Rijken and D. Collen, *J. Biol. Chem.*, 256 (1981) 7035–7041.
14 L. Holmberg, B. Bladh and B. Åstedt, *Biochim. Biophys. Acta*, 445 (1976) 215–222.
15 P. G. Gaffney and A. D. Curtis, *Thromb. Haemostasis*, 53 (1985) 134–136.

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Note

Gas chromatographic analysis of tautomerizing amidines

Retention indices of N,N'-diphenylformamidines on a non-polar column*

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In the course of our studies on the applicability of gas chromatography to the analysis of amidines[1–6], the relationships between the structure and retention indices of trisubstituted amidines, $R_xN=CR_zN(R_y)_2$, have been investigated. We have found that in the series of amidines containing variable substituent R_x at the imino nitrogen atom the retention indices correlate well with those of corresponding simple model compounds such as substituted hydrocarbons, R_xH, or primary amines, R_xNH_2, taken as standards (Std), and that the correlations with primary amines are of much higher quality[2–4,6]:

$$I(\text{amidine}) = a \cdot I(\text{Std}) + b \tag{1}$$

where I are the Kováts[7,8] retention indices.

In the series of amidines containing variable substituents R_y at the amino nitrogen atom, their retention indices can be correlated with those of corresponding secondary amines, $R_yR'_yNH$. The conclusion was drawn that for the prediction of the retention indices of trisubstituted amidines a diparameter linear regresssion (eqn. 2) can be used[4].

$$I(\text{amidine}) = a_1 \cdot I(R_xNH_2) + a_2 \cdot I(R_yR'_yNH) + b \tag{2}$$

It was also found that, in most instances, the regression coefficients a are different from unity[2–4,6] and that they depend to some extent on the substituent R_z at the amidino carbon atom.[3] Therefore, the use of additivity rules may lead to erroneous results in many instances.

The above results concerned trisubstituted amidines, but N,N'-disubstituted amidines display tautomerism[9,10]:

$$R_xN=CR_zNHR_y \rightleftharpoons R_xNHCR_z=NR_y \tag{3}$$

* Amidines, Part XXVII; for Part XXVI, see ref. 6.

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If the substituents R_x and R_y are not identical, the tautomers have different physical and chemical characteristics[9,11,12], so it would be expected that their retentions would also be different. On the other hand, it is known[9,10,13,14] that such amidines may form hydrogen-bonded cyclic dimers (I). It might be expected that the formation of the dimers would cause an increase in boiling points and a consequent increase in retention indices.

R_x, R_y, R_z, N, H, C

I

Thus the question arose of whether a diparameter regression of the type in eqn. 2 can be applied for the prediction of the retention indices of N,N'-disubstituted amidines, and how far the regression parameters differ from those for trisubstituted amidines. The value of *a* and *b* in eqn. 2 may serve as an indication of whether amidines in the gas phase exist as monomeric species or as hydrogen-bonded dimers, analogous to those of carboxylic acids.

In this work the retention indices of 38 disubstituted N,N'-diphenylformamidines were determined on a non-polar GE SE-30 column. The compounds investigated have the general formula II.

$$[XC_6H_4N{=}CHNHC_6H_4Y \rightleftharpoons XC_6H_4NHCH{=}NC_6H_4Y]$$

II

The substituent X and Y are listed in Table I.

EXPERIMENTAL

Materials

N,N'-Diphenylformamidines were synthesized in our laboratory according to known procedures[15–17]. C_{12}–C_{24} *n*-alkanes were purchased from Applied Science Labs. (State College, PA, U.S.A.).

Gas chromatography

A Chromatron Model GCHF 18.3.4 gas chromatograph (VEB Chromatron, Berlin, G.D.R.) equipped with a flame ionization detector and 1 m × 3 mm I.D. column filled with 15% GE SE-30 silicone gum rubber on Chromosorb W AW (60–80 mesh) was used. The column temperature was maintained at 240°C. The carrier gas (nitrogen) flow-rate was 25 ml/min. Samples of approximately 0.1 *M* solutions in methanol (or pentane for hydrocarbons) were injected by means of a 10-μl Hamilton syringe.

Retention indices and dead times were determined by regression analysis by the method of Grobler and Bálizs[18], as improved by Haken *et al.*[19], and modified

by us to permit the use as standards of a series containing only *n*-alkanes with an even number of carbon atoms. The retention times were recorded by means of a Cobrabid (Poland) KB 5503 electronic integrator with an accuracy of 0.5 s.

RESULTS AND DISCUSSION

The retention indices of the N,N'-diphenylformamidines with confidence intervals at a significance level of 0.05, calculated from at least five measurements, are given in Table I. As the retention index depends to a small extent on temperature, we determined the retention indices at the same temperature as for previously studied trisubstituted formamidines[4], *i.e.*, 240°C. Under these conditions only one peak was observed for each unsymmetrically substituted ($X \neq Y$) N,N'-diphenylformamidine, with no evidence that the two tautomers have different retentions.

We correlated the retention indices of the N,N'-diphenylformamidines with those of corresponding anilines[2]. Calculations were made by means of the least-squares method. The regression coefficients a and b with confidence intervals calculated at a significance level of 0.05 and the estimators of correlation, *i.e.*, the correlation coefficient (r) and Exner's ψ function[20], are given in Table II.

The regression parameters are very close to those calculated for the series of trisubstituted N^1,N^1-dialkyl-N^2-phenylformamidines[4], limited to compounds containing the same set of substitutents X on the phenyl ring:

$$I(\text{amidine}) = (1.078 \pm 0.059) \cdot I(R_xNH_2) + (1.080 \pm 0.035) \cdot I(R_yR'_yNH) - 127.7 \quad (2a)$$

TABLE I

RETENTION INDICES OF N,N'-DIPHENYLFORMAMIDINES ON A GE SE-30 NON-POLAR COLUMN AT 240°C

X	*Y*	*I*	*X*	*Y*	*I*
H	H	1933 ± 1	*p*-OCH_3	*p*-Br	2543 ± 1
H	*p*-CH_3	2122 ± 3	*p*-OC_2H_5	*p*-OC_2H_5	2619 ± 5
H	*p*-OCH_3	2266 ± 3	*p*-OC_2H_5	*p*-Cl	2509 ± 5
H	*p*-OC_2H_5	2319 ± 3	*p*-Cl	*p*-Cl	2419 ± 2
H	*p*-Cl	2215 ± 2	*p*-Cl	*p*-Br	2535 ± 1
H	*p*-Br	2321 ± 4	*p*-Br	*p*-Br	2633 ± 2
H	*m*-CH_3	2095 ± 2	*m*-CH_3	*m*-CH_3	2180 ± 2
H	*m*-OCH_3	2230 ± 1	*m*-CH_3	*m*-OCH_3	2315 ± 1
H	*m*-OC_2H_5	2291 ± 3	*m*-CH_3	*m*-OC_2H_5	2384 ± 4
H	*m*-Cl	2200 ± 3	*m*-CH_3	*m*-Cl	2296 ± 2
H	*m*-Br	2294 ± 2	*m*-CH_3	*m*-Br	2404 ± 8
p-CH_3	*p*-CH_3	2199 ± 4	*m*-OCH_3	*m*-OCH_3	2477 ± 3
p-CH_3	*p*-OCH_3	2326 ± 1	*m*-OCH_3	*m*-OC_2H_5	2550 ± 3
p-CH_3	*p*-OC_2H_5	2409 ± 3	*m*-OCH_3	*m*-Cl	2458 ± 0
p-CH_3	*p*-Cl	2304 ± 1	*m*-OC_2H_5	*m*-OC_2H_5	2586 ± 6
p-CH_3	*p*-Br	2406 ± 1	*m*-OC_2H_5	*m*-Cl	2509 ± 1
p-OCH_3	*p*-OCH_3	2485 ± 3	*m*-Cl	*m*-Cl	2399 ± 2
p-OCH_3	*p*-OC_2H_5	2539 ± 3	*m*-Cl	*m*-Br	2518 ± 1
p-OCH_3	*p*-Cl	2443 ± 1	*m*-Br	*m*-Br	2604 ± 6

TABLE II

MULTIPLE REGRESSION PARAMETERS FOR CORRELATION OF RETENTION INDICES OF N,N'-DIPHENYLFORMAMIDINES *VS.* RETENTION INDICES OF CORRESPONDING ANILINES

Standard	$a_1 = a_2$	*b*	*r*	ψ	*Eqn.*
$XC_6H_4NH_2$ and $YC_6H_4NH_2$	1.028 ± 0.025	−42.7	0.9902	0.1132	4
$XC_6H_4NH_2$	1.039 ± 0.106	−72.6	0.9910	0.1480	5

indicating that the N,N'-diphenylformamidines are not dimerized in the gas phase.

The regression coefficients a_1 and a_2 obtained for N,N'-diphenylformamidines are identical because the retentions of both tautomers are indistinguishable. Hence the equation for the prediction of retention indices of N,N'-diphenylformamidines (FDPh) takes the form

$$I(\mathrm{FDPh}) = a[I(XC_6H_4NH_2) + I(YC_6H_4NH_2)] + b \qquad (4)$$

This equation implies that the retention index of an unsymmetrically disubstituted formamidine is the mean of the retention indices of the two symmetrically disubstituted compounds.

The preparation of symmetrically N,N'-disubstituted amidines ($X = Y$ in eqn. 4 or $R_x = R_y$ in eqn. 3) is less complicated than the preparation of unsymmetrically disubstituted compounds, as in the latter instance considerable amounts of side products are formed. Therefore, it seems reasonable to find the parameters of the equations for the prediction of the retention indices of various types of amidines (various

TABLE III

DISTRIBUTION OF ERRORS OF PREDICTIONS OF RETENTION INDICES BASED ON EQNS. 2a, 4 AND 5

I(calc) − I(exp)	*Equation*		
	2a	*4*	*5*
0–10	4	12	15
10–20	2	16	13
20–30	8	3	4
30–40	3	4	3
40–50	12	0	0
50–60	5	1	1
60–70	1	1	2
70–80	2	1	0
80–90	1	0	0
Mean accuracy of prediction	39.6 ± 6.4	18.0 ± 5.4	17.4 ± 5.5

R_z) on the basis of correlations of retention indices of symmetrically disubstituted compounds:

$$I(R_xN{=}CR_zNHR_x) = 2a \cdot I(R_xNH_2) \quad (5)$$

The parameters of such an equation for the N,N'-diphenylformamidines (Table II), although they were obtained from smaller amount of compounds, were within the confidence intervals the same as based on all the compounds studied.

We compared the retention indices calculated with the use of the three sets of parameters (eqns. 2a, 4 and 5) with the experimental values and the distributions of errors are summarized in Table III. As can be seen, in most instances the error does not exceed 20 retention index units (i.u.) and, if parameters obtained for trisubstituted amidines are used, it does not exceed 50 i.u.

CONCLUSIONS

The results presented show that tautomerizing amidines can be analysed by gas chromatography. Their retention indices can be predicted with satisfactory accuracy using a linear regression in the form of eqn. 4.

REFERENCES

1 J. Oszczapowicz, *Pol. J. Chem.*, 52 (1978) 1311.
2 J. Oszczapowicz, J. Osek and E. Dolecka, *J. Chromatogr.*, 315 (1984) 95.
3 J. Oszczapowicz, J. Osek, K. Ciszkowski, W. Krawczyk and M. Ostrowski, *J. Chromatogr.*, 330 (1985) 79.
4 J. Osek, J. Oszczapowicz and W. Drzewiński, *J. Chromatogr.*, 351 (1986) 177.
5 J. Oszczapowicz, J. Osek, W. Krawczyk and B. Kielak, *J. Chromatogr.*, 357 (1986) 93.
6 J. Oszczapowicz, K. Ciszkowski and J. Osek, *J. Chromatogr.*, 362 (1986) 383.
7 E. Kováts, *Helv. Chim. Acta*, 41 (1958) 1915.
8 L. S. Ettre, *Chromatographia*, 6 (1973) 489.
9 R. L. Shriner and F. W. Neumann, *Chem. Rev.*, 35 (1944) 351.
10 G. Häfelinger, in S. Patai (Editor), *The Chemistry of Amidines and Imidates*, Wiley, London, New York, Sydney, Toronto, 1975, Ch. 1, pp. 1–84; J. Ševčík and H. Grambal, Ch. 12, pp. 565–617.
11 R. Burtles and F. L. Pyman, *J. Chem. Soc.*, 123 (1923) 361.
12 E. Raczyńska and J. Oszczapowicz, *Tetrahedron*, 41 (1985) 5175.
13 L. Hunter and J. A. Marriott, *J. Chem. Soc.*, (1941) 777.
14 P. Sohár, *Acta Chim. Acad. Sci. Hung.*, 54 (1967) 91.
15 L. Claisen, *Justus Liebigs Ann. Chem.*, 287 (1895) 366.
16 J. Oszczapowicz, R. Orliński and E. Hejchman, *Pol. J. Chem.*, 53 (1979) 1259.
17 J. Oszczapowicz and R. Orliński, *Pol. J. Chem.*, 44 (1971) 2327.
18 A. Grobler and G. Bálizs, *J. Chromatogr. Sci.*, 12 (1974) 57.
19 J. K. Haken, M. S. Wainwright and R. J. Smith, *J. Chromatogr.*, 133 (1977) 1.
20 O. Exner, *Collect. Czech. Chem. Commun.*, 31 (1966) 3222.

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CHROM. 19 012

Note

Behaviour of synthetic corticoids in ointment on 3-cyanopropyltrichlorosilane in high-performance thin-layer chromatography

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In previous papers[1–4] we have considered the effect of the methylene chain length of amino chemically bonded silicas in high-performance thin-layer chromatography (HPTLC). Whereas there have been many reports on high-performance liquid chromatography (HPLC) on silica gels with chemically bonded cyanoalkyl groups, there are few reports on the effectiveness of cyanoalkyl bonded silicas in HPTLC[5–7]. Therefore, we have now studied the HPTLC separation of synthetic corticoids in ointment on 3-cyanopropyltrichlorosilane (3CPTS)-treated thin-layer plates.

EXPERIMENTAL

Chemical reagents and materials

Commercially available 10 cm × 10 cm thin-layer plates pre-coated with silica gel (silica gel 60, Art. 5631) were obtained from Merck (Darmstadt, F.R.G.). 3CPTS was obtained from Aldrich (Milwaukee, WI, U.S.A.). Most sample synthetic corticoids were from Tokyo Kasei (Tokyo, Japan) (Table I); other chemical reagents were from Wako (Osaka, Japan).

TABLE I

CORTICOIDS USED

No.	*Corticoid*
1	Prednisolone
2	Prednisolone valerate acetate
3	Hydrocortisone acetate
4	Dexamethasone
5	Betamethasone valerate
6	Betamethasone dipropionate
7	Fluocinolone acetonide
8	Beclometasone dipropionate
9	Prednisolone acetate
10	Hydrocortisone
11	Flumetasone pivalate

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Apparatus

The HPTLC measurements were carried out using a Shimadzu CS-910 TLC scanner equipped with a C-R1A Shimadzu Chromatopac, and a Shimadzu CS-930 TLC scanner equipped with a DR-2 Shimadzu Chromatopac.

Cyanoalkyl-treated plates

As described previously[8,9], eight dried plates of silica gel were immersed in 260 ml of a 1.92% toluene solution of 3CPTS. After standing for 72 h at room temperature, the plates were washed several times with toluene, chloroform and methanol, and then dried *in vacuo* at 70°C for 2 days, finally producing 3CPTS ($\equiv SiCH_2CH_2CH_2CN$)-treated plates for HPTLC.

Chromatography

Sample solutions dissolved in methanol were spotted 1.5 cm from one edge of the plate. The plate was developed to a distance of 6 cm from the origin at room temperature in a Camag Twin Through Chamber (10 cm × 10 cm). After drying, visualization of the corticoid spots with sulphuric acid was carried out according to the procedures of Carstensen[10] and Heftmann[11]. The plate was thoroughly dried, sprayed uniformly with 10% sulphuric acid solution and heated at 100°C for 10 min in an oven. The corticoids appeared as fluorescent violet spots on a dark white background, under UV irradiation with a Camag Reprostor system.

Densitometry

Quantitation of the fluorescence intensity of corticoid spots was carried out

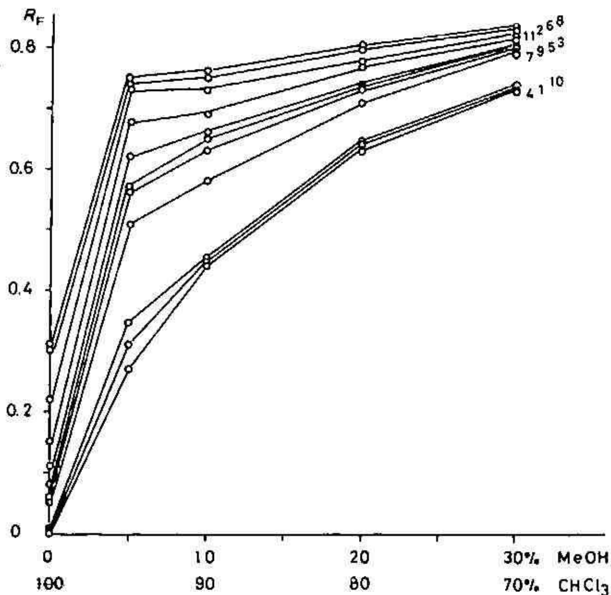

Fig. 1. The dependence of R_F on the mobile phase composition in the normal-phase mode. Stationary phase: 3CPTS-treated HPTLC plate. Migration distance; 6.0 cm in an unsaturated chamber. Sample: corticoids numbered as in Table 1.

directly on the 3CPTS-treated plates with a Shimadzu CS-910 TLC scanner or a CS-930 TLC scanner. Integrated values of the fluorescence intensity were derived by use of a Shimadzu C-R1A Chromatopac or an DR-2 Chromatopac.

Procedure

A 1-g amount of ointment was blended four times using 30 ml of methanol, warmed in a hot-bath at 50°C for 5 min and then cooled in the icebox for 30 min at 5°C. The organic phase was dried and made up to volume with methanol in a 30-ml volumetric flask.

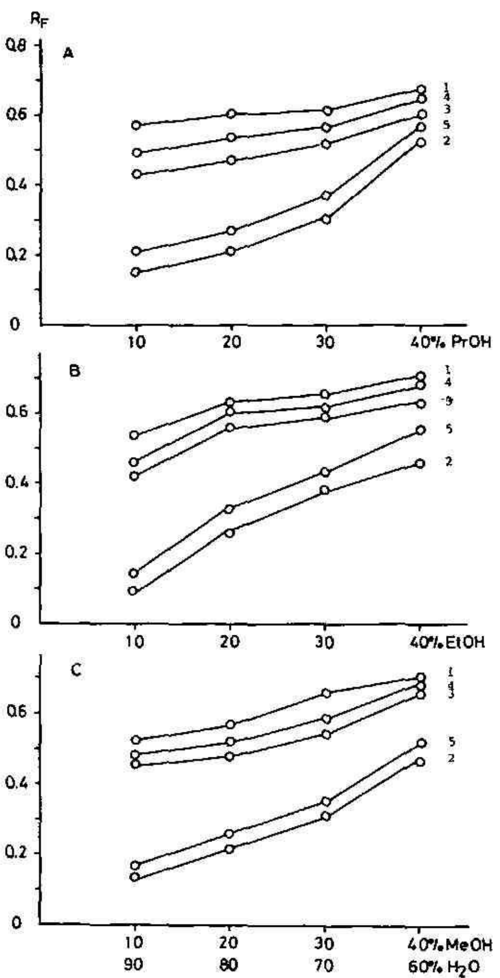

Fig. 2. The dependence of R_F on the mobile phase composition for corticoids on the 3CPTS-treated HPTLC plate in reversed-phase systems. Migration distance: 6.0 cm in an unsaturated chamber. Mobile phases: (A) propanol–water; (B) ethanol–water; (C) methanol–water. Sample: corticoids numbered as in Table I.

RESULTS AND DISCUSSION

Using the densitometric recordings of the fluorescence intensity, the relationship between the treatment temperature and the treatment time for each corticoid on the 3CPTS-treated plate was studied. A temperature of 100°C and a time of 10 min were found to be the most suitable. Synthetic corticoids were then investigated on the 3CPTS-treated HPTLC plate, using chloroform and chloroform–methanol as eluents. Fig. 1 shows the dependence of R_F on the mobile-phase composition. The 3CPTS-treated HPTLC plate, having a weakly polar stationary phase, may be suitable for separating strongly polar substances under mild elution conditions.

Fig. 2 shows the dependence of R_F for corticoids on the 3CPTS-treated HPTLC plate in reversed-phase systems, using methanol–water, ethanol–water or propanol–water as eluent. Because of the pK value of the cyano group in aqueous

TABLE II

RECOVERY OF CORTICOIDS FROM COMMERCIAL OINTMENTS ACCORDING TO THE DESCRIBED METHOD

C.V. = Coefficient of variation.

Sample No. (n = 5)	*Amount (g)*	*Corticoid added (mg)*	*Corticoid found (mg)*	*Recovery (%)*	*Corticoid content (%)*	*C.V. (%)*
1	1.01	0	4.99			
		5.01	9.92–9.95	98.4–99.0	0.49	0.33
2	0.99	0	2.98			
		3.00	5.91–5.97	97.7–99.7	0.30	1.10
3	1.02	0	9.99			
		10.02	19.85–19.98	98.4–99.7	0.98	0.71
4	1.01	0	0.49			
		0.51	0.99–1.00	98.0–100.0	0.05	1.10
5	1.02	0	1.20			
		1.20	2.43–2.39	95.0–99.2	0.12	2.30
6	1.00	0	0.63			
		0.64	1.25–1.26	96.9–98.4	0.06	0.79
7	1.03	0	0.24			
		0.25	0.48–0.49	96.0–100.0	0.02	2.19
8	0.99	0	0.24			
		0.25	0.48–0.49	96.0–100.0	0.02	2.17
9	1.01	0	5.00			
		5.03	9.91–10.00	97.6–99.4	0.50	0.99
10	1.01	0	9.98			
		10.01	19.85–19.93	98.6–99.4	0.99	0.44
11	1.02	0	0.19			
		0.22	0.40–0.41	95.5–100.0	0.02	2.42

media, the 3CPTS-treated HPTLC plate may be regarded as a weakly basic ion exchanger.

Table II shows the recovery of corticoids from commercial ointments according to this method.

REFERENCES

1 M. Okamoto, F. Yamada and T. Omori, in A. Zlatkis (Editor), *Advances in Chromatography 1982*, University of Houston, Houston, TX, 1982, pp. 177–181.
2 M. Okamoto, F. Yamada and T. Omori, *Chromatographia*, 16 (1982) 152.
3 M. Okamoto, K. Matsui, F. Yamada and M. Noguchi, *Yakugaku Zasshi*, 102 (1982) 1099.
4 M. Okamoto, F. Yamada and T. Omori, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 5 (1982) 163.
5 T. Omori, M. Okamoto and F. Yamada, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 6 (1983) 47.
6 W. Jost and H. E. Hauck, in R. E. Kaiser (Editor), *Proceedings of the 3rd International Symposium on HPTLC, Wuerzburg, April 1985*.
7 M. Okamoto and M. Noguchi, *Yakugaku Zasshi*, 106 (1986) 836.
8 M. Okamoto and H. Kishimoto, *J. Chromatogr.*, 212 (1981) 251.
9 M. Okamoto, *J. Chromatogr.*, 202 (1980) 55.
10 H. Carstensen, *Eur. J. Steroids*, 1 (1966) 233.
11 E. Heftmann, *Chromatography of Steroids*, Elsevier, Amsterdam, 1976, p. 18.

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Note

Derivatization of the *Fusarium* mycotoxin moniliformin for gas chromatography–mass spectrometry analysis

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The *Fusarium* mycotoxin moniliformin (3-hydroxycyclobut-3-ene-1,2-dione) has been found to occur naturally in samples of fungally contaminated maize[1,2], and has been implicated as a causative agent of animal diseases[3], resulting from the feeding of moulded materials. The extraction and analysis of moniliformin is difficult and methods have relied upon high-performance liquid chromatography (HPLC) with UV detection[1] or thin-layer chromatography (TLC) with colourimetric[3–5] or fluorescence quenching[6] for visualisation. Identification has thus been based entirely on agreement of retention times (or R_F values) with authentic moniliformin and has relied on the efficacy of lengthy clean-up procedures to remove potential interferences. In this laboratory we have adopted a similar approach, and recently reported an ion-pair HPLC procedure for the analysis of moniliformin in maize[7], but this again of necessity has relied on extensive clean-up and utilised non-specific UV detection.

In order to pursue a surveillance programme to establish the incidence of occurrence of moniliformin in maize intended for human consumption (and thus to assess its significance as a mycotoxin in the food supply), it is thought essential to develop an adequate confirmatory procedure; for this purpose mass spectrometry (MS) was investigated. The relatively low molecular weight of moniliformin (MW = 98) and the absence of characteristic ions in its mass spectrum, coupled with its lack of amenability to gas chromatography (GC) indicated the need for derivatization. Trimethylsilylation, methylation (diazomethane) and trifluoroacylation were not a suitable basis for a GC–MS confirmatory procedure. However, reaction with N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) containing 1% *tert.*-butyldimethylchlorosilane (TBDMCS) produced a compound with a molecular weight of 453 which had a characteristic mass spectrum, was formed quantitatively and in selected ion GC–MS gave good linear calibration at low levels. This paper reports the isolation, purification by high-performance size-exclusion chromatography and structural identification by nuclear magnetic resonance (NMR) spectrometry and MS of this derivative of moniliformin, and demonstrates its potential for utilisation for a quantitative GC–MS confirmatory procedure.

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EXPERIMENTAL

Materials

Moniliformin was purchased as its sodium salt from Sigma (St. Louis, MO, U.S.A.) and the free acid was custom synthesized by Lancaster Synthesis (Morecambe, U.K.), and stored at −36°C at all times. MTBSTFA containing 1% TBDMCS was obtained from Regis Chemicals (Phase Separations, Deeside, U.K.).

Derivatization

A solution of moniliformin (free acid) in acetone (1 mg/ml) was used throughout, from which aliquots (50 μl) were withdrawn and evaporated to dryness in a vial. A volume of 50 μl of derivatizing reagent (MTBSTFA–TBDMCS) in acetonitrile (1:1) was added and the mixture heated at 100°C for 15 min in a fan-circulating oven. The derivatized moniliformin was stable for several days when stored in excess derivatizing reagent, but when purified was prone to decomposition on protracted storage in solvent.

Mass spectrometry

Electron ionizaton (EI) mass spectra were obtained on a VG 7070H mass spectrometer using direct insertion probe introduction, with a water cooled probe heated from 50°C to 250°C at 2°C/s. Chemical ionization (CI) spectra were obtained using ammonia reagent gas at an indicated source housing pressure of $5 \cdot 10^{-5}$ mbar. The mass spectrometer source was held at 200°C. Ionization was at 70eV, with a 200-μA trap current. The mass spectrometer was scanned from m/z 500 to 25 at 1 s/decade, and the spectra were acquired and processed with a VG 11/250 data system.

GC–MS was carried out with a Carlo Erba 4160 GC instrument directly coupled to the above mass spectrometer, using a 25 m × 0.22 mm I.D. fused-silica CP SIL 5CB column (Chrompack, U.K.) programmed from 150°C to 250°C at 10°C/min after an initial 2-min delay. The helium carrier gas pressure was 0.9 bar, and the injection of samples (1.7 μl) was in a split mode (20:1) with the injector temperature set at 250°C.

HPLC (size-exclusion) purification

High-performance size-exclusion LC was carried out using a Waters 6000A pump (Waters Assoc., Milford, MA, U.S.A.) and Rheodyne 7010 injection system (50 μl loop). The column was 300 × 7.7 mm PL gel 5-μm poly(styrene–divinylbenzene) of nominal pore size 50 Å obtained from Polymer Lab. (Church Stretton, Salop, U.K.). The chloroform solvent was pre-purified by passage through a column of neutral alumina to remove methanol stabiliser, and was employed at a flow-rate of 0.5 ml/min. Detection was at 260 nm with a Pye LC–UV detector. The component was collected beginning 14 min after injection thus collecting material eluted after the peak maxima to minimise trapping of the tail of the derivatizing reagent. Repetitive trapping was carried out and the isolated derivatized component was bulked, and finally re-examined by HPLC to test for the absence of derivatizing reagent.

NMR analysis

Solvent was removed from the collected HPLC fraction by small scale frac-

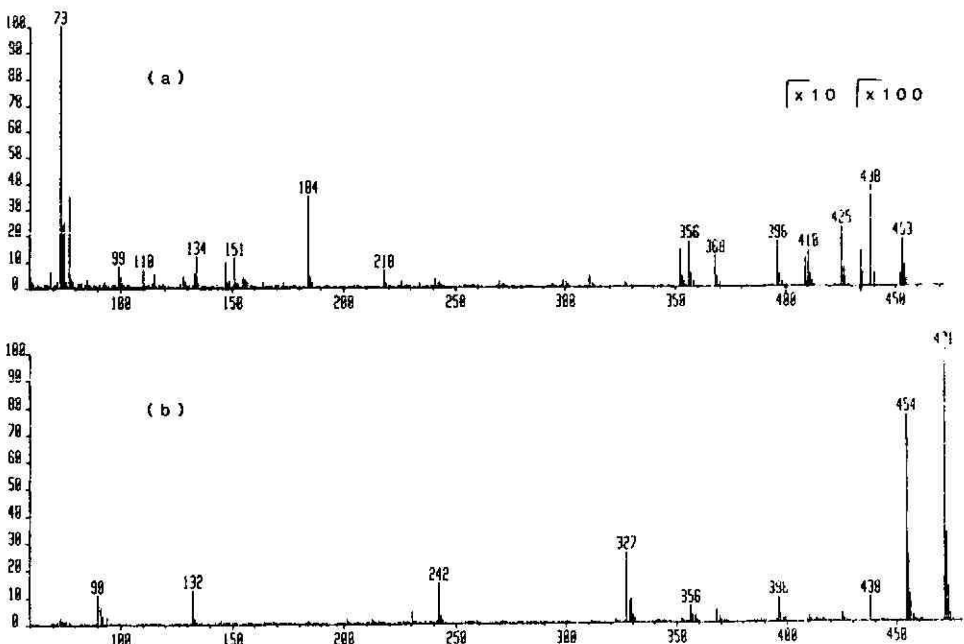

Fig. 1. Probe spectra of purified moniliformin derivative of molecular weight = 453. (a) Electron ionization. (b) Ammonia chemical ionization. Probe heated from 50°C to 250°C at 2°C/s. Mass spectrometer source at 200°C, ionization at 70 eV with scanning from *m/z* 500 to 25 at 1 s/decade.

tional distillation. The product was dissolved in deuterochloroform, which was also removed by distillation and finally the product re-dissolved in deuterochloroform for analysis. Proton NMR spectra were recorded at 200 MHz on a Nicolet NTC Fourier transform (FT)-NMR spectrometer, shifts being reported in parts per million relative to tetramethylsilane internal standard. Carbon-13 spectra were obtained at 50 mHz on a Nicolet NTC FT-NMR spectrometer, and at 22 MHz on a Jeol FX-90Q spectrometer.

RESULTS AND DISCUSSION

Both the electron and chemical ionization spectra of the derivatized moniliformin are shown in Fig. 1. The spectra were obtained by fractionation of the product from excess derivatizing reagent during thermal elution from the probe. Identical spectra to those produced by probe introduction were obtained by capillary GC–MS for the peak which eluted at 8 min under the conditions given in the Experimental section, and which proved to be the quantitatively formed product of the derivatization reaction.

To allow studies by NMR the derivatized product was separated from excess reagent by high-performance size-exclusion chromatography (HPSEC). Fig. 2a shows the HPSEC chromatogram of the derivatized reaction mixture. The derivatized moniliformin peak eluted on the tail of the derivatizing reagent. The component was

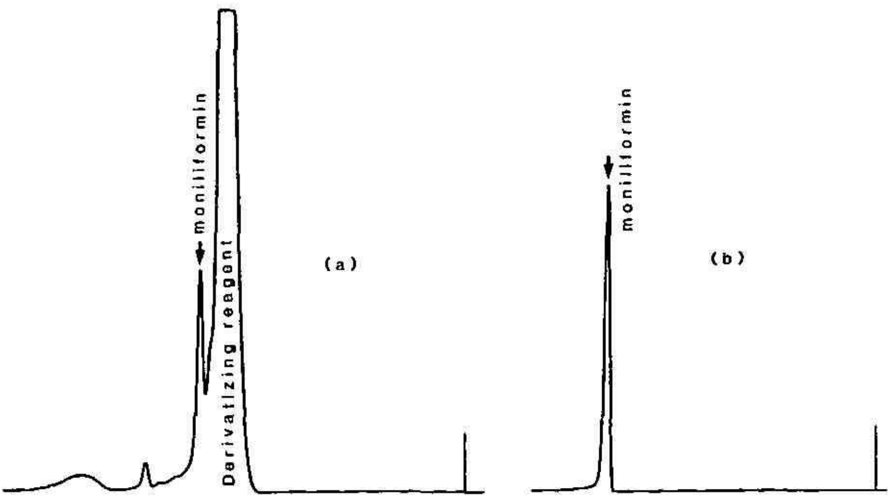

Fig. 2. High-performance size-exclusion chromatographic purification of derivatized moniliformin. (a) Derivatized reaction mixture. (b) Isolated moniliformin derivative re-chromatographed. Column: 300 × 7.7 mm PL gel 5 μm poly(styrene–divinylbenzene) of 50 Å pore size operated with chloroform solvent at a flow-rate of 0.5 ml/min. UV detection at 260 nm.

trapped slightly after elution of the peak maxima and on re-chromatographing (as shown in Fig. 2b) the isolated derivatized component was found to be free of excess reagent. Re-analysis by probe MS confirmed that the derivatized component had been isolated with high purity and that no reaction or decomposition had taken place during the chromatographic stage.

The proton and carbon-13 NMR results for the derivatized moniliformin are shown in Table I. The signals can be assigned to a structure formed from monili-

TABLE I

PROTON AND CARBON-13 ASSIGNMENTS FOR MONILIFORMIN DERIVATIVE

Protons	*Total number*	*Shift relative to TMS (ppm)*	*Carbons*	*Total number*	*Shift relative to TMS (ppm)*
tert.-Butyl	18	0.91	*tert.*-Butyl	8	26.3
Dimethylsilyl	12	0.10	Dimethylsilyl	3	− 2.9
			Dimethylsilyl	3	0.6
N–CH_3	3	3.13	N–CH_3	1	68.3
H_x	1	5.32			

formin by *tert.*-butyldimethylsilylation of the free hydroxyl group, and additional *tert.*-butyldimethylsilylation combined with N-methyl-trifluoroacetamide adduct formation having occurred at one of the keto groups. Such addition products from N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) reagent with carbonyl groups have recently been observed in the case of aliphatic aldehydes[8], and the proposed product from the reaction with moniliformin is of an analogous structure, and consistent with the spectroscopic evidence reported herein. In the proton NMR spectrum one major *tert.*-butyl resonance was observed at 0.91 ppm and a major dimethylsilyl at 0.10 ppm. The N–CH_3 protons are assigned to a broad signal at 3.13 ppm, the broadness resulting from hindered rotation about the N–CO amide bond which is normal for N-substituted systems. A resonance at 5.32 ppm is assigned as the olefinic proton of the silylated moniliformin which is consistent with the proposed structure in both observed shift positions and in relative intensity.

On protracted carbon-13 scanning which was necessary to accumulate sufficient NMR data for assignment on the relatively small amount of derivative available, progressive decomposition was found to occur during analysis in deuterochloroform solution. Although the products of this decomposition led to some signals being obscured, nevertheless the carbons from both the *tert.*-butyl groups produced a signal at 26.3 ppm. Two dimethyl resonances were observed at -2.9 and 0.6 ppm, the separation reflecting the close proximity of the N-methylacetamide to one of these, whilst the carbon of the N–CH_3 produced a weak signal at 68.3 ppm. The EI mass spectrum shows a weak ion at m/z 453, assigned as the molecular ion which is supported by the presence of fragment ions at $M-15$ and $M-57$. The molecular weight of 453 is confirmed in CI by the presence of an intense $M+1$ ion at m/z 454, together with a $M+18$ base peak at m/z 471.

The intense fragment ion at m/z 184 in the EI spectrum is assigned to:

$$\left[Me{-}N{=}C(CF_3){-}O{=}Si(Me)_2 \right]^{+\cdot}$$

formed by elimination and rearrangement of geminally placed *tert.*-butyldimethyl and N-methyl-N-trifluoroacetamide groups and subsequent elimination of a *tert.*-butyl group from the derived product. This fragment ion was observed in the MSTFA adduct products of aliphatic aldehydes[8], and is direct evidence in this case of the presence of a similar adduct. Ions at m/z 73, 110 and 134 are typically those seen in TMS and MTBSTFA derivatized products.

The mechanism of formation of the proposed derivative is envisaged as taking place through initial *tert.*-butyldimethylsilylation of the alcohol to form a monoderivatized product, followed by attack on one of the two carbonyls resulting in an allyl stabilised cation:

This is followed by N-methyl-N-trifluoroacetamide nitrogen anion trapping, that could occur at either the site of carbonyl derivatization (1,2-trapping) or at the delocalised allyl position (1,4-trapping). The proposed product, as opposed to other isomeric arrangements of these groups within the moniliformin molecule is sterically favoured, with the bulky *tert.*-butyl groupings being on opposite rather than adjacent carbons. Mass spectral evidence also supports geminally placed silyloxy-acetamide

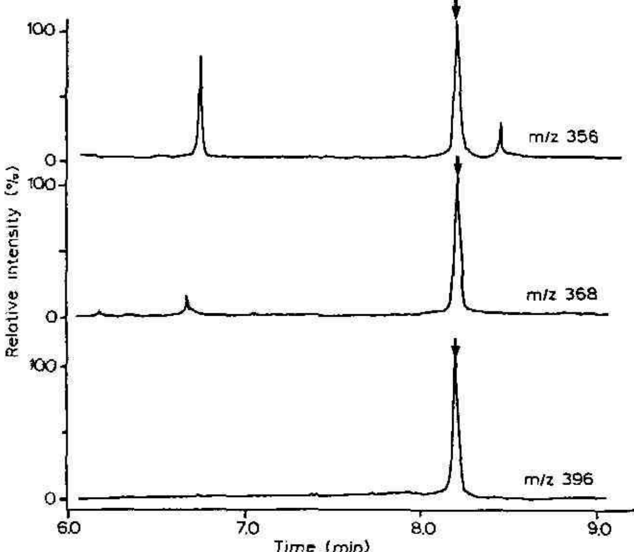

Fig. 3. Selected-ion monitoring chromatograms for derivatized moniliformin. GC–MS conditions: 25 m × 0.22 mm I.D. fused-silica CP SIL 5CB column, temperature programmed from 150°C to 250°C at 10°C/min after an initial 2-min delay (split injection). Selected-ion monitoring of *m/z* 396, 368 and 356 with an 80-ms dwell time per ion and a 20-s interscan delay.

groups, but unfortunately none of the spectroscopic evidence is capable of unequivocally establishing the substitution pattern. The derivative characterised in this paper could clearly be employed for confirmation of the presence of moniliformin in extracts from biological materials by obtaining full scan GC–MS spectra. We were however additionally interested in the possibilities of both improved sensitivity achievable through use of selected-ion monitoring, and in the possibility of direct MS quantification. Although the yield of the derivatization reaction was not determined, probe analysis did not show any evidence of underivatized moniliformin, no additional products were detected by GC, and it was thus presumed that the reaction was quantitative. In Fig. 3 selected-ion monitoring chromatograms are shown for the analysis of derivatized moniliformin standard; the monitoring of three ions enabling a check on the specificity of analysis. Quantification was carried out by monitoring m/z 396, on the basis of peak heights. Good linear calibration was demonstrated with a regression coefficient of 0.998 (standard error = 3.74) over the range 5–500 picograms of derivatized moniliformin injected. At the lower limit this would allow sub-μg/kg sensitivity on typical gram equivalent amounts produced in concentrated and cleaned-up extracts, obtained from contaminated cereals.

REFERENCES

1 P. G. Thiel, C. J. Meyer and W. F. O. Marasas, *J. Agric. Food Chem.*, 30 (1982) 308.
2 A. von Thalmann, S. Matzenauer and S. G.-S. Karlsruhe, *Ber. Landwirtsch.*, 63 (1985) 257.
3 C. J. Rabie, A. Lubben, A. I. Ouw, E. B. Rathbone, P. S. Steyn and R. Vleggaar, *J. Agric. Food Chem.*, 26 (1978) 375.
4 H. Kamimura, M. Nishijima, K. Yasuda, K. Saito, A. Ibe, T. Nagayama, H. Ushiyama and Y. Naoi, *J. Assoc. Off. Anal. Chem.*, 64 (1984) 1067.
5 J. Jansen and K. Dose, *Fresenius' Z. Anal. Chem.*, 319 1984) 60.
6 H. R. Burmeister, A. Ciegler and R. F. Vesonder, *Appl. Environ. Microbiol.*, 37 (1979) 517.
7 M. J. Shepherd and J. Gilbert, *J. Chromatogr.*, 358 (1986) 415.
8 M. Ende and H. Luftmann, *Tetrahedron*, 40 (1984) 5167.

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Note

Simultaneous determination of the alditol acetate derivatives of amino and neutral sugars by gas–liquid chromatography

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Gas–liquid chromatography (GLC) of alditol acetates has frequently been used for the identification and quantification of neutral sugars in biological materials. Amino sugars are important components of the carbohydrate portion of glycoproteins, glycolipids, mucopolysaccharides and the free oligosaccharides of milk and urine, which are usually present together with neutral sugars. N-Acetylalditol acetates of amino sugars have too long retention times and poor responses on gas chromatography[1,2]. The use of a column of Poly A-103[3] gives better results for the alditol acetates of amino sugars, but the alditol acetates of neutral sugars are not resolved. Hence an efficient method for the simultaneous determination of amino and neutral sugars as alditol acetates has not yet been established, although the derivative is stable and the alditol derivatives give fewer GLC peaks than do the trimethylsilyl derivatives of aldose, possessing two anomers of the pyranose and furanose rings.

To overcome these problems, we selectively methylated amino groups in the alditols of amino sugars by reaction with formaldehyde and sodium cyanoborohydride[4]. The mixture of amino sugars (glucosamine, galactosamine and mannosamine) and neutral sugars was reduced with sodium borohydride, and amino groups were treated with formaldehyde and sodium cyanoborohydride, then acetylated. The resulting derivatives were used as samples for GLC.

EXPERIMENTAL

Reagents

All chemicals were of analytical-reagent grade. Sodium cyanoborohydride was purchased from Kanto Chemicals (Tokyo, Japan), and the other reagents were obtained from Nakarai Chemicals (Kyoto, Japan).

Apparatus

GLC was performed with a Shimadzu 4CM chromatograph equipped with a hydrogen flame ionization detector, using a glass column (2 m × 0.3 cm I.D.) packed with 2% EGSS-X on Chromosorb W AW DMCS (60–80 mesh) at 195°C and a

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flow-rate of nitrogen of 45 ml/min. Peak areas and retention times were measured by use of a Shimadzu Chromatopac-E1A integrator. GLC–mass spectrometry (GLC–MS) was conducted with a JEOL JMS-D 300 instrument equipped with a glass column (1 m × 0.2 cm I.D.) packed with 3% ECNSS-M on Gas-Chrom Q (100–120 mesh) as described in a previous paper[5].

Evaporation

All evaporations were conducted under reduced pressure at bath temperatures not exceeding 40°C.

Preparation of amino and neutral sugars for gas chromatography

Monosaccharides (0.1–2 mg) were reduced with sodium borohydride (3 mg) in water (2 ml) for 2 h at room temperature, and the pH of the solution was adjusted to *ca.* 6 with 1 *M* acetic acid. Formaldehyde (0.5 ml of 0.7% aqueous solution) and sodium cyanoborohydride (3 mg) were added to the alditol solution and the mixture was allowed to stand for 3 h at room temperature. Excess of cyanoborohydride was destroyed by the addition of a few drops of concentrated hydrochloric acid and the solution was concentrated. Methanol (1 ml) was added and the solution was evaporated to remove the resulting boric acid as trimethyl borate, and then to dryness. After the last step had been repeated three times, the sample was stored *in vacuo* over potassium hydroxide pellets.

Peracetylation was carried out with acetic anhydride–pyridine (1:1) (2 ml) at 100°C for 2 h. Toluene (1 ml) was then added to the reaction mixture, which was evaporated to dryness. By this treatment, the residual acetic anhydride and pyridine were removed as an azeotropic mixture with toluene. The residue was dissolved in chloroform (5 ml) and the solution was washed twice with water (5 ml). The chloroform layer was evaporated to dryness, the residue was redissolved in chloroform (50 μl) and the solution was applied to the gas chromatograph.

RESULTS AND DISCUSSION

Fig. 1 shows the reaction scheme for an amino sugar. In the procedure, amino and neutral sugars were first converted into alditols with sodium borohydride, then the amino group of the alditols of amino sugars was methylated with formaldehyde and sodium cyanoborohydride at pH *ca.* 6. As an excess of formaldehyde causes by-products in the methylation, the aldehyde should be used as a dilute aqueous solution. Hydrochloric acid was added to the reaction mixture to destroy excess of cyanoborohydride, and the resulting boric acid was evaporated as trimethyl borate

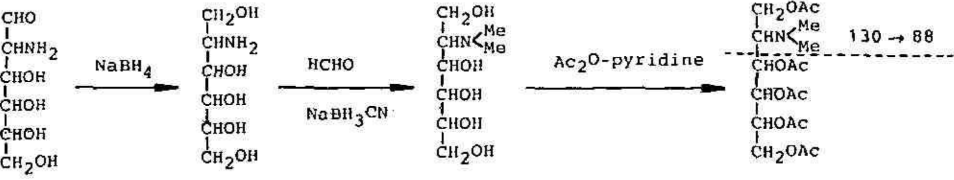

Fig. 1. Reaction scheme for an amino sugar. Me = methyl; Ac = acetyl.

by adding excess of methanol *in vacuo*. The N-methylated alditols thus obtained were acetylated together with neutral alditols in the mixture of acetic anhydride and pyridine. After acetylation, acetic anhydride and pyridine in the reaction mixture were readily evaporated as an azeotropic mixture[6] by adding excess of toluene *in vacuo*, and the resulting residue was dissolved in chloroform. The chloroform layer was washed with water to remove a small amount of by-products arising from formaldehyde, and applied to the gas chromatograph for analysis.

On a column of 3% ECNSS-M[7,8], used for the alditol acetates of neutral sugars, the peaks of the three amino sugar derivatives in the sugar mixture were detected between those of xylitol and mannitol acetates, which had much shorter retention times than those of N-acetylalditol acetates. However, glucosamine and mannosamine derivatives gave almost the same retention time, so we made further investigations.

On a column of 2% EGSS-X[9], the derivatives of neutral sugars (L-rhamnose, L-fucose, D-ribose, L-arabinose, D-xylose, D-mannose, D-galactose and D-glucose), D-glucosamine and D-galactosamine were separated satisfactorily, and the derivatives of D-glucosamine and D-mannosamine were able to be detected as a shoulder peak, as shown in Fig. 2. Table I gives the retention times. Amino sugar derivatives were shown to be in the N-dimethyl form (compound I in Fig. 1) by the characteristic mass fragments at *m/z* 130 and 88 ($130-CH_2=C=O$) using GLC–MS.

The response factors (peak area of each sample/peak area of internal standard)

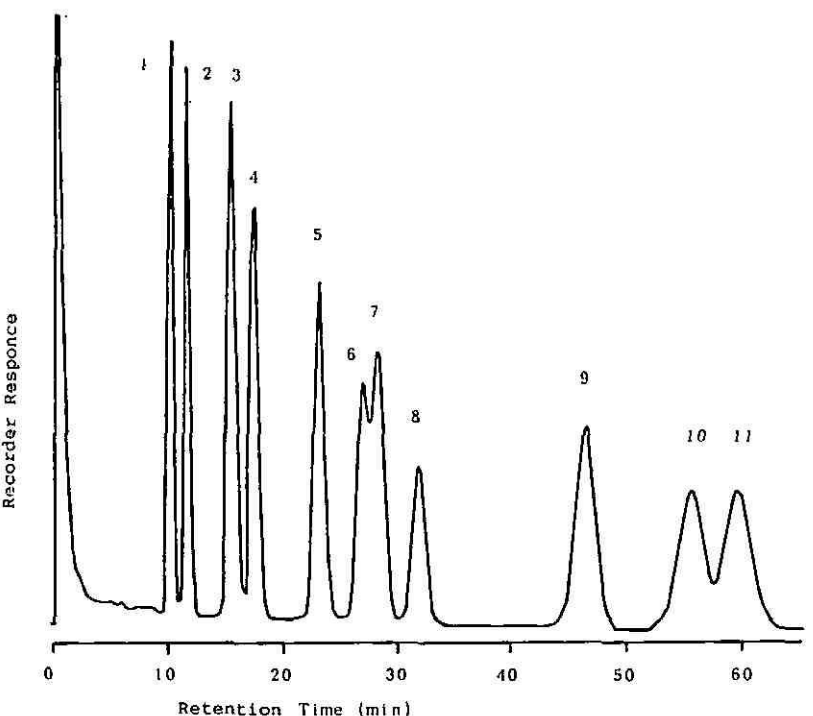

Fig. 2. Separation of the derivatives of amino and neutral sugars by GLC. Peaks as in Table I.

TABLE 1

RETENTION TIMES OF ALDITOL ACETATES OF AMINO AND NEUTRAL SUGARS ON A 2% EGSS-X COLUMN

*Peak No.**	*Neutral sugar (as alditol acetate)*	*Retention time (min)*	*Peak No.**	*N-Dimethyl amino sugar (as alditol acetate)*	*Retention time (min)*
1	Rhamnitol	9.64	6	Glucosaminitol	24.81
2	Fucitol	10.98	7	Mannosaminitol	25.88
3	Ribitol	14.41	8	Galactosaminitol	29.08
4	Arabinitol	16.28			
5	Xylitol	21.34			
9	Mannitol	42.21			
10	Galactitol	50.08			
11	Glucitol	53.94			

* See Fig. 2.

of glucosamine, galactosamine and mannosamine to xylose as an internal standard were 0.97, 1.03 and 0.97, respectively.

In conclusion, the proposed method is suitable for the determination of mixtures of neutral sugars, glucosamine, galactosamine and mannosamine.

REFERENCES

1 M. B. Perry and A. C. Webb, *Can. J. Biochem.*, 46 (1968) 1163.
2 L. J. Griggs, A. P. E. R. White, J. A. Finkelstein, W. E. Moeckel, K. G. Holden, J. E. Zarembo and J. A. Weisbach, *Anal. Chem.*, 43 (1971) 369.
3 W. Niedermeier and M. Tomana, *Anal. Chem.*, 57 (1974) 363.
4 C. F. Lane, *Synthesis*, (1975) 135.
5 S. Ukai, S. Yokoyama, C. Hara and T. Kiho, *Carbohydr. Res.*, 105 (1982) 237.
6 L. H. Horsley, *Azeotropic Data—III, Adv. Chem. Ser.*, No. 116, American Chemical Society, Washington, DC, 1973.
7 J. S. Sawardeker, J. H. Sloneker and A. Jeanes, *Anal. Chem.*, 37 (1965) 1603.
8 T. Kiho, C. Hara and S. Ukai, *Chem. Pharm. Bull.*, 33 (1985) 270.
9 D. H. Shaw and G. W. Moss, *J. Chromatogr.*, 41 (1969) 350.

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Note

Quantitative analysis of olefins in a light hydrocarbon mixture by bromination and gas chromatography using a methylsilicone column

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Because of their ability to polymerize over solid catalysts and adsorbents, olefins are undesirable impurities in many petrochemical feedstocks. The analysis of trace olefins in alkanes is an important procedure in the chemical laboratories of many petrochemical plants. The classical methods used to separate olefins are time-consuming, requiring long columns and the calculation of the bromine index[1]. The results obtained are somewhat uncertain because an average molecular weight must be assumed for the olefins in order to obtain their percentage from the bromine index. Furthermore, as the retention times of paraffins and olefins do not greatly differ, the small olefin peaks are usually overlapped by the larger paraffin signals. Thus, the accuracy of the analysis is impaired.

In the present note we report a fast, convenient technique for the separation of trace amounts ($<0.5\%$, w/w) of olefins in light hydrocarbon cuts. This method includes bromination of the double bonds in the sample, followed by temperature-programmed chromatography. The technique has been tested with several solutions of olefins in alkanes. The results are promising.

EXPERIMENTAL

An Hewlett-Packard 5880A gas chromatograph equipped with a flame ionization detector and an on-column injection port was employed with nitrogen as the carrier gas. The column was fused silica (25 m × 0.2 mm I.D.) with methylsilicone (cross-linked phase, Ultra, Hewlett-Packard) as stationary phase. Both the injector and detector were kept at 200°C. Samples were injected with a 5-μl Hamilton syringe.

The reagents were sodium sulphite, bromine, carbon tetrachloride, *n*-hexane (olefin-free, confirmed by using the ASTM bromine index technique), *n*-hexane (technical grade, contaminated with alkanes and traces of alkenes) and a C_5–C_{11} paraffin

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raffinate consisting of about 85%, w/w of C_5 and C_6 alkanes. A solution of bromine in carbon tetrachloride (*ca.* 0.25 mol/kg) was prepared to avoid handling liquid bromine.

Solutions containing between 0.05 and 0.4% (w/w) olefins in different alkanes were prepared for trial separations. A small portion (10 ml) of each solution was brominated with about the same volume of the bromine solution. The bromine added was always enough for the solution to remain pale yellow throughout the bromination. The solution was then kept in the dark for 10 min. This was essential to avoid photochemical reactions leading to bromination of the alkanes. After bromination, the excess of bromine was eliminated by adding 20 ml of saturated sodium sulphite solution and stirring for 1 min. Once the organic phase had separated, a 0.4-μl sample was injected into the gas chromatograph. In a typical experiment the oven was kept at 40°C for 6 min, then heated at a rate of 25°C/min to a final temperature of 130°C. The oven was kept at this temperature for 11 min. The complete experiment lasted about half an hour.

RESULTS AND DISCUSSION

For each group of olefins investigated (C_5 and C_6), the olefins brominated in positions 1,2 were the last to elute. Since 1,2-dibromopentane was eluted before any dibromohexane, separation of the two groups of peaks was simple. Although bromination of the double bond in branched olefins might result in the formation of enantiomeric pairs, these are not separable on a non-chiral column. Thus, the number of dibromoalkane peaks in our chromatograms should correspond to the number of olefins in our samples.

The results obtained are summarized in Table I. The response factor, *F*, was

TABLE I

RESPONSE FACTORS AND EFFECTIVE CARBON NUMBERS FOR C_5 AND C_6 OLEFINS

Values quoted for *F* are averages of five determinations. Deviations from the mean value were less than ±0.01 in all cases.

Olefin	*Solvent (%, w/w)*	*Olefin (%, w/w)*	*% area of dibromoalkane*	*Response factor, F*	*ECN*
1-Hexene	*n*-Hexane (pure)	0.096	0.0866	0.90	5.40
1-Hexene	*n*-Hexane (pure)	0.360	0.323	0.90	5.40
1-Hexene	*n*-Hexane (technical)	0.160	0.145*	0.91	5.44
2-Methyl-1-pentene	C_5–C_{11}	0.122	0.110*	0.90	5.40
2-Methyl-1-butene	*n*-Hexane (pure)	0.013	0.0113	0.87	4.35
2-Methyl-1-butene	*n*-Hexane (technical)	0.075	0.066	0.88	4.40

* Overlapping of the dibromoalkane and an unidentified paraffin (probably C_{10} or C_{11}) peak. The area of the former was obtained by subtraction of the area of the unwanted peak.

calculated as the ratio of the percentage area under a dibromoalkane peak to the olefin content (%, w/w). The effective carbon number (ECN) is defined as nF, where n is the number of carbon atoms in the olefin. Very small amounts of monobromoalkanes (formed because of unavoidable exposure to light) may be eluted during the chromatography, but the area under their peaks is negligible compared with the dibromoalkane signals.

The experimental results show that, for the C_5 and C_6 olefins investigated, *both the response factor and the effective carbon number depend only on the number of carbon atoms*. Branched and linear olefins with the same number of carbon atoms exhibit the same values for F and ECN. This suggests that the response factor measured for one C_5 or C_6 olefin can be used for all C_5 or C_6 olefins.

It is obviously tempting to try to extrapolate our results to olefins with chain lengths other than C_5 and C_6. We note that, for the olefins investigated, bromination of the double bond reduces *the effective carbon number by a constant factor equal to 0.6 units*. Stenberg *et al.*[2] found that a variety of substituent groups reduce the ECN of a paraffin series by a constant factor. If those results hold for the dibromine substitution, the response factor for any dibromoalkane can be calculated as $F = (n - 0.6)/n$, where n is the number of atoms in the olefin.

We have employed the described technique to measure the olefin content in a petrochemical feedstock consisting of C_5–C_6 alkanes containing C_6 (mainly) and C_7 olefins. The olefin content determined in five samples was 2.66 ± 0.05% (w/w) olefins.

Research is currently underway in our laboratory to check the equation for F with a large number of olefins and to extend the technique to solutions containing higher percentages of heavier olefins.

CONCLUSIONS

We have presented a technique for the determination of olefins in light hydrocarbon cuts. The results obtained compare well with those from the classical bromine index technique. Our method is suitable for use in quality-control laboratories where a rapid analysis of olefins is required.

REFERENCES

1 *ASTM Standards, Bromine Index D-2710-77*, ASTM Committee D-2 Petroleum Products and Lubricants, Philadelphia, PA, 1977, Part 24, p. 604.
2 J. C. Stenberg, W. S. Gallaway and D. T. L. Jones, *Preprints, International Gas Chromatography Symposium, Lansing, MI, 1961*, pp. 159–184.

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CHROM. 19 046

Note

High-performance liquid chromatographic, stability indicating assay for disodium EDTA in ophthalmic preparations

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The use of the chelating agent ethylenediaminetetraacetic acid (EDTA) is widespread in the pharmaceutical industry. It is used as a treatment of heavy metal poisoning[1] as well as an antioxidant in many pharmaceutical preparations. EDTA is generally added as the disodium salt and functions by chelating heavy metals which often catalyze oxidations. The current analytical methodology for EDTA in formulations is titrimetric. It involves titration of a preparation of chelometric standard calcium carbonate using the EDTA formulation as titrant with hydroxy naphthol blue indicator (for sterile EDTA solution for injection)[2] or titration of the EDTA solution with standard magnesium solution using Arsenazo I as the indicator and tris(hydroxymethyl)aminomethane as a buffer[3]. Both of these methods are time-consuming and non-specific. Other methods for determining EDTA are reported in the literature including thin-layer chromatography, gas chromatography[4] and high-performance liquid chromatography (HPLC)[5], however, in these methods EDTA has to be either derivatized or converted to a photochemically unstable iron complex prior to analysis. In 1981 Parkes *et al.*[6] developed an HPLC method for the determination of the impurity nitrilotriacetic acid in bulk EDTA. This system has been modified and adapted in our laboratories to the analysis of EDTA in ophthalmic preparations and has provided a direct stability indicating assay for EDTA in a variety of media. The method has increased sample throughout by approximately 300%. Although the method has only been fully validated for ophthalmic preparations it has also been successfully applied to both serum and feces samples.

EXPERIMENTAL

The nitrilotriacetic acid disodium salt used in these studies was obtained from Aldrich. The EDTA was a USP reference standard.

The HPLC system comprised a Waters Model 6000A chromatographic pump, a DuPont variable-wavelength detector with UV lamp operated at 254 nm and a Waters μBondapak C_{18} column, 30 cm × 4 mm I.D. Data were collected and chromatograms displayed on a Hewlett-Packard 3388 recording integrator. The injection volume was 30 μl.

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Mobile phase

Ten ml of 1 *M* tetra-*n*-butylammonium hydroxide solution was added to 910 ml of water and the pH of the solution was adjusted to 7.5 with phosphoric acid. An 80-ml volume of methanol was added, the solution mixed thoroughly, filtered through a 0.45-μm filter and deaerated for approximately 10 min.

Sample solvent (0.2% in copper sulfate)

Cupric sulfate pentahydrate (3 g) is dissolved was 1 l water.

Nitrilotriacetic acid internal standard solution

A solution containing approximately 200 mg of nitrilotriacetic acid disodium salt was prepared by dissolving and diluting to volume with water to 100 ml (approximately 2 mg/ml).

EDTA standard

Approximately 200 mg of disodium EDTA was weighed accurately into a 100-ml volumetric flask and dissolved and diluted to volume with distilled water. A 5.0-ml volume of this solution was pipetted into a 25-ml volumetric flask and 5.0 ml of internal standard solution was added. The mixture was then diluted to volume with 0.2% copper sulfate solution (approximately 0.4 mg/ml).

Sample preparation

A 5.0-ml volume of sample was pipetted into a 25-ml volumetric flask. A 5.0-ml volume of internal standard solution was added and the mixture was diluted to volume with 0.2% cupric sulfate solution.

RESULTS AND DISCUSSION

The comparative precision data presented (Table I) demonstrate that the HPLC method is more reproducible than the titrimetric method. A typical chromatogram is shown in Figure 1. The EDTA response is linear and gave a correlation coefficient of 0.99999 in the range of 0.2 to 2.1 mg/ml. The analysis is applicable to

TABLE I

COMPARISON OF PRECISION FOR EDTA ANALYSES

	Precision for EDTA assay by HPLC (%)	*Precision for EDTA titrimetic assay (arsenazo I method) (%)*
	100.05	97.90
	98.75	98.00
	99.58	99.80
	99.62	98.90
	100.58	101.00
	99.65	100.20
Mean	99.71	99.3
S.D.	0.603	1.24
R.S.D.	0.605	1.25

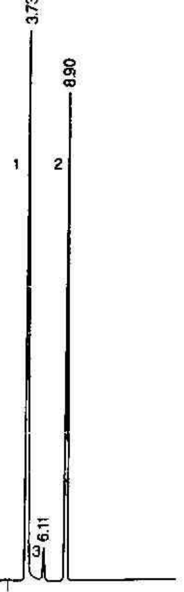

Fig. 1. Typical chromatogram of EDTA in opthalmic preparation. Peaks: 1 = EDTA; 2 = nitrilotriacetic acid.

a variety of ophthalmic solutions (Table II) and has been successfully used to quantitate EDTA in recalcified blood serum, EDTA solution and human feces.

The HPLC assay presented constitutes a direct, stability indicating analysis for

TABLE II

OPTHALMIC PREPARATIONS ASSAYED BY HPLC METHOD

All samples were purchased retail.

Product	*Label claim (mg/ml)*	*Found (mg/ml)*
Decongestants		
Clear Eyes	1.0	1.008
Murine	0.5	0.504
Murine Plus	1.0	0.988
Visine	1.0	1.000
Moisture Drops	*	1.082
Vaso Clear A	*	0.262
Collyrium	1.0	0.991
Tear Gard	1.0	1.021
Degest 2	*	0.196
Vasocon	*	0.291
Lens care		
Lensineyes	1.0	1.002
Sensitive eyes	*	0.248
Soft-mate	2.0	1.923

* No label claim available.

a very common antioxidant used to chelate metals in topical occular decongestants. The technique is more reproducible than the present titrimetric assay and does not suffer from the interferences possible in the titrimetric assay. The HPLC method presented can be easily automated and allows analysis of a variety of EDTA containing solutions on a single system.

REFERENCES

1 J. Wynn, *Toxicol. Appl. Pharmacol.*, 16 (1970) 807.
2 *United States Pharmacopeia*, 21st rev., U.S. Pharmacopeial Convention, Rockville, MD, 1985, p. 368.
3 E. E. Kaminski and D. Pacenti, *J. Pharm. Sci.*, 63 (1974) 1133–1136.
4 M. Blank and F. Snyder, *J. Chromatogr.*, 170 (1979) 379–383.
5 J. Harmsen and A. van den Toorn, *J. Chromatogr.*, 249 (1982) 379–384.
6 D. Parkes, M. Caruso and J. Spradling, III, *Anal. Chem.*, 53 (1981) 2154–2156.

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Note

Rapid chromatographic purification of the mitochondrial isoenzyme of beef heart malate dehydrogenase

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Malate dehydrogenase (MDH, E.C. 1.1.1.37) occurs in virtually all eukaryotic cells in at least two forms (isoenzymes) identified as mitochondrial (m-MDH) and cytosolic (c-MDH) according to their location[1]. It plays important metabolic rôles, being a necessary component of the tricarboxylic acid cycle and of the malate shuttle[1], and is used in analytical biochemistry as an indicator enzyme for the determination of the activity of aspartate aminotransferase (AST, GOT), ATP citrate lyase and citrate synthase[2–5]. MDH is also applied for the determination of oxaloacetate and malate and of other substances, *e.g.*, acetate, acetyl-coenzyme A (CoA) and citrate[6].

The purification of beef or pig heart MDH involves mostly ammonium sulphate fractionation and several chromatographic steps[7–9] (using cation and anion exchangers, molecular sieves or hydroxyapatite). The ion-exchange methods depend on the fact that c-MDH has an acidic isoelectric point, whereas m-MDH has a basic one[1].

The aim of the present paper is to describe a quick semi-preparative purification using two high-performance procedures (hydrophobic chromatography and cation-exchange chromatography). A combination of these methods yields homogeneous m-MDH.

EXPERIMENTAL

Materials

Beef hearts were obtained from a slaughter-house and were stored at −60°C. NADH and sodium pyruvate were obtained from Reanal (Budapest, Hungary) and Lachema (Brno, Czechoslovakia), respectively. The materials used for electrophoretic separations were mostly from Serva (Heidelberg, F.R.G.).

Enzyme preparation

Beef heart tissue (50 g) was homogenized with 100 ml of 20 m*M* sodium phosphate buffer (pH 7) and centrifuged at 6000 *g* for 30 min. The crude homogenate was precipitated with ammonium sulphate, most of the MDH activity being salted out between 40 and 75% saturation. These initial procedures were carried out at 4°C; the subsequent chromatographic steps proceeded at room temperature, the eluted fractions being kept in an ice-bath.

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The supernatant containing MDH activity (15 ml) was separated in two stages on a glass column (120 mm × 12 mm I.D.) packed with Spheron-Micro 300 (12 μm, Lachema). The column was attached to two pumps (P-500) and a gradient programmer (GP-250) from Pharmacia (Uppsala, Sweden). As starting and terminating buffers, 0.1 *M* sodium phosphate (pH 7) 30% saturated with ammonium sulphate and 0.1 *M* sodium phosphate (pH 7) were used (flow-rate 4 ml/min). The samples were injected with a V-7 valve equipped with a 10-ml superloop (Pharmacia). The separations were evaluated by an UV-1 monitor (λ = 280 nm) and a FRAC-100 collector (Pharmacia). The fractions containing m-MDH were diafiltrated and concentrated with an ultrafiltration cell (Amicon, Danvers, MA, U.S.A.) with a YM-10 membrane. The sample was divided into two portions and applied to a Mono S HR 5/5 column (50 mm × 5 mm I.D.) from Pharmacia or to a column of the same dimensions packed with Spheron-Micro SB 300 (12 μm, Lachema). The columns were attached to the above-mentioned chromatographic system. As starting and terminating buffers, 20 m*M* sodium phosphate (pH 7) and the same buffer with 0.3 *M* sodium chloride were used (flow-rate 1.5 ml/min).

Enzyme analysis

The activity of malate dehydrogenase was assayed spectrophotometrically in the presence of NADH and pyruvate at 25°C[10]; the protein concentration was calculated from the absorbance at 280 nm. The activity of aspartate aminotransferase (AST, GOT) was determined using a GOT opt. Monotest (Boehringer, Mannheim, F.R.G.). The spectrophotometric measurements were performed in a Cary 118 apparatus (Varian, Palo Alto, CA, U.S.A.). Gel permeation chromatography was carried out on a Superose 12 column (Pharmacia) attached to the above-mentioned chromatographic system, using 0.1 *M* sodium phosphate (pH 7) with 0.15 *M* sodium chloride as the mobile phase (flow-rate 0.7 ml/min). Electrophoretic analysis of MDH was carried out in 15% starch gels using the separation and staining conditions described[11]; polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was performed as described previously[12].

RESULTS AND DISCUSSION

The conventional ammonium sulphate fractionation was carried out as the first purification step in the described isolation procedure. It brought about a nearly four-fold purification of MDH and a reduction of the sample volume (Table I). Moreover, it was compatible with the subsequent hydrophobic chromatography (the salted-out homogenate could be used directly without diafiltration).

The chromatography on a Spheron-Micro column was a convenient method for further purification of MDH. The unsubstituted Spherons show moderate hydrophobicity and can be used for hydrophobic chromatography of several proteins[13]. The elution with a decreasing salt concentration resulted in a relatively good separation of the main MDH isoenzymes from each other and from most of the contaminants (Fig. 1). The isoenzymes were identified by means of starch electrophoresis; c-MDH (the prevailing form in the first peak with MDH activity in Fig. 1) migrated to the anode at the pH value used[11], whereas m-MDH (the most important constituent of the second peak with MDH activity in Fig. 1) moved in the opposite direction.

TABLE I

PURIFICATION OF THE MITOCHONDRIAL ISOENZYME OF MALATE DEHYDROGENASE FROM BEEF HEART

Details of the procedure as described in the text. The activity of m-MDH is given (it corresponds to *ca.* 60% of the total MDH activity in the initial steps); m-MDH and c-MDH were separated nearly completely on a Spheron-Micro column (Fig. 1).

Fraction	*Protein (mg)*	*Total activity (U)*	*Specific activity (U/ml)*	*Purification*	*Recovery (%)*
Crude supernatant	2650	4000	1.5	(1)	(100)
Ammonium sulphate fractionation (40–75%)	540	2900	5.4	3.6	73
Hydrophobic chromatography (Spheron-Micro)*	53	2800	53	35	70
Cation-exchange chromatography (Mono S)*	2.7	2450	915	610	62

* The separations were carried out in two stages.

The purification of m-MDH was approximately ten-fold (Table I), that of c-MDH was slightly worse.

Chromatography on columns containing strong cation exchangers (with sulphonyl groups) was chosen for the final step in the purification of m-MDH. Both materials tested (Mono S and Spheron-Micro SB) gave comparable results. Homogeneous m-MDH was eluted with a sodium chloride concentration gradient (Fig.

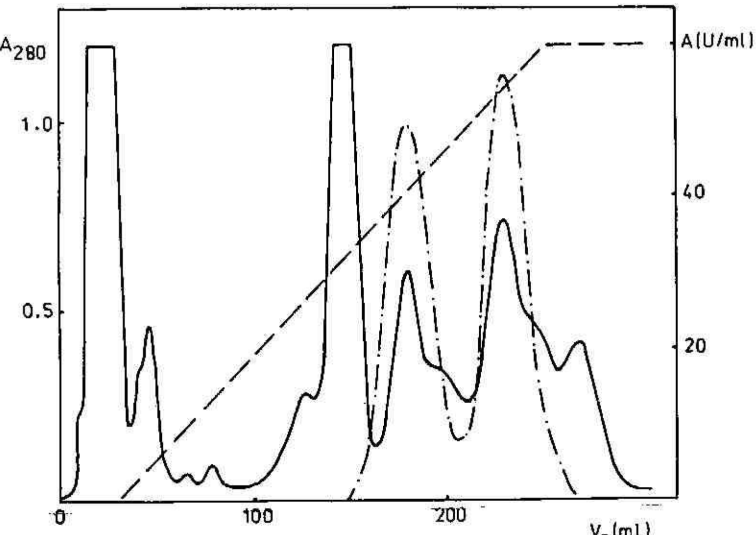

Fig. 1. Chromatography of crude malate dehydrogenase on a Spheron-Micro column. Buffers: A, 0.1 *M* sodium phosphate (pH 7) 30% saturated with ammonium sulphate; B, the same but without ammonium sulphate. V_e = Elution volume; ——, absorbance at 280 nm (A_{280}); -----, gradient; –·–·–, MDH activity (U/ml). Approximately 270 mg of protein were applied to the column.

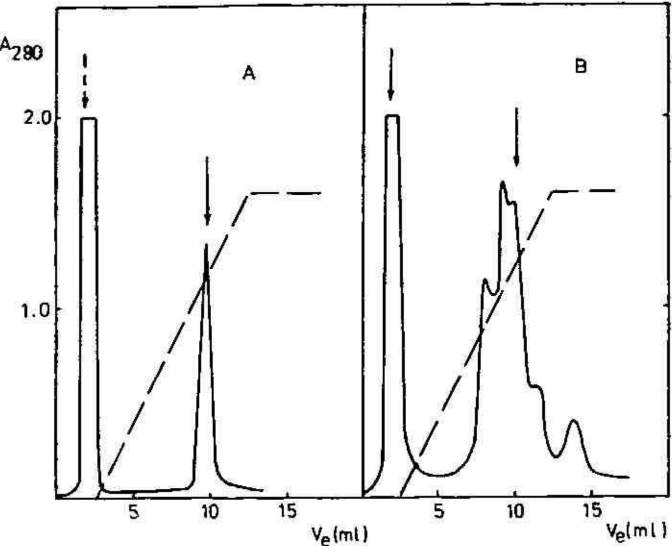

Fig. 2. Chromatography of malate dehydrogenase on a Mono S column. Buffers: A, 20 mM sodium phosphate (pH 7); B, the same but with 0.3 M sodium chloride. Full and broken arrows correspond to high and low MDH activities, respectively; other symbols as in Fig. 1. (A) Separation of m-MDH purified by hydrophobic chromatography (see Fig. 1 and Table I); *ca.* 25 mg of protein with *ca.* 1400 units of m-MDH were injected. (B) Separation of crude MDH (salted-out with ammonium sulphate); a comparable MDH activity to that in (A) was injected.

2A). The purification of m-MDH was nearly twenty-fold in this step (see Table I). The chromatography on cation exchangers was also carried out with the crude homogenate (after ammonium sulphate fractionation and diafiltration), *cf.*, Fig. 2B. The increase in specific activity was nearly 100-fold in this case, however, the m-MDH obtained was not homogeneous. This means that the hydrophobic chromatography removed some impurities which could not be separated on the cation exchangers (compare Fig. 2A and B). The increase in specific activity achieved by the combination of hydrophobic and ion-exchange chromatography was nearly 200-fold (Table I). The homogeneity of the final product (Table I) was shown by re-chromatography on a Mono S column (after diafiltration), by chromatography on a Superose 12 column and by SDS-PAGE.

The rapid chromatographic procedure described is suitable for the preparation of several milligrams of the homogeneous mitochondrial isoenzyme of malate dehydrogenase. Larger columns for high-performance hydrophobic and cation-exchange chromatography can be used for the preparation of m-MDH on a preparative scale. The purified m-MDH can be used for exact enzymatic studies as well as for analytical purposes (it does not contain detectable activities of aspartate aminotransferase or of other enzymes). The amount of m-MDH prepared on the semi-preparative scale (Table I) was sufficient for more than 1000 assays of aspartate aminotransferase in biological materials (under the conditions given in ref. 2). On the other hand, non-negligible AST activities were found in the partially purified MDH preparations. The MDH/AST activity ratio in the m-MDH fraction obtained in the first chromatographic step, *i.e.*, after hydrophobic chromatography, was approximately

400. However, this partially purified enzyme can also be used for AST activity assay in most biological samples, giving the blank AST activity of 1–2 U/l (which is one order of magnitude lower than the upper limit for the normal values of AST in human blood serum[14] and is comparable with the standard error of the AST assay[2]).

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REFERENCES

1 L. J. Banaszak and R. A. Bradshaw, in P. D. Boyer (Editor), *The Enzymes*, Vol. 11, Academic Press, New York, 3rd ed., 1975, p. 369.
2 H. U. Bergmeyer, *Methoden der enzymatischen Analyse*, Vol. 1, Akademie-Verlag, Berlin, 2nd ed., 1970, p. 685.
3 P. A. Srere, *J. Biol. Chem.*, 234 (1959) 2544.
4 P. A. Srere, *J. Biol. Chem.*, 236 (1961) 50.
5 S. Ochoa, *Methods Enzymol.*, 1 (1955) 685.
6 J. R. Stern, *Methods Enzymol.*, 1 (1955) 559.
7 R. K. Gerding and R. G. Wolfe, *J. Biol. Chem.*, 244 (1969) 1164.
8 S. Englard, L. Siegel and H. H. Breiger, *Biochem. Biophys. Res. Commun.*, 3 (1960) 323.
9 F. C. Grimm and D. G. Doherty, *J. Biol. Chem.*, 236 (1961) 1980.
10 H. U. Bergmeyer, *Methoden der enzymatischen Analyse*, Vol. 1, Akademie-Verlag, Berlin, 2nd ed., 1970, p. 446.
11 I. H. Fine and L. A. Costello, *Methods Enzymol.*, 6 (1963) 958.
12 I. Matysková, J. Kovář and P. Racek, *Biochim. Biophys. Acta*, 839 (1985) 300.
13 P. Štrop, F. Mikeš and Z. Chytilová, *J. Chromatogr.*, 156 (1978) 239.
14 *GOT opt. Monotest*, Boehringer, Mannheim, 1980.

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Note

High-performance liquid chromatographic determination of taurine in formulations as the dansyl derivative

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Since retinal degeneration in the cat has been shown to be due to dietary deficiency of taurine (Tau) (2-aminoethanesulphonic acid)[1], there has been increasing evidence of its important role in the body of other mammalian species, including man. Hence the Tau requirement in primate nutrition has been reviewed[2] and its presence in formula diets has been studied[3].

Apart from a multi-step procedure involving gas chromatography[4], liquid chromatography was initially the technique mostly chosen for the determination of the ionic compound Tau. Procedures based on the amino acid analyser have been applied[5] but, in addition to problems of overlapping problems in the Tau zone of the chromatographic profile, the need for a complex apparatus appears excessive with respect to the simplicity of the analytical object. Pre-column derivatization and reversed-phase high-performance liquid chromatography (HPLC) have been the techniques mostly used for Tau determinations in recent years. In particularly, treatment with *o*-phthaldehyde (OPA) has been applied in different procedures and chromatographic systems[6–10]. In addition to the difficulty of resolving Tau from interfering compounds[6,9], the major disadvantage of the OPA derivative is its low stability, which necessitates rigorous control of the reaction and injection times in order to obtain high reproducibility[7].

The aim of this work was to test another conventional amino acid reagent, 5-dimethylaminonaphthalenesulphonyl chloride (dansyl chloride, DNS-Cl), for the determination of Tau in formulations by a rapid, simple and reliable procedure suitable for routine analysis.

EXPERIMENTAL

Materials

Tau and cysteic acid (CysA) were supplied by Sigma (St. Louis, MO, U.S.A.), DNS-Cl and HPLC-grade methanol by Merck (Darmstadt, F.R.G.) and all other reagents and solvents by Carlo Erba (Milan, Italy). DNS-Cl solution (1.5 mg/ml) in acetonitrile was maintained at −20°C and always kept wrapped with aluminium foil in reaction vials to exclude light. The tested formulations were powdered and capsule preparations containing 2% (w/w) and 250 mg per capsule of Tau, respectively.

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Chromatographic conditions

A Jasco (Tokyo, Japan) HPLC system equipped with a Twincle pump, Uvidec-100-III-detector set at 254 nm and VL-611 injector was used. The separations were performed on a cartridge (25 cm × 4 mm I.D.) filled with a LiChrosorb RP-8 (7 μm) mounted on a Manu-Fix holder. Methanol–water (35:65) containing acetic acid (0.6%, v/v) and triethylamine (0.008%, v/v) was used as the eluent at a flow-rate of 0.5 ml/min.

Sample preparation

A 1-g amount of powdered formulation and 0.1 mmol of CysA, used as an internal standard, or the content of a capsule and 1 mmol of CysA were added to water (100 ml total volume), shaken for 5 min sonicated for 30 min, and filtered. An aliquot (5 ml) was withdrawn, washed with *n*-hexane (5 ml), treated with 10% trichloroacetic acid (1 ml) and centrifuged. The supernatant was neutralized with sodium hydroxide solution (5 mol/l) and brought to volume (50 ml) with 0.05 mol/l lithium carbonate buffer (pH 9.5). The capsule sample was again diluted 1:10 with carbonate buffer.

Derivatization

In PTFE-lined screw-capped vials an aliquot (1 ml) of the final buffered sample was treated with DNS-Cl solution in acetonitrile (0.5 ml). The mixture was maintained at 40°C for 10 min and, after cooling, the formed dansyl-Tau (DNS-Tau) and dansyl-CysA (DNS-CysA) were analysed by injecting 10-μl volumes of the sample into the chromatograph.

Quantitative analysis

Different volumes of 1 mmol/l aqueous Tau solution containing 10–200 nmol (*i.e.*, 1.2–25 μg) and always a constant volume (50 μl) of 2 mmol/l aqueous CysA solution, were mixed, brought to 1 ml with the above-mentioned carbonate buffer and treated according to the already described derivatization procedure. A calibration graph was obtained by plotting ratios between the peak heights of DNS-Tau and DNS-CysA (R_h) *versus* the amount of Tau. During formulations analysis the contents of Tau in the final samples were obtained from the R_h values.

RESULTS AND DISCUSSION

By exploiting the latest improvements in the dansylation of amino acid suitable for HPLC analysis[11], a derivatization technique alternative to that involving OPA was tested. In order to reduce the procedure time, the reaction temperature was brought to 40°C; under these conditions 10 min were sufficient to give the same maximum yields as obtained at room temperature in more than 30 min. At higher temperatures the reaction time became critical, owing to possible DNS-Cl hydrolysis and competing decomposition of DNS derivatives.

Fig. 1. shows a typical HPLC separation of DNS derivatives of Tau and CysA as internal standard. Under the described conditions the retention time of DNS-Tau relative to DNS-CysA was 1.12 and the overall instrumental analysis time was slightly more than 10 min. The stability of the DNS derivatives was tested by determining

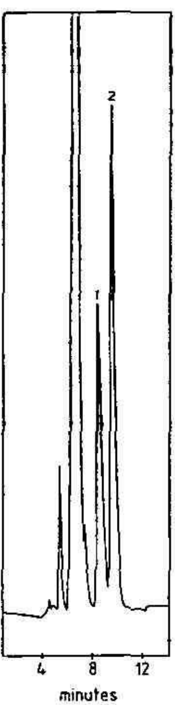

Fig. 1. Typical HPLC profile obtained from a sample analysis. Peaks: 1, DNS-CysA; 2, DNS-Tau.

the peak height of aliquots from the same sample solution kept at room temperature for different periods; no decrease was found for over 8 h. The only precaution necessary is to keep the reaction vials wrapped in aluminium foil to protect the samples from photodegradation, hence eliminating the need to analyse them immediately after derivatization step. The relationship between the DNS-Tau/DNS-CysA peak-height ratio and amount of Tau was linear. The intercept and correlation coefficient were −0.0120 and 0.9998, respectively, and the slope was 0.1672 and 0.0207 for amounts of Tau in micrograms and nanomoles, respectively. Assays performed in duplicate on at least three specimens of each formulation were gave recoveries of 98.5% and 97.6% (relative standard deviation 1.4% and 1.8%) for powdered and capsule formulations, respectively.

In conclusion, the procedure described here can be considered to be useful alternative to the previously reported methods and, with regard to its reliability and simplicity, it appears to be suitable for the routine determination of Tau in formulations. Further experiments are needed in order to assess the applicability of the proposed derivatization procedure to the determination of Tau in biological fluids.

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REFERENCES

1 K. Knopf, J. A. Sturman, M. Armstrong and K. C. Hayes, *J. Nutr.*, 108 (1978) 773.
2 K. C. Hayes, *Nutr. Rev.*, 43 (1985) 65.
3 H. F. Erbersdobler, E. Trautwein and H. G. Greulich, *Eur. J. Pediatr.*, 142 (1984) 133.
4 H. Kataoka, S. Yamamoto and M. Makita, *J. Chromatogr.*, 306 (1984) 61.
5 H. F. Erbersdobler, H.-G. Greulich and E. Trautwein, *J. Chromatogr.*, 254 (1983) 332.
6 J. D. Stuart, T. D. Wilson, D. W. Hill, F. H. Walters and S. Y. Feng, *J. Liq. Chromatogr.*, 2 (1979) 809.
7 B. R. Larsen, D. S. Grosso and S. Y. Chang, *J. Chromatogr. Sci.*, 18 (1980) 233.
8 G. H. T. Wheler and J. T. Russel, *J. Liq. Chromatogr.*, 4 (1981) 1281.
9 M. Eslami and J. D. Stuart, *J. Liq. Chromatogr.*, 7 (1984) 1117.
10 L. L. Hirschberger, J. De La Rosa and M. H. Stipanuk, *J. Chromatogr.*, 343 (1985) 303.
11 Y. Tapuhi, D. E. Schmidt, W. Lindner and B. L. Karger, *Anal. Biochem.*, 115 (1981) 123.

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Note

Isolation and purification of phorbol from croton oil by reversed-phase column chromatography

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The diesters of the diterpene alcohol phorbol (Fig. 1A) have generated much interest as pharmacological probes in cancer research and for their diverse biological effects at extremely low concentrations[1–3]. One of these diesters, 12-O-tetradecanoylphorbol-13-acetate (TPA, Fig. 1B), is the most potent tumor promoter known in the two stages mouse skin model of carcinogenesis[4,5]. In addition to its tumor promoting activity, TPA also produces a variety of biological effects *in vivo* and *in vitro*[6,7]. Croton oil, the seed oil of *Croton tiglium* L., is a valuable source of the diesters of phorbol and related diterpene alcohols.

Recently, we became interested in preparing some analogues of TPA for use as probes in the study of the mechanism of action of the phorbol esters. These synthetic studies required large quantities of the phorbol alcohol. The relatively high cost of the commercially available product prompted us to explore alternative methods of obtaining phorbol from croton oil which are more efficient than published methods[1,2,8]. In this report we describe a simple, reliable and inexpensive method for the isolation and purification of phorbol from croton oil.

MATERIALS AND METHODS

Melting points were determined in open capillary tubes with a Thomas-Hoover melting point apparatus and are not corrected. Elemental analyses were performed by M-H-W Laboratories (Phoenix, AZ, U.S.A.) and were within ±0.4% of the theoretical values. IR spectra were obtained in KBr pellets using a Perkin-Elmer 281 instrument. UV measurements were obtained on a Beckman Model DU-8 spectrophotometer. Proton nuclear magnetic resonance spectra were recorded at ambient temperature on a Jeol-FX90Q instrument or a Nicollet NT300WB spectrometer with

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A $R_1, R_2 = H$

B $R_1 = CH_3(CH_2)_{12}CO—$, $R_2 = CH_3CO—$

Fig. 1. Structure of (A) phorbol and (B) 12-O-tetradecanoylphorbol-13-acetate.

tetramethylsilane (TMS) as the internal standard. Mass spectra were obtained on a Finnigan 4000 instrument in the chemical ionization (CI) mode using isobutane as the reagent gas.

Croton oil (Fisher Scientific, New York, NY, U.S.A.) was stored in amber glass bottles at 4°C until used. Reference sample of phorbol was obtained from Chemical Carcinogenesis (Eden Prairie, MN, U.S.A.). All other chemicals used throughout the work were of reagent grade. The solvents used in the chromatographic separation were of HPLC grade.

Preparation of a crude extract of phorbol form croton oil

Croton oil (200 g) was mixed with a solution of $Ba(OH)_2 \cdot 8H_2O$ (22 g) in methanol (1 l) in a 2-l round bottom flask under an atmosphere of nitrogen. The reaction vessel was wrapped in an aluminum foil to protect the contents from light and was shaken for 10–12 h. The reaction mixture was filtered and the filtrate was concentrated *in vacuo* at a temperature below 40°C. The oily residue was mixed with distilled water (1 l) and extracted with diethyl ether (3 × 200 ml). The aqueous phase was adjusted to pH 5 with 2 *N* sulfuric acid and treated with a saturated solution of sodium sulfate (16 ml). The mixture was stored at 4°C overnight and filtered. The filtrate was adjusted to pH 7 with 2 *N* sodium hydroxide and concentrated under reduced pressure. The oily residue was digested with hot absolute ethanol (40–50 ml) and filtered rapidly. The precipitate was washed repeatedly with hot absolute ethanol. The filtrate was concentrated and stored at 4°C for several weeks. Few crystals of phorbol alcohol were added to induce crystallization and the mixture was stored at 4°C for additional several weeks. The mixture was concentrated under reduced pressure to provide crude phorbol fraction as an oily residue. The crude fraction was stored under nitrogen at 4°C until it was subjected to purification by reversed-phase column chromatography.

Thin-layer chromatography (TLC)

Normal phase analytical TLC was performed on silica gel GF plates (250 µm, 10 × 20 cm uniscored, Analtech). Reversed-phase analytical TLC was performed on

TABLE I

R_F VALUES OF PHORBOL IN VARIOUS SOLVENT SYSTEMS ON SILICA GEL AND REVERSED-PHASE RPS-F TLC PLATES (ANALTECH)

Solvent system (v/v)	R_F	*TLC plate*
Ethyl acetate–methanol (10:1)	0.23	Silica gel
Methanol–chloroform (1:9)	0.70	Silica gel
Acetic acid–chloroform (2:5)	0.26	Silica gel
Pyridine–chloroform (2:5)	0.31	Silica gel
Chloroform–acetic acid (1:1)	0.42	Silica gel
Ethyl acetate–acetic acid (5:2)	0.80	Silica gel
Ethyl acetate–methanol (1:1)	0.66	Silica gel
Chloroform–acetonitrile–trifluoroacetic acid (7:6:0.05)	0.12	Silica gel
Acetonitrile–chloroform (95:5)	0.15	Silica gel
Acetonitrile–methanol (10:1)	0.36	Silica gel
Methanol–acetonitrile–water (1:1:5)	0.8	RPS-F
Methanol–acetonitrile–water (1:1:10)	0.70	RPS-F
Methanol–acetonitrile–water (1:1:20)	0.56	RPS-F
Methanol–acetonitrile–water (1:1:40)	0.40	RPS-F

reversed-phase RPS-F plates (250 μm, 10 × 20 cm uniscored, Analtech). A number of solvent systems were used (Table I). The TLC plates were visualized under short-wave ultraviolet light followed by spraying with a vanillin–sulfuric acid–absolute ethanol (3:0.5:100) spray reagent and heating over a hot plate at 120°C.

Purification of phorbol by preparative reversed-phase column chromatography

Phorbol was obtained from the crude extract by reversed-phase column chromatography using a system composed of metering pump (Model FMI, Fluid Metering, Oyster Bay, NY, U.S.A.), a PTFE rotary valve (Type 50, Rheodyne, Cotati, CA, U.S.A.), a multiple wavelength absorbance–fluorescence detector set at 254 nm in the absorbance mode (Model VA-5, ISCO, Lincoln, NE, U.S.A.) and fraction collector (Retriever II, ISCO). Columns prepacked with LiChroprep RP-8, 40–63 μm were used (size A, 240 × 10 mm I.D. and size B, 310 × 25 mm I.D., Merck, Darmstadt, F.R.G.). A linear gradient of methanol–acetonitrile–water (1:1:5) and methanol–acetonitrile–water (1:1:40) was used as the mobile phase at a flow-rate of 10 ml/min.

The crude phorbol fraction was dissolved in distilled water to give a total volume of 40 ml. When a size A column was used, a 1-ml aliquot of the solution of the crude fraction was loaded onto the column and the gradient elution was started. The UV absorbance of the eluate was monitored and 10-ml fractions were collected (Fig. 2). Phorbol typically eluted within 15 min. After 45 min the column was eluted with methanol and after 60 min the column was re-equilibrated with methanol–acetonitrile–water (1:1:40) in preparation for the next separation.

When the size B column was used, a 4-ml aliquot of the solution of the crude phorbol fraction was loaded on the column and eluted with the same linear gradient as that used with the size A column at a flow-rate of 10 ml/min. Fractions (10 ml) were collected and phorbol typically eluted within 1 h.

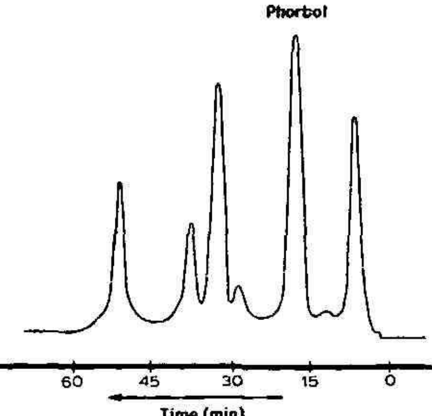

Fig. 2. Preparative chromatographic separation of phorbol from the crude extract of saponified croton oil. Column: LiChroprep RP-8, 40–63 μm (size A). Solvent: linear gradient of methanol–acetonitrile–water (1:1:5) and methanol–acetonitrile–water (1:1:40) over 45 min. Flow-rate: 10 ml/min. Detector: UV at 254 nm. Phorbol eluted at 15 min. At 45 min the column was eluted with methanol and at 60 min it was re-equilibrated with methanol–acetonitrile–water (1:1:40).

Fractions containing phorbol were pooled and the solvent was removed under reduced pressure at a temperature below 60°C. Acetone was added to the residue to precipitate the phorbol. The precipitate wass filtered and dried in a vacuum dessicator over phosphorous pentoxide for 24 h. An amount of 3.26 g of pure phorbol was obtained from 200 g of croton oil (yield 1.6%). A sample of the phorbol was recrystallized from ethyl acetate, m.p. 233–234°C [lit. 234°C (ref. 9)]. *IR* (KBr) 3400, 2900, 1710 and 1640 cm^{-1}. UV (absolute ethanol) λ_{max} nm (ε), 210 (6870), 233 (4340). ^{1}H NMR (d_4-methanol): 0.72 (d, J = 5, 1H), 1.1 (d, J = 6, 3H), 1.2 (s, 6H), 1.75 (m, 3H), 1.9 (m, 1H), 2.5 (q, AB pattern, 2H), 3.1 (bm, 1H), 3.2 (bm, 1H), 3.9 (s, 2H), 4.1 (d, J = 11, 1H), 5.60 (m, 1H), and 7.60 (bs, 1H) ppm. Elemental analysis: calculated (found) for $C_{20}H_{28}O_6$: C, 65.90 (65.83); H, 7.75 (7.88).

RESULTS AND DISCUSSION

Phorbol and other diterpene alcohols are present in croton oil in the form of esters of fatty acids. These alcohols are sensitive to light and are easily oxidized by atmospheric oxygen. Therefore, croton oil was treated with barium hydroxide under a nitrogen atmosphere and in the absence of light to saponify the esters. This resulted in the formation of the barium salts of the fatty acids, which are insoluble in methanol, and the free diterpene alcohols. The barium salts were removed by filtration and the filtrate was concentrated to remove the solvent. The residue was mixed with water to solubilize the hydrophilic diterpene alcohols and the hydrophobic constituents were removed by ether extraction. The excess barium hydroxide was removed from the aqueous solution by the addition of sulfuric acid to form the insoluble barium sulfate. After the filtration of barium sulfate the filtrate was concentrated to remove water. The crude phorbol alcohol was extracted from inorganic impurities with hot absolute ethanol. Concentration of the ethanol extract is reported to result

in the formation of phorbol crystals[1,2]. However, we were not able to obtain phorbol crystals from the concentrated solution even after the addition of few crystals of phorbol to induce crystallization.

Examination of the alcoholic solution indicated the presence of a major component which had the same chromatographic mobility on TLC as that of the phorbol reference standard (Table I). Therefore, we explored the utility of reversed-phase column chromatography in the isolation and purification of phorbol from the crude extract. Octylsilane bonded to silica gel (40–63 μm) was used as the stationary phase. A variety of solvents were attempted. A mixture of methanol–acetonitrile–water (1:1:40) in the isocratic mode provided a good separation of phorbol from the other impurities in the extract. The use of a linear gradient of methanol–acetonitrile–water (1:1:5) to methanol–acetonitrile–water (1:1:40) over 45 min resulted in faster elution of phorbol without affecting the chromatographic resolution of the various components in the extract. Also changing the flow-rate between 6 and 10 ml/min did not affect the resolution and maintaining the flow-rate within this range resulted in reproducible results. After the separation with the linear gradient was completed it was essential to wash the column with methanol to remove dark material that was retained at the bottom of the column. Approximately 50 mg of phorbol could be purified in each run using the size A column. A run required approximately 1 h. The crude phorbol fraction obtained from 200 g of croton oil was purified in 30–40 injections.

The phorbol obtained after column purification was of sufficient purity to use for subsequent synthetic work. The purity of the product was ascertained from its homogeneity on examination in a number of TLC systems. In these systems only one product was detected which had the same chromatographic mobility as the reference sample of phorbol. The identity of the product was further confirmed by examining its IR, UV, CI-MS and ^{1}H NMR spectra. These spectra were identical to those obtained from the reference standard and were consistent with the structure of phorbol.

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REFERENCES

1 E. Hecker and R. Schmidt, *Fortschr. Chem. Org. Naturst.*, 31 (1974) 377.
2 E. Hecker, in H. Busch (Editor), *Methods in Cancer Research*, Vol. 6, Academic Press, New York, 1971, p. 439.
3 E. Hecker, *Cancer Res.*, 28 (1968) 2338.
4 E. Hecker, in T. J. Slaga, A. Sivak and R. K. Boutwell (Editors), *Mechanisms of Tumor Promotion and Cocarcinogenesis*, Vol. 2, Raven Press, New York, 1978, pp. 1–588.
5 R. K. Boutwell, *C.R.C. Crit. Rev. Toxicol.*, 2 (1974) 419–443.
6 P. M. Blumberg, *C.R.C. Crit. Rev. Toxicol.*, 8 (1980–81) 153, 199.
7 E. Hecker, N. E. Fusenig, W. Kunz, F. Marks and H. W. Thielman (Editors), *Carcinogenesis —A Comprehensive Survey*, Vol. 7, Raven Press, New York, 1982.
8 Gary T. Marshall and A. D. Kingdom, *J. Chromatogr.*, 206 (1981) 421.
9 L. Crombie, M. L. Games and J. J. Pointer, *J. Chem. Soc. C*, 1347 (1968).

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Note

Determination of mofebutazone and its 4-hydroxy metabolite in plasma and urine by high-performance liquid chromatography

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Mofebutazone (MPB), the monophenyl analogue of phenylbutazone (DPB), is an anti-inflammatory drug which is said to be tolerated better than phenylbutazone. In man, its anti-inflammatory activity and toxicity are less than those of phenylbutazone[1,2]. In equine practice, however, it appears that mofebutazone is less effective than phenylbutazone. This can be attributed either to its limited solubility or to the low bioavailability when given orally. In order to study the pharmacokinetics of MPB in the horse, a method is needed which distinguishes between MPB and its metabolites or degradation products.

MPB can be rapidly oxidized to the 4-hydroxy compound, 1-phenyl-3,5-dioxo-4-hydroxy-4-*n*-butylpyrazolidine (4-OH-MPB), as shown in Fig. 1. The same product can also be formed during the analysis of MPB or even in pharmaceutical preparations, in which the percentage decomposition of MPB ranges between 5.2 and 10.2% and in some cases 76 and 82% of 4-OH-MPB is formed[3]. The method used for the determination of MPB and 4-OH-MPB in these pharmaceutical preparations[3] was based on thin-layer chromatography (TLC) followed by spectrophotometry, and is considered as unsuitable for the determination of both substances in biological fluids. Mofebutazone alone has been analysed qualitatively in screening methods by high-performance liquid chromatography (HPLC)[4,5]. In disposition studies in the rat[6,7] and man[8], mofebutazone was quantified using the compound [4-^{14}C]mofebutazone. However, due to the minor differences in TLC R_F values between MPB and 4-OH-MPB in these studies, the measured radioactivity could be related to the MPB concentration only if the metabolism was neglected. However, by taking appropriate measures to prevent oxidation, MPB and 4-OH-MPB can be simultaneously determined by HPLC using the method described here.

C_6H_5 C_6H_5 O N NH (O) O N NH C_4H_9 O HO C_4H_9 O

MPB 4-OH-MPB

Fig. 1. Chemical structure of mofebutazone (MPB) and its oxidation product, 4-hydroxymofebutazone (4-OH-MPB).

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EXPERIMENTAL

Reagents and materials

Mofebutazone was obtained from Laboratoria Flandria (Zwijnaarde, Belgium) and its purity checked by HPLC. 4-OH-MPB was prepared by passing air through a solution of MPB in a mixture of acetone and toluene[9]. Its purity was checked by melting point (179°C) and HPLC analysis. The internal standard phenylbutazone was a gift from Byk Gulden (Konstanz, F.R.G.). All other chemicals were of reagent grades. Methanol was of HPLC grade obtained from Merck (Darmstadt, F.R.G.).

Instrumentation

The HPLC system was a Varian instrument with a variable wavelength UV detector set at 240 nm. Chromatography was done on a Nucleosil 5 C_{18} cartridge system (10 cm × 3 mm I.D.) from Chrompack (Antwerpen, Belgium). An appropriate disposable guard column (1 cm × 2.1 mm I.D.) was used.

Chromatographic conditions

The mobile phase for the analysis of MPB and 4-OH-MPB comprised 60% methanol and 40% water–acetic acid (30:1). The flow-rate was 0.4 ml/min.

Sample analysis

To 1 ml plasma in a screw-caped tube, 1 ml of 1 *M* acetate buffer (pH 5.3) and 0.1 ml of the internal standard solution [150 μg/ml phenylbutazone in ascorbic acid–methanol (1:100, w/v)] was added. The use of vitamin C was necessary to prevent oxidation of MPB during the extraction step. Extraction was performed by rolling on a commercial rolling apparatus with 5 ml diethyl ether for 5 min. After centrifugation (5 min), the organic phase was separated and evaporated under a nitrogen atmosphere at 40°C. The residue was redissolved in 0.1 ml methanol and 10 μl were injected into the liquid chromatograph.

Urine (0.5 ml) was analysed in a similar manner except that 0.2 ml of internal standard were added. Centrifugation was not necessary.

Calibration curve

The linearity of the calibration was determined by adding 1, 2.5, 5, 8, 10, 15, 20 and 25 μg of MPB and 4-OH-MPB per ml to blank plasma samples. In a similar way, samples containing 5, 10, 20, 25, 50, 75 and 100 μg of MPB and 4-OH-MPB per ml urine were analysed. Four aliquots of each concentration were analyzed as described. The coefficients of variation were determined using replicate samples of plasma standards containing 5, 10 and 20 μg of MPB and 4-OH-MPB per ml, and of urine standards containing 10, 20 and 50 μg of MPB and 4-OH-MPB per ml.

Determination of extraction efficiency

The recoveries of the drugs were estimated from the changes in the peak-height ratios when the drugs were added to the plasma (urine) and the internal standard was added to the final extract compared to the peak height ratios when both the drugs and internal standard were added to the final extract.

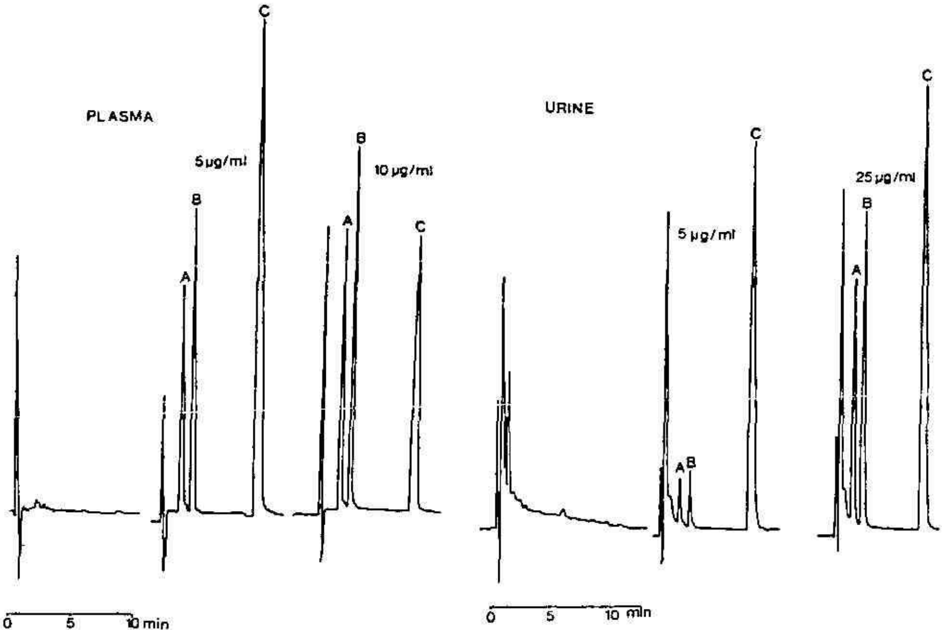

Fig. 2. Chromatograms obtained from residues of spiked plasma and urine. Peak: A = 4-OH-MPB; B = MPB; C = internal standard, DPB.

RESULTS AND DISCUSSION

Under the described chromatographic conditions, baseline resolution was achieved between MPB and 4-OH-MPB in plasma and urine extracts. Extracts from drug-free plasma and urine were found to be free from interfering peaks. Representative chromatograms from plasma and urine spiked with MPB and 4-OH-MPB are shown in Fig. 2. The retention times were 2.75, 3.60 and 8.50 min respectively for MPB, 4-OH-MPB and the internal standard DPB.

The peak-height ratio of MPB and 4-OH-MPB *versus* DPB was chosen as the quantitative measure of the detector response. Regression analysis of these data resulted in a correlation coefficient, r, greater than 0.998 for both MPB and 4-OH-MPB either in plasma or in urine.

The average recoveries (± S.D.) of MPB and 4-OH-MPB from plasma samples to which drug standard had been added at concentrations ranging from 5 to 20 µg/ml were determined to be 80.0 ± 3.7 and 67.7 ± 1.8% respectively (Table I). The extraction recovery from urine, however, was markedly higher giving values of 91.8 ± 3.5 and 95.2 ± 3.7% for MPB and 4-OH-MPB respectively. Compared to plasma, either a better separation between the organic phase and the urine or the lack of urinary protein binding could contribute to these higher recoveries. The data in Table I also suggest that there was practically no recovery dependence on concentration over the range of drug levels investigated.

The precision of the assay was assessed by analysis of plasma and urine samples containing known concentrations of MPB and 4-OH-MPB. The results of these de-

TABLE I

PERCENTAGE RECOVERY OF MPB AND 4-OH-MPB FROM PLASMA AND URINE

$n = 4$.

MPB and 4-OH-MPB concentration (μg/ml)	*Percentage recovery (means ± S.D.)*	
	MPB	*4-OH-MPB*
5*	79.0 ± 4.1	66.2 ± 2.0
10*	78.1 ± 3.4	68.5 ± 1.6
20*	82.9 ± 1.7	68.2 ± 1.5
5**	94.3 ± 2.8	95.9 ± 4.5
10**	89.4 ± 2.7	92.4 ± 1.7
20**	92.3 ± 3.9	92.4 ± 1.7

* Plasma.
** urine.

TABLE II

ASSAY PRECISION FOR MPB AND 4-OH-MPB IN PLASMA

$n = 4$.

	MPB (μg/ml)			*4-OH-MPB (μg/ml)*		
	5	*10*	*20*	*5*	*10*	*20*
Mean observed concentration	4.86	9.91	19.79	4.81	10.01	20.05
Standard deviation	0.28	0.18	0.35	0.18	0.20	0.35
Coefficient of variation (%)	5.8	1.8	1.7	3.7	2.0	1.6

TABLE III

ASSAY PRECISION FOR MPB AND 4-OH-MPB IN URINE

$n = 4$.

	MPB (μg/ml)			*4-OH-MPB (μg/ml)*		
	10	*20*	*50*	*10*	*20*	*50*
Mean observed concentration	10.04	19.96	48.77	9.87	20.07	49.57
Standard deviation	0.20	0.07	0.47	0.18	0.10	1.05
Coefficient of variation (%)	2.0	0.3	1.0	1.9	0.5	2.1

terminations (Tables II and III) show that, exept for the lowest MPB and 4-OH-MPB plasma concentrations, the estimates of the drug concentration are highly reproducible. The precision of the method is well within acceptable limits for both compounds over the concentration range investigated.

Concerning the stability of MPB, it should be noted that the use of a 1% (w/v) ascorbic acid solution in which the internal standard is dissolved was sufficient to prevent the oxidation of MPB to 4-OH-MPB, at least during the extraction and evaporation step. Furthermore, by redissolving the residue only immediately before chromatographing the sample, further decomposition was avoided. When not to be analyzed, dry residues could be stored deep-frozen for at least 12 h without any degradation. Antioxidants should be added when blood and urine samples are collected.

To minimize chromatographic interferences due to phenolic and other amphoteric compounds[10–12], screening methods for non-steroidal anti-inflammatory drugs (NSAIDs), especially for doping analysis in horses, often use a washing of the organic layer with 0.1 *M* sodium bicarbonate. However, such a washing of the organic layer containing MPB resulted in a complete transfer of MPB from the organic to the aqueous phase and could consequently give rise to false negatives.

CONCLUSIONS

An HPLC method for the quantitation of MPB and its metabolite 4-OH-MPB in plasma and urine has been validated for concentrations ranging from 1 to 25 μg/ml in plasma and from 5 to 100 μg/ml in urine. The method is rapid, precise and accurate. It is currently applied to plasma and urine samples from horses after intravenous and oral administration of 8.8 mg/kg MPB.

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REFERENCES

1 S. Thune, *Acta Reumatol. Scand.*, 13 (1967) 63.
2 J. F. L. Woodbury, W. A. Turner and R. Tiongson, *Can. Med. Assoc. J.*, 101 (1969) 801.
3 O. E. Schultz and M. Mocha, *Pharm. Ztg.*, 122 (1977) 1305.
4 T. Daldrup, F. Susanto and P. Michalke, *Fresenius' Z. Anal. Chem.*, 308 (1981) 413.
5 F. Nielsen-Kudsk, *Acta Pharmacol. Toxicol.*, 47 (1980) 267.
6 V. M. Bass, R. I. Mrongovius and K. E. Schulte, *Eur. J. Drug. Metab. Pharmacokin.*, 5 (1980) 201.
7 R. Mrongovius, V. M. Bass and K. E. Schulte, *Eur. J. Med. Chem.*, 17 (1982) 275.
8 M. A. Kassem and K. E. Schulte, *Eur. J. Drug Metab. Pharmacokin.*, 9 (1984) 223.
9 S. Veibel, S. Linholter and P. Sorensen, *Acta Chem. Scand.*, 12 (1958) 1359.
10 J. P. Hunt, P. E. Haywood and M. S. Moss, *Eq. Vet. J.*, 11 (1979) 259.
11 F. T. Delbeke, M. Debackere and N. Desmet, *Chromatographia*, 17 (1983) 381.
12 F. T. Delbeke and M. Debackere, in G. H. Johnsson (Editor), *Control of the Use of Drugs in Racehorses*, Canadian Member Laboratories of the AORC, Toronto, 1983, p. 34.

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Note

Dosage de la bromadiolone (rodonticide anticoagulant) dans le plasma, le foie et le rein du rat

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(Reçu le 2 juillet 1986; manuscrit modifié reçu le 23 août 1986)

La bromadiolone, 3-{3-[4'-bromo(1,1'-biphenyl)-4-yl]-3-hydroxy-1-phenylpropyl}-4-hydroxy-2H-1-benzopyran-2-one, est un rodonticide anticoagulant dérivé de l'hydroxy-4-coumarine et synthétisé par la société Lipha (France) en 1975. Son efficacité contre les rongeurs sauvages ainsi que sa toxicité pour les différentes espèces ont été largement étudiées[1–5]. Aucun travail n'a jusqu'à présent fait état de sa cinétique.

Hunter[6,7] a décrit deux méthodes analytiques pour la détermination des rodonticides anticoagulants, dont la bromadiolone, dans des tissus animaux en vue du diagnostic toxicologique. La première méthode emploie la réaction acide–base en post-colonne tandis que la seconde utilise l'appariement d'ion. Offrant une grande sensibilité, ces techniques sont difficulement applicables pour un grand nombre d'analyses, car la purification préalable se fait à l'aide d'une chromatographie liquide nécessitant un collecteur de fraction. Cette dernière méthode a été améliorée par le même auteur[8] pour la détermination toxicologique de sept rodonticides anticoagulants en utilisant différentes méthodes de purification dont l'usage des cartouches Sep-Pak Si-60 après la chromatographie sur colonne.

Une méthode rapide, utilisant les cartouches Sep-Pak Si-60, pour la détermination des rodonticides anticoagulants autre que la bromadiolone dans les tissues animaux, a été décrite par Mundy et Machin en 1982[9]. Fasco *et al.*[10] ont employé les cartouches Sep-Pak C_{18} pour la quantification du coumafène et ses métabolites dans le plasma.

Dans le présent travail, nous décrivons une méthode pour la quantification de la bromadiolone dans le plasma, le foie et le rein de rat, par utilisation des cartouches Sep-Pak C_{18} pour la purification et la chromatographie phase inverse avec appariement d'ion et une détection fluorimétrique.

PARTIE EXPERIMENTALE

Les animaux

Rattus norvegicus souche sauvage capturé en milieu naturel. Après anéstesie à l'éther le sang est prelevé par ponction intracardiaque dans un tube de 5 ml contentant 0,1 ml de citrate de soudium. Le foie et reins sont prélévés sur le même animal.

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Matériel et produits

Les solvants, acétonitrile, acétate d'ethyle, hexane, chloroforme, acétone, sont de qualité "Spectrosol" (S.D.S., Villeurbanne, France). Les cartouches Sep-Pak C_{18} ont été fournies par Millipore (St. Quentin en Yvelines, France). L'hydroxyde de tétrabutylammonium (40%, p/v) a été fourni par Aldrich Chimie (Strasbourg, France). Tous les autres réactifs sont de qualité analytique.

La bromadiolone, mélange de deux diastéréoisomères, a été fournie par la société Lipha (Lyon, France).

Appareillage:

Le chromatographe est un appareil Hewlett-Packard HP 1080 équipé d'un injecteur automatique à volume variable et d'une colonne analytique, LiChrosorb C_{18}, 250 × 4,6 mm, granulométrie 10 μm (Merck). Le détecteur est un spectrofluorimètre Jobin-Yvon JY 3D avec une cuve à quartz de 20 μl à fente horizontale reliée à la sortie de la colonne.

Extraction et purification

Plasma. 1 ml de plasma est extrait trois fois avec 3 ml d'acétate d'éthyle. Les extraits sont regroupés et évaporés à sec sous courant d'azote à 40°C.

Le résidu sec est repris par 3 ml d'acétonitrile et 3 ml d'hexane. Après agitation et centrifugation, l'hexane est éliminé. L'acétonitrile est lavé une deuxième fois avec 3 ml d'hexane qui sont à nouveau éliminés après centrifugation. L'acétonitrile est évaporé à sec sous azote. L'extrait sec est repris dans 0,5 ml de la phase mobile.

Foie et rein. 1-2 g de tissu sont homogénéisés avec 5 g de sulfate de sodium anhydrde et 30 ml du mélange acétone-chloroforme (1:1, v/v) à l'aide d'un Ultra Turrax. L'extrait est filtré sur un filtre en papier. Le résidu est reextrait avec 30 ml de melange acétone-chloroforme. Les extraits sont regroupés et évaporés à sec sous vide avec un évaporateur rotatif. Le résidu sec est dissous dans 1 ml d'acétone et 5 ml d'un tampon phosphate 0,025 *M*, pH 7,4 (par passage dans une cuve à ultrason),

TABLEAU I

RENDEMENT D'EXTRACTION DANS LE PLASMA, LE FOIE ET LE REIN DE *RATTUS NORVEGICUS* SUPPLÉMENTÉS AVEC DIFFÉRENTES QUANTITÉS DE BROMADIOLONE (m ± S.D.; n = 4)

	Niveau de supplémentation (ppm)	*Recuperation (%)*
Plasma	0,02	102 ± 8
	0,2	92 ± 12
	2,0	87 ± 3
Foie	0,05	76 ± 2
	0,50	85 ± 6
	5,00	84 ± 4
Rein	0,05	93 ± 3
	0,50	89 ± 6
	5,00	93 ± 5

puis purifié sur une cartouche Sep-Pak C_{18}, préalablement rincée avec 4 ml de méthanol et 4 ml du tampon phosphate, à raison de 5 ml/min. La bromadiolone est éluée avec 1 ml du méthanol.

Analyse chromatographique

La phase mobile contient 75% de méthanol et 25% du tampon phosphate 0,025 *M*, pH 7,4. 0,32% de l'hydroxide de tétrabutylammonium sont ajoutés au mélange et le pH est a justé à 7,5 avec HCl 6 *N*.

Pour un débit de 1 ml/min, le temps de rétention de la bromadiolone est de 7,2 min.

La détection fluorimétrique se fait à 320 nm (excitation) et 390 nm (émission).

RESULTAT ET DISCUSSION

Le rendement d'extraction de la bromadiolone dans le plasma, le foie et le rein de rat, supplémentés avec des quantités de 0,02–2 µg/ml pour le plasma et 0,05–5 µg/g pour le foie et le rein est de 76–101% (Tableau I).

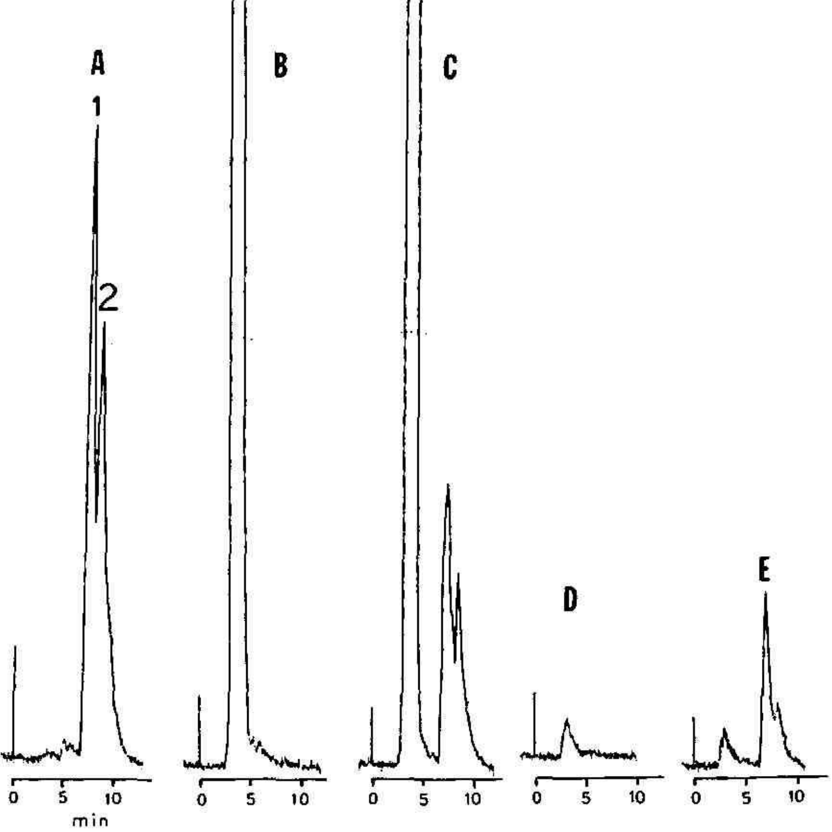

Fig. 1. Exemples de chromatogrammes chromatographie liquide à haute performance. (A) Étalon de bromadiolone 0,4 ppm 20 ng injectés: (1) isoméré majoritaire, (2) isoméré minoritaire. (B) Extrait de foie témoin. (C) Extrait de foie supplémenté à 0,2 ppm (10 ng injectés). (D) Extrait de plasma témoin. (E) Extrait de plasma supplémenté à 0,05 ppm (5 ng injectés).

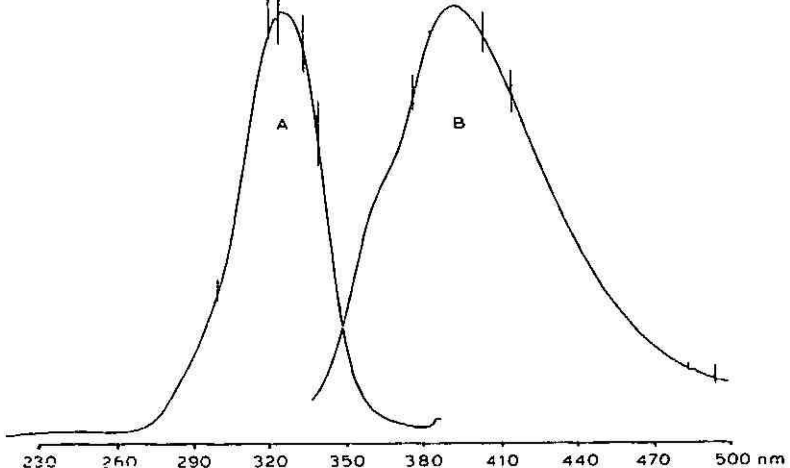

Fig. 2. Spectre de fluorescence de la bromadiolone. (A) Spectre d'excitation (émission à 390 nm); (B) spectre d'émission (excitation à 320 nm).

Le choix de longueurs d'onde d'excitation et d'émission (320 et 390 nm respectivement) correspond à la sensibilité maximale (Fig. 2). Hunter[6] rapporte que l'excitation de la bromadiolone à 310 nm donne une réponse peu satisfaisante par rapport à celle obtenue à 250 nm.

L'utilisation du partage acétonitrile–hexane pour la purification des extraits plasmatiques permet d'éliminer un grand nombre de composés lipophiles, qui dans nos conditions chromatographiques, ne présentent pas d'interférence avec la bromadiolone, mais leur élimination permet de préserver la colonne analytique. Ce système de partage, utilisé pour purifier les extrait hépatiques et rénaux a donné peu de satisfaction au niveau du rendement d'extraction. L'emploi des cartouches Sep-Pak C_{18} a permis d'éliminer des composés co-extraits avec la bromadiolone et qui sont susceptibles de se fixer de facon irréversible sur la colonne analytique et aussi de réaliser un grand nombre d'analyses avec rapidité et sans recourir à des appareils de chromatographie pour la purification.

Cette méthode a été utilisée pour l'étude de la cinétique plasmatique, hépatique et rénale de la bromadiolone chez *Rattus norvegicus* sauvage, et par la suite, pour la quantification plus précise des deux diastéréoisomères de la bromadiolone.

REFERENCES

1 M. Grand, *Phytiatr. Phytopharm.*, 25 (1976) 69.
2 G. Lorgue, Ch. Mally et K. Nahas, *Rec. Med. Vet.*, 4 (1985) 329.
3 G. Lorgue, K. Nahas, G. Keck et M. Rampaud, *12th Vertebrate Pest Conference, San Diego, CA, March 1986.*
4 R. E. Marsh, W. G. Howard et W. B. Jackson, *Pest Control*, 48 (1980) 22.
5 C. G. J. Richards, *J. Hyg.*, 86 (1981) 363.
6 K. Hunter, *J. Chromatogr.*, 270 (1983) 267.
7 K. Hunter, *J. Chromatogr.*, 270 (1983) 277.
8 K. Hunter, *J. Chromatogr.*, 321 (1985) 255.
9 D. E. Mundy et A. F. Machin, *J. Chromatogr.*, 234 (1982) 427.
10 M. J. Fasco, M. J. Cashin et L. S. Kaminsky, *J. Liq. Chromatogr.*, 2 (1979) 565.

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Note

High-performance liquid chromatographic determination of several monoamines in brain tissue of DBA/2 mice during a single run of 20–25 minutes without prior clean-up of samples

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Norepinephrine (NE), dopamine (DA) and serotonin (5HT) control many brain functions and are important modulators of behavior. Measurements of these monoamine transmitters and their principal metabolites in brain tissue is thus critical to the progress of a number of neuroscience projects. High-performance liquid chromatography (HPLC) with amperometric detectors is now a method of choice for the determination of monoamine levels in the central nervous system, other tissues and body fluids because of its sensitivity and accuracy[1–11].

However, it has been difficult to resolve several monoamines and metabolites during a single, rapid HPLC run. Moreover, the neutral metabolite of NE, 3-methoxy-4-hydroxyphenylglycol (MHPG), has been hard to capture because it is eluted from the column quickly[1]. If measures are taken to delay its elution, other compounds of choice are also retarded and the run becomes uncomfortably long[10].

Recently, we have developed a HPLC system which surmounts these limitations[3]. It permits the simultaneous analysis of thirteen catecholamines and indoleamines during a run of less than 20 min. The thirteen compounds resolved are: NE, MHPG, DL-normetanephrine (NM), epinephrine (EPI), 3-methoxytyramine (3MT), DL-metanephrine (MN), L-β-3,4-dihydroxyphenylalanine (DOPA), DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxy-L-tryptophan (5HTP), 5-hydroxytryptamine or serotonin (5HT) and 5-hydroxyindole-3-acetic acid (5HIAA). Through ongoing development, we are adding additional compounds.

The present study was undertaken to apply this HPLC technique to the analysis of these catecholamines and indoles in discrete regions of brain tissue. We have been able to identify and resolve the majority of the compounds in various regions of mouse brain without prior clean-up of samples during a run of less than 20–25 min, depending on the chromatographic conditions. However, many of the compounds listed above are minor metabolites or precursors of monoamine transmitters

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which normally exist at very small concentrations in vertebrate brain. They are near the limits of detection for our system unless a higher sensitivity setting is selected or unless they are increased by experimental manipulations. Sporadic values obtained for these compounds are not reported.

For this initial work, we opted for a lower sensitivity setting and to concentrate on the following seven compounds: NE and its major metabolite, MHPG, DA and its metabolites, DOPAC and HVA, and serotonin and its metabolite, 5HIAA. Data are reported for the thalamus of male DBA/2 mice.

EXPERIMENTAL

Instrumentation

The HPLC system consists of a Waters (Bedford, MA, U.S.A.) 590 programmable solvent delivery pump and a refrigerated Waters Intelligent Sample Processor (WISP) connected to a Bioanalytical Systems (West Lafayette, IN, U.S.A.) Biophase ODS 5 μm, C_{18}, 250 × 4.6 mm column with a Bioanalytical Systems Biophase ODS 5 μm, C_{18}, 30 × 4.6 mm guard column and a Bioanalytical Sytems LC4B amperometric detector with glassy carbon electrode. The integrating recorder is a Shimadzu C-R3A data processor equipped with a floppy disk drive and cathode ray tube (Shimadzu Scientific Instruments, Columbia, MD, U.S.A.).

Chemicals

The following chemicals were purchased from Sigma (St. Louis, MO, U.S.A.): 3-methoxy-4-hydroxyphenylglycol (hemipiperazine salt) (MHPG), arterenol bitartrate (norepinephrine bitartrate) crystalline (NE), epinephrine bitartrate (EPI), L-β-3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxyphenylacetic acid (DOPAC), DL-normetanephrine hydrochloride (NM), dopamine (3-4-dihydroxyphenylethylamine HCl) (DA), DL-metanephrine hydrochloride (MN), 5-hydroxyindole-3-acetic acid (free acid) (5HIAA), 3-methoxytyramine (3-methoxy-4-hydroxyphenethylamine) HCl (3MT), 5-hydroxytryptamine hydrochloride (serotonin) (5HT), 5-hydroxytryptophan (5HTP), 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid) (HVA), L-(−)-isoproterenol (IP), L-cysteine free base and L-ascorbic acid. In addition perchloric acid (PCA) (70% in water) was purchased from Eastman Kodak (Rochester, NY, U.S.A.), 1-heptanesulfonic acid (HSA) was purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.) and acetonitrile was purchased from J. T. Baker (Philipsburg, NJ, U.S.A.).

Chromatographic conditions

The mobile phase was a mixture of 0.10 *M* citric acid, 0.06% diethylamine, 0.05 m*M* Na_2EDTA, 225 ml/l HSA and 3% acetonitrile at pH 2.55. These components were dissolved in deionized water (< 1 MΩ resistance), then filtered through a 47-mm, 0.2-μm filter (Rainin Instrument, Woburn, MA, U.S.A.). The solvent was sparged with helium gas to deaerate it. All separations were performed isocratically at a flow-rate of 1.5 ml/min at room temperature, a detector setting of 0.85 V and a sensitivity setting of 5 nA.

Standards

Fresh standard compounds were ordered in brown bottles and were kept in a dessicator in the freezing compartment. The stock solutions were prepared in deionized water at a concentration of 1 mg/ml then diluted serially with 5% PCA to give the working solution of 20 ng/ml. Aliquots of 50 μl of this were injected into the HPLC system from the refrigerated sample compartment.

Tissue preparation

Brain tissue was obtained from 5–7 weeks old DBA/2 male mice. They were killed by cervical dislocation and the brains were rapidly removed and frozen in liquid nitrogen. Dissections were carried out over liquid nitrogen on a frosted plate. The thalamus was obtained from appropriate coronal sections. The tissues were then weighed and homogenized (Tekmar Tissumizer, Cincinnati, OH, U.S.A.). The extractions were performed in 5% PCA containing the internal standard (20 ng/ml IP) and 1% cysteine. Then the homogenate was centrifuged through Isolab (Norton, OH, U.S.A.) QS-GS filter columns. Aliquots of 50 μl of this material were then injected into the HPLC system. The brains from eight animals were analysed.

RESULTS AND DISCUSSION

The standard chromatogram is illustrated in Fig. 1 and a brain tissue chro-

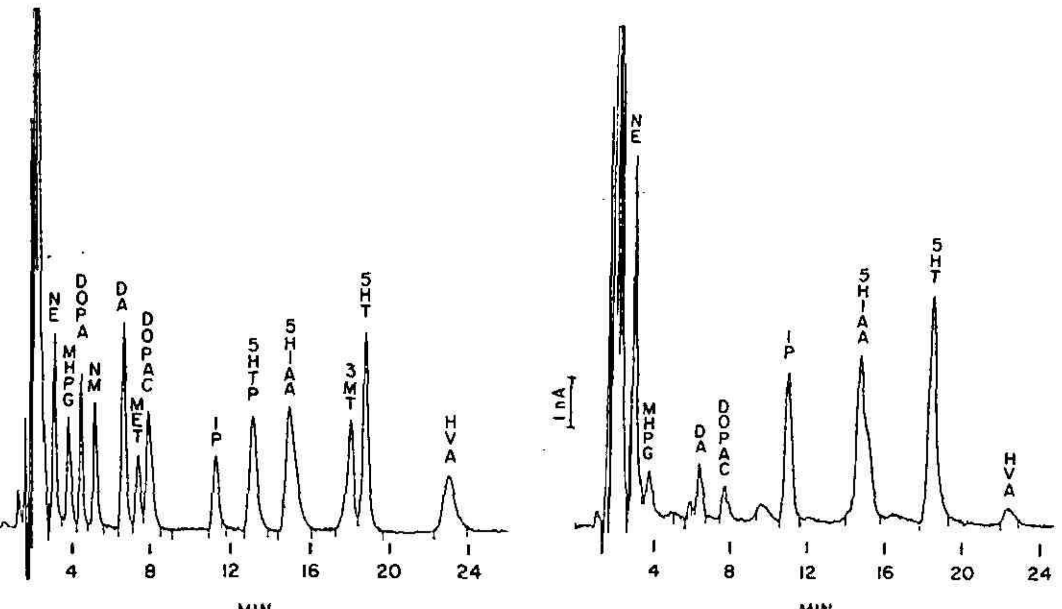

Fig. 1. Chromatogram of standard compounds showing elution times in minutes. The chromatographic conditions and abbreviations are given in the Experimental section.

Fig. 2. Representative chromatogram of thalamic tissue from the brain of a DBA/2 mouse. The elution times of endogenous monoamines are shown in minutes along with the internal standard, (20 ng/ml IP). The chromatographic conditions and abbreviations are found in the Experimental section.

TABLE I

HPLC VALUES [pg/mg (ORIGINAL TISSUE) ± S.E.] FOR MONOAMINES IN THE THALAMUS OF DBA/2 MICE

n = 8. See Experimental for abbreviations. ND = not detected.

Monoamine	*pg/mg original tissue*
NE	250 ± 17.5
MHPG	22.5 ± 5
DA	45 ± 5
DOPAC	27.5 ± 2.5
HVA	25 ± 2.5
3MT	ND
5HT	218 ± 12.5
5HIAA	183 ± 10

matogram for the thalamus is illustrated in Fig. 2 as a representative case. The mean monoamine levels ± standard error (S.E.) obtained from this brain region are given in Table I. The significance of these results is discussed below.

The rapid separation of so many acid, neutral and basic compounds, with varying polarities, is demanding on the chromatographic system. The principal factor responsible for this resolution is the constitution of the mobile phase. The ionic strength of the buffer, the concentration of ion-pairing reagent and organic solvent as well as pH were all varied to obtain optimal separation. As a result, the mobile phase is quite rich in solutes and rather acidic. The ionic strength of the citrate buffer enhanced retention as, in general, did HSA, thus permitting adequate separation of peaks and freedom from the solvent front. The high concentration of acetonitrile dramatically shortened the chromatogram to keep it within reasonable time constraints. The low pH also enhanced separation of certain compounds.

The reversed-phase ODS column is a powerful one for catecholamine separations and the 25-cm one used here is high in theoretical plates. However, we have found that individual columns of this manufacturer (Bioanalytical Systems) may sometimes produce a larger front than desired. This can be mitigated to some extent by changing to a Supelco (Bellafonte, PA, U.S.A.) 15-cm, 3-μm ODS column which has higher resolution properties. Minor adjustments in concentration of acetonitrile (3–4%) and HSA (175–225 mg/l) or in pH (2.45–2.65) may then be required to fine-tune the system. One should begin first with a simple pH change in increments of 0.05. Once a perfect separation is effected, one can expect 3–6 months of column life despite heavy use, the low pH and the syrupy mobile phase. But the column should be washed daily in deionized water for 1 h and stored in 30% methanol when not in use (always preceded and followed by a water wash). At the point in time when the column appears to decay, it can sometimes be reconstituted by passing 50% isopropanol through it overnight.

To prevent breakdown of standards and sample compounds or to identify them better, we take additional precautions. We use cysteine as an antoxidant to circumvent the use of ascorbate which produces a larger front (Bioanalytical Systems, personal communication). We also refrigerate the sample compartment of the automatic injector. Diethylamine helps prevent tailing of peaks and EDTA is used to

chelate heavy metallic ions such as Ca^{2+}. It is not possible to use the optimal potential for each compound but the compromise chosen was 0.85 V. This provides much greater sensitivity for MHPG, for example, while still maintaining high sensitivity for other catechols.

We have tested a number of internal standards to use with the present chromatogram. The results are reported elsewhere[2]. IP is the standard of choice for catecholamines and if a double internal standard is desired, a second one for the indoles, 5-hydroxyindole can be added. It elutes in mid-run near IP yet is completely separated from IP and the other compounds.

In the present study, we report recovery of seven monoamines from the thalamus of DBA/2 mice (Table I). The values are given in pg/mg original tissue ± standard error (S.E.). Please note the recovery in the low picogram range for several compounds including MHPG. The values are similar to those obtained by other methods (see refs. 1–11). Quality control was carried out by mass spectrometry and by other HPLC methods to determine purity of those peaks which we thought could possibly conflict with other compounds. Depending on the tissue, the front may be larger or smaller. If it tends to conflict with NE, the run can be prolonged by adding more HSA or decreasing the amount of acetonitrile. Better still, the brain tissue can be extracted using an acetate buffer containing ascorbate oxidase[2] instead of the PCA extraction reported here. Refinement using this latter technique and the recovery of additional monoamines in other brain tissues will be reported elsewhere.

CONCLUSIONS

This HPLC technique permits the rapid determination of several catecholamines and indoleamines, including metabolites and precursors during a single run. It is valuable for brain tissue analysis for the following reasons: It measures most desired monoamines found in the central nervous system, it permits MHPG to be determined along with the other compounds, it requires no prior cleanup of samples, it generates a vast amount of data in a short period of time and it allows for simultaneous comparisons of several neurotransmitters and metabolites in the same tissues under identical experimental conditions.

REFERENCES

1 G. L. Diggory and W. R. Buckett, *J. Pharmacol. Methods*, 11 (1984) 207.
2 M. Frankfurt, K. Renner, E. Azmitia and V. Luine, *Brain Res.*, 340 (1985) 127.
3 M. G. Hadfield, P. Crane, M. E. King, E. A. Nugent, C. Milio and N. Narasimhachari, *J. Liq. Chromatogr.*, 8 (1985) 2689.
4 J. M. P. Holly and H. L. J. Makin, *Anal. Biochem.*, 128 (1983) 257.
5 W. A. Hunt and T. L. K. Dalton, *Anal. Biochem.*, 135 (1983) 269.
6 A. M. Krstulovic, *J. Chromatogr.*, 229 (1981) (.
7 I. N. Mefford, *J. Neurosci. Methods*, 3 (1981) 207.
8 W. Rathburn and M. J. Druse, *J. Neurochem.*, 1 (1985) 57.
9 E. Shohami, M. Segal and D. M. Jacobwitz, *J. Neurosci. Methods*, 8 (1983) 275.
10 C. van Valkenburg, J. van Krust, P. Moleman, H. van Berkum, U. Tjaden and U. de Jong, *J. Neurosci. Methods*, 11 (1984) 29.
11 M. Warnhoff, *J. Chromatogr.*, 307 (1984) 271.

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Book Review

Quantitative chemical analysis, by Stanley E. Manahan, Brooks/Cole Publishing Co., Monterey, CA, 1986, XXII + 701 pp., price £ 13.95, ISBN 0-534-05538-9.

Students of chromatography or of instrumental methods of analysis should stay away from this book. It purports to be a textbook in quantitative chemical analysis but it is only partly that: classical quantitative inorganic analysis is well and thoroughly presented in the first fifteen chapters but organic analysis is ignored (except for a 5 pages of introduction to organic chemistry in Chapter 1!).

Instrumental methods (including chromatography) are much less satisfactorily presented and seem to be second-hand knowledge with all its inaccuracies and half-truths. The examples that abound in previous chapters suddenly become rare. Diagrams and figures that were simple and clear become less clear (Figs. 16.2 and 17.2) or even awkward, as is the analogy of swimmers with chromatography (Fig. 17.4). Inaccuracies here are not only historical (Martin and Synge won their Nobel prize in 1952, not 1954 as stated), but more seriously technical and scientific: For example, it is claimed that separation on thin-layer "phases" is purely adsorption (p. 447, corrected and completed in the next chapter); that silica TLC plates should be activated by heating them at 150–200°C (p. 500), which is the best way to degrade them!; and R_F is defined as a "retardation value" (p. 501), an unfortunate confusion perpetuated by books such as this one.

I doubt if anybody could use a chromatographic technique with this book as a guide.

Separation techniques are followed by three chapters dealing with spectrophotometric techniques and one chapter about nuclear and radiochemical analysis. Some confusion arises owing to the use of improper terminology such as fluorescence for photoluminescence, or a lack of depth, *e.g.*, radiochemical methods are dealt with in less than 15 pages and RIA in 12 lines. Other relatively common techniques in the fields of biochemistry or clinical chemistry such as EMIT and ELISA do not even receive a mention.

Finally, in the analysis of "real" samples, it is stated that "the analytical chemist may have to consult books to see if an analysis is feasible" (p. 629); I would suggest consulting an analyst who knows!

Lausanne P. MARGOT

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Errata

J. Chromatogr., 356 (1986) 220–226

Page 222, 6th line from the bottom should read: "(3:2). The column was eluted with methanol–water (3:2) at 25 ml/min into fractions".

J. Chromatogr., 366 (1986) 343–350

Page 346, Table II, 2nd row, "Na_2CO_3" should read "Na_2SO_4".

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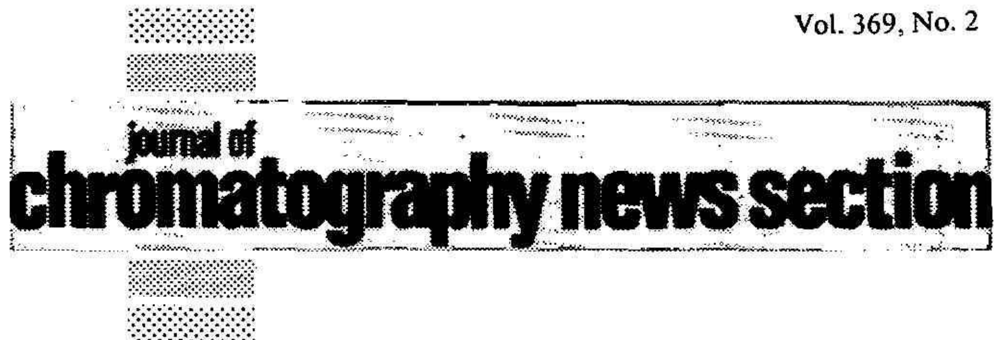

NEW BOOKS

Reaction detection in liquid chromatography (*Chromatographic Science Series, Vol. 34*), edited by I.S. Krull, Marcel Dekker, New York, Basel, 1986, X + 377 pp., price US$ 79.75 (U.S.A. and Canada), US$ 95.50 (rest of world), ISBN 0-8247-7579-1.

Quantitative analysis of catecholamines and related compounds, edited by A. Krstulović, Ellis Horwood, Chichester, 1986, *ca.* 250 pp., price *ca.* US$ 84.15, ISBN 0-85312-824-3.

Computer applications in the polymer laboratory (*ACS Symposium Series* No. 313), edited by T. Provder, American Chemical Society, Washington, DC, 1986, X+323 pp., price US$ 69.95 (U.S.A. and Canada), US$ 83.95 (rest of world), ISBN 0-8412-0977-4.

Rückstandsanalytik von Pflanzenschutzmitteln, by H.-P. Thier and H. Frehse, Thieme, Stuttgart, New York, 1986, X+325 pp., price DM 98.00, ISBN 3-13-666601-1.

ANNOUNCEMENTS OF MEETINGS

3rd PREPARATIVE-SCALE LIQUID CHROMATOGRAPHY SYMPOSIUM, WASHINGTON, DC, U.S.A., MAY 4–5, 1987

The above-mentioned symposium will take place in the Washington Sheraton Hotel, 2660 Woodley Road, Washington, DC 20008, U.S.A.

The success of the first two symposia in this series, the fact that preparative liquid chromatography has specific problems which will take time and many discussions to solve, the observation that these problems are generally not addressed in detail at chromatography symposia as well as the growing interst in the field, have triggered the Washington Chromatography Discussion Group to decide to run this symposium on a yearly basis.

In addition to invited lectures, oral contributions and poster presentations will be accepted.

Abstracts of papers submitted for inclusion in the programme are requested by November 15, 1986. The scientific committee will meet soon afterwards to organize the programme which will be published early December, 1986.

Submitted abstracts and all correspondence should be sent to: Symposium Manager, 3rd WSPLC, Barr Enterprises, P.O. Box 279, Walkersville, MD 21793, U.S.A. Tel.: (301) 898-3772.

8th INTERNATIONAL SYMPOSIUM ON CAPILLARY CHROMATOGRAPHY, RIVA DEL GARDA, ITALY, MAY 19–21, 1987

The 8th International Symposium on Capillary Chromatography will be held at Palazzo dei Congressi in Riva Del Garda, Italy, May 19–21, 1987. Both format and atmosphere will be similar to the previous successful meetings (Hindelang 1975, 1977, 1979, 1981; Riva del Garda 1983, 1985; Gifu 1986).

Scientific programme. The programme will cover basic and practical aspects of capillary gas chromatography, capillary liquid chromatography, capillary supercritical fluid chromatography and capillary electrokinetic chromatography with emphasis on: stationary phases and columns, instrumentation (sampling, detection, multidimensional and ancillary techniques), applications (petrochemistry, environment, biomedical, food and drug analysis, etc.)

The symposium will consist of: review papers by leading scientists in the field on the latest developments in column technology, sampling, applications, instrumentation, etc.; invited papers by young scientists; submitted papers presented in poster sessions in order to achieve intensive discussion.

Plenary and parallel discussion sessions on special topics will serve to augment the formal presentation. In "workshop type seminars" scientists of the instrument manufacturers will present and discuss the latest developments in capillary instrumentation. One-day short courses will be organized on May 22. Topics will include CGC and CSFC. Information on request.

Submission of papers. Authors intending to submit papers for the symposium will be required to adhere to the following schedule: abstract (300 words) before December 20, 1986. Notification of acceptance will be mailed to the authors by December 31, 1986; manuscripts ready for direct reproduction before February 1, 1987, to the address given below. A book of the Symposium Proceedings will be available at the registration desk. The papers will be published in *Journal of High Resolution Chromatography and Chromatographic Communications* after the usual reviewing.

Scholarships. Ten scholarships for young scientists are available. Proposals are welcome.

Exhibition. In conjunction with the symposium there will be an exhibition of capillary chromatography instruments and accessories. Companies interested in participation in this exhibition should direct their inquiries to Dr. P. Sandra at the address given below. The instrument exhibition will be held jointly with the poster sessions.

Registration. The registration fee (including the symposium proceedings and the social programme) will be: prior to April 1, 1987: delegates (from outside Italy) US$ 200, Italian delegates 200.000 lira; after April 1, 1987: delegates (from outside Italy) US$ 220, Italian delegates 220.000 lira; students: 50% reduction of above fees.

Accommodation will be available in a limited number of reasonably priced hotels within walking distance from the conference hall.

For further details or submission of papers, please contact: Dr. P. Sandra, Research Institute for Chromatography, P.O. Box 91, B-8610 Wevelgem, Belgium.

4th INTERNATIONAL CONFERENCE ON CHEMOMETRICS IN ANALYTICAL CHEMISTRY, AMSTERDAM, THE NETHERLANDS, MAY 18–20, 1988

The conference is organized under the auspices of the KNCV (Royal Netherlands Chemical Society, Analytical Division), VCV (Flemish Chemical Society) and the Chemometrics Society.

The scientific programme will include invited plenary lectures, keynote lectures, and submitted research papers. The following topics will be covered: application and development of formal techniques for design, optimization and evaluation of analytical procedures and results; application of systems theory, operations research, information theory, statistics and other chemometric techniques in analytical chemistry; computerized signal-, image-, and data processing, optimum filtering, parameter estimation, time series analysis, calibration, curve resolution; multivariate data analysis, pattern recognition, cluster analysis, principal components analysis, factor analysis, etc.; expert systems/artificial intelligence, data retrieval/library searching, management systems; mathematical

modelling and computer simulation of analytical processes; robotics and automation; and education in chemometrics.

Papers presented at the conference will be refereed for publication in a special issue of *Analytica Chimica Acta*.

For further information, please contact: International Conference on Chemometrics in Analytical Chemistry, CAC-88, Laboratory for Analytical Chemistry, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV Amsterdam, The Netherlands. Tel.: (020)-5223541 (Dr. Smit).

CALENDAR OF FORTHCOMING MEETINGS

Dec. 8–12, 1986 Kent, OH, U.S.A.	**Kent State University's Chromatographic Course: Fundamentals of Chromatographic Analysis** Contact: Carl J. Knauss, Chromatographic Course Coordinator, Chemistry Department, Kent State University, Kent, OH, U.S.A. Tel.: (216) 672-2327. (Further details published in Vol. 366.)
Dec. 15–17, 1986 Nottingham, U.K.	**An Introductory Short Course on HPLC Analysis of Small Molecules** Contact: Dr. G.W. Bennett, Department of Physiology and Pharmacology, Queen's Medical Centre, Clifton Boulevard, Nottingham NG7 2UH, U.K., Tel.: (0602) 70011, ext. 3297/3949.
March 9–13, 1987 Atlantic City, NJ, U.S.A.	**38th Pittsburgh Conference and Exposition of Analytical Chemistry and Applied Spectroscopy** Contact: Mrs. Alma Johnson, Program Secretary, 12 Federal Drive, Suite 322, Pittsburgh, PA 15235, U.S.A. (Further details published in Vol. 360, No. 2.)
March 11–12, 1987 Brighton, U.K.	**International Symposium on Thin-Layer Chromatography** Contact: The Executive Secretary, The Chromatographic Society Secretariat, Trent Polytechnic, Burton Street, Nottingham NG1 4BU, U.K. (Further details published in Vol. 363, No. 2.)
March 24–June 5, 1987 Uppsala, Sweden	**Biochemical Separation Methods, Uppsala Separation School** Contact: Secretary Ulrika Jansson, Institute of Biochemistry, University of Uppsala, Biomedical Center, P.O. Box 576, S-751 23 Uppsala, Sweden. (Further details published in Vol. 366.)
April 3–9, 1987 Beijing, China	**ChinaChem '87, International Exhibition on Chemical and Petrochemical Industries** Contact: Adsale Exhibition Services, 21/F Tung Wai Commercial Building, 109–111 Gloucester Road, Hong Kong. Tel.: (5) 8920511, telex: 63109 ADSAP HX, Fax: (5) 731709.
April 7–10, 1987 Stockholm, Sweden	**International Symposium on Analysis of Neurotransmitters** Contact: The Swedish Academy of Pharmaceutical Sciences, P.O. Box 1136, S-111 81 Stockholm, Sweden.
April 27–May 1, 1987 Sydney, Australia	**9th Australian Symposium on Analytical Chemistry** Contact: The Secretary 9AC, Mr. John ames, P.O. Box 137, North Ryde, N.S.W. 2133, Australia. Tel.: (020) 887-8688. (Further details published in Vol. 350, No. 2.)

May 4–5, 1987 Washington, DC, U.S.A.	***3rd Preparative-Scale Liquid Chromatography Symposium*** Contact: Symposium Manager, 3rd WSPLC, Barr Enterprices, P.O. Box 279, Walkersville, MD 21793, U.S.A. Tel.: (301) 898-3772.
May 11–14, 1987 Ghent, Belgium	**2nd International Symposium on Quantitative Luminescence Spectrometry in Biomedical Sciences** Contact: Dr. W. Bayens, State University of Ghent, Laboratory of Pharmaceutical Chemistry and Drug Quality Control, Harelbekestraat 72, B-9000 Ghent, Belgium. (Further details published in Vol. 354.)
May 13–15, 1987 Amsterdam, The Netherlands	**Scientific Computing and Automation (Europe)** Contact: K. Foley, Scientific Computing and Automation, P.O. Box 330, *1000 AH Amsterdam, The Netherlands.* Tel.: (020) 5862 828. (Further details published in Vol. 362, No. 3.)
May 19–21, 1987 Riva Del Garda, Italy	**8th International Symposium on Capillary Chromatography** Contact: Dr. P. Sandra, Research Institute for Chromatography, P.O. Box 91, B-8610 Wevelgem, Belgium.
May 24–27, 1987 Columbia, SC, U.S.A.	**1st International Symposium on the Interface between Analytical Chemistry and Microbiology** *Contact: 1st International* Symposium on the Interface between Analytical Chemistry and Microbiology, P.O. Box 7126, Columbia, SC 29202, U.S.A. (Further details published in Vol. 360. No. 2.)
May 27–29, 1987 Budapest, Hungary	**ARCH '87, Automated Reasoning in Chemistry** Contact: ARCH '87 Conference, Mrs. O. Enyedy, Secretary, Institute of Isotopes of the Hungarian Academy of Sciences, P.O. Box 77, H-1525 Budapest, Hungary.
June 1–4, 1987 Loen, Norway	**Euro Food Chem IV, 4th European Conference on Food Chemistry** Contact: Euro Food Chem IV, Conference Secretariat, Norwegian Food Research Institute, P.O. Box 50, N-1432 Ås-NLH, Norway. (Further details published in Vol. 363, No. 2.)
June 12–15, 1987 Lund, Sweden	**International Symposium on Titration Techniques** Contact: "Symposium on Titration Techniques", c/o The Swedish Chemical Society, Wallingatan 26 A, S-111 24 Stockholm, Sweden.
June 21–26, 1987 Toronto, Canada	**XXV Colloquium Spectroscopium Internationale** Contact: Mr. L. Forget, Executive Secretary CSI XXV, National Research Council of Canada, Ottawa, K1A 0R6 Canada. Tel.: (613) 993-9009, telex: 053-3145. (Further details published in Vol. 330, No. 2.)
June 28–July 4, 1987 Amsterdam, The Netherlands	**HPLC '87, 11th International Symposium on Column Liquid Chromatography** Contact: Organisatie Bureau Amsterdam bv, Europaplein, 1078 GZ Amsterdam, The Netherlands. Tel.: (31) 20-440807, telex: 13499 raico nl. (Further details published in Vol. 331, No. 2 and Vol. 366.)

Aug. 12–19, 1987 Budapest, Hungary	**World Congress of Theoretical Organic Chemists.** Contact: E.A. Lang, WATOC CONGRESS, Hungarian Chemical Society, Anker köz 1, H-1061 Budapest, Hungary.
Aug. 17–21, 1987 Oberammergau, F.R.G.	**7th International Symposium on Affinity Chromatography and Interfacial Macromolecular Interactions** Contact: Prof. Dr. H.P. Jennissen, Institut für Physiologie, Physiologische Chemie und Ernährungsphysiologie, Universität München, Veterinärstr. 13, D-8000 München 22, F.R.G.
Aug. 25–30, 1987 Beijing, China	**8th International Conference on Computers in Chemical Research and Education** Contact: Cheng Qian, 345 Lingling Road, 200032 Shanghai, China. Telex: 33354 SIOC CN.
Sept. 7–11, 1987 Paris, France	**Euroanalysis VI, European Conference on all Aspects of Analytical Sciences** *Contact: G.A.M.S., 88 Boulevard Malsherbes*, 75008 Paris, France. (Further details published in Vol. 357, No. 3.)
Sept. 22–25, 1987 Selvino, Italy	***4th International Symposium on Instrumental* Thin-Layer Chromatography (Planar Chromatography)** Contact: Dr. Helmut Traitler, NESTEC Ltd., Nestlé Research Department, CH-1800 Vevey, Switzerland. Tel.: (021) 51 01 11. (Further details published in Vol. 363, No. 2.)
Sept. 23–25, 1987 Barcelona, Spain	**International Symposium on Pharmaceutical and Biomedical Analysis** Contact: Dr. Emilio Gelpi, Symposium Secretariat, International Symposium on Pharmaceutical and Biomedical Analysis, Palau de Congressos, Avgda. Reina M^a. Cristina s/n, 08004 Barcelona, Spain. Tel.: (325) 30 00-223 99 40, telex: 53.117 foimb-e.
Sept. 28–30, 1987 Barcelona, Spain	**International Symposium on Applied Mass Spectrometry in the Health Sciences** Contact: Dr. Emilio Gelpi, Symposium Secretariat, International Symposium on Applied Mass Spectrometry in the Health Sciences, Palau de Congressos. Dept. de Convencions, Avgda. Reina M.ª Cristina s/n, 08004 Barcelona, Spain. Tel.: (325) 30 00-223 99 40, telex: 53.117 foimb-e.
Sept. 28–Oct. 1, 1987 Gaithersburg, MD, U.S.A.	***Accuracy in* Trace *Analysis – Accomplishments, Goals, Challenges*** Contact: Harry Hertz, A309 Chemistry Building, National Bureau of Standards, Gaithersburgh, MD 20899, U.S.A. Tel.: (301) 921 2851.
Sept. 28–Oct. 2, 1987 Amsterdam, The Netherlands	**2nd Amsterdam HPLC Summercourse** Contact: Dr. J.C. Kraak, Laboratory for Analytical Chemistry, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV Amsterdam, The Netherlands.
Oct. 12–16, 1987 Sils-Maria, Switzerland	**5th Symposium and Workshop on Ion Chromatography** Contact: Workshop Office IAEAC, M. Frei-Hausler, Postfach 46, CH-4123 Allschwil 2, Switzerland. (Further details published in Vol. 366.)

Oct. 20–22, 1987
Sopron, Hungary

3rd Symposium on the Analysis of Steroids
Contact: Prof. S. Görög, c/o Hungarian Chemical Society, Anker köz 1, H-1061 Budapest, Hungary.

April 18–21, 1988
Las Vegas, NV, U.S.A.

Flow Analysis IV, An International Conference on Flow Analysis
Contact: Dr. Gilbert E. Pacey, Department of Chemistry, Miami University, Oxford, OH 45056, U.S.A. (Further details published in Vol. 357, No. 3.)

May 18–20, 1988
Amsterdam, The Netherlands

CAC–88, 4th International Conference on Chemometrics in Analytical Chemistry
Contact: CAC–88, Laboratory for Analytical Chemistry, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV Amsterdam, The Netherlands. Tel.: (020)-5223541 (Dr. Smit).

June 19–24, 1988
Washington, DC, U.S.A.

HPLC '88, 12th International Symposium on Column Liquid Chromatography
Contact: Symposium Manager, Barr Enterprises, P.O. Box 279, Walkersville, MD 21793, U.S.A. Tel.: (301) 898 3772. (Further details published in Vol. 363, No. 2.)

Aug. 28–Sept. 1, 1989
Wiesbaden, F.R.G.

11th International Symposium on Microchemical Techniques
Contact: Gesellschaft Deutscher Chemiker, Abt. Tagungen, P.O. Box 900440, D-6000 Frankfurt/Main 90, F.R.G. Tel.: (069) 79 17-366/360, telex: 4170497 gdch d.

Aug. 27–31, 1990
Vienna, Austria

7th European Conference on Analytical Chemistry, "Euroanalysis 7"
Contact: Prof. Robert Kellner, Austrian Society for Microchemistry and Analytical Chemistry, Institute for Analytical Chemistry, Technical University of Vienna, Getreidemarkt 1, A-1060 Vienna, Austria.

PUBLICATION SCHEDULE FOR 1986

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	1985	J 1986	F	M	A	M	J	J	A	S	O	N	D
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INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 362, No. 3, pp. 461–464. A free reprint can be obtained by application to the publisher.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For review articles, see page 2 of cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography.* Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (*e.g.,* Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc., should be on separate sheets.

Introduction. Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

Summary. Full-length papers and Review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes are published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet.* If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. Please check a recent issue for the layout of the reference list. Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts.* Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication".

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A New International Journal **CHEMOMETRICS AND INTELLIGENT LABORATORY SYSTEMS**

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★ **developments in statistical theory and mathematics with application to chemistry**

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