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JOURNAL OF LIQUID CHROMATOGRAPHY, 17(11), 2301-2318 (1994)

DONOR-ACCEPTOR CHIRAL CENTRIFUGAL PARTITION CHROMATOGRAPHY: COMPLETE RESOLUTION OF TWO PAIRS OF AMINO-ACID DERIVATIVES WITH A CHIRAL II DONOR SELECTOR

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ABSTRACT

Two racemic compounds, namely N-(3,5-dinitrobenzoyl)-*tert*-butylvallnamide, and N-(3,5-dinitrobenzoyl)-*tert*-butylleucinamide, have been successfully completely resolved by centrifugal partition chromatography, using N-dodecanoyl-L-Proline-3,5-dimethylanilide as a chiral selector, and a biphasic system made with heptane, ethyl acetate, methanol and water. This opens the way to a better understanding of this class of separation by centrifugal partition chromatography.

INTRODUCTION

While resolution of optical isomers by liquid column chromatography using chiral stationary phases becomes more and more popular and diversified, with more than 100 commercial chiral solid phases[1], very few works have been published concerning this kind of separation by countercurrent chromatography or by centrifugal partition chromatography. Both techniques refer to support-free liquid-liquid chromatography with two immiscible liquids prepared by mixing two or more solvents or solutions; an instrument keeps one liquid stationary while the other liquid is pumped through it, and the chromatographic process occurs between the two liquid phases

Partial resolution of ± Norephedrine, using (R,R)-di-5-nonyltartrate as a chiral selector, and the biphasic system 1.2-dichloroethane / 0.5 M $NaPF_6$ in water, pH 4, has been obtained in around 4 days at 2 or 8 ºC, using a Rotary Locular countercurrent chromatographic system (RLCCC) with 592 locules[2]. For this separation, the tartrate appears as an enantioselective ionophore toward the Norephedrine, ammonium salt[3].

Complete resolution of D,L-Isoleucine was obtained using N-dodecyl-L-Proline as a chiral selector, with the biphasic system 1-Butanol / Water, pH 5.5, and Cu^{II} ions as the metal center for Ligand Exchange Chromatography. Separation was performed with a droplet countercurrent chromatograph (DCCC) in approximately 2 days[4]. A racemic carboxylic acid was resolved using R-2-aminobutanol as a chiral selector and the biphasic system Chloroform / Methanol / Water, pH 7, in about 40 hours, with a DCCC[5]. For these two separations, interactions are mainly ionic.

A few partial resolution of amino-acids or drug enantiomers using aqueous two phase systems and a protein as the chiral selector have also been published [6,7].

Combination of an enzyme reactor with a centrifugal partition chromatograph (CPC) has been used to resolve racemic amino-acid esters into the L free amino acid and the D amino-acid ester; incubation time was 5 hours, and CPC separation time was 3 hours[8].

Using a high performance centrifugal partition chromatograph (HPCPC)[9], we undertook some experiments in our laboratory, and before the successful separation described in "Results and Discussion", we had some unsuccessful experiences that we would like to summarize, as we think these experiences could help other groups working in this field.

1/ β-Cyclodextrin (β-CD) as a chiral selector :

β-CD stationary phases for column chromatography has been developed by D. W. Armstrong *et al.* [10]. β-CD has been reported to be very soluble in mixtures of water and dimethyl sulfoxide[11], and is readily soluble in the lower phase of any two-phase system WDT, made with Water / Dimethyl sulfoxide / Tetrahydrofuran[12] (up to 0.1 M, *i.e.* ≈57 g/l). We were using the system WDT 5 (water / DMSO / THF, 24.5 / 16.2 / 59.3, v/v/v, with (β-CD) = 0.05 M) to resolve the Trogger Base (2,8-dimethyl-6*H*,12*H*-5,11-methanodibenzo[*b*,*f*][1,5] diazocine[13], using the upper phase as the mobile phase. With this system the partition coefficient of the Trogger Base ($D \frac{\text{stationary}}{\text{mobile}}$) is 0.17 without the β-CD, and 0.42 with the β-CD, which seems to prove an interaction between the β-CD and the Trogger Base. But no detectable resolution occurred.

2/ (2R,3R)-di-n-butyl tartrate (DBT) as a chiral selector :

Chiral separations on reversed phase columns with DBT added to the mobile phase have been fully studied by C. Pettersson *et al.*[14]. Optimum selectivity is found at high loading of DBT on the support. Based on these results, we tested the ternary system Heptane / DBT / phosphate buffer in water,(PB), the ternary diagram of which is not available. Starting from the system DBT / PB, for which the organic phase is heavier than the aqueous phase, we added aliquots of heptane; the resulting liquid system is always biphasic, which is not surprising, but around the ratio HEP / DBT / PB 1/4/4 (v/v/v), the two phases have the same densities ($\Delta\rho$ = 0), and there is no settling; adding more heptane results in a DBT rich upper phase and a water rich lower phase, which can be used in CPC, despite the rather high viscosities of the two phases.

We selected the system HEP / DBT / PB 0.1 M, 25/25/50, v/v/v, which corresponds roughly to a solution 2 M of DBT in heptane as the organic upper phase, and an aqueous lower phase; adding hexafluorophosphate 0.1 M as a

counterion in the lower phase results in a triphasic system, part of the heptane being excluded from the DBT rich upper phase. After removing this heptane, the final biphasic system is then more than 2 M in DBT in the upper phase, which is then used as the stationary phase in CPC, and is quite stable under normal operating conditions. We tried to resolve ± Norephedrine with this system, but, due to the rather high viscosities of the two phases, we just observed a broad peak coming out, even at higher rotational speed[15] (1700 rpm). The flow rate was limited to 0.5 ml/min, because of the resultant hydrodynamic pressure[16].

3/ First attempt of charge transfer chiral chromatography :

The first charge transfer chiral selector tested in our laboratory was the N,N'-bis-[N-(3,5-dinitrobenzoyl)-(S)-phenylalanyl]-3,6-dioxa-1,8-octanediamine, (**1**).(Figure 1)(see Experimental). Racemic compounds similar to (**1**) display very high selectivity factors (α > 100) when chromatographed on a (S)-N-(2-naphtyl)alanine silica stationary phase[17].

Solubility of (**1**) is very low in most solvents, except in DMSO, and this is why we tested (**1**) with the biphasic system called WDT 3, *i.e* water / DMSO / THF, 16.7 / 25.1 / 58.2, v/v/v. In this system, up to 10 g/L of [I] can be dissolved in the upper phase (THF rich).

The system WDT 3, (**1**) = 10 g/L in upper phase, has been used in the descending mode to try to resolve the ± Lorazepam, which is known to be easily resolved on a N-(3,5-dinitrobenzoyl)-L-phenylalanine silica stationary phase[18]; the rotational speed was 1400 rpm and the flow rate was 3 ml/min. Even with the high partition coefficient observed for (**1**), on-line UV detection is not possible in these experiments because of the presence of amount of (**1**) in the mobile phase; TLC monitoring of the collected fraction was more convenient, but we did not observe a chiral resolution for the racemate, which had a partition coefficient of ≈3.6 in this system.

From experiment 1 and 2, we concluded that transposition of HPLC experiments where the observed selectivity factors are in the range 1.1 to 1.5, to CPC result in a chiral defeat, maybe because the chiral selector is not linked to a rigid matrix, which is known to play a role upon the enantioselectivity[19,20]. Experiment 3 will be further investigated, as its failure can be due to a poor choice of the racemate to be resolved.

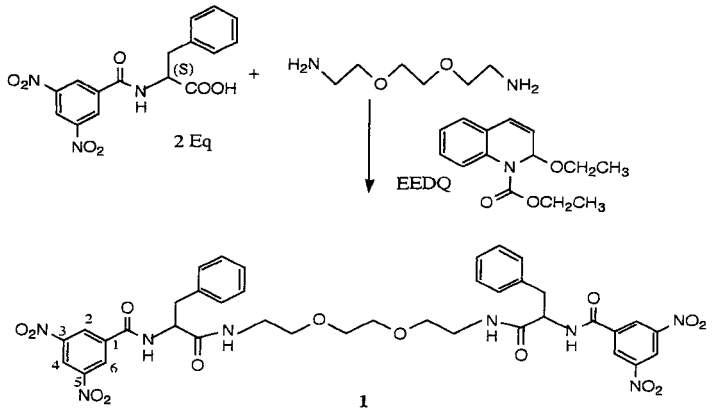

Figure 1 Synthesis of *N,N'-bis-[N-(3,5-dinitrobenzoyl)-(S)-phenylalanyl]-3,6-dioxa-1,8-octanediamine* (**1**).

This paper describe experiment 4, which was a success, and where the chiral selector is similar to a selector recently described by W.H. Pirkle *et al.*[21], which display high recognition for specific racemic compounds.

EXPERIMENTAL SECTION

Chemicals

N,N'-bis-[N-(3,5-dinitrobenzoyl)-(S)-phenylalanyl]-3,6-dioxa-1,8-octanediamine (**1**). To a solution of 3.6 g (25 mmol) of 3,6-dioxa-1,8-octanediamine and 17.9 g (50 mmol) of N-(3,5-dinitrobenzoyl)-(S)-phenylalanine[22] in 400 ml of tetrahydrofuran (THF), a solution of 12.4 g (50 mmol) of 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) in 50 ml of THF was added at room temperature. The mixture was refluxed during 3.5 hours, then the solution was evaporated. The residue was diluted with dichloromethane and washed with 1% ortophosphoric acid, 0.2M potassium hydroxide and distilled water. After drying over sodium sulfate, the solution was evaporated and the residual solid collected. Recrystallization from ethanol-water gave 10.4 g (51% yield) of a solid, m.p. 214°C. ^{1}H-NMR (200 MHz): δ

(DMSO-d_6) 2.9-3.6 (m, 16H, $2CH_2Ar$, $2CH_2N$ and $4CH_2O$), 4.80 (m, 2H, 2CH), 7.1-7.4 (m, 10H, $2C_6H_5$), 8.33 (t, 2H, $2NHCH_2$), 8.94 (m, 2H, $2C^4H$), 9.02 (m, 4H, $2C^{2,6}H$), 9.45 (d, 2H, 2NHCH). IR (KBr): 3289 cm^{-1} (NH st), 1639 cm^{-1} (CO st, Amide I), 1537 cm^{-1} (N-C=O st si, Amide II and NO_2 st as), 1343 cm^{-1} (NO_2 st si), 1078 cm^{-1} (C-O st as). $[\alpha]_D^{22}$=-5.6° (c=1.0, THF). Analysis calculated for $C_{38}H_{38}N_8O_{14}$, C 54.94, H 4.61, N 13.49; found, C 55.04, H 4.55, N 13.15 %.

N-dodecanoyl-L-proline (**2**). L-Proline (11.6 g, 100 mmol) was dissolved in 150 ml of 1M sodium hydroxide solution and cooled in an ice-bath. The dodecanoyl chloride (24.1 g, 110 mmol) and 200 ml of 1M sodium hydroxide solution were added simultaneously over a period of 20 min. The solution was stirred at room temperature for 45 min and acidified with concentrated hydrochloric acid (pH 2-3). The solution was extracted with diethyl ether and the organic phase washed with a 12 % sodium chloride solution. After drying over sodium sulfate it was evaporated and 30 g (100% yield) of an oil collected (Figure 2).

N-dodecanoyl-L-proline-3,5-dimethylanilide (**3**). To a solution of 6.5 g (54 mmol) of freshly distilled 3,5-dimethylaniline and 15 g (50 mmol) of N-dodecanoyl-L-proline in 200 ml of THF, a solution of 12.6 g (50 mmol) of EEDQ in 100 ml of THF was added at room temperature. The mixture was allowed to react 24 Hrs at room temperature, then the solution was evaporated. The residue was diluted with dichloromethane and washed with 1% orthophosphoric acid, 0.2M sodium hydroxide and distilled water. After drying over sodium sulfate, the solution was evaporated and the residual collected. Recrystallization from ethanol-water gave 17.6 g (88% yield) of a white solid, m.p. 63°C. ^{1}H-NMR (200 MHz): δ ($CDCl_3$) 0.88 (t, 3H, CH_3), 1.25 (m, 18H, $9CH_2$), 1.67 (m, 2H, C^3H_2-Pro), 2.07 (m, 1H. C^2Ha-Pro), 2.27 (s, 6H, $ArCH_3$), 2.30 (m, 2H, CH_2CO), 2.60 (m, 1H, C^2Hb-Pro), 3.47 (m, 2H, CH_2N), 4.80 (d, 1H, CH), 6.70 (s, 1H, $C^{4'}$H), 7.17 (s, 2H, $C^{2',6'}$H), 9.62 (s, 1H, NH). IR (KBr): 3325 cm^{-1} (NH st), 1626 cm^{-1} (CO st, Amide I). $[\alpha]_D^{22}$=-66.9° (c=1.2, ethanol 96°). Analysis calculated for $C_{25}H_{40}N_2O_2.H_2O$, C 71.73, H 10.11, N 6.69; found, C 71.78, H 9.64, N 6.53 %. (Figure 2).

(±) N-(3,5-dinitrobenzoyl)amino acids. The appropriate racemic amino acid (23 mmol) was dissolved in 50 ml of 1M sodium hydroxide solution and

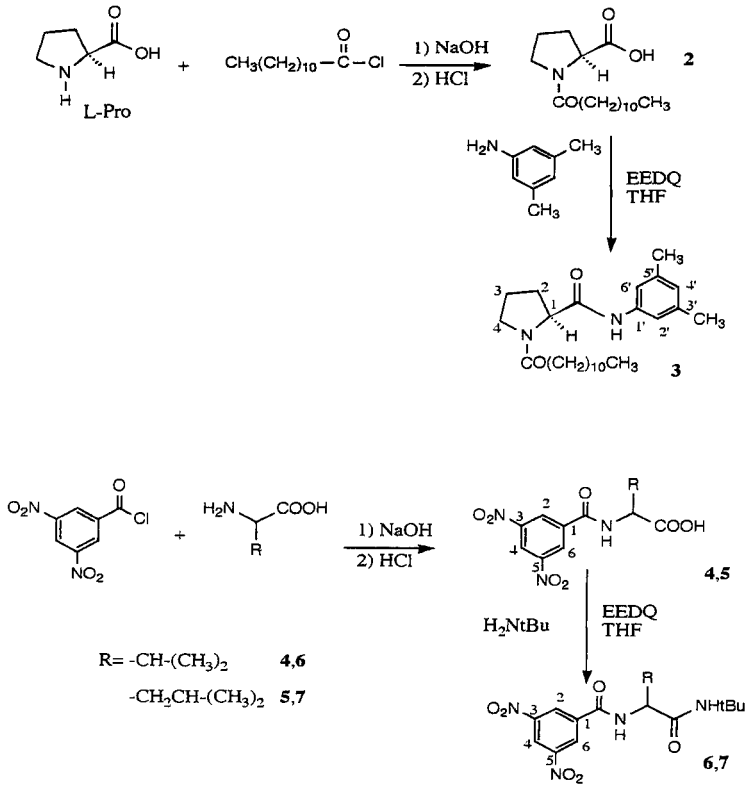

Figure 2 Synthesis of the chiral selector and of the racemics used in centrifugal partition chromatography.

cooled in an ice-bath. The 3,5-dinitrobenzoyl chloride (5.3 g, 23 mmol) and 50 ml of 1M sodium hydroxide solution were added simultaneously over a period of 20 min. The solution was stirred at room temperature for 90 min and acidified with concentrated hydrochloric acid (pH 2-3). The resulting solid was collected by filtration and washed with water. Recrystallization from ethanol-water gave the corresponding N-(3,5-dinitrobenzoyl)amide.

(±) N-(3,5-dinitrobenzoyl)valine (**4**). Yield 41%. m.p. 187°C. ^{1}H-NMR (200 MHz): δ ($CDCl_3/CD_3OD$) 1.06 (d, 6H, $2CH_3$), 2.36 (m, 1H, CH), 4.74 (d, 1H, CHN), 9.09 (d, 2H, $C^{2,6}H$), 9.17 (d, 1H, $C^{4}H$). ^{13}C-NMR (50.3 MHz): δ ($CDCl_3/CD_3OD$) 17.8 and 18.9 ($2CH_3$), 30.9 (CH), 58.2 (CHN), 121.0 ($C^{4}H$), 127.6 ($C^{2,6}H$), 137.3 (C^{1}), 148.4 ($C^{3,5}$), 163.3 (CONH), 173.7 (COOH). IR (KBr): 3323 cm^{-1} (NH st), 1714 cm^{-1} (CO st, COOH), 1633 cm^{-1} (CO st, Amide I), 1538 cm^{-1} (N-C=O st si, Amide II and NO_2 st as), 1343 cm^{-1} (NO_2 st si) (Figure 2)

(±) N-(3,5-dinitrobenzoyl)leucine (**5**). Yield 67%. m.p. 181°C. ^{1}H-NMR (200 MHz): δ ($CDCl_3/CD_3OD$) 0.98-1.03 (m, 6H, $2CH_3$), 1.80 (m, 3H, $CHCH_2$), 4.77 (m, 1H, CHN), 8.54 (d, 1H, NH), 9.14 (m, 3H, ArH). ^{13}C-NMR (50.3 MHz): δ ($CDCl_3/CD_3OD$) 20.7 and 22.4 ($2CH_3$), 24.6 (CH), 39.7 (CH_2), 51.4 (CHN), 120.5 ($C^{4}H$), 127.4 ($C^{2,6}H$), 137.0 (C^{1}), 148.1 ($C^{3,5}$), 163.5 (CONH), 174.2 (COOH). IR (KBr): 3375 cm^{-1} (NH st), 1722 cm^{-1} (CO st, COOH), 1644 cm^{-1} (CO st, Amide I), 1538 cm^{-1} (N-C=O st si, Amide II and NO_2 st as), 1346 cm^{-1} (NO_2 st si) (Figure 2).

(±) N-(3,5-dinitrobenzoyl)-tert-butylamido derivatives of amino acids. To a solution of 9.6 mmol of N-(3,5-dinitrobenzoyl)amino acids and 10 mmol (2.5 g) of EEDQ in 50 ml of THF, 10 mmol (0.7 g) of *tert*-butylamine were added at room temperature. The mixture was refluxed during 18 hours and the resulting solution was evaporated. The residue was diluted with dichloromethane and washed with 5% sodium hydrogen carbonate, 1M hydrochloric acid and distilled water. After drying over sodium sulfate, the solution was evaporated and the residual solid collected. Recrystallization from ethanol-water gave the corresponding amide.

(±) N-(3,5-dinitrobenzoyl)-tert-butylvalinamide (**6**). Yield 89%. m.p. 235°C. ^{1}H-NMR (200 MHz): δ ($CDCl_3$) 1.01 (t, 6H, $2CH_3$), 1.38 (s, 9H, tBu), 2.25 (m, 1H, CH), 4.35 (t, 1H, CHN), 5.82 (s, 1H, NH), 7.78 (d, 1H, NHCOAr), 9.04 (d, 2H, $C^{2,6}H$), 9.17 (m, 1H, $C^{4}H$). ^{13}C-NMR (50.3 MHz): δ ($CDCl_3/CD_3OD$) 18.7 and 19.0 ($2CH_3$), 28.3 (tBu), 31.0 (CH), 51.6 (CqtBu), 60.6 (CHN), 121.0 ($C^{4}H$), 127.7 ($C^{2,6}H$), 137.3 (C^{1}), 148.4 ($C^{3,5}$), 163.0 (NHCOAr), 170.6 (CONH). IR (KBr): 3324 cm^{-1} (NH st), 1639 cm^{-1} (CO st, Amide I), 1544 cm^{-1} (N-C=O st si, Amide II and NO_2 st as), 1341 cm^{-1} (NO_2 st si) (Figure 2).

(±) N-(3,5-dinitrobenzoyl)-tert-butylleucinamide (**7**). Yield 84%. m.p. 238°C. ^{1}H-NMR (200 MHz): δ ($CDCl_3$) 0.92 (t, 6H, $2CH_3$), 1.37 (s, 9H, tBu), 1.63 (m, 3H,

$CHCH_2$), 4.60 (m, 1H, CHN), 5.97 (s, 1H, NH), 8.00 (d, 1H, NHCOAr), 9.08 (d, 2H, $C^{2,6}H$), 9.17 (m, 1H, C^4H). ^{13}C-NMR (50.3 MHz): δ ($CDCl_3/CD_3OD$) 21.6 and 22.4 ($2CH_3$), 24.6 (CH), 28.0 (tBu),40.7 (CH_2), 51.3 (CqtBu),53.0 (CHN), 120.8 (C^4H), 127.6 ($C^{2,6}H$), 137.1 (C^1), 148.3 ($C^{3,5}$), 162.9 (NHCOAr), 171.5 (CONH). IR (KBr): 3294 cm^{-1} (NH st), 1644 cm^{-1} (CO st, Amide I), 1556 cm^{-1} (N-C=O st si, Amide II and NO_2 st as), 1342 cm^{-1} (NO_2 st si) (Figure 2).

Centrifugal Partition Chromatography :

Apparatus. A series 1000 HPCPC (Sanki Eng. Limited, Nagaokakyo, Kyoto, Japan) was used[9]. It is a bench top CPC (30 x 45 x 45 cm, ≈ 60 kg); the column is a stacked circular partition disk rotor which contains 2136 channels with a total internal volume of around 240 ml. The column is connected to the injector and the detector through two high pressure rotary seals containing a drilled sapphire rod passing through two toroidal seals similar to those used with HPLC pump pistons. The partition disks are engraved with 1.5 x 0.28 x 0.21 cm channels connected in series by 1.5 x 0.1 x 0.1 cm ducts. A 4-port valve included in the series 1000 allows the HPCPC to be operated in either the descending or ascending mode. The HPCPC was connected to an HPLC system gold (Beckman, San Ramon, CA, USA), including a solvent delivery pump model 126 and a manual sample injector.

The solvent system was a mixture of Heptane / Ethyl acetate / Methanol / Water (see Results and Discussion); the flow rate was 5 ml/min, with a rotational speed of 1200 rpm. The CPC run was performed in the descending mode, and the backpressure was around 3 MPa.

HPLC

A HPLC Waters workstation (Waters Chromatography Division, Millipore Corp., Milford, MA) was used to control the optical purity of the CPC collected fractions. The column was a 10 x 0.46 cm, packed with N-(3,5-dinitrobenzoyl)-L-phenylalanine bonded to 5 μm silica (Chirachrom A1, Interchim, Montluçon, France), and the mobile phase a mixture of Heptane / 2-Propanol / Methanol, 63 / 27 / 10, v/v/v. The flow rate was 1 ml/min, and UV detection was performed at 254 nm.

TLC Monitoring

CPC experiments were monitored by TLC on Kieselgel 60 F 254 spezial (Riedel de Haën, Seelze, Germany), with Heptane Ethyl Acetate (1:1).

Recovering of Enantiomers

The CPC fractions were concentrated individually under reduced pressure to obtain an aqueous suspension which was then extracted with Heptane / Ethyl acetate (1:1). The organic phase was dried over sodium sulfate and the solution evaporated. The enantiomer was separated from the chiral selector by conventional column chromatography on silica gel (60-200 μm), using an Heptane / Ethyl acetate (1:1) mixture as eluant.

RESULTS AND DISCUSSION

Selection of the Solvent System.

As it is usual in centrifugal partition chromatography when a new mixture has to be studied, the first thing to do is to find a class of solvents where the mixture is freely soluble, then to achieve a biphasic system by adding non miscible solvents in which the selected solvents and the mixture will partition[23]. In our case both the Chiral selector (CS) and the racemic compounds (R) need to be freely soluble, and more, CS has to be mainly in one phase, which will be the stationary phase, while R have better to be well partitioned, and in favor of the other phase, if possible, and which will be the mobile phase.

We found CS and R to be readily soluble in Ethyl acetate (EtOAc) and in Methanol (MeOH), and we tested their partitioning in the quaternary system Heptane / EtOAc / MeOH / Water, which has been extensively described [23,24].

The system Heptane / EtOAc / MeOH / Water, 3 / 1/ 3 / 1 (v/v/v/v) allows CS to be more in the upper phase (which will be the stationary phase), with a partition coefficient, $D = \frac{C_{stationary}}{C_{mobile}}$, of around 3.1, and R more in the lower phase, with a partition coefficient (when CS is not present) of around 0.3 for the Valine derivative, and around 0.5 for the Leucine derivative (D was estimated by HPLC).

In presence of CS, the apparent partition coefficient of R, D', varies greatly, and it is not the same for the (+) and the (-) isomers, as shown on Table I.

It appears that one isomer has little or no affinity for the chiral selector, while the other isomers show a rather strong affinity for it.

The apparent partition coefficient, D', results from a quadratic equilibrium scheme (Figure 3). On this Figure, R means either (+) or (-) isomer, and :

$$D_{CS} = \frac{(\overline{CS})}{(CS)}, \quad D_R = \frac{(\overline{R})}{(R)}, \quad D_{RCS} = \frac{(\overline{RCS})}{(RCS)} \qquad \text{(Partition Coefficients)}$$

$$\overline{K} = \frac{(\overline{RCS})}{(\overline{R})(\overline{CS})}, \quad K = \frac{(RCS)}{(R)(CS)} \qquad \text{(Complex Formation Constants)}$$

(overlined term means : in the stationary phase).

The apparent coefficient for R is :

$$D' = \frac{(\overline{R}) + (\overline{RCS})}{(R) + (RCS)} = D_R \times \frac{1 + \overline{K} D_{CS} (CS)}{1 + K(CS)} \qquad [1]$$

Assuming the (+) isomer to be the most retarded, the selectivity factor, $\alpha_{+,-}$ is then (since $D_{R+} = D_{R-}$)[25]:

$$\alpha_{+,-} = \frac{D'_+}{D'_-} = \frac{1 + \overline{K}_+ D_{CS} (CS)}{1 + \overline{K}_- D_{CS} (CS)} \times \frac{1 + K_- (CS)}{1 + K_+ (CS)} \qquad [2]$$

Simplification occurs when $K_- = \overline{K}_- = 0$ (no complex formation between the (-) enantiomer and CS, which is roughly our case for the Valine der., see Table I). We have then :

$$\alpha_{+,-} = \frac{1 + \overline{K}_+ D_{CS} (CS)}{1 + K_+ (CS)} \qquad [3]$$

When 1 can be neglected, compared to the other terms, we get :

$$\alpha_{+,-} = \frac{\overline{K}_+}{K_+} D_{CS} \qquad [4]$$

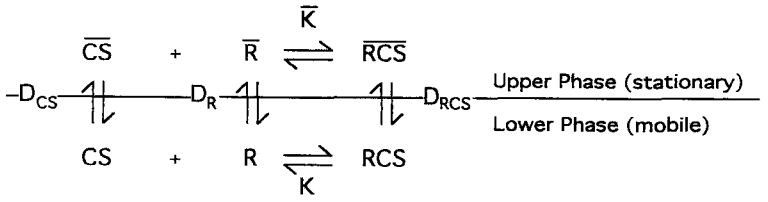

Figure 3 Quadratic scheme describing the equilibrium involved in donor-acceptor chiral centrifugal partition chromatography. D is a partition coefficient, and K is a complex formation constant.

Table I: Apparent partition coefficients, D', of (±) Val and (±) Leu Derivatives

	D' when (CS) = 0	D' when $(CS)_{upper\ phase} \approx 0.019$ M, $(CS)_{lower\ phase} \approx 0.006$M ($D_{CS} \approx 3.1$)
(-) Val Der.	≈ 0.3	0,45
(+) Val Der.		1.5
(+) Leu Der.	≈ 0.5	0,5
(-) Leu Der.		5.5

Equation [4] means that the chiral separation will be better if the chiral selector is mainly in the stationary phase (D_{CS} large), and if complex formation is favored in the stationary phase too, *i.e.* electron donor acceptor interaction more important in the stationary phase than in the mobile phase, which means that the stationary phase should have a lower dielectric constant than the mobile phase (which is our case).

CPC Runs

Collected fractions of the CPC runs were monitored by TLC, since on-line UV detection is not possible, due to the presence of CS in the mobile phase.

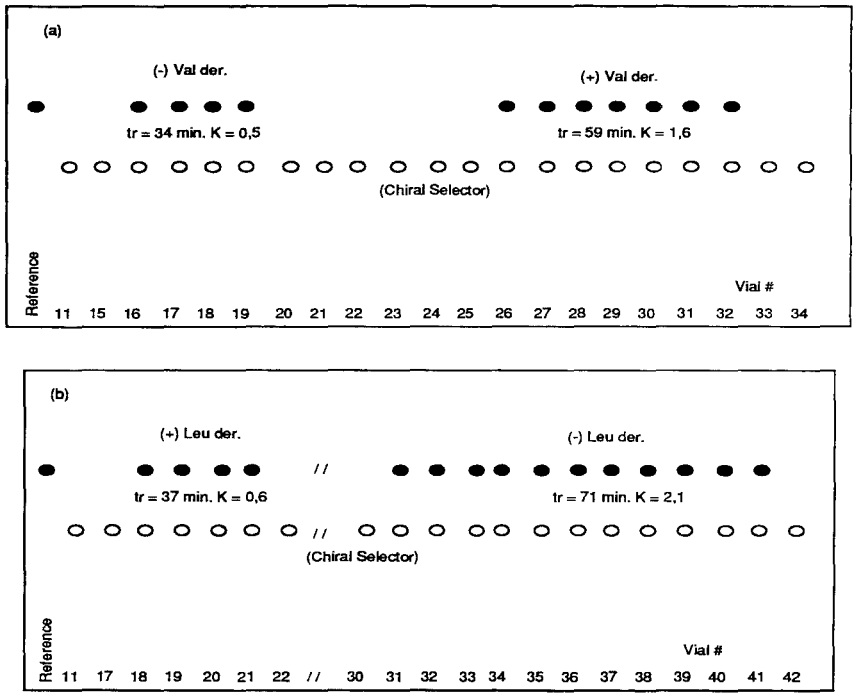

Figure 4 TLC control of the CPC runs.

CPC conditions : heptane / ethyl acetate / methanol / water 3/1/3/1
Descending mode, Flow rate = 5 ml/min
Rotational speed : 1200 rpm; Backpressure ≈ 3 MPa
$V_{stat}/V_{mob} \approx 1$; (CS) = 0.01M, in upper phase
Sample : 2 ml of a 0.001 M aminoacid der.
Collected fractions every 2 min (10 ml).

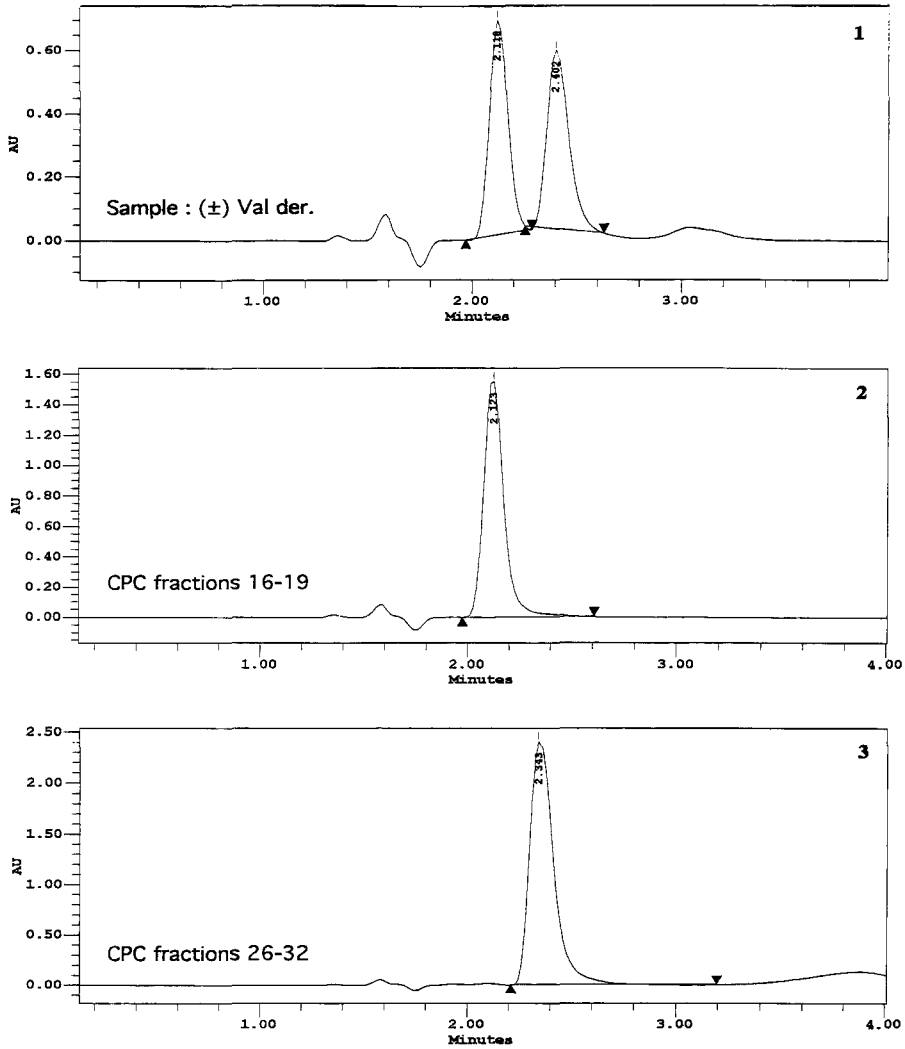

Figure 5 HPLC control of the CPC separation of the Val der. enantiomers : •1 original sample ; •2 CPC fractions 16-19, after removal of CS, and corresponding to (-) Val der.; •3 CPC fractions 26-32, after removal of CS, and corresponding to (+) Val der.

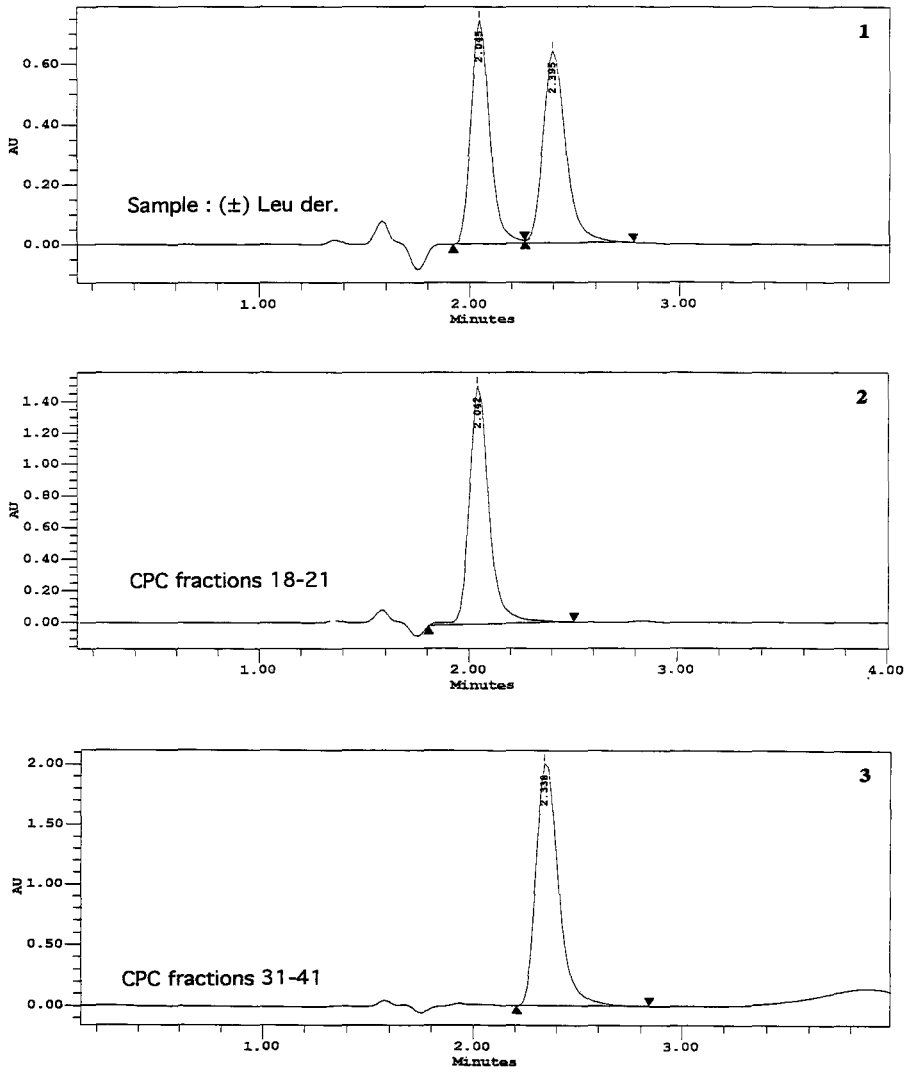

Figure 6 HPLC control of the CPC separation of the Leu der. enantiomers : •1 original sample ; •2 CPC fractions 18-21, after removal of CS, and corresponding to (+) Leu der.; •3 CPC fractions 31-41, after removal of CS, and corresponding to (-) Leu der.

Figure 4 shows two typical TLC control and CPC conditions, one is for the resolution of the (±) Val derivative, the other for the resolution of the (±) Leu derivative. There is more than 70 ml between the end of the first peak and the beginning of the second peak, for (±) Val der., and more than 120 ml for (±) Leu der.. The first peaks correspond to the (-) Valine der. (α=-6.9º , c = 1.0, chloroform), and to the (+) Leucine der. (α=+9.0º , c = 1.1, chloroform). The approximate selectivity factors (estimated from the middle of the black dots (Fig. 4) are 3.2 for (±) Val der., and 3.5 for (±) Leu der.. CS, which is in every fraction, is easily removed by simple column chromatography on silica (see experimental); the pure enantiomer comes first, and CS remains on the column, and is recovered by washing with EtOAc as eluant.

Remark : The elution orders of R(±) and CS, both in TLC and open column chromatography, are opposite to the one expected from the relative partition coefficients of the studied species in the biphasic system Heptane / EtOAc / MeOH /Water 3/1/3/1, where CS appears as "less polar" than the racemics. This highlights the unique selectivity of centrifugal partition chromatography compared to silica based chromatography, and which is achieved by a fine tuning of the solvent system in order to get a particular and often narrow specificity. In this particular case, we observed that CS was totally in methanol in the system heptane / methanol, and was precipitating if a small amount of water was added, which means that CS does not "like" neither heptane nor water. When ethyl acetate is added, then CS redissolves, mainly in the heptane/ethyl acetate upper phase. This mechanism could be called preferential solvation and desolvation.

Figure 5 & 6 show the HPLC chromatograms corresponding to the two CPC runs.

CONCLUSION

Total separation of two pairs of optical isomers in less than one and a half hour by Donor Acceptor Chiral Centrifugal Partition Chromatography (DAC-CPC) is a real encouragement in finding some new selectors specially tailored for CPC resolution of specific racemic mixtures. CPC generally display a higher α than HPLC does for a given pair of molecules differing only slightly, but it seems

that this is not the case for chiral differences. We are now undertaking systematic studies to find the rules which will direct us to new and powerful selectors. To our knowledge, this is the first time a complete resolution of non-ionic racemics has been acheived in countercurrent chromatography.

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REFERENCES

1 S. Ahuja, "Chiral Separations by Liquid Chromatography", ACS Symposium Series 471, Washigton DC, 1991, Chap. 1.

2 B. Domon, K. Hostettmann, K. Kovacevic, V. Prelog, J. Chromatogr., 250, 149 (1982)

3 V. Prelog, M. Kovacevic, M. Egli, Angewante Chem. Int., 28, 1147 (1989)

4 T. Takeuchi, R. Horikawa, T. Tanimura, J. Chromatogr., 284, 285 (1984)

5 S. Oya, J.K. Snyder, J. Chromatogr., 370, 333 (1986)

6 B. Ekberg, B. Sellergren, P-Å. Albertsson, J. Chromatogr., 333, 211 (1985)

7 T. Arai, H. Kuroda, Chromatographia, 32, 56 (1991)

8 D. W. Armstrong, R. Menges, I.W. Wainer, J. Liq. Chromatogr., 13, 3571 (1990)

9 A.P. Foucault, O. Bousquet, F. Le Goffic, J. Liq. Chromatogr., 15, 2721 (1992)

10 D.W. Armstrong, W. DeMond, A. Alak, W.L. Hinze, T. E. Riehl, K.H. Bui, Anal. Chem., 57, 234 (1985)

11 A.K. Chatjigakis, C. Donzé, A.W. Coleman, P. Cardot, Anal. Chem., 64, 1632 (1992)

12 A.P. Foucault, P. Durand, E. Camacho-Frias, F. Le Goffic, Anal. Chem., 65, 2150 (1993)

13 The Trogger base was synthetized by P. Durand, in our laboratory, following a procedure described in I. Sucholeiki, V. Lunch, L. Phan, C.S. Wilcox, J. Org. Chem., 53, 98 (1988)

14 C. Pettersson, E. Heldin, H.w. Stuurman, J. Chrom. Sci., 28, 413 (1990)

15 A.P. Foucault, E. Camacho-Frias, C.G. Bordier, F. Le Goffic, J. Liq. Chromatogr., 17, 1 (1994)

16 D.W. Armstrong, G.L. Bertrand, A. Berthod, Anal. Chem., 60, 2513 (1988)

17 W.H. Pirkle, T.C. Pochapsky, J. Chromatogr., 369, 175 (1986)

18 L. Oliveros, C. Minguillón, C. Billaud, J. Pharm. Biomed. Anal., 10, 925 (1992)

19 W.H. Pirkle, M.H. Hyun, B. Banks, J Chromatogr., 316, 585, (1984)

20 W.H. Pirkle, R.J. Dappen, J. Chromatogr., 404, 107 (1987)

21 W.H. Pirkle, P.G. Murray, J. Chromatogr., 641, 11 (1993)

22 L. Oliveros, M. Cazau, J. Chromatogr., 409, 357 (1987)

23 A. Foucault, Handbook of Centrifugal Partition Chromatography, A. Foucault Editor, Marcel Dekker Publishers, in preparation,.chapter 4

24 W.D. Conway, Countercurrent Chromatography, W.D. Conway, Ed., VCH Publishers, New York, 1990, chapter 7

25 if the (-) isomer is the most retarded, all the discussion remains the same by just inverting (+) for (-)

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STRUCTURE RETENTION RELATIONSHIP FOR STEROID HORMONES. FUNCTIONAL GROUPS AS STRUCTURAL DESCRIPTORS

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ABSTRACT

Quantitative Structure Retention Relationship was investigated for a group of thirty steroid hormones. Structure of every compound was represented as a combination of the estrane skeleton and three to nine elements of a set of eighteen molecular fragments. Capacity factors of these compounds were represented as a sum of contributions due to the individual molecular fragments. From the temperature dependence of capacity factors the enthalpy and entropy of the retention process of these compounds were determined. It was shown that the enthalpy can also be considered as additive. Contributions to the capacity factor and to the enthalpy due to individual molecular fragments were calculated by the multiple regression method.

INTRODUCTION

Correlation of the molecular structure with physicochemical properties and biological activity seemed attractive since long ago [1, 2]. In chromatography, this correlation was named quantitative structure - retention relationship (QSRR) [3]. There are two ways of approaching this problem. In one of them the retention parameters are expressed as a function of the properties belonging to the molecule

as a whole, e. g. dipole moments, molecular refractivity, hydrophobicity, topological indexes and many others [2, 4-8]. In the second, contributions of the individual substituents to retention are considered. Additivity of substituent effects can be interpreted on the basis of linear Gibbs energy (or linear free energy relationship - LFER) [1, 3, 4, 9, 10] which was extensively used for explaining the effects of structural parameters on chemical rates and equilibria [11].

If the assumption on LFER is applicable, the following thermodynamic relationships for the capacity factor k′:

$$k' = K \cdot \varphi \qquad (1)$$

where K is the partition coefficient of the solute molecules between stationary and mobile phases, φ is the volume phase ratio of the stationary to the mobile phase, and

$$\ln k' = -\frac{\Delta G}{RT} + \ln\varphi \qquad (2)$$

where ΔG - molar Gibbs energy, R - the molar gas constant, T - absolute temperature, lead to the expression

$$\ln k' = \sum_i \ln k_i' + \ln\varphi \qquad (3)$$

where k_i' denotes contribution from the i^{th} fragment of a given compound to k′. For a set of capacity factors of a number of compounds we can find contributions k_i' using the least squares method for solving the system of linear equations. The method was comprehensively outlined in the paper of Chen and Horváth [3]; details being available in textbooks of statistics, e.g. [12]. The results of such QSRR investigation for a large number of aromatic - aliphatic acids (experimental data from [13], paper chromatography) were presented in [3]. Papers on QSRR using RPLC data for catecholamine derivatives [3], cardiac glycosides and steroid hormones [14] as well as benzodiazepines [15] were also concerned with the estimation of contributions of various molecular fragments (atomic groups, substituents) to retention.

QSRR was also investigated in a somewhat simplified way, i. e. by comparison of retention parameters of two compounds differing in only one molecular feature,

present in one compound, and absent in another one. This method was used for steroid hormones in [16] (normal phase LC) and in [17] (RPLC).

Such a simplified approach does not provide simultaneous contributions of all substituents, whereas the method treating a group of compounds as composed of a set of "building blocks" can in principle give contributions from all the elements of the set.

The group of compounds selected in this study consisted of 30 steroid hormones. Every compound can be represented by combining three to nine elements of the set of eighteen characteristic atomic groups and the estrane skeleton. As a matter of fact, in the beginning the number of characteristic groups was 19; then it was reduced to 18: the reasons will be given later on. We anticipated that the LFER assumption holds in this case, i. e. for every compound:

$$\ln k' = \text{const.} + \sum_{i=1}^{18} a_i \ln k_i' \qquad (4)$$

where the constant is the contribution from the estrane skeleton and includes also $\ln\varphi$. All the coefficients a_i take only two values: 1 (when the corresponding atom group is present in a given molecule) or 0 (when it is missing).

Eqn. 2 can be also written as [10]:

$$\ln k' = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} + \ln\varphi \qquad (5)$$

where ΔH, ΔS - denote enthalpy and entropy of the process of transfer of a solute molecule from mobile to stationary phase, respectively. Van't Hoff plots of ln k' vs 1/T offer the possibility of the estimation of ΔH and ΔS.

In this way ΔH and ΔS values were found. Next, it was checked if the values can also be treated as additive; if so, the following equations should be true:

$$\Delta H = \text{const.} + \sum_i a_i h_i \qquad (6)$$

$$\Delta S = \text{const.} + \sum_i a_i s_i \qquad (7)$$

where h_i and s_i - the increments to ΔH and ΔS of the characteristic atomic groups.

The main goal of this work was to verify the three relations 4, 6 and 7 experimentally.

EXPERIMENTAL

Materials

The names and CAS Registry Numbers of the steroids selected in this work are given in Table I. Solutions were prepared by diluting concentrated stock solutions (about 1 mg/ml, methanol) with mobile phase to the concentration about 25 μg/ml.

HPLC system

The HPLC system used was a Pye - Unicam PU 4100 (pump, oven and UV detector), Shimadzu RCA integrator, and a Varian fluorescence detector (for the measurement of void volume V_o). The chromatograms of steroid compounds were monitored at 210 nm (3, 9, 18, 22, 23, 25, 27, 28), 280 nm (10-12) and 240 nm (the rest). The column used were Partisil 10 ODS and Partisil 10 ODS-1, both 250 x 4.6 mm. Methanol-water was used as a mobile phase (55:45), flow rate was 1,5 ml/min. Capacity factors k' were measured on the column 1 for 30 steroids as a function of temperature at 35, 40, 45, 50, 55 and 60°C (only for lynestrenol at 40-65°). They were also measured on the column 2 at constant temperature, 40°C. Void volume V_o was determined as proposed by Neidhart et al [18], their method amounts to the doping of the mobile phase with a fluorophore (quinine sulphate), injection of undoped mobile phase, and measuring time of the decrease of fluorescence. Phase ratio φ was evaluated as the column inner volume minus V_o, divided by V_o. At 40°C φ was equal 0,48.

Statistics

Statistical analysis of data was performed with Stratgraphics Plus, version 6.0.

RESULTS AND DISCUSSION

Capacity factors k' were measured for 30 steroids as a function of temperature on the column 1. The measurements were also carried out on the column 2 at

TABLE 1
Names and CAS Registry Numbers of the Studied Set of Compounds

Compound		**CAS Registry Number**
1	Cortisone	[53-06-5]
2	Medroxyprogesterone acetate	[71-58-9]
3	Lynestrenol	[52-76-6]
4	Testosterone	[58-22-0]
5	Nandrolone	[434-22-0]
6	Progesterone	[57-83-0]
7	Prednisone	[53-03-2]
8	Prednisolone	[50-24-8]
9	Androsterone	[53-41-8]
10	Ethinyloestradiol	[57-63-6]
11	Estradiol	[50-28-2]
12	Estrone	[53-16-7]
13	Hydrocortisone	[50-23-7]
14	Methyltestosterone	[58-18-4]
15	Methylprednisolone	[83-43-2]
16	Ethisterone	[434-03-7]
17	Norethisterone	[68-22-4]
18	Methylandrostenediol	[521-10-8]
19	Methandienone	[72-63-9]
20	Methylprednisolone acetate	[53-36-1]
21	Prednisolone acetate	[52-21-1]
22	5α-androstane-3,17-dione	[846-48-8]
23	5α-androstane-17α-methyl-17β-ol-3-one	[521-11-9]
24	Cortexolone	[152-58-9]
25	Pregnenolone	[145-13-1]
26	1,4-androstadien-3,17-dione	[897-06-3]
27	5α-androstan-17β-ol-3-one	[521-18-6]
28	5α-androstan-3β-ol-17-one	[481-29-8]
29	Hydroxyprogesterone	[68-96-7]
30	Medroxyprogesterone	[520-85-4]

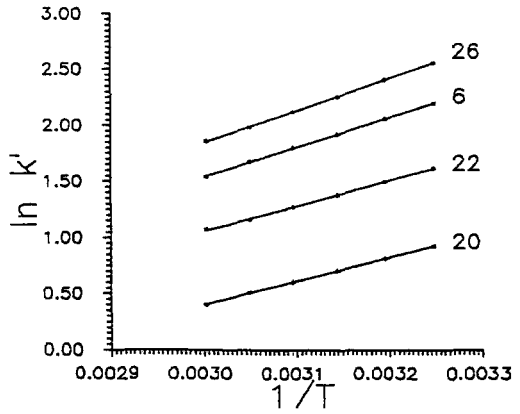


Figure 1 Plot of lnk' vs 1/T for several steroids. Column: Partisil ODS-1, mobile phase, methanol - water (55:45). See Table I for the names of compounds.

constant temperature of 40°C. Several plots of lnk' vs 1/T are shown in Figure 1. Correlation coefficients of the linear regression lnk' vs 1/T are very near to 1, so the assumption that the mechanism of the process is invariant over the temperature range investigated and the enthalpy is constant is justified. Values of ΔH and ΔS calculated from eqn. 5 are listed in Table II along with the measured values of k'.

In order to verify if the LFER can be accepted in this investigation, the linear regression of lnk' on ΔH was calculated. The correlation coefficient of this regression was high, R = - 0.9774, so the relations given by eqns. 4,6 and 7 should be true. Hence the sets of lnk', ΔH and ΔS were used to calculate the increments $\ln k_i'$ to lnk', the increments h_i to ΔH and the increments s_i to ΔS by means of multiple linear regression.

During this calculation a difficulty was encountered. In the matrix of coefficients a_i one or more columns appeared to be linear combinations of others. To overcome this problem it was necessary to delete the column corresponding to the C(20)=O group. As a consequence the character of neighbouring groups was changed:

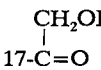
TABLE 2

Values of k', ΔH and ΔS of the Studied Steroids. Column: Partisil ODS-1, mobile phase: methanol-water (55:45), temp. 40°C (for k'). The enthalpy and entropy units are kcal·mol^{-1} and cal·mol^{-1}·deg^{-1}, respectively.

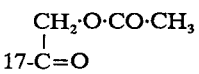
Compound	k'	ΔH	ΔS
1	0.9437	-3.13	-8.60
2	6.712	-5.17	-11.3
3	28.30	-6.52	-12.7
4	4.146	-4.50	-10.1
5	3.573	-4.40	-10.1
6	7.649	-5.29	-11.4
7	0.8779	-3.01	-8.36
8	1.033	-3.26	-8.84
9	6.810	-4.88	-10.4
10	3.204	-4.73	-11.3
11	3.462	-4.73	-11.2
12	2.749	-4.32	-10.3
13	1.094	-3.27	-8.78
14	5.413	-4.74	-10.3
15	1.509	-3.66	-9.37
16	3.495	-4.34	-9.91
17	3.033	-4.23	-9.85
18	6.803	-5.14	-11.1
19	3.802	-4.26	-9.47
20	2.189	-4.23	-10.5
21	1.519	-3.79	-9.77
22	4.353	-4.47	-9.89
23	7.675	-4.80	-9.89
24	1.935	-3.76	-9.22
25	10.86	-5.69	-12.0
26	4.774	-4.76	-10.6
27	5.834	-5.11	-11.3
28	5.844	-4.89	-10.6
29	2.175	-3.84	-9.21
30	3.430	-4.40	-10.1

instead of (21)CH_3 we have

instead of (21)CH_2OH we have



whereas 21(CH_2·O·CO·CH_3) was converted to



Taking the value of the coefficient of determination, R^2, as a criterion, one can see that the additivity of lnk′ is good (R^2 equals to 0.9693 and 0.9778 respectively for columns 1 and 2) and the additivity of ΔH is seen to be fairly good (R^2 = 0.9328). However, one can hardly agree that such an additive dependence for ΔS (R^2 = 0.6672) exists. Contributions h_i to ΔH are given in Table 3. The greatest positive partial enthalpies for C(3)=0 and 3-OH are measures of the heat consumption by these atomic groups during the transfer from the mobile to stationary phases. This heat is probably needed for the breaking of hydrogen bonds between these groups and the alcoholic - aqueous environment. Interestingly enough, the same groups in other positions (C(11)=0, C(17)=0, 11-OH and 17-OH) have far smaller partial enthalpies. Such functional groups as C = O and C-OH have essentially the same values when situated at the same site.

The contributions from double bonds between different atoms are also different. The large negative constant can be interpreted in such a way that the basic core of all these compounds, the estrane skeleton, has far greater affinity to hydrocarbon chains of the stationary phase than to the polar species of the mobile phase.

Apart from the relations based on the LFER assumption we have also tried to determine the increments to capacity factor according to:

$$k' = \text{constant} + \sum_{i}^{18} a_i \, k_i' \qquad (8)$$

Values of R^2 for such a multiple regression were equal to 0.9786 and 0.9855 for columns 1 and 2 respectively. Comparison with the values of R^2 for multiple linear regression using eqn. 4 shows that eqn. 8 describes the contributions of individual

TABLE 3

The Enthalpy Increments h_i and the Capacity Factor Increments k_i' due to the Individual Molecular Fragments.

	Molecular fragment	Enthalpy increments h_i	Capacity factor increments k_i'
1	1(2)C=C	0.01±0.1	-0.04±0.5
2	4(5)C=C	0.2±0.2	-1.8±0.6
3	C(3)=O	2.3±0.3	-26±1
4	C(11)=O	0.7±0.3	-1±1
5	C(17)=O	1.6±0.3	-6±1
6	3-OH	2.1±0.3	-25±1
7	11-OH	0.5±0.2	-1±0.9
8	17-OH	1.3±0.2	-5.8±0.9
9	(19)CH_3	-0.3±0.2	0.6±0.7
10	CH_3 17-C=O	0.8±0.2	-1.7±0.8
11	6-CH_3	-0.5±0.2	0.8±0.6
12	CH_2OH 17-C=O	0.9±0.2	-2.2±0.9
13	6π electrons	0.1±0.3	-2.7±1
14	17-C≡CH	0.2±0.2	-0.5±0.6
15	5(6)C=C	0.06±0.3	-0.2±1
16	17-CH_3	0.1±0.2	0.8±0.6
17	17-O·CO·CH_3	-0.8±0.3	3.6±1
18	CH_2·O·CO·CH_3 17-C=O	-0.5±0.2	0.5±0.7
	constant	-8.3±0.4	37±1

groups to retention better than eqn. 4. The F values point to the same conclusion. Contributions k_i' to k', according to eqn. 8, are given in Table 3. It can be seen that the same groups which have the largest positive increments to ΔH show the largest negative contributions to k'. Nevertheless, the correlation between k' and ΔH (R = -0.8188) is rather poor. It can also be seen in Table 3 that not all the contributions are statistically significant. According to the results of the Student's test one could exclude variables (contributions from fragments) 1, 4, 7, 14, 15, 18 by

taking significance level equal to 0.3. After elimination of these variables R^2 increased from 0.9786 to 0.9829.

It is known from the published data [19, 20] that in the class of steroid compounds the retention is not only determined by the kind of substituents but also depends on their stereochemical position. Thus the analysis of additive contributions should discern not only different substituents but also their stereochemistry, e. g. 3α-OH, 3β-OH and aromatic 3-OH ought to be treated separately. So we have tried to do so but an attempt to follow this idea resulted in the increase of linearly dependent columns and the calculations had to be abandoned.

In order to determine the actual predictive value of the procedure the method of cross - validation was applied [21]. The correlation coefficient of these cross - validated vs. observed values was 0.9038.

CONCLUSIONS

The capacity factor of a steroid hormone can be represented as a sum of increments due to individual molecular fragments (atomic groups). The linear representation given by eqn. 8 seems to be more reliable than the logarithmic relation of eqn. 4. The possibility of the additive representation for retention enthalpy and entropy was also checked and it was found that only the enthalpy can be considered as additive. Nevertheless the high value of the coefficient of determination in the multiple linear regression of lnk' indicates that LFER is a good empirical rule in this case. So correlation coefficients of regresion insufficiently descriminate between theoretical models [22, 23].

The knowledge of contributions from various atomic groups can be used to predict chromatographic behaviour of other compounds and to understand better interactions between solute and its environment. The investigation can be continued taking into account the discrimination of substituents with regard to their geometric isomerism.

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REFERENCES

1 E. Tomlinson, J. Chromatogr., 113: 1-45 (1975).

2 R. Kaliszan, J. Chromatogr., 220: 71-83 (1981).

3 Bor-Kuan Chen, C. Horváth, J. Chromatogr., 171: 15-28 (1979).

4 R. Kaliszan, Quantitative Structure-Chromatographic Retention Relationships, Wiley, New York, 1987.

5 K. Valkó, P. Slégel, J. Liq. Chromatogr., 14: 3167-3179 (1991).

6 S. V. Galushko, J. Chromatogr., 552: 91-102 (1991).

7 R. Kaliszan, K. Ośmiałowski, S. A. Tomellini, Shih-Hsien Hsu, S. D. Fazio, R. A. Hartwick, J. Chromatogr., 352: 141-155 (1986).

8 T. Hamoir, B. Bourguignon, D. L. Massart, H. Hindriks, J. Chromatogr., 633: 43-56 (1993).

9 C. Reichardt, Solvents and Solvent Effects in Organic Chemistry, VCH Verlagsgesellschaft mbH, Weinheim, 1988.

10 W. Melander, D. E. Campbell, C. Horváth, J. Chromatogr., 158: 215-225 (1978).

11 J. E. Leffler, E. Grunwald, Rates and Equilibria of Organic Reactions, Wiley, New York, 1963.

12 J. H. Zar, Biostatistical Analysis, Prentice Hall Inc., Englewood Cliffs, N.J., 1974, Chapter 19.

13 M. Kuchăr, V. Rejholec, M. Jelinková, O. Němeček, J. Chromatogr., 150: 419-428 (1978).

14 V. Ya. Davydov, G. N. Filatova, E. Smolkova-Keulemansova, Yu. Zima, Chromatographia, 25: 1059-1066 (1988).

15 N. Dimov, M. Moskovkina, J. Chromatogr., 552: 59-69 (1991).

16 Shoji Hara, Shigeki Ohnishi, J. Liq. Chromatogr., 7: 69-82 (1984).

17 J. M. Di Bussolo, W. R. Nes, J. Chromatogr. Sci., 20: 193-202 (1982).

18 B. Neidhart, K. P. Kringe, W. Brockmann, J. Liq. Chromatogr., 4: 1875-1886 (1981).

19 Kazutake Shimada, Michiru Nonaka, J. Liq. Chromatogr., 14: 2109-2117 (1991).

20 M. Olsson, L. C. Sander, S. A. Wise, J. Chromatogr., 537: 73-83 (1991).

21 G. Rum, W. C. Herndon, J. Am. Chem. Soc., 113: 9055-9060 (1991).

22 S. Ehrenson, J. Org. Chem., 44: 1793-1797 (1979).

23 N. Sadlej-Sosnowska, I. Śledzińska, J. Chromatogr., 595: 53-61 (1992).

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF MECLOFENAMATE SODIUM IN PLASMA

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ABSTRACT

A reversed phase high-performance liquid chromatographic method for the analysis of meclofenamate sodium in plasma was developed. Diclofenac was used as the internal standard. The chromatography was performed using a resolve C_{18} column; the mobile phase consisted of 60:40 % methanol to water and adjusted to pH 3.0 using acetic acid; a flow rate of 1.5 ml/min; and UV detection at 270 nm. Retention times were 3.6 and 5.9 min for diclofenac and meclofenamate, respectively. The mean (±SD) absolute and relative recovery of meclofenamate were found to be 96.49±0.59 and 100.48±0.73, respectively. The minimum detectable concentration of meclofenamate by this method was 150 ng/ml sample. The sensitivity obtained should enable the use of the method in future bioequivalency and/or pharmacokinetic studies.

INTRODUCTION

Meclofenamate sodium is a nonsteroidal anti-inflammatory drug (NSAID) administered orally and is used for the treatment of acute and chronic rheumatoid arthritis and osteoarthritis (1-5). The recommended adult dose is 200 to 400 mg/day in 3 to 4 divided doses. The drug is also used for monorrhagia in doses of 100 mg three times daily (6).

A number of analytical procedures were developed for the determination of meclofenamate sodium. These include a fluorometric assay (7), gas-liquid chromatography and a thin layer chromatography (8). Recently an HPLC method was developed for meclofenamate sodium in both urine and plasma (9). The GLC method requires highly sophisticated equipment and is not technically feasible for routine use in bioequivalency study. The HPLC method previously reported is time consuming.

This study describes a simple, sensitive, and reproducible high-performance liquid chromatographic method for the quantitative determination of meclofenamate sodium in plasma using diclofenac sodium as internal standard. A single extraction step is followed by reversed-phase chromatography, eliminating the tedious and time-consuming procedures required by the previously reported method (9). Meclofenamate sodium

and diclofenac sodium can be internal standards for each other during either assay.

MATERIALS

Meclofenamate sodium and the internal standard diclofenac (Sigma Chem. Co., St. Louis, MO, U.S.A.), glacial acetic acid and hydrochloric acid (BDH Chemicals Ltd., Poole, U.K.) were analytical grade and used without further purification. Methanol and chloroform (BDH Chemicals Ltd., Poole, U.K.) were HPLC grade.

METHODS

Instruments

The following instruments were utilized:
A Model LC-10AD solvent delivery pump, a Model SPD-10AV UV-Vis detector, a Model CTO-10A column oven, and a Model C-R4A chromatopac computing integrator (Shimadzu Corporation, Koyato, Japan), a Model 7010 Rheodyne injector (Rheodyne Inc., Catati, CA, U.S.A.), stainless steel column (Resolve C_{18}, 150 mm length x 3.9 mm i.d., 5 μm particles, Waters Associates, Milford, MA, U.S.A.), and a Model CFC-301 Gallenkamp centrifuge (Gallenkamp, Louchborough, England).

Standard Stock Solution

An accurately weighed sample of 10 mg meclofenamate and 10 mg diclofenac were dissolved in methanol in two separate 100 ml volumetric flasks to give standard stock solution of 100 μg/ml.

Chromatographic Conditions

The mobile phase was a mixture of 60% methanol and 40% HPLC water adjusted to pH 3.0 by using acetic acid. It was degassed daily by passing it through 0.45 μm membrane filter (Millipore, Bedford, MA, U.S.A.).

The mobile phase was pumped at flow rate of 1.5 ml/min, which produced a backup pressure of about 219 kgf/Cm^2. The column oven temperature was adjusted at 35°C. The effluent was monitored at 270 nm and attenuation at 0.002 AUFS. The chart speed was 1.5 mm/min.

Analytical Procedure

Drug-free blank plasma (0.5 ml) were spiked with known amounts of meclofenamate in 15 ml glass stoppered test tube. To each tube 7.5 ml chloroform, 0.2 ml 6N HCl and 50 μl of diclofenac (I.S.) were added. The samples were shaken for 2 minutes and the chloroform layer, containing the drug, was transferred to 10 ml quick fit test tube and evaporated to dryness under vacuum at 45°C. The residue was dissolved in 0.5 ml of

the mobile phase, vortexed for one minute and then an aliquot of 20 µl was directly injected into the loop.

RESULTS AND DISCUSSION

The mobile phase used for the assay provided good separation of meclofenamate and diclofenac (I.S.) and sharp peaks with no interference from plasma substances. Figure 1 represents a typical chromatogram of: blank plasma (A), plasma containing the drug (B), and plasma containing meclofenamate and the internal standard (C). Using the developed assay procedure the retention time for the internal standard and meclofenamate were 3.6 and 5.9 minutes, respectively. Heating the column to 35°C gave sharp peaks.

Quantification

Peak-height ratios of the drug to the internal standard were used in constructing four different standard calibration lines in plasma and mobile phase by spiking 0.5 ml drug-free plasma and 0.5 ml mobile phase samples with the drug standard to produce a final concentration of 0.5, 1.0, 2.0, 4.0 and 10.0 µg/ml meclofenamate. The standard curves were prepared in a period of three weeks. Least squares linear regression analysis of the mean standard calibration plots for mobile

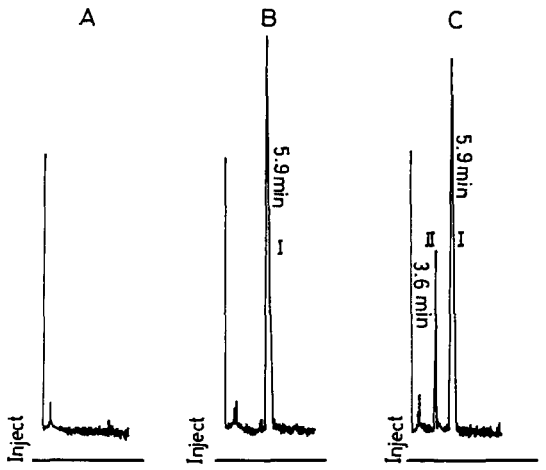

FIGURE 1: Chromatograms of blank plasma (A), plasma containing meclofenamate (B), and plasma containing meclofenamate and internal standard (C).

Key: I; Meclofenamate, II; Internal standard

phase and plasma samples resulted in the following equations:

$$Y = 0.234 x - 0.01525, r = 0.999 \text{ (Plasma)}$$

and $$Y = 0.260 x + 0.002, r = 0.999 \text{ (Mobile phase)}$$

Analysis of variance of the slopes, intercepts and correlation coefficients of the four standard plots from plasma indicated non-significant difference ($F=2.83$, $P>0.05$). These results confirm the linearity of the standard curves and the excellent reproducibility of the assay method.

Recovery

The absolute recovery of meclofenamate and the internal standard (diclofenac) were determined by comparing the peak-height of the drug obtained from plasma with the peak-height obtained by the direct injection of pure aqueous drug standard at three different concentrations (0.75, 3.0 & 8.0 μg/ml). The relative recovery of the drug was calculated by comparing the concentration obtained from the drug-supplemented plasma to the actual added concentrations. The results of the recovery studies are shown in Table 1. The average absolute and relative recovery of meclofenamate were found to be 96.49+0.59 and 100.48+0.73, respectively.

Precision

The intraday precision was evaluated by replicate analysis of plasma samples containing meclofenamate at three different concentrations (low, medium and high). The intraday precision showed coefficient of variations (CV) of 2.00 to 3.95 (Table 2). The interday precision was similarly evaluated over 3-weeks period. The interday CVs ranged from 1.65 to 2.97 (Table 2).

Sensitivity

The limit of quantification for this method was 150 ng/ml plasma. If additional sensitivity is needed,

Table 1 : Absolute and Relative Recovery of Meclofenamate from Plasma *.

Conc. (µg/ml)	Mean Peak Heights (cm) Aqueous	Plasma	Absolute Recovery %	Relative Recovery % Mean ± SD
0.75	1.05 ± 0.08	1.02 ± 0.07	97.14	99.67 ± 1.85
3	3.83 ± 0.07	3.69 ± 0.04	96.34	100.71 ± 1.03
8	9.99 ± 0.49	9.59 ± 0.37	95.99	101.07 ± 2.41
Diclofenac (I.S.) 1	4.63 ± 0.08	4.60 ± 0.1	99.35	

* Eight replicate analyses of each concentration.

injection volume could be increased or reconstitution in small volume of the mobile phase could be done.

Application

A typical mean plasma concentration-time profile for meclofenamate in beagle dogs (n=5) following intravenous administration of 40 mg dose is shown in

Table 2 : Intraday and Interday Precision of Meclofenamate in Plasma.

Intraday*			Interday**		
Added Conc. (μg/ml)	Measured Conc. (μg/ml)	Bias %	Added Conc. (μg/ml)	Measured Conc. (μg/ml)	Bias***
0.75			0.75		
Mean	0.76	1.33	Mean	0.74	-1.33
S.D.	0.03		S.D.	0.02	
CV %	3.95		CV%	2.7	
3			100		
Mean	3	0	Mean	3.03	1
S.D.	0.06		S.D.	0.05	
CV%	2		CV%	1.65	
8			8		
Mean	7.98	-0.25	Mean	8.09	1.13
S.D.	0.22		S.D.	0.24	
CV%	2.76		CV%	2.97	

* Mean values represent eight different plasma samples for each concentration.

** Interday precision was determined from 23 different runs over 3-weeks period at the three concentrations.

*** Bias = 100x (measured concentration-added concentration) / added concentration.

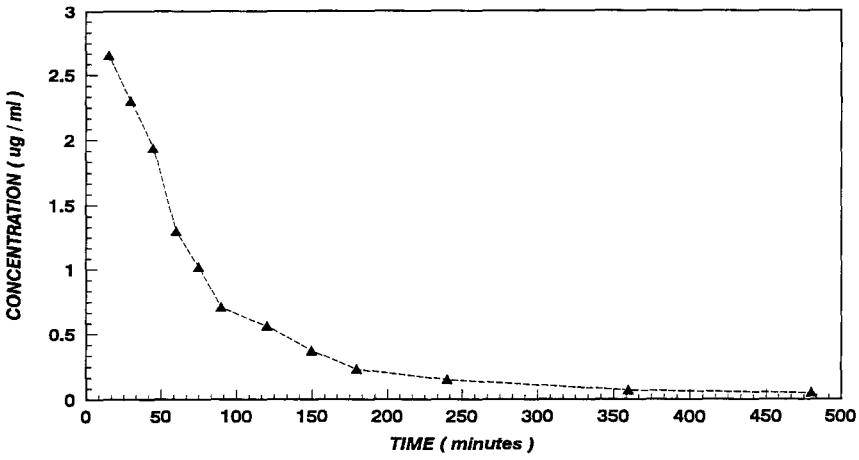

FIGURE 2: Average plasma concentration versus time profile for meclofenamate in five dogs after intravenous administration of 40 mg dose.

Fig. 2. The method was sufficiently sensitive to enable meclofenamate quantification from plasma upto 8 hours post dose.

Conclusion

The HPLC method developed in this study has the sensitivity, simplicity, reproducibility and rapidity which makes it versatile and valuable in many applications, specifically in drug level monitoring, drug-drug interactions, pharmacokinetic and bioequivalence studies.

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REFERENCES

1. R.F. Willkens, Curr. Ther. Res., 23 (Suppl): 81-89 (1978).

2. R.Eberl, Curr. Ther. Res., 23 (Suppl.): 131-137 (1978a)

3. J.R. Ward, J.A. Bolzan, C.L. Brame, Curr. Ther. Res., 23 (Suppl.): S60 (1978).

4. J.Zuckner, A. Baldssare, G. Harris, Curr. Ther. Res., 23 (Suppl.): S66-S71 (1978).

5. R.F. Willkens, P. Vreed, Pharmatherapeutica, 2: 587-596 (1981).

6. J.M. Vargyas, J.D. Campeau, D.R. Mishell, Am. J. Obstet. Gynecol., 157: 944-950 (1987).

7. A.J. Glazko, Ann. Phys. Med. Suppl., 9: 24-36 (1967).

8. A.J. Glazko, T. Chang, P.E. Borondy, W.A. Dill, R. Young, L. Croskey, 23 (Suppl.): S22-S41 (1978).

9. J.R. Koup, E. Tucker, D.J. Thomas, A.W. Kinkel, A.J. Sedman, R.Dyer, M. Sharoky, Biopharm. Drug Disposi., 11: 1-15 (1990).

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EFFECT OF ELUENT pH ON THE IONIC AND MOLECULAR FORMS OF THE NON-STEROIDAL ANTI-INFLAMMATORY AGENTS IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

High-performance liquid chromatographic conditions for the best separation of some non-steroidal anti-inflammatory agents were described. The dependence of eluent pH on the ionic (protonated) and molecular (non-protonated) forms of analysed compounds have been investigated. This paper is the study of the retention behavior of some anti-inflammatory agents depending of the eluent pH.

Some derivatives of phenol (acetaminophen, aspirin, salicylamide, phenacetin and salicylic acid), pyrazolidinedione (sulfinpyrazone, oxyphenbutazone, phenylbutazone and ketazone),

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amino-2-nicotinic acid (nixylic and niflumic acid) and amino-2-benzoic acid (mefenamic and flufenamic acid) were analysed with the isocratic RP-HPLC separation.

The different variations of mobile phase methanol-water v/v, containing 1% acetic acid were performed in order to obtain the best conditions of the separation. For all analysed substances the pH, pKa and the fitted pKa values were calculated in different mixtures of methanol-water using the graphical method given by G.Charlot and B.Trémillon. The capacity factors (k') and the separation factors (α) were calculated for all of them.

INTRODUCTION

In this paper are described the compounds from the non-steroidal anti-inflammatory drugs (NSAIDs), agents which possess analgesic and anti-inflammatory properties and often antipyretic properties. They are used in the treatment of rheumatic deseases.

Lechat (1) classified analgesic-antipyretic agents in two groups, which possesses anti-inflammatory properties and which does not. Analgesic-antipyretics which possess the anti-inflammatory properties are:

- phenolic derivatives
- pyrazolidinedione derivatives
- propionic acid derivatives
- amino-2-nicotinic acid derivatives
- amino-2-benzoic acid derivatives

The numbers of paper describe the high-performance liquid chromatographic method for assaing the mentioned non-steroidal anti-inflammatory agents. Some phenolic derivatives (acetaminophen, aspirin, salicylamide, phenacetin and salicylic acid) were determined in different pharmaceutical preparations

(2, 3), in multicomponent analgesic tablets (4, 5), in elixir formulations (6) and in cough mixtures (7). A group of non-steroidal anti-inflammatory agents were analysed by thin-layer chromatography (8). Aspirin in analgesic tablets was determined by high-performance reversed-phase TLC (9) and using second derivative UV-spectrophotometry (10). The simultaneous spectrophotometric assay of the active consituents of phenolic derivatives in multicomponent analgesics, using Kalmanfiltering, (11) have been developed. The mixtures of certain phenolic and pyrazolidinedione derivatives were analysed by spectrophotometric method using the different coloured or complexometric reactions (12, 13).

Pyrazolidinedione derivatives were separated and determined in bulk drug by HPLC and potentiometry (14). Stability control and determination of degradation products were performed in injectable formulations (15) by HPLC. The mentioned substances were determined in pharmaceutical preparations using the caracteristic reactions of functional groups by spectrophotometric method (16-18). Coulometric titration, with potentiometric determination of the end point, was used for the determination of pyrazolidinedione derivatives (19). Oxyphenbutazone was determined in tablets and ophtalmic ointments using the biamperometric titration (20). Phenylbutazone in tablets was determined by nuclear magnetic resonance (21).

The purity assay of amino-2-nicotinic acid derivatives have been devolped using HPLC method (22).

Mefenamic acid from the amino-2-benzoic acid derivatives was analysed in different pharmaceutical mixtures by means of semimicro liguid chromatography (23), ion-pair partition chromatography (24) and spectrophotometry (25, 26). Colorimetric

determination of two fenamates in antirheumatic drugs have been developed (27-30).

EXPERIMENTAL

Chemicals

All chemicals and reagents used were of an analytical reagent grade. Methanol "Normapur" Prolabo was used as eluent. Acetic acid "Normapur" Prolabo was added to eluent. The solvents were degased and after mixing filtered through a millipore DA 0.65μm filter. Double distilled water was used. All investigated pharmaceutical substances were obtained from commercial sources. Their identity was checked by IR and NMR spectra and their purity by TLC.

Apparatus

The chromatographic system consisted of a Shimadzu LC-6A pump, a Shimadzu SPD-6A detector and Shimadzu C-R3A data handling device. Separations were performed on Lichrosorb RP 18 column 250 x 4.6mm, with particles of 7μm sizes (Hibar, Merck). Samples were introduced through a Rheodyne injector valve with a 10μl sample loop. A mixture of methanol-water containing 1% acetic acid was used as a mobile phase with fixed flow rate 1.0ml/min, at room temperature and with a detection wavelength of 230 and 254nm.

Chromatographic conditions

The standard substances were dissolved in such a way that 1ml of the mobile phase contained 0.05mg of analysed substance.

Eleven different mixtures of mobile phase methanol/water v/v such as: 30/70; 35/65; 40/60; 45/55; 50/50; 55/45; 60/40; 65/35; 70/30; 75/25 and 80/20 containing 1% acetic acid were prepared.

The methanol-water levels containing 1% acetic acid were changed to obtain the acceptable separations.

An isocratic elution was made at room temperature with a flow rate 1ml/min and 10μl portions of the preparations were injected. The detector wavelength was usualy set up at 230nm. Pyrazolidinedione derivatives were detected at 254nm.

RESULTS AND DISCUSSION

The analysed non-steroidal anti-inflammatory agents ionize and can exist in a mobile phase in molecular and ionic forms. The increase in the ionization of the component causes an increase in dissolution in the water phase and reduces the retention time. In that case the ionic form of the analysed substance passes through the column without retaining. For that reason, the chromatogram may show that the peak has been coused to split.

High-performance liquid chromatography analysis of hidrosoluble and ionizing substances requires the ionization of molecules to be avoided. Only in that case the RP-HPLC method can be used in qualitative and quantitative analyses.

In our experiments of HPLC analysis the anti-inflammatory agents which belongs to acidic substances, such as phenolic, pyrazolidinedione, amino-2-nicotinic acid and amino-2-benzoic acid derivatives, have been investigated There is no acidic functional group in molecul of phenacetin, but it was analysed in combination with substances from phenolic derivatives because of the structural similarity with aminophenazone. The ionization has been avoided by the addition of acetic acid. An acetic acid belongs to the HA/A^- type of acid/base pair.

Eleven mixtures of methanol and water in various ratios containing 1% acetic acid were analysed. The Charlot-Trémillon graphical method (31) was used to determine the pKa values of acetic acid in various proportions of methanol and water, which are

TABLE I. pH mobile phase

MOBILE PHASE * (v/v)	pKa (corrected)	Δ pKa ($pKa_{cor.}$ - pKa)	pH
30/70	5.20	0.45	2.98
35/65	5.30	0.55	3.02
40/60	5.40	0.65	3.08
45/55	5.50	0.75	3.12
50/50	5.55	0.80	3.15
55/45	5.65	0.90	3.20
60/40	5.85	1.10	3.30
65/35	6.00	1.25	3.37
70/30	6.25	1.50	3.50
75/25	6.60	1.85	3.68
80/20	6.80	2.05	3.78

* mobile phase containing 1% acetic acid

$pKa_{acetic\ acid}$ = 4.75 (in water)

necessary for the calculation of pH eluants (Figure 1 and Table I). This graphical method gives the relation of pKa in the function of $1/\varepsilon$ (ε - dielectric constant of mobile phase). The values of pKa of acetic acid in pure methanol and water are 9.7 and 4.75 respectively. That makes possible the determination of the pKa of acetic acid in the mobile phases (pKa corrected). For example, for the mobile phase 60/40 v/v, pKa corrected for acetic acid is 5.85 (ΔpKa = 5.85 - 4.75 = 1.10). For all variations of mobile phases, it have been calculated the ΔpKa values and those value factors were used for the calculations of pH (Table I) from the equation: pH = 1/2 pKa - 1/2 log c.

For all the analysed substances, the fitted pKa (pKa corrected) in mobile phases were calculated respectively. For

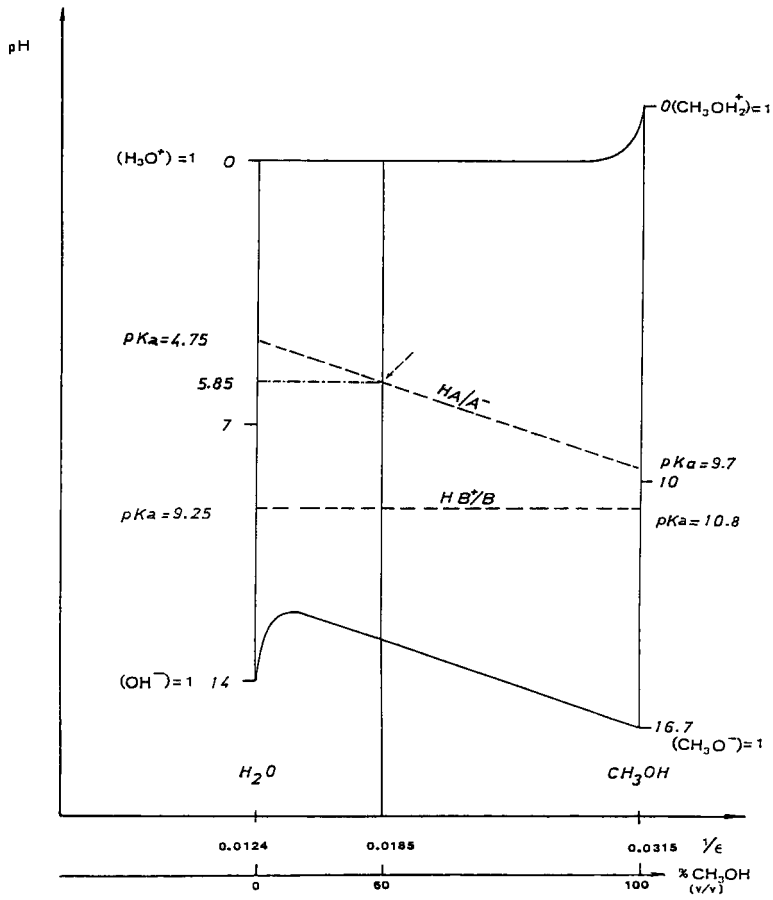

Figure 1. Graphical method Charlot-Trémillon

TABLE II. Values of pKa in mobile phase

	pKa (in water)	30/70	35/65	40/60	45/55	50/50	55/45	60/40	65/35	70/30	75/25	80/20
		pKa (corrected)										
A	3.5	3.95	4.05	4.15	4.25	4.30	4.40	4.60	4.75	5.00	5.35	5.55
B	3.0	3.45	3.55	3.65	3.75	3.80	3.90	4.10	4.25	4.50	4.85	5.05
C	8.2	8.65	8.75	8.85	8.95	9.00	9.10	9.30	9.45	9.70	10.05	10.25
D	9.5	9.95	10.05	10.15	10.25	10.30	10.40	10.60	10.75	11.00	11.35	11.55
E	3.7	4.15	4.25	4.35	4.45	4.50	4.60	4.80	4.95	5.20	5.55	5.75
F	4.7	5.15	5.25	5.35	5.45	5.50	5.60	5.80	5.95	6.20	6.55	6.75
G	2.8	3.25	3.35	3.45	3.55	3.60	3.70	3.90	4.05	4.30	4.65	4.85
H	4.4	4.85	4.95	5.05	5.15	5.20	5.30	5.50	5.65	5.90	6.25	6.45
I	5.0	5.45	5.55	5.65	5.75	5.80	5.90	6.10	6.25	6.50	6.85	7.05
J	5.0	5.45	5.55	5.65	5.75	5.80	5.90	6.10	6.25	6.50	6.85	7.05
K	3.9	4.35	4.45	4.55	4.65	4.70	4.80	5.00	5.15	5.40	5.75	5.95
L	4.2	4.65	4.75	4.85	4.95	5.00	5.10	5.30	5.45	5.70	6.05	6.25

A - Acetylsalicylic acid
B - Salicylic acid
C - Salicylamide
D - Acetaminophen
E - Ketazone
F - Oxyphenbutazone
G - Sulfinpyrazone
H - Phenylbutazone
I - Niflumic acid
J - Nixylic acid
K - Flufenamic acid
L - Mefenamic acid

Table III. Eluent pH dependence on molecular form percent

	MOBILE PHASE (METHANOL/WATER)										
%	30/70	35/65	40/60	45/55	50/50	55/45	60/40	65/35	70/30	75/25	80/20
pH	2.98	3.02	3.08	3.12	3.15	3.20	3.30	3.37	3.50	3.68	3.78
SUBSTANCE	MOLECULAR FORM IN PERCENT										
A	90	91	92	93	93	94	95	96	97	98	98
B	74	77	79	81	82	83	86	88	91	94	95
C	100	100	100	100	100	100	100	100	100	100	100
D	100	100	100	100	100	100	100	100	100	100	100
E	94	94	95	95	96	96	97	97	98	98	99
F	99	99	100	100	100	100	100	100	100	100	100
G	65	68	70	72	75	77	80	82	86	91	92
H	99	99	100	100	100	100	100	100	100	100	100
I	100	100	100	100	100	100	100	100	100	100	100
J	100	100	100	100	100	100	100	100	100	100	100
K	96	96	97	97	98	98	99	99	100	100	100
L	98	98	98	98	99	99	99	99	99	100	100

example: pKa for acetylsalicylic acid in water is 3.5; in the mobile phase 60/40 pKa fitted is 3.5 + 1.1 = 4.6 (Table II). Acetaminophen belongs to HB^+ type of acids and it does not necessery to correct the pKa value.

Using the values of pKa fitted and the pH calculated, it was possible to calculate the ratio of the molecular (non-protonated) and ionic (protonated) percent of the substances (Table III) from the equations:

$$\% \text{ ionization} = \frac{100}{1 + 10^{(pK_a - pH)}} \qquad \text{for HA acids}$$

$$\% \text{ ionization} = \frac{100}{1 + 10^{(pH - pK_a)}} \qquad \text{for } HB^+ \text{ acids}$$

TABLE IV. HPLC separation factors

SAMPLE	λ (nm)	$E^{1\%}_{1cm}$	k'	α
Acetaminophen	230	486	1.65	1.94
Salicylamide	230	464	3.20	1.28
Acetylsalicylic acid	230	474	4.09	1.45
Phenacetin	230	473	5.94	1.12
Salicylic acid	230	486	6.63	
Ketazone	254	472	3.17	1.51
Oxyphenbutazone	254	492	4.79	1.66
Sulfinpyrazone	254	467	7.94	1.83
Phenylbutazone	254	471	14.57	
Niflumic acid	230	485	4.30	2.09
Nixylic acid	230	491	9.00	
Flufenamic acid	230	486	7.48	1.12
Mefenamic acid	230	488	8.36	

k' - capacity factor
α - separation factor
mobile phase: methanol/water v/v containing 1% acetic acid
flow rate: 1ml/min

Table IV presents the values of the capacity factor k' and separation factor α for all the substances in the mobile phase which consists methanol-water in various proportions containing 1% acetic acid. For all of the applied mixtures of methanol-water in the mobile phase, salicylamide, acetaminophen, oxyphenbutazone, phenylbutazone, niflumic acid, nixylic acid and mefenamic acid, which pKa > 4.0, were in 98-100% molecular form. These experimental conditions enable a quantitative HPLC separation.

Acetylsalicylic acid, ketazone and flufenamic acid, which 3.5 < pKa < 4.0, exist in the applied mobile phase in 90-100% molecular form.

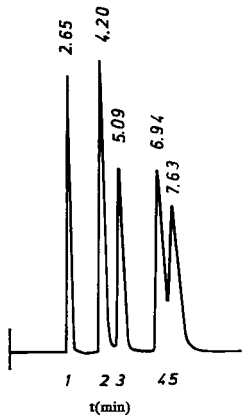

Figure 2. Chrtomatogram of Acetaminophen (1), Salicylamide (2), Acetylsalicylic acid (3), Phenacetin (4) and Salicylic acid (5)
mobile phase: methanol/water 45/55 v/v containing 1% acetic acid

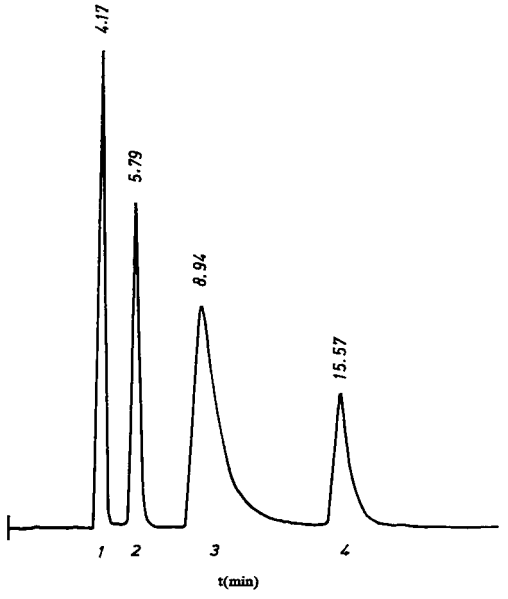

Figure 3. Chrtomatogram of Ketazone (1), Oxyphenbutazone (2), Sulfinpyrazone (3) and Phenylbutazone (4)
mobile phase: methanol/water 50/50 v/v containing 1% acetic acid

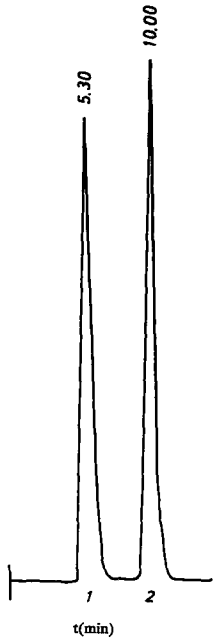

Figure 4. Chrtomatogram of Niflumic acid (1) and Nixylic acid (2) mobile phase: methanol/water 70/30 v/v containing 1% acetic acid

For the salicylic acid and sulfinpyrazone, which pKa < 3.5, it is necessery to increase the acetic acid percent (> 1%) to get the acceptable percent of molecular form.

The capacity factors, k' were calculated using the equation: $k' = (t_R - t_o) / t_o$, where t_R is the retention time of the analyte and t_o is the retention time of the non-retained peak (taken as a first deviation of the baseline following the injection of 100 μl of methanol).

Separation factors, α were calculated using the equation: $\alpha = k'_2 / k'_1$.

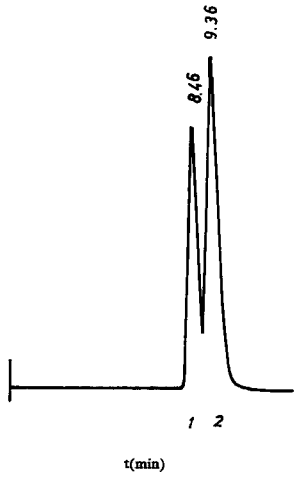

Figure 5. Chromatogram of Flufenamic acid (1) and Mefenamic acid (2)
mobile phase: methanol/water 75/25 v/v containing 1% acetic acid

Representative chromatograms of a mixed standard solution are shown in Fig. 2-5. UV detections have been done at 230 and 254nm. The eluent consists of methanol-water in various proportions containing 1% acetic acid with a flow rate 1ml/min at room temperature.

REFERENCES

1. P.Lechat, *Abrégé de Pharmacologie Médical*, Masson, Paris, 226, (1981).
2. Y.Matsushima, Y.Nagata, K.Takakusagi, M.Niyomura, *J.Chromatogr.*, 332, 265, (1985).
3. V.D.Gupta, A.R.Heble, *J.Pharm.Sci.*, 73, 1553, (1984).
4. D.J.Krieger, *J.Assoc.Off.Anal.Chem.*, 67, 339 (1984).

5. A.Verstraeten, E.Roets, J.Hoogmartens,
J.Chromatogr., 388, 201, (1987).
6. W.R.Sisco, C.T.Rittenhouse, L.A.Everhart, A.M.McLaghlin,
J.Chromatogr., 354, 355, (1986).
7. L.Carnevale, *J.Pharm.Sci.*, 72, 196, (1983).
8. P.R.Kamath, N.M.Sanghavi, *Indian Drugs*, 22(6), 336, (1985).
9. J.Sherma, S.Stellmacher, T.J.White,
J.Liq.Chromatogr., 8(16), 2961, (1985).
10. K.Kitamura, M.Mabuchi, T.Fukui, K.Hozumi,
Chem.Pharm.Bull., 35(9), 3914, (1987).
11. A.Van Loosbroek, H.J.G.Debets, P.M.J.Coenegracht,
Anal.Lett., 17(B9), 779, (1984).
12. A.A.El Kheir, S.Belal, M.El Sadek, A.El Shanwani,
Analyst, 111(3), 319, (1986).
13. A.A.El Kheir, S.Belal, A.El Shanwani,
Pharmazie, 40(1), 62, (1985).
14. C.Herrenknecht, D.Ivanović, E.Guernet-Nivaud, M.Guernet,
J. Pharm. Biomed. Anal., 8, 1071, (1990).
15. H.Fabre, N.Hussam-Eddine, B.Mandrou,
J.Pharm.Sci., 73(12), 1708, (1984).
16. E.M.El-Kommos, *Analyst*, 108(1290), 1144, (1983).
17. S.Belal, A.A.El Kheir, A.Afaf, A.El Shanwani,
Analyst, 110(2), 205, (1985).
18. A.S.Issa, Y.A.Beltagi, M.G.Kassem, H.S.Daabees,
Talanta, 32(8A), 657, (1985).
19. C.Herrenknecht, E.Guernet-Nivaud, M.Guernet, C.Guetin,
Ann.Pharm. Fr., 43, 557, (1985).
20. D.Sebkova, L.Safarik, *J.Assoc.Off.Anal.Chem.*,
69(2), 191, (1986).
21. R.Stromberg, *J.Pharm.Sci.*, 73(11), 1653, (1984).

22. C.Guechot, P.Nicolle, *J.Chromatogr.*, 303, 440, (1984).

23. Y.Matsushima, Y.Nagata, M.Niyomura, K.Takakusagi, *J.Chromatogr.*, 332, 269, (1985).

24. W.M.Adams, *J.Assoc.Off.Anal.Chem.*, 66(5), 1178, (1983).

25. C.S.P.Sastry, D.S.Mangala, K.Ekambareswara, *Indian J.Pharm.Sci.*, 47(3), 123, (1985).

26. S.Zommer-Urbanska, H.Bajorowicz, *J.Pharm.Biomed.Anal.*, 4(4), 475, (1986).

27. M.S.Mahrous, M.M.Abdel-Khalek, M.E.Abdel-Hamid, *Talanta*, 32(8A), 651, (1985).

28. S.T.Hassib, H.M.Safwat, R.I.El-Bagry, *Analyst*, 111(1), 45, (1986).

29. S.M.Hasan, S.A.M.Shaaban, *Pharmazie*, 39(10), 691, (1984).

30. A.S.Issa, Y.A.Beltagi, M.G.Kassem, H.S.Daabees, *Talanta*, 32(3), 209, (1985).

31. Charlot, G., Trémillon, B., Les réactions chimiques dans les solvants et les sels fondus, Masson, Paris, 78, (1963).

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SEPARATION OF C_{60} AND C_{70} FULLERENES WITH A TRIPHENYL BONDED SILICA PHASE IN MICROCOLUMN LIQUID CHROMATOGRAPHY

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ABSTRACT

C_{60} and C_{70} fullerenes were chromatographically separated with triphenyl, diphenyl and monophenyl bonded silica phases in microcolumn liquid chromatography. The results indicate that the triphenyl bonded phase having the narrowest pore size possesses the best separation performance among the evaluated phases. The retention power of the triphenyl bonded phase for C_{60} and C_{70} was much greater than that of typical octadecylsilica (ODS) phases, although the separation factor between C_{60} and C_{70} was almost comparable to that of ODS phases. With the triphenyl bonded phase, a smaller temperature dependence than ODS phases was observed for the separation of C_{60} and C_{70}.

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INTRODUCTION

Retention behaviours of fullerenes in high performance liquid chromatography (HPLC) have been studied concentrically in recent years [1-6], because the separation and purification of fullerenes are inevitable procedures to characterize those interesting compounds to use in various wide fields. Hawkins *et al.* [1,2] reported the liquid chromatographic separation of C_{60} and C_{70} on a phenylglycine-derivartized bonded phase ("Pirkle Column"), and Cox *et al.* [3] used a dinitroanilinopropylsilica stationary phase to separate them. Pirkle *et al.* [4] have synthesized a new stationary phase, the so-called "buckyclutcher", and obtained an excellent separation of C_{60} and C_{70}. For this separation, pyrenylethyl [5] and nitro-derivartized phenylethyl [5,6] bonded phases have been introduced by Kimata and Tanaka *et al.* All of the phases described above include phenyl ring(s) in their bonded phase structures and some of them possesses nitro group(s) on the ring in order to enhance the interaction between bonded phase ligands and fullerene molecules.

In our previous investigations we studied the retention behaviour of fullerenes with various stationary phases such as commercially available monomeric and polymeric ODS phases [7-12], multi-legged phenyl bonded phases [12,13] and methoxyphenylpropyl bonded stationary phases [12,14]. This is because the fact that fullerene molecules can be considered to be very big polycyclic aromatic hydrocarbons (PAHs) with specific shape and size, and therefore, they are regarded as good sample probes to investigate the retention

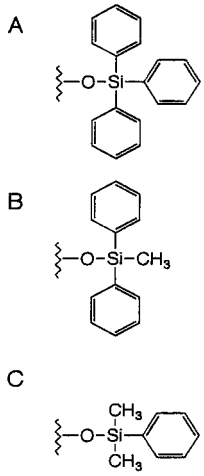

FIGURE 1 Chemical structures of triphenyl (Triph; A), diphenyl (Diph; B) and monophenyl (Monoph; C) bonded phases.

behaviour of PAHs in liquid chromatography. On the other hand, if the chromatographic separation mechanism of fullerenes with the stationary phases is elucidated, a new design of the stationary phase to effectively separate them can be developed by the concept based on the mechanism.

In this work, three types of stationary phases, triphenyl (Triph), diphenyl (Diph) and monophenyl (Monoph) bonded silica phases were evaluated as stationary phases to separate C_{60} and C_{70} fullerenes in microcolumn liquid chromatography, since microcolumns are ideal for use in these studies because of limited amounts of experimentally synthesized bonded phases.

EXPERIMENTAL

Bonded Phase Synthesis

The syntheses of these bonded phases were described previously [15]. The basic characteristics of these bonded phases are summarized in Table 1.

Chromatographic Measurements.

The bonded phases were packed using a slurry method into a fused-silica capillary (Tokyo Kasei, Tokyo, Japan) of 0.53 mmi.d. x 150

TABLE 1

Basic Characteristics of the Bonded Phases Investigated in This Study.

bonded phase	pore size (Å)	carbon content (%)	surface coverage (μmol/m^2)
Triph	70	11.2	1.14
Triph	150	4.79	1.19
Triph	300	1.64	0.94
Diph	70	13.7	2.20
Diph	150	6.11	2.16
Diph	300	2.57	2.23
Monoph	70	10.8	3.40
Monoph	150	5.35	3.81
Monoph	300	3.28	5.77

mm length. The microcolumn HPLC system consisted of a microfeeder MF-2 pump (Azuma Electric, Tokyo, Japan), a Rheodyne 7520 injector (Cotati, CA, USA) with a 0.2 μL injection volume and a Uvidec 100-III UV detector (Jasco, Tokyo, Japan) set at 320 nm. The mobile phase was guaranteed reagent grade n-hexane (Kishida Chemical, Osaka, Japan). The typical flow-rate was 2 μL/min., and a mixture of toluene and cyclohexane was used as the sample solvent. The chromatographic measurements were done at least three times. The column temperature was controlled using a modified Hewlett-Packard Model 5820-II Gas Chromatographic oven (Yokogawa Analytical Systems, Mitaka, Tokyo, Japan).

RESULTS and DISCUSSION

The Separation Performance of Triphenyl, Diphenyl and Monophenyl Bonded Stationary Phases.

Figs.2a, 2b and 2c show chromatograms of the separation of C_{60} and C_{70} on Triph, Diph and Monoph bonded phases prepared with different pore size silica gels. These retention data are also summarized in Table 2. With the phases having a 70 Å pore size, the Triph phase exhibited the best retention power and separation performance, although good separation was obtained with all phases, as shown in Fig.2a. In the cases of 150 Å (Fig.2b) and 300 Å (Fig.2c) pore size, the retentions of C_{60} and C_{70} are shorter than those of the 70 Å phases.

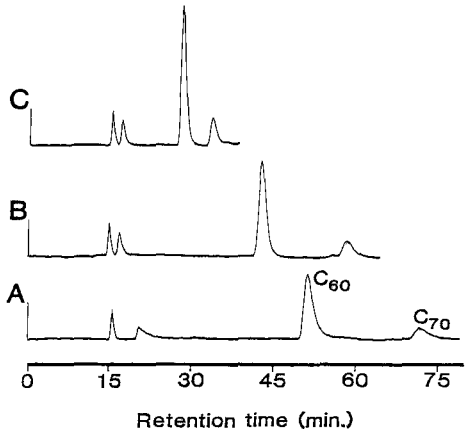

FIGURE 2a Chromatograms for the separation of C_{60} and C_{70} with Triph (A), Diph (B) and Monoph (C) bonded phases. Pore size, 70 Å; mobile phase, n-hexane; column temperature, 30 °C.

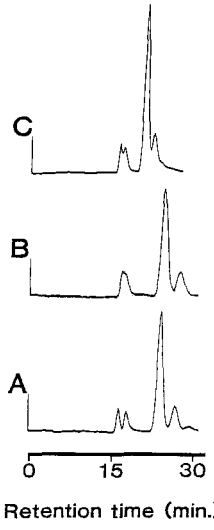

FIGURE 2b Chromatograms for the separation of C_{60} and C_{70} with Triph (A), Diph (B) and Monoph (C) bonded phases. Pore size, 150 Å; mobile phase, n-hexane; column temperature, 30 °C.

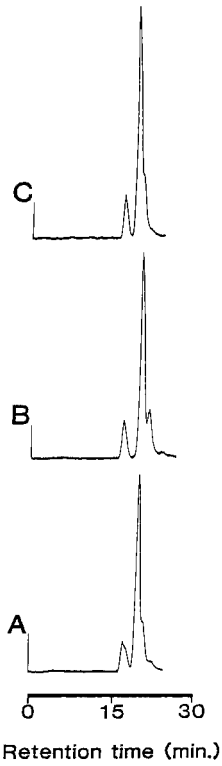

FIGURE 2c Chromatograms for the separation of C_{60} and C_{70} with Triph (A), Diph (B) and Monoph (C) bonded phases. Pore size, 300 Å; mobile phase, n-hexane; column temperature, 30 °C.

This can be attributed to the lower carbon loadings of these wider pore phases.

It can be said from the above results that the three phenyl rings of Triph bonded phases' ligands possess a strong retentivity based on the π-π interaction with fullerene molecules, because these bonded phases

TABLE 2

Retention Data for the Separation of C_{60} and C_{70} with Triphenyl (Triph), Diphenyl (Diph) and Monophenyl (Monoph) Bonded Phases. Mobile Phase, n-hexane; Column Temperature, 30°C.

bonded phase	pore size (Å)	capacity factor (k')		separation factor α (C_{70}/C_{60})
		C_{60}	C_{70}	
Triph	70	2.28	3.59	1.57
Diph	70	1.85	2.89	1.56
Monoph	70	0.863	1.24	1.44
Triph	150	0.454	0.633	1.39
Diph	150	0.452	0.639	1.41
Monoph	150	0.273	0.370	1.36
Triph	300	0.157	0.217	1.38
Diph	300	0.203	0.266	1.31
Monoph	300	0.152	0.194	1.28

indicate the best retention capability in spite of the lowest surface coverage value among the three phases having the same pore size. Furthermore, the chromatographic characteristics of these three phases (i.e. Triph, Diph and Monoph) are most evident in 70 Å silica gel phases.

Table 3 shows the retention data for C_{60} and C_{70} with various bonded stationary phases. In this table, the BP phase is a multi-legged

TABLE 3

Retention Data for C_{60} and C_{70} with Five Different Bonded Phases. Mobile Phase, n-hexane; Column Temperature, 30°C.

bonded phase	capacity factor (k')		separation factor α (C_{70}/C_{60})
	C_{60}	C_{70}	
Tiph 70 Å	2.28	3.59	1.57
BP	2.76	5.25	1.90
DMPP	3.09	5.77	1.87
Develosil ODS-5	0.87	1.60	1.84
Capcell Pak C18 SG120*	0.34	0.53	1.56

*Conventional monomeric ODS column with polymer coated silica (4.6 mmi.d. x 250 mm; Shiseido, Tokyo, Japan); flow rate, 1mL/min.

bonded silica phase [12,13] and DMPP bonded phase is a multi-methoxy phenyl stationary phase [12,14], and these two phases have been found to have an excellent separation performance for fullerene mixtures in our previous investigations. In addition, Develosil ODS-5 (Nomura Chemical, Seto, Japan) is the phase that exhibited the best separation factor of C_{60} and C_{70} among various commercially-available ODS phases in our previous evaluation [9].

Consequently, Table 3 demonstrates that the Triph 70 Å phase has a much higher retention power than commercially-available ODS phases, although the retentivity of the triphenyl phase is somewhat less than those of BP and DMPP bonded phases.

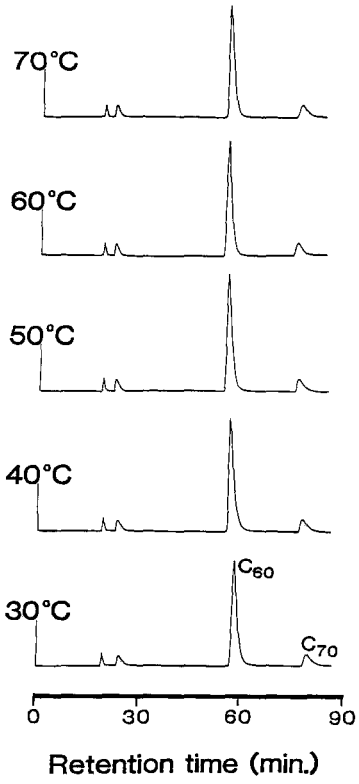

FIGURE 3 Chromatograms for the separation of C_{60} and C_{70} with the Triph 70Å phase at different column temperatures. Mobile phase, n-hexane.

Effect of Temperature on the Separation of C_{60} and C_{70} with the Triphenyl Bonded Phase.

Fig.3 depicts chromatograms for the separation of C_{60} and C_{70} with the Triph 70 Å bonded phase at different column temperatures. These chromatograms indicate the small temperature dependence of

TABLE 4

Capacity Factors of C_{60}, C_{70} and Five PAHs with Triph 70 Å Phase at Different Column Temperatures. Mobile Phase, n-hexane.

	column temperature (°C)				
	30	40	50	60	70
naphthalene	0.763	0.707	0.634	0.582	0.538
phenanthrene	1.52	1.38	1.20	1.07	0.971
o-terphenyl	2.32	2.07	1.77	1.57	1.40
pyrene	1.66	1.54	1.41	1.30	1.21
triphenylene	3.02	2.77	2.44	2.22	2.02
C_{60}	1.98	1.97	1.94	1.93	1.93
C_{70}	3.10	3.09	3.03	3.02	3.02

this separation with the triphenyl phase. For comparison, retention data for five PAHs were also measured and summarized in Table 4. Because the capacity factors of PAHs decrease with increasing the column temperature, the slight variation in the retention data of C60 and C70 may be specific for fullerenes.

Separation factors of C60 and C70 with various phases at different temperatures were tabulated in Table 5 and these data were also plotted in Fig.4 with some additional data. The separation factors with ODS phases, especially in the case of the polymeric phase, decrease significantly when the column temperature is elevated. However, as reported previously [12], the BP phase indicates a very small temperature dependence for the separation of C60 and C70, and

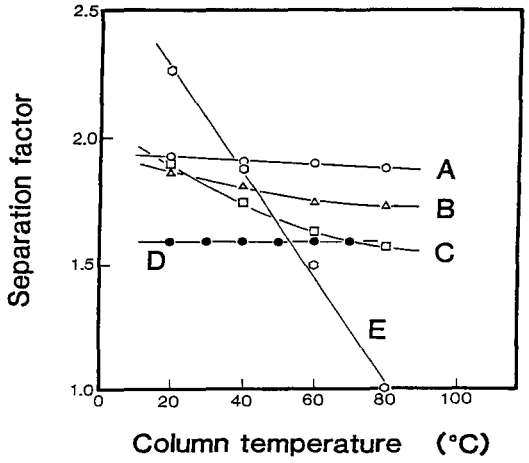

FIGURE 4 Relationships between separation factors for C60 and C70 with five different bonded phases versus column temperature. (A) BP, (B) DMPP, (C) monomeric ODS, (D) Triph 70 Å and (E) polymeric ODS. In this figure plots (A), (B), (C) and (E) were reported in our previous paper [12]. Mobile phase, n-hexane.

TABLE 5

Separation Factors of C_{60} and C_{70} with Five Bonded Phases at Different Column Temperatures. Mobile Phase, n-hexane.

	separation factor α (C_{70}/C_{60})		
bonded phase	20 ℃	40 ℃	60 ℃
Triph 70 Å	1.57	1.57	1.56
BP	1.92	1.90	1.89
DMPP	1.86	1.80	1.74
Develosil ODS-5	1.89	1.74	1.62
Wakosil-II 5C18AR*	2.26	1.87	1.49

*Conventional polymeric ODS column (4.6 mmi.d. x 250 mm; Wako Pure Chemical, Osaka, Japan).

the DMPP stationary phase also exhibits a relatively smaller variation than the ODS phases, because of conformational rigidity of the BP and DMPP phases.

Therefore, it can be assumed from the above results that the Triph 70 Å phase also possesses a conformational rigidity which is not witnessed using the ODS phases.

REFERENCES

1)J.M.Hawkins, T.A.Lewis, S.D.Loren, A.Meyer, J.R.Heath, Y.Shibato and R.J.Saykally., J.Org.Chem., 55, 6250(1990).

2)W.H.Pirkle, C.J.Welch, J.Org.Chem., 56, 6873(1991).

3)D.M.Cox, S.Behal, M.Disko, S.M.Gorun, M.Greaney, C.S.Hsu, E.B.Kollin, J.Millar, J.Robbins, W.Robbins, R.D.Sherwood and P.Tindall, J.Am.Chem.Soc., 113, 2940(1991).

4)C.J.Welch and W.H.Pirkle, J.Chromatogr., 609, 89(1992).

5)K.Kimata, K.Hosoya, T.Araki and N.Tanaka, J.Org.Chem., 58(1), 282(1993).

6)J.F.Anacleto and M.A.Quillian, Anal.Chem., 65, 2236(1993).

7)K.Jinno, T.Uemura, H.Nagashima and K.Itoh, J.High Resolut. Chromatogr., 15, 627(1992).

8)K.Jinno, H.Nagashima, K.Itoh, M.Saito and M.Bounoshita, F.J.Anal.Chem., 344, 435(1992).

9)K.Jinno, T.Uemura, H.Nagashima and K.Itoh, Chromatographia, 35, 38(1993).

10)K.Jinno, T.Uemura, H.Ohta, H.Nagashima and K.Itoh, Anal.Chem., 65, 2650(1993).

11)K.Jinno, T.Uemura, H.Ohta, H.Nagashima, K.Itoh and N.Nagae, Chimica Oggi, in press.

12)K.Jinno, H.Ohta, Y.Saito, T.Uemura, H.Nagashima, K.Itoh, Y.-L.Chen, G.Luehr, J.Archr, J.C.Fetzer and W.R.Biggs, J.Chromatogr., 648, 71(1993).

13)K.Jinno, K.Yamamoto, T.Ueda, H.Nagashima, K.Itoh, J.C.Fetzer and W.R.Biggs, J.Chromatogr., 594, 105(1992).

14)K.Jinno, Y.Saito, Y.-L.Chen, G.Luehr, J.Archer, J.C.Fetzer and W.R.Biggs, J.Microcol.Sep., 5, 135(1993).

15)K.Jinno and M.Okamoto, Chromatographia, 18, 495(1984).

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A SIMULTANEOUS ASSAY FOR ACADESINE (AICA-RIBOSIDE) AND ACADESINE 5′-MONOPHOSPHATE USING ION-PAIR REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A reverse phase (C_{18}) high performance liquid chromatographic method using an ion-pair reagent has been developed for the simultaneous quantification of the cardioprotective drug, acadesine (AICA-riboside), and a major nucleotide metabolite, acadesine 5′-monophosphate (ZMP), in perchloric acid (PCA) extracts of mouse heart tissue. The limit of quantification (LOQ) was 0.5 μM for both acadesine and its 5′-monophosphate.

INTRODUCTION

The cardioprotective properties of acadesine (AICA-riboside) (Figure 1) are currently being evaluated in Phase 3 clinical trials involving patients undergoing coronary artery bypass graft surgery (CABG) (1). Acadesine is a substrate for adenosine kinase and acadesine 5′-monophosphate (ZMP) is a major cellular metabolite (2). Separate HPLC assays for acadesine and its 5′-monophosphate have been developed (3). This paper describes a

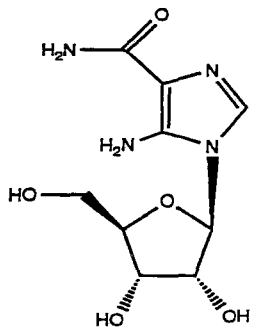

FIGURE 1. Structure of Acadesine (AICA-riboside)

HPLC assay for the simultaneous determination of both compounds in tissue extracts using ion-pair reverse phase chromatography.

MATERIALS

Tetrabutylammonium phosphate was purchased from Sigma Chemical (St. Louis, IL). Tetraethylammonium hydroxide and tetrapropylammonium hydroxide were purchased from Aldrich Chemical (Milwaukee, WI). The pH of the mobile phases was adjusted with phosphoric acid (Curtin Matheson Scientific, Houston, TX). All buffers were filtered through Millipore 0.45-μm HVAP-filters (Bedford, MA) and were degassed using a vacuum pump. Standards were prepared from acadesine (AICA-riboside) from Gensia (San Diego, CA) and reagent grade ZMP purchased from Sigma Chemical (St. Louis, IL) using Milli-Q (Millipore, Bedford, MA) deionized water. All other compounds were reagent grade.

METHODS

Thirty minutes after i.p. administration of 500 mg/kg acadesine to mice, the animals were killed, their hearts

removed, blotted dry of extraneous blood, and freeze-clamped using liquid nitrogen-cooled Wollenberger clamps. Each frozen heart was weighed and homogenized at 4° C in 3 ml ice-cold 0.6 N perchloric acid with a single 10 sec burst at setting 8 using a Brinkmann Model PT 10/35 Polytronic homogenizer. The homogenate was then centrifuged at 2000 x g for 10 min at 4° C and 1 ml of the supernatant was added to 75 μl of K_2CO_3. The neutralized sample (checked by full-range pH paper) was re-centrifuged and the supernatant passed through a 0.45 μm nylon filter (Western Analytical, Temecula, CA).

The extracts were analyzed using a Waters HPLC system consisting of a model 510 pump, satellite WISP auto injector Model 700 and an adjustable UV absorbance detector Model 484 set at 270 nm. The data were collected and analyzed using a Maxima 820 computer based software (Waters Millipore, Milford, MA). Samples were chromatographed on a Beckman Ultrasphere C-18, 4.6 x 150 mm, 5 μ column (Alltech Associates, Deerfield, IL) at a flow rate of 1.5 ml/min using an injection volume of 50 μl. The column was equilibrated for at least 30 minutes with mobile phase before sample analysis.

RESULTS & DISCUSSION

Three ion-pair reagents were tested to determine the optimal conditions for elution of acadesine and ZMP. Blank neutralized PCA extracts of mouse heart tissue and extracts spiked with both acadesine and ZMP were analyzed using each of the three ion pair buffers. The retention times of acadesine and ZMP using a mobile phase of 20 mM tetraethylammonium phosphate (pH 3.6) were 6.5 and 7.3 min, respectively. With a mobile phase consisting of 20 mM tetrabutylammonium phosphate pH 4.7, acadesine and ZMP had

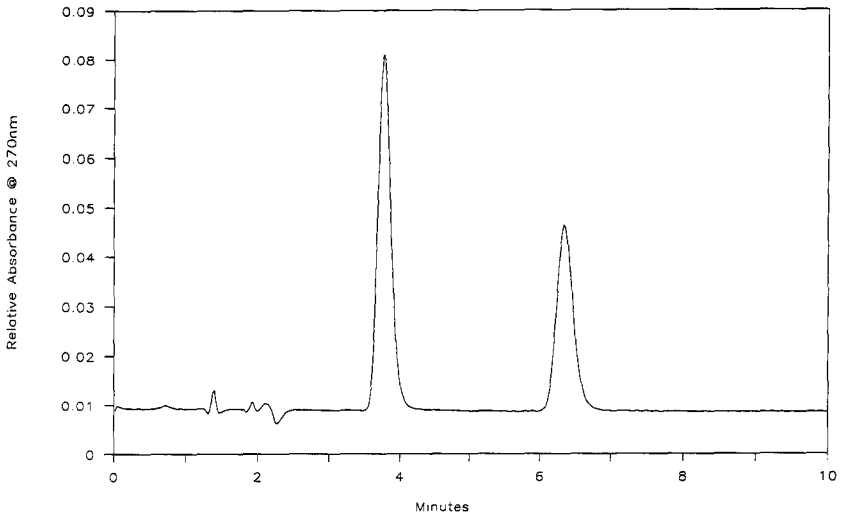

FIGURE 2. HPLC chromatogram of standard acadesine (retention time = 3.7 min) and standard ZMP (retention time = 6.3 min) using a 100 mM tetrapropylammonium phosphate pH 3.5 mobile phase

corresponding retention times of 3.4 and 26.1 min. The mobile phase selected consisted of 100 mM tetrapropylammonium phosphate pH 3.5 in which acadesine and ZMP were eluted in 3.7 and 6.3 min, respectively (Figure 2). The tetrapropylammonium phosphate buffer yielded the best results since the run time was 10 minutes with no endogenous peaks coeluting with either acadesine or ZMP. A typical chromatogram of acadesine and ZMP measured in heart tissue from a rat administered acadesine i.p. is shown in Figure 3.

Intra-assay precision (Table 1) for the analysis of ZMP from 0.5 to 100 μM had a mean %c.v.=1.71 (n=3) and the mean relative percent error was 3.1 (n=3). The inter-assay precision for ZMP

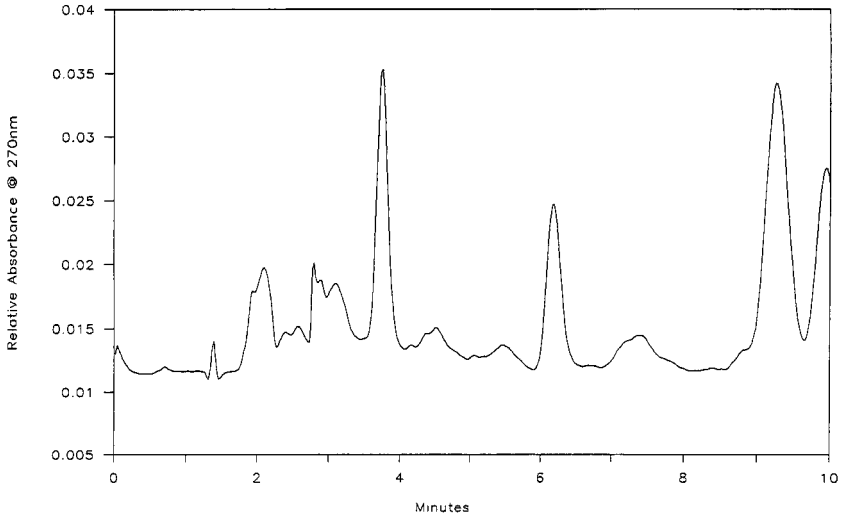

FIGURE 3. HPLC chromatogram of extract from heart of a mouse administered acadesine i.p. (acadesine retention time = 3.7 min; ZMP retention time = 6.3 min)

from 0.5 to 100 μM (Table 2) had a mean %c.v.=1.64 and the mean relative percent error was 2.7 (n=3).

Intra-assay precision (Table 1) for the analysis of acadesine from 0.5 to 100 μM had a mean %c.v.=3.27 and the mean relative percent error was 4.72 (n=3). The inter-assay precision (Table 2) had a mean %c.v.=2.47 and the mean relative percent error was 4.5 (n=3).

The limit of quantification (LOQ) for acadesine and ZMP was determined to be 0.5 μM.

The linearities of the concentration-time plots for both acadesine and ZMP were determined by variance stabilized transformation regression (4). The linearities of repeated runs of standards were found to be constant with a mean slope of 22308

TABLE 1

Intra-Assay Precision for the Analysis of Aqueous Acadesine and ZMP

Number of Runs	Amount Acadesine Added (μM)	Amount Acadesine Found (μM)	$\pm$s.d.	%c.v.	Relative Error %
3	0.5	0.51	0.04	8.55	+2.0
3	1	1.08	0.08	7.58	+8.0
3	5	5.20	0.11	2.12	+4.0
3	10	10.58	0.30	2.84	+5.8
3	25	24.12	0.03	0.13	-3.5
3	50	47.84	0.44	0.91	-4.3
3	100	94.62	0.70	0.74	-5.4
			mean =	3.27	4.72

Number of Runs	Amount ZMP Added (μM)	Amount ZMP Found (μM)	$\pm$s.d.	%c.v.	Relative Error %
3	0.5	0.49	0.01	2.34	-2.0
3	1	1.01	0.04	4.32	+1.0
3	5	5.19	0.10	1.93	+3.8
3	10	10.56	0.04	0.34	+5.6
3	25	23.97	0.22	0.90	-4.1
3	50	48.35	0.51	1.05	-3.3
3	100	98.07	1.04	1.06	-1.9
			mean =	1.71	3.1

TABLE 2

Inter-Assay Precision for the Analysis of Aqueous Acadesine and ZMP

Number of Runs	Amount Acadesine Added (μM)	Amount Acadesine Found (μM)	$\pm$s.d.	%c.v.	Relative Error %
3	0.5	0.49	0.01	2.04	-2.0
3	1	1.03	0.03	2.98	+3.0
3	5	5.30	0.11	2.09	+6.0
3	10	10.68	0.44	4.13	+6.8
3	25	24.10	0.55	2.28	-3.6
3	50	47.83	0.86	1.80	-4.3
3	100	94.43	1.85	1.96	-5.6
			mean =	2.47	4.5

Number of Runs	Amount ZMP Added (μM)	Amount ZMP Found (μM)	$\pm$s.d.	%c.v.	Relative Error %
3	0.5	0.49	0.01	1.17	-2.0
3	1	1.03	0.04	3.94	+3.0
3	5	5.13	0.07	1.35	+2.6
3	10	10.39	0.17	1.65	+3.9
3	25	24.46	0.33	1.33	-2.2
3	50	48.77	0.14	0.29	-2.5
3	100	97.17	1.73	1.78	-2.8
			mean =	1.64	2.7

TABLE 3

Standard Curve Statistics

Aqueous Acadesine

Date	Slope	Y-intercept	Correlation Coefficient
04/08/93	24448.228	3062.690	0.9999
04/08/93	24206.710	3008.784	1.0000
04/08/93	23771.813	5828.875	0.9999
04/09/93	24660.553	10901.537	0.9998
04/12/93	23831.858	513.597	1.0000
n =	5	5	5
mean =	24183.832	4663.097	0.9999
s.d. =	384.49	3962.12	0.000
C.V.% =	1.59	84.97	0.008

Aqueous ZMP

Date	Slope	Y-intercept	Correlation Coefficient
04/08/93	22275.137	309.267	0.9999
04/08/93	22118.305	-486.342	0.9999
04/08/93	21808.841	636.863	0.9999
04/09/93	23514.643	1008.210	1.0000
04/12/93	21824.880	-1124.333	1.0000
n =	5	5	5
mean =	22308.361	68.733	0.9999
s.d. =	702.78	865.27	0.000
C.V.% =	3.15	1258.88	0.005

± 703 (3.15% c.v.) for ZMP and a mean slope of 24184 ± 384 (1.59% c.v.) for acadesine (Table 3). The correlation coefficient was 0.9999 for ZMP and 0.9999 for acadesine (Table 3).

In summary, this HPLC assay for the simultaneous quantification of acadesine and ZMP eliminates the need for separate and time-consuming assays. The assay can be applied

toward measuring concentrations of acadesine and ZMP in biological samples.

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REFERENCE

1 J. Leung, T. Stanley, J. Mathew, P. Curling, P. Barash, M. Salmenpera, G. Reves, M. Hollenberg, and D. Mangano, J. Am. Coll. Cardiol., 19: 112A (1992)

2 R. L. Sabina, D. Patterson, and E. W. Holmes, J. Biol. Chem., 260: 6107-6114 (1985)

3 R. Dixon, J. Fujitaki, L. Chrastil, and J. Lee, Res. Comm. Chem. Pathol. Pharmacol., 65: 405-408 (1989)

4 A. M. McLean, D. A. Ruggirello, C. Banfield, M. A. Gonzalez, and M. Bialer, J. Pharm. Sci., 75: 1005-1008 (1990)

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USE OF TRIETHYLAMINE AS AN ION-PAIRING REAGENT

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ABSTRACT

Evidence that triethylamine (TEA) functions as an ion pairing reagent (IPR) in a mobile phase system (20 mmol/L acetic acid, 20 mmol/L phosphoric acid, 30 mmol/L TEA, pH 7.0) under both gradient and isocratic elutions is presented. The retention of anions (nicotinic, xanthurenic, anthranilic, and 3-hydroxyanthranilic acids) was increased with increasing TEA (cations) concentration; that of cations (benzylamine) was decreased and that of zwitterions or neutrals (kynurenine, 3-hydroxykynurenine, and aniline) exhibited no changes. TEA is shown to be a useful IPR in gradient elution due to its rapid equilibration time with the column. Proper selections of detection wavelength and gradient program are important to eliminate the interference of impurities in many commercial TEA preparations.

INTRODUCTION

Ion pair chromatography (IPC) is a powerful tool in the separation of ionic or ionogenic compounds (1-5). Although the actual mechanism involved in IPC is still not clear the phenomena seen are that the retention of compounds of the same charges are increased, while those of opposite charges are decreased and neutrals are not affected by increasing IPR concentrations within certain ranges (2,6,7). These phenomena make the concentration of the IPR an important parameter to control the retention and selectivity of ionic compounds in IPC (8-11).

Classified by their size, there are two types of IPR: small and large molecules (1,3). Large IPRs have one or more long hydrophobic tails (e.g. trioctylamine and heptanesulfonate). They are more strongly retained by the column, and are not easily flushed out of the column. Small counter ions (e.g. perchlorate, tetra-methylammonium) are more water soluble and are therefore easily eliminated from the column. Advantages of small IPRs are rapid column (re)equilibration time which makes them practical in gradient elution and the possibilities of mixed chromatographic behavior (1).

Triethylamine (TEA) is often added to mobile phases as a modifier or as a competing base to improve peak shapes and control the retention of amines (12-14). In addition, it has been used successfully as an IPR in the separations of nucleotides (15) and tryptophan metabolites in our laboratory (16) by reversed-phase liquid chromatography.

In the previous study (16), there are two points that may affect the relationship of retention and TEA concentration: 1) that the gradient elution may mask some retention changes of zwitterions (tryptophan, kynurenine and 3-hydroxykynurenine) when the TEA concentration is increased, and 2) the concentration of TEA was decreasing to some extent during the gradient elution as the TEA was not included in the acetonitrile reservoir. In addition, the effect of the TEA concentration on the retention of cations with increasing TEA concentration was not known since only anions and zwitterions were present in the mobile phase of the previous study (16).

This study is aimed to clarify these uncertainties and provide more evidences that TEA functions as an IPR in the optimized mobile phase system of the previous study (16). Aniline and benzylamine were included as model cation and neutral compounds respectively. The advantages and limitations of using TEA as an IPR in gradient elution are discussed.

MATERIALS

The HPLC system (Waters, Milford, MA, USA), reference standards and reagents used were as described in Chuang et al (16). Aniline (AN) and benzylamine (BA) were purchased from Aldrich Chemical Co. Chromatographic separation was performed on a Nova-Pak C_{18} steel column (150 mm x 3.9 mm i.d., 4 um).

METHODS

A) Gradient elution study

The chromatographic conditions were as described in previous study [16]. Duplicate runs of eleven reference standard mixture were analyzed by using two mobile phase systems. The first one is the optimized mobile phase developed in the previous study (16). This mobile phase (MP) consisted of a binary linear gradient of MP-A solution (20 mmol/L phosphoric acid, 20 mmol/L acetic acid, and 30 mmol/L TEA, pH 7.0 adjusted with 2 mol/L sodium hydroxide) and MP-B solution (acetonitrile). TEA (30 mmol/L) was included in MP-B acetonitrile reservoir in the second mobile phase system. The gradient program was started from 100%A to 20%A and 80%B within 20 minutes with a flow rate of 0.8 mL/minute.

B) Isocratic elution study

a) The effect of TEA concentration on the retention of anions and neutral compounds (Tryptophan metabolites of the kynurenine pathway):

The mobile phase was a 99:1 (v/v) mixture of buffer solution (20 mmol/L phosphoric acid, 20 mmol/L acetic acid, and 30 mmol/L TEA, adjusted to pH 7.0 with 2 mol/L NaOH) and acetonitrile. The column was equilibrated with a new mobile phase for at least 20 column volumes for each mobile phase change. The detector wavelength was 254 nm, with a sensitivity of 0.1 or 0.2 AUFS. Ten microliters of a six-reference mixture, containing kynurenine (KN), 3-hydroxykynurenine (HK), nicotinic (NA), xanthurenic (XA), anthranilic (AA)

and 3-hydroxyanthranilic (HA) acids were injected in duplicate and all chromatographic runs were performed at ambient temperature (22-24°C). The capacity factor (k') was calculated as follows: $k' = (k'/t_0)-1$, where t_0 is the column void time.

b) The effect of TEA concentration on the retention of cations and neutral compounds (Aniline and benzylamine):

The experimental conditions were as described above except that a buffer:acetonitrile mixture of 97:3 (v/v) was chosen as the mobile phase. The sensitivity was set at 0.010 or 0.050 AUFS. Ten microliters of AN and BA mixture (125 ng each) were injected for analysis.

RESULTS AND DISCUSSION

A) Gradient elution study

In order to keep the concentration of TEA constant during the gradient run, 30 mmol/L TEA was added to the reservoir containing acetonitrile (MP-B) for the gradient elution. In TABLE 1, the retention times of 11 compounds analyzed by two MP systems (with and without TEA added to MP-B) were shown and the difference in retention times for each compound was calculated.

The data shows that the differences, in the retention times of the eleven compounds, were negligible. This indicates that the effect of the inclusion of TEA in the acetonitrile reservoir on retention is minimal. This is probably due

TABLE 1. The effect of including TEA in MP-B (acetonitrile) on retention time

Compound	Retention time (min) Without TEA (A)	With TEA (B)	(B)-(A)
QA	3.01	3.03	+0.02
HK	3.65	3.62	-0.03
HA	4.69	4.63	-0.06
PA	5.97	5.95	-0.02
NA	6.70	6.67	-0.03
KN	7.74	7.66	-0.08
AA	10.85	10.95	+0.10
TRP	12.03	12.06	+0.03
XA	13.27	13.20	-0.07
KA	14.83	14.81	-0.02
QN	16.98	16.97	-0.01

The data shown above are average retention times of duplicate runs.

to the narrow gradient changes (0-15%) of acetonitrile (or TEA) during the chromatographic run.

B) Isocratic elution studies

a) The effect of TEA concentration on the retention of anions and neutral compounds:

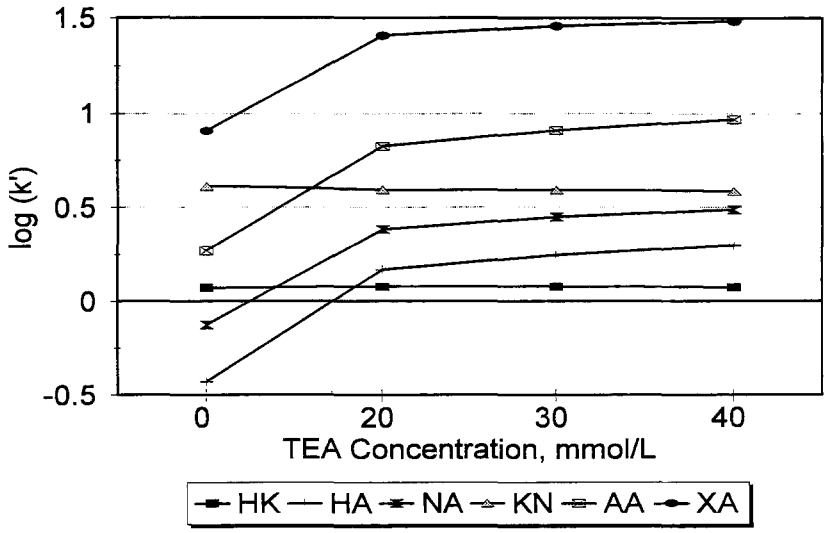

Figure 1. The effect of TEA concentration on the retention of six TRP metabolites. HK, 3-hydroxykynurenine; HA, 3-hydroxy- anthranilic acid; NA, nicotinic acid; KN, kynurenine; AA, anthranilic acid; XA, xanthurenic acid.

In FIGURE 1, the effect of TEA concentration on retention of the six compounds analyzed by isocratic elution is shown. The retention is expressed as the log(k') to show the curves of early eluted peaks clearly. The retention of four compounds (HA, NA, AA, and XA) increases along with the increase in TEA concentration, while those of HK and KN are not affected up to 40 mmol/L TEA, the highest concentration used in this study.

The isocratic study was performed to validate the findings of the gradient elution. The data shows no difference compared to the gradient analysis (16): the retention of anions (HA, AA, NA, and XA, containing opposite charged ions

vs TEA cation) is increased with TEA concentration increase and the retention of zwitterions (HK and KN) is not affected, indicating that TEA is acting like an IPR in this analytical system.

b) The effect of TEA concentration on the retention of cations and neutral compounds (Benzylamine and aniline):

Benzylamine (BN) and aniline (AN) were used as model cation and neutral compound in contrast to tryptophan metabolites which are anions or neutrals. The effect of the TEA concentration on the retention of AN and BN analyzed by isocratic elution is shown in FIGURE 2. The retention time of BN decreases when TEA is added to the mobile phase, and reaches a minimum at a TEA concentration of 10 mmol/L. However, the retention of AN is only slightly affected by changes in TEA concentration up to 40 mmol/L. The aromatic amine functional group of AN (pKa 4.6, ref. 17) is not in the ionized form under the conditions (pH 7.0) of the mobile phase, so that the retention of AN is not affected by TEA addition and TEA concentration changes. However, the aliphatic amine functional group of BN (pKa 9.35, ref. 18) is cationic under the same conditions, therefore the retention of BN is decreased by addition of cationic TEA ions.

These data show that under both gradient and isocratic elutions the retention of anions is increased with increasing TEA concentration; that of cations is decreased and that of zwitterions or neutrals, no changes, and thus support that TEA functions as an IPR under this mobile phase system.

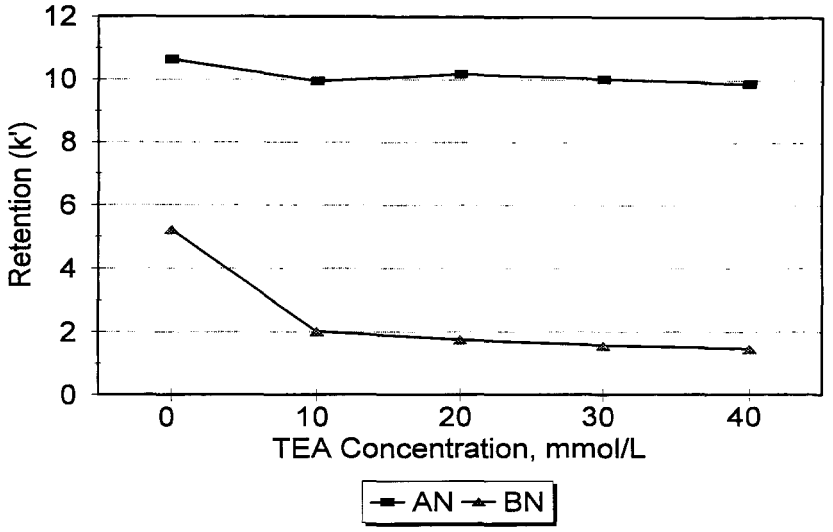

Figure 2. The effect of TEA concentration on the retention of aniline (AN) and benzylamine (BN).

It has been suggested that TEA does not have a deleterious effect on the stationary phase like that of tetrabutylammonium and is very water soluble and also has a low absorbance in the UV range (15). According to Gloor and Johnson's classification (1), TEA is a small IPR since it has three short hydrophobic alkyl chains. Therefore, TEA is not only rapidly (re)equilibrated in the column but is also easily eliminated from the column, making it practical for use in gradient elution.

From our experience with TEA, it equilibrates and re-equilibrates (during gradient elution) with the column very rapidly. However, it was found that

some TEA impurities were trapped at low acetonitrile concentrations (0-5%) in the gradient elution and then eluted at higher concentrations of acetonitrile, which perturbs the chromatographic baseline. We have tested on three brands of TEA preparation, all preparations have this problem. This problem is most apparent when a high sensitivity setting and a low wavelength (254 nm) were used in the detection. Most of these impurity peaks were not observed at a detection wavelength of 340 nm (19).

CONCLUSION

TEA functions as an IPR in an optimized mobile phase system on the observations that the retention of compounds with opposite charges (anions) was increased with TEA (cations) concentration increase; retention of compounds with same charges (cations) was decreased and that of neutrals (or zwitterions), no changes. In gradient elution TEA is a practical IPR due to its short (re)equilibration time. A precaution of using TEA in gradient is that many impurities are present in the commercial preparations. Proper selections of detection wavelength and gradient program are important to eliminate the interference of impurities.

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REFERENCES

1. R. Gloor, E. L. Johnson, J. Chromatogr. Sci., 18: 413-423 (1977)

2. B. A. Bidlingmeyer, J. Chromatogr. Sci., 18: 525-539 (1980)

3. C. F. Poole, S. A. Schuette, Contemporary Practice of Chromatography, Elsevier, Amsterdam, 1984, p. 526.

4. T. Dzido, J. Chromatogr., 439: 257-266 (1988)

5. L. R. Snyder, J. L. Glajch, J. J. Kirkland, Practical HPLC Method Development, John Wiley & Sons, Inc., New York, 1988, p. 106.

6. B. A. Bidlingmeyer, S. N. Deming, W. P. Price, Jr., B. Sachok, M. Petrusek, J. Chromatogr., 186: 419-434 (1979)

7. J. H. Knox, R. A. Hartwick, J. Chromatogr., 204: 3-21 (1981)

8. R. C. Kong, B. Sachok, K. Johansson, J. Chromatogr., 199: 307-316 (1980)

9. W. Lindberg, E. Johansson, K. Johansson, J. Chromatogr., 211: 201-212 (1981)

10. P. M. J. Coenegracht, N. V. Tuyen, H.J. Metting, P.J.M. Coenegracht-Lamers, J. Chromatogr., 389: 351-367 (1987)

11. A. H. Billiet, J. Vuik, J. K. Strasters, L. D. Galan, J. Chromatogr., 384: 153-162 (1987)

12. L. R. Snyder, J. L. Glajch, J. J. Kirkland, Practical HPLC Method Development, John Wiley & Sons, Inc., New York, 1988, p. 60.

13. J. S. Kiel, S. L. Morgan, J. Chromatogr., 320: 313-323 (1985)

14. R. W. Roos, C. A. Lau-cam, J. Chromatogr., 370: 403-418 (1986)

15. L. S. Folley. S. D. Power, R. O. Poyton, J. Chromatogr., 281: 199-207 (1983)

16. C-Z. Chuang, F. A. Ragan, Jr., C. Prasad, J. Chromatogr., 534: 13-21 (1990)

17. A. E. Martell, R. M. Smith, Critical Stability Constants, Vol 2, Plenum Press, New York, 1982, p. 7.

18. A. E. Martell, R. M. Smith, Critical Stability Constants, Vol 5, Plenum Press, New York, 1982, p. 133.

19. C-Z. Chuang, Studies on the Simultaneous Analysis of Multiple Tryptophan Metabolites of the Kynurenine Pathway in the Rat Brain by Gas Chromatography/Mass Spectrometry and High-Performance Liquid Chromatography. Ph.D. dissertation, 1991.

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PAIRED ION REVERSED-PHASE HPLC ASSAY FOR THE SIMULTANEOUS DETERMINATION OF IOTHALAMIC ACID AND PARA AMINOHIPPURIC ACID IN PLASMA

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ABSTRACT

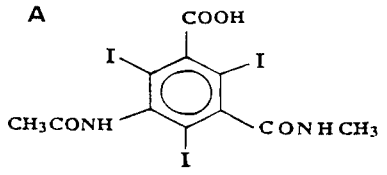
An ion paired reversed - phase high performance liquid chromatographic assay for iothalamic acid and para aminohippuric acid in the same sample of plasma is described. The analysis uses one internal standard for both drugs. Sample preparation consists of precipitating plasma proteins with methanol and centrifuging to settle the proteins. The supernatant is evaporated and the residue reconstituted with mobile phase for injection. For HPLC a C8 column and a mobile phase consisting of potassium phosphate buffer with dodecyl triethyl ammonium phosphate IP reagent, 22.5 % methanol and 2.5 % acetonitrile with UV detection at 254 nm was used. Coefficients of variation for the assay were in the range of 1.6 - 12.1% for iothalamic acid and 4.3 - 17.7% for para aminohippuric acid for four levels of concentration. Limits of quantitation were 3.0 μg/mL for iothalamic acid and 5.0 μg/mL for para aminohippuric acid. This isocratic HPLC assay is simple, rapid and relatively inexpensive.

INTRODUCTION

Iothalamic acid (Io, Figure 1A, 3-(acetylamino)-2,4,6,triiodo-5-[(methylamino) carbonyl]-benzoic acid) is a well known radiographic contrasting agent used in renal and urological procedures (1). It is also used as a marker, to replace inulin for measuring glomerular filtration rate (GFR). Para aminohippuric acid (PAH, Figure 1B, N-(4-aminobenzoyl)-glycine) is used to determine the effective renal plasma flow (ERPF) and as an ideal marker for renal blood flow (RBF) (2-4). The current methods used for these measurements are considered relatively invasive requiring IV infusion of markers and accurate urine collections. Measurements of creatinine clearance though widely used is prone to errors and can not detect minor changes in GFR (5).

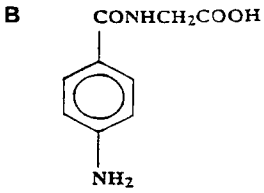
Studies employing Io clearance for estimating GFR indicate, good correlation with inulin clearance and that this method is well tolerated by children and infants. Since the use of radiolabled iothalamic acid in patients is risky and subject to strict health and safety guidelines during usage, a method for measuring "cold" - Io as well as PAH in plasma and urine is advantageous.

Among the numerous methods reported for determining Io and PAH, those using radiolabled drugs are not suitable. Most other methods determine only one of these compounds (1,6-8). Many of the methods use colorimetry for PAH and several of the HPLC methods use immiscible organic solvent extractions to isolate the compounds from plasma and urine (1,6,8,9). While two methods used paired - ion mobile phases with reversed-phase columns for HPLC separation of either Io or PAH only one method reports the simultaneous assay for Io and PAH using reversed-phase HPLC (4,5,10). One significant problem with isocratic elution using the mobile phases reported in the earlier method, where the organic phase is below 10 % is that residual plasma and urine constituents are strongly retained on the column. Over time, this could cause column performance to deteriorate. Usually under these conditions a second pump



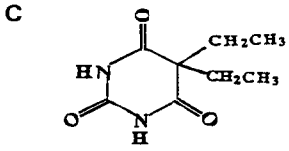
IOTHALAMIC ACID

$C_{11}H_9I_3N_2O_4$
MW. 613.9



PARA AMINOHIPPURIC ACID

$C_9H_{10}N_2O_3$
MW. 194.2



BARBITAL

$C_8H_{12}N_2O_3$
MW. 184.2

FIGURE 1. Chemical structures of (A) iothalamic acid, (B) p-aminohippuric acid and (C) 5,5 diethyl barbituric acid (barbital, internal standard).

coupled through a gradient controller is used to flush the column with a polar mobile phase for 2-5 minutes after each sample run followed by equilibration with the running mobile phase for at least 10 minutes. This technique requires either two pumps and a controller or a switching valve and a single pump coupled to a controller. A simple and less costly method was desirable to determine Io and PAH simultaneously. It was decided to increase the retention time of the compounds beyond 10 minutes and an ion-paired mobile phase was selected in order to achieve this. However p-aminobenzoic acid (PABA) was not adequately resolved from the two compounds, therefore barbital was used as the internal standard .

We decided to work in an isocratic mode with higher amounts of organic modifier in order to eliminate build up of a plasma residue on the column. An octyl C_8 reversed-phase column was selected in place of a C_{18} and the detector wavelength was fixed at 254 nm.

A complete assay validation, reported in this paper was performed. This was followed by determination of Io and PAH in a set of clinical samples .

EXPERIMENTAL

Apparatus

The HPLC system used consisted of a Waters M-510 HPLC pump, a model 712 WISP autoinjector, and a model 481 Lambda Max UV detector (Waters Assoc., Milford, MA). An HP 3396A-integrator (Hewlett Packard, Avondale,PA) was used for collecting the chromatographic data. The detector wavelength was 254 nm and the absorbance was set at 0.01 aufs. Separation was achieved with an Octyl (C-8) ,5µm particle size, 4.6 mm (i.d.) x 25cm reversed-phase column (Rainin Instrument Co. Inc., Woburn, MA).

Reagents

All solvents were of HPLC grade [Fischer Scientific Co., Fair Lawn, NJ]. Dodecyl triethylammonium phosphate [(Q-12, Ion pair reagent, 0.5M solution), Regis Chemical Co., Morton Grove, Il], potassium diacid phosphate, potassium mono acid phosphate [Fischer Scientific Co., Fair Lawn,NJ], iothalamic acid [Mallinckrodt Chemicals Inc., St.Louis, MO], p-aminohippuric acid sodium salt and barbital [Sigma Chemicals Co., St. Louis, MO], were used as received. Deionized distilled water was obtained from "Barnstead Nanopure" purification system (Barnstead Co., Boston, MA). Drug free human plasma was obtained from the Irwin Memorial Blood Center, San Francisco and Long Hospital Blood Bank, UC San Francisco, CA.

Mobile Phase

The mobile phase was composed of 22.5% methanol, 2.5% acetonitrile, and 1.75mM IP reagent in 10 mM potassium phosphate. It was prepared dissolving 6.44 g KH_2PO_4, 7.04 g K_2HPO_4 and 14 mL 0.5M Q-12 IP reagent solution in 4 L of deionized water and the pH was adjusted to 7.50. Three liters of the prepared buffer was filtered through a 0.22μm filter and mixed with 900 mL of methanol and 100 mL of acetonitrile. The mixture was degased by sonication under vacuum. The isocratic flow rate of the mobile phase was 1 mL/min.

Sample preparation

Calibration curve samples were prepared by spiking iothalamic acid, p-aminohippuric acid and the internal standard (barbital) into plasma. The plasma proteins in these samples, quality assuarance (QA) controls and clinical samples (100 μL each) were precipitated out with 500 μL aliquots of HPLC grade methanol. Samples were vortexed for 15 seconds and centrifuged for 10 minutes at 2500 rpm. The supernatants were concentrated under nitrogen and residues were reconstituted in running buffer. Fifteen to twenty micro liter samples were injected onto the column for analysis.

All clinical samples, QA samples and stock solutions of compounds were stored at -20°C until analysis. Spiked samples for calibration curves and controls at four different drug concentrations were prepared with drug free plasma and were spiked with separately prepared drug solutions.

RESULTS

Figure 2a and 2b represent typical chromatograms of the internal standard in blank plasma and the two compounds and internal standard in plasma, respectively. The mean retention times of p-aminohippuric acid, barbital and iothalamic acid are 12.0, 15.9 and 18.2 minutes

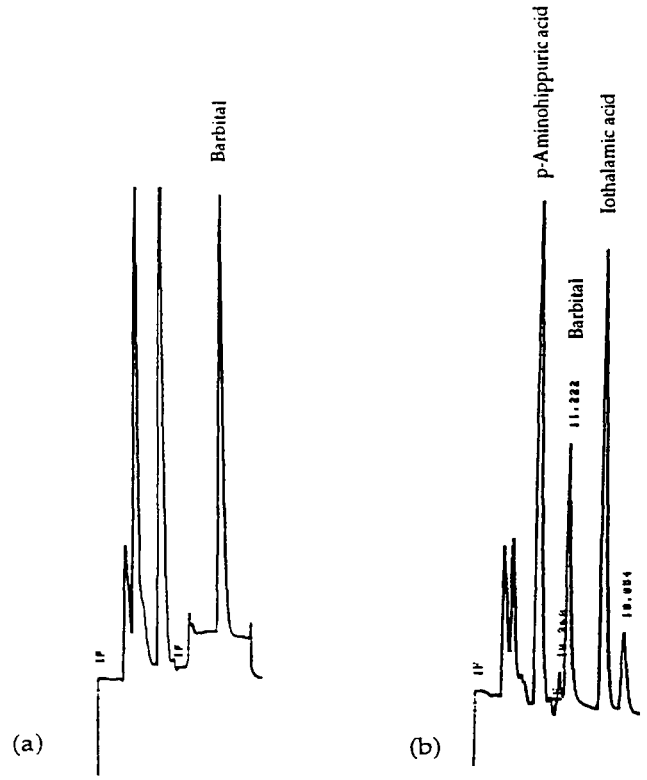


FIGURE 2. (a). Chromatogram of blank plasma with internal standard.

(b). Chromatogram of iothalamic acid, p-aminohippuric acid and the internal standard in plasma.

respectively. (However, fluctuations of these retention times are observed due to the variation of temperature and column performance). The concentration data used for the calibration curves are presented in Table 1. (5.0 to 200.0 μg/mL for Io and 10.0 to 380.0 μg/mL for PAH). Even though the limits of quantitation were 3.0 μg/mL (CV% 10.03) and 5.0 μg/mL (CV% 9.56) for these two drugs, these lowest points were omitted from the calibration curves.

TABLE 1

Data Used for Calibration Curves of Iothalamic Acid and p-Aminohippuric Acid in Plasma.

Iothalamic Acid			p-Aminohippuric Acid		
Conc. Io. µg/mL	Peak Height Ratic (Io/Barb.)	Calc. Conc. Io,µg/mL	Conc. PAH µg/mL	Peak Height Ratio (PAH/Barb)	Calc. Conc. PAH,µg/mL
0.00	0.00	0.00	0.00	0.00	0.00
5.00	0.16	4.72	10.00	0.41	9.27
10.00	0.29	9.75	25.00	0.98	23.23
20.00	0.55	20.10	50.00	2.04	49.45
40.00	1.03	39.39	100.00	4.20	102.30
80.00	2.06	80.28	200.00	8.19	200.48
140.00	3.62	142.54	300.00	12.43	304.41
200.00	5.01	198.26	380.00	15.34	375.87

Two typical calibration graphs used for the calculation of concentration of the two compounds are presented in Figures 3 and 4. Linear regression of peak height ratio vs. compound - concentration gives typical coefficients of determination (r^2) of 0.999 or better for iothalamic acid and p-aminohippuric acid.

Variability Studies

Interday and intraday variability was studied using frozen controls at four concentratons, extra-low, low, medium and high. Five samples from each concentration were assayed for both interday and intraday studies. For interday variability, six calibration curves on six different days were used and one calibration curve was used for the intraday variability study. The ranges for the coefficients of variation were 6.33 to 12.10% for iothalamic acid and 4.30 to 17.72% for p-aminohippuric acid for the interday studies as shown in Tables 2 and 3, respectively. Coefficients of variation ranged from 1.60 to 5.00 % for iothalamic acid and 7.00 to 8.41 % for p-aminohippuric acid for the intraday study as shown in Tables 4 and 5, respectively.

Recovery

Assay recovery was measured by comparing the peak height ratios of the compounds to barbital, at four different compound-concentrations in plasma and in aqueous compound solutions spiked at the same concentration. The internal standard was added to the plasma samples only after the supernatant was decanted from the precipitated proteins. Both sets of samples were then evaporated and reconstituted in running buffer for injection. The mean % recovery was calculated as follows:

$$\% \text{ Recovery} = \frac{\text{peak height ratio of compound in plasma}}{\text{peak height ratio of compound in water}} \times 100$$

The mean recovery was 91.1 % for iothalamic acid and 79.2 % for p-aminohippuric acid.(Tables 6 and 7)

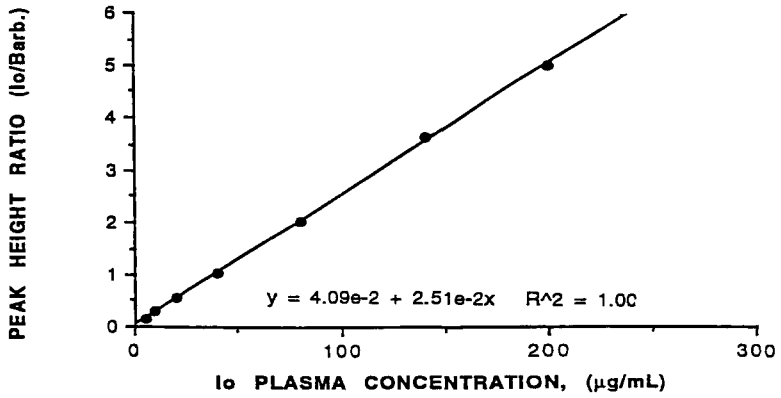


FIGURE 3. Calibration curve of iothalamic acid in plasma.

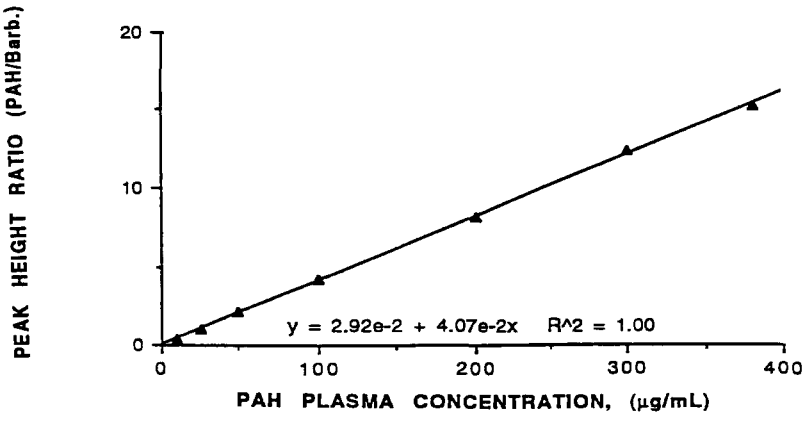


FIGURE 4. Calibration curve of p-aminohippuric acid in plasma.

TABLE 2
Interday Variation of Iothalamic Acid in Plasma

	CONCENTRATION* (μg/mL)			
	EXTRA-LOW [12.0]	LOW [35.0]	MEDIUM [85.0]	HIGH [150.0]
1	12.92	38.03	81.84	154.92
2	13.21	37.52	82.17	148.17
3	12.48	37.41	82.23	150.23
4	12.44	37.22	81.08	149.15
5	13.13	37.22	79.43	145.89
Mean	12.84	37.50	81.35	149.67
SD	1.55	2.37	5.79	14.46
%CV	12.10	6.33	7.14	9.63

*Each value represents mean of n=5

TABLE 3
Interday Variation of p-Aminohippuric Acid in Plasma

	CONCENTRATION* (μg/mL)			
	EXTRA-LOW [25.0]	LOW [70.0]	MEDIUM [180.0]	HIGH [320.0]
1	25.12	73.74	186.13	333.58
2	23.87	75.43	188.87	336.69
3	23.88	73.47	186.57	339.78
4	25.21	73.86	179.83	340.79
5	24.76	74.56	190.68	339.01
Mean	24.57	74.21	186.41	337.97
SD	4.34	3.19	22.32	34.12
%CV	17.72	4.30	11.96	10.11

*Each value represents mean of n=5

TABLE 4
Intraday Variation of Iothalamic Acid in Plasma

	CONCENTRATION* (µg/mL)			
	EXTRA-LOW [12.0]	LOW [35.0]	MEDIUM [85.0]	HIGH [150]
1	14.03	37.30	78.49	141.50
2	14.12	37.05	78.33	132.15
3	14.12	35.65	72.29	135.88
4	13.77	36.29	75.72	138.17
5	12.85	36.53	80.11	142.98
6	13.47	36.39	75.16	124.51
Mean	13.73	36.54	76.68	135.87
SD	0.50	0.59	2.84	6.79
%CV	3.62	1.60	3.70	5.00

*Each value represents mean of n=5

TABLE 5
Intraday Variation of p-Aminohippuric Acid in Plasma

	CONCENTRATION (µg/mL) (n=5)			
	EXTRA-LOW [25.0]	LOW [70.0]	MEDIUM [180.0]	HIGH [320.0]
1	21.72	65.85	180.88	298.95
2	20.09	67.48	172.34	282.84
3	17.90	55.77	176.21	281.33
4	20.08	63.95	165.54	280.74
5	17.22	58.32	155.18	295.06
6	19.63	61.85	163.43	298.97
Mean	19.44	62.20	163.93	289.65
SD	1.64	4.49	12.26	19.68
%CV	8.41	7.22	7.48	7.00

TABLE 6
Recovery of Iothalamic Acid from Plasma

Conc. Io	Peak Height Ratio (Plasma)		Peak Height Ratio (Water)		Recovery %
µg/mL	Mean*	Std.Dev.	Mean*	Std. Dev.	
12	0.34	0.0004	0.36	0.0239	96.6
35	0.81	0.0098	0.95	0.0060	85.7
85	1.92	0.0421	2.09	0.0782	92.0
150	3.97	0.0980	4.40	0.0373	90.1
Overall recovery of Iothalamic Acid					**91.1.%**

* n=5

TABLE 7
Recovery of p-Aminohippuric Acid from Plasma

Conc. PAH	Peak Height Ratio (Plasma)		Peak Height Ratio (Water)		Recovery %
µg/mL	Mean*	Std. Dev.	Mean*	Std. Dev.	
25	0.86	0.0021	1.20	0.0230	71.39
70	2.42	0.0225	3.11	0.0321	77.77
180	6.30	0.1040	7.50	0.2546	84.05
320	11.53	0.3022	14.48	0.0785	79.60
Overall recovery of p-aminohippuric acid					**78.2.%**

* n=5

Table 8
Storage Stability (24 hours at RT) of Iothalamic Acid and p-Aminohippuric Acid in Plasma

Conc.,µg/mL	Percent Change Io*	Conc.,µg/mL	Percent Change PAH*
12.0	13.78	25.0	-19.27
35.0	10.09	70.0	1.63
85.0	9.12	180.0	4.53
150.0	1.67	320.0	0.37

*Each value represents mean of n=5

Table 9
Heat Deactivation Stability of Iothalamic Acid and p-Aminohippuric Acid in Plasma

Conc.,µg/mL	Percent Change Io*	Conc.,µg/mL	Percent Change PAH*
12.0	4.92	25.0	4.16
35.0	1.48	70.0	-12.02
85.0	-2.85	180.0	-15.70
150.0	-3.74	320.0	-4.10

*Each value represents mean of n=5

Stability

The stability of the two compounds in plasma during storage at room temperature for 24 hours and during heat deactivation at 56-58°C for 55 minutes were evaluated (Tables 8 and 9 respectively). Five samples each from the four controls were used for both stability studies. Further, we investigated the stability of the two compounds during three freezing and thawing cycles. Our results showed that there was a loss of both compounds to the extent of 3% for Io and 10.2 % for PAH after the third cycle (Table 10).

Table 10

Freeze - Thaw Stability of Iothalamic Acid and p-Aminohippuric Acid in Plasma

	Percent Change Io Conc.				Percent Change PAH Conc.		
	Freeze-Thaw Cycle				Freeze-Thaw Cycle		
Conc.,µg/mL	1	2	3	Conc.,µg/mL	1	2	3
12.0	0.00	24.84	6.75	25.0	0.00	28.49	-9.77
35.0	0.00	-1.94	-5.02	70.0	0.00	-3.92	-4.52
85.0	0.00	-2.03	-5.46	180.0	0.00	-2.73	-3.83
150.0	0.00	-7.50	-8.46	320.0	0.00	-6.96	-22.92
Mean	**0.00**	**3.34**	**-3.05**		**0.00**	**3.72**	**-10.26**

DISCUSSION

The forgoing method is the first reported, for determining iothalamic acid and p-aminohippuric acid in the same plasma sample using an isocratic paired ion reverse phase HPLC assay. The use of barbital to replace PABA as the internal standard has the advantage of eliminating interferences by metabolites of these drugs, including PABA. The recently marketed ion pairing reagent dodecyl trimethylammonium phosphate is needed only in small quantities. The long 12- carbon chain, bonds more effectively to the C-18 group on the column packing thus giving better retention properties for acidic drugs. The main advantage of the method is that gradient - elution or dual pump operations are eliminated by having a polar mobile phase containing 20-25% methanol. This results in improved column performance over long periods of time without frequent washing of the column to remove interfering substances usually present in bio-matrices.

The assay method has a lower limit of quantitation of 3.0μg/mL for iothalamic acid and 5.0μg/mL for p-aminohippuric acid. The recovery of the compounds during extraction were very satisfactory and their stability in plasma was within acceptable limits.

Using this method, plasma and urine samples from twenty-four subjects with normal and impaired renal function were analysed for Io and PAH. During the course of a pharmacokinetic study the renal clearances of iothalamic acid and p-aminohippuric acid were determined (11).

The validation of this assay was carried out according to the guidelines put forward by the conference on Analytical Methods Validation; Bioavailability, Bioequivalence and Pharmacokinetic Studies sponsored by FDA, AAPS and AOAC among others [12]. It is noteworthy that, this reversed -phase ion pair HPLC method with isocratic elution is a simple, rapid, relatively inexpensive and accurate method for simultaneous determination of these two compounds in human plasma.

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REFERENCES

1. F.Gaspari, L.Mainardi, P.Ruggenenti and G.Remuzzi, J.Chromatogr., 570(2), (1991), 435.

2. H.Chassis, H.A.Ranges, W.Goldrig and H.W.Smith, J.Clin.Invest., 17, (1938), 683.

3. H.W.Smith, N.Finkelstein, L.Aliminosa, B.Cranford and M.Graber J.Clin.Invest., 24, (1946), 388.

4. P.D.Jenny, A.Weber and A.L.Smith, J.Chromatogr., 490,(1989), 213.

5. T.Prueksaritanont, M.Chen and W.L.Chiou, J.Chromatogr., 306, (1984), 89.

6. R.N.Dalton, M.J.Wiseman, C.Turner and G.Viberti, Kidney Int., 34, (1988), 117.

7. L.B.Schwartz, B.L.Gewertz and M.G.Bissell, Clin.Chem., 34/1, (1988) 165.

8. M.M.Reidenberg, B.J.Lorenzo, D.E.Drayer, J.Kluger, T.Nestor, J.C.Regnier, B.A.Kowal and I.Bekersky,Ther.Drug Monit., 10, (1988), 434.

9. M.Kiguchi and J.Sudo , J.Toxicol.Sci., 12, (1987),30

10. S.Boschi and B.Marchesini, J.Chromatogr., 224, (1981) ,139.

11. Drug Study (1992) #DSU 87-45/PK6 of the Drug Studies Unit, School of Pharmacy, University of California, San Francisco, CA 94143.

[12].V.P.Shah, K.K. Midha, S. Dighe, I.J.McGilveray, J.P.Skelly, A.Yakobi, T.Layloff, C.T.Wiswanathan, C.Edgar Cook, R,D.McDowall, K.A.Pittman, and S. Spector. *J.Pharm. Sci.*, 81-3, 309, (1992).

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A SENSITIVE AND FAST METHOD FOR THE DETERMINATION OF POLYAMINES IN BIOLOGICAL SAMPLES. BENZOYL CHLORIDE PRE-COLUMN DERIVATIZATION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A sensitive, rapid pre-column derivatization HPLC procedure for determination of biologically important polyamines is described. Benzoylated putrescine, cadaverine, norspermidine, spermidine, norspermine and spermine were separated on reverse-phase column using 42:58 acetonitrile:water as mobile phase and detected by UV absorption at 198 nm. The detection limits were: 0.8 pmol for putrescine, 1 pmol for spermidine and 1.3 pmol for spermine. The method was successfully applied to the analysis of polyamines in small samples of unicellular organisms.

INTRODUCTION

Our recent studies [1] on the biological function of polyamines led us to investigate whether the concentration of polyamines in cultures of algae and other unicellular organisms depends on the time in their cell cycle, hence to seek a fast and sensitive polyamine assay.

Many polyamine analysis methods are based on those for amino acids, using both pre- and post-column derivatization high-performance liquid chromatography [2,3]. Post-column derivatization uses ion-exchange resins as stationary phases; this method suffers from long retention time and interference from amino acids [3,4]. Pre-column derivatization using dansyl chloride or tosyl assays [2,5,6] are highly sensitive, but again interference from amino acids and poor separation of derivatives on reverse phase column prove to be problems. Redmond and Tseng

[7] reported a benzoyl chloride pre-column derivatization method based on the Schotten-Baumann reaction combined with ether extraction to eliminate interference from amino acids; it uses 60: 40 methanol: H_2O as mobile phase and 254 nm absorption detection. This method has found applications in body fluid [8], mammal tissue [9] and plant [10] analysis. Although this method has the advantage of fast separation, its low sensitivity has limited its application in our studies. In the present paper, we report a sensitive and fast quantitative method for polyamines based on the benzoylated polyamine separation on reversed phase column, using 42:58 acetonitrile: H_2O as mobile phase and 198 nm absorption detection, which allows faster separation and more sensitive detection.

EXPERIMENTS

Materials and Equipments

Materials 1,3-diaminopropane, putrescine and norspermidine (3,3'-iminobispropylamine) were from Sigma. Cadaverine, 1,6-hexanediamine, spermidine and spermine were from Aldrich. Norspermine (N,N'-bis[3-aminopropyl]-1,3-propanediamine) was a gift from Dr. S. Marvin Friedman. Benzoyl chloride (99%, A.C.S.reagent, Aldrich) was used without further purification. Anhydrous ethyl ether (A.R.) was from Mallinckrodt. Methanol, acetonitrile and water used for separation were all of HPLC grade.

Equipment A Beckman HPLC equipped with Model 110B high-pressure pump and Model 163 variable wavelength absorbance detector was used throughout the study, with a Kipp & Zonen recorder (Model BD41). A Beckman 5μm C_8 reverse-phase column (25cm x 4.6mm) was used to separate the benzoylated polyamines. Sample injection was carried out using either 20 μl sample loop (Beckman) or 25 μl micro-syringe (Hamilton).

Sample Treatment, Polyamine Derivatization and HPLC Separation

Chlamydomonas reinhardtii, Gonyaulax polyedra, Pyrocystics lununa and *Plasmodium falciparum* were grown and treated as described previously [1a]. Briefly, after addition of a polyamine internal standard to 10~500 mg wet cell pellets (cadaverine as internal standard for *Chlamydomonas reinhardtii* and *Plasmodium falciparum* ; 1,6-hexanediamine for *Gonyaulax polyedra* and *Pyrocystics lununa*), the cells were broken by sonicator or beadbeater in 1~3 ml water (check under microscope to make sure all the cells were broken), extracted with 2 ml 5% trichloroacetic acid and centrifuged at 8,000 *g* at ca. 4°C for 20 min. The pellets were resuspended in 1 ml water and 2 drops of 2 N NaOH and recentrifuged in 2 ml 5% trichloroacetic acid.

To *ca.* 5 ml pooled supernatant of sample, 1.5 ml 2N NaOH was added to adjust the pH >13, followed by 5 μl benzoyl chloride. The mixture was magnetically stirred for 20 minutes for complete reaction. 2 ml NaCl (sat.) was added and the resulting solution was extracted twice with 1ml ether. Pooled ether fractions were evaporated by a stream of nitrogen and the residue (benzoylated polyamines) was dissolved in 0.2 ml 42% acetonitrile/water (for acetonitrile/water mobile phase) or 60% methanol/water [8] (for methanol/water mobile phase). The resulting solution was subjected to HPLC separation. Polyamines standards, dissolved in distilled water, were derivatized with NaOH and benzoyl chloride and separated by the same procedure.

RESULT AND DISCUSSION

It has been shown [7] that the benzoylated derivatives of putrescine, cadaverine, spermidine and spermine are well separated on reversed-phase column using 60:40 methanol: H_2O as the mobile phase. We confirmed this. Fig. 1 shows a typical chromatogram of these benzoylated polyamines, together with derivatives of norspermidine and norspermine. However, when we used this method to study the time dependence of the polyamine level in *Chlamydomonas reinhardtii* and *Gonyaulax polyedra* as a function of the cell cycle, requiring frequent collection of samples of limited amount, it became important to increase both detection sensitivity and separation speed. It was even more imperative when assaying for possible traces of norspermine in very small samples of the parasitic *Plasmodium falciparum*, which is phylogenetically related to dinoflagellates.

We found that the absorbance of benzoylated polyamines increases *ca.* 50 times from 254 to 198 nm in acetonitrile as solvent, which means that the detection sensitivity of the method could be greatly increased by a change of detection wavelength. However, the use of methanol/water as mobile phase precluded this possibility because of the UV cut-off point of methanol at 205nm.

Our result showed that benzoylated polyamines are well separated on a RP-C_8 column when using 42% acetonitrile/water as mobile phase (Fig. 2). This allows detection at 198 nm without significant background noise, and only an overlap of the peaks of hexanediamine and spermidine

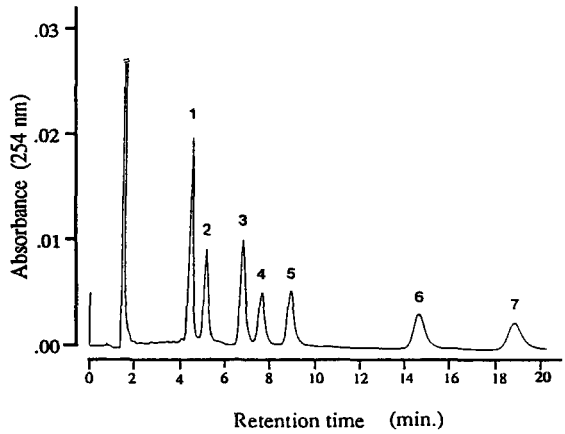

Fig. 1 A chromatogram of a standard mixture of benzoylated polyamines (containing *ca.* 2 nmol each), with methanol/water 60:40 as a mobile phase and detection at 254 nm.. 1. putrescine, 2. cadaverine, 3. hexanediamine, 4. norspermidine, 5. spermidine, 6. norspermine and 7. spermine.

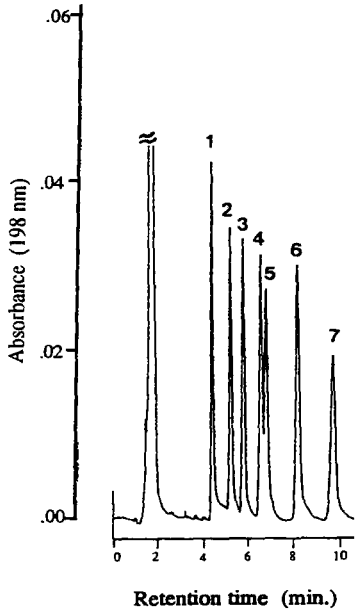

Fig.2 Separation of a standard mixture of benzoylated polyamines with 42% acetonitrile/water as mobile phase and detection at 198 nm. Each peak contains *ca.* 0.1 nmol polyamines. 1. putrescine, 2. cadaverine, 3. norspermidine, 4. spermidine, 5. hexanediamine, 6. norspermine and 7. spermine.

as drawback. Since hexanediamine is not a natural polyamine, but serves only as potential internal standard, this overlap is of no consequence. By using this mobile phase, the retention time of spermine is about a half of its value in methanol/water. The detection limits are: 0.8 pmol for putrescine, 1 pmol for spermidine and 1.3 pmol for spermine, therefore 60 times less than with the methanol/water system [8]. In both mobile phase systems, however, diaminopropane overlaps with putrescine and they are difficult to separate by simply changing the ratio of mobile phases.

Improper use of dissolving solvent may cause poor resolution [8]. Our experiment showed that 42% acetonitrile/water is suitable for benzoylated polyamines. The solution can be kept for up to six months at room temperature without significant change.

Per gram of wet weight, we found that *Chlamydomonas reinhardtii* cells contained 5.5 μmol putrescine, 0.9 μmol norspermidine and 0.2 μmol spermidine in average; *Gonyaulax polyedra* and *Pyrocystics lununa* contained only norspermine (0.06 and 0.016 μmol, respectively). Polyamine concentrations in *Plasmodium falciparum* were 0.01 for putrescine, 0.03 for spermidine and 0.005

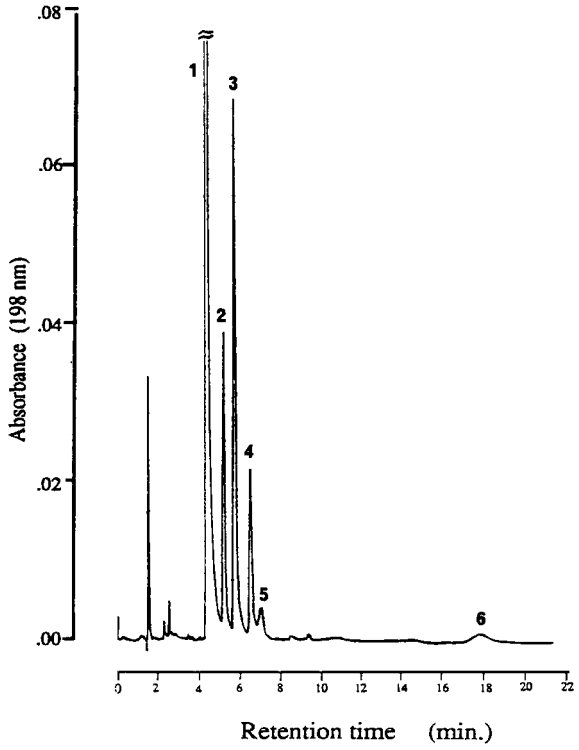

Fig. 3 Chromatogram of a sample of *Chlamydomonas reinhardtii* (treatment of sample and derivatization, see text). Mobile phase: 42% acetonitrile in water. Absorption detection wavelength: 198 nm. 1.putrescine, 2. cadaverine (internal standard), 3. norspermidine, 4. spermidine; 5 and 6. unknown.

for spermine (all in nmol per 10^6 cells); no norspermine was found above our detection limit. The recovery rates of polyamines were satisfactory for the samples (>90%). A typical chromatogram of *Chlamydomonas reinhardtii* polyamine separation is shown in Fig. 3.

CONCLUSION

By using 42% acetonitrile/water as mobile phase and UV detection at 198 nm, benzoylated putrescine, cadaverine, norspermidine, spermidine, norspermine and spermine can be satisfactorily

separated on C_8 reverse-phase column in 10 minutes, with detection limit of less than 1.3 pmol. The mobile phase also serves as dissolving solvent. This method can been used in the polyamine analysis in some unicellular prokayotes (*Chlamydomonas reinhardtii, Gonyaulax polyedra, Pyrocystics lununa* and *Plasmodium falciparum*) and would work well, we expect, in other biological samples.

ACKNOWLEDGEMENTS

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REFERENCES

1. a). Y.-H. Mei, A. U.Khan, T. Wilson, *Quimica Nova*, 16, 000(1993) b). A. U. Khan, Y.-H. Mei, T. Wilson, *Proc. Natl. Ca. Sci. USA*, 89, 11426-7(1992).
2. For reviews: a) N. Seiler, *Methods in Enzymology*, Vol. 94: *Polyamines*. H. Tabor and C. W. Tabor Ed., Academic Press, New York, 1983, pp 3-54. b) R. Durie, *Progress in Cancer Research and Therapy*, Vol. 8: *Polyamines as Biochemical Markers of Normal and Malignant Growth*, Raven Press, New York, 1978, pp 121-127.
3. V. R. Villanueva, R. C. Adlakha, *Anal. Biochem.*, 91, 264-75(1978).
4. K. Hamana, S. Matsuzaki, *J. Biochem.*, 91, 1321-8(1982).
5. N. Seiler, B. Knodgen, F. Eisenbeiss,, *J. Chromatogr.*, 145, 29-32(1978).
6. T. Hayashi, T. Sugiura, S. Kawai and T. Ohno, *J. Chromatogr.*, 145, 141-6(1978).
7. J. W. Redmond, A. Tseng, *J. Chromatogr.* 170, 479-81(1979).
8. S. Wongyai, P. J. Oefner, G. K. Bonn, *J. Liq. Chromatogr.*, 12, 2249-61(1989).
9. R. Porta, R. L. Doyle, O. Sellinger, S.B. Tatler, T.E. Wilens, R.A. Schatz and O.Z. Sellinger, *J. Neurochem.*, 37, 723-9(1981).
10. D. R. Roberts, M. A. Walker, E. B. Dumbroff, *Phytochem.*, 24, 1089-90(1985).

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A SENSITIVE HPLC METHOD FOR THE DETERMINATION OF TERFENADINE AND ITS METABOLITE IN HUMAN PLASMA

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ABSTRACT

A sensitive and selective HPLC assay with fluorescence detection was developed for the analysis of terfenadine and its acid metabolite in human plasma. The compounds were isolated from plasma by liquid extraction with methyl-t-butyl ether:isopropyl alcohol (95:5 % v/v). The chromatographic separation was carried on cyanopropylsilane column (15 cm X 4,6 mm) with a mobile phase consisting of 0.001 M acetate buffer, pH 4.0 : acetonitrile (25:75 % v/v). The eluent was monitored at 230 nm excitation and 300 nm emission wavelengths with a 270 nm cut-off filter. The range of quantification was 2 to 1000 ng/ml for terfenadine and 5 to 1000 ng/ml for acid metabolite, respectively. The assay showed linearity over the range of quantification ($r^2 > 0.998$). This method has been applied to the analysis of human plasma samples.

INTRODUCTION

Terfenadine (TRF) is a non-sedating H1-receptor antagonist used in the treatment of allergic rhinitis[1]. Terfenadine undergoes extensive first-pass metabolism (over 99% of absorbed dose) resulting in two metabolites[2,3]. The major metabolite, a carboxylic acid analogue of terfenadine, has approximately one third of the antihistaminic activity of terfenadine. The peak plasma levels of terfenadine are below 10 ng/ml after administration of single or twice daily doses of 60 mg tablet[4]. However, the active metabolite levels are much higher and readily detectable for approximately 16 hours. Few analytical methods for the determination of terfenadine and its acid metabolite have been reported[5-7]. However, these methods lack good sensitivity or specificity[7]. The present paper describes a simple, sensitive, and selective HPLC method for determination of terfenadine and its acid metabolite in human plasma.

MATERIALS AND METHODS

Chromatographic System

Chromatographic determination was performed using a Hewlett Packard model 1050 liquid chromatograph equipped with a model 1046 A HP fluorescence detector, an autosampler, and a 3390 a integrator. The chromatography was carried out using a Microsorb 5 μm cyanopropylsilane (15 cm X 4.5 mm ID) column (Rainin, CA, USA) maintained at ambient temperature. The eluent was monitored at 230 nm excitation and 300 nm emission wavelengths with a cut-off filter at 270 nm.

Reagents

Terfenadine and acid metabolite were obtained from KV Pharmaceuticals and Teva Pharmaceuticals, respectively. Propranolol was obtained from Sigma (St.Louis, MO, USA). Methyl-t-butyl ether, isopropyl alcohol, and acetonitrile (all HPLC grade) were obtained from EM Science. All other chemicals were of analytical grade.

Drug Solutions

A 200 μg/ml stock solutions of terfenadine and its acid metabolite were prepared in methanol. Working solutions were prepared from the stock solution.

Internal Standard (IS) and Extraction Solutions

A 100 μg/ml stock solution of propranolol was prepared in methanol. The final extraction solution consisted of 50 ng of propranolol per ml of methyl-t-butyl ether:isopropyl alcohol (95:5 % v/v).

Mobile Phase

Sodium acetate buffer 0.001 M was prepared in deionized distilled water, and the pH was adjusted to 4.0 with acetic acid. The mobile phase consisted of acetonitrile and buffer (75:25 % v/v).

Preparation of Plasma Standards

To 1 ml of plasma in a 15 ml screw capped centrifuge tube was added an aliquot of drug solution containing 2 to 1000 ng of terfenadine and acid metabolite, 0.5 ml of 0.1 N HCl, and 5 ml of extraction solution containing internal standard. The tubes were vortexed for 20 seconds and centrifuged for 15 min at 3000g. The organic layer was transferred to clean evaporating tube. The aqueous phase was re-extracted with another 5 ml of extraction solution and the pooled organic fraction was evaporated to dryness under gentle stream of nitrogen at 40^0 C. The residue was dissolved in 100 μl methanol and 40 μl was injected onto the column.

Quantification

Standard curves for plasma were constructed using four replicates at each concentration (2 - 1000 ng/ml). The peak height ratios of standard to the IS were plotted against concentration (ng/ml).

TABLE I
Absolute Recovery of Terfenadine and Acid Metabolite from Plasma

Concentration (ng/ml)	% Recovery	
	Terfenadine	Acid metabolite
50	100.9	85.33
100	92.64	92.55
250	95.06	85.1
500	94.20	81.12

Absolute Recovery

The absolute recovery of terfenadine and acid metabolite from plasma was determined at final concentrations of 50 - 500 ng/ml plasma. The recoveries were calculated by comparing the peak heights of extracted spiked sample to the peak heights of methanolic standard solutions of the same concentration.

RESULTS AND DISCUSSION

The chromatographic column and conditions were chosen to provide complete resolution of terfenadine, acid metabolite, and internal standard. Figure 1 shows the chromatograms obtained from blank plasma and plasma spiked with 100 ng/ml of terfenadine and its acid metabolite. The peaks are sharp and well resolved from each other without any interference from endogenous plasma constituents. Retention time for acid metabolite, propranolol(IS), and terfenadine were 9.5, 12.2, and 15.1 minutes, respectively.

The absolute recovery results are presented in Table I. The present extraction procedure resulted in absolute recoveries ranging from 81.12 to 92.55 % for acid metabolite and from 92.24 to 100.90 % for terfenadine. This method produced superior absolute recoveries for terfenadine and comparable or better recoveries for acid metabolite than those reported in the previous method[5]. Propranolol was chosen as the internal standard since it extracts with the same extraction solution as the drug and metabolite. Propranolol also separates well from the drug and metabolite, and can be detected by fluorescence under the same conditions.

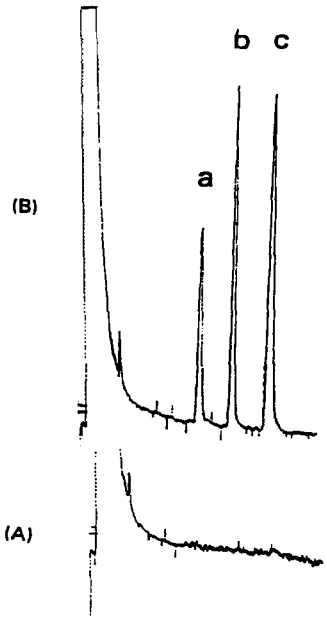

FIGURE 1. Chromatograms of (A) blank plasma (B) extract of plasma containing 100 ng/ml of tefenadine and acid metabolite.
Peaks: a - acid metabolite(9.5 min) , b - propranolol (12.2 min) c - terfenadine(15.1 min).

The ratio of the peak height of terfenadine or its metabolite to the peak height of internal standard was plotted against drug concentration spiked plasma. Standard curves were linear over the concentration range 2 to 1000 ng/ml for terfenadine and 5 to 1000 ng/ml for acid metabolite, with correlation coefficients (r^2) consistently greater than 0.998. The sensitivity of the assay was 2 ng/ml for terfenadine and 5 ng/ml for the acid metabolite. Intra-day and inter-day accuracy and precision are presented in Table II and Table III,

TABLE II

Intra-day Validation of the Assay for Terfenadine and its Acid Metabolite (n = 4).

TERFENADINE

Amount added	Amount found	Accuracy	CV
2	1.95 ± 0.11	97.50	5.60
5	4.17 ± 0.10	83.41	8.50
10	8.66 ± 0.30	86.67	3.40
25	24.7 ± 1.40	98.80	5.20
50	48.03 ± 1.31	96.06	2.71
100	87.34 ± 1.52	87.34	1.38
250	267.08 ± 9.06	106.80	3.40
500	536.08 ± 8.08	107.20	1.50
1000	980.5 ± 23.22	98.10	2.40

TERFENADINE ACID METABOLITE

Amount added	Amount found	Accuracy	CV
5	5.69 ± 0.1	113.7	2.0
10	9.33 ± 1.0	93.3	10.7
25	25.95 ± 1.0	103.8	3.9
50	49.15 ± 7.8	98.30	15.9
100	91.33 ± 0.5	91.33	1.0
250	256.34 ± 7.9	102.50	3.1
500	522.25 ± 7.9	102.50	3.1
1000	989.3 ± 12.5	98.93	1.3

respectively. The intra-day precision showed a CV range of 1.38 to 8.5 % for terfenadine and 1.0 to 15.9 % for acid metabolite. The Inter-day precision CVs varied from 1.8 to 14.3 % for terfenadine and 1.0 to 8.7% for acid metabolite.

A variety of drugs such as pseudoephedrine, ibuprofen, aspirin, acetaminophen, and tricyclic antidepressants which might be coadministered were tested for interference. The results indicated no

TABLE III

Inter-day Validation of the Assay for Terfenadine and its Acid Metabolite (n = 4).

TERFENADINE

Amount added (ng/ml)	Amount found (ng/ml)	Accuracy (%)	CV (%)
2	1.71 ± 0.14	85.5	7.8
5	5.712 ± 0.4	114.2	6.5
10	9.36 ± 0.4	93.6	4.2
25	26.3 ± 0.9	105.0	3.2
50	54.04 ± 7.7	108.08	14.3
100	89.22 ± 1.67	89.20	1.8
250	267.63 ± 6.1	107.10	2.2
500	483.79 ± 17.9	96.80	3.7
1000	1004.5 ± 13.9	100.50	8.7

TERFENADINE ACID METABOLITE

Amount added (ng/ml)	Amount found (ng/ml)	Accuracy (%)	CV (%)
5	5.59 ± 0.4	111.4	6.7
10	9.24 ± 0.5	92.4	4.3
25	26.14 ± 1.0	104.6	3.2
50	53.26 ± 4.6	106.50	8.7
100	94.19 ± 7.5	94.19	7.9
250	247.77 ± 14.9	99.19	7.9
500	510.87 ± 4.6	102.00	1.0
1000	995.83 ± 4.8	99.50	1.0

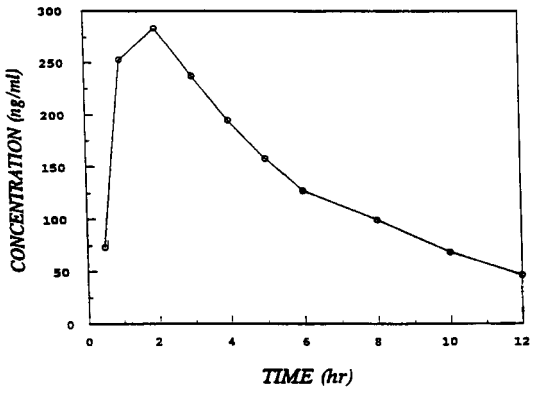

FIGURE 2. Mean plasma concentration profile of acid metabolite

interference from these compounds with the peaks of the drug, metabolite, or internal standard.

This method has been successfully applied to the analysis of plasma samples of five subjects. A typical mean plasma concentration-time profile for acid metabolite following oral administration of 60 mg of terfenadine is shown in Figure 2.

CONCLUSION

The HPLC method described in this paper offers excellent separation of terfenadine, its acid metabolite, and internal standard under isocratic conditions. The method provides excellent recoveries, linearity, and reproducibility. The sensitivity, selectivity, and linearity of this method makes it applicable for bioavailability or pharmacokinetic studies.

REFERENCES

1. D. Mctavish, K.L. Goa, M. Ferril, Drugs, 39(4):552-574(1990).
2. D.A. Garteiz, R.H. Hook, B.J. Walker, R.A. Okerholm, Arzneim. Forsch., 32:1185-1190(1988).
3. G.A. Leeson, K.Y. Chan, W.C. Knapp, S.A. Biedenbach, G.J. Wirght, R.A. Okerholm, Arzneim. Forsch., 32:1173-1178(1982).
4. J.K. Woodward, Cutis, 42:5-9(1988).
5. J.E. Coutant, P.A. Westmark, P.A. Nardella, S.M. Walter, R.A. Okerholm, J. Chromatgr., 570:149-156(1991).
6. F.E.R. Simmons, W.T.A. Watson, K.J. Sommons, J. Allergy Clin. Immunol., 80(6):884-889(1987).
7. C.E. Cook, D.L. Williams, M. Myers, C.R. Tallent, G.A. Leeson, R.A. Okerholmm, G.J. Wright, J. Pharm Sci., 69:1419-1423(1980).

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HPLC DETERMINATION OF RESIDUAL IVERMECTIN IN CATTLE DUNG FOLLOWING SUBCUTANEOUS INJECTION

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ABSTRACT

Residual ivermectin in cattle dung was determined in order to measure its rate of excretion and persistence under field conditions. The drug was extracted into methanol and subsequently determined by reversed-phase high performance liquid chromatography using a 300 × 3.9 mm Bondclone C_{18} column, 47:33:20 acetonitrile/methanol/water as the mobile phase and UV detection at 245 nm. The determination limit for 5 g of sample was 0.020 mg kg^{-1}. HPLC analyses for aminoacids in the dung were suggestive of changes in their contents with time, which may account for the differential behaviour of insects attracted by the dung.

INTRODUCTION

Many different helminthicidal products used to treat animals are eventually found in their excrement (urine and faeces). Some of the products only have a limited action on the coprophagous fauna, but others destroy virtually all insects attracted to the excrement over a more or less long period (1). Products recently introduced to the market have a wide spectrum of activity.

Ivermectin (22,23-dihydroavermectin B_1), one of the most commonly used substances in preventative treatments, is a highly effective systemic antiparasitic against both internal (gastrointestinal and respiratory) parasites and external parasites (flies, ticks) (2). This drug is usually administered via subcutaneous injections in typical doses of 200 $\mu g\ kg^{-1}$ body weight; most of the injected substance is subsequently excreted as such in faeces depending on the particular animal or administration route (3). Ivermectin in faeces preserves its insecticide action, which has fostered studies on its potential environmental impact, *i.e.* its effect on non-target organisms in the soil (5–10). Many such studies were carried out in laboratory conditions and provided results that differed markedly from those obtained under real conditions, as we have recentlky shown the potential impact of the drug depends on the particular region, climate, insect fauna and —probably— year season (11).

Deriving a factual cause–effect correlation entails the accurate measurement of residual amounts of the drug in animal excretions. Initially, this was done by using high performance liquid chromatography (HPLC) with derivatization and subsequent fluorescence detection of the derivative obtained, this derivatization

procedure, based on various reactions, is usually labour-intensive (12–16), so UV detection procedures have also been used , usually at 245 nm (17–23). These procedures have so far been most often applied to such samples as serum, plasma, milk, vegetables and swine tissues, using a prior extraction–cleanup procedure with acetonitrile, methanol/acetonitrile, methanol or ethyl acetate and the aid of sonication or maceration, or, alternatively, solid–liquid extraction cartridges and later the extracts are preferably analyzed on a C_{18} column and with ternary mobile phases ($CH_3CN/CH_3OH/H_2O$).

To extract the drug from the faeces two procedures have been mainly proposed: extraction with acetone (24) or a Soxhlet (6 h) with an ethylacetate/methylene chloride mixture(25).

In this work, a HPLC procedure for the determination of residual ivermectin in cattle dung was developed in order to quantify the drug excretion by cattle and its environmental persistence under field conditions. At a subsequent stage, dung samples were used to determine aminoacids, by using a procedure originally developed for rat faeces (26), in order to study the anomalous behaviour of insects in the field.

EXPERIMENTAL

Reagents

Procaine and aminoacid standards were supplied by Sigma Aldrich Química S.A. (Madrid, Spain). An ivermectin standard and Ivomec^R were supplied by Merck Sharp & Dohme de España S.A. (Madrid, Spain). HPLC gradient-grade methanol

and acetonitrile were purchased from Scharlau (Barcelona, Spain). Finally, nanopure water obtained from a Milli-Q apparatus (Millipore Ibérica, Madrid, Spain) was used throughout.

Apparatus and chromatographic conditions

Ivermectin analysis

The experimental setup used for this purpose was composed of a CM4000 gradient pump, a variable-wavelength SM4000 detector, a CI4000 recorder–integrator and a Dynamixer eluent mixer, all from LDC Analytical (Riviera Beach, FL), in addition to a Marathon Injector from Spark Holland (Emmen, The Netherlands) and a 300 × 9 mm Bondclone 10C18 column from Phenomenex (Torrance, CA). The mobile phase used consisted of 47:33:20 acetonitrile/methanol/water and was passed at a flow-rate of 1 ml/min. The injected volume was 20 μl and degassing was done with helium.

Aminoacid analysis

The assembly used for aminoacid analyses consisted of a mechanical grinder and a Cryodos lyophilizator from Teistar (Terrassa, Barcelona, Spain), a Heidolph tube shaker from Selecta (Barcelona, Spain), and a Pico Tag workstation composed of two 510 pumps, a Satellite WISP autoinjector, a TCH column oven, a UV detector, a System Interface Module and a Maxima Chromatographic workstation, all from Waters Associates (Millipore, Milford, MA). The injected volume used was 8 μl.

Sampling

Faeces were collected from a group of 7 Morucha steers (276 ± 18.6 kg body weight) which had been treated with a subcutaneous injection of ivermectin (Ivomec^R) at 200 μg kg^{-1} body weight. Samplings were done at different times (days) after injection. Additional faeces samples were collected from another control group consisting of 100 animals that were given no Ivomec^R.

Several experiment series were carried out in order to determine the drug excretion rate and its degradation under the prevailing field conditions.

Samples were divided into portions that were analysed by the three participating workgroups (11). All the samples were stored frozen prior to analysis.

Procedures

Ivermectin analysis

An amount of 5 g of thawed sample was added 25 ml of methanol and the mixture was stirred for 25 min, after which it was centrifuged at 1500 G for 15 min. The supernatant was then concentrated to 7 ml in a rotary evaporator at 80°C and centrifuged under the same conditions as the initial mixture. The resulting extract was added the internal standard at a concentration of 50 ppm and the mixture was made to 10 ml with methanol and filtered through PTFE (Ø = 13 mm, 0.50 μm mesh). Aliquots of 20 μl of the filtrate were used for injection into the chromatograph.

Aminoacid analyses

An amount of 8 g of frozen dung was lyophilized. The lyophilizate was then ground and a portion of 50 mg was weighed and placed in a Corning tube that was introduced into the Pico Tag workstation, where it was subjected to hydrolysis, derivatization and chromatographic analysis.

RESULTS AND DISCUSSION

Extraction of ivermectin from cattle dung

Experiments were initially carried out by using raw dung and then dung + ivermectin and afterwards dung + Ivomec^{R}. Water, water/methanol, methanol and the mobile phase were assayed as extractants and the influence of the solvent volume and extraction time and temperature was studied in order to develop a straightforward, expeditious extraction procedure for the samples.

All the solvents tested gave rise to a common front at 4 min in the experiments involving raw dung. Methanol gave an additional, small peak at 5.80 min which increased in size with the increasing alcohol content in water/methanol mixtures. The mobile phase also gave a further peak at 8.00 min (Fig. 1). Because such peaks would make the chromatogram background and pure ivermectin eluted at 12 min, an internal standard not affected by the background was required. Several compounds were assayed and procaine, which eluted at 7 min, was chosen for this purpose.

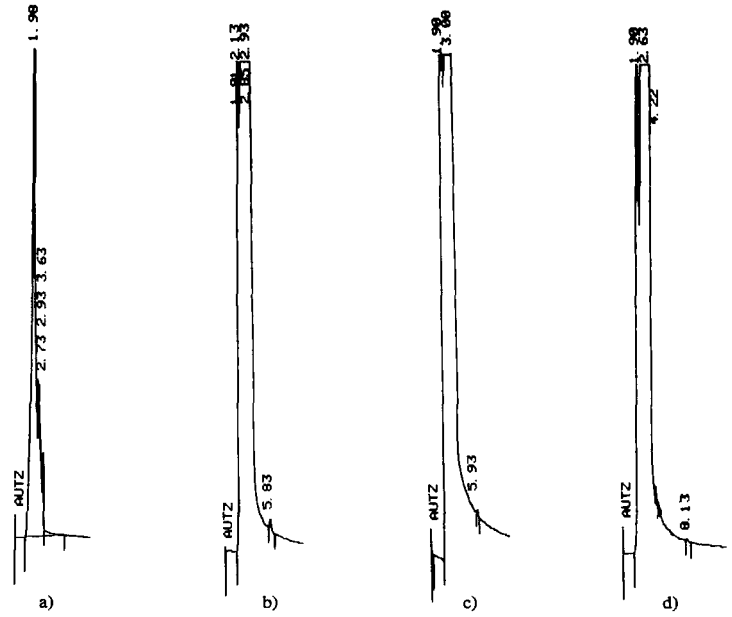


Figure 1.- Chromatograms obtained from raw samples extracted with: a) water, b) methanol, c) water-methanol mixtures, d) mobile phase.

As the analyses of dung samples fortified with ivermectin and Ivomec^{R} provided identical results, in the next paragraphs are described in a common way.

Variable water volumes (10–40 ml) used at different temperatures (20–100°C) and shaking times (10–40 min) provided chromatographic backgrounds that were similar to that described above, the peak for ivermectin did not appear. This suggests that water does not leach ivermectin from dung.

Adding methanol to the water allowed some of the drug to be extracted. However, the extraction yield never exceded 60% at any methanol/water ratio

—the effect of the alcohol content in the mixture was much more marked than that of the time, temperature or extractant volume used.

By using methanol or the mobile phase as extractant, drug recoveries over 90% were obtained at room temperature.

Figure 2 summarizes the results provided by the different solvents that extracted the ivermectin , of which methanol was finally chosen in order to reduce analytical costs and avoid the appearance of the above-mentioned small peak at 8 min. The nature of the matrix implies two centrifugation steps in order to remove solid residues.

Quantitative analysis

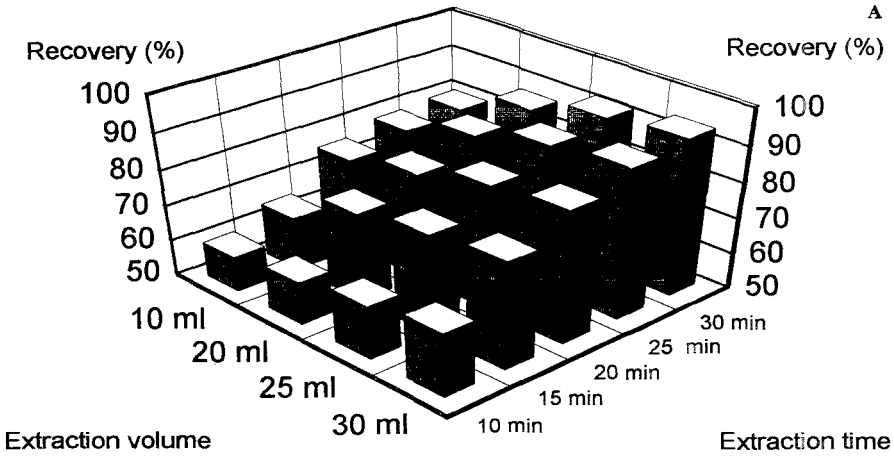
Application of the proposed procedure to the cattle dung samples provided chromatograms such as that shown in Fig. 3.

The calibration graph run from fortified dung samples was linear and fitted the equation

$$(A_i/A_s)C_s = 4.09\ C_i - 0.126$$

where A_i and A_s denote the peak areas for ivermectin and procaine, C_s the internal standard concentration and C_i the ivermectin concentration. The variance was 3.680×10^{-4}.

The detection and determination limits obtained for 5 g of sample were 0.010 and 0.020 mg kg^{-1} dung, respectively.



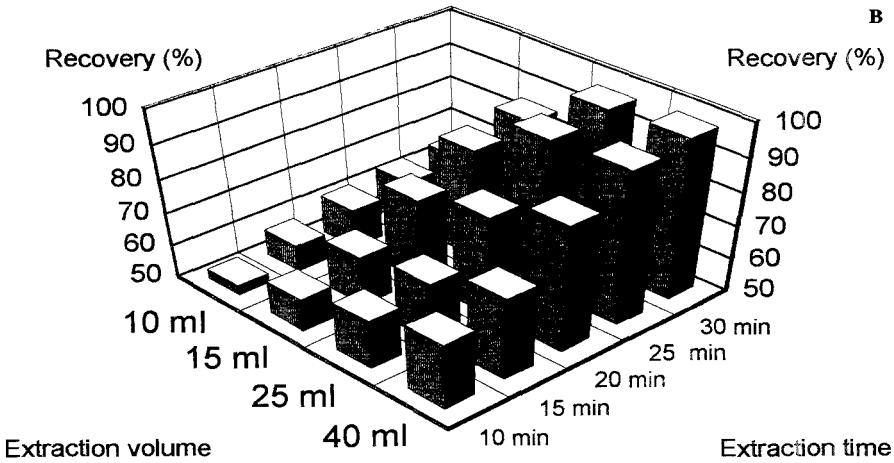


Figure 2.- Ivermectin recovery % vs shaking time and extraction volume with: a) methanol, b) mobile phase, c) 75% water-25% methanol, d) 50% water-50% methanol e) 25% water-75% methanol.

(continued)

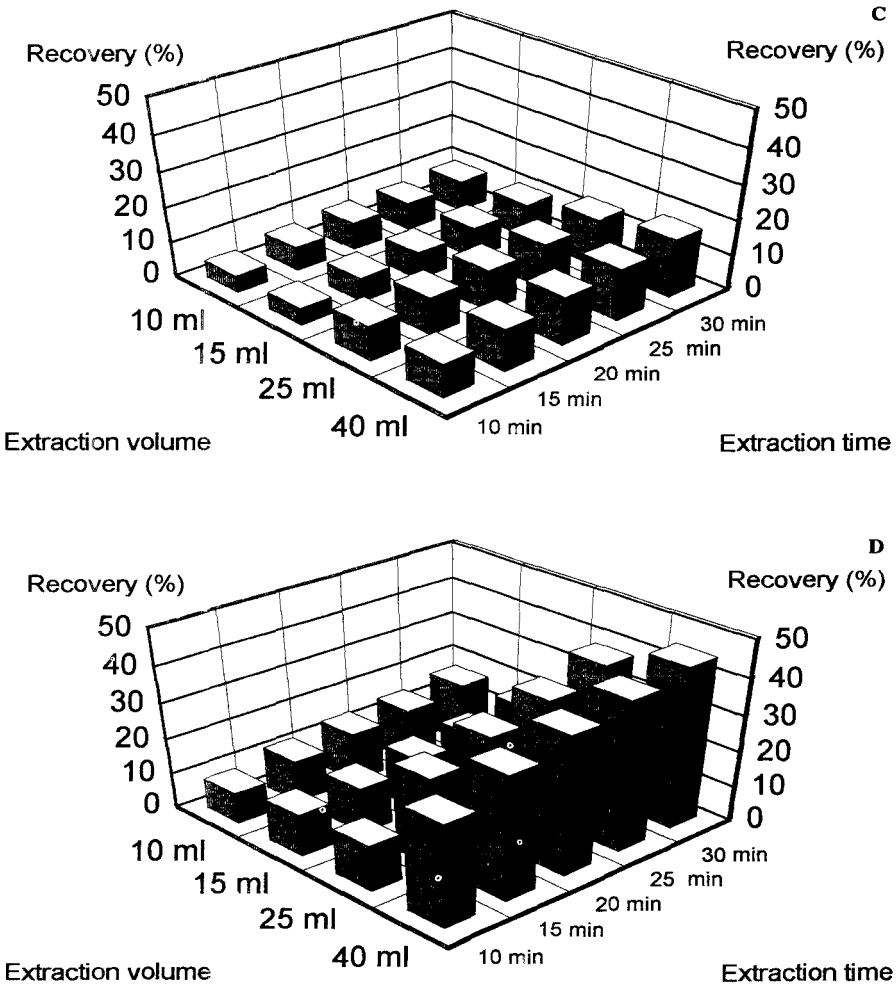


Figure 2 *(continued)*.

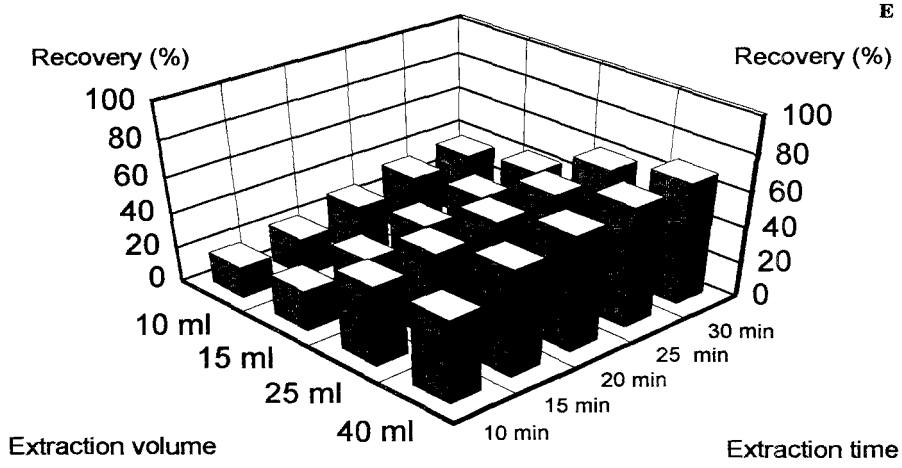


Figure 2 *(continued)*.

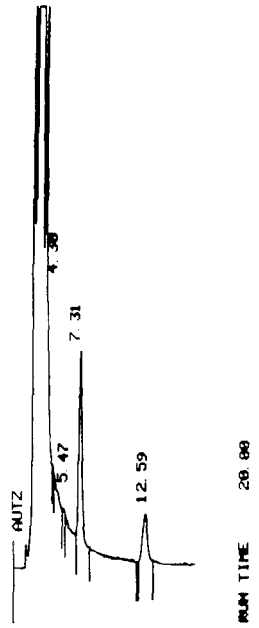


Figure 3.- Chromatogram of a sample fortified with internal standar.

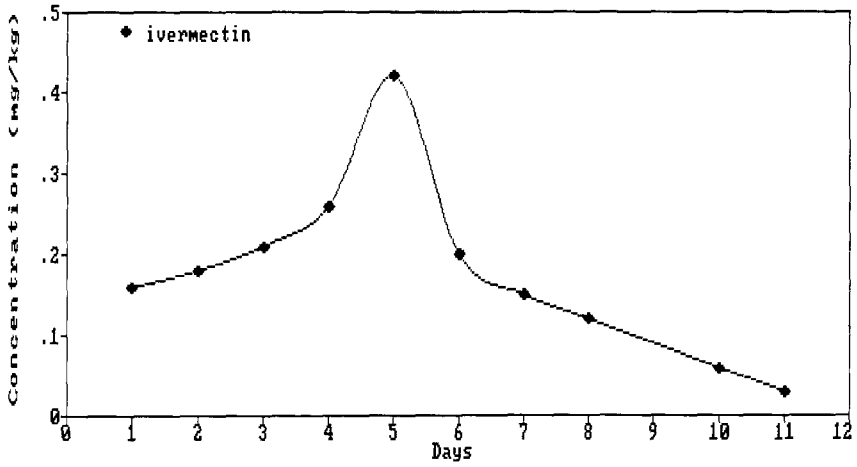


Figure 4.- Ivermectin excretion profile in cattle dung after injection.

Excretion of ivermectin by cattle

Figure 4 shows the variation of the amount of ivermectin found in the dung with time. As can be seen, such an amount peaked *ca.* 5 days after injection and decreased sharply afterwards to levels below the detection limit at 12 days.

A series of dung samples were exposed to field conditions for up to 30 days; none of them was found to contain any ivermectin after 6–7 days; however, the absence of the drug was followed by an increased attractiont of beetles, which were collected from pitfall traps baited with dung. These results (11) suggest that some biochemical process have led to *in situ* modifications in the composition of dung from treated animals,resulting in increasing attractiveness, as reflected in the fetid odour of the dung, similar to that of faeces from omnivores. This led us to

analyse the dung for aminoacids during the exposure period in order to detect any differences in the dung composition before and after treatment.

Aminoacid analyses

As can be seen from Fig. 5, the aminoacid profiles for the treated and untreated samples were indeed different. Analysis on samples previously subjected to ivermectin determinations also revealed the aminoacid contents to change with time after injection. The changes, however, varied from compound to compound. Thus, the concentrations of glutamic and aspartic acid rose up till the fourth day, and later they dropped abruptly. On the other hand, the histidine and methionine concentrations increased slightly for 7 days and then decreased gradually. The alanine, valine and leucine give the highest peaks in the fourth and seventh day, whereas that proline increased throughout the period studied and the other aminoacids assayed (serine, arginine, threonine, tyrosine, isoleucine, phenylalanine and lysine) remained essentially constant.

CONCLUSIONS

Extraction of ivermectin with ethanol is a straightforward procedure that provides drug recoveries close to 100%.

Ivermectin is rapidly excreted by cattle. Its concentration in dung increased daily in pats dropped on days one to four after injection. A peak of elimination was observed for day 5 followed by a quick decrease. After the twelfth day, the drug concentration is below the detection limit of the used procedure.

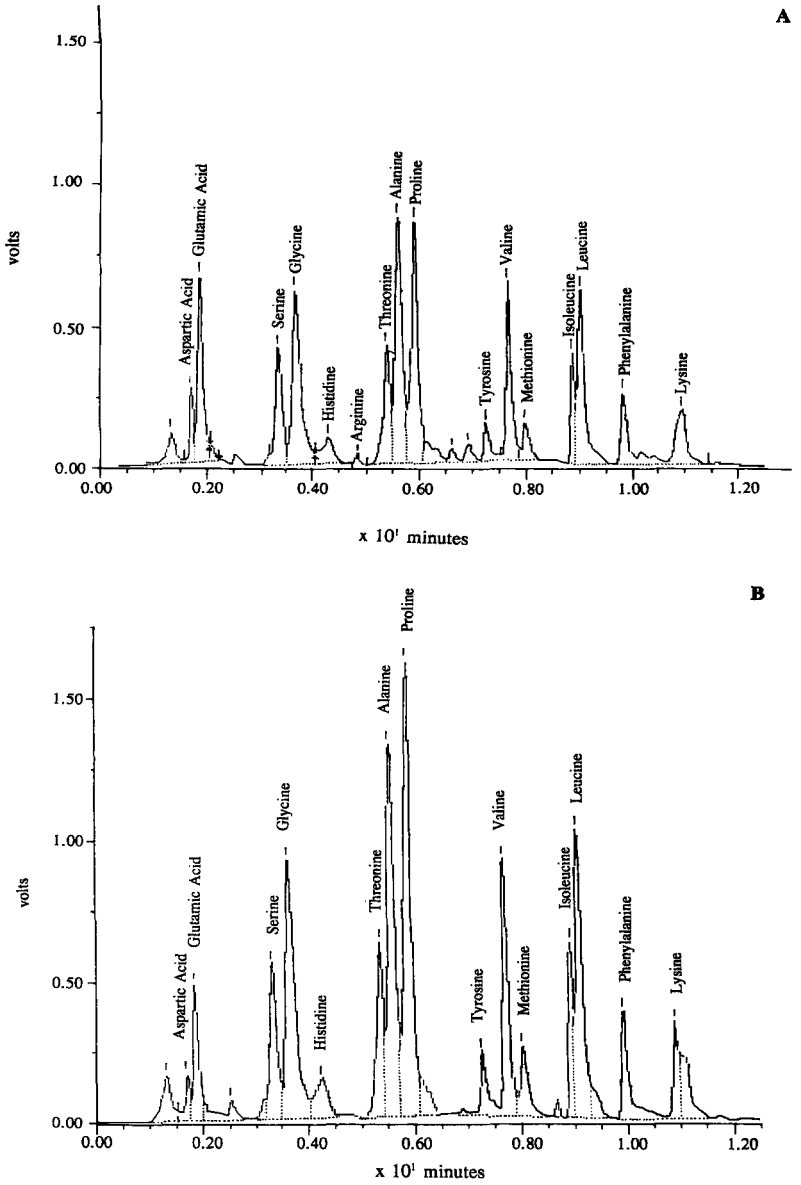


Figure 5.- Chromatograms showing the aminoacids found in cattle dung before (a) and after (b) drug administration.

The residual concentration of ivermectin in dung exposed to field conditions decreases rapidly to undetectable levels after 6 days, when the dung whitens and develops a fetid odour. Also, the aminoacid concentrations decrease after 6–7 days, which might explain the anomalous attraction of the beetles.

REFERENCES

1. J.P. Lumaret, Acta Oecologia Oecol. Applic. 7(4), 313-324 (1985).

2. W.C. Campbell, M.H. Fisher, E.O. Stapley, G. AlbersSchönberg and T.A. Jacob. Science, 221, 823-828 (1983).

3. W.C. Campbell, Ivermectin and Abamectin. Springer-Verlag. New York. 1989.

4. C.D. Schmidt, Environmental Entomology, 12, 455-457 (1983).

5. J.A. Miller, S.E. Kunz, D.D. Oehler and R.W. Miller, Journal of Economic Entomology, 74, 608-611 (1981).

6. T.J. Ridsdill-Smith, Journal of the Australian Entomology Society, 27, 175-178 (1988).

7. K.G. Wardhaugh and H. Rodriguez-Menendez, Journal of Applied Entomology 106, 381-389 (1988).

8. R. Wall and L. Strong, Nature 327, 418-421 (1987).

9. B. Houlding, T.J. Ridsdill-Smith and W.J. Bailey, Australian Veterinary Journal 68, 185-186 (1991).

10. K.G. Wardhaugh and R.J. Mahon, Bulletin of Entomological Research 81, 333-339 (1991).

11. J.P. Lumaret, E. Galante, C. Lumbreras, J. Mena. M. Bertrand, J.L. Bernal, J.F. Cooper, N. Kadiri and D. Crowe, Journal of Applied Ecology (corrected proofs) 1993.

12. J.W. Tolan, P. Eskola, D.W. Fink, H. Mrozik and L.A. Zimmerman, J. Chromatogr. 190, 367-376 (1980).

13. P.C. Toway, J.S. Wood and G.V. Downing, J. Agric. Food Chem. 29, 1051-1060 (1981).

14. J.D. Stong, Anal. Chem. 59, 266-270, (1987).

15. R. Chiou, R.J. Stubbs and W.F. Bayne, J. Chromatogr. Biomed. Appl. 60 (416), 196- 202 (1987).

16. I. Nordlander and H. Johnsson, Food Addit. Contam. 7 (1), 79-82 (1990).

17. H.J. Schnitzerling and J. Nolan, Anal. Chem. 68, 37-40 (1985).

18. S.J. Doherty, A. Fox and D.W. Fink, J. Assoc. Anal. Chem. 73 (6), 931-934 (1990).

19. D.D. Oehler and J.A. Miller, J. Assoc. Anal. Chem. 72 (1), 59-65 (1989).

20. S.H.L. Chiu, E. Sestokas and R. Taub, J. Chromatogr. Biomed. Appl. 77, 433, 217-224 (1988).

21. M. Alvinerie, J.F. Sutra, P. Galtier and P.L. Toutain, Ann. Rech. Vet. 18 (3), 269-274 (1987).

22. C.M. Dickinson, J. Chromatogr. Biomed. Appl. 93 (528), 250-257 (1990).

23. J. Vuik, J. Agric. Food. Chem. 39 (2), 303-305 (1991).

24. B.A. Halley, R.J. Nessel and A.Y.H. Lu, Ivermectin and Abamectin (ed. N.C. Campbell). pp. 162-172, Springer-Verlag. New York. 1989.

25. J.F. Cooper, Recherches menees dans le cadre du contrat SRTIE/MERE/90038 Operation n° 237.01.90.40037. France (1991).

26. G. Sarwar, H.G. Botting and R.W. Peace, J. Assoc. Off. Anal. Chem. 71 (6), 1172-1175 (1988).

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A PROCEDURE FOR THE SEPARATION AND QUANTITATIVE ANALYSIS OF ASCORBIC ACID, DEHYDROASCORBIC ACID, ISOASCORBIC ACID, AND DEHYDROISOASCORBIC ACID IN FOOD AND ANIMAL TISSUE*

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ABSTRACT

A procedure is presented for the direct and simultaneous determination of ascorbic acid (AA) and isoascorbic acid (IAA) in food products and animal tissues by reverse phase high-performance liquid chromatography. Two PLRP-S columns in series were used with a pH 2.2 mobile phase containing 20 mM phosphate buffer and 0.17% metaphosphoric acid. An amperometric detector set at 0.7 volt and 20 mA was used. As little as 0.5 ng of each compound could be detected. When the same samples were incubated with homocysteine to reduce dehydroascorbic acid (DHAA) and dehydroisoascorbic acid (DHIAA) to AA and IAA respectively and reinjected into the system, the values for total AA and IAA were obtained. The concentration of the oxidized forms, DHAA and DHIAA, could then be calculated by substraction.

INTRODUCTION

D-Isoascorbic acid (IAA), also known as erythorbic acid or D-araboascorbic acid, is a C-5 epimer of L-ascorbic acid (AA) and has 5% of the vitamin activity of AA

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(1,2). In spite of this, for economic reasons, IAA is frequently used as an antioxidant in foods (3,4).

There are several highly sensitive high-performance liquid chromatography (HPLC) methods for the direct measurement of AA in foods and biological fluids (5). Some of these methods are also able to measure dehydroascorbic acid (DHAA) (6-8) and/or IAA (8-12). The method of Kutnink et al. (10) has a high sensitivity for the measurement of AA, IAA and uric acid in human plasma; however, it does not measure DHAA. On the other hand, the method of Vanderslice and Higgs (8,9) separates and quantifies AA and IAA and their corresponding oxidised forms, but is not as sensitive as the aforementioned method because it requires a post-column derivatization.

We reported a procedure for the direct and simultaneous determination of AA and IAA in food products by paired-ion reverse-phase high performance liquid chromatography with electrochemical detection (13). When the same samples were incubated with homocysteine to reduce DHAA and dehydroisoascorbic acid (DHIAA) to AA and IAA respectively and reinjected into the system, the values for total AA and IAA were obtained. The concentration of the oxidized forms could then be calculated by subtraction. The procedure is highly sensitive but was subject to interferences when it was applied to biological fluids and tissues. Modifications to this procedure are reported here and include the use of polystyrene divinyl benzene polymer (PLRP-S) columns as proposed by Vanderslice and Higgs (9) but conserving our approach of treating the sample with homocysteine (7,14) in order to estimate AA, IAA, DHAA and DHIAA by a double injection into the HPLC system.

MATERIALS AND METHODS

HPLC System

A chromatographic system (Spectra-Physics, San Jose, CA) consisting of a SP8800 pump, SP8760 autosampler cooler, SP8780 autosampler and a Chromjet

integrator was used. A pulse dampener (SSI, Supelco, Oakville, ONT) was incorporated into the system. The amperometric detection system (BAS, West Lafayette, IN) included an LC-4B controller and an electrode flow cell consisting of a glassy carbon electrode, a stainless steel electrode top, and an Ag/AgCl reference electrode. The applied potential was +0.7 volts (oxidative) and the sensitivity range was 20 mA. Two 25 cm x 4.6 mm, 5 μm particle size, PLRP-S columns (Polymer Laboratories Inc., Amherst, MA) connected in series were used to separate AA and IAA. For food analysis, the columns were kept at 22°C and for biological fluids and tissue analysis they were cooled to 5°C. The mobile phase was 20 mM sodium phosphate monobasic, monohydrate containing 0.17% metaphosphoric acid at a final pH 2.2. The mobile phase was filtered through a 0.22 μm GS filter (Millipore Corp., Bedford, MA), degassed for half hour using a water aspirator and followed by bubbling helium continuously. For food analysis the flow was 0.7 ml/min at 22°C, but for tissues the flow was set at 0.6 ml/min and the columns cooled at 5°C. Samples and standards in a volume of 20 μl were injected into the chromatograph using the autosampler, maintained at 4-5°C. A calibration curve of at least 5 standards was run daily. Peak area for these standards were stored and the integrator automatically calculated a quadratic fit through the levels. Sample peaks were automatically compared with the calibration curve in order to calculate sample concentration.

Sample Preparation

Samples (0.5-3.0 g) were homogenized with a polytron homogenizer in enough cold 17% metaphosphoric acid (J.T. Baker Inc. Phillipsburg, NJ) to give a final concentration of 0.85% (w/v) as described by Pelletier and Brassard (15). Homogenates were centrifuged at 30,000 x g in a refrigerated centrifuge for 30 min. The supernatant was filtered through a Millex-GS 0.22 μm filter unit (Millipore, Bedford, MA). Usually 500 μl of clear supernatant was mixed with 115 μl 45% K_2HPO_4 buffer

pH 9.8, to give a final pH of 7.1. After maintaining the mixture at 25°C for 30 min, 0.85% metaphosphoric acid was added to bring the final volume to 2 ml. A 100 µl aliquot of the treated supernatant was diluted to 10 ml with mobile phase buffer. A 20 µl aliquot of this was injected into the system. This procedure allowed for the determination of AA and IAA. For the determination of DHAA and DHIAA, a second aliquot of clear supernatant was mixed with 45% K_2HPO_4 buffer, pH 9.8, containing 1% homocysteine (Sigma Chemical Co., St. Louis, MO) and kept at 25°C for 30 min. The rest of the procedure was identical to that used for AA and IAA. A second 20 µl injection in the HPLC system resulted in values for AA + DHAA and IAA + DHIAA. Therefore the concentrations of DHAA and DHIAA could be calculated by subtraction.

Preparation of Standards

AA (BDH Chemicals Ltd. Poole, England) and IAA (Sigma Chemical Co, St. Louis, MO) stock standards were prepared at a concentration of 2.5 mg/ml with 0.85% metaphosphoric acid. Intermediate standards (50 µg/ml) were prepared by diluting the stock standards 1:50 with metaphosphoric acid. The intermediate standards were used to prepare a calibration curve. Usually, 500 µl aliquots of several diluted intermediate standards were treated with 45% K_2HPO_4 buffer, pH 9.8, containing 1% homocysteine and kept at 25°C for 30 min. After diluting the standard in the same form as the sample, 20 µl aliquots containing 0.5 to 2.5 ng of AA and IAA, were injected into the HPLC system.

Samples

Processed meats and other foods were obtained from local supermarkets. Plasma and tissues were obtained from five male Wistar rats (Charles River, Canada, St. Constant, Quebec) that were fed an AIN-76 diet (16) for three months. After this period, they were fed for 5 days the same diet but containing 100 g IAA/kg diet.

Tissues were collected from animals anesthetized with halothane (2% in oxygen) (Fluothane, Ayerst Laboratory, Montreal). Plasma and samples of liver, adrenal gland and spleen were stored at -75°C until analysis.

RESULTS AND DISCUSSION

A chromatogram of standards is shown in figure 1. The AA and IAA peaks, 1 ng each, were well resolved on a stable baseline. Usual retention times were 15.6 min for AA and 18.3 min for IAA. These retention times were reproducible and changed slightly with different batches of mobile phase. Nevertheless, the separation of the AA and IAA peaks remained 1.7 to 1.8 min apart. In our previous method (13), the retention times were 27.3 for AA and 28.6 min for IAA, therefore two PLRP-S columns in this case performed much better than three C18 reverse-phase columns even in more adverse conditions. The columns were run at 0.6 ml/min because they were cooled down to 5°C to allow for a better separation of AA and IAA. As will be shown later, when the columns are used for food analysis the flow was 0.7 ml/min and the temperature was kept at 22°C and the retention times were shorter.

The observed detector response of the integrator for standard solutions of AA and IAA, in the range of 0.5 to 2.5 ng, revealed an almost linear relationship. Figure 2 shows a calibration graph for AA and IAA. The lowest detection level for AA or IAA was set at 0.5 ng; at this concentration the chromatograph characteristics were still far from the generally accepted definition of chromatography detection limit, namely the amount of analyte which produces a peak height more than two times the noise level.

In our previous method (13) the detector response for AA and IAA was almost equal, however with the present system the response for IAA was smaller. As recoveries for both were the same (97-102%) this has to be attributed to a different mobile phase or to an unknown factor.

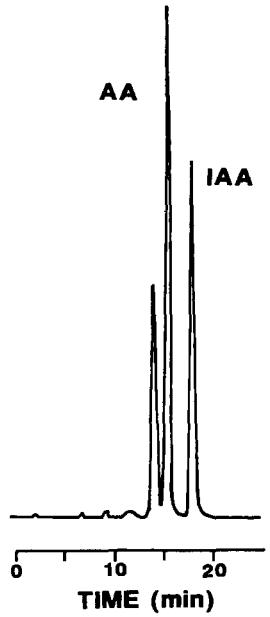

Figure 1. Chromatogram of standards. AA: ascorbic acid and IAA: isoascorbic acid (1 ng each). The retention times were 15.6 min for AA and 18.3 min for IAA. The peak at 14.0 min corresponds to homocysteine.

Feeding rats a diet containing 10% IAA for five days produced high levels of this compound in plasma and the selected tissues, liver, spleen and adrenal gland (Figure 3). As can be seen in the chromatograms, the peaks of AA and IAA were well separated on a stable baseline and no interfering peaks were observed, except in the case of adrenal gland where an unidentified peak with a retention time of 17.5 min was close to IAA (18.3 min), however this was not a problem for the quantification of IAA. This interfering peak required the cooling of columns at 5°C. The values for AA, IAA, DHAA and DHIAA obtained after feeding rats a diet containing 10% IAA for 5 days, are presented in Table 1. As expected, the adrenal gland accumulated the highest

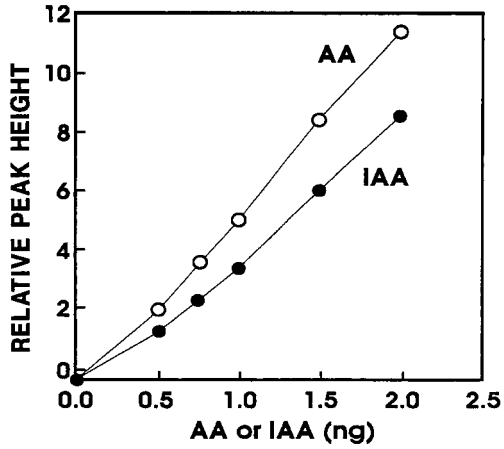

Figure 2. Calibration curve for standard solutions of ascorbic acid (AA) and isoascorbic acid (IAA) in the range of 0.5 to 2.0 ng.

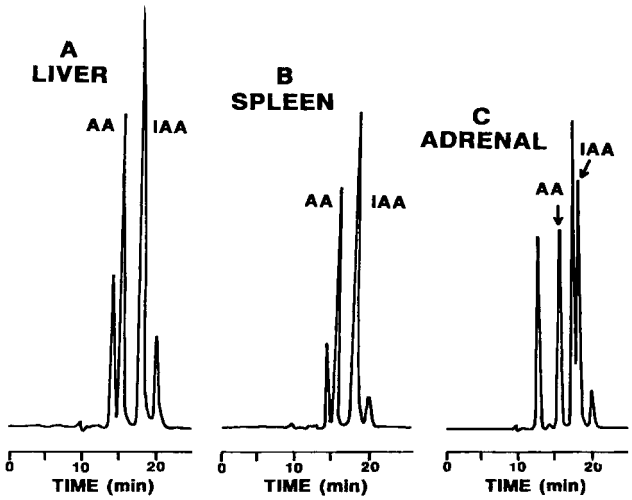

Figure 3. Chromatograms of tissues from rats fed a diet a diet containing 10% of isoascorbic acid (IAA). Retention times were almost identical to those in Fig. 1. In panel A is liver, in panel B is spleen and in panel C is adrenal. In the latter tissue there was an unidentified peak with a retention time of 17.5 min.

TABLE 1

Ascorbic Acid, Dehydroascorbic Acid, Isoascorbic Acid and Dehydroisoascorbic Acid Content of Plasma and Selected Tissues of Rats Fed a Diet Containing 10% of Isoascorbic Acid.

Tissue	Total AA	AA	DHAA	Total IAA	IAA	DHIAA
Plasma	9.09* ±1.65	6.48 ±1.27	2.61 ±0.52	53.52 ±14.69	40.38 ±12.11	13.14 ±6.89
Spleen	376.6 ±56.8	257.8 ±32.0	118.6 ±31.1	456.6 ±116.4	299.4 ±89.9	157.2 ±30.7
Liver	114.8 ±33.8	101.6 ±38.0	13.2 ±7.7	123.20 ±42.8	102.2 ±31.7	21.2 ±15.2
Adrenal	1794.4 ±418.9	1505.2 ±530.0	289.0 ±156.6	1983.6 ±433.0	1465.4 ±558.8	518.2 ±512.9

* The values are mean ± SD, and are expressed in μg/g of tissue, or ml of plasma. There were 5 rats in the group.

levels of AA. Interestingly, the levels of IAA are equal to those of AA under our dietary conditions. Total AA and Total IAA were present mainly in the reduced forms (AA and IAA) with the exception of spleen in which the oxidized forms (DHAA and DHIAA) represented approximately 50% of the total vitamin. A complete study of the effect of dietary IAA (and DHIAA) on the tissue levels of AA (and DHAA) will be presented later.

As indicated above, food analysis was performed with the two PLRP-S columns at 22°C, because no interfering peaks were observed in any of the samples selected. Chromatograms (not shown) showed AA and IAA well resolved on a stable baseline. Because of the higher temperature and a higher flow (0.7 ml/min), retention times were 11.9 min. for AA and 13.4 min. for IAA. The calibration curve (not shown) was similar to that in the tissue analysis. Recoveries studies were performed with all food samples and the values were in the range 94-104%.

Table 2 shows a sample of selected processed foods, in which meat products, without exception, presented variable levels of IAA and DIAA. In some products, such

TABLE 2

The Content of Ascorbic Acid and Isoascorbic Acid and their Corresponding Oxidized Forms in Selected Foods.

Food	Total AA	AA	DHAA	Total IAA	IAA	DHIAA
skimmed 1% milk	nd	—	—	nd	—	—
Condensed milk	1.47*	0.79	0.68	nd	—	—
Evaporated milk (2%)	19.60	16.80	2.80	nd	—	—
Cherry cheesecake	10.55	4.40	6.15	nd	—	—
Diet product, chocolate	22.75	10.34	12.41	nd	—	—
Iced Tea	nd	—	—	10.43	7.34	3.09
Cooked Ham	tr	—	—	72.16	68.99	3.17
Sausage	1.44	0.63	0.81	12.81	7.63	5.18
Chicken spread	5.13	4.24	0.89	42.13	29.46	12.67
Fruit Preservative	8433.0	7767.0	667.0	nd	—	—

* Values are expressed as mg/100 g or ml and represent the average of duplicate determinations, individual values did not differ by more than 3% from the average value.
nd: not detected, tr: traces.

as condensed milk, cheesecake and a diet product, DHAA was as high or higher than AA. On the other hand, products that contained IAA, the oxidized form was in small proportion. A fruit preservative, a product to be used in home fruit canning, contained only AA and DHAA at very high levels. These values represent a selected sample of a more widespread survey that is underway in our laboratory.

The method described here allows for the quantification of AA, IAA, DHAA and DHIAA in food samples and in tissues of animals, and is highly sensitive. The amperometric detection permits quantification at levels of 0.5 ng/20 μl injection, but

requires a second injection in the chromatograph, after treatment with homocysteine, to determine the oxidized forms. On the other hand, the method of Vanderslice and Higgs (8,9) only requires one injection; however, after separation on the column, the compounds are converted into fluorescence derivatives, which require a more complex HPLC system and makes the method more difficult and less sensitive.

REFERENCES

1. J. Fabianek, and H. Hepr. Antiscorbutic activity of d-araboascorbic acid, Proc. Soc. Exp. Biol. Med., 125: 462-465 (1967).

2. O. Pelletier and C. Godin. Vitamin C Activity of D-isoascorbic acid for the guinea-pig, Can. J. Physiol. Pharmacol., 47: 985-991 (1969).

3. J.C. Bauernfeind and D.M. Pinkert. Food processing with added ascorbic acid, Adv. Food Res., 18: 220-315 (1970).

4. B. Borenstein. The role of ascorbic acid in foods, Food Technol., 41: 98-99 (1987).

5. L.A. Pachla, D.L. Reynolds, and P.T. Kissinger. Analytical methods for determining ascorbic acid in biological samples, food products and pharmaceuticals, J. Assoc. Off. Anal. Chem., 68: 1-12 (1985).

6. R.C. Rose and D.L. Nahrwold. Quantitative analysis of ascorbic acid and dehydroascorbic acid by high-performance liquid chromatography, Anal. Biochem., 114: 140-145 (1981).

7. W.A. Behrens and R. Madère. A highly sensitive high-performance liquid chromatography method for the estimation of ascorbic and dehydroascorbic acid in tissues, biological fluids, and foods, Anal. Biochem., 165: 102-107 (1987).

8. J.T. Vanderslice and D.J. Higgs. Chromatographic separation of ascorbic acid, isoascorbic acid, dehydroascorbic acid and dehydroisoascorbic acid and their quantitation in food products, J. Micronutr. Anal., 4: 109-118 (1988).

9. J.T. Vanderslice and D.T. Higgs. Separation of ascorbic acid, isoascorbic acid, dehydroascorbic acid and dehydroascorbic acid in food and animal tissue, J. Micronutr. Anal., 7: 67-70 (1990).

10. M.A. Kutnink, J.H. Skala, H.E. Sauberlich and S.T. Omaye. Simultaneous determination of ascorbic acid, isoascorbic acid 9erythorbic acid) and uric acid in human plasma by high-performance liquid chromatography with amperometric detection, J. Liquid Chromatog., 8: 31-46 (1985).

11. L.L. Lloyd, F.P. Warner, J.F. Kennedy and C.A. White. Quantitative analysis of vitamin C (L-ascorbic acid) by ion suppression reversed phase chromatography, Food Chem., 28: 257-268 (1988).

12. W. Schüep and E. Keck. Measurement of ascorbic acid and erythorbic acid in processed meat by HPLC, Z. Lebensm Unters. Forsch., 191: 290-292 (1990).

13. W.A. Behrens and R. Madère. Quantitative analysis of ascorbic acid and isoascorbic acid in foods by high-performance liquid chromatography with electrochemical detection, J. Liquid Chromatog., 15: 753-765 (1992).

14. R.E. Hughes. The use of homocysteine in the estimation of dehydroascorbic acid, Biochem. J., 64: 203-208 (1956).

15. O. Pelletier and R. Brassard. Determination of vitamin C (L-ascorbic acid and dehydroascorbic acid) in food by manual and automated photometric methods, J. Food Sci., 42: 1471-1477 (1977).

16. American Institute of Nutrition. Report of the American Institute of Nutrition Ad Hoc Committee on standards for nutritional studies, J. Nutr., 107: 11340-1348 (1977).

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THIN LAYER AND HIGH PRESSURE LIQUID CHROMATOGRAPHIC DETERMINATION OF HISTAMINE IN FISH TISSUES

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ABSTRACT

TLC and HPLC techniques have been examined for histamine determination in fish. In case of HPLC determination a comparison of the performance characteristics of UV-Vis and Fluorescence detection is also described. Regression analysis was performed between the two sets of data, in order to examine the relative efficiency of the two techniques for the determination of histamine in fish sample extracts. Either of the two techniques proved sufficient for the determination of histamine in common concentration ranges in fish extracts although fluorescence detection had better sensitivity. The recovery of histamine from fish samples was decreased in comparison to the standard solutions but it was still sufficient for routine analysis of the fish samples.

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INTRODUCTION

Histamine [4-(2-aminoethyl)-imidazole] is produced mainly by kidneys, liver and intestinal mucosa because of histidine decarboxyliosis by the enzyme histidine decarboxylase (1-2). Abnormal secretion and neurotransmitters metabolism (histamine being a by-product) are correlated with several diseases such as pheochromocytoma (3), neuroblastoma (4), schizophrenia (5), malignant hyperthermia (6) and probably hypertension (7). It is generally accepted that histamine acts as endotoxin and plays part in allergic shock (8).

However, besides indigenous production of histamine, it is possible to be noticed elevation of its level in plasma up to 2.6 ppb (9) after consuming foodstuff, especially fish, where histamine is present because of micro-organism action. The excretion of histamine products produced during its metabolism is only 2-3% in the original form while 4-8% is excreted in the form of methylistamine, 9-11% as imidazoloacetic acid, 42-47% as methylimidazoloacetic acid, 16-32% as imidazoloacetic acid ribozide and less than 1% as acetylistamine (10). According to EEC guidelines 91/493, histamine acceptable upper limit in fish is 100 ppm and several investigators have reported that levels vary with the kind of fish and their freshness, for example, in salmon 0-50 ppm, in tuna canned 16-74 ppm, and in anchovy 3.1-13.8 ppm (11-12).

For histamine determination, several techniques have been developed : electrophoretic, radioimmuno-enzymatic, fluorescent and chromatographic (TLC, GC/MS, HPLC) (13-21). In particular, HPLC has been reported as the method of choice for the determination of histamine in case of pig hypothermia and beef stress, while in medicine in case of brain tumour.

In the present study, thin layer and high performance liquid chromatographic techniques have been investigated for histamine determination in fish samples. A comparison of the performance characteristics of UV-Vis and fluorescence detection is, also, described in case of HPLC detection. Also the stability and repeatability of the derivatization procedure for the preparation of the fluorescent derivative of histamine was examined.

MATERIALS AND METHODS

Equipment and Instruments

-Thin layer chromatographic plates 10x10 cm (MERCK No 3156)
-UV chamber 254-366 nm (CAMAG)

-Liquid chromatograph (GILSON, model 802)
-UV-Vis detector (GILSON) at 210 nm
-Fluorescence detector (GILSON model 121)
-Chromatography column (a.Lichrospher RP-18 5μm and b.Hamilton PRP-X 200
-Fluorescence filters, excitation 305-395 nm, emission 430-470 nm.

Solvents and Solutions

For TLC:

The developing solvent consisted from ethanol and NH_4OH (80/20 v/v)
The spraying reagents used for colour development were prepared as follows:
1.Iodoplatinate (0.25 g $PtCl_6$ and 5g KI diluted to 100 ml with a solution containing 2 ml c.HCl in deionized water).
2.Ninydrine (0.5 g ninydrine diluted to 100 ml with acetone).
3.Fluorescamine (0.0125 g fluorescamine diluted to 100 ml with acetone).

For HPLC:

The mobile phase used for the absorbance detection was an aqueous solution of 0.4 M KH_2PO_4, pH=4.5.
- Mobile phase for the fluorescence detector 0.05 M NaH_2PO_4 70% and acetonitrile 30%, pH=4.5.
- Derivatization solvents : O-phthalaldehyde (OPA) 0.1% in methanol ,2M NaOH ,1N H_2SO_4.
Standard histamine stock solution of 1000 ppm was prepared in water (169.1 mg histamine hydrochloric/100 ml) and working standards of 0.1, 0.5, 1, 2, 5, 8, 10 ppm in 0.2 N HCl.

Specimen Pretreatment

Histamine isolation was performed according to Mietz and Karnas (11). Five grams of tissue with 10 ml of 5% CCl_3COOH solution were treated and purified with a mixture of n-butanol, n-heptane and chloroform. Histamine is finally isolated with 3 ml 0.2N HCL. From these 3 ml, a) 1.3 and 5μl were injected on TLC plates, b) 20 μl were injected in HPLC with UV detector and c) 100 μl were derivatized for fluorescence detection.

Derivatization Procedure for Fluorescence Detection

A volume of 100 μl from the above hydrochloric solution of histamine was diluted with 900 μl of 0.2 N HCl, treated with 200 μl of 2M NaOH and 100 μl of methanolic solution 0.1% OPA, and were kept in darkness for 4 min. Finally, 200 μl of 1N H_2SO_4 were added and the solution kept for 2 hours. At this time, the specimen was ready for injection. The analysis of the specimens was then executed in the same day.

RESULTS AND DISCUSSION

TLC Identification of Histamine

Histamine was separated from aqueous standard solutions by thin layer chromatography. Three colour developing substances were tested as spraying reagents: iodoplatinate, ninydrine and fluorescamine. The mobile phase employed was a mixture of ethanol/aqueous ammonia (80/20 v/v) and the R_f value was 0.62. The injected volume was 1 μl in all the experiments.

In table 1, the results obtained from the above experiment are presented. All the spraying reagents were proved equally efficient in refer to the detection limit obtained (50 ng absolute), although fluorescamine gave a more clear chromatographic picture. When 0.2 N HCl was used as diluent for the standard solutions the spots were stable for 24 hours only but when HCl was replaced with methanol the stability of the colour was better for longer periods of time. Iodoplatinate reagent coloured the plate with a light brown colour and the spots became dirty. Ninydrine developed a red violet colour and the rate of colour development increased by heating the plates to 60°C.

Performance Characteristics of the Absorption and Fluorescence Determination of Histamine by HPLC

Histamine could be determined after the elution from the chromatographic column either directly by absorbance measurement or by measurement of fluorescence after derivatization. This procedure was described at the experimental section.
In figures 1 and 2, four typical chromatograms obtained from histamine determinations

TABLE 1.
Characteristics of TLC Histamine Determination with Various Spraying Reagents (R_f = 0.62).

Spraying reagents	Developed colour	Remarks
a. Iodoplatinate	Brown	Dirty spots because of light brown colour on the plate surface
b. Ninydrine	Red violet	Heating at 60°C accelerates the colour development
c. Fluorescamine	Yellowish-green	Stable colour when MeOH is used as diluent, well defined spots

are given. In each figure the first chromatogram correspond to standard solutions while the second correspond to fish samples (sardines). In figure 1 the chromatograms were produced by absorption measurements and in figure 2 by fluorescence measurements. It was obvious that the later were almost free from interfering peaks while the picture by absorption detection appeared to be more confused because of the existence of other peaks due to the matrix of the samples.

Regression analysis was performed for both of the determination techniques. The calibration curve by ultraviolet detection was linear up to 10 ppm of histamine. However, the calibration curve by fluorescence detection was extended just to 2 ppm because of its higher sensitivity. The sensitivity expressed as the slope of the calibration curve was five times greater with fluorescence detection than with absorption detection.

In table 2 the two procedures are given in a comparative form as well as the regression data of each working curve.

Critical Comparison of the Absorption and Fluorescence Calibrations in the Determination of Histamine by HPLC

The histamine content of ten different fish samples (anchovy) was determined following the two prescribed techniques and referring to the corresponding calibration

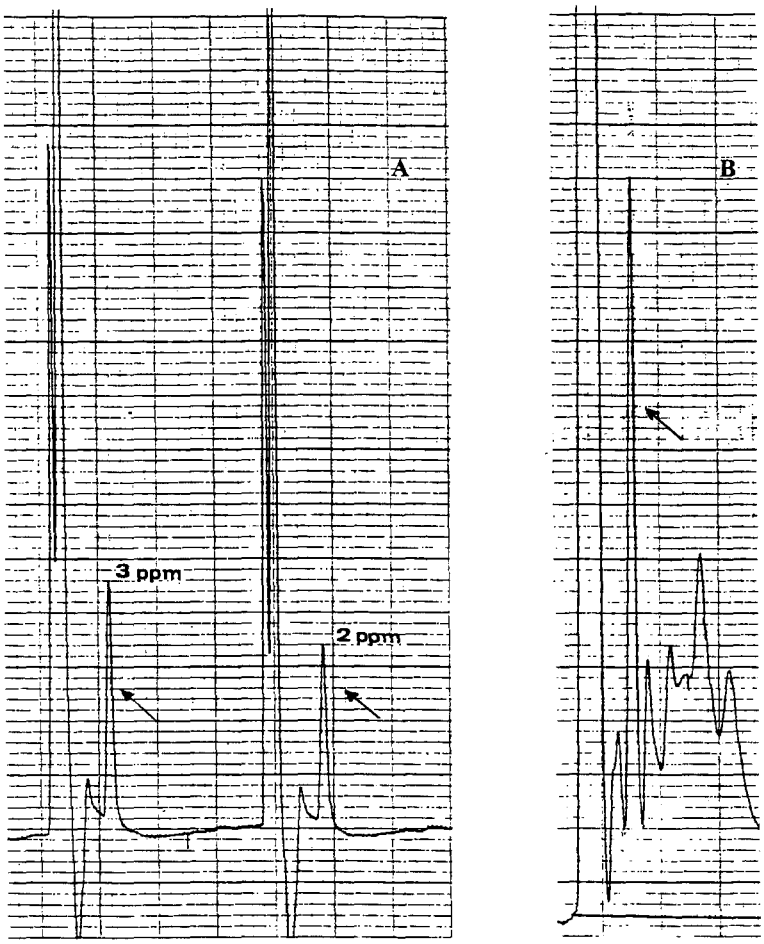

FIGURE 1. Typical chromatograms from HPLC determinations of histamine in standard solutions (A) and in sardine samples (B). The detection was based in absorbance measurements, 0.005 AUFS.

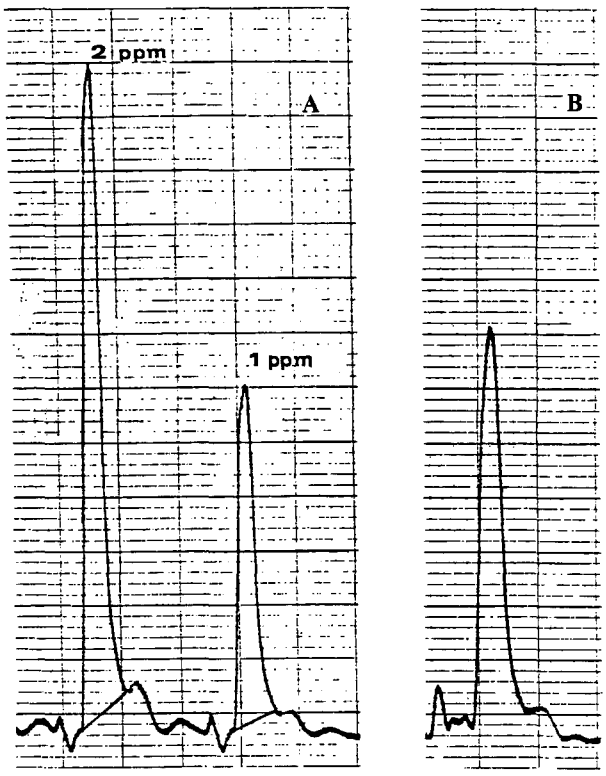

FIGURE 2. Typical chromatograms from HPLC determinations of histamine in standard solutions (A) and in sardine samples (B). The detection was based in fluorescence measurements, 0.02 AUFS. The solutions were diluted 1:10 to 1:15 before their injection, as it is described in the text.

curves (Table 2). Regression analysis was performed between the two sets of data, in order to examine the relative efficiency of the two techniques for the determination of histamine in fish sample extracts. The extracts of fish samples which were measured for fluoresence, needed to be diluted 1:10 to 1:15 with the extraction mixture but for the absorbance measurement they were used directly. The results of these determinations are listed in table 3.

The concentrations calculated by UV-Vis detection were scaled on Y-axis against the concentration calculated by fluoresence detection which were scaled on

TABLE 2.
Comparison of Sensitivity of the two Techniques for the Determination of Histamine in Standard Solutions after High Performance Liquid Chromatographic Elution.

Parameter	Absorbance	Fluorescence
Chromatographic Data		
Detectors	UV-Vis, 210 nm	Excitation 305-395nm Emission 430-470nm
Chromatographic column	Hamilton, PRP-X200 No 79441 Cationic	Lichrospher RP-18, 5μm
Mobile phase	0.4M KH_2PO_4 100% (pH=4.5)	0.05M NaH_2PO_4 70% Acetonitrile 30% (pH=4.5)
Flow rate	0.5 ml/min	0.5 ml/min
Pressure	0.26 kpsi	1.10 kpsi
Development time	4 min	4 min
Regression Data		
Concentration Range	0 to 10 ppm	0 to 2 ppm
Intercept value (a)	0.42	-2.31
Confidence Interval of the intercept (95%)	(-5, 6)	(-4.9, 0.25)
Standard deviation of the intercept	2.19	1.26
Slope value (b)	11.756	56.083
Confidence Interval of the slope (95%)	(10.8, 12.7)	(53.5, 58.7)
Standard Deviation of the slope	0.38	1.29
Correlation Coeff.(r)	0.9974	0.9901
Detection limit	10 ng	1 ng

TABLE 3.
Concentration of Histamine in Anchovy Samples Analysed by HPLC and UV-Vis or Fluorescence Detectors.

Sample No.	Concentration(ppm) from absorbance	Concentration (ppm) from fluorescence
1	5.50	5.60
2	7.50	7.35
3	4.50	4.87
4	6.00	5.61
5	3.00	3.36
6	2.25	2.17
7	3.34	3.35
8	3.50	3.55
9	3.75	3.60
10	3.54	3.50

X-axis. In case of equal efficacy between the two techniques (i.e. same sensitivity and precision), the slope of the linear curve should be equal to unit (in this case +1) and the intercept equal to zero. Moreover when the reproducibility of the two techniques is comparable, the correlation coefficient should also be very close to unit (in this case +1). The results of this regression analysis are listed in table 4.

Thus, it was proved that the identical values laid inside the confidence intervals for a 95% significance level. So either of the two techniques could be used for the determination of histamine in such concentration range in fish extracts.

Reproducibility of Derivatization Procedure

The procedure described in the experimental section for the production of a fluorescent product of histamine was repeated six times in the same extract and the variation of the peak heights obtained during the HPLC elution was examined. The mean height of the six determinations was 109.5 ± 16.9 at 99% significant level and the relative standard deviation was 9.38%. Thus, the derivatization procedure was proved sufficiently reproducible.

The next step was to examine the day-to-day stability of the derivative product. Six repetitions of the derivatization procedure were done in a day and the extracts were measured by HPLC. They left stand for 48 hours and then they were analysed

TABLE 4.
Regression Data for the Comparison of the Efficacy of the Two Techniques for the Determination of Histamine in Fish Extracts.

Statistics	Experimental Value	
Overall mean	4.29 ppm	
Experimental Error	0.155	
Relative Experimental Error	3.61 % (<5%)	
Regression Equation	Y = - 0.24 +	0.95X
Standard Deviation	of intercept 0.22	of slope 0.05
Confidence Limits (95%)	-0.27, 0.74	0.85, 1.06
Identical Values	0.0	1.0

again. The two sets of data were statistically tested for their homogeneity. The coefficient of variation was 0.563 and 0.792 for the two days and the pooled coefficient was 0.678. The calculated t-value was 2.46 and the critical values for 95 and 99% significant levels are 2.23 and 3.17 respectively. Thus, for a 99% significance the two sets are homogeneous, although in lower significance they might be different. The conclusion coming from these results is that the fluorescence product must be measured in day in order to avoid probable differentiation in the results.

Recovery Study

The recovery efficiency of the extraction procedure was tested either in standard solutions and in fish samples by absorbance detection. Six different standard solutions and three sardine samples were analysed each time and the results are shown in details in Table 5. The standard solutions were spiked in duplicate with 5, 25 and 50 μg of histamine. One part from each of the three fish samples was spiked with 5 μg of histamine and the other equal part was analysed directly without any addition.

As it can be seen from the above table, although the recovery of histamine from fish samples was decreased in comparison to the standard solutions, it was still sufficient for routine analysis of the fish samples.

TABLE 5.
Recovery of Histamine after Extraction from Standard Solutions and Fish Samples.

Statistics	Standard Solutions	Sardine Samples
Mean Recovery(%)	88.25	75.44
Range of values	80.5-94.5	64.68-82.35
Standard Deviation	5.13	9.44
Standard Error	2.09	5.45
99% Confid. Limits	82.85-93.65	61.38-89.5

CONCLUSIONS

TLC application by three spraying reagents proved sufficient for histamine determination. Fluorescamine as development reagent gave a slightly clearer picture lasting only 24 hours when the standard solution was diluted with 0.2 N HCl but being stable for a longer period when the standard solutions were prepared in methanol. This technique was proved sufficient for the detection of histamine in solutions with concentration of 50 ppm.

Absorbance detection was less sensitive than fluorescence detection. However, for the routine analysis of histamine in fish samples both techniques could be applied with reliable performance characteristics.

Although fluorescence detection was proved very sensitive, it should be kept in mind that it is more time consuming because of an extra procedure needed for the derivatization of histamine.

REFERENCES

1. R. W. Schayer, O. H. Ganley, Am. J. Physiol., 197: 721 (1959)

2. S. S. Sumner, S. Taylor, J. Food Protec., 52: 105 (1989)

3. F. V. Leung, J. Griggiths, Can. Med. Ass. J., 111: 1321 (1974)

4. D. W. Wooley, E. Shaw, Brit. Med. J., 1: 122 (1954)

5. C. H. Williams Pers, Biol. Med., 20: 120 (1976)

6. K. Imai, M. S. Wang, S. Vo, Z. Tamura, Clin. Chim. Acta, 43: 248 (1973)

7. P. Holtz, K. Credner, G. Kroneberg, Arch. Exp. Pathol. Pharmakol., 204: 228 (1947)

8. D. F. Devereux, S. A. Rice, S. N. Giri, Proc. West. Pharmacol. Soc., 18: 114 (1975)

9. P. P. VanArsdel, G. N. Beall, Arch. Intern. Med., 106: 192 (1960)

10. K. Lindell, R. W. Shayer, H. Westling, Clin. Sci., 13: 313 (1959)

11. J. L. Mietz, E. Karmas, J. AOAC, 61: No 1 (1978)

12. S. Taylor, E. R. Lieber, M. Leatherwo, J. Food Science, 43: 247 (1978)

13. L. Vear Foo, J. AOAC, 60: No 1 (1977)

14. R. C. Lundstrom, L. D. Racicot, J. AOAC, 66: 5 (1983)

15. G. Skofitsch, A. Saria, P. Holzez, F. Lembeck, J. Chromatogr., 226: 53 (1981)

16. J. C. Robert, J. Vatier, B. K. Nguyen Phnoc, S. Bonfils, J. Chromatogr., 273: 275 (1983)

17. F. J. Salquero, I. M. Mackie, Intern. J. Food Sci. Technol., 22: 409 (1987)

18. J. L. Roberts, A. Ken Aulsebrook, J. A. Oates, J. Chromatogr., 338: 41-49 (1985)

19. M. Harulusa, H. Vasueda, T. Shida, J. Chromatogr., 181: 153 (1980)

20. M. Harulusa, H. Vasueda, T. Shida, J. Chromatogr., 221: 1 (1980)

21. Official methods of analysis, 11th Ed., AOAC, Washington DC, 18.039-18.045, (1970)

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IMPROVED METHODS OF OLIGOSACCHARIDE ANALYSIS FOR GENETIC STUDIES OF LEGUME SEEDS

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ABSTRACT

The analysis of low molecular weight carbohydrates in single seed of lentils has been carried out using two different high performance liquid chromatography (HPLC) methods. One used a reversed-phase column coupled to a refractive index detector (HPLC-RI), while the other utilised an anion-exchange phase column coupled to a triple-pulsed amperometric detector (HPAC-PAD). The latter was found to be more sensitive and could be used for the analysis of very small samples, hence allowing parts of a seed to be analysed and then grown and used for genetic studies.

INTRODUCTION

Legume seeds are an important source of protein for human and animal nutrition. Their value as a food, however, is limited by the presence of considerable amounts of α-

galactosides of the raffinose family, which have been implicated as the main cause of flatus (Calloway, 1971; Rackis, 1975; Reddy *et al.* 1984, Price *et al.* 1988). In consequence, the determination of the oligosaccharide content in pulses has been the subject of many investigations, many of which are linked to improving the quality of pulses for human nutrition (Price *et al.* 1988; Adsule, 1989).

Different methods have been proposed to remove raffinose oligosaccharides, including soaking, cooking, germinating or fermenting the seeds, or the meal, prior to incorporation into human diets (Rao and Belavady, 1978; Iyer *et al.* 1980; Reddy and Salunkhe, 1980; Jood *et al.* 1986, Kataria *et al.* 1990; Vidal-Valverde and Frias, 1992; Vidal-Valverde *et al.* 1992, 1993a,b). An alternative approach is to seek genetic variation for the α-galactosides and to use this in the development of improved varieties which have acceptable low levels of this compounds. If variants are found then it will become necessary to follow the segregation of the oligosaccharides in a breeding programme, and for this to accur it is necessary to develop semi-micro analytical methods which allow single seeds to be analysed non-destructively. In addition, it may be necessary to analyse the seed coat (testa) and the embryo of each seed separately, since the two can be genetically differents.

A number of methods have been used for the analysis of soluble carbohydrates in foods. Although they can be quantified by gas chromatography, their lack of volatility requires a time-consuming derivatization procedure (Knapp, 1971). High performance liquid chromatography (HPLC) methods, incorporating micro-particle silica-amino based columns and refractive index (RI) detection, are used widely and have given excellent results (Knudsen, 1986; Muzquiz *et al.* 1992; Arentoft and Sørensen, 1992; Vidal-Valverde *et al.* 1992; 1993a,b). Neither of these methods, however, has been carried out previously on the small amounts of sample required for single seed analysis.

It has been reported that high performance anion-exchange chromatography with pulsed amperometric detection (HPAC-PAD) will separate and quantify small amounts of sugars in a mixture of standard solutions (Rocklin and Pohl, 1983; Townsend *et al.* 1988; Wang and Zopf, 1989). Low-molecular weight sugars having pK values ranging from 12 to 14 can be separated by anion exchange, which allows their separation on a strongly basic hydroxide-form anion exchange column with highly alkaline eluents (Rendleman, 1973). HPAC-PAD equipped with a gold electrode is sensitive only to compounds containing

oxidizable functional groups including hydroxyl, amine and sulfide moieties; carboxylic acids and inorganic species do not interfere (Rocklin and Pohl, 1983).

The object of the present investigation was to assess the effectiveness of HPLC-RI and HPAC-PAD for determining the content of low-molecular weight oligosaccharides in individual lentil seeds.

MATERIALS AND METHODS

Reagents.

Standard sugars (fructose, sucrose, raffinose and stachyose) were obtained from Sigma Chemical Co. Poole, Dorset, UK.

Sample preparation.

The Syrian Local Large (SLL) genotype of lentil (*Lens culinaris*) used in this study was obtained from the John Innes Institute germplasm collection. Single seeds were separated into the testa and the embryo components. Single embryos were ground in a Glen Creston grinder (type 31-700) and the resultant flour was used to extract the sugars.

Extraction procedure.

For the extraction of the α-galactosides, mono- and disaccharides, a range of weights of flour from single embryos were suspended in 5 ml of 80% ethanol, the sample boiled under reflux for 15 min, cooled and then centrifuged at 5000 rpm. The residue was extracted twice more, and washed with distilled water until no carbohydrate was detected (Molisch's test, Pearson, 1975). The supernatants were combined, concentrated in a Buchner Vortex evaporator and the residue used for chromatographic analysis.

HPLC determination.

The HPLC-RI analysis was performed on an HPLC chromatograph (Philips Analytical) equipped with a model PU-4100 pump, a Rheodyne Model 7000 sample injector (Berkeley, Ca) fitted with a 100μl stainless steel injection loop, and a model 132 optical reflection type differential refractometer detector (Gilson Associates). The chromatographic system was controlled by an IBM PC with 715 HPLC system controller software (Gilson Associates). A pre-column (3.2 mm i.d. x 4.0 cm) packed with C_{18} Porasil B and a μBondapack/Carbohydrate column (3.9 mm i.d. x 30 cm) (Waters Associates) at 35°C were employed, with a mobile phase of acetonitrile/water (75:25) which had previously been filtered through a millipore FH (0.45 μm) membrane (Waters Associates) and degassed with helium. The flow rate was 2 ml min^{-1}.

The HPAC-PAD analysis was performed on a Dionex (Sunnyvale, CA) LC gradient pump module and model PAD-II detector equipped with a solvent-compatible electrode. Sample injection was *via* a Dionex autosampler equipped with a 25 μl sample loop. Carbohydrates were separated on a CarboPac PA-100 pellicular anion-exchange resin column (4.0 x 250 mm) and a CarboPac PA-100 guard column (3 x 25 mm) (Dionex, Sunnyvale, CA) with a flow rate of 1 ml min^{-1} at ambient temperature. The mobile phase consisted of 145 mM sodium hydroxide solution, prepared with degassed water and 50% NaOH (BDH) solution. The mobile phase was degassed to prevent absorption of carbon dioxide and subsequent production of carbonate, which would act as a displacing ion and shorten retention times. Detection was by triple-pulsed amperometry with a gold electrode. The following working pulse potentials and durations were used for detection of low molecular weight sugars: E_1 = 0.05 V (t_1 = 300 msec); E_2 = 0.60 V (t_1 = 120 msec) and E_3 = -0.60 V (t_3 = 300 msec). The CHOH secondary hydroxyl groups are oxidized at E_1, E_2 which removes the reaction products, while E_3 cleans the electrode at a negative potential (Rocklin and Pohl, 1983). The response time of the PAD was set to 1 sec and the output range was set at 10000nA. Chromatographic data were collected and plotted using Dionex AutoIon 450 software.

For both HPLC-RI and HPAC-PAD systems, quantification of each sugar was accomplished by plotting the peak areas of the sample against those of standard solutions. Commercial ciceritol and verbascose standards were not available. Verbascose was isolated

from peas (Price, 1981) and ciceritol from lentils (Bernabe et al., 1993) and their identification confirmed on TLC silica gel plates as indicated previously (Vidal-Valverde and Rojas-Hidalgo, 1976). Insufficient amounts of these oligosaccharides were prepared for use as standards for HPLC, therefore, ciceritol (trisaccharide) and verbascose (pentasaccharide) were identified by their retention time and quantified, using raffinose (trisaccharide) and stachyose (tetrasaccharide) as standards.

RESULTS AND DISCUSSION

A prerequisite for studying the inheritance of low-molecular weight carbohydrates in legumes is to optimize the conditions for analysing the minimum amount of sample. The embryo and testa can be separated from single seeds and analysed separately; a single lentil seed weighs between 20 and 100 mg, with 5-15% of this weight corresponding to the testa. High performance liquid chromatography with a refractive index detector (HPLC-RI) and high performance anion-exchange chromatography with a triple-pulsed amperometric detector (HPAC-PAD) were used to quantify the monosaccharide, fructose; the disaccharide, sucrose and the oligosaccharides of the raffinose family (raffinose, ciceritol, stachyose and verbascose) in the embryos from seeds of the lentil genotype SLL. The results from the two methods were compared to establish the optimum conditions for analysing the composition in single embryos.

Table 1 shows the retention times (t_R, capacity factor (k') and number of theoretical plates (N) of the fructose, sucrose, raffinose and stachyose detected by HPLC-RI and HPAC-PAD. Figure 1 shows the chromatograms of these sugars in a standard mixture (a) and SLL lentil embryo sample (b) analysed by HPLC-RI, whilst Figure 2 shows the standard mixture (a) and SLL lentil embryo sample (b) analyzed by the HPAC-PAD system.

A standard curve was plotted out for each available standard. The equation constant, the correlation coefficient, the concentration range and the detection limit for each sugar are illustrated in Tables 2 and 3. The correlation coefficients of all standard sugars were always greater than 0.99. The concentration range analysed and the detection limit were between 10- and 100-fold higher using the HPAC-PAD system compared with HPLC-RI. This system also showed a good detection limit for fructose, sucrose, raffinose and stachyose, again with a higher sensitivity than for HPLC-RI.

TABLE 1

Chromatographic parameters in the detection of low molecular weight sugars by HPLC-RI and HPAC-PAD[a]

Parameters						
	HPLC-RI			HPAC-PAD		
Sugars	t_R	k'[b]	N[c]	t_R	k'	N
Fructose	2.90	1.38	1098	5.40	1.97	5184
Sucrose	5.00	3.10	1600	7.90	3.34	3994
Raffinose	9.00	6.38	2304	15.10	7.30	8635
Stachyose	16.50	12.52	3951	17.00	8.34	6400

[a] HPLC-RI: μBondapack/Carbohydrate column (3.9 x 300 mm) (Waters Associates) at 35°C; eluent acetonitrile/water (75:25). HPLC-PAD: CarboPac PA100 (4.0 x 250 mm) (Dionex); eluent, 145 mM NaOH.

[b] $k' = (t_R - t_M)/t_M$, where t_R = retention time of solute and t_M = retention time of solvent front.

[c] N, number of theoretical plates = $16\,[t_R/W]^2$, where t_R represents retention time and W represents peak width.

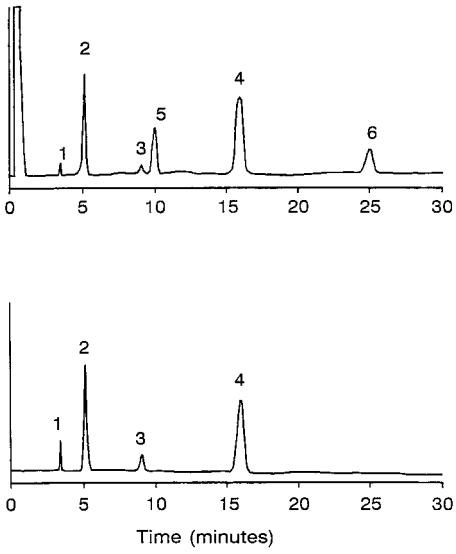

FIGURE 1.- Chromatograms obtained using HPLC-RI system: μBondapack/ Carbohydrate column (3.9 x 300 mm) (Waters Associates) at 35°C; eluent acetonitrile/water (75:25). (a) Standard solution; (b) SLL cotyledon lentil sample, where: 1.- Fructose; 2.- Sucrose; 3.- Raffinose; 4.- Stachyose; 5.- Ciceritol; 6.- Verbascose.

The amount of fructose, sucrose, raffinose, ciceritol, stachyose and verbascose in lentil embryo meal was analyzed by HPLC-RI system using different sample sizes (from 10 mg to 100 mg) (Figure 3). The results obtained showed good agreement for samples ranging between 40 mg and 100 mg. When the weight of the sample was in the range 20 mg to 40 mg the amount determined for each sugar was slightly lower, and with 10 mg samples the amount found was at the limit of the detection level. Previous reports have shown excellent resolution and quantification of low molecular weight carbohydrates in legumes by HPLC-RI techniques working with large amounts of sample. Knudsen (1986) determined the content of the raffinose oligosaccharide family in soya beans, chickpeas, garden peas and kidney beans using 10 g of sample. Arentoft and Sørensen (1992) and Muzquiz *et al.* (1992) analyzed the content of raffinose, stachyose and verbascose in pea and lupin species working with 0.5 g of initial sample, and Vidal-Valverde *et al.* (1992, 1993a) with 2.0 g and 10 g

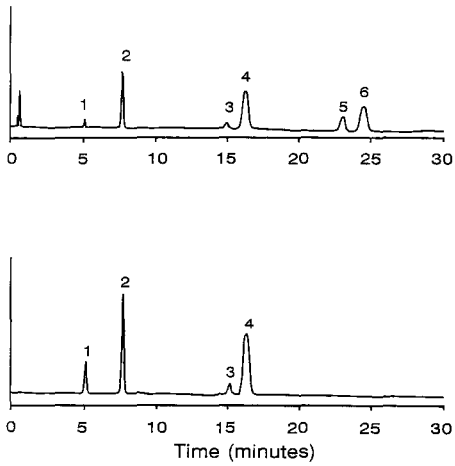

FIGURE 2.- Chromatograms obtained using HPLC-PAD system: CarboPac PA100 (4.0 x 250 mm) (Dionex); eluent, 145 mM NaOH. (a) Standard solution; (b) SLL cotyledon lentil sample, where: 1.- Fructose; 2.- Sucrose; 3.- Raffinose; 4.- Stachyose; 5.- Ciceritol; 6.- Verbascose.

samples, respectively. The conditions that we have established here, with the HPLC-RI system, allowed the determination of a very small amount of sample. In the case of fructose and verbascose their contents were quantified totally when the sample size was 20 mg. For sucrose, raffinose and ciceritol the amount of sample required was higher (30 mg), and 40 mg of lentil flour was required to accurately determine the stachyose content (Figure 3). These results, therefore, demonstrate that the limit of the amount of sample required to quantify all of the low molecular weight sugars in lentil meal using the HPLC-RI method is 40 mg.

Figure 4a and 4b show the content of monosaccharides, disaccharides and α-galactosides using high performance anion-exchange chromatography with the pulsed amperometric detector (HPAC-PAD) system. In this case, all the sugars were accurately analysed using sample sizes from 10 - 100 mg (Figure 4a). In addition, sample weights between 1 and 10 mg were also successfully analyzed and quantified using this method (Figure 4b). We are not aware of any other data regarding the analysis of soluble carbohydrates in legumes using such small amounts of sample. Several workers have

TABLE 2

Chromatographic constants of HPLC-RI system

Sugar	equation y = a + bx		r	Range (μg/mL)	Detection limit (μg/mL)
Fructose	5469	17981	0.999	4100-680	1300
Sucrose	22576	24517	0.999	50600-6325	450
Raffinose	22015	10092	0.999	5900-980	170
Stachyose	86237	10981	0.999	23900-3980	1380

TABLE 3

Chromatographic constants of HPAC-PAD system

Sugar	equation y= a + bx		r	Range (ng/mL)	Detection limit (ng/mL)
Fructose	-13607	11342	0.994	17-170	28
Sucrose	656750	4943	0.997	126-1260	138
Raffinose	-45919	4413	0.999	23-295	16
Stachyose	-235627	8517	0.996	30-230	43

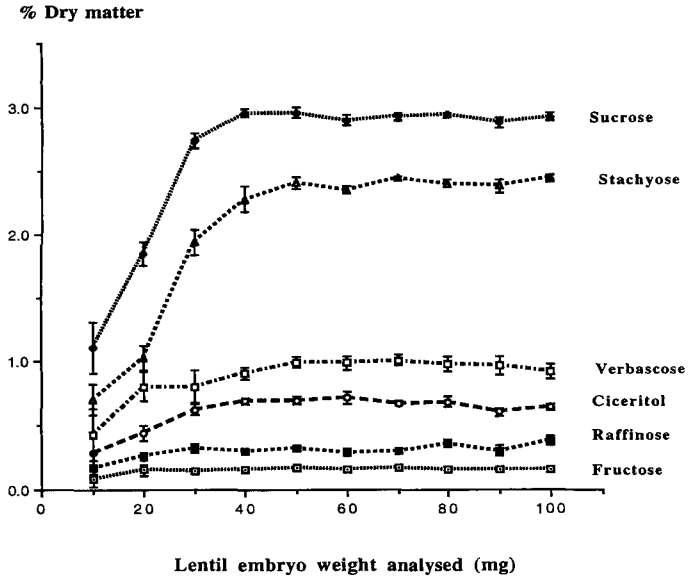

FIGURE 3.- Monosaccharide, disaccharide and α-galactoside content in g/100g dry weight of SLL lentil embryos using HPLC-RI system: μBondapack/ Carbohydrate column (3.9 x 300 mm) (Waters Associates) at 35°C; eluent acetonitrile/water (75:25). Values are the mean of four determinations and the bars record the standard deviation.

separated different sugar standards previously with an HPAC-PAD analytical system. Rocklin and Pohl (1983) separated alcohols, monosaccharides, disaccharides and other oligosaccharides such as maltose oligomers and the detection limits were found to be as low as 30 ppb for sugar alcohol and monosaccharides and about 100 ppb for maltose oligomers. They determined the lactose content of flavoured potato chips, containing a high concentration of potentially interfering salts, and found this to be about 70 ppm. Similar results were obtained by Townsend *et al.* (1988), who used the same method to separate a variety of neutral, silylated and phosphorylated oligosaccharides. Other authors for example Hardy *et al.* (1989), have analyzed different carbohydrates in biological samples and quantified at the subnanomole level neutral and amino sugars using pulsed amperometric detection. Wang and Zopf (1989), separated and quantified different lacto-N-fucopentoses in

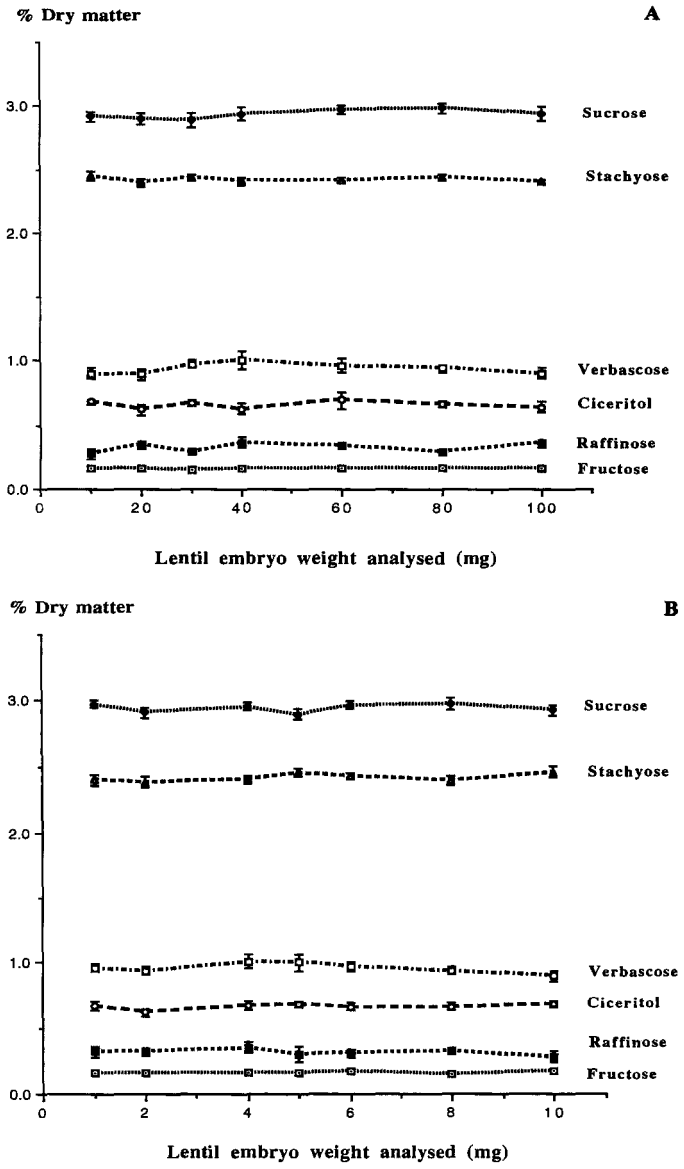

FIGURE 4.- Monosaccharide, disaccharide and α-galactoside content in g/100g dry weight of SLL lentil embryos [(a) between 10 - 100 mg; (b) between 1 - 10 mg] using HPLC-PAD system: CarboPac PA100 (4.0 x 250 mm) (Dionex); eluent, 145 mM NaOH. Values are the mean of four determinations and the bars record the standard deviation.

50 μl of human milk. The method presented here allowed us to analyse 1mg of lentil flour and to detect 8ng of fructose, 150 ng of sucrose, 34 ng of raffinose, 120 ng of ciceritol, 120 ng of stachyose and 48 ng of verbascose.

CONCLUSION

The HPLC-IR and HPAC-PAD analytical procedures described here for the quantification of low molecular weight carbohydrates allow the separation and detection of these sugars in legume seeds. This study shows that both methods are reliable when the seed size is not a limiting factor. The anion exchange column with triple-pulsed amperometric detection, however, results in a higher sensitivity for detecting carbohydrates and will allow parts of seeds, embryos and testas of lentils to be analysed individually, which is a prerequisite for genetic studies.

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REFERENCES

Adsule, R.N.; Kadam, S.S. and Leung, H.K. Lentil. In CRC *Handbook of World Legumes: Nutritional Chemistry, Processing Technology, and Utilization*. Vol II. Salunkhe, D.K. and Kadam, S.S. CRC Press. Inc. Boca Raton, Florida. 1989, pp. 132-152

Arentoft, A-M and Sørensen, H. 1992. α-Galactosides and dietary fibres in relation to pea quality: Methods of oligosaccharide analyses. *1st Conference for Grain Legumes,* 457-458.

Bernabe, M.; Fenwick, R.; Frias, J.; Jimenez-Barbero, J.; Price, K.; Valverde, S. and Vidal-Valverde, C. 1993. Determination, by NMR spectroscopy, of the structure of ciceritol, a pseudotrisaccharide isolated from lentils. *J. Agric. Food Chem.* 41, 870-872.

Calloway, D.H.; Hickey, C.A. and Murphy, E.L. 1971. Reduction of intestinal gas-forming properties of legumes by traditional and experimental food processing methods. *J. Food Sci.* 36, 251-255.

Iyer, V.; Salunkhe, D.K.; Sathe, S.K. and Rockland, L.B. 1980. Quick-cooking beans (*Phaseolus vulgaris* L.). II. Phytates, oligosaccharides, and antienzymes. *Qual. Plant. Plant Foods Hum. Nutr.* 30, 45-52.

Jood, S.; Metha, U. and Singh, R. 1986. Effect of processing on available carbohydrates in legumes. *J. Agric. Food Chem.* 34, 417-420.

Kataria, A.; Chauhan, B.N. and Punia, D. 1990. Effect of domestic processing and cooking methods on the content of carbohydrates of amphidiploids (black gram x mung bean). *Food Chem.* 36, 63-72.

Knapp, D.R. *Handbook of Analytical Derivatization Reactions*, Wiley, N.Y. 1971, p.539.

Knundsen, I.M. 1986. High-performance liquid chromatographic determination of oligosaccharides in legume seeds. *J. Sci. Food Agric.* 37, 560-566.

Muzquiz, M.; Rey, C.; Cuadrado, C. and Fenwick, G.R. 1992. Effect of germination on the oligosaccharide content of lupin species. *J. Chrom.* 607, 349-352.

Pearson, D. Sugar and preserves. In: *The Chemical Analysis of Food.* Churchill Livingstone. (Eds). London, 1975, p. 107.

Price, K.R. 1986. A simple method for the extraction and isolation of verbascose from pea. *Chemistry and Industry*, 3, 180-181.

Price, K.R.; Lewis, J.; Wyatt, G.M. and Fenwick, G.R. 1988. Flatulence -causes, relation to diet and remedies. *Die Nahrung.* 32, 609-626.

Rao, P.U. and Belavady, B. 1978. Oligosaccharides in pulses. Varietal differences and effects of cooking and germination. *J. Agric. Food Chem.* 26, 316-319.

Rackis, J.J. 1975. In: *Physiological Effects of Food Carbohydrates.* Allen, J., Heilge, J. Eds., American Chemical Society, Washington, 207-222.

Reddy, N.R. and Salunkhe, D.K. 1980. Changes in oligosaccharides during germination and cooking of black gram and fermentation of black gram/rice blends. *Cereal Chem.* 57, 356-360.

Reddy, N.R.; Pierson, M.D.; Sathe, S.K and Salunkhe D.K. 1984. Chemical, nutritional and physiological aspects of dry bean carbohydrates - A review. *Food Chem.* 13, 25-68.

Rendleman, J.A. Ionization of carbohydrates in the presence of metal hydroxides or oxides. Carbohydrates in Solution, *Advances in Chemistry*, Series 117, American Chemistry Society, 1973, p. 51.

Rocklin, R.D. and Pohl, A. 1983. Determination of carbohydrates by anion exchange chromatography with pulsed amperometric detection. *J. Liq. Chrom.* 6, 1577-1590.

Townsend, R.R.; Hardy, M.R.; Hindsgaul, O. and Lee, Y.C. 1988. High-performance anion-exchange chromatography of oligosaccharides using pellicular resins and pulsed amperometric detection. *Anal. Biochem.* 174, 459-470.

Vidal-Valverde, C. and Rojas-Hidalgo, E. 1976. A chromatography-polarimetric method for the quantitative determination of some sugars. *Anal. Quim.* 72, 275-279.

Vidal-Valverde, C.; Frias, J. and Valverde, S. 1992. Effect of processing on the soluble carbohydrate content of lentils. *J. Food Protection* 55, 301-304.

Vidal-Valverde, C. and Frias, J. 1992. Changes in carbohydrates during germination of lentils. *Z. Lebensm. Unters. Forsch.* 194, 461-464.

Vidal-Valverde, C; Frias, J. and Valverde, S. 1993a. Changes in the carbohydrate composition of legume after soaking and cooking. *J. Am. Dietetic Assoc.* 93, 547-550.

Vidal-Valverde, C.; Frias, J.; Prodanov, M.; Tabera, J.; Ruiz, R. and Bacon, J. 1993b. Effect of natural fermentation on carbohydrates, riboflavin and trypsin inhibitor activity of lentils. *Z. Lebensm. Unters. Forsch.*, (in press).

Wang, W.T. and Zopf, D. 1989. Liquid ion-exchange chromatography under pressure of milk oligosaccharides using a pulsed amperometric detector. *Carboh. Res.* 189, 1-11.

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ANALYSIS OF DIETHYLENETRIAMINE IN WATER AND SOIL AT PPB LEVELS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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ABSTRACT

A fast and sensitive method for the determination of diethylenetriamine in water and soil by High Performance Liquid Chromatography (HPLC) with Fluorescence Detection is described. Diethylenetriamine is converted to its fluorescamine derivatives through pre-column derivatization and is separated by an isocratic reverse phase chromatographic system (Nucleosil C18) with acetonitrile and borate buffer at pH 8 as the mobile phase. Water samples at concentrations of diethylenetriamine from 20 - 100 μg/L were derivatized with fluorescamine and chromatographed directly through direct aqueous injections. Recoveries averaged 94 $\pm$ 7% for a population of nine samples. Soil samples at concentrations of diethylenetriamine from 0.24 - 1.0 μg/g were extracted with $CaCl_2$ solution (2 M) followed by derivatization with fluorescamine. Derivatized soil extracts were then analyzed by HPLC. Recoveries averaged 21 $\pm$ 2% for a population of nine samples.

INTRODUCTION

Diethylenetriamine is commonly used as a chemical intermediate for reactive polyamide resins, fatty aminoamides, fatty imidazolines. It is also used as a solvent for sulfur, acid gases, various resins, and dyes. During its manufacture and commercial use, diethylenetriamine can enter the environment through emissions in air or in waste water. Because of its chemical and physical properties, diethylenetriamine

is expected to leach into the ground once it is released on to land. To date, the environmental fate of diethylenetriamine is still unknown largely due to the lack of an adequate analytical methodology. To study the environmental fate of diethylenetriamine, a rapid and sensitive analytical method is essential in order to identify and quantify diethylenetriamine at relevant environmental concentrations.

Fluorescamine, an important fluorogenic reagent, has been used extensively to label primary and secondary amines, amino acids, peptides, and proteins[1,2]. It also has been used to derivatize aliphatic diamines and polyamines through both pre- and post-column derivatization[3,4]. This non-fluorescent reagent reacts rapidly with primary amines in an aqueous environment to form pyrrolinones which fluoresce at 475-500 nm upon excitation at approximately 390 nm[5]. The resulting fluorescence is proportional to the amine concentration and can be used for quantitation. The derivatization reaction and the hydrolysis of excess reagent generally take place at pH $\geq$ 7 and at room temperature. The reaction rate depends strongly on the pH, the nature of the organic co-solvent, and the buffer selected. Diethylenetriamine contains two primary and one secondary amine groups that are susceptible to fluorescamine derivatization. This paper describes a quick and highly sensitive method for the determination of diethylenetriamine in water and soil as its fluorescamine derivative by HPLC with fluorescence detection.

MATERIALS AND METHODS

Chemicals:

Diethylenetriamine (lot # 00722J2) was obtained from Aldrich (Milwaukee, Wisconsin) with a chemical purity of 99%. Fluorescamine (lot # 08808E2) was also obtained from Aldrich with a chemical purity of 98%. Calcium chloride and boric acid (crystal) were obtained from Mallinckrodt (Paris, Kentucky) and were analytical grade. All other chemicals were obtained from commercial sources and were at a minimum reagent grade. All solvents were HPLC grade.

Water and soil:

Water recovery samples were prepared in deionized water collected in the state of Kentucky. Soil recovery samples were prepared in soil also collected in the state of Kentucky. The textual classification of the soil was clay.

Recovery sample preparation and fluorescamine derivative formation:

1. Water. Water recovery samples were prepared in deionized water. They were fortified with a stock solution of diethylenetriamine prepared in methanol. The fortification levels produced were 20.4, 61.2 and 102 μg/L (three replicates at each concentration). An additional three deionized water samples were left unfortified to be utilized as control samples. To 1.00 mL of sample solution was added 20 μL of dilute NaOH solution (0.1 N). Exactly 100 μL of fluorescamine stock solution (5 mg/mL prepared in

acetone) was then added to the mixture at room temperature. Rapid addition and mixing were essential to achieve optimal fluorescence. An aliquot of the reaction mixture was injected directly onto the HPLC column.

2. *Soil.* Soil recovery samples were prepared in Kentucky soil. Approximately 2 g of soil was weighed into a glass centrifuge tube and fortified with a stock solution of diethylenetriamine prepared in $CaCl_2$ (2M). The fortification levels produced were approximately 0.24, 0.50 and 1.0 μg/g (three replicates at each concentration). An additional three soil samples were left unfortified to be utilized as control samples. Each soil sample was extracted with 4.00 mL of $CaCl_2$ solution (2 M) by vortexing vigorously for approximately 3 minutes. The extract was separated via centrifugation for approximately 10 minutes at 2000 rpm. To exactly 1.00 mL of soil extract was added 20 μL of dilute NaOH solution (0.1 N). Exactly 400 μL of fluorescamine stock solution (2 mg/mL prepared in methanol) was then added to the mixture at room temperature. Rapid addition and mixing were essential to achieve optimal fluorescence. An aliquot of the reaction mixture was injected directly onto the HPLC column.

High Performance Liquid Chromatography (HPLC):

HPLC was performed on an isocratic reverse phase system with fluorescence detection. Instruments included a Hewlett Packard 1050 solvent pump, a Hewlett Packard 1050 autosampler, a Waters 470 fluorescence detector, and a Hewlett Packard 3396A integrator. A Nucleosil C18 (10 μm, 250 X 4.6 mm) column was used to separate diethylenetriamine-fluorescamine derivatives. The HPLC mobile phase was 50% methanol and 50% borate buffer (0.1 M, pH 8) at 1 mL/minutes for water samples, and 40% methanol and 60% borate buffer at 1.8 mL/minutes for soil samples. The borate buffer was prepared by titrating 0.1 M boric acid with 1 N sodium hydroxide. Fluorescence detection (excitation: 390 nm, emission: 475 nm) was used to monitor the eluted derivatives. The injection volume was 20 μL for all samples.

Quantitation:

The quantification of diethylenetriamine was achieved using an external calibration method. External calibration standards were prepared in deionized water and $CaCl_2$ solution (2 M) for water samples and soil samples, respectively, using stock solutions of diethylenetriamine prepared in methanol. The concentrations of the standards were 20, 40, 60, 80 and 100 μg/L. Each standard was subjected to derivatization with fluorescamine following procedures described above. Two complete sets of standards were analyzed with each sample set, one prior to analysis of the samples and one immediately following the samples. A standard curve was constructed by plotting the peak area (for water samples) or peak height (for soil samples) of diethylenetriamine-fluorescamine derivative versus the concentration (μg/L) of the standard injected using linear regression. The correlation coefficient, slope, y-intercept and minimum detectable limits were calculated. The concentrations of diethylenetriamine in each sample were

determined using the linear regression and the diethylenetriamine-fluorescamine derivative peak area or height of the samples.

For soil samples, since external calibration standards were prepared in pure $CaCl_2$ solution, the matrix effect on detector responses was examined in order to obtain accurate quantification. An experiment was conducted to determine the differences in detector response between a sample in pure $CaCl_2$ and a sample in soil extract ($CaCl_2$ extract). In this experiment, two 100 μg/L diethylenetriamine samples were prepared, one in pure $CaCl_2$ solution (2 M) and one in control soil extract ($CaCl_2$ extract). These two samples were derivatized with fluorescamine and analyzed by HPLC simultaneously. Four repetitive HPLC injections were performed for each sample alternately. The mean peak height of each sample was calculated. A detector response factor of 0.667 was calculated as the ratio of the peak height of the sample prepared in soil extract to the one prepared in pure $CaCl_2$. This detector response factor was used to correct the method recovery.

RESULTS AND DISCUSSION

Under slightly basic conditions (pH 8), the derivatization reaction between fluorescamine and diethylenetriamine in water resulted in at least three fluorescent derivatives, presumably as products of both the primary amino-group and the secondary amino-group. The separation of these derivatives was achieved using an isocratic reverse phase C18 (Nucleosil) column with a borate buffered (pH 8) mobile phase. Under the derivatization conditions, a major fluorescent derivative was formed and reached its highest fluorescent intensity almost instantaneously. This derivative (retention time ~ 3.7 minutes) was found to be stable for at least few hours and used for quantitation. Identification of the three major derivatives was beyond the scope of this work and was not pursued. Prior to achieving these experimental conditions, various experiments were conducted to optimize the derivatization reaction and the separation of various fluorescent products. The derivatization reactions were conducted at various pHs (pH 6, 7, 8 and 9) and in various buffers (NaOH, borate buffer, and phosphate buffer). It was found that the rate of formation as well as the intensity of the fluorescent derivatives increased with increasing pH. Although these fluorescent products were most intense at pH 9, the degradation (presumably via hydrolysis) of these products at pH 9 was significantly faster than at pH 8. Running the reaction at pH 8 was, therefore, more desirable. The effect of different buffers on the reaction rate was also examined. Borate buffer (pH 8) was preferable than phosphate buffer (pH 8). The reaction in phosphate buffer resulted in several fluorescent products with similar intensities, while a single predominant product was formed in borate buffer. Adjusting pH with a small amount of dilute NaOH solution produced similar results as to using borate buffer at pH 8. Instead of using borate buffer at pH 8, NaOH was used to adjust the pH of water samples as well as soil extracts in order to minimize the dilution of analyte. The water method was validated by fortification of deionized water with diethylenetriamine at nominal concentrations ranging from 100 -20 μg/L with a mean recovery of 94 $\pm$ 7%. The limit of quantitation was defined as one-half of the signal response of the lowest concentration calibration standards. Based on this

TABLE 1

Analytical Results for the Recovery of Diethylenetriamine From Water and Soil.

Sample Type	Concentration Fortified (μg/L or μg/g)	Concentration Recovered (μg/L or μg/g)	Recovery %	Number of Replicates	Overall Mean ± STD (n=9)
Water	20.4	18.5 ± 1	91 ± 5%	3	94.7 ± 7%
Water	61.2	57.2 ± 4	93 ± 6%	3	
Water	102	104 ± 4	102 ± 4%	3	
Water Control	NA	< 13.5	NA	3	
Soil	0.246	0.0337 ± 0.002	13.7 ± 0.8 %	3	13.8 ± 1%
Soil	0.499	0.0638 ± 0.002	12.8 ± 0.5%	3	21 ± 2%*
Soil	0.996	0.150 ± 0.002	15.0 ± 0.4%	3	
Soil Control	NA	<0.0186	NA	3	

* Corrected for detector response factor

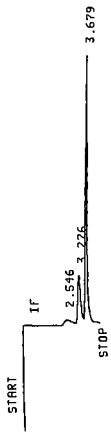

Figure 1. **An HPLC chromatogram of a water sample at 102 μg/L using the reverse phase C18 system. (Peak of interest = 3.679 minutes)**

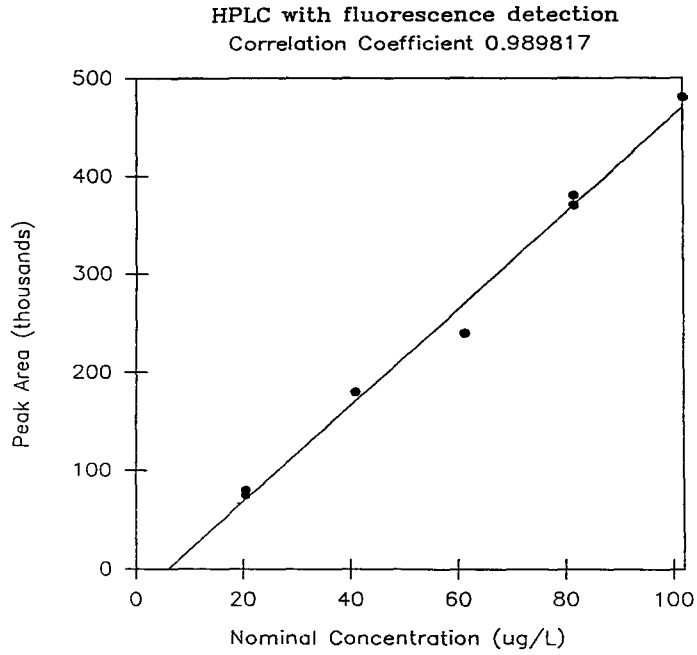

Figure 2. An external calibration curve for water method.

definition, a limit of quantitation for this method was calculated to be 13.5 μg/L. However, based on the signal-to-noise ratio, detector sensitivities, and injection volumes, a ten-fold lower quantitation limit (~ 1 μg/L) could easily be achieved. The recovery data for this water method is presented in Table 1. A representative chromatogram showing the analysis of diethylenetriamine at 102 μg/L is shown in Figure 1. A typical linear regression analysis for calibration standards is presented in Figure 2.

Development of a soil method presented much greater challenges owing to the difficulties in extracting diethylenetriamine from a soil matrix. The strong adsorption of diethylenetriamine to soil has been studied extensively[6]. Diethylenetriamine has a high water solubility and a relatively low vapor pressure. It contains two primary and one secondary amine groups, with pK_a values of 9.4 and 10.1. These functional groups will be partially protonated at pH ranges found in most soils and ground water. The ionic or electrostatic interactions between amines and the charged soil surface were considered the predominant mechanism in regard to soil adsorption[7]. Because of these interactions, the mineral content and the cation exchange capacity of a soil are also considered as important parameters influencing soil

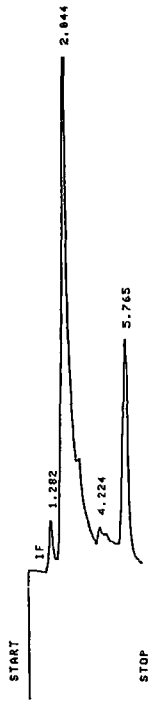

Figure 3. An HPLC chromatogram of a soil sample at 0.98 μg/L using the reverse phase C18 system. (Peak of interest = 5.765 minutes)

adsorption[8]. Various solvents were used to extract diethylenetriamine from soil during the method development. They included: $CaCl_2$ (1 M and 2 M), KCl (1 M and 2 M), NH_4OH (3.4%), HCl (0.1 M and 1 M), sodium borate buffer (0.1 M, pH 8), water, and mixtures of acetonitrile, methanol, and water. Among these solvents, $CaCl_2$ at a 2 M concentration proved to be the most promising solvent. The extraction procedures were also examined extensively. It was found that extended shaking (e.g. 2 hours, overnight and three days) did not improve extraction efficiencies, indicating that equilibrium was achieved after vigorously vortexing for 3 minutes. To achieve a lower quantitation limit, experiments were also conducted to optimize the solvent-to-soil ratio. Higher ratios would produce higher extraction efficiency but analytes would be more dilute if no concentration steps were involved. The optimal ratio was determined to be 2-to-1 for this method.

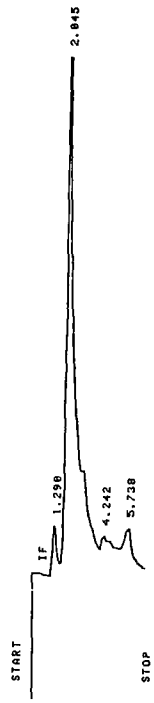

Figure 4. An HPLC chromatogram of a control soil sample. (Peak of interest = 5.738 minutes)

Similar to water samples, at least three fluorescent derivatives (with one major) were formed under the derivatization conditions. These three derivatives, however, could not be separated under the HPLC conditions described above. The co-elutions observed were due to the high ionic strength of the sample matrix (2 M $CaCl_2$). This matrix effect was significantly decreased when the injection volume was at or below 2 μL. Unfortunately, the sensitivity required for this method could not be achieved using a 2 μL injection volume. Various LC conditions were tested in an attempt to resolve these co-eluting peaks. These included various gradient elutions, various stationary phases (C18, C8, CN, NH_2, phenyl, etc.), many different buffered mobile phases at different pHs (pH 3.5, 5, 6, 7, 8, and 9), and with different organic modifiers (acetonitrile, methanol, THF, etc.). None of the conditions could successfully resolve these co-eluting peaks. The isocratic HPLC condition, slightly modified from the one used in the water method, was therefore chosen. As a result, the quantitation of diethylenetriamine in soil extract

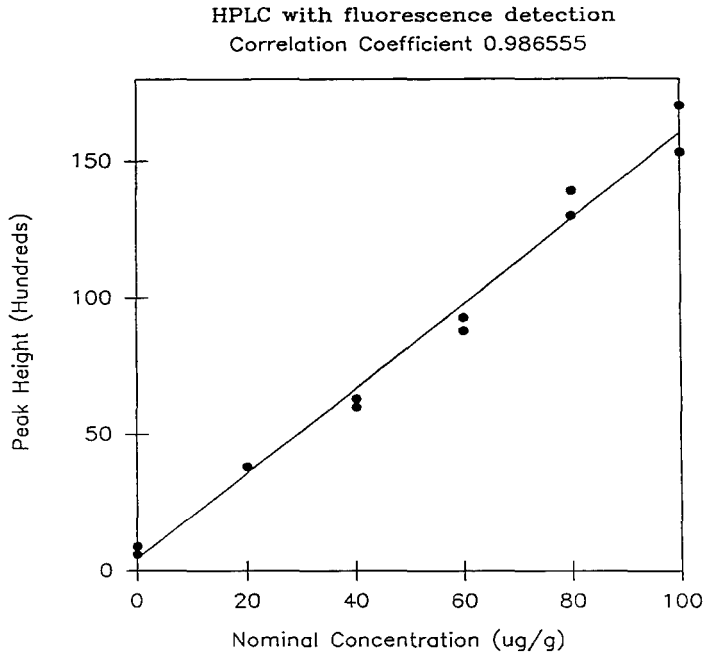

Figure 5. An external calibration curve for soil method.

was based on the peak height of the three co-eluting components. One of the three co-eluting components was also present in blank $CaCl_2$ solution which resulted in a relatively high intercept for the calibration curve. The fluorescent derivative peak (retention time ~ 5.7 minutes) reached its maximum within 20 minutes after the mixing with fluorescamine and was stable for a few hours. This peak could therefore be used for quantitation. The decision to use methanol to prepare the fluorescamine stock solutions was made after the observation that the use of acetone resulted in high background fluorescence in soil extracts. The use of methanol minimized the background interference, and therefore, maximized the sensitivity for diethylenetriamine.

The soil method was validated by fortification of matrix soil with diethylenetriamine at nominal concentrations ranging from 1.0 - 0.24 μg/g with a mean recovery of 13.8 $\pm$ 1.1% using external calibration standards prepared in pure $CaCl_2$ solution. The mean absolute method recovery, after correction for the detector response factor, was 21% $\pm$ 2%. The limit of quantitation was 0.13 μg/g after correction for method recovery (13.8%). Analytical results for the recovery of diethylenetriamine from

the matrix soil are presented in Table I. Representative chromatograms of a fortified soil sample and a soil control sample are shown in Figures 3 and 4, respectively. A typical linear regression analysis for diethylenetriamine standards is presented in Figure 5. These results demonstrated that diethylenetriamine was extremely difficult to extract once it was applied to soil. Although the method recovery of diethylenetriamine from soil was low (21%), the precision of the method was very good with a relative standard deviation of 8% for a population of 9 samples.

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REFERENCES

1. H. Nakamura, J. J. Pisano, Arch. Biochem. Biophys., 177: 334-335 (1976)

2. K. Imai, J. Chromatogr., 105: 135-140 (1975)

3. K. Samejima, J. Chromatogr., 96: 250-254 (1974)

4. K. Samejima, M. Kawase, S. Sakamoto, M. Okada, Y. Endo, Anal. Biochem., 76: 392-406 (1976)

5. A. H. M. T. Scholten, U.A.Th. Brinkman, R.W. Frei, J. Chromatogr., 218: 3-13 (1981)

6. J.W. Davis, Environ. Toxicol. Chem., 12: 27-35 (1993).

7. R. T. Podoll, K. C. Irwin, S. Brendlinger, Environ. Sci. Technol., 19, 196-200 (1985)

8. J. Dragun, "Soil Chemistry of Hazardous Materials" in Hazardous Material Control Research Institute, Silver Springs, MD. 1988.

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TEST PROCEDURE VALIDATION FOR THE TLC ASSAY OF A DEGRADATION PRODUCT IN A PHARMACEUTICAL FORMULATION

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ABSTRACT

An experimental and statistical approach for validating the TLC assay of a degradation product in a pharmaceutical formulation is given. This validation approach is intended to take into account the main particular aspects of the technique.

INTRODUCTION

We recently proposed a design for validating the assay of an active ingredient in a pharmaceutical formulation using spectrophotometry (1) and thin layer chromatography (2). UV spectrophotometry served as a basis for comparing various statistical approaches. Quantitative thin layer chromatography (TLC) illustrated the case of a complex technique in which the plate and mobile phase are renewed after each chromatographic development, the number of samples which can be analyzed in a same run is limited.

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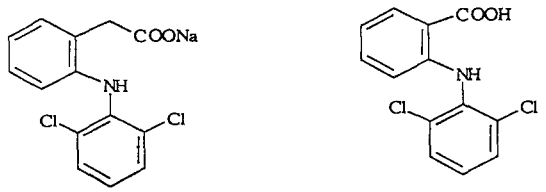

D IV

FIGURE 1. Structure of Diclofenac sodium and compound IV used in the present study.

The present work concerns the particular aspects of a validation assay for an impurity using TLC with a special focus on the detection and quantitation limits (LOD and LOQ). At the LOQ, in addition to the accuracy and repeatability tests, a ruggedness test has been carried out to take into account the major sources of variations of the technique : the influence of some critical factors of the method (plate batch number, mobile phase composition and temperature) on the assay results, resolution and Rf values have been investigated.

The study has been carried out on one of the degradation products (see degradation scheme in ref. 2) of diclofenac sodium (D) in a tablet formulation. For the purpose of the work, the mobile phase used is such as the degradation product (compound IV, chosen as a model compound, Fig. 1) is located in the tailing of the active drug (present in large amount) as this illustrates a case which often arises in stability-indicating assays.

PRELIMINARY EXPERIMENTS

None of the solvent systems reported in the literature for D (3-6) could achieve a simultaneous separation of D and its degradation compounds. Different mobile phases have been investigated. The solvent system dichloromethane : methanol (92 : 8, v/v) allows the separation of D from IV with respective Rf values of 0.27 and 0.38, whilst compound VI does not migrate and other compounds are eluted near the solvent front (Rf > 0.83). Fig.2 shows the separation of IV at a 0.5 % level (with respect to D). Another solvent system, toluene : ethyl acetate (100 :3, v/v), should be used for the separation of the less polar compounds, which gives Rf values of 0.18 (II), 0.24 (I), O.29 (VII), 0.55 (V), 0.60 (III), while D, IV, and VI do not migrate.

Methanol was found to be the most suitable solvent both for extraction and on-plate application.

Quantitation was carried out at 280 nm, on-plate maximum absorbance wavelength of IV.

EXPERIMENTAL

Reagents, materials and apparatus.

All chemicals were of analytical grade. D, decomposition products and Voltarene L.P. tablets (containing 100 mg of D) were kindly supplied by Ciba-Geigy laboratories (Basel, Switzerland). For the chromatography, silica gel Merck 60 F 254, 20x10 cm HPTLC plates, a Camag twin-trough chamber (20 x 20 cm) and a Linomat IV (Muttenz, Switzerland) band applicator were used. A Camag TLC Scanner II chromatogram densitometer (Muttenz, Switzerland) with a deuterium lamp, connected to a Merck-Hitachi D-2000 integrator (Merck, Darmstadt, Germany) was used for densitometric measurements.

Test procedure submitted to validation.

-Standard solutions.

Use a reference batch of D free from compound IV .

Stock solution of D (2 g l^{-1}) : weigh accurately about 200 mg of D into a 100 ml calibrated flask, add about 90 ml of methanol, dissolve by sonication for 10 min, allow to cool and add methanol to the mark.

Stock solution of IV (50 mg l^{-1}) : weigh accurately about 5 mg of IV into a 100 ml calibrated flask, add about 90 ml of methanol, dissolve by sonication for 10 min, allow to cool and add methanol to the mark.

Working solutions (corresponding to 0.5%, 1%, and 1.5% of IV with respect to D) : place 25 ml of stock solution of D and respectively 5, 10, and 15 ml of stock solution of compound IV in three calibrated flasks of 50 ml. Make up to the mark with methanol.

-Test solution.

Place a tablet with about 90 ml of methanol in a 100 ml calibrated flask, sonicate for 10 min, allow to cool and add methanol to the mark. Centrifuge at 4000 rev min^{-1}. for 30 min. The supernatant is the test solution containing potentially compound IV.

-Sample application.

Before use, pre-wash the plates with methanol for 1 h, then dry at 80°C for 30 min and cool down. Apply in triplicate with the bandwise applicator 5 μl of each solution as bandlengths of 5 mm, at a delivery speed of 0.1 μl s^{-1}.

-Chromatography.

The chromatography should be carried out at about 20° ± 5°C.

Pre-equilibrate the plate with dichloromethane-methanol (92 : 8, v/v) vapors for 1 h. Then start the development and allow the eluent to migrate up to a distance of about 60 mm. The spots can be visualized under UV light at 254 nm : the respective Rf values are about 0.27 and 0.38 for D and IV. The resolution R, evaluated by the ratio a/b (Fig. 2) should not be lower than 0.70 at the 0.5% level.

-Densitometry.

Perform zero adjustment above the band of the analyte. Scan each track twice at 280 nm in the reflectance mode, using a 10 nm monochromator bandwith, a 0.4 x 3 mm slit dimension and a 0.3 mm s^{-1} scanning speed. Record the peak height. The repeatability between three loadings at 0.5% level should not be higher than 10%. Calculate the percent of compound IV in the tablet from the regression equation of the calibration line.

Test procedure validation.

Throughout the validation procedure, tablets were spiked using compound IV in methanolic solution. Unless otherwise stated, triplicate applications of a 5 μl volume of solution were performed as indicated in the routine test procedure.

-On-plate stability of the compound.

A standard solution containing 0.5% of IV (with respect to D) was applied on the plate; after 1 h the same solution was applied on the same plate and the plate was immediately developed. The response factors were compared.

-Stability of the solutions.

A standard solution (0.5% of IV) aged for 24 h at ambient temperature was applied on the plate. Its response factor was compared to that of a freshly prepared standard solution.

-Specificity.

The specificity of the technique for the determination of IV was tested by

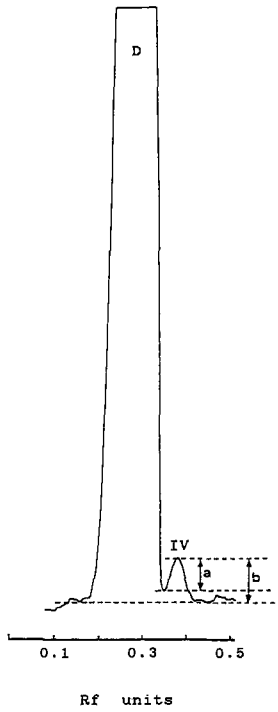

FIGURE 2. Chromatogram showing the limit of quantitation for compound IV (0.5% with respect to D).

applying the degradation compounds as indicated in the paragraph preliminary experiments. The non-interference of the formulation ingredients was assessed on an analytical placebo and a placebo stressed at 60°C for 7 h.

-Linearity and accuracy.

. Standard solutions.

The linearity of the response of the standard solutions was tested with five solutions containing 0.5%, 0.75%, 1%, 1.25%, and 1.5% of compound IV with respect to D (1 g l^{-1}) applied on the same plate.

Test solutions.

The linearity and accuracy of the test procedure were tested by spiking five tablets with compound IV (0.5%, 0.75%, 1%, 1.25%, and 1.5% with respect to D). The recovery was calculated by reference to the graph obtained from five standard solutions of the same concentrations, applied on the same plate. A single application of each test and standard solution was carried out on each of three plates issued from a same batch.

-Limit of detection.

The LOD was evaluated from a standard solution and subsequently confirmed on a tablet spiked at the same concentration (0.15% of IV) on plates from different batches and at either ends of the plates.

-Limit of quantitation.

The repeatability and accuracy of the test procedure at the LOQ were tested on six tablets spiked with 0.5% of IV (with respect to D). The recoveries were calculated from two series of standard solutions containing 0.5%, 0.75%, and 1% of IV (with respect to D). A single application of each solution was carried out on each of three plates issued from a same batch.

-Repeatability of the chromatographic system.

The repeatability of the chromatographic system was assessed by applying onto a same plate seven replicates of each of two standard solutions (0.5% and 1.5%).

-Ruggedness.

The ruggedness of the method was tested at the LOQ on a test solution prepared from a spiked tablet (0.5%); the recoveries were calculated by reference to a series of standard solutions (0.5%, 0.75%, and 1.0%). A Plackett and Burman experimental design was used (7). Each of the critical factors chosen (plate batch, mobile phase composition and developing temperature) was tested at three levels. The factor levels were as follows : three batch numbers; ratio dichloromethane : methanol (93 : 7, 92 : 8, or 91 : 9, v/v); temperature (15°C, 20°C, or 30°C). For the convenience of manipulation, the test was carried out in two blocks (higher + and basic 0 levels; lower - and basic 0 levels), each comprising four experiments.

RESULTS AND DISCUSSION

-On-plate stability.

It has been shown that some compounds are easily oxidized on the plate, the oxidation being catalyzed by the presence of metal ions in the silica coating, the acidity of the

silica, or the solvent system used (8). Therefore, the first test carried out was the assessment of on-plate stability of both the impurity and the active principle (present in large amount). The test was performed at the LOQ. No artefact was observed and no difference was found ($p = 0.05$) within the detection limits of the method, in the response factors for a solution applied at 1 hour interval. This delay allows to cover the time for band application. The on-plate stability of compound IV after development was also tested : no significant difference in the response was found between two scannings carried out at 90 min interval; this is sufficient to cover the measurement time. A two-dimensional chromatography was not carried out in this study because no degradation was noted during the chromatographic process; it should be performed if a degradation occurs during the chromatographic development.

-Solution stability.

The stability of the solutions containing 0.5% of IV was tested : no difference in the response factors was found ($p = 0.05$) between a solution aged for 24 h and a freshly prepared one. The solutions can be used within this delay without the results being affected.

-Specificity.

Under the conditions used, compound IV was well separated from the other degradation compounds and partially resolved from D. No interference of the formulation ingredients was observed.

-Linearity and accuracy.

Linearity of the calibration curve.

The test procedure uses standard solutions containing increasing amounts of IV in the presence of D (100% of the theoretical content) to imitate tablet solutions. The addition of the parent compound is particularly recommended when the impurity is located in the tailing of the parent compound as it allows the on-plate separation to be checked and a similar mode of measurement to be performed for standard and test solutions. In addition, it takes into account a possible change in the Rf values of the degradation product at trace levels in the presence of a large amount of the active drug. The linearity should be assessed from the LOQ to 150% of the tolerated limit (taken as 1% with respect to D for the purpose of the work).

The linearity of the calibration curve (peak height *vs* applied amount of IV) was first examined by a graphic plot : a straight line not passing through the origin was obtained (Fig.3).

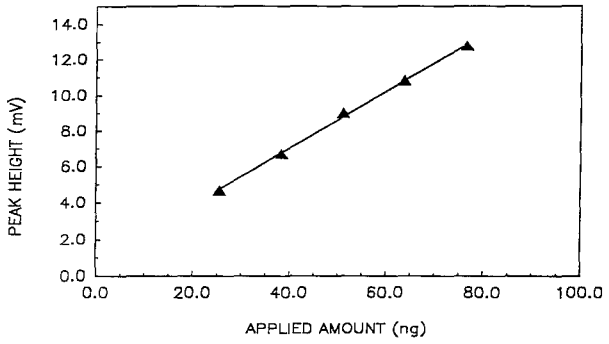

FIGURE 3. Calibration line for compound IV.

The regression line, calculated from the least-squares method was :

Peak height (μV) = (159.503 $\pm$ 6.070) applied amount (ng) + (698.933 $\pm$ 328.361), with the confidence intervals calculated at p = 0.05.

The correlation coefficient was 0.998. The linearity of the regression was assessed by ANOVA with F_{cal} of regression = 3221 ($p < 0.01$) and F_{cal} of non-linearity = 2.36 ($p > 0.05$).

Due to the fact that the regression line did not pass through the origin and that the analyte was not at a target concentration, a multi-level calibration was needed on each plate. For routine analysis, a graph constructed with three concentration levels was found to be sufficient.

Linearity and accuracy of the test procedure.

It should be noted that throughout this study, tablets were spiked with compound IV in solution. This can be accepted (9) when the analyte is not available in sufficient amount (in solid form) to prepare homogeneous samples simulating degraded tablets. In addition, it was assumed that by using a simple extraction procedure in a non-complex matrix together with a solvent having a high solubility *vs* the compound of interest, the major source of variations could be ascribed to the technique itself (sample application, plate-to-plate variations, development and densitometric evaluation). The proposed experimental approach is intended to take into account these variations by performing a single application of each solution on each of three plates, which

TABLE 1. Linearity and accuracy of the test procedure.

Amount added (ng)	Amount found (ng)	Recovery (%)	Bias (%)
	24.69	96.82	-3.18
25.50	23.99	94.08	-5.92
	26.75	104.90	4.90
	36.63	95.76	-4.24
38.25	35.78	93.54	-6.46
	37.95	99.22	-0.78
	51.90	101.76	1.76
51.00	49.21	96.49	-3.51
	49.66	97.37	-2.63
	67.19	105.40	5.40
63.75	62.17	97.52	-2.48
	62.60	98.20	-1.80
	79.13	103.43	3.43
76.50	71.60	93.59	-6.41
	78.29	102.34	2.34

allows the chromatographic system reproducibility to be evaluated. The results are given in TABLE 1.

The maximum experimental bias on each point was lower than 7%; a bias of $\pm$ 10% can be largely accepted for an impurity at these levels.

Linearity and accuracy were assessed by plotting the graph of amount found *vs* amount applied resulting from the three experiments. As a Barlett test showed that the recovery dispersions were not significantly different, one-way ANOVA was carried out on the recovery data. No significant difference between the plates ($p = 0.05$) was found and the graph was constructed using for each added amounts X the data from the three plates Y. The linear regression equation obtained was :

Amount found = (1.016 ± 0.068) amount added - (1.335 ± 3.709) with confidence intervals calculated at $p = 0.05$. The correlation coefficient was 0.994. The linearity was assessed by ANOVA with F_{cal} of regression = 837 ($p < 0.01$) and F_{cal} of non-linearity = 0.02 ($p > 0.05$). The t-tests showed that the slope of the line was not significantly different from unity ($t_{cal} = 0.517$) and that the line passed through the origin ($t_{cal} = 0.777$) corresponding to $p > 0.05$. The procedure could be considered as accurate and linear within the range investigated.

From the three experiments, an estimation of the overall repeatability (RSD = 3.24%, n = 15) and inter-plate reproducibility of recoveries (RSD = 4.32%, n = 15) was calculated.

-Repeatability.

The chromatographic system repeatability was 2.57% and 1.88% (n = 7 loadings) at respective levels of 0.5 and 1.5% of compound IV. The scanning repeatability was found to be 0.16% (n = 7 scans).

-Limit of detection.

The LOD is usually defined as the amount of substance which gives a signal-to-noise ratio of 2 or 3. In the case of an analyte eluted in the tailing of a parent compound, the background due to this latter is the limiting factor together with the noise and the amount which gives visually a shoulder can be considered as the LOD. The LOD was estimated to be 7.5 ng of IV in the presence of 5 μg of D, which corresponds to 0.15% of IV with respect to D (Fig.4). This evaluation was confirmed with tablets spiked at this level, on different plates and at either ends of the plates. Increasing the applied volume of solution (from 5 to 8 μl) did not give a noticeable change of the LOD due to a correlated loss of resolution by spot diffusion.

-Limit of quantitation.

The LOQ was estimated by the amount of compound which gave a signal equal to about 3 times that of the LOD (about 0.5%). This limit was subsequently validated (repeatability and accuracy) by the analysis of six extracts from tablets spiked with compound IV at this level on each of three plates (Fig.2). The recoveries are given in TABLE 2.

As the Bartlett test was not significant ($X^2 = 0.37$), one-way ANOVA was carried out which showed no significant effect of the plates on the results. The total mean recovery from the three plates was (98.98 $\pm$ 2.48)% with confidence limits on the RSD between 3.77% and 7.54%. The maximum experimental bias was 9.02%, which is largely tolerated at the LOQ. Although triplicate loadings are indicated in the test procedure for an accurate determination, the present data show that a single application of the solutions could comply with the assay requirements for a degradation product.

-Ruggedness.

In the United States Pharmacopeia (10), ruggedness is very similar to reproducibility. The ruggedness can be evaluated by studying the influence of critical factors of the

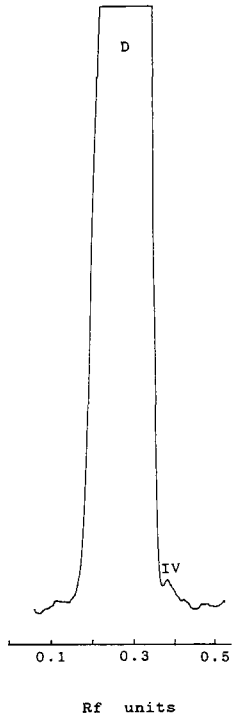

FIGURE 4. Chromatogram showing the limit of detection for compound IV (7.5 ng) in the presence of D (5 μg).

procedure successively or in combination through an experimental design (7). In the present study, selected factors were those which could be critical for separation, identification and quantitation : plate batch number, eluent composition and temperature. For quantitative factors, the variations used were in the range of order of those which could be found between laboratories. The influence of the different factors was tested at the LOQ on the assay, resolution (R) and Rf value, which are of major interest for identification and quantitative analysis. The results are given in TABLE 3.

A factor was considered as significant when the difference | D | between the average of results at extreme and nominal levels was larger than $\sqrt{2}$ times the SD at the nominal level (7, 11). Two-way ANOVA on reproducibility data for the procedure gave a SD of 5.69 and

TABLE 2. Repeatability and accuracy of the procedure at the limit of quantitation.

	Plate 1	Plate 2	Plate 3
	94.24%	97.25%	95.25%
	96.08%	96.35%	109.02%
	105.14%	99.10%	93.41%
	98.78%	104.55%	98.00%
	94.24%	108.20%	95.25%
	100.59%	96.35%	99.84%
m_i	98.18%	100.30%	98.46%
sd	4.24%	4.95%	5.65%
m		98.98%	
SD_r		4.98%	
SD_R		4.98%	

SD_r : SD of overall repeatability
SD_R : SD of inter-plate reproducibility

0.07 for assay and R respectively. Moreover, the SD between the Rf values of three plates was 0.01.

From TABLE 3, it can be seen that the plate batch number is only significant on the Rf (at a minor extent) and that a change in the mobile phase composition or temperature influence both resolution and Rf. However none of these factors has a significant effect on the assay results.

-Sensitivity.

It is commonly expressed as the slope of the calibration line but can also be assimilated to the resolution power of the method, i.e. the minimum variation of concentration or amount which gives a significant variation of the response with α and ß risks (9) :

Sensitivity = $(t_{(1-\alpha/2)} + t_{(1-\beta)})\, s\sqrt{2}/b$, where b is the slope of the calibration line, s the standard deviation of repeatability and t the Student t-value. The sensitivity was found to be 7.25 ng at LOQ and 6.13 ng on the whole range of linearity ($\alpha = \beta = 0.05$). These results are very close to the limit of detection.

TABLE 3. Results of the ruggedness test.

NOMINAL LEVEL(0) AND LOWER LEVEL(-)

Exp.	Factors			Results			
	a	b	c	Assay	R	Rf	
1	0	0	0	93.73%	0.96	0.38	y_1
2	-	0	-	92.80%	0.82	0.46	y_2
3	0	-	-	94.13%	0.98	0.36	y_3
4	-	-	0	94.07%	1.00	0.32	y_4

Differences due to each factor for the respective results (%, R, Rf) :

Da = $[(y_1+y_3)-(y_2+y_4)] / 2$ = 0.50, 0.06,-0.02
Db = $[(y_1+y_2)-(y_3+y_4)] / 2$ = -0.84,-0.10, 0.08
Dc = $[(y_1+y_4)-(y_2+y_3)] / 2$ = 0.44, 0.08,-0.06

NOMINAL LEVEL(0) AND UPPER LEVEL(+)

Exp.	Factors			Results			
	a	b	c	Assay	R	Rf	
1	0	0	0	104.43%	0.98	0.40	y_1
2	+	0	+	97.14%	0.76	0.39	y_2
3	0	+	+	94.85%	0.62	0.42	y_3
4	+	+	0	101.53%	0.82	0.47	y_4

Da = $[(y_1+y_3)-(y_2+y_4)] / 2$ = 0.31, 0.01,-0.02
Db = $[(y_1+y_2)-(y_3+y_4)] / 2$ = 2.60, 0.15,-0.05
Dc = $[(y_1+y_4)-(y_2+y_3)] / 2$ = 6.99, 0.21, 0.03

CONCLUSION

This paper was not intended to cover all the aspects of TLC validation for a degradation product but to point out some particular aspects related to the determination at trace levels. Particular attention has been paid to some critical factors which may affect the results : within-batch and inter-batch plate variations, modification of the mobile phase composition and temperature. Other factors could be also relevant to study (applied volume, saturation time of the chamber, etc.). It should be noted that the proposed design is complicated by the fact that a multi-

level calibration is needed since the calibration graph does not go through the origin and the level of analyte is not assumed to be known.

TLC is mainly used in pharmaceutical industry as a qualitative or semi-quantitative method. However, this study shows that it gives reliable results for analytes at low levels and may be a useful alternative to HPLC (12), in particular when a small number of samples has to be analyzed.

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REFERENCES

1. Fabre H., Sun S.W., Maillols H. and Mandrou B.- Assay validation for an active ingredient in a pharmaceutical formulation : practical approach using spectrophotometry, Analyst, in press, July, 1993.
2 Sun S.W. and Fabre H.- Practical approach for validating the TLC assay of an active ingredient in a pharmaceutical formulation, J. Liq. Chromatogr., in press, 1993.
3. Schumacher A., Geissler H. E. and Mutschler E.- Quantitative Bestimmung von Diclofenac-Natrium aus Plasma durch Absorptionsmessung mit Hilfe der direkten Auswertung von Dünnschichtchromatogrammen, J. Chromatogr., 181, 512, 1980.
4. Stead A.H., Gill R., Wright T., J.P. Gibbs J.P. and Moffat A.C.- Standard thin-layer chromatographic system for the identification of drugs and poisons, Analyst, 107, 1106, 1982.
5. Moffat A.C.(Ed.)- Clarke's Isolation and Identification of Drugs, 2nd edn, p. 169, The Pharmaceutical Society of Great Britain, London, 1986.
6. Sarbu C.- Detection of some non-steroidal anti- inflammatory agents on thin-layer chromatographic plates coated with fluorescent mixtures, J. Chromatogr., 367, 286, 1986.
7. Youden W.J. and Steiner E.H.- Statistical Manual of the A.O.A.C., pp. 33-36, 70-71, 82-83, A.O.A.C., Arlington, VA, 1975.

8. Fabre H. and Mandrou B.- Quality control of phenylbutazone I: Analysis of phenylbutazone and decomposition products in drugs by TLC, J. Pharm. Sci., 70, 460, 1981.

9. Rapport d'une Commission SFSTP, Guide de validation analytique, S.T.P. Pharma Pratiques, 2, 205, 1992.

10. The United States Pharmacopeia, XXII Rev., p. 1712, The United States Pharmacopeial Convention, Rockville, MD, 1990.

11. Massart D.L., Vandeginste B.G.M., Deming S.N., Michotte Y. and Kaufman L.- Chemometrics : a textbook, p.105, Elsevier, Amsterdam, 1988.

12. Beaulieu N., Lovering E.G., Lefrançois J. and Ong H.- Determination of diclofenac sodium and related compounds in raw materials and formulations, J. Assoc. Off. Anal. Chem., 73, 698, 1990.

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SEPARATION OF ORGANOPHOSPHONATES BY ION CHROMATOGRAPHY WITH INDIRECT PHOTOMETRIC DETECTION

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ABSTRACT

An ion chromatographic method which utilizes lithium trimesate to separate three diphosphonate drugs and two process related organophosphonates is described. The lack of a chromophore on the five species and the high absorptivity of the trimesate anion at 254 nm allows for facile detection using indirect photometry. The effects of mobile phase concentration on the capacity factor of the species were investigated and a logarithmic relationship was established which was found to be dependent on the charge of the analyte anion and the mobile phase anion.

INTRODUCTION

A number of diphosphonates have found applications in the pharmaceutical industry for treatment of abnormal calcium metabolism such as osteoporosis,

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ectopic calcification, and various bone diseases. Diphosphonates are known to bind strongly to calcium hydroxyapatite, the major constituent of the mammalian skeleton, through chelation of calcium. This chelation inhibits bone resorption, as found with osteoporosis or various bone diseases (1), or at higher levels inhibits crystal growth through the prevention of further addition of calcium, orthophosphate, or hydroxide into the crystal lattice, leading to calcification in joints, arteries and various organs (2,3). Endogenous inorganic pyrophosphate performs such a role in mammals but tends to be hydrolysed quickly in the body (4).

Etidronate, pamidronate, and clodronate are some of the diphosphonates which are currently marketed. Their development was based on the observation that diphosphonates are more effective due to their increased stability towards hydrolysis in the body as compared to endogenous pyrophosphate (1-5). This extra stability is due to the presence of P-C-P bonds in these drugs as opposed to P-O-P bonds found in endogenous pyrophosphate. Alendronate, an amino diphosphonate (Fig. 1), is a new member of the class of diphosphonates which is specifically targeted for inhibition of bone resorption.

Routine chromatographic analysis of diphosphonates is complicated due to their high ionic character and general lack of a strong UV chromophore. Current methods of analysis include derivatization prior to chromatographic separation (6-9), post column reaction (10-13), ion conductivity detection (14), flame photometric detection (15), or inductively coupled plasma detection (16).

Previous chromatographic analysis for alendronate (9) consisted of pre-column derivatization of the amine group with 9-fluorenylmethyl chloroformate (FMOC-Cl) at pH 9 followed by elution from a polymeric phase (Hamilton PRP-1). FMOC-Cl reacts rapidly with primary and secondary amines to produce very stable derivatives. This derivatization is routinely used for reverse phase

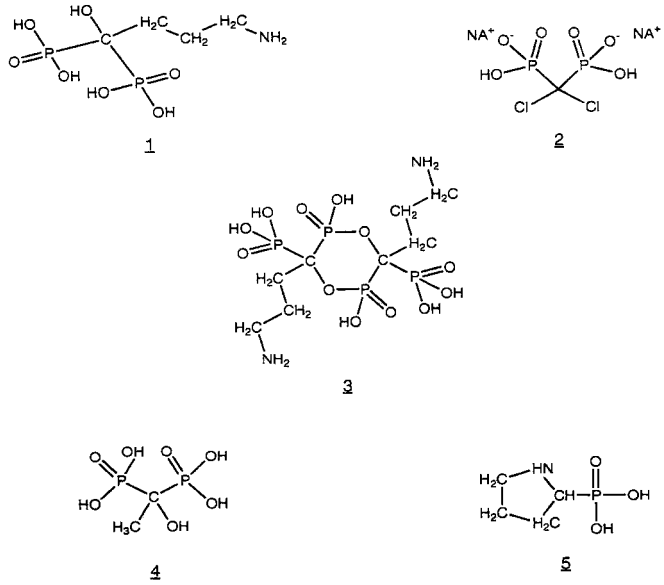

Fig. 1: Structures of the organophosphonates. 1 - Alendronate; 2 - Clodronate; 3 - Alendronate Dimer; 4 - Etidronate; 5 - 2-Phosphonopyrrolidine

chromatography of amino acids (17,18). The added FMOC group allows for better retention in the reverse phase mode and provides a strong chromophore suitable for UV or fluorescence detection.

There are two major disadvantages associated with the use of FMOC derivatization however. First considerable time and effort are devoted to sample preparation. Secondly, FMOC derivatization would be specific only for species which contain a primary or secondary amine group available for derivatization. The synthesis of alendronate (19) generates ionic by-products such as alendronate dimer, 2-phosphonopyrrolidine, chloride, phosphite, and methanesulfonate and could potentially generate de-aminated phosphonates as well. Some of these by-products would not be detected by the FMOC derivatization method.

In response to the above-mentioned disadvantages to the FMOC-HPLC method an ion chromatography method was developed. The use of ion chromatography with indirect photometric detection allows for separation and detection of diphosphonates with standard HPLC pumps and UV detectors and requires minimal sample preparation.

Indirect photometric detection (IPD) was introduced by Schill in 1981 (20) and has subsequently found wide application (21-29). It requires a mobile phase species which absorbs highly at a wavelength that the analyte does not. If the mobile phase species has equilibrated with the stationary phase, the detector senses a strong steady absorbance. When a non-absorbing analyte is injected onto an ion exchange stationary phase, it displaces mobile phase ions from charged sites on the stationary phase resulting in a localized zone of higher mobile phase ion concentration. This zone is manifested in the detector as a region of higher absorbance and produces a positive system peak. When the analyte ion is eventually eluted from the stationary phase, its transparency results in a localized region of lower absorbance and is manifested in the detector as a negative analyte peak.

Salts of carboxylic acids such as phthalic acid, citric acid and succinic acid tend to be good displacing anions for anion exchange chromatography and have been widely utilized. Salts of aromatic carboxylic acids such as phthalic acid, benzoic acid, trimesic acid, and p-hydroxybenzoic acid are suitable for ion chromatography with IPD because of their displacing strengths and their ability to strongly absorb light due to the presence of the aromatic ring. Lithium trimesate was chosen as the mobile phase for this particular separation.

An assay for etidronate using nitric acid as an additive in the mobile phase and IPD has been previously reported (30). The method does not, however, address the issue of impurities in the bulk drug. The method reported here, using

trimesate ion, can be used as an impurity profile as well as an assay. As a strong displacing agent, trimesate elutes both low-charged weakly retained analytes and high-charged strongly retained analytes without having to resort to a gradient method. Separation was achieved for three diphosphonate drugs (alendronate, etidronate, and clodronate) and two organophosphonates (2-phosphonopyrrolidine and alendronate dimer) which are by-products of the synthesis of alendronate (structures are given in Fig. 1). The analytes were detected by IPD. The retention characteristics of the organophosphonates were investigated as a function of eluent ion concentration, pH, and hydrophobicity. Results were found to closely match theoretical considerations.

EXPERIMENTAL

Chromatographic Equipment

The HPLC Equipment consisted of a Spectra Physics SP8800 ternary pump, a Spectra Physics SP8775 autosampler (Spectraphysics, Piscataway, New Jersey) and a Kratos Spectroflow 757 absorbance detector (ABI Analytical, Foster City, California) . The chromatograms were processed using PE Nelson Access Chrom version 1.7 software (PE Nelson, Cupertino, California). The column used was a Hamilton PRP-X100, 250 x 4.1 mm (Hamilton Co., Reno, Nevada) which consists of 10 μm spherical particles of poly(styrene-divinylbenzene) trimethylammonium base ion exchanger. This column is a low capacity (0.2 meq/g) ion exchanger.

Chromatographic Conditions

The mobile phase was trimesic acid (1,3,5-benzenetricarboxylic acid, Sigma, St Louis, Missouri) in water. Adjustments of pH were made with lithium

hydroxide (Baker analyzed, J.T. Baker, Philipsburg, NJ). Samples were prepared by dissolution in deionized water and were introduced into the chromatographic system through a 10 μL loop.

The column was equilibrated with the mobile phase until a steady baseline was obtained. The detector was zeroed at a higher wavelength than that used for detection such that the baseline absorbance at the detection wavelength is around 0.5 absorbance units. All chromatographs were performed at ambient conditions with a flow rate of 1.0 mL/min unless otherwise stated. Capacity factors were determined as defined by:

$$k' = (t_r - t_0)/t_0$$

where t_r is the retention time of the analyte peak and t_0 is the first perturbation in the baseline after injection of water.

pK_a Determinations

The pK_a's of alendronate dimer and 2-phosphonopyrrolidine were determined through potentiometric titrations using a Metrohm 665 dosimat and a 670 titroprocessor (Brinkmann, Westbury NY).

RESULTS AND DISCUSSION

System Optimization

The high ionic character of the diphosphonates render them incapable of being retained strongly on a reversed phase column without some form of derivatization. Adjustment of pH in order to protonate the phosphonate groups generally require pH conditions of less than 2 which is usually unacceptable for

these columns. They can be retained on silica based mixed mode columns but with very poor efficiency which is manifested in the form of very broad peaks. Polymer based anion-exchange columns provide mixed mode interactions without the tailing observed with silica based mixed mode columns. There are two types of sites available on polymer based anion exchange columns - hydrophobic and ion exchange sites. Separation is affected by ion exchange and by interaction of the organic analyte ion with the non-polar polymeric backbone provided the analyte ion possesses a hydrophobic moiety (31,32).

When using IPD, both the retention and the detection capabilities are dependent upon the nature of the mobile phase anion. Sensitivity is dependent upon the concentration of the highly absorbing mobile phase anion (28,33). The noise is directly proportional to the absorbance of the mobile phase anion and the absorbance increases with increasing mobile phase anion concentration. The signal is proportional to the concentration of the analyte anion and the difference in absorptivity between the analyte and the mobile phase anions. The signal to noise ratio is therefore inversely proportional to the concentration of the mobile phase anion. Specifically, sensitivity increases with decreasing concentration of the mobile phase anion. It is advantageous therefore to have a relatively strong displacing anion in the mobile phase so that one can obtain short elution times at lower concentrations while maximizing sensitivity for the analyte ions.

The optimal wavelength for detection is determined by two factors. First, it is best to choose a wavelength where the difference in the molar absorptivity of the mobile phase and the analyte anions is at its greatest (ideally at a maximum for the mobile phase anion and a minimum for the analyte anion). Secondly, the absorbance at this wavelength should not be too high as to saturate the detector.

Preliminary experiments using sulfobenzoic acid and p-hydroxybenzoic acid, which are strong displacing agents with strong chromophores, exhibited poor

selectivity for the separation of the organophosphonates. Trimesate ion, which is also a strong displacing agent with a strong chromophore, was chosen because it exhibited better selectivity.

The retention times of the five organophosphonates were monitored as a function of pH and trimesate concentration. The optimum mobile phase conditions were found to be 1 mM trimesic acid adjusted to pH 5.5 (Fig. 2). The samples were chromatographed under ambient conditions with a flow rate of 1 mL/min . There is minimal difference in the efficiency of the analyte peaks for flow rates varying from 0.25 to 1 mL/min. Detection was at 254 nm. Under these conditions, the method also resolves chloride, phosphite and methanesulfonate (byproducts from the synthesis of alendronate sodium) along with the five organophosphonates being studied (Fig. 3). The method also resolves unidentified thermal degradates from alendronate (Fig. 4).

Under the above conditions a linear detector response was observed from 0.1 mg/mL to 2 mg/mL of the five organophosphonates (correlation coefficients

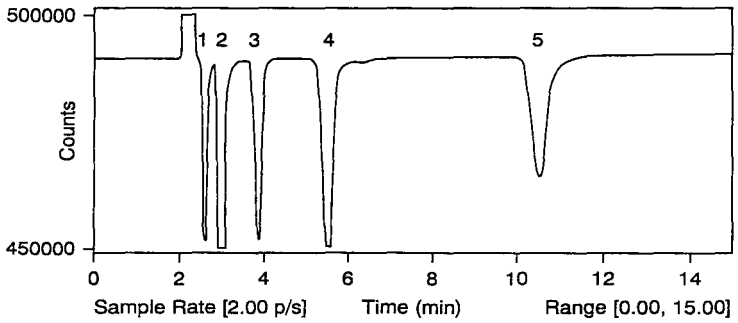

Fig. 2: Separation of the organophosphonates. Chromatographic conditions are 1 mM trimesic acid adjusted to pH 5.5 with detection at 254 nm. 1 - 2-phosphonopyrrolidine; 2 - alendronate; 3 - alendronate dimer; 4 - etidronate; 5 - clodronate.

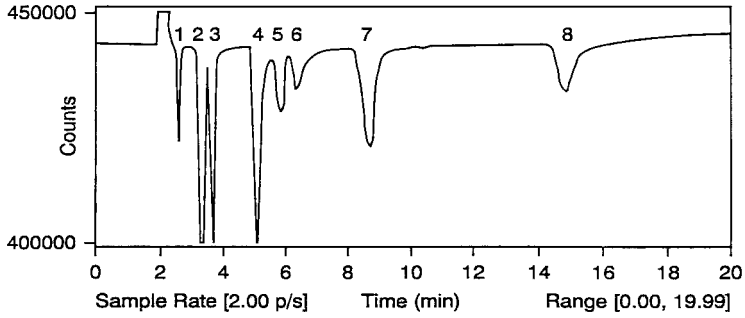

Fig. 3: Separation of the organophosphonates and process related components from the synthesis of alendronate. Chromatographic conditions are 1mM trimesic acid adjusted to pH 5.5 with detection at 254 nm. 1 - 2-phosphonopyrrolidine; 2 - alendronate; 3 - phosphite; 4 - chloride; 5 - methanesulfonate; 6 - alendronate dimer; 7 - etidronate; 8 - clodronate.

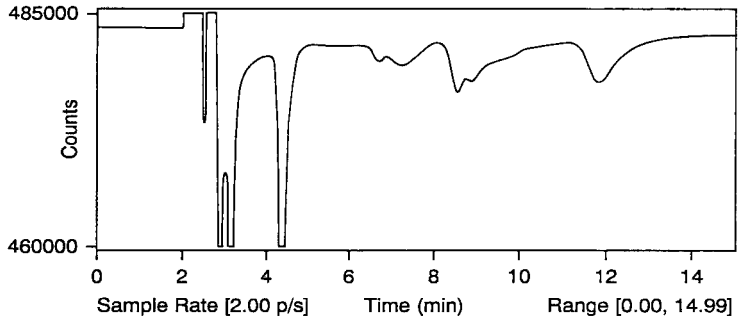

Fig. 4: Separation of alendronate from its thermal degradates. Chromatographic conditions are 1 mM trimesic acid adjusted to pH 5.5 with detection at 254 nm.

were all greater than 0.9997) and the limits of detection ranged from 50 ng for 2-phosphonopyrrolidine to 250 ng for alendronate dimer. The detection limits of the organophosphonates are an order of magnitude greater than that of species possessing small ionic masses such as Cl^- (20 ng) and this is a reflection of their larger ionic masses. If the analyte and displacing ion are of the same charge, each analyte ion replaces one ion of the mobile phase ion in the localized zone (in order to preserve electroneutrality) . Consequently, the response on a molar ionic basis should be the same for any analyte irrespective of any other properties of the analyte and the detection limit, on a mass basis, will be lower for analytes of small ionic mass.

Effect of Mobile Phase Anion Concentration on the Retention of the Analytes

The interaction between the mobile phase displacing anion and the analyte anion and their distribution between the stationary phase and the mobile phase can be described by:

$$yS_xM + xA \rightleftharpoons xS_yA + yM \quad \text{---------- K1} \quad \{1\}$$

where

y = charge on the analyte anion
x = charge on the mobile phase anion
M = mobile phase anion
S = stationary phase cation
A = analyte anion

Small and Miller (22) has previously derived the relation between the capacity factor and the mobile phase anion concentration at constant pH which is expressed as:

$$[S_yA]^x/[A]^x = K1[S_xM]^y/[M]^y \qquad \{2\}.$$

Assuming that the stationary phase is saturated with respect to the mobile phase anion, its concentration can be assumed to be constant and:

$$[S_yA]/[A] = B/[M]^{y/x} \quad \{3\}$$

and

$$\log k' = -(y/x)\log[M] + \log C \quad \{4\}$$

where B and C are constants. Thus a logarithmic plot of capacity factor versus mobile phase ion concentration should give a straight line with a slope that is the ratio of the charges on the analyte and the mobile phase ion. This equation applies to a number of species eluted by various mono- and multivalent mobile phase ions (22,28,34).

The capacity factors of the five organophosphonates along with chloride ion were measured as a function of trimesate ion concentration at a constant pH of 5.0. At this pH the organophosphonates are each present as predominantly one solute species (Table 1). The logarithmic plot of capacity factor versus trimesate ion concentration proved to be linear for all six species (Fig. 5).

A slope of -0.5 was observed for chloride. Since chloride possesses a charge of negative one, the slope indicated (from Equation 4) that the displacing ion was the double charged trimesate species rather than the triple charged species at this pH. However, the pKa's for trimesic acid (Table 1) indicates that, at pH 5, the triple charged species should be the dominant trimesate species. One would thus anticipate that the triple charged species would be the displacing ion. The observed behavior can be explained by elaborating on Equation 4. Assuming the presence of a protonated species HM in the system, its interaction with the analyte can be expressed similarly to Equation 1:

$$yS_{x-1}HM + (x-1)A \rightleftharpoons (x-1)S_yA + yHM \quad \text{---------- K2} \quad \{5\}$$

TABLE 1

Acid Dissociation Constants

	pK1	pK2	pK3
Trimesic Acid (36)	3.10	3.90	4.70
Alendronate (37)	< 2	6.2	9.9
Alendronate Dimer	< 3	4.1	> 9
2-Phosphonopyrrolidine	< 3	5.6	
Etidronate (38)	< 2	2.5	6.89
Clodronate (38)	< 2	2.3	5.82

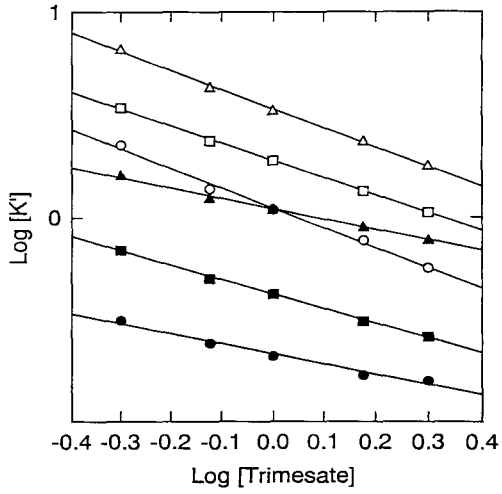

Fig. 5: Plot of log capacity factor vs log trimesate (mmolar) concentration at pH 5.0. Δ - clodronate; □ - etidronate; ○ - alendronate dimer; ▲ - chloride; ■ - alendronate; ● - 2-phosphonopyrrolidine.

In addition the interaction between the two anions, M and HM, in the mobile phase and in the stationary phase can be expressed as:

$$x\text{-}1S_xM + xH^+ + M \rightleftharpoons xS_{x-1}HM \text{ ---------- } K3 \quad \{6\}$$

and

$$H^+ + M \rightleftharpoons HM \text{ ---------- } K4 \quad \{7\}$$

Equation 7 indicates that at a fixed pH, the ratio of M to HM remains constant and is independent of total mobile phase anion (M_T). However such a linear relationship does not exist between S_xM and $S_{x-1}HM$. It is evident that even at constant pH, the relative amounts of S_xM and $S_{x-1}HM$ will vary in a non-linear fashion relative to the concentration of M_T.

Equations 1 and 5 can be combined such that:

$$K5 = K1 * K2 = ([S_yA]^{2x-1} [M]^y [HM]^y) / ([A]^{2x-1} [S_xM]^y [S_{x-1}HM]^y) \quad \{8\}$$

and

$$[S_yA]/[A] = (D [S_xM] [S_{x-1}HM]/[M] [HM])^{y/(2x-1)} \quad \{9\}$$

Where D is a constant. If the stationary phase is saturated with respect to S_xM only, then S_xHM and HM can be expressed in terms of S_xM and M by using Equation 3 and 4 which leads to:

$$k' \propto ([S_xM]/[M])^{y/x} \quad \{10\}$$

Since the stationary phase is saturated with respect to S_xM, its concentration can be considered constant and thus:

$$\log k' = -y/x\log[M] + E \quad \{11\}.$$

Similarly, if the stationary phase is saturated with respect to S_xHM only, it can be shown that

$$k' \alpha ([S_{x-1}HM]/[HM])^{y/(x-1)} \quad \{12\}$$

and

$$\log k' = -y/(x-1)\log[HM] + F \quad \{13\}$$

where E and F are constants. If the stationary phase is saturated with respect to both S_xM and $S_{x-1}HM$, then:

$$\log k' = 2y/(2x-1)\log[M] + G \quad \{14\}$$

where G is a constant.

The slope observed for 2-phosphonopyrrolidine was -0.54 and for etidronate, clodronate and alendronate dimer -0.83, -0.93 and -0.95. Taking into account the pKa's of these species, the observed slopes are more in line with the theory that the doubly charged trimesate species is the species which is being exchanged with the analyte ion on the stationary phase. The only deviation was noted for alendronate which possessed a slope of -0.70 despite the fact that it would be predominantly singly charged at this pH.

Further evidence is provided by the data generated by Motimizu et al (28) using trimesate ion as the displacing ion. They observed that, using a IC-Anion PW column, slopes of -0.40, -0.39, and -0.73 were obtained for chloride, nitrate, and sulfate ion respectively. Similarly, using a IC Anion SW column, slopes of -0.46, -0.42, and -0.75 were obtained for chloride, nitrate, and sulfate ion respectively. These results are more compatible with the assumption that the

stationary phase is saturated with both negative two and three charged trimesate species than the assumption they made (saturation with respect to only a negative three charged trimesate ion). Saturation with respect to only the negative three charged trimesate ion would have given slopes of -0.33 for chloride and nitrate and a slope of -0.67 for sulfate. Saturation with respect to both the negative two and three charged trimesate would give slopes of -0.4 for chloride and nitrate and -0.8 for sulfate. No pH data was presented for their work, but their experimental section does indicate that they used just enough base to neutralize the trimesic acid which would place their pH range from 5 to 7. The authors also performed studies with many other carboxylate ion additives in the mobile phase and found similar results.

The dependence of the capacity factor on the mobile phase concentration was then investigated at a higher pH (6.0). The analytes used were chloride, alendronate dimer and etidronate as they would be present as predominantly one species at this pH. The slopes were found to be similar to those observed at pH 5.0 (Table 2).

Logarithmic plots of capacity factor versus displacing ion concentration can be applied to facilitate method development for the separation of ionic species. The retention time of the ionic species for which separation is desired can be monitored at two different concentrations and their retention times can be predicted from the line which joins the two points. The approximate region where separation is optimized (the concentration at which the species do not co-elute and are at maximal separation) can then be determined.

Effect of pH on the Retention of the Analytes

The dependence of the capacity factor of the organophosphonates on the pH of the mobile phase at constant mobile phase anion concentration was

TABLE 2

Slopes For log Trimesate Concentration vs log Capacity Factor

	pH 5.0	pH 6.0
Chloride	-0.50	-0.50
2-Phosphonopyrrolidine	-0.54	
Alendronate	-0.70	
Alendronate dimer	-0.95	-0.96
Etidronate	-0.83	-0.91
Clodronate	-0.93	

investigated. The capacity factor was monitored while varying the pH from 3.3 to 10 (Fig. 6). The capacity factor initially decreased with increasing pH indicating that deprotonation of the trimesic acid led to stronger displacing capability due to higher ionic strengths. A minima was observed at pH 4.5 after which the capacity factors again increased. This minima lends credence to the theory that the double charged species is the displacing agent since it occurs in the region where the double charged species is at its maximum concentration. The increase in the capacity factor can also be attributed to the deprotonation of the analyte ions which would cause them to become more tightly bound to the stationary phase. However such a correlation requires that the area where a minimum occurs should vary with the analyte and should also appear at a higher pH than 4.5. This factor thus appears to be minimal.

Effect of Organic Modifier on the Retention of the Analytes

Since the stationary phase is polymer, there are two possible types of interactions - ion exchange and adsorption. If adsorption plays a major role in the interaction with analyte ions, the addition of an organic modifier would affect the separation. Specifically the retention time of an analyte ion should decrease if

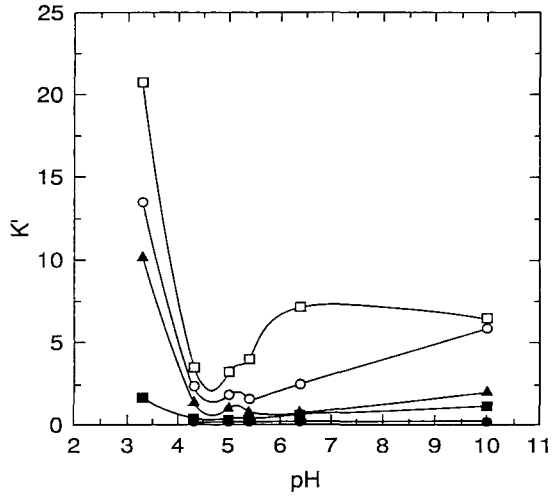

Fig. 6: Influence of pH on capacity factor. □ - clodronate; ○ - etidronate; ▲ - alendronate dimer; ■ - alendronate; ● - 2-phosphonopyrrolidine.

there was some type of adsorption interaction in effect. The retention time of an analyte ion could also increase if its retention is due primarily to ion exchange. As the mobile phase becomes more nonpolar the ion exchange sites would become more polar relative to the mobile phase increasing its attraction for species with little or no hydrophobic character and thus increasing their retention (35). It was indeed observed that chloride, which would have no hydrophobic interaction with the stationary phase, is increasingly retained with increasing percentage of acetonitrile in the mobile phase. The addition of 10% acetonitrile to the mobile phase decreased the retention of 2-phosphonopyrrolidine and alendronate and increased the retention of alendronate dimer and etidronate affecting a separation of the previously co-eluting alendronate and alendronate dimer (Figure 7). Increased amounts of acetonitrile up to 40% results in increased retention for 2-phosphonopyrrolidine, alendronate, alendronate dimer, and etidronate.

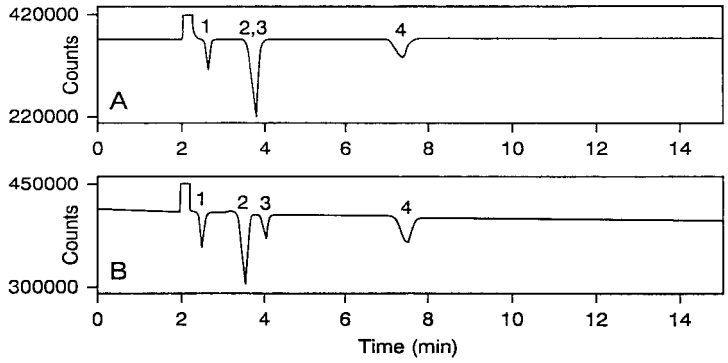

Fig. 7: Influence of organic modifier on retention of analytes. 1 mM trimesic acid adjusted to pH 6.5. 1 - 2-phosphonopyrrolidine; 2 - alendronate; 3 - alendronate dimer; 4 - etidronate. A: No organic modifier. B: Addition of 10% acetonitrile.

CONCLUSION

An ion chromatographic method has been developed for the separation of organophosphonates. This method utilizes trimesate ion as the displacing ion with indirect UV detection. It is suitable for the separation and detection of organic species which possess high ionic character and which do not possess an appreciable chromophore. It has also been demonstrated that a linear relationship exists between the log capacity factor of the analyte species and the log concentration of the displacing ion. This relationship is dependent on the charge on the analyte anion and the displacing anion and can be utilized to facilitate method development for the optimum separation of ionic species.

ACKNOWLEDGEMENTS

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REFERENCES

1. G.R. Mundy, *Bone*, 8, S9-S16, 1987.

2. M.D. Francis and R.R. Martodam, *The Role of Phosphonates in Living Systems*, R.L. Hilderbrand, ed, CRC Press, Boca Raton, FL, 1983, 55-96.

3. F.J. Schoen, H. Harasaki, K.M. Kim, H.C. Anderson, and R.J. Levy, *J. Biomed. Mater. Res.*, 22, 11-36, 1988.

4. G. Golomb, A. Schlossman, H. Saadeh, M. Levi, J. Van Gelder, E. Breuer, *Pharmaceutical Research*, 9, 143-148, 1992.

5. H. Fleish, *Bone*, 8, 523-528, 1987.

6. Z. Ismail, S. Aldous, E.J. Triggs, B.A. Smithhurst, and H.D. Barry, *J. Chromatogr.*, 404, 372-377, 1987.

7. M.L. Rueppel, L.A. Suba, and J.T. Marvel, *Biomed Mass. Spectrom*, 3, 28-31, 1976.

8. G. Flesch and S.A. Hauffe, *J. Chromatogr. Biomed. Appl.*, 489, 446-451, 1989.

9. J.D. DeMarco, S.E. Biffar, D.G. Reed, and M.A. Brooks, *J. Pharm Biomed. Anal.*, 7, 1719-27, 1989.

10. S.E. Meek and D.J. Pietrzyk, *Anal. Chem.*, 60, 1397-1400, 1988.

11. H. Waldoff and P. Sladek, *Fresenius Z. Anal. Chem.*, 320, 163-168, 1985.

12. E. Kwong, S. McClintlock, and M. Cotton, *American Association of Pharmaceutical Sciences National Meeting*, Atlanta, GA, September, 1989.

13. C.R. Hoggarth, L.A. Gifford, and P.T. Daley-Yates, *Analytical Proceedings*, 27, 18-19, 1990.

14. E.W. Tsai, D.P. Ip, M.A. Brooks, *J. Chromatogr.*, 596, 217-224, 1992

15. T.L. Chester, C.E. Lewis, J.J. Benedict, R.J. Sundberg, and W.C. Tettenhorst, J Chromarogr., 225, 17-25, 1981.

16. K.A. Forbes, J. Vecchiarelli, P.C. Uden, R.M. Barnes, *Advances in Ion Chromatography*, P. Jandik and R.M. Cassidy, eds , Century Int. Inc., Franklin, MA, 1989, 487-502.

17. S. Einarsson, B. Josefsson, and S. Lagerkvist, *J. Chromatogr.*, 282, 609-618, 1983.

18. H.A. Moye and A.J. Boning Jr., *Analyt. Lett.*, 12, 25-35, 1979.

19. G.R. Kieczykowski, U.S. Patent 5,019,651, May 28, 1991.

20. M. Denkert, L. Hackzell, G. Schill, and E. Sjorgen, *J. Chromatogr.*, 218, 31-43, 1981.

21. J.E. Parkin and H.T. Lau, *J. Chromatogr.*, 314, 488-494, 1984.

22. H. Small and T.E. Miller Jr., *Anal. Chem.*, 54, 462-469, 1982.

23. J.R. Larson and C.D. Pfeiffer, *J. Chromatogr.*, 259, 519-521, 1983.

24. J.E. Parkin, *J. Chromatogr.*, 303, 436-439, 1984.

25. P.J. Naish, *Analyst*, 109, 809-812, 1984.

26. T.A. Walker, T.U. Ho, and N. Akbari, *J. Liq. Chromatogr.*, 12 (7), 1213-1230, 1989.

27. T.A. Walker, T.U. Ho, and N. Akbari, *J. Liq. Chromatogr.*, 14 (7), 1351-1366, 1991.

28. S. Motomizu, M. Oshima, and T. Hironaka, *Analyst*, 116, 695-700, 1991.

29. N. Chauret and J. Hubert, *J. Chromatogr.*, 469, 329-338, 1989.

30. E.W. Tsai, D.P. Ip, M.A. Brooks, *J. Pharmaceut. & Biomed. Anal.*, 11, 513-516, 1993.

31. S. Afrashtehfar and F.F. Cantwell, *Anal. Chem.*, 54, 2422-2427, 1982.

32. R.A. Hux and F.F. Cantwell, *Anal. Chem.*, 56,1258-1263, 1984.

33. H. Small, *Ion Chromatography*, Plenum Press, NY, 1989,196-200.

34. Y. Miura and J. Fritz, *J. Chromatogr.*, 482, 155-163, 1989.

35. T. Walker, *J. Liq. Chromatogr.*, 11(7), 1513-1530, 1988.

36. A. Diop, A. Jardy, M. Caude and R. Rosset, *Analusis*, 14(2), 67-73, 1986.

37. Merck Internal Communication, R. Criddle to R. Bergstrom, 1987.

38. P. Diesch, T. Gunther, and M. Rohnelt, *Z. Naturforsch*, 31 C, 661-663, 1976.

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LIQUID CHROMATOGRAPHY CALENDAR

1994

JULY 31 - AUGUST 5: American Society of Pharmacognosy Annual Meeting, International Research Congress on Natural Products, joint meeting with the Assoc. Français pour l'Enseignement et la Recherche en Pharmacognosie, and the Gesellschaft fur Arzneipflanzenforschung, and the Phytochemical Society of Europe, Halifax, Nova Scotia, Canada. Contact: D. J. Slatkin, P. O. Box 13145, Pittsburgh, PA 15243, USA.

AUGUST 14 - 17: Summer National Meeting & Particle Technology Forum, AIChE, Denver, Colorado. Contact: AIChE Express Service Center, 345 East 47 Street, New York, NY 10017, USA.

AUGUST 21 - 23: Australasian Plastics & Rubber Inst. 7th Technology Convention, Melbourne, Australia. Contact: APRI, P. O. Box 241, Mont Albert 3127, Australia.

AUGUST 21 - 26: 208th ACS National Meeting, Washington, DC. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 29 - SEPTEMBER 2: Synthetic Membranes in Science & Industry, University of Tubingen, Germany. Contact: Dechema e.V., Exhibitions & Congresses, Theodor-Heuss-Allee 25, P. O. Box 150104, D-60486 Frankfurt am Main, Germany.

SEPTEMBER 4 - 9: 4th European Rheology Conference, Seville, Spain. Contact: C. Gallegos, Dept. de Ingenieria Quimica, Universidad de Seville, 41071 Seville, Spain.

SEPTEMBER 5 - 7: 25th International Symposium on Essential Oils, Grasse, France. Contact: Assoc. des Ingenieurs et Techniques de la Parfumerie (A.I.T.P.), 48 ave. Riou-Blanquet, 06130 Grasse, France.

SEPTEMBER 8 - 10: Surfaces in Biomaterials 1994, Scottsdale, Arizona. Contact: Surfaces in Biomaterials Foundation, c/o Ardel Management, P. O. Box 26111, Minneapolis, Minn, USA.

SEPTEMBER 12 - 15: 3rd International Conference on Separations in Biotechnology, University of Reading, U.K. Contact: SCI, Conference Office, 14/15 Belgrave Square, London SW1X 8PS, U.K.

SEPTEMBER 25 - 28: Future of Spectroscopy: From Astronomy to Biology, Ste.-Adele, Que., Canada. Contact: Doris Ruest, Conference Mgr., NRC Canada, Ottawa, Ont., Canada K1A 0R6.

SEPTEMBER 27 - 29: CCPC International Conference on Chemical Process Automation, Houston, Texas. Contact: AIChE Express Service Center, 345 East 47th Street, New York, NY 10017, USA.

OCTOBER 3 - 4: Course on Capillary Electrophoresis, Budapest, Hungary. Contact: Dr. F. Kilar, Central Research Laboratory, Medical School, University of Pecs, Szigeti ut 12, H-7643 Pecs, Hungary.

OCTOBER 5 - 7: 9th Inetrnational Symposium on Capillary Electrophoresis, Budapest, Hungary. Contact: Dr. F. Kilar, Central Research Laboratory, Medical School, University of Pecs, Szigeti ut 12, H-7643 Pecs, Hungary.

OCTOBER 9 - 14: 186th Meeting of the Electrochemical Society. Contact: Electrochem. Soc., 10 S. Main St., Pennington, NJ 08534-2896, USA.

OCTOBER 16 - 19: 46th Southeastern Regional Meeting, ACS, Birmingham, Alabama. Contact: L. Kispert, Chem Dept, Univ of Alabama, Box 870336, Tuscaloosa, AL 35115, USA.

OCTOBER 17 - 19: 3rd Int'l Symposium on Supercritical Fluids, Strasbourg, France. Contact: Int'l Soc for the Advancement of Supercritical Fluids, 1 rue Grandville, B. P. 451, F-54001 Nancy Cedex France.

OCTOBER 18 - 22: 30th ACS Western Regional Meeting, Sacramento, California. Contact: S. Shoemaker, Calif. Inst of Food & Agricultural Res., Univ. of Calif-Davis, 258 Cruss Hall, Davis, CA 95616-8598, USA.

OCTOBER 23 - 27: Annual Meeting of the American Association of Cereal Chemists, Nashville, Tennessee. Contact: J. M. Schimml, AACC, 3340 Pilot Knob Road, St. Paul, MN 55121-2097, USA.

OCTOBER 24 - 26: 2nd Internat'l Conference on Chemistry in Industry, Bahrain, Saudi Arabia. Contact: SAICSC-ACS, P. O. Box 1723, Dhahrain 31311, saudi Arabia.

NOVEMBER 2 - 5: 29th Midwestern Regional Meeting, ACS, Kansas City, Kansas. Contact: M. Wickham-St. Germain, Midwest Res. Inst, 425 Volker Blvd, Kansas City, MO 64110, USA.

NOVEMBER 3: Anachem Symposium, Dearborn, Michigan. Contact: Paul Beckwith, Program Chairman, Detroit Edison Co., 6100 W. warren, Detroit, MI 48210, USA.

NOVEMBER 7 - 10: 13th Int'l Conference on the Application opf Accelerators in Research & Industry, Denton, Texas. Contact: J. L. Duggan, Univ. of N. Texas, Dept. of Physics, P. O. Box 5368, Denton, TX 76203, USA.

NOVEMBER 10 - 11: 17th Int'l Conference on Chemistry, Biosciences, Pharmacy & Environmental Pollution, New Delhi, India. Contact: V. M. Bhatnagar, Alena Chem of Canada, P. O. Box 1779, Cornwall, Ont., Canada K6H 5V7.

NOVEMBER 11 - 12: 3rd Conference on Current Trends in Computational Chemistry, Jackson, Mississippi. Contact: J. Leszczynski, Jackson State University, Dept. of Chem., 1400 J. R. Lynch St., Jackson, MS 39217, USA.

NOVEMBER 13 - 16: 50th Southwest Regional Meeting, ACS, Fort Worth, Texas. Contact: H. C. Kelly, Texas Christian Univ, Chem Dept, Ft. Worth, TX 76129, USA.

NOVEMBER 13 - 18: Annual Meeting of the A.I.Ch.E., San Francisco, California. Contact: AIChE Express Service Center, 345 East 47th St., New York, NY 10017, USA.

NOVEMBER 14 - 17: 33rd Eastern Analytical Symposium, Somerset, New Jersey. Contact: Sheri Gold, EAS, P. O. Box 633, Montchanin, DE 19710, USA.

NOVEMBER 16 - 18: Envirosoft'94, 5th Int'l Conference on Development and Application of Computer Techniques to Environmental Studies, San Francisco, California. Contact: Computational Mechanics, Inc., 25 Bridge St., Billerica, MA 01821, USA.

DECEMBER 4 - 6: IBEX'94, International Biotechnology Expo & Scientific Conference, Moscone Center, San Francisco, California. Contact: Cartlidge & Associates, Inc., 1070 Sixth Avenue, Suite 307, Belmont, CA 94002, USA.

1995

MARCH 6 - 10: PittCon'95: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, New Orleans, Louisiana. Contact: Pittsburgh

Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

APRIL 2 - 7: 209th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

APRIL 25 - 28: Biochemische Analytik '95, Leipzig. Contact: Prof. Dr. H. feldmann, Inst. fur Physiologische Chemie der Universitat, Goethestrasse 33, D-80336 Munchen, Germany.

MAY 28 - JUNE 2: HPLC'95, 19th International Symposium on Column Liquid Chromatography, Convention Center, Innsbruck, Austria. Contact: HPLC'95 Secretariat, Tyrol Congress, Marktgraben 2, A-6020 Innsbruck, Austria.

MAY 31 - JUNE 2: 27th Central regional Meeting, ACS, Akron Section. Contact: J. Visintainer, Goodyear Research, D415A, 142 Goodyear Blvd, Akron, OH 44236, USA.

JUNE 6 - 8: 28th Great Lakes Regional ACS Meeting, LaCrosse-Winona Section. Contact: M. Collins, Chem. Dept., Viterbo College, La Crosse, WI 54601, USA.

JUNE 14 - 16: 50th Northwest/12th Rocky Mountain Regional Meeting, ACS, Park City, Utah. Contact: J. Boerio-Goates, Chem Dept, 139C-ESC, Brigham Young Univ, Provo, UT 84602, USA.

JULY 9 - 15: SAC'95, The University of Hull, UK, sponsored by the Analytical Division, The Royal Society of Chemistry. Contact: The Royal Society of Chemistry, Burlington House, Picadilly, London W1V 0BN, UK.

AUGUST 20 - 25: 210th ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 12 - 15: 5th International Symposium on Drug Analysis, Leuven, Belgium. Contact: Prof. J. Hoogmartens, Inst. of Pharmaceutical Sciences, Van Evenstraat 4, B-3000 Leuven, Belgium.

OCTOBER 18 - 21: 31st Western Regional Meeting, ACS, San Diego, Calif. Contact: S Blackburn, General Dynamics, P. O. Box 179094, San Diego, CA 92177-2094, USA.

OCTOBER 22 - 25: 25th Northeastern Regional Meeting, ACS, Rochester, New York. Contact: T. Smith, Xerox Corp, Webster Res Center, M/S 0128-28E, 800 Phillips Rd, Webster, NY 14580, USA.

NOVEMBER 1 - 3: 30th Midwestern Regional ACS Meeting, Joplin, Missouri. Contact: J. H. Adams, 1519 Washington Dr., Miami, OK 74354-3854, USA.

NOVEMBER 1 - 4: 31st Western Regional ACS Meeting, San Diego, California. Contact: T. Lobl, Tanabe Research Labs, 4450 Town Center Ct., San Diego, CA 92121, USA.

NOVEMBER 5 - 7: 30th Midwestern Regional Meeting, ACS, Joplin, Missouri. Contact: J. H. Adams, 1519 Washington Dr, Miami, OK 74354, USA.

NOVEMBER 29 - DECEMBER 1: Joint 51st Southwestern/47th Southeastern Regional Meeting, ACS, Peabody Hotel, Memphis, Tenn. Contact: P.K. Bridson, Chem Dept, Memphis State Univ, Memphis, TN 38152, USA.

DECEMBER 17 - 22: 1995 International Chemical Congress of Pacific Basin Societies, Honolulu, Hawaii. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1996

FEBRUARY 26 - MARCH 1: PittCon'96: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, Chicago, Illinois. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

MARCH 24 - 29: 211th ACS National Meeting, New Orleans, LA. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

JUNE 16 - 21: "HPLC '96: Twentieth International Symposium on High Performance Liquid Chromatography," San Francisco Marriott Hotel, San Francisco, California. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

AUGUST 18 - 23: 212th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

OCTOBER 16 - 19: 52nd Southwest Regional ACS Meeting, Houston, Texas. Contact: J. W. Hightower, Dept. Chem. Eng., Rice University, Houston, TX 77251, USA.

OCTOBER 24 - 26: 52nd Southwestern Regional Meeting, ACS, Houston, Texas. Contact: J. W. Hightower, Chem Eng Dept, Rice Univ, Houston, TX 77251, USA.

NOVEMBER 6 - 8: 31st Midwestern Regional Meeting, ACS, Sioux Falls, South Dakota. Contact: J. Rice, Chem Dept, S. Dakota State Univ, Shepard Hall Box 2202, Brookings, SD 57007-2202, USA.

NOVEMBER 9 - 12: 48th Southeast Regional ACS Meeting, Greenville, South Carolina. Contact: H. C. Ramsey, BASF Corp., P. O. Drawer 3025, Anderson, SC 29624-3025, USA.

1997

APRIL 6 - 11: 213th ACS National Meeting, San Antonio, Texas. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 7 - 12: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1998

MARCH 29 - APRIL 3: 215th ACS National Meeting, St. Louis, Missouri. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 23 - 28: 216th ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1999

MARCH 21 - 26: 217th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 22 - 27: 218th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2000

MARCH 26 - 31: 219th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 20 - 25: 220th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2001

APRIL 1 - 6: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 19 - 24: 222nd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2002

APRIL 7 - 12: 223rd ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 8 - 13: 224th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

The Journal of Liquid Chromatography will publish, **at no charge**, announcements of interest to liquid chromatographers in every issue of the Journal. To be listed in Meetings & Symposia, we will need to know: Name of the meeting or symposium, sponsoring organization, when and where it will be held, and whom to contact for additional details. Incomplete information will not be published. You are invited to send announcements to **Dr. Jack Cazes, Editor, Journal of Liquid Chromatography, P.O. Box 2180, Cherry Hill, NJ 08034-0162, USA.**

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2. C. T. Mant, R. S. Hodges, "HPLC of Peptides," in HPLC of Biological Macromolecules, K. M.

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