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COMPUTATIONAL CHEMICAL ANALYSIS OF NEWLY DEVELOPED GUANIDINO-PHASE FOR QUANTITATIVE ANALYSIS OF SACCHARIDES IN LIQUID CHROMATOGRAPHY

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ABSTRACT

A guanidino-phase was synthesized from a propylamine bonded phase. This new bonded phase had higher retention capacity for saccharides than the original amino phase, and saccharides were quantitatively recovered. The computational chemical analysis of the retention mechanism indicated that electrostatic forces may contribute to the retention on the guanidino phase, and that a combination of electrostatic force and hydrogen-bonding may contribute to this on amino phase. The poor recovery from amino phase may be due to the formation of glycosides.

INTRODUCTION

Frequently used packing materials for separation of saccharides are ion-exchangers in borate buffer,[1] aqueous sodium hydroxide,[2] or aqueous acetonitrile,[3] propylamine-bonded packing materials in aqueous acetonitrile,[4] and amide-bonded packing materials in aqueous acetonitrile.[4] Among them, propylamine-bonded packing materials have been the most commonly used because they are easily handled, but the recovery of saccharides from the packing material is very poor, probably due to formation of N-glycosides or their Amadori rearrangement products. This can be explained by the chromatographic behavior and reactivity of 2-deoxysaccharides easily converted to N-glycosides in vitro.[5]

Protamine-bonded packing material has been synthesized from propylamine-bonded packing materials and protamine, which is a basic protein containing about 70% arginine.[6] The recovery of saccharides is excellent from the column, but the retention capacity was not satisfactory. Therefore, we directly modified the amino-phase to synthesize the guanidino-bonded phase. In biochemical research, amino-groups of proteins have been converted to guanidino groups at moderate conditions.[7] This reaction method was applied for synthesis of the guanidino phase; the guanidino bonded phase was studied for liquid chromatographic behavior towards saccharides. The retention of saccharides on the guanidino phase was further analyzed by computational chemistry.

EXPERIMENTAL

Reagents and Materials

An NH2P-50 column (150 x 4.6 mm I.D., 5μm) packed with a pentaethylenehexamine-bonded vinyl alcohol copolymer gel[8] was purchased from Showa Denko (Tokyo, Japan). S-Methylisothiourea sulfate was obtained from Fluka Chemie AG. Monosaccharides were obtained from Wako (Osaka, Japan). HPLC-grade acetonitrile was obtained from Kanto-Kagaku (Tokyo, Japan). Water was Milli-Q grade water.

Preparation of the Guanidino-Bonded Column

A guanidino-bonded column was prepared by delivery of a 0.5M S-methylisothiourea sulfate solution. The flow rate was 0.5 mL/min and the

reaction temperature was 80°C. The reaction process was monitored from the retention time of glucose in aqueous acetonitrile. The retention time of glucose reached a constant after two days of reaction. Therefore, 1400 mL S-methylisothiourea solution was necessary.

Chromatography of Saccharides

The liquid chromatograph consisted of a DGU3A degassing unit, an LC-9A pump, a LC-10A RID, a CR6A integrator (Shimadzu, Kyoto, Japan) and a Model 7125 injector (Rheodyne, CA, USA). The water bath, Model Minder H, was from Taiyo Kagaku, Tokyo, Japan.

Computational Chemical Calculation

The computer used for the calculations was a Macintosh 8100/100, and the software was CAChe™ from Sony-Tektronix (Tokyo, Japan). The chemical calculation was performed without modification of the programs. The geometry of a molecule, created using CAChe™ molecular editor, was first optimized using molecular mechanics calculations.

The properties used for the calculation were bond stretch, bond angle, dihedral angle, improper torsion, van der Waals, electrostatic and hydrogen bond. The cut-off distance for van der Waals interaction was 9Å.[9] Cricket-Graph™ from Computer Associates was used for data handling.

RESULTS AND DISCUSSION

The retention times and recoveries of saccharides listed in Table 1 were measured in aqueous 80% acetonitrile before and after modification of an NH2P-50 column. The effect of anions was measured, after modification, to phosphate, sulfate and borate forms.

The retention capacity of the hydroxy form of the guanidino column was about three times that of the original amino column, and that of the phosphate and sulfate-forms was about 110%. The capacity of the phosphate form was 120% of that of the amino phase. However, the saccharides were quantitatively recovered from the guanidino column. Such recovery was not achieved from the amino column. An example of chromatography on the phosphate form guanidino column is shown in Figure 1.

Table 1

Retention Factors (k) and Relative Recovery (r, %) On and From Amino Acid and Guanidino Phases

Saccharides	Amino-Phase			Guanidino-Phase									
	k	r Fru*,1	r Glc*,2	k OH*,3	r OH*,3	k COOH*,4	r COOH*,4	k SO_4*,5	r SO_4*,5	k PO_4*,6	r PO_4*,6	k B_2O_4*,7	r B_2O_4*,7
Arabinose	2.07	10.1	12.0	5.91	92.1	2.28	75.2	2.24/2.85	84.9	2.82	82.9	2.32	93.6
Deoxyglucose	8*	8*	8*	2.71	107.2	2.22	57.3	1.79	94.6	2.54	86.3	1.89	102.7
Deoxyribose	8*	8*	8*	1.29	59.1	1.22	13.9	1.00	65.5	1.36	74.3	3.25	9*
Fructose	2.61	100.0	118.4	10.54	58.6	2.94	101.5	3.04	97.1	3.55	102.0	5.28	9*
Galactose	3.79	21.1	25.0	10.30	92.0	3.74	83.0	4.08/5.06	88.6	5.52	81.3	3.70	93.7
Glucose	4.02	84.4	100.0	9.94	100.0	4.30	100.0	4.21/4.84	100.0	5.56	100.0	3.73	100.0
Maltose	9.47	58.0	68.7	---	---	---	---	---	---	---	---	---	---
Mannose	3.35	15.5	18.4	9.57	103.8	3.23	84.4	3.54	89.0	4.27	91.5	3.82	92.4
Ribose	1.78	10.3	12.2	5.18	90.7	1.64	82.0	1.65	85.7	1.82	89.9	2.64	79.0
Sucrose	7.21	106.1	125.7	---	---	---	---	---	---	---	---	---	---
Xylose	2.21	42.1	49.8	6.99	95.5	2.43	87.5	2.18/2.69	87.1	2.79	87.2	2.68	86.8

*All measurements were made on a 15 cm x 4.6 mm ID column in 80% aq. acetonitrile, flow rate 1.0 mL/min using an RI detector; however, eluent for borate-form guanidino-phase[7] was 70% aq. acetonitrile and the flow rate for amino column was 0.6 mL/min. [1] Recovery was calculated as fructose standard. [2] Recovery was calculated as glucose standard. [3] Hydroxy-form guanidino-phase; [4] Carboxy-form guanidino-phase; [5] Sulfate-form guanidino-phase, some saccharides showed two peaks; [6] Phosphate-form guanidino-phase; [7] Borate-form guanidino-phase; [8] Peaks were too small and retention times were close to system peaks; [9] Peaks were too flat.

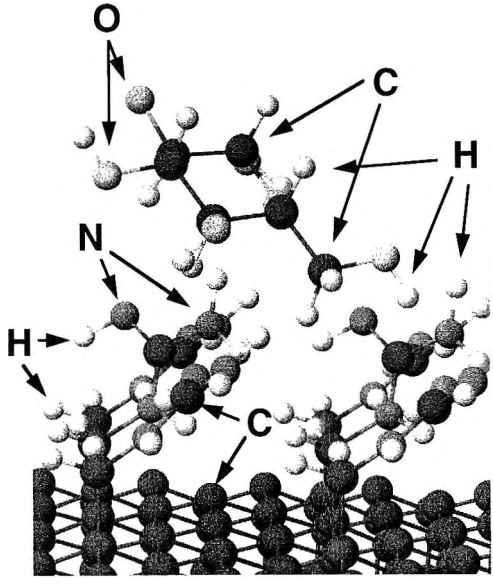

Figure 1. Glucose adsorbed on guanidino phase, optimized by MM2 calculation.

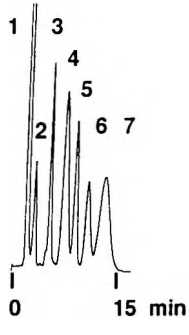

Figure 2. Chromatogram of saccharides in 82% aqueous acetonitrile. 1 & 2: solvent peak, 3: ribose, 4: xylose + arabinose, 5: fructose, 6: mannose, 7: glucose + galactose.

Previously, the elution order of saccharides from an amino column was successfully analyzed by molecular mechanics calculation of computational chemistry. The retention factors were basically related to summation of calculated van der Waals and hydrogen bonding energies after subtraction of

Table 2

Energy Values Optimized by Molecular Mechanics Calculation

Compounds	Monosaccharides FIN[1]	HB[2]	ES[3]	VW[4]	Complex With Amino-Phase ES[6]	HB+VW[6]
Arabinose	16.02	-2.81	6.05	1.87	-0.37	-16.54
Deoxyribose	8.48	-1.69	-1.03	1.29	---	---
Fructose	20.64	-2.28	12.08	1.31	-0.58	-17.59
Galactose	17.26	-2.39	10.22	3.64	1.28	-25.92
Glucose	16.09	-2.62	9.28	3.60	2.39	-28.12
Mannose	16.15	-3.01	9.21	4.00	-0.18	-21.97
Ribose	17.03	-1.96	7.41	1.38	-0.74	-22.83
Xylose	16.67	-1.93	6.41	1.44	0.757	-16.56

	Complex With Guanidino-Phase FIN[1]	HB[2]	ES[3]	VW[4]	ES[5]	HB+VW[5]
Arabinose	1590.3	-12.92	-272.65	253.32	-0.18	-10.62
Deoxyribose	1582.56	-10.56	-280.34	251.74	-0.78	-10.37
Fructose	1593.63	-12.94	-267.57	252.99	-0.12	-10.94
Galactose	1588.33	-13.15	-271.41	255.53	-3.11	-10.83
Glucose	1587.03	-12.47	-270.94	252.77	-1.69	-12.64
Mannose	1587.18	-13.92	-270.73	253.61	-1.42	-13.26
Ribose	1589.26	-11.80	-273.42	254.36	-2.30	-8.81
Xylose	1589.69	-11.81	-273.93	252.82	-1.82	-10.47
Guanidino-Phase	1584.93	-5.92	-278.52	257.87	---	---

[1] Final energy, Kcal/mol; [2] Hydrogen bond energy, Kcal/mol; [3] Electrostatic energy, Kcal/mol; [4] van der Waals energy, Kcal/mol; [5] Complex form electrostatic and hydrogen plus van der Waals energy, Kcal/mol; [6] Complex form electrostatic and hydrogen plus van der Waals energy, Kcal/mol on amino-phase.

the individual energy of saccharides from the molecular interaction energy.[10] The same method was applied to study the retention mechanism of saccharides on the guanidino column. The original amino phase consisted of 368 carbons, 30 nitrogens, and 318 hydrogens. The molecular weight was 5,154. The amino groups were converted to guanidino groups. The final guanidino phase consisted of 316 carbons, 36 nitrogens and 71 hydrogens, due to the computer capacity and the size of guanidinyl group. The molecular weight was 4367. The adsorption form of glucose on the guanidino phase is shown in Figure 2.

The retention factors were related to their final, hydrogen bonding, electrostatic, and van der Waals energy values as calculated by MM2. After subtraction of the individual energy of saccharides from the molecular interaction energy listed in Table 2, the retention factor showed a high correlation with the electrostatic energy values of saccharides, with a correlation coefficient of 0.892 (n = 8). Although aminoguanidine has been reported to react with D-Glucose,[11] the weak interaction in chromatographic condition resulted in a high recovery of saccharides from this guanidino column. Some saccharides showed two peaks. This may be due to on-column isomerization of saccharides in liquid chromatography.[12] The retention time on the borate-form column was too long and the theoretical plate number was poor, due to formation of borate-esters.[13] Therefore, the borate-form column can be used for only specific separation. If the retention form in chromatography and the surface structure of adsorbent are known, the difference of retention factors can be predicted from energy values calculated by computational chemistry.

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DEVELOPMENT OF AN HPLC/DIODE-ARRAY DETECTOR METHOD FOR SIMULTANEOUS DETERMINATION OF 5-HMF, FURFURAL, 5-*O*-CAFFEOYLQUINIC ACID AND CAFFEINE IN COFFEE

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ABSTRACT

This paper describes an interference-free reversed-phase HPLC procedure for the simultaneous quantification of 5-HMF, furfural, 5-*O*-caffeoylquinic acid and caffeine in green and roasted coffee samples. The sample preparation was simple, only involving a boiled water extraction followed by filtration. The chromatographic separation was achieved using a reversed-phase column Spherisorb ODS2 (5μm; 25.0 x 0.46 cm). Gradient elution was carried out using water+acetic acid (0.2%) (A) and methanol (B). The effluent was monitored by a diode-array detector and chromatograms were recorded at 280 nm. The determinations were performed in the linear ranges 0.25-10 μg/mL, 0.1-10 μg/mL, 2.5-100 μg/mL, 0.25-200 μg/mL for 5-HMF, furfural, 5-*O*-caffeoylquinic acid, and caffeine,

respectively. Extensive quality assurance of the method was performed by the standard additions method in both coffee matrices (green and roasted coffee). For roasted coffee, the precision obtained (n=10) was better than CV% 0.52, 0.62, 0.14, and 0.50 for 5-HMF, furfural, 5-*O*-caffeoylquinic acid, and caffeine, respectively; for green coffee, it was better than 0.76 for 5-*O*-caffeoylquinic acid and 0.89 for caffeine.

For roasted coffee samples, recovery values were between 85 and 104%, 87 and 103%, 88 and 94%, and 74 and 77% for 5-HMF, furfural, 5-*O*-caffeoylquinic acid, and caffeine, respectively. For green coffee sample, recovery values were between 73 and 74% for 5-*O*-caffeoylquinic acid and between 94 and 98% for caffeine.

INTRODUCTION

Green and roasted coffee are matrices of the same food, although with very diversified chemical composition, aromatic and textural properties. Mechanisms of roasting reactions have been largely studied, yet too much remains to be understood.[1] Therefore, in spite of the enormous effort undertaken until now to chemically discriminate between *Coffea arabica* and *Coffea canephora* var. *robusta*, once roasted, no unquestionable marker has been found.[2,3] Simultaneous monitorization of furanic aldehydes, such as furfural (2-furaldehyde) and hydroxymethylfurfural (5-hydroxy-2-furaldehyde, 5-HMF), 5-*O*-caffeoylquinic acid and caffeine in coffee, among others, can be particularly helpful, namely, as a guarantee of authenticity of coffee varieties, in the characterization of its geographic origins, to ensure the quality of roasted coffee and indirectly the quality control of the roasting process itself. Individual methodologies to quantify these four compounds are quite spread in literature,[1,4] although multiparametric techniques are quite scarce.[5-10] Furthermore, these latter methodologies do not involve all the above mentioned compounds.

To fulfil this gap and reach the proposed aims of contributing to help the quality control of coffee in all steps, viz. from geographic origin until sailing to the final consumer, this simple, expeditious, and economic methodology herein presented was developed and its validation, accuracy, and reproducibility were assessed in both green and roasted coffee matrices.

MATERIALS AND METHODS

Coffee Samples and Standards

Green Robusta (India) coffee beans were supplied by the coffee industry, and one portion was roasted at 205°C, during 15min. in a stove. The green or roasted beans were ground in a hammer mill to pass 0.8 mm.

5-HMF, furfural, 5-*O*-caffeoylquinic acid, and caffeine were obtained from Sigma Chemical Co.

Extraction of Compounds from Coffee

A 1 g portion of powdered coffee bean samples, was blended with 150 mL of water and boiled during 2 min. This solution was transferred to a 200 mL volume flask and immediately diluted to the volume mark. The mixture was filtered and 20 μl were analysed by HPLC.

HPLC Analysis

Separation of compounds was achieved with an analytical HPLC unit (Gilson), using a reversed-phase Spherisorb ODS2 (5μm, particle size; 25.0 x 0.46 cm) column. The solvent system used was a gradient of water+acetic acid (0.2%) (A) and methanol (B). The gradient was as follows: 0'-7.5% B, 10'-20% B, 12'-30 % B, 20'-35% B, 28'-40% B. Elution was performed at a solvent flow rate of 1 mL/min. Detection was accomplished with a diode-array detector, and chromatograms were recorded at 280 nm.

The mentioned compounds were identified by chromatographic comparisons with authentic standards and by their specific UV spectra. Quantification was based on the external standard method.

RESULTS AND DISCUSSION

Analytical Curve and Detection Limit

For the sample preparation, we observed that previous clarification with Carrez solutions[8] or a passage through a Sep-pack C-18 cartridge[11] were not

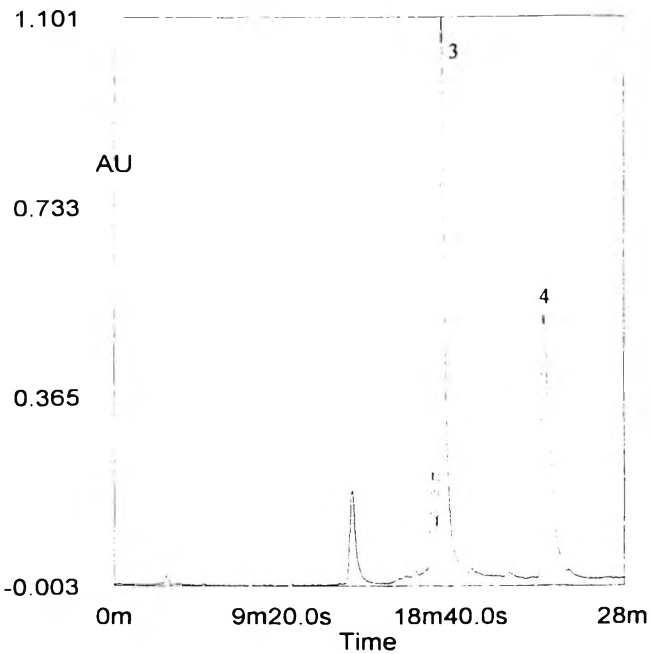

Figure 1. HPLC profile of a green coffee sample from India. Detection at 280 nm. (3) 5-*O*-caffeoylquinic acid; (4) Caffeine.

necessary and caused losses of 5-HMF and furfural. Under the assay conditions described, a linear relationship between the concentration and the UV absorbance at 280 nm was obtained. This linearity was maintained over the concentration range 0.25-10 μg/mL, 0.1-10 μg/mL, 2.5-100 μg/mL, 0.25-200 μg/mL for 5-HMF, furfural, 5-*O*-caffeoylquinic acid and caffeine, respectively. The correlation coefficient for each standard curve invariably exceeded 0.99 for all compounds under study.

The calibration curves were obtained by triplicate determinations of each of the calibration standards, the peak area values (arbitrary units) were plotted as average values. The relative percent average deviations of triplicates were less than 2% in all cases. The average regression equation for 5-HMF, furfural, 5-*O*-caffeoylquinic acid, and caffeine were $y = 69433x + 2883.8$, $y = 66225x + 2721.0$, $y = 12751x + 4301.7$, $y = 21124x + 23423.0$, respectively. The detection limit values for 5-HMF, furfural, 5-*O*-caffeoylquinic acid, and caffeine were calculated as the concentration corresponding to three times the standard deviation of the background noise and were 0.25 μg/mL, 0.10 μg/mL, 2.50 μg/mL, and 0.25 μg/mL, respectively.

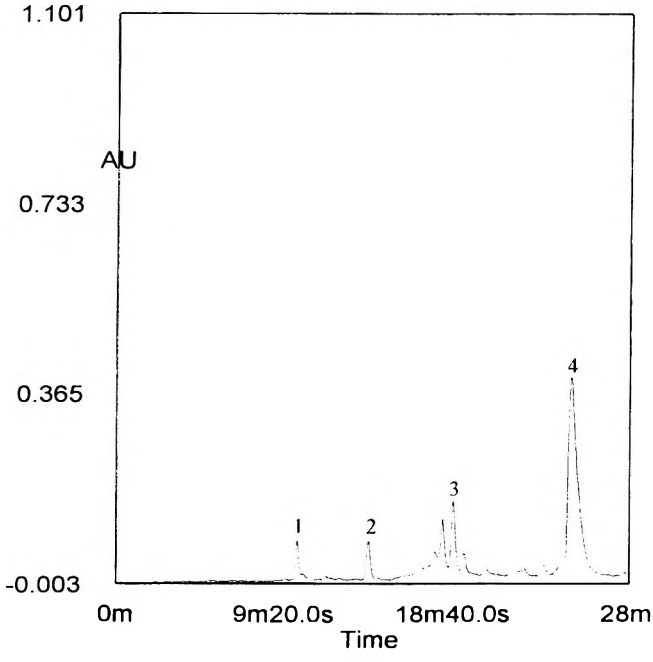

Figure 2. HPLC profile of a roasted coffee sample from India. Detection at 280 nm. (1) 5-HMF; (2) Furfural; (3) 5-*O*-caffeoylquinic acid; (4) Caffeine.

Validation of the Method

The chromatograms obtained for a green and a roasted coffee samples from India are shown in Figures 1 and 2. The retention times (RT) obtained for compounds under study were: RT 9m53s for 5-HMF, RT 13m49s for furfural, RT 18m27s for 5-*O*-caffeoylquinic acid, and RT 24m49s for caffeine. The use of a diode-array detector proved to be very helpful to observe the peak purity and chemical nature of each peak. The unidentified compounds had identical UV spectra when recorded with a diode-array detector, with identical shape and maximum at 320 nm, which suggested that they could be hydroxycinnamic acids.

Results of quantification applied to one sample are shown in Table 1. During the roasting process, compositional changes occurred, with the decomposition of naturally 5-*O*-caffeoylquinic acid and appearance of 5-HMF and furfural, which agrees with previous works.[1,11,12]

Table 1

5-HMF, Furfural, 5-*O*-Caffeoylquinic Acid and Caffeine Content in Green and Roasted Coffee Sample[a] From India (*Coffea Canephora* var. *Robusta)*

	Green Coffee mg/kg ± sd	Roasted Coffee mg/kg ± sd
5-HMF	-----	61.6 ± 0.776
Furfural	-----	19.9 ± 1.270
5-*O*-caffeoylquinic acid	$46.5x10^3$ ± 0.350	$7.3x10^3$ ± 0.115
Caffeine	$29.4x10^3$ ± 0.263	$27.5x10^3$ ± 0.251

[a] Values are expressed as mean ± sd of three determinations.

Table 2

Recovery of 5-*O*-Caffeoylquinic Acid and Caffeine from a Spiked Green Coffee Sample[a] From India (*Coffea Canephora* var. *Robusta)*

	Added (mg/kg)	Found (mg/kg)	Standard Deviation	CV%	Recovery %
Chlorogenic acid*	10.0	7.28	0.438	6.02	72.8
	25.0	18.53	0.919	4.96	74.1
	40.0	29.00	0.177	0.61	72.5
Caffeine	10.0	9.43	0.148	1.58	94.3
	25.0	24.53	0.156	0.63	98.1
	50.0	48.90	0.007	0.01	97.8

[a] Mean value found for 3 assays for each studied concentration.
* Corresponds to 5-*O*-caffeoylquinic acid.

The precision of the analytical method was evaluated by measuring the peak chromatographic area of the compounds 10 times on the same sample. In the roasted coffee sample the standard deviation was 0.697, 0.768, 0.189, 0.622, and the coefficient of variation was 0.52%, 0.62%, 0.14%, and 0.5%, for 5-HMF, furfural, 5-*O*-caffeoylquinic acid, and caffeine, respectively. For the green coffee sample, the standard deviation was 0.351, 0.263, and the

Table 3

Recovery of 5-HMF, Furfural, 5-*O*-Caffeoylquinic Acid and Caffeine from a Spiked Green Coffee Sample[a] from India (*Coffea Canephora* var. *Robusta)*

	Added (mg/`kg)	Found (mg/kg)	Standard Deviation	CV%	Recovery %
5-HMF	0.5	0.43	0.007	1.7	85.0
	1.0	0.92	0.21	2.3	91.0
	2.5	2.61	0.141	5.4	104.4
Furfural	0.5	0.44	0.078	7.8	87.0
	1.0	1.03	0.156	5.0	103.0
	2.0	2.04	0.092	4.5	101.8
Chlorogenic acid	10.0	9.40	0.325	3.5	94.0
	25.0	22.43	0.198	0.9	89.7
	40.0	35.24	0.170	0.5	88.1
Caffeine	10.0	7.71	0.120	1.6	77.1
	25.0	17.09	0.255	1.5	68.4
	50.0	37.11	0.834	2.3	74.2

[a] Mean value found for 3 assays for each studied concentration.

* Corresponds to 5-*O*-caffeoylquinic acid.

coefficient of variation was 0.76%, 0.89%, for 5-*O*-caffeoylquinic acid and caffeine, respectively. In order to study the recovery of the procedure, one roasted coffee sample was added to known quantities of 5-HMF, furfural, 5-*O*-caffeoylquinic acid and caffeine, and one green coffee sample was added to known quantities of 5-*O*-caffeoylquinic acid and caffeine. The samples were analysed in triplicate before and after the addition of these compounds in order to demonstrate the effectiveness of the extraction and the accuracy of the proposed method.

The results are listed in Tables 2 and 3. For the roasted coffee sample, recovery values were between 85 and 104%, 87 and 103%, 88 and 94%, and 74 and 77% for 5-HMF, furfural, 5-*O*-caffeoylquinic acid, and caffeine, respectively. For the green coffee sample, recovery values were between 73 and 74% for 5-*O*-caffeoylquinic acid and between 94 and 98% for caffeine.

In conclusion, this study suggests that the technique proposed herein is quite useful for the simultaneous analysis of 5-HMF, furfural, 5-*O*-caffeoylquinic acid, and caffeine in coffee samples. Despite the complexity of the matrix, the sample pre-treatment is simple and this approach only requires an HPLC/diode-array detector. This technique could also be indirectly helpful in the roasting industry.

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SEPARATION OF D AND L ENANTIOMERS OF [(PHENYLSULFONYL)AMINO]-3-OXO-1-OCTADECANOL BY CHIRAL HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A convenient and rapid method has been developed for the separation of the D and L enantiomers of [(phenylsulfonyl)amino]-3-oxo-octadecanol using chiral High Performance Liquid Chromatography (HPLC). An amylose tris(3,5-dimethylphenylcarbamate) chiral HPLC column was used for the separation. The two enantiomers were fully resolved from each other with approximate retention times of 11 and 13 minutes for the L and the D forms respectively. The total run time per sample was 30 minutes.

INTRODUCTION

The chiral separation of the two enantiomers of [(phenylsulfonyl)amino]-3-oxo-1-octadecanol was necessary in order to obtain information regarding the enantiomeric purity of the product during synthesis.[2] Chiral HPLC was found

to be an appropriate analytical technique to monitor the synthesis and to determine the enantiomeric purity of the final product. The synthetic challenge was to synthesize exclusively the L enantiomer. The analytical challenge was to develop a method for the separation of the two enantiomers. The developed analytical method provided the required information for the optimization of synthetic conditions as well as the basis to demonstrate the enantiomeric purity of the final product.

Purified enantiomers and a racemic mixture of [(phenylsulfonyl)amino]-3-oxo-octadecanol[1] were provided by our Chemical Development group for method development. The HPLC method is very simple, fast, and convenient for the intended purposes. The structures of the two enantiomers are presented in Figure 1.

MATERIALS AND METHODS

Equipment

The high performance liquid chromatograph (HPLC) consisted of a Spectra-Physics P2000 pump, a Thermo Separations Products AS3000 autoinjector, and a Spectra-Physics UV2000 variable wavelength ultraviolet detector. The analytical column was an amylose tris(3,5-dimethyl-phenylcarbamate) or Chiralpack AD, 25 cm x 4.6 mm I.D., obtained from Regis Chemical Company, Morton Grove, Illinois 60053, USA.

Reagents

Hexane and isopropanol, both Liquid Chromatography Grade, were obtained from EM Science, 480 S. Democrat Road, Gibbstown, New Jersey 08027, USA., and used to make the mobile phase. The D and L enantiomers of [(phenylsulfonyl)amino]-3-oxo-1-octadecanol[3] were made by our Chemical Development group, and the structures confirmed by high resolution nmr, mass spectroscopy, and infrared spectroscopy.

Mobile Phase and Analysis

The mobile phase consisted of a mixture of hexane and isopropanol at a ratio of 9:1 respectively. The mobile phase was pumped at a flow rate of 1.0 mL per minute. The column temperature was maintained at approximately 25

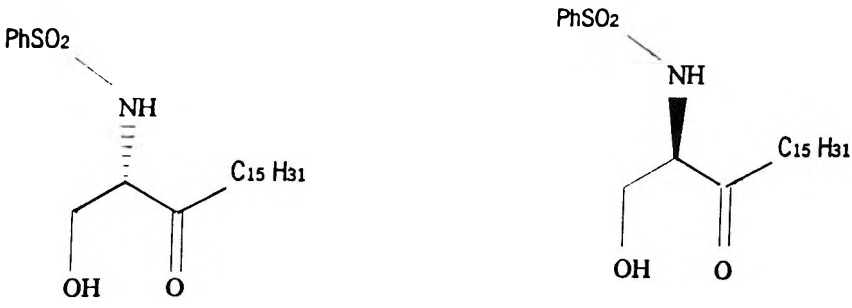

Figure 1. Structures of the two enantiomers of [(phenylsulfonyl)amino]-3-oxo-1-octadecanol.

degrees Centigrade using a temperature controlled HPLC column heater. Injections into the chromatographic system were performed using the HPLC auto-injector with a set injection volume of 50 microliters. The detection was made at 254 nm using the ultraviolet HPLC detector at a range of 0.1 AUFS. The sample was prepared by weighing 100 mg of sample into a 100 mL volumetric flask, dissolved, and then diluted to the mark with mobile phase. Injections were made from this preparation. Sample chromatograms are shown in the Results section below.

RESULTS AND DISCUSSION

During method development, several Pirkle concept type of chiral columns (Phenylglycine, Naphthylleucine, and Naphthylalanine obtained from Regis Technologies, Inc.) were evaluated without success. There were restrictions on the composition of the mobile phase due to the solubility of the compound. At this point, derivatized cellulose and amylose (3,5-dimethylphenylcarbamate) chiral columns were evaluated. The cellulose type gave us some retention, but no separation, while the amylose type column fully resolved the two enantiomers under the same conditions. Theoretically, the difference in results between the cellulose and the amylose type of columns may be attributed to structural differences between cellulose and the amylose. Cellulose and amylose differ in the way glucose is linked together, and the type of conformation which results from this linking. In cellulose, the linkage is of the $\beta(1\rightarrow4)$ type, while in amylose the linkage is of the $\alpha(1\rightarrow4)$ type.[3]

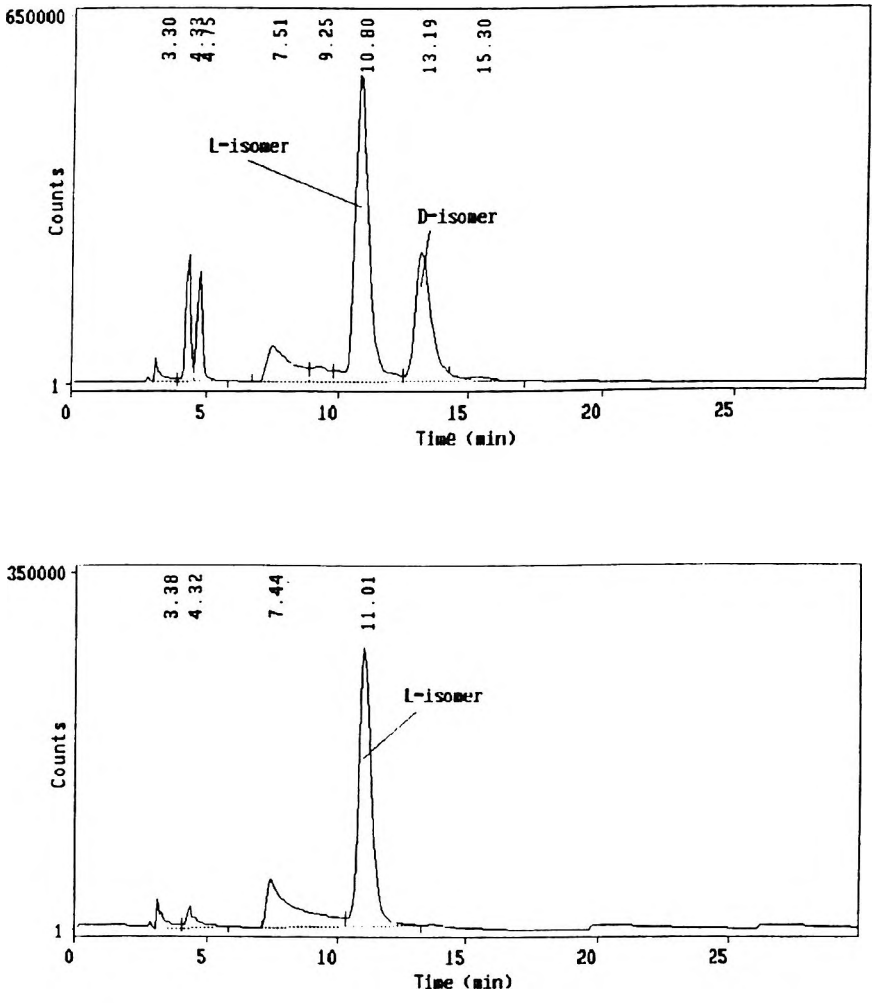

Figure 2. Sample chromatograms of a racemic mixture and the L-isomer.

This difference accounts for the difference in conformation: cellulose has a linear type of conformation, while amylose has a helical type of conformation. Since both types of columns are derivatized as the 3,5-dimethylphenylcarbamate, it is then the type of conformation which provides the basis for the separation of [(phenylsulfonyl)amino]-3-oxo1-octadecanol. In addition, both enantiomers contain a 15 carbon aliphatic chain with a phenylsulfonyl end. It is likely that the helical conformation of the amylose

provides the right geometry for the aliphatic chain of the enantiomers to interact with the helical frame of amylose, while the phenylsulfonyl end of the enantiomers interacts with the dimethylphenyl portion of the derivatized amylose. The phenylsulfonyl portion of the molecule interacts with the dimethylphenylcarbamate portion of the solid substrate through a π-π type of interation of the corresponding benzene rings. It is therefore, the orientation of the phenylsulfonyl group of the particular enantiomer, with respect to the dimethylphenyl group of the derivatized amylose, that determines which enantiomer is retained more or less than the other.

Based on the chromatographic retention times, one concludes that the D enantiomer's phenylsulfonyl portion has stronger interaction than the L enantiomer's. It also appears that for this interction to take place, the helical conformation is required as the cellulose counterpart failed to achieve resolution.

CONCLUSIONS

A convenient and rapid chiral chromatographic method has been developed (Figure 2) for the separation of the D and L enantiomers of [(phenylsulfonyl)amino]-3-oxo-1-octadecanol. Full baseline resolution was achieved. The absolute retention times for the two enantiomers were 11 and 13 minutes for the L and D forms respectively.

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CHEMILUMINESCENCE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF CORTICOSTEROIDS AND *p*-NITROPHENACYL ESTERS BASED ON THE LUMINOL REACTION

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ABSTRACT

Reducing agents, especially compounds having an α-hydroxycarbonyl group, give an intense chemiluminescence with luminol in the presence of catalyst in alkaline solution. Based on this reaction, the chemiluminescence detection of corticosteroids and *p*-nitrophenacyl esters of carboxylic acids has been developed. These compounds were separated by high performance liquid chromatography on a reversed phase column (Prodigy ODS) for corticosteroids with 30% acetonitrile and for *p*-nitrophenacyl esters with 50% acetonitrile as eluent, and detected by the chemiluminescence reaction with luminol-sodium hydroxide solution containing the catalyst as the post-column reagent. Hexacyanoferrate (III) and hexacyanoferrate (II) were used as catalyst of the reaction. The detection limits of cortisone and *p*-nitrophenacyl hippurate were 1.1 pmol and 0.8 pmol.

INTRODUCTION

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) has been a well-known organic chemiluminescence reagent, since it was first reported in 1928 by Albrecht.[1] The chemiluminescence has been mainly used to determine hydrogen peroxide, other oxidants, and metal ions. Recently, the chemiluminescence analytical methods with immobilized enzyme reactor, based on luminol reaction, have been reported and used to determine clinically important organic compounds such as glucose, ammonia, urea, uric acid, etc.[2-5] In all these methods, hydrogen peroxide produced by oxidizing enzymes has been detected.

We found that reducing agents, such as glucose, ascorbic acid, uric acid, corticosteroids, hydroxylamine, phenacyl alcohol etc., give a chemiluminescence with luminol in the presence of catalyst in an alkaline solution. Based on this finding, the chemiluminescence flow injection analysis (FIA) for the determination of reducing agents was developed. In these reducing agents, corticosteroids, and phenacyl alcohol produced intense light with luminol in alkaline solution.[6]

Carboxylic acids are widely distributed in nature and important as nutritional substrates and metabolites in living organisms. Therefore, there is a widespread interest in the isolation and quantitation of these compounds and a need for more convenient methods that are simple and reliable. Recently, several papers have been reported for the tagging of carboxylic acids with reagents that afford chromophores having UV and Vis absorbance.[7] Among those reagents, phenacyl bromide, and substituted phenacyl bromides[8-12] have been widely accepted for the determination of free fatty acids. We also found that these phenacyl bromides give the chemiluminescence.

In this paper, we describe the chemiluminescence detection system for corticosteroids and *p*-nitrophenacyl esters of carboxylic acids after separation by HPLC.

EXPERIMENTAL

Reagents

Luminol and all steroids were purchased from Tokyo Chemical Industry (Tokyo, Japan). Phenacyl bromide and its derivatives were also purchased from Tokyo Chemical Industry. All carboxylic acids and 18-Crown-6

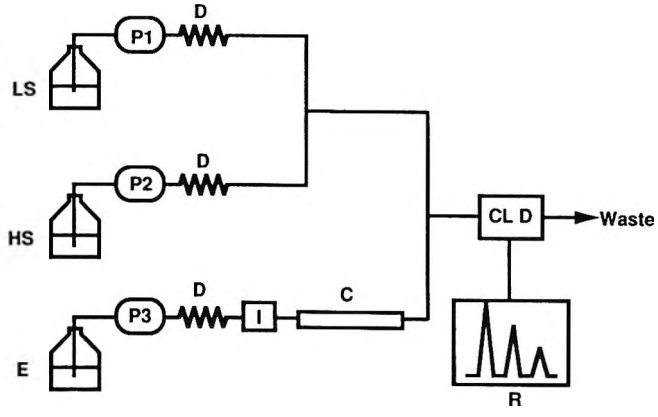


Figure 1. Flow diagram of chemiluminescence HPLC system. LS: luminol solution, HS: hexacyanoferrate (III) solution, E: eluent, P1, P2, P3 : pump, D: damper, I: injector, C: column, CLD: chemiluminescence detector, R : recorder.

(1,4,7,10,13,16-hexaoxacyclooctadecane) were purchased from Wako (Osaka, Japan). All other chemicals were of analytical-reagent grade. Standard solutions were prepared by dissolving in 30 % acetonitrile to give concentration of 200 μmole/L for corticosterone, 100 μmole/L for cortisone and phenacyl alcohol, and by dissolving in acetonitrile to give concentration of 50 μmole/L *p*-nitrophenacyl acetate and *p*-nitrophenacyl benzoate.

Preparation of Phenacyl Esters of Carboxylic Acids

Methanol solutions of the carboxylic acids were neutralized with a KOH-methanol solution. The solvent was removed under aspirator vacuum to give the potassium salts of the carboxylic acids. An excess solution of the phenacyl bromide / 18-Crown-6 (20 : 1 in acetonitrile) was then added and the mixture was refluxed for 30 min. The reaction mixture was evaporated under aspirator vacuum. The precipitated crude product was isolated by filtration and then recrystallized from ethanol. These esters were identified by NMR and MS spectra.

Apparatus

Figure 1 illustrates the flow diagram for a HPLC system. The pumps used were a Model 576 HPLC pump (GL Sciences, Tokyo, Japan) for the luminol

solution, a Hitachi Model L-6200 (Hitachi, Tokyo, Japan) for the hexacyanoferrate (III) solution, and a Model 501G HPLC pump (Nihon Waters Co., Tokyo, Japan) for the eluent. Each pump was attached to the damper. The injection device was U6K injector (Nihon Waters Co., Tokyo, Japan) and the column was a Prodigy 5μ ODS (3) (150 × 4.6 mm i.d., Phenomenex Co., California, USA).

The chemiluminescence detector equipped with a spiral type flow cell having a volume of 100 μL and with a photomultiplier tube biased at 750 V was a Soma S-3400 (Soma Optics LTD., Tokyo, Japan). The recorder was an Unicorder U-228 chart recorder (Nihon Denshi Kagaku Co., Kyoto, Japan).

Chromatographic Conditions

Eluents were 30 % acetonitrile and 50 % acetonitrile for the separation of corticosteroids and *p*-nitrophenacyl esters, respectively. Luminol solution was prepared in 0.4 M sodium hydroxide solution containing 1.0 mM luminol and 250 mM hexacyanoferrate (II). Hexacyanoferrate (III) solution was 0.2 mM hexacyanoferrate (III) aqueous solution. The flow rate of the eluent was 1.0 mL/min, and that of the luminol solution and the hexacyanoferrate (III) solution were 0.5 mL/min and 0.3 mL/min, respectively.

RESULTS

Conditions for Chemiluminescence in the FIA System

In order to determine the optimal conditions for the chemiluminescence detection system, concentrations of reagents and flow-rates were tested. The concentrations of reagents are shown in Figure 2, 3, 4, and 5. The optimal conditions of luminol, hexacyanoferrate (III), sodium hydroxide, and hexacyanoferrate (II) for all of the standard compounds were 1.0 mM, 0.2 mM, 0.4 M, and 250 mM, respectively.

Figures 6 and 7 illustrate the effect on varying the flow-rates of the luminol solution and hexacyanoferrate (III) solution with the eluent flow-rate constant at 1.0 mL/min. The maximum responses were obtained at 0.5 mL/min for the luminol solution and 0.3 mL/min for the hexacyanoferrate (III) solution.

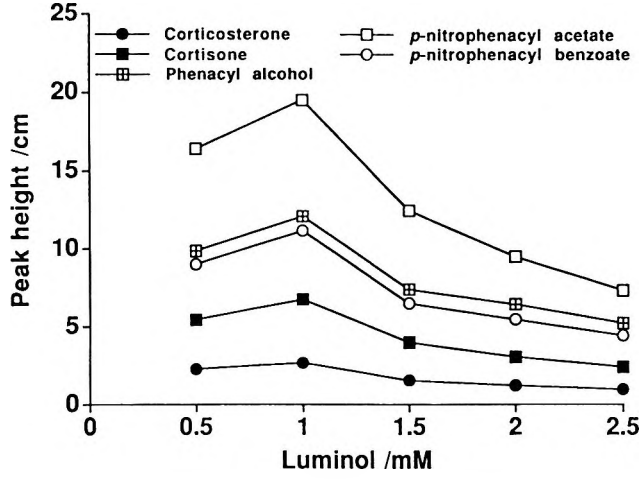


Figure 2. Effect of luminol solution. Conditions: luminol solution containing 0.4 M NaOH and 250 mM $K_4Fe(CN)_6$, hexacyanoferrate (III) solution containing 0.2 mM $K_3Fe(CN)_6$.

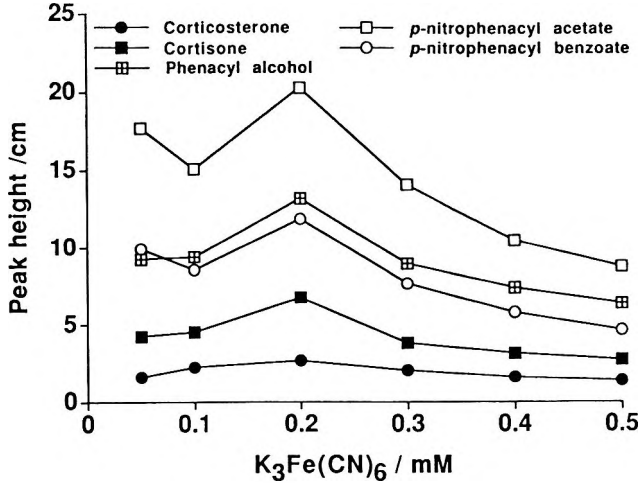


Figure 3. Effect of hexacyanoferrate (III) concentration. Conditions: luminol solution containing 1.0 mM luminol, 0.4 M NaOH and 250 mM $K_4Fe(CN)_6$.

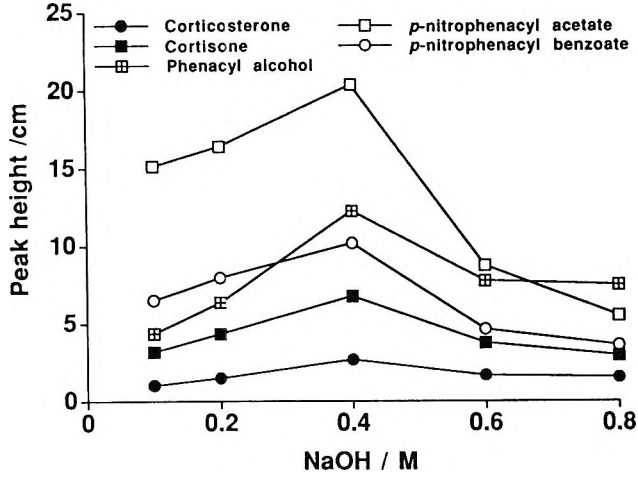


Figure 4. Effect of sodium hydroxide concentration. Conditions: luminol solution containing 1.0 mM luminol and 250 mM K4Fe(CN)6, hexacyanoferrate (III) solution containing 0.2 mM $K_3Fe(CN)_6$.

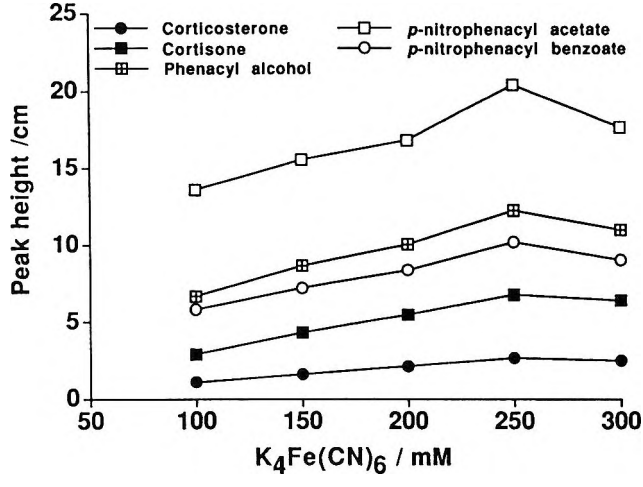


Figure 5. Effect of hexacyanoferrate (II) concentration. Conditions: luminol solution containing 1.0 mM luminol and 0.4 M NaOH, hexacyanoferrate (III) solution containing 0.2 mM $K_3Fe(CN)_6$.

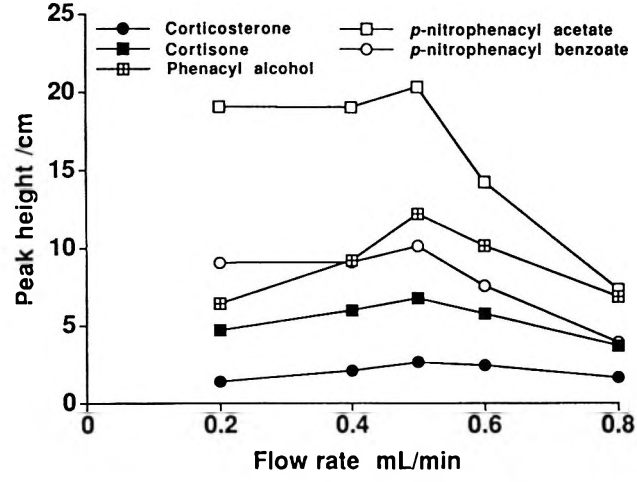


Figure 6. Effect of flow-rate of luminol solution. Conditions: luminol solution containing 1.0 mM luminol, 0.4 M NaOH and 250 mM $K_4Fe(CN)_6$, hexacyanoferrate (III) solution containing 0.2 mM $K_3Fe(CN)_6$.

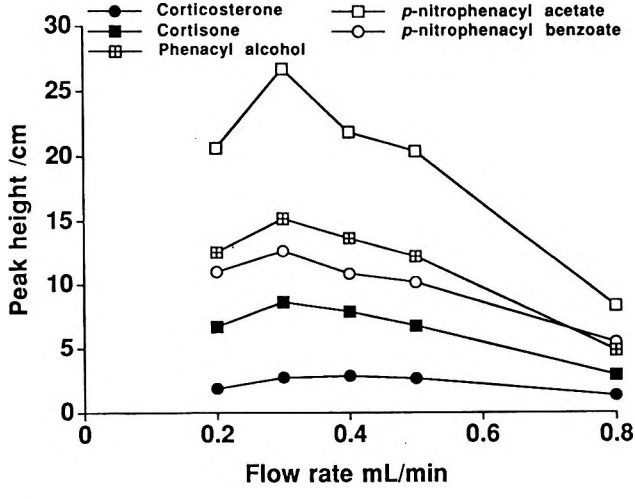
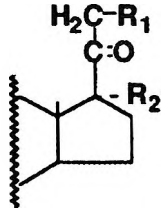


Figure 7. Effect of flow-rate of hexacyanoferrate (III) solution. Conditions: luminol solution containing 1.0 mM luminol, 0.4 M NaOH and 250 mM $K_4Fe(CN)_6$, hexacyanoferrate (III) solution containing 0.2 mM $K_3Fe(CN)_6$.

Table 1

Chemiluminescence Intensities of Steroids



Steroids	Relative Intensity	R_1	R_2
Cortisone	2.62	OH	OH
Methylprednisolone	1.45	OH	OH
Dexamethasone	1.38	OH	OH
Hydrocortisone	1.00	OH	OH
Tetrahydrocortisol	0.99	OH	OH
Corticosterone	0.65	OH	H
Deoxycorticosterone	0.20	OH	H
Betamethasone	0.16	OH	OH
Progesterone	0.00	H	H
11α-Hydroxyprogesterone	0.00	H	H
17α-Hydroxypregnenolone	0.00	H	OH

Chemiluminescence HPLC for Corticosteroids

Table 1 lists the chemiluminescence intensities obtained by injecting standard steroids solutions into the FIA. The intensities are normalized so that the signal of hydrocortisone has a value of 1.00.

Figure 8 shows a chromatogram from a standard mixture of corticosteroids. The separation of the compounds was obtained within 20 min and detected by the proposed detection system.

A linear regression analysis of the working curves which were obtained in the range 0.1 ~ 3 nmol per injection yielded the equation Y = 17.376X - 0.086 (r = 0.997) for predonisolone, Y = 15.664X - 0.100 (r = 0.996) for hydrocortisone, Y = 47.017X - 0.340 (r = 0.996) for cortisone, Y = 11.458X - 0.097 (r = 0.996) for methylpredonisolone, Y = 1.349X - 0.008 (r = 0.997) for betamethasone, Y = 8.643X - 0.044 (r = 0.999) for dexamethasone and Y =

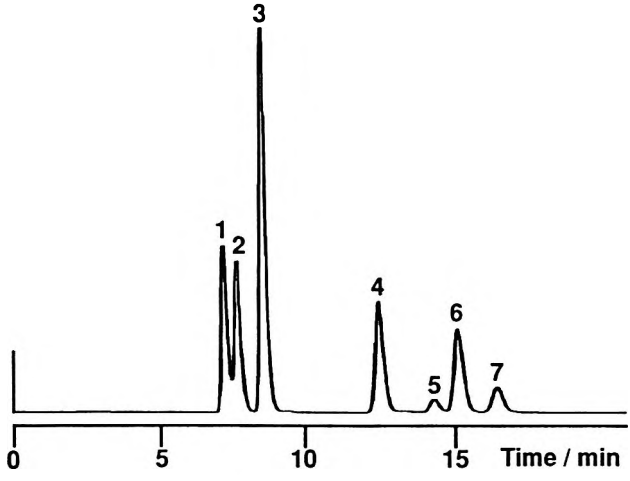


Figure 8. Chromatogram obtained with a mixture of corticosteroids. Peaks: 1 = prednisolone, 2 = hydrocortisone, 3 = cortisone, 4 = methylprednisolone, 5 = betamethasone, 6 = dexamethasone and 7 = corticosterone, 1 mg each.

2.566X - 0.024 (r = 0.994) for corticosterone. The chemiluminescence, peak height (Y), was related to the moles (X) with high linearity. The relative standard deviation was 1.0 ~ 2.5 % for the injection analysis of standard solution repeated 10 times. The limits of detection (S/N = 3) for predonisolone, hydrocortisone, cortisone, methylpredonisolone, betamethasone, dexamethasone, and corticosterone were 2.9 pmole, 3.2 pmole, 1.1 pmole, 4.3 pmole, 34.9 pmole, 5.5 pmole, and 20.5 pmole, respectively.

Chemiluminescence HPLC for Carboxylic Acids

The chemiluminescence of four phenacyl esters with luminol was examined by the FIA system. The results are shown in Table 2. *p*-Nitrophenacyl esters gave a higher chemiluminescence than other phenacyl esters. Therefore, *p*-nitrophenacyl bromide was selected as the pre-column reagent for chemiluminescence HPLC of carboxylic acids.

Figure 9 shows a chromatogram obtained from a standard mixture of synthesized *p*-nitrophenacyl esters of various carboxylic acids. The separation of these esters was achieved within 30 min.

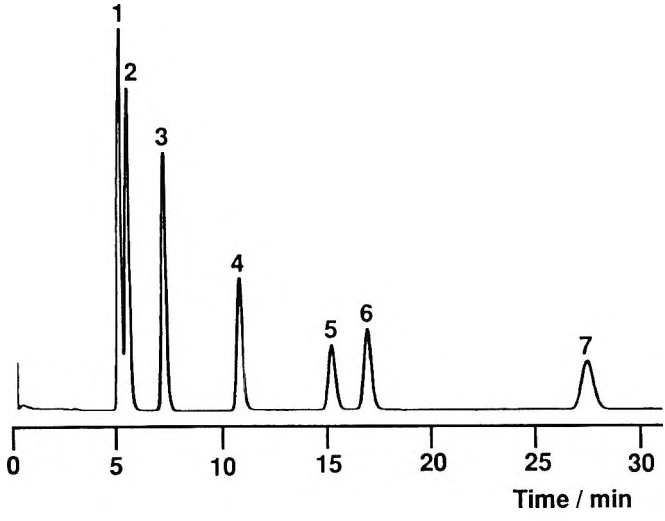
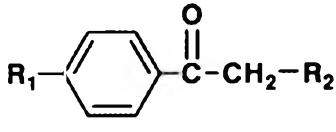


Figure 9. Chromatogram obtained with a mixture of p-nitrophenacyl esters of carboxylic acids. Peaks: 1 = acetic acid, 2 = hippuric acid, 3 = propionic acid, 4 = butyric acid, 5 = benzoic acid, 6 = valeric acid and 7 = caproic acid, 100 ng each.

Table 2

Relative Chemiluminescence Intensities of Phenacyl Esters



R_1	R_2	Relative Intensity
H	OH	1.00
H	$OCOCH_3$	1.09
Br	$OCOCH_3$	2.07
NO_2	$OCOCH_3$	3.61
C_6H_5	$OCOCH_3$	1.11

A linear regression analysis of the working curves which were obtained in the range 10 ~ 350 pmol per injection yielded the equation Y = 153.091X + 0.329 (r = 0.999) for acetic derivative, Y = 130.364X + 0.266 (r = 0.999) for hippuric derivative, Y = 104.427X + 0.214 (r = 0.999) for propionic derivative, Y = 50.969X + 0.110 (r = 0.999) for buthyric derivative, Y = 25.748X + 0.051 (r = 1.000) for benzoic derivative, Y = 31.298X + 0.048 (r = 1.000) for valeric derivative, and Y = 19.551X - 0.024 (r = 1.000) for caproic derivative. The chemiluminescence, peak height (Y), was related to the moles (X) with high linearity. The relative standard deviation was 1.0 ~ 2.5 % for the injection analysis of standard solution repeated 10 times. The limits of detection (S/N = 3) for acetic derivative, hippuric derivative, propionic derivative, butyric derivative, benzoic derivative, valeric derivative, and caproic derivative were 1.0 pmole, 0.8 pmole, 1.5 pmole, 2.9 pmole, 5.0 pmole, 4.5 pmole, and 6.8 pmole, respectively.

DISCUSSION

In the proposed system, hexacyanoferrate (III) as cooxidant was used as the catalyst for the chemiluminescence reaction. The chemiluminescence intensity is very high, but the background intensity is large in the presence of hexacyanoferrate (III). Shevlin and Neufeld[13] have reported that the addition of hexacyanoferrate (II) onto the luminol reaction in the presence of hexacyanoferrate (III) decreases the chemiluminescence intensity. Therefore, the background intensity could be controlled by the addition of hexacyanoferrate (II) to the luminol solution.

Among the previously observed reducing agents for the chemiluminescence intensity with luminol, there was a different relative response for each compound. There was no obvious correlation potential for each compounds. In previous papers,[14-18] the chemiluminescence reaction of lucigenin with reducing agents was reported. Maeda et.al.[19,29] described that α-hydroxycarbonyl group is essential for the chemiluminescence and α-hydroxycarbonyl compounds convert easily to the 1, 2-enediol tautomer, which are strong reductants for reacting with lucigenin. Our results of the chemiluminescence of luminol with reducing agents were similar to their results. The agreement between the chemiluminescence spectra produced with the reducing agents and the fluorescence spectrum of an aminophthalic acid indicated that aminophthalic acid is chemiluminescent species.

As shown in Table 1, there is a different relative response for each corticosteroids. Cortisone, methylprednisolone, dexamethasone and hydrocortisone, having 17, 21-diol-20- one side-chain, gave an intense

chemiluminescence. 17 α-Hydroxypregnenolone, having a hydroxy group at C-17 and progesterone and 11 α-hydroxyprogesterone, having no hydroxy group at the α- position of the carbonyl group, gave no chemiluminescence with luminol. Although 17 α-hydroxypregnenolone has an α-hydroxycarbonyl group in the side - chain, the hydroxy group at C-17 is tertiary and therefore cannot be converted to a 1, 2- enediol structure.

As mentioned above, the phenacyl esters of carboxylic acids gave an intense chemiluminescence with luminol. Phenacyl alcohol is obtained by the hydrolysis of the phenacyl esters in the alkaline medium and the phenacyl alcohol gives a chemiluminescence.

The proposed chemiluminescence detection system for HPLC with luminol in the presence of catalyst in alkaline solution proved to be applicable to the determination of corticosteroids without derivatization, and of carboxylic acids following pre-column derivatization with *p*-nitrophenacyl bromide.

The detailed mechanisms are not yet elucidated at present about the luminol reaction by the reducing agents. Further studies on the application of this system to the assay of other reducing agents are in progress in our laboratory.

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SEPARATION OF METALLATED PETROPORPHYRIN MODELS USING MICELLAR ELECTROKINETIC CAPILLARY CHROMATOGRAPHY

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ABSTRACT

Some percentage of the total metals found in crude oils is present as organometallic porphyrin complexes, and petroporphyrin separations are of interest in geochemical sciences, environmental monitoring, and process control. Various chromatographic techniques including GC, SFC, and HPLC have been used to resolve mixtures of petroporphyrins. In this work, a micellar electrokinetic capillary chromatographic method has been developed and applied to the separation of petroporphyrin model compounds, and using the MECC method Ni (II) and V(IV)O Etio I and Octaethyl type porphyrins are completely resolved in 30 to 50 minutes. The MECC technique offers several advantages over a similar HPLC separation including smaller mobile phase and sample volumes required, milder aqueous based solvents, and less expensive columns.

Furthermore, the peak capacity of the MECC separation is expected to be large because less hydrophobic, neutral species can migrate in the 6 to 30 minute separation time window, suggesting that the technique when coupled to an ICP/MS detection system might prove extremely useful for metal speciation studies.

INTRODUCTION

Metals including As, Co, Fe, Mn, Ni, and V are found at concentrations ranging from < 1 to >1000 ppm in crude oils. Some 27 to 100% of the total metals may be present as organometallic porphyrin complexes which are associated with the heavier crude oil fractions.[1] These petroporphyrin complexes, first identified by Treibs in 1934, are derived from heme or chlorophyll, appear red to brown, and are thermally labile. Petroporphyrins usually contain Ni (II) or V(IV)O and are distinguished by various ring types (Etio, DPEP, Rhodo analogs) and different substituents (H, *n*-alkyl).[2] Complex and informative petroporphyrin profiles are used to study the geochemistry of oil formation, maturation, and migration,[3] to identify crude oils for environmental monitoring purposes,[4] and to aid in metal speciation for process control.[5]

Various high resolution chromatographic techniques have been used for multi-component analysis of petroporphyrin mixtures. Capillary GC[6] offers high resolution and efficiency, but the samples must be volatile and mass spectral detection is normally required. Before GC analyses, petroporphyrins are demetallated and derivatized, and this preparation may alter the profiles. SFC[7,8] is less efficient and less common than GC, but it does not require sample demetallation and visible absorbance detection may be used. Unfortunately, however, metalloporphyrins can adsorb onto packed columns sometimes used for SFC separations. Porphyrin adsorption, evidenced by peak tailing and slow bleed off, can foul columns necessitating replacement. HPLC,[9-12] which also uses packed columns, is even less efficient than SFC; furthermore, method development can be time consuming and organic solvents such as hexane, methylene chloride, and tetrahydrofuran may be required.

Capillary electrophoresis,[13] a separation technique used for less volatile and ionic materials, offers rapid analysis times and high efficiencies along with minute sample size requirements and reagent consumption. Capillary zone electrophoresis resembles both HPLC and electrophoresis in that a high voltage potential is applied across the ends of a capillary column filled with an aqueous buffer solution, thereby allowing for migration of charged species and

electroosmotic flow which moves neutral components past the detector. UV-VIS, LIF, and MS detectors are often interfaced to CE systems. In one CE mode called micellar electrokinetic capillary chromatography, a surfactant is added to the separation buffer, and species are separated based upon differential partitioning between the aqueous buffer and psuedo-stationary phase created by the surfactant micelles. Researchers have used CZE and MECC to separate free base[14] and metallated[15] anionic porphyrins, as well as polar and high molecular weight fuel-related materials.[16] In this work, we have examined the applicability of MECC to the separation of metallated petroporphyrin models and compared our results to a similar HPLC analysis.[12]

MATERIALS

Apparatus

Porphyrin absorbance measurements were made using either an HP 8452a photodiode array or Cary 3E spectrophotometer. Buffer pH was measured with a Corning Digital 110 meter and combination glass membrane pH electrode, following a 2-point calibration with certified standard pH 7 and 10 reference buffers. MECC separations were accomplished using a Beckman P/ACE 2210 automated capillary electrophoresis system equipped with a fused silica capillary and an absorbance detector which included a deuterium lamp and bandpass filters. MECC data were analyzed using Beckman System Gold™ software.

Reagents

The Ni (II) and V(IV)O derivatives of etioporphyrin I and octaethylporphyrin were obtained from Midcentury Chemicals (Posen, IL). A.C.S. reagent grade sodium tetraborate, boric acid, and other inorganic substances were obtained from Fisher, along with HPLC grade acetone and other organic solvents. ~99% sodium dodecyl sulfate (SDS) was obtained from Sigma. Sudan III and 1-(2-pyridylazo)-2-napthol (PAN) dye markers were obtained from Aldrich.

METHODS

For capillary electrophoresis separations buffers were prepared using 18M Ω water from a Barnstead NANOpure II™ system. Aqueous buffer pH was

measured and adjusted prior to vacuum degassing and filtering through 0.45μm filters. Organic modifiers were added to aqueous buffer solutions immediately prior to use, and modified buffers were sonicated for a few minutes but pH was not readjusted. Samples were prepared by adding aliquots of metalloporphyrin stock solutions in acetone to aqueous buffers. Porphyrin sample solutions could be centrifuged to remove particulate matter, but filtration through Acrodisc membrane filters resulted in porphyrin adsorption and loss.

New fused silica capillaries were validated by rinsing for 5 min. each with 100mM NaOH, water, and 50mM borate buffer (pH 8.35) then separating a Beckman test mixture containing benzoic acid derivatives. Surface activation was completed by rinsing again with base, water, and borate buffer which was allowed to remain in the capillary for at least 8 hours. Before the first porphyrin separations, the capillary was rinsed again with base, water, and run buffer which was allowed to remain in the capillary for at least 30 minutes. 2 min. run buffer pre-rinses and 5 min. water post-rinses were initially programmed into the automated run sequences; however, this rinsing procedure was modified as described later in the text.

Initial separations conditions used were as follows: Run buffer - 40mM SDS, 22.5mM total borate, pH 8.5; Porphyrins - 1 to 4 μM each of metalloporphyrins in 20% acetone, 80% run buffer; Markers - acetone or methanol for electoosmotic flow and Sudan III or PAN for the micelles; Capillary - 57 cm L_t (50 cm L_d) x 75 μm i.d. fused silica, 2518 nL V_t; Injections - pressure, 2 sec, ~12 nL; Voltage - constant, 24 kV, normal polarity (+ injection to - detection side); Temperature - 30°C; Detection - absorbance, 400+5nm. Changes to the initial separation conditions are as described later in the text.

RESULTS AND DISCUSSION

Porphyrin Solubility and Absorbance Data

Structures of the nickelated petroporphyrin model compounds are depicted in Figure 1. Freeman and co-workers[17] have previously discussed nickel and vanadyl petroporphyrin derivatives and suggested that they exhibit maximum solubilities in solvents with Hildebrand solubility parameters, δ, of 9.5. For use in MECC separations, organic solvents must be miscible with aqueous buffers and must also exhibit suitable UV cut-offs if absorbance detection is to be used.

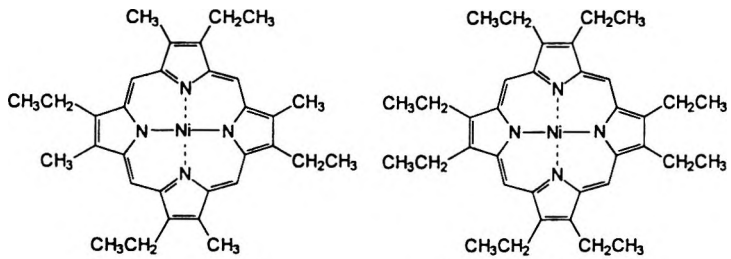

Figure 1. Structures of the Ni (II) derivatives of the petroporphyrin model compounds.

Table1

Solvent Parameters and Porphyrin Solubilities[a]

Solvent	δ[b]	UV Cut Off (nm)[c]	Porphyrin Solubilities (μg/mL) VO-EtioI	VO-Octa-ethyl	N-EtioI	Ni-Octa-ethyl
CH_2Cl_2	9.70	235			≥1500	
$(CH_3)_2CO$	9.90	330	≥220	≥220	≥270	≥150
CH_3CN	11.7	190	≥46		≥2	
CH_3OH	14.4	210	≥3		≥1	

[a] All porphyrin solubility values taken from Reference 17 except those in $(CH_3)_2CO$ which were measured in this work using methods described in that literature.

[b] Taken from Reference 18.

[c] Taken from Reference 19.

Various solvent parameters and sample solubility information is listed in Table 1. Absorbance maxima and extinction coefficients measured for the petroporphyrins in acetone, a common solvent which met the criteria discussed above, are listed in Table 2. For MECC separations, stock solutions of petroporphyrins in acetone were prepared and mixed in varying proportions with the run buffers.

Table 2

Porphyrin Absorbance Data in Acetone at Ambient Temperatures

	Soret Band		α Band	
Porphyrin	**λmax (nm)**	**ε(L/mol-cm)**	**λmax (nm)**	**ε(L/mol-cm)**
VO-Etio I	404	3.34E5±0.01	568	2.98E4±0.02
VO-Octaethyl	404	3.25E5±0.01	568	2.87E4±0.02
Ni-Etio I	388	1.95E5±0.02	550	3.33E4±0.01
Ni-Octaethyl	390	2.05E5±0.03	550	2.70E4±0.07

Initial MECC Separations

Shigeru Terabe[20] has suggested a scheme for optimization of MECC separations which includes running under a standard set of conditions and calculating capacity factors, k'. If $k' < 0.5$, the surfactant concentration is increased; if $k' > 10$, buffer additives such as organic modifiers, cyclodextrins, or bile salts are utilized. Initial trials using conditions listed in the experimental section resulted in average migration times of 2.5±0.1 and 6.2±0.2 min. for the electroosmotic flow (t_0) and micellar (t_{mc}) markers; respectively. The analyte mixture was not resolved and also passed the detector window in 6.2 min., indicating complete retention of the porphyrins within the micelles such that $k' >> 10$ and buffer additives would be required.

Before buffer additives were attempted, however, the surfactant concentration was varied from 20 to 100 mM with the same net results - no resolution of the mixture and porphyrin co-migration with the micelles. As buffer SDS concentration and conductivity increased, the t_0 changed from 2.5 to 3.1 min., while t_{mc} lengthened from 6.2 to 10 min. Power levels were also increased from 1.2 to 3.8 W/m over this range of SDS concentrations, making Joule heating of the buffer a concern.

Interestingly, the average peak area increased by a factor of 29 going from 20 to 100 mM SDS indicating that more porphyrin was being solubilized in the aqueous buffer. At 20 mM SDS the peak area %RSD was extremely high due to low signal-to-noise resulting from low porphyrin solubility and low deuterium lamp energy at 400 nm; at $\geq$ 40 mM SDS peak area %RSD was $\leq$ 10. Migration time precision was typically $\leq$ 1.3 %RSD in these early separations.

Table 3

Separation as a Function of Acetone Fraction in the Buffer

Acetone (% v/v)	SDS (mM)	Borate Avg. (mM)	M_t (min.)	%RSD (n=3)	Signal Identification
0	40	22.5	2.45	0.35	EOF
			5.81	1.04	Micelles/Porphyrins
5	38	21.4	2.55	0.06	EOF
			6.30	0.04	Micelles/Porphyrins
10	36	20.3	3.12	0.22	EOF
			9.32	0.90	Micelles
			9.54	0.85	Porphyrins
15	34	19.1	3.49	0.35	EOF
			11.04	1.66	Micelles
			12.07	1.51	Porphyrins
20	32	18	4.04	0.14	EOF
			11.38	1.59	Micelles
			15.79	0.59	VO-Porphyrins
			16.07	0.65	Ni-Porphyrins
30	28	15.8	6.09	0.37	EOF
			22.02	0.05	VO Etio I
			24.95	0.30	VO Octaethyl
			27.32	0.33	Ni Etio I
			30.63	0.25	Ni Octaethyl

Organic Modifier Studies

Various organic modifiers including acetone, acetonitrile, dimethyl sulfoxide, ethanol, ethylene glycol, methanol, and 2-propanol have been used in CZE[21] and MECC[22] separations; although, the two most common modifiers are probably acetonitrile and methanol. These buffer additives effect MECC resolution and efficiency by altering capacity factors and selectivities of the analytes, and the electroosmotic flow and electrophoretic mobilities are effected

Table 4

Separation as a Function of Organic Modifier at 30% v/v in the Buffer[a]

Organic Modifier	M_t (min)	%RSD	n	Signal
Methyl ethyl ketone	4.95	1.68	6	EOF
	16.20	3.87		Porphyrin 1
	16.83	3.56		Porphyrin 2
	17.10	4.12		Porphyrin 3
	17.46	4.00		Porphyrin 4
Acetonitrile	5.03	2.05	6	EOF
	16.59	9.85		Porphyrins
Methanol	6.21	0.37	2	EOF

[a] Final concentrations of 28mM SDS - 16 mM Borate in the Buffer.

by changes in the dielectric constant and viscosity of the buffer medium, as well as in the zeta potential. In addition, the surfactant critical micelle concentration (CMC) and aggregation number may be modified by ions and organic solvents present in aqueous solutions.[23]

Organic modifier studies were conducted using the same initial conditions listed in the experimental section except that samples were 1 to 2 μM of each porphyrin in 30% acetone / 70% 40 mM SDS- 22.5 mM Borate-pH 8.5 buffer and sample injections were increased to 5 sec (~29 nL or 1% V_t).

In the first study, the fraction of acetone in the separation buffer was increased from 0 to 30%, and in the second study, acetone was compared to other organic modifiers each at 30% (v/v) added. Results are given in Tables 3 and 4; representative electropherograms are shown in Figures 2 and 3.

With acetone as the organic modifier in the run buffer, below 10% (v/v) added, no porphyrin separation occurred and species eluted with the micellar marker. At acetone concentrations $\geq$ 10% the micellar marker actually eluted before the petroporphyrins, indicating that migration of the metallated porphyrins was related to factors other than micellar partitioning alone. At 20% acetone added, vanadyl porphyrins were separated from nickel complexes

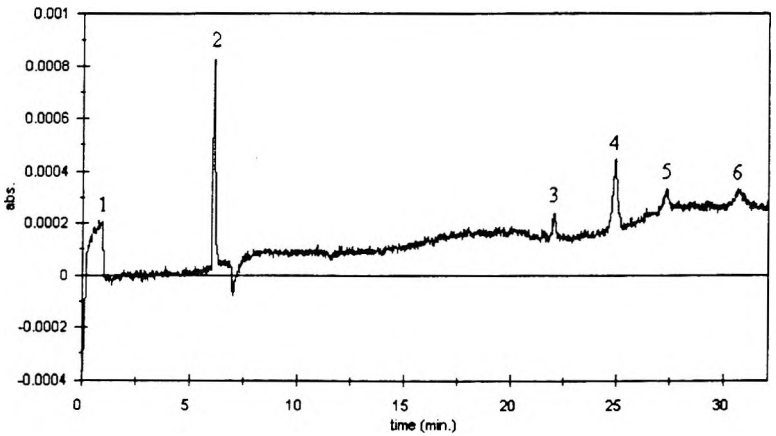

Figure 2. MECC separation of a mixture of vanadyl and nickel petroporphyrins using run buffer which contained 28 mM SDS - 16 mM Borate - 30 % v/v Acetone. A 5 sec sample injection was used and other conditions are as listed in the experimental section. Events or peaks are identified as: 1) baseline zero, 2) EOF, 3) VO Etio I, 4) VO Octaethyl, 5) Ni Etio I, and 6) Ni Octaethyl porphyrin.

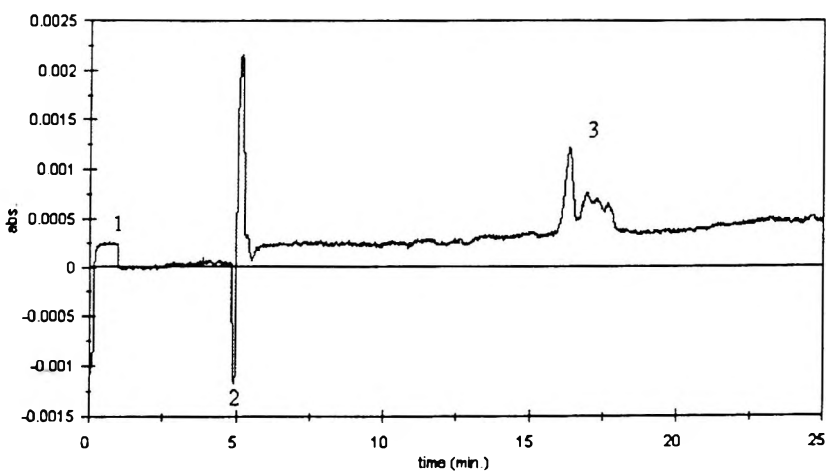

Figure 3. MECC separation of a mixture of vanadyl and nickel petroporphyrins using run buffer which contained 30 % v/v Methyl Ethyl Ketone as the organic modifier. Other conditions are as listed for Figure 2. Events or peaks are identified as: 1) baseline zero, 2) EOF, and 3) incompletely resolved porphyrins.

and, at 30%, all four porphyrins resolved in under 31 minutes. Porphyrins migrated in the order VO Etio I, VO Octaethyl, Ni Etio I, and Ni Octaethyl, and some porphyrin interaction occured with the fused silica capillary surface.[24,25]

In comparing porphyrin separations using different organic modifiers at 30% (v/v), it was concluded that acetone was better than methylethyl ketone, acetonitrile, or methanol in resolving the mixture. Although methylethyl ketone demonstrated some promise in separating the analytes, porphyrins could not be resolved with acetonitrile, and absolutely no porphyrin peaks were observed when methanol was used in the buffer. The final concentrations of buffer components were 28 mM SDS-16 mM Borate-30% Organic Modifier for this comparison.

Reproducibility and Treatment of the Capillary Surface

The porphyrin separation results, obtained with 30% acetone added to the run buffer, appeared very promising at first, but subsequent runs were not very reproducible as evidenced by increases in migration times and decreases in peak areas. Sample degradation, changes in buffer composition, and porphyrin adsorption to the capillary surface were thought likely causes of irreproducibility.

In an attempt to alleviate these problems, porphyrin stock solutions were deoxygenated and stored in amber bottles, and porphyrin samples were prepared immediately prior to use. Organic modifiers were added to the aqueous buffers fresh each day, and buffer solutions in the instrument vials were replaced frequently (after 3 runs). The capillary post-run rinsing procedure was modified to include a 15 sec 1M HF rinse followed by washing with water, and this proved useful in creating a reproducible capillary surface and EOF, as discussed by Schwer and Kenndler.[21] The effect of this capillary treatment was examined by including the HF rinsing procedure in the separation of the Beckman test mixture of benzoic acid derivatives; after 31 runs peak migration time reproducibilities were less than 2.5% RSD.

Although migration times were more consistent, porphyrin peak area reproducibility was still a challenge. Sample degradation and/or precipitation appeared to transpire as evidenced by loss of solution color. de Waal and co-workers[11] noted that poor separations are obtained when precipitation of vanadyl and nickel porphyrins in oil extracts occurs in the starting mobile phase. For the MECC separations, porphyrin solubilization might be effected if

surfactant aggregation in the modified run buffer was significantly different than that in aqueous solution; therefore, the critical micelle concentration of SDS was determined in a solution containing 16 mM Borate-30% Acetone using a fluorescence technique.[26] However, at ambient temperature a CMC value of 7 mM, well below the 28 mM SDS present in the modified run buffer, was indicated by a curve fit of the data.

Increased SDS Concentrations

At constant 30% (v/v) acetone and reduced total borate (12mM) concentrations, the amount of SDS in the run buffer was varied from 15 to 42 mM. Over this range, the EOF and power level increased from 5.2 to 6.1 min. and 0.83 to 2.1 W/m; respectively. At 15 mM SDS, two small porphyrin peaks were resolved and migration times were near 10 minutes. With 42 mM SDS present, peak areas were substantially larger and all four porphyrins were resolved in a migration time window of 36 to 50 min. Representative data are shown in Figure 4. It should be noted that injection times were increased to 10 sec (~59 nL, 2% V_t) for the sample and a secondary buffer plug added to push the porphyrins further up into the capillary before application of the separation voltage.

At constant SDS and total borate concentrations of 42 and 12 mM; respectively, the acetone fraction was varied from 20 to 30% by volume. Over this range, the EOF increased from 4.6 to 6.3 min. At 20% acetone, two incompletely resolved porphyrin signals migrated with times of 29 and 31 min.; while, at 30% acetone, all four porphyrin signals were completely resolved in a migration time window of 32 to 47 min. as shown in Figure 5. Reproducibility data for the petroporphyrin separation in 42 mM SDS-12mM Borate-30% Acetone over different days and with different capillaries is summarized in Table 5.

Results obtained by varying the organic modifier composition are in agreement with studies[21] which demonstrate that the aprotic solvent acetone causes decreases in the electroosmotic flow velocity due to decreases in the dielectric constant and increases in the solution viscosity coefficient over the 20 to 30% v/v range. The zeta potential depends on the apparent pH of the buffer solution, and, in low ionic strength phosphate buffer containing 50% v/v acetone, it is approximately constant above pH' ~9.[21] For the 42 mM SDS-12 mM Borate-30% v/v Acetone buffer used in this study, the measured pH' was 9.6; however, the effect of this higher ionic strength SDS buffer on the deprotonation equilibria of silanol groups on the capillary surface is not known.

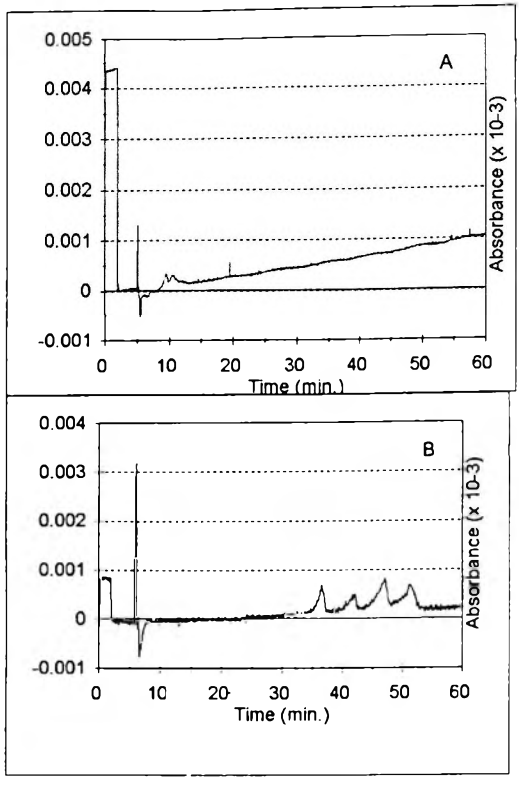

Figure 4. Petroporphyrin separation as a function of SDS concentration in the run buffer which contained 12 mM Borate - 30 % v/v Acetone. 10 sec primary and secondary injections of sample and buffer were used along with an HF rinse. Other conditions are as listed in the experimental section. A) 15 mM SDS, and B) 42 mM SDS.

Slight differences in buffer composition may lead to pronounced effects on solution properties and surface charge which combine to alter observed electrophoretic mobilities. This may partially explain variations in migration time behavior, in addition to other effects previously discussed.

Quantitative irreproducibility is related to low D_2 lamp energy at 400 nm and variable porphyrin concentrations which combine to make absorbance detection less than ideal in this case. Weinberger and co-workers[14] have demonstrated that a tungsten lamp provides better sensitivity than a deuterium lamp (LOD = 0.7-1.7 x 10^{-12}M) for absorbance detection of urinary porphyrins in CE separations. The petroporphyrin samples are estimated to be in the 1 to

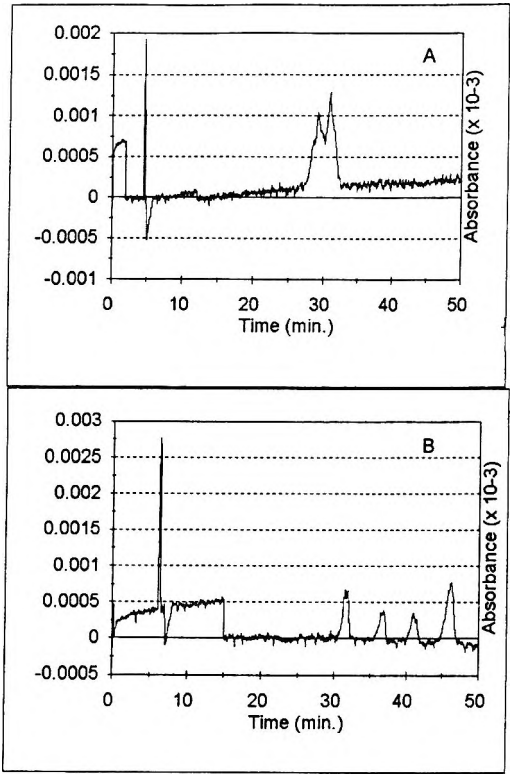

Figure 5. Petroporphyrin separation as a function of Acetone fraction in the run buffer which contained 42 mM SDS - 12 mM Borate. Other conditions are as listed for Figure 4. A) 20 % v/v Acetone, and B) 30 % v/v Acetone.

2 x 10^{-6}M range when first prepared; however, some sample precipitation, decomposition, and/or wall adsorption occurs before analytes reach the detector window. Average peak base widths of 2.5 min. in the MECC method are indicative of zone spreading which reduces the peak height and detectability. Various attempts to focus sample zones by field amplification methods did not improve detection, and porphyrins could not be separated on a linear polyacrylamide coated capillary. Thus, future plans include utilization of a tungsten radiation source or a different type of detector. Sample solubility and stability will be improved by using less polar organic modifiers and/or more surfactant in the buffer, and methyl ethyl ketone is currently being re-examined at higher SDS concentrations.

Table 5

Separation with 42mM SDS -12mM Borate - 30% Acetone Buffer[a]

Signal	M_t (min.)	%RSD	Peak Area	%RSD
EOF	6.26	0.80		
VO Etio I	32.32	3.30	758	6.33
VO Octaethyl	37.84	3.80	473	4.86
Ni Etio I	42.60	4.82	508	28.2
Ni Octaethyl	47.72	4.80	1092	9.71

[a] Data are from Day 1, Capillary 1; other migration time results are summarized below:

	Day 2, Capillary 1	Day 1, Capillary 2	Day 2, Capillary 2
EOF	6.14	6.04	6.05 [min]
Porphyrins	29.86 - 42.62	28.31 - 46.55	34.09 - 51.16 [min.]

Provided that detection can be improved, the MECC method presented here compares favorably to the HPLC separation of petroporphyrin compounds by Xu and Lesage.[12] The MECC method utilizes fused silica capillaries which are more cost effective (~$10) than the aminopropyl HPLC column (~$350). Aqueous based buffers are used in the MECC separation as opposed to hexane, toluene, and methylene chloride HPLC mobile phases. 60 nL MECC sample injections are two orders of magnitude smaller than those used in the HPLC work. MECC run times of 60 min. are comparable to 50 min. HPLC separation times plus column reconditioning. The MECC migration order was VO Etio I, VO Octaethyl, Ni Etio I, and Ni Octaethyl porphyrins, the reverse of that observed in the HPLC separation. The MECC method gave an average peak resolution of 2.2; whereas, the HPLC method appeared to range from 2.8 (two vanadyl porphyrins) to 36 (Ni Etio I and VO Octaethyl porphyrins). Average efficiency for the MECC method was 4450 theoretical plates compared to 6545 calculated from the HPLC chromatogram. Column lifetime and reproducibility were not discussed in the HPLC work.

Due to different separation mechanisms being operative, the elution order for the petroporphyrin model compounds in MECC is opposite to that in the HPLC work. These differences are important in terms of the total peak capacity. Although Xu and Lesage could separate similar porphyrins in a 19 minute window, it is doubtful that very polar compounds can be separated using

the gradient elution scheme they described. The MECC separation, on the other hand, offers a "window of opportunity" which stretches from the EOF time (~ 6 min.) to the porphyrin migration time (~ 30 min.). During this period, compounds which are much less hydrophobic than the petroporphyrins may elute, allowing the separation mechanism to be exploited for metal speciation studies by MECC. Researchers have sucessfully interfaced micellar LC to ICP/MS detection systems for the analysis of various organometallic compounds,[27,28] and efforts are ongoing to develop CE systems for speciation of metalloporphyrins and metalloproteins.[29]

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RETENTION CHARACTERISTICS OF TITANIUM DIOXIDE AND POLYETHYLENE-COATED TITANIUM DIOXIDE AS REVERSED-PHASE SUPPORTS

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ABSTRACT

The retention of 19 solutes markedly differing in their physicochemical characteristics was determined on titanium dioxide and polyethylene-coated titanium dioxide columns in water and water-methanol eluent mixtures. Principal component analysis was used for the elucidation of the relationship between retention behavior and physicochemical parameters. Polyethylene-coated titanium dioxide showed stronger retention than titanium dioxide did, proving the retention increasing effect of polyethylene coating. Retention parameters formed a loose cluster with the excess molar refraction and the effective hydrogen bond acidity of solutes on the two-dimensional nonlinear map of principal component loadings, indicating the involvement of steric conditions and electronic interactions in the retention.

INTRODUCTION

The majority of high performance liquid chromatographic (HPLC) analyses is carried out in reversed-phase (RP) separation mode.[1] The wide-spread application of RP-HPLC may be due to its versatility and the environmental and toxicological safety of the eluent components.[2] Silica supports with covalently bonded hydrophobic ligands are extensively used in RP-HPLC,[3] however, the successfull application of silica-based supports in RP-HPLC is limited by the low stability of silica at high pH values,[4] and by the undesirable electrostatic interactions between the polar substructures of solutes and the free silanol groups not covered by the hydrophobic ligand.[5,6] To increase the pH range of application, many other supports have been developed, such as alumina,[7,8] zirconia,[9,10] and various polymer-based supports.[11,12]

More recently, polymer-coated supports have become popular since they combine the advantageous mechanical properties of support with the dynamic chemical properties of polymers.[13,14] Polymer coating improves not only selectivity but also the chemical stability of stationary phases.[15] The study of the retention behavior of barbituric acid derivatives[16] and nonionic surfactants[17,18] on polyethylene-coated supports have recently been reported. Due to its advantageous mechanical properties and high stability of extreme pH values,[19] titanium dioxide is a promising substitute for silica, specially, in the separation of basic compounds.[20] Octadecyl-coated titanium dioxide was also prepared,[21] and its retention characteristics was compared with those of other reversed-phase supports.[22] It was established, that, the separation capacity of octadecyl-coated titanium dioxide for basic compounds is superior to that of octadecyl-coated silica.[23]

Principal component analysis (PCA)[24] has been frequently used in chromatography for the evaluation of retention data matrices of considerable dimensions.[25] The main advantages of PCA in chromatography are as follows:

a) Clustering of variables according to their relationship (clustering chromatographic systems or solutes according to their retention characteristics),

b) The possibility of extraction of one or more background variables having concrete physicochemical meaning for the theory and practice of chromatography,

c) Reduction in the number of variables (a reduction in the number of chromatographic systems or solutes to the minimum necessary for solution of a problem),

d) Combination with nonlinear mapping or cluster analysis facilitates the visual evaluation of the resulting multidimensional matrices of PC loadings and variables.

PCA has been successfully used in chromatography for the characterization of hydrophobic interaction chromatography media,[26] the clustering of solutes,[27] the elucidation of the role of various molecular parameters in the retention,[28] the classification of antihistamine drugs in various HPLC systems,[29] etc.

The objectives of our investigations were to determine the retention behavior of solutes with widely different physicochemical parameters on a titanium dioxide and a polyethylene-coated titanium dioxide column, and to elucidate the relationship between retention characteristics and physicochemical parameters of solutes by means of principal component analysis.

MATERIALS AND METHODS

The chemical name of solutes are compiled in Table 1. They were selected to minimize the intercorrelations between their physicochemical parameters included in PCA: excess molar refraction (further parameter I), dipolarity/polarizability (II), effective hydrogen bond acidity (III), effective hydrogen bond basicity (IV), the characteristic volume of McGovan (V), total dipole moment (VI), maximum electron access charge (electron deficiency) on an atom (VII), and a solvent (water) accessible molecular volume (VIII). Titanium dioxide support was prepared by the research group of Dr.M. Zaharescu (Institute of Physical Chemistry "I.G.Murgulescu", Romanian Academy, Bucharest, Romania). Polyethylene-coated titanium dioxide was prepared as described earlier.[16] Columns of 250 x 4 mm I.D. was filled with titanium dioxide and polyethylene-coated titanium dioxide using a Shandon (Pittsburgh, PA, USA) analytical pump. The eluents were the eluent systems proposed for the filling of adsorption and reversed-phase columns, respectively.[30]

The HPLC system consisted of a Merck-Hitachi apparatus (Model L-6000A pump, L-4000A UV detector and D-2500A integrator, Merck, Darmstadt, Germany). Detection wavelength varied between 210 and 254 nm, depending on the absorption maximum of the solute. Water and water-methanol mixtures were used as eluent, the flow-rate being 1 mL/min. Columns were not thermostated, each determination being run at room temperature (22±2° C). Dead volumes were determined by injecting 1 % $NaNO_3$. The use

Table 1

Chemical Name, Log k_0', and Relative Standard Deviation (R.S.D.%) of Solutes on Titanium Dioxide (TiO_2) and Polyethylene-Coated Titanium Dioxide (PE-TiO_2) Supports

No.	Chemical Name	TiO_2		PE-TiO_2	
		Average	R.S.D. %	Average	R.S.D. %
1	n-Hexylbenzene	-0.114	1.28	0.544	0.85
2	1,3,5-Triisopropylbenzene	-0.126	0.43	1.212	1.36
3	1,4-Dinitrobenzene	-0.101	0.76	0.038	1.39
4	3,5-Dichlorophenol	0.221	0.84	1.347	0.58
5	4-Iodophenol	-0.234	1.08	0.389	0.70
6	Benzamide	-0.291	0.87	0.179	1.41
7	Benzene	-0.017	1.32	0.823	0.86
8	Chlorobenzene	-0.223	0.92	0.749	0.74
9	Cyclohexanone	-0.184	1.53	0.349	0.83
10	Phenol	0.604	0.66	0.023	1.27
11	Hexachlorobutadiene	0.041	1.37	1.672	1.53
12	Indazol	-0.345	1.19	0.690	0.89
13	Caffeine	-0.057	0.96	1.187	1.34
14	4-Nitrobenzoic acid	-0.136	0.81	1.190	0.98
15	N-Methyl-2-pyrrolidinone	-0.237	1.04	0.402	0.92
16	Naphtalene	0.195	1.11	1.215	0.78
17	4-Chlorophenol	0.293	1.42	1.049	1.04
18	Piperidine	-0.132	0.91	-0.889	0.59
19	Benzonitrile	0.014	1.387	-0.562	1.21

of titanium dioxide as reversed-phase support was motivated by the finding that it showed negligible retention capacity in adsorption separation mode, even in n-hexane eluent. Solutes were dissolved in the eluent at a concentration of 0.1 mg/mL.

Each determination was run in quadruplicate and the capacity factor in water (log k_0') or extrapolated to water in the case of more hydrophobic compounds and its relative standard deviation (R.S.D.%) was calculated.

PCA was applied to find the similarities and dissimilarities between the retention capacity of titanium dioxide and polyethylene-coated titanium dioxide

columns and the physicochemical parameters of solutes listed above. The logarithm of the capacity factor, determined in water or extrapolated to water ($\log k_0'$), of solutes determined on titanium dioxide and on polyethylene-coated titanium dioxide and the physicochemical parameters listed above were the variables (altogether 10 variables) and the solutes the observations. The limit of the variance explained was set to 99.9%.

As the visual evaluation of the multidimensional matrices of PC loadings and variables is difficult, their two-dimensional nonlinear maps were also calculated.[31] The iteration was carried out to the point when the difference between the two last iterations was lower than 10^{-8}. Softwares for PCA and two-dimensional nonlinear mapping was prepared by Dr. Barna Bordás (Plant Protection Institute of Hungarian Academy of Sciences, Budapest, Hungary) and were run on an IBM AT computer.

In order to compare the retention characteristics of titanium dioxide and polyethylene-coated titanium dioxide with those of other polyethylene-coated supports, linear correlations were calculated between the $\log k_0'$ values in Table 1 and the similar values determined on polyethylene-coated silica and polyethylene-coated zirconia. The corresponding $\log k'$ values were taken from Ref. 32.

RESULTS AND DISCUSSION

Chromatogram of some solutes on polyethylene-coated titanium dioxide is shown in Fig. 1. The peaks are symmetric, even at the higher retention times, indicating that this support can be successfully used for the separation of these class of solutes without buffering the eluent.

The logarithm of the capacity factors and the relative standard deviations are compiled in Table 1. The low values of the relative standard deviation indicate the good stability of the HPLC system and that of the polyethylene-coating.

The data in Table 1 clearly show that titanium dioxide support has a low retention capacity, even in aqueous eluent, indicating that the binding strength of the adsorption centers on the surface of titanium dioxide is fairly low. This finding suggests, that, in the case of any reversed-phase support prepared from titanium dioxide, the undesirable side effect of the original adsorption centers on the retention is negligible.

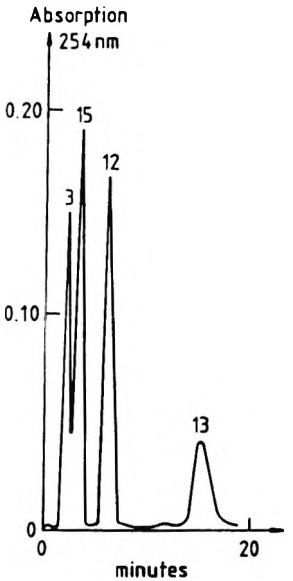

Figure 1. Separation of some solutes on polyethylene-coated titanium dioxide. Eluent: water. Number refer to solutes in Table 1.

The results of PCA are compiled in Table 2. The overwhelming majority of the information contained in the original data matrix can be described by three background variables. In other words, a few theoretical variables are sufficient to describe the relationship between the physicochemical parameters and retention characteristics of these solutes. Unfortunately, PCA does not define these variables as concrete physical or physicochemical entities, only indicates their mathematical possibility.

The retention parameters of solutes have high loadings in different PC components, indicating that titanium dioxide and polyethylene-coated titanium dioxide expose different retention characteristics. The fact that the majority of physicochemical parameters, but not the log k_0' values, have high loadings in the first PC, suggests that the correlation between the two groups of variables is not strong enough for the reliable prediction of the retention of solutes in these RP-HLPC systems. It can be further assumed, that other molecular parameters not included in the calculation may also have a considerable impact on the relationship between retention and molecular characteristics.

Table 2

Similarities and Dissimilarities Between the Physiochemical Parameters and Retention Characteristics of Various Solutes on Titanium Dioxide ($\log k_0'$ TiO_2) and Polyethylene-Coated Titanium Dioxide ($\log k_0'$ PE-TiO_2) Supports[a]

No of Principal Component	Eigenvalues	Variance Explained %	Total Variance Explained %
1	3.16	31.55	31.55
2	2.35	23.46	55.01
3	1.86	18.62	73.63
4	0.70	7.00	80.62
5	0.67	6.70	87.33
6	0.58	5.81	93.13

Principal Component Loadings

Variables	No. of Principal Component					
Retention parameters:	1	2	3	4	5	6
$\log k_0'$ TiO_2:	-0.22	-0.18	**0.64**	**0.68**	-0.21	-0.06
$\log k_0'$ PE-TiO_2'	-0.33	**0.62**	0.37	-0.04	0.08	**0.52**
Physiocochemical Parameter						
I	0.31	**0.62**	**0.52**	-0.17	-0.30	0.06
II	**0.84**	0.35	0.15	-0.05	-0.21	0.02
III	0.31	0.03	**0.73**	-0.01	**0.56**	-0.18
IV	**0.69**	0.21	**-0.52**	0.34	-0.09	0.10
V	**-0.52**	**0.78**	-0.26	0.13	0.12	-0.14
VI	**0.78**	0.01	-0.27	0.23	0.37	0.28
VII	**0.71**	0.42	0.06	-0.05	-0.04	-0.37
VIII	**-0.50**	**0.77**	-0.29	0.15	0.13	-0.16

[a] Results of principal component analysis. For symbols see **MATERIALS AND METHODS.**

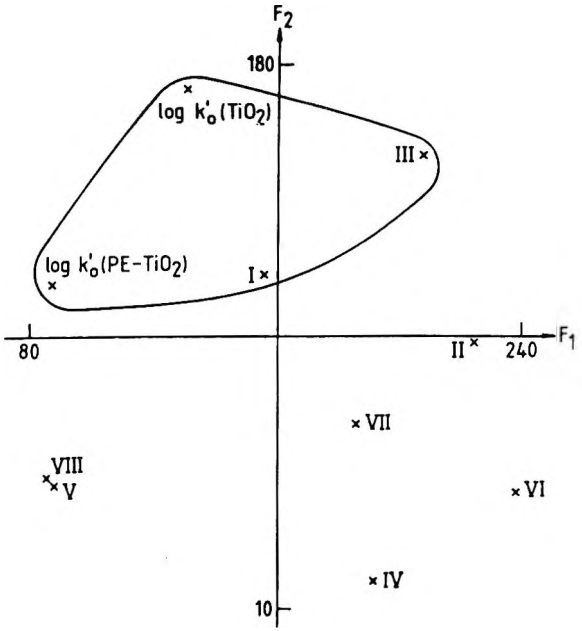

Figure 2. Relationship between the various physicochemical parameters of solutes and their retention behaviour on tita- nium dioxide and polyethylene-coated titanium dioxide supports. Two-dimensional nonlinear map of principal component loadings (number of iterations: 234, maximum error: $4.21.10^{-2}$). For symbols see **MATERIALS AND METHODS**.

The two-dimensional nonlinear map of principal component loadings is shown in Fig 2. Chromatographic parameters form a loose cluster with the excess molar refraction (I) and with the effective hydrogen bond acidity (III), indicating that more than one physicochemical parameter influences the retention of solutes on both titanium dioxide and polyethylene-coated titanium dioxide supports in reversed-phase separation mode. The cluster formation of excess molar refraction and effective hydrogen bond acidity with the retention characteristics suggests that steric correspondence between solutes and the surface of the support, as well as the electronic interactions between them, exert the highest impact on the retention. Solutes do not form separate clusters on the two-dimensional nonlinear map of principal component variables (Fig. 3). This finding supports our previous conclusions that the physicochemical parameters of the solutes are really different, resulting in different retention.

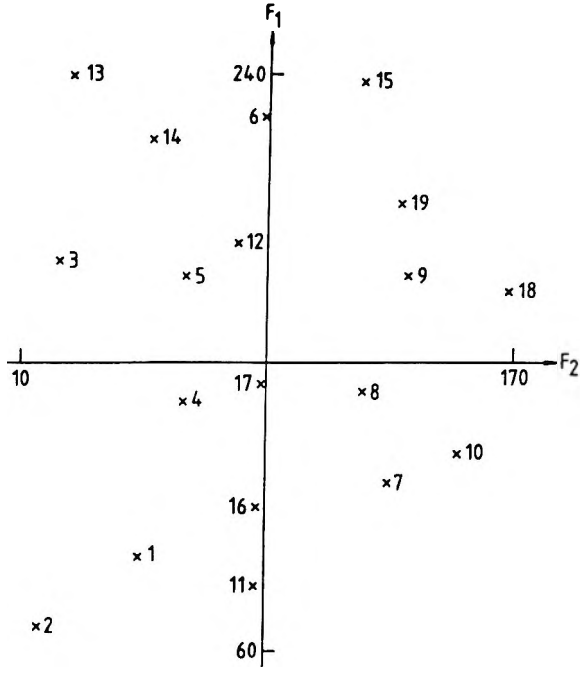

Figure 3. Distribution of solutes according to their retention behaviour on titanium dioxide and polyethylene-coated titanium dioxide supports. Two-dimensional non-linear map of principal component variables (number of iterations: 208, maximum error: $5.55.10^{-2}$). Numbers refer to solutes in Table 1.

The coefficients of linear correlations between the various log k values were not significant (titanium dioxide - polyethylene-coated silica: r = 0.0235; titanium dioxide -polyethylene-coated zirconia: r = 0.0264; polyethylene-coated titanium dioxide - polyethylene-coated silica: r = 0.3688; polyethylene-coated titanium dioxide - polyethylene-coated silica: r = 0.4245). These finding indicates that the retention behavior of titanium dioxide based supports may be different from those of other polymer-coated supports, which advocates their future use in both pharmaceutical and environmental analysis.

It can be concluded from the data, that the retention capacity of titanium dioxide and polyethylene-coated titanium dioxide differ considerably under reversed-phase conditions. Titanium dioxide shows negligible retention strength, which makes it a promising support in reversed-phase chromatography. Principal component analysis suggested that steric conditions and electronic interactions exert the highest impact on the retention.

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DETERMINATION OF METYROSINE AND ITS METABOLITE IN SERUM BY REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY USING SOLID PHASE EXTRACTION AND FLUORESCENCE DETECTION

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ABSTRACT

A novel and rapid method for the separation and determination of metyrosine and its major metabolite alpha-methyldopa in serum by high performance liquid chromatography with fluorescence detection is reported. The methods involved a solid-phase extraction of the two analytes and the internal standard dopamine using a Bond-Elut strong cation-exchange (SCX) column. The eluate obtained from the SCX column is then chromatographed on a reversed phase octadecylsilane column (Spherisorb 0DS2, 250 x 4.6 mm I.D.) with a 92.5:5:2.5 v/v/v 0.1 M aqueous phosphate buffer pH 3-acetonitrile - methanol mobile phase containing EDTA and heptane sulfonate. The flow rate was 1.0 mL/min with excitation at 282 nm and a 370 nm emission filter. The detection and quantitation limits were 1.0 μg/mL and 0.1 μg/mL for metyro-

sine and alpha-methyldopa, respectively, using 1 mL of serum. Linear calibration curves of 5-35 μg/mL and 0.2-2.5 μg/mL for metyrosine and alpha-methyldopa, respectively, show coefficients of determination of more than 0.9995. Precision calculated as %RSD and accuracy calculated as % error were within 2.5 - 6.5% and 2.8-4.2%, respectively, for metyrosine and 4.1-6.3% and 1.3 - 1.5%, respectively, for alpha-methyldopa.

INTRODUCTION

Metyrosine is an orally active inhibitor of catecholamine synthesis peripherally as well as centrally. It is used to control hypertension in patients with phaeochromocytoma and may be given as a pre-operative preparation to those patients for whom surgery is contra-indicated.[1] Its major serum metabolite alpha-methyldopa has similar activity. [2] A review of the literature revealed that metyrosine is determined in biological fluids and tissues principally by gas chromatography-mass spectrometry.[3] It has also been determined fluorometrically in biological fluids using a liquid-liquid extraction procedure.[4] It has been determined in dosage forms, either polarographically through treatment with nitrous acid [5] or colorimetrically via its reaction with 4-aminoantipyrine in the presence of an alkaline oxidizing agent.[6] The USP 23 recommends a non-aqueous titration method, with potentiometric detection of the end point for the evaluation of the bulk drug material.[7] Thus far, no HPLC methods have been reported for the determination of metyrosine and alpha-methyldopa in serum.

This paper describes a reversed phase HPLC method using fluorescence detection and solid phase extraction (SPE) to measure low μg/mL concentrations of metyrosine and its metabolite in serum, with good sensitivity, selectivity and fast chromatographic run time. The assay procedure possesses the required sensitivity to be useful for monitoring blood levels of metyrosine at 1 gm doses.

EXPERIMENTAL

Reagents and Chemicals

Metyrosine (alpha-methyl-L-tyrosine) was obtained from Fluka (Biochemika, AS, USA). Alpha-methyldopa [3-(3,4-dihydroxy phenyl)-2-methyl-L-alanine] was purchased from Sigma (St. Louis, MO, USA). The

internal standard dopamine hydrochloride was obtained from USP (Rockville, MD, USA). Blank bovine serum (Cat # 3160-34) was obtained from Instrumentation Lab (Lexington, MA, USA). Acetonitrile and methanol were obtained from J.T. Baker (Phillipsburg, NJ, USA) and were HPLC grade. All chromatographic solutions were filtered through a 0.45 μm filter (Alltech, Deerfield, IL. USA)and degassed prior to use.

HPLC Conditions

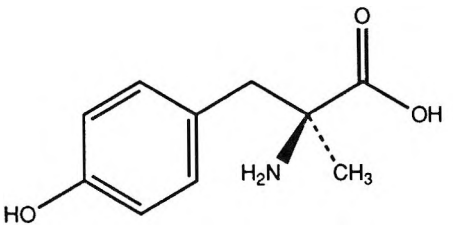
Chromatography was performed on an isocratic HPLC system consisting of a Beckman Model 110A solvent delivery module (Beckman, San Ramon, CA, USA) and a Spectroflow 980 fluorescence detector (Kratos Analytical, Ramsey, NJ, USA) with excitation at 282 nm and a 370 nm emission filter. Data acquistion was performed on a HP Model 3290 integrator (Hewlett Packard, Avondale, PA, USA). The stationary phase was a 250 X 4.6 mm id Spherisorb ODS2 5μ column (Metachem Technologies, Inc., Torrance CA., USA) at ambient temperature (23°C). The mobile phase was prepared by dissolving 30 mg of 1-heptanesulphonic acid sodium salt-1-hydrate, 10 mg of the disodium salt of EDTA, 1.379 gm of NaH_2PO_4x H_2O, 2.5 mL methanol and 5 mL acetonitrile in 100 mL water. The pH of the mobile phase was adjusted to 3.0 with 0.1M phosphoric acid and the flow rate was set at 1.0 mL/min. Quantitation was based on linear regression analysis of analyte peak height versus analyte concentration.

Preparation of Standard Solutions

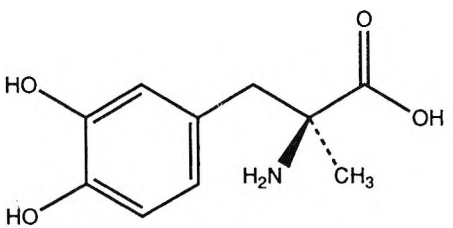
Individual stock solutions of metyrosine, alpha-methyldopa and the internal standard dopamine were prepared in methanol to give concentrations of 100 μg/mL. Appropriate volumes of the two analytes and the internal standard were placed into 1 mL volumetric flasks and drug free serum added to volume to give final serum concentrations of 5, 10, 15, 20, 25, 30, and 35 μg/mL for metyrosine, 0.2,0.5, 1.0, 1.25, 1.5, 2.0, and 2.5 μg/mL for alpha-methyldopa and 2 μg/mL of the dopamine.

Assay Procedure and Preparation of Standard Curve

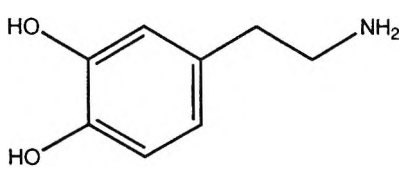
A 1-mL volume of distilled water was added to 1-mL serum samples containing metyrosine, its metabolite and internal standard. For an unknown sample, 20 μL of the internal standard solution was added to 1 mL of serum



Metyrosine (I)



alpha-Methyldopa (II)



Dopamine (III)

Figure 1. Chemical structures of metyrosine (I), alpha-methyldopa (II) and dopamine (internal standard, III).

sample followed by the addition of 1-mL of distilled water. The samples were vortex-mixed for 2 min and then passed through a 1-mL SCX Bond-Elut SPE column attached to a vacuum manifold (Vac-Elut, Varian Sample Preparation Products, Harbor City, CA, USA) which was previously conditioned with 2-mL of methanol followed by 2-mL of 0.1 N hydrochloric acid. The column was washed with 2-mL water and allowed to air dry for 3 min. The analytes of interest were eluted with 4 x 250 μL of 1 M diibasic potassium phosphate (pH adjusted to 5.0 with 1 M phosphoric acid) and a 100-μL aliquot was injected into the HPLC system.

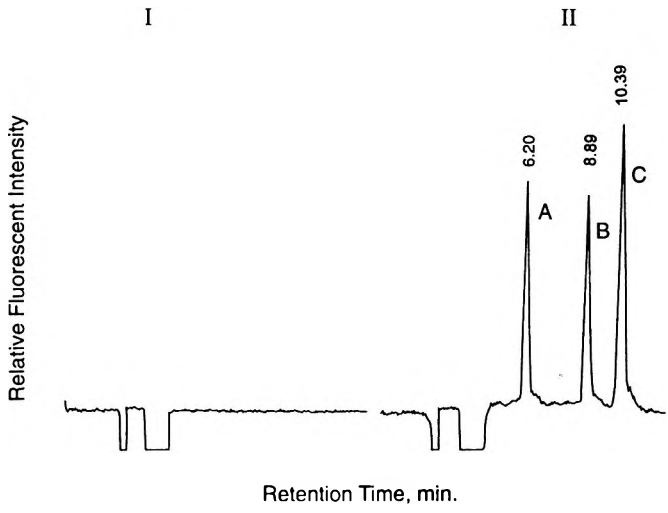


Figure 2. Representative chromatograms of (I) serum blank and (II) serum sample with alpha methyldopa (2 μg/mL), (B) dopamine (IS, 2 μg/mL) and (C) metyrosine (30 μg/mL). See Experimental section for HPLC conditions.

Linear calibration curves were constructed in the range of 5-35 μg/mL and 0.2-2.5 μg/mL for metyrosine and alpha-methyldopa, respectively. Linear regression analysis of drug/internal standard peak height ratios versus concentration gave slope and intercept data for each analyte which were used to calculate the concentration of each analyte in the serum samples.

For absolute recovery experiments of each analyte, spiked samples were compared to unextracted stock solutions. Drug peak height ratios were used to calculate the recoveries.

RESULTS AND DISCUSSION

The chemical structures of metyrosine, alpha-methyldopa, and dopamine (internal standard I.S.) are shown in Figure 1. Metyrosine and alpha-methyldropa show native fluorescence at 370 nm with excitation at 282 nm. Attempts were made to separate metyrosine and alpha-methyldopa on a cyanopropyl column with mobile phases consisting of various portions of acetonitrile-methanol and water. None of these mobile phases were successful in our laboratory due to the lack of stability of the cyanopropyl column. However, an octadecylsilane column enabled the analytes to be separated within

Table 1

Accuracy and Precision Data for Serum Samples with Added Metyrosine (M) and Alpha-Methyldopa

Compound	Concn. Add (μg/mL)	Concn. Found (μg/mL)[a,b]	Error (%)	R.S.D. (%)
Intra-day				
M	8.0	7.99 ± 0.530	0.13	6.63
	32.0	32.59 ± 1.11	1.84	3.41
A	0.7	0.72 ± 0.047	2.85	6.35
	2.2	2.15 ± 0.056	2.27	2.61
Inter-day				
M	8.0	8.23 ± 0.544	2.87	6.57
	32.0	30.65 ± 0.770	4.22	2.51
A	0.07	0.68 ± 0.043	1.57	6.32
	2.2	2.17 ± 0.090	1.36	4.14

[a] Based on n=3 for intra-day assay.
[b] Based on n=9 for inter-day assay.

a reasonable chromatographic run time at a 1 mL/min flow rate. The final mobile phase composition for the analysis of metyrosine and alpha-methyldopa was 92.5:5:2.5 v/v/v 0.1 M aqueous phosphate buffer pH 3-acetonitrile-methanol containing EDTA and heptanesulfonate. Figure 2 shows representative chromatograms of a serum blank and serum spiked with the two analytes and dopamine internal standard.

An SPE procedure was developed for sample cleanup to decrease the sample preparation time normally seen in liquid-liquid extraction. Octadecylsilane and octylsilane SPE columns were initially investigated and they gave very low recoveries of the two analytes. There were several advantages to the use of a strong cation-exchange (SCX) column for serum cleanup. In addition to an almost interference-free chromatographic analysis, the extraction procedure can be adapted for batch processing by using a Vac-

Elut chamber, which allowed the processing of ten serum samples simultaneously in less than 15 min. Under the SPE process described in this paper, the SCX column can withstand two column volumes of water washes, so that the non-polar components are washed off the column without affecting the recoveries of metyrosine and alpha-methyldopa.

The calibration curves showed good linearity in the ranges 5-35 μg/mL and 0.2-2.5 μg/mL for metyrosine and alpha-methyldopa, respectively. The coefficients of determination were more than 0.9995 for metyrosine and alpha-methyldopa, respectively. Representative linear regression equations obtained for metyrosine and alpha-methyldopa were $y = 0.0546x + 0.02821$ and $y= 0.78683x + 0.00027$, respectively, where y and x are the D/IS peak-height ratios and concentration of each analyte, respectively. The intra-day precision (n=3) as expressed by percent RSD and percent error (accuracy) was 3.41-6.63% and 0.13 -1.84% for metyrosine and 2.61-6.53% and 2.27-2.85% for alpha-methyldopa. The inter-day precision and accuracy (n=9, over three days) were 2.51-6.57% and 2.87-4.22% for metyrosine and 4.14-6.32% and 1.36-1.57% for alpha-methyldopa, respectively. The detailed data is listed in Table 1.

In summary, a precise, accurate and rapid HPLC method using isocratic conditions and employing solid-phase extraction and fluorescence detection has been developed for the analysis of metyrosine and its major metabolite alpha-methyldopa. The method is sensitive to 1 μg/mL and 0.1 μg/mL for metyrosine and alpha-methyldopa, respectively (S/N = 2). The total chromatographic run time of the isocratic method was less than 11 min.

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DETERMINATION OF FLUOXETINE AND NORFLUOXETINE IN HUMAN PLASMA BY ION-INTERACTION RP-HPLC

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ABSTRACT

The paper reports a sensitive Ion-Interaction Reverse Phase HPLC-UV method for the separation and determination of fluoxetine ((±)N-methyl-(4-(trifluoromethyl)phenoxy) benzene-propanamine) in human plasma. An ODS silica-based column is used as the stationary phase and the mobile phase consists of 5.00 mM octylamine in water/acetonitrile (60/40 v/v), at pH = 6.4, with UV detection at 230 nm. The method is selective towards potentially interferent drugs, such as paroxetine, amylsulpride, fluvoxamine, mianserin, imipramine, amitryptiline, cromipramine, maprotiline, haloperidol, flunitrazepam and diazepam. The proposed method was successfully applied to the determination of fluoxetine and norfluoxetine in plasma of some patients being treated for obsessive-compulsive disorders.

INTRODUCTION

The use of selective serotonine-reuptake inhibitors (SSRI's) is widely prescribed in therapy for depression, obsessive-compulsive disorder, panic attack disorder, bulimia, social phobia, and post-traumatic stress disorder.[1-13] SSRI's are non-tricyclic antidepressants that enhance serotoninergic neurotrasmission process, through selective inhibition of neuronal reuptake of serotonine in presynaptic neurons. The chronic inhibition of serotonine reuptake leads to downregulation of serotonergic 5-HT1 presynaptic inhibitory autoreceptors and to increased serotonine release.[1] In particular, fluoxetine ((±)N-methyl-γ-(4-trifluoromethyl-phenoxy)benzene-propanamine), (prozac), has received widespread popularity in everyday clinical practice and is preferred with respect to classic tricyclic antidepressants, such as imipramine and amitryptiline.[2]

Fluoxetine, if administrated orally, exhibits few side effects in terms of frequence and severity. The major problem, as for other antidepressants, is its great inter-individual variability in clinical response which makes it difficult to value the correct posology. Fluoxetine posology ranges within 20 and 80 mg/day. The maximum concentration in plasma is generally attained after 4-8 hours, the elimination half-life varies between 1 and 9 days and steady-state plasma concentrations are achieved after 2-4 weeks.[1-4]

Metabolic processes in the liver convert some fluoxetine to norfluoxetine ((±)γ-(4-trifluoromethyl)phenoxybenzenepropanamine) which inhibits serotonine reuptake and significantly impacts to the overall clinical efficacy of fluoxetine. Norfluoxetine has a elimination half-life ranging between 3 and 10 days. Peak plasma concentrations are attained 3 days after the administration of the parent compound and steady-state plasma concentrations are reached after 4-5 weeks of fluoxetine administration.[1-2]

In order to determine whether the individual clinical response is correlated with the plasma concentrations, a sensitive and specific analytical method for determination of fluoxetine and norfluoxetine in plasma is required.

Some gas chromatographic[14-16] and HPLC methods (both normal-[7] and reversed phase modes[13,17-24] have been reported for determination of fluoxetine. Most methods employ derivatization reactions with dansyl chloride[21] and fluorescence detection[21,22] in order to have the desired sensitivity. A major problem[13,18,19,23] arises from interferences from other drugs prescribed as antidepressant (often in association with fluoxetine), which could be present in the plasma.

This paper reports an ion-interaction RP-HPLC method with UV detection (230 nm) which permits detection levels as low as 4.5 μg/L for fluoxetine and 2.3 μg/L for norfluoxetine in human plasma. The procedure does not require any derivatization reaction or pretreatment step. Moreover the method does not suffer any interference from the following drugs: paroxetine, amilsulpride, fluvoxamine, mianserin, imipramine, amitryptiline, cromipramine, maprotiline, haloperidol, flunitrazepam, and diazepam.

EXPERIMENTAL

Apparatus

The chromatographic analyses were carried out with a Merck-Hitachi (Tokyo, Japan) Lichrograph Chromatograph Model L-600, interfaced with a model L-4200 UV-Vis and a model L-3720 conductometric detectors.

A Metrohom 654 pH-meter equipped with a combined glass-calomel electrode was employed for pH measurements. A Hitachi (Tokyo, Japan) model 150-20 spectrophotometer was utilized for absorbance measurements.

A Minifuge T from Heraeus Sepatech (Hanau, Germany) centrifuge and a Minishaker (Duroni, Torino, Italy) rotator were used for sample preparation.

Chemicals and Reagents

Ultrapure water from a Millipore Milli-Q system (Millipore Corporation, Bedford, MA,USA) was used for the preparation of all the solutions. Octylamine and acetonitrile were Fluka (Buchs, Switzerland) analytical grade chemicals; NaOH and HCl were from Merck (Darmstadt, Germany); n-hexane and isoamylic alcohol from Farmitalia Carlo Erba (Milano, Italy).

(±)Fluoxetine hydrochloride and norfluoxetine maleate were kindly provided by Eli Lilly Co.(Indianapolis, In, USA). Fluoxetine hydrochloride is a white to off-white crystalline solid which is soluble at concentrations of 14 mg/mL in water, 250 mg/mL in methanol, and 125 mg/mL in chloroform. Less than 1% is soluble in benzene, ethyl acetate, and hexane. 10.0 mg/mL aqueous standard solutions of fluoxetine and norfluoxetine were prepared with the working aqueous solutions prepared by serial dilution.

Two sets of standard plasma respectively containing 0.0, 50.0, 100.0, 200.0, 400.0, 600.0, 800.0, and 1000.0 μg/L of fluoxetine and norfluoxetine were prepared by addition of the proper amount of standard solutions to 2.0 mL aliquots of drug-free plasma samples.[13]

Chromatographic Conditions

A 250 x 4.6 mm, 5 μm ODS-2 Phase Separations (Spherisorb) fully endcapped column with a carbon load of 12% (0.5 mmol/g), together with a 15.0 x 4.6 mm LiChrospher RP-18 5 μm guard pre-column were used. The mobile phase consisted of a mixture of 5.00 mM n-octylamine water solution / acetonitrile (60:40 v/v) brought to an "operational" pH value of 6.4±0.2 by the addition of o-phosphoric acid.[25,26] The chromatographic system was conditioned by passing the eluent through the column until a stable baseline signal was obtained; a minimum of 1 h at a flow-rate of 1.0 mL/min was necessary. After use, the stationary phase was washed by flowing water (1.0 mL/min for 15 min), a 50/50 (v/v) water/acetonitrile mixture (1.0 mL/min for 15 min), and acetonitrile (1.0 mL/min for 5 min) through it.[25,26] Dead times were evaluated by injection of $NaNO_3$ solutions (10 mg/L) and conductometric detection of the unretained Na^+ ion.

Sample Collection and Extraction Procedure

Blood samples (10 mL) were collected from patients diagnosed with Obsessive-Compulsive Disorder (OCD) according to DSM-III-R criteria,[17] who were undergoing fluoxetine therapy at daily doses ranging from 20 to 60 mg. Blood was collected in evacuated tubes containing lithium-eparine; the tubes were centrifuged (3000 g, 10 min., 4°C) and plasma transferred to polypropylene tubes (Eppendorf, Hamburg, Germany) and kept frozen (-80°C) until assayed.

The extraction procedure, a modification of a previously published report,[20] consisted of three steps: alkalinization, organic extraction, and back-extraction. 2.0 mL aliquots of plasma were transferred to polycarbonate screw-capped tubes (Falcon, Becton Dickinson Labware, Lincoln Park, NJ, USA) and 400 μL of 0.33 M NaOH solution were added. The samples were shaken for 5 sec and then extracted by rotating (MSA Clinomixer, Duroni, Torino, Italy) with 14 mL of n-hexane-isoamylic alcohol (985/15 V/V) for 20 min.

After centrifugation (2500 g, 5 min., 20°C) the organic phases were collected, adjusted to pH < 2.0 with 400 μL of HCl, shaken for 1 min, and centrifuged for 10 min (1500 g T=20°C). The organic phase was discarded and 100 mL of the aqueous phase was injected into the HPLC system. Each analysis was performed in triplicate. For routine analyses, only two injections were generally required with 1.0 mL of total blood samples necessary. When extracted according to the procedure described, the samples of plasma were stable for at least 24 h when stored at 4°C, and for at least 1 year when stored at -70°C.

RESULTS AND DISCUSSION

The different hydrophilicity of the aminic group present in the structures of fluoxetine and norfluoxetine (reported in Fig.1) suggests than ion-interaction HPLC method may prove useful for their separation.

This technique offers the advantage of good versatility since retention can be optimized as a function of many factors, as IIR (ion-interaction reagent) concentration, IIR alkyl chain lenght, acetonitrile percentage, pH. The effects of all these parameters have been systematically studied and reported in previous works (25,26).

Different compositions of the mobile phase were compared, ranging the percentage of acetonitrile between 30% and 50%, the octylamine concentration between 3.0 mM and 7.0 mM and the pH between 4 and 8. The optimized conditions resulted to be : 5.00 mM octylamine aqueous solution / acetonitrile (60/40 v/v) mixture, at pH = 6.4, that lead to a good resolution of the analytes within a reasonable total analysis time and minimize the interference of the matrix.

The proposed method was proved to be robust, being the resolution satisfactory with acetonitrile percentage ranging between 35% and 45%, octylamine concentration between 4.0 mM and 6.0 mM and pH between 6.0 and 7.0.

The separation obtained for a mixture of norfluoxetine and fluoxetine at concentration of 0.1 mg/L each is shown in Fig.1. The intra-day repeatibility was within 2% and the intra-day reproducibility for different mobile phase preparations was always within 5%. Detection limits (s/n = 3) were 4.5 μg/L and 2.3 mg/L, respectively for fluoxetine and norfluoxetine.

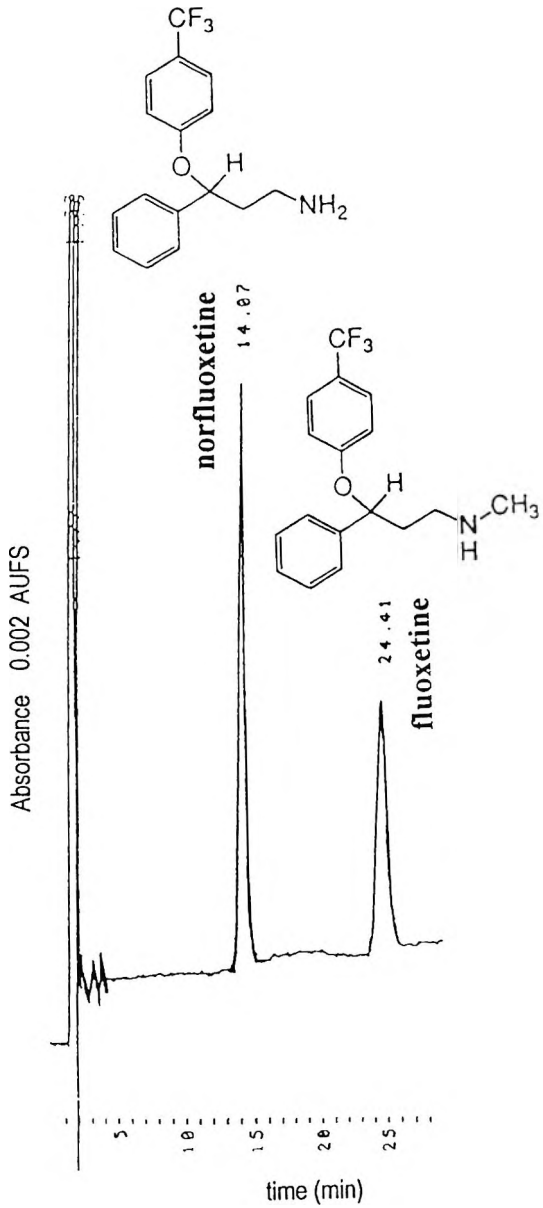

Figure 1. Separation of a mixture of norfluoxetine and fluoxetine (0.10 mg/L each). Stationary phase: Phase Separations ODS-2, (250 x 4.6 mm), endcapped, 5 μm. 100 μL injected. Mobile phase: 5.00 mM octylammonium o-phosphate in water-acetonitrile (60/40 v/v), pH 6.4. Flow-rate 1.50 mL/min. Spectrophotometric detection at 230.

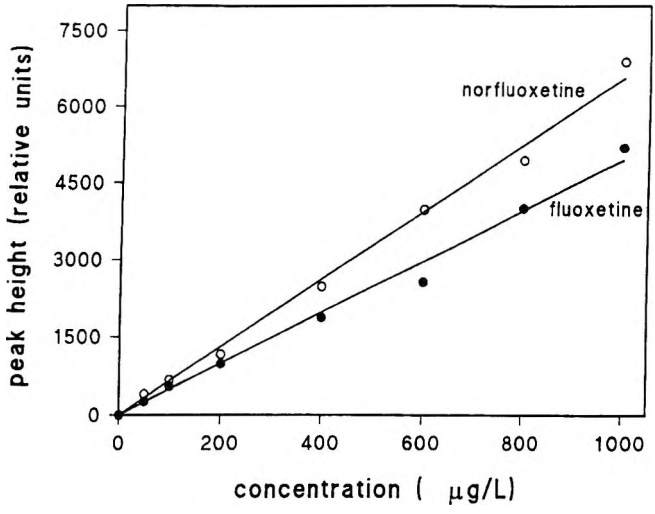

Figure 2. Peak height (relative units) vs. standard concentration in plasma for fluoxetine and norfluoxetine.

Calibration Plots and Recovery Data

Peak heights (relative units as given by the integrator) vs. standard concentration calibration plots for fluoxetine and norfluoxetine in drug-free plasma were constructed for concentrations ranging between 0.0 and 1000.0 µg/L. The plots (Fig.2), show very good linearity and were fitted by the following equations (*95%* confidence limits):

for fluoxetine: $y = 4.9518\ (\pm 0.1242)\ x$; $r^2 = 0.9906$

for norfluoxetine: $y = 6.5701\ (\pm 0.1288)\ x$; $r^2 = 0.9943$

where: y= peak height (relative units), x = the standard concentration (µg/L) and r^2 = correlation coefficient.

Some recovery tests were then performed on spiked plasma samples containing both analytes. Recoveries for both fluoxetine and norfluoxetine always greater than 92% were obtained.

Table 1

Capacity Factors k' (t_0 = 1.46) for Some Potentially Interfering Drugs

Drug	k'
Fluoxetine	14.89
Norfluoxetine	8.50
Paroxetine	13.80
Amylsulpride	1.60
Fluvoxamine	12.73
Mianserin	>70
Imipramine	58.67
Amitryptiline	>70
Clomipramine	>70
Maprotiline	20.86
Haloperidol	9.68
Flunitrazepam	3.95
Diazepam	6.94

Interference

In order to evaluate the matrix interference and recovery yield, experiments were performed for a fluoxetine-free plasma sample voluntarily offered by one of the authors. The sample was treated as described above and the chromatogram recorded under the optimized experimental conditions as in Fig.1, showed that the time-window corresponding to the retention of fluoxetine and norfluoxetine is interferent-free from plasma components.

In the therapy of obsessive-compulsive disorders, other drugs can be prescribed, both in co-diagnosis or in association with fluoxetine. To consider possible interferences in plasma analysis, under the optimized experimental conditions, the chromatographic retention of some common drugs, such as paroxetine, amysulpride, fluvoxamine, mianserin, imipramine, amitryptiline, clomipramine, maprotiline, haloperidol, flunitrazepam, and diazepam were determined (Table 1). The capacity factors indicate that the drugs can be separated well enough to guarantee very good resolution of the analytes of interest.

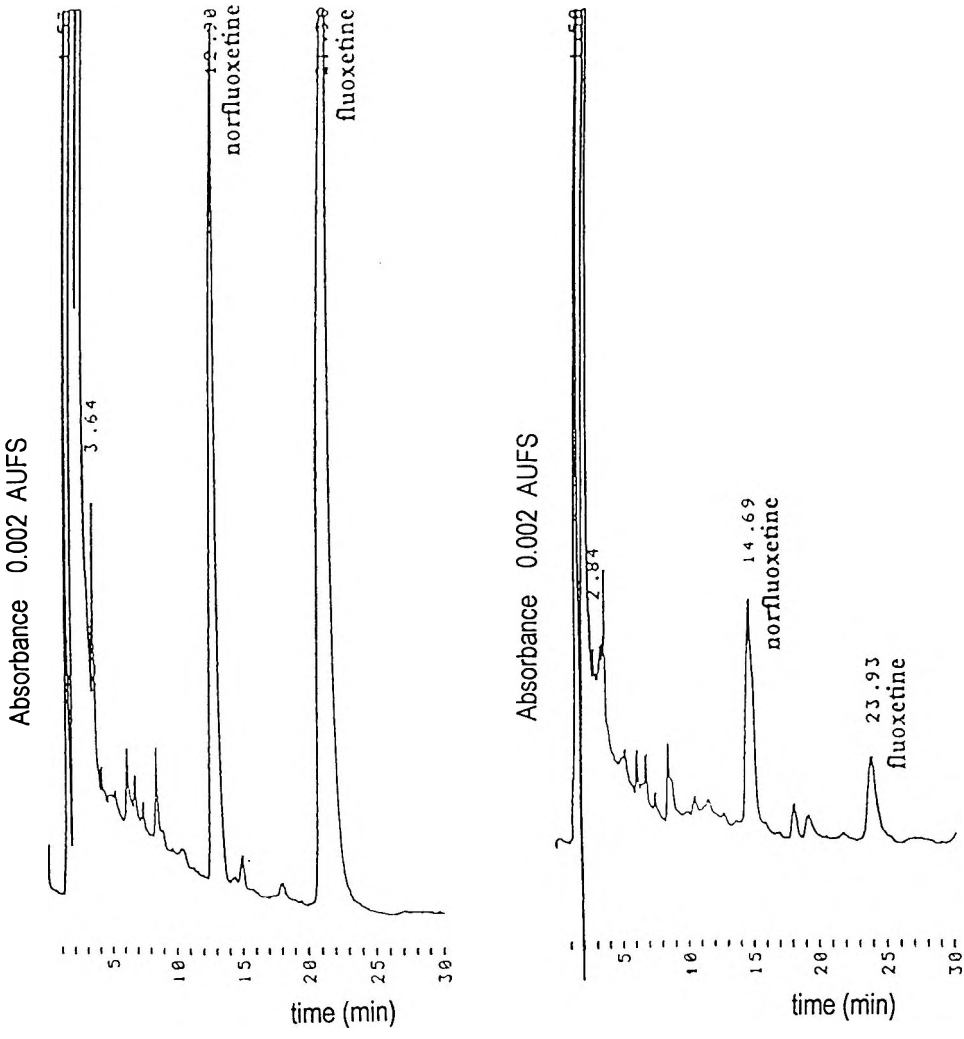

Figure 3. Examples of separation of norfluoxetine and fluoxetine in patient serum samples. Conditions as in Fig. 1.

Patient Plasma Analysis

In order to apply the method to real cases, samples of some patients plasma were analyzed. Typical chromatograms recorded for two patients

Table 2

Concentration of Fluoxetine and Norfluoxetine Found in Patient Plasma

Patient	Age (Years)	Sex	Daily Dosage (mg)	Duration of Therapy (Months)	Indication	FLX (μg/L)	NFLX (μg/L)	FLX/ NFLX Ratio
1	24	M	20	3	OCD	149±2	177±3	0.84
2	25	M	60	2	OCD	92±1	134±2	0.69
3	42	M	40	2	OCD	38±1	177±3	0.21
				3 later		30±1	153±2	0.19
4	24	F	40	4	OCD+GAD	620±3	318±4	1.95
5	25	F	40	3	OCD	87±1	155±3	0.56
6	45	F	20	1	OCD	321±3	98±1	3.27
				2 later		340±3	117±2	2.90

OCD = Obsessive Compulsive Disorder
GAD = Generalized Anxiety Disorder

(Fig.3) show that the plasma concentrations of fluoxetine and norfluoxetine are quite different despite the fact that the amount of time elapsed since drug administration were the same for both the patients. Furthermore, not only was the concentration of the two analytes different, but also the absolute values.

The fluoxetine/norfluoxetine ratio, appears to depend on the individual metabolism. The average results and standard deviations are reported in Table 2. As can be seen, the large variation of concentration was found in patient plasma, as well as different absolute concentration ratios.

Each analysis was repeated in triplicate and repeatibility was always within 2%. The quantitative determination was performed by means of the calibration curve. Some results were also obtained using the standard addition method. Such data confirmed the negligible matrix effects (correlation coefficients r^2 always > 0.99) and gave quantitative results which agreed within 5% with those obtained from the regular calibration plot method. As a conclusion, the IIR-HPLC technique proved to be suitable for the separation of fluoxetine and norfluoxetine, leading to satisfactory separation and detection limits lower than 5 μg/L. The analytical method described here has been transferred to laboratories of the hospital where studies are in progress.

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RETENTION IN RP-HPLC: LIPOPHILICITY DETERMINATION OF SUBSTITUTED BIPHENYLS BY REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The RP-HPLC capacity factors (k') of a series of substituted biphenyls were determined on a C_{18} column with methanol/water as the mobile phase. A linear relationship was found between log k' and the volume fraction of methanol (φ) for each of the 25 tested compounds with the correlation coefficient > 0.99. High correlation was found between log k'_w and S, which are intercept and slope of plot of log k' vs. φ, respectively. The values of S and log k'_w were quantitatively correlated with the solvatochromic parameters of the solutes and two statistically significant equations were established which showed that solute volume and hydrogen bond basicity were of major importance in influencing the retention of these studied compounds. The obtained log k'_w was chosen as a measure of the solute hydrophobicity and will be used for the on-going Quantitative Structure-Activity Relationship (QSAR) studies of the substituted biphenyls.

INTRODUCTION

The lipophilicity character of a chemical has long been shown to play a basic role in determining distribution phenomena, such as the adsorption on soil or sediment and the bioconcentration by aquatic organisms.[1,2] In addition, it influences the ecotoxicity of a chemical.[3] Lipophilicity is generally defined as the tendency of a chemical to distribute between an immiscible nonpolar solvent and water. The logarithm of the partition coefficient of a chemical in the n-octanol/water system (Kow), which is usually measured by "shake flask" method, is widely used because of its simplicity and some similarity between n-octanol and biological membranes. However, the conventional "shake flask" method has limited application range up to log Kow = 4, and it is time-consuming and requires considerable amounts of pure stable compounds.[4] It has been proven that the retention capacity factor (k') of a compound in a reversed-phase high performance liquid chromatography (RP-HPLC) system, is a reliable indirect descriptor of the lipophilicity of a compound.[5-11] Moreover, some studies have shown that log k'_w, the retention capacity factor which is extrapolated from a binary phase to 100% water in a RP-HPLC system, is an even better descriptor of lipophilicity than the isocratic factor because it is independent of any organic modifier effects and it reflects polar-non-polar partitioning in a manner similar to shake-flask measurements.[6,12-13]

The 4-alkyl or alkoxy-4'-cyanobiphenyls is an important group of liquid crystal materials.[14] With their more and more extensive uses, these compounds, along with their precursors, become environmentally and toxicologically important. However, there have been few investigations on their environmental behavior.[15,16] Our interest in lipophilicity resulted from a need to develop quantitative structure-activity relationships (QSARs) for their toxicities to *Daphnia magna* and Alga. These compounds are generally very non-polar; for example, by applying the fragmental method of Hansch and Leo,[17] the calculated log Kow for 4-n-butyl-4'-cyanobiphenyl is higher than 5. Direct measurement of their n-octanol/water partition coefficients by the conventional 'shake flask' method is difficult because of their highly lipophilic characteristics.

In this paper, first we are reporting a systematic study of the lipophilicity of these compounds by using a RP-HPLC method, and the measured data will be used in the on-going research of quantitative structure-activity relationships (QSARs) for the compounds. Second, based on the linear solvation energy relationships (LSERs), we are using solvatochromic parameters to study the major solute factors governing the retention of these studied compounds in RP-HPLC.

MATERIALS AND METHODS

Instruments

The HPLC system (Shimadzu, Japan) consisted of a SCL-8A system monitor, a LC-8A pump, a C-R4A integrator, and a SPD-6AV ultraviolet spectrophotometer as the detector. A C_{18} reversed-phase Nucleosil 7 (Dalian Institute of Chemical Physics, Academic Sinica) (15 x 4.6 mm i.d.) column was used.

Chemicals

The structures of the 25 substituted biphenyls are shown in Table 1. These compounds were supplied by the Yantai Valiant Fine Chemicals Co. Ltd., Yantai, P. R. China. The purity for each of the chemicals is greater than 99%. The water used as a mobile phase component was double-distilled. Methanol was analytical grade and redistilled before use. Sodium nitrate was also analytical grade.

Measurement of Log k'

The log k' values were determined for each compound. The mobile phases were made by mixing methanol with water in the proportions 100:0, 90:10, 85:15, 80:20, and 75:25 (v/v). The flow rate was 1.0 mL/min. All measurements were made in, at least, duplicate. The average reproducibility of each determination was better than 1.0% relative. The capacity factors (k') were determined using $k' = (t_r-t_0)/t_0$, where t_r is the retention time of the compound, and t_0 is the void volume or the dead time. An aqueous solution of sodium nitrate was used for the measurement of t_0.

The regression analysis was performed using the "Statgraphics" program (STSC, Inc., 1987).

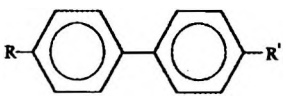
RESULTS AND DISCUSSIONS

The relationship between log k' and methanol concentration in mobile phase given by Snyder et al.[18] is :

$$\log k' = \log k'_w - S\,\varphi \qquad (1)$$

Table 1

The Structure of the Substituted Biphenyls



No.	R	R′
1	H	H
2	$CH_3C(O)$	H
3	CH_3CH_2	H
4	CH_3CH_2	CN
5	CH_3CH_2	I
6	$CH_3H_7C(O)$	H
7	C_4H_9	H
8	C_4H_9	CN
9	C_4H_9	I
10	$C_4H_9C(O)$	H
11	C_5H_{11}	H
12	C_5H_{11}	CN
13	C_5H_{11}	I
14	CH_3CH_2O	CN
15	$CH_3(CH_2)_2O$	CN
16	$CH_3(CH2)_3O$	CN
17	$CH_3(CH_2)_4O$	CN
18	CH_3CH_2O	I
19	$CH_3(CH_2)_2O$	I
20	$CH_3(CH_2)_3O$	I
21	$CH_3(CH_2)_4O$	I
22	OH	CN
23	OH	I
24	Br	H
25	Br	Br

where k'_w represents the k' value for a compound if pure water is used as eluent, S is the slope of the regression curve, and φ is the volume percentage of methanol in the mobile phase. For each studied compound, linear correlation was found between log k' and φ, and the correlation coefficients were all > 0.99. The values of S and the extrapolated log k'_w are given in Table 2.

Table 2

The Values of Solvatochromic Parameters for the Studied Compounds

No.	R	R′	S[a]	Log K′$_w$[a]	V_x/100[b]	π^*_2[b]	α_2[b]	β_2[b]
1	H	H	3.897	3.663	0.920	1.20	0.00	0.28
2	$CH_3C(O)$	H	3.178	2.887	1.116	1.55	0.06	0.67
3	CH_3CH_2	H	5.032	4.899	1.116	1.14	0.00	0.30
4	CH_3CH_2	CN	4.351	4.036	1.215	1.34	0.22	0.67
5	CH_3CH_2	I	6.162	6.168	1.297	1.36	0.10	0.32
6	$C_3H_7C(O)$	H	4.270	4.044	1.312	1.51	0.06	0.67
7	C_4H_9	H	5.487	5.462	1.312	1.10	0.00	0.30
8	C_4H_9	CN	4.798	4.711	1.411	1.30	0.22	0.67
9	C_4H_9	I	6.554	6.707	1.493	1.32	0.10	0.32
10	$C_4H_9C(O)$	H	4.972	4.765	1.410	1.49	0.06	0.67
11	C_5H_{11}	H	6.312	6.332	1.410	1.08	0.009	0.30
12	C_5H_{11}	CN	6.221	6.028	1.509	1.28	0.22	0.67
13	C_5H_{11}	I	7.185	7.393	1.591	1.30	0.10	0.32
14	CH_3CH_2O	CN	3.859	3.514	1.260	1.44	0.28	0.89
15	$CH_3(CH_2)_2O$	CN	4.542	4.227	1.358	1.42	0.28	0.89
16	$CH_3(CH_2)_3O$	CN	5.239	4.951	1.456	1.40	0.28	0.89
17	$CH_3(CH_2)_4O$	CN	5.803	5.571	1.554	1.38	0.28	0.89
18	CH_3CH_2O	I	5.570	5.525	1.342	1.42	0.16	0.54
19	$CH_3(CH_2)_2O$	I	6.227	6.223	1.440	1.40	0.16	0.54
20	$CH_3(CH_2)_3O$	I	6.515	6.577	1.538	1.38	0.16	0.54
21	$CH_3(CH_2)_4O$	I	6.839	6.907	1.636	1.36	0.16	0.54
22	OH	CN	2.467	1.885	1.064	1.53	0.82	0.88
23	OH	I	4.158	3.784	1.146	1.55	0.70	0.53
24	Br	H	4.729	4.576	1.051	1.35	0.10	0.24
25	Br	Br	5.431	5.344	1.182	0.70	0.20	0.20

[a] S and log k′$_w$ are the slope and intercept values of plot of log k′ vs. φ (the volume percentage of methanol in the mobile phase), respectively.
[b] Solvatochromic parameters of the solutes calculated according to references 23, 24.

The relationship between the slope S and the intercept values (log k'w) was investigated for the tested compounds, and a good linear correlation was observed:

$$S = (0.770 \pm 0.062) + (0.876 \pm 0.012) \log k'_w \qquad (2)$$

$$n = 25,\ S.D. = 0.078,\ r = 0.9979$$

In the above equation, and elsewhere, n is the number of the data points, while S.D. and r indicate the average standard deviation and the correlation coefficient of the fit, respectively. It is clear that for the studied compounds, S and log k'_w are highly correlated and this may be a reflection of the suitability of the methanol/water system for estimating the lipophilicy of the compounds.[6]

In order to investigate the main factors controlling the retention of these compounds in RP-HPLC, the methodology of linear solvation energy relationships (LSERs) in conjunction with solvatochromic parameters, was used to describe the retention in RP-HPLC.[19-22] According to this approach, variation in capacity factors with solute structure can be related to their potential for intermolecular interactions as follow:

$$\log k' = SP_0 + mV_x/100 + s\pi^*_2 + a\,\alpha_2 + b\,\beta_2 \qquad (3)$$

where V_x, π^*_2, α_2, and β_2 are parameters that describe the solute, and m, s, a, and b are the corresponding regression coefficients. SP0 is the intercept of the relationship defined by eqn. 3. The V_x term measures the intrinsic molar volume; π^*_2 is a measure of solute dipolarity/polarizability, α_2 is the hydrogen bond donor ability (HBD) or HBD acidity, and β_2 is the hydrogen bond acceptor ability (HBA) or HBA basicity. The values of solvatochromic parameters for the studied compounds were calculated according to the references[23,24] and are given in Table 2.

Many studies have shown that S is dependent not only on the HPLC system but rather on solute structure.[25-28] Therefore both log k'w and S can be described by the same solute parameters used to describe log k' and the general equations are as follows:[26]

$$\log k'_w = SP_w + m_wV_x/100 + s_w\pi^*_2 + a_w\alpha_2 + b_w\beta_2 \qquad (4)$$

$$S = SP_s + m_sV_x/100 + s_s\pi^*_2 + a_s\alpha_2 + b_s\beta 2 \qquad (5)$$

where the subscripts w and s designate fitting coefficients for log k'_w and S, respectively. By regression analysis, the LSER equations of S and log k'_w for the data listed in Table 2 are as shown in eqns. 6 and 7:

$$S = (-0.681\pm0.458) + (5.666\pm0.346)\,V_x/100 - (2.984\pm0.279)\,\beta_2 \quad (6)$$

$$n = 25,\ S.D. = 0.312,\ R = 0.9648$$

$$\log k'_w = (-1.40\pm0.459) + (6.380\pm0.347)\ V_x/100 - (3.606\pm0.280)\ \beta_2 \quad (7)$$

$$n = 25,\ S.D. = 0.313,\ R2 = 0.9728$$

In the above two equations, the π^*_2 and α_2 terms are not included because they are not significant at the 95% confidence level, and V_x and β_2 are the most important parameters in the equations. So, it is clear that for the studied compounds, solute size and hydrogen bond basicity are the two major retention governing factors in RPLC, and this is in accordance with other studies.[19-22,29] Eqn. 6 shows that increasing hydrogen bond basicity results in a decrease in S, whereas increasing the size of the solute leads to an increase in S.

Therefore, as the solute becomes increasingly hydrophobic, S will become increasingly positive; in contrast, as the solute becomes more hydrophilic and more polar, S will decrease when other conditions remain the same. Eqn. 7 shows that increasing solute size and/or decreasing hydrogen bond basicity will increase solute lipophilicity.

CONCLUSION

The retention capacity factors (log k') for the 25 substituted biphenyls were measured at five different compositions of the eluent, and the log k'_w was extrapolated from the linear relationship between log k' and the volume fraction of methanol in the mobile phase (φ) for each studied compound. The log k'_w may be an advantageous lipophilic parameter used in QSAR studies.

LSER equations were established for S and log k'_w, which are slope and intercept of plot of log k' vs. φ, respectively. The results show that solute size and hydrogen bond basicity are the two major factors influencing the retention of these studied compounds in RP-HPLC.

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DETERMINATION OF GLUTAMATE IN RAT BRAIN MICRODIALYSATES BY MICROBORE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

A highly sensitive microbore liquid-chromatographic method with electrochemical detection has been developed for the determination of glutamate in rat brain dialysates. Isocratic separation of glutamate was achieved using a microbore reversed phase C_{18} column and a 100 mM Na_2HPO_4-methanol (60:40, v/v, adjusted to pH 5.0 with orthophosphoric acid) mobile phase. A hydrodynamic voltamogram was generated (300-900 mV) from which an optimal working potential of +600 mV was established. The absolute detection limit of glutamate was 17 fmol (signal-to-noise ratio of three). This system offers the significant

advantages of small sample consumption and improved detection limits compared with conventional liquid chromatographic applications. These factors permit greater sampling frequency and better temporal resolution between *ex-vivo* brain neurochemistry and measures of behavioural performance.

INTRODUCTION

Glutamate is a fast-acting excitatory neurotransmitter in the central nervous system[1,2] with defined actions in modulating neuronal excitability, synaptogenesis[3,4] and cerebral ischemic injury.[5,6]

The *in-vivo* cerebral microdialysis technique is currently restricted by the relatively large sample volume required for analyte detection by conventional chromatographic methods. In order to correlate changes in *ex-vivo* brain neurochemistry with behavioural output over time, it is necessary to develop highly sensitive analytical techniques. Currently, microbore column high performance liquid chromatography (HPLC) has the significant advantages of very high sensitivity and relatively small sample volume consumption compared to that of conventional analytical column applications. Microbore column technology has been exploited in the analysis of both platelet[7] and plasma[8] serotonin and in the determination of acetylcholine and biogenic amines by on-line brain microdialysis.[9]

Glutamate is neither fluorescent nor electroactive but can be made so by pre-[10] and post-column[11] o-phthalaldehyde (OPA) derivatization. In the present study, we have developed a highly sensitive pre-column OPA derivatization assay employing microbore column liquid chromatography and electrochemical detection (ED) to determine glutamate content in rat brain microdialysates with a high sampling frequency.

MATERIALS AND METHODS

Chemicals and Reagents

Glutamate was purchased from Sigma (St. Louis, MO, USA). The glutamate stock solution (0.1 mg/mL) was prepared in double deionised water and kept at 4°C. o-phthalaldehyde (OPA) and ß-mercaptoethanol (ß-ME) were supplied by Fisher Scientific (Loughborough, UK).

Liquid Chromatography

The HPLC-ED (BAS, LC-4C, Bioanalytical System, West Lafayette, IN, USA) system consisted of a Rheodyne 9125 injector (5 μL loop), a solvent delivery system (BAS, PM-80) and a reversed phase C_{18} microbore column (BAS UniJet, 150 x 1 mm I.D., particle size 5 μm). A high-efficiency pulse damper was incorporated into the system to reduce background noise. The derivatized glutamate product was oxidised using a glassy carbon working electrode (+600 mV vs. Ag/AgCl) with a special thin-layer gasket for microbore LC-ED. In order to minimize dead volume, the microbore column was connected directly between the injector and the working electrode. The detector output current was monitored using an integrator (Chromatocorder Sic, Tokyo, Japan). Isocratic separation was achieved at room temperature (23°C) with a flow rate of 40 μL/min.

The mobile phase consisted of 100 mM Na_2HPO_4 and methanol (60:40 v/v) adjusted to an apparent pH of 5.0 with orthophosphoric acid. Water and methanol were HPLC grade (BDH, Poole, UK). Before use, the mobile phase was filtered through a 0.22 μm filter (Millipore) using vacuum assistance.

Derivatization

The derivatization reaction was carried out at room temperature. OPA (27 mg) was dissolved in 1 mL methanol, 9 mL 0.4 M potassium tetraborate (adjusted to pH 10.4 with 5 M NaOH) and 5 μL ß-ME. The OPA stock reagent was covered in foil and stored at 4°C for 1 week. The OPA working solution was prepared each day by diluting 1 mL of the OPA stock reagent with 3 mL of potassium tetraborate (0.4 M). The derivatising agent (6 μL) was reacted with an equal volume of dialysate or glutamate standard for 2 min before injection onto the analytical column.

Microdialysis Procedures

The experiments were carried out in adult, Lister hooded male rats (OLAC, Bicester, UK), weighing between 280 and 350 g. The rats were anaesthetized with urethane (1.2 g/kg, i.p.) and placed in a Kopf stereotaxic frame. A microdialysis probe (BAS, dialysing length 2 mm, diameter, 0.32 mm) was placed into the right nucleus accumbens with its tip located at the following coordinates, taken from the atlas of Paxinos and Watson:[12]: AP +1.7 mm from the bregma, ML -1.5 mm, DV -7.5 from dura. Body temperature was

maintained at 37 °C with a heating pad. Implanted probes were perfused at 1.0 μL/min with an artificial CSF composed of (mM): NaCl 147; KCl 3; $MgCl_2$ 1; $CaCl_2$ 1.3; NaH_2PO_4 0.20; Na_2HPO_4 1.30; pH 7.4. Brain dialysates were collected every 5 min into 2 μL perchloric acid (0.2 M) and immediately analysed for their glutamate content. The position of the probe was verified by standard histological procedures at the end of the experiment.

Recovery and Precision

The relative recovery of glutamate (Ri) in-vitro, defines the ratio of the dialysate concentration of a substance (C_{out}) to its concentration in the medium surrounding the probe (C_{in}).

$$Ri = C_{out} / C_{in}$$

To determine intra-assay variance, quadruplicate assays were carried out on the same sample concentration at different times during the day. Inter-assay variance was determined by the same sample concentration on days one, two, four, and six. Coefficients of variation (C.V.s) were calculated from these values.

RESULTS

Figure 1 shows representative chromatograms of the lowest concentration of glutamate detectable (17 fmol), a higher concentration (850 fmol) of a standard sample (Figure 1B), and a baseline dialysate sample collected from the nucleus accumbens 60 mins after probe implantation, which contains 587 fmol glutamate (Figure 1C). The retention time for glutamate was approximately 8.0 min. Samples could be injected every 15 min without significant interference from late eluting substances. The relative recovery of glutamate (flow rate of 1.0 μL/min) was 39.1 ± 1.4% (n=5). The hydrodynamic voltammogram (Figure 2) shows the detector response of glutamate as a function of the applied voltage. The current-voltage relationship was non-linear with a clearly defined plateau region between 500 and 700 mV.

The reproducibility of the method was defined by examining both intra- and inter-assay variabilities. The intra- and inter-assay variation for the determination of glutamate at concentrations of 85, 170, 850, and 1710 fmol were acceptable with C.V.s of less than 10% (Table 1).

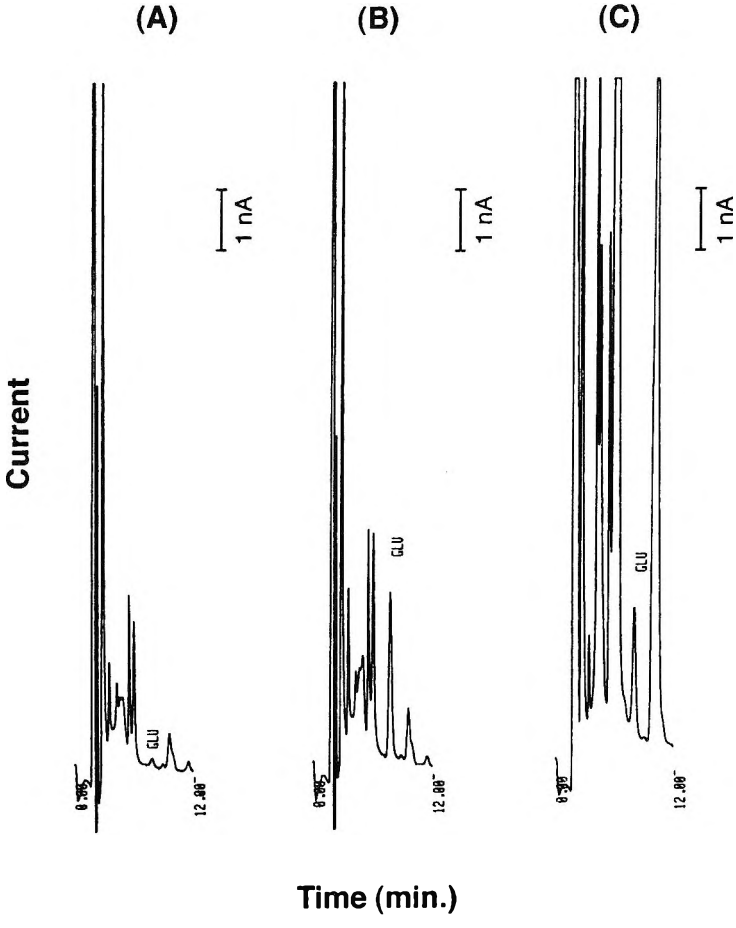

Figure 1. Chromatograms of (A) spiked glutamate (17 fmol) in artificial CSF, (B) spiked glutamate (850 fmol) in artificial CSF, and (C) a baseline dialysate sample collected from the nucleus accumbens 60 mins after probe implantation which contains 587 fmol glutamate.

To determine assay linearity, four different concentrations of glutamate (85-1710 fmol) were analysed. The peak-areas were linearly related to the concentration of glutamate and the equation for the regression line for glutamate was found to be $y = 0.57 x + 1.31$ ($r=0.999$). The slopes of the calibration graphs (n=4) were reproducible throughout the study: 0.55 ± 0.011 (mean ± S.D.) with a C.V. of 4.01 %.

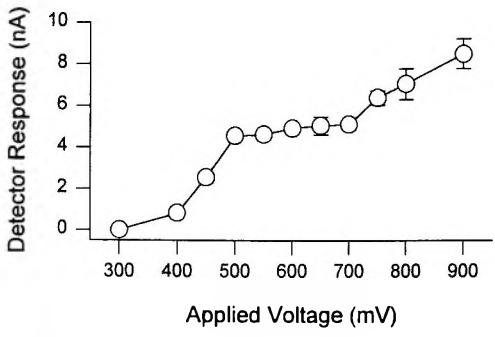

Figure 2. Hydrodynamic voltammogram of an OPA-derivatised glutamate standard (100 ng/mL). The detector response at each potential was recorded on 4 successive trials (± SEM).

Table 1

Intra- and Inter-Assay Precision and Relative Error in Glutamate Determination for Spiked Concentrations of 85, 170, 850 and 1710 fmol (n=4)

Spiked Concentrations (fmol)	Measured Concentrations (mean ± SEM)	C.V. (%)	Relative Error (%)
Intra-assay			
85	80 ± 4	4.9	-6.2
170	166 ± 14	8.2	-2.6
850	849 ± 71	8.4	-0.12
1710	1689 ± 59	3.5	-1.25
Inter-assay			
85	82 ± 4	4.8	-.32
170	172 ± 9	5.4	1.4
850	876 ± 40	4.6	3.1
1710	1708 ± 81	4.7	0.1

Precision [C.V. %] = [standard deviation (S.D.)/mean] × 100.
Relative Error (%) = [(spiked conc. - measured conc.)/spiked conc.] x 100.

The limit of detection, as defined by the lowest concentration of a standard that can be measured with acceptable precision (C.V. less than 20%), was 17 fmol (signal-to-noise ratio of three). The lowest practical limit of quantification was 51 fmol.

DISCUSSION

Microbore column liquid chromatography systems invariably exhibit lower detection limits compared with conventional HPLC-ED systems. This advantageous feature arises from (1) reduced peak broadening, (2) a smoother baseline due to reduced mobile phase consumption, and (3) higher coulometric yields due to more prolonged contact with the working electrode.

Additionally, microbore HPLC-ED systems require smaller sample volumes, making them ideal for high frequency sampling and improved temporal resolution in in-vivo brain dialysis.

In order to reduce baseline interference from the OPA-alkylthiol derivative during the electrochemical detection of excitatory, inhibitory, and other amino acids in rat brain microdialysates, the OPA-sulphite derivative has been used. In this setting, a significant improvement in interference was observed, however, the applied potential remained high at +700 mV[11] and +850 mV.[13]

In the present study, we have shown that, provided the applied potential is reduced to +600 mV, the OPA-alkylthiol derivative can be oxidised without significant baseline interference. Since no loss in detector response was encountered at this potential (Figure 2) the detection limit was lowered to just 17 fmol on column. This is a considerable improvement over a recent microbore fluorescent-based detection system which reported a detection limit for glutamate of only 67 fmol.[11]

In summary, a highly sensitive isocratic microbore HPLC-ED method has been developed for the determination of glutamate in rat brain microdialysates. This methodology offers improved detection limits and greater stability compared with conventional gradient elution LC-ED systems, and consequently will be of considerable utility for behavioural studies with well-defined events in time, such as learning.

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DETERMINATION OF PENTOXYVERINE IN COUGH PREPARATIONS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A simple liquid chromatographic method for quantification of pentoxyverine in cough preparations is described. The LCseparation of the drug from the complex matrices of the dosage formulations was undertaken on a reversed phase 100RP-18 Lichrosphere (5 μm) column, 15 cm × 4.6 mm i.d. The mobile phase used was methanol and 25% w/w ammonia in the ratio 99.2 : 0.8, v/v isocratically at a flow rate of 1.2 mL min^{-1} with U.V. detection at 258 nm. at ambient temperature. Good percentage assay and mean added recovery results were obtained with relative standard deviations (RSD) less than 2%.

INTRODUCTION

Pentoxyverine (carbetapentane), 1-phenylcyclopentane carboxylic acid 2-(2-diethylaminoethoxy)ethyl ester, is available as the citrate or HCl salt. It is a non-narcotic antitussive with selective action on cough center.[1-4] A number

of methods have been reported for the assay of pentoxyverine citrate in bulk form,[5-7] in tablet formulation[8-15] and in syrup form.[16] The cited methods, which are all in Chinese, included flourimetric,[5] potentiometric,[6] titrimetric with different titrants,[7-12] colorimetric,[13] first-derivative U.V.-spectrophotometric,[14] a neutralisation extraction technique,[15] and a derivative spectrophotometry-damping factor matrix method.[16]

As there is no method reported for the determination of pentoxyverine in cough preparations containing it, it was deemed useful to develop an HPLC method for the routine and accurate determination of the drug in presence of various excipients, sugar bases and other combinations encountered in such preparations. The developed HPLC method was successfully applied to two cough preparations marketed in Saudi Arabia.

EXPERIMENTAL

Waters liquid chromatograph 600E, equipped with Waters-U6K Millipore Injector, Waters-486 tunable absorbance detector, and Waters-746 data module was used. The column used was stainless steel, 15 cm × 4.6 mm i.d., packed with 5 μm Lichrosphere 100RP-18 bonded material. The mobile phase was composed of methanol (HiperSolv™ BDH Chemicals Ltd., Pool-UK) and 25% w/w ammonia (Analar BDH) in the ratio 99.2 : 0.8, v/v, pumped isocratically at a flow rate of 1.2 mL min^{-1}.

Degassing of the mobile phase was carried out by purging pure helium into the solvent reservoir at a rate of 10 mL min^{-1}, U.V. setting was at 258 nm and twenty microlitre volumes were injected into the column at room temperature.

Materials and Reagents

Reference pentoxyverine citrate was kindly provided by the central laboratory in Riyadh and it was used, as received, without further treatment. Toclase® syrup (BN 94G29 MFD 07/94) containing 7.5 mg pentoxyverine hydrochloride in each 5 mL and Toclase® + expectorant oral solution (BN 94F09 MFD 06/94) containing pentoxyverine citrate 10.65 mg, terpine hydrate 15 mg, and sodium citrate 65 mg in each 5 mL, were collected from local pharmacies in Riyadh. Chlorpromazine hydrochloride (Winlab) was used as internal standard.

Standard Solutions and Samples Preparation

Reference pentoxyverine citrate stock solution

0.2000 % w/v in methanol.

Internal standard

Chlorpromazine hydrochloride stock solution 0.020% w/v in methanol was diluted with methanol to give 10 $\mu g\ mL^{-1}$ solution.

Standard series

1-8 mLs of the reference drug stock solution were transferred into 25 mL volumetric flasks; to each flask was added 4.0 mLs of internal standard solution (10 $\mu g\ mL^{-1}$) and volume completed with methanol.

Triplicate injections of each dilution were made and regression analysis of concentration *vs* peak ratio of drug against internal standard was obtained.

The slope consistency of the prepared standard was checked at different days; within day (n = 5) and between day runs (n = 15) for three different concentrations at low, medium, and high levels of the standard curve were done and relative standard deviations were calculated to check for the reproducibility and precision of the method.

Sample preparation

2 mL, 3 mL, and 4 mL of each preparation were transferred into 25 mL volumetric flasks and 4.0 mL of internal standard was added; volume completed with methanol and 3 to 5 runs were done for each sample solution. Calculations for concentration were either computed from regression analysis data or from a direct comparison of sample to an equivalent standard solution.

When using the direct comparison method the following formula can be adopted for calculation:

$$\text{C mgs / 5 ml as citrate} = \frac{A}{B} \times C\ (mg) \times \frac{5}{V}$$

$$\text{or as HCl} = \frac{A}{B} \times C\ (mg) \times \frac{5}{V} \times 0.70377$$

where A = sample peak ratio, B = standard peak ratio, C = mgs of standard in the volume of standard used and V = volume of cough preparation taken.

Recovery Experiment

To 2 mL of either sample was added 2 mL of standard solution (0.200 %, w/v) and 4.0 mL of internal standard (10 μg mL^{-1}) and volume completed to 25 mL with methanol. Triplicate injections were made for each solution. On the other hand, separate 2 mL volumes of each sample and separate 2 mL standard solution (0.200% w/v) were transferred into 25 mL volumetric flasks and to each flask was added 4.0 mL of internal standard (10 μg mL^{-1}), volume completed with methanol and triplicate injections were made for each solution; recovery was calculated as follows:

$$\frac{P_{(ad)} - P_{(sp)}}{P_{(st)}} \times 100$$

where $P_{(ad)}$ = peak ratio for added solution, $P_{(sp)}$ peak ratio for sample solution and $P_{(st)}$ peak ratio for standard solution.

RESULTS AND DISCUSSION

Different mobile phases and columns were tried for the separation and quantification of pentoxyverine in Toclase® syrup and Toclase® + expectorant oral solution. Mobile phases containing water were found to cause delayed and badly tailing peaks for the drug with the columns tried. No elution occurred when using acetonitrile alone or in combination. Methanol, on the other hand, was promising and needed ammonia to improve peak shape and retention time. This was found satisfactory at a ratio of 99.2 : 0.8, v/v methanol : ammonia; where the capacity factor and tailing factor of the eluting peak were compared for the different columns used. With phenyl column capacity factor (K_1) was about 0.6 (fast). For C18 Bondapak K_1 value was about 2 but with tailing factor exceeding 2.0. Lichrosphere 100RP-18 (5 μm) 12.5 cm × 4.0 mm, i.d. and Lichrosphere 100RP-18 (5 μm) 15 cm × 4.6 i.d. were the best to use with K_1 values of about 1.35 and 1.95, respectively at a flow rate of 1.2 mL min^{-1}; however, for the syrup and oral solution only Lichrosphere 15 cm × 4.6 mm, i.d. resolved the drug from the sugar bases and other excipients encountered in

Table 1

Comparative Study of Chromatograms of Solutions in Water and in Methanol*

Solvent	Solvent Peak	Tailing Factor	Capacity Factor (K_1)	Resolution Between Drug and Internal Standard
Water	Negative	Drug ≈ 1.90 Internal Standard ≈ 1.5	Drug 2.06 Internal Standard ≈ 2.91	2.15
Methanol	-----	Drug ≈ 1.6 Internal Standard ≈ 1.5	Drug ≈ 1.95 Internal Standard ≈ 2.78	2.33

* Refer to Figure 1 (a,b).

these preparations. The lowest tailing factor obtained was about 1.6 which is considered within acceptable practically found values (≤ 1.6).[17] Internal standard was used, so as to minimize possible errors.[17] Several drugs were tested for selection of a suitable internal standard and testing possible interference as indicative for specificity. Chlorpromazine hydrochloride (K_1 = 2.78) was found to be a suitable internal standard for this study. Also during the first trials to obtain optimal conditions for this study, solutions of reference drug, internal standard and samples were prepared and diluted with water as a cheap solvent, before injecting onto the column. The obtained chromatograms were compared with those obtained for similar solutions prepared in methanol.

Table 1 summarizes the comparative study which indicated better conditions when methanol was used throughout the assay. Also, a peak response of about 25% increase in height was obtained with methanol solutions Fig. 1 (a, b). The slight difference in resolution (2.33 and 2.15) was significant since it gave more reproducible peak-ratios. Sugar in Toclase® syrup tend to crystallize slowly from methanol solutions on standing for about three hours or on vigorous shaking of the solution without any effect on the single peak areas of the drug or internal standard and hence on the peak ratio. Fig. 2 (a, b) shows typical chromatograms for both preparations.

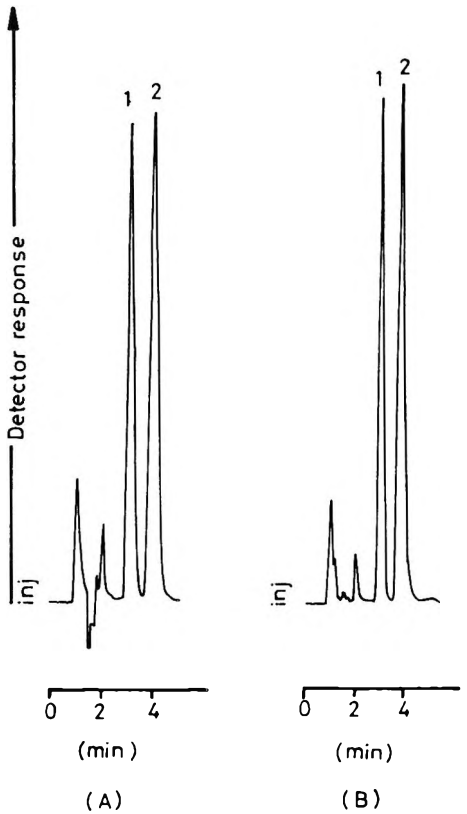

Figure 1. a) Typical chromatogram of injected aqueous solutions of pentoxyverine citrate (1) (0.04% w/v) and chlorpromazine HCl (internal standard) (2) (2×10^{-4}% w/v). b) Typical chromatogram of injected methanolic solutions of pentoxyverine citrate (1) (0.032%, w/v) and chlorpromazine HCl (internal standard) (2) (1.6×10^{-4} %, w/v).

Good system suitability and column efficiency were reflected by the acceptable data obtained for capacity factor (K_1), resolution, tailing factor, number of theoretical plates, height equivalent to theoretical plate (HETP)[17,18] and the relative standard deviation as in Table 2.

Pentoxyverine weekly absorbs in U.V. region as it contains only a phenyl group as the effective chromofore with typical benzenoid absorption at 262 nm, 258 (λ_{max}) and 254 nm. The calculated molar absorptivity has a log $\epsilon$of 2.27 in

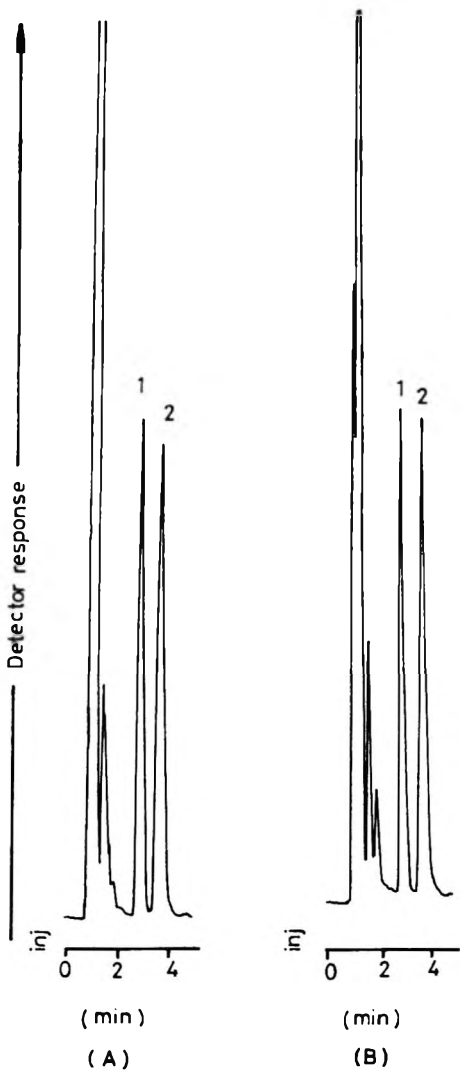

Figure 2. a) Typical chromatogram of resolved pentoxyverine HCl (1) (0.03408 %, w/v, citrate) and chlorpromazine HCl (internal standard) (2) (1.6×10^{-4}, w/v) from Tolcase® syrup. b) Typical chromatogram of resolved pentoxyverine citrate (1) (0.03408 %, w/v) and chlorpromazine HCl (internal standard) (2) (1.6×10^{-4} %, w/v) from Tolcase® + expectorant oral solution.

Table 2

Parameters for Resolved Pentoxyverine Citrate and Chlorpormazine Hydrochloride (Internal Standard)

Parameter	Pentoxyverine Citrate	Chlorpromazine Hydrochloride
Detection wavelength (λ_{max} nm)	258	
Flow rate (mL min^{-1})	1.2	
Capacity factor (K')	1.95	2.78
Resolution (Rs)	2.33	
Tailing factor	≈ 1.65	≈ 1.50
Number of theoretical plates N/m	20408	
HETP mm	0.049	
Relative standard deviation (RSD%)	< 2.0	
Analysis time	4.5 min	

methanol and 2.24 in water at 258 nm. With this low absorptivity pentoxyverine is considered a good candidate for the highly sensitive HPLC U.V. detectors; the absorption was monitored at 258 nm (λ_{max}) so as to get maximum sensitivity.[17]

Regression analysis of the calibration curve (conc. *vs* peak-ratio) indicated a linear relationship between peak-ratio (Y) and conc. ($\mu g\ mL^{-1}$) for pentoxyverine citrate (range 80 $\mu g\ mL^{-1}$ to 640 $\mu g\ mL^{-1}$).

$$Y = 1.2 \times 10^{-3} + 2.2775 \times 10^{-3}\ C$$

with a correlation coefficient, r = 0.9999. The detection limit was 8 $\mu g\ mL^{-1}$ at a signal to noise ratio of 3:1.

The reproducibility and precision of the method was assessed by the follow up of within day, between day data for low, medium, and high concentrations within the standard curve, and also by the follow up of the slope consistency for data of standard curve injected for four days. RSDs in all three cases were less than 2% indicating good reproducibility and precision of the method (Table 3). Table 4 collects the obtained results of assay of pentoxyverine in Toclase® syrup and Toclase® expectorant oral solution, as well as the recovery testing of added amount of pentoxyverine citrate to the two preparations. The obtained results reflected excellent precision and accuracy.

Table 3

Reproducibility and Precision as Evaluated by RSD% for Within-Day, Between-Day and Slope Consistency

Concentration ($\mu g\ mL^{-1}$)	Within-day RSD %	Between-day (n=15) RSD %	Slope Consistency $\bar{x}$ B ± SD (n=20) RSD %
Low (80)	1.38	1.5	$2.2798 \times 10^{-3} \pm$
Medium (400)	1.14	1.00	1.8807×10^{-5}
High (640)	0.92	0.64	0.82

n: Number of independent determinations.
RSD: Relative standard deviation.
$\bar{x}$ B: Average peak ratio per unit concentration ($\mu g\ mL^{-1}$).

Table 4

Assay and Added Recovery of Pentoxyverine in Pharmaceutical Formulations

Preparation	Volume Taken (mLs)	Claimed Content/ Volume Taken (mg)	n	Found Average for (n) Expts	Assay (%)	Added Recovery X± SD CV (n)
Tolcase®	2	3.0	3	3.01	100.70	
syrup	3	4.5	5	4.508	100.18	
(pentoxyverine	4	6	5	6.146	102.44	100.82 ± 1.25
HCl)						1.24 (3)
		Pooled Assay Result (n = 13) X ± SD 101.17 ± 1.39				
Tolcase® +	2	4.26	3	4.1995	98.58	
expectorant	3	6.39	5	6.296	98.53	
(pentoxyverine	4	8.52	5	8.36	98.18	100.43 ± 0.78
HCl)						0.78 (3)
		Pooled Assay Result (n = 13) X ± SD 98.38 ± 0.83				

X = Arithmetic mean. SD = Standard deviation. CV = Coefficient of variation. n = no of independent determinations.

Pentoxyverine is marketed as the hydrochloride salt in Toclase® syrup and as the citrate in Toclase® + expectorant oral solution. In this study, both were determined in terms of the citrate using reference pentoxyverine citrate and an adopted formula given for the calculation of content per 5 mL preparation either as the citrate or as the hydrochloride (see Experimental).

Although the method was applied to Toclase® syrup and Tolcase® + expectorant oral solution only, it is expected to be applicable for Tolcase® tablets which were not available in Riyadh local pharmacies.

It's worth noting that all cited methods in the literature were for the drug in bulk form[5-7] and in tablet formulation[8-15] and only one method was for a syrup form with combination different from those of the formulations[16] studied. Also, all the cited methods were in Chinese, with the exception of the potentiometric method,[6] used for the determination of the drug in bulk form.

CONCLUSION

The HPLC method described in this study is the only method for the separation and quantification of pentoxyverine in the formulations of cough preparation studied. The sensitivity, reproducibility, simplicity, and short analysis time (4.5 min) of the method makes it valuable in the routine analysis of pentoxyverine in its available formulations.

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THE VALIDATION OF A NEW STABILITY-INDICATING HPLC TECHNIQUE DEVELOPED FOR THE QUANTITATIVE ANALYSIS OF ROOPEROL TETRA-ACETATE

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ABSTRACT

A high performance liquid chromatographic method was developed for the quantitative analysis of rooperol tetra-acetate in the aqueous receptor phase of *in vitro* diffusion cells. System validation of the developed HPLC technique was carried out through the determination of the precision, limit of quantitation, sensitivity, linearity, and range of the system. In each of the above tests mentioned, an isocratic mobile phase of acetonitrile/water facilitated the elution of rooperol tetra-acetate dissolved in acetonitrile. Experimental variations were controlled through the use of the internal standard, prazepam. The precision results, expressed as the percent relative standard deviation, ranged from between 1.35% to 4.23%.

The results obtained in all other tests performed were acceptable thus verifying that the analytical method used is of good design. Further investigations on rooperol tetra-acetate involved performing stability tests, thus chromatographically characterizing the degradation process of the drug. Heat degradation of rooperol tetra-acetate, when stored in a glass vial and a plastic holder, was assessed over a twelve week period. Data evaluation involved the application of the t-distribution test, used to determine if a significant difference, at a 95% confidence level, exists between the drugs stored under heat and a sample stored at room temperature.

INTRODUCTION

Various clinical studies on the aglycone of hypoxoside, namely rooperol, have successfully demonstrated the application of the agent as a cytotoxic substance.[1,2,3] The hypoxoside, which has shown potential in the treatment of solar keratoses, is first extracted from the corms of *Hypoxis rooperi* and *H. latifolia*[4] before it is deconjugtated to rooperol using beta glucosidase. Rooperol, which is susceptible to oxidation, was stabilised by acetylating the compound to give rooperol tetra-acetate. The mechanism by which the drug effects its cytotoxic activity is believed to be through the disruption of chromosomal structures during the mitotic stage of cell development.[2] Problems associated with the delivery of clinical amounts of rooperol tetra-acetate into skin from a topical vehicle, necessitated the development of a quantitative assessment technique for the drug in diffusion assessment procedures. In order to achieve the release of rooperol tetra-acetate into the skin from a topical base, an effective drug delivery system needs to be developed. Before such a delivery system can be designed, a suitable quantitative assay method for the analysis of rooperol tetra-acetate is essential. Validation of the developed HPLC method involved performing precision, limit of quantitation, and range assays of the active drug combined with the internal standard and the assessment of replicate injections of this standard solution into the HPLC system. This method is useful in confirming the reliability and reproducibility of analytical applications selected for the study of rooperol tetra-acetate. Limited HPLC work has been previously performed on rooperol tetra-acetate and these studies concentrated on chromatographic analysis of the biotransformation of hypoxoside and rooperol analogues in man through the use of an in-line sorption enrichment HPLC technique. As system validation is an integral part of liquid chromatographic methods it was necessary to, firstly, design a suitable validated analytical method for the HPLC separation of rooperol tetra-acetate and, secondly, to establish the stability

profile for the drug in the analytical system.[5] Investigations on the stability of rooperol tetra-acetate involved subjecting the drug to elevated temperatures. The United States Pharmacopoeia guidelines for stability testing were used to determine the conditions under which accelerated tests were to be conducted.[6] The aim of this study is to describe the analytical technique used for validating the quantitative analysis of rooperol tetra-acetate and to generate a stability profile for the drug.[7]

MATERIALS

Reagents and Chemicals

A mobile phase of spectral grade acetonitrile obtained from Burdick and Jackson, USA and HPLC grade water purified through a Milli-Q system (Millipore, USA) was filtered through a 0.45μm membrane filter (type BD, Millipore, USA) and simultaneously degassed under vacuum at ambient temperature. Rooperol tetra-acetate was obtained from the Department of Pharmacology at Stellenbosch University, South Africa, and the prazepam was obtained from Parke-Davis (South Africa).

Apparatus

A solvent pump, (Model SP8810, Spectra-Physics, California, USA) was connected to a manual injection valve (Model 7126, Rheodyne, California, USA) equipped with a 20μL injection loop. A 10μm C_{18} analytical column which was custom packed with octadecylsilane was used. The variable U.V. detector (Linear 200 model, Spectra-Physics, California, USA) which was set at a wavelength of 260nm and sensitivity of 0.002 A.U.F.S. was connected to a datajet integrator (Model SP4600, Spectra-Physics, California, USA). A hot box oven (Model 4B-5599-E, Gallenkamp, England) was used to carry out the accelerated stability tests at elevated temperatures.

HPLC Conditions

The mobile phase was prepared by mixing 70 parts acetonitrile with 30 parts water in a stoppered flask. The mixture was equilibrated to room temperature before being filtered and degassed. The flow rate was maintained at 1.5mL/min and all operations were carried out at ambient laboratory temperature.

METHODS

Precision Studies

At the beginning of each of the three precision runs performed, three individual samples of 0.01g rooperol tetra-acetate were weighed out and each sample was dissolved in acetonitrile in a 10mL volumetric flask. Each rooperol tetra-acetate sample was then diluted to a concentration of 0.1μg/mL in acetonitrile. The internal standard solution, which was diluted to a concentration of 1μg/mL in acetonitrile, was prepared by dissolving 0.025g prazepam in acetonitrile in a 25mL volumetric flask. A 2mL sample of the internal standard was added to a 2mL sample of rooperol tetra-acetate and 10μL of this final standard solution was injected into the HPLC system. A total of ten replicate injections, of each of the three standard solution samples prepared, were made on each of three occasions and the data obtained was used to calculate the percent relative standard deviation.

Limit of Quantitation Studies

A 1000μg/mL solution of rooperol tetra-acetate dissolved in acetonitrile was prepared. Using the 1000μg/mL stock solution, six dilutions ranging in concentration from 0.501μg/mL to 1.002μg/mL were made. A 2mL aliquot of each dilution solution was mixed with 2mL of internal standard solution. A total of six 10μL replicate injections of the standard solution were introduced into the HPLC system. From the data obtained, the ratio of areas was determined and was used to calculate the mean and percent relative standard deviation. Data from the limit of quantitation studies was also used to plot a calibration curve for rooperol tetra-acetate and to determine the sensitivity and linearity.

Range Studies

Range studies were performed by preparing six samples of rooperol tetra-acetate in acetonitrile, the concentration of which covered the extremes of the range concerned. The masses of rooperol tetra-acetate weighed ranged from 14mg to 30.6mg. Suitable dilutions of each solution were made and a 2mL aliquot of the diluted solution was mixed with 2mL internal standard. Up to six replicate injections measuring 10μL each of standard solution were introduced into the HPLC system. The ratio of the areas was calculated from the data

obtained. The corresponding drug concentrations were calculated by solving the regression equation for concentration and substituting the peak areas ratio for the sample.

Stability Studies

A 1g sample of rooperol tetra-acetate was measured into a teflon plastic holder and the same amount was measured into a glass vial. Both containers were placed in the oven set at 40°C. At weekly intervals, a 0.1μg/mL solution of rooperol tetra-acetate in acetonitrile was prepared from each of the two samples stored in the oven. A 2mL measure of the rooperol tetra-acetate solutions was mixed with 2mL internal standard and six replicate injections of the mixture, each measuring 10μL, were introduced into the HPLC system. For the purpose of comparison, a 0.1μg/mL solution of rooperol tetra-acetate in acetonitrile was freshly prepared from a sample maintained at room temperature. Experimental manipulation of the fresh sample was identical to that of the samples stored at higher temperatures.

The retention times and areas of each peak produced were recorded. Statistical evaluation of the results collected over twelve weeks made it possible to assess the storage stability of rooperol tetra-acetate.

RESULTS AND DISCUSSION

Precision Studies

Precision studies are concerned with the repeatability and reproducibility of a given method.[8] Most reported chromatographic methods have percent relative standard deviations of between 5% and 10% . However, the commonly accepted limit for inter-day RSD values for a given HPLC assay is less than 5%.

The percent relative standard deviation for the nine lots of injections had an overall average of 2.4% for all ninety injections. The percent relative standard deviation values for day one were 2.71%, 4.23%, and 1.42%; day two, 2.25%, 3.36%, and 1.45% and day three, 1.89%, 2.10%, and 2.33%. It is evident from the results that the assay method is sufficiently precise for quantitative analysis.

Limit of Quantitation Studies

Limit of quantitation studies are used to quantify the lowest concentration of drug that may be quantitatively assessed in a given sample.[9] Results for this test were determined by calculating the percent relative standard deviation of the instrument response for progressively lower concentrations of rooperol tetra-acetate. The lowest concentration quantified from the ratio of the areas was 0.0501μg/mL. However, at these low concentrations the percent RSD value was as high as 15.2%, thus indicating that the reliability of the system at this level is significantly reduced. The limit of quantitation was calculated to be 50.1ng.

Sensitivity Studies

Sensitivity studies are dependent upon the degree of response, by the system, to concentrations of analyte covering the entire range of analysis.[9] The slope of the analytical calibration curve provides a measure of the sensitivity of the system. In the analysis of rooperol tetra-acetate, the slope and therefore, the sensitivity of the system was calculated as being 0.5727 (Figure 1). In addition, the width of the vertical bars of the calibration curve are an indication of the accuracy with which the system is able to respond to decreasing concentrations of analyte. The presence of narrow vertical bars indicates that the sensitivity of the system is satisfactory.

Linearity Studies

When examining the calibration curve, it is evident that the ratio of areas is proportional to the concentration of rooperol tetra-acetate present in the samples analysed. The correlation coefficient of the slope of the regression line which gives a measure of linearity was calculated to be 0.99765 (Figure 1). A variance value of one indicates a linear slope and, therefore the value obtained experimentally, certifies that the system is acceptably linear.

Range Studies

The results obtained during this test clearly indicate that both the system and method employed are reliable. Acceptable precision was obtained when the method used was applied to samples containing concentrations of analyte which encompass the entire range of analysis.[5] The percent deviations of the masses weighed range from -0.09% to +2.6% (Table 1).

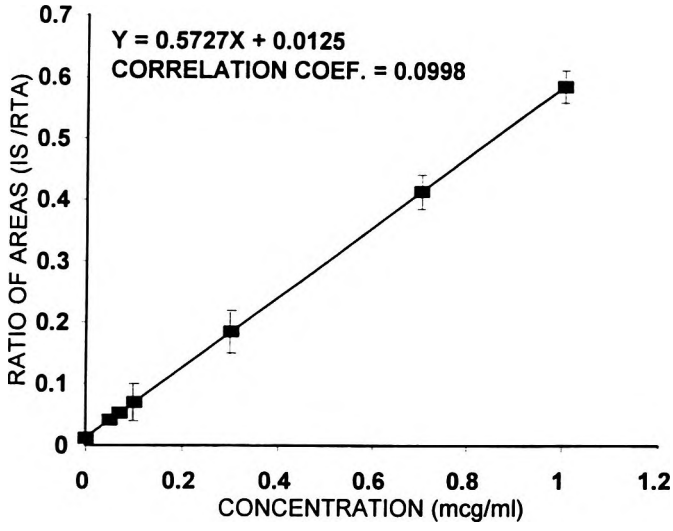


Figure 1. Calibration Curve for Rooperol Tetra-Acetate.

Table 1

Range Data

Mass RTA Weighed (g)	Mass RTA Calculated (g)	Percent Standard Deviation
0.00014	0.000143	-2.1
0.00177	0.001798	-1.6
0.002276	0.002246	-0.037
0.00042	0.000409	+2.6
0.02209	0.021587	+2.3
0.03057	0.030596	-0.09

Stability Studies

The overall stability test results indicate that there was significant rooperol tetra-acetate degradation of both drug samples prepared following exposure to high temperatures during the three month period of analysis.

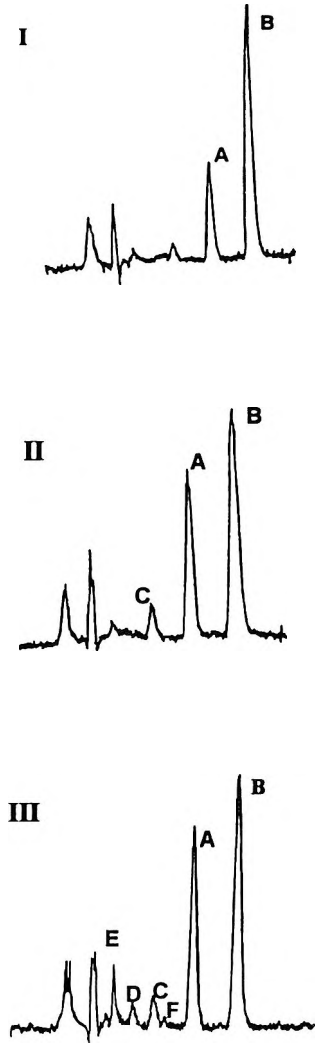


Figure 2. Typical Chromatograms of Rooperol Tetra-Acetate. (A) represents rooperol tetra-acetate; (B) represents the internal standard, prazepam; (C), (D) (E) and (F) are all degradation peaks. Chromatogram (I) was taken after two weeks of analysis, (II) and (III) after four and ten weeks respectivly.

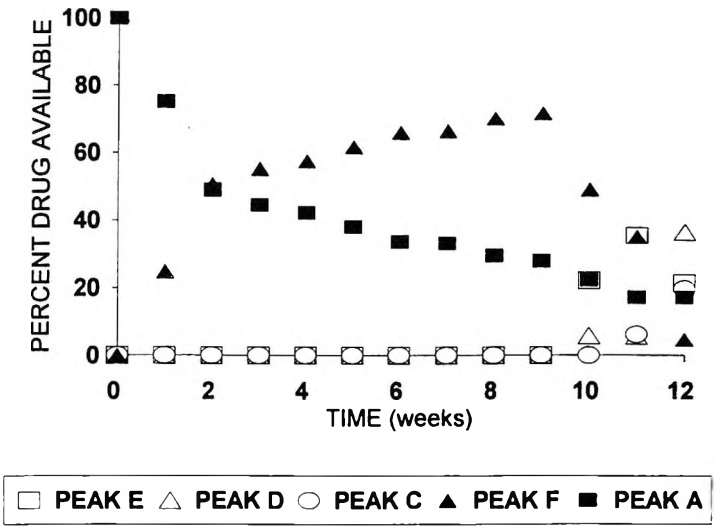


Figure 3. Stability profile illustrating the degradation of rooperol tetra-acetate in a glass vial. The graph shows the changes brought about by degradation of the sample during heat exposure. Initially, a single peak A was detected. With the progression of time, the appearance of additional peaks (C to F) were noted.

Peaks C, D, E, and F (Figure 2) in both the plastic- and glass-stored samples were not present in the chromatograms of the freshly prepared samples, but began to appear in the degrading sample following three weeks of exposure of the plastic holder to elevated temperatures and ten weeks of exposure of the glass vial. Initially, a single peak (A) was detected (Figure 2[I]). However, with the progression of time, the appearance of additional peaks (C, D, E, F) signified the onset of the degradation process (Figure 2 [II and III]).

The stability profile of rooperol tetra-acetate displays a significant percentage mass decrease in peak A and a percentage mass increase in peaks C, D, E, and F in both the glass vial (Figure 3) and the plastic holder (Figure 4). Towards week twelve the baseline of the chromatogram was very rugged, thus, suggesting the emergence of several additional degradation products whose concentrations were far too low for the detector to quantitate. In addition, slight variations from week to week in the mobile phase composition, injection volume, pH, and temperature during chromatographic analysis could well have contributed to any decrease in the precision of the method. The more rapid onset of the degradation process observed with the plastic-stored sample may

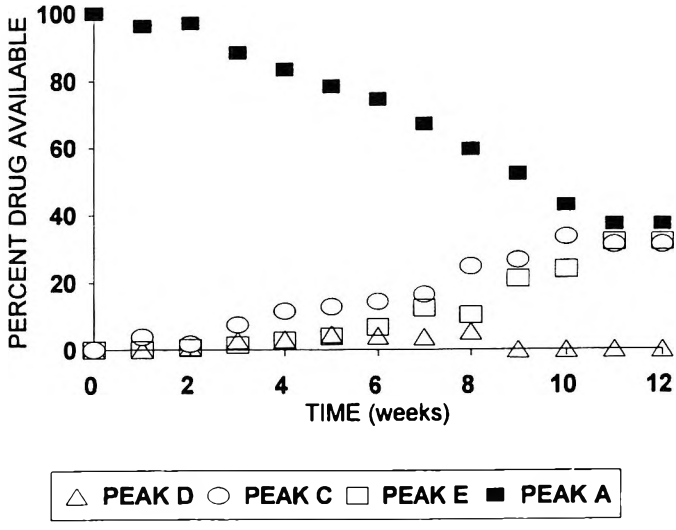
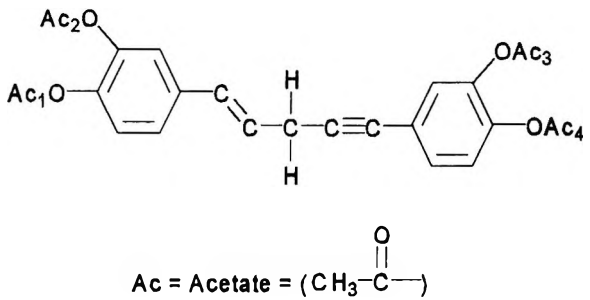


Figure 4. Stability profile illustrating the degradation of rooperol tetra-acetate in a plastic holder.

possibly be due to unfavourable interaction of the drug with plasticizers released from the holder upon heat exposure. The glass vial used in the comparative study was inert and as a result, the rate of heat degradation of the drug was slower.

The student's *t* distribution test was applied to the peak area ratios obtained for all peaks and the results showed that at a 95% level, there is a significant difference in composition between the freshly prepared samples and the heated samples, thus confirming the instability of rooperol tetra-acetate in heat.

From the study of the structure of rooperol tetra-acetate (Figure 5), it is safe to presume that the metabolites formed when the drug is stored under elevated temperatures arise from successive cleavage of the acetate chemical groups. These acetate groups, situated on the periphery of the structure, experience decreased electronic attraction from the strong pi and triple bonds present at the core of the molecule, resulting in relatively easier detachment of the acetate groups from the core structure. Subsequent degradation may arise as a result of cleavage of the bonds which bind the two aromatic compounds of the rooperol tetra-actate structure.



ROOPEROL TETRA-ACETATE

Figure 5. The Chemical Structure of Rooperol Tetra-Acetate (MW = 450.45g/mol).

CONCLUSION

The precision, linearity, sensitivity, and range studies data indicate that the method selected is suitable for quantitative analysis of rooperol tetra-acetate. Furthermore, the technique has proven to be both reliable and reproducible. The method developed and validated can now be used to determine the diffusion characteristics of rooperol tetra-acetate through membrane systems.

The stability profile of rooperol tetra-acetate shows that the drug is significantly unstable and its degradation upon exposure to elevated temperatures for extended periods of time, is a significant property of the drug. The inherent instability of rooperol tetra-acetate at elevated temperatures, impacts on the manufacturing and diffusion processes where heat application may be necessary. For this reason, it is essential that the possible emergence of degradation peaks is acknowledged, particularly during the analysis of the diffusion properties of the drug.

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SEPARATION AND DETERMINATION OF DYES BY ION-PAIR CHROMATOGRAPHY

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ABSTRACT

A reverse phase ion-pair high performance liquid chromatographic method is presented to determine six food dyes (E-110, E-120, E-122, E-123, E-124 and E-127). The tetra-butylammonium ion (TBA) is used as counter-ion in the mobile phase. The separation was accomplished with a Nova-pack C_{18} column using methanol -NaH_2PO_4/Na_2HPO_4 pH=7 buffer solution 0.1M containing TBA 0.05 M as mobile phase. The chromatograms were monotorized by measuring the absorbance at 520 nm. The influence of methanol content, counter ion concentration, pH and flow-rate of the mobile phase were investigated. In the chromatographic conditions selected, the total chromatogram was obtained in seven minutes. The calibration graphs were established by measuring the peak area in the chromatograms. Determination limits ranging from 1 to 7.8 ng were obtained. Samples containing the six dyes in different proportions were analyzed through the proposed method, obtaining good recoveries in all cases. Finally, the method was successfully applied to the analysis of dyes in several commercial products.

INTRODUCTION

Synthetic colors are added to foods to replace natural color lost in processing, to reduce batch to batch variation, and to produce products with consumer appeal where no natural color exists. In recent years, food additives have increasingly come under investigation for evaluation of their safety in use.

Toxicological investigation of food colors is underway in many countries.[1,2] These toxicological data result in repeated revisions of a number of permitted food dyes.

In the European Community, a Scientific Committee has reviewed the safety in the use of all compounds proposed for inclusion in a Community List of coloring matters, authorized for use in foodstuffs.[3] Special attention was paid to the sulfonated azodyes. Systematic studies on chronic toxicity have led pharmacologists and nutritionists to define acceptable daily intakes (ADI) for each of the permitted dyes.

The dyes studied in this paper contain sulfonic or carboxylic acid function and are present as anions over a large pH range. In a reverse phase HPLC system, anionic substances have very short retention times. An ion-pair chromatographic system would be a way of achieving adequate retention. When a hydrophobic ion of opposite charge (counter ion) is added, the retention of the ionized substance in a nonpolar stationary phase is enhanced. Because the ion-pair formed is more hydrophobic than the ionized molecule, it will be retained for a longer time inside the column.

The main problem in the quantitative determination of dyes in food is at the extraction step. In this way, the colors have been isolated from food by dyeing the color onto wool,[4] by polyamide columns,[5] ion-exchange resins,[6,7] and by liquid extraction using an ion-pair reagent.[8,9] Several other methods involving the use of C_{18}[10,11] or amine quaternary[12] columns have been described more recently.

High performance liquid chromatography (HPLC) has became a popular technique to determine synthetic food dyes. For instance, although ion exchange[13] and reverse phase HPLC[14,16] has both been used, it is the reverse phase ion-pair HPLC[17-20] that has been found particularly useful for the separation and detection of several dyes. In the ion-pair chromatography of anionic substances, several counter ions such as cetrimide,[21,22] a cationic detergent, and tetra-alkylammonium ion[23-25] have been used.

In this report, a reverse phase ion-pair HPLC method is proposed for the separation and quantification of six food colors. Tetrabutylamoniun ion (TBA) was chosen as counter-ion in the mobile phase. The optimal conditions were selected and the method was applied to the quantification of these substances in commercial products.

EXPERIMENTAL

Apparatus

The chomatographic system consisted of Waters variable wavelength UV-Vis detector model 486, a quaternary gradient pump Waters series 35 equipped with a solvent programmer, a Rheodyne Model 7125 injector with a 20 µL sample loop, and a NEC 386/25 computer fitted with Water Baseline software were used for all the measurement and treatment of data.

The detection wavelength was 520 nm. The analytical column was a Nova-Pack C18 (3.9 mm x 150 mm's ID, partic'e size 3 µm).

The pH values were measured with a Crison, model 2001 with a combined glass electrode.

Reagents

All solvents and reagents were of analytical grade unless indicated otherwise. Solutions were made with deionized water (Milli-Q quality).

- Dyes standards solutions: Pure samples of Amaranth (E-123), Ponceau 4R (E-124), Carmoisine (E-122), Sunset Yellow (E-110), and Erythrosine (E-127) were obtained from Aldrich Company and Carminic acid (E-120) from Sigma Company. The stock solutions (200 mg/L) were prepared by dissolving pure samples in deionized water, except E-127, that was dissolved in ethanol.

- Tetra-n-butylammonium hydroxide 40% aqueous solution (Sigma) diluted to 10% with deionized water. This solution was used as a TBA stock solution to prepare mobile phases.

- Buffers: Composition for 2 L and ionic strength 0.1 M: pH = 7.0 ; 5.780 g $NaH_2PO_4.H_2O$, 9.380 g $Na_2HPO_4.2H_2O$. pH = 4.5 ; 0.9 mL 1M H_3PO_4 , 27.598 g $NaH_2PO_4.H_2O$. pH = 3.0; 32 mL 1M H_3PO_4, 27.598 g $NaH_2PO_4.H_2O$.

- Methanol was HPLC grade from Panreac.

RESULTS AND DISCUSSION

Six food dyes were investigated in this paper: Amaranth (E-123), Ponceau 4R (E-124), Carmoisine (E-122), Sunset Yellow (E-110) and Erythrosine (E-127). As commented before, these compounds are anionic species in a large pH range and low retention times are observed in a reverse phase HPLC system. Retention and selectivity can be controlled by the formation of an ionic- pair, and by the selection of the type and concentration of the organic solvent in the mobile phase. Addition of inorganic electrolytes or adjustment of its pH can also be used to improve the separation.

In this work, we have used the ion-pair chromatography to study the separation of these dyes on a Nova-Pack C18 column with the chromatographic system above described.

The mobile phase consisted of methanol - phosphate buffer solution mixture containing tetra-n-buthylammonium ion (TBA) as the ion-pairing reagent.

The flow-rate was fixed at 1 mL/min unless indicated otherwise. Before use, the eluents were filtered through Millipore filter and dissolved gases were removed by purging with helium for 10 min. The wavelength selected to monotorize the chromatogram was 520 nm.

The influences of methanol percent, counter ion concentration and pH value in the mobile phase as well as the mobile phase flow-rate in the separation procedure will be discussed in the next sections.

Effect of Methanol Content in the Mobile Phase on the Retention

In these experiments, the reservoirs of the solvent system were filled with the following two eluents:

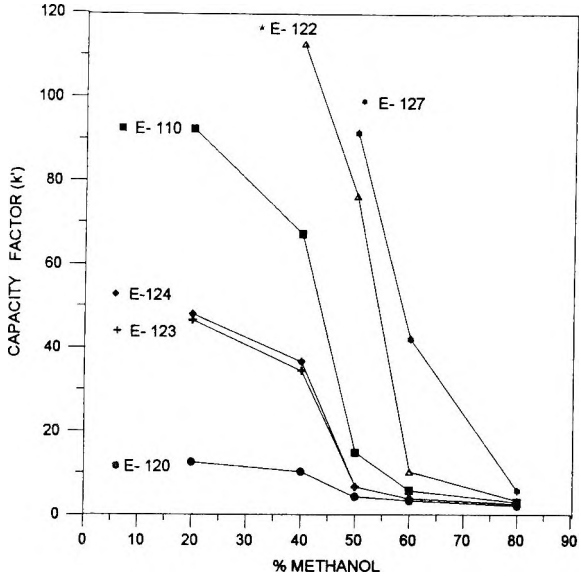

Figure 1. Influence of methanol percent in the mobile phase on capacity factor of various dyes using a reverse phase ion -pair HPLC system.

-Eluent A consisted of methanol (20%), phosphate buffer solution 0.1M pH=7 (80%) and TBA 0.005M.
-Eluent B consisted of methanol (80%), phosphate buffer solution 0.1M pH=7 (20%) and TBA 0.005M.

Both eluents were mixed and pumped to the column with the solvent programmer.

For this study 20 μL of each dye solution were injected individually into the column and chromatograms were obtained with a different methanol percentage in the mobile phase.

Figure 1 shows the influence of methanol percentage in the mobile phase on retention (capacity factor) of this group of dyes. Retention decreases with higher methanol concentrations in the mobile phase because the polarity of the eluent also decreases. Indeed, the retention times are spread over a wide range. E-110, E-122 y E-127 exhibit long retention times, and need high a methanol percentage to be eluated. However E-123, E-124 and E-120 are hardly retained with this methanol percentage. The very different retention properties of the dyes requires a methanol gradient elution system to achieve an adequate separation.

Table 1

Chromatographic Parameters of a Group of Dyes*

Dye	Retention Time RT	Capacity Factor k′	Resolution R_s
E-120	1.57	5.28	----
E-123	3.33	12.32	4.63
E-124	4.93	18.72	4.57
E-110	5.48	20.92	2.29
E-122	6.13	23.52	3.02
E-127	7.67	29.68	4.74

* See text

$k' = (RT_1 - RT_0) / RT_0$

$R_s = 2(RT_2 - RT_1) / (W_1 + W_2)$

RT_2 and RT_1 : retention time of solutes 2 and 1 which are successively eluated. RT_0 : retention time of an unretained solute. W_1 and W_2 : peak width of solutes 1 and 2

Gradient Elution Optimized

Using the solvent programmer, it is possible to change the proportion of eluent A and B, and also the methanol percent, during the cromatography.

Several methanol gradients were assayed and a gradient profile consisting of a linear change from the initial eluent composition (60% eluent A , 40% eluent B) to the final composition (100% eluent B) was selected as follows:

t_0 (init.) 60% eluent A, 40% eluent B
t_1 (1 min.) 50% eluent A, 50% eluent B
t_2 (3 min.) 100% eluent B
t_3 (8 min.) 100% eluent B

Therefore, methanol concentration varied from 44% to 80%.

In Table 1 the retention time (RT) and capacity factor (k′) of each dye are summarized. In addition, the chromatographic resolution (R_s) between two adjacent peaks were calculated and are showed in the same table. The gradient profile selected permits an excelent separation, as can be ascertained from $R_{s,,}$ in a short time, about eigth min.

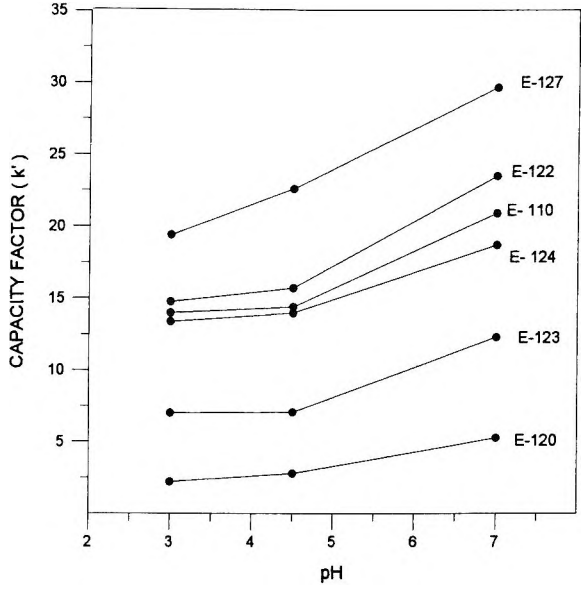

Figure 2. Influence of pH in the mobile phase on capacity factor of various dyes using a reverse phase ion -pair HPLC system.

Effect of pH in the mobile phase on the retention

The influence of pH of the mobile phase on the retention propertes was studied. For this purpose, the pH value was fixed in the eluents A and B by using different phosphate buffer solutions. Chromatograms of a solution with all dyes were obtained with the gradient elution profile selected before. Figure 2 shows, graphically, the behaviour of this group of dyes at three pH values. As can be seen, the retention increases when pH also increases. A pH value of 7.0 was selected to ensure ionisation of dyes and, also, the ion pair formation with the TBA, without the eluents damaging the silica packing material. An NaH_2PO_4/Na_2HPO_4 solution of pH 7 (ionic strength 0.1) was used for buffering.

Effect of counter ion concentration

With the aim to study the influence of counter-ion in the mobile phase on capacity ratio, the chromatograms were obtained, according to the gradient profile selected, with different TBA concentration in the eluents.

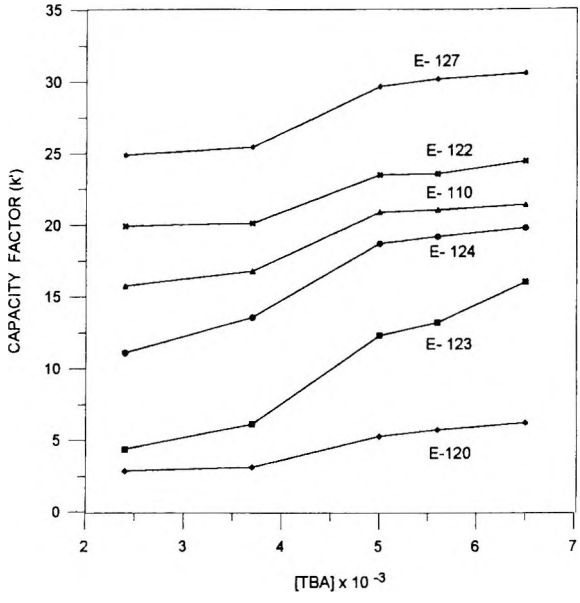

Figure 3. Influence of TBA concentration in the mobile phase on capacity factor of various dyes using a reverse phase ion -pair HPLC system.

As can be seen in Figure 3, the retention times for all dyes increase when the counter ion concentration increase. The chromatographic resolutions (R_s) were calculated in each chromatogram. A concentration of TBA 0.005 M was selected because it provided R_s values higher than 1.5.

Effect of flow-rate of the mobile phase

Finally, the influence of the flow- rate of the mobile phase was studied. The retention times and chromatographic resolution between peaks decrease when the flow-rate of mobile phase increase. A flow-rate of 1.5 mL/min was selected. In all cases satisfactory chromatographic resolution between peaks (R_S) were obtained.

CHROMATOGRAPHIC PROCEDURE SELECTED

From the studies carried out before, we propose a chromatographic procedure to determine six food dyes, wich is described in this section.

Two eluents (eluents A and B) were mixed to form the mobile phase:
Eluent A: methanol (20%), NaH_2PO_4/Na_2HPO4 buffer solution 0.1M pH=7 (80%) and TBA 0.005M.
Eluent B: methanol (80%), NaH_2PO_4/Na_2HPO_4 buffer solution 0.1M pH=7 (20%) and TBA 0.005M.

Before use, the eluents were filtered through Millipore filter and dissolved gases were removed by purging with helium for 10 min.

The proportions of eluent A and B were varied during chromatography by using the solvent programmer. The gradient profile consisted of a 1 min. linear change from 60% eluent A and 40% eluent B to 50% eluent A, 50% eluent B , then a second 2 min. linear change to the final composition (100% eluent B) and finally, a isocratic period (5min.) using the final composition. After chromatography, the mobile phase was returned to initial conditions using a 2 min. linear gradient.

The flow-rate of the mobile phase in the column was set at 1.5 mL/min. The column was conditioned by pumping initial eluent composition at least 5 min. before injection of the sample.

20 μL volume of a dye solution was injected in the chromatographic system. The gradient elution was initiated and absorbance monitorized at 520 nm. Duplicate chromatographic separation were made for all dye solutions. The chromatographic conditions optimized are summarized in Table 2 and the chromatogram corresponding of a standard of the six dyes is shown in Figure 4. From this chromatogram, it can be ascertained that the chromatographic procedure selected is excellent for the separation of the solutes.

QUANTITATIVE DETERMINATION

Calibration Graphs

The linearity of the assay was checked by running, in duplicate, a set of standards through the chromatographic procedure decribed above.

Two calibration graphs were obtained for each dye by plotting the peak area and the peak height versus the concentration. A good linearity was obtained for all dyes in the range studied: 8-40 mg/L for E- 120 and 2-10 mg/L for the rest of them.

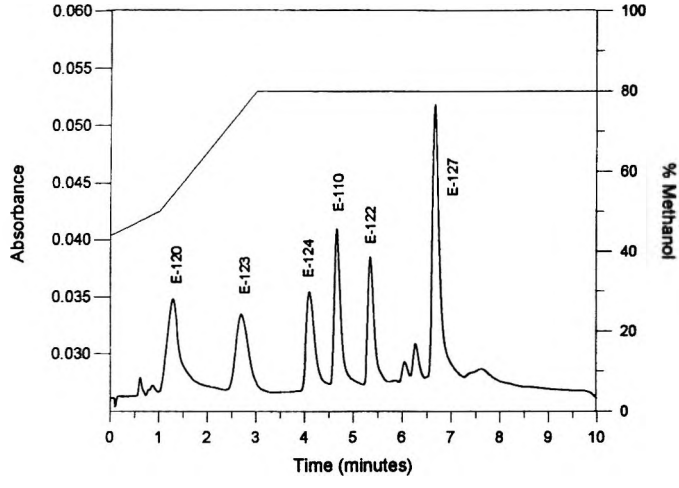

Figure 4. Chromatogram of dyes mixture obtained by the proposed method and the gradient profile selected (see text and Table 2).

Table 2

Chromatographic Conditions Selected

Column:	**Nova-Pak C18**
Mobile phase	
Eluent A	methanol (20%), phosphate pH=7 buffers (80%), TBA (0.005 M)
Eluent B	methanol (80%), phosphate pH=7 buffers (20%), TBA (0.005 M)
Gradient profile	
	t_0 (initi) 60% eluent A, 40% eluent B
	t_1 (1 min.) 50% eluent A, 50% eluent B
	t_2 (3 min.) 100% eluent B
	t_3 (8 min.) 100% eluent B
Flow-rate	1.5 mL/min
Injection volume	20 μL
Detection	520 nm

Table 3

Statistical Parameters

Dye		Standard Deviation (mg/L)	Relative Standard Deviation	Determination Limit[1] (ng)
E-120	Area	0.853	± 8.29	7.8
	Height	1.351	± 12.85	52.0
E-124	Area	0.029	± 1.87	1.0
	Height	0.053	± 3.55	16.6
E-110	Area	0.071	± 4.22	1.4
	Height	0.037	± 2.43	15.6
E-123	Area	0.036	± 2.21	7.6
	Height	0.036	± 2.26	17.0
E-122	Area	0.039	± 2.32	7.6
	Height	0.023	± 1.28	26.0
E-127	Area	0.060	± 3.61	5.6
	Height	0.038	± 2.20	14.0

[1] $C_Q = 10\ S_B / m$: C_Q = determination limit; S_B = standard deviation of blank; m = slope of calibration graph.

Statistical

Precission of the assay was checked by performing nine replicate runs of mixtures of six dyes. The standard deviation and the relative standard deviation were calculated. The determination limits were also obtained for each dye.[26] In Table 3 the statistical data obtained are summarized.

Analysis of synthetic mixtures

To test the validity of the proposed method, several synthetic mixtures containing all dyes in different proportions were prepared and analyzed using the chromatographic procedure described.

Table 4

Recoveries Obtained in the Analysis of Synthetic Mixtures Using the Peak Area

Composition of Mixtures (mg/L)						Recoveries (%)					
C.A.	P	AN	AM	C	E	C.A	P	AN	AM	C	E
20	9.6	3.2	3.2	9.6	3.2	95.92	97.75	105.5	96.00	101.75	94.00
32	6.4	6.4	6.4	4.8	4.8	100.68	97.60	99.75	101.00	95.75	95.25
24	4.8	9.6	9.6	3.2	9.6	101.86	91.25	100.40	101.00	98.50	99.00
16	3.2	4.8	4.8	6.4	6.4	103.77	87.75	96.50	104.60	103.75	98.00
8	1.6	1.6	1.6	1.6	1.6	111.17	87.75	93.37	94.62	97.75	92.75
	Statistical Parameters*										
	$\bar{X}$					100.55	95.53	99.10	99.44	99.54	95.80
	S					3.34	3.71	4.54	4.08	3.18	2.64
	S_m					1.67	2.14	2.03	1.82	1.42	1.18

C.A.: E-120, P: E-124, C: E-122, AN: E-110, AM: E-123, E: E-127/

* Values of recoveries higher than 110% or smaller than 90% have not been considered for calculating these parameters.

Table 5

Recoveries Obtained in the Analysis of Synthetic Mixtures Using the Peak Height

Composition of Mixtures (mg/L)						Recoveries (%)					
A.C.	P	AN	AM	C	E	C.A	P	AN	AM	C	E
32	9.6	9.6	4.8	6.4	3.2	95.51	81.25	88.25	82.50	92.55	91.50
24	6.4	4.8	6.4	4.6	1.6	106.16	83.40	81.00	85.75	89.00	90.25
16	4.8	6.4	1.6	3.2	4.6	81.26	70.75	85.45	78.50	91.75	93.50
20	3.2	1.6	9.6	4.8	4.8	112.02	68.75	81.50	90.60	91.75	92.36
8	1.6	4.8	3.2	9.6	6.4	142.62	67.50	83.25	85.52	96.00	93.25
	Statistical Parameters*										
	$\bar{X}$					107.51	73.48	83.90	89.10	92.25	92.20
	S					22.84	6.96	2.67	4.15	2.50	1.34
	S_m					10.21	3.11	1.19	1.86	1.12	0.60

C.A.: E-120, P: E-124, C: E-122, AN: E-110, AM: E-123, E: E-127/

* Values of recoveries higher than 110% or smaller than 90% have not been considered for calculating these parameters.

The results obtained using the area and height peak are summarized in Table 4 and 5 respectively. The recoveries (%) obtained show that the determination of all dyes are suitable, with best results when we used the peak area as an analytical signal of measurement.

Analysis of commercial samples

The method described was applied to analyze commercial products containing two or more of the dyes studied. Thus, we have analyzed three beverages (Bitter Kas, Bitter Kalty and grenadine), gelatin desserts, and syrup with strawberry flavor. The amount of dye in each sample was calculated by using the calibration graph established and by using the standard addition method.

The samples were prepared as follows:

A) Beverages (Bitter Kas, Bitter Kalty and grenadine).

1) Quantitative determination by direct preparation using the calibration graphs established: 5 mL of the sample was transferred to a 25- mL flask and diluted with deionized water to the mark.

2) Quantitative determination by standard addition: to 5mL of the beverage sample were added different amounts (2, 4, 6, 8 mg/L) of the dye to determine and proceed as before.

B) Gelatin and syrup.

1) Quantitative determination by direct preparation using the calibration graphs established: 1g product was diluted with 10 mL of deionized water in beaker over low heat with gentle swirling until dissolved. The solution was filtered and transferred to a 25-mL flask and diluted with deionized water to the mark.

2) Quantitative determination by standard addition: to 1g product were added different amounts (2, 4, 6, 8 mg/l) of dye to determine and proceed as before.

The chromatograms of all solutions were recorded according to the chromatographic procedure, and the peak area of each compound was obtained. The results obtained, using the calibration graph and the standard addition method, were very similar and are summarized in Table 6.

Table 6

Analysis of Commercial Samples

	Bitter Kas		Bitter Kalty			Grenadine	
	C(mg/L)	P(mg/L)	C(mg/L)	AM(mg/L)	AN(mg/L)	P(mg/L)	AM(mg/L)
Direct measurement	34.3 ± 0.1	10.3 ± 0.2	16.7 ± 0.3	38.3 ± 0.4	6.9 ± 0.2	50.8 ± 0.3	29.5 ± 0.3
Standard addition	35.0 ± 0.1	9.9 ± 0.1	16.7 ± 0.3	39.9 ± 0.3	8.8 ± 0.2	50.4 ± 0.2	28.6 ± 0.2

	Gelatine		Syrup	
	AM(mg/Kg)	P(mg/Kg)	P(mg/Kg)	C(mg/Kg)
Direct measurement	160.0 ± 0.4	275.0 ± 0.3	118.8 ± 0.1	146.2 ± 0.3
Standard addition	161.2 ± 0.3	278.7 ± 0.3	119.1 ± 0.2	143.6 ± 0.3

CONCLUSIONS

The experimental results obtained in this paper, demonstrate that the chromatographic method proposed is sufficiently specific, sensitive, accurate, and rapid to analyze red and orange dyes, authorized for use in food in Spain, in commercial products.

ACKNOWLEDGMENT

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J. LIQ. CHROM. & REL. TECHNOL., 20(18), 3089–3093 (1997)

THE BOOK CORNER

HANDBOOK OF CAPILLARY ELECTROPHORESIS APPLICATIONS, H. Shintani, J. Polonsky, eds., Blackie Academic & Professional Publishers. This book can also be ordered from Chapman & Hall, 115 Fifth Avenue, New York, NY 10003, 1997, 737 pp., $229.95.

Capillary Electrophoresis was first introduced by Hjerten in 1967 and popularized in 1981 by Jorgenson. The first commercial HPCE instrument was introduced in 1988. Since then, many companies have introduced CE instruments, and one can safely say that we are now looking at third generation instruments. CE has grown tremendously in the last ten years. There are at least 15 books, 2 handbooks, and 2 application books (including Handbook of CE Applications) other than those published by instrument companies. There are over 1000 published research papers. In short, CE is fully accepted as a high resolution microseparation technique which has been applied in every field of research, and for different types of ions and molecules, small organic to large biomolecules.

The present book, " Handbook of CE Applications," is a welcome addition to the analyst's library. It is well written, comprehensive, and up to date. The Hanbook is divided into seven main parts, in addition to the Introduction, Chapter 1, and Appendices.

Part One is Equipment Systems (Chapters 2-11); Part Two is Biochemistry Applications (Chapters 12-21); Part Three is Pharmaceutical Science Applications (Chapters 22-27); Part Four is Bioscience Applications (Chapters 28-33); Part Five is Ion Analysis Applications (Chapters 34-37); Part Six is Food Analysis Applications (Chapters 38-40); Part Seven is Environmental Science Applications (Chapters 41-43).

The Handbook is written by many authors. The organization of all chapters is similar (a credit to the editors), almost each has an introduction and a table summarizing the experimental conditions, along with the references.

This is a nice arrangement which makes it easy for the analyst to follow and reproduce the experiment without having to read the original article. I found the book to be both comprehensive and useful. I also found some omissions, for example, in Table 7.1 on LIF detection, there is no mention of the KrF UV laser which has been used in many CE applications. As mentioned earlier, the book is recommended to analytical chemists in diversified fields.

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Reviewed by
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J. LIQ. CHROM. & REL. TECHNOL., 20(18), 3095 (1997)

EDUCATION ANNOUNCEMENT

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The instructor, Dr. Jack Cazes, is founder and Editor-in-Chief of the Journal of Liquid Chromatography & Related Technologies, Editor of Instrumentation Science & Technology, and Series Editor of the Chromatographic Science Book Series. He has been intimately involved with liquid chromatography for more than 35 years; he pioneered the development of modern HPLC technology. Dr. Cazes was Professor-in-Charge of the ACS Short Course and the ACS Audio Course on GPC, and has taught at Rutgers University. He is currently Visiting Scholar at Florida Atlantic University.

Details may be obtained from Dr. Jack Cazes, P. O. Box 970210, Coconut Creek, FL 33097. Tel.: (954) 570-9446; E-Mail: cazes@worldnet.att.net.

J. LIQ. CHROM. & REL. TECHNOL., 20(18), 3097–3105 (1997)

LIQUID CHROMATOGRAPHY CALENDAR

1997

OCTOBER 5 - 8: Conference on Formulations & Drug Delivery, La Jolla, California, sponsored by the ACS Div. of Biochem. Technol. Contact: ACS Meetings, 1155 16th St, NW, Washington, DC 20036. Tel: (202) 872-6286; FAX: (202) 872- 6013; Email: miscmtgs@acs.org.

OCTOBER 6 - 10: Validation d'une Procedure d'Analyse, Qulification de l'Appareillage; Application a la Chromatographie Liquide, Montpellier, France. (Training course given in French) Contact: Prof. H. Fabre. Tel: (33) 04 67 54 45 20; FAX: (33) 04 67 52 89 15; Email: hfabre@pharma.univ-montpl.fr.

OCTOBER 19 - 22: 49th ACS Southeast Regional Meeting, Roanoke, Virginia. Contact: J. Graybeal, Chem Dept, Virginia Tech, Blacksburg, VA 24061, USA. Tel: (703) 231-8222; Email: reglmtgs@acs.org.

OCTOBER 21 - 23: Sensors Expo: Conference on Exposition of Sensors, Detroit, Michigan. Contact: Expocon Mgmt. Assoc., 3363 Reef Rd, P. O. Box 915, Fairfield, CT 06430-0915, USA. Tel: (203) 256-4700; Email: sensors@expo.com.

OCTOBER 21 - 23: Biotechnica Hannover '97: Int'l. Trade Fair for Biotechnology, Hannover, Germany. Contact: D. Hyland, Hannover Fairs USA, Inc., 103 Carnegie Center, Princeton, NJ 08540, USA.

OCTOBER 21 - 24: 152nd Fall Technical Meeting & Rubber Expo'97, Cleveland, Ohio, sponsored by ACS Div. of Rubber Chem. Contact: ACS Meetings, 1155 16th St, NW, Washington, DC 20036. Tel: (202) 872-6286.

OCTOBER 21 - 25: 33rd ACS Western Regional Meeting, Irvine, California. Contact: L. Stemler, 8340 Luxor St, Downey, CA 90241, USA. Tel: (310) 869-9838; Email: reglmtgs@acs.org.

OCTOBER 25 - 30: 24th Annual Conference of the Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Providence, Rhode Island. Contact: ACS Div. of Anal. Chem., Tel: (301) 846-4797; FAX: (301) 694-6860.

OCTOBER 26 - 29: ISPPP'97 – 17th International Symposium on the Separation of Proteins, Peptides, and Polynucleotides, Doubletree Hotel, Washington, DC. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279. Walkersville. MD 21793. USA. FAX: (301) 898-5596.

OCTOBER 26 - 29: 8th Symposium on Handling of Environmental & Biological Samples in Chromatography and the 26th Scientific Meeting of the Group of Chromatography and Related Techniques of the Spanish Royal Society of Chemistry, Almeria, Spain. Contact: M. Frei-Hausler, IAEAC Secretariat. Postfach 46, CH-4123 Allschwill 2, Switzerland. FAX: 41-61-4820805.

OCTOBER 29 - NOVEMBER 1: 32nd ACS Midwest Regional Meeting, Lake of the Ozarks, Osage Beach, Missouri. Contact: C. Heitsch, Chem Dept, Univ of Missouri-Rolla, Rolla, MO 65401, USA. Tel: (314) 341-4536; FAX: (314) 341-6033.

NOVEMBER 2 - 6: AAPS Annual Meeting & Expo, Boston, Mass. Contact: AAPS, 1650 King Street, Alexandria, VA 22314-2747, USA. Tel: (703) 548-3000.

NOVEMBER 2 - 6: 11th International Forum on Electrolysis in the Chemical Industry, Clearwater Beach, Florida. Contact: P. Klyczynski, Electrosysthesis Co., 72 Ward Rd., Lancaster, NY 14086, USA.

NOVEMBER 6 - 8: 2nd North American Research Conference on Emulsion Polymers/Polymer Colloids, Hilton Head Is., SC. Contact: A. V. Patsis, SUNY New Paltz, Inst. of Mater. Sci., New Paltz, NY 12561, USA.

NOVEMBER 7 - 8: 6th Conference on Current Trends in Computational Chemistry, Jackson, Miss. Contact: J. Leszcynski, Jackson State Univ., Chem. Dept., 1400 J. R. Lynch St., Jackson, MS 39217, USA. Tel: 601) 973-3482; Email: jersy@iris5.jusms.edu.

NOVEMBER 11 - 15: 5th Chemical Congress of North America, Cancun, Mexico. Contact: ACS Meetings, 1155 16th St. NW, Washington, DC 20036-4899, USA. Tel: (202) 872-6286; FAX: (202) 872-6128.

NOVEMBER 16 - 21: AIChE Annual Meeting, Westin Bonaventure/Omni Los Angeles, Los Angeles, California. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA. Tel: 1-800-242-4363.

NOVEMBER 16 - 21: Eastern Analytical Symposium, Garden State Convention Center, Somerset, New Jersey. Contact: S. Good, EAS, P. O. Box 633, Montchanin, DE 19710-0635, USA. Tel: (302) 738-6218.

NOVEMBER 20 - 21: New Horizons in Chemistry Symposium, Los Angeles. Contact: J. May, Tel: (213) 740-5962; Email: jessy@methyl.usc.edu.

1998

FEBRUARY 26 - 28: Sample Handling and Analysis of Organic Pollutants, Archamps, France. Contact: ACTIVE Assoc. Office, c/o Pharmapeptides, CUR Business Park, 74166 Archamps, France.

FEBRUARY 28 - MARCH 1: 28th Annual Spring Prog. in Polymers: Degradation & Stabilization of Polymers, Hilton Head Is., South Carolina. Contact: A. V. Patsis, SUNY New Paltz, Inst. of Mater. Sci., New Paltz, NY 12561. Email: ims@mhv.net.

MARCH 1 - 5: 28th International Symposium on Environmental Analytical Chemistry (ISEAC 28), University of Geneva, Switzerland. Contact: ACTIVE Assoc. Office, c/o Pharmapeptides, CUR Business Park, 74166 Archamps, France.

MARCH 1 - 6: PittCon '98, New Orleans, Louisiana. Contact: PittCon '98, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA. Tel: (800) 825-3221; FAX: (412) 825-3224.

MARCH 8 - 12: AIChE Spring National Meeting, Sheraton, New Orleans. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA.

MARCH 15 - 18: 14th Rocky Mountain Regional Meeting, Tucson, Arizona. Contact: R. Pott, Univ of Arizona, Tucson, AZ 85721.

MARCH 23 - 25: 5th Meeting on Supercritical Fluids: Materials and Natural Products Processing, Nice, France. Contact: F. Brionne, Secretariat, ISASF, E.N.S.I.C., 1 rue Grandville, B.P. 451, F-54001 Nancy Cedex, France. Email: brionne@ensic.u-nancy.fr.

MARCH 29 - APRIL 2: 215th ACS National Meeting, Dallas, Texas. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036, USA.

MAY 3 - 8: HPLC'98 – 22nd International Symposium on High Performance Liquid Phase Separations and Related Techniques, Regal Waterfront Hotel, St. Louis, Missouri. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

MAY 20 - 22: 32nd Middle Atlantic ACS Regional Meeting, Wilmington, Delaware. Contact: G. Trainor, DuPont Merck, P. O. Box 80353, Wilmington, DE 19880-0353. Email: reglmtgs@acs.org.

MAY 26 - 29: VIIIth International Symposium on Luminescence Spectrometry in Biomedical and Environmental Analysis: Detection Techniques and Applications in Chromatography and Capillary Electrophoresis, Las Palmas de Gran Canaria (Canary Islands), Spain. Contact: Dr. Jose Juan Dantana Rodriguez, University of Las Palmas of G. C., Chem. Dept., Faculty of Marine Sci., 35017 Las Palmas de G. C., Spain. Tel: 34 (9) 28 45.29.15 / 45.29.00; FAX: 34 (9) 28 45. 29.22; Email: josejuan.santana@quimica.ulpgc.es.

MAY 27 - 29: 30th Central Regional ACS Meeting, Ann Arbor, Michigan. Contact: D. W. Ewing, J. Carroll Univ., Chem. Dept., Cleveland, OH 44118. Tel: (216) 397-4241. Email: ewing@jevaxa.jeu.edu.

JUNE 1 - 3: 31st Great Lakes Regional ACS Meeting, Milwaukee, Wisconsin. Contact: A. Hill, Univ. of Wisc., Chem. Dept., Milwaukee, WI 53201, USA. Tel: (414) 229-4256; Email: reglmtgs@acs.org.

JUNE 2 - 5: Third International Symposium on Hormone and Veterinary Drug Residue Anbalysis, Congres Centre Oud Sint-Jan, Bruges, Belgium. Contact: C. Van Peteghem, Dept. of Food Analysis, University of Ghent, Harelbekestraat 72, B-9000 Ghent, Belgium. Tel: (32) 9/264.81.34.

JUNE 10 - 12: 53rd ACS Northwest Regional Meeting, Columbia Basin College, Pasco, Washington. Contact: K. Grant, Math/Science Div, Columbia Basin College, 2600 N 20th Ave, Pasco, WA 99301, USA.

JUNE 13 - 19: 26th ACS National Medical Chemistry Symposium, Virginia Commonwealth Univ/Omni Richmond Hotel, Richmond, Virginia. Contact: D. J. Abraham, Virginia Commonwealth Univ, Dept of Med Chem, P. O. Box 581, Richmond, VA 23298, USA. Tel: (804) 828-8483.

JULY 12 - 16: SFC/SFE'98: 8th International Symposium & Exhibit on Supercritical Fluid Chromatography, St. Louis, Missouri. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.
AUGUST 23 - 28: 216th ACS National Meeting, Boston, Massachusetts. Contact: ACS, 1155 16th Street, NW, Washington, DC 20036, USA.

AUGUST 31 - SEPTEMBER 3: AIChE Conference on Safety in Ammonia Plants & Related Facilities, Charleston Place, Charleston, South Carolina. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA. Tel: 1-800-242-4363.

SEPTEMBER 7 - 11: 15th International Symposium on Medicinal Chemistry, Edinburgh, Scotland. Contact: M. Campbell, Bath University School of Chemistry, Claverton Down, Bath, BA2 7AY, UK. Tel: (44) 1225 826565; FAX: (44) 1225 826231; Email: chsmmc@bath.ac.uk.

SEPTEMBER 13 - 18: 22nd International Symposium on Chromatography, ISC'98, Rome, Italy. Contact: F. Dondi, Chem. Dept., Universita di Ferrara, Via L. Borsari, 46, I-44100 Ferrara, Italy. Tel: 39 (532) 291154; FAX: 39 (532) 240707; Email: mo5@dns.Unife.it.

SEPTEMBER 23 - 25: International Symposium on Preparative and Industrial Chromatography and Allied Techniques (SPICA '98), Strasbourg, France. Contact: Mlle. Francoise Brionne, E.N.S.I.C., 1, rue Grandville, B.P. 451, F-54001 Nancy Cedex, France.

SEPTEMBER 24 - 26: XIVth Conference on Analytical Chemistry, sponsored by the Romanian Society of Analytical Chemistry (S.C.A.R.), Piatra Neamt, Romania. Contact: Dr. G. L. Radu, S.C.A.R., Faculty of Chemistry, University of Bucharest, 13 Blvd. Reepublicii, 70346 Bucharest - III, Romania.

NOVEMBER 15 - 20: AIChE Annual Meeting, Fontainbleu/Eden Roc, Miami Beach, Florida. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA.

1999

MARCH 7 - 12: PittCon '99, Orlando, Florida. Contact: PittCon '99, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA.

MARCH 21 - 25: 217th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 22 - 26: 218th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

OCTOBER 8 - 13: 51st ACS Southeast Regional Meeting, Knoxville, Tennessee. Contact: C. Feigerle, Chem Dept, University of Tennessee, Knoxville, TN 37996, USA. Tel: (615) 974-2129; Email: reglmtgs@acs.org.

2000

MARCH 5 - 10: PittCon 2000, Chicago, Illinois. Contact: PittCon 2000, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA.

MARCH 26 - 31: 219th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036. Email: natlmtgs@acs.org.

JUNE 25 - 30: HPLC'2000 – 24th International Symposium on High Performance Liquid Phase Separations, Seattle, Washington. Contact: Barr Enterprises, P.O.B. 279, Walkersville, MD 21793, USA.

AUGUST 20 - 25: 220th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Email: natlmtgs@acs.org.

2001

APRIL 1 - 6: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036. Email: natlmtgs@acs.org.

AUGUST 25 - 31: 222nd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Email: natlmtgs@acs.org.

2002

APRIL 7 - 12: 223rd ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Email: natlmtgs@acs.org.

SEPTEMBER 8 - 13: 224th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Email: natlmtgs@acs.org.

2003

MARCH 23 - 28: 225th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Email: natlmtgs@acs.org.

SEPTEMBER 7 - 12: 226th ACS National Meeting, New York City. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2004

MARCH 28 - APRIL 2: 227th ACS National Meeting, Anaheim, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC.

AUGUST 22 - 27: 228th ACS National Meeting, Philadelphia, Pennsylvania. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128.

2005

MARCH 13 - 18: 229th ACS National Meeting, San Diego, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 28 - SEPTEMBER 2: 230th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2006

MARCH 26 - 31: 231st ACS National Meeting, Atlanta, GA. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

SEPTEMBER 10 - 15: 232nd ACS National Meeting, San Francisco, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2007

MARCH 25 - 30: 233rd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 19 - 24: 234th ACS National Meeting, Boston, Massachusetts. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs @acs.org.

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b) Sponsoring organization,
c) When and where it will be held, and
d) Whom to contact for additional details.

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Text Discussion
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F. D. Pierce, H. R. Brown

Utah Biomedical Test Laboratory
520 Wakara Way
Salt Lake City, Utah 84108

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Following are acceptable reference formats:

Journal:

1. D. K. Morgan, N. D. Danielson, J. E. Katon, Anal. Lett., 18, 1979-1998 (1985).

Book:

1. L. R. Snyder, J. J. Kirkland, **Introduction to Modern Liquid Chromatography**, John Wiley & Sons, Inc., New York, 1979.

Chapter in a Book:

1. C. T. Mant, R. S. Hodges, "HPLC of Peptides," in **HPLC of Biological Macromolecules**, K. M. Gooding, F. E. Regnier, eds., Marcel Dekker, Inc., New York, 1990, pp. 301-332.

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