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DETERMINATION OF PARTITION COEFFICIENTS OF 2-AMINO-2-OXAZOLINES BY RP-HPLC: APPLICATION TO HYDROPHOBICITY STUDIES

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ABSTRACT

A comparative study of the hydrophobicity for a series of bioactive 5-aryloxy-methyl-2-amino-2-oxazolines is reported. Octanol-water partition coefficients were measured using the classical shake-flask method. For two lipophilic compounds, high-performance liquid chromatography (HPLC) was used as a detector for the shake-flask method. The capacity factors (log k') were determined by an HPLC method using methanol-water as a mobile phase and a C_{18} column as stationary phase. The influence of the mobile phase composition was examined, allowing the determination of log k'_w values through extrapolation to 100% water from capacity factors data. The relationship between the pH eluent and the capacity factor was studied by working at different pH values.

INTRODUCTION

The 1-octanol/water partition coefficient ($P_{o/w}$) and its related properties continue to provide a major means of studying drug activities.[1,2] For a number of years, the chromatographic methods, especially RP-HPLC, have been proposed to determine the lipophilicities of drugs.[3-6] An interrelationship between the partition coefficient *P* and the chromatographic column capacity factor *k'* in RP-HPLC has been established in a Collander-type equation.[7]

We recently developed such a chromatographic approach of the lipophilicity, applied to series of potentially active 2-amino-2-oxazolines.[8] The aim of the present work was to study the lipophilicities of 16 new 5-aryloxymethyl-2-amino-2-oxazolines by mean of a RP-HPLC technique. First, we measured log $P_{o/w}$ using the classical shake-flask method. For two lipophilic compounds, the log $P_{o/w}$ measurement was made using HPLC as a detector. The capacity factors (log k') were determined by a high-performance liquid chromatography method using methanol-water as a mobile phase and a C_{18} column as stationary phase. The reliability of the chromatographic methodology was checked by the correlation of the k'_w data, obtained through extrapolation to 100% water from capacity factors data, with the 1-octanol/water partition coefficient.

The influence of the pH eluent on the capacity factor was studied by working at three different pH values. The observed variations were discussed in relation to the pKa's of the 5-aryloxymethyl-2-amino-2-oxazolines, determined by a potentiometric method.

MATERIALS AND METHODS

Apparatus and Chromatographic Conditions

For the capacity factor determination, chromatography was performed with a Waters Assoc. (Milford, MA, USA) apparatus equipped with a Model 501 pump, a Model 455 ultraviolet detector operating at 254 nm and a U6K manual injector. The compounds were chromatographied on a C_{18} Novapak column, 3.9 mm x 150 mm, 4 μm particle size, (Waters).

The mobile phase composition ranged from 30 to 50 % (v/v) methanol with 0.06 M phosphate buffer at various pH's (5.4; 6.4 ; 7.4). The flow rate was 1.5 mL/min. The detector output was recorded with a Model 746 Data Module integrator.

A specific procedure was developed for the log $P_{o/w}$ determination of the lipophilic compounds **15** and **16**. In this way, HPLC was used as the detector. The same apparatus, equipped with the C_{18} Novapak column was used, but the mobile phase was a mixture of acetonitrile/water with 0.06 M phosphate buffer at pH 7.4. The flow rate was 1.5 mL/min.

Standards and Reagents

New 5-aryloxymethyl-2-amino-2-oxazolines were synthesized by a previously described method.[9] Their structures were supported by elemental analysis, IR, ^{1}H and ^{13}C NMR spectral data. Stock solutions, containing 1mg/mL of each drug, were prepared in methanol and stored at -20°C.

HPLC-grade methanol and acetonitrile (Prolabo) were used without further purification to prepare the mobile phases. Water was deionized and double distilled. To prepare the phosphate buffer solutions, potassium dihydrogen phosphate and dipotassium hydrogen phosphate trihydrate (Merck) were used. The mobile phases were filtered through a 0.45 μm membrane filter.

Measurement of Log k'

The column dead-time of the system (t_o) was measured as the time from injection to the first distortion of the baseline after injection of pure water. Consequently, the stock solutions of tested compounds were diluted with water to the final injected concentrations of 50 μg/mL.

According to their chromatographic behaviour, the retention time (t_r) of each compound was determined at five different methanol-phosphate buffer mixtures ranged from 30 to 50%. The compounds were injected separately from each other three times and the mean value of the retention time was retained.

The log k'_w value for each compound was obtained by regression analysis of log k' data, expressed from the retention times t_r, through the formula: $k' = (t_r - t_o) / t_o$, and extrapolation to 0% methanol content.

The correlation/regression analyses were carried out with a statistical program on a Vectra computer (Hewlett Packard).

Measurement of pKa

Except for compounds **15** and **16**, which are not soluble enough in aqueous solution, the pKa determinations were performed using a classical potentiometric method described elsewhere.[10]

Determination of Log P

For all compounds, the octanol-water partition coefficients were determined by the classical "shake-flask" technique using a conventional methodology. Samples in a weight range of 5-10 mg were partitioned between 5 mL of 1-octanol saturated with water and 50 mL of water saturated with n-octanol. The pH of the water phase was adjusted at 1[illegible], ensuring that all compounds were more than 99% un-ionized.

For the two compounds **15** and **16**, $P_{o/w}$ was determined using HPLC as the detector, from the ratio of peak areas in the octanol and buffer phases, respectively.[11] Four independent measurements were performed for each sample.

RESULTS AND DISCUSSION

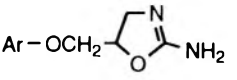
The chemical formulae of the tested 5-aryloxymethyl-2-amino-2-oxazolines are given in Figure 1.

Determination of Log k'_w and S (Slope of the regression analysis)

In this study, we have chosen to measure the log k' value extrapolated to 0% of the organic modifier in the mobile phase, log k'_w (polycratic method). For all compounds, linear relationships ($r > 0.98$) were proven to exist between the log k' values and methanol concentrations, allowing the calculation of log k'_w and S through extrapolation (Table 1). The slopes, S, for the equations were mostly constant, which is related to the structural similarity of the molecules.

Effect of the pH of the Eluent on Log k'_w

The influence of the solute ionization on RP-HPLC determination of capacity factors has often been discussed.[12-14] In general, for the determination



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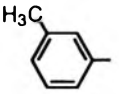
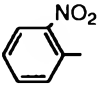
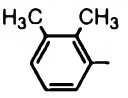
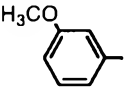
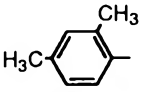
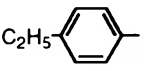
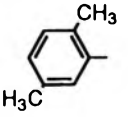
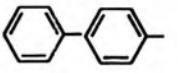
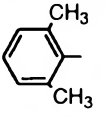
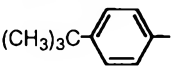
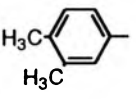
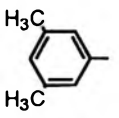
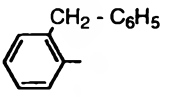
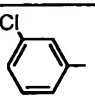
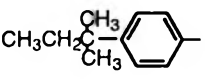
1		9	
2		10	
3		11	
4		12	
5		13	
6		14	
7		15	
8		16	

Figure 1. Structural formulae of 5-aryloxymethyl-2-amino-2-oxazolines.

of hydrophobicity, the unionized form of the solutes is taken as the reference state.[1,4] For basic compounds, it is necessary to work at high pH. As aqueous mobile phases above pH 8 often cause premature column failure, this can be a limiting factor for the HPLC applications.[15]

Table 1

Analytical Data for 5-Aryloxymethyl-2-amino-2-oxazolines

Cpd No.	Log k'_w pH = 5.4	Slope	Log k'_w pH = 6.4	Slope	Log k'_w pH = 7.4	Slope	pK_a	Log $P_{o/w}$
1	1.793	-0.04	1.799	-0.04	1.309	-0.02	8.48	1.75
2	2.202	-0.04	2.264	-0.04	1.710	-0.02	8.81	2.25
3	2.494	-0.05	2.397	-0.04	2.105	-0.03	8.23	2.23
4	2.267	-0.04	2.298	-0.04	1.531	-0.02	8.92	2.29
5	2.115	-0.04	2.015	-0.04	2.136	-0.04	8.44	2
6	1.992	-0.03	2.207	-0.04	1.720	-0.02	8.61	2.1
7	2.342	-0.04	2.664	-0.05	1.818	-0.03	8.80	2.25
8	1.969	-0.04	2.139	-0.02	1.296	-0.02	8.32	2.09
9	0.937	-0.03	1.343	-0.04	0.839	-0.03	8.86	0.8
10	1.427	-0.04	1.449	-0.04	1.176.	-0.02	8.86	1.34
11	2.331	-0.04	2.442	-0.04	2.012	-0.03	8.51	2.27
12	3.014	-0.05	3.234	-0.05	2.781	-0.04	9.14	2.8
13	3.364	-0.05	3.175	-0.05	2.793	-0.03	8.46	2.84
14	2.053	-0.04	2.703	-0.05	1.924	-0.03	8.88	2.35
15	3.723	-0.06	3.415	-0.05	3.174	-0.04	---	3.05*
16	4.346	-0.06	3.924	-0.06	3.562	-0.04	---	3.69*

* HPLC determination.

In order to study the variation of log k'_w in terms of ionization, the pKa determinations of compounds **1-14** have been performed (Table 1). As the 5-aryloxymethyl-2-amino-2-oxazolines are basic molecules (pKa = 8.67 ± 0.27, variation coefficient 3.1%), the ionization percentage is near 100% at pH 5.4, 99% at pH 6.4 and 89% at pH 7.4. So, at pH 7.4, besides the cation form, the neutral (uncharged) species are present.

Table 1 shows the variations of the log k'_w values with the pH of the eluent. For most compounds, the capacity factors are almost comparable at pH 5.4 and 6.4, which is related to similar ionization effects. But, the log k'_w values are very often minimum at pH 7.4. This last result seems paradoxical, because it takes no account of the contribution of the less polar species in developping the final retention of the solute.[13]

On the other hand, as a function of the mobile-phase apparent pH, the properties and the capacities of the modified stationary phase can, therefore, vary.[14]

Correlation Between Lipophilic Indices

For all compounds, the log k'_w values correlated with log $P_{o/w}$ according to the equations **1, 2, and 3** for the experimental data listed in Table I (where n is the number of data, r is the correlation coefficient, s is the standard error of the estimate, F is a measure of the significance of the correlation, and p is the probability level).

$$pH = 5.4 \log k'_w = 1.235\ (\pm 0.09) \log P - 0.389\ (\pm 0.211) \quad (1)$$
$$(n = 16,\ r = 0.965,\ s = 0.232,\ F = 189.021,\ p < 0.0001)$$

$$pH = 6.4 \log k'_w = 1.021\ (\pm 0.07) \log P + 0.163\ (\pm 0.164) \quad (2)$$
$$(n = 16,\ r = 0.969,\ s = 0.181,\ F = 214.617,\ p < 0.0001)$$

$$pH = 7.4 \log k'_w = 1.044\ (\pm 0.116) \log P - 0.362\ (\pm 0.271) \quad (3)$$
$$(n = 16,\ r = 0.924,\ s = 0.298,\ F = 81.602,\ p < 0.0001)$$

If the log $P_{o/w}$ values of compounds **15** and **16** are omitted from the analysis, then the correlation coefficient r always decreases (0.947, 0.945 and 0.863 at pH 5.4, 6.4 and 7.4 respectively).

As judged from the regression analysis, a significant correlation was established among the studied hydrophobic parameters, especially at pH 5.4 and 6.4, when the ionization percentage of all compounds is almost comparable.

This confirms the choice of log k'_w as a lipophilic index. However, at pH 7.4, near the pKa values, the correlation decreases slightly. Based on the knowledge of the pKa, the apparent partition coefficient D has been calculated at pH 7.4 from the log $P_{o/w}$ value.[16] Nevertheless, the substitution of log D instead of log $P_{o/w}$ did not improve the correlation.

Finally, we would like to develop the determination of log $P_{o/w}$ using HPLC as a detector, for lipophilic compounds. It is hoped that this technique will find widespread application due to its simplicity and reliability.

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DETERMINATION OF PORPHYRINS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH CHEMILUMINESCENCE DETECTION

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ABSTRACT

The peroxyoxalate chemiluminescence reaction, based on the oxidation of bis (2,4 dinitrophenyl) oxalate with hydrogen peroxide, was developed as a new detection method for the determination of porphyrins after separation by reverse phase high performance liquid chromatography. To optimize signal to noise ratio, experimental parameters such as design of the detection flow-cell and the effects of pH and organic solvents were investigated. Significant enhancements in detectability, selectivity and linearity were demonstrated for the determination of various hydrophilic and hydrophobic porphyrins when compared to absorbance and fluorescence methods (e.g., the detection limits (S/N = 3) for uroporphyrin and coprophorphyrin were about 40 and 5 fmol, respectively). Application of this method for the sensitive and selective determination of urinary porphyrins was also demonstrated.

INTRODUCTION

Porphyrins are naturally occurring tetrapyrrole derivatives with a porphine structure and play a key role in biological processes.[1] Disorders arising from inherited or acquired defects in biosynthetic pathway of heme in the bone marrow and liver are known as porphyrias.[2] Measurement of porphyrins present in biological materials is important for the diagnoses of these diseases.[3] Earlier methods of determination included liquid extraction,[4] ion exchange[5] and column chromatography,[6] followed by UV absorbance or fluorimetric determination. These methods were laborious and lacked sensitivity and selectivity. Thin layer chromatography (TLC) has improved the separation and quantitation of porphyrins[7] and the technique of high performance liquid chromatography (HPLC) has further enhanced the analysis speed, sensitivity and selectivity for the determination of porphyrins.[8]

Although UV-Vis absorbance detection has been widely used for HPLC, its detection limit was inadequate for the determination of minor porphyrin components.[9-11] Several authors have used fluorescence detection to improve sensitivity; however only a few publications have appeared in the literature so far[9-14] and background interferences from sample matrices appear to be a problem for the quantitation of various porphyrins present in real samples.

Peroxyoxalate chemiluminescence(PO-CL) has been demonstrated to be a highly sensitive and selective detection technique for the detection of biological molecules.[15] Albrecht et al.[16] have applied PO-CL for the screening of total porphyrin content in urine and the detection limit was about 250 μg/L. Recently, determination of porphyrins with PO-CL by coupling with TLC,[17] cloud point extraction,[18] and flow injection analysis[19] have been reported. In the present work, we further establish PO-CL as a new detection method for porphyrin determination by combining with HPLC separation.

EXPERIMENTAL

Pure standards of uro-, copro-, hemato-, proto-, zinc proto-porphyrins and hexa-, penta-carboxylic acids were obtained from Porphyrin Products (Logan, UT USA). HPLC grade acetonitrile, hydrogen peroxide (30%) were purchased from Fisher. High purity of bis(2,4 dinitrophenyl) oxalate (DNPO) was purchased from Fluka (Ronkonkoma, NY). All other reagents were of analytical grade obtained from Fisher and Aldrich. Doubly distilled deionized water was used.

The porphyrin standards were dissolved in a small amount of 1 M NaOH and were then diluted with doubly deionized water. Except for proto- and zinc proto-porphyrins, all other porphyrin standard solutions were stable for at least one week when kept in the dark. Proto- and zinc proto-porphyrin standard solutions have to be prepared just before use. DNPO with concentrations of 1.0-4.0 mmol/L in ethyl acetate and H_2O_2(3%-5%) in acetonitrile were used respectively and were made up just before each experiment and used immediately.

Instrumentation

The HPLC system consisted of a Varian 5500 liquid chromatograph(Houston, TX, USA), a model 7125 sample injector with a 10 μL injection loop (Rheodyne, Cotati, CA, USA), and a Perkin-Elmer model 610-S fluorescence detector (Norwalk, CT, USA) equipped with a 10 μL flow cell. Fluorescence excitation was performed at 400 nm with a bandpass of 20 nm and emission was collected at 620 nm with a bandpass of 20 nm. Chromatograms were recorded on a Perkin Elmer LCI-100 integrator or on a strip-chart recorder.

A model 7611-00 Cole-Parmer Ismatec Schlauch pump (Chicago, IL, USA) was used to deliver the chemiluminescence reagents for postcolumn reaction. The detection system consisting of a 10 μL quartz-tube micro flow-cell, a EMI-Gencom INC. model RFI/B-258B 211 photomultiplier (Plainview, NY, USA), a current amplifier, a high-voltage supply and a Perkin Elmer LCI-100 Integrator.

The HPLC analytical column was a 5 μm Econosphere C18 (150 x 4.6 mm I.D.), (Alltech, Deerfield, IL, USA). This column was protected by a 3 cm refillable pellicular C18 guard column (Alltech, Deerfield, IL, USA).

Chromatographic Conditions

HPLC grade acetonitrile and doubly-distilled deionized water were vacuum filtered through a 0.45 μm filter. Eluent A was a mixture of 95% acetonitrile with 5% 0.02 M phosphate solution (pH = 7.0). Aqueous mobile phase (eluent B) was prepared by first dissolving sodium phosphate monobasic in water to give a 0.02 M solution and adjusted to pH 3.5 with phosphoric acid. Acetonitrile was added to give a 5% mixture (V/V). Both eluents were degassed further by sonication for five minutes. Table 1 shows the linear gradient conditions employed for the separation of various porphyrins.

Table 1

Linear Gradient Conditions

Time (min)	Eluent A*	Eluent B**
0.00	20	80
5.00	60	40
10.0	62	38
10.5	100	0
17.0	100	0
17.5	20	80
20.0	20	80

* Eluent A was 95% CH_3CN, 5% 20 mM phosphate buffer (pH = 7.00).

**Eluent B was 5% CH_3CN, 95 % 20 mM phosphate buffer (pH = 3.50).

Sample Preparation

Urine samples were collected from healthy adults over a twenty four hour period and were preserved with sodium carbonate (2g/L urine) and ethylenediaminetetraacetate (3g/L urine) and stored under refrigeration in the dark at 4°C. Before injection, urine was filtered through a 0.20 mm filter membrane and readjusted to its previous pH (~6.8).

RESULTS AND DISCUSSION

Design of Post-Column Detection System

Although TCPO is a more stable oxalate reagent and is widely used in PO-CL HPLC systems, it does not offer sufficient sensitivity for the determination of minor porphyrin components in urine without preconcentration.[18] On the other hand, DNPO could offer much higher sensitivity as a result of shorter CL half-life when compared to TCPO, provided that the detection system is capable of exploiting the fast kinetics of the DNPO system. Using a FIA system[19] we have demonstrated that detection limits in the 10^{-9} M range can be achieved for the DNPO-CL detection of various porphyrins under optimized conditions; in the present HPLC system, a slight modification of the FIA detection flow-cell was carried out to maximize S/N.

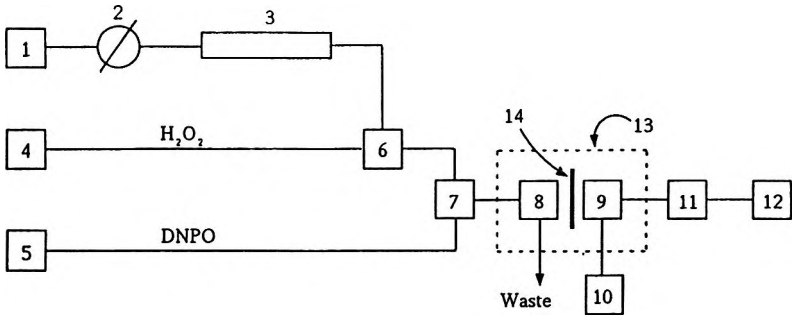

Figure 1. Schematic diagram of HPLC system with postcolumn chemiluminescence detection of porphyrins. 1 - LC pump; 2 - injection valve; 3 - HPLC column; 4,5-pumps for DNPO and H_2O_2, 6,7 - mixing fee; 8 - detection flow-cell; 9 - photomultiplier tube; 10 - high voltage power supply; 11 - current amplifier; 12 - integrator or recorder; 13 - dark box; 14 - 570 nm cut-off filter.

Figure 1 shows a schematic diagram of the postcolumn PO-CL detection system for porphyrins. The eluent from the HPLC column first mixed with H_2O_2 within a low dead-volume stainless-steel tee and then the solution mixed with DNPO inside another tee that was connected to the detection flow-cell via a PTFE tubing (see Fig. 2). The distance between the point at which the eluent, H_2O_2 and DNPO converged (mixing point) and the detection window was found to be critical in maximizing the CL signal intensity.

To optimize this particular distance, the length of the PTFE tubing which connected the second mixing tee and the detection flow-cell was varied and a distance of ~5 cm was found to provide the best S/N.

Optimization of PO-CL Conditions

The effect of flow rate on the CL intensity were investigated and the relative CL intensity was found to reach a maximum and then decreased gradually when the flow rate was increased from 0.5 to 2.5 mL/min. In the range of 0.75-1.50 mL/min, relatively high CL signals, low noise, and reproducible peaks were observed, and the CL signal decreased about 20% when the flow rate exceeded 2 mL/min. We chose 1.25 mL/min as the optimal flow rate for our experiments, since it provided the best S/N while minimizing peak broadening.

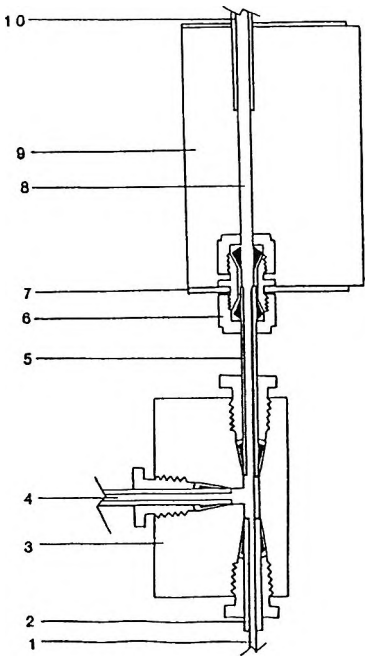

Figure 2. Diagram of detection flow-cell interfaced to the mixing-tee. 1 - 0.32 mm I.D. capillary column for DNPO; 2 - 0.4 mm I.D. PTFE tubing; 3 - mixing tee; 4 - 0.32 mm I.D. PTFE tubing for HPLC eluent mixed with H_2O_2; 5 - flow-cell connecting tubing (0.32 mm I.D. PTFE tubing); 6 - 1/16 in. swagelok union; 7 - light seal (darkened metal plate); 8 - location of the detection window; 9 - flow cell holder; 10 - waste outlet.

The effect of DNPO concentration was also examined. The CL signal increased with an increase in DNPO concentration; however, from 2 mmol/L to 4 mmoll/L, the signal intensity increased only about 10%, and at higher concentrations, the reaction rates appeared to occur too fast for the detection system to capture and gave poorer S/N noise. So, the optimum DNPO concentrations fall in the range of 1.5 mmol/L to 2.0 mmol/L.

Effect of pH and Organic Solvent

An important consideration in adapting PO-CL detection to HPLC is that the pH and solvents used for efficient/selective HPLC separation should be compatible with those employed for providing optimum CL detection.[20]

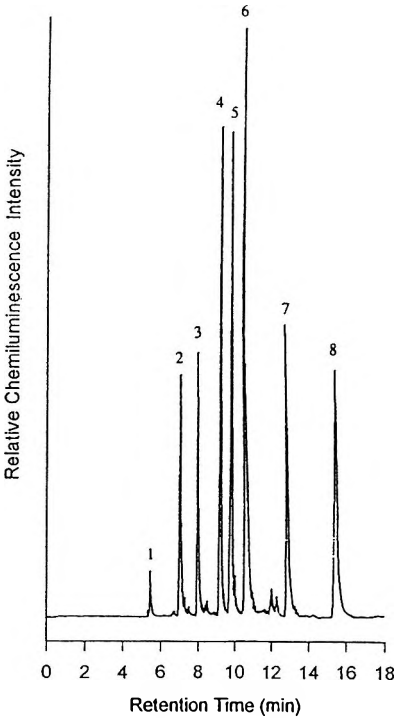

Figure 3. Chromatogram of porphyrin standards (each at 250 μg/L). u = uroporphyrin; 6: hexacarboxylic acid porphyrin; 5: pentacarboxylic acid porphyrin; C1: coproporphyrin I; C3: coproporphyrin III; HP: hematoporphyrin IX; Znpp: zinc protoporphyrin and PP: protoporphyrin.

Based on our previous studies,[19] DNPO can be used in a solvent mixture of up to 35% H_2O with no significant effect on the CL intensity for most porphyrins. Thus, it is possible to use a mobile phase consisting of a relatively high percentage of an aqueous buffer while maintaining optimum conditions for PO-CL detection of porphyrins. Also, based on our earlier work,[19] we found that the optimum pHs for performing CL detection are as follows: uroporphyrin (pH = 4 to 4.5); copro- and other porphyrins (pH = 5 to 7), and fortunately, these pHs happened to fall within the pH range of mobile phases employed for efficient/selective porphyrin separations in HPLC.[9-14] Table 1 shows the linear gradient conditions employed in our experiments for achieving optimum HPLC separation and PO-CL detection of porphyrins.

Table 2

Linearity, Detection Limits and Precision Data

Paramter	Uro-	Hexa	Penta	CoproI	CoproIII	Hemato	Znpro-	Proto
Detection Limit (μg/L)	3.0	0.71	0.63	0.43	0.43	0.35	0.62	0.78
Linear Range (μg/L)	$0\text{-}10^3$	$0\text{-}10^3$	$0\text{-}10^3$	$0\text{-}10^3$	$0\text{-}10^3$	$0\text{-}10^3$	$0\text{-}10^3$	$0\text{-}10^3$
Interception	1.21	0.66	2.99	3.49	3.90	4.57	2.84	1.87
Slope	0.05	0.14	0.19	0.47	0.46	0.64	0.19	0.13
Correlation Coefficient	0.997	0.999	0.999	0.998	0.997	0.998	0.998	0.998
R.S.D. (%) (n = 6)	1.56	2.78	0.96	0.43	0.40	0.46	0.37	0.42

Figure 3 illustrates a chromatogram of porphyrin standards which consisted of 250 μg/L each of uro-, hexa-, penta-, copro-I and III, hemato-, zinc proto- and proto-porphyrins, showing complete baseline separation for all porphyrins within 18 minutes. Table 2 shows the results of calibration data, detection limits and precision. Importantly, when compared to absorbance detection,[11] an improvement factor ranging from about 50 to 100 was obtained with PO-CL detection for the porphyrins investigated in this study. When compared to fluorescence detection,[9,11,14] an improvement factor of about 5 to 10 was obtained.

Fig. 4 shows a chromatogram of a urine sample from a healthy individual using PO-CL detection. When compared to chromatograms obtained with fluorescence detection,[9-14] an important difference is that when fluorescence detection is employed, large amounts of non-porphyrin fluorescent substances are usually eluted ahead of the porphyrins and lead to the appearance of a large, broad band near the column void volume; however, such potential background interference is clearly absence from the chromatogram shown in Fig. 4, thus demonstrating the superior selectivity of PO-CL detection. By comparing with retention times of porphyrin standards as shown in Fig. 3 and chromatograms from urine samples spiked with various porphyrin standards, peak assignments in Fig. 4 can be made as follows: Peak 1 = uroporphyrin: peak 2 = hexacarboxylic acid porphyrin; peak 3 = pentacarboxylic acid porphyrin; peak 4 = coproporphyrin I and peak 5 = coproporphyrin III. It can be seen that peak 1 is actually comprised of two small

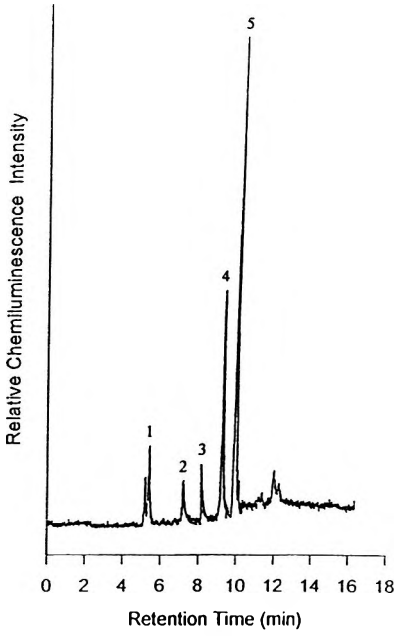

Figure 4. Chromatogram of urinary porphyrins from a healthy adult female.

peaks, arising possibly from uroporphyrin I and III isomers. Unfortunately, a standard of uroporphyrin III was not available during our experiments and the identity of the latter peak at the location of peak 1 is not certain. However, very similar peaks, in terms of relative elution order and peak area, have been reported for the HPLC separation of uroporphyrin I and III isomer standards using fluorescence detection.[14] Concentration of uroporphyrin I and coproporphyrin I and III as shown in Fig. 4 were found to be 48 μg/L, 10 μg/L and 25 μg/L, respectively, which are within the normal concentration range found in urine of healthy individuals.[1] For recovery tests, various known concentrations of porphyrin standards were spiked into urine samples and recoveries fall between 96 to 101%.

In summary, the PO-CL reaction has been successfully coupled with HPLC using DNPO as the oxalate reagent for the HPLC determination of various hydrophilic and hydrophobic porphyrins. Significant improvements in limits of detection and selectivity were demonstrated for the measurement of micro-trace amounts of porphyrins in urine samples when compared to conventional detection and selectivity methods. Such improved analytical capabilities should be valuable to clinical and biomedical scientists who engage in porphyrin research.

ACKNOWLEDGMENTS

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CHIRAL RESOLUTION OF SEVERAL PHENOTHIAZINE COMPOUNDS AND TRIMIPRAMINE, A DIBENZAZEPINE DRUG ON CHIRALCEL OJ-R

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ABSTRACT

The Chiralcel OJ-R phase was tested on its discriminative properties towards six phenothiazine compounds, namely promethazine, oxamemazine, thiazinamium, ethopropazine, trimeprazine, and dixyrazine, besides one dibenzazepine compound, trimipramine. The effect of changes of the mobile phase upon the resolution of the enantiomers was investigated.

Acetonitrile supports a symmetrical peak shape while methanol promotes chiral interactions of the analytes on the stationary phase. Consequently the highest resolutions were mostly found for analyses with ternary mixtures of acetonitrile, an alcohol and aqueous sodium perchlorate solutions. Only the drugs with small substituents on side-chain nitrogen showed generally a satisfactory chiral interaction; promethazine and thiazinamium, the two most resembling analytes, could be baseline separated.

INTRODUCTION

The Chiralcel OJ-R column is a recently developed member of the successful family of polysaccharide polymer phases.[1] Racemic phenothiazine drugs are a group of medicines that belong to different pharmacological classes. Promethazine, oxamemazine, trimeprazine, and thiazinamium have antihistaminic properties; dixyrazine is an antipsychotic and ethopropazine an anticholinergic drug. Trimipramine, a dibenzazepine compound, is an antidepressant. The chemical structures of these drugs are given in Fig. 1.

HPLC analysis on different chiral stationary phases has already been envisaged for most of these drugs. Direct chiral resolution was successfully accomplished for trimipramine with α_1-glycoprotein added tc the mobile phase[2] or immobilised on silica, described for several phenothiazine-related drugs.[3-5] β-Cyclodextrin stationary phases have proved to be also appropriate for this type of compounds.[6,7]

Ponder *et al.* examined the resolution of promethazine, ethopropazine, trimeprazine and trimipramine enantiomers on a variety of columns, including the Chiralcel OJ phase using n-hexane mixed with ethanol or isopropanol.[8]

This study investigates whether this similar tris(4-methylbenzoyl) ester of cellulose (Fig. 2) that is to be used under reversed phase conditions, allows the chiral discrimination of the cited drugs. The Chiralcel OJ-R phase can be applied with a greater variety of eluents.

For the aqueous portion, sodium perchlorate was dissolved up to a concentration range of 0.1 M to 1.0 M. Other components of the mobile phase that were tested included acetonitrile, methanol, ethanol, propanol and isopropanol.

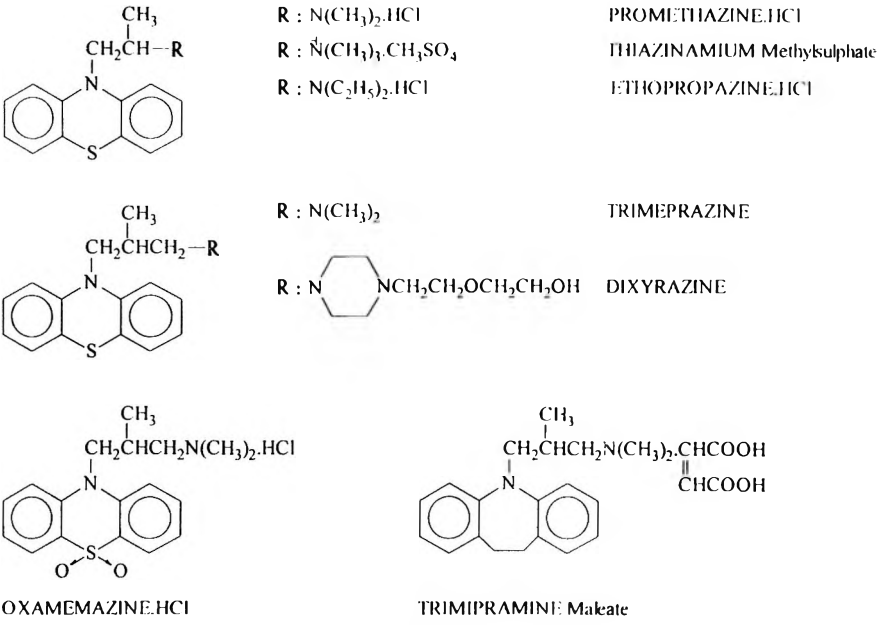

Figure 1. Chemical structures of the chiral compounds used in this study.

MATERIALS

Chemicals

The racemic drugs promethazine hydrochloride, trimipramine maleate, oxamemazine hydrochloride and thiazinamium methylsulphate were provided by SPECIA (Paris, France). Dixyrazine was obtained by extraction from Esucos® tablets (UCB Pharma, Belgium). Ethopropazine hydrochloride and trimeprazine hemi-tartrate were purchased from Sigma-Aldrich (Bornem, Belgium).

The latter drug was treated to set free the base form. It was also transformed into its hydrochloric salt but no significant difference was observed

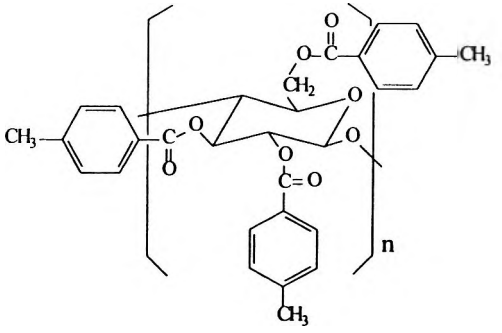

Figure 2. Monomer unit of the Chiralcel OJ-R cellulose derivative, coated on a silica support.

between the chromatograms of the base form and the salt under several conditions tested.

All solvents were of analytical or HPLC quality; sodium perchlorate was purchased from Merck (Darmstadt, Germany). Deionised and distilled water was used throughout.

Apparatus and Chromatographic Conditions

Chromatography was performed on a 15 cm x 4.6 mm I.D. Chiralcel OJ-R column (Daicel Co., Tokyo, Japan) at ambient temperature. The different constituents of the mobile phase were mixed instantly in the appropriate proportions by a Varian 9010 SDS pump (Varian Associates Inc., Walnut Creek, CA, USA) and pumped at a constant flow of 0.5 mL min^{-1}. Analytes were dissolved in either acetonitrile:water 30:70 or in methanol, since pure acetonitrile as a solvent gave rise to bad peak shapes (fronting due to its high elution strength) and the use of methanol generally resulted in comparable peak shapes, as when taking the mobile phase as solvent. The solutions contained 100 µg mL^{-1} of racemic drug and were kept in the dark. The volume injected was 20 µl (Rheodyne injector).

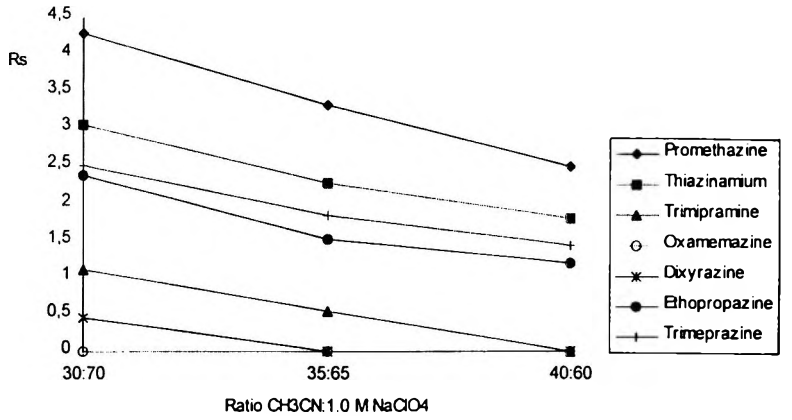

Figure 3. Influence of the change in volume ratio CH_3CN:1.0 M $NaClO_4$ of the mobile phase upon the resolution.

Detection was achieved at two wavelengths simultaneously (230 and 254 nm) with a Hewlett Packard 1050 Diode Array Detector (Waldbronn, Germany). Integration of the more intense chromatogram was made with the Hewlett Packard software package (1990), being at 254 nm for all analytes except for oxamemazine. The following parameters were measured:

k'1 : capacity factor of the first eluted enantiomer: $(t_1-t_0) / t_0$.

k'2 : capacity factor of the second eluted enantiomer: $(t_2-t_0) / t_0$.

The elution time of methanol was used to determine the t_0-value on Chiralcel OJ-R.

α : selectivity factor: k'2 / k'1.

Rs : resolution factor: $Rs = 1.18 (t_2-t_1) / (w_1+w_2)$; w is the width at half-height of the peak based on peak area and height.

Rp : Kaiser's peak separation index: the ratio of valley height between two peaks and the peak height, which rises to 1 for perfectly separated peaks. This factor was therefore preferred to Rs factors for enantioms that were not completely resolved.

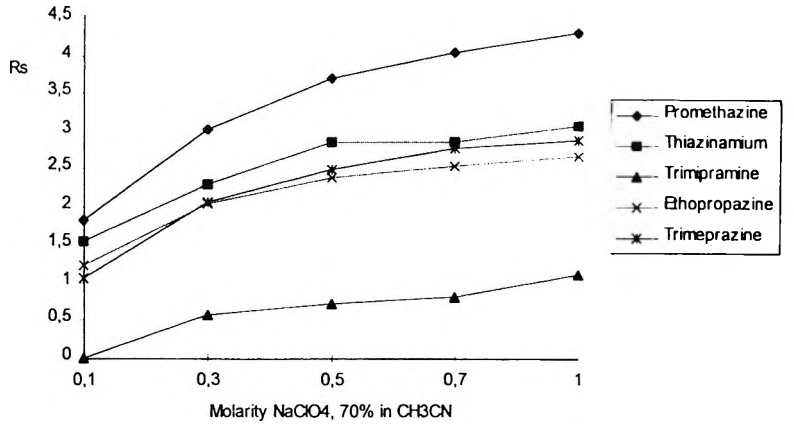

Figure 4. Influence of the molarity of the $NaClO_4$ solution mixed with acetonitrile as mobile phase upon the resolution.

RESULTS

At first stage, a binary mixture of acetonitrile and aqueous $NaClO_4$ solutions were applied since this mobile phase has proved very useful in other RP-Chiralcel systems. Similarly, as seen with the commonly used reversed phase HPLC systems, rendering the mobile phase more polar enhances the retention times of the analytes on the cellulose-based stationary phase; therefore the greater the portion of acetonitrile the quicker the compounds elute. By increasing the aqueous portion, better resolutions can be obtained at the expense of higher retention times. Substitution of 5% acetonitrile by aqueous fraction, nearly doubles elution times. In Fig. 3, the effect of different ratios of acetonitrile:1.0 M $NaClO_4$ upon the resolution is displayed.

Higher molarity of sodium perchlorate slightly enhanced retention times of the compounds and clearly improved chiral interactions. The salt concentration of sodium perchlorate was increased from 0.1 M to 1.0 M and the effect on the resolution examined (Fig. 4). Oxamemazine could not be chirally separated and dixyrazine enantiomers were only slightly resolved (Rs = 0.5). Further analyses were performed with 1.0 M salt solution. The use of perchlorate buffer solutions (acidified with concentrated perchloric acid to pH 2) had merely a small effect on the peak shape; peak broadening was reduced a little.

Table 1

Chiral Resolution on the Chiralcel OJ-R Column using Ternary Mixtures as Mobile Phase

Drug	Mobile Phase Composiiton (v/v)					Rs(Rp)*	k'_1	α
	CH_3CN	$NaClO_4$ 1.0M (aq)	CH_3OH	C_2H_5OH	C_3H_7OH			
Promethazine	20	40	40			3.18	2.04	1.98
	15	40	45			3. 7	4.49	2.20
	15	50		35		1.67(0.98)	2.95	1.64
	15	65			20	1.81(0.98)	2.40	1.52
Thiazinamium	20	40	40			1.93	2.47	1.86
	15	40	45			2.98	5.49	1.98
	15	50		35		1..30(0.93)	2.52	1.78
	15	65			20	1.29(0.91)	1.90	1.54
Ethopropazine	20	40	40			1.30(0.84)	2.44	1.33
	15	40	45			1.47(8.89)	2.90	1.38
	15	50		35		1.06(0.64)	2.64	1.34
	15	65			20	0	0.38	1
Trimeprazine	20	40	40			1.35(0.87)	3.54	1.32
	15	40	45			1.50(0.86)	4.68	1.34
	15	50		35		0.91(0.40)	3.85	1.23
	15	65			20	0	0.36	1
Trimipramine	20	40	40			0	2.22	1
	15	40	45			0.89(0.30)	5.02	1.20
	15	50		35		0	2.98	1
	15	65			20	0	2.45	1
Dixyrazine	20	40	40			0	3.71	1
	15	40	45			1.02(0.48)	9.33	1.36
	15	50		35		0	4.11	1
	15	65			20	0	2.81	1

* The Kaiser peak resolution factor is given between brackets for the not completely baseline separated enantiomers.

The addition of methanol as a third constituent of the mobile phase had an advantageous influence on the chiral interaction. The resulting resolutions however were not as high as could be expected from the improved selectivity factors due to peak broadening when using alcohols. Table 1 lists the chromatographic parameters for various ternary mixtures using different primary alcohols. Oxamemazine is not included as its enantiomers could not

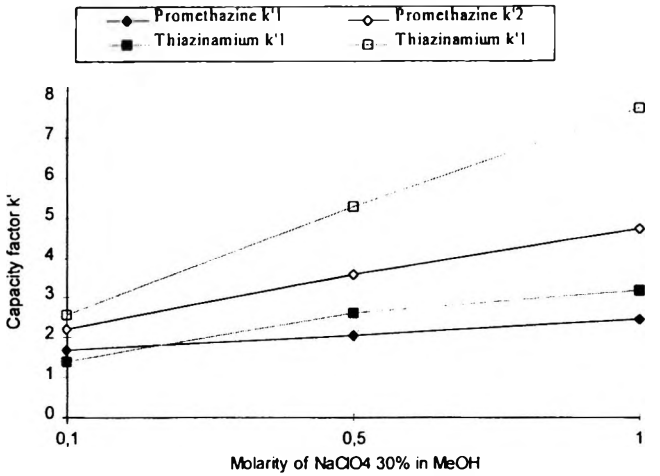

Figure 5. Influence of the molarity of the $NaClO_4$ solution mixed with methanol as mobile phaseupon the capacity factors of promethazine and thiazinamium.

be separated under any of the applied conditions. Dixyrazine and trimipramine only showed chiral interaction if the elution time of the less retained enantiomer was prolonged up to at least 20 minutes. Substitution of 1-propanol by 2-propanol interfered with the chiral interaction of the analytes. No chiral discrimination was observed using this secondary alcohol.

From Table 1, it is clear that only promethazine and thiazinamium, the two most similar compounds tested, interact satisfactorily with the Chiralcel OJ-R column and that methanol is the preferred alcohol. Therefore methanol was added in various proportions to 1.0 M $NaClO_4$. The peak broadening effect prevented easy baseline separations despite the enhanced selectivity factors compared to acetonitrile composed eluents. Plate numbers however amount to less than 10 % for methanol compared to acetonitrile composed mobile phases. No chiral discrimination was observed for trimipramine, oxamemazine and dixyrazine. The influence of the concentration of sodium perchlorate was striking in combination with methanol as well. This is represented in Figure 5 showing the improved separations for the promethazine and thiazinamium enantiomers.

Figure 6 illustrates the similar effect of mobile phase modification on the chromatographic behaviour of promethazine and thiazinamium. Figure 7 shows chromatograms of the analyses of ethopropazine and trimeprazine and Figure 8 gives an idea of the poor chiral interaction of trimipramine and dixyrazine on the Chiralcel OJ-R column.

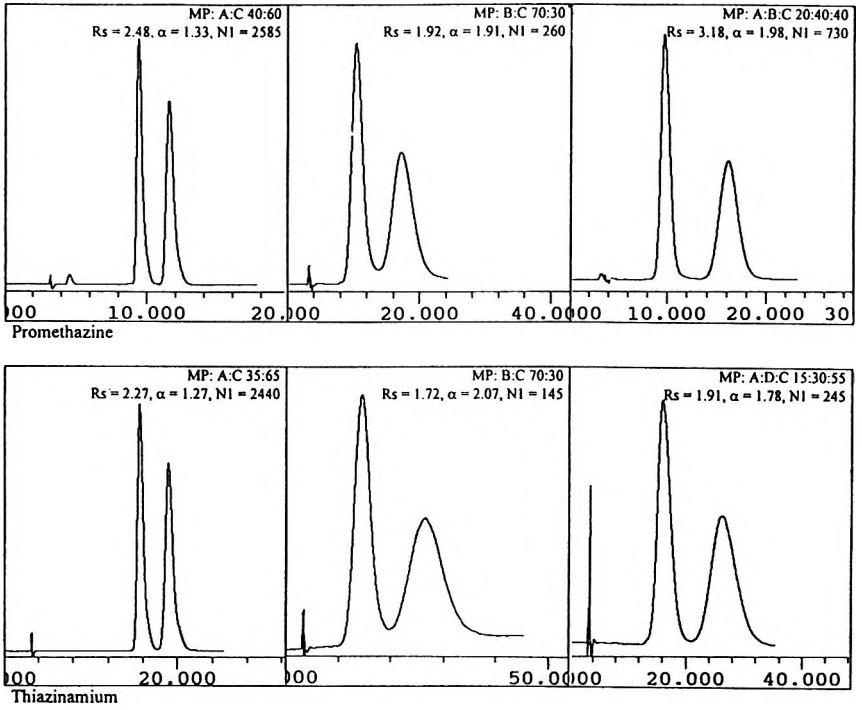

Figure 6. Chiral resolution of promethazine and thiazinamium using varied mobile phase compositions.
MP: mobile phase; A: acetonitrile; B: methanol; C: 1.0 M sodium perchlorate solution; D: ethanol. Other parameters: cf. Materials section

DISCUSSION

Cellulose tris (4-methylbenzoate) is a synthetic polymer which exists as a β-polymeric chain of derivatized D-(+) glucose residues in β-1,4-linkage. These chains lie side by side and possess a certain degree of rigidity and assume an extended helical structure into which the enantiomers of the analytes can interact stereospecifically. The chiral recognition process may thus proceed through interactive forces such as H-bonding, dipole, and π–π interactions between the analytes, and the 4-methylbenzoate moieties of the cellulose polymer, but is also determined by the formation of appropriate interstrand and intrastrand inclusion complexes between the chiral cavities in these cellulosic polymers and the cyclic groups in the drugs investigated in this study. A detailed adsorption mechanism of the chiral solutes versus the polymer has not been established in detail thus far.[9-11]

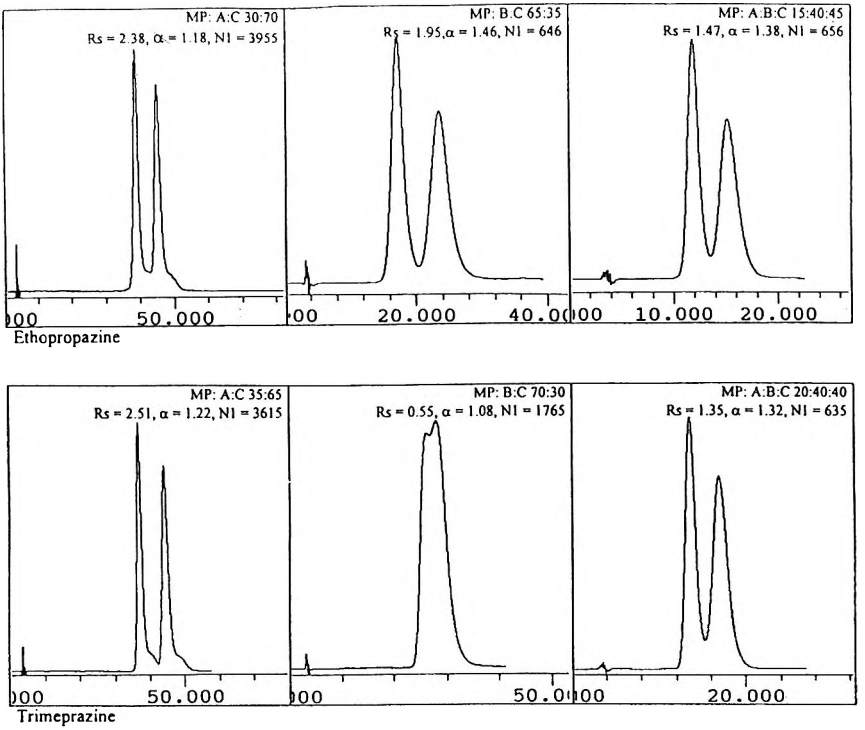

Figure 7. Chiral resolution of ethopropazine and trimeprazine using varied mobile phase compositions.
MP: mobile phase; A: acetonitrile; B: methanol; C: 1.0 M sodium perchlorate solution. Other parameters: cf. Materials section

Francotte *et al.*[12] postulated that the chiral cavities on the cellulose polymer have a high affinity for the aromatic groups. Aromatic compounds would accordingly interact with the substituted derivatized cellulose, the 4-methylbenzoate group in this case, through π–π interactions. Therefore, a suitable sterical fit of (part of) the enantiomer into these cavities plays an essential role in effective resolution of the drug racemates. These multiple interactions, along with solvent effects, contribute to the resolution process for a chiral CSP of this type.

Strict stereospecific requirements are set for a sufficient selective interaction of both enantiomers, which is illustrated by the analyses of this limited group of compounds. Promethazine and thiazinamium only differ in the nature of the nitrogen in the side chain.

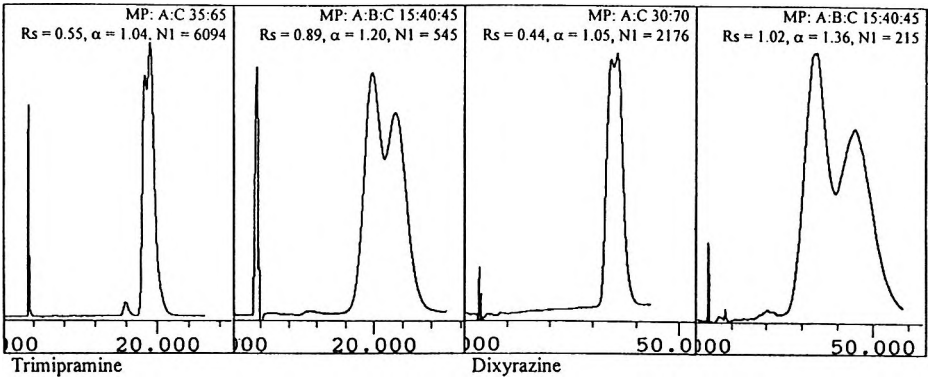

Figure 8. Chiral resolution of trimipramine and dixyrazine using varied mobile phase compositions.
MP: mobile phase; A: acetonitrile; B: methanol; C: 1.0 M sodium perchlorate solution. Other parameters: cf. Materials section

The chiral interaction got only slightly worse due to the additional methyl substituent on the nitrogen. If both methyl groups on this very nitrogen moiety of promethazine are replaced by ethyl groups (rendering it more lipophilic) as in ethopropazine, chiral interaction is greatly negatively affected.

While the latter three drugs have the hydrogen bonding site (-N-) in α-position to the chiral centre, in all the other compounds this site is distanced by one methylene group more (β). Although one could conclude out of the similar results obtained for promethazine and thiazinamium, that the hydrogen binding possibility of that nitrogen can be considered of minor importance, the additional length of one of the substituents on the chiral carbon atom interferes with adequate chiral interaction sites on the polymer; for the results are quite less satisfactory for trimeprazine compared to promethazine.

Moreover, any chiral discrimination is prevented when the sulfur moiety is oxidized (trimeprazine vs. oxamemazine). This indicates that the sulfur moiety with its free electron pair plays an essential role in the interaction phenomena towards the cellulose polymer. The negative effect of large substituents of the nitrogen moiety is confirmed comparing the results for trimeprazine and dixyrazine.

Replacing the sulphur bridge of trimeprazine by an ethylene group as in trimipramine will result in an increasing degree of non-planarity of the two aromatic rings i.e. the two aromatic rings in trimipramine become more folded along the central azepine heterocyclic ring. An enhanced degree of twisting of the aromatic ring thus probably prevents an advantageous stereopositioning of the tricyclic system towards the cellulose cavities.

Compared to the results on the Chiralcel OJ column as published by Ponder *et al.*,[8] the best chiral resolutions were also noted for promethazine, and near baseline separation was found for ethopropazine as well as for trimeprazine. The latter therefore clearly interacted more favourably on the normal phase analogue of the cellulose ester.

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FREE SOLUTION CAPILLARY ELECTROPHORETIC SEPARATION OF BASIC PROTEINS AND DRUGS USING CATIONIC POLYMER COATED CAPILLARIES

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ABSTRACT

A simple capillary coating which exhibits reversed (anodic) electroosmotic flow (EOF) has been developed for the separation of cationic compounds by free solution capillary electrophoresis. Poly(2-aminoethyl methacrylate hydrochloride) (PAEM) was chemically bound onto the fused-silica capillary inner wall. Capillaries modified by this method exhibit an essentially pH-independent EOF in the pH range from 3 to 5.5, and have been applied to the improved separation of basic proteins and drugs (e.g., beta-adrenergic blocking drugs). High efficiency and precise migration (relative standard deviation was less than 1%) were observed.

INTRODUCTION

Capillary electrophoresis (CE) has been developed into a new powerful analytical separation technique.[1-3] Samples ranging from small inorganic ions to biopolymers, *e.g.*, proteins, have been successfully separated by CE. CE separation is based on the different electrophoretic mobility of charged species. It offers the advantages of fast and efficient separation, high resolution, small sample and running buffer volumes, and simple instrumentation. The small sample size requirement makes it to be the preferred choice for bioanalysis. However, since its debut, practitioners of CE have soon realized that capillary electrophoretic separation of proteins with fused-silica capillaries is hampered by solute adsorption onto the capillary inner wall. This phenomena is primarily the result of electrostatic attraction between positively charged proteins and the negatively charged silanol groups on the capillary inner wall, and the hydrogen bonding between the solute and the capillary inner surface. Other types of interactions, i.e., van der Waals force, hydrophobic interaction, etc., may also contribute to this problem. These solute-wall interactions often lead to band broadening, peak asymmetry, nonreproducible migration, and low recovery of separated species. In extreme cases, positively charged analytes may irreversibly adhere onto the capillary inner wall. Although these interactions are sometimes observed with small molecules, the relevant forces increase with the size of molecule so that large molecules, such as proteins are most severely affected and present unique challenges to the separation method.

Several attempts have been taken to eliminate or minimize these solute-wall interactions. Generally, they can be grouped into three main categories: (1) capillary surface modification; [4-22] (2) manipulation of running buffer pH and ionic strength,[23-27] or use of buffer additives;[28-32] and (3) use of a radial potential field.[33] Capillary modification has demonstrated to be the most flexible for the separation of proteins. Extreme acidic or basic buffers have successfully been employed to minimize the undesired solute-wall interactions. Acidic buffers reduce the ionization of silanol groups on the capillary surface and thus diminish the ionic interactions between solutes and the capillary surface. Basic buffers reverse the negative charges on the proteins to positive charges, thus reducing the undesired ionic interactions.

Limitations of running CE under these extreme pH conditions include: a) a more restricted pH operating range; b) the capillary may be degraded under these pH conditions; c) the resolution may be reduced as all the solutes have the similar ionization status; and d) some proteins may be denatured under extreme pH conditions.

High ionic strength buffers are effective in minimizing solute-wall interactions, but they also increase the electricity current and thus increase the Joule heating which may degrade the efficiency and reproducibility of the separation. The use of a radial potential field could directly control the zeta potential and showed the power to reduce solute-wall interaction in acidic pH range, but it did not work well at alkaline pH conditions and it also led to complicated instrumentation. The use of buffer additives is an effective method to reduce the solute-wall interactions. For example, fluorosurfactant[29, 30] has been successfully used to separate basic proteins.

Capillary modification by both covalent bounding and physical adsorption chemicals to its inner surface has been proven powerful strategy in reducing the solute-wall interactions. In recent years, many chemicals have been used as capillary modification reagents, such as: polyacrylamide,[4] poly(vinyl-pyrrolidinone),[6] polyethyleimine (PEI),[8] vinyl-bound polyacrylamide,[9] poly-ethylene glycol,[7] glycerolglycidoxypropyl,[6] poly(methylglutamate),[12] poly-ether,[13] chitosan,[19] polymers with quaternary ammonium groups,[14,15,18] cryptand,[18] polyvinyl alcohol (PVA),[28] and poly(diallyldimethyl-ammonium chloride) (PDADMAC).[22] Among the listed coatings, PEI, PDADMAC, chitosan, and other positive polymer coatings reverse the charge and zeta potential on the capillary inner wall, and produce a reversed electroosmotic flow (EOF). They are effective in reducing the adsorption of basic proteins onto the capillary inner wall.

In the present paper we report a method to chemically modify fused-silica capillary with Poly(2-aminoethyl methacrylate hydrochloride) (PAEM). Capillaries modified by this method were used to improve the separation of cationic proteins and drugs. Efficient and symmetric peaks were observed using PAEM coated capillaries. High precision of protein migration was also observed. The coating reproducibility was excellent.

EXPERIMENTAL

Instrument

All separations were performed on a laboratory-constructed capillary electrophoresis instrument enclosed in a plexiglas box, which consists of a HPCE high power supply (Spellman, Plainview, NY, U.S.A.), a high power supply local control (Chamnoix Industries, Johnson City, NY, U.S.A.), a

Spectra 100 UV detector (Thermal Separations, Freemont, CA, U.S.A.) and a LCI 100 integrator (The Perkin Elmer, Norwalk, CT, U.S.A.). Injections were accomplished hydrodynamically by elevating the sample reservoir to a height of approximately 5 cm above the other buffer reservoir for approximately 5 seconds.

Reagents and Materials

Fused-silica capillary of 75 μm I.D. was purchased from Polymicro Technology (Phoenix, AZ, U.S.A.). Protein standards, ammonium persulfate, and N,N,N',N'-tetramethylethylene-diamine (TEMED) were purchased from Sigma (St. Louis, MO, U.S.A.). All beta-blockers were gifts from Professor R. Kaliszan (Medical Academy and University of Gdansk, Poland).

Methacryloxypropyltrimethoxysilane was obtained from Hüls American Inc. (Bristol, PA, U.S.A.). 2-aminoethyl methacrylate hydrochloride and all other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.).

Capillary Modification

Fused-silica capillaries were first conditioned with 1 M sodium hydroxide for one hour, then rinsed with deionized water for thirty minutes. A solution of 1% bifunctional compound, methacryloxypropyltrimethoxysilane (adjusted to pH 3.5 by acetic acid), was placed into a capped vial to which one end of the capillaries was immersed, and a syringe was used to apply a pressure to the vial to push the solution into capillaries.

The bifunctional compound was allowed to react with the capillary inner wall for two hours at room temperature. Capillaries were then rinsed with deionized water.

A solution of *ca.* 10% 2-aminoethyl methacrylate hydrochloride (AEM), containing 1 to 3 mg/mL TEMED and *ca.* 1 mg/mL ammonium persulfate was drawn into the capillaries and allowed to react with the bifunctional compound until polymerization was completed. The excess unbound polymer and monomer were removed from the capillaries by rinsing capillaries with deionized water and the capillaries were left to dry at 30 to 40°C overnight.

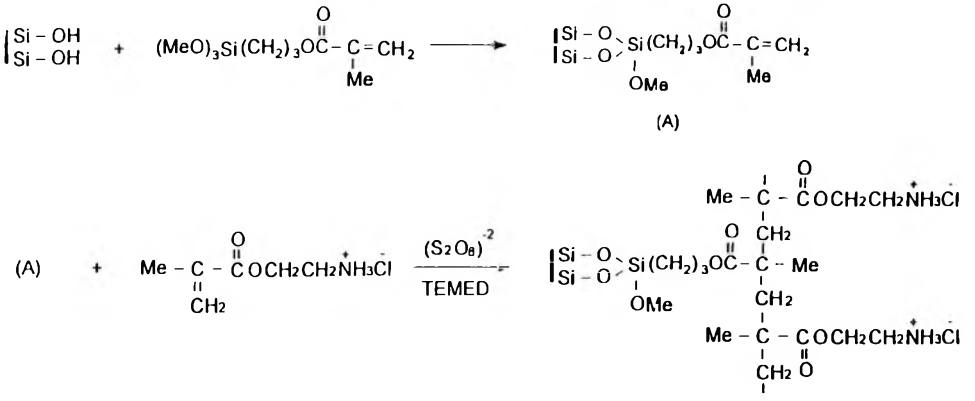

Figure 1. Reaction scheme for the preparation of PAEM chemically coated capillaries.

RESULTS AND DISCUSSION

Capillary Modification

The reaction of bifunctional compound, methacryloxypropyl-trimethoxysilane, with fused-silica capillary inner wall was discussed by Hjerten.[4] The polymerization of 2-aminoethyl methacrylate hydrochloride was previously discussed by Smith and co-workers.[34] Figure 1 shows the chemical reaction scheme for capillary surface modification. When chemically binding PAEM to the capillary inner wall, one must be careful to maintain the monomer solution in acidic condition. It is known that in neutral or basic conditions the monomer may rearrange before polymerization takes place.[34] After polymerization, the polymer chain and the dipole-dipole interaction or hydrogen bonding between the amine and its neighboring ester group prohibit the formation of the cyclic intermediate, therefore no rearrangement takes place after polymerization.[34]

It is interesting to note the potential to further modify the PAEM coated capillary via the active amino groups on PAEM. For example, it should be possible to immobilize proteins or other reagent on PAEM via a carbodiimide linkage to the free amino groups. It may also be possible to tune the EOF by copolymerizing AEM with neutral monomers, such as acrylamide, during the synthesis process.

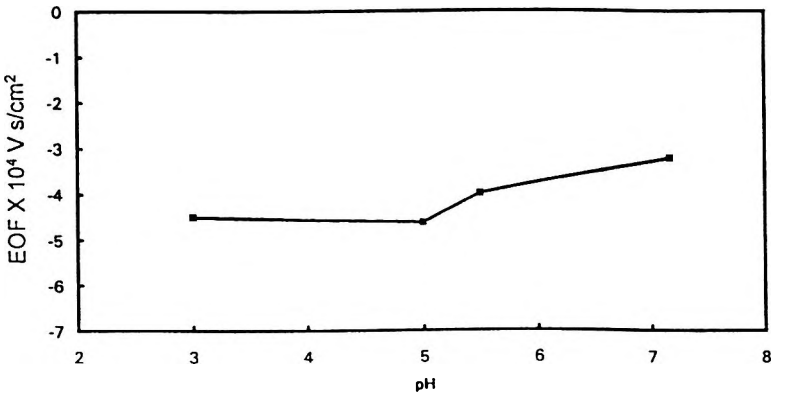

Figure 2. Plot of EOF of PAEM coated capillaries as a function of buffer pH. 75 μm I.D. × 360 μm O.D. PAEM coated capillary, 62.2 cm (45.1 to detector). Hydrodynamic injection at cathode. Acetone was the neutral marker. Detection wavelength was 205 nm. 15 kV applied voltage. Buffers were 10 mM phosphate (pH 3.0 and 7.2) and 17 mM acetate (pH 4.0 and 5.5).

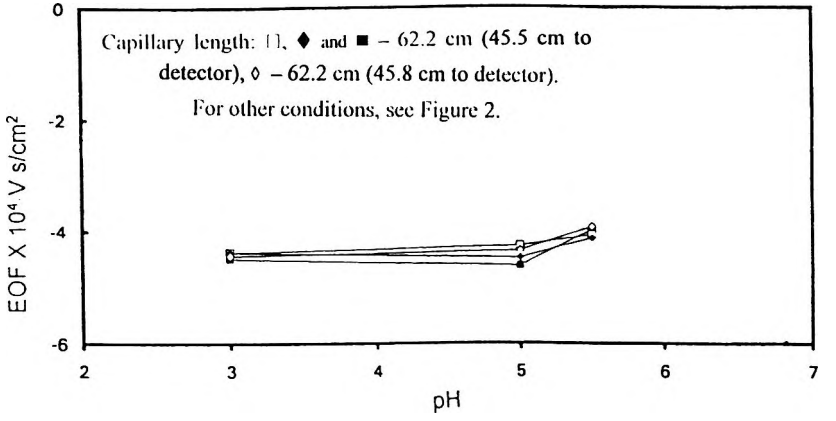

Figure 3. Reproducibility of PAEM coating. EOF of four independently prepared capillaries at selected buffer pH. Capillary length:. □, ◆ and ■ - 62.2 cm (45.5 cm to detector), ◇ - 62.2 cm (45.8 cm to detector). For other conditions, see Figure 2.

Electroosmotic Flow of Capillaries Modified with PAEM

The positive charges on the polymer, poly(2-aminoethyl methacrylate hydrochloride) (PAEM), reverse the charges on the capillary inner wall and the zeta potential of the capillary inner surface in acidic pH range, thus reversing the electroosmotic flow (EOF). Figure 2 shows the EOF as a function of running buffer pH. Acetone was used as the neutral marker when measuring the EOF. As shown in Figure 2, at pH values below 5.5, the EOF remained quite constant, because the amino groups on the polymer were almost fully protonized at this pH range. At pH values higher than 5.5, EOF changed with pH as the amino groups become partially ionized. There may be some active silanol groups remaining on the capillary surface, thus at higher pH their dissociation also affects the EOF.

Reproducibility and Stability of PAEM Coating

To test the reproducibility of PAEM coating, EOF of four independently prepared capillaries was measured at various pH values. The results are shown in Figure 3. As observed from Figure 3, the reproducibility was excellent. The relative standard deviation of EOF ranged from 1% to 3%.

To determine the stability of PAEM coated capillaries, a PAEM coated capillary was used for one week. During this period of time, the capillary was subjected to an electric field of *ca.* 250 V/cm under different pH conditions (in the range from 3 to 7.2) for a total of *ca.* 15 hours. Afterward, the capillary was subject to a continued electric field of 250 V/cm at pH 4.5 (25 mM acetate buffer) for 30 hours. No significant change in the EOF was observed (from μ_{eo} = 4.68×10^{-4} $cm^2V^{-1}s^{-1}$ initially to μ_{eo} = 4.66×10^{-4} $cm^2V^{-1}s^{-1}$). When an electric field of 250 V/cm was applied to this capillary for additional 18 hours, the EOF changed significantly (from 4.68×10^{-4} to *ca.* 2.6×10^{-4} $cm^2V^{-1}s^{-1}$). Thus the total lifetime for this capillary was more than 45 hours but less than 63 hours, under continuous high field conditions.

Separation of Basic Proteins and Drugs

It is usually difficult to separate basic proteins using bare fused-silica capillaries due to the solute-wall interactions. When PAEM coated capillaries were used, the separation of basic proteins was dramatically improved. As

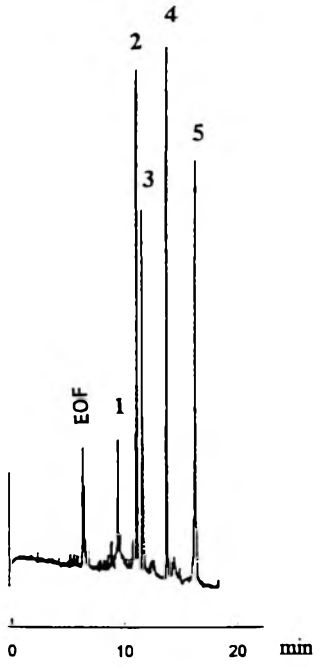

Figure 4. Separation of 5 proteins using PAEM coated capillary. 62.1 cm (44.6 cm to detector). 25 mM acetate pH 4.5 buffer. 15 kV applied voltage. Hydrodynamic injection at cathode. Analytes: 1- myoglobin, 2- ribonuclease A, 3- trypsin, 4- cytochrome C, 5- lysozyme. All analyte concentrations were *ca.* 1 mg/mL. Detection wavelength was 210 nm.

mentioned above there are many interactions that contribute to the problem of solute adsorption onto the capillary inner surface. Hydrogen bonding, Van der Waals force, and hydrophobic interactions may play an important role in the interaction of fused-silica surface with the solute. For the PAEM modified capillary, the authors believe that ionic interactions is the primary contribution to the reduction of solute adsorption. The positive charges on PAEM reversed the charge and the zeta potential of capillary inner wall, and eliminated the electrostatic attraction between capillary inner wall and the basic proteins. Furthermore, basic proteins were repelled by the capillary inner wall by means of electrostatic repulsion. Therefore, the peak shape and efficiency were improved. Figure 4 shows the separation of 5 proteins using a PAEM coated capillary.

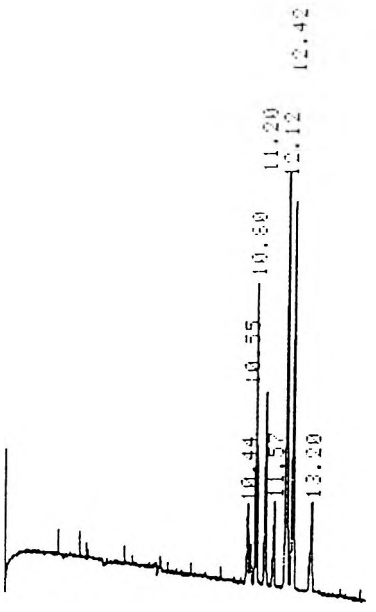

Figure 5. Separation of 8 beta-blockers using a PAEM coated capillary. 62.1 cm (44.6 cm to detector). 25 mM acetate pH 4.7 buffer. 15 kV applied voltage. Hydrodynamic injection at cathode. Numbers above the peaks are migration times in minutes. Peak identification: 10.44 min —acebutolol, 10.55 min —betaxolol, 10.80 min —dilivalol, 11.20 min —carteolol, 11.57 min —atenolol, 12.12 min — propanolol, 12.42 min — pindolol, 13.20 min —nifenalol.

The sample was injected at the cathode. Because PAEM coated capillary has a reversed EOF which was faster than the electrophoretic flow of all analytes, all proteins migrated from the cathode to the anode. As observed from Figure 4, highly efficient (N=400,000 plates per meter) and symmetric peaks were obtained. The relative standard deviation (%RSD) of migration time of proteins was less than 1% with five measurements. No concentrated buffer solution was required for the separation of basic proteins when PAEM coated capillaries were used.

The separation of small cationic compounds, for example, beta-blockers, by bare capillary could also encounter the solute-wall interaction problem.[35] The peaks could be tailing and wide. Using PAEM coated capillaries, the separation of beta-blockers was also improved. Figure 5 shows the separation

Table 1

Reproducibility of Migration Times (in Minutes) of 8-Beta-blockers using a PAEM Coated Capillary*

Run	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7	Peak 8
1	10.47	10.59	10.84	11.24	11.61	12.15	12.45	13.24
2	10.43	10.55	10.80	11.18	11.54	12.18	12.36	13.14
3.	10.44	10.55	10.80	11.20	11.57	12.12	12.42	13.20
%RSD	0.20	0.22	0.21	0.27	0.30	0.25	0.37	0.38

* For peak identification and separation conditions, see Figure 5 and the text.

of 8 beta-blockers at pH 4.7, 25 mM acetic buffer. Numbers above the peaks are migration times in minutes. As observed from Figure 5, highly efficient and symmetric peaks were obtained. Table 1 shows the reproducibility of migration times in minutes of these beta-blockers. As shown in Table 1, highly reproducible migration was obtained.

The pH-independent EOF coating in the pH range from 3 to 5.5 should also expand the applications of micellar electrokinetic chromatography (MEKC). It is well known that bare capillaries exhibit long and poor reproducible MEKC migration in this pH range. The fast anodal EOF should shorten the migration in MEKC. The pH-independent EOF should produce more reproducible migration in MEKC.

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ANALYSIS OF ε-N-2-FUROYLMETHYL-L-LYSINE (FUROSINE®) IN CONCENTRATED MILK BY REVERSED PHASE CHROMATOGRAPHY WITH A MICROBORE COLUMN

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ABSTRACT

This paper reports the results of a study aimed to develop a rapid, accurate and sensitive HPLC method for routine analysis of ε-N-2-furoylmethyl-L-lysine (furosine) in acid hydrolyzed of processed food products. The method uses a microbore reversed phase HPLC column packed with a polymer coated silica-based octadecyl sorbent (5 μm, 250 x 1 mm I.D) eluted with a mobile phase consisting of 20 mM phosphate buffer, at pH 2.5. The identification of furosine was performed by UV detection at 280 nm in a micro flow cell of 300 nL. The limit of detection was 0.3 ng based on a signal to noise ratio of 3 with a sample volume of 0.5 μL. The separation was achieved by isocratic elution within 15 min. This method was successfully applied to the identification and quantitative determination of furosine in a sample of concentrated milk hydrolyzed with hydrochloric acid. Dosage was performed by the method of external standard.

INTRODUCTION

The Maillard reaction (non-enzymatic browning) represents a complex series of degradative reactions involving carbonyl and amino functionalities which lead to the production of polymeric pigments, many flavor and aroma constituents and other degradation products. In milk and dairy products, as well as other food products, the Maillard reaction is initialized by the reaction between the carbonyl group of a reducing carbohydrate and the amino functions of a amino acid or protein with the formation of a glycosamine which is then transformed to a ketosamine by Amadori rearrangement. One of the most stable Amadori compounds in these food products is represented by ε-deoxy-ketosyl-lysine.[1] The lysine blocked in this Amadori compound is not nutritionally available. Current analytical methods to evaluate the extent of the Maillard reaction and the level of nutritionally unavailable lysine in processed food require the transformation of ε-deoxy-ketosyl-lysine into ε-N-2-furoylmethyl-L-lysine (furosine) by acid hydrolysis and subsequent determination of furosine.[2] The level of furosine has been accepted as an indicator of the Maillard reaction and as an indirect parameter to study possible changes in food nutritional quality by evaluation of the concentration of reactive and blocked lysine.

Chromatographic methods have been proposed for the determination of furosine in the acid hydrolyzed samples. These techniques include gas chromatography,[3-5] ion-exchange chromatography (ICE) with commonly used amino acid analyzers,[6-7] and ion pair reversed phase HPLC.[8-11] These methods differ in sensitivity and reproducibility, are not free from interferences, and some of them are time-consuming due to pre- or post column derivatization, pretreatment of sample and gradient elution.

In recent years increasing attention has been given to the use of micro-HPLC columns in a variety of applications in food analysis.[12-15] This interest is due to several advantages that micro-HPLC has compared to conventional HPLC. Recognized advantages using miniaturized HPLC techniques are the increased mass sensitivity, the higher peak efficiency, the low consumption of mobile phase, the low heat capacity and the smaller sample volume required for analysis.[16-19]

This paper reports the results of a study performed to develop an accurate and straightforward chromatographic method for the identification and dosage of furosine in food employing a reversed phase microbore column with isocratic condition and multiwavelength UV detection. In order to select the optimum

chromatographic elution to perform a rapid and reproducible separation, the effect of pH and temperature on the retention time and selectivity were examined. The optimized method was applied to the identification and dosage of furosine in a sample of concentrate milk hydrolyzed with hydrochloric acid.

MATERIALS AND METHODS

Instrument and Column

The chromatographic experiments were carried out using an HPLC system equipped with a Model 421A microprocessor controller and a Model 114M single-piston reciprocating pump with the capability of delivering micro flow rates, all from Beckman Instruments, Inc. (Fullerton, CA, USA); a 7520 Rheodyne (Cotati, CA, USA) microsample injector with a 0.5 μL sample rotor in peek and a Model 433 variable-wavelength detector with a standard micro flow cell (300 nL), in conjunction with a Data System 450 software, both from Kontron Instruments (Milan, Italy). A circulating water bath (Haake, Berlin, Germany) with variable temperature control was used to thermostat the temperature of the column and the eluent reservoir, which were enclosed in 500 mL and 1 L water jacked, respectively.

The column employed in the experiments was a SGE (Ringwood, Victoria, Australia) glass lined tubing (250 x 1 mm I.D.) packed with 5 μm C-18/P-8/5 polymer coated silica-based octadecyl sorbent.

Chemicals

Furosine of purity >99% was supplied by Neosystem Laboratoire (Strasbourg, France). Reagent-grade phosphoric acid, acetic acid, hydrochloric acid, sodium hydroxide and HPLC-grade water were obtained from Carlo Erba (Milan, Italy). Sample of concentrated milk was purchased from a local store.

Mobile phases were prepared by adding the correct volume of either phosphoric or acetic acid to a volumetric flask containing HPLC grade water. The pH was adjusted to the appropriate value with 0.1 M sodium hydroxide solution and measured with a glass electrode, Model HI 1131, and Model HI 9017 Microprocessor pH-meter, both from Hanna Instruments (Woonsocket, RI). All solutions were filtered through a type HA 0.45 μm membrane filter (Millipore, Vimodrome, Italy) and degassed by sparging with He before use.

Sample Preparation

A weighted aliquot of concentrated milk corresponding to 50 mg of protein (determined by Kjeldhal method) was submitted to acid hydrolysis by adding, in a screw-cap glass tube, 8 mL of 8 N hydrochloric acid. After purging with nitrogen for 2 min, the glass tube was capped and heated at 110°C for 23 h.[9] The hydrolyzed sample was then filtered through a type HA 0.22 μm membrane filter (Millipore, Vimodrome, Italy) and injected prior to diluting 50-fold with water.

Quantitative Analysis

A stock standard solution was prepared by dissolving in water the appropriate amount of furosine. Working standard solutions at five different concentrations ranging from 1.19 to 23.8 μg/mL were prepared by diluting the stock standard solution with water. The calibration graph was obtained by the peak-area method analyzing each working standard solution in quintuplicate.

RESULTS AND DISCUSSION

The goal of this study was to develop a method for the rapid, selective and accurate analysis of furosine by using a microbore reversed phase column packed with a polymer coated silica-based octadecyl stationary phase. In order to find optimal isocratic conditions, a study of the influence of the mobile phase composition and of the temperature on the chromatographic behavior of furosine was undertaken. Experiments were performed under isocratic conditions using as eluent neat aqueous buffer solutions at various pH values ranging from 2.5 to 5.8. In order to cover this range, either sodium phosphate or acetate buffer was employed in the pH region where they posses adequate buffering capacity.

The effect of varying the pH of the mobile phase on the retention behavior of furosine is depicted in Figure 1. It is observed that the retention time of furosine is not greatly affected up to pH 4.5. Above this pH value, the retention time dramatically increases in accordance with a corresponding lower degree of protonation of the lysine residue of furosine at higher pH values which is aspected to improve the hydrophobicity of the analyte.

The effect of the temperature on the chromatographic retention of furosine was investigated in order to find optimal analysis time with mobile phases containing 20 mM phosphate buffer at pH 2.5 3.5 or 5.8. Under this

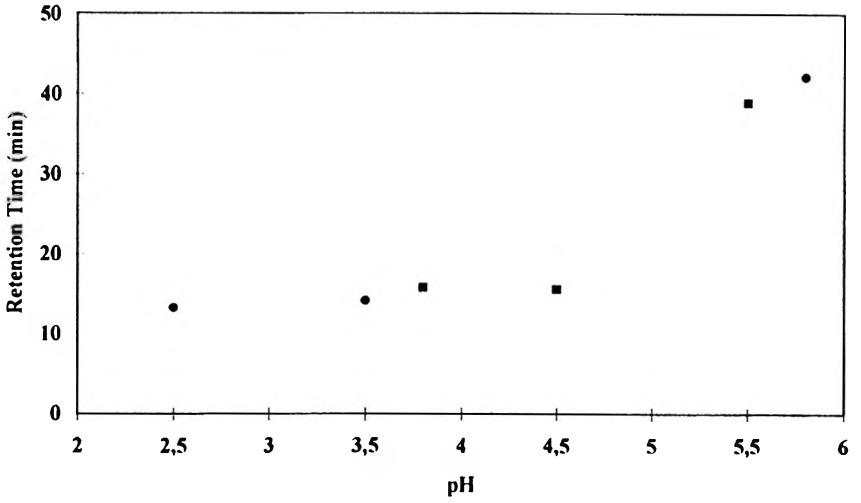

Figure 1. Dependence of furosine retention time on the pH of the mobile phase consisting of net aqueous solutions containing either 20 mM phosphate (●) or 20 mM acetate (■) buffer. Column SGE 5 μm C_{18}/P-8/5 (250 x 1 mm I.D.); flow rate 40 μl/min; temperature 25°C; detection, 280 nm.

experimental conditions the retention time decreased with increasing temperature and plots of the logarithmic retention factor versus reciprocal temperature yielded straight lines with correlation coefficients better than 0.9996, as it is shown in Figure 2.

In reversed phase chromatography the enthalpy changes associated with the reversible binding of the hydrophobic analyte by the hydrocarbonaceous stationary phase are in most cases aspected to be temperature independent and can be evaluated by the slope of the plots in Figure 2 which are referred to as van't Hoff plots.[20] Despite the relatively narrow temperature range (15°C) over which the van't Hoff plots of Figure 2 were obtained, we calculated the enthalpy of binding as reported above in order to gain some informations on the energetics of the furosine retention in the reversed phase column employed in this study. The values of the enthalpy of binding for the analyte eluted at pH 3.5 and 5.8 resulted to range from 1.52 to 1.54 kcal/mol, respectively. Such low values of enthalpy of binding are in accordance with the hydrophilic character of furosine which is eluted from the hydrophobic stationary phase with neat aqueous mobile phases. On the other hand, the almost identical value of the enthalpy of binding cannot explain the relatively large differences in the logarithmic retention factor obtained at pH 5.8. This may be related to changes

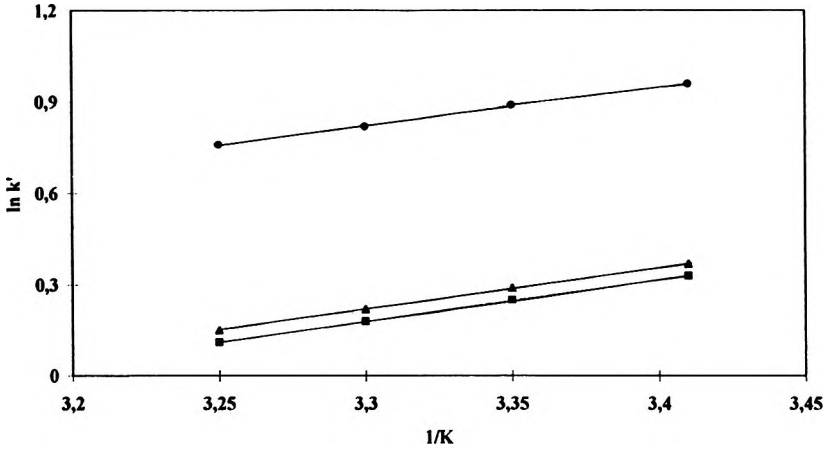

Figure 2. van't Hoff plots of furosine eluted at pH 2.5 (■), 3 8 (▲) and 5.8 (●). Experimental condition as in Figure 1.

in the enthalpy of binding which accompany the corresponding changes in the entropy of binding and that may be caused by the variation in the pH of the mobile phase. However, the evaluation of the corresponding variations of the entropy of binding is not possible owing to the unknown value of the phase ratio.

Further experiments were performed with the neat aqueous mobile phase containing 20 mM phosphate buffer at pH 2.5 and with column temperature controlled at 25°C. The precision of the method was evaluated in intraday and interday measurements of the retention time by repeated injections of an aqueous solution containing 0.01 mg/mL of standard furosine, using the same apparatus and column (see Table 1). Data obtained by two different operators were used for the evaluation of the interday precision. The interday data were acquired over a period of 10 days. The results presented in Tables 1 and 2 show that the intraday repeatability was 0.37 % whereas the interday precision was better than 0.75 %.

The same retention time with comparable repeatability was found in samples of concentrated milk subjected to acid hydrolysis, either with or without the addition of known amounts of standard furosine, ensuring sufficient peak identification. Dosage was performed by the method of external standard. The repeatability of the determination and linearity of the calibration graph were determined by analyzing solutions of standard furosine

Table 1

Retention Time, Standard Deviation (S.D.), and Relative Standard Deviation (R.S.D.) of Multiple Injections of a Standard Solution of Furosine. Chromatographic Conditions as in Figure 3.

Retention Time (min)				Mean (min)	S.D. (min)	R.S.D. (%)
13.21	13.18	13.24	13.38			
13.22	13.24	13.25	13.26			
13.26	13.21	13.31	13.30			
13.31	13.28	13.33	13.36	13.265	0.049	0.37

Table 2

Retention Time, Standard Deviation (S.D.), and Relative Standard Deviation (R.S.D.) of Multiple Injections of a Standard Solution of Furosine Performed in Ten Days. Chromatographic Conditions as in Figure 3.

Day	Mean Retention Time* (min)	S.D. (min)	R.S.D. (%)
1	13.427	0.100	0.75
2	13.219	0.052	0.39
3	13.235	0.031	0.23
4	13.224	0.048	0.36
5	13.237	0.054	0.41
6	13.360	0.047	0.35
7	13.392	0.082	0.62
8	13.371	0.073	0.56
9	13.332	0.086	0.65
10	03.307	0.036	0.27

* Mean of eight injections

at five different concentrations ranging from 1.19 to 23.8 μg/mL. The sample concentration was limited to the above range in order to avoid peak tailing and retention time shifting which occur when the sample size approaches the column sample load capacity,[21] which is a critical parameter for microbore columns.

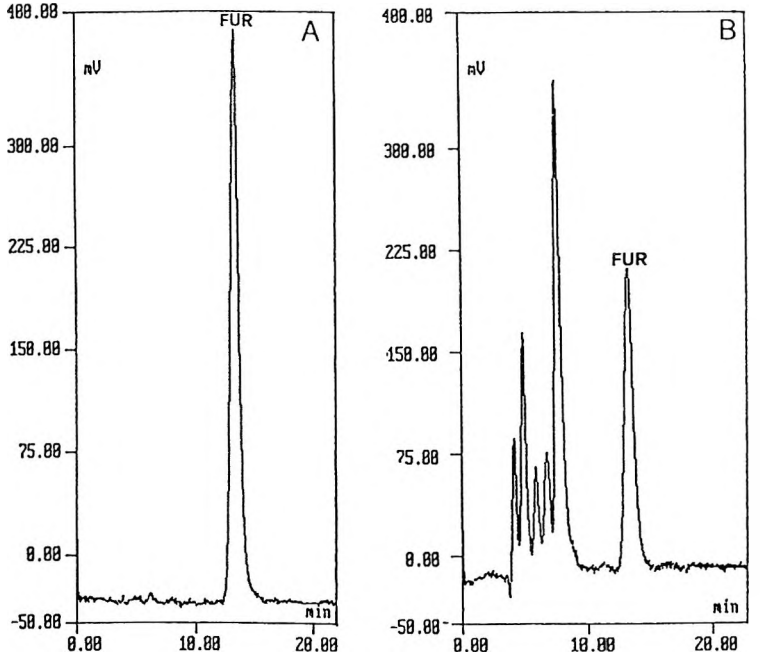

Figure 3. Chromatogram of a solution of standard furosine (panel A) and of an acid hydrolyzed sample of concentrated milk (panel B). Mobile phase, 20 mM phosphate buffer in water (pH 2.5); other conditions as in Figure 1.

Table 3

Reproducibility of Peak-Area and Peak-Height Mode

Concentration Furosine μg/mL	Mean Peak-Area mV×min	S.D. mV×min	R.S.D. (%)	Mean Peak-Height mV	S.D. mV	R.S.D. (%)
1.19	42.09	1.246	2.96	52.50	3.073	5.85
2.38	87.32	1.588	1.82	103.5	3.144	3.05
5.94	213.56	3.228	1.51	250.86	4.661	1.86
11.90	412.46	1.935	0.47	485.48	6.400	1.32
23.80	779.54	18.779	2.41	900.73	17.104	1.90

Table 4

Recovery of Furosine from a Concentrated Milk

Amount in Sample (ng)	Added (ng)	Found (ng)	Recovery (%)	R.S.D. (%)
2.484	0.476	2.948	99.58	0.256
	0.952	3.550	103.31	0.113
	1.428	4.062	103.84	0.112

The peak area mode gave a higher repeatability than the peak height mode (see Table 3) and for this reason was shown as the method of analysis. The linear regression analysis provided the equation $y = 12.9771 + 32.5167x$ with correlation coefficient of 0.9992, where y is the peak area and x is the concentration of furosine in μg/mL.

The limit of detection was defined as the amount of injected sample which gave a signal to noise ratio of 3 and was determinated to be 0.3 ng.

The accuracy of the method was evaluated by determining the recovery of furosine in a sample of concentrated milk of a known level of furosine. Three different amounts of standard furosine were added to the sample which was subjected to the chromatographic analysis. The recovery was calculated based on the difference between the total concentration determinated in the spiked samples and the concentration dose in the non spiked samples.

Results with the relative standard deviations are reported in Table 4. The relative standard deviation of the determinations for each concentration was better than 0.256 % and the mean recovery ranged from 99.6 to 103.8, indicating a high degree of accuracy.

The method was applied to the analysis of furosine in sample of concentrated milk subjected to acid hydrolysis. A typical chromatogram is displayed in Figure 3 and shows that unknown peaks detected in the sample did not interfere with the identification and dosage of furosine. The analysis in triplicate resulted in an average dosage of 1.38 g /100 g of proteins with repeatability of data better than 0.61 % (RSD).

CONCLUSIONS

This study has proven that furosine can be efficiently and selectively analyzed in samples of concentrated milk subjected to acid hydrolysis by a simple, rapid and straightforward method employing a polymer coated silica-based octodecyl stationary phase packed in a microbore column.

The microbore format of the column advantageously resulted in detecting furosine, which elutes as a very efficient and resolved peak with highly repeatable retention time and mass recovery, at level as low as 0.3 ng. It is also notable that no column pretreatment is necessary.

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NEURAL NETWORK CAPABILITY FOR RETENTION MODELING IN MICELLAR LIQUID CHROMATOGRAPHY WITH HYBRID ELUENTS

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ABSTRACT

Until date no papers have been reported about modeling studies in Micellar Liquid Chromatography by means of neural networks, only classical statistical methods have been used. In this work an overview into the capabilities of neural networks for modeling retention data in Micellar Liquid Chromatography is presented. To solve the problem of capacity factor modeling some parameters have been evaluated: type of activation function, number of neurons in the hidden layer and the use of some input and/or output data transformations. These studies have been carried out with the retention data for twenty-three compounds (benzene derivatives and polycyclic aromatic compounds) in an octyl silica column using micellar mobile phases containing CTAB (hexadecyltrimethylammonium bromide) as the surfactant and modified with n-propanol.

From the results obtained some considerations can be drawn. The selection of the activation function is very important to get good results and the best ones have been obtained when a linear activation function, a recurrent network and a logarithm transformation (logarithm of the capacity factor) have been used.

INTRODUCTION

Micellar Liquid Chromatography (MLC) with hybrid eluents is an attractive separation technique due to the versatility that surfactants confer to the chromatographic system and the possibilities of controlling selectivity and analysis time modifying both, the surfactant and/or the alcohol concentration in the mobile phase (alcohols are generally used as the organic modifier to avoid the efficiency lost obtained in its absence).

Moreover, some advantages can be cited when this technique is compared to conventional Reversed Phase Liquid Chromatography (RPLC): low cost and non-toxicity of surfactants *versus* expensive and flammable solvents of chromatographic grade,[1-4] unique selectivity,[4-9] compatibility of mobile phases with salts and water-insoluble compounds,[7] shorter equilibration times for gradient elution,[10] and detection enhancement.[11-15]

Until now, modeling of retention data has been performed by means of classical statistical methods such as multiple regression. Several equations that relate capacity factors with total surfactant and alcohol concentration have been evaluated. Thus, Torres Lapasió *et al.*[16] reported that for the catecholamines studied for them in mobile phases containing SDS (sodium dodecylsulphate) and propanol the best equation was as follows:

$$1 / k' = A\mu + B\varphi + C\mu\varphi + D \qquad (1)$$

where k' is the solute capacity factor, μ the total surfactant concentration, φ the alcohol volume fraction and A, B, C and D the equation parameters. Later, our investigation group[17] studied the capacity of retention prediction of some empirical equations when mobile phases containing CTAB or SDS as the surfactant and modified with n-propanol or n-butanol were used. Our results suggested that the following equation

$$1 / k' = A\mu + B\varphi^2 + C\varphi + D\mu\varphi + E \qquad (2)$$

was of more general applicability that the former (eq. 1) at least for the compounds studied for us (benzene derivatives and polycyclic aromatic compounds).

Although artificial neural networks have been developing for nearly 50 years[18] they were not implemented until now. These artificial neural networks can be applied to the resolution of a great variety of problems,[18] such as classification, modeling, association and mapping, as well as classical statistical methods or pattern-recognition methods (regression analysis, clustering methods, principal component methods, etc.); the main advantages are that the relationship between input and output data need not be specified in mathematical form and they are capable of modeling even nonlinear relationships. Moreover, some nets can associate; this means that they can recognize information although it is partial or distorted.

In this work, we are interested in investigating the capability of neural networks to model retention data in micellar liquid chromatography as a function of surfactant and alcohol concentrations, as a preliminary step to study their capacity of prediction and to compare with the statistical methods implemented earlier.

Our purposes in this paper are to study the influence of the architecture of the net (that is the way the individual neurons are connected), in particular, the node activation function, the layers number, and of some data transformations on the retention of twenty-three compounds (benzene derivatives and polycyclic aromatic compounds) in a C_8 column with micellar mobile phases containing CTAB as the surfactant and modified with n-propanol.

EXPERIMENTAL SECTION

Chromatographic Data

In this work retention data for twenty-three compounds obtained earlier,[17,19] in a C_8 column have been used. The solute capacity factors were determined in micellar mobile phases containing CTAB as the surfactant and modified with n-propanol. The mobile phases composition to obtain the experimental retention data used have been summarized in Figure 1.

Benzene derivatives and polycyclic aromatic hydrocarbons were as follows: (1) benzene, (2) benzylic alcohol, (3) benzamide, (4) toluene, (5) benzonitrile, (6) nitrobenzene, (7) phenol, (8) 2-phenylethanol, (9) chlorobenzene, (10) phenylacetonitrile, (11) 3,5-dimethylphenol, (12) naphthalene, (13) 1-naphthol, (14) 2-naphthol, (15) 1-naphthylamine, (16) pyrene, (17) phenanthrene, (18) 2,3-benzofluorene, (19) fluorene, (20) fluoranthene, (21) acenaphtylene, (22) acenaphthene, and (23) anthracene.

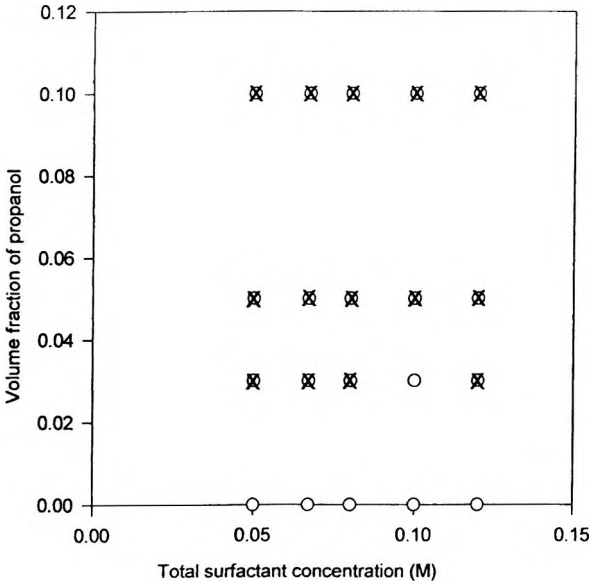

Figure 1. Mobile phase compositions for compounds 1 to 15 (O) and 16 to 23 (X).

Data Manipulation

Data manipulation was carried out by means of Microsoft Excel[20] and Neural Network Development Tool[21] software.

To study the capability of neural networks to model solute retention data in micellar liquid chromatography with hybrid eluents some parameters have been evaluated: (a) activation functions in the nodes of the hidden layer, (b) number of neurons in the hidden layer (one to three) and comparison with a recurrent link, and (c) data transformations (inverse and logarithm of capacity factors and the use of micellized surfactant concentration as input variable instead of total surfactant concentration).

Input data and output data files were created or transformed by means of Microsoft Excel and the neural network analysis was replicated five times because in nonlinear optimization studies often multiple minima can be found. The maximum number of iterations was fixed in 10000. Comparisons are always made by calculating the mean over capacity factor relative error (in absolute value) for all the mobile phases considered.

RESULTS AND DISCUSSION

The results will be presented in three different sections, although we must keep in mind that the results of the previous studies condition the latter. That is, in a first step some activation functions have been evaluated, so in the second section we have used the best function and then we have studied the influence of the number of neurons in the hidden layer and the use of a recurrent link. The third step comprises the studies achieved with data transformations, both output data transformations (k', 1/k', log k') and input data transformation (micellized surfactant concentration instead of total surfactant concentration).

A. Studies on the Activation Function in the Nodes of the Hidden Layer.

The activation functions in the nodes of the hidden layer that have been evaluated are the following:

$$y = \frac{1}{1+e^{-x}} \tag{3}$$

$$y = \frac{x}{|x|}\ln(1+|x|) \tag{4}$$

$$y = x \tag{5}$$

$$y = -1 + \frac{2}{1+e^{-x}} \tag{6}$$

where y means the node output signal and x the total node input signals. These functions will be identified as 1, 2, 3, and 4, respectively, in the discussion of the results obtained. In these studies, the linear function (eq.(5)) has been the activation function in the input and the output layer.

The input data for the network are the total surfactant concentration in the mobile phase (M) and the volume fraction of n-propanol in the mobile phase and the output data in this section is the capacity factor (k'). The network in this step of the study consisted of an input layer, a hidden layer (with one neuron) and an output layer.

In Figure 2 the mean relative error (in absolute value)(calculated as $((k'_{cal}-k'_{exp})/k'_{exp})*100$) is plotted for every compound and for the four activation functions. As can be observed the lowest errors have been obtained with the activation function

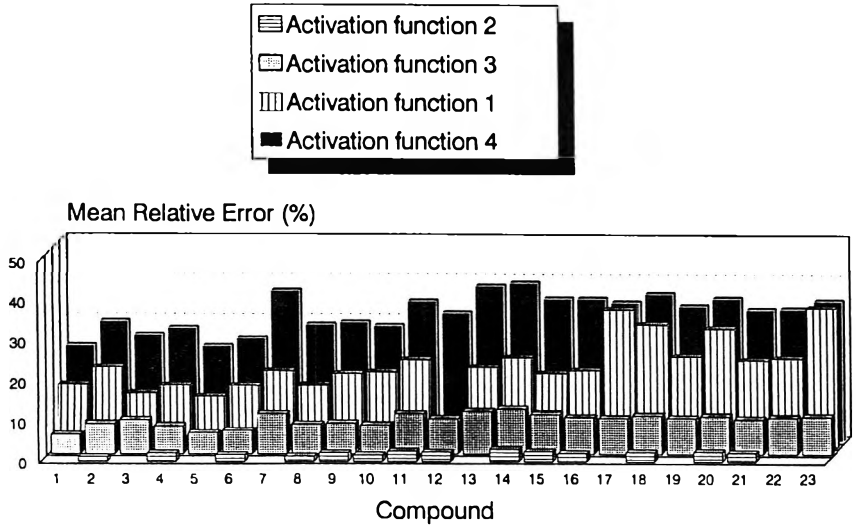

Figure 2. Mean relative errors (in absolute value) for every compound using different activation functions in the nodes of the hidden layer.

2 but it has not been considered the best function because for many compounds the parameter convergence has not been produced after 10000 iterations. The compounds for which there was not a solution are 1, 3, 5, 7, 13, 17, 19, 22 and 23.

The activation function that has been considered as the best is the linear function ($y = x$) because although greater errors than with function 2 have been obtained it is always possible to find a solution (parameter convergence). For the other functions (1 and 4) the errors are very important and at the same time of the five assays with the network and the same data, the solutions obtained are different. For example, it can be cited that for the compound number 4 and the activation function 1, the mean relative errors (in absolute values) are respectively 19.66 %, 24.61 %, 17.52 %, 15.38 % and 19.22 %. These data have been obtained after 5, 4, 10, 7 and 5 iterations respectively.

B. Number of Neurons in the Hidden Layer.

In Figure 3 the different networks that have been evaluated are represented. It can be observed that the input and the output layers are the same and the differences between them consist in the number of neurons in the hidden layer (Figures 3a, 3b and 3c) and the use of a recurrent link in Figure 3d.

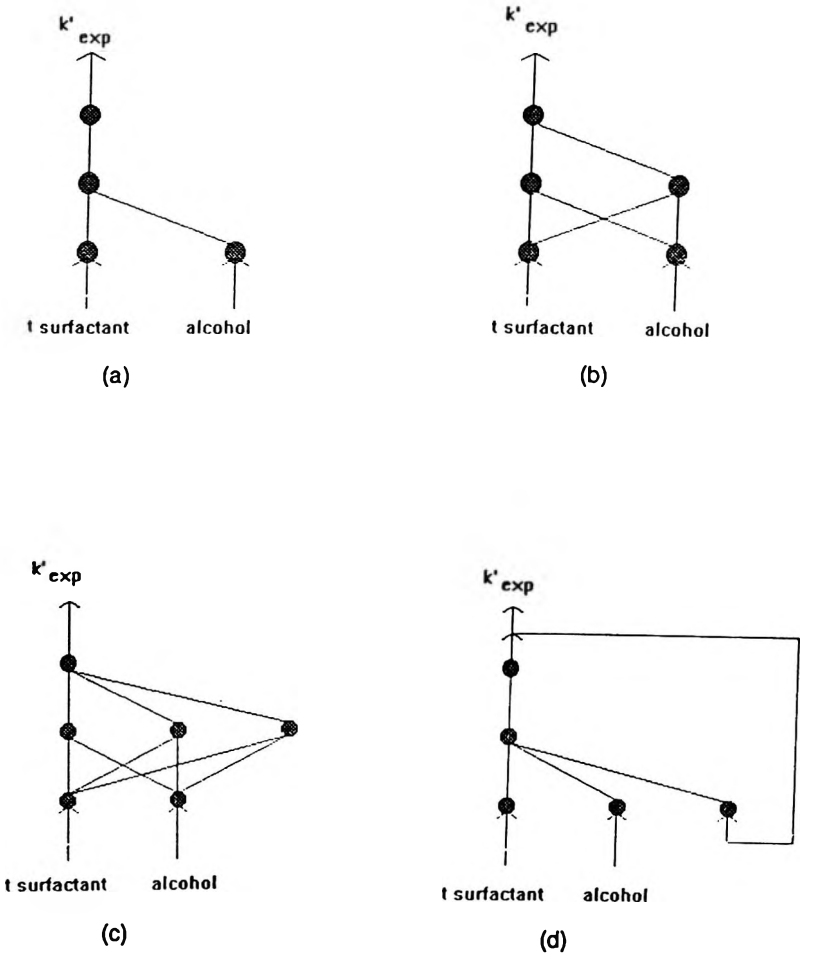

Figure 3. Architecture of the networks employed.

For all the assays a linear activation function in the different layers is used. The results of these studies are shown in Table 1. The mean relative errors for compounds are tabulated for the networks shown in Figure 3 (with one, two and three neurons in the hidden layer and with the recurrent link).

Table 1

Mean Relative Error (%) for Every Compound and for the Networks Shown in Figure 3

Compound	1 Neuron	2 Neurons	3 Neurons	Recurrent Link
1	4.96	4.96	4.96	3.22
2	7.52	7.52	7.52	4.78
3	8.54	8.54	8.54	3.64
4	7.04	7.04	7.04	4.50
5	5.39	5.39	5.39	4.00
6	5.94	5.94	5.94	4.13
7	10.12	10.12	10.12	7.74
8	7.49	7.49	7.49	5.36
9	7.68	7.68	7.68	4.82
10	7.23	7.23	7.23	5.35
11	10.16	10.16	10.16	7.74
12	8.84	8.84	8.84	5.50
13	10.70	10.70	10.70	8.25
14	11.32	11.32	11.32	8.40
15	9.96	9.96	9.96	6.86
16	9.18	9.18	9.18	5.31
17	9.02	9.02	9.02	5.19
18	9.59	9.59	9.59	5.60
19	8.99	8.99	8.99	5.14
20	9.46	9.46	9.46	5.29
21	8.76	8.76	8.76	4.95
22	9.10	9.10	9.10	5.22
23	9.38	9.38	9.38	5.38

The mean relative errors for networks with one, two and three neurons in the hidden layer are the same, so it seems that the number of neurons does not influence the errors obtained. These errors ranged from 4.96 % (for compound 1) to 11.32 % (for compound 14).

When a network with a recurrent link is used for the capacity factor modeling an important decrease in the mean relative error is obtained. Thus, the ratio between the relative errors obtained with the network with one neuron and the recurrent link ranged from 1.3 (for compound 5) to 2.35 (for compound 3).

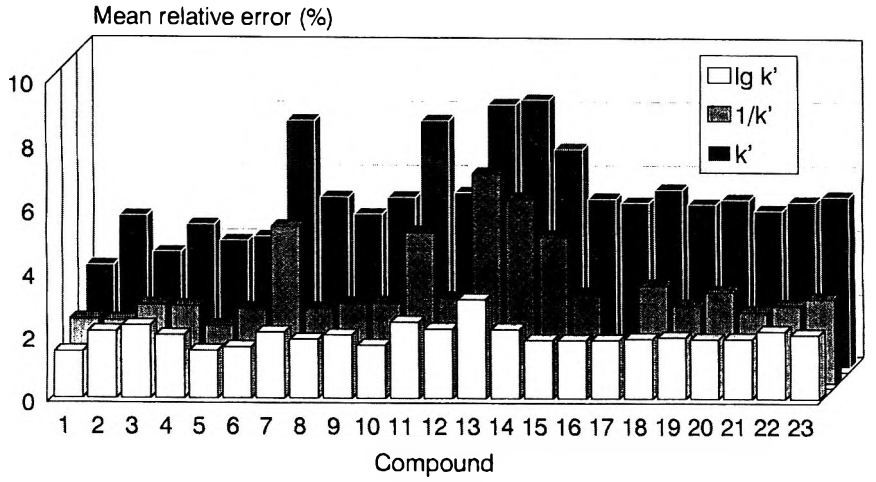

Figure 4. Mean relative errors (in absolute value) for the different compounds studied when k', 1/k' and log k' are used as the output data.

From the results presented in this section it can be concluded that by means of networks with recurrent links a great improvement in the capability of retention modeling is obtained.

C. Data Transformations.

The studies of data transformations have been carried out by using the linear activation function in the nodes of the hidden layer and the network shown in Figure 3d.

In a first step some transformations of the output data have been evaluated, that is, k', 1/k' and log k' are the output of the net. In Figure 4 the mean relative errors for compounds one to twenty-three are shown. It can be observed that both, inverse and logarithm transformations significantly improve the errors obtained with respect to that shown when k' is the output of the net. The best results were achieved when the logarithm of the capacity factors is used as the output data. Thus, it can be cited that the errors are clearly low and ranged from 1.46 % (for compound 1) to 3.13 % (for compound 13).

For the inverse transformation, errors ranged from 1.80 % to 6.58 %, that is, sometimes a ratio of more than two is found when inverse and logarithm transformation are compared.

Table 2

Comparison of Mean Relative Errors for Compounds when Total Surfactant Concentration in the Mobile Phase and Micellized Surfactant Concentration are used as the Input Data

Compound	Total Surf. Concentration (M)	Micellized Surf. Concentration (M)
1	1.46	1.47
2	2.11	2.13
3	2.30	2.31
4	1.99	1.99
5	1.49	1.51
6	1.61	1.63
7	2.09	2.10
8	1.86	1.88
9	2.00	2.00
10	1.67	1.68
11	2.42	2.44
12	2.20	2.19
13	3.13	3.14
14	2.19	2.19
15	1.84	1.85
16	1.84	1.84
17	1.84	1.85
18	1.89	1.88
19	1.94	1.95
20	1.88	1.88
21	1.89	1.90
22	2.14	2.14
23	2.03	2.04

Then, by using the logarithm of the capacity factor as the output data we have compared the results obtained when the input data are micellized surfactant concentration (total surfactant concentration *minus* the critical micellar concentration) and the alcohol volume fraction to the previous data (in which total surfactant concentration and alcohol volume fraction have been used as the input data).

Table 2 groups the mean relative errors for compounds, when total surfactant concentration in the mobile phase and micellized surfactant concentration are used as the input data. The error values show that there are not significative differences when both total surfactant concentration and micellized surfactant concentration are used as the input data. Equal or slightly greater errors are obtained when micellized surfactant concentration is used, so we propose the use of total surfactant concentration as the input data together with the n-propanol volume fraction.

From the results presented in the discussion, we propose the use of the linear activation function (y = x) in the nodes of the hidden layer, the network with the recurrent link and the logarithm transformation of the output data to achieve the retention modeling in micellar liquid chromatography, at least for the compounds studied, and the mobile phases considered.

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SOLID PHASE EXTRACTION OF BIOGENIC AMINES FROM WINE BEFORE CHROMATOGRAPHIC ANALYSIS OF THEIR AQC DERIVATIVES

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ABSTRACT

This paper describes a method for determining the most important biogenic amines in wines. It uses reversed phase high performance liquid chromatography (RP-HPLC) with gradient elution and fluorimetric detection, performed on the amines after automatic precolumn derivatization with 6-aminoquinolyl-n-hydroxysuccinimidyl carbamate (AQC). Solid phase extraction (SPE) with strong cation exchanger (SCX) cartridges was used

prior to derivatization to improve the selectivity and to reduce the detection limits (LOD) of the method. The overall method was used to determine the aforementioned amines in red wines from the Tarragona region.

INTRODUCTION

Biogenic amines are alkaline, aliphatic and heterocyclic compounds whose negative presence in food has been well documented.[1-6] These compounds can be found in the raw material but, above all, they are found in the fermented products as a result of the decarboxylation of their precursor amino acids.[7,8]

In the last two decades, the determination of the content of biogenic amines in food products has become more and more important. Increasing interest in producing processed foods that meet the quality that complies with import and exportation laws have encouraged research into these areas.

Biogenic amines are compounds formed during the fermentative processing of foods. Several authors have proved that, for grapes and wines, they appear during the malolactic fermentation, which is the main reason why red wines have higher quantities than white wines.

Historically, a major problem with biogenic amines' identification and quantitation in wines has been the lack of a sensitive analytical technique. Many reports have introduced derivatizing agents and/or other sample treatments to overcome the problems that wines, especially red wines, show because of their matrix complexity. So, many analytical reagents have become popular among wine researchers. Orthophthaldialdehyde (OPA),[7,9-21] the derivatizing agent that enhances both the selectivity as well as the sensitivity of the method when working with HPLC, has been adopted as the best of the agents used, and is at present the method adopted by the *Office International de la Vigne et du Vin* (OIV) for the standard validation of an analytical method.[22] Nevertheless, various shortcomings are also described in all of the reports written on this subject, as we have pointed out in a previous paper.[23] A new derivatizing agent, AQC, overcomes most of these limitations, although its use with different samples is still being studied.[24]

Furthermore, solid-phase extraction is a useful technique that commonly cleans-up and concentrates the analytes by reducing the sample pretreatment which is normally necessary when using other techniques such as liquid-liquid extraction (LLE). To enrich aqueous amine samples, many sorbents including C_{18} and strong and weak cation exchangers, have been tested and compared.[26]

Although C_{18} is the most popular sorbent used for extracting organic compounds from aqueous samples, when amines have to be isolated, the best results are obtained by using weak cation exchangers such as Amberlites.[10,18,27]

Nevertheless, C_{18} recoveries can be improved by modifying the polarity of the amines, either by derivatizing[28] or by reacting them with suitable ion pair reagents.[25]

The aim of this work is to provide a fast analytical method for the simultaneous determination of eight undesirable biogenic amines such as histamine, cadaverine, putrescine, iso-amylamine, tyramine, β-phenethylamine, ethylamine and methylamine, all of them correlated with each other in terms of synergistic toxicity. They are isolated from the main matrix with a strong cation exchanger (SCX), eluted with an optimized solvent mixture and finally chromatographed after AQC automatic derivatization in order to test an alternative procedure to the well-known OPA derivatization.

This method has been used to determine the above-mentioned amines in several red wines from the Tarragona region.

EXPERIMENTAL

Chemicals and Reagents

The eight amines studied were: methylamine, histamine, ethylamine, tyramine, putrescine, cadaverine, β-phenethylamine, and 3-methylbutylamine (iso-amylamine), all of which were supplied by Aldrich-Chemie (Beerse, Belgium). An individual standard solution of 2000 mg L^{-1} of each amine was prepared in HPLC-grade methanol (Scharlau, Barcelona, Spain) and stored in darkness at 4°C. More dilute solutions used in the calibration and SPE studies were prepared by diluting these standard solutions with Milli-Q purified water.

The methanol (MeOH), acetonitrile (ACN) and tetrahydrofuran (THF) used in the chromatographic and the extraction method were HPLC grade (Scharlau). The water used for more diluted solutions was of Milli-Q quality (Millipore, Bedford, MA, USA). The sodium acetate reagent (NaAc, HPLC grade) was also supplied by Scharlau. For the automatic derivatization method, the AccQ·Fluor Reagent Kit (Waters, Milford, USA) was used.

Equipment

A Hewlett-Packard (Waldbronn, Germany) model 1050 HPLC equipped with an HP 1046A programmable fluorescence detector was used to perform the chromatographic experiments. The samples were derivatized and injected with an HP Series 1050 automatic injector. Separation was performed using a thermostatted Spherisorb ODS-2 cartridge (250 x 4.6 mm I.D., particle size 5 μm) preceded by a guard-column, both heated to 65°C and supplied by Hewlett-Packard. Chromatographic data was collected and recorded on an HP ChemStation version A.01.01.

SPE experiments were performed using a Visiprep™ DL Disposable Liner Solid Phase Extraction Vacuum Manifold with 12 individual flow control valves from Supelco (Bellefonte, USA).

High-Performance Liquid Chromatographic Method

Two solvent reservoirs containing (A) 1% THF and 0.05M NaAc in Milli-Q water and (B) MeOH were used to perform the optimized gradient programme which began with 5 min of isocratic elution at 35% of MeOH followed by a 7 min linear gradient from 35 to 100% (v/v) methanol. Then, the programme had three additional minutes at 100% of MeOH as a clean-up step and then 2 min to reach the initial conditions and stabilise the corresponding mobile phase. Determination was at a flow-rate of 1 mL min^{-1}. The AQC-derivatives eluted were detected by monitoring their fluorescence using 250 nm and 395 nm as the wavelengths of excitation and emission respectively. Under these conditions, all 8 amines were eluted in less than 13 min.

Derivatization

The derivative reagent was formed by reconstituting the 6-aminoquinolyl-n-hydroxy-succinimidyl carbamate powder with 1 mL of acetonitrile. The alkalinity needed to perform the derivatization was obtained with the borate buffer supplied by Waters in its Fluor Reagent Kit (AccQ·Tag™).

The derivatization was fully automated by means of an injector program. The injection system mixed the reagents automatically, drawing the AQC reagent, the borate buffer, and the sample sequentially into the injection needle. The steps in the derivatization sequence have been specified in a previous paper.[23]

Solid-Phase Extraction

In order to simultaneously clean up and concentrate the sample, solid-phase extraction was carried out with the sulphonic acid cation exchanger (SCX cartridges, 500 mg) supplied by Varian (Harbor City, CA, USA). The solid-phase extraction consisted of a prior step to condition the cartridge by rinsing it with two fractions of 3 mL of methanol. Then, it was rinsed with two fractions of 3 mL of 0.1 mM HCl. The sample was then passed through the conditioned cartridge, washed with 3 mL of 0.1mM HCl and eluted with two fractions of 1 mL of 75 mM borate in MeOH 50 % (v/v) (2.86 g of sodium tetraborate decahydrate was dissolved with distilled water in a 100 mL volumetric flask and then 50 mL of this solution was diluted to 100 mL with MeOH). This step has been optimized according to the procedures described in the following section.

RESULTS AND DISCUSSION

After sample derivatization according to the procedure previously reported,[23] the chromatographic gradient elution was optimized to obtain good resolution between the peaks in a shorter analysis time, with no interference from the more polar compounds usually present in the analysis of real samples. Figure 1 shows the optimum separation of a 5 mg L^{-1} standard mixture of the 8 amines studied, after derivatization with the AQC reagent. All of the amines were well resolved and there was no interference from the peaks corresponding to the derivatization reagents and other system peaks. Ten identical standard samples were derivatized using this procedure. Between-day reproducibility for retention times was excellent (always lower than 1%, average R.S.D. = 0.46%).

In order to verify the linearity of the response of the different derivatives at the previously specified wavelengths for the working concentration, standard solutions of amines that ranged between 0.1 and 15 mg·L^{-1} were prepared and injected. Calibration graphs of each amine were constructed by plotting the amine peak-area against the amine concentration. Linear least-squares regression was used to calculate the slope, the intercept, the correlation coefficient (r^2) and the LODs. All the calibration lines showed ranges of linearity ($r^2 > 0.995$) between 0.20-0.50 and 10-20 mg L^{-1}. The LOD's were calculated by direct injection of the amines and ranged between 20 μg L^{-1} (β-phenethylamine and 3-methylbutylamine) and 60 μg L^{-1} (methylamine).

The method was then applied to real samples by fortifying a red wine with standard solutions. The linearity, the LODs, the repeatability, and the reproducibility were determined. Good linearities ($r^2 > 0.995$) were obtained

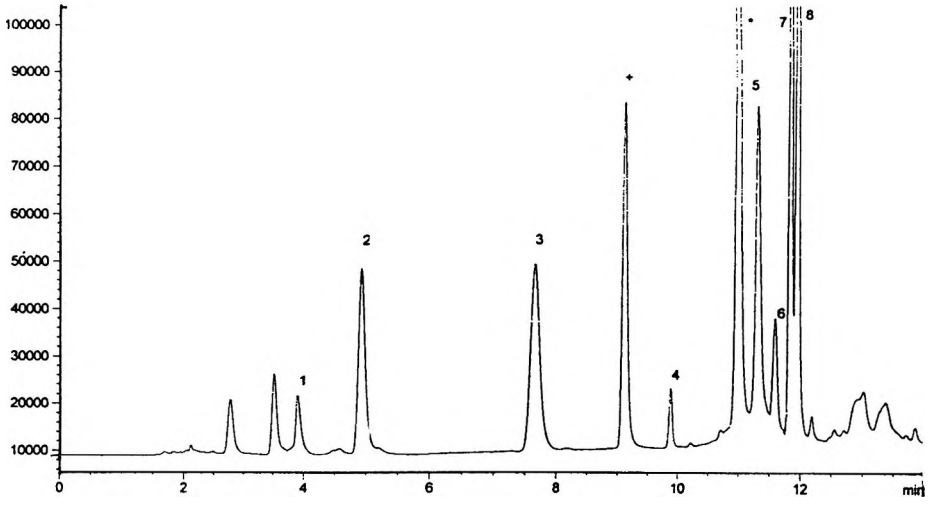

Figure 1. Optimum chromatographic separation of the AQC-amine derivatives. The standard solution of the biogenic amines was about 5 mg L^{-1}: 1 = methylamine, 2 = histamine, 3 = ethylamine, 4 = tyramine, 5 = putrescine, 6 = cadaverine, 7 = β-phenethylamine, 8 = 3-methylbutylamine. Experimental conditions given in text. (+) = peak corresponding to the excess of AQC; (*) Unknown.

between 0.5-1.0 and 10-15 mg l^{-1} with detection limits between 25 μg L^{-1} for β-phenethylamine and 3-methylbutylamine and 500 μg L^{-1} for methylamine and ethylamine. Reproducibility and repeatability of the method were determined at two concentration levels by adding 2 and 10 mg L^{-1} of a standard solution to a red wine. In both cases repeatability was lower than 8% and reproducibility less than 10%.

After many successive analyses, the peak widths of the amine derivatives increased, mainly for histamine and ethylamine, although this was temporarily avoided by flushing the chromatographic system with ACN after every ten injections.

The method was first applied to the analysis of wines which had not been subjected to any special treatment. In this case, they were only filtered through a 0.45 μm nylon membrane.

Figures 2a and 2b show an example of a red wine which was analyzed with and without standard addition about 2 mg·L^{-1} of each of the biogenic amines. It can be seen that the most polar compounds, such as amino acid

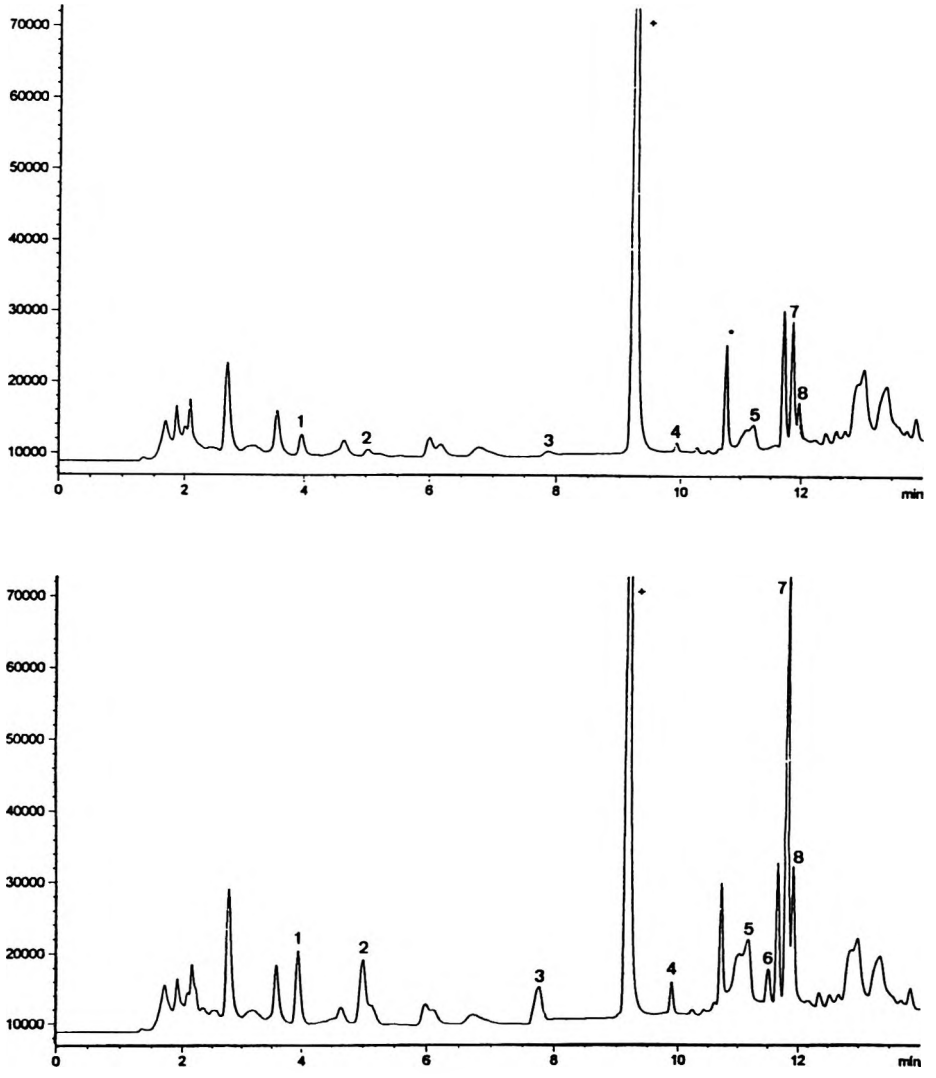

Figure 2. Chromatogram of red wine and the same sample spiked with a standard solution at a final concentration of 2 mg L^{-1} of the amines studied: a) red wine and b) red wine spiked with the standard solution. For peak assignments, see Fig. 1.

derivatives, did not interfere with respect to the peaks corresponding to the compounds studied. Figure 2a shows that histamine can be determined directly without interferences from amino acids. This is highly interesting since it is one of the limitations of the commonly used OPA. However, the rest of the

Table 1

Recovery (R) and Relative Standard Deviation (R.S.D.) of Eight Biogenic Amines Extracted from Red Wine by Solid Phase Extraction[a]

	R (%)	R.S.D. (%)
methylamine	70	8.7
histamine	100	4.1
ethylamine	85	5.1
tyramine	100	4.4
putrescine	100	4.0
cadaverine	101	3.9
b-phenethylamine	80	5.7
3-methylbutylamine	100	5.7

[a] Results from triplicate analysis of 15 ml of red wine spiked with 500 $\mu g \cdot L^{-1}$ of the amines. The baseline is determined from the same wine spiked with methanol instead of amines.

compounds can only be identified because their concentrations are near the detection limits of the method. On the other hand, tyramine cannot be identified in this sample. In order to decrease the matrix effect and to determine these amines at low concentration levels, a solid-phase extraction technique was studied. In previous papers, SPE with C_{18} cartridges was used to this end, but some tedious steps such as pH adjustment and the use of an ion-pair reagent[25] were necessary in order to obtain good results.

We reported in a previous study[21] that biogenic amines in wines can be preconcentrated using SPE with SCX as strong cation exchanger and HCl in MeOH as eluent. The recoveries ranged between 75 and 100% for all the amines except for two of the most important amines present in wines, putrescine and cadaverine, the recoveries of which were 35% and less than 10%, respectively.

To improve this extraction method, several buffer solutions and organic solvents were tested. The best results were obtained with 75mM borate solution in MeOH 50/50 v/v. Attempts to increase the percentage of methanol failed because the borate was not very soluble in methanol at concentrations higher than 50%. Furthermore, lower borate concentrations (25 mM and 50 mM) gave poor amine recoveries, even if the percentage of methanol was increased. So, the above mentioned concentration was chosen as a compromise between

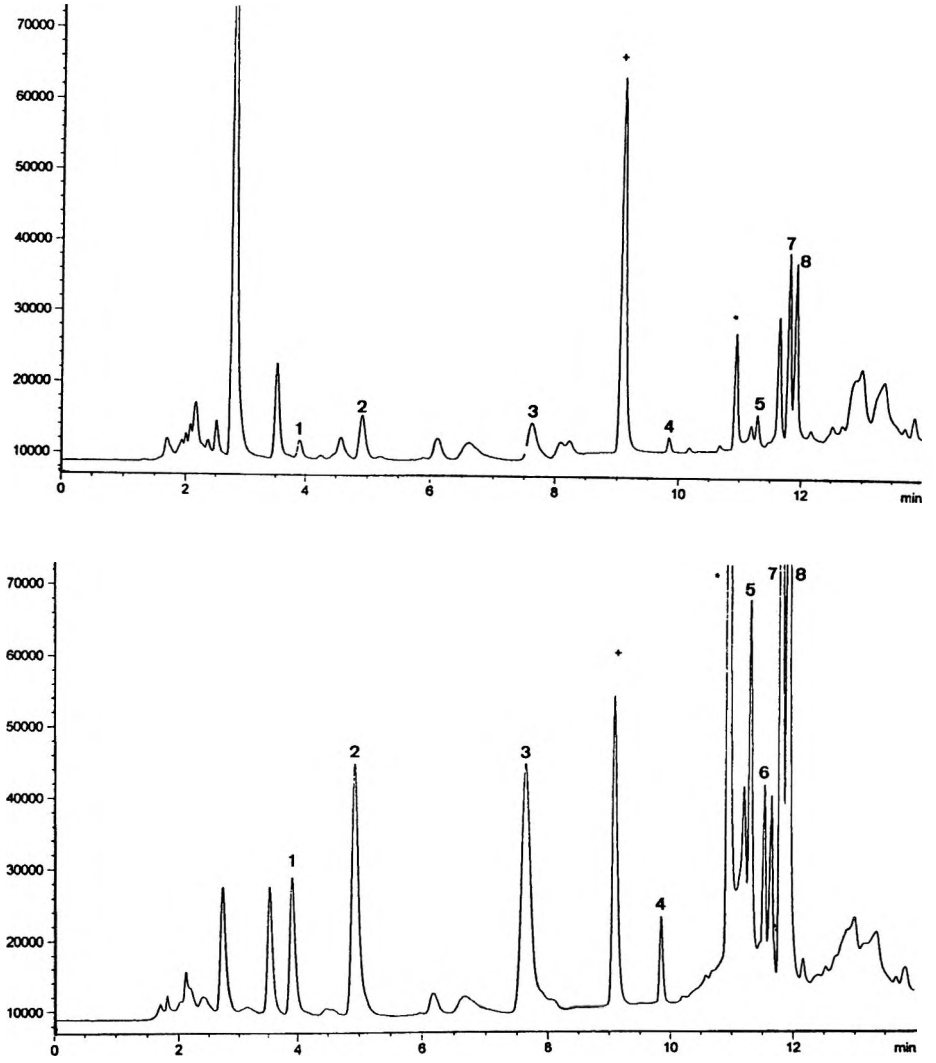

Figure 3. Chromatograms showing the difference when the SPE procedure is applied. 3*a*) Wine given in Fig. 2a after SPE; 3*b*) Same wine spiked with 0.3 mg L^{-1} and processed with the analytical procedure described. For peak assignments, see Fig. 1.

the percentage of organic solvent and the ionic strength, since neither of them can be increased without precipitating the salt. Under these conditions, the results obtained after preconcentrating 15 mL of a red wine fortified with 0.3 mg L^{-1} of each of the biogenic amines and eluted with 1 mL of the optimized

eluting solution are shown in Table 1. As can be seen, putrescine and cadaverine were now well recovered (100%). The recovery of the rest of the amines varied between 80% and 100%, except for methylamine, whose recovery was 70%. So, as well as 3-methylbutylamine and cadaverine, this method also recovered histamine, tyramine and putrescine, which are present in larger quantities in wines.

Figure 3a shows the results of the SPE applied to the wine the chromatogram of which can be seen in Fig. 2a, whereas Fig. 3b gives the chromatogram resulting from the same treatment of the fortified wine. There is a decrease in the most polar peaks that appeared at the beginning of the chromatogram as well as the decrease in other system peaks that interfere in the determination of the analytes. In this wine, in addition to the histamine, methylamine, putrescine, cadaverine, β-phenethylamine and 3-methylbutylamine could also be easily determined after SPE. On the other hand, ethylamine coelutes with another peak, which this treatment removes, although tyramine cannot be identified.

The LOD's of the method were determined using the S/N ratio = 3 for all compounds in a fortified red wine.[23] The LOD's determined ranged between 20 μg L^{-1} for β-phenethylamine and 3-methylbutylamine and 0.1 mg L^{-1} for methylamine. The repeatability was close to 10% for all the amines and the reproducibility was close to 15%.

Attempts to decrease the detection limits result made sample preparation more difficult because an off-line concentration method, such as rotary evaporation of the eluates or concentration under nitrogen steam, had to be added. Experiments to evaporate samples under vacuum failed to recover most of the amines analysed. Neither did concentration under nitrogen steam increase the efficiency of the treatment.

This method was applied to fifteen red wines belonging to different varieties from Tarragona. Results obtained for the SCX concentration are shown in Table 2.

CONCLUSIONS

The HPLC system described enables biogenic amines to be determined in wines with good sensitivity and specificity. By means of automatic pre-column AQC-derivatization and fluorescence detection, appropriately coupled with a prior SCX extraction, eight amines can be quantified at low levels. SCX cartridges again proved to be adequate to clean up the wine samples, while

Table 2

Analysis of 15 Red Wines[a]

Name	Concentration Range	Median
histamine	5.3 - 7.8	6.3
putrescine	3.3 - 4.8	4.4
3-methylbutylamine	0.1 - 0.2	0.1
methylamine	0.7 - 0.8	0.8
ethylamine	0.2 - 0.6	0.5
cadaverine	0.5 - 0.7	0.6
b-phenethylamine	0.5 - 0.6	0.6
tyramine	ND	-

[a] Concentrations in mg L^{-1}. Results from SCX extraction of the wines.

retaining biogenic amines, so enabling the samples to be simultaneously concentrated. Recoveries have been improved in comparison with other studies and the concentration step has been optimized, allowing these compounds to be detected at low levels with no interference from other compounds such as amino acids.

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DETERMINATION OF CHENODEOXYCHOLIC ACID IN PHARMACEUTICAL PREPARATIONS OF URSODEOXYCHOLIC ACID BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH COULOMETRIC ELECTROCHEMICAL DETECTION

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ABSTRACT

1-(2,5-Dihydroxyphenyl)-2-bromoethanone (2,5-DBE) has been used as an electroactive labelling reagent for high performance liquid chromatographic (HPLC) analysis of chenodeoxycholic acid impurity in pharmaceutical formulations of ursodeoxycholic acid.

The preparation of this reagent has been performed in a single step by bromination of 2,5-dihydroxyacetophenone (2,5-DAP), with remarkable improvement as regards the synthesis previously reported. Several experiments have been performed in order to give maximum conversion of the bile acids to their electroactive esters.

The determination of the derivatized compounds has been carried out on an Adsorbosphere column with a methanol-acetonitrile-acetate buffer eluent and detected by a porous graphite electrode set at an oxidation potential of + 0.6 V. The high sensitivity and specificity

shown by this novel method allows the determination of low concentrations of the cited impurity in capsule and tablet formulations containing the above therapeutic agent.

INTRODUCTION

Cholesterol is the precursor of bile acids and is normally present in the bile in high concentrations. The alteration of the bile composition, that is due to an excessive quantity of cholesterol with respect to the bile acids and to the phospholipids, causes the formation of gall-stones.

For the treatment of cholesterol gall-stones disease, two of bile acids present in human, chenodeoxycholic acid (CDCA) and the ursodeoxycholic acid (UDCA) are used.[1,2] However, because of the side-effects associated with CDCA therapy, UDCA is the most commonly administered compound.[3] Since the pharmaceutical preparations of UDCA could contain impurities of CDCA because the above drug is produced from bovine bile, a novel method of analysis of both the compounds has been investigated.

Recently, different procedures for the determination of bile acids in pharmaceutical and biological matrices have been reviewed.[4] These include especially chromatographic techniques, but also electrochemical, enzymatic and immunological methods.

The HPLC technique is the method of choice due to its precision and simplicity. But, the conventional UV detection system has a limited sensitivity for the unconjugated bile acids because they have weak molar absorptivities and short UV wavelengths must be used.[5-7] This results in an increase of the interferences from matrix components.

In order to improve the sensitivity for the HPLC determination of free bile acids, more convenient revelation methods have been used. Alternating voltage polarographic detection,[8] pulsed amperometric detection,[9] direct analysis by electrochemical detection (ED)[10] and direct combination with a mass spectrometer[11,12] represent some significant examples that have appeared in literature. A chemical label should permit measurement at low concentrations and minimize the effect of interfering substances present in complex matrices. In fact, precolumn labeling of bile acids allows sensitive UV and also fluorescence and electrochemical detection.[13-17] Particularly, HPLC coupled with ED represents a very sensitive method providing enhanced selectivity as a result of the limited number of substances which can undergo redox reactions under certain conditions.[18]

This paper describes a highly sensitive and selective method for the determination of impurity of CDCA in tablets and capsules of UDCA by HPLC-ED after pre-column derivatization. To this purpose we established a novel reaction of derivatization of these two bile acids with 2,5-DBE to form electroactive esters measurable by HPLC with ED. Moreover, a conventional on-line UV detector was present. The electrochemical probe 2,5-DBE was previously synthesized from 1,4-dimethoxybenzene,[19] or from 2,5-diacetoxy-α-diazoacetophenone[20] with different steps of reaction and low yields.

We have synthesized the 2,5-DBE with more satisfying results and in a single step of reaction from 2,5-DAP by direct bromination with phenyltrimethyammonium bromide tribromide ($PTMABr_3$). The brominating agent we have used has the advantage to reduce the forming of byproducts and to exploit a less toxic reagent than the ones generally used.[21]

EXPERIMENTAL

Apparatus

The HPLC apparatus comprises two Model 510 pumps, a Model 712 WISP auto-injector and a Model 490E absorbance detector (Waters Assoc., Milford, MA, USA) set at 257 nm and 0.05 absorbance units full scale. The UV detector was connected in series with the electrochemical detector (Model 5100A Coulochem; ESA, Bedford, MA, USA) which consisted of a control module and an analytical cell (Model 5010) containing two on-line porous graphite coulometric electrodes.

The analysis was performed in the oxidative mode. The ED sensitivity range and response time were set at 100 nA and 10 s, respectively. Signals from the detectors were converted to chromatographic traces and integrated by an APC IV computer system (NEC, Boxborough, MA, USA) using Maxima 820 software (Waters Assoc., Milford, MA, USA).

Mass spectra were obtained on a model Kratos MS 25 RF. IR spectra were recorded on a Perkin-Elmer 1600 Fourier transformed spectrometer as KBr disks. The ^{1}H-NMR spectra were recorded at 80 MHz on a Bruker WP instrument for $CDCl_3$ solutions with tetramethylsilane (TMS) as internal standard. Chemical shifts are expressed in ppm (δ). Elemental analysis for C,

H, N were obtained on a Carlo Erba 1106 analyzer (Milan, Italy) and agreed with theoretical values to within ± 0.4%. UV absorption spectra were recorded on a Uvikon 860 (Kontron, Zurich, Switzerland) spectrometer in CH_3CN/MeOH 9:1 solution. Analytical thin layer chromatography (TLC) was performed on Merck 60 F_{254} silica gel plates.

Chemicals

2,5-Dihydroxyacetophenone, phenyltrimethylammonium bromide tribromide, ursodeoxycholic acid, and chenodeoxycholic acid were obtained from Fluka (Buchs, Switzerland). HPLC-grade methanol, acetonitrile and water were from Carlo-Erba (Milan, Italy). Other chemicals used were of reagent grade or better.

Chromatographic Conditions

Separations were performed on a 3 μm Adsorbosphere column (100x4.6 mm i.d.; Alltech, Deerfield, IL, USA) fitted with a guard column (Hypersyl ODS RP-18, 5 μm particles, 4x4 mm i.d.; Policonsult, Rome, Italy) and eluted, isocratically, with methanol:acetonitrile:sodium acetate buffer 0.1 M (60:20:20, v/v) at pH 6.5. The mobile phase was filtered through GS-type filters (0.22 μm, Millipore, Bedford, MA, USA) and on-line degassed with a Model ERC-3311 solvent degasser (Erma, Tokyo, Japan). Chromatography was performed at room temperature (21°C) and a flow-rate of 1.0 mL/min.

Synthesis

1-(2,5-Dihydroxyphenyl)-2-bromoethanone(2,5-DBE)

Phenyltrimethylammonium bromide tribromide ($PTMABr_3$), (2.5 g, 6.6 mmol) was slowly added to a solution of 2,5-dihydroxyacetophenone (2,5-DAP) (1g, 6.6 mmol) in 20 mL of dry THF. The mixture was kept under stirring overnight at room temperature (21°C) and checked by TLC with eluent cyclohexane:ethyl acetate (7:3 v/v). The precipitate formed was removed by suction filtration, and then rotaryevaporated to dryness. The residue was purified by flash chromatography (cyclohexane:ethyl acetate 7/3 v/v) giving

960 mg of 2,5-DBE (yield 63%). UV: λmax 255 nm, ε = 10,356 M^{-1} cm^{-1} I.R. (KBr cm^{-1}) λ_{max}: 3335 (OH), 1620 (C=O) cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 11.4 (s, 1H, OH); 11.1(s, 1H, OH); 7.3-6.7(m, 3H, ArH); 4.4(s, 2H, CH_2). MS (m/z): 232 (M^+ +2), 230, 150, 136, 108.

1-(2,5-Dihydroxyphenyl)-2-ethanone-2-chenodeoxycholate (2,5-DE-UDC)

The solution of UDCA (196 mg, 0.5 mmol) in 5 mL of dry CH_3CN was added to 2,5-DBE (120 g, 0.5 mol), 100 μL of triethylamine (0.71 mmol), and heated at 70°C for 2 h. The mixture was diluted with 20 mL of H_2O and was extracted three times with diethyl ether. Then the organic layer was washed with saturated $NaHCO_3$ and water, dried (Na_2SO_4), evaporated and purified by flash chromatography (hexane:ethyl acetate 7:3 v/v) giving 185 mg of product (yield 70%). UV: λmax 257nm, ε = 15,890 M^{-1} cm^{-1}; IR (KBr, cm^{-1}) νmax: 3335 (OH), 2931 (CH_2), 1729 (CO), 1660 (Ph-CO); MS (m/z): 542 (M+), 507, 415, 373, 341, 150, 137, 109.

1-(2,5-Dihydroxyphenyl)-2-ethanone-2-ursodeoxycholate (2,5-DE-CDC)

This compound was obtained by the same procedure described for the 2,5-DE-UDC.

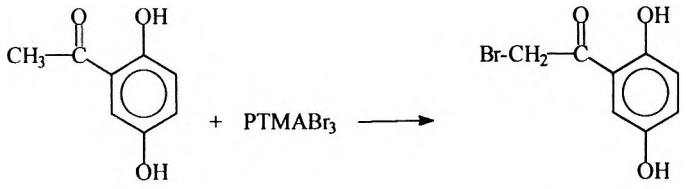
UV: λmax 257 nm, ε=15,503 M^{-1} cm^{-1}; I.R. (KBr, cm^{-1}) νmax: 3445(OH), 1732(CO), 1657(Ph-CO); MS (m/z): 542(M^+), 507, 357, 150, 137, 109.

RESULTS AND DISCUSSION

Derivatization

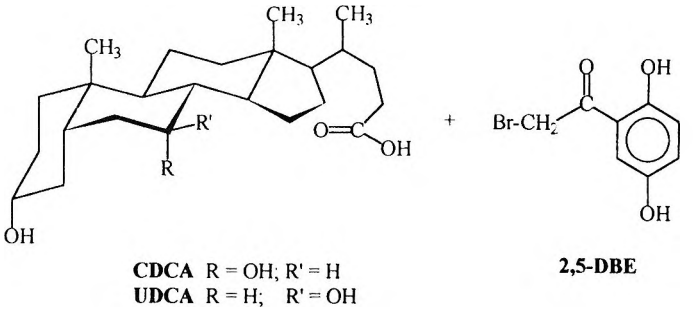
Scheme 1 illustrates the novel synthesis of 2,5-DBE. The preparation of this labeling reagent has been performed in one step. In fact, the treatment of 2,5-DAP with $PTMABr_3$ has given the brominated product immediately.

Scheme 2 represents the CDCA and UDCA reaction of esterification with 2,5-DBE to give the derivatized compounds 2,5-DE-CDC and 2,5-DE-UDC by nucleophylic substitution. The experiments have been performed to determine optimum derivatization time in order to give maximum conversion of the bile acids to their electroactive derivatives.



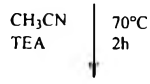
2,5-DAP 2,5-DBE

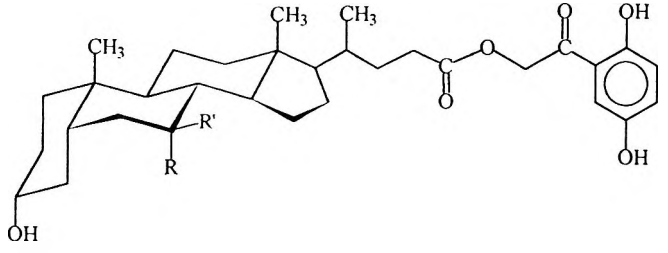
Scheme 1: Reaction of 2,5-DAP with $PTMABr_3$ to give labelling reagent 2,5-DBE.



CDCA R = OH; R' = H
UDCA R = H; R' = OH

2,5-DBE





2,5-DE-CDC R = OH; R' = H
2,5-DE-UDC R = H; R' = OH

Scheme 2: Derivatization reaction of CDCA and UDCA with 2,5-DBE to give the electroactive esters 2,5-DE-CDC and 2,5-DE-UDC.

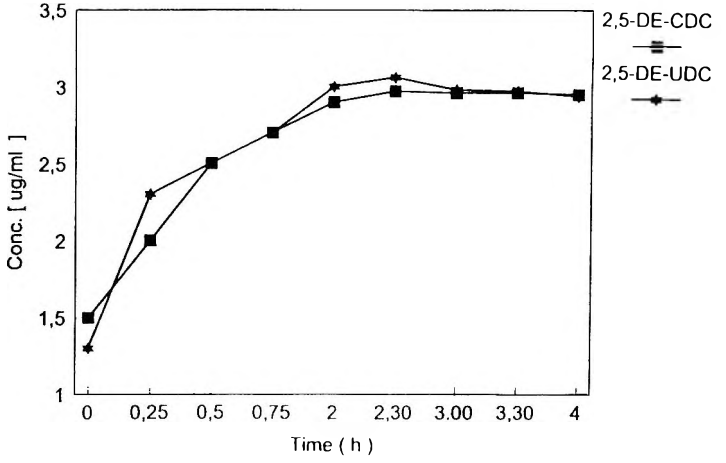


Figure 1. Concentration of derivatized esters 2,5-DE-CDC and 2,5-DE-UDC *versus* time curve.

In fact, figure 1 shows the trend of the derivatization procedure which is complete after 2 h with 70% yield. The synthesized esters show stability in the reaction mixture up to 12 h after the optimum.

Optimization of Detection

Several parameters has been examined in order to optimize the electrochemical detection of the above compounds. Under the chromatographic conditions, which are soon after described, the ester derivatives responded at the ED oxidation potentials higher than +0.2 V. The enhanced signals were obtained as the working electrode potential has been increased from + 0.2 V to +1.0 V. With the additional applied potential, no further increases in ester peak heights occurr and a rise in the background current can be observed. Electroactive properties of the compounds 2,5-DE-CDC and 2,5-DE-UDC have been examined by their hydrodynamic voltammograms (figure 2). The figure indicates that the best potential is +0.6 V, because for superior potentials the response would be an amplified one only for the derivatization reagent 2,5-DBE.

The ED performance has been markedly influenced by the ionic strength but not by the pH of the mobile phase. With increasing concentrations of the sodium acetate buffer (from 0.05 to 0.1 M), an increase of the bile

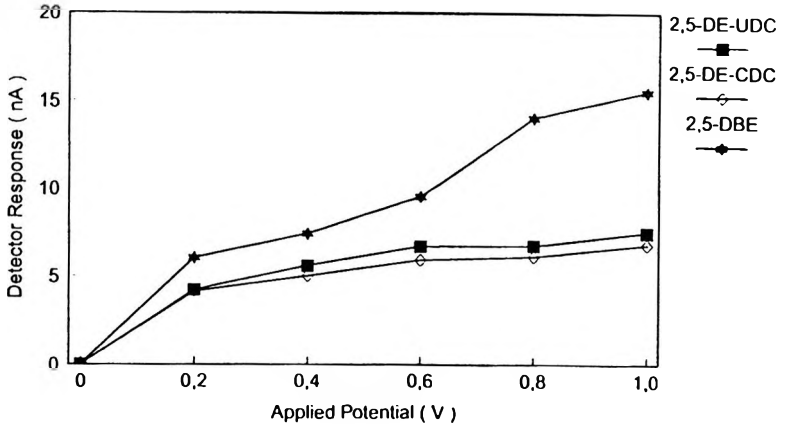


Figure 2. Hydrodynamic voltammograms of the electroactive compounds.

esters electrochemical response has been observed. No significant improvement in the detector response has been achieved by further increasing the sodium acetate molarity, which was consequently fixed at 0.1 M and the pH at 6.5.

Chromatography

Figure 3 shows a representative HPLC chromatogram with ED detection of standards. Chromatographic separations have been carried out under isocratic reversed phase conditions on a 3 μm Adsorbosphere column. The mobil phase consists of the ternary mixture methanol:acetonitrile: sodium acetate 0.1 M (60:20:20, v/v), at flow-rate of 1 mL/min.

The analysis is complete within 9 minutes and the retention times are 4.07 for 2,5-DE-UDC and 8.71 for 2,5-DE-CDC, at concentrations of 4.4 and 3.9 nmol/mL, respectively. The injected volume is 5 μL.

In the described analysis conditions, from the conventional UV detector connected on-line and set at 257 nm there was no signal. When the concentration values of the bile esters are increased by about twenty times, we observe only the relative peak to the derivatized drug. So, the UV detection is also applicable but only for concentrations superior to 80/90 pmol/mL and for the assay of the principal compound.

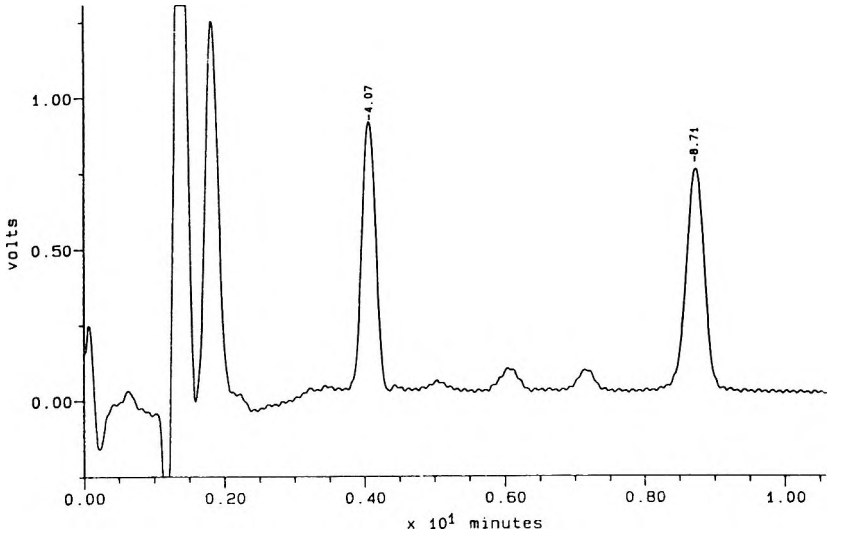


Figure 3. Chromatogram obtained by HPLC with ED detection of 5 μL of the standard solutions of electroactive esters 2,5-DE-UDC (4.4 nmol/mL) and 2,5-DE-CDC (3.9 nmol/mL).

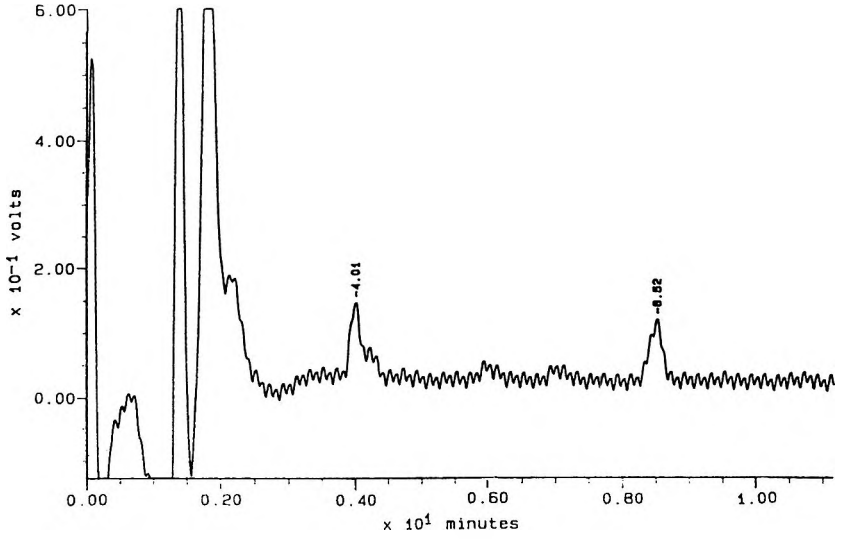


Figure 4. Chromatographic recording of detection limit of electroactive esters 2,5-DE-UDC (0.88 pmol/mL) and 2,5-DE-CDC (0.78 pmol/mL).

Linearity and Detection Limit

The linearity of response has been examined for both esters 2,5-DE-CDC and 2,5-DE-UDC in the range 0.2-2.0 μg/mL. The coefficients of linear regression of the standard curves have been consistently greater than 0.99.

Detection limit (figure 4) is determined by five runs and it is in the range of 0.88 and 0.78 pmol for 2,5-DE-UDC and 2,5-DE-CDC respectively (S/N= 5).

Analysis of Pharmaceutical Formulation

Four different commercial formulations containing UDCA have been subjected to the derivatization and HPLC analysis using the proposed ED system. The high sensitivity achieved by ED monitoring permits an accurate quantification of the trace of the CDCA impurity present in the pharmaceutical preparations. Figure 5 shows a representative HPLC chromatogram of a tablet formulation.

Under the same analysis conditions as the impurity traces there was an excessively high signal due to 2,5-DBE and 2,5-DE-UDC. This has made it impossible to quantify both compounds in the same chromatogram. Then the active principle can be determined by dilution or by an on-line UV detection system.

Figure 6 shows the HPLC chromatogram of the same formulation using the UV detector. Other potential bile acid impurities or other ingredients of the formulations do not interfere with the analysis of both compounds when the detection is carried out with a UV detection system and with ED.

The results, presented in Table 1, are in agreement with the label claim and demonstrate the precision of the method.

CONCLUSION

The derivatization of bile acids CDCA and UDCA with 2,5-DBE yields stable and highly sensitive electroactive esters, which are measurable by HPLC. The derivatization procedure developed in this study allows the detection of CDCA impurities at a level of less than 1 pmol.

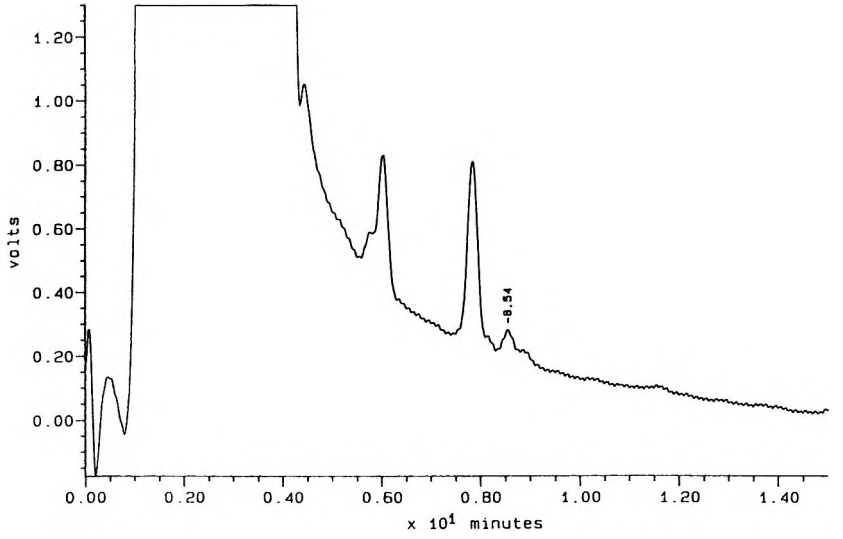


Figure 5. Chromatographic recording of derivatized CDCA impurity present in a tablet preparation.

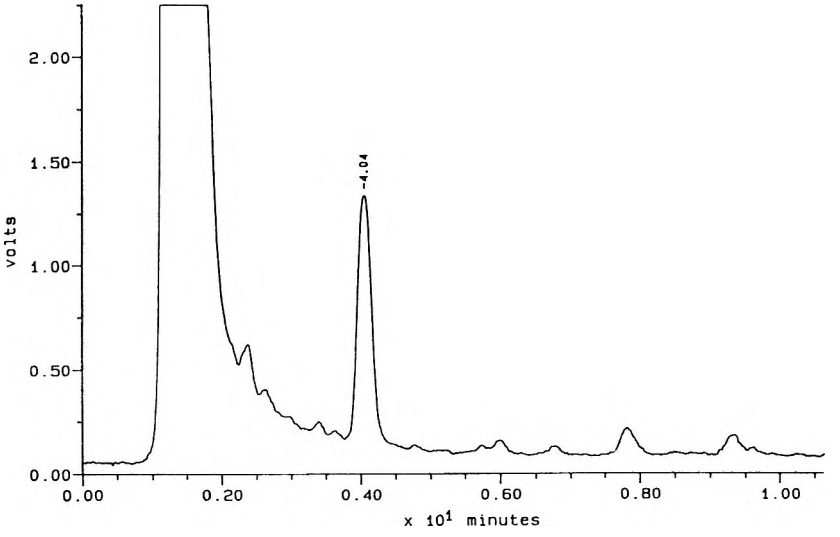


Figure 6. Chromatographic recording of UDCA obtained by HPLC with UV detection.

Table 1

Assay Results for the HPLC Determination of CDCA Impurity in Commercial Pharmaceutical Formulations of UDCA

Formulation	Label Claim (mg)	% Found*	%CDCA*
Tablet 1	UDCA 300	99.7 (0.3)	1.2 (2.5)
Tablet 2	UDCA 150	101.0 (0.8)	1.3 (4.5)
Capsule 1	UDCA 450	101.6 (1.4)	0.6 (3.9)
Capsule 2	UDCA 250	100.2 (1.1)	0.8 (5.4)

* Mean (RSD) of six determinations

The sensitivity obtained is higher than that produced by the electrochemical methods reported in the literature.[8-10] The applied potential permits the selective oxidation of the esters derivatives without interference because of the limited number of substances which can undergo redox reactions under this condition.

Because of the selectivity achieved, no interference from the commercial product matrix was observed by the method described here and consequently complex extraction procedure are not required. Since this method offers a means of enhancing the selectivity and sensitivity, it can be used to determine the trace amounts of the electroactive compound CDCA in quality control assays of commercial pharmaceutical formulations containing the UDCA drug.

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DETERMINATION OF ORGANOPHOSPHORUS PESTICIDE RESIDUES IN CARROT USING GEL PERMEATION CHROMATOGRAPHY

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ABSTRACT

To investigate the application of gel permeation chromatography to the determination of organophosphorus pesticides in carrot, phorate, dimethoate, pirimiphos-methyl, chlorfenvinphos, quinalphos and triazophos were chosen. Samples were extracted with ethyl acetate and the extracts clean-up by gel permeation chromatography using Bio-Beads SX-3 gel. The high pigment content of samples caused band broadening on the column.

INTRODUCTION

The extraction and partition steps of standard multi residue methods for pesticides provide a degree of clean-up in food matrix. However, food samples contain a wide variety of compounds that are extractable and may give rise to interferences or other problems. The most universally applicable clean-up procedure is gel permeation chromatography or size exclusion chromatography. It uses a macroreticular resin matrix that progressively retards the elution of

compounds based on the inverse of molecular size. Large lipids, and polymeric co-extractives, elute earlier than most pesticides. The technique was first applied by Stalling et al.[1] to the cleanup of organochlorine and polychlorinated biphenyl residues in fatty foods.

Specht and Tillkes[2] developed a GPC system (column: 25mm ID x 40cm long) using polystyrene resin Biobeads SX-3 with cyclohexane-ethyl acetate (1:1) as mobile phase, which was suitable for clean-up of a wide range of pesticides in various foods.

The wide applicability of Bio-Beads SX-3 is indicated by its use in the determination of organophosphorus pesticides residues in fruit and vegetables,[3] in cereals, cereal products and animal feed[4] and in quantitative detection of the pesticides in plant and animal tissues.[5]

The main disadvantages are; -lower molecular weight co-extractives often elute in the pesticide fraction and the separation of the large pesticides is incomplete. For example, interferences from the food matrix can be reduced by increasing the dump fraction, but large pesticides, which elute early, are also progressively removed.[6]

The aim of this work was to investigate the application of gel permeation chromatography to the determination of organophosphorus pesticides in carrots, six pesticides were chosen. Compounds representative of the pesticides which have been used on carrot were; phorate, dimethoate, pirimiphos-methyl, chlorfenvinphos, quinalphos and triazophos.

MATERIALS AND METHODS

The pesticides used as test substances were obtained from Dr. Ehrenstorfer, Augsburg (Germany). Bio-Beads SX-3 (200-400 mesh) was purchased from Bio-Rad Laboratories (UK). Samples of the organically-grown carrots were provided by a health food shop in local market.

Gel Permeation Chromatography (GPC)

Column: Anachem glass preparative chromatography column (45cm x 1cm ID) fitted with a polytetrafluoroethylene (PTFE) bed support and adjustable plunger.

Sample introduction: Rheodyne PTFE rotary valve fitted with a 1mL sample loop.

Solvent delivery system: Kontron Model 420 HPLC pump, flow rate 1mL/min.

Elution solvent: Cyclohexane-Ethyl acetate (1:1).

Column preparation: An appropriate amount (30g) of Bio-Beads SX-3 was placed in a 500mL conical flask and an adequate volume of elution solvent (200mL) was added. The gel was left to stand for 24h and fully swollen gel was packed into the column. After the gel had settled, the plunger was depressed until a bed height of 35cm was obtained. Elution solvent was pumped through the column at a flow rate of 1mL/min for 2h prior to use.

Gas Chromatography (GC)

A Carlo Erba 4200 gas chromatograph equipped with a flame ionization detector (FID) and 25m x 0.32mm ID fused silica column containing a 0.5 micron film thickness of BP-1 non-polar bonded phase was operated under the following conditions; Helium carrier gas flow rate was 2mL/min, injector and detector temperatures were 250°C and 260°C respectively, the oven temperature programmes were; 1) initial temperature isothermal at 150°C for 2 min, and from 150°C to 230°C at 15°C/min, then 20 min at 230°C, 2) initial temperature isothermal at 100°C for 5 min, and from 100°C to 260°C at 5°C/min, then 20 min at 260°C. The samples were injected in the split mode with a split ratio of 30:1. Results were collected using a Spectra Physics 4270 integrator.

Preparation of Samples

Extraction

Chopped carrot (50g) were placed in the spark-proof blender and ethyl acetate (50mL) and sodium sulphate (30g) added and then homogenized at high speed for 3min. The homegenate was filtered and re-extracted with further ethyl acetate (20mL). This step was repeated and the extracts were combined. The combined extracts were concentrated tc approximately 20mL in vacuo using a rotary evaporator.

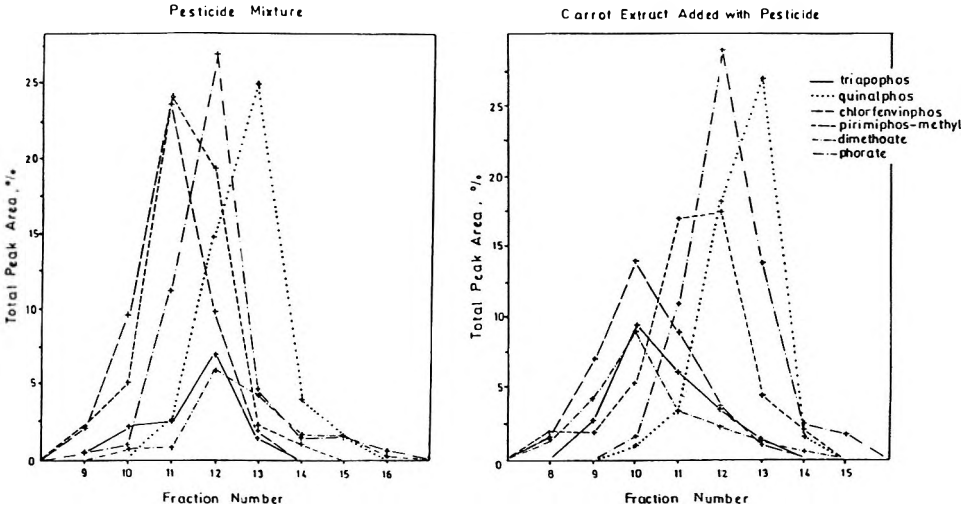

Figure 1. Elution Profiles of the Pesticides from the Bio-Beads SX-3 column.

Fortification

Carrot extract (20 mL), which contained no pesticide, was fortified by adding a known volume of the standard mixture solution of the pesticides. The concentration of the pesticides was 0.2 mg/mL.

Pesticide Elution Profile

A standard mixture solution (0.2mg/mL) of pesticides prepared in cyclohexane:ethyl acetate (1:1). A 1 mL aliquot of the standard mixture solution was injected onto the column and twenty 2mL fractions of the column eluate were collected. The GPC column was washed for a further 10 min before the next sample was introduced. The total run time was 50 min.

Each fraction was reduced to 0.1 mL using a stream of N_2 and then analysed using GC to determine the amount of pesticide pesent. The same procedure was carried out on the fortified carrot extract.

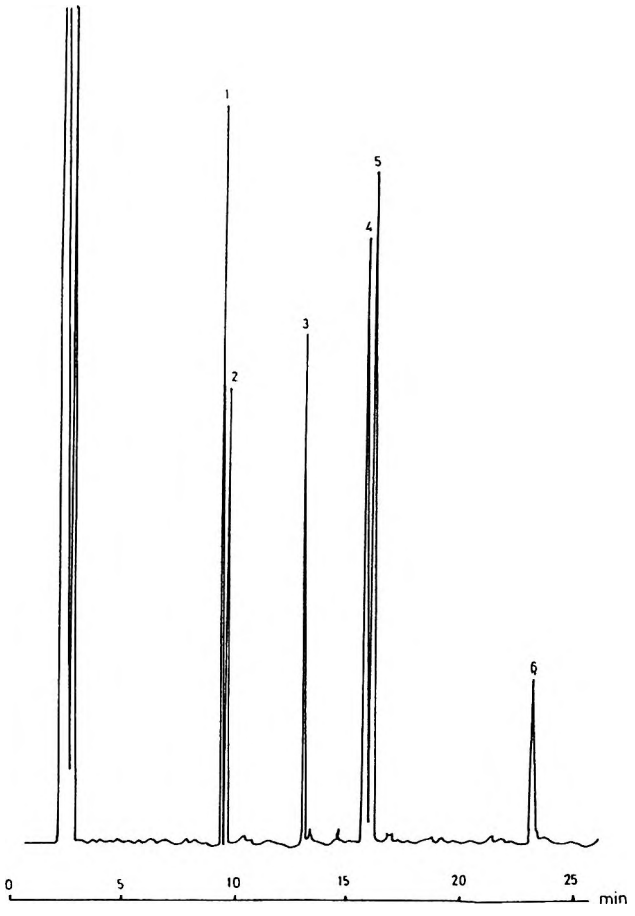

Figure 2. Separation of six OP pesticides on a BP-1 fused silica capillary column. Temp. prog: 150°C for 2 min., 15°C min^{-1} to 230°C, Peaks: 1. Phorate, 2. Dimethoate, 3. Pirimiphos-methyl, 4. Chlorfenvinphos, 5. Quinalphos, 6. Triazophos.

RESULTS AND DISCUSSION

The fractions from the GPC column containing pesticides are shown in Figure 1. The peak areas of the pesticides, which were obtained from the GC integrator, were plotted against the fraction number. The GC chromatogram of the pesticide mixture obtained with a 25m BP-1 non-polar column is given in Fig. 2.

Table 1

Elution Profile of the OP Pesticides from a Bio-Beads SX-3 GPC Column

Pesticide	Elution Volume for Pesticides mL	Volume Fraction No	Elution Volume for the Fortified Carrot Extract, mL	Volume Fraction No
Phorate	15 - 33	8 - 17	17 - 31	9 - 16
Dimethoate	17 - 33	9 - 17	13 - 29	7 - 15
Pirimiphos-methyl	15 - 29	8 - 15	13 - 29	7 - 15
Chlorofenvinphos	15 - 27	8 - 14	13 - 27	7 - 14
Quinalphos	19 - 31	10 - 16	17 - 29	9 - 15
Thiazophos	15 - 27	8 - 14	145 - 29	8 - 15

The elution volume required from the Bio-Beads SX-3 column for each of the pesticides in the standard mixture solution and in fortified carrot extract are given in Table 1. For each pesticide, there was a volume range in which the compound eluted from the GPC column. The separation between the pesticides was incomplete since their molecular weights were close to each other. Chamberlain (1990) has reported a similar elution profile for some organophosphorus pesticides for cereals using a Bio-Beads SX-3 GPC column.

The 'dump volume' was determined as 15 mL and the 'collect volume' was determined as 20 mL from the graph in which is shown the elution profile of pesticides from carrot extracts. The elution volumes of pesticides from the GPC column demonstrated a small difference between the pesticide mixture alone and the carrot extract which is fortified by pesticides. Separation between the co-extractives and the pesticides was incomplete and the co-extractives from the carrot caused band broadening on the column.

This effect arose from relatively high pigment content of carrot, particularly β-carotene which is the dominant pigment in carrot. To reduce the pigment effect, a less concentrated carrot extract, equivalent to 1 g/mL (50g. of carrot was homogenised with 50 mL ethyl acetate and the homogenate was washed twice with 20 mL of ethyl acetate, then the extract was concentrated to ca. 40 mL instead of 20 mL), was injected onto the column, but the broadening was not completely prevented.

Interferences from the carrot could be reduced by increasing the dump fraction but large pesticides, such as chlorfenvinphos, which elute early, would also be progressively removed. Separation mechanisms other than size exclusion, i.e. adsorption and partition, may also be involved. The prevalence of one type of the mechanism over the other is largely determined by the mobile phase and packing pore size. With Bio-Beads SX-3 gel (2000 molecular weight exclusion limit), both size exclusion and adsorption occurred in the presence of the poorly solvating mobile phase. The technique provided adequate clean-up and further clean-up was not required before applying onto the gas chromatography column.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF TRAMADOL IN PHARMACEUTICAL DOSAGE FORMS

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ABSTRACT

A simple, specific and accurate high performance liquid chromatographic (HPLC) method for determination of tramadol in pharmaceutical dosage forms has been developed. Reversed phase chromatography was conducted using μ-Bondapak C_{18} column (3.9 x 150 nm) with an isocratic mobile phase consisting of 0.005 M triethylamine in 0.01 M sodium phosphate buffer (pH 5.5) containing 17% acetonitrile. The effluent was monitored on a UV detector at 230 nm. Each analysis required no longer than 8 minutes. Quantification was achieved by the measurement of the peak-area ratio of the drug to the internal standard (metoclopramide) and the detection limit was 75 ng/mL. Linear response ($r > 0.999$) was observed over the range of 0.1 - 10 μg/mL and was run on 6 different occasions. There was no significant difference ($p < 0.05$) between inter- and intra- day studies for tramadol determined for two different concentrations

(0.5 and 5.0 mg/mL). The mean relative standard deviations (RSD%) of the results of within-day precision and accuracy of the drug was < 7%. The stability of tramadol at different temperatures indicated that the drug is stable at 4, 25, and 50°C for at least 4 weeks. The effect of light, 1 N HCl, and 1 N NaOH on the stability of tramadol has also been investigated.

INTRODUCTION

Tramadol hydrochloride ((±)-trans-2-(dimethylaminomethyl)-1-(m-methoxyphenyl)- cyclohexanol hydrochloride; Tramal®) is shown in Figure 1. It is a centrally acting analgesic drug.[1] Tramadol has opioid agonist properties and activates monoaminergic spinal inhibition of pain. In healthy volunteers with experimentally induced pain, oral tramadol exhibited analgesic activity similar to that of dextropropoxyphene and was more effective than flupirtine[2] or dipyrone (metamizole)[3] but less effective than tildine.[4] Tramadol could be administered orally, rectally, intravenously or intramuscularly. Intravenous Tramadol 50-100 mg was equivalent in analgesic efficacy to morphine 5 to 15 mg/kg in patients with moderate pain following surgery.[5] Orally administered tramadol 100 mg was superior to that of placebo in a double blind crossover study in 12 healthy volunteers with pain induced by selective transcutaneous stimulation of the sural nerve.[6]

Most of the analytical methods available to quantitate tramadol utilize Gas Chromatographic assays with mass spectrometric detection (GC-MS).[7-8] To our knowledge, there is no up to date HPLC method available for the quantitation of tramadol in dosage forms for quality control purposes. Bioanalytical assays of tramadol in plasma and urine are available in the literature, however, they are written in Chinese.[9-10] This investigation describes a simple, specific, and sensitive HPLC method for the determination of Tramadol in pharmaceutical dosage forms.

EXPERIMENTAL

Chemicals and Reagents

Tramadol hydrochloride was a gift from Grunenthal (Stolberg, Germany). Metochlopramide was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A). All other reagents and chemicals were of HPLC grade and used as received.

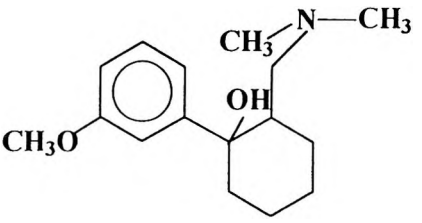

Figure 1. Structural formula of tramadol.

Chromatography

A Waters chromatographic system was used in the current method and consisted of the following: Waters 501 solvent delivery system, Waters 717 autosampler, Model 484 tunable absorbance UV detector set at 230 nm. Chromatograms were recorded on a Waters 746 Data Module integrator chart. The analytical separation was achieved on a μBondapak C-18 (5 mm particle size, 3.9 x 150 mm ID) equipped with a guard column (5 μm particle size, 25 x 4 mm ID).

The mobile phase consisted of 17% acetonitrile in 0.01 M sodium phosphate buffer (pH 5.5) containing 0.005 M triethylamine. Degassing of mobile phase was achieved by filtration through a 0.22 μm membrane filter.

The mobile phase was pumped isocratically at a flow rate of 1.2 mL/ min. The HPLC system was operated at ambient temperature. The chart speed was 0.25 cm/min. The injection volume ranged from 25 to 50 μL. Each sample run time required no longer than 8 min.

Standard Solutions

Stock solution of tramadol HCl (as 1 mg/mL tramadol) and metochlopramide (1 mg/mL), internal standard (IS), were prepared in deionized water (HPLC quality water). Tramadol stock solution was diluted to 100 and 10 mμg/mL to be used as working solutions. Weekly dilutions were made in deionized water to give tramadol concentrations of 0.1 - 10 μg/mL, and a constant concentration of 0.5 μg/mL of the IS. An aliquot of each standard was injected onto the HPLC in triplicates to be used as the standard curve.

Analysis of Dosage Forms

Capsules

The content of each capsule (50 mg labeled) was emptied in 200 mL of HPLC deionized water. Following vigorous shaking for 10 min, the undissolved contents were allowed to settle down. A ten mL of the supernatant was transferred to a 15 mL screw capped tube and centrifuged at 6000 rpm for 5 min. A one mL of the above solution was diluted with water to a final concentration of 10 μg/mL. An aliquot of 150 μL was mixed with 150 μL of the internal standard and a volume of 50 μL was injected in triplicates onto the HPLC for chromatography. Six replicates of commercial capsules were analyzed using the same method.

Recovery was calculated using a reference standard containing 50 mg (n = 3) subjected to the same treatment as described above for the capsules.

Intravenous ampoules

An aliquot of 100 μL from the ampoule (100 mg/2 mL ampoule) was diluted 100 fold with deionized water for a theoretical final concentration of 50 μg/mL. In a ten mL volumetric flask, a one mL of the above solution was mixed with 2.5 mL of the IS and brought to volume with deionized water. Aliquots of 75 μL of the solution were injected in triplicates onto the HPLC. A total of 6 vials were analyzed using this method.

Stability Study

The stability of tramdol solution was determined under different conditions of temperature, light, and pH for a period of 4 weeks. Different amber vials of tramadol solution at a concentration of 5 μg/mL were kept at 4, 25 and 50 ^{o}C and the stability was determined on day 0, 7, 14, 21, and 28.

Also the effect of extreme pH conditions on the stability of tramdol was investigated by using 1 N HCl and 1N NaOH. The effect of light was examined by exposing clear glass vials containing the drug solution to the day light for 4 weeks. Samples were taken for analysis once a week for the different conditions.

Drug Analysis

The peak area ratios of tramadol to IS were plotted against tramadol concentrations. To assess the accuracy and precision of the within-day and between-day assay, aqueous samples of tramadol at two different concentrations of 0.5 and 5 μg/mL on six different occasions over a four weeks period. Least square linear regression analysis was used to determine the slope, the intercept, and the correlation coefficients (r) of the standard curves. Each point on the calibration curve was based on at least 6 determinations.

The standard curves were constructed over a three month period to determine the day to day variability of the slopes and the reproducibility of the assay. The amount of tramadol in each dosage form was determined after considering the dilution factor for each dosage form.

Statistical Analysis

All results are expressed as mean ± standard deviations (SD). The relative standard deviation (RSD%) was calculated for all values. The *t*-test was used to examine the concentration difference at each day, and one-way analysis of variance (ANOVA) was employed to evaluate the reproducibility of the assay. The level of confidence was 95%.

RESULTS AND DISCUSSION

Figure 2 shows a typical chromatogram obtained following the analysis of tramadol capsule. Using the above chromatographic conditions, tramdol and metoclopramide were eluted at about 6.2 and 4.4 minutes, respectively. The current method provided optimum resolution of the drug and the internal standard.

The quantitation of the chromatograms were performed using peak-area ratios (Y) of the drug to the IS *versus* drug concentrations (X). Least-squares regression calibration curves were found to be linear over the serum concentration range of 0.1 to 10 μg/mL of tramadol. The mean linear regression equation was as follows: $Y = 0.58 X + 0.011$. The mean correlation coefficients, r, was generally > 0.999. The detection limit of the assay was 100 ng/mL at a signal to noise ratio of >3.

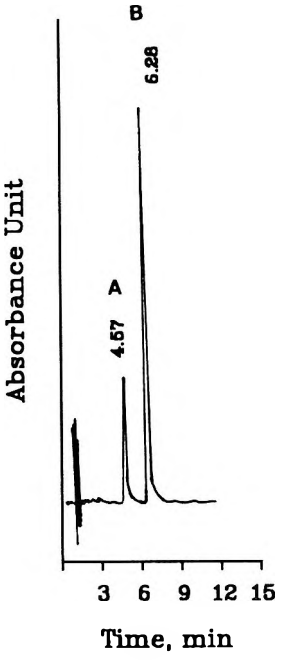

Figure 2. A typical chromatogram of tramadol capsule. A: metoclopramide and B: tramadol

Table 1 represents the results obtained for intra- and inter-day variability study of tramdol samples. The within-day precision for the studied concentrations (0.5 and 5 μg/mL) showed RSD% of 4.3 and 6.4%, respectively. The evaluated between-day precision evaluated over a 4 weeks period varied from 5.3 to 6.9%. This outcome shows the accuracy of the assay.

The reproducibility was evaluated by comparing the linear regressions of six standard plots carried out on 6 different occasions over a three month period. The mean correlation coefficient was > 0.999. The RSD% of the slopes, of the six lines, was 6.8%. Analysis of variance of the data indicated no significant difference in the slopes ($p < 0.05$), within-day and between-day, of the calibration curves of tramadol. The results confirmed the reproducibility of the assay method.

The stability of tramadol (5 μg/mL) was studied under extreme conditions of temperature and pHs. Table 2 represents the percentage recovery of tramadol at different sampling time throughout the study. Tramdol was shown

Table 1

Intraday and Interday Precision of Tramadol Standards

Theoretical Concentration (ug/mL)	Intraday[a] Measured Concentration (μg/mL) (Mean SD)	RSD%	Interday[b] Measured Concentration(μg/mL) (Mean SD)	RSD%
0.5	0.51 (0.02)	4.3	0.48 (0.03)	6.3
5.0	5.32 (0.34)	6.4	5.40 (0.37)	6.9

[a] Mean values represent six different tramadol standards for each concentration.
[b] Interday reproducibility was determined from six different runs over a 4 week period.

Table 2

Stability of Tramadol Solution Under Different Storage Conditions (at 5μg/mL)

Condition	Percent Recovery Days 0	7	14	21	28
Temperature °C					
4	102	98	99	101	96
25	103	102	97	95	93
50	98	101	99	102	99
1N HCl	103	102	104	98	101
1N NaOH	99	101	103	95	100

to be stable for at least 4 weeks at different temperatures even up to 50°C. Although extreme pH's conditions were used, the drug seemed to be stable for up to 4 weeks. When the drug was exposed to the light (in clear glass vials) at room temperature, tramdol did not show any sensitivity to light.

The amount of tramadol in each dosage form was calculated after using the dilution factor. Statistical analysis was used to detect any significant differences between capsules. One-way analysis of variance of six replicates of each capsule (six capsules were analyzed). Results indicated that no significant difference exists between the six capsules ($p < 0.05$). The same trend was observed for the intravenous dosage form ($n = 6$) subjected to the same treatment. The measured amount of each of the studied dosage forms was within the manufacturer's claimed one.

CONCLUSION

The HPLC method described herein provides a simple, rapid, and reproducible determination of tramadol in dosage forms which makes it potentially valuable in quality control of the drug's dosage forms.

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RAPID AND SENSITIVE PROCEDURE FOR THE QUANTITATION OF PIMETHIXENE IN HUMAN MILK AND PLASMA BY SOLID PHASE EXTRACTION (SPE) USING HPLC

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ABSTRACT

A method is described for the quantitative determination of pimethixene from plasma and milk samples by reversed-phase high performance liquid chromatography using diphenylpyraline as an internal standard.

The sample matrix was processed by a solid phase extraction (SPE) technique using 3 mL C_{18} Supelco cartridges. The plasma and milk samples were deproteinized using acetonitrile and the liquid layer was combined with water (1:2 v/v), throughly mixed and subsequently passed through pre-conditioned cartridges. The compounds were eluted with 6 mL of methanol. Then the methanolic solution was evaporated to dryness, reconstituted with 1 mL of methanol and analysed with an octyl-ODP column (150 X 6 mm I.D).

The mobile phase consisted of acetate buffer 0.051 M - methanol (62:38 v/v). Detection was perfomed at 254 nm with the sensitivity set at 0.002 AUFS. The overall recovery of pimethixene was 86.82% in plasma and 80.39% in milk. The limit of detection, corresponding to 2 times signal to noise ratio, was estimated as 15 ng/mL in both plasma and milk samples.

INTRODUCTION

Pimethixene, 9-(1-methyl-4-piperidylidene) thioxanthene, is a potent antagonist of histamine and serotonin. It has the general properties and uses of antihistamines, and has been reported to have sedative properties.[1] Pimethixene has been used in the treatment of respiratory disorders in children[2] and are readily available over the counter under the following proprietary names: calmixene, muricalm and sedosil. Although there are several reports related to HPLC analysis of antihistamines as a class of drugs[3,4,5] but is non-specific for pimethixene. The present report describes a high performance liquid chromatographic procedure for the quantitative determination of pimethixene in milk and plasma samples by solid phase extraction, using diphenylpyraline as the internal standard.

EXPERIMENTAL

Chemicals and Solutions

Pimethixene maleate and diphenylpyraline hydrochloride were supplied by Sigma Company (St. Louis, MO. USA) and used without further purification. Ammonium acetate and glacial acetic acid, analytical reagent grade, were obtained from Merck (Darmstadt, Germany). HPLC grade methanol and water were used throughout.

Stock solutions were prepared by accurately weighing appropriate quantities of pimethixene maleate and diphenylpyraline hydrochloride, and dissolving each, separately, in a 50 mL volumetric flask to prepare a 1 mg/mL solution in methanol. The working standards of pimethixene in the concentration range between 53 and 740 ng/mL, and containing 250 ng/mL of diphenylpyraline as internal standard, were prepared by diluting the appropriate quantities of stock solution with methanol.

Apparatus

A solvent delivery systen with two Model LC-6A high pressure pumps, coupled with a model SPD-6AV UV spectrophotometric detector, operated at 254 nm were controlled by a system programmer, model SCL-6B (Shimadzu Instruments, Kyoto, Japan). The reversed-phase octyl-ODP column (150 X 6 mm I.D), product of Asahi Chemical Company, Kawasaki, Japan, was placed in a model CTO-6A oven at 40°C, and equipped with a Rheodyne 7161 injector fitted with a 20 μL loop, was operated isocratically. Chromatograms were recorded on a chart paper with a Chromatopac model C-R 6A thermal printer-plotter (Shimadzu) at a speed of 2 cm/min. Supelclean C_{18} cartridges and vacuum glass manifold were obtained from Supelco, Bellefonte, PA, USA).

Chromatographic Conditions

The mobile phase was acetate buffer, ionic strength 0.051 M - methanol (62:38 v/v). The pH was adjusted to 3.5 using glacial acetic acid. The column was equilibrated with the eluting solvent by pumping the mobile phase at a flow rate of 0.3 mL/min for 24 h and degassed by slowly bubbling with helium gas throughout the analysis. The flow rate was set at 1.4 mL/min during analysis and the detection performed at 254 nm with a sensitivity of 0.002 absorbance unit full scale (AUFS).

Extraction Procedure

Stock pimethixene methanolic solution, 1 mL, of concentrations ranging from 53-740 ng/mL and containing a fixed amount (250 ng/mL) of diphenylpyraline used as an internal standard was evaporated to dryness in a 15 mL glass centrifuge tube under nitrogen. Plasma or milk sample, 1 mL, and 3 mL of acetonitrile were added into the tubes, vortexed at high speed, briefly, and centrifuged at 2000 rpm for 5 min to precipitate the protein particles. The upper clear layer was collected and transferred to another clean tube. Water, 6 mL, was added to the tube, thoroughly mixed and subsequently treated by solid phase extraction using 3 mL C_{18} Supelco cartridges. The mixture was slowly passed through the cartridges that had been conditioned with 2 mL of methanol and washed with 2 mL of water. The cartridges were fitted to a vacuum glass manifold system that helped to control the flow rate of solvent through the particles. The sorbents were washed once with 2 mL of water before the final elution of the compounds with 6 mL of methanol. It was observed that the addition of 6 mL of water to the sample before processing by solid phase extraction was necessary, as the water helps to hold back the compounds on the

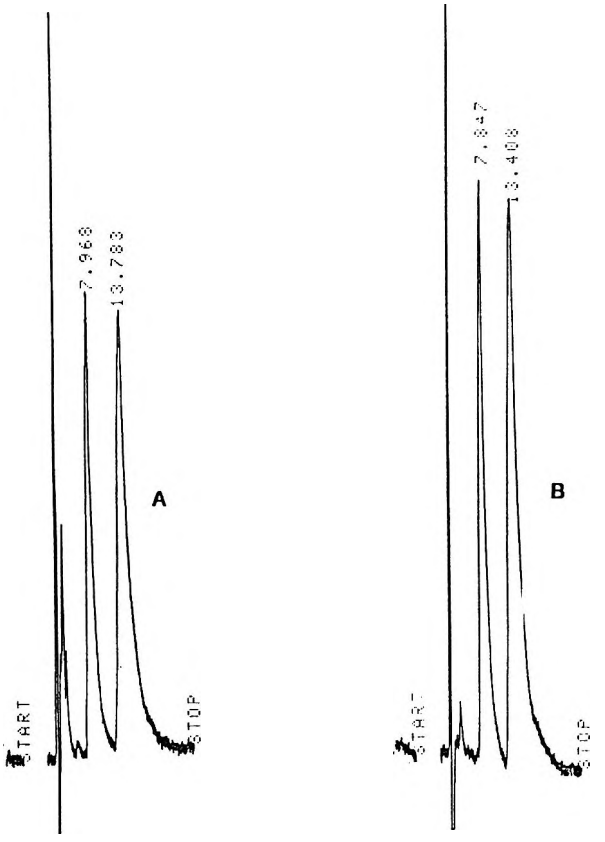

Figure 1. Representative chromatograms of (A) plasma and (B) human milk extracts spiked with pimethixine and diphenylpyraline as internal standard at different concentrations.

particles by reducing the concentration of acetonitrile which has the ability to elute the compounds together with unwanted materials from the packings. Therefore, washing the packings with 2 mL of water helps to remove the unwanted materials while holding back the desired compounds on the packing before the final elution with 6 mL of methanol. The methanolic solution was evaporated to dryness with an evaporation unit (Pierce Reacti-Therm, Model 18780) under a stream of nitrogen at 45°C. The residue was redissolved in 1 mL of methanol and aliquots of 20 μL were injected onto the analytical column.

RESULTS AND DISCUSSION

Figures 1A and 1B represent typical chromatograms of pimethixene and diphenylpyraline extracts from spiked plasma and milk samples processed by solid phase extraction. The retention time of pimethixene was 13.4 min and that of diphenylpyraline 8.0 min, with a resolution of R_s=3.67.

The calibration plot of peak height ratio of pimethixene to diphenylpyraline against concentration of pimethixene, in the range of 53-740 ng/mL was linear. The linear regression and correlation analyses were found to be y=0.0049x + 0.0044 (r=0.9999) and y=0.0046x - 0.0033 (r=0.9998) for pimethixene from plasma and milk samples, respectively.

The recovery efficiency of pimethixene from plasma and milk was determined by making a standard calibration curve of the peak height ratio of pimethixene to the internal standard after direct injection of the methanolic solution containing known quantities of pimethixene (53, 106, 211, 317, 422, 528, 634, and 740 ng/mL) against their concentrations and, by using the characteristics of this standard curve, the recovered amount of the drug after extracting spiked plasma or milk samples containing an equivalent amount of the drug was calculated. At each of the eight pimethixene concentrations used, four replicate samples were measured; results are presented in Tables 1 and 2.

The overall recovery of pimethixene was estimated by plotting a standard calibration of the "added" versus the "found" concentrations of pimethixene in both plasma and milk samples, which gave linear relationships with y=0.8682x + 9.63 (r=0.9996) for plasma and y=0.8039x + 8.33 (r=0.9998) for milk.

Therefore the slopes (0.8682 for plasma and 0.8039 for milk) of these regression lines were used as estimates of the overall recovery for pimethixene, 86.82% for plasma and 80.39% for milk samples, respectively.

Precision and accuracy of the method were assessed by spiking plasma and milk samples with pimethixene standards at two concentration levels (106 and 422 ng/mL) and each containing 250 ng/mL of diphenylpyraline as internal standard. Extraction and HPLC analysis were done as described above. The intra-day precision for each concentration level resulted from four determinations n=4, of samples of same concentration while the overall accuracy and precision for each concentration level resulted from twelve determinations, n=12, of samples of the same concentration at three different days (4X3) within one month.

Table 1

Standard Calibration and Recovery Data of Pimethixene from Spiked Plasma Samples Treated by SPE (n=4)

	Added Conc. (ng/mL)	Mean Peak Ht. Ratio	Found Conc. (ng/mL)	S.D.	C.V. %	Recovery %
1	53	0.268	55.91	0.57	1.02	105.50
2	106	0.521	100.50	0.98	0.98	94.77
3	211	1.056	194.50	1.95	1.00	92.17
4	317	1.578	286.20	2.85	1.00	90.29
5	422	2.085	375.30	3.90	1.04	88.94
6	528	2.592	464.40	4.55	0.98	87.96
7	634	3.141	560.90	5.38	0.96	88.48
8	739	3.662	652.50	6.72	1.03	88.30

$y = 0.0049x + 0.0044$ ($r = 0.999$); Average recovery = 92.05%; Average C.V. = 1.00%.

Table 2

Standard Calibration and Recovery Data of Pimethixene from Spiked Milk Samples Treated by SPE (n=4)

	Added Conc. (ng/mL)	Mean Peak Ht. Ratio	Found Conc. (ng/mL)	S.D.	C.V. %	Recovery %
1	53	0.242	51.50	0.49	0.95	97.13
2	106	0.470	91.40	1.06	1.16	86.26
3	211	0.939	174.00	1.80	1.04	82.46
4	317	1.485	269.90	2.51	0.93	85.14
5	422	1.939	349.70	3.36	0.96	82.86
6	528	2.394	429.60	4.17	0.97	81.37
7	634	2.909	520.20	5.48	1.05	82.04
8	739	3.364	600.10	6.12	1.02	81.21

$y = 0.0046x - 0.0033$ ($r = 0.9998$); Average recovery = 84.81%; Average C.V. = 1.01%.

Table 3

Accuracy and Precision Data for the Determination of Pimethixene from Spiked Plasma Samples

Spiked Amount (ng/mL)	Day	n	Accuracy Found Conc.	Precision S.D.	Precision C.V.%	Overall Mean Conc. ± S.D.
106	1	4	100.50	1.40	1.39	
	2	4	98.77	1.28	1.30	99.33±1.35
	3	4	98.68	1.37	1.39	
422	1	4	378.10	3.98	1.05	
	2	4	376.80	3.91	1.04	376.95±39
	3	4	375.90	4.01	1.07	

Table 4

Accuracy and Precision Data for the Determination of Pimethixene from Spiked Milk Samples

Spiked Amount (ng/mL)	Day	n	Accuracy Found Conc.	Precision S.D.	Precision C.V.%	Overall Mean Conc. ± S.D.
106	1	4	92.08	1.16	1.26	
	2	4	92.16	1.08	1.17	92.0±1.15
	3	4	91.77	1.22	1.33	
422	1	4	350.60	3.77	1.08	
	2	4	351.50	3.56	1.02	350.69±3.66
	3	4	350.00	3.65	1.04	

Therefore for the assessment of the overall precision within one month at two concentration levels, 24 plasma and milk samples each were treated. The results are presented in Tables 3 and 4. The detection limit of the assay, defined as the minimum drug concentration to produce 2 times signal to noise ratio at 0.002 AUFS, was found to be approximately 15 ng/mL for both plasma and milk samples.

CONCLUSION

The technical simplicity, speed and specificity of the method guarantee a reliable procedure for routine analysis of pimethixene in biological fluids.

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J. LIQ. CHROM & REL. TECHNOL., 20(5), 797-809 (1997)

DETERMINATION OF DESMETHYL-SELEGILINE, METHAMPHETAMINE AND AMPHETAMINE - THE MAIN METABOLITES OF SELEGILINE IN PLASMA BY HPLC AFTER DERIVATIZATION

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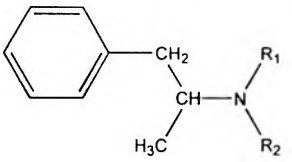
pharm-analyt Lab. GmbH.
Ferdinand Pichler Gasse 2
A-2500 Baden, Austria

ABSTRACT

Determination of desmethylselegiline, methamphetamine and amphetamine in plasma requires a very sensitive and selective method. These substances are metabolites of the MAO-inhibitor selegiline. This is the first published HPLC method which is able to determine these three substances down to a limit of quantification of 0.2 ng/mL human plasma. After extraction as free bases into an organic solvent and reextraction as salts into an inorganic acid all three substances and an internal standard (phentermine) are derivatized for fluorescence detection after reversed phase chromatography. The recovery is better than 90 % for all substances. The linearity in the range tested (0.2 - 15 ng for each substance/mL plasma) is very good indeed. The precision and accuracy is usually smaller than 5 %. Pharmacokinetic results are presented.



	R_1	R_2
Selegiline	CH_3	CH2CCH
Desmethylselegiline	H	CH2CCH
Methamphetamine	CH_3	H
Amphetamine	H	H

Figure 1. Structures of selegiline and ist amine metabolites.

INTRODUCTION

Selegiline (Deprenyl) is a selective and irreversible inhibitor of monoamino oxidase B. Therefore it is used in treatment of Parkinson's disease. Selegiline is readily absorbed from the gastrointestinal tract. As a lipophilic substance selegiline is showing a high volume of distribution and also a high rate of biotransformation. Therefore the plasma concentrations of selegiline are very low and the half-life of eliminination is very short with about 9 minutes. Selegiline is converted via metabolism to desmethylselegiline (DMS), methamphetamine (MA) and amphetamine (A). These metabolites have much longer elimination half-lifes than selegiline (see Figure 1).

Amphetamines have been of considerable interest in forensic science and toxicology and some methods for their analysis exist. Few of them, however, allow for determination at low ng/mL or pg/mL plasma.

Recently published methods for determining selegiline with GC-MS[1] or its metabolites DMS, MA and A in plasma were using GC-ECD after derivatization[2] or GC-negative ion CI-MS.[3] No more published methods can be found in the last 5 years.

This study describes the determination of DMS, MA and A in plasma down to determination values of 0.25 ng/mL for DMS and 0.20 ng/mL for MA and A. HPLC with fluorescence detection was used after extraction into an organic solvent and reextraction into an anorganic acid with following derivatization of the secondary amino group (DMS and MA) and the primary amino group (A) with the same derivatization agent.

EXPERIMENTAL

Amphetamine and methamphetamine was from the university institute of organic chemistry (Vienna/Austria). Desmethylselegiline was synthesized. The internal standard phentermine was a gift from Gerot (Vienna/Austria). Reagents of GR quality were supplied by E. Merck (Darmstadt, FRG) and by Rathburn (Scotland). The derivatization agent (AQC) was first published for amino acids by the Millipore Corporation (Milford, USA)[4,5] and was synthesized in our laboratory.

AQC: 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate

Apparatus and Chromatographic Equipment

The chromatographic system consisted of a HP-1090 (Hewlett Packard, USA) with a fluorescence detector FP-920 (Jasco, Japan) connected to a PE-Nelson 900 software for integration.

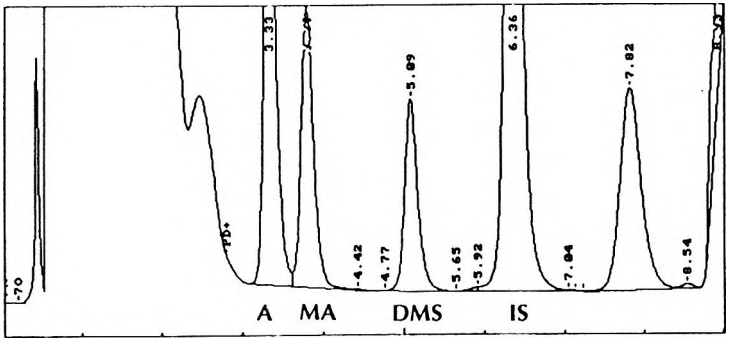
The analytical column 125 x 4 mm i. d. (SRD, Vienna, Austria) was filled with Supersphere RP 18 e μm. The mobile phase was a step gradient mixture of :

A. 19 % acetonitrile/27 % methanol/54 % buffer (v/v)
B. 75 % acetonitrile, 25 % buffer (v/v)

The step to mixture B was after 8 minutes for 2 minutes in order to clean the column. The flow rate was 2.0 mL/min at 60°C column temperature.

Preparation of Plasma Samples

1.0 mL plasma was mixed with internal standard solution (20 μL) and 50 μL 2 M sodium hydroxide. After adding 4 mL of extraction solvent (mixture of ethylacetate/hexane [1:1]) the samples were shaken for 1 minute and then centrifuged. 3 mL of the organic phase was reextracted by 0.3 mL of diluted hydrochloric acid. 0.2 mL of the acid phase was mixed with diluted sodium hydroxide solution and buffer. After adding 100 μ of derivatization agent (3 mg AQC/mL acetonitrile) the samples were mixed. After 10 minutes at room temperature, the samples could be injected.


3.33
5.09
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A
MA
DMS
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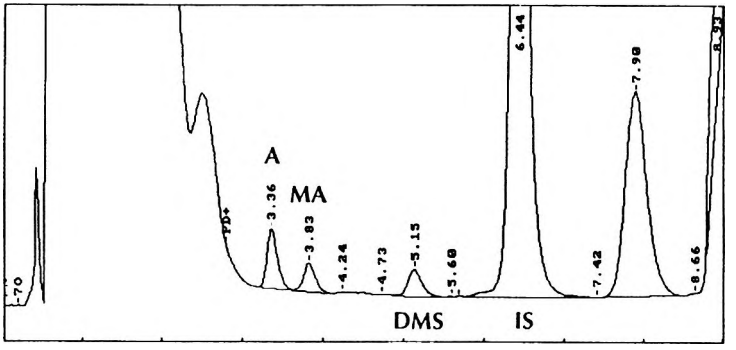
A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DMS
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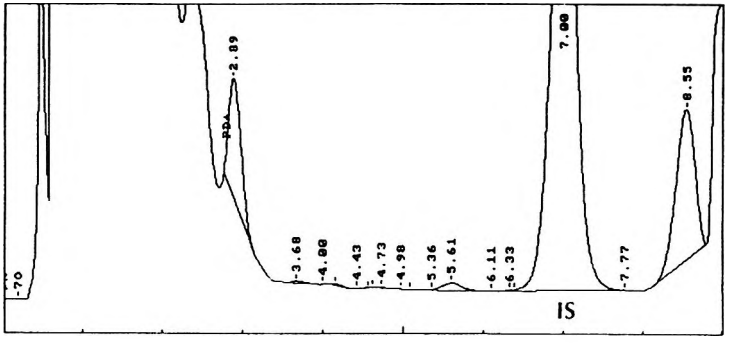
B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C

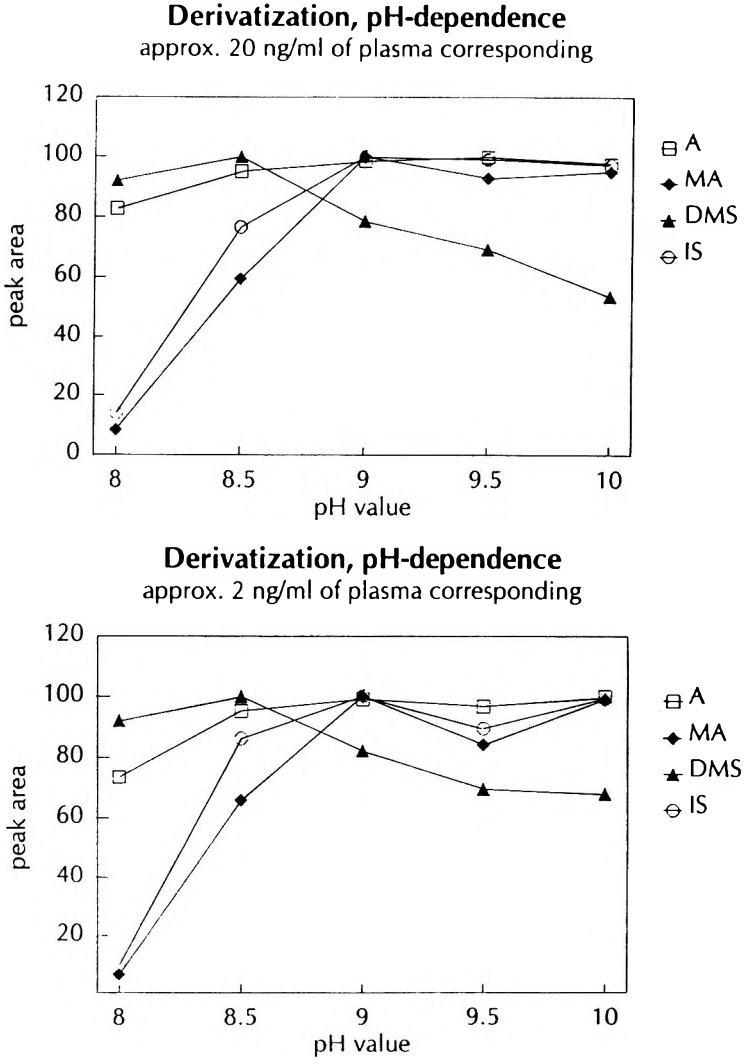

Figure 3. pH-dependence of the derivatization at 2 different concentration levels for A, MA, DMS and the Internal Standard (phentermine).

Figure 2. (left) Plasma calibration samples:
A: 5.22 ng A, 5.12 ng MA and 5.45 ng DMS / ml of plasma.
B: 0.42 ng A, 0.41 ng MA and 0.45 ng D MS / ml of plasma.
C: Blank pool-plasma.

Validation

The method was validated by adding various different quantities of DMS, MA and A to pooled human plasma. The resulting concentrations were between 0.2 and 15 ng/mL pool-plasma. These calibration series were subjected to the entire analytical procedure, so as to test the linearity, precision and accuracy of the method.

RESULTS AND DISCUSSION

Extraction and Reextraction

The absolute recovery from plasma at 4 - 9 ng/mL were 94.3 % for DMS (± 1.90 %, n = 4), 103.3 % for MA (± 0.85 %, n = 4) and 92.2 % for A (± 0.79 %, n = 4).

Chromatographic Separation

Our investigations showed some problems with endogenous plasma substances. Therefore we tried different reversed phase columns from different suppliers.

Also we changed the composition of the mobile phase with different percentages of acetonitrile and methanol. Figure 2 shows plasma calibration samples.

Derivatization Conditions

Different compositions of the reaction mixture were tried for derivatization. Fig. 3 shows the pH-dependence at 2 ng/mL and 20 ng/mL of the 3 substances and the internal standard.

Linearity, Precision, and Accuracy

In the calibration series, the linear regression between the spiked plasma concentrations and the peak area was determined after analysis of the calibration samples (Table 1).

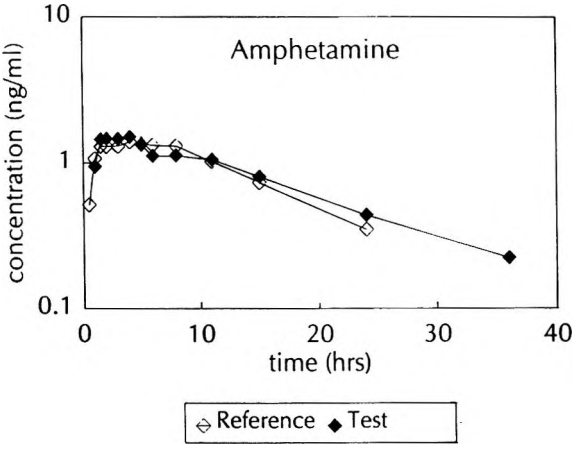

Figure 4. Plasma levels of A of a selected volunteer (vol. 7) after oral administration of 5 mg of selegiline.

Table 1

Linear Regression of A, MA, and DMS

	Slope	Intercept	Corr. Coeff	Range	Number
A	0.9545	0.0252	0.9989	0.21 - 15.29	18
MA	0.5397	0.0000	0.9981	0.20 - 14.97	18
DMS	0.3522	0.0989	0.9987	0.22 - 15.95	18

Table 2

Limit of Quantification for A, MA, and DMS

	Conc. -spiked/ mL Plasma	Precision (CV%)	Accuracy
amphetamine	0.17 ng	± 8.50 %	- 2.25 %
methamphetamine	0.18 ng	± 5.30 %	+ 12.90 %
desmethylselegiline	0.25 ng	± 12.17 %	- 8.30 %

Table 3

Amphethamine: Linearity, Precision, and Accuracy in Plasma

Sample	Conc-Obs ng/mL	Mean ng/mL	±CV%	Conc-Calc ng/mL	Accuracy %
St 0	0.013 -0.012 -0.025				
St 1	0.217 0.226 0.222	0.222	2.14	0.211	4.87
St 2	0.427 0.416 0.408	0.417	2.24	0.423	-1.32
St 3	0.848 0.843 0.807	0.832	2.68	0.844	-1.41
St 4	2.091 2.156 2.144	2.130	1.63	2.105	1.22
St 5	4.948 4.918 5.032	4.966	1.19	5.223	-4.92
St 6	15.390 15.402	15.529 15.795	1.49	15.291	1.56

Table 4

Methamphethamine: Linearity, Precision, and Accuracy in Plasma

Sample	Conc-Obs ng/mL	Mean ng/mL	±CV%	Conc-Calc ng/mL	Accuracy %
St 0	0.068 0.018 0.018				
St 1	0.232 0.223 0.218	0.224	3.25	3.207	8.38
St 2	0.425 0.403 0.383	0.404	5.14	0.414	-2.40
St 3	0.816 0.853 0.774	0.814	4.91	0.827	-1.55
St 4	2.051 2.085 2.000	2.045	2.09	2.061	-0.77
St 5	4.903 4.629 4.924	4.819	3.42	5.115	-5.79
St 6	15.513 15296 15.067	15.292	1.46	14.975	2.12

Table 5

Desmethylselegiline: Linearity, Precision, and Accuracy in Plasma

Sample	Conc-Obs ng/mL	Mean ng/mL	±CV%	Conc-Calc ng/mL	Accuracy %
St 0	-0.006				
	-0.274				
	0.020				
St 1	0.241	0.230	15.42	0.221	4.28
	0.190				
	0.259				
St 2	0.448	0.442	1.39	0.441	0.33
	0.436				
	0.444				
St 3	0.849	0.862	3.40	0.881	-2.19
	0.895				
	0.841				
St 4	2.221	2.205	1.01	2.196	0.39
	2.179				
	2.2`4				
St 5	5.170	5.217	2.19	5.449	-4.26
	5.347				
	5.133				
St 6	16.500	16.185	2.15	15.953	1.45
	16.243				
	15.812				

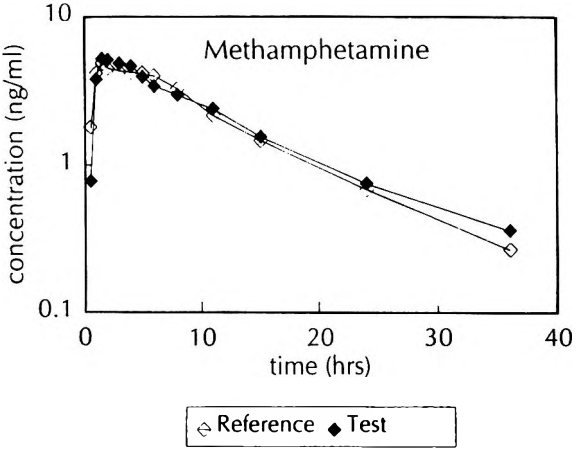

Figure 5. Plasma levels of MA of a selected volunteer (vol. 7) after oral administration of 5 mg of selegiline.

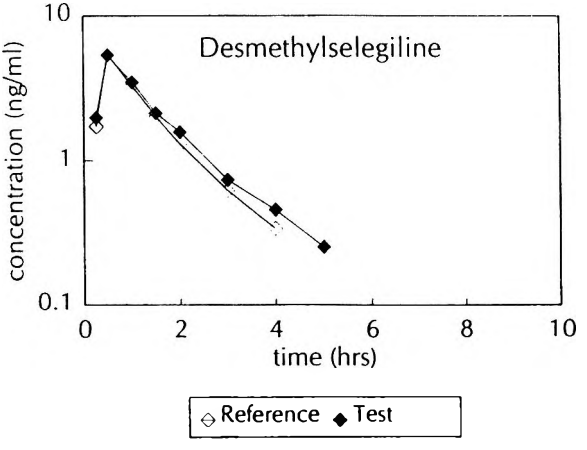

Figure 6. Plasma levels of DMS of a selected volunteer (vol. 7) after oral administration of 5 mg of selegiline.

The values for precision and accuracy[6] are listed in Table 3 for amphetamine, A, Table 4 for methamphetamine, MA, and Table 5 for desmethylselegiline, DMS.

Limit of Quantification

6 spiked plasma samples at low levels were analyzed. The following results were obtained (Table 2).

Stability

The stability was tested in spiked plasma and injection solution. A, MA and DMS were stable in plasma at room temperature over 4.5, 8 and 24 hours. The same results were obtained after two freeze and thaw cycles. The derivatized A, MA and DMS was stable in the injection solution over 27 hours at room temperature and was also stable after freezing and thawing.

Pharmacokinetics

After oral application of a single dose of selegiline the following results (see Figures 4, 5 and 6) were obtained. More detailed pharmacokinetic results from bioequivalence studies are published elsewhere.[7]

CONCLUSION

To sum up, it can be said that the analytical method described herein is the first HPLC method for measuring A, MA and DMS together to a very low level of about 200 pg/mL plasma. Only one published method is able to measure all 3 substances to such a low level and this method used GC-negative-ion CI-MS.[3]

ACKNOWLEDGEMENT

The authors wish to thank Mr. A. Meyer for his invaluable technical assistance.

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J. LIQ. CHROM. & REL. TECHNOL., 20(5), 811-814 (1997)

THE BOOK CORNER

RETENTION AND SELECTIVITY IN LIQUID CHROMATOGRAPHY. edited by R. M. Smith. Journal of Chromatography Library, Volume 57, Elsevier, Amsterdam, 1995, 457 pp., Price: $242.75.

It is not a secret that optimization in liquid chromatography is a function of the stationary phase, the mobile phase and the sample properties. Most published optimization procedures to date concentrate on the mobile phase composition to achieve the separation of a mixture. This is justified because it is easier and cheaper to change the composition of the mobile phase rather than the stationary phase. It is also a fact that the mobile and stationary phases are selected based on the properties of the mixture to be resolved. However, not many studies dealt with that aspect until the publication of this book, which brings together a number of studies which examine the ways in which the retention and selectivity of separations in high performance liquid chromatography are dependent on the chemical structure of the analytes and the properties of the stationary and mobile phases.

It is refreshing to see that the editor (Dr. Smith) has had an active part in this book; in addition to editing he wrote three chapters. I have seen books in the past where the editor did not write any of the chapters. The book is divided into twelve chapters, each written by an authority in the field.

The first chapter deals with prediction of retention, based on solute molecular structure (R. Smith). This is a comprehensive chapter which discusses different groups of compounds, both aliphatic and aromatic. Chapter 2 (K. Valko) is a continuation, in a way, of Chapter 1, and discusses retention prediction of compounds of pharmaceutical interest, based on molecular parameters, hydrophobicity and topological matrix and information theory.

Chapters 3 and 4 (R. Smith) deal with retention indices and their application in HPLC. Chapter 5 (M. Bogusz) and Chapter 6 (P. Kuronen) deal with retention indices in normal and reversed phase and gradient HPLC for the identification of compounds. P. Jandera continues the theme of indices in Chapters 7 and 8 by discussing interaction and polar indices and their effect on retention and selectivity.

S. West discusses solvent selectivity in Chapter 9, with emphasis on steroids and benzene derivatives. Polycyclic aromatic hydrocarbons' selectivity and retention in RP-HPLC are discussed in Chapter 10 (L. Sander and S. Wise), which deals with phase type, pore size, bonding density, bonded phase length, and other topics related to the stationary phase.

Chapter 11 (J. Pesek and E. Williamsen) compare novel stationary phases such as non-C_{18} alkane phases, chiral phases and non-silica based phases, while Chapter 12 (A. Bolck and A.K. Smilde) deals with the characterization of RP-HPLC stationary phases.

This book, overall, is very well written and discusses topics which have not been discussed in detail previously. The book is also well organized and avoids repetition which can be annoying. The overall discussion is concise and to the point. I found the book to be both interesting and helpful. It is recommended for all those who use HPLC.

ELECTROANALYTICAL CHEMISTRY, A SERIES OF ADVANCES, edited by A. J. Bard and I. Rubinstein, Marcel Dekker, Inc., New York, Volume 57, 1996, 525 pp., Price: $175.00.

In their introduction to the series the editors stated, "This series is designed to provide authoritative reviews in the field of modern electroanalytical chemistry, defined in its broadest sense. Coverage is comprehensive and critical. Enough space is devoted to each chapter of each volume so that derivations of fundamental equations, detailed descriptions of apparatus and techniques, and complete discussions of important references can be provided, so that the chapters may be useful without repeated reference to the periodical literature. Chapters vary in length and subject area. Some are reviews of recent developments and applications of well-established techniques, whereas others contain discussions of the background and problems in areas still being investigated extensively and in which many statements may still be tentative. Finally, chapters on techniques generally outside the scope of electroanalytical chemistry, but which can be applied fruitfully to electrochemical problems, are included."

This is Volume 57 of the series which is well established. The book is made up of three reviews (see Table of Contents, below). Each chapter deals with a different aspect of electroanalytical chemistry. These reviews are each up-to-date, comprehensive, well written, and thoroughly illustrated. The scope of each, however, is limited to a specific group and not really generalized to all analytical chemists, because the material is specific and highly technical.

Table of Contents

Reviewed by
Dr. Haleem J. Issaq
SAIC Frederick
NCI-Frederick Cancer Research & Development Center
Frederick, Maryland 21702-1201

J. LIQ. CHROM. & REL. TECHNOL., 20(5), 815 (1997)

EDUCATION ANNOUNCEMENT

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- Gradient Elution Techniques
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- Logical HPLC System Troubleshooting

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 Gel Permeation Chromatography for many years.

Details may be obtained from Dr. Jack Cazes, P. O. Box 970210, Coconut Creek, FL 33097, USA. (954) 973-8516; E-Mail: jcazes@icanect.net.

J. LIQ. CHROM. & REL. TECHNOL., 20(5), 817–823 (1997)

LIQUID CHROMATOGRAPHY CALENDAR

1997

MARCH 16 - 21: PittCon '97, Atlanta, Georgia. Contact: PittCon '97, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA. Tel: (800) 825-3221; FAX: (412) 825-3224.

APRIL 13 - 17: 213th ACS National Meeting, San Francisco, California. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-6059; FAX: (202) 872-6128.

APRIL 14 - 19: Genes and Gene Families in Medical, Agricultural and Biological Research: 9th International Congress on Isozymes, sponsored by the Southwest Foundation for Biomedical Research, Hilton Palacio del Rio, San Antonio, Texas. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

MAY 5 - 9: 151st ACS Rubber Div Spring Technical Meeting, Anaheim, California. Contact: L. Blazeff, P. O. Box 499, Akron, OH 44309-0499, USA.

MAY 23 - 24: ACS Biological Chemistry Div Meeting, San Francisco, California. Contact: K. S. Johnson, Dept of Biochem & Molec Biol, Penn State Univ, 106 Althouse Lab, University Park, PA 16802, USA.

MAY 27 - 28: IInd Miniaturisation in Liquid Chromatography versus Capillary Electrophoresis Conference, University of Ghent, Ghent, Belgium. Contact: Prof. Dr. W. Baeyens, Faculty of Pharmaceutical Analysis, University of Ghent, Harelbekestraat 72, B-9000 Ghent, Belgium.

MAY 27 - 30: ACS 29th Central Regional Meeting, Midland, Michigan. Contact: S. A. Snow, Dow Corning Corp., CO42A1, Midland, MI 48686-0994.

MAY 28 - 30: 31st ACS Middle Atlantic Regional Meeting, Pace Univ, Pleasantville, NY. Contact: D. Rhani, Chem Dept, Pace University, 861 Bedford Rd, Pleasantville, NY 10570-2799, USA. Tel: (914) 773-3655.

MAY 28 - JUNE 1: 30th Great Lakes Regional ACS Meeting, Loyola University, Chicago Illinois. Contact: M. Kouba, 400G Randolph St, #3025, Chicago, IL 60601, USA. Email: reglmtgs@acs.org.

JUNE 1 - 4: 1997 International Symposium, Exhibit & Workshops on Preparative Chromatography: Ion Exchange, Adsorption/Desorption Processes and Related Separation Techniques, Washington, DC. Contact: J. Cunningham, Barr Enterprises, 10120 Kelly Road, Box 279, Walkersville, MD 21793, USA. (301) 898-3772; FAX: (301) 898-5596; Email: Janetbarr@aol.com.

JUNE 16 - 19: Capillary Electrophoresis, Routine Method for the Quality Control of Drugs: Practical Approach. L'Electrophorese Capillaire, Methode de Routine pour le Contrôle Qualité des Medicaments: Approche Pratique, Monpellier, France. (Training course given in two languages) Contact: Prof. H. Fabre, Tel: (33) 04 67 54 45 20; FAX: (33) 04 67 52 89 15; Email: hfabre@pharma.univ-montpl.fr

JUNE 22 - 25: 27th ACS Northeast Regional Meeting, Saratoga Springs, New York. Contact: T. Noce, Rust Envir & Infrastructure, 12 Metro Park Rd, Albany, NY 12205, USA. Tel: (518) 458-1313; FAX: (518) 458-2472.

JUNE 22 - 26: 35th National Organic Chemistry Symposium, Trinity Univ., San Antonio, Texas. Contact: J. H. Rigby, Chem Dept, Wayne State Univ., Detroit, MI 48202-3489, USA. Tel: (313) 577-3472.

SEPTEMBER 7 - 11: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (703) 231-8222.

SEPTEMBER 21 - 26: Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Cleveland, Ohio. Contact: J. A. Brown, FACSS, 198 Thomas Johnson Dr, Suite S-2, Frederick, MD 21702, USA. Tel: (301) 846-4797; FAX: (301) 694-6860.

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 - 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 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; Email: reglmtgs@acs.org.

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: 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.

1998

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 29 - APRIL 2: 215th ACS National Meeting, Dallas, Texas. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

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. Email: reglmtgs@acs.org.

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; FAX: (804) 828-7436.

AUGUST 23 - 28: 216th ACS National Meeting, Boston, Massachusetts. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6218; Email: natlmtgs@acs.org.

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 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 4 - 7: 50th ACS Southwest Regional Meeting, Res. Triangle Pk, North Carolina. Contact: B. Switzer, Chem Dept, N Carolina State University, Box 8204, Raleigh, NC 27695-8204, USA. Tel: (919) 775-0800, ext 944; Email: switzer@chemdept.chem.ncsu.edu.

1999

MARCH 7 - 12: PittCon '99, Orlando, Florida. Contact: P:ttCon '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 - 30: 219th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899.

AUGUST 20 - 24: 220th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2001

APRIL 1 - 5: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 19 - 23: 222nd 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.

2002

APRIL 7 - 11: 223rd ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 8 - 12: 224th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899.

2003

MARCH 23 - 27: 225th 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.

SEPTEMBER 7 - 11: 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 1: 227th ACS National Meeting, Anaheim, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128.

AUGUST 22 - 26: 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 - 17: 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 1: 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.

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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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