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DETERMINATION OF SUFENTANIL IN HUMAN PLASMA BY CAPILLARY ELECTROPHORESIS AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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ABSTRACT

A fast capillary electrophoretic method and a gas chromatographic - mass spectrometric method were developed for the determination of sufentanil in human plasma. Spiked plasma samples and patient plasma samples were prepared by liquid-liquid extraction. In the capillary electrophoretic analysis, both inorganic and organic buffers were tested with and without surfactants, at various pHs. The final separation with fast analysis time was performed with organic 3-[N-morpholino]propanesulfonic acid solution at pH 7.00, with a bubble cell capillary and UV detection at 195 nm. The gas chromatographic separation was performed without sample derivatization, in a nonpolar column material with fentanyl as the internal standard. The mass spectrometric identification was done in electron impact mode. The limit of detection for sufentanil was lower in the gas chromatographic - mass spectrometric technique than in capillary electrophoresis with UV, therefore more sample was required in the capillary

electrophoretic analysis. Comparison of the techniques was done with spiked plasma samples. Both techniques were found to be excellent for the determination of sufentanil. Advantages of the capillary electrophoretic analysis over the gas chromatographic method are better efficiency and the faster elution.

INTRODUCTION

Sufentanil (N-[4-(methoxymethyl)-1-[2-(2-thienyl)ethyl]-4-piperidinyl]-N-phenylpropanamide) and fentanyl (N-phenyl-*N*-[1-(2-phenylethyl)-4-piperidinyl] propanamide) are potent short-acting opioid analgesics used as anesthetic drugs (Figure 1). Comparison shows that sufentanil is five to ten times more potent than fentanyl, although both opioids have short half-lives in blood. Because sufentanil has no seriously adverse haemodynamic effects, not even at high doses, it has been preferred in long-durational cardiac surgical procedures such as coronary artery bypass grafting (CABG). During these procedures, sufentanil (or fentanyl) is administered intravenously as citrate complex. The given dose is dependent on patient's weight and his diseases.[1]

In human plasma of patients undergoing non-cardiac surgery, about 92 per cent of sufentanil is complexed with proteins and other plasma compounds, while the rest of it, which is the pharmacologically active moiety of the drug, exists free.[2] By contrast, the free fraction of sufentanil, and therefore, also the therapeutic level of the free drug, is not reliably known for cardiac surgical patients.

In routine clinical laboratory work sufentanil is quantified with a radioimmunoassay (RIA) technique, a sensitive method with a determination limit as low as 50 pg/mL.[3] However, there are a number of disadvantages to the use of radioactive labels, including an analysis time of several hours and incapability of separating the free active drug from the inactive protein-bound one and from metabolities. In view of these drawbacks, other methods, gas chromatographic-mass spectrometric methods in particular, have been developed for the determination.[4,5,6] Capillary electrophoresis (CE) offers a further choice, as the separation efficiency of the technique is excellent.[7] Although detectability is poor with CE, especially with the UV detector, this disadvantage can be overcome by developing fast electrophoretic methods for quantitation that reduce the width of the sample zones. As well, on-line coupling with a mass spectrometer can be expected to improve the detectability at very low concentrations, as achieved in many other applications.[8]

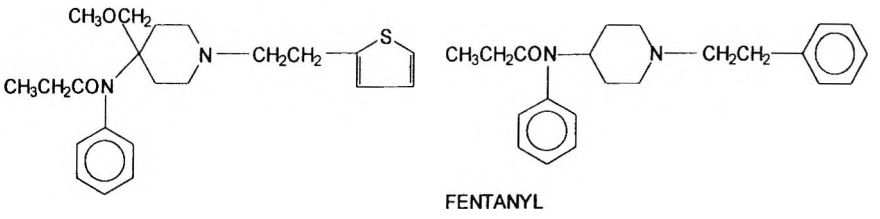


Figure 1. Chemical structures of sufentanil and fentanyl.

We have developed a fast capillary zone electrophoretic (CZE) method and a gas chromatographic mass spectrometric (GC-MS) method for determination of the free and total sufentanil in plasma samples. The capillary zone electrophoretic method is based on the separation of analytes under a strong electric field in an aqueous electrolyte solution made of organic buffer (pH 7.00), whereas the gas chromatographic - mass spectrometric method depends on the separation of components with a retention gap and nonpolar stationary phase column. In both cases the method development included sample pretreatment with liquid-liquid extraction (LLE) and thorough validation of the separation methods.

EXPERIMENTAL

Chemicals and Reagents

Sufentanil (MW 386.6 g/mol) and fentanyl (MW 336.5 g/mol) were purchased from Janssen Pharmaceutica, Beerse, Belgium (Sufenta forte 50 μg/mL and Fentanyl 50 μg/mL). Ethyl acetate (Rathburn Chemicals Ltd., Walkerburn, Scotland) and methanol (E.Merck, Darmstadt, Germany) were HPLC grade, and *n*-heptane was spectroscopy quality (Merck). 3-[N-Morpholino]propanesulfonic acid (MOPS, MW 209.3 g/mol), sodium dodecylsulfate (SDS, MW 288.4 g/mol), and N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES, MW 238.2 g/mol) were purchased from Sigma (99.5%, Sigma Ultra, St. Louis, MO, USA). Betaine was from Fluka (Buchs, Switzerland) and sodium dihydrogen phosphate, disodium hydrogen phosphate, and glycine were from Merck.

Water was distilled, ion-exchanged (Water-I, Gelman, Ann Arbor, MI, USA) and filtered with 0.45 μm membrane filters (Millipore, Molsheim, France). Helix pomatia juice was from Sepracor (France). Pooled and freeze-dried plasma was from Labquality Ltd. (Finland). All chemicals were used without further purification.

Instrumentation

Capillary electrophoretic analysis was done on an HP Chemstation 3D CE (Hewlett-Packard, Palo Alto, California, USA). The final separations were achieved by using inside uncoated bubble cell capillaries (length 58 cm, i.d. 50 μm, L_{det} 50 cm, Hewlett-Packard). Injections were made hydrostatically within 30 s (50 mbar pressure). The temperature and applied voltage were 22°C and 20 kV, respectively. Detection was accomplished with a diode array detector (DAD) at wavelength 195 nm.

GC-MS analyses were performed on a Hewlett-Packard 5890A gas chromatograph interfaced to an HP 5989A MS Engine quadrupole mass spectrometer (Hewlett-Packard). The final analytical column was HP-5 fused silica capillary column (length 11 m, internal diameter 0.32 mm, film thickness 0.17 μm, Hewlett-Packard). A DPTMDS-deactivated retention gap (3 m * 0.32 mm i.d., HNU-Nordion, Helsinki, Finland) was connected in front of the analytical column. The helium flow rate was 1.5 mL/min (calculated at 150°C). Injections of 1 μL were made with an HP 7673 autosampler (Hewlett-Packard) in splitless mode. The temperature was kept at 60°C for 1.5 min, after which it was programmed at 30°C/min to 265°C, at 15°C/min to 280°C, and finally at 20°C/min to 300°C, where it was kept for 2 min. The injector, interface, ion source and quadrupole temperatures were 290°C, 290°C, 280°C, and 120°C, respectively.

The mass spectrometer was operated in EI-mode (70 eV, multiplier 2500 V) by using selected ion monitoring (SIM) with m/z 245 for fentanyl and m/z 140 and 289 for sufentanil. Data were recorded and evaluated with an HP 9000/345 data system.

A Jenway 3030 pH meter and an electrode (Jenway, Felsted, England) containing 3 M KCL in saturated AgCl solution were used to adjust the pH of the electrolyte solutions. The pH meter was calibrated with CONVOL standard buffer solutions purchased from BDH (BDH Chemicals Ltd., Poole, England).

Preparation of Electrolyte Solution

Phosphate, MOPS, HEPES, phosphate - betaine (5:3 v/v), glycine - SDS, phosphate - SDS and MOPS buffers with concentrations varying from 10 mM to 3 M were used to validate the separation method. The pHs of the electrolyte solutions ranged from 6.50 to 10.60. The final running electrolyte solution used for the fast separations was 100 mM MOPS of pH 7.00 adjusted with 10 mL of 0.1 M NaOH.

Sample Pretreatment

Preparation of reference solutions for quantitation

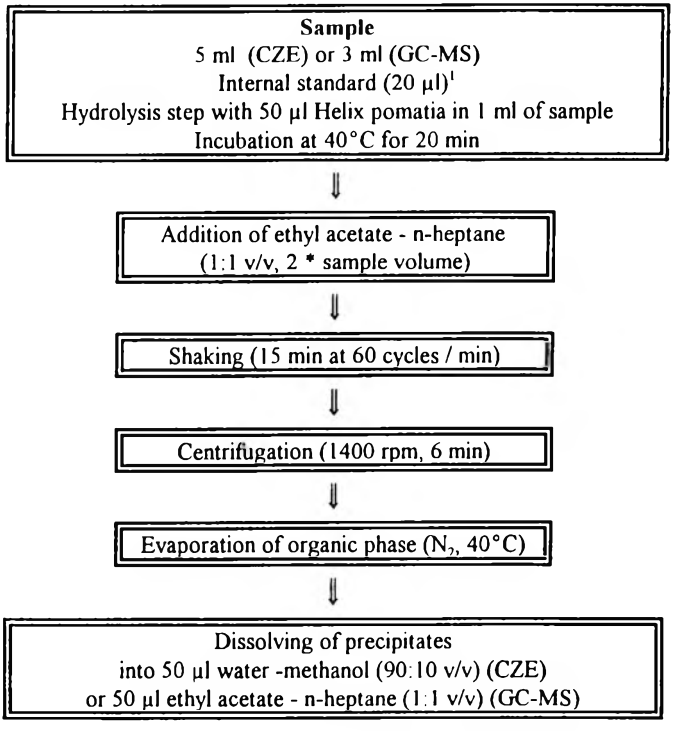
Pooled blank plasma was spiked with Sufenta forte at concentrations varying from 1 to 100 ng/mL. The concentrations of sufentanil in spiked plasma samples were 1, 2, 5, 10, 20, 50, and 100 ng/mL. In CZE, methanol was used to control the electroosmosis during the runs. For the GC-MS analyses, each standard plasma sample also contained fentanyl as internal standard.

Plasma samples

The plasma samples from coronary artery bypass grafting (CABG) patients (five patients, both sexes, unknown ages and weights) were taken three times during surgical operations: 1. before induction of anesthesia, 2. after induction of anesthesia, but before administration of heparin, and 3. after administration of heparin, before the start of cardiopulmonary bypass. Arterial blood for the determination of sufentanil concentrations was drawn into pre-chilled EDTA tubes, and the plasma was separated and stored frozen before analysis.

Liquid-Liquid Extraction

Free sufentanil was determined from test plasma samples spiked with Sufenta forte in different concentrations by isolating with liquid-liquid extraction (Figure 2). Sample volumes were 3 mL for GC-MS analysis and 5 mL for CZE analysis. Sample preparation was based on an earlier reported method.[9]



1= fentanyl in the determination sufentanil by GC-MS
2= hydrolyser excluded in analyses of free sufentanil

Figure 2. Preparation of plasma samples.

The extraction method was also tested for patient plasma samples. The final aim was to separate the free and the conjugated fractions of patient plasma samples. This was done by adding a hydrolysis step into the extraction procedure (Figure 2). The hydrolysis was done after extraction of free sufentanil with enzymatically with Helix pomatia solution.

RESULTS

Optimization of the Separation in CZE and GC-MS

The capillary electrophoretic separations were optimized with sufentanil

and fentanyl standards, by choosing a pH value and buffer concentration that would give the fastest electrophoretic separation and quantitation of sufentanil from patient plasma. The electrolyte solutions tested were neutral or basic buffers of different concentration and content. In the basic region sufentanil was neutral and could be separated from the endogenic and other exogenic compounds only with electrolyte solutions containing SDS, but at neutral pH it was in cationic form and migrated fast within five minutes. The basic electrolyte solution was excluded, since the migration times were increased in SDS solution decreasing the detectability of sufentanil at pg level in patient plasma samples (Figure 3). Buffers without additives allowed the fastest analyses and, owing to the low currents at high voltages, the organic buffers were better than inorganic buffers for fast separation since they could be used at high concentrations. MOPS solution at 100 mM concentration was accordingly chosen for further optimization in CZE. However, only 30 mM phosphate buffer - 50 mM SDS in CE could provide separation of sufentanil and fentanyl (Figure 3), and concentrations should then be above 0.1 μg/mL. Accordingly, in this study we used MOPS buffer to obtain very sharp peaks and UV-responses at low concentration levels.

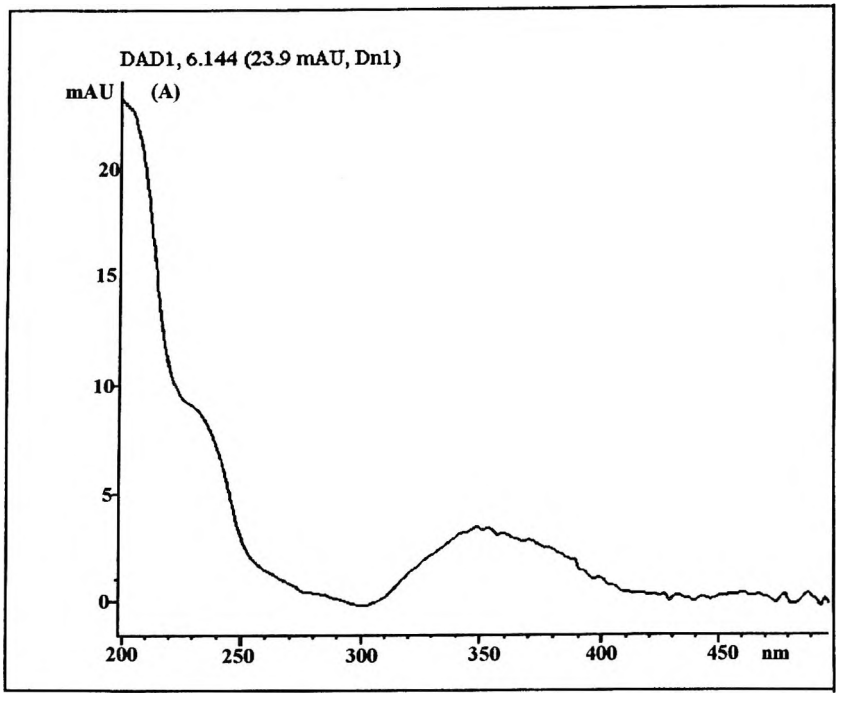
In the case of GC-MS, the optimization was carried out to give good separation of sufentanil and fentanyl, and the method was optimized to give baseline separation for both.

Also, the instrumental parameters were optimised. The capillary lengths in CZE the separation of sufentanil from endogenic and exogenic compounds was optimized by using 40 to 70 cm capillaries. In GC-MS the capillary lengths of 10 to 18 metres were tested. The final capillary lengths were 50 cm and 11 metres in CZE and GC-MS, respectively.

The optimised separations of spiked test samples with both techniques are presented in Figures 4 and 5.

Interferences

In CZE the plasma proteins interfered with the separation, especially when aqueous inorganic buffers with additives were used; with MOPS, however, they migrated after electroosmosis and offered no interference in the analyses. Figures 6 and 7 show the electropherograms of the analyses of plasma samples from CABG patient. In Figure 6 sufentanil has determined by CZE. The negative peak interference was due to the high ionic strength of the sample after 200-fold concentration. Figure 7 shows that also in the MEKC method tested in our studies during the method development procedures, the



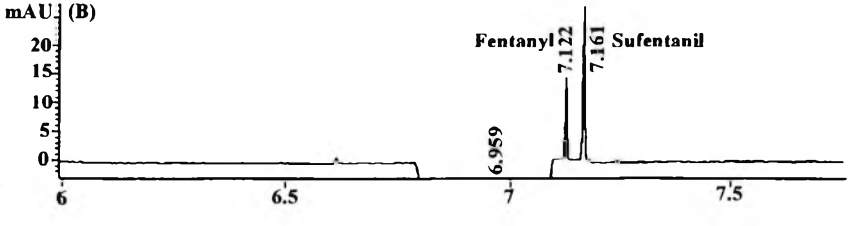
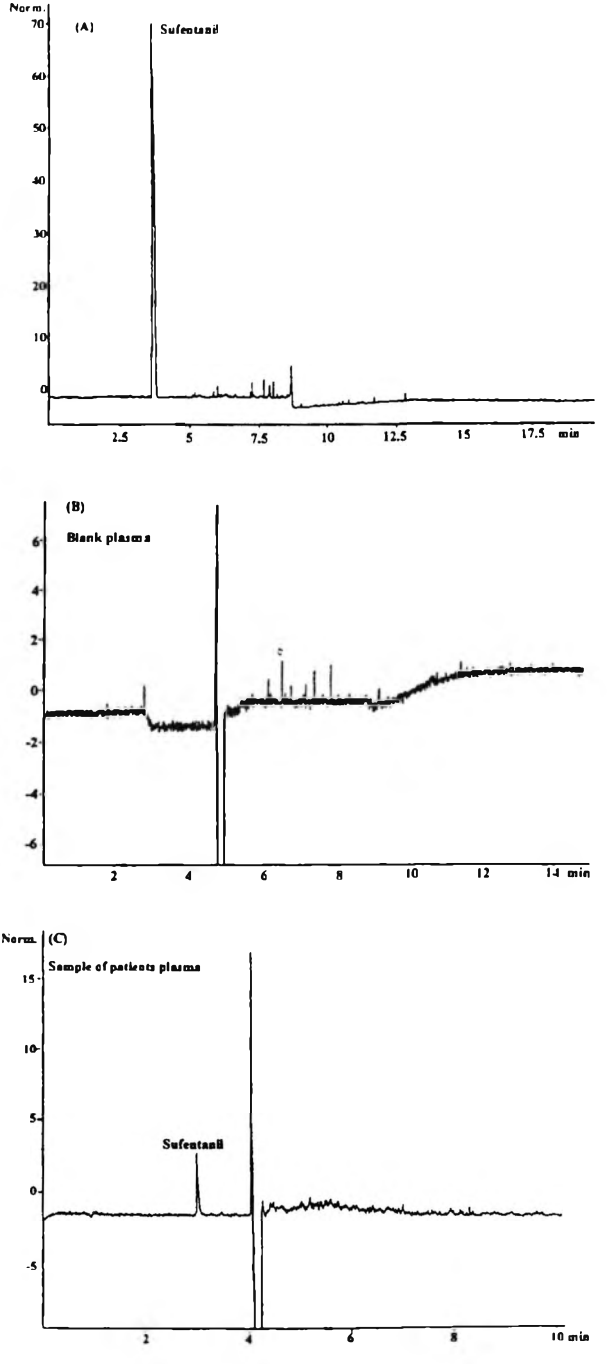


Figure 3. (A) The UV spectrum of sufentanil obtained with DAD, (B) electropherogram of fentanyl and sufentanil separation in 30 mM phosphate - 50 mM SDS electrolyte solution. Concentration of standards 20 μg/mL.

Figure 4 (right). Electropherograms obtained for the quantitation of sufentanil in patient plasma. (A) Standard sufentanil spiked to pooled blank plasma, (B) blank plasma and (C) plasma sample from CABG patient.


Norm.
70
60
50
40
30
20
10
0
(A)
Sufentanil
2.5
5
7.5
10
12.5
15
17.5 min
(B)
Blank plasma
6
4
2
0
-2
-4
-6
2
4
6
8
10
12
14 min
Norm.
(C)
Sample of patients plasma
15
10
5
0
-5
Sufentanil
2
4
6
8
10 min

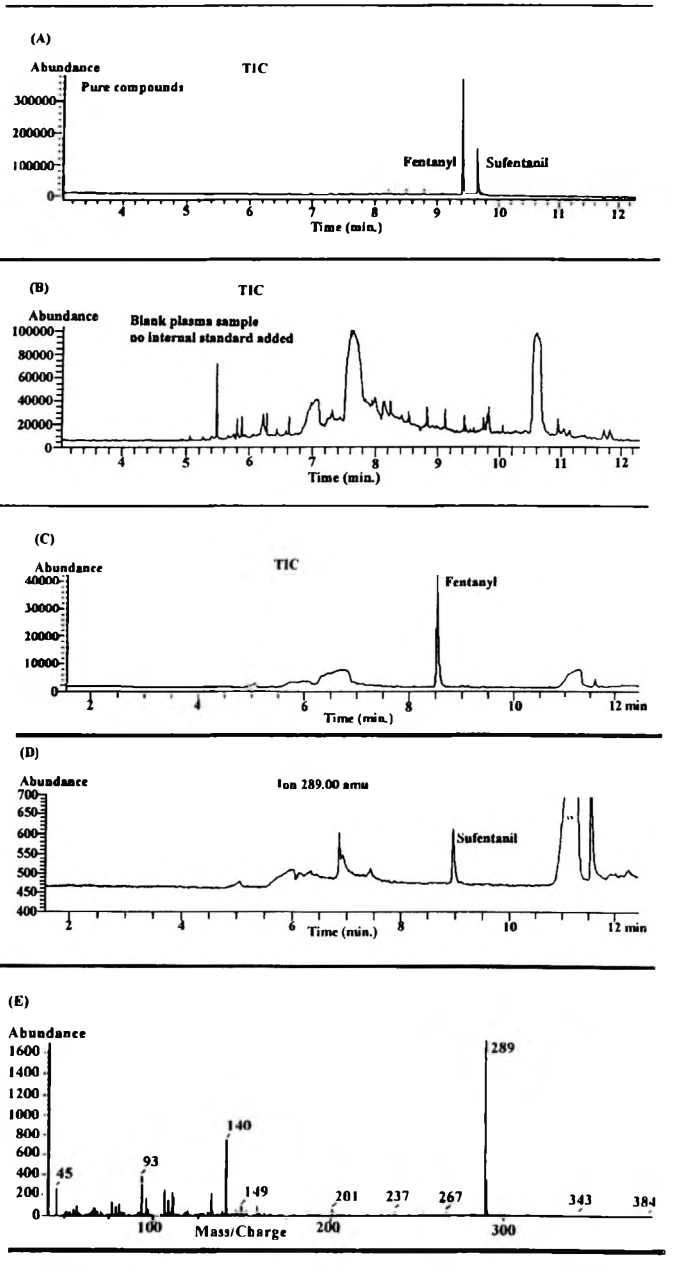


Figure 5. (A) Total ion chromatogram (TIC) of pure fentanyl and sufentanil after liquid -liquid extraction, (B) TIC of blank plasma, (C) TIC of free drug fraction of patients plasma sample, (D) ion 289 from extraction of free drug fraction of patients plasma sample and (E) mass spectrum of sufentanil.

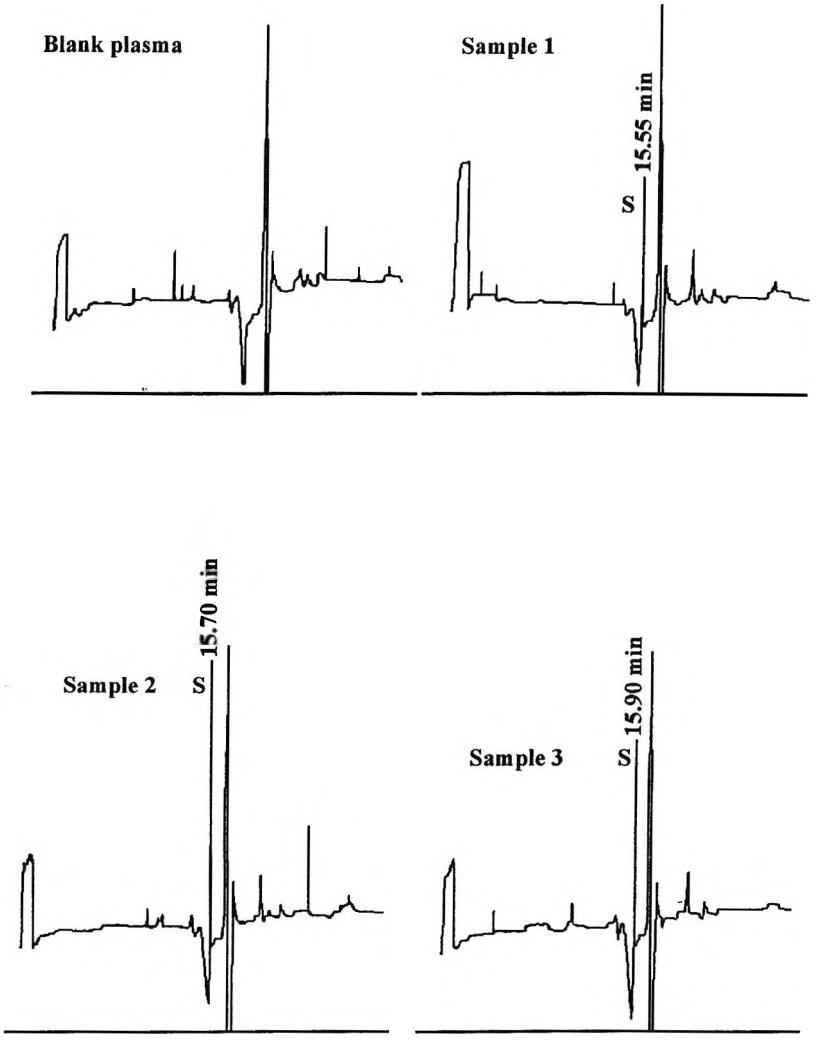


Figure 6. Electropherograms recorded from samples of CABG patient X in electrolyte solution 50 mM phosphate - 3 M betaine (5:3 v/v, pH 7.0). (S) sufentanil. Samples 1, 2 and 3 as described in the Experimental section.

quantifying of sufentanil was not repeatable, because of the broad peak due to endogenic compounds remaining in the sample after the extraction step. In the GC-MS analyses, however, endogenous compounds in plasma which may not be isolated during the pretreatment steps, did not interfere with the analysis.

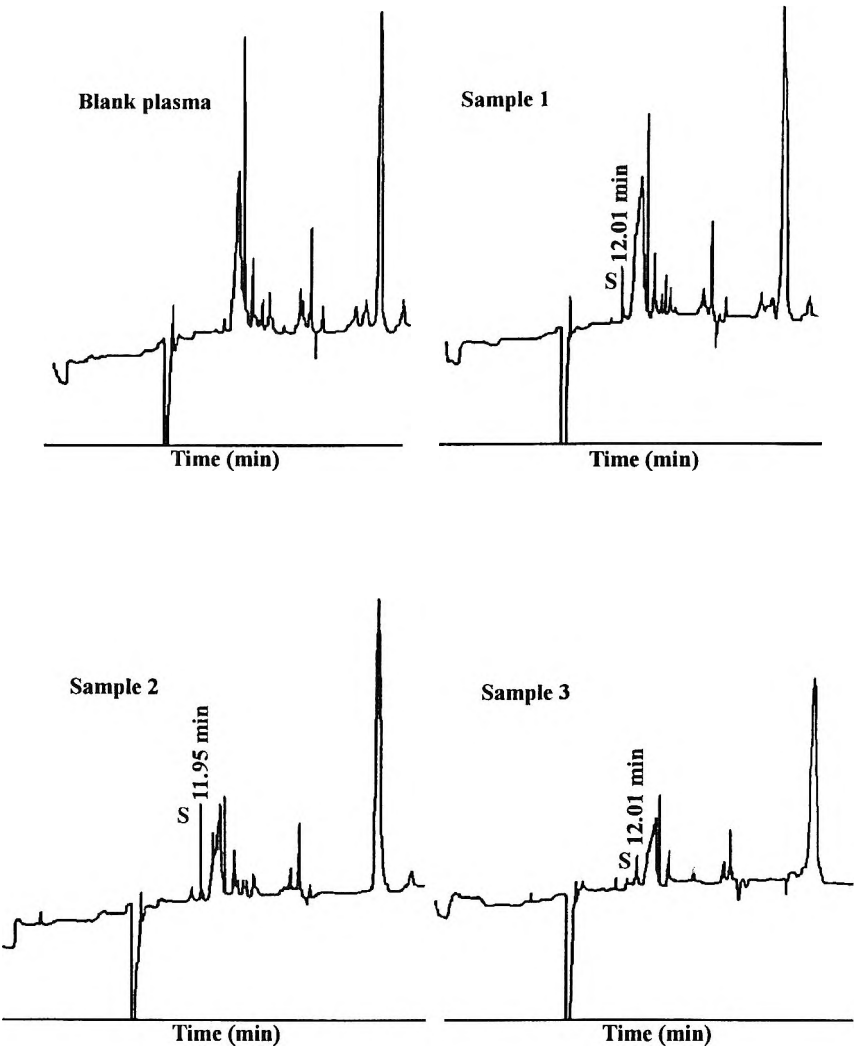


Figure 7. Electropherograms recorded from samples of CABG patient X in electrolyte solution 30 mM glycine - 50 mM SDS (pH 10.5). (S) sufentanil. Samples 1, 2 and 3 as described in the Experimental section.

Linearity

Calibration curves were determined, both in GC-MS and in CZE, on the basis of samples of pooled drug-free plasma spiked with reference compounds.

Table 1

The Concentration of Sufentanil of One Patient During CABG Operations

Sample	CZE-DAD (conc./sample amount)	GC-MS (conc./sample amount)
0 (before induction of anesthesia)	-----[1]	-----[1]
1 (after induction of anesthesia and before administration of heparin), free sufentanil	10.0 ng/3 mL	3 ng/1 mL
1, free + conjugated sufentanil	12.5 ng/3 mL	4 ng/1 mL
2 (after administration of heparin, just before start of cardiopulmonary bypass) free sufentanil	0.003 ng/3 mL	0.0009 ng/ 1mL
2, free + conjugated sufentanil	4 ng/3 mL	1.2 ng/1 mL

1 = sufentanil not added to the sample.

The results showed linear correlations (r=0.997) for peak height (CZE) and peak area (GC-MS) within the concentration range 1 ng/mL to 100 ng/mL. With CZE the detection limit was 3 ng/mL and with GC-MS 20 pg/mL (S/N=3, n=5). The limit of determination was 8 ng/mL in CZE and 50 pg/mL (n=5) in GC-MS.

The free and bound sufentanil fractions were determined from the patient samples. The amount of conjugated sufentanil varied from 3 to 24 per cent of the amount of free sufentanil, which means an amount of bound sufentanil in plasma between 1 and 24 ng/mL.

Table 2

Recoveries of the Spike Plasma Samples at Two Concentration Levels

Sample, Quality (free/conjugated sufentanil)	CZE-DAD (conc. /sample amount)	GC-MS (conc. /sample amount)
0.1 μg/mL, free sufentanil	0.167 μg/5 mL	0.179 μg/3 mL
0.1 μg/mL, conjugated sufentanil	-----[1]	0.020 μg/3 mL
1 μg/mL, free sufentanil	0.987 μg/5 mL	1.02 μg/3 mL
1 μg/mL, conjugated	0.110 μg/5 mL	0.105 μg/3 mL

1 = not quantified

The plasma concentration of sufentanil is dependent on the time of sampling: highest concentrations of both free and bound sufentanil were measured in samples taken before the administration of heparin. In addition, the doses are individual which reflects the concentrations in the patient samples.

Quantitation

The quantitation of sufentanil in patients plasma samples was made with help of a calibration curve. Table 1 shows data on the concentrations of sufentanil in one patient before and during CABG operation.

Compatibility of the Results

To check the agreement between the CZE and GC-MS results, we used plasma samples spiked at concentration levels of 1 μg/mL and 0.1 μg/mL. These samples were extracted as described in Experimental. As expected, almost all sufentanil was in the fraction containing the free form of the drug (Table 2).

DISCUSSION

The liquid-liquid extraction gave good recoveries, and no cross-contamination was observed between the free and bound fractions. Our studies also showed that the sample preparation step needs still to be less time-consuming, however, and more specific for the isolation of sufentanil especially when low concentrations of the drug is to be determined.

Both capillary zone electrophoresis and gas chromatography - mass spectrometry are excellent methods for the determination of sufentanil. Both methods are rapid and repeatability is good. More sample was needed for CZE than for GC-MS because of the low concentration levels.[1] However, the total analysis time was shorter in CZE than in GC-MS. The fast separation and overlapping with fentanyl means that sufentanil and fentanyl cannot be determined simultaneously in CZE. Our studies nevertheless suggest that the CZE method is also suitable for individual fentanyl analysis, though this was not specifically investigated. The GC-MS technique is excellent for the identification of both sufentanil and fentanyl, especially when they are present in plasma samples at picogram to femtogram level.

The best injection technique for sufentanil and the matrix in GC-MS analyses was splitless injection, since the concentrated samples were still contaminated with proteins and sugars. That was also the reason for using a retention gap, which by retaining the contaminants protects the analytical column. However, elution of compounds coextracted with the analyte can never entirely be avoided and produces noise in GC-MS analyses.

A drawback in CZE technique was the low response of the UV detector to the analyte, which could nevertheless be enhanced by concentrating the samples. The disturbance caused by the simultaneous increase in the concentration of endogenous compounds could be avoided by fast analysis under high voltage while the ionic strength was kept high. In summary, CZE is a highly promising technique for the opioid analysis from body fluids and can be used as a reference method for gas chromatographic-mass spectrometric methods.

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MICELLAR ELECTROKINETIC CHROMATOGRAPHY ESTIMATION OF CRITICAL MICELLAR CONCENTRATION OF SODIUM DODECYL SULPHATE SYSTEMS IN SALINE MEDIA

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ABSTRACT

Micellar Electrokinetic Chromatography was applied to the estimation of the critical micellar concentration of sodium dodecyl sulphate in saline media of different nature (2-(N-cyclo-hexylamino)-ethanesulphonic acid and Ammonium Acetate) and concentration. The linear variation of the capacity factor for two series of compounds (11 benzene and naphthalene derivatives and 17 1,4-dihydropyridines) as a function of sodium dodecyl sulphate concentration in the electrolytic solution allowed this estimation.

INTRODUCTION

The introduction of a surfactant at a concentration above its critical micelle concentration (c.m.c.) in the electrolyte solution in Capillary Electrophoresis has given rise to the Micellar Electrokinetic Chromatography (MEKC).[1-4] In this technique, solutes are distributed between the aqueous and micellar phases according to their association constants with the micelles. If an anionic surfactant is employed, micelles which have an anodic electrophoretic mobility will migrate to the cathode if a strong enough electroosmotic flow exists towards the cathode.

This means that if a neutral solute is introduced in the system, it will elute from the separation capillary at a time somewhere between the migration time of the electroosmotic flow marker, t_0, and the migration time of the micelle, t_m. From these two parameters, the capacity factor (k') of a solute in MEKC can be defined as follows: [1,2]

$$k' = (t_r - t_0) / t_0 [1 - (t_r / t_m)] \quad (1)$$

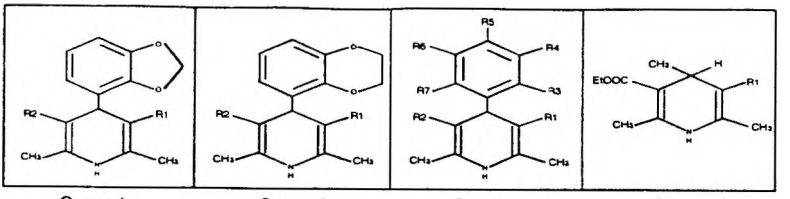
where t_r is the migration time of the solute.

If the micellar concentration in the buffer is low, the solute capacity factor of a solute can be related to the total surfactant concentration in the buffer using the following equation:[2,5]

$$k' = (K_2 + v) (C - c.m.c.) \quad (2)$$

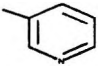
where K_2 is the solute-micelle association constant per surfactant monomer, v is the molar volume of the micelle, and C is the total concentration of surfactant in the buffer. From eq. (2), it can be observed that the c.m.c. of the micellar system can be estimated from the intercept/slope ratio of the straight line of the variation of the capacity factor of a solute in MEKC as a function of the total concentration of the surfactant in the electrolyte solution.[5] Despite this possibility, MEKC has seldom been used in order to determine c.m.c. values in this manner.

Another method of applying MEKC to the estimation of the c.m.c. of a micellar system is to measure the variation of the effective electrophoretic mobility of a neutral compound as a function of the total concentration of surfactant in solution. By this method, the c.m.c. of sodium dodecyl sulphate (SDS) in a electrolytic solution 0.005 M in borax was achieved[6] by using naphthalene as a test solute and SDS concentrations ranging from 0.003 to 0.008 M.



GROUP 1	$-R_1$	$-R_2$
1	$-COOCH_2-$ (1)	$-COOCH_3$
2	$-COOCH_3$	$-COOCH_3$
3	$-COOCH_2CH_2OCH_3$	$-COOCH_2CH_2OCH_3$
5	$-COOCH_2CH_3$	$-COOCH_2CH_3$
6	$-COOCH_2CH_2OCH_3$	-COOIsp
10	$-COOCH_2CH_3$	$-COOCH_3$
14	-COOIsp	$-COOCH_3$

(1)



GROUP 2	$-R_1$	$-R_2$
4	$-COOCH_2CH_3$	$-COOCH_3$

GROUP 3	$-R_1$	$-R_2$	$-R_3$	$-R_4$	$-R_5$	$-R_6$	$-R_7$
7	$-COOCH_2CH_3$	$-COOCH_3$	-H	$-NO_2$	-H	-H	-H
8	$-COOCH_2CH_3$	$-COOCH_3$	$-OCH_2OCH_3$	-H	-H	-H	-H
9	$-COOCH_2CH_3$	$-COOCH_3$	-H	$-OCH_3$	-H	$-OCH_3$	-H
11	$-COOCH_2CH_3$	$-COOCH_3$	-H	-H	-H	-H	-H
12	$-COOCH_2CH_3$	$-COOCH_3$	-H	$-OCH_3$	$-OCH_3$	-H	-H
13	$-COOCH_2CH_3$	$-COOCH_3$	$-OCH_3$	$-OCH_3$	-H	-H	-H
17	-COOIsp	$-COOCH_2CH_2OCH_3$	-H	$-NO_2$	-H	-H	-H

GROUP 4	$-R_1$	$-R_2$	$-R_3$
15	$-COOCH_3$	$-COOCH_2CH_3$	-H
16	$-COOCH_2CH_3$	$-COOCH_2CH_3$	-H

Figure 1. Structures of 1,4-dihydropyridines and their identification numbers.

In this work, MEKC has been used to estimate the c.m.c. of SDS in saline media which consisted of two buffers, 2-(N-cyclo-hexylamino)-ethanesulphonic acid (CHES) (pH = 10) (at three different concentrations) and Ammonium Acetate pH = 9). Data for the capacity factors of these two groups of solutes (11 benzene and naphthalene derivatives and 17 1,4-dihydropyridines) previously measured for different SDS concentrations in solution[7,8] have been used.

EXPERIMENTAL

Figure 1 shows the structure of the 1,4-dihydropyridines studied and the identification numbers used throughout this paper. The benzene and naphthalene derivatives were the following: 1) benzene, 2) benzyl alcohol, 3) benzamide, 4) toluene, 5) benzonitrile, 6) nitrobenzene, 7) 2-phenylethanol, 8) chlorobenzene, 9) phenylacetonitrile, 10) naphthalene, 11) 1-naphthylamine.

Experimental micellar electrokinetic chromatographic data used in this work was previously determined to obtain solute-micelle association constants of the compounds studied as indicated in References.[7,8]

Briefly: a) For the benzene and naphthalene derivatives, a P/ACE System 2050 capillary electrophoresis (Beckman, Fullerton, CA, USA) with UV detection (214 nm) and 25 μm I.D. capillaries (Polymicro Technologies, Phoenix, AZ, USA) were used. Ammonium Acetate (pH 9) and 2-(N-cyclo-hexylamino)-ethanesulphonic acid (CHES) (pH = 10) buffers were used. Sudan III or benzo(a)pyrene and dimethylformamide were used to determine micelle migration time and electro-osmotic flow time respectively. The washing routine employed for the capillary prior to each injection, in order to determine solutes capacity factors, was the following: Milli-Q water for 3 min, 0.1 M sodium hydroxide for 3 min, Milli-Q water for 2 min and separation buffer for 3 min. Working temperature was 25 °C and the electrical field strength applied was 15 kV.

b) For 1,4-dihydropyridines, the instrument used consisted of a Prince programmable injector, a Lambda 1000 UV-detector (238 nm) and a high voltage power supply, all purchased from Lauer Labs (The Netherlands). The integrator employed was a HP3394 from Hewlett Packard (Avondale, PA, USA). A 25 μm I.D. capillary (Polymicro Technologies, Phoenix, AZ, USA) was employed. CHES (pH 10) buffers were used. Sudan III and dimethylformamide were used to determine micelle migration time and electro-osmotic flow time respectively. The washing routine employed was the following: Milli-Q water for 2 min, 0.1 M sodium hydroxide for 2 min, Milli-Q water for 2 min and separation buffer for 2 min. Working temperature was 31 °C and the electrical field strength applied was 15 kV.

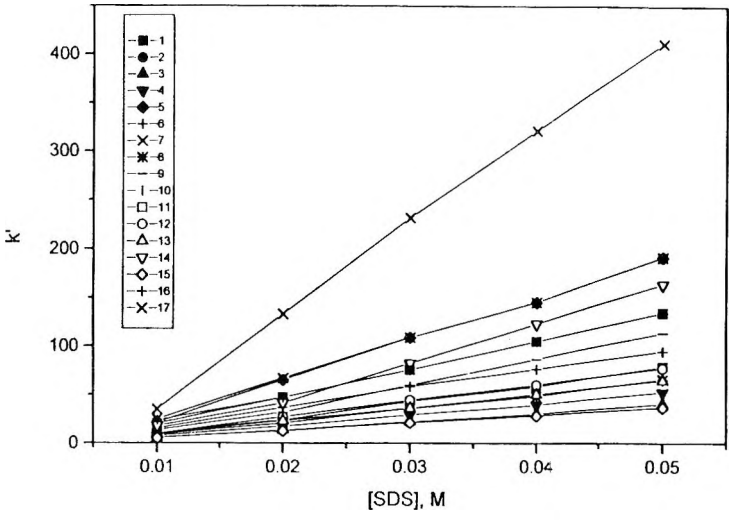


Figure 2. Variation of the capacity factor for a group of 17 1,4-dihydropyridines as a function of the SDS concentration in a 0.05 M CHES buffer (pH=10).

Data Manipulation

A Wilcoxon matched-pair test was carried out using a SOLO Statistical System.[9]

RESULTS AND DISCUSSION

The determination of the variation of capacity factors for a group of benzene and naphthalene derivatives and 1,4-dihydropyridines in a MEKC system as a function of total SDS concentration in the buffer solution[7,8] should enable estimation of the c.m.c. of SDS in different saline media. Such variation is a straight line (eq. (2)) except for very hydrophobic solutes, for which the variation of the capacity factor with the SDS concentration was shown to be not linear, due to the error in the determination of the capacity factor for these compounds whose migration times are very similar to the micelle migration time (eq.(1)).[7] If a straight line is obtained for a solute, the ratio between the intercept and the slope of this straight line should allow for the estimation of the c.m.c. of the micellar system in the buffer employed.

Table 1

Critical Micelle Concentration for SDS Calculated from Benzene and Naphthalene Derivatives Capacity Factors Data in Different Electrolyte Solutions by MEKC

Compound	c.m.c. (0.05 M CHES)	c.m.c. (0.10 M CHES)	c.m.c. (0.05M NH_4OAc)
1	$5.27\ 10^{-3}$	$1.71\ 10^{-3}$	$1.67\ 10^{-3}$
2	$3.82\ 10^{-3}$	$1.01\ 10^{-3}$	$1.25\ 10^{-3}$
3	$5.57\ 10^{-3}$	$1.35\ 10^{-3}$	$1.50\ 10^{-3}$
4	$4.67\ 10^{-3}$	$1.71\ 10^{-3}$	$2.34\ 10^{-3}$
5	$5.01\ 10^{-3}$	$1.75\ 10^{-3}$	$2.78\ 10^{-3}$
6	$4.33\ 10^{-3}$	$1.46\ 10^{-3}$	$2.22\ 10^{-3}$
7	$3.23\ 10^{-3}$	$2.00\ 10^{-3}$	$2.54\ 10^{-3}$
8	$3.52\ 10^{-3}$	$2.04\ 10^{-3}$	$2.37\ 10^{-3}$
9	$4.37\ 10^{-3}$	$2.16\ 10^{-3}$	$2.70\ 10^{-3}$
10	$1.27\ 10^{-3}$	$1.49\ 10^{-3}$	$1.97\ 10^{-3}$
11	$3.54\ 10^{-3}$	$3.14\ 10^{-3}$	$2.52\ 10^{-3}$
c.m.c. $\pm\ \sigma_{N-1}$	$4.05\ 10^{-3} \pm 1.19\ 10^{-3}$	$1.80\ 10^{-3} \pm 5.55\ 10^{-4}$	$2.17\ 10^{-3} \pm 5.07\ 10^{-4}$

Figure 2 shows, as an example, the variation of the capacity factor of a group of 17 1,4-dihydropyridines as a function of the SDS concentration in a 0.05 M CHES buffer (pH = 10).[8] Similar plots were obtained for the other buffer used (0.08 M CHES) and the other group of compounds studied in this work (11 benzene and naphthalene derivatives), in the different experimental conditions studied (0.05 M and 0.10 M CHES and 0.05 M Ammonium Acetate). Good linearity was observed in all cases ($r>0.99$) and this enabled the employment of eq. (2) in order to estimate the c.m.c. values for the SDS micellar systems in the different saline conditions.

Table 1 groups the c.m.c. values obtained for the SDS micelles with the group of 11 benzene and naphthalene derivatives when 0.05 M and 0.10 M CHES buffers (pH=10) and 0.05 M Acetate buffer (pH=9) were used. This table provides the c.m.c. values obtained with each solute as well as the average values (and standard deviation) for the c.m.c. obtained for all solutes in each buffer. Table 2 gives the c.m.c. values obtained with the group of 17 1,4-dihydropyridines and 0.05 M and 0.08 M CHES buffers (pH = 10).

Table 2

Critical Micelle Concentration for SDS Calculated from 1,4-Dihydropiridines Capacity Factor Data in Different Electrolyte Solutions by MEKC

Compound	c.m.c. (0.05 M CHES)	c.m.c. (0.08 M CHES)
1	$3.22\ 10^{-3}$	$2.31\ 10^{-3}$
2	$3.17\ 10^{-3}$	$2.10\ 10^{-3}$
3	$3.88\ 10^{-3}$	$2.27\ 10^{-3}$
4	$4.04\ 10^{-3}$	$1.73\ 10^{-3}$
5	$4.77\ 10^{-3}$	$1.81\ 10^{-3}$
6	$1.46\ 10^{-3}$	$2.33\ 10^{-3}$
7	$3.91\ 10^{-3}$	$1.54\ 10^{-3}$
8	$4.65\ 10^{-3}$	$1.67\ 10^{-3}$
9	$5.88\ 10^{-3}$	$1.83\ 10^{-3}$
10	$2.98\ 10^{-3}$	$2.21\ 10^{-3}$
11	$4.94\ 10^{-3}$	$1.52\ 10^{-3}$
12	3.6310^{-3}	$2.12\ 10^{-3}$
13	$2.97\ 10^{-3}$	$2.21\ 10^{-3}$
14	$6.90\ 10^{-3}$	$1.87\ 10^{-3}$
15	$3.18\ 10^{-3}$	$2.03\ 10^{-3}$
16	$3.22\ 10^{-3}$	$1.31\ 10^{-3}$
17	$5.96\ 10^{-3}$	$2.01\ 10^{-3}$
c.m.c. $\pm \sigma_{N-1}$	$4.05\ 10^{-3} \pm 1.35\ 10^{-3}$	$1\ 93\ 10^{-3} \pm 3.06\ 10^{-4}$

From these tables, it can be observed that all solutes provide similar SDS c.m.c. values for a given buffer, being the highest standard deviation obtained corresponding to the lowest CHES concentration (0.05 M); this is true for the benzene and naphthalenes derivatives as well as for the group of 1,4-dihydropyridines. This result can be observed in Figure 3 which shows the box plot for the SDS c.m.c. values obtained with the two groups of compounds studied in the different experimental conditions.

The box plot is defined in terms of percentiles and gives a quick overview of the median and spread of the data, and also the mean, minimum, and maximum values for the variable studied. The length of the upper and lower lines pertaining to the box shows how stretched the tails of the distribution are.

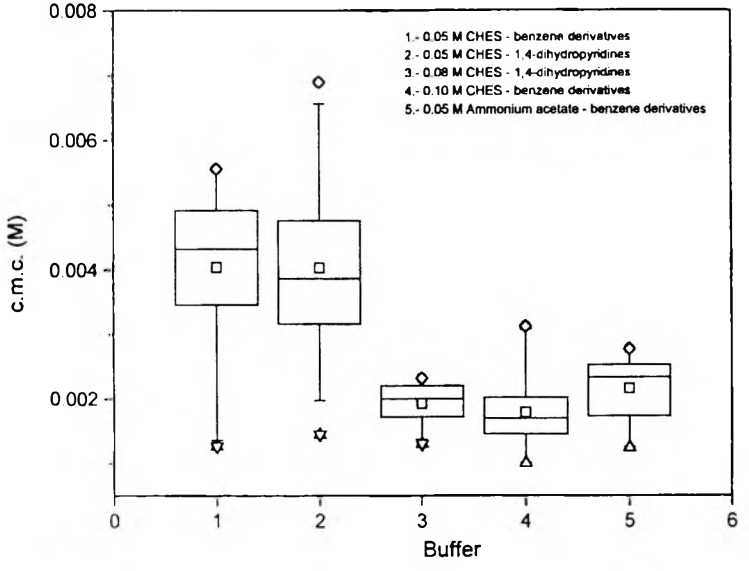


Figure 3. Box plot for the SDS c.m.c. values obtained with the two groups of compounds studied (benzene and naphthalene derivatives and 1,4-dihydropyridines) in different experimental conditions.

All c.m.c. average values obtained for SDS micellar systems were lower than the c.m.c. of this micellar system in pure water ($8 \cdot 10^{-3}$ M),[10] and also lower than the c.m.c. of SDS obtained in a 0.005 M borax buffer ($5.29 \cdot 10^{-3}$ M), by using a MEKC method (variation of the effective electrophoretic mobility of a neutral compound as a function of the total concentration of surfactant in solution).[6] This could be expected because the introduction of a salt in a micellar medium generally decreases the c.m.c. value.[11] Good agreement was obtained for the c.m.c. average value obtained for the same buffer (0.05 M CHES (pH =10)) and the two different groups of compounds studied. Values were statistically similar; Wilcoxon test, prob. 0.8589 for the 11 benzene and naphthalenes derivatives and 1,4-dihydropyridines numbers 1-11.

Figure 3 also shows that the c.m.c. for SDS decreases when increasing the buffer concentration in solution (0.05 M, 0.08 M and 0.10 M CHES buffers). Values were statistically different when comparing 0.05 M and 0.08 M CHES (Wilcoxon test, prob. 0.0044) and when comparing 0.05 M and 0.10 M CHES (Wilcoxon test, prob. 0.0044); values were statistically similar when comparing 0.08 M and 0.10 M CHES for the 11 benzene and naphthalene derivatives and 1,4-dihydropyridines numbers 1-11 (Wilcoxon test, prob. 0.3739).

When comparing the c.m.c. values obtained for a CHES (pH =10) and an Acetate buffer (pH =9) at the same concentration (0.05 M), it can be observed that a minor value is obtained for this last buffer being statistically significantly different (Wilcoxon test, prob. 0.0058).

The c.m.c. value obtained for SDS in a 0.10 M CHES buffer was close to that measured by a spectrophotometric method, which gave a value of 3 10^{-3} M.[12] A similar value was also obtained when the c.m.c. was measured by a conductrimetric method in the presence of another organic buffer (N,N'-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid) at a 0.10 M concentration (pH=7) (3.1 10^{-3} M).[13]

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CAPILLARY ELECTROPHORESIS: A GOOD ALTERNATIVE FOR THE SEPARATION OF CHIRAL COMPOUNDS OF ENVIRONMENTAL INTEREST

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ABSTRACT

Capillary electrophoresis potential for the separation of the enantiomers of chiral compounds of environmental interest is described. Applicability of capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) to achieve these separations is discussed.

INTRODUCTION

The importance of chirality in biological phenomena, chemical synthesis, toxicology, environmental control, and design of pharmaceutical agents has given rise to the search for new and effective means to resolve enantiomeric mixtures.[1] These separations have become particularly interesting in pharmacological and environmental fields. In the case of the pharmaceutical industry, many of the most widely prescribed drugs contain at least one chiral center, and an important percentage are marketed as racemates; thus, the study of the properties of individual drug enantiomers is important.[2]

Also, an enantiomer of a given drug may be physiologically active, whereas the other enantiomer may be totally useless, or even toxic.[3] Concerning chiral compounds detrimental to the environment, enantiomers may have different toxicities or one of them may not be toxic at all.[4] On the other hand, many herbicides and pesticides are produced as racemic mixtures and, often, only one enantiomer is biologically active. The extent to which various agricultural chemicals are being applied in the environment will soon make it urgent to monitor such potential ecological hazards, enantioselectively.[5]

Chromatographic techniques are important tools to carry out enantiomeric separations, since chiral stationary phases are easily accessible and commercially available today. High performance liquid chromatography (HPLC) and gas chromatography (GC) are the most common techniques used routinely and, in complementary form, in these types of separations. Various gas and liquid chromatographic methods have been used to resolve numerous enantiomeric mixtures.[6-10] Nevertheless, in recent years, another instrumental technique, —considered an interesting alternative to HPLC and GC techniques.— has been developed, i.e., capillary electrophoresis (CE).[11] The utilization of capillary tubes, of inside diameter generally ranging from 10 to 200 μm to achieve electrophoretic separations, has enabled obtaining high efficiencies and an enormous resolving power.[12-14] Even though CE was initially limited to the separation of charged compounds, the introduction of a micellar system into the separation buffer in 1984[15], which resulted in what is generally called micellar electrokinetic chromatography (MEKC), enabled the application of CE to the separation of uncharged compounds.[16-20] The limitations of GC techniques to the separation of volatile compounds and the poor performance that HPLC techniques sometimes provide, causing loss in sensitivity, have led one to consider CE techniques as an interesting alternative. In fact, they combine the high performance of GC techniques with the versatility of HPLC techniques as to selectivity and range of application.[21] The use of the principles and additives originally developed for the separation of chiral mixtures in HPLC has enabled the wide use of CE to perform enantiomeric separations.[1,2,5,22-26] Aside from all other considerations mentioned here, which show the great potential of CE to achieve enantiomeric separations, CE can also be considered a part of the so-called group *clean analytical techniques*. This is due to the small volumes of mobile phase (electrolyte solution) and sample required to perform separations by this technique. This property confers to CE a great environmental interest.

CE applications to the separation of chiral mixtures have been described mainly for drugs.[27-35] The aim of this work is to describe this technique's potential for the separation of the enantiomers of chiral compounds of

environmental interest. Although other CE techniques have been used to achieve the separation of chiral compounds (isotachophoresis, gel capillary electrophoresis and electrochromatography),[23] only the applicability of capillary zone electrophoresis (CZE) and MEKC will be discussed in this work, since they are primarily used to achieve chiral separations of environmental interest.

CHIRAL SEPARATIONS BY CAPILLARY ZONE ELECTROPHORESIS (CZE)

CZE is the easiest mode of CE to use since, in this working mode, the capillary contains only the electrolyte solution. Assuming that the capillary surface is negatively charged, there will be an electroosmotic flow which, if strong enough, will sweep the negatively charged substances towards the cathode.[36] The different electrophoretic migrations of the analytes enable their separation in the capillary, eluting, initially, the positively charged substances, later the neutral ones (not separated) and, finally, the negatively charged solutes.

Chiral separations are usually performed by CZE by adding a chiral selector to the electrolyte solution. The complexation reaction between enantiomer and chiral selector can be compared to the partition of an analyte between a mobile phase and a pseudophase. Whereas, in HPLC, the efficiency is limited by the profile of the laminar flow, transfer mass term, and possible additional interaction with the residual silanol groups of the stationary phase, in CE, high efficiencies are generally obtained. This is due to the flat profile originated and to an homogeneous partition of the chiral selector in the electrolyte which, in turn, minimizes the mass transfer term.[22]

Although several CZE chiral separation approach principles exist,[37] the most frequently used in the chiral separation of pollutants is the formation of inclusion complexes. They are molecular compounds of specific structural arrangements, in which one compound (the host molecule) spatially encloses another (the guest molecule), or at least part of it. The inclusion phenomena have found the widest use in separation methods such as chromatographics.[38] Two types of compounds are basically used in CZE to form inclusion complexes with enantiomers: cyclodextrins or their derivatives, and chiral crown ethers being the cyclodextrins the ligands most widely employed in the separations of compounds of environmental interest. Cyclodextrins are cyclic oligo-saccharides that consist of six, seven, or eight glucopyranose units and which are named α-, β-, and γ-cylodextrins, respectively. Their structure is unique; it resembles a truncated cone with both ends open. The top of the torus corresponds to the more open side which is rimmed with secondary hydroxyl

groups on carbons 2 and 3 of each glucose unit, all rotated to the right. The smaller opening of the cones is rimmed with the more polar primary hydroxyl groups on carbon 6 of the glucose unit. The interior of the cyclodextrin cavity contains two rings of C-H groups with a ring of glycosidic oxygens in between.[37] Their surface is relatively hydrophilic, whereas their cavity is of a hydrophobic nature. The exceptional properties of cyclodextrins have been described,[38-40] and their ability to selectively include a wide variety of guest molecules into their hydrophobic cavity is remarkable. Derivatives of the cyclodextrins have been synthesized in order to enhance their water solubility and to modify their cavity size.[41-50] Chiral recognition is based on the inclusion of an aryl or alkyl group in the cavity, in addition to hydrogen-bonding between secondary hydroxyl groups of the cyclodextrin and substituents of the enantiomer.

The separation of different phenoxy acids into their respective enantiomers was done by CZE.[51-53] Phenoxy acids are widely used in agriculture as selective herbicides. Phenoxy-propionic acid herbicides are racemic mixtures and d-isomers are the only active ingredients. Chiral separations of these herbicides are required in order to assess the enantiopurity of formulations and to optimize enantioselective production processes. The experimental conditions in which separations were achieved are grouped in Table 1. Results obtained in the separation of different herbicides (chiral and non chiral) with three types of cyclodextrins (α-cyclodextrin, β-cyclodextrin and a derivative of the latter) were compared. The addition of heptakis-(2,6-di-O-methyl)-β-cyclodextrin to the electrolyte solution improves the results obtained with β-cyclodextrin, since it enables the separation of all herbicides studied and the chiral separation of two of them (2-(2-methyl-4-chlorophenoxy)propionic acid and 2-(2,4-dichlorophenoxy)propionic acid). The use of α-cyclodextrin further improves the results and changes the selectivity obtained entirely. A 0.01 M concentration of this cyclodextrin allows the separation of all herbicides and the chiral separation of three of them (those mentioned above and 2-(2-methyl-4,6-dichlorophenoxy)propionic acid). Excessively high concentrations of α-cyclodextrin in the separation buffer resulted in the co-migration of several peaks close to the electroosmotic flow marker peak. The results obtained concerning the identification and determination of impurities are comparable to those obtained by chromatographic methods. However, the authors found CZE simpler, more flexible and cost-effective. By changing the chiral selector in the electrolyte solution, complete changes in selectivity can be obtained which may be used as a criterion to confirm impurities. The methods developed were successfully applied to the analysis of real production samples and the determination of their enantiopurities.[51]

Table 1

Experimental Conditions used on Enantiomeric Separations by CZE

Compounds	Chiral Selector	Buffer	Voltage	Injection	Detection	Observations	Ref.
2-(2-methyl-4-chlorophenoxy) propionic acid;	α-cyclodextrin	0.05 M LiOAc	30 kV	Hydrodynamic (pressure) (20 mbar x 6 s)	UV, 200 nm	Determination of impurities in production samples at levels as low as 1 mg/g relative to the	51
2-(2-methyl-4,6-dichlorophenoxy) propionic acid;	β-cyclodextrin					main component.	
2-(2,4-dichlorophenoxy) propionic acid	heptakis-(2,6-di-O-methyl-β-cyclodextrin						
2-(2-methyl-4-chlorophenoxy)-propionic acid; (MCPP)	heptakis-(2,6-di-O-methyl)-β-cyclodextrin	0.03 M LiOAc (pH = 4.8)	30 kV	Hydrodynamic (pressure) (20 mbar x 6 s)	UV, 200 nm	Chiral sep'n in river and drinking water samples spiked with 5 ppb MCPP and DP. Preconcentration by field	52
2-(2,4-dichlorophenoxy)-propionic acid (DP)						amplification (sample volume, 20 mL or 40 mL).	

(continued)

Table 1 (continued)

Experimental Conditions used on Enantiomeric Separations by CZE

Compounds	Chiral Selector	Buffer	Voltage	Injection	Detection	Observations	Ref.
2-(2-methyl-4-chlorophenoxy) propionic acid;	α-cyclodextrin	0.05 M NaOAc (pH = 4.5)	20 kV	Hydrodynamic (pressure)	UV, 230 nm	Study of the influence of cyclodextrin nature and the addition of organic midifiers	53
2-(2,4-dichlorophenoxy) propionic acid;	di-O-methyl-β-cyclodextrin					upon the sep'n of phenoxy acid herbicides.	
2-(2,4,5-trichlorophenoxy) propionic acid	tri-O-methyl-β-cyclodextrin						
1,1'-bi-2-naphthol; 1,1'-binaphthyl-2,2'-dicarboxylic acid;	α-cyclodextrin	0.01 M Phosphate + 0.006 M Borate (pH = 9)	20 kV	Hydrodynamic (gravity) (10 cm x 10 s)	UV, 254 nm	Study of the influence of the nature and conc'n of the cyclodextrin on enantiomeric resolution. Molecular modeling is	54
	β-cyclodextrin						
1,1'-binaphthyl-2,2'-diyl hydrogen phosphate	γ-cyclodextrin					used to calculate interaction energies between enantiomers and cyclodextrins	

Table 1 (continued)

Experimental Conditions used on Enantiomeric Separations by CZE

Compounds	Chiral Selector	Buffer	Voltage	Injection	Detection	Observations	Ref.
1,1'-bi-2-naphthol (BN); 1,1'-binaphthyl-2,2'-dicarboxylic acid (BNC); 1,1'-binaphthyl-2,2'-diyl hydrogenphosphate (BNP)	α-cyclodextrin (α-CD), β-cyclodextrin (β-CD), γ-cyclodextrin (γ-CD), heptakis-(2,3,6-tri-O-methyl)-β-cyclodextrin (TM-β-CD)	0.025 M Phosph. (pH=10.5) cntng 0.010 M α-CD + 0.010 M TM-β-CD (BN, BNP, and BNC)	15 or 20 kV	Hydrodynamic (pressure) (30 mbar x 3-7 s)	UV, 214 nm	Successful separation of binaphthyl enantiomers is achieved by using >2 chiral selectors.	55
	hydroxypropyl-α-cyclodextrin (HP-α-CD), hydroxypropyl-β-cyclodextrin (HP-β-CD), hydroxypropyl-γ-cyclodextrin (HP-γ-CD), dextran, α-CD-phosphate, β-CD-phosphate, γ-CD-phosphate	0.025 M Phosph. (pH=7.0) cntng 3% dextran (BNP & BNC) 0.025 M Phosph. (pH=9) and 0.01 M β-CD phosphate + 0.01 M α-CD 0.025 M Phosph. (pH=10.5) and 0.005 M β-CD Phosph. + 0.010 M α-CD + 0.010 M TM-β-CD					

(continued)

Table 1 (continued)

Experimental Conditions used on Enantiomeric Separations by CZE

Compounds	Chiral Selector	Buffer	Voltage	Injection	Detection	Observations	Ref.
1,1'-binaphthyl-2-2'-dicarboxylic acid (BNC); 1,1'-binaphthyl-2,2'-diyl hydrogen phosphate (BNP); 2,2'-dihydroxy-1,1'-binaphthyl-3,3'-dicarboxylic acid (HBNP)	Non-cyclic-oligosaccharides (α-1,4-dextrins)	0.04 M Carbonate (pH = 9)	15 kV	Electromigration (15 kV x 10 s)	UV 225 nm (BNC) 215 nm (BNP) 235 nm (HBNC)	Hydrogen bonding as well as hydrophobic interaction ins suggested as an essential force for enantioselective complexation between saccharide and anionic binaphthyl.	56

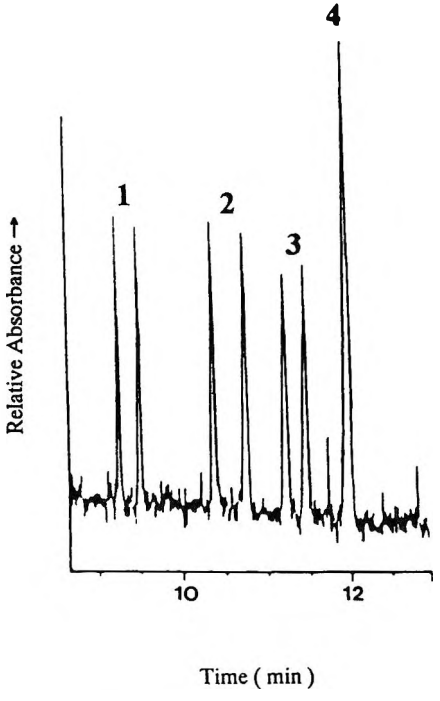

Figure 1. Chiral separation of three optically active phenoxy acid herbicides and another non-chiral phenoxy acid. Buffer: 0.05 M acetate, pH = 4.45, 0.025 M tri-O-methyl-β-cyclodextrin. Detection: 230 nm. Capillary: 50 cm (to the detector) x 0.075 mm I.D., fused silica. Separation voltage/current: 20 kV (400 V/cm)/41 μA. Hydrodynamic injection: 5 s. Temperature: 30 °C. Concentration of each analyte: 1 μg/mL. 1: 2-(2,4,5-trichlorophenoxy)propionic acid. 2: 2-(4-chloro-2-methylphenoxy)-propionic acid. 3: 2-(2, 4-dichlorophenoxy)propionic acid. 4: 2,4-dichlorophenoxyacetic acid. Reproduced with permission from ref. 53.

On the other hand, a comparison of the results obtained in the chiral separation of three phenoxy acid herbicides with different native and modified cyclodextrins by CZE[53] has shown that tri-O-methyl-β-cyclodextrin added to an acetate separation buffer is what ensures good separation of the two enantiomers of each of the three optically active herbicides, separately and in mixtures of the three. Di-O-methyl-β-cyclodextrin or α-cyclodextrin separated the enantiomers of two of the herbicides, β-cyclodextrin provided very little separation and γ-cyclodextrin gave no separation. Finally, it was shown that separations improved when methanol was added to the electrolyte solution, but due to the considerable increase in the analysis time, its use was not considered

helpful. Figure 1 shows the chiral separation of the three optically active phenoxy acid herbicides (2-(2, 4, 5-trichlorophenoxy)-propionic acid, 2-(4-chloro-2-methylphenoxy)propionic acid, and 2-(2, 4-dichlorophenoxy)-propionic acid) into their six enantiomers by CZE using a 0.05 M acetate buffer (pH = 4.45), 0.025 M in tri-O-methyl-β-cyclodextrin. They are separated from other non-chiral phenoxy acid (2,4-dichlorophenoxyacetic acid) at 1 μg/mL levels in an analysis time close to 12 min.

Enantiomeric separations of binaphthyl compounds have also been achieved by CZE[54-56] under the conditions detailed in Table 1. These solutes can be taken as typical examples of the separation of aromatic compounds. The effect of the type and concentration of cyclodextrin on the separation of the enantiomers of 1,1'-bi-2-naphthol, 1,1'-binaphthyl-2,2'-diyl hydrogen phosphate and 1,1'-binaphthyl-2,2'-dicarboxylic acid has been studied.[54] Non-cyclic oligosaccharides have also been used as chiral selectors for the separation of the enantiomers of these compounds.[56]

Several works have appeared dealing with the optimization of enantiomeric separations by CZE using chiral mobile-phase additives. The elution order of enantiomeric pairs in CZE can be optimized by reversal of the migration order.[57-60] Three different approaches can be used to achieve migration reversal for cationic and anionic enantiomers:

i) electroosmotic flow can be reversed using different additives in the electrolyte buffer,

ii) migration order can be reversed selecting different cyclodextrins as chiral additives due to the change in the separation and complexation mechanisms, and

iii) through the variation of the pH value using chargeable cyclodextrins in the separation buffer.

The application of various radial electric potential gradients across the capillary wall has been proposed to control the zeta potential and the electroosmotic flow, which enhance chiral resolution in cyclodextrin-modified CZE.[61] Finally, several models have recently been developed to explain separation in chiral CE[62] or to describe the effect of different factors on the separation selectivity of enantiomers.[63-66] However, in spite of the interesting results obtained in these works, the optimization approaches and models were tested in every case with drugs and were not applied to compounds of environmental interest.

CHIRAL SEPARATIONS BY MICELLAR ELECTROKINETIC CHROMATOGRAPHY (MEKC)

The addition of a surfactant at a concentration above its critical micellar concentration (c.m.c.) to the separation buffer in CE has given origin to the micellar electrokinetic chromatography (MEKC). A surfactant is a molecule possessing two zones of very different polarity, therefore resulting in solutions of special characteristics. There is a non-polar zone, of a hydrophobic nature, constituted by a hydrocarbon chain. The other zone can be polar or even ionic and permits classification of the surfactants into three principal classes: ionic (cationic and anionic), non-ionic, and zwitterionic.

When surfactant molecules are present in solution at low concentrations, they exist as monomers, but, at a given temperature and concentration (c.m.c.), they associate spontaneously to form submicroscopic aggregates called micelles.[67-70] As a result of the combination of hydrophobic and hydrophilic properties in the surfactant molecules, micellar systems have exhibited very interesting properties, such as the capacity to solubilize hydrophobic solutes into aqueous solutions or the possibility of improving sensitivity and selectivity of different analytical methodologies.[40,71,72]

MEKC enables separations of neutral and ionic compounds with the advantages of CE.[73] The most widely used micellar systems in MEKC have been those of anionic nature, such as sodium dodecyl sulphate (SDS). In this case, if the capillary generates a cathodic electroosmotic flow whose velocity is superior to that of the migration of anionic micelles towards the anode, these will migrate towards the cathode, but at a velocity lower than that of the electroosmotic flow. Neutral solutes are distributed between the micellar and aqueous phases according to their solute-micelle association constants.

Thus, they elute at a time somewhere between the migration time of a solute that moves with the electroosmotic flow and the elution time of a very hydrophobic solute, always associated with the micelle (micelle migration time).[15,20,74] The separation selectivity in MEKC can be controlled through a great number of parameters, such as the buffer and surfactant concentrations in the electrolyte solution and the nature and concentration of organic additives such as alcohols.[75]

The two methods most frequently used in MEKC to perform enantiomeric separations are the employment of chiral surfactants and the addition of chiral selectors to the micellar solution.[76]

Chiral Separations by MEKC with Chiral Surfactants

Chiral surfactants originate chiral aggregates. Most analytes are adsorbed onto the surface of the micelle or interact with the polar groups of the surfactants. Therefore, surfactants with chiral polar groups can be used for chiral dis-crimination.[76] Although many chiral surfactants are available, only a few have been found useful for enantiomeric separations by MEKC. Included are some amino acid derivatives, bile salts, glycosides, and saponins.[76] Recently, some novel chiral surfactants based on (R,R)-tartaric acid and long-chain aliphatic amines have been synthesized and have been used to achieve some chiral separations.[77] In some cases, chiral surfactants are used with achiral micelles (mixed micelles) in order to enhance the selectivity of the chiral separation.[78,79] Mixed micelles of bile salts and polyoxyethylene ethers have also been used.[80]

Bile salts have been considered as promising pseudo-phases in MEKC. Their structures and aggregation behaviour allow chiral recognition and the reduction of capacity factor values with respect to those obtained with SDS. These systems respond to organic modifiers in the same general manner as the SDS system.[81] In spite of these interesting properties, the usefulness of chiral surfactants in enantiomeric separations of pollutants has been investigated in very few works.[82] Separation of the two optical isomers of a silvex phenoxy acid herbicide (2-(2,4,5-trichlorophenoxy)propionic acid) was achieved by using a novel "in-situ" charged micelle, having chiral selectivity, named N,N-bis-(3-D-gluconamidopropyl)deoxycholamide, and using a high borate concentration in the electrolyte solution (0.4 M) (pH = 10) and a relatively high surfactant concentration (0.05 M). The addition of organic modifiers did not improve the chiral separation of the silvex phenoxy acid but, rather, decreased it.[82] This surfactant, together with others containing a cholic moiety (N,N-bis-(3-D-gluconamidopropyl)-cholamide), were evaluated in MEKC for the separation of enantiomers of other compounds.[82] These chiral, steroidal glycoside surfactants combine both the structural features of bile salts (chiral surfactants) and glycosidic surfactants (in-situ charged, and also chiral surfactants) through the steroidal portion and the polyolic polar groups, respectively. These two neutral surfactants could be charged readily via borate complexation.

As an example of enantiomeric separation of aromatic compounds, the chiral separation of bi-naphthyl derivatives can be cited.[80-83] The experimental conditions in which these separations were achieved are presented in Table 2, along with the results obtained by MEKC with chiral selectors.

Table 2

Experimental Conditions used on Enantiomeric Separations by MEKC

Compounds	Chiral Selector	Buffer	Voltage	Injection	Detection	Observations	Ref.
1,1'-bi-2-naphthol	Sodium deoxycholate Sodium deoxycholate + Polyoxyethylene ethers	0.016 M NaCl + Methanol (pH = 8.1-8.3)	20 kV	Hydrodynamic (gravity) (15 cm x 1-6 s)	UV, 210 nm	Determination of the effect of three variables on chiral resolution: (1) the type of ether; (2) the mole fraction of ether in solutions with bile salt; and (3) the percentage of methanol in the mobile phase.	80
1,1'-bi-2-naphthol; 1,1'-binaphthyl dicarboxylic acid; 1,1'-binaphthyl diyl hydrogen phosphate	Sodium cholate Sodium deoxycholate Sodium tauro-deoxycholate	0.01 M Phosphate + 0.06 M Borate + Methanol (pH = 9) Sodium acetate (pH = 4.7)	15-20 kV	Syphoning	UV (Laser-etched flow cells and a modified commercial detector)	Optimiztion of the enantiomer separation and study of poss. mechanism of chiral recognition	81

(continued)

Table 2 (continued)

Experimental Conditions used on Enantiomeric Separations by MEKC

Compounds	Chiral Selector	Buffer	Voltage	Injection	Detection	Observations	Ref.
Silvex phenoxy acid herbicide (2-(2,4,5-)-trichlorophenoxy propionic acid	N,N-bis-(3-D-gluconamidopropyl deoxycholamide	0.4 M Na Borate (pH = 10)	20 kV	Hydrodynamic (pressure) (34 mbar for various lengths of time	UV, 240 nm	Chiral separation is not improved by add'n of organic modifiers	82
1,1'-bi-2-naphthol 1,1'-binaphthyl-2, 2-diyl hydrogen phosphate;	N,N-bis-(3-D-gluconamidopropyl)-cholamide (Big Chap)	0.025 M-0.200 M Na borate (pH=8-11)	20 kV	Hydrodynamic (pressure) (34 mbar for various lengths of time	UV, 240 nm	Surface charge density of micelles is adjusted by varying the brate conc'n and the pH of the electrolyte. Resolution incr5eases with increasing pH and borate conc'n and decreasing temperature. Deoxy Big Chap micelles were more enantioselective than Big Chap micelles.	82
1,1'-binaphthyl-2, 2'-diamine	N,N-bis-(3-D-gluconamidopropyl)-deoxycholamide (Deoxy Big Chap)	Na borate + 15% methanol					

Table 2 (continued)

Experimental Conditions used on Enantiomeric Separations by MEKC

Compounds	Chiral Selector	Buffer	Voltage	Injection	Detection	Observations	Ref.
1,1'-bi-2-naphthol; 1,1'-binaphthyl-2, 2'-diyl hydrogen phosphaye	n-Dodecyl-β-D-glucopyranoside-4,6-hydrogen phosphate, Na salt n-Dodecyl-β-D-glucopyranoside-6-hydrogen sulfate, mono Na salt	0.03 M Na dihydrogen phosphate + 0.01 M Na borate (pH = 8)	20 kV or 28 kV	Hydrodynamic (pressure) 30 mbar x 1 s)	UV, 214 nm 254 nm	The 2 chiral micelles allows resolution of the enantiomers of 1,1'-binaphthyl-2,2'-diyl hydrogen phosphate. Chiral separation of 1,1'-bi-2-naphthol is only possible with the phosphate derivative.	55
1,1'-bi-2-naphthol; 1,1'-binaphthyl-2, 2'-dicarboxylic acid; 1,1'-binaphthyl-2, 2-diyl hydrogen phosphate	Na deoxycholate (SDC) Na deoxycholate + α-cyclodextrin	0.025 M Phosphate (pH = 8)	15 kV	Hydrodynamic (pressure) (30 mbar x 3-7 s)	UV, 214 nm	Enantioseparation of the three compounds is achieved with two chiral selectors (SDC + α-cyclodextrin.	

(continued)

Table 2 (continued)

Experimental Conditions used on Enantiomeric Separations by MEKC

Compounds	Chiral Selector	Buffer	Voltage	Injection	Detection	Observations	Ref.
Diniconazole; Uniconazole	γ-Cyclodextrin + 5% 2-Me-2-propanol	0.1 M SDS* 2 M Urea 0.1 M Borate (pH = 9)	15 kV	Hydrodynamic (pressure)	UV, 254 nm	Study of the influence of the nature and conc'n of cyclodextrin and add'n of organic modifiers on enantiomeric separation.	88
Polychlorinated biphenyls (PCB's) (45, 84, 88, 91, 95, 132, 136, 139, 149, 171, 183, & 196)†	γ-Cyclodextrin	0.10 M CHES‡).11 M SDS 2 M Urea (pH = 10)	15 kV	Hydrodynamic (pressure) (20 mbar x 1.2 s)	UV, 235 nm	Optimization of the chiral separation of a mixture of 9 PCB's. (18 enantiomers)	90
Polychlorinated biphenyls (PCB's) (45, 64, 88, 91, 95, 132, 136, 139, 149, 171, 183, & 196)	β-Cyclodextrin γ-Cyclodextrin	0.09 M CHES 0.11 M SDS 2 M Urea (pH = 10)	15 kV	Hydrodynamic (pressure) (20 mbar x 1.2 s)	UV, 235 nm	Optimization of the chiral separation of a mixture of 8 PCB's (16 enantiomers)	92

Table 2 (continued)

Experimental Conditions used on Enantiomeric Separations by MEKC

Compounds	Chiral Selector	Buffer	Voltage	Injection	Detection	Observations	Ref.
1,1'-bi-2-naphthol; 2,2,2-trifluoro-1-(9-anthryl)ethanol; 1,1'-binaphthyl-2,2'-diyl hydrogen phosphate	α-Cyclodextrin β-Cyclodextrin γ-Cyclodextrin 2,6-di-O-methyl-β-cyclodextrin 2,3,6-tri-O-methyl-β-cyclodextrin	0.05 M SDS 0.02 M Phosphate/Borate (pH = 9) Na d-camphor-10-sulphonate	20 kV		UV, 220 nm	Study of the influence of the add'n of different cyclodextrins, organic modifiers, and chiral additives.	93
1,1'-bi-2-naphthol; 1,1'-binaphthyl-2,2'-diyl hydrogen phosphate	γ-Cyclodextrin and poly-(Na N-undecylenyl-D-valinate)	0.025 M Borate (pH = 9)	12 kV		UV, 280 nm	A synergistic effect on the enentioselectivity was observed by use of both chiral selectors. The add'n of acetonitrile to the buffer reduces the enentioselectivity. The effect of the add'n of methanol is solute-dependent.	94

* SDS: Sodium dodecylsulphate. † Ballschmiter nomenclature.[89] ‡ CHES: 2-(N-cyclohexylamino)-ethanesulphonic acid)

Chiral Separations by MEKC with Chiral Selectors

Enantiomeric separation by MEKC with achiral micelles is possible if a chiral selector is added to the separation buffer. Chiral selectors most used in this MEKC method are cyclodextrins; this technique has been called "cyclodextrin modified micellar electrokinetic chromatography (CD-MEKC). Enantiomeric separation in CD-MEKC is based on the chiral recognition of cyclodextrins being important the choice of the type of cyclodextrin and its concentration. A mixture of different cyclodextrins may be helpful in order to increase selectivity. Addition of methanol also affects, not only the width of the migration time window and the capacity factors, but also selectivity.[84]

This technique is also considered very interesting for the separation of high hydrophobic compounds tending to be totally incorporated into the micelle and, hence, migrate at the same velocity as that of the micelle. The partition of the solutes among the micelle and the aqueous phase is affected by the presence of the cyclodextrin, which increases the fraction of the solute in the non-micellar aqueous phase, enhancing the resolution.[85-87] Likely for these reasons, CD-MEKC has been the technique mostly used to achieve enantiomeric separations of pollutants, especially those of a highly hydrophobic character.

The enantiomers of diniconazole and uniconazole, which are vinyl triazoles, were separated by CD-MEKC. They have fungicidal and plant growth regulating activities and their enantiomers are known to differ significantly in their biological properties. In both cases, the R-enantiomer demonstrates stronger fungicidal activity than the S-enantiomer, whereas the S-enantiomer is more active than the R-enantiomer with regard to plant growth regulating activity. Furthermore, uniconazole has a higher plant growth regulating activity than diniconazole, but it is less active as a fungicide. Consequently, diniconazole-M, containing a high proportion of the R-enantiomer, and uniconazole-P, containing a high proportion of the S-enantiomer, have been developed as a high activity fungicide and an effective plant growth regulator, respectively.[88] This production requires an efficient analytical method to separate the enantiomers. This was found to be possible using CD-MEKC with SDS micelles.

The study of the influence of the nature and concentration of the cyclodextrin and the addition of organic modifiers to the electrolyte solution has shown that the optimum conditions for the separation of enantiomers pertain to the use of γ-cyclodextrin at a 0.05 M concentration in the presence of a 5% 2-methyl-2-propanol as organic modifier.

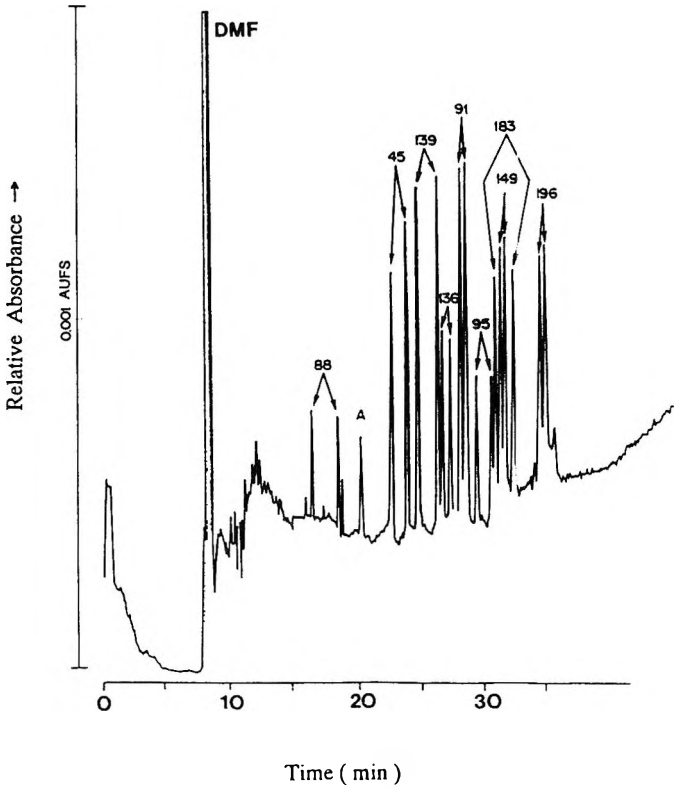

Figure 2. Electropherogram of the separation of a mixture of nine chiral PCB's. Each pair of enantiomers is identified by a number according to Ballschmiter nomenclature.[89] A: unknown peak. Separation buffer: 0.10 M CHES (pH = 10), 2 M urea, 0.11 M SDS and 0.05 M γ-cyclodextrin. Injection by pressure, 0.02 min at 20 mbar. Temperature, 45 °C. UV detection, 235 nm. Capillary, 65 cm length x 50 μm I.D. Applied voltage, 15 kV, current, 56 μA. Reproduced with permission from ref. 90.

CD-MEKC has also enabled the chiral separation of twelve polychlorinated biphenyls (PCB's) (45, 84, 88, 91, 95, 132, 136, 139, 149, 171, 183, and 196, Ballschmiter nomenclature[89]) that were individually separated into their two enantiomers by using γ-cyclodextrin as chiral selector in the separation buffer, which contained 2-(N-cyclohexylamino)ethanesulphonic acid (CHES) and SDS micelles. The multicomponent separation of the eighteen enantiomers of a mixture of nine chiral PCB's (containing one octachlorinated biphenyl) was also performed in an analysis time close to 35 min. (Figure 2).[90] This analysis time can be considered excellent, taking into account that the separation of a mixture of one hexachlorinated biphenyl and four

heptachlorinated biphenyls in their enantiomers by GC required about 110 min.[91] Chiral separation of PCB's was also studied by using mixtures of β- and γ-cyclodextrins in the separation buffer. The same twelve PCB's were individually separated into their two enantiomers but, in the separation of multicomponent mixtures, only the separation of a mixture of the sixteen enantiomers of an eighth PCB mixture was reported.[92] The only change in selectivity observed when using mixtures of β- and γ-cyclodextrins to achieve multicomponent separations of PCB's with respect to the use of γ-cyclodextrin alone in the separation buffer,[90] is the reversal in the elution order for PCB's 136 and 139. The use of β and γ-cyclodextrin mixtures reduces the cost of the technique with respect to γ-cyclodextrin-modified MEKC. However, the use of γ-cyclodextrin alone as modifier in MEKC to perform chiral separation of PCB's seems to have more potential in the separation of multicomponent mixtures or the separation of some individual, highly hydrophobic PCB's in their enantiomers.[90]

The effect of the addition of a chiral additive to the electrolyte solution in CD-MEKC with SDS micelles has been studied. The presence of sodium d-camphor-10-sulphonate in the SDS solution containing γ-cyclodextrin or 2,3,6-tri-O-methyl-β-cyclodextrin improved enantioselectivity in the separation of aromatic compounds such as 1,1'-bi-2-naphthol, 2,2,2-trifluoro-1-(9-anthryl)ethanol and 1,1'-binaphthyl-2,2'-diyl hydrogen phosphate.[93] Also, chiral separation of the enantiomers of 1,1'-bi-2-naphthol and 1,1'-binaphthyl-2,2'-diyl hydrogen phosphate by use of a combination of a polymerized chiral micelle (poly-(sodium N-undecylenyl-D-valinate)) and γ-cyclodextrin was superior to the use of either chiral selector alone. A synergistic effect on the enantioselectivity was observed when using both chiral selectors. Since surfactant monomers do not exist in the covalently bonded polymerized micelle, the interference of surfactant monomers on the enantioselectivity of γ-cyclodextrin is eliminated. Therefore, the use of a combination of both chiral selectors was considered to be a promising alternative for enantiomeric separations.[94]

The experimental conditions concerning all mentioned CD-MEKC separations are detailed in Table 2.

APPLICATION TO REAL SAMPLES

In spite of the enormous possibilities that CE techniques possess as applied to the separation of pollutants, the principal drawback, in view of the analysis of these compounds in environmental samples, is their lack of

sensitivity, particularly when trace analysis is required. Commercial CE instrumentation is equipped with UV detectors capable of detecting quantities in the order of 600 femtograms. However, in CE, injection volumes are generally in the nanolitre range to avoid the loss of the high separation efficiency that this technique ensures. This implies low sensitivity, expressed in concentration terms (about 10^{-6} M). Capillary geometry and improved detector cell design influence sensitivity in CE since, according to Beer's law, the optical absorbance of a sample is directly proportional to the optical pathlength through which the absorbance measurement is performed. Bent capillaries (Z-cell), alternative capillary shapes (rectangular), multi-reflection flow cells or end-column detection[96] have been used in order to increase the sensitivity obtained. A sensitivity enhancement ranging from 3- to 40-fold has been obtained.

Since the determination of lower and lower levels of pollutants in environmental samples is necessary nowadays, other methods to increase the sensitivity in CE have been developed. Detection limits in CE can be improved by using more sensitive detection systems, or by performing on-line concentration methods.[52,95,96] Laser-induced fluorescence, mass spectrometry and electrochemical or radiochemical detection have been employed. Excellent results have been obtained with laser-induced fluorescence which gives a 10^{-12} M sensitivity under favourable conditions. The problem is that this detection method cannot be used in a general form for environmental analysis. Methods based on indirect fluorescence have more applicability, but a 10^{-7} M sensitivity is obtained.

Different methods exist to achieve on-line concentration in CE. Sample *stacking* with discontinuous buffer systems has been used extensively. When a sample is dissolved in a solvent with electrical conductivity lower than that of the electrophoresis running buffer, a concentration, or *stacking*, occurs upon electrokinetic sample injection. The electric field strength in the low conductivity sample medium is higher than that in the running buffer, and ions rapidly migrate to the interface between the lower and higher conductivity zones. Upon reaching the interface, the analytes then slow (stack), causing contraction of the sample zone.[96] When *field amplification* is used, a large sample plug is hydrodynamically injected onto the column in a low-conductivity buffer. Then, the sample is focused at the cathodic end of the capillary, at the sample buffer–running buffer interface, using a voltage polarity opposite to that employed for the electrophoresis. Finally, when focusing is complete (as indicated by a change in current), the polarity is again reversed and the separation is performed. For a negatively charged silica surface, anions tend to stack at the back end of the sample buffer plug. Using a polarity opposite to the separation mode will result in driving out the sample buffer

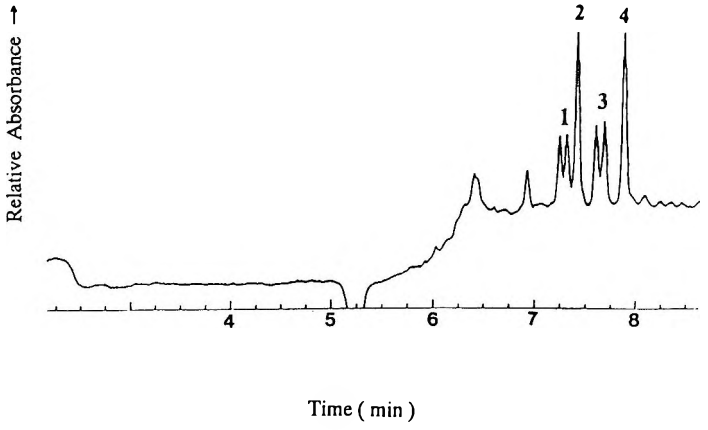

Figure 3. Chiral separation of a Rhine river water sample, spiked with 5 ppb of four herbicides and using cyclodextrin-modified CZE and C_{18} disks. Buffer: 0.03 M lithium acetate (pH = 4.8) with 20 g L^{-1} heptakis-(2,6-di-O-methyl)-β-cyclodextrin. Sample volume 20 mL. Voltage: 30 kV. Temperature: 30° C. UV detection: 200 nm. 1: 2-(2-methyl-4-chlorophenoxy)-propionic acid. 2: 2-methyl-4-chlorophenoxyacetic acid. 3: 2-(2,4-dichlorophenoxy)-propionic acid. 4: 2,4-dichlorophenoxyacetic acid. Reproduced with permission from ref. 52.

ahead of the negatively charged analytes. This method can be used to separate cationic analytes by coating the capillary.[96] On-column concentration of neutral molecules was achieved in MEKC by using field-amplification sample stacking. Neutral analytes were dissolved in a low-concentration micellar solution that was still above the critical micelle concentration. The lower total ionic strength in the sample buffer, compared to the electrophoresis buffer, allowed the negatively charged micelles to migrate rapidly into the boundary between the sample and the running buffer where they slowed down. The technique was performed by using normal or reverse electrode polarity and enabled a 75-85-fold increase in sensitivity for 1,2,4,7- and 1,2,4,8-tetrachlorodibenzo-p-dioxins.[97] *Isotachophoresis* may act as a concentration method for dilute samples, as their concentration is adapted to that of the leading zone according to Kohlrauch's regulating function.[98] This preconcentration step can be performed either in a dual- or single-column mode. Isotachophoretic concentration is performed in dual-column mode in a pre-capillary. The sample ions, which are concentrated in a sharp, narrow zone, are transferred to the analytical capillary where they are separated by CZE. In single-column mode, the use of buffers in a discontinuous system allows the use of this method with commercially available CE instruments.[95]

Another method for on-line concentration implies loading large volumes of solutes onto microcolumns of *chromatographic* material followed by elution onto a CE capillary.[96] The determination of phenoxy acid herbicides in drinking and river water samples at sub-ppb levels was performed by using field-amplification and sample pretreatment with C_{18} membrane disks for simultaneous filtering and solid phase extraction. Desorption from disks directly into the CZE vials was performed by employing acetonitrile-buffer mixtures, thus providing a sample matrix with a sufficiently low and constant conductivity.[52] Figure 3 shows the electropherogram corresponding to the chiral separation of a Rhine river water sample spiked with 5 ppb of four herbicides, two of them chiral, by using CZE and a derivative of β-cyclodextrin as chiral selector in the separation buffer (sample volume 20 mL). Changes in selectivity of the four herbicides were observed when using α-cyclodextrin; this change in elution order can be useful as confirmation criteria for the presence of these herbicides.

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AFFINITY CHROMATOGRAPHY OF GLYCOENZYMES AND GLYCOPROTEINS ON CONCANAVALIN A–BEAD CELLULOSE

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ABSTRACT

Concanavalin A immobilized on chlorotriazine bead cellulose was applied to affinity purification of glycoenzymes and glycoproteins. Enzymes such as invertase from baker's yeast, endopolygalacturonase (Rohament P) and exopolygalacturonase from carrot roots, as well as extracellular mannoproteins from the yeast *Cryptococcus laurentii* were examined. Chromatography was performed on minicolumns filled with Concanavalin A-triazine bead cellulose gel with the content of immobilized Concanavalin A within the range 1.2 – 8.2 mg per mL of gel. The specifically bound glycoenzymes or glycoproteins were eluted with a solution of the corresponding counter-ligand α-methyl mannopyranoside. Individual degrees of purification, estimated from the measurements of specific activity of crude and purified glycoenzymes, were 14.5-fold for invertase, 93-fold for polygalacturonase and 3.9-fold for exopolygalacturonase. The yeast mannoprotein was isolated from the heteroglycoprotein fraction. The purified mannoprotein contained mainly mannose, with traces of glucose. The purification effect was verified by FPL-chromatography.

INTRODUCTION

Lectins are proteins (glycoproteins) that specifically bind particular carbohydrate structures and have been shown to be widely distributed in plants, animals and bacteria.[1,2] The ability of lectins to interact with glycoconjugates has been evaluated for isolation and fractionation of glycoproteins or oligosaccharides by affinity chromatography. Affinity chromatography, with immobilized lectins, seems to be quite an effective technique, because it can achieve not only fractionation of the glycoproteins or oligosaccharides, but also their structural assessment on the basis of the elution profile from an immobilized lectin column. Most of the well-characterized lectins and their immobilized derivatives on agarose are commercially available. Sepharose carrier with immobilized Concanavalin A (Con A) is widely used in affinity chromatography of many saccharide compounds from the biological sources.[3] Con A is a lectin with an ability to bind, specifically, the nonreducing terminal mannosyl or glucosyl residue of saccharide chains. Con A-Sepharose was evaluated in the purification procedure of glycoenzymes (glucanases, glucosidase, mannosidase or cellobiase),[4-7] glycoproteins from human sera and from cellular membranes,[8,9] antibodies and antigens,[10,11] lysozymes,[12] heteropolysaccharides from plants,[13] and many other compounds.[14]

In the present work, a conjugate of Con A with macroporous bead cellulose was prepared and investigated as an affinity chromatography matrix for glycoenzymes and extracellular glycoproteins. Mild conditions of lectin immobilization were chosen to avoid impairment of its mannose/glucose binding activity. The prepared Con A-triazine bead cellulose was used in affinity purification of the glycoenzymes containing mannosyl units, such as invertase, polygalacturonase and exopolygalacturonase, as well as extracellular heteromannoproteins from yeast.

EXPERIMENTAL

Materials

Concanavalin A-triazine bead cellulose (Con A-TBC) was prepared according to the procedure described in our previous work.[15] Con A was provided by Lectinola (Charles University, Prague, Czech Republic), Perlose MT 100 (bead cellulose) with the size of particles 0.100-0.250 mm was supplied by Lovochemie (Lovosice, Czech Republic), 2,4,6-trichloro-1,3,5-triazine and invertase (INV) (EC.3.2.1.26, β-D-fructofuranosidase) grade V: practical from baker's yeast (2.3 U/mg) were obtained from Sigma (St.Louis,

MO., USA), α-methyl-D-mannopyranoside (α-MMP) from Fluka (Buchs, Switzerland) and polygalacturonase Rohament P (PG) lyophilizate (1.86 U/mg) was from Rohm GmbH (Darmstadt, Germany). Exopolygalacturonase (exoPG) lyophilizate (0.085 U/mg) was prepared from carrot roots[16] and extracellular glycoproteins (GPCL) produced by *Cryptococcus laurentii* were isolated by a procedure described by Masler et al.[17]

Methods

Affinity chromatography on Con A-TBC

Minicolumn filling of 1g Con A-TBC (2.0 x 1.0 cm), containing 1.2 - 8.2 mg Con A/mL of gel, was equilibrated with 50 mM acetate buffer pH 4.7 or 5.8 containing 100 mM NaCl, 0.1 mM $CaCl_2$ and 0.1 mM $MnCl_2$. 4 H_20. After loading of solution of one of the above mentioned glycoenzymes or glycoproteins in equilibration buffer (1 mL), the non-bound proteins were washed out with the some buffer but without $CaCl_2$ and $MnCl_2$. $4H_20$. For elution of specifically bound glycoenzymes/glycoproteins, a solution of α-MMP (as counter-ligand) was used with the concentration dependent upon the strength of Con A–glycoenzyme/glycoprotein interaction. Flow rate was within the range of 12-18 mL/hod. In the collected fractions (1-3 mL), protein content[18] and enzyme activity of INV[19] or PG, exoPG[20] or saccharide content[21] were determined.

Fast protein liquid chromatography (FPLC)

Purity of all glycoenzymes/glycoproteins was checked by size-exclusion chromatography. FPLC equipment from Pharmacia (Uppsala, Sweden) with standard prepacked columns of Superose 12^{TM} or 6^{TM}, both HR 10/10 were used. The elution buffer was 50 mM phosphate buffer (pH 7) with 150 mM NaCl, flow rate was 0.5 mL/min or 0.3 mL/min. UV absorbance of effluent was monitored at 280 nm. The fractions of volume 0.5 or 0.3 mL were tested also for enzyme activity or saccharide content.

Gas chromatography (GC)

Glycoproteins were hydrolysed with 1 M HCl for 8 hours, then reduced with $NaBH_4$ and acetylated in pyridine-acetic anhydride (1:1) 16 hours at room temperature. The resulting alditol acetates were analysed by GC using a Hewlett-Packard Model 5890. Temperature was programmed to hold at 125°C for 2 min, then to increase to 165°C for 20 min; a column. PAS 1701 (250 cm x 0.32 cm), was used. The carrier gas was H_2 (15 $cm^3 . min^{-1}$).

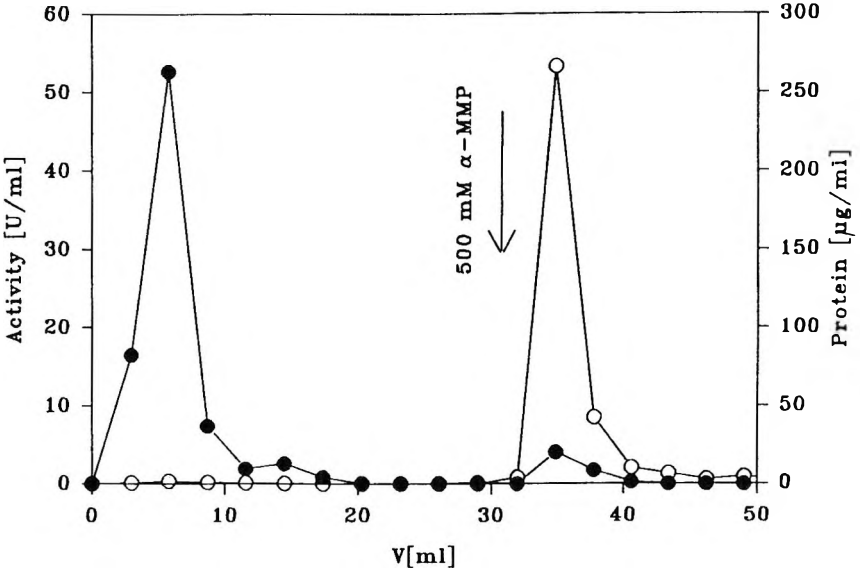

Figure 1. Affinity elution of invertase. Equilibration buffer - pH 4.7, loading: 1mL solution of INV (80mg of lyophilizate); ● - protein, ○ - activity.

RESULTS AND DISCUSSION

In our previous work[15] we described ability of Con A-TBC matrix to bind baker's yeast invertase and quantitative parameters (binding capacity and dissociation constants) of this interaction were determined. Because of a very strong interaction between invertase and Con A, the elution reagent of high concentration (500 mM α-MMP) have left to act at least 2 h (incubation time) in order to obtain a substantial improvement of the elution characteristics.

The purpose of this work is to investigate the possibility of using of this sorbent for purification of glycoenzymes and separation of glycoproteins containing mannosyl units. The sorbent was prepared by a described procedure[15] where Con A was bound to chlorotriazine bead cellulose (CHTBC). The content of immobilized Con A was within the range of 1.2 – 8.2 mg/mL of gel. The minicolumns containing of 1g of wet Con A-TBC were used for affinity purification of the glycoenzymes (INV, PG, exoPG) and isolation of mannoproteins from the glycoproteins (GPLC).

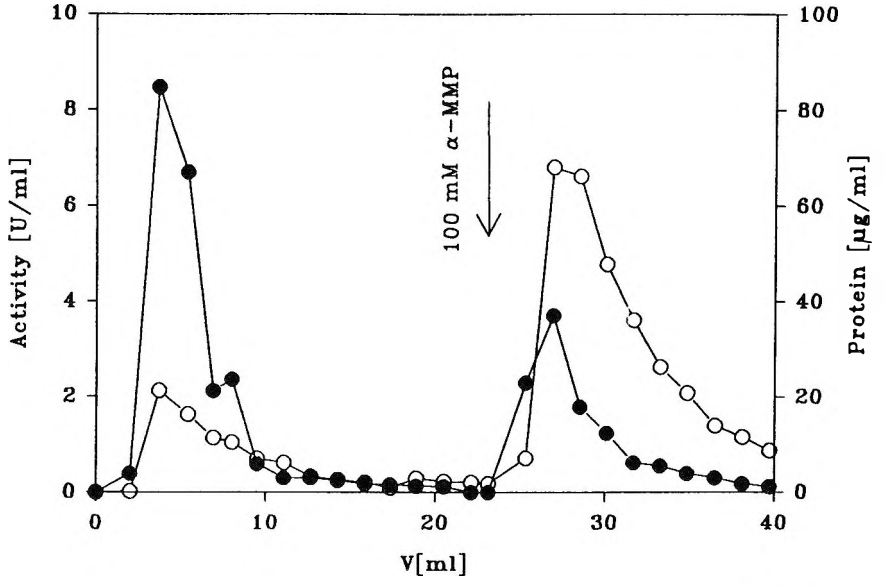

Figure 2. Affinity elution of polygalacturonase. Equilibration buffer - pH 4.7, loading: 1 mL solution of PG (1mg of lyophilizate); ● - protein ❍ -activity.

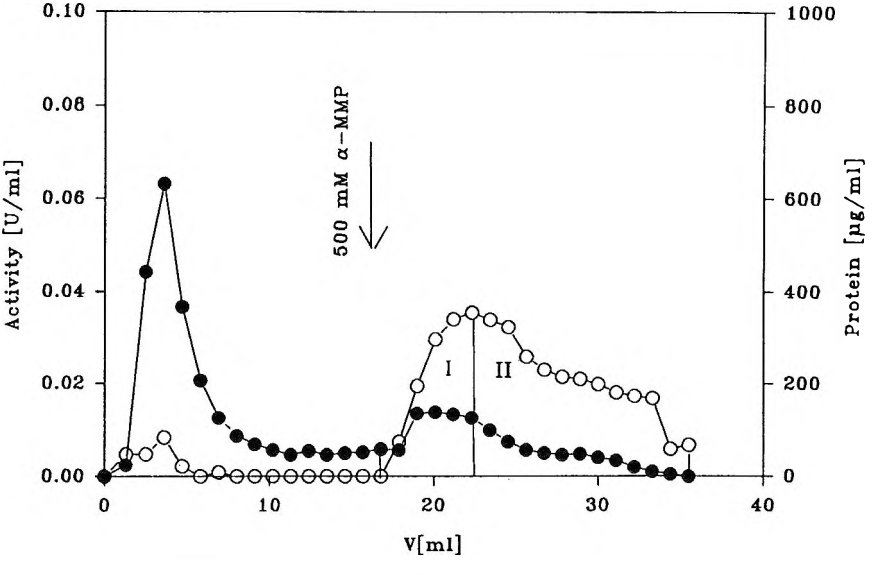

Figure 3. Affinity elution of exopolygalacturonase. Equilibration buffer - pH 4.7, loading: 1 mL solution of exoPG (10 mg of lyophilizate); Collected fractions : I - first fraction , II - second fraction, ● - protein, ❍ - activity.

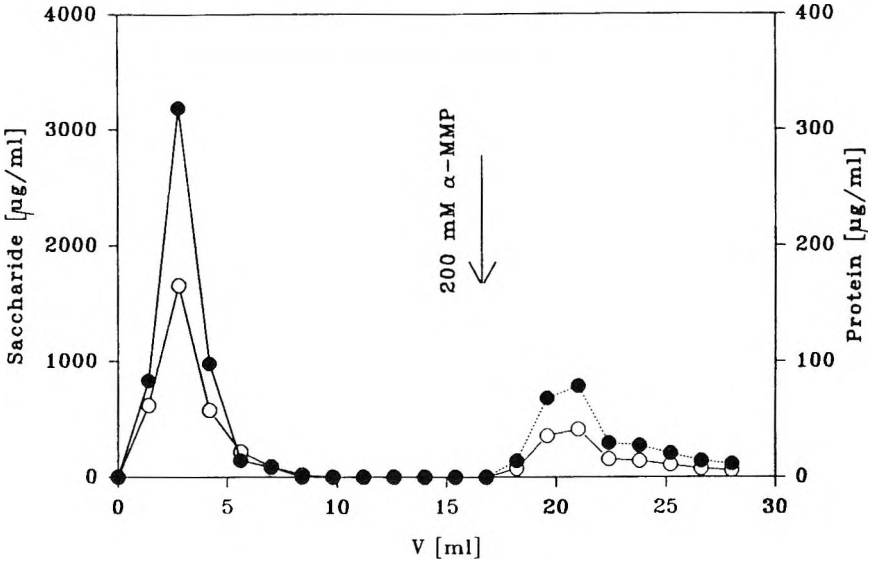

Figure 4. Affinity elution of extracellular glycoproteins. Equilibration buffer - pH 5.8, loading: 1 mL solution of GPLC (10 mg of lyophilizate); ● - saccharide, ○ - protein.

The elution course of the INV is shown in the Figure 1. Sorbent containing 1.2 mg of Con A per mL of gel was used. For more effective elution of the sorbed enzyme, prolonged treatment with α-MMP solution (20 h) was used. The recovery of purified invertase was 71.4 %.

The purification courses of polygalacturonase and exopolygalacturonase on Con A-TBC matrix are presented in Figures 2 and 3. Due to the lower interaction of the polygalacturonases with Con A-TBC, sorbent with 5 mg per mL of gel of immobilized Con A was used. The satisfactory elution of purified PG was reached already with 100 mM solution of α-MMP.

Exopolygalacturonase with very low specific activity (0.142 U/mg) was loaded on the column with the same content of immobilized Con A (5 mg/mL of gel) as polygalacturonase. Interaction with the immobilized Con A was observed, but the enzyme specifically eluted with 500 mM α-MMP was not so successfully purified as INV or PG.

Table 1

Purification of Glycoenzymes on Con A/TBC Minicolumns

Glycoenzyme	Content of Con A (mg/mL Gel)	Total Activity (U)	Specific Activity of Crude Enz. (U/mg)	Recovery (%)	Specific Activity of Purif. Enz. (U/mg)	Purification Factor
INV	1.2	281.0	147. 90	71.4	2154.0	14.5
PGA	5.0	1.86	2.88	68.6	268.1	93.1
e-PGA	5.0	0.85	0.14	I.fr.* 19.6	0.267	1.9
				II.fr. 27.5	0.554	3.9

*The fractions collected after elution with α-MMP were pooled in two main parts (Fract. I & II).

Table 2

Molar Content of Monosaccharides (Man = 1) in Heteroglycoprotein (A) and Purified Mannoprotein (B)

Saccharide	Molar Ratio X_R* A	B
Arabinose	0.07	---
Glucose	0.10	0.06
Galactose	0.13	---
Mannose	1.00	1.00
Xylose	0.02	---

*$X_R = X_i / X_{(mann)}$; X_i = molar content of monosaccharide; $X_{(mann)}$ = molar content of mannose.

Figure 4 shows affinity chromatography of the extracellular heteroglycoproteins on Con A-TBC with content of immobilized Con A of 8.2 mg/mL of gel. In this case, the fraction of bound glycoprotein, obtained with 200 mM α-MMP elution, contained the same saccharide : protein ratio as the original sample of GPLC (95:5).

The results of glycoenzymes purification on Con A - affinity column are shown in Table 1. A significant increase of invertase and polygalacturonase specific activities was observed. In the case of polygalacturonase, the high purification effect can be caused, also, by removal of the inhibitors present in the original sample.

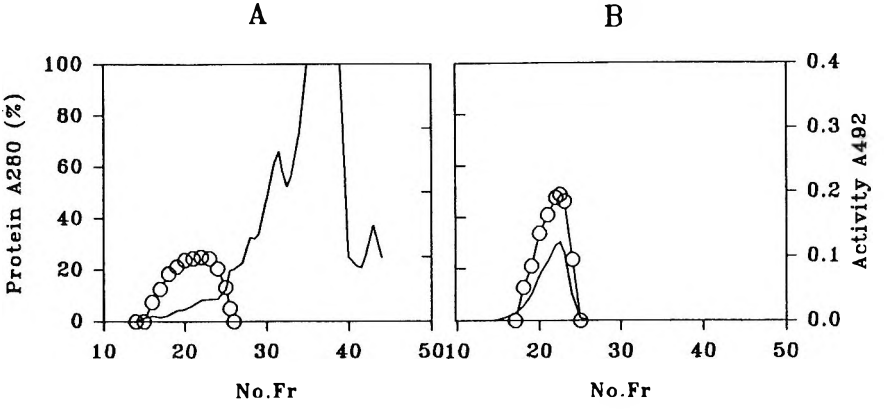

Figure 5. FPLC of invertase on Superose 6™. A - original sample, B - sample after purification; —— protein, ❍ - activity.

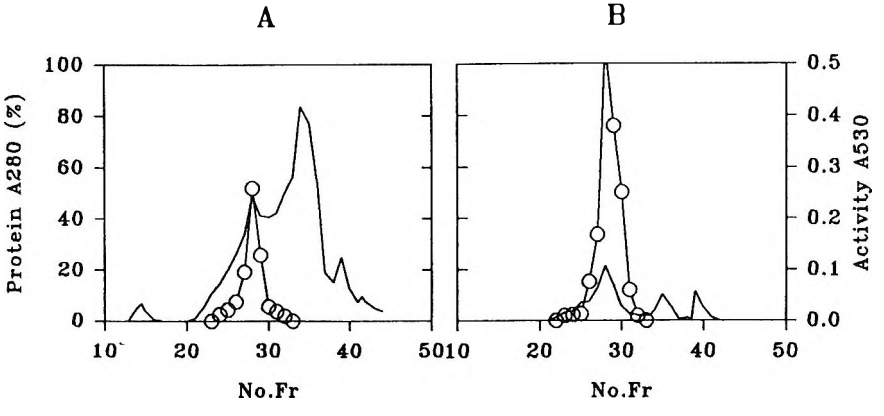

Figure 6. FPLC of polygalacturonase on Superose 12™. A. original sample, B. sample after purification; —— protein, ❍ - activity.

By purification of exopolygalacturonase, the eluted enzyme was divided into two fractions with different specific activities. The efficiency of purification was very low in both fractions, as was the recovery of bound enzyme. This could have been caused by deactivation of enzyme during the purification procedure.

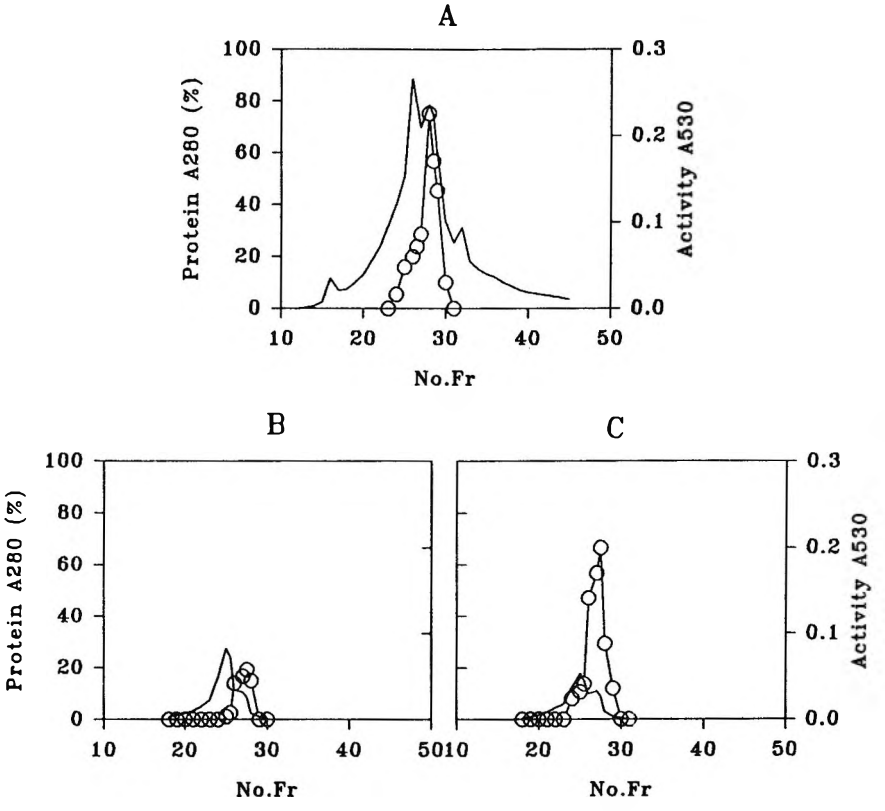

Figure 7. FPLC of exopolygalacturonase on Superose 12™. A. original sample, B. sample after purification (I), C. sample after purification (II); —— protein, ○ - activity.

The purification of the studied glycoenzymes was verified by FPLC chromatography. Superose 6™ or Superose 12™ column was used to determine purity and molecular distribution of the samples separated by affinity chromatography on Con A-TBC. Samples before and after purification were analysed, whereby the sensitivity of protein detection was usually at purified sample higher than at original one.

The significant invertase purification is demonstrated in Figure 5B. The position of the peak in FPLC corresponds to a molecular mass of 26–27 x 10^4. Interestingly, the invertase as protein was not identified in FPLC of the crude sample (Figure 5A), but instead the activity in the fractions was measured.

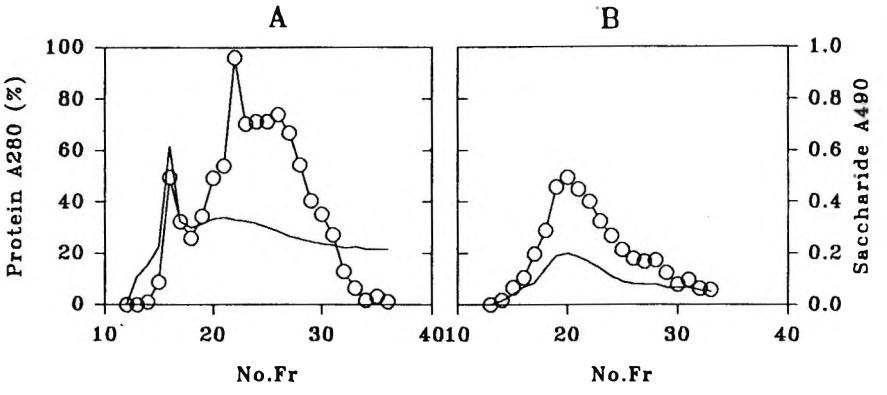

Figure 8. FPLC of extracellular glycoproteins on Superose 12™. A. original sample B. sample after purification; —— protein, ○ - saccharide.

Using affinity chromatography accompanying proteins have been almost completely removed and, in Figure 5B, one can observe, mainly, the peak of the invertase proteins. The results of FPLC chromatography of polygalacturonase (Fig.6 A,B) showed the purification effect of Con A-TBC on this glycoenzyme.

Similarly, as with invertase, a large amount of accompanying proteins was removed and the purity of the eluted enzyme with molecular mass about 35.000 was enriched 93.1-fold.

The lyophilizate from carrot root extract contained exopolygalacturonase of very low specific activity. Some of the accompanying glycoproteins can also interact with Con A immobilized on bead cellulose. Removal of these proteins by Con A-affinity chromatography was, therefore, more complicated than in the previously described experiments. The fractions collected after elution with α-MMP (Fig. 3) were pooled into two main parts (fraction I and II) with different specific activities. Purity of the obtained fractions was examined by FPLC and compared with that of the original sample (Figure 7A, B, C).

Fraction I, with molecular mass of the main peak of inactive proteins Mw ≅ 68,000, has lower specific activity than fraction II with molecular mass of the main enzyme peak Mw ≅ 48,000. Molecular distribution of the extracellular glycoproteins, before and after Con A-affinity chromatography, revealed by FPLC, is presented in Figure 8 A, B.

The separation of a fraction with broad distribution of molecular mass was observed. Changes in the composition of saccharides in glycoproteins were determined by GC (after hydrolysis, reduction and acetylation) (Table 2).

CONCLUSION

Con A-bead cellulose was successfully used as affinity sorbent for purification of two glycoenzymes: invertase and polygalacturonase. One-step chromatography procedure provided high purification factors estimated from the measurements of specific activity of the crude and purified glycoenzymes: 14.5-fold for invertase and 93.1-fold for polygalacturonase. The good increase of the specific activity of described enzymes was achieved. The results of the enzyme recovery were satisfactory as well. By FPLC monitoring of their purities, it was found that the final products of Con A-affinity chromatography were practically homogeneous in molecular weight. Purification of exopolygalacturonase was less successful because it contained accompanying glycoproteins with affinity to Con A-TBC. Good results were obtained in the separation of mannoproteins from heteroglycoproteins. The purified fraction contained mainly mannose with traces of glucose without contaminating saccharides such as arabinose, galactose and xylose.

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RELATIONSHIP STUDY BETWEEN REVERSED-PHASE HPLC RETENTION DATA AND THE pKa VALUES IN A SERIES OF 2-AMINO-2-OXAZOLINES

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ABSTRACT

In reversed-phase liquid chromatography, the retention behaviour of ionogenic compounds is strongly related to the pH mobile phase. An application is presented for three basic 5-substituted 2-amino-2-oxazolines. The pH dependence of their capacity factor values, expressed as log k'_W, was investigated in a RP-HPLC procedure using a column packed with poly-methacrylate. The plot of capacity factor *vs* pH was described by a sigmoidal curve, leading us to approach the ionization constants of the molecules. Finally, the pKa values have been discussed in regard to chromatographic parameters and to experimental conditions.

INTRODUCTION

At different pH values, the drugs containing ionogenic functions may exist as a mixture of dissociated and undissociated forms. Their retention behaviour, expressed as the capacity factor, k', in a reversed-phase high-performance liquid chromatographic system (RP-HPLC), is dependent on both the ionized and the unionized species.[1] Consequently, the mobile phase pH is an important tool to accomplish a complete separation between the different solutes in terms of selectivity and of column efficiency.[2,3] In RP-HPLC, as the eluent is a mixture organic modifier/buffer, the aqueous phase pH is chosen in regard to the degree of ionization (pKa) of the studied compounds. If the solute is a weak base, at pH < pKa, the molecule is completely ionized and the retention is low. Conversely, at pH > pKa, the ionization is suppressed and the retention is enhanced.

In the present study, the dependence of the capacity factor (expressed as log k'_W) on the aqueous phase pH was investigated for three potentially active basic drugs using a RP-HPLC procedure (Table 1). This experiment may lead to an approach to the measurement of pKa.

The theoretical basis of the pKa determination using liquid chromatographic retention data was first proposed by Horvath *et al.*[1] Then, others studies related to acidic compounds showed the ability of curve fitting to measure pKa values from capacity factors.[4,5]

In many ways, the RP-HPLC methods, using an octadecyl-modified silica column (ODS), have been found useful only for neutral and acidic compounds, since column degradation occurs with mobile phases of pH above 8.[6] Consequently, in order to investigate the pH dependence of the capacity factor values for basic 2-amino-2-oxazolines, we chose a column packed with hydrophilic polymethacrylate.

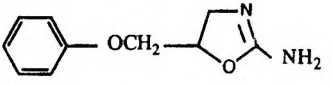
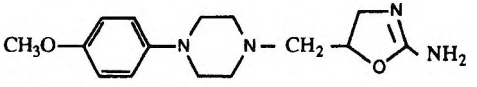
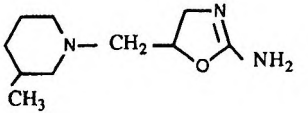
EXPERIMENTAL

Apparatus and Chromatographic Conditions

Chromatography was performed with a Waters Assoc. apparatus equipped with a Model 590 pump, a WISP Model 717 *plus* automatic injector and a Lambda Max Model 480 ultraviolet detector operating at 214 nm.

Table 1

Chemical Structures of Studied 2-Amino-2-oxazolines

Compound No.	Structure
1	
2	
3	

The compounds were chromatographed on a Shodex Pak polymeric column (RS pak D18-613, 151 mm X 6 mm i.d., Asahi). The mobile phase composition ranged from 25% to 60% acetonitrile with 0.067M phosphate buffer (v/v) at various pH values (from 4 to 9). The 9.5 pH value was obtained from an appropriate dilution of sodium hydroxide.

The flow rate was 2.5 mL/min. The detector output was recorded on a Data Jet integrator (Spectra Physics, France).

Chemicals

The synthesis of the 5-substituted 2-amino-2-oxazolines, characterized from spectral data (IR, ^1H and ^{13}C NMR), is described elsewere.[7,8]

All chemicals and solvents were of analytical or HPLC grade. Acetonitrile HPLC gradient was purchased from SDS (Peypin, France). Water was deionized, doubly glass-distilled. 1-Octanol was obtained from Merck (Darmstadt, Germany). Buffer solutions in pH range 4 - 9 were prepared by mixing the proper volume of 0.067 M aqueous solution of potassium dihydrogen phosphate and di-sodium hydrogen phosphate (KH_2PO_4; Na_2HPO_4, 12 H_2O, Prolabo, France). Their pH's were tested by pH-metry.

Measurement of Log k'_W

Stock solutions containing 1mg/mL of 2-amino-2-oxazolines in methanol were diluted with water to the final injected concentrations (50 µg/mL). According to their chromatographic behaviour, the retention time of each compound was determined in triplicate at six different acetonitrile-phosphate buffer mixtures ranging from 25% to 60%. For each compound, the log k'_W value was obtained by a regression analysis of the log k' data, expressed from the retention time, t_r, through the formula : $k' = (t_r - t_0 / t_0)$, and extrapolation to 0% acetonitrile content. The column dead-time of the system (t_0) was measured as the time from injection to the first distortion of the baseline after injection of each molecule in water. The correlation/regression analyses were carried out with a statistical program (StatView II) on a Power Macintosh computer. This method was previously described for other 2-amino-2-oxazolines.[9,10]

Calculation of pKa Values

The plot of log k'_W *vs* pH is a sigmoidal curve with a midpoint pH corresponding to the pKa value of the tested compound.[11] These graphical measurements were compared to pKa values obtained using a classical potentiometric method.[12]

In the same way, the pKa at different acetonitrile concentrations was determined by plotting the log k' *vs* pH.

RESULTS AND DISCUSSION

The studied compounds are weak bases with different H-accepting sites. A previously reported X-ray crystallographic study showed that the *endo* nitrogen atom of the amidine moiety represents the first protonation site (pKa_1).[13]

For compounds **2** and **3**, a second basic center (pKa_2) was found on a piperazine nitrogen atom and on the piperidine nitrogen atom, respectively. The pKa values, determined by a classical potentiometric technique, are listed in Table 2.

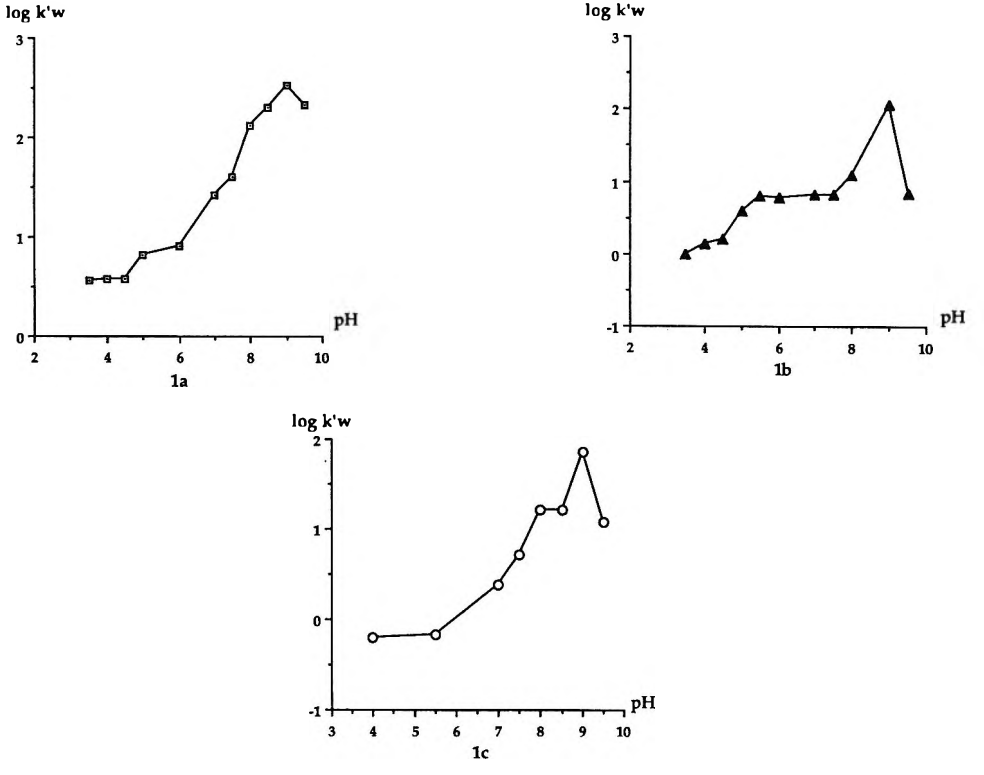


Figure 1. Plots of the capacity factors (expressed as log k'_W) *vs* the aqueous phase pH of the eluent. (1a: compound **1**; 1b: compound **2**; 1c: compound **3**).

Table 2

pKa Values of Studied 2-Amino-2-oxazolines

Chemicals		**pKa Measured by Potentiometry**	**pKa Estimated by HPLC**
Compound **1**	pKa 1:	8.59	7.06
Compound **2**	pKa 1:	8.05	8.24
	pKa 2:	5.22	4.20
Compound **3**	pKa 1:	9.23	not determined
	pKa 2:	7.13	8.76

pH Dependence of the Capacity Factor Values

The pH dependence of the capacity factor values is illustrated in Figure 1a (compound **1**) and in Figures 1b and 1c (compounds **2** and **3**), respectively. For bases, already published studies have shown that the retention variation with pH is typically described by a sigmoidal function.[3,14] The pKa value can be determined at the midpoint of the the plot of log k' *vs* pH. In such experiments, a sufficient number of experimental data are necessary for the measurement of pKa with a good accuracy.

For the compounds **1** and **2**, the curve outlines almost agree with the analytical parameters (one and two H-accepting sites, respectively). But for **3**, as it will be further discussed, only one midpoint was observed.

The pKa values measured from the sigmoidal plots are depicted in Table 2. The observed differences may be related to the nature of the column, to the nature of the organic modifier, to the ionic strength of the buffer, or to the existence of ion-pair interactions.

Reversed-phase packings are the most widely used types of stationary phases in HPLC. Generally, the columns are filled with microparticule, porous silicas treated by means of silanization, using appropriate alkylsilanes. These packings suffer from a limited pH stability, and the measurement of k' *vs* pH is less accurate for basic solutes than for acidic ones.[2,15] This may be due to silanol effects, which play an important role with bases. By using a polymeric column, we avoided the change in the ionization state of the surface silanols, which may otherwise produce potential errors.[16] We chose a column packed with hydrophilic polymethacrylate gel having octadecyl (C_{18}) groups.

Hanson *et al.*[15] have recently reviewed the synthesis and properties of polymer-coated RP stationary phases. On this type of polymer-coated RP column, the retention mechanism differs from that observed with classical silanized silicas. The difference is due to the existence of polar groups in polymethacrylate and, generally, excellent separations of basic compounds have been reported.[15]

Acetonitrile exhibits a more complex behaviour towards the acid-base system than methanol.[16] Acetonitrile is an aprotic solvent with a high dielectric constant and, so, in this medium, bases are not completely dissociated. In this work, the use of acetonitrile leads to sharp peaks, permitting measurement of the capacity factor, k', with a sufficient accuracy.

Table 3

Effect of the Medium on the Acidity Constant

Acetonitrile (%)	Compound 1 pKa_1	Compound 2 pKa_1	Compound 2 pKa_2
30	7	8.71	4.63
35	7.05	9.61	4.54
40	7.24	10.29	4.47
45	7.57	10.75	5.45

The deviations observed between the pKa values (Table 2) may be explained in terms of dielectric constant difference between the aqueous/organic mobile phase and the water.[4] As the 2-amino-2-oxazolines are weak bases, they are protonated at pH < pKa. In this ionization state, they could be mixed with the phosphate ions from the buffer in ion-pair interactions. When the 2-amino-2-oxazolines are in an un-ionized state (pH above the pKa's values), these ion-pair interactions are not involved, and the capacity factors diminish. This phenomenon is illustrated by the decrease of the log k'_W values, always observed at pH 9.5 (Figures 1a, 1b and 1c). Consequently, for the compound **3**, we cannot measure the pKa_1 value, this ionization constant being close to 9.5 (Table 2).

Effect of the Mobile Phase Composition on the Acidity Constant

The effect of the mobile phase composition on the ionization equilibrium was studied by measuring the pKa at different acetonitrile concentrations, ranging from 30 to 45%. The ionization constant values obtained for the compounds **1** and **2** are shown in Table 3.

The pKa_1 values increase with increasing the organic modifier percentage in the mobile phase. This effect, already observed by Li *et al.*,[4] could be explained by a change of the mixture dielectric constant. According to Coulomb's law, the attraction force is inversely proportional to the dielectric constant. For acetonitrile, the dielectric constant is 38.8 at 20°C, whereas it is 78.5 for water. Thus, an increase of the acetonitrile concentration leads to a decrease of the dielectric constant of the mixture. Consequently, the acetonitrile percentage and the acidity constant varied in the same way. Our results (Table 3) agree with this observation.

CONCLUSION

In this work, the dependence of the capacity factor (expressed as log k'_W) on the aqueous phase pH was investigated for three 2-amino-2-oxazolines using a RP-HPLC procedure. In order to avoid the change in the ionization state of stationary phase surface silanols, we chose a column packed with hydrophilic polymethacrylate having octadecyl groups.

The acidity constants of these basic compounds were determined from the sigmoidal curve drawn by plotting the pH variation upon the log k'_W, at its midpoint. They were compared to the values measured using a potentiometric method.

In a second part, we studied the effect of the mobile phase composition on the acidity constants, permitting us to observe the relationship between the two parameters in the retained RP-HPLC system.

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A RAPID AND SIMPLE ASSAY TO DETERMINE TOTAL HOMOCYSTEINE AND OTHER THIOLS IN PEDIATRIC SAMPLES BY HIGH PRESSURE LIQUID CHROMATOGRAPHY AND FLUORESCENCE DETECTION

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ABSTRACT

A rapid and simple method, using pre-column fluorescent labeling, followed by high pressure liquid chromatography (HPLC), for the determination of total concentration of thiols (homocysteine, cysteine, cystinylglycine, and glutathione) in small-volume, pediatric samples, is presented. Thiols in plasma were first reduced with tri-n-butylphosphine, and the resulting free —SH groups were labeled with the fluorescent compound 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F). The ABD-thiols were desorbed from plasma proteins by precipitating proteins with TCA, and then fractionated by reverse-phase chromatography. Peak areas were used to determine concentrations in the unknowns.

Baseline resolution of all four thiols was obtained in about 12 minutes. This method requires a maximum of 75 μL or less of the sample. It is specific to thiols, and chromatograms do not show any other peaks. It is sensitive to about 500 pmol/L, and is linear to at least 300 μmol/L of each of the thiols. Within- and between-day CV's were less than 4%. Reference ranges for children up to 18 years of age (mean ± SD), were: 8.9 ± 2.9, and 232 ± 56 umol/L for total homocysteine, and total cysteine, respectively. No sex- or age- dependent changes were observed in children up to 18 years of age of reference values for total homocysteine or total cysteine.

INTRODUCTION

Elevated plasma homocysteine is an independent risk factor for development of a variety of vascular occlusive diseases, including carotid, coronary, and peripheral arteries.[1-5] Increased plasma homocysteine can be due to either genetic defects or secondary to either drugs or nutritionally related from deficiency of vitamins B6, B12 or folate.[6] Atherosclerosis has its inception in childhood;[7] therefore, ability to prevent or delay this process depends on ability to identify and alter these factor(s) responsible early in life.[8] Thus, determination of homocysteine levels in pediatric populations may provide information for the role of this amino acid in pathogenesis of atherosclerosis.[6] Further, since difference between normal and clinically abnormal levels for homocysteine is at the most 2x above the upper normal range, and, most of the time, it is only 1.3 to 1.5 times of the normals,[1] a method with greater sensitivity and precision to suit pediatric samples is highly desired.

Several different methods are available to determine homocysteine in plasma.[9] Of these, use of pre-column fluorescent labeling agents specific to sulfhydryl groups, followed by high pressure liquid chromatography (HPLC) and fluorescence detection, offer high specificity and sensitivity. Fluorobenzoxadiazoles are specific fluorescent labeling agents for thiol groups and yield high fluorescence.[10] Two of these compounds, ammonium-7-fluoro-2,1,3,-benzoxadiazole-4-sulfonate (SBD-F) and 4-(aminosulfonyl)- 7-fluoro-2,1,3-benzoxadiazole (ABD-F) are particularly useful in the assay of thiols.[11,12] Both ABD-F and SBD-F are nonfluorogenic, provide stable and sensitive adducts, thereby allowing detection at picomolar levels.[10] Although SBD-F has been used for the determination of homocysteine and other thiols in plasma,[12-16] it's reaction with sulfhydril compounds is relatively slow and requires drastic conditions (pH 9.5, 60 minute incubation at 60°C) for

completion. These harsher conditions, in general, are not conducive and possible degradation of thiols occurs. Unlike SBD-F, ABD-F is 30X more reactive and requires milder conditions, (viz., pH 8.0, 5-10 minute incubation at 50°C) and, therefore, problems related to re-oxidation can be avoided.[11]

Araki and Sako[14] are the first to present a protocol to determine total thiols in plasma samples using tri-n-butylphosphine as the reducing agent, and the SBD-F as the labeling agent. They have separated and quantitated individual thiols by HPLC, using gradient elution, in about 20 minutes. Vester and Rasmussen[15] modified Araki and Sako's protocol, using an isocratic buffer system and smaller sample size. However, this procedure still required 20 minutes for completion. Ubbink et al.[16] further modified this method and obtained a complete elution of thiols in less than 10 minutes. However, this procedure requires an extremely acidic buffer of pH 2.1. Further, it is very sensitive to buffer pH and even a slight change in pH affected the resolution of cysteine, homocysteine and cystinylglycine.

In spite of several advantages associated with the use of ABD-F, the possibility of using this procedure routinely for the determination of sulfhydryl groups in clinical laboratories has not yet been explored. Hence, we report a simple procedure to quantitate various thiols in plasma, using ABD-F as a fluorescent labeling agent. Conditions for reduction and desorption of all thiols in plasma from the plasma proteins, labeling of the desorbed thiols with ABD-F, and fractionation of individual thiols by HPLC (i.e., columns, buffers, etc.) are also thoroughly evaluated. In addition, the use of this protocol in pediatric setting is explored to obtain a baseline separation of cysteine, homocysteine, cystinylglycine and glutathione in about 10 minutes, using a sample volume of less than 100 μL.

EXPERIMENTAL

Materials

4-(Aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F) was purchased from Waco Chemicals (Richmond, VA, USA). D,L-homocystine, L-cystine, cystinylglycine (oxidized form), glutathione, tri-n-butylphosphine and other routine chemicals were obtained from Sigma Chemicals (St. Louis, MO. USA). HPLC grade methanol was from Scientific Products (Deerfield, IL. USA). A RP-18, 4.6 x 250 mm column, packed with 5 μm diameter particles, was

obtained from the Supelco Corp. (Bellefonte, PA, USA). Cartridges (3.2 x 40 mm size) with various packing materials were from Applied Biosystems (Foster City, CA, USA).

Apparatus

An HPLC unit from Thermo-Separation Products (Spectra-Physics Division, Freemont, CA) was used in this study. This unit (model #AS3000) was equipped with a ternary gradient pump, a refrigerated autosampler, and a fluorescence detector. Operation of the HPLC unit, as well as data collection and calculations, were accomplished through a PC unit. All solvents were filtered through a 0.45 μm filter (Type HA, Millipore, Bedford, MA, USA) before use. Solvents were degassed by continuous sparging with helium gas. Fractionation was carried out at room temperature (20 ± 2°C). Effluents were monitored for their fluorescence intensities at an excitation wavelength of 386 nm and an emission wavelength of 516 nm. Signal detection and quantitation of peak areas were accomplished using automated data acquisition software from the Thermo-Separation Products.

Specimens

Whole blood was collected into tubes containing either EDTA or heparin. Cells were separated within 60 minutes after collection to avoid influx of thiols from red blood cells[9] by centrifugation for 10 minutes at 3500 RPM (2500 x g). The resulting plasma was used for analysis, either immediately, or stored at -70°C.

To establish a pediatric reference range, samples were collected from 0.5- to 18-year-old male and female children. Fifteen samples each were collected from four different age groups, ranging from 0.1 to 4, 4.1 to 9.0, 9.1 to 14.0 and 14.1 to 18.0 years, with an equal number of samples each for males and females in each age group. To test for possible interference from different drugs, samples were obtained from patients undergoing therapy with a variety of drugs for infections, epilepsy, cardiac problems, cancer and other indicated conditions.

Sample Preparation

The general concepts to determine total thiols in plasma after reduction with tri-n-butylphosphine at pH 8.0, separation of the reduced forms from

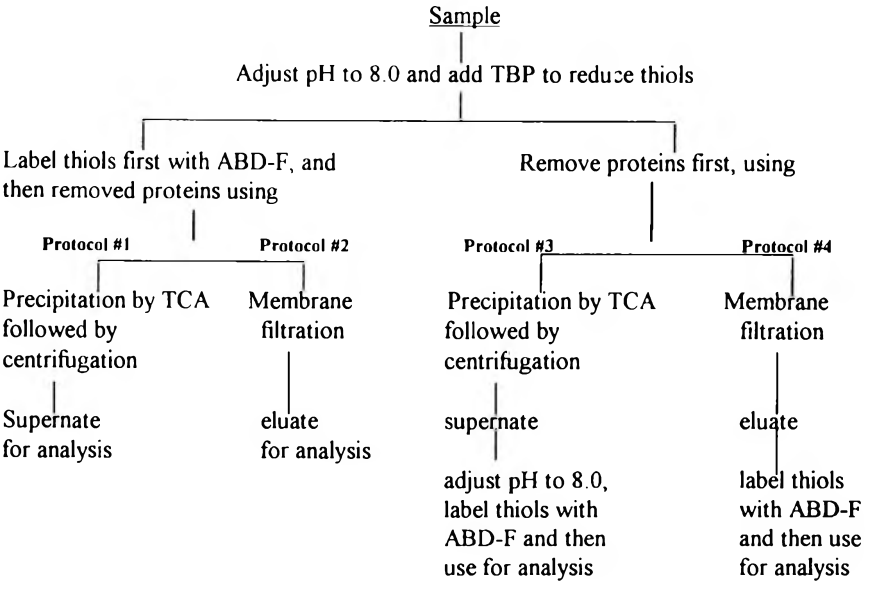

Figure 1. Summary of four different protocols that were used for sample preparation.

plasma proteins, and pre-column labeling with the ABD-F[10-13] were essentially followed. However, each individual step as well and it's sequence were evaluated to obtain results in a shorter time with high sensitivity using smaller sample volumes. For the separation of reduced thiols from plasma proteins and then labeling with ABD-F, four different protocols were used, and a summary of these different ones are given in Figure 1. Briefly, after reduction with tri-n-butylphosphine, the reduced thiols were derivatized, either before, or after removal of plasma proteins. Removal of proteins was accomplished either by acid precipitation using trichloroacetic acid (TCA) followed by centrifugation or by membrane filtration.

Calibration and Calculation

Individual stock solutions of cysteine (2 mM), homocysteine (0.5 mM), cystinylglycine (0.5 mM), (oxidized form), and glutathione (reduced form, 1 mM) were prepared by dissolving, first, in about 2 mL of 0.1 N HCl and then making up to volume with water. Stock solutions were stored at 4°C. A working standard mixture containing 100 µmol/L of homocysteine,

cystinylglycine, glutathione, and 400 μmol/L of L-cysteine was prepared using 0.2 M borate buffer, pH 8.0. For calibration, working standards, to have different levels in the range of 0 to 100 μmol/L, were prepared either in 0.2 M borate buffer, pH 8.0 containing 2 mM EDTA or in pooled plasma. Standards were reduced and derivatized similarly as the patient samples.

Each standard was analyzed in triplicate. From the resulting peak areas, a linear regression line was calculated by plotting concentrations on the abscissa and area on the ordinate. Concentration in the unknown sample was obtained using peak area of the sample from the slope of the calibration curve.

The detection limits were estimated, using aqueous standards, by injecting successively lower concentrations until a signal-to-noise ratio of 3:1 was obtained. Recoveries were determined by spiking plasma samples with known amounts from stock standards and by comparing the obtained values to the expected values after spiking. For this, standards at two different concentrations were used. Precision of the method was evaluated by calculating within-run and between-run coefficients of variation at three different concentrations.

Statistical Analysis

Data are presented as means ± SD. Linear regression analysis was performed using Spearman rank correlation. Student's t-test was used for comparisons, and a p value of < 0.05 was considered significant.

RESULTS AND DISCUSSION

Fluorescent Labeling

ABD-F was used as labeling agent for thiols, since it is highly reactive, even under mild conditions. It reacts faster with the —SH groups than with the other labeling agents, (e.g., SBD-F). As a result, the time required for completion was reduced to a maximum of 10 minutes. Further, ABD-F requires a reaction temperature of 50°C and, therefore, side reactions with other plasma compounds can be minimized. In this regard, this is the first report where ABD-F is used for a routine clinical work in a hospital setting.

Table 1

Comparison of Results Obtained after Using Four Different Sample Preparation Protocols

Protocol	Concentration (μmol/L,* Mean ± SD) Cysteine	Homocysteine
1	288 ± 11	10.8 ± 0.9
2	280 ± 8	10.9 ± 1.1
3	272 ± 16	10.1 ± 1.9
4	291 ± 13	11.1 ± 1.3

* Mean ± ISD for three different experiments.

Sample Preparation

The objectives here were to optimize conditions for reduction of the oxidized thiols, derivatization with ABD-F in the shortest possible time, and to use a minimum number of steps to minimize sample dilution. The pH for reduction of thiols with TBP, as well as the labeling of free —SH groups by the ABD-F reagent, is found to be optimal above pH 7.0.[10,12] However, ABD-adducts have maximum fluorescence at acidic pH.[10] To facilitate these two diametrically opposite optimal requirements, we have evaluated four derivatization protocols for labeling and removal of plasma proteins. A brief summary of these protocols and their ensuing results are given in Figure 1 and Table 1. Here, the following sample preparation protocols were evaluated:

1) Removal of proteins by precipitation with TCA, or

2) by membrane filtration; and

3) labeling of thiols before, or

4) after removal of plasma proteins.

Among these four different possibilities, labeling of thiols before the removal of plasma proteins, and removal of proteins by acid precipitation were found to be effective and convenient. This is due to the following reasons:

Both reduction and labeling require an optimum pH of above 7.0[10] and, therefore, reduction and labeling can be accomplished simultaneously without any need to manipulate the pH of these two reactions. Further, an added advantage here is that the normal plasma pH is around 7.4. By this, both reduction and labeling can be accomplished simultaneously at the same pH. Consecutive addition of TBP followed by ABD-F proved to be more convenient, since it minimized the number of steps. During the labeling step, any interference due to the presence of plasma proteins was not observed. However, it is possible that the ABD-F might also be reacting with the free —SH groups of these proteins, but it does not seem to be a problem here since the concentration of the ABD-F used here is 100 times higher than is required. Therefore, removal of proteins prior to the labeling of the —SH groups appears to be unnecessary.

Membrane filtration and acid precipitation, followed by centrifugation, were evaluated to remove proteins from the samples. Membrane filtration offered an advantage due to the absence of sample dilution. However, this procedure was found to be cumbersome for handling large numbers of samples, relatively expensive, and sample recovery was never more than 80%. This relatively poor recovery was found to be not ideal for small-volume, pediatric samples. Also, whatever advantage the membrane filtration procedure has offered, mainly in avoiding the sample dilution, at the end, it was nullified since it required pH adjustment to below 7.0 to obtain high fluorescence yield. Therefore, removal of proteins by acid precipitation using TCA was found to be convenient, easy and economical.

Based on these observations, the final optimized protocol for reduction and labeling, using smaller sample and reagent volumes, is as follows: To a 2.0 mL plastic centrifuge tube, 80 μL of sample or standards, 20 μL of 0.23 M borate buffer, pH 8.0, containing 4 mM Na_2 EDTA, were added, and the contents were mixed. To this, 10 μL of 10% (V/V) tri-n-butylphosphine in dimelthylformamide were added, mixed and incubated for 30 minutes at 4-6°C for reduction and decoupling of thiols from plasma proteins.

The —SH groups of these reduced and free thiols were labeled with ABD-F by adding 50 μL of ABD-F (5 mg/mL of 0.2 M borate buffer, pH 8.0 containing 2 mM Na_2 EDTA) and incubated for 10 minutes at 50°C in a circulating water bath. Proteins were precipitated by adding 40 μL of ice cold 20% trichloroacetic acid. Supernatants were obtained by centrifugation at 4°C for 10 minutes at 15,000 x g. About 120 μL of supernatants, containing the labeled homocysteine and other thiols, were carefully transferred to conical

autosampler vials, capped and analyzed immediately or stored at 4°C until analyzed, within 24-48 hours. Twenty-five microliters of each sample were used for the analysis.

The advantages of this protocol are: final dilution is only 2.5 X of the starting sample and, therefore, resulted in increased sensitivity, and combination of reduction and labeling steps. This procedure was simplified by avoiding the manipulation of sample pH. This, in turn, resulted in the use of small sample volumes for analysis.

Chromatography

The main objective, here, is to optimize the chromatographic conditions to obtain results in a shorter time, employing an isocratic elution system. A variety of parameters relating to columns, buffers, solvents, flow rates, sample-size, etc., were evaluated to obtain better resolution and to complete the analysis in a shorter time period.

1) Columns and Packing Material. Of the packing materials that were evaluated, a C_{18}, reverse phase, 5μ particle size was found to be superior to the others. In spite of the advantages offered by the cartridges (e.g., low cost, high flow rate, low back pressure, etc.), with cartridges peaks were never properly resolved and a "bunching" of the peaks was always seen. This is probably due to the weak binding of the ABD-adducts in presence of the solvents employed. Therefore, use of a long, standard size column was necessary for the proper resolution.

2) Buffers and Solvents. Several different buffers (e.g., biphthalate, pH 4.0; phosphate, pH 2.1 or pH 6.0; acetate, pH 4.0) and solvents of either methanol or acetonitrile were employed before for the isocratic resolution of ABD[11] and SBD[10,15,16] adducts of thiols in plasma. Phosphate buffer (0.1 M, pH 2.1) with 4% (v/v) ACN[16] did resolve peaks well, but resulted in poor precision. Under the acidic conditions, the column's effectiveness was poor beyond about 50 analyses. Acetate buffer, pH 4.0, required a longer time for completion.

Both ACN and methanol were effective, but methanol is preferred, due to its relatively low cost. Among the different concentrations, a methanol content of 8% gave a superior resolution. The concentration

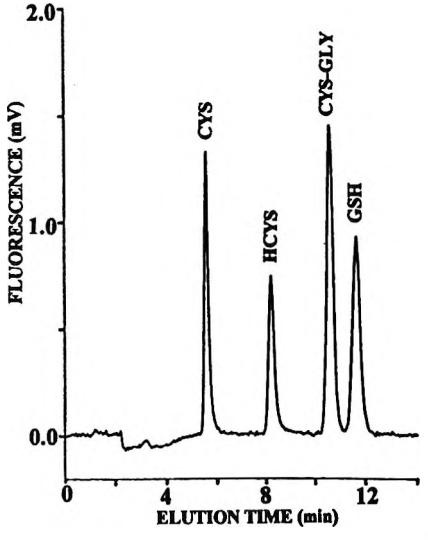

Figure 2. A typical elution pattern of a mixture of standards containing 200 μmol/L of cysteine (CYS), and 50 μmol/l each of homocysteine (HCYS), cystinylglycine (CYS-GLC) and glutalthione (GSH). Each peak represents 4 μmoles of cysteine and 1 μmole each of homocysteine, cystinylglycine and glutathione.

of methanol appears to be critical in terms of the time required for completion and for resolution. Thus, a phosphate buffer (0.1 M, pH 6.0) with 8% methanol (v/v) offered the best resolution and short period for completion of the analysis.

3) Flow Rate. A 2 mL/min flow rate was necessary to obtain sharper peaks. Although this high flow rate resulted in higher back pressure, it was necessary, since a flow rate of 1 mL/min resulted in peaks that were too broad and overlapping.

4) Sample Size. When different volumes of sample were injected, a 25 μL sample injection per analysis provided the best resolution and peak size which, in turn, gave a better precision in quantifying the peak areas.

Based on these observations, the following chromatographic conditions were found to be optimal: column size, 4.6 x 250 mm, with a packing of RP18 and 5 μm size particles; a 0.1 M phosphate, pH 6.0, buffer containing 8% (v/v) methanol; a 25 μL sample volume per injection; and an isocratic elution system with a 2.0 mL/min flow rate.

Under these conditions, the total analysis required only 12 minutes for completion, and it was able to analyze samples, in succession, every 12 to 14 minutes.

A typical chromatogram, showing an elution pattern of a standard mixture containing cysteine, homocysteine, cystinylglycine and glutathione, is presented in Figure 2. Using the above-described optimized chromatographic conditions, the elution times for these amino acids are: cysteine = 5.62 ± 0.16, homocysteine = 8.32 ± 0.23, cystinylglycine = 10.7 ± 0.5 and glutathione = 11.88 ± 0.47, minutes (mean ± SD, n = 20).

These elution times are relatively consistent and did not vary appreciably over a period of 6 to 8 weeks of continuous assays. Sometimes, an occasional drift in the elution time was seen with cystinylglycine and glutathione, but not with cysteine and homocysteine. This drift in elution time with cystinylglycine and glutathione was easily corrected by changing the methanol concentration in the elution buffer. During this work, it was observed that the elution, as well as clear separation of cystinylglycine from glutathione, are dependent on the methanol concentration in the buffer. Increase in percent of methanol resulted in faster elution times but poorer resolution; and an opposite effect (viz. better resolution and longer elution times) with the decreased percent of methanol. However, methanol concentration of the buffer has no significant influence on the resolution of cysteine and homocysteine.

Figure 3 represents a typical chromatogram showing different thiols that are commonly present in "normal" human plasma. Based on elution of standards, these were identified as cysteine, homocysteine, and cystinylglycine. Only three adducts of ABD-F (cysteine, homocysteine, and cystinylglycine) are seen in a typical plasma. However, ABD-glutathione is seen in samples with hemolysis only. Two additional unidentified peaks, labeled peak A and B in Figure 4, are always seen in samples from patients with renal failure. They are present at both pre- and post-dialysis and, therefore, dialysis did not specifically remove these ABD-reacting compounds.

Dialysis did decrease their concentrations, especially peak B. Although these two unidentified compounds are present in patients with renal failure, they did not interfere with resolution of homocysteine and cystinylglycine, since they are eluted between homocysteine and cystinylglycine. Except for these two, no other ABD-adducts were seen in over 400 samples analyzed, from patients with a variety of diseases and medications, indicating a high degree of specificity of the ABD-F.

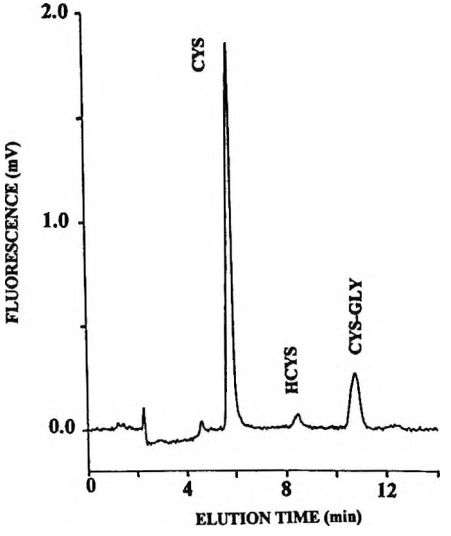

Figure 3. Elution pattern of various thiols that are seen in a "normal" human plasma sample, showing cysteine (CYS), homocysteine (HCYS) and cystinylglycine (CYS-GLY).

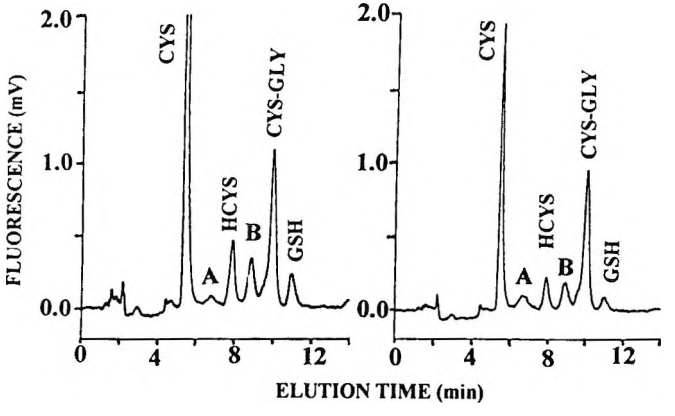

Figure 4. Pattern of various thiols that are seen in samples from patients with renal failure at pre- (Fig. 4A, left) and post- (Fig. 4B, right) dialysis, showing cysteine (CYS), homocysteine (HCYS), cystinylglycine (CYS-GLY), glutathione (GSH) and two unidentified compounds (peaks A and B).

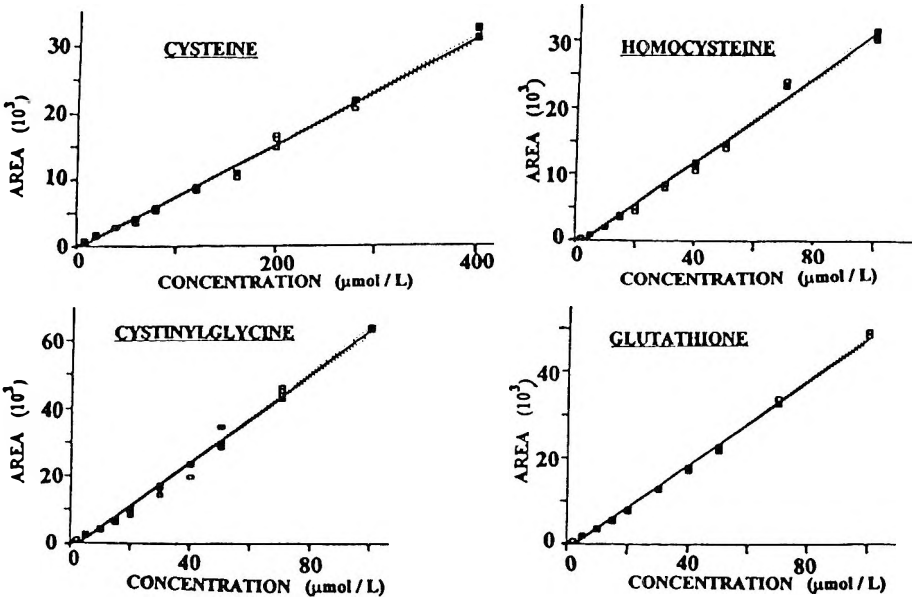

Figure 5. Calibration Curves for cysteine, homocysteine, cystinylglycine, and glutathione. The obtained regression equations were, cysteine: Y = -428 + 78 X, r^2 = 0.9970, X-intercept = 5.0; homocysteine: Y = -1061 + 326 X, r^2 = 0.9916, X-intercept = 3.3; cystinylglycine: Y = - 1860 + 647 X, r^2 = 0.9964, X-intercept = 2.9; glutathione: Y = - 1220+488 X, r^2 = 0.9979, X -intercept = 2.5 (X=concentration, μmol/L; Y= peak area).

Correlation

The present method, using ABD-F as the labeling agent, together with the modifications in sample preparation and fractionation protocol by the HPLC, was compared to the previously published procedure of Araki and Sako[13] as modified by Ubbink et al.[15] (see Table 2). It provided slightly lower values for cysteine and homocysteine. In spite of several modifications to the sample preparation and chromatography, reasonably good, comparable, data were obtained between these two methods.

Linear regression analysis of the data yielded good agreement between these two assays, and the obtained regression equations are: for cysteine, y = -4.2 + 1.012 X, r^2 = 0.9382; and for homocysteine, y = -0.18 + 1.001 X, r^2 = 0.9954.

Table 2

Validation of the Modified Derivatization Protocols to Determine Total Cysteine and Homocysteine

Amino Acid	n	Method	Concentration (μmol/L) Mean	SD	Range	p*
Cysteine	20	Ubbink et al.	291	68	152-380	---
		Present	280	72	141-364	0.0904
Homocysteine	20	Ubbink et al.	11.6	7.2	5.9-28.0	---
		Present	11.0	7.3	5.1-26.0	0.0325

* Paired t-test, two tailed. The regression analysis provided the following: Cysteine, $Y = -4.2 + 1.012X$, $r^2 = 0.9382$; Homocysteine, $Y = -0.18 + 1.001X$, $r^2 = 0.9954$. (Y = present method, and X = method of Ubbink et al.[15]

Table 3

Regression Analysis of Concentration of the Amino Acid and Obtained Peak Areas

Amino Acid	Regression Equation	r^2	X-intercept
Cysteine	y = -428 + 78X	0.9941	5.0
Homocysteine	y = -1061 + 326X	0.9893	3.3
Cystinylglycine	y = -1860 + 647X	0.9927	2.9
Gluthathione	y = -1220 + 488X	0.9958	2.5

Linearity

Excellent linearity to all the amino acids, when tested at 0 to 400 μmoles/L cysteine and 100 μmoles/L for others, was seen (see Fig. 5). We did not test these compounds at higher concentrations. Linear regression analysis was performed by plotting the observed peak area (Y) versus concentration in μmoles/L (X) of each thiol. The obtained linear regression equations showed a high degree of correlation between peak area and concentration (Table 3).

Table 4

Recoveries of Analysis

Compound	Concentration (μmol/L) Expected	Obtained	Recovery (%)
Cysteine	40.0	37.6	94
	200.0	210.0	105
Homocysteine	10.0	9.2	92
	50.0	52.0	104
Cystinylglycine	10.0	9.6	96
	50.0	52.4	105
Glutathione	10.0	9.6	96
	50.0	52.4	105

Recovery

Recoveries of analysis at two different concentrations are given in Table 4. They were obtained by analyzing samples with a known addition of amino acids to a freshly pooled human plasma. Excellent recoveries, with a maximum of ± 6% mean, were always obtained.

Detection Limits

Limits of quantitation (detection) were calculated from regression lines that were obtained with calibration curves from Table 3. The lowest quantifiable (detectable) limit, in μmoles/L, are: cysteine = 4.3, homocysteine = 3.3, for cystinylglycine = 2.9, and glutathione = 2.5.

In this assay, the minimal amount of detection, at a signal-to-noise ratio of 3:1, is about 5 pmol of each amino acid. With a starting plasma sample 80 μL and 25 μL for each analysis, the calculated sensitivity is at least 10 pmoles for each amino acid.

Table 5

Reproducibility at Within- and Between-Days, and at Three Different Concentrations

Compound	Conc'n	n	Reproducibility (% CV ± SD) Within-Day	Between-Days
Cysteine	20	5	5.4 ± 2.1	5.8 ± 2.8
	80	5	4.2 ± 1.8	5.8 ± 2.0
	200	5	3.1 ± 1.0	4.7 ± 1.8
Homocysteine	5	5	4.6 ± 2.0	4.9 ± 2.0
	20	5	3.7 ± 1.7	4.2 ± 1.8
	50	5	2.8 ± 0.8	3.6 ± 1.0
Cystinylglycine	5	5	3.8 ± 1.6	5.7 ± 2.0
	20	5	2.7 ± 1.0	4.5 ± 1.4
	50	5	1.9 ± 0.8	3.9 ± 1.0

Reproducibility

The method was tested at 200 μmol/L for cysteine and 50 μmol/L, each, for other amino acids. Each sample was tested at least 5 times on each day and on three different days. The CV's for reproducibility within-day was found to vary 1.9 to 3.8%, and between-days from 3.6 to 5.7%. (Table 5), indicating that this is a highly reproducible protocol.

Reference Range

Using the present protocol, reference ranges for total cysteine and homocysteine were calculated for pediatric populations ranging from 0.1 to 18.0 years of age, representing both sexes. The obtained ranges (mean ± 2SD) for total homocysteine and cysteine are: 3.3 to 14.5 and 122-346, μmoles/L, respectively. No significant sex- or age-dependent differences in reference values for total cysteine and homocysteine were observed in children up to 18 years of age. This is in contrast to the data obtained for adults,[1,17] where males tend to have higher homocysteine levels compared to females.

We have presented, here, a modified protocol to determine total homocysteine and total cysteine using ABD-F as the fluorescent labeling agent. Sample preparation and fractionation conditions of the ABD-adducts by the HPLC were optimized for small-volume, pediatric samples.

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SIMULTANEOUS RESOLUTION AND DETECTION OF A DRUG SUBSTANCE, IMPURITIES, AND COUNTER ION USING A MIXED-MODE HPLC COLUMN WITH EVAPORATIVE LIGHT SCATTERING DETECTION

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ABSTRACT

An alternative approach to developing individual potency, impurity, and counter ion methods is the simultaneous resolution and detection of the drug substance, impurities, and the counter ion in a single chromatogram. LY326315 hydrochloride was used as a model compound to demonstrate this concept. The separation was achieved using a conventional HPLC system with an Alltech mixed-mode column, a reversed phase eluant, and evaporative light scattering detection (ELSD). The mixed-mode column, which has both reversed phase and ion chromatography functionalities (e.g. phenyl/cation, C8/anion), coupled with ELSD offers a novel approach to simultaneously resolving and detecting pharmaceutical compounds and counter ions in a single chromatogram.

INTRODUCTION

Pharmaceutical compounds are routinely evaluated for drug substance purity, including quantitation of the counter ion, as well as, content of possible impurities and degradation products. Typically, three separate methods are often independently developed and used to analyze a drug substance for potency, non-volatile impurities, and the counter ion. The two methods for potency and impurities often employ reversed phase high performance liquid chromatography (HPLC) in conjunction with ultraviolet (UV) spectrophotometry detection. A third method for the counter ion has traditionally been performed by titration methods or ion chromatography (IC) with conductivity detection. An alternative approach using HPLC with a mixed-mode column and an evaporative light scattering detector (ELSD) would have the advantage of accomplishing these separations in a single chromatogram.

Mixed-mode (or mixed-interaction) stationary phases offer a rational approach to improving selectivity as compared to largely unimodal, conventional HPLC stationary phases.[1] In mixed-mode chromatography, multiple reaction mechanisms are employed to enhance selectivity. The Alltech mixed-mode column series combine reversed phase and ion-exchange phase capability in a single support. This multifunctional support consists of a high purity, 100 angstrom pore, spherical silica substrate which has been bonded with either anionic (amine) or cationic (carboxylate) functionalities in addition to conventional reversed phase (C_8, C_{18}, or phenyl) functionalities. Applications which previously required special conditions, such as ion-pair reagents or base deactivated supports, can be developed by alternately controlling the state of ionization via eluant pH adjustments along with buffer strength and organic modifier composition.[2] The current literature demonstrates the applicability of the mixed-mode columns for use with inorganic ions and carboxylic acids,[3] sulfonated azo dyes,[4] nucleic acid constituents,[5] and oligomers.[6]

The ELSD has gained acceptance as a sensitive universal detector.[7-8] The ELSD operates by nebulizing the volatile effluent from the HPLC column into a fine mist. The mist is then carried through a temperature controlled drift tube where the volatile components (mobile phase) are vaporized. A fine cloud of non-volatile solute particles is carried through a light beam causing light scattering where this scattered light is detected by a photomultiplier. Response is a function of the amount of light scattered and is proportional to the concentration.

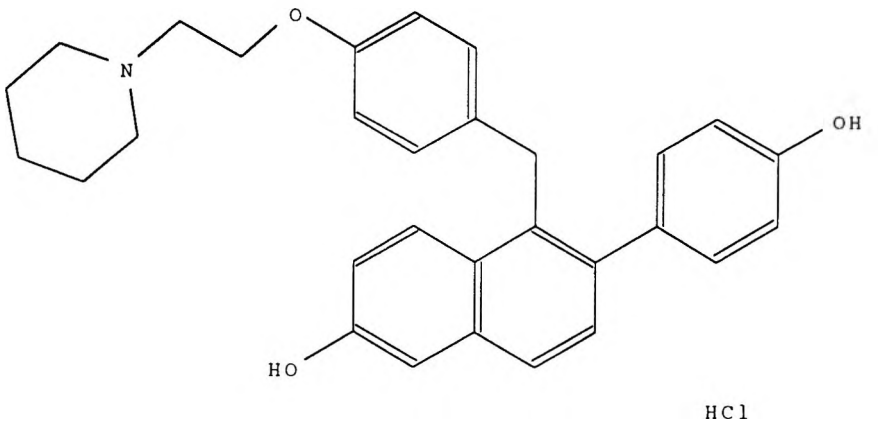


Figure 1. Structure of LY326315 hydrochloride.

The ELSD is not influenced by the UV spectral properties of solvents used for mobile phases, therefore the ELSD is not subject to baseline drift from gradient elution. The choice of acceptable solvents is expanded since the spectral background is not an issue with the ELSD. The ELSD is not affected with sample solvent interferences and sample response is independent of the chemical structure or optical characteristics of the solute and is therefore capable of detecting inorganic ions such as sodium[9] and chloride in addition to organic compounds.

A limitation of the ELSD requires the complete volatilization of all mobile phase components. Addition of nonvolatile components to the mobile phase would cause an elevated background by the continuous generation of solid particles into the light source. The elevated background decreases the sensitivity of the detector for the sample components.

The current literature demonstrates the applicability of the ELSD for use with phospholipids,[10-16] triglycerides, fats and fatty esters,[17-22] carbohydrates,[23-24] synthetic polymers,[25] steroids,[26] inorganic counter ions,[9,27-29] and pharmaceutical compounds.[9,28]

The purpose of this research was to demonstrate the concept of simultaneously resolving and detecting a pharmaceutical compound and its counter ion in a single chromatogram. The intent of this paper is to show the applicability of using mixed-mode HPLC columns in combination with ELSD

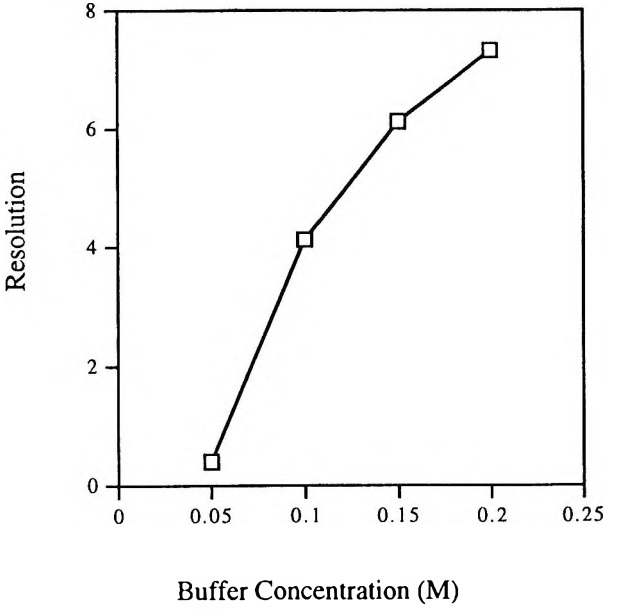


Figure 2. Effect of ammonium acetate buffer concentration on resolution between LY326315 and chloride using mixed-mode phenyl/cation column. Mobile phase: 50% methanol / 50% ammonium acetate buffer pH 4.0.

to resolve and detect a drug substance, impurities, and counter ion in a single chromatogram. LY326315 hydrochloride, a new selective estrogen receptor modulator (SERM), was selected as the model compound for this effort. The structure of LY326315 hydrochloride is shown in Figure 1.

EXPERIMENTAL

The LY326315 hydrochloride drug substance and related impurities were synthesized at Eli Lilly and Company (Indianapolis, IN). Sodium chloride was purchased from Mallinckrodt Chemicals, Inc. (Paris, KY). Ammonium acetate and glacial acetic acid were purchased from EM Science (Gibbstown, NJ). Chempure™ brand methanol was purchased from Curtin Matheson Scientific, Inc. (Houston, TX). The water was deionized and filtered through a Millipore Milli-Q™ water purification system (New Bedford, MA).

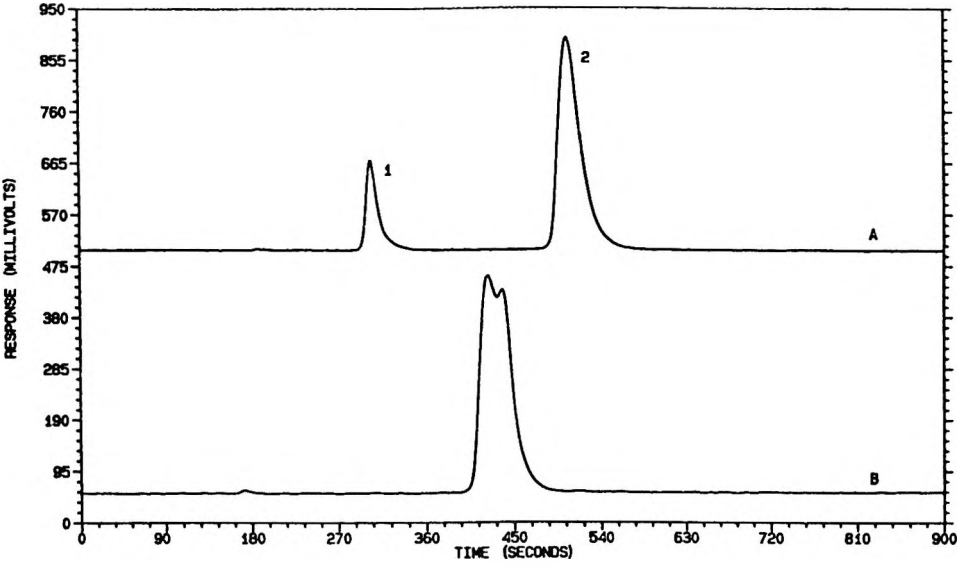


Figure 3. Chromatograms showing the effect of buffer concentrations at 0.05 M and 0.15 M on resolution between the LY326315 and chloride peaks using mixed-mode phenyl/cation column. Mobile phase: 50% methanol / 50% ammonium acetate buffer pH 4.0. (A) = 0.15 M buffer, (B) = 0.05 M buffer. Peak 1 = chloride, peak 2 = LY326315.

The mixed-mode HPLC columns - phenyl/cation, phenyl/anion, C_8/cation, and C_{18}/anion (250 mm x 4.6 mm, 7 µm particle size) - were obtained from Alltech Associates, Inc. (Deerfield, IL). A Shimadzu (Kyoto, Japan) series 10A autoinjector and pump were used with a Sedex 55 evaporative light scattering detector (Richard Scientific, Novato, CA). NF grade nitrogen (Air Products and Chemicals, Inc., Allentown, PA) was used as carrier gas for the ELSD.

The chromatography parameters examined were: ammonium acetate buffer strength (0.05 M - 0.20 M), buffer pH (4.0 - 5.5), organic modifier composition (0% - 55%), and detector temperature (28°C - 90°C). A mobile phase flow rate of 1.0 mL/minute was used.

Samples were prepared in 50% methanol / 50% water. Injection volume was 100 µL. The column temperature was ambient. The ELSD detector was set at a nitrogen pressure of 1 bar.

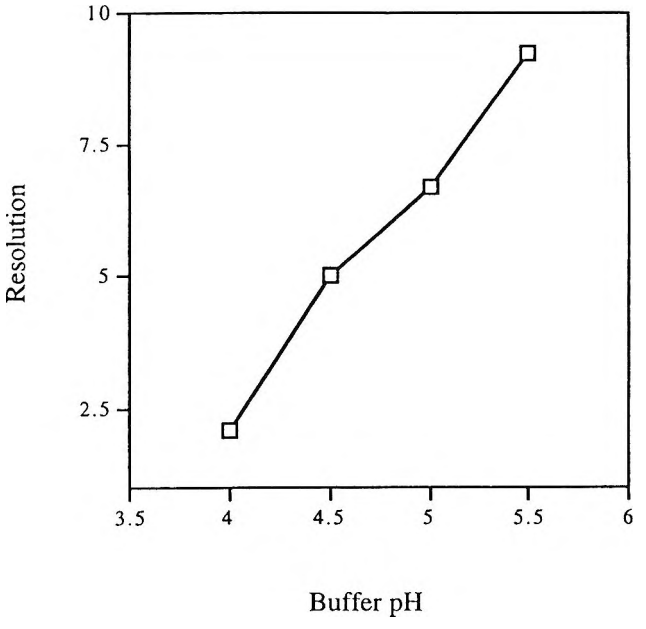


Figure 4. Effect of buffer pH on resolution of LY326315 and chloride using mixed-mode phenyl/cation column. Mobile phase: 50% methanol / 50% 0.1 M ammonium acetate buffer.

RESULTS AND DISCUSSION

Effect of Buffer Strength

Ammonium acetate was the only buffer tested, in concentrations ranging from 0.05 M to 0.20 M, for the evaluation of the phenyl/cation mixed-mode column. Ammonium acetate buffer was selected because it is volatile and therefore compatible with the ELSD. Ammonium acetate is also necessary to enhance the response of chloride. LC/MS studies have been previously conducted indicating the formation of ammonium chloride clusters as the moiety being detected by the ELSD.[29] Increasing the buffer concentration had a significant effect by increasing the resolution between the LY326315 and chloride peaks (Figure 2). At the low concentration of 0.05 M, the two peaks overlapped (Figure 3). Adequate resolution was obtained with a 0.10 M buffer concentration. The retention of chloride decreased while the retention of LY326315 increased as buffer concentration increased. Buffer strength had

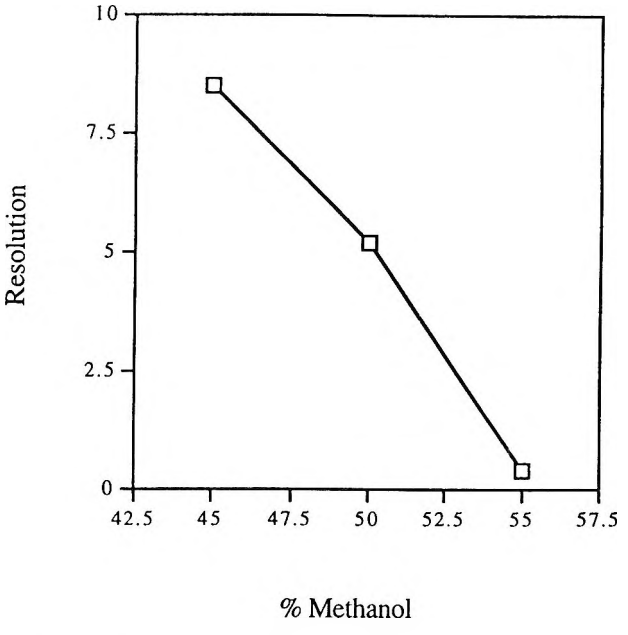


Figure 5. Effect of percent methanol in mobile phase on resolution between LY326315 and chloride using mixed-mode phenyl/cation column. Mobile phase: methanol / 0.1 M ammonium acetate buffer pH 4.5.

little effect on the peak shape in the chromatography. The amount of additives should be kept to a minimum when using an ELSD to maintain maximum sensitivity, less baseline noise, and less preventative maintenance; therefore a mobile phase containing ammonium acetate buffer at a strength of 0.10 M was used for further optimization of other parameters.

Effect of Buffer pH

Glacial acetic acid was used to attain the various pH adjustments between 4.0 and 5.5 in increments of 0.5 for the 0.10 M ammonium acetate buffer solutions. The resolution between the LY326315 and the chloride peaks was sensitive to changes in pH. The resolution increased with increasing pH as illustrated in Figure 4. Similar to the effect with buffer concentration, the retention of chloride decreased while the retention of LY326315 increased as the buffer pH increased. However, unlike buffer concentration changes,

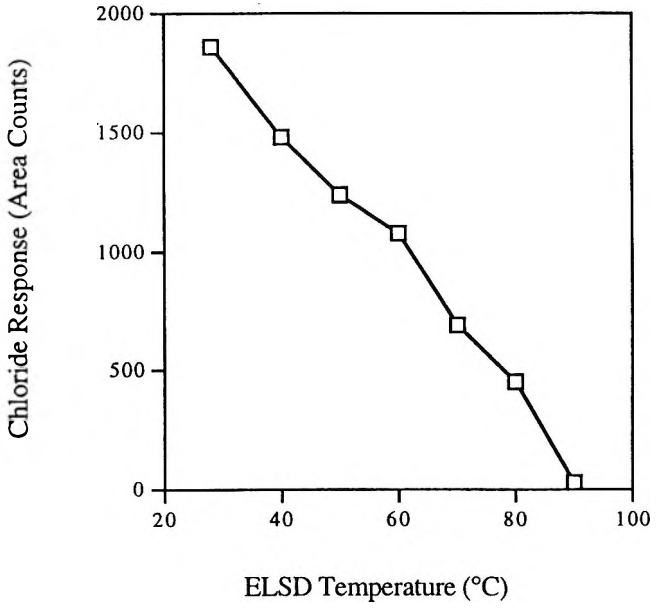


Figure 6. Effect of ELSD temperature on chloride response.

chromatographic peak shape was compromised as pH increased. More peak tailing was observed for LY326315 as the buffer pH was increased. Thus, a mobile phase containing 0.1 M ammonium acetate buffer at pH 4.5 was used for further optimization of other parameters.

Effect of Organic Modifier Composition

Methanol was used as the organic modifier for these experiments, in mobile phase concentrations ranging from 45% to 55% in increments of 5%. The resolution between the LY326315 and chloride peaks was sensitive to changes in methanol concentration. The resolution decreased with increasing methanol concentration as illustrated in Figure 5.

The percent methanol had little effect on ion retention but significant effect on LY326315 retention. A mobile phase consisting of 50% methanol / 50% 0.1 M ammonium acetate buffer at pH 4.5 was used for further work.

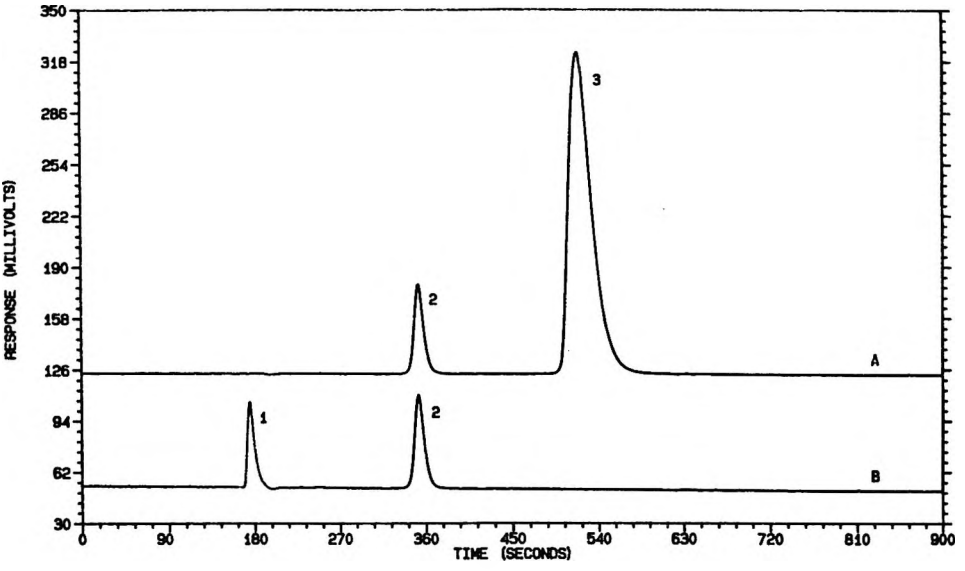


Figure 7. Chromatogram of LY326315 hydrochloride (A) as resolved with the mixed-mode phenyl/cation column and detected by ELSD and a chromatogram of a sodium chloride standard (B). Peak 1 = sodium, peak 2 = chloride, peak 3 = LY326315.

Effect of Detector Temperature

The Sedex ELSD detector permits the drift tube to be temperature controlled. With the pressure set at 1.0 bar and detector gain at 7, the temperature was increased to determine if it had an effect on peak sensitivity. Figure 6 shows the effect of ELSD temperature on chloride response. Significant chloride peak area loss was observed as the detector temperature was increased. This decrease in response is attributed to a breakdown in the ammonium chloride cluster moiety with increasing temperatures. The response of LY326315 was not significantly affected by detector temperature. Detector temperature was set at 28°C to maximize peak area response for chloride.

Optimized Conditions

Figure 7 was obtained using the following optimized conditions on the mixed-mode phenyl/cation column: the isocratic mobile phase consisted of 50% methanol/50% 0.1 M ammonium acetate buffer at pH 4.5 (adjusted with acetic acid). The ELSD settings were: temperature at 28°C, 1.0 bar, and gain of 7.

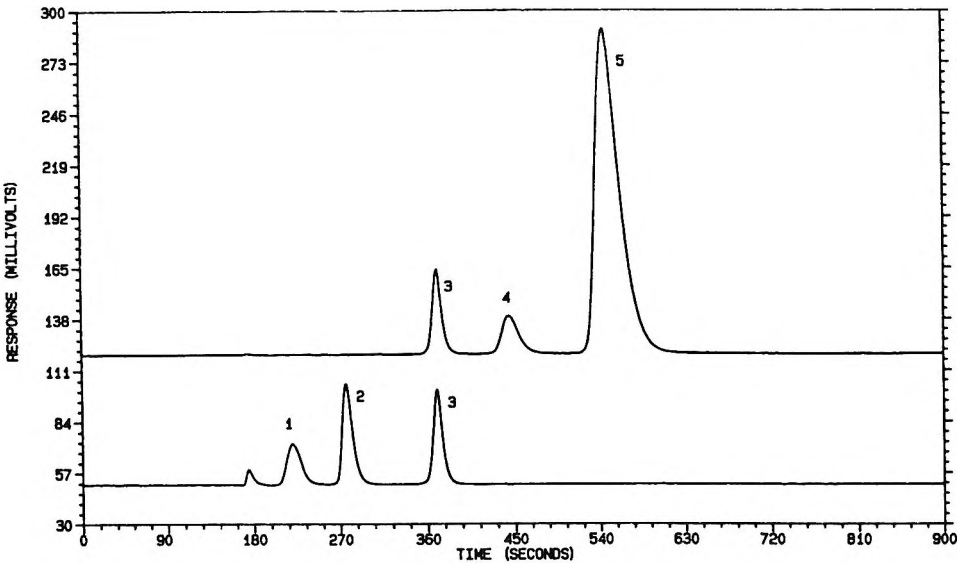


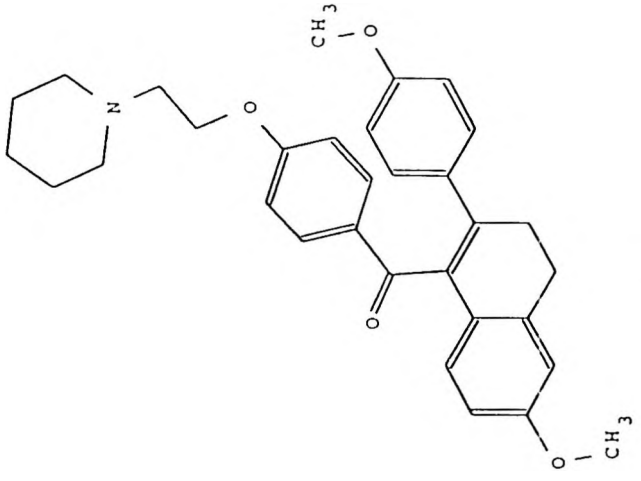
Figure 8. Chromatograms of LY326315 hydrochloride and spiked impurities (process intermediates) as resolved from the mixed-mode phenyl/cation column and detected by ELSD. Peak 1 = compound 174266, peak 2 = compound 151630, peak 3 = chloride, peak 4 = compound 317695, peak 5 = LY326315. Peak at approximately 180 seconds is undetermined.

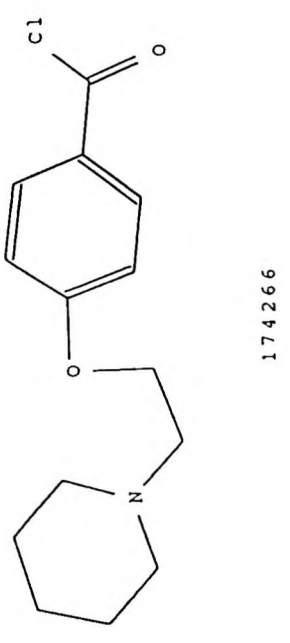
The chromatograms in Figure 8 show where process related impurities, compounds 174266, 151630, and 317695 elute in relation to the LY326315 and chloride peaks. These impurities are resolved from each other, the drug substance, and chloride. The structures of these impurities are shown in Figure 9.

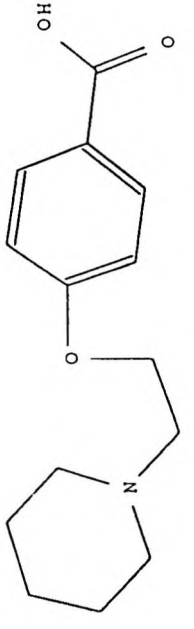
Other Mixed-Mode Columns

The mixed-mode cation columns were designed to retain cations and the mixed-mode anion columns likewise retain anions.[2] Therefore in these experiments with the mixed-mode phenyl/cation column, the greater retention of chloride compared to sodium was an unexpected result.

Figure 9. (right) Structures of process-related impurities, compounds 174266, 151630, and 317695.


317695


HCl
174266


HCl
151630

Three other Alltech mixed-mode columns - phenyl/anion, C_8/cation, and C_{18}/anion - were tested using the same mobile phase established for the mixed-mode phenyl/cation column. The buffer pH at 4.5 was ideal since the recommended pH is <5 for the anion columns and >4 for the cation columns. The mixed-mode anion columns retained chloride and LY326315 considerably longer than the cation columns. However, on all the mixed-mode anion and cation columns tested, chloride was retained more than sodium.

Literature and manufacturer reports indicate no previous use of mixed-mode columns to analyze small ions such as chloride and sodium. A possible explanation for the chloride retention on both the anion and cation mixed-mode columns involves the mobile phase buffer component which is ammonium acetate. On the mixed-mode phenyl/cation column, which has the carboxylic acid functionality, sodium and chloride are reacting with ammonium acetate forming a sodium acetate complex and an ammonium chloride complex. The ammonium chloride is adsorbing like an ion-pair reagent on the phenyl portion of the column. Sodium acetate has a lower affinity for the stationary phase and therefore has little retention. On the mixed-mode phenyl/anion column, which has the amine functionality, both mechanisms are occurring - ion exchange and ion-pairing for ammonium chloride - therefore creating longer retention when compared to the phenyl/cation column. Other possible explanations include ion interactions with exposed silanols on the column's silica support, size exclusion effects, or a combination of some of the above. Further experiments would be needed to determine the primary contributors to chloride or sodium ion retention.

CONCLUSION

The applicability of a mixed-mode HPLC column in combination with an evaporative light scattering detector for the simultaneous resolution and detection of a drug substance, impurities, and the counter ion has been demonstrated. This novel approach demonstrates a means to simultaneously resolve and detect pharmaceutical compounds, impurities, and counter ions in a single chromatogram, in contrast to developing three separate methods. The mixed-mode column can be used with typical reversed phase mobile phases and with conventional HPLC systems. Since the ELSD is capable of detecting many types of solutes, regardless of functional groups, its versatility was demonstrated here for both organic compounds and inorganic ions. In combination, the mixed-mode column with ELSD provides a new alternative for the analytical chemist performing method development.

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LIQUID CHROMATOGRAPHIC ASSAY OF AMINOCARB AND FENITROTHION IN PESTICIDE FORMULATIONS

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ABSTRACT

A simple, rapid and robust liquid chromatographic method for the analysis of oil-based and emulsion spray-mixes containing aminocarb and fenitrothion insecticides is reported. The extracts of the spray-mixes, after necessary method optimization, were analysed using an HP RP-C8 column (200 x 4.6 mm ODS, 5 μm) with UV detection and methanol/water (85/15 v%) as the mobile phase. The linear concentration ranges for aminocarb and fenitrothion were 0.05 to 5.0 μg and 0.10 to 4.2 μg, respectively. Limit of detection and limit of quantification were 0.04 and 0.08 μg (aminocarb) and 0.05 and 0.10 μg (fenitrothion), respectively, in 20-μL injection volume. Analysis of different oil-based and emulsion spray-mixes of the two insecticides gave reproducible values with low CV, and agreement between the expected and measured values was good. The method could be modified and adapted for the trace analysis of the analytes from forestry matrices.

INTRODUCTION

Fenitrothion [O, O-dimethyl O-(3-methyl-4-nitrophenyl) phosphorothioate] and aminocarb [4-(dimethylamino)-3-methylphenyl methylcarbamate] are insecticides of economic significance used extensively in Canadian forestry since the early 1970's to control the defoliating lepidopteran insect pests.[1] Both insecticides are effective, economical, easy to handle, and are applied aerially as aqueous emulsions or as oil solutions. Several gas chromatographic (GC), high pressure liquid chromatographic (HPLC), thin layer chromatographic (TLC) and spectrophotometric methods for the determination of aminocarb and fenitrothion residues found in various matrices, and the contents of active ingredient (AI) present in their formulations, have been reported.[2-8] Among them, the traditional GC method is very sensitive; nevertheless, it is problematic because of the heat-labile character of aminocarb,[9-11] and instability and isomerization potential of fenitrothion above 200°C, especially while using metal columns in GC.[7] Time consuming derivatizations, accompanied by solvent partitions and column cleanup, are often required to overcome these problems.

Quality assurance and quality control of the spray-mixes used in forestry have become very high priorities during spray operations. Usually, the spray-mixes used in forestry spraying contain a single AI mixed with solvents, stickers, surfactants and other adjuvants to enhance target deposition and coverage. The company methods are usually suitable for the pure AI and for the commercial formulation concentrates, rather than for the spray-mixes containing a variety of additives and solvents added to the concentrates prior to the spray application, and these additives are normally prone to interfere either in the GC or HPLC analysis. Furthermore, the spray season in forestry is usually very short and a good number of emulsion and oil-based spray-mixes have to be analysed on time and on short notice. Therefore, robust, rapid and reliable methods to analyse and quantify the AI in the spray-mixes are required. Herein, we report a simple and sensitive reversed-phase HPLC method with UV detection to analyse the AI components present in the spray-mixes containing either aminocarb or fenitrothion insecticide.

MATERIALS AND METHODS

Analytical Standards

Analytical grade aminocarb (> 98% purity) and fenitrothion (> 97% purity) were supplied by Chemagro Chemical Co., Toronto, ON, and Sumitomo Chemical Co., Osaka, Japan, respectively. Both insecticides (100.0 mg) were dissolved separately in ethyl acetate and methanol and diluted to 100.0 mL in volumetric

flasks to give 1000 μg/mL solutions. The ethyl acetate stock solution of either insecticide was stable for the entire study period (> 7 weeks), whereas the methanolic solution of aminocarb developed a light brown colour after prolonged storage. To circumvent the problem, fresh solutions of the insecticides in methanol were prepared every two weeks. A 30.0-mL aliquot of each stock solution was diluted to a 100-mL volume in a volumetric flask with the same solvent to give a 300 μg/mL stock standard solution of each insecticide. Solutions of different concentrations were prepared by diluting this stock standard solution. All standards were kept in tightly sealed volumetric flasks in darkness at 1°C and filtered prior to injection into the HPLC system. Twenty-μL volumes of the standard solution of each insecticide at different concentrations were injected, in triplicate, into the HPLC and the detector response was measured for each in terms of peak area. Calibration curves were prepared by plotting the average peak area (y-axis) against the mass of the analytes (x-axis). Quantitation was done by comparing the peak area of the test material to that of the standard in the calibration curve and computing the concentration therefrom.

Solvents

Spectroquality solvents of ethyl acetate, acetonitrile, and methanol (J.T. Baker) were obtained from Canlab, Toronto, ON. Pure water, used throughout this study, was prepared by passing distilled water through a Milli-Q® water purification system (Millipore Co., Bedford, MA). Solvents were filtered using 0.20-μm pore size Nylaflo filters (Gelman Sciences Inc., Rexdale, ON). The mobile phases (acetonitrile/water and methanol/water) were filtered (0.20-μm pore size) and degassed prior to use.

Spray-Mixes

The selected spray-mixes of aminocarb and fenitrothion used in the present study are given in Table 1 along with the approximate AI concentrations, the generic names of the additives known to be present in them, and the suppliers. The composition and exact chemical nature of the additives in spray-mixes are proprietary information of the manufacturers.

Instrumentation

All chromatography was carried out using a Hewlett-Packard (HP) (Palo Alto, CA), model 1084B HPLC, equipped with a UV variable wavelength (190 to 600nm) detector, dual solvent systems and associated gradient pumps, an HP 79849

Table 1

Composition of the Spray-Mixes

Spray-Mix	Composition of Spray-Mix	Expected AI Concentration (g/mL)	Dosage (g AI/ha)	Application Rate (L/ha)
A-O[a]	Metacil 180 FO[b] + I.D. 585[c]	0.048	70	1.46
A-EC[d]	Metacil 180 F.E[e] + I.D. 585 + Atlox[f] + Water	0.048	70	1.46
F-O[g]	Sumithion-O[h] + I.D. 585	0.110	210	1.50
F-EC[i]	Sumithion-EC[j] + Triton X-100[k] + Water	0.110	210	1.50

[a] Aminocarb-oil
[b] Aminocarb, oil-based formulation (Chemagro Chem. Co., Toronto, ON)
[c] Petroleum distillate consisting of aromatics (Shell Canada Ltd., Toronto, ON)
[d] Aminocarb-emulsion
[e] Aminocarb,emulsion formulation
[f] Emulsifier (Atlas Chem. Industries, Brantford, ON)
[g] Fenitrothion-oil
[h] Fenitrothion, oil-based formulation (Sumitomo Chem. Co., Osaka, Japan)
[i] Fenitrothion, emulsion
[j] Fenitrothion, emulsion formulation
[k] Emulsifier, sticker and spreader (Rohm and Haas Canada Inc., West Hill, ON)

auto-sampler and variable volume Rheodyne® injector. All instrument control and data collection were done using a microprocessor controlled electronic integrator linked to an LC terminal (HP 79850 B). A full description of the instrument was given in an earlier publication.[12] The operating parameters were as follows:

Columns:	(1) Zorbax C-18, 250 x 4.6 mm ODS, 10-μm diam. (2) HP C-8, 200 x 4.6 mm ODS, 5-μm diam. (3) Whatman Partisil C-18, 250 x 4.6 mm ODS, 10-μm diam. (4) Regis C-18, 150 x 4.6 mm ODS, 5-μm diam.
Column pressure:	1.6×10^3 to 11.6×10^3 kPa
Mobile phase:	Acetonitrile/water, methanol/water (5, 10 or 15 v% of water, isocratic for the first 8 min followed by 100% acetonitrile or methanol for the next 10 min to flush out the late eluters).
Flow rate:	0.5 and 1.0 mL/min
Oven temperature:	30 and 50°C
Injection volume:	20 μL
Concentration:	Aminocarb standard, 2.5 to 250 μg/mL (0.05 to 5.0 μg per injection); Fenitrothion standard, 5.0 to 210 μg/mL (0.10 to 4.2 μg per injection)
Attenuation:	5.12×10^{-2} AU/cm
Chart speed:	0.5 cm/min
Wavelength:	Aminocarb, 248:430 nm (sample:reference); Fenitrothion, 270:430 nm (sample:reference)
Run time:	18 min

Sample Preparation

The spray-mixes of aminocarb and fenitrothion formulations used in the aerial spray programs were shaken well on a wrist-action shaker for 0.5 h. Aliquots (0.3-0.5 mL) were weighed separately into 50-mL volumetric flasks and made up with methanol. Each solution was shaken for 0.5 h for complete analyte extraction, allowed to settle, and an aliquot was filtered (Nylaflo filter, 0.20-μm pore size) to remove particulates. The filtrate was transferred quantitatively to a graduated, stoppered centrifuge tube and the volume was adjusted either by concentration under

N_2 (Meyer N-Evap®) or by dilution with methanol, so that the concentration of the desired analyte in the test sample was within the concentration range of the calibration curve prepared using the standard. A 20-μL volume of the methanolic extract of each spray-mix was injected several times (n = 6) into the HPLC. The average peak area was calculated and the concentration of each analyte was computed from the respective calibration curves.

Method Optimization

A systematic approach to methods development task has been to research and find reliable assay conditions to separate and quantify aminocarb and fenitrothion from their respective spray-mixes. During the initial stages, column selection and the choice of mobile phase with its proper composition were done by trial and error, in order to get good resolution of the peaks of the target analytes. The resolution pattern of each insecticide was studied by using, successively, the four columns listed in the instrumentation section and injecting into each of them, several times, 20-μL volumes of the standards. Different ratios of acetonitrile/water and methanol/water were tried as mobile phases, and their resolution patterns were examined. The same procedure was repeated using sample solutions prepared from the individual spray-mixes.

Consistently efficient, good and reproducible resolution of the peaks of aminocarb and fenitrothion from the respective spray-mixes was possible only by using the HP C-8, 200 x 4.6 mm ODS, 5-μm column with methanol/water (85/15 v%) as the mobile phase. The use of C-8 column gave good resolution of the analyte peak and separation was relatively fast (run time, 18 min) with good efficiency (sharp and narrow peak), thus permitting a high output of sample analyses. As an added benefit, column deterioration after prolonged use was found to be minimal and the column life was excellent. Because the column packing was efficient, no guard column was used (there was some concern that such a pre-column would reduce the efficiency of the analytical column). The C-18 columns used in this study were more hydrophobic; therefore, their separation, selectivity and resolution potentials were poor and retention times (RTs) were longer, thus increasing the analysis time. Also, columns with 10-μm particle size had higher run times compared to 5-μm particle size columns. The peak resolution and efficiency were poor with the shorter Regis C-18 150 x 4.6 mm column, and plugging of the frits occurred occasionally which negated the separations. Although Zorbax and Whatman columns were similar in their size and bonded-phase packing, poor peak resolution and variability in RTs were observed during the preliminary assay studies, which resulted in abandoning further trials with them.

In optimizing the mobile phase selection, 85% methanol and 15% water mixture was the primary choice because of good resolution of the analytes in the test samples, producing distinct, sharp and narrow peaks with peak asymmetry factors around 0.85 to 1.15. However, trial studies showed that acetonitrile/water mixtures could also serve as an excellent substitute for methanol/water as mobile phase because of their lower viscosity (reduced back pressure) and higher solvent strength. Nevertheless, the relatively higher cost of acetonitrile, and its reported toxicity and related disposal problems, precluded its use in the present study. Although methanol/water did serve as an excellent mobile phase to separate the analyte peak, some band overlapping of impurity peaks was observed, especially while analysing the oil-based spray-mixes. Since there was no interest in those impurity peaks, no attempts were made to separate the bands.

Band spacing and baseline noise (*ca* 0.0001 AU) were found to be good at the column temperature of 30°C. However, at 50°C the RTs of peaks were decreased, reducing their band spacings, which in turn negatively affected the peak resolutions, especially in the oil-based spray-mixes; also, there was enhanced background noise. Similarly, using the mobile phase flow rate of 0.5 mL/min increased the RTs, which increased the analysis time. On the other hand, a flow rate of 1.0 mL/min not only reduced the analysis time but also enhanced the separation efficiency. Finally, to avoid the back pressure build-up with time, especially while using methanol/water as mobile phase, and to improve system reliability, an optimum column pressure of 3.5×10^3 kPa was chosen and used during the study. Prolonged use of the HP RP C-8, 5-μm column occasionally produced detectable impurities (adjuvants in spray-mixes, additives from the column packing and their degradation products etc.) as late eluters, and this effect was reduced by washing the column daily with methanol.

RESULTS AND DISCUSSION

Linearity of UV Detectors

The linearity of the UV detector to aminocarb and fenitrothion was checked by injecting 20-μL aliquots of each analyte standard in triplicate. Detector response (average peak area) was plotted against the concentration of each insecticide standard. The calibration curve was linear in the concentration range of 0.05 to 5.00μg for aminocarb and 0.10 to 4.20 μg for fenitrothion, in 20-μL injection volume. The curve passed through the origin with "r" values of 0.998 for aminocarb and 0.990 for fenitrothion.

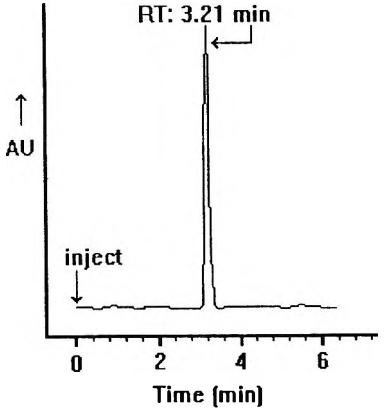

Figure 1. Liquid chromatogram of aminocarb after injecting 20 µL of a 50 µg/mL standard.

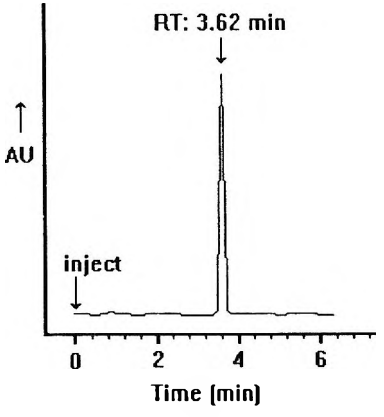

Figure 2. Liquid chromatogram of fenitrothion after injecting 20 µL of a 50 µg/mL standard.

The reproducibility in the peak area measurements at the above concentration ranges of aminocarb and fenitrothion were 96 and 92%, respectively. The average percent standard deviation (SD) observed in the peak area measurements for all the concentrations of the standards injected was 7 for aminocarb and 11 for fenitrothion.

Limit of Detection and Limit of Quantification

In the analysis of pesticide formulations and spray-mixes, the emphasis is placed on the content of the analyte in the test sample, as opposed to trace analysis where the emphasis is primarily to find the chemical in the matrix at residue levels (μg to ng/g). In the former case, the AI is usually a major component, the sample size is large and higher quantities of the prepared sample can be injected into the liquid chromatograph if the analyte has a weak chromophore. It is a macromethod, and of necessity, accuracy and precision are of fundamental importance. In such situations, the concept of limit of detection (LOD) and limit of quantification (LOQ) may not be applicable. However, from the observed linearity over the concentration ranges studied (0.05 to 5.00 μg in 20 μL for aminocarb and 0.10 to 4.20 μg in 20 μL for fenitrothion) and from the baseline noise of the chromatograms of the two insecticide standards, the LOQ values for aminocarb and fenitrothion were conservatively established as 0.08 and 0.10 μg, respectively. LOD values were 2 orders lower than the LOQs, i.e., 0.04 μg for aminocarb and 0.05 μg for fenitrothion (in 20 μL).

HPLC Chromatograms of the Standards

Typical chromatograms of aminocarb and fenitrothion standards, obtained by injecting 1.0 μg in 20 μL onto the HP C-8, 200 x 4.6 mm ODS, 5-μm column, are given in Figures 1 and 2. Each analyte peak was well resolved, narrow and symmetrical, showing that the selection of column, mobile phase and maximum absorption wavelength (248 nm for aminocarb and 270 nm for fenitrothion) were appropriate for the two insecticides. The RTs of aminocarb and fenitrothion were 3.21 and 3.62 min, respectively.

HPLC Chromatograms of the Spray-Mixes

The commercial formulations of aminocarb (Matacil) and fenitrothion (Sumithion) contained aromatic hydrocarbons, especially polyalkylated benzenes, as solvents.[7,8,13] In addition, the spray-mixes contained I.D. 585, Atlox and Triton as additives (Table 1), all of which are aromatic in origin. These materials also absorb in the UV region[13] and were eluted along with the analytes of interest. Fortunately, all the aromatics present in the spray-mixes emerged from the column either before or after the components of interest, as seen in Figures 3 to 6. The peaks corresponding to each analyte were distinct, fairly symmetrical, free from interference and resolution was quite satisfactory.

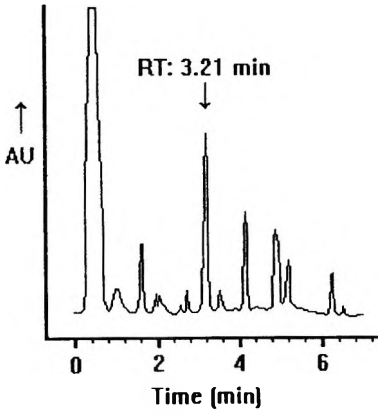

Figure 3. Liquid chromatogram of A-O spray-mix of aminocarb after 20-μL injection (see Table 1 for spray-mix details).

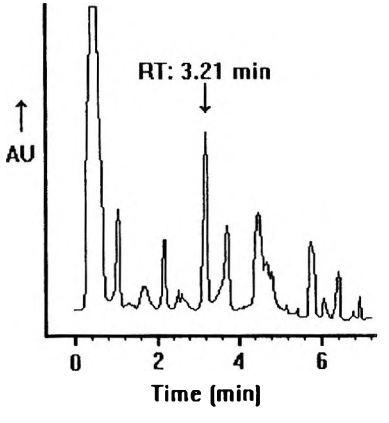

Figure 4. Liquid chromatogram of A-EC spray-mix of aminocarb after 20-μL injection (see Table 1 for spray-mix details).

Aminocarb and Fenitrothion Contents in the Spray-Mixes

Replicate analysis (n = 6) of the aminocarb (Matacil) spray-mix, A-O, showed that it contained, on average, 4.64% of the insecticide (range 4.51 to 4.86%) or 97% of the expected value (Table 1). The corresponding value for the A-EC spray-mix

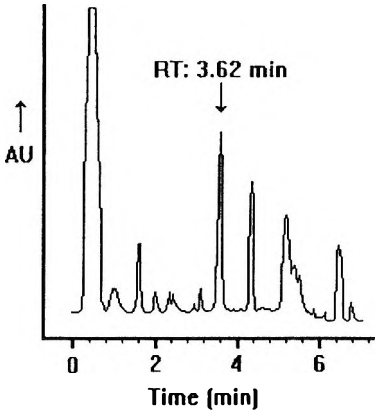

Figure 5. Liquid chromatogram of F-O spray-mix of fenitrothion after 20-µlL injection (see Table 1 for spray-mix details).

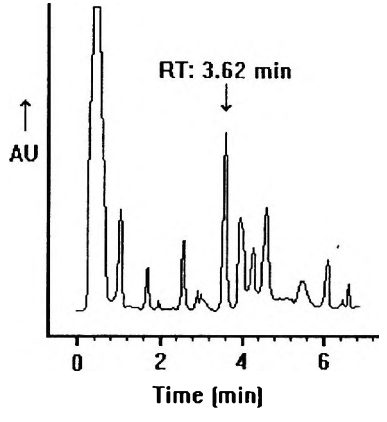

Figure 6. Liquid chromatogram of F-EC spray-mix of fenitrothion after 20-µL injection (see Table 1 for spray-mix details).

was 4.53% (range 4.48 to 4.71%) or 94% of the expected value. The F-O spray-mix of fenitrothion contained 11.2% AI (range 10.9 to 11.8%), corresponding to 102% of the expected value. On the other hand, the value for F-EC spray-mix was only 9.41% AI (range 8.30 to 9.91%) or 86% of the expected value, indicating possible hydrolysis of the fenitrothion ester in the aqueous mixture.

To determine the precision of the method, each spray-mix was stored at 4°C in darkness and re-analysed each day for the following two days. The mean AI (%), ± SD and CV (%) for the spray-mixes were 4.61, 0.06 and 0.71 (A-O); 4.51, 0.07 and 0.63 (A-EC); 11.1, 0.08 and 0.92 (F-O); and 9.2, 0.11 and 1.12 (F-EC), respectively, indicating good precision.

The HPLC method reported in this paper was simple, sensitive and rugged, and the data on analyte contents showed good agreement between the expected and measured values. Based on the results of this study, it is apparent that the method is useful to analyse aminocarb and fenitrothion contents in forestry spray-mixes used in insect control programs. With necessary modifications, such as solvent extraction, partitioning, column cleanup and sensitivity optimization, the method could be extended to trace analysis of aminocarb and fenitrothion residues found in various forestry matrices following aerial spray applications.

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COMPARATIVE STUDY OF THE SEPARATION AND DETERMINATION OF ASPARTAME AND ITS DECOMPOSITION PRODUCTS IN BULK MATERIAL AND DIET SOFT DRINKS BY HPLC AND CE

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ABSTRACT

The direct separation of aspartame (α-L-aspartyl-L-phenylalanine methyl ester) LL-α-APM, and several decomposition products namely LL-β-aspartame (LL-β-APM), L-α-aspartyl-L-phenylalanine (α-AP), L-β-aspartyl-L-phenylalanine (β-AP), and diketopiperazine (DKP) was accomplished by high performance liquid chromatography (HPLC) using a Chirobiotic T (Teicoplanin) column, and capillary zone electrophoretic (CZE) methods. The presence of any of these decomposition products in diet soft drinks labeled to contain the sweetener, also in coffee containing the artificial sweetener LL-α-APM, were investigated.

INTRODUCTION

Aspartame (LL-α-APM) is widely used as an artificial sweetener in both dry powder form and in aqueous solution, as in the case of various diet soft drinks and other beverages. In dry powder LL-α-APM is relatively stable while, in aqueous solution it undergoes decomposition and racemization forming a variety of degradation products which is reported to be related to the length of storage, temperature and pH of the diet foods and beverages containing the sweetener.[1,2,3]

Witt[4] indicated that the analysis of a diet beverage (pH 2.55), after 50 weeks of storage at 20°C, had approximately 20% of the original LL-α-APM converted to DKP, another 20% was converted to α-AP, and 15% was converted to β-AP and LL-β-APM. Gaine and Bada[5] also identified six diastereomeric products after heating LL-α-APM sample for several hours at pH 8.8 - 9.8.

To help in evaluating the health significance of these decomposition products, various methods for monitoring the concentrations of aspartame and these products has been developed, including fluorescence,[6] gas chromatography[7] and the most frequently used high performance liquid chromatography,[8,9,10,11] and recently capillary zone electrophoresis.[12] In this study, an HPLC method using Chirobiotic T (Teicoplanin) column, and a capillary zone electrophoretic (CZE) method were used for the separation of aspartame and four of its decomposition products in a direct single run. A calibration curve for each of these compounds was individually constructed, and the presence of these products in diet soft drink and coffee sweetened with aspartame, were investigated.

EXPERIMENTAL

HPLC Apparatus

The HPLC system consisted of the following (all were products of Waters, Milford, MA, USA): a 501 solvent delivery pump, a Lambda Max 481 variable wavelength detector, a 746 data module, and a U6K injector. Chirobiotic T (Teicoplanin) column serial no. 8615 (250 x 4.6 mm I.D., particle type 5μm spherical) was obtained from Advanced Separation Technologies, Inc., Whippany, New Jersey, USA.

Capillary Electrophoresis Apparatus

The work was carried out on Waters Quanta 4000E Capillary Electrophoresis System equipped with a UV detector (214 nm), and a positive power supply deliver upto 30KV (Waters Corporation, Milford, MA, USA). All analyses were performed on Accusep polyamide fused-silica capillaries (60cm x 75 μm I.D.)obtained from (Waters, Milford, MA, USA). The detection time constant was set at 0.3 second, all analyses were for 20 second injections with hydrostatic mode. A constant applied voltage of +15 KV was used in all experiments. Electrophoretic conditions and data acquisition were controlled by the Millennium 2010 Chromatographic Manager (Waters, Milford, MA, USA).

Chemicals

LL-α-Aspartame (Lot:E-403240), LL-β-aspartame (Lot:672-45), L-α-aspartyl-L-phenylalanine (Lot:N941-122A), L-β-aspartyl-L-phenylalanine (Lot:490-135-A), and DKP (Lot: 7R-1) were kindly supplied by NutraSweet AG, Zug, Switzerland. Ethanol was purchased from Merck (Darmstadt, Germany), di-sodium hydrogen orthophosphate and di-sodium tetraborate was obtained from BDH Chemicals (Dorset, England).

Buffer and Solutions

Stock solutions of 4 mg/mL of all compounds (except for DKP, for which a stock solution of 1 mg/mL was used was made due to the limited solubility); all were prepared daily with Milli-Q purified water. To establish a calibration curve for each compound, five concentrations in the range of 5 -100 μg/mL for HPLC and in the range of 250 - 4000 μg/mL for CZE (except for DKP for which the range was 50 - 1000 μg/mL); all preparations were made in Milli-Q purified water.

Sample Preparations

Diet Seven-Up® soft drink was degassed in an ultrasonic bath then diluted 5 and 20 fold in Milli-Q-purified water and filtered through a 0.45 μm Millipore filter for the determination by CZE and HPLC respectively. To a 100mL of warm coffee , 500 mg of aspartame (commercially known as Equal® NutraSweet, Deerfield, Il, USA) was added.

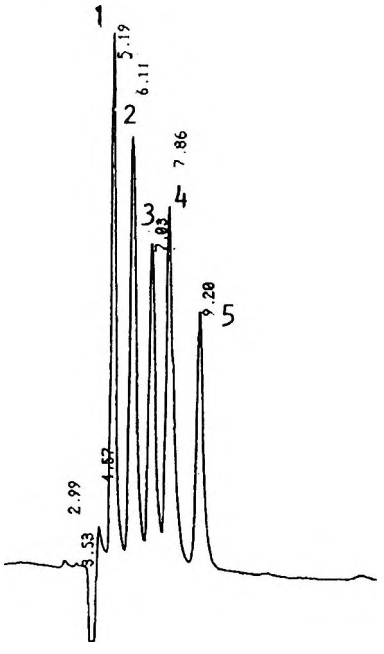

Figure 1. Chromatogram of a mixture containing aspartame and decomposition products (100 μg/each) Peaks: 1=DKP; 2=L-β-AP; 3=LL-β-APM; 4=L-α-AP; 5=LL-α-APM. Chromatographic conditions: column: Chirobiotic T (Teicoplanin) 250 x 4.6 mm I.D. Mobile phase: Ethanol:H_2O (55:45 V/V) pH 3.85. Flow rate: 0.6 mL/min. Detection: 215 nm, sensitivity=0.01 a.u.f.s.; attenuation: 32.

The solution was heated to a temperature of 70°C for 10 minutes, immediately diluted 10 and 50 fold in Milli-Q purified water then filtered through a 0.45 μm Millipore filter for the determination of CZE and HPLC respectively.

RESULTS AND DISCUSSION

The chromatographic baseline separation of LL-α-APM and four of its degradation products which includes LL-β-APM, L-α-AP, L-β-AP, and DKP were achieved in a single run using an HPLC method with Chirobiotic T (Teicoplanin) column in less than 10 min (Fig. 1). The separation of the same compounds were also accomplished by capillary zone electrophoresis method in less than 18 min run (Fig. 2).

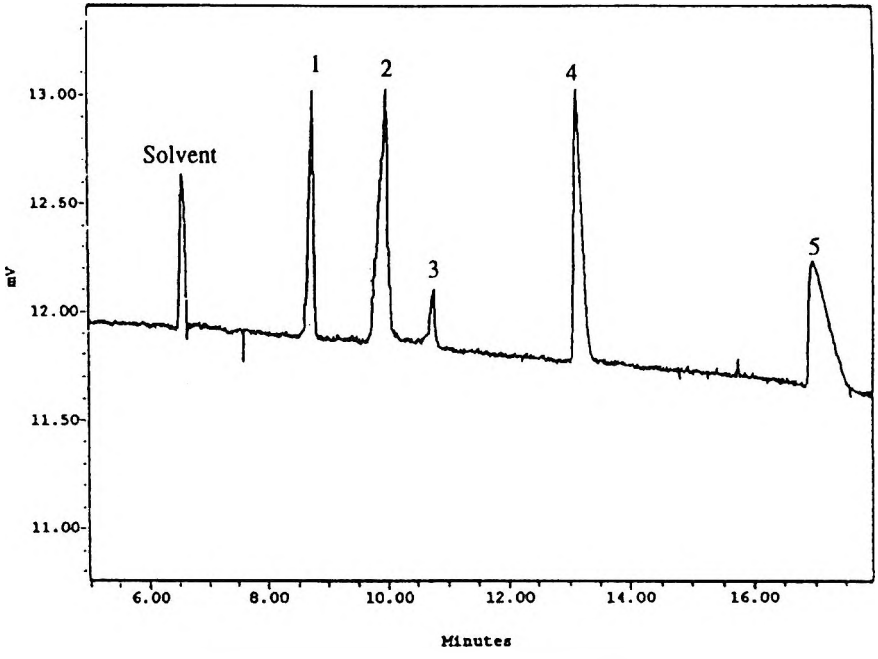

Figure 2. Electropherogram (2 mg mixture), Peaks: 1=LL-β-APM; 2=LL-α-APM; 3=DKP; 4=L-β-AP; L-α-AP. Condition: Buffer=25mM phosphate/25 mM Borate (1:1) pH 9.0; run voltage=15 Kv; injection mode=hydrostatic for 20 sec.

Standard curves for the quantitative determination of each of these compounds were constructed individually using both methods. The regression analysis of these curves (Table 1) indicated a linear relationship between the peak area (Y), and the concentration (C) over the range of 5-100 μg/mL for all the compounds when the HPLC method was used (Fig. 3), while a higher linear range of 250-4000 μg/mL was obtained by CZE method. (Fig. 4) Except for DKP, the linear range was 100-1000 μg/mL owing to the lower detection limit of HPLC.

These methods were also applied to the analysis of LL-α-APM and the degradation products in Diet Seven-Up® soft drink, and after the addition of commercially available aspartame dry powder (Equal NutraSweet) to coffee. The results of the analyses of diet soft drink samples by both methods indicated the presence of 4-5% DKP of the total amount of LL-a-APM present in the

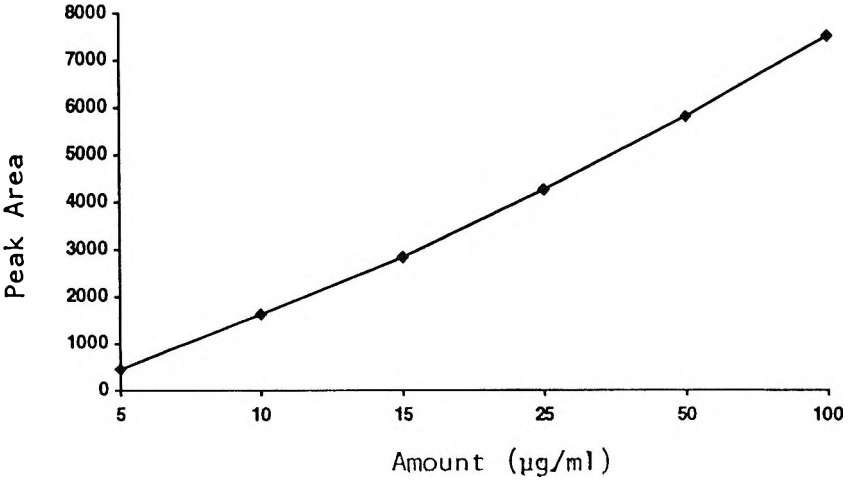

Figure 3. Sample standard curve for the quantitative determination of LL-α-APM in a bulk powder form by HPLC method.

Table 1

Results of Standard Curves Regression Analysis for LL-α-APM and Degradation Products

Compound	Method	Slope	r
LL-α-APM	HPLC	113.395	0.998
	CZE	0.566	0.998
LL-β-APM	HPLC	117.670	0.999
	CZE	0.568	0.999
α-AP	HPLC	142.206	0.998
	CZE	549.7 + 3.5x	0.998
β-AP	HPLC	96.750	0.998
	CZE	1.067	0.999
DKP	HPLC	142.056	0.997
	CZE	9.929	0.999

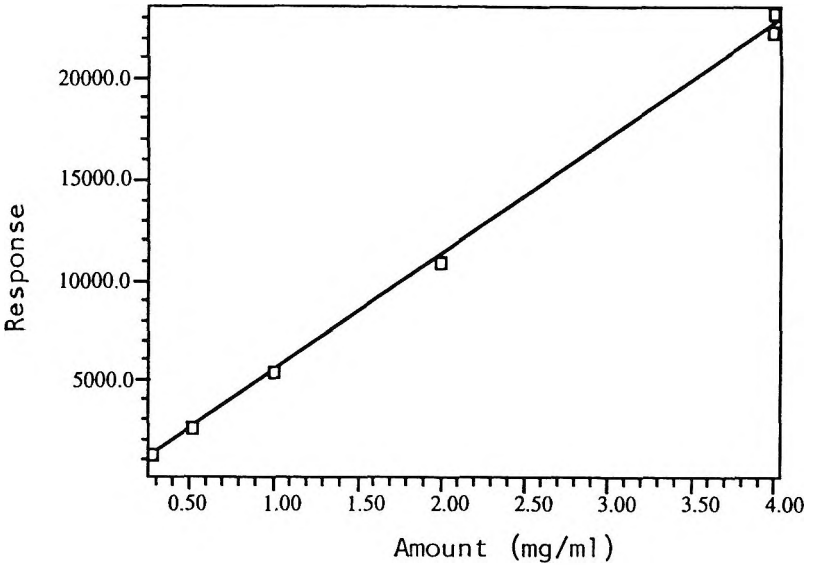

Figure 4. Sample standard curve for the quantitative determination of LL-α-APM in a bulk powder form using capillary zone electrophoresis.

sample. The analysis did not show any of the other degradation products which were analyzed in our standard samples. On the other hand, the analyses of the coffee samples showed only LL-α-APM with mean recovery of 99.6±1.2 and no degradation products were detected.

The HPLC method appears to have some advantage when compared with CZE method. The limit of detection (the injection amount which give 3 times of the baseline to noise ratio) was lower by a factor of 20, and injections reproducibility was better for all the compounds used in this work, while the separation efficiency (N) of these compounds was higher in CZE method. Both methods gave good linearity. These findings were in agreement with the previous work done by Jimidar et al.[12]

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SERUM INORGANIC IODIDE DETERMINED BY PAIRED-ION REVERSED-PHASE HPLC WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

We propose an automated method for the analysis of serum inorganic iodide (SII), using paired-ion reversed phase HPLC with electrochemical detection and a silver working electrode. Assay conditions include a flow rate of 1.0 mL/min and an operating potential of 0.10 V. The retention time for iodide is 5.3 min. Sample preparation consists in protein removal by ultrafiltration and concentration of the ultrafiltrate, because of the very low levels of SII, especially in iodine deficient areas. Ultrafiltration separation is achieved by pouring 2mL of a serum sample into a filter cup (membrane cutoff: 5kD) and then using a centrifugal force of 14000G over 90 min. A 1200μL aliquot of the ultrafiltrate is concentrated by a factor of 10 to a volume of 120μL in a centrifugal vacuum concentrator (Speed-Vac). A 100μL aliquot of the concentrate is injected into the HPLC. Due to concentration the detection threshold (signal-to-noise ratio of 3) could be lowered to 0.004μmol/L. The recovery of iodide during concentration of the ultrafiltrate tested with ^{123}I was 102.2%.

The within and between-run precision (CV) for a serum sample with 0.04μmol/L are 1.9% and 4.2%, respectively. For comparison with a standard method based on the isotope dilution principle serum samples from 26 patients who underwent thyroid scintigraphy with ^{123}I were measured by both HPLC(y) and the standard technique(x). The data obtained show a high correlation (r=0.99; y=0.94x + 0.007; S_{yx}=0.0179). Levels of SII typical of an iodine deficient area are measured in sera from 27 patients with low urinary iodine excretion (44μmol/molCrea): SII=0.023μmol/L (mean); range: 0.01- 0.036μmol/L.

INTRODUCTION

Iodine deficiency is the most common cause of thyroid enlargement and goiter is, indeed, the obvious and familiar feature.[1] But it is only a visible mark of an inadequate iodine supply. Iodine deficiency disorders[2] is the term now used to denote all the effects of functional and developmental abnormalities, including thyroid disorders, which occur when the physiological requirements of iodine are not met in a given population. Evaluation of the status of iodine nutrition is therefore highly desirable.[3] As most of iodine is excreted in the urine, urinary iodine excretion is currently the most convenient laboratory marker of iodine deficiency.[4] The present recommendation[5] is to evaluate the iodine intake of a given population by the measurements of iodine concentrations in a representative number of casual urine samples.[6,7] The most precise estimation of the iodine supply of an individuum, however, is the determination of the urinary iodine in 24-hour collections.[8,9] The feasibility and the completeness of 24-hour collections however, are often in doubt, since accurate urine collections are notoriously difficult to obtain. The measurement of serum inorganic iodide (SII) therefore might be a more convenient alternative for demonstrating iodine deficiency of an individuum. Furthermore many aspects of iodine metabolism, especially the effects of iodine on the thyroid gland,[10] can only be fully understood if the concentration of SII is known. The determination of SII is, therefore, of great value in pathophysiologic studies of thyroid function, particularly for the calculation of the absolute iodine uptake.[11,12,13]

Here, we present a relatively simple automated method for the direct determination of inorganic iodide in serum using paired-ion, reversed phase HPLC and ultrafiltration for removal of proteins. The required sensitivity was achieved by concentrating the iodide content of the ultrafiltrates with a centrifugal vacuum concentrator (Speed-Vac).

MATERIALS AND METHODS

Apparatus

We assembled a modular HPLC system comprising a Model 510 HPLC pump, a Model 717 autosampler, a Model 460 electrochemical detector, and a temperature-control system consisting of the temperature-control module and a column heater (all from Waters Chromatography Div., Millipore, Milford, MA). Specific subcomponents of the electrochemical detector included a Ag/AgCl reference electrode, a 50μ gasket defining an analytical cell volume of 2.5μL and a silver working electrode.

The autosampler and detector were connected on-line to a Power Mate 386/25 personal computer(NEC Technologies, Boxborough, MA) operating with 810 Baseline chromatography software from Waters. The chromatographic column was a Resolve™ C_{18} reversed phase column, 3.9 x 150 mm, 90Å (9 nm), 5μm (Waters) held at a temperature of 35°C in the column heater.

Chemicals

Analytical-grade di-sodium hydrogen phosphate dodecahydrate ($Na_2HPO_4.12H_2O$), ethylene dinitrilo tetraacetic acid (Titriplex™ III) and H_3PO_4 were obtained from Merck(Darmstadt, Germany); di-n-butylamine was from Sigma Chemical CO. (St.Louis, MO). The ion-pairing reagent tetrabutylammonium phosphate(TBAP) was purchased from Waters and analytical-grade potassium iodide from Serva (Heidelberg, Germany) was used to prepare calibration solutions.

Mobile Phase

The mobile phase consisted of 10 mmol/L $Na_2HPO_4.12H_2O$, 1 mmol/L Titriplex III, 10 mmol/L TBAP, and 6 mmol/L di-n-butylamine in HPLC-grade water. The pH of the resulting mobile phase was adjusted to 7.0 with 85% orthophosphoric acid (H_3PO_4). Before use, the mobile phase was filtered through a 0.45-μm-pore membrane filter (Waters) and degassed under reduced pressure.

Serum Samples

Serum samples were collected from 27 euthyroid children (age 9-17y, mean 12.8y), living in an iodine deficient area with low urinary iodine excretion: 44 μmol/mol creatinine (median). For comparison with the standard method based on the isotope dilution principle (s.below), sera from 26 euthyroid patients referred to our clinic for scintigraphic evaluation of thyroid nodules were obtained.

Separation of proteins by ultrafiltration[14] was achieved by pouring 2mL of a serum sample into a filter cup (UFC4LCC25 from Nihon Millipore Kogyo,Japan) with a membrane cutoff of 5kD and, then, using a centrifugal force of 14000G over 90 min. A 1200μL aliquot of the ultrafiltrate is concentrated by a factor of 10 to a volume of 120μL in a Speed-Vac-concentrator. A 100μL aliquot is injected into the HPLC.

HPLC Analysis

We used isocratic HPLC at a flow rate of 1.0 mL/min with electrochemical detection at a potential difference of 0.10 Volt vs Ag/AgCl. The full-scale integrator sensitivity was 1.0 Volt, corresponding to a full-scale detector sensitivity of 50 nA. Concentrations were calculated from peak areas by using the integrator.

Comparison Method Based on the Isotope Dilution Principle

According to Stanley,[15] the calculation of serum inorganic iodide(SII) is based on the assumption that the specific activity of SII equals the specific activity of urinary inorganic iodide. Applying a simplified procedure with one blood sample only SII can, therefore, be determined from the following formula:[13,15,16]

SII = Serum ^{127}I= Serum^{123}I(1hr) x (Urinary^{127}I(1-2hr) / Urinary^{123}I(1-2hr)) where ^{127}I is stable iodine and ^{123}I is a radioactive isotope of iodine frequently used in thyroid scintigraphy.

Patients, 26, referred for scintigraphic evaluation of nodular goiter were studied. After each patient had emptied his bladder, a tracer dose of 10MBq (270μCi) of ^{123}I was administered intravenously. A blood sample was taken one hour after injection of ^{123}I for measurement of serum ^{123}I and urine collection was extended over two hours after ^{123}I administration for determination of urinary ^{123}I and urinary ^{127}I. The radioactivity of ^{123}I was

Table 1

Within and Between-Run Precision (Coefficients of Variation=CV) for Determination of Serum Inorganic Iodide Concentrations Using HPLC

Serum Sample (μmol/L)	Intraassay-CV (%)	Interassay-CV (%)
0.12	1.13	5.35
0.04	1.88	4.19
0.02	1.91	8.36

measured in a well-type counter, urinary ^{127}I was determined by HPLC.[17] SII was calculated according to the above mentioned formula and compared with the value obtained by measurement of SII in the same blood sample, using HPLC.

Statistical Analysis

Correlations between the results by HPLC and the reference method based on the isotope dilution principle were assessed with ordinary least-squares linear regression techniques. Intra- and interassay SDs and CVs (Table 1) were calculated from 10 measurements of three serum samples with different iodide concentrations. Differences between slopes measured by adding increasing amounts of potassium iodide to different serum specimens were investigated by analysis of covariance. Data management and computations were performed with the statistical software package STATISTICA/w from Statsoft (Tulsa, OK).

RESULTS

Application and Detection Limit of HPLC

Typical chromatograms obtained by HPLC are shown in Fig.1 for two concentrations of calibrator representing the highest and lowest points of the calibration curve and for a serum sample containing 0.14μmol/L iodide. The retention time of iodide is 5.3 min; the total analysis time for one sample is 7.5

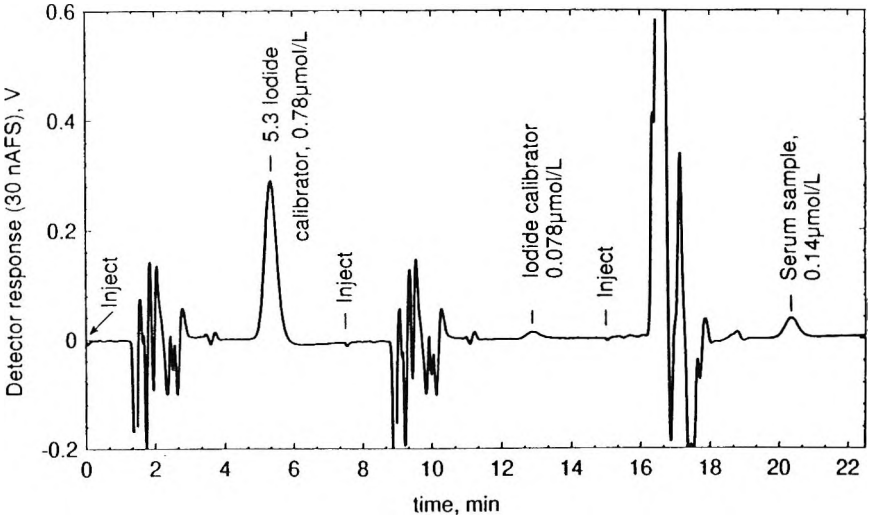

Figure 1. Chromatograms of calibrators containing 0.78 and 0.078 µmol/L potassium iodide and of a serum sample with 0.14 µmol/L inorganic iodide. Electrochemical detection was with 0.6 V integrator sensitivity corresponding to 30 nA full-scale (nAFS) detector sensitivity

min. The detector response shows a quadratic relationship with the iodide concentrations in the lower range between 0.078 and 0.78µmol/L (Fig.2). According to concentration of the ultrafiltrate, the usual detection limit of the HPLC assay of 0.04 µmol/L[17,18] based on a signal to noise ratio of 3, could be lowered by a factor of ten to 0.004 µmol/L.

Precision

The within and between-run precision (CV) for various serum iodide concentrations are given inTable 1. The intraassay-CV of each serum sample is based on 10 measurements within one run, the interassay-CVs were calculated from 10 determinations of the serum specimens over a period of 4 weeks.

Recovery of Iodide During Sample Preparation

To test the recovery of iodide during preparation of samples 2mL aliquots of 12 different serum specimens each spiked with 1kBq of ^{123}I were placed

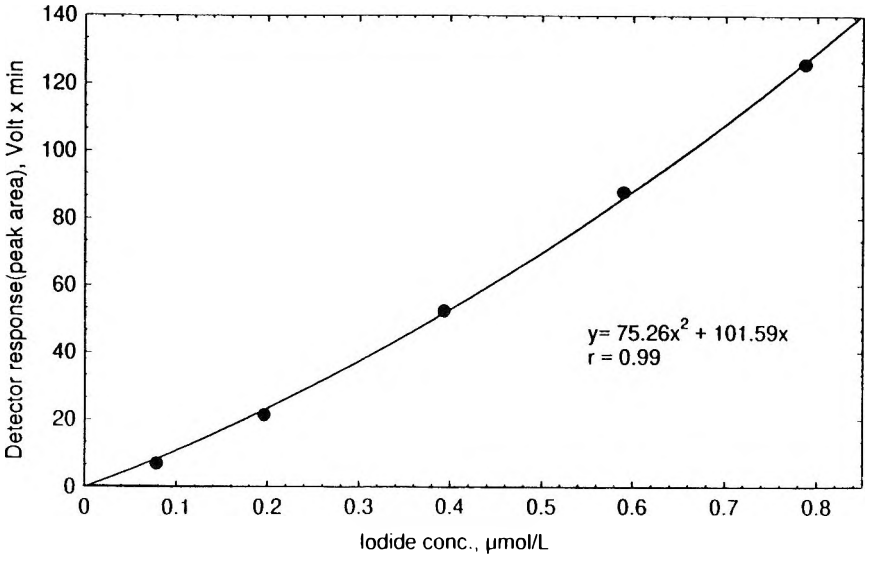

Figure 2. The calibration curve based on aqueous solutions of potassium iodide shows a quadratic relationship with the iodide concentrations in the lower range

onto filter cups and ultrafiltrated as described earlier. The radioactivity of the serum samples and of the ultrafiltrates was measured in a well-type counter. The calculated recovery was 90.8% ± 4.6%. 1200µL aliquots of the same radioactive ultrafiltrates were then concentrated in a Speed-Vac-concentrator by a factor of 10. The radioactivity of the ultrafiltrates before and after concentration was determined in the well-type counter, yielding a recovery of 102.2% ± 5.6%. The loss of iodide during ultrafiltration and concentration is, therefore, very low and negligible.

Recovery of Iodide in Serum

To detect possible interference effects by unknown compounds in serum, we supplemented four serum samples with iodide concentrations of 17 to 155 nmol/L before preparation with increasing amounts of potassium iodide and analyzed. The slope between the amount of iodide measured and the amount added was calculated for each sample. The results and the corresponding regression lines are shown in Fig.3.

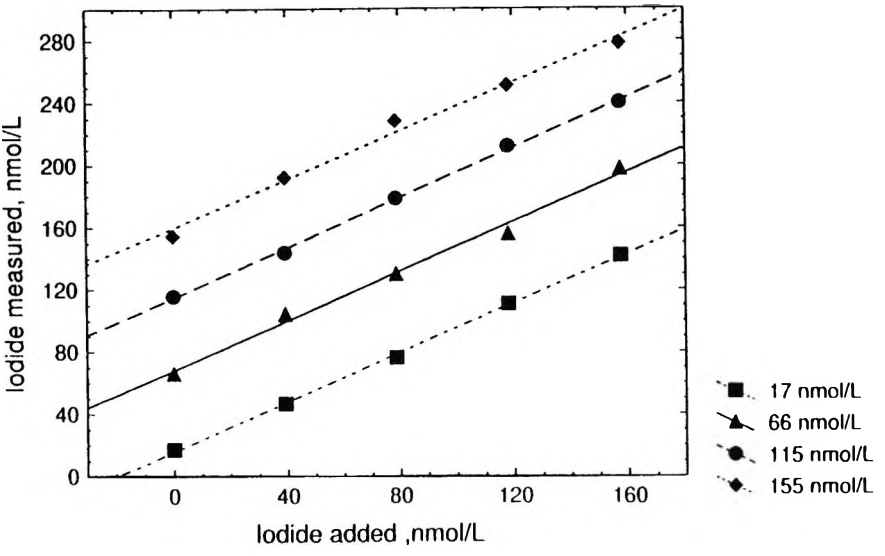

Figure 3. Recovery of iodide added to four different serum samples before preparation. Each point represents the mean of two experiments

There was no significant difference between slopes (P = 0.41). The mean slope was 0.80, suggesting an absence of interference effects and indicating that recovery of iodide was almost complete, which is in agreement with the calculated average recovery of 89.3%.

Comparison with the Reference Method

For comparison, the iodide content of 26 serum samples was determined by both HPLC and the reference method based on the isotope dilution principle. The calculated regression coefficient is 0.91 and the intercept does not differ significantly from 0 (Fig.4).

SII Levels in Iodine Deficiency

To determine the range of SII levels typical of an iodine deficient area, sera from 27 euthyroid children with low urinary iodine excretion (range: 14.5 -126.1 μmol/molCreatinine, median: 44 μmol/molCreatinine) were measured by HPLC. The results are given in Table 2 and are well comparable with those published in the literature for different iodine deficient areas (see Table 2).

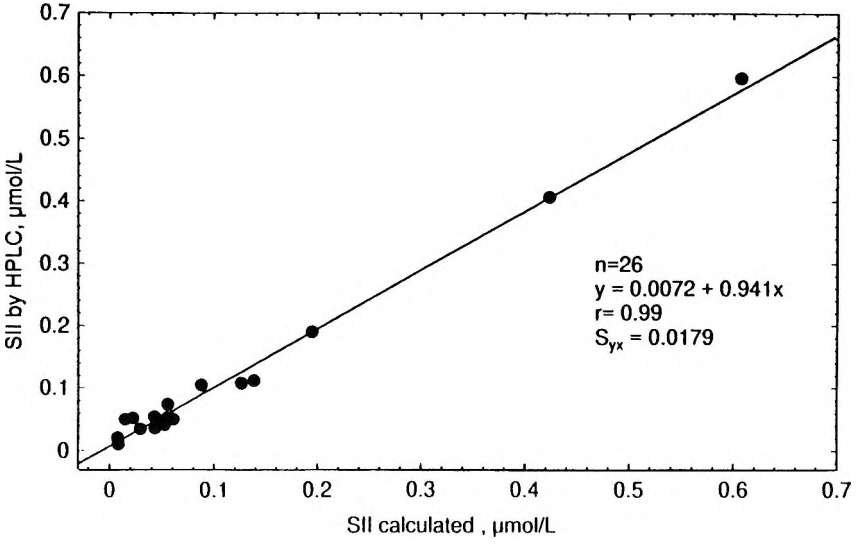

Figure 4. Comparison of SII concentrations in sera from 26 patients measured by HPLC with calculated SII values obtained from a tracer (^{123}I) method based on the isotope dilution principle.

Table 2

Levels of Serum Inorganic Iodide in Iodine Deficiency: Comparison of Own Data with those Published in the Literature for Euthyroid Subjects

Author	Ref.	n	Mean (µmol/L)	Range (µmol/L)
Koutras	13	13	0.020	0.008 - 0.080
Mantzos	21	10	0.016	0.006 - 0.031
Postmes	16	27	0.021	0.008 - 0.041
this study		27	0.023	0.010 - 0.036

DISCUSSION

In contrast to the measurement of urinary iodine, comparatively little attention has been given to the determination of serum inorganic iodide, possibly because of the technical difficulties of directly measuring SII, since very small quantities are involved. Especially in iodine deficient areas, levels

of SII significantly below 0.04μmol/L are usually found.[13,19,20] Furthermore, direct methods for the chemical determination of SII[16,21,22] use the colorimetric ceric-arsenic assays, which are based on the catalytic effect of iodide in the Sandell and Kolthoff reaction.[23,24] These methods, however, are subject to various potential sources of error, mainly due to the fact, that even slight contaminations from the PBI (protein bound iodine) fraction or from free thyroid hormones may greatly increase the SII value. In addition, the concentration of iodine in the reagents used for the separation and direct determination of iodine is usually several times higher than that of SII.[21] For that reason, most investigators used indirect methods for the estimation of SII, based on the isotope dilution principle and the determination of iodine in urine[10,13,15,19,25-28] or saliva.[20,29-32] SII has been determined, satisfactorily by applying these methods, but they are very cumbersome and associated with the administration of radioactive tracers. They exhibit additional disadvantages as Wayne[33] and Fitting[34] pointed out and therefore a direct method with the required accuracy, precision and sensitivity for measuring SII would be highly desirable.

As recently shown,[17] an alternative approach for determining iodide in biological fluids is afforded by paired-ion, reversed phase HPLC with electrochemical detection. This technique offers several advantages over the above-mentioned direct chemical methods. Unlike the chemical assays, which are sensitive to any iodine containing compound, the HPLC method showed almost complete absence of interfering substances due to the electrochemical detection in combination with the chromatographic technique.[17] The HPLC, therefore, measures selectively unbound iodide and its specificity is not impaired by contaminations from organically bound iodine.

Hurst et al.[35] described a HPLC method using electrochemical detection for the determination of iodide in serum, whereas Buchberger[14] measured SII by ion chromatography with post-column reaction detection. The least iodide concentrations detectable by the HPLC methods have been reported to range from 0.03 to 0.06 μmol/L,[17,18,35] the detection limit of the post-column reaction was 0.008μmol/L.[14] Compared to a normal range of about 0.004 to 0.04 μmol/L usually found for SII values in iodine deficient areas,[13,16,19-21] the chromatographic techniques are also too insensitive to detect such minute concentrations.

In our present study, we describe a relatively simple automated method for the direct determination of inorganic iodide in serum using paired-ion, reversed phase HPLC and ultrafiltration for removal of proteins. The required sensitivity was achieved by concentrating the iodide content of the ultrafiltrates with a Speed-Vac-concentrator. The recovery of iodide during preparation of

samples and during concentration of the ultrafiltrates was 90.8% and 102.2%, respectively. Therefore, the loss of iodide by ultrafiltration and concentration is very low. The data concerning accuracy and precision (s.Table 1) clearly document that the proposed method is an accurate, precise and effective alternative to the current procedures for determining serum inorganic iodide. Due to the relatively short retention time of iodide (5.3min) the total analysis time for one sample is 7.5min which gives a turnaround rate of eight samples per hour.

The present study compares, for the first time, data from serum samples measured for free inorganic iodide by both HPLC and a reference method based on the isotope dilution principle. There is almost complete agreement between the amount of inorganic iodide in the serum samples as measured by HPLC and the amount calculated according to the isotope dilution principle.

The SII values determined by HPLC, however, are slightly lower than the calculated ones (see Fig.4). These calculations are based on the assumption that the specific activity of serum inorganic iodide equals the specific activity of total urinary iodine. There is evidence, however, that the kidneys excrete into urine not only inorganic serum iodide but also organic iodine compounds in small amounts.[36-40] Furthermore, some of the inorganic iodide in the urine is derived from thyroidal hormones and from other organic iodine compounds metabolized and deiodinated during their passage through the kidneys.[38,40-42] Consequently, the specific activity of neither total urinary iodine nor inorganic urinary iodide equals exactly the specific activity of serum inorganic iodide. The calculated values of SII are, therefore, slightly higher than the values determined by HPLC or by saliva/plasma methods.[20,43]

Due to variations in the iodine intake values of SII reported by various authors differ widely. However, in iodine deficiency our results are well comparable with those published in the literature (s.Table 2), with a mean SII concentration of about 0.02 μmol/L. In contrast to this very low SII level, Hurst et al.[35] from Pennsylvania in the U.S., measured SII concentrations in 6 euthyroid subjects. As expected, according to the adequate iodine supply in the U.S., the mean value was significantly higher (0.70 μmol/L) than the mean SII concentration in iodine deficient areas.

In conclusion, the present study clearly demonstrates that paired-ion reversed phase HPLC with electrochemical detection, is an accurate and precise method for determination of SII. Comparison with a reference method based on the isotope dilution principle shows nearly complete agreement.

In contrast to these indirect methods, which are very cumbersome and associated with the administration of radioactive tracers to patients, or compared with direct chemical methods being subject to various potential sources of error, the HPLC assay is a relatively simple automated procedure for the direct determination of SII.

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

J. LIQ. CHROM. & REL. TECHNOL., 20(9), 1463–1469 (1997)

LIQUID CHROMATOGRAPHY CALENDAR

1997

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.

JUNE 2 - 4: Advanced HPLC Short Course, sponsored by the Chromatography Forum of the Delaware Valley, Widener University, Chester, PA. Contact: Jim Alexander, Rohm & Haas Labs, 727 Norristown Rd., Spring House, PA 19477, USA. Tel: (215) 619-5226; Email: rsrjna@rohmhaas.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, Lab. de Chimie Analytique, Fac. de Pharmacie, F-34060 Montpellier Cedex 2, France. Tel: (33) 04 67 54 45 20; FAX: (33) 04 67 52 89 15; Email: hfabre@pharma.univ.montp1.fr.

JUNE 20: Enantiomeric Separation in Capillary Electrophoresis, a short course given by Dr. K. Altria, Glaxo-Wellcome, Ware, UK, in Montpellier, France. Contact: Prof. H. Fabre, Lab. de Chimie Analytique, Fac. de Pharmacie, F-34060 Montpellier Cedex 2, France. Tel: (33) 04 67 54 45 20; FAX: (33) 04 67 52 89 15; Email: hfabre@pharma.univ.montp1.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.

AUGUST 2 - 9: 39th Rocky Mountain Conference on Analytical Chemistry, Hyatt Regency Denver, Denver, Colorado. Contact: Patricia Sulik, Rocky Mountain Instrumental Labs, 456 S. Link Lane, Ft. Colleins, CO, USA. (303) 530-1169; Email: gebrown@usgs.gov.

AUGUST 11 - 13: 10th International Symposium on Polymer Analysis and Characterization (ISPAC-10), University of Toronto, Canada. Contact: Prof. S. T. Balke, Dept. of Chem. Engg & Appl. Chem., Univ. of Toronto, Toronto, Ont., Canada, M5S 1A4. Tel/FAX: (416) 978-7495; Email: balke@ecf.toronto.edu.

SEPTEMBER 7 - 11: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036, USA.

SEPTEMBER 14 - 17: International Ion Chromatography Symposium, Westin Hotel, Santa Clara, California. Contact: Janet Strimaitis, Century International, P. O. Box 493, Medfield, MA 02052-0493, USA. Tel: (508) 359-8777; FAX: (508) 359-8778; Email: century@ixl.net.

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: ISPPP'97 – 17th International Symposium on the Separation of Proteins, Peptides, and Polynucleotides, Doubletree Hotel, Washington, DC. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA. Tel: (301) 898-3772; FAX: (301) 898-5596; EMail: Janetbarr@aol.com.

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.

MAY 3 - 8: HPLC'98 – 22nd International Symposium on High Performance Liquid Phase Separations and Related Techniques, Regal Waterfront Hotel, St. Louis, Missouri. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA. Tel: (301) 898-3772; FAX: (301) 898-5596; EMail: Janetbarr@aol.com.

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: PittCon '99, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA

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

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

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

2000

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

MARCH 26 - 30: 219th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899.

JUNE 25 - 30: HPLC'2000 – 24th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Seattle, Washington. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA. Tel: (301) 898-3772; FAX: (301) 898-5596.

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.

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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d) Whom to contact for additional details.

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One complete original manuscript and two (2) clear copies, with figures, must be submitted for peer review. After all required revisions have been completed, and the final manuscript has been accepted, the author will be asked to provide, if possible, a 3½" or 5¼" PC-Compatible computer diskette containing the complete manuscript. Microsoft Word, Word for Windows, WordPerfect, WordPerfect for Windows and ASCII are preferred formats. Text, including tables, and figures, if in electronic format, should be saved in separate files on the diskette. Label the diskette with the corresponding author's last name, the title of the manuscript and the file number assigned to the manuscript.

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   Use Times Roman 12 point font, if possible.

The general organization of the manuscript should be:

Title
Author(s)' names and full addresses
Abstract
Text Discussion
References

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F. D. Pierce, H. R. Brown
Utah Biomedical Test Laboratory
520 Wakara Way
Salt Lake City, Utah 84108

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INTRODUCTION
MATERIALS
METHODS
RESULTS
DISCUSSION
ACKNOWLEDGMENTS

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5. The **first line of each paragraph** within the body of the text should be indented a half inch.

6. **Acknowledgments**, sources of research funds and address changes for authors should be listed in a separate section at the end of the manuscript, immediately preceding the references.

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