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QUANTITATIVE ANALYSIS OF A BIOCIDE IN SILICONE EMULSIONS USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Traditional high performance liquid chromatographic (HPLC) methods are not typically used for characterization of silicones, due to a lack of absorption in the ultraviolet (UV) region of the spectrum. However, many additives in water-based, silicone formulations absorb in the UV and can be quantified using an HPLC method. Direct injection of a silicone emulsion can lead to irreversible deposition of siloxane polymer on an HPLC column and should be avoided.

This paper describes a sample preparation procedure in which the silicone emulsion is "broken" and the polymer precipitated prior to chromatographic analysis of the aqueous phase. A reverse-phase, HPLC method, originally developed by Rohm and Haas, was modified and used to quantify the two active components of Kathon® CG Biocide in Dow Corning® 1784 Emulsion using this sample preparation procedure. This

procedure was also shown to be effective for quantitation of Kathon® LX in Dow Corning® Antifoam B and may be applicable to analysis of Kathon® in a wide variety of silicone emulsions.

INTRODUCTION

High performance liquid chromatography (HPLC) with ultraviolet (UV) detection is not commonly used to characterize silicones, due to lack of absorption for most siloxane polymers in the UV spectrum. However, many additives in water-based, silicone emulsions absorb in the UV and can be quantified using an HPLC method.

The method described in this paper was specifically developed to quantify the active components of Kathon® CG Biocide in Dow Corning® 1784 Emulsion. The method had to be compatible with an HPLC system already in place in a Quality Assurance (QA) Laboratory, which imposed some limitations on development and optimization of the method. Since the QA HPLC system did not have gradient capability, the method for Kathon® analysis had to use an isocratic solvent system. The method also had to leave no residual siloxane polymer or other contamination in the system, have no detrimental effect on the column, and otherwise cause no conflicts with the other analyses being run on the system.

Most analyses of water soluble components in silicone emulsions start with separation of the siloxane polymer and aqueous phases in order to avoid any potential interference caused by the polymer.[1] However, in an effort to simplify the HPLC method, initial analysis of Kathon® in silicone emulsions was attempted using direct injection of the emulsion. Upon injection of the neat emulsion, the aqueous phase containing the Kathon® components passed through the chromatographic system, while the siloxane portion of the emulsion adsorbed onto the column.

The siloxane polymer was theoretically removed at the end of each run by flushing the system with tetrahydrofuran (THF). This early work only involved a few samples and the THF flush appeared to be removing the polymer from the column in between samples. However, once actual method development was initiated, involving a large number of samples, steadily increasing column back pressure indicated that the THF flush was not removing the siloxane from the column. A sample preparation procedure was developed in which the siloxane portion of the emulsion is precipitated by addition of methanol/acetic acid to the

sample followed by centrifugation, leaving the water soluble components in the aqueous phase. The aqueous phase can then be injected and analyzed without depositing polymer on the column.

MATERIALS

Dow Corning® silicone emulsions are widely used as additives in a variety of markets, including personal care products and food packaging. Water-based silicone emulsions include many application-specific formulations which contain a range of weight percent siloxane polymer as well as a variety of water soluble additives. These emulsions are complex mixtures and are challenging to characterize qualitatively or quantitatively.

Biocides are typically added to water-based silicone emulsions to inhibit bacterial growth with time. "Kathon" is a proprietary name for a family of microbiocides manufactured and marketed by the Rohm and Haas Company. Kathon® formulations all contain two active constituents: 2-methyl-4-isothiazolin-3-one (A) and 5-chloro-2-methyl-4-isothiazolin-3-one (B). The chemical structures of these compounds are shown in Figure 1. For the sake of brevity, they will be referred to as compounds or components A and B throughout the rest of this paper.

The formulation Kathon® CG (CG = cosmetic grade) is sold for use as a preservative in a very wide range of applications, including skin and body creams, shampoos, bubble bath, sun-screens, and mascara. Kathon® CG consists of 0.35% compound A, 1.15% compound B, 25% magnesium nitrate, and 73.5% water (percent by weight). According to the EEC (European Economic Community) Council Directive regarding cosmetic products, Kathon® CG is permitted for use as a preservative in cosmetic formulations with a maximum authorized concentration of 0.0015% (15 ppm) of a mixture of compound A and B in the ratio 1:3. An evaluation of a variety of analytical methods to quantify Kathon® CG in fully-formulated cosmetics was performed in 1989 at the request of the Commission of the European Communities.[2] The study concluded that a high performance liquid chromatographic method developed previously by Rohm and Haas[3] was the best method available at that time. This reverse-phase, HPLC method was modified and used with the sample preparation procedure described herein to quantify the active components of Kathon® CG in Dow Corning® 1784 Emulsion. This same method was also used to quantify Kathon® LX (food contact grade) actives in Dow Corning® Antifoam B. Both Kathon® CG and Kathon® LX contain the same concentrations of components A and B.

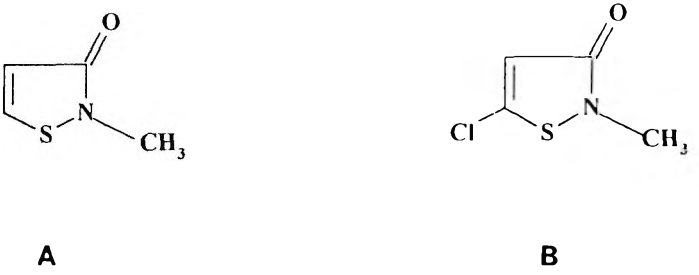

Figure 1. Chemical structure of active components in Kathon® CG and LX biocides.

EXPERIMENTAL

HPLC Instrumentation and Conditions

A Perkin-Elmer HPLC Series 4 liquid chromatograph was used for all sample and standard analyses included in this report. The HPLC configuration used for analysis of 1784 Emulsion samples included the components/conditions as listed. Injections were performed manually.

Mobile Phase :	65/35 0.4% acetic acid in H_2O / methanol (v/v); (Millipore® H_2O, glacial acetic acid, HPLC-grade methanol).
Pump:	Perkin-Elmer Series 4, single head, gradient HPLC pump; Solvent Flow Rate : 1 mL/min.
Injector :	Rheodyne® Model 7126 Syringe-Loading Sample Injector (manual); Injection Volume (via injection loop) = 50 microliters.
Column Temp:	Ambient.
Columns :	Alltech Spherisorb® ODS-1 Classic 5 micron Analytical, 250mm x 4.6mm, catalog # 8364; Alltech PRP-1 Reversed-Phase Guard Column (Spherisorb® ODS-1, 5 micron), 25mm x 2.3mm with stainless steel holder, catalog # 79447; (total run time = 15 min.).

Detection :	Waters® M-490 programmable, multi-wavelength UV Detector wavelength = 280 nm, range = 0.1 AUFS; Sampling Rate : 0.2 points per second; Temperature = ambient.

Note that the original HPLC method developed by Rohm and Haas[3] does not include acetic acid in the eluent mixture. However, of the different eluent systems evaluated by de Kruijf,[2] water with acetic acid, combined with methanol, gave the best results. The pH of Kathon® CG, as received, is low relative to Dow Corning® 1784 Emulsion (emulsion pH = 6.0 - 8.0). The lot of Kathon® used for spiking DC® 1784 Emulsion and making calibration standards had a pH of 2.6 (per Rohm and Haas). Maintaining a low pH by adding acetic acid to the HPLC eluent greatly improved the resulting chromatography. A 65/35 pure water/methanol eluent mixture was attempted during this study and the resulting peaks for Kathon® in the aqueous phase of a DC® 1784 Emulsion sample, prepared without acetic acid, were small and poorly shaped relative to an injection of the same sample using eluent and sample preparation with acetic acid. Acetic acid is a common modifier added to reverse-phase HPLC eluent systems and, in this case, may be preventing interaction of the active Kathon® components with free silanols present on the stationary phase.[4]

Sample Preparation

Approximately 5 gm of the emulsion sample was weighed accurately into a 1/2 oz glass bottle using an analytical balance. The weight was recorded to four decimal places. The aliquot of emulsion sample was then diluted to approximately 10 gm with methanol, containing 0.6% glacial acetic acid (v/v). The weight of diluted emulsion was also recorded to four decimal places. The dilution factor for the sample was calculated by dividing the total (diluted) sample weight by the original emulsion aliquot weight. The bottle was capped with a polyethylene-lined lid and placed on a wrist-action shaker for 30 minutes. The sample was then centrifuged for 30 minutes at 3000 rpm. The siloxane precipitated from the emulsion and was visible as a clear layer on the bottom of the bottle while the aqueous layer remained on top. An HPLC syringe was used to collect a portion of the aqueous phase for injection. Sometimes a "skin" was observed on the surface of the aqueous layer, which could be easily pushed aside before inserting the syringe needle.

ppm A+B	Area A+B
2.03	370703
4.02	745572
6.9	1279517
12.67	2356010
25.28	4692792
Summary Output	
Regression Statistics	
Multiple R	0.99999837
R Square	0.99999674
Observations	5
	Coefficients
Intercept	-2819.9633
Slope	185828.955

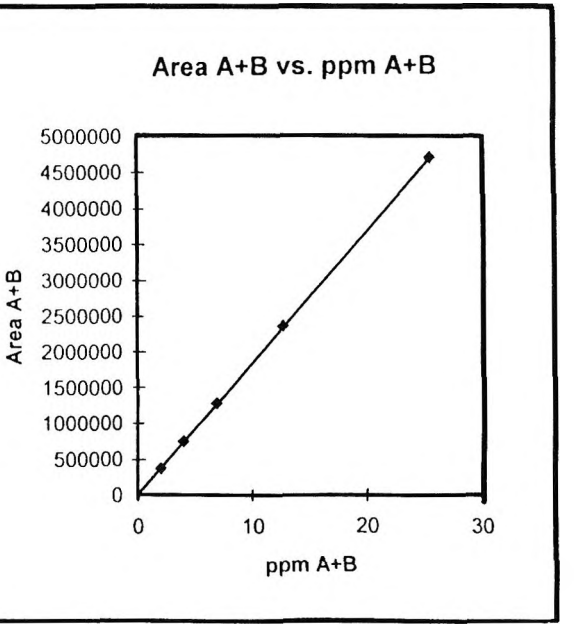

Figure 2. Calibration curve for Kathon® active components : area A+B *vs* ppm A+B.

Preparation of Standards and Calibration

A sample of Kathon® CG, containing 1.54%, by weight, active Kathon®, (A+B) was used to prepare a stock solution of 1526 ppm active, by dilution in the HPLC eluent. A total of 5 calibration standards were prepared by dilution with HPLC eluent, from this stock solution, at concentrations which bracketed the expected Kathon® concentration of the samples. The standards were analyzed using the conditions listed previously and a calibration curve was generated using the raw peak areas for each standard (A+B) *vs* the solution concentration for each standard (A+B).

A typical calibration curve is shown in Figure 2. Solution concentrations of the active Kathon® components in the aqueous phase of prepared emulsion samples were experimentally determined using raw peak areas and linear regression of the calibration curve. Multiplying the solution concentration of a sample by its dilution factor gave the concentration of active Kathon® present in the original emulsion sample.

Data Collection and Chromatographic Method of Analysis

Chromatographic data acquisition and measurement of raw peak areas was performed using PE Nelson® Access*Chrom software, version 1.8. Most chromatography software packages allow use of external and internal standard methods as well as normalized area percent for quantitative analysis. An external standard method was chosen for the analysis of Kathon® CG in DC® 1784 Emulsion in order to confirm the linearity of response *vs* concentration for the two active Kathon® components. Because the two active components have different detector responses (see Discussion), response factors would have to be experimentally determined for each component in order to generate accurate concentration values using an internal standard or normalized area percent method.

A standard additions method could also be used, in which the analyte of interest is spiked into the sample, with the level of analyte originally contained in the sample being determined by extrapolation.[5] The method of standard additions is especially useful if matrix effects interfere with analyte detection. The use of a standard additions method in this case could potentially help minimize the impact of the chromatographic interferences observed with DC® 1784 Emulsion analysis (see Discussion). However, most chromatography software does not currently include standard additions as a method option and the required calculations must be done manually. Since this method was intended for implementation in a QA laboratory, it was desirable to have a method which could eventually be automated.

RESULTS

Extraction Efficiency

The sample preparation procedure developed during this study, to enable HPLC analysis of silicone emulsions, includes both precipitation of the siloxane and extraction of the Kathon® components into the aqueous phase. Before quantitative analysis could be performed, it was necessary to determine to what extent the active Kathon® components were extracted into the aqueous phase. Extraction methods typically do not result in 100% recoveries, due to partitioning of the analyte between phases.[6] However, due to the hydrophobic, nonpolar nature of the bulk siloxane polymer, high recoveries of the active Kathon® components were expected.

Table 1

Percent Recoveries of Active Kathon® Components from Spiked Silicone Emulsion

Active Spiked (A+B)	Active Detected (A+B)	% Active Recovered (A+B)
7.67 ppm	7.36 ppm	96%
11.57 ppm	12.22 ppm	106%
15.49 ppm	14.68 ppm	95%

Three DC® 1784 Emulsion samples, spiked with a typical range of Kathon ® concentrations, were used to determine the efficiency of the extraction. The percent recovery was estimated using the difference between the amount spiked and the amount detected by the method after sample preparation (single injection). The percent Kathon® CG actives recovered from the three spiked DC® 1784 Emulsion samples are shown in Table 1. These analyses were performed on the same day that the samples were spiked.

A recovery of 95% to 106% is a broader range than that reported previously for determining active Kathon® CG in cosmetic samples using a similar reverse-phase HPLC method, in which the cosmetics were emulsified in mobile phase and then filtered prior to injection.[2] This method reported 98% to 102% recoveries in a 0.1 to 40 ppm active Kathon® range, but it is unknown if these values were based on single injection results or averages of multiple injections. The repeatability of this previously reported method is, therefore, unknown and it is possible that the cosmetic samples analyzed did not experience the baseline fluctuation and peak interferences observed with the DC® 1784 Emulsion samples.

Repeatability and Accuracy

In order to determine the repeatability of the entire method, including sample preparation, data was generated using 11.57 ppm and 15.49 ppm active Kathon®-spiked samples. Five solutions each, of the two spiked samples, were prepared and analyzed separately on five different days. Data was generated using both combined and separate calibration curves for components A and B. The same calibration standards were used for all the analyses and were refrigerated when not in use. A new calibration curve was generated for each day of analysis.

Table 2

Repeatability of HPLC Results for Spiked Samples using Combined Calibration Curve (Conc. A+B *vs* Area Response A+B)

Kathon® Spike Conc'n. =	11.57 ppm active (A+B)	15.49 ppm active (A+B)
Sample Number		
1	12.22 ppm active (A+B)	14.68 ppm active (A+B)
2	11.67 " " "	14.72 " " "
3	11.96 " " "	15.22 " " "
4	12.34 " " "	14.70 " " "
5	12.24 " " "	14.72 " " "
Mean =	12.09	14.81
S.D. =	0.2716	0.2309
R.S.D =	2.2%	R.S.D. = 1.6%

The repeatability results for the method are listed in Tables 2 and 3. The relative standard deviation values listed represent ± one standard deviation. The linearity of all the calibration curves generated during this work was excellent, with correlation coefficients of 0.9999+.

Note the excellent agreement between the spiked concentrations and the experimentally determined levels of active Kathon® listed in Tables 2 and 3. In all cases the results are within the percent recovery range, i.e., 95% to 106% active. Since the average percent recovery is approximately 100% for the levels of spiked Kathon® evaluated, this method of sample preparation and quantitative analysis of DC® 1784 Emulsion using HPLC is estimated to be accurate to within 6% relative, in the range of 7 ppm to 15 ppm active Kathon®.

Detection Limit

The limit of detection is the analyte concentration which gives an instrument signal significantly different from the blank or background signal.[5] There are several ways of calculating detection limit but, for comparative purposes, the definition stated by de Kruijf,[2] i.e., 2 x baseline noise, will be used here.

Table 3

Repeatability of HPLC Results for Spiked Samples Using Separate Calibration Curves for Components A and B

1. 11.57 ppm Active Kathon® Spike: (actual conc'n. = 2.927 ppm A, 8.640 ppm B)

Sample No.	ppm Component A	ppm Component B	ppm Component A+B
1	3.117	9.092	12.21
2	2.999	8.648	11.65
3	2.907	9.110	12.02
4	3.205	9.095	12.30
5	3.090	9.146	12.24
	$\overline{\text{Mean}} = 3.064$	$\overline{\text{Mean}} = 9.018$	$\overline{\text{Mean}} = 12.08$
	S.D. = 0.1143	S.D. = 0.2081	S.D. = 0.2643
	R.S.D. = 3.7%	R.S.D. = 2.3%	R.S.D. = 2.2%

2. 15.49 ppm Active Kathon® Spike: (actual conc'n. = 3.919 ppm A, 11.57 ppm B)

Sample No.	ppm Component A	ppm Component B	ppm Component A+B
1	3.962	10.59	14.55
2	4.004	10.57	14.57
3	4.265	10.75	15.02
4	4.061	10.46	14.52
5	3.891	10.74	14.64
	$\overline{\text{Mean}} = 4.037$	$\overline{\text{Mean}} = 10.62$	$\overline{\text{Mean}} = 14.66$
	S.D. = 0.1419	S.D. = 0.1228	S.D. = 0.2060
	R.S.D. = 3.5%	R.S.D. = 1.2%	R.S.D. = 1.4%

The detection limit for the active Kathon® components, using this HPLC method, is very good in the absence of any baseline disturbances or peak interference. Using peak heights, the detection limits for components A and B were estimated to be 0.013 ppm and 0.045 ppm, respectively, for a 50 microliter injection of a standard solution containing 0.59 ppm A and 1.75 ppm B. However, due to the presence of interfering peaks and baseline drift in chromatograms of the aqueous phase of DC® 1784 Emulsion samples, the detection limit in the actual sample matrix is not as low. Again, using peak heights, the detection limit for a 50 microliter injection of aqueous phase from

the 7.67 ppm spiked sample (1.94 ppm A + 5.73 ppm B) was estimated to be 0.50 ppm for component A and 1.4 ppm component B. A detection limit of 2 ppm total active Kathon® CG in DC® 1784 Emulsion, using this method, compares well with the 1 ppm detection limit reported by Rohm and Haas for analysis of Kathon® CG actives, using their recommended HPLC method for water-dilutable samples.[3]

DISCUSSION

Detector Response

The two active components in Kathon® CG do not respond equally with UV detection at 280 nm. In Table 4 are listed the peak area values for components A and B from chromatograms of five standard solutions. The area values have been normalized by dividing the raw peak area by the component concentration so that the values listed represent area response per ppm component. The decimal point was added to the area values to aid in visual comparison of the data.

The normalized data in Table 4 clearly show that component A responds more strongly at 280 nm than does component B. However, as long as the ratio of component A to B remains relatively constant at 1:3 in the sample, the total peak area response (A+B) can be used to calculate the ppm active Kathon®. If the ratio of the two components does not remain constant, for example, if one component is consumed or otherwise degraded with time relative to the other component, then each component must be quantified separately. This can be accomplished by using a separate calibration curve for each component or by using a calculated response factor for each component to correct for their difference in response. Response factors are instrument-specific and should be determined experimentally on the same system to be used for the actual analysis.

At no time during this work was a significant change in the ratio of the normalized area percent for the two Kathon® components in emulsion samples observed. Therefore, unless specified otherwise, all the sample concentrations of active Kathon® listed in this paper, determined using HPLC, were calculated using calibration curves of concentration A+B *vs* total area response. For a comparison of the repeatability of results using a calibration curve for A+B *vs* separate calibration curves for the two components, see Tables 2 and 3.

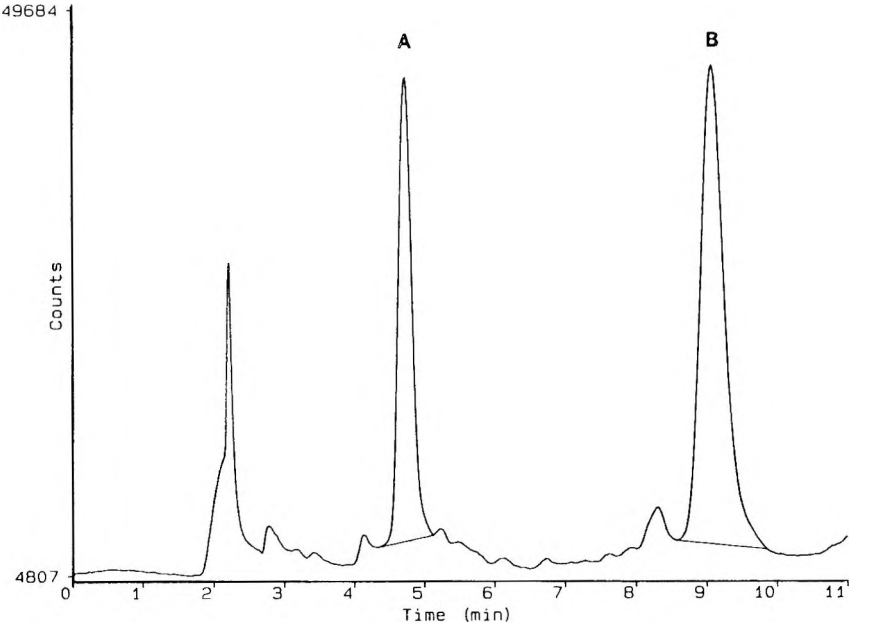

Figure 3. Active Kathon® CG components in Dow Corning® 1784 Emulsion after precipitation of siloxane.

Table 4

Normalized Area Response Values for Components A, B and A+B

	ppm A	Area% A / ppm A	ppm B	Area % B / ppm B	ppm A+B	Area % A+B / ppm A+B
Std 1	0.51	250.076	1.52	163.291	2.03	185.094
Std 2	1.02	247.392	3.00	167.358	4.02	187.665
Std 3	1.75	251.256	5.15	165.754	6.90	187.440
Std 4	3.21	247.677	9.46	166.116	12.67	186.780
Std 5	6.40	249.064	18.88	166.367	25.28	187.303

Chromatographic Interference

All of the data listed in this report for DC® 1784 Emulsion was generated by injection of the aqueous phase of the sample after precipitation of the siloxane polymer. Unfortunately, other water soluble components in DC® 1784 Emulsion remain in the aqueous phase and cause minor interference with elution of the Kathon® components. Figure 3 is a chromatogram of the aqueous phase of a production lot of DC® 1784 Emulsion (15.5 ppm active Kathon®) after precipitation of the siloxane. The chromatographic peaks representing these other components tended to shift in position, relative to the Kathon® peaks, from injection to injection. This made manual selection of the endpoints of the active component peaks necessary. The methanol/acetic acid solution added to the emulsion was adjusted to try to match the pH of the aqueous phase of the sample to the HPLC solvent in order to minimize baseline disturbances and fluctuating retention times. The use of an autosampler, so that the time between sample injections is constant, and/or a gradient elution system could potentially make the retention times of all the eluting peaks more repeatable and enable the chromatographic software system to automatically analyze the samples. However, at the time this method was developed, the QA HPLC system did not include an autosampler or gradient elution capability, so these two options were not pursued. Another option would be to remove the interfering components from the aqueous phase before injection. This could be very difficult and would most likely result in further dilution of the active Kathon® components, which would, in turn, reduce the sensitivity of the method. In the interest of keeping sample preparation as simple as possible with minimal dilution and handling, cleanup of the aqueous phase of the sample was not attempted.

The sample preparation procedure developed for Dow Corning® 1784 Emulsion was also found to work very well for HPLC analysis of Kathon® LX in Dow Corning® Antifoam B. DC® Antifoam B is a more complex formulation than DC® 1784 Emulsion, containing a greater number and concentration of water soluble components in addition to silicone. Some of these other components are visible in the chromatogram, but the baseline variability and retention time fluctuations observed in the DC® 1784 Emulsion analyses were not seen with the DC® Antifoam B samples. A chromatogram of the aqueous phase from a DC® Antifoam B sample (19 ppm active Kathon® LX) is shown in Figure 4. The fact that this sample preparation procedure allows HPLC analysis of the aqueous phases of these two, very different emulsions demonstrates that this method may be applicable to a wide variety of silicone emulsions containing Kathon® as a preservative.

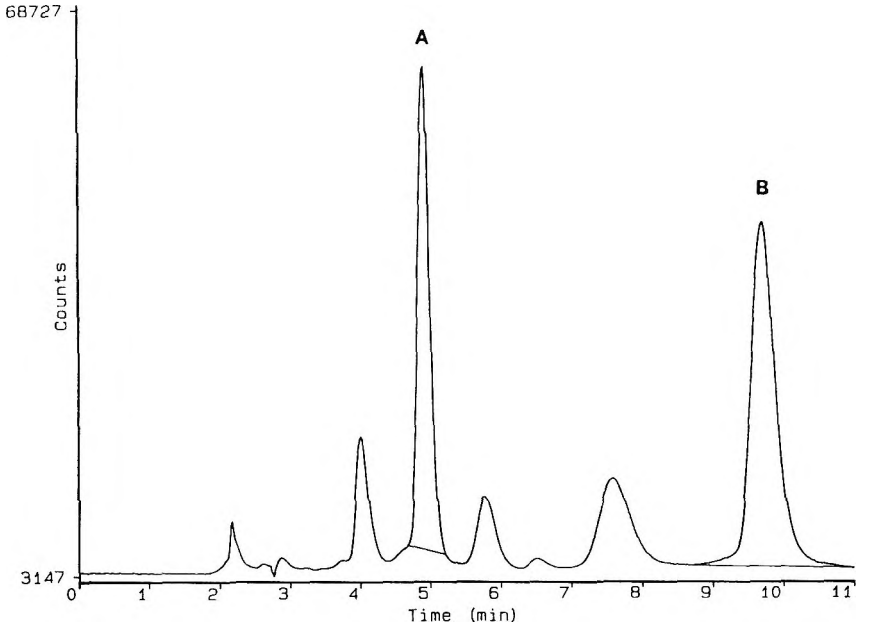

Figure 4. Active Kathon® LX components in Dow Corning® Antifoam B after precipitation of siloxane.

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SIMULTANEOUS QUANTITATION OF SOME PHENOTHIAZINE DRUG SUBSTANCES AND THEIR MONOSULPHOXIDE DEGRADES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY(HPLC)

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ABSTRACT

A simple liquid chromatographic procedure is presented for the simultaneous quantitation of three phenothiazine drugs, namely, perphenazine, trifluoperazine and triflupromazine and their sulphoxide degrades. The LC-separation was performed on a Novapak-phenyl-4 (150 x 3.9 mm id) column and a mobile phase composed of methanol + 1.5 x 10^{-2} M sodium acetate buffer pH 6.5 (81:19, v/v). Isocratic elution and UV-detection were adopted. Satisfactory percent assay and mean recoveries with low relative standard deviations were obtained for each drug and its sulphoxide in bulk and in tablet dosage form.

The stability-indicating characteristics of the presented LC-procedure prove its advantage over the pharmacopeial methods used to limit the sulphoxides.

INTRODUCTION

The phenothiazine drugs are clinically used as tranquillizers for the treatment of moderate and servere mental and emotional disturbances.[1] Chemically, these drugs are unstable as they decompose under the effect of various factors such as light, heat, oxygen and metal ions, particularly when existing in solution. Official monographs direct that preparations of phenothiazine drugs be protected from exposure to light and air, and ampoules filled under nitrogen.

It is believed that under mild changes of conditions,[2,3] the decomposition oxidation products are mainly the monosulphoxides some of which are found to be significantly less potent than the parent compounds.[4] However, mesoridazine, which is a side-chain monosulphoxide metabolic product of thioridazine, is well known to be psychoactive.[5-8] More serious is chlorpromazine sulphoxide which is as active pharmacologically as the parent compound, but it is suspected of causing photo-toxic side effects associated with chronic use of chlorpromazine.[9,10,11] It is this reason that has investigated many workers to explore reliable methods for the simultaneous quantitation of phenothiazine drugs and their sulphoxide degrades in bulk form and in pharmaceutical formulations.

Several quantitative analytical procedures based on ordinary spectrophotometry[12-15] were reported for the determination of phenothiazine drugs and their sulphoxides. But the ordinary spectrophotometric method lacks sensitivity and selectivity. Furthermore, second derivative spectrofluorometry alone[16] or combined with TLC,[17] second derivative and third-derivative UV-spectrophotometry,[18] and GLC[19] were also proposed.

Although the derivative UV-spectrophotometric procedures are more selective than the ordinary ones, yet the sensitivity is not improved. On the other hand, GLC exhibits adequate sensitivity and selectivity, but the phenothiazine intact molecule breaks down readily under the relatively high temperature utilized in the analysis.

For the three phenothiazine drugs investigated in this work, namely, perphenazine (PR), trifluperazine (TF) and trifluopromazine (TP), the British Pharmacopoeia (BP)[20] and the United States Pharmacopeia (USP),[21] adopt titrimetry and ordinary spectrophotometry for the assay of the raw drug and their tablet formulations, respectively.

In the present work, a high performance liquid chromatography (HPLC) is elaborated for the simultaneous assay of each of PR, TP and TM and their sulphoxides. The work clearly indicates the superiority of HPLC in the accurate determination of trace amounts of sulphoxide impurities that may contaminate the parent phenothiazine drug.

EXPERIMENTAL

Materials

Reference perphenazine (Lot No. P-4320) and trifluoperazine (Lot No. T-4333) were purchased from Winlab (U.K.). Triflupromazine reference material was purchased from Sigma Chemical Co. (U.S.A.). The powder materials of the three phenothaizine drugs were used without further treatment.

Perphenazine tablet formulations (Trilafon®-4 mg, Lot No. 91 L 160413) and trifluoperazine hydrochloride (Apo-Trifluoperazine-5 mg tablets, BN S-8260), were obtained from Schering (U.S.A.) and Apotex (Canada), respectively, through their Medical Representative Offices in Riyadh, Saudi Arabia.

Trifluoperazine hydrochloride (Stelazine®-5 mg tablet, BN 5800 and Stelazine®-1 mg tablet, BN 3030) were the products of SK & K (U.K.) and were kindly received from King Khalid University Hospital, King Saud University, Riyadh.

Water employed was all-glass doubly distilled. Methanol and acetonitrile (BDH) and chloroform (Fluka) used in chromatographic investigations were HPLC grade.

For the preparation of different buffer systems, sodium acetate trihydrate (Merck-AnalaR), boric acid (BDH-AnalaR), 85% orthophosphoric acid (Merck) and citric acid (Hopkin & William-AnalaR), were utilized.

Dodecyl sodium sulphate which served as ion-pairing reagent in ion-pair chromatographic experiments and hydrogen peroxide (Perhydrol-30% H_2O_2)

used for the preparation of peroxyacetic acid needed for the oxidation of parent phenothiazine drugs to their respective monosulphoxides, were the products of Merck.

Apparatus

The liquid chromatograph employed for the preliminary chromatographic investigations was Varian Model 5000 equiped with single piston reciprocating pump, pulse damper and high pressure injector Rheodyne 7125 of 20 μL loop. Varian variable wavelength UV-Visible detector model UV 50, CDS 111L integrator and recorder model 9176 were attached.

After fine-tuning of method development, the system used consisted of Waters 600 E liquid chromatograph equiped with injector U6K and Waters 486 tunable absorbance detector.

The column utilized was chemically bonded Novapak-phenyl-4, 150 x 3.9 mm id (Waters) and the mobile phase consisted of methanol and 1.5×10^{-2} M sodium acetate buffer of pH 6.5 (81:19, v/v). The optimum flow rate of the mobile phase and the UV detection were determined for each compound.

Measurements and adjusments of pH for solutions were made using the pH-meter Model 523 WTW (Germany).

Reagents

Peroxyacetic acid (CH_3COOOH)

Prepared by diluting 5 mL 30% H_2O_2 to 500 mL of glacial acetic acid and allowing to stand overnight at room temperature. This reagent when added to parent phenothiazine drug in methanol yields quantitatively the respective monosulphoxide.

Standard solutions of reference phenothiazine drug substance (025% w/v)

For each of the three phenothiazine drugs 25 mg of reference material was accurately weighed, transferred into 100 mL calibrated flasks and dissolved in methanol. All the flasks should be wrapped with aluminium foil since the phenothiazine derivatives are prone to photodegradation.

Methods

Preparation of the phenothiazine drug monosulphoxide

Based on Davidson's procedure (3,12) 5 mL-volume of peroxyacetic acid was added to 5 mL of the reference phenothiazine drug solution. The reaction mixture was left to stand at room temperature for 15 min. The completeness of the reaction was checked by TLC using silica plates. To remove excess glacial acetic acid and alcohol, the reaction mixture was evaporated under *vacco* at room temperature using oil pump. The residue of the sulphoxide obtained was dissolved in the appropriate solvent to yield a standard solution of the sulphoxide required for further work.

Establishment Of calibration curves

Standard series

From the stock methanolic solution of perphenazine (25 μg mL^{-1}) 1, 2,..., 6 mL portions were transferred into six 25-mL volumetric flasks followed by 1, 2,..., 6 mL portions of the standard perphenazine-sulphoxide solution. The internal standard solution was added (final concentration 3 μg mL^{-1}) to each flask before completion to volume with the mobile phase. The standard ratios of peak-area responses for perphenazine and perphenazine sulphoxide to the internal standard using triplicate injections of 10 μL were calculated for each solution. The mean ratios were plotted versus concentration of the parent drug and the sulphoxide. Alternatively, linear regression equations could be worked out using the least squares method. The procedure was repeated for trifluoperazine and triflupromazine and their sulphoxides.

Sample preparations

Twenty perphenazine tablets (Trilafon®-4 mg) were accurately weighed and finely powdered. The mean mass of material per tablet was calculated and a portion of powder containing about 4 mg of the active ingredient was accurately weighed. About 5 mL of water was added and the mixture was heated for 3 min on a water-bath. After cooling, approximately 50 mL of water was added and the mixture was shaken for about 15 min before completion of volume to 100 mL with methanol. The mixture was filtered and 5 mL volume of the filtrate was transferred into a 50 mL calibration flask, followed by the internal standard and adjustment of volume with the mobile phase.

The procedure was exactly repeated for trifluoperazine and triflupromazine tablets.

Procedure

Triplicate volumes of 10 μL each of the standard and sample preparations of perphenazine were injected into the chromatograph. The mean peak-area response ratios (Y) of perphenazine to the internal standard for the standard and sample preparations were calculated. The amount of perphenazine per tablet was computed from the expression:

$$\text{Amount of perphenazine (mg / tablet)} = \frac{A_1}{A_2} \times C \times \frac{W_1}{W_2}$$

where A_1 is the peak-area ratio of the sample, A_2 is the peak-area ratio of the standard, W_2 is the mass of portion of powder taken and C is the concentration of the final diluted standard solution.

Similarly the contents per tablet for trifluoperazine and triflupromazine determined.

Analysis Of Binary Mixtures Of The Parent Phenothiazine Drug And Its Sulphoxide

To investigate the capability of the elaborated HPLC method for the simultaneous quantitation of perphenazine, trifluoperazine and triflupromazine in presence of their respective sulphoxides, a set of HPLC experiments were performed using synthetic mixtures of each phenothiazine and its sulphoxide and powdered tablet samples spiked with the appropriate standard sulphoxide solution.

The ratio (m/m) for the binary mixtures and spiked samples was varied in such a way that the percentage of sulphoxide to parent compound spanned the range from 25 down to 0.25%.

The BP method[20] of assay was applied to the same solution analysed by the elaborated HPLC method and the results obtained were compared (See conclusion).

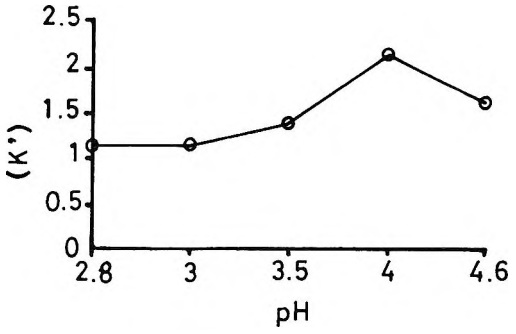

Figure 1: Capacity Factor (k') versus pH.

RESULTS AND DISCUSSION

The elaboration of an adequate liquid chromatographic method to address a given separation is usually performed by undertaking preliminary investigations involving various chromatographic systems. In this study of the three phenothiazine drugs and their sulphoxides, experiments were carried out using TLC, reverse phase ion-pair and reverse phase liquid chromatography. The results of the TLC on silica plates yielded acceptable R_f values (~0.5) for each drug and its sulphoxide employing a mobile phase consisting of methoanol and 1.5 x 10^{-2} M ammonium acetate buffer of pH 6.5 (75:25, v/v). This indicates that the methanol-acetate buffer system is potentially a suitable mobile phase.

Systematic investigations by adopting reverse phase liquid chromatography using μ-Bondapak C_{18}-10 (300 x 3.9 mm id) and Novapak phenyl-4 (150 x 3.9 mm id) columns with a mobile phase consiting of methanol, water, acetonitrile and 1.5x 10^{-2} M-sodium acetate buffer of pH 6.5, in varying proportions. Excellent separation was obtained on Novapak-4 column and methanol + 1.5 x 10^{-2} M-sodium acetate, pH 6.5 (81:19, v/v).

Similar studies for the suitability of reverse-phase ion-pair chromatography with μ-Bondapak C_{18}-10 (300 x 3.9 mm id) column, methanol + 1.50 x 10^{-2} M-sodium acetate mobile phase and sodium dodecyl sulphate as ion-pair forming reagent i.p.f.r. were conducted. Typical results of the effects of parameters such as pH and concentration of i.p.f.r. on capabcity factor (k') for perphenazine are demonstrated in Figs. 1 and 2.

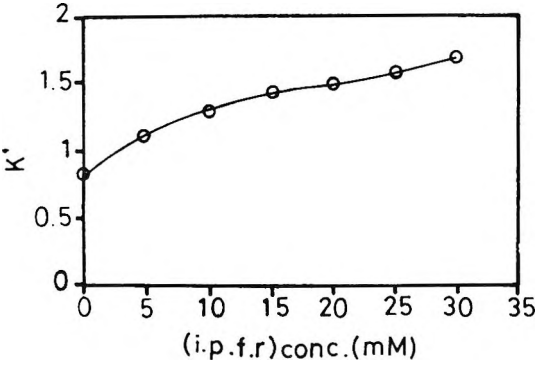

Figure 2: Capacity factor (k') versus concentration of ion pair forming reagent (i.p.f.r.).

As shown in Fig. 1, the increase in the pH of the mobile phase from 2.8 to 4.0 leads to an increase in the capacity factor (k') which decreases above pH 4.0. It was observed that at pH ≥ 6.5 the peak of perphenazine chromatogram was too broad leading to low sensitivity.

The effect of concentration of the i.p.f.r. was studied by adding varying amounts of sodium dodecyl sulphate to perphenazine in the mobile phase at pH 4.0. As a result of formation of an adduct of lower polarity than that of perphenazine alone, a better separation was expected. Figure 2 depicts typical findings for perphenazine where a steady increase of capacity factor, k', with the increase in the concentration of i.p.f.r. at pH 4.0 is observed.

The effect of buffer concentration on the chromatographic separation by ion-pair chromatography was investigated by utilizing increasing concentration of the acetate in the range 0.05 to 0.7 M at constant pH and ionic strength. A substantial reduction of the peak height occurred at buffer concentration above 0.1 M. This reduction might be due to the decreased formation of the ion-pair adduct owing to competition between dodeceyl sulphate and acetate ions.

Although separation parameters were worked out by adopting both reverse phase and ion-pair reverse phase chromatography, the former mode of liquid chromatography proved to be more satisfactory. Table 1 assembles the final chromatographic parameters worked out by employing HPLC in the reverse-phase mode. Figures 3-5 represent typical chromatograms for the three phenothiazine drugs studied, their respective sulphoxides and the internal standard.

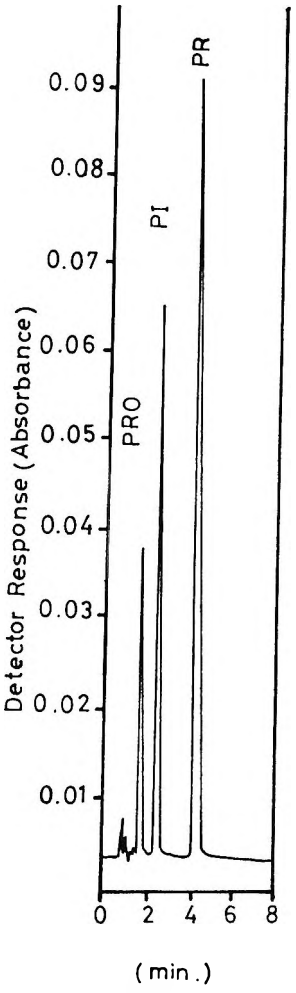

Figure 3: Chromatogram of perphenazine (PR), perphenazine sulphoxide (PRO) and pindolol (PI) as internal standard. AUFSD = 0.1, 10 μL injected. Retention times are 4.4, 1.7 and 2.5 minutes for PR, PRO and PI respectively. UV-detection at 254 nm, mobile phase flow rate 1.0 mL/min.

To test the suitability of the proposed HPLC system and to validate its performance characteristics such as precision, accuracy, linearity, limit of detection and ruggedness, a number of experiments were performed using the

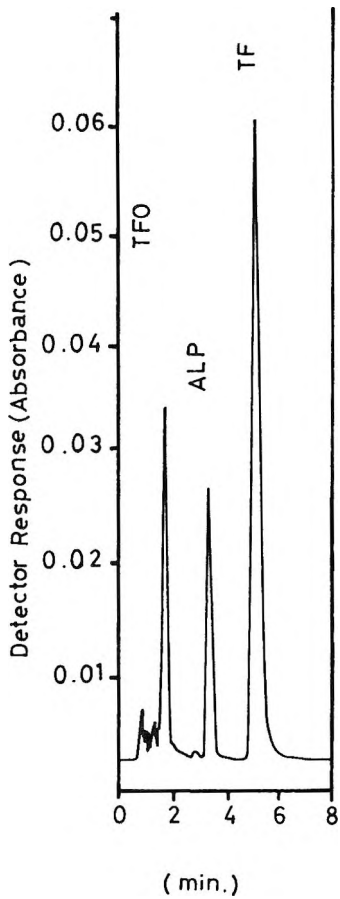

Figure 4: Chromatogram of trifluoperazine-2HCl (TF), trifluoperazine-sulphoxide (TFO) and alprenolol (ALP) as internal standard. AUFSD = 0.1, 10 μL injected. Retention times are 5.5, 1.8 and 3.4 minutes for TF, TFO and ALP respectively. UV-detection at 254 nm, mobile phase flow rate 1.2 mL/min.

three phenothiazine drugs (in bulk and in dosage form) and their sulphoxides. The entries of Tables 2 (a, b, c) summarize the ranges of concentrations employed and average peak-area response ratios (Y), for each phenothiazine drug and its sulphoxide. The intercept (A), the slope (B) and the correlation coefficient (r) of the best fit line was determined by least squares regression analysis. The detection limit (DL), which is the lowest detectable

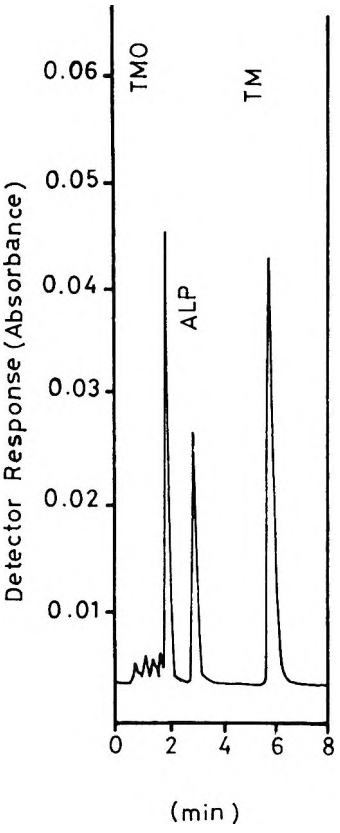

Figure 5: Chromatogram of triflupromazine-HCl (TM), triflupromazine-sulphoxide (TMO) and alprenolol (ALP) as internal standard. AUFSD = 0.1, 10 μL injected. Retention times are 6.1, 2.0 and 3.2 minutes for TM, TMO and ALP respectively. UV-detection at 254 nm, mobile phase flow rate 1.2 mL/min.

concentration, was determined for each compound by comparing test results from samples of known concentration of analyte with those of blank samples taking 3:1 as the signal-to-noise ratio.

To assess the reproducibility of the proposed HPLC system, at least six replicate 10 μL injections of standard solutions for each of perphenazine, trifluoperazine and triflupromazine and their sulphoxides were performed at

Table 1

The Chromatographic Parameters Established by RP-HPLC

*P' C':	PRO & PRO	TF & TFO	TM & TMO
Retention time (t_R) in minute	4.4 (PR) 1.7 (PRO) 2.5 (PI)	5.5 (TF) 1.8 (TFO) 3.4 (ALP)	6.1 (TM) 2.0 (TMO) 3.2 (ALP)
Capacity factor (k')	3.8 (PR) 1.12 (PRO) 2.2 (PI)	4.3 (TF) 1.2 (TFO) 2.5 (ALP)	4.6 (TM) 1.28 (TMO) 2.78 (alp)
Resolution (Rs)	3.7 (PR-PI) 1.82 (PRO-PI)	2.15 (TF-ALP) 2.28 (TFO-ALP)	4.1 (TM-ALP) 1.97 (TMO-ALP)
Column efficiency (N) plate/m	21333	20983	19987
Selective factor (α) ($=k'_2/k'_1$)**	1.70	1.72	1.65
Tailing factor, at 10%	1.20 (PR)	1.12 (TF)	1.25 (TM)

PR = Perphenazine, PR = Perphenazine sulphoxide.
TF = Trifluoperazine, TFO = Trifluoperazine sulphoxide.
TM = Triflupromazine, TMO = Triflupromazine sulphoxide.
PI Pindolol and (ALP) Alprenolol are internal standards.
*P' = Chromatographic Paramers, C' = Compound.
**refers to capacity factors for the parent phenothiazine and the internal standard.

low, medium and high concentrations of their linear ranges. The relative standard derivation (RSD) in all cases was less than 2% [Table 2 (c)], suggesting adequate reproducibility.

To examine the interday and intraday precision of the proposed HPLC method, as a measure of its ruggedness, a two-way analysis of variance (ANOVA) was performed using five replicates average peak-area ratios of

Table 2

The Parameters of the Standard Curves Using the Proposed HPLC Method (a), the Linear Regression equations, (b) and the Reproducibility Measured by R.S.D. %, (c)

Part (a)

Average Peak Area Response Ratios (Y)

PR/is	PRO/is	TF/is	TFO/is	TM/is	TMO/is
0.610	0.460	0.602	0.217	0.668	0.247
1.210	0.920	1.210	0.324	1.327	0.490
1.810	1.390	1.800	0.434	2.009	0.741
2.420	1.840	2.410	0.542	2.675	0.997
3.060	2.370	3.000	0.648	3.279	1.229
3.630	2.740	3.610	0.760	3.970	1.478
				4.680	1.719
				5.308	1.954

Part (b)

Regression equation Y = A + BC

C' P'	PR	PRO	TF	TFO	TM	TMO
A	-2.667×10^{-2}	0.000	3.333×10^{-3}	-4.286×10^{-4}	-3.773×10^{-3}	-6.679×10^{-3}
B	0.607	0.241	0.3000	0.054	0.664	0.1222
r	0.9999	0.9994	1.0000	1.0000	0.9999	0.9999
R	0.5-6	0.5-12	1-12	2-14	1-10	1-6
n	6	6	6	6	6	6
D.L.	0.1	0.2	0.15	0.15	0.1	0.2

(continued)

Table 2 (continued)

The Parameters of the Standard Curves Using the Proposed HPLC Method (a), the Linear Regression equations, (b) and the Reproducibility Measured by R.S.D. %, (c)

Part (c)

Reproducibility, R.S.D. %

C' / C	PR	PRO	TF	TFO	TM	TMO
Low	1.37	1.83	0.58	0.59	0.42	1.37
Medium	1.08	1.20	1.54	0.46	0.23	0.32
High	0.91	0.54	0.97	0.35	0.75	1.26

PR = Perphenazine, PR = Perphenazine sulphoxide.
TF = Trifluoperazine, TFO = Trifluoperazine sulphoxide.
TM = Triflupromazine, TMO = Triflupromazine sulphoxide.
is = Internal standard
A = Intercept
B = Slope
n = Number 0f determination
C = Concentration
D.L. = Lower detection limit in ng.
C' = Compound
R = Range of concentration in μgmL^{-1}
P' = Parameter
r = Correlation coefficient

medium concentration for each compound for five consecutive days. Typical ANOVA results for trifluoperazine dihydrochloride and its sulphoxide are displayed in Tables 3 (a, b). It is evident that at 95% confidence level, the tabulated variance ratios (F) at the specified degrees of freedom are greater than those observed with regard to between- and within-days variations. It can therefore be concluded that the precision is satisfactory based on the non-significant difference between the observed $F^{4,16}$ and the tabulated $F_{0.95}.^{4,16}$

To evaluate the accuracy of the proposed HPLC method, added recovery experiments were carried out by spiking known quantities of standard drug substance to the sample preparations. Typical and acceptable results for

Table 3 (a, b)

Two-way Analysis of Variance (ANOVA)

Part (a)
Daily Replicate Responses of 8 μg/mL Solution of Trifluoperazine 2HCl (TFO) and Two-way ANOVA

Day				
1	**2**	**3**	**4**	**5**
0.41	0.44	0.42	0.44	0.43
0.42	0.42	0.44	0.43	0.42
0.44	0.43	0.42	0.41	0.44
0.42	0.43	0.44	0.43	0.42
0.45	0.41	0.42	0.43	0.43

Part (b)
Daily Replicate Responses of 4μg/mL Solution of Triflupormazine Hcl (TM) and Two-way ANOVA

Day				
1	**2**	**3**	**4**	**5**
2.70	2.68	2.67	2.71	2.69
2.69	2.71	2.68	2.67	2.70
2.68	2.67	2.70	2.69	2.68
2.70	2.69	2.68	2.71	2.67
2.67	2.70	2.69	2.68	2.69

Source of Variation	**Degree of Freedom**	**Sum of Squares**	**Mean of Square**
Between days:	4	1.831055×10^{-4}	4.577637×10^{-5}
Within days:	4	2.136231×10^{-4}	5.340576×10^{-5}
Error	16	3.808699×10^{-3}	2.380437×10^{-4}
Total	24	4.205427×10^{-3}	

-Observed $F^{4,16}$ for between days variation = 0.1923024
-Observed $F^{4,16}$ for within days variation = 0.22243528
-Tabulated $F_{0.95}{}^{4,16}$ for the analytical error = 3.06

Table 4

Results of Determinations of Perphenazine (Trilafon®-4mg), Trifluoperazine 2HCL (Stelazine®-5mg) Tablets and Added Recovery Experiments by the Proposed HPLC Method

Perphenazine Tablets		Trifluoperazine Tablets	
mg/Tablet Found	%Added Recovery	mg/Tablet Found	%Added Recovery
3.97	97.7	4.94	98.3
4.00	99.1	4.98	98.0
4.05	99.8	4.94	98.8
4.08	100.1	4.92	99.2
4.05	100.4	4.93	100.0
4.05	99.7	4.91	100.5
4.06	----	4.92	----
4.06	----	----	----
4.01	----	----	----
4.00	----	----	----
****4.03	99.6%	****4.94	99.1%
***±0.04	±0.6%	***±0.023	±1.0%
**±0.88%	----	±0.46%	----
*4.03 ± 0.025	----	*4.94 ± 0.21	----

**** Mean mg per tablet
*** Standard deviation
** Relative standard deviation
* True mean at 95% confidence level

recovery experiments using perphenazine and trifluoperazine tablets were 99.6% ± 0.6 and 99.1% ± 1.0, respectively (Table 4).

CONCLUSION

The results summarized in Tables (5, 6 and 7) indicate that the elaborated HPLC method, unlike the BP[20] method, is capable of quantitating precisely and accurately perphenazine and trifluoperazine and their coexisting sulphoxides. The results obtained by the BP method are clearly for total recovered quantities of the parent compound and its sulphoxide. The BP method for the assay of the

Table 5

Results of the Analysis of Perphenazine and Its Sulphoxide

Part (a)

Results of the Analysis of PR & PRO Synthetic Mixture by the Proposed HPLC and BP Methods

Proposed HPLC Method				BP Method	
C. of PR μg/ml	C of PRO μg/ml	% m/m PRO:PR	% of PR recovered	% of PRO recovered	% of total recovered as PR
80	20	25.00	98.56	99.95	108.10
90	10	11.11	99.00	99.60	107.56
190	10	5.26	98.98	99.50	107.20
495	5	1.01	99.01	98.60	106.60
497.5	2.5	0.50	99.50	97.60	105.40
997.5	2.5	0.25	99.80	96.40	102.98

Part (b)

Results of the Analysis of PR Tablets Powder Spiked With PRO by the Proposed HPLC and BP Methods

Proposed HPLC Method				BP Method	
C. of PR μg/ml	C of PRO μg/ml	% m/m PRO:PR	% of PR recovered	% of PRO recovered	% of total recovered as PR
80	20	25.00	98.10	99.00	107.88
90	10	11.11	98.50	98.80	107.00
190	10	5.26	98.20	98.20	106.80
495	5	1.01	97.98	98.00	06.20
497.5	2.5	0.50	99.99	97.60	105.10
997.5	2.5	0.25	99.40	96.40	104.00

PR = Perphenazine
PRO = Perphenazine sulphoxide
C = Concentration

Table 6

Results of the Analysis of Trifluoperazine. 2HCl and its Sulphoxide

Part (a)

Results of the Analysis of TF & TFO Synthetic Mixture by the Proposed HPLC and BP Methods

Proposed HPLC Method				BP Method	
C. of PR μg/ml	C of PRO μg/ml	% m/m PRO:PR	% of PR recovered	% of PRO recovered	% of total recovered as TF
80	20	25.00	98.88	99.20	110.90
90	10	11.11	96.40	99.10	109.20
190	10	5.26	97.40	98.80	107.60
495	5	1.01	98.80	97.00	105.80
497.5	2.5	0.50	98.70	96.00	104.70
997.5	2.5	0.25	99.00	95.20	102.90

Part (b)

Results of the Analysis of TF Tablets Powder Spiked With TFO by the Proposed HPLC and BP Methods

Proposed HPLC Method				BP Method	
C. of PR μg/ml	C of PRO μg/ml	% m/m PRO:PR	% of PR recovered	% of PRO recovered	% of total recovered as TF
80	20	25.00	95.00	98.90	109.00
90	10	11.11	95.80	98.60	108.60
190	10	5.26	96.95	98.50	108.10
495	5	1.01	98.00	96.80	106.99
497.5	2.5	0.50	98.20	96.40	106.00
997.5	2.5	0.25	98.60	95.80	104.50

TF = Trifluoperazine. 2HCL
TFO = Trifluoperazine sulphoxide
C = Concentration

Table 7

Results of the Analysis of TM & TMO Synthetic Mixture by the Proposed HPLC Method

Proposed HPLC Method

C. of PR μg/mL	C. of PRO μg/mL	% m/m PRO:PR	% of PR recovered	% of PRO recovered
80	20	25.00	95.88	99.60
90	10	11.11	96.67	99.40
190	10	5.26	98.00	99.00
495	5	1.01	98.99	96.00
497.5	2.5	0.50	99.99	95.80
997.5	2.5	0.25	99.80	95.00

TM = Triflupormazine. HCl
TMO = Triflupormazine sulphoxide
C = Concentration

tablets is a spectrophotometric procedure which measures the absorbance of the two compounds additively, and hence the content per tablet will be for total recovered as parent compound. The superiority of the elaborated HPLC method over that of the BP stems from the ability of the former to separate and then quantitate.

It can be seen from Tables 5 and 6 that the elaborated HPLC method is sensitive to low levels of sulphoxide since accurate results can be achieved down to 0.25% m/m contamination. The sensitivity can be improved further if fluorometric or electrochemical rather than UV detection is used.

The results obtained for triflupromazine (USP official drug) in synthetic mixtures, (Table 7) confirm the suitability of the HPLC method for accurate and precise determination of triflupromazine sulphoxide in the presence of the parent compound.

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A REVERSE PHASE HPLC METHOD FOR QUANTIFICATION OF PEROXISOMICINE AND OTHER ANTHRACENONIC COMPOUNDS

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ABSTRACT

An HPLC analytical system is described for the separation, identification, and quantification of Peroxisomicine A_I (T-514) and related anthracenonic compounds.

Peroxisomicine is regarded as a potential antineoplastic agent. The system employed uses reverse phase (both C_8 and C_{18}) and diode array detection. The resolution, sensitivity, and reproducibility achieved make the system suitable to be employed for the analysis of plant extracts as well as biological fluids.

INTRODUCTION

Peroxisomicine A_I (3,3'-dimethyl-3,3',8,8',9,9'-hexahydroxy-3,3' ,4,4'-tetrahydro-(7,10')-bianthracen-1,1'-(2H,2'H)dione) also known as T-514 (Fig.1) is a dimeric anthracenonic compound isolated from plants of genus *Karwinskia.*[1] The plants from this genus are characterized by the toxicity of its fruit upon ingestion.[2,3,4] Peroxisomicine A_I exhibits selective *in vitro* toxicity on tumor cells,[5] suggesting that this compound could have a potential antineoplastic effect, and is currently under preclinical screening.

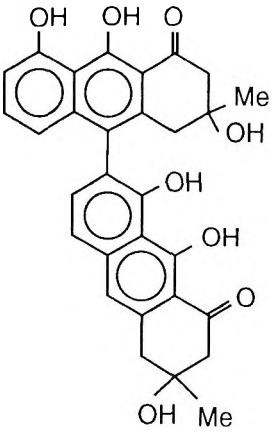


Figure 1. Structure of Peroxisomicine or T-514.

Peroxisomicine A_1 is found in the seeds of some species of the genus, especially in *K. humboldtiana* and K *parvifolia*.[6] From these plants, other secondary metabolites were isolated and identified. All of them are dimeric 9,10- dihydroxy anthracenones, which have been named according to their respective molecular weights. In *K. parvifolia* we reported five dimeric anthracenones besides Peroxisomicine A_1, namely: T-496 and T-516, previously described in *K. humboldtiana*, a diastereoisomer of Peroxisomicine A_1 (Diast T-514 or Peroxisomicine A_2), and, more recently, a third stereoisomer and a positional isomer of Peroxisomicine A_1, called Y and X respectively in this work. Details of the structures of these two compounds will be published elsewhere.

In order to solve the problems inherent to the production and quality control of this substance, we have considered necessary to have an analytical technique, with resolution, precision, sensitivity, and reproducibility. The proposed technique would be useful in investigating the pharmacokinetic studies of Peroxisomicine A_1.

Previous to this work, we reported the use of TLC (silica gel) and quantification by means of a densitometer,[7] taking advantage of the strong absorbance, in the visible range, of these type of compounds. Although this method proved to be useful for quantification of this and other anthracenones present in seeds and biological fluids,[8,9] we found severe difficulties when we tried to separate Peroxisomicine A_1 from other substances with similar Rf,

present also in the extracts. The errors inherent to quantification were not acceptable for our analytical requirements; the sensitivity was not adequate for studies on the mechanism of action of Peroxisomicine A_I; detection limit was 0.1 μg and calibration curves were linear in the range of 0.2-2 μg.

A spectroscopic method has also been reported to quantify another hydroxyanthracenone isolated from *K. humboldtiana* (the so called T-544 or tullidinol). This procedure has many interferences and the linearity reported is between 0.5 and 5 mM.[10]

Based on the physical properties, as well as spectral characteristics of Peroxisomicine A_I, we propose, in the present work, the use of the HPLC to solve the analytical problem. There is no previous report in the literature about the use of HPLC to analyze or separate dimeric anthracenonic compounds of this type.

EXPERIMENTAL

Chemicals

Acetonitrile, methanol, and distilled water were HPLC grade from Aldrich Co. Acetic acid was purchased from Merck. Standards of Peroxisomicine A_I and other anthracenonic compounds were isolated, purified, and identified in our laboratory by the procedures previously described.[6,7] Purity and identity was checked by chromatography, melting points, and spectroscopic data. Standards from these substances were prepared by diluting stock solutions of each compound (1 mg/mL in methanol) with methanol, HPLC grade. Due to instability of the compounds under investigation, the stock solutions were frozen at -20 °C and the dilutions were prepared just prior to the analysis.

Apparatus and Chromatographic Conditions

The HPLC system used was a Hewlett-Packard HP-1090 Series II/L. Samples of 5 μL were injected by an autoinjector onto one of the columns and conditions detailed as follows: a) Silica column (5μm, 300 x 0.4 mm); eluent: benzene/acetone (80:10),0.1% acetic acid; Flow 1 ml/ min.; b) bonded-phase CN-propyl column (5μm, 300 x 0.4 mm); eluent: CCl_4/ACN (98:2),0.1% acetic acid. Flow 1 mL/min; c) ODS-Hypersil column (5μm, 100 mm x 2.1 mm I.D.). Elution was accomplished as follows: 4 minutes isocratic with 35% solvent A, consisting of acetonitrile/water/acetic acid (30:70: 1.6) and 65% solvent B

(methanol); then a linear gradient was applied for 2 minutes to reach 100% B; during 2 minutes was maintained in 100% B and then return to the initial conditions in 2 minutes; an additional minute was left before applying the next injection. The flow was 0.4 mL/min; d) MOS Hypersil column (5 μm, 100 x 2.1 mm). Solvent systems were the same as in the ODS column, but the initial conditions were 50% B. All other parameters were similar. The temperature of the column was kept at 23° C in all cases.

All the mobile phases were filtered prior to use through compatible membrane filter (0.45 μm, Millipore). Signals were monitored by an HPLC 1090 photodiode-array detector with a main sample wavelength of 410 nm and a bandwidth of 10 nm. Signals were also recorded at 269 nm and 310 nm. with the same bandwidth. The reference wavelength was 550 nm with a bandwidth of 50 nm. Data were collected and analyzed on a HP HPLC 3D Chem Station, DOS series.

Preparation of Samples

For analysis of plant extracts, air dried ground fruits from *K. parvifolia* (0.5 g), were successively extracted with petroleum ether and ethyl acetate at room temperature by means of a stirrer, until the solutions appeared colorless. The ethylacetate extract containing the dimeric anthracenones was evaporated to dryness under N_2 (to prevent decomposition) and redissolved in methanol.

Total blood samples obtained from drug-free volunteers with EDTA were spiked with the adequate volume of a solution of Peroxisomicine A_1 (1 mg/mL), in order to obtain final concentrations of 5, 10, and 20 μg/mL; 0.3 mL of acetonitrile was added to 0.2 mL of these solutions, mixing thoroughly during 1 minute and centrifuged at 3000 rpm for 1 minute. The supernatant was isolated and filtered through Millipore filter, porosity 0.45 μm.

Analysis of Data

Results are expressed as mean ± SD and were statistically evaluated using the program "Mystat" for Macintosh. Linearity was assessed by means of regression square analysis for the calculation of correlation coefficients, slopes and intercepts.

Table 1

Retention Times of Dimeric Anthracenones from Genus *Karwinskia*

Compound	C_8 Column	C_{18} Column
Peroxisomicine A_2	1.66	1.64
Peroxisomicine A_1	2.65	2.43
T-516	2.85	2.42
X	2.55	2.85
Y	3.00	3.17
T-496	6.92	6.99

RESULTS

Analytical Separation

In order to choose an appropriate chromatographic system, we tested different stationary phases. Separation of the analyses required stationary phases that exhibited good resolution for qualitative and quantitative determination of the compounds of interest. Using silica, good resolution was obtained, but after several experiments, asymmetry in the peaks was observed, appearing from an excessive retention of compounds. With the normal bonded-phase (CN-propyl), good resolution was also obtained, both under analytical and in saturation conditions. The toxicity and cost of the solvents used were an inconvenience for their further use. With the reverse phase (Fig. 2) resolution, both under analytical and saturation conditions, was good and the time of analysis was only 10 minutes. We chose this system to continue our work; however, in order to identify some components in the plants, a C_8 phase was also needed, run under the same system of eluents as the C_{18}, but in different proportions. Retention times of the diverse anthracenonic compounds under analysis in both phases, are shown in Table 1.

Detection

The λ_{max} for these compounds lies between 260 and 270 nm, but we used another maximum at 410 nm to avoid interferences present in the biological matrix.

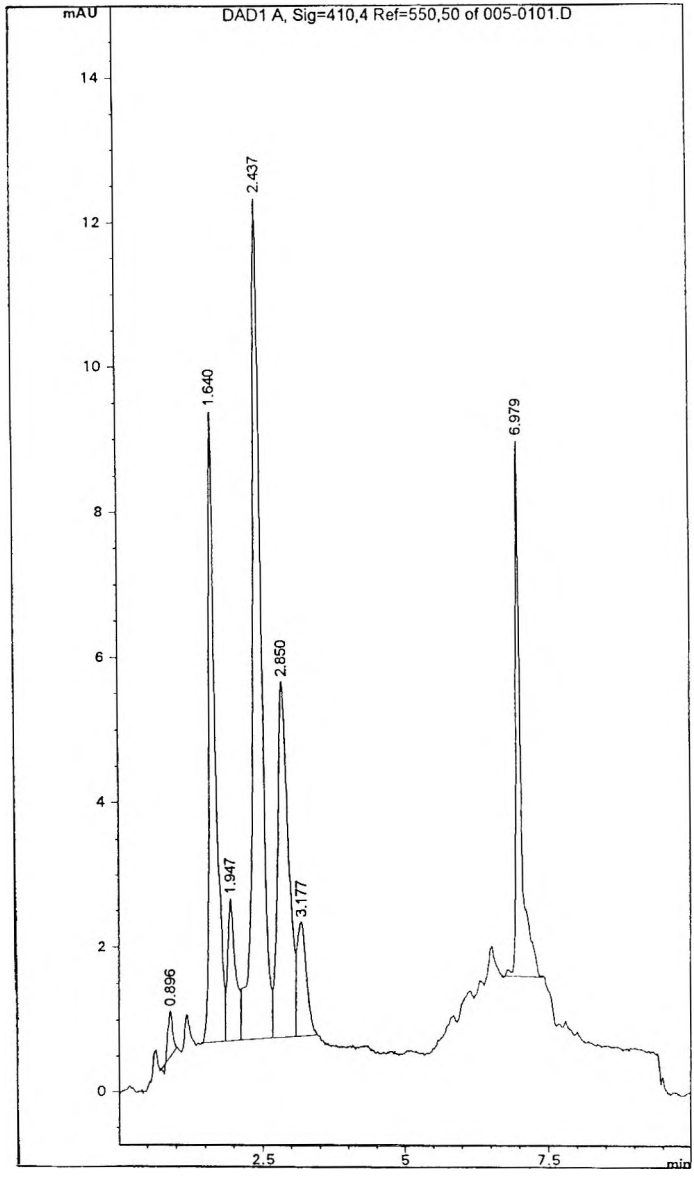
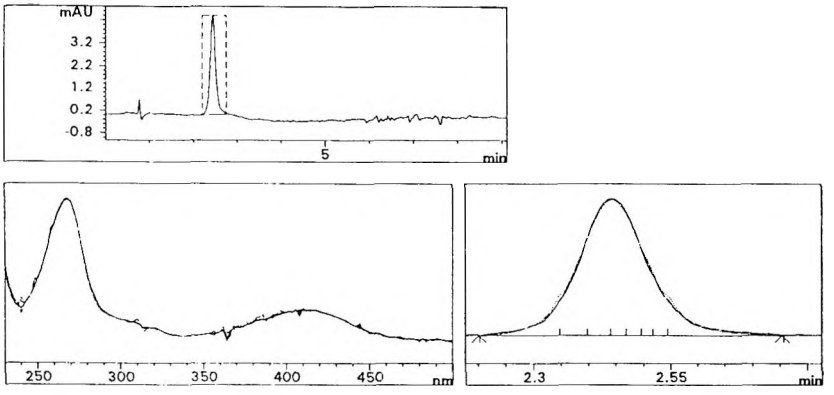


Figure 2. Chromatogram of an extract from seed of *K. parvifolia;* column ODS; peroxisomicine A_1 appears at retention time 2.43 min., peroxisomicine A_2 at 1.64 min; compound X at 2.85 min; compound Y at 3.17 min and T-496 at 6.98 min. Elution conditions are in the text.

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--> Spectral Data contains no impurity <--

Purity level   : 997.569  (mean, threshold 990)
Reference      : Nearest Peak baseline spectra (integrated)
PeakStart/End  : BaseLine / BaseLine
Spectra        : 7 (Range 5.4/21.3 mAU) (Selection set by user, 7)
Spectra        : 0 % below threshold of 990
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Figure 3. Spectral purity analysis of peroxisomicine A_1, using diode array detector. Spectral range: 220-600 nm. Absorbance ratios were displayed at 269, 310 and 410 nm. The level of purity obtained for this fraction was 99.97%

In order to verify the purity of the standards used, the spectral purity was tested by a diode array system, by superposition of spectra in 7 different points of the chromatogram and calculating the absorbances ratios at 269, 310 and 410 nm. Fig. 3 shows the spectral purity analysis of the Peroxisomicine A_1

In some instances, we complemented this analysis with the use of the C_8 system (Fig 4).

Precision

The precision of the chromatographic system was evaluated by injecting constant volumes (5 μL) of standards of Peroxisomicine A_1 in concentrations ranging from 1 μg/mL to 250 μg/mL. The coefficient of variation (CV) of retention times, areas, and heights are summarized in Table 2.

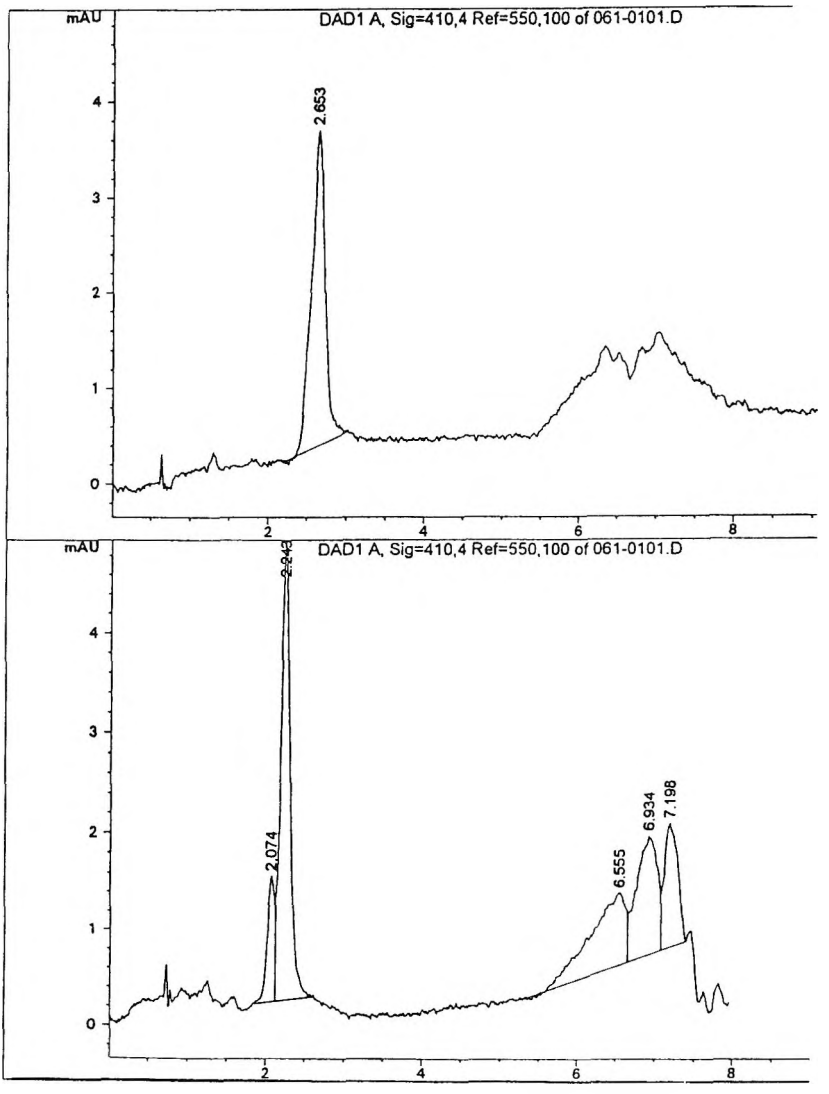

Figure 4. Chromatograms of the same fraction in C_{18} (upper) and C_8 (lower). Conditions as in text.

Linearity and Sensitivity

A linear relationship was obtained between area and concentration, within a range of 5-250 ng of injection. The assay linearity was checked by linear regression analysis of independent calibration curves. Using peak areas,

Table 2

Precision of Areas, Heights and Retention Times for Standards of Peroxisomicine A_1

n	Mass (ng)	Area X	Area CV	Height X	Height CV	Ret. Time (min) X	Ret. Time (min) CV
6	5	11.82	10.8	--	--	2.41	0.2
10	30	54.13	8.8	5.96	10.4	2.41	0.1
9	50	71.78	5.2	8.10	6.0	2.39	0.2
10	100	126.62	1.8	14.16	2.4	2.38	0.1
6	400	791.56	1.4	76.29	1.1	2.41	0.1
6	1000	2132.49	1.0	167.43	0.5	2.43	0.1

Table 3

Recovery Rate of Peroxisomicine A_1 Added to Blood Samples

Conc (μg/mL)	n	Recovery (%)	S.D.	C.V.
5	5	81.84	4.38	6.56
10	3	83.58	2.55	3.05
20	3	82.60	2.87	3.51

correlation coefficients were computed as ranging between 0.998 and 0.999. The curves intercepts were -2.75 ± 1.05; slopes were 0.478 ± 0.02.

Recovery

Samples of blood, spiked with peroxisomicine A_1 in concentrations of 5, 10, and 20 μg/mL, were processed as described in Experimental. Percents of recovery obtained are shown in Table 3.

DISCUSSION

At present, there are no previous reports about HPLC analysis of hydroxyanthracenones. The method herein reported has the aim of achieving the requirements of sensitivity and resolution required for the analysis of the

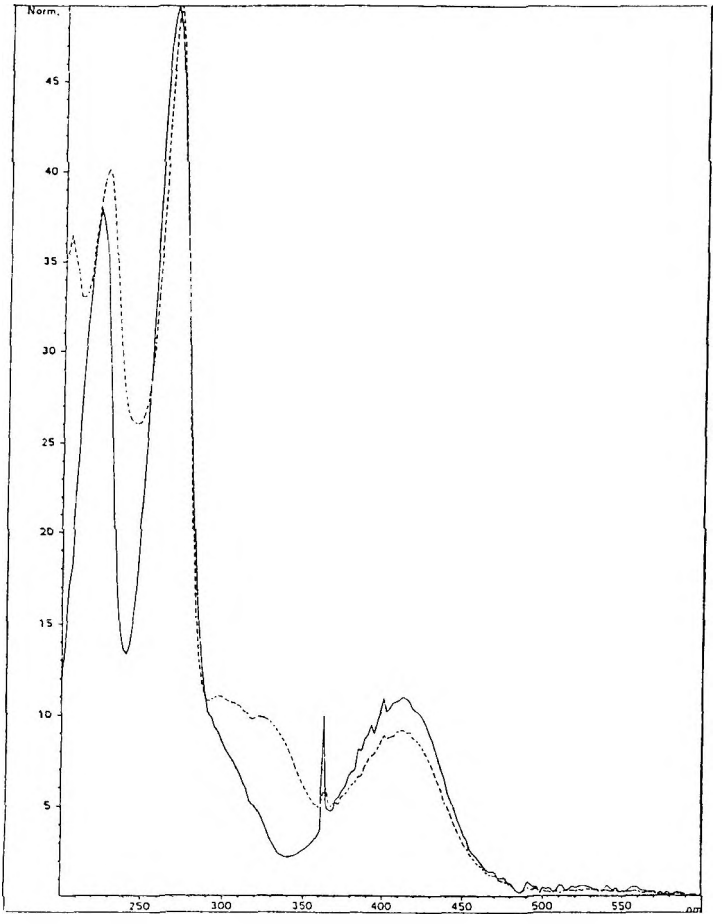

Figure 5. Spectra taken at two sites of the peak appearing in the upper chromatogram of Figure 4 (C_{18}) show the impurity present.

compounds of interest, as well in biological fluids as in plant extracts. It is also rapid, simple, accurate, and robust in application.

Separation

During the development of the methodology, we tried different solvent systems as well as different columns. Only the reverse phase systems (both C_{18} and C_8) exhibited satisfactory properties. It was necessary to use gradients for

the analysis of plant extracts because some of the compounds showed long retention times when the elution was isocratic. This is the case for one of the main components in seeds, the so called T-496, which can be eluted only when we achieve 100% methanol.

Symmetry, as well as width of the peaks, were significantly improved with the use of acetonitrile and acetic acid. The lengths of the columns used and the low flow rates (0.4 mL/min) increased sensitivity by using less solvent.

We also emphasize the importance of using two different chromatographic systems in order to separate all the components present. As is shown in Table 1, the compound X appears between Peroxisomicine A_1 and compound Y in C_{18}, while, in C_8, it appears before Peroxisomicine A_1; the order of elution of Y does not change. Similarly, T-516 is not well resolved from Peroxisomicine A_1 in C_{18}, but, in C_8, both compounds are properly separated.

Detection

Although satisfactory results can be obtained at a fixed λ, the diode array system presents the advantage of detecting at various λ simultaneously, helping us to assure identity and purity of the peaks. The relationships of areas and heights of the peaks at three different λ (410, 269 and 310 nm) as well as the analysis in two different systems, allowed the detection of impurities which elute sometimes near the compounds of interest (Figs. 4 and 5).

Quantification

We only report the results of quantification for the Peroxisomicine A_1. This is the compound of more interest in our laboratory due to its biological activity.

We tried to use an internal standard, and for that purpose we thought that Peroxisomicine A_2 was a good option, but the recovery was substantially less when we used this compound; for this reason we decided to use the external standard method. The lower limit of quantification was 5 ng for the Peroxisomicine A_1. If necessary, this limit can be easily improved by using UV detection or fluorescence instead of visible light. However, in this work we prefer the wavelength 410 nm because there are no interferences from the biological material.

A high recovery extraction procedure was a prerequisite to achieve the results required. We observed that the method proposed for the preparation of the sample prior to analysis and precipitation of proteins is advantageous due to its low cost, rapidity, small sample volume, and precision in the results.

We examined mixtures of acetonitrile/sample of 2:1 and 1.5:1. The results obtained by both methods were not significantly different, so we chose the last one which meant less dilution of the sample.

When applying the steps considered as optimal for sample extraction and chromatographic separation to blood analysis, all blank samples tested revealed no interferences with endogenous substances.

Applicability

The method described here is used currently to analyze plant extracts from genus Karwinskia (aerial and subterranian parts) as well as monitoring the purification procedures of Peroxisomicine A_1 and similar compounds.

The diode array detector, coupled with the chromatography system, is of great utility to assure purity of the different lots of peroxisomicines used in biological and clinical trials (Phase I).

We also propose this methodology as an alternative to the TLC method already described in order to analyze blood from people intoxicated with these plants.

Implementation of this method to the pharmacokinetics and metabolic studies of Peroxisomicine A_1 are currently in progress.

We are currently working on the conditions to scale up the procedure for the isolation and purification of Peroxisomicine A_1.

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EVALUATION OF THE CHROMATOGRAPHIC BEHAVIOUR OF FLUOXETINE AND NORFLUOXETINE USING DIFFERENT CYCLODEXTRINS AS MOBILE PHASE ADDITIVES AND FLUORIMETRIC DETECTION

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ABSTRACT

The principal goal of this work was to investigate the liquid chromatographic retention behaviour of fluoxetine and norfluoxetine using HPLC with respect to mobile phase composition, pH, flow rate and the amount of the native β-cyclodextrin (β-CD) or the β-hydroxypropyl-cyclodextrin (HP-β-CD), added to the mobile phase. Further, it was of interest to evaluate the effectiveness of β-CD and HP-β-CD to enhance fluorescence detection of these compounds.

Another purpose of this study, was to calculate the formation constants of the inclusion complexes (K_f) of fluoxetine and norfluoxetine within the HP-β-CD, in different mobile

phase compositions. Based on these findings, the log K_f values of these two compounds referred to pure aqueous mobile phase (log K_{fw}), can be determined by extrapolation.

INTRODUCTION

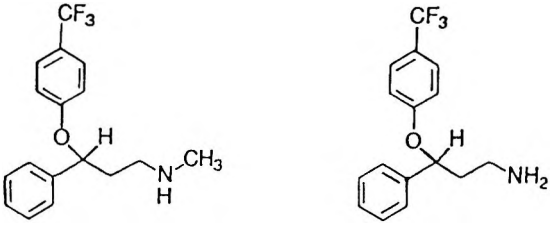
Fluoxetine is an important new antidepressant drug for the treatment of unipolar mental depression. Both fluoxetine (FL) and its N-desmethylated metabolite norfluoxetine (NR) (fig. 1), enhance serotoninergic neurotransmission through potent and selective inhibition of presynaptic serotonin reuptake.[1-3]

FL, as well as NR, are two compounds of great pharmacological and analytical importance. Several HPLC methods have been reported for the determination of FL and NR in serum or plasma.[4-9] Since FL is marketed as a racemic mixture, a number of methods have been reported for the indirect[10-12] and direct[13] chromatographic separation of the enantiomers of FL and NR.

Cyclodextrins (CDs) are cyclic oligosaccharides composed of six or more glycopyranose moieties bonded together via α-(1,4)-linkages. Generally, the external part of the CD molecule is hydrophilic compared to its cavity. The hydrophilic nature of the molecule's external portion is due to the primary and secondary hydroxyl groups, being located on the smaller and larger sides of the CD molecule, respectively. CDs have the ability to form inclusion complexes with a variety of molecules. The formation of an inclusion complex depends on the shape, size and spatial geometry of the solute, the diameter of the CD cavity and other factors.[14]

In this paper, the liquid chromatographic retention behaviour of FL and NR was investigated by using a reversed phase column (RP-Spherisorb-phenyl) with respect to mobile phase composition, pH, flow rate and the amount of the β-cyclodextrin (β-CD) or the 2-hydroxypropyl-β-CD (HP-β-CD) added to the mobile phase. Moreover, the effectiveness of β-CD and HP-β-CD, to serve as fluorescence enhancement agents on the chromatographic detection of these compounds was studied.

Generally, considerable attention has been focused on the use of CDs in luminescence applications.[15-17] The presence of CDs can dramatically enhance the fluorescence signal of complexed solutes. The factors thought of being responsible for such intensified luminescence, include shielding of the CD-



Fluoxetine **Norfluoxetine**

Figure 1. Molecular structure of Fluoxetine and Norfluoxetine.

complexed analyte molecule from quenching by water molecules or solvent-borne quenchers. In addition, the included solute molecule experiences a less polar and more rigid local microenvironment.[15,18]

Chromatographic separation, using CDs, is mainly the result of variation in the stability of inclusion complexes of the analytes with the CD. The elution time of a solute is a function of the stability of these complexes. Several intermolecular interactions are responsible for the formation of these complexes. These driving forces act synergistically and are related to the physicochemical properties of the guest molecule. Since the formation constant (K_f) of the complex is dependent on many factors the use of CDs as mobile phase additives provides a high degree of selectivity.

From this point of view, in this work special attention was devoted to determine the apparent formation constants of FL and NR complexes (K_f) within the HP-β-CD. The effect of the organic modifier, e.g., acetonitrile, on the inclusion process has been investigated by using mobile phases with different amounts of the organic solvent. Finally, the K_f values of these two compounds referred to pure aqueous mobile phase (K_{fw}) have been found experimentally by extrapolation based on the above mentioned data.

MATERIALS

All solvents were of HPLC-grade and were purchased from Tech-line (Athens, Greece). Triethylamine and glacial acetic acid were of analytical grade and were purchased from Aldrich. Fluoxetine and Norfluoxetine in the form of their hydrochloride salts were kindly provided by Eli Lilly and used as received.

METHODS

Apparatus

The liquid chromatographic system consisted of a Waters model 501 pump, a Rheodyne model 7125 injector with 5μL, 20μL and 100μL loops, as well as a Perkin Elmer LS30 fluorimetric detector with an 8μL flow cell. The chromatograms were obtained by using a Hewlett Packard integrator model HP3394A.

A Spherisorb-phenyl S5 column (150 X 4.6-mm I.D.) was obtained from Hellamco (Athens, Greece). When not in use, the column was stored in 100% methanol. pH readings were obtained by using a Metrohm Herisau pH-meter (model 654). All experiments were performed at room temperature (about 25°C). The compounds were detected at an excitation wavelength of 235nm and an emission wavelength of 315nm.

Chromatographic Conditions

The mobile phases, consisting of triethylammonium acetate buffer and the appropriate amount of the organic modifier, were freshly prepared, filtered and degassed under vacuum by using a Millipore system.

Buffers were prepared using triethylamine solutions of different concentrations (i.e., 0.1%-2.5%) which were adjusted by addition of glacial acetic acid to the desired pH (i.e., 3.5-7.0).

The effect of the organic modifier in this study was examined by preparing organic/aqueous mobile phase systems. The organic modifiers used were: methanol, acetonitrile, tert-butanol, cyclopentanol.

Solutions

The stock standard solutions of the compounds (1.00mg/mL) were accurately prepared by dissolving an appropriate amount of the compound in HPLC-water and kept in amber-coloured bottle in a refrigerator and renewed every week. Working standard solutions of each compound (1.00μg/mL) were prepared every day in mobile phase. Typically a volume of 5μL of each solution was injected. The void volume of the column was determined by injecting 5μl. of pure methanol. To evaluate the reproducibility of the retention times each run was performed three times.

A standard curve and five validation samples at five concentrations of each compound were assayed, i.e., 50-, 100-, 200- 350- and 500 ng/mL. The standards were prepared by diluting the appropriate aliquots of the stock solution with mobile phase. Typically, a volume of 20 μL or 100 μL of each standard was injected. The standard curves were generated by a linear least-squares regression analysis of the peak height of each compound versus its concentration.

RESULTS AND DISCUSSION

Preliminary studies

The effect of mobile phase composition on the retention time and the resolution of FL and NR, using CDs as mobile phase additives, was investigated by changing the organic modifier/buffer ratio in the mobile phase from 10:90 to 50:50. Acetonitrile, MeOH, t-BuOH and cyclopentanol have been used as organic modifiers in binary or ternary mobile phase mixtures. Further, the presence of the organic modifier in the mobile phase, leads to an enhancement or a reduction of the fluorescence signal, with respect to its ability to compete with solutes for the β-CD cavity. The best results were obtained by using ACN as organic modifier.

The effect of the ionic strength on the retention behaviour of FL and NR was studied by varing the concentration of triethylammonium acetate (TEAA) from 0.1 - 2.5% (w/v) at different pH values. The decrease of salt concentration results in an increase of retention and resolution.

On the other hand, the effect of pH in this study was investigated by changing the pH of the aqueous content of the mobile phase from 3.5 to 7.0 (respecting column limitations). As pH increases, the solutes become more retained and hence, the column's inclusion selectivity and solutes' resolution also increase.

Furthermore, the effect of the flow rate on the retention of FL and NR was investigated by decreasing the flow rate (v) from 2.0 to 0.2 mL/min.

The presence of CDs in the mobile phase enhances the fluorescence signal which depends on the CD concentration. This dependence is no doubt due to the increased proportion of the solute molecule which is included in the CD cavity. Excited states of analyte molecules possessing a -NH moiety, as FL and NR, are efficiently quenched by water molecules.[15] Thus, fluorescence enhancement, observed for the molecules of FL and NR can be due to the elimination, or the reduction, of their exposure to water molecules with the added CDs media.

Typically, addition of β-CD and HP-β-CD in the mobile phase always results in an enhanced fluorescence compared to that observed in bulk water; the relative magnitude of this enhancement was found to be dependent upon the specific β-CD system used. This is presumably due to their different complexing tendencies.

It has been reported that HP-β-CD exhibits an equal or worse complexing ability than native β-CD does; nevertheless, the use of HP-β-CD has two main advantages compared to the β-CD: it presents lower solution viscosity and greater water solubility at each concentration level .

Moreover, it appears that for the molecules of FL and NR, HP-β-CD exhibits greater fluorescence enhancement ability than native β-CD does. Figure 2 presents the typical chromatograms by injecting equal amounts of FL and NR, under the same chromatographic conditions, without β-CD (fig. 2a) and in the presence of 10mM β-CD (fig. 2b) and 10mM HP-β-CD (fig. 2c), respectively. With the addition of native β-CD, as it is presented in Table I, the fluorescence signal has been increased by 22% and 51% for the molecule of NR and FL, respectively. The addition of the same amount of HP-β-CD results in greater fluorescence output and the signal has been increased by 40% and 110% for the molecule of NR and FL, respectively.

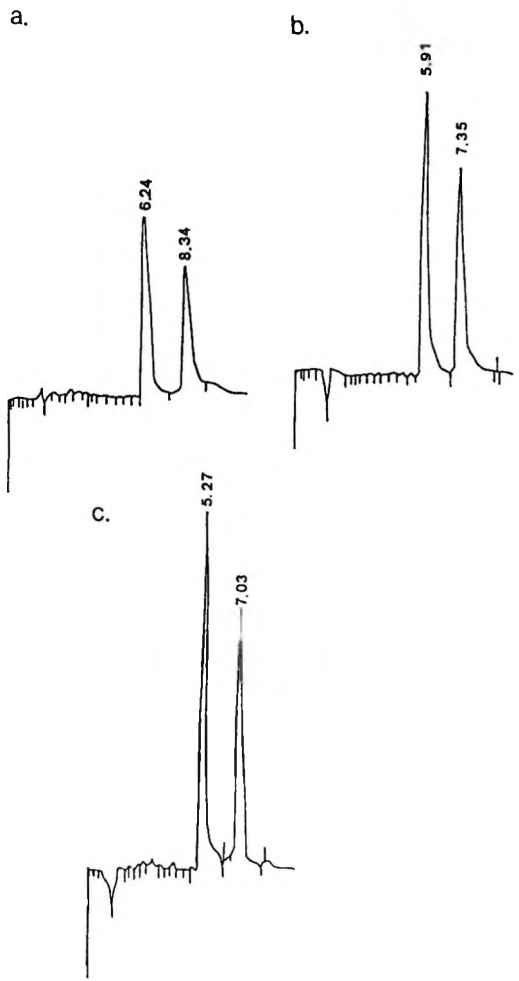


Figure 2. Chromatographic resolution of NR and FL: a. without β-CD b. in the presence of 10mM β-CD, c. in the presence of 10mM HP-β-CD. Chromatographic conditions: 0.5 %TEAA (pH:6.5) /ACN, 60/40, flow rate : 1.5ml/min.

Table 1

Enhancement of Fluorescence* of Norfluoxetine and Fluoxetine in the Presence of Different Cyclodextrins Media.

Compound	Without CD	With 10mM β-CD	With 10mM HP-β-CD
NR	26257 (±78)	32244 (±66)	45108 (±67)
FL	18086 (±56)	27367 (±45)	39708 (±85)

*Representative peak height corresponding to 5μL injections of 1μg/ml of each compounds; integrator attenuation 4; n=5 (runs performed).

Table 2

Equations Relating Peak Height (H) with β-CD Concentration
$H = a + b \cdot [\beta\text{-CD}]$

Compound	a	b	r	n
NR	9094 (±2377)	2547040 (±228170)	0.990	5
FL	9547 (±1099)	1863680 (±105522)	0.995	5

a : intercept, b : slope, r : correlation coefficient, n : number of points

Fluorescence Chromatographic Detection of FL and NR by Using β-CD as Mobile Phase Additive

It has already been mentioned that the enhancement of the fluorescence signal with the addition of CDs media depends on the CD concentration. As it is shown in Table 2, the peak height of FL and NR increases linearly by increasing the β-CD concentration from 5mM to 17mM, under the same chromatographic conditions (i.e.,mobile phase: 0.5% TEAA (pH:6.5)/ACN: 60/40, flow rate : 1.7mL/min).

Table 3

Equations Relating Peak Height (H) with Flow Rate (v) :
$1/H = a + b \cdot v$

Compound	a	b	r	n
NR	$5.84 \cdot 10^{-6}$ ($\pm 1.26 \cdot 10^{-6}$)	$3.78 \cdot 10^{-5}$ ($\pm 1.62 \cdot 10^{-6}$)	0.997	5
FL	$5.29 \cdot 10^{-6}$ ($\pm 1.74 \cdot 10^{-6}$)	$4.49 \cdot 10^{-5}$ ($\pm 2.23 \cdot 10^{-6}$)	0.996	5

a : intercept, b : slope, r : correlation coefficient, n : number of points.

Moreover, an increase of the fluorescence signal (H) has been observed when the flow rate (v) of the mobile phase decreases from 1.3 to 0.3mL/min, keeping the other chromatographic conditions constant, i.e., mobile phase:0.5% TEAA (pH:6.5)/ACN, 60/40 and β-CD 10mM. Table 3 shows the dependence of peak height on flow rate.

On the other hand, a considerable decrease of flow rate (v) results in an approximately 3.5-fold enhancement of fluorescence which leads to a better sensitivity. The difference in slope values presented in Table 3 indicates that the influence of flow rate is greater for the molecule of FL.

It seems that by decreasing the flow rate the molecules have greater opportunity to interact with the CD cavity and thus fluorescence signal is further intensified, possibly due to a greater tendency of solutes to be encapsulated into the protective microenvironment of CD cavity.

Fluorescence Chromatographic Detection of FL and NR by Using HP-β-CD as Mobile Phase Additive

HP-β-CD presents greater water solubility than β-CD and the use of more concentrated HP-β-CD medium in lieu of native β-CD, leads to a better sensitivity. As shown in tables 4 and 5, the peak height of FL and NR increases as the concentration of HP-β-CD increases from 5mM to 50mM under the same

Table 4

Equations Relating Peak Height (H) of NR with [HP-β-CD]
$H = a + b \cdot [HP\text{-}\beta\text{-}CD]$

Buffer/ ACN	a	b	r	n
90/10	24759 (±2401)	1243870 (±87680)	0.992	5
80/20	39235 (±1431)	1521310 (±52289)	0.998	5
70/30	51595 (±939)	2226310 (±34301)	0.999	5
60/40	101219 (±3872)	2188430 (±141413)	0.994	5
50/50	159686 (±1885)	1838690 (±68866)	0.998	5

a : intercept, b = slope, r = correlation coefficient, n = number of points

Table 5

Equations Relating Peak Height (H) of FL with [HP-β-CD]
$H = a + b \cdot [HP\text{-}\beta\text{-}CD]$

Buffer/ ACN	a	b	r	n
90/10	19633 (±1242)	934473 (±45384)	0.996	5
80/20	29020 (±1399)	1477040 (±51109)	0.998	5
70/30	46655 (±1660)	2341910 (±60644)	0.999	5
60/40	81486 (±1084)	1822680 (±39583)	0.999	5
50/50	135523 (±2099)	1101420 (±76658)	0.993	5

a : intercept, b = slope, r = correlation coefficient, n = number of points.

chromatographic conditions (e.g., flow rate : 0.8mL/min, 0.5% TEAA (pH : 5.5)) using different mobile phase ratios.

In reversed-phase HPLC it is common to use aqueous/organic mobile phase systems. In addition to the dilution effect, the presence of the organic modifier in the mobile phase leads to another complication with the CDs

Table 6

Equations Relating the Peak Height (H) with flow rate (v) :
$1/H = a + b \cdot v$

Compound	a	b	r	n
NR	$7.22 \cdot 10^{-6}$ ($\pm 7.44 \cdot 10^{-7}$)	$7.70 \cdot 10^{-6}$ ($\pm 8.70 \cdot 10^{-7}$)	0.981	5
FL	$1.41 \cdot 10^{-5}$ ($\pm 7.61 \cdot 10^{-7}$)	$9.18 \cdot 10^{-6}$ ($\pm 8.89 \cdot 10^{-7}$)	0.986	5

a : intercept, b : slope, r : correlation coefficient, n : number of points.

media, since the organic solvent will compete with the analyte molecules for the CD cavity binding sites, which reduces the percentage of complexed (and protected) fluorophore. In fact, as shown in Tables 4 and 5, as the ACN concentration in the mobile phase increases, the fluorescence intensity observed also increases.

Using HP-β-CD as a fluorescence enhancement agent, the intensity increases tremendously by decreasing flow rate (v) from 1.3 to 0.3mL/min, keeping the other chromatographic conditions constant i.e., mobile phase:0.5% TEAA (pH: 5.5)/ ACN : 90 / 10, HP-β-CD 15mM. Table 6 describes the dependence of peak height (H) of FL and NR on flow rate.

After studying the liquid chromatographic retention behaviour of NR and FL by using CDs as mobile phase additives and with respect to mobile phase composition, pH, ionic strength, a set of isocratic conditions was chosen for the simultaneous separation of these compounds. The conditions are as follows: acetonitrile / buffer, 60/40 (v/v); buffer 0.5% TEAA (w/v); pH 7.0; flow rate : 1.7 mL/min; detection Ex: 235nm, Em: 310nm, attenuation factor : 3 (fluorimetric detector).

The calibration curve for each compound was constructed from a linear-squares regression of peak-height of the standards versus the concentrations. Typical correlation coefficients were r : 0.9998. The linearity of the curve has

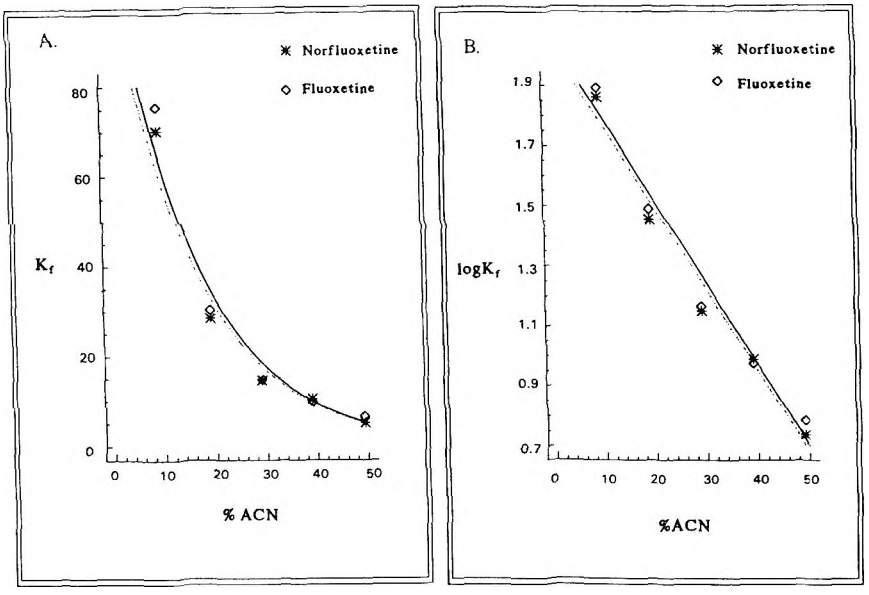


Figure 3. Dependence of the capacity factor (k') of NR (A) and FL (B) on HP-β-CD concentration.

been demonstrated from 50 ng to 500 ng/mL. These concentrations have been chosen according to the produced plasma concentrations at steady state for both compounds which, after a daily oral dose of 60mg, are between 200 and 500 ng/mL for fluoxetine and 180 and 450 ng/mL for norfluoxetine.[10] The proposed method was tested by analysing five replicates of a series of standard solutions: 50-, 100-, 200-, 350-, 500 ng/mL. The limit of quantitation of the method is 5ng/mL for FL (i.e.,500 pg injected amount) and 4ng/mL for NL (i.e.,400 pg injected amount).

Calculation of the Formation Constants of the Inclusion Complexes (K_f) of Fluoxetine and Norfluoxetine

Another interesting observation is that the capacity factor (k') of FL and NR, in all mobile phase compositions, decreases in the presence of CDs media. In reversed-phase liquid chromatography it is known that the hydrophobic interactions i.e., dispersion forces, between the bonded alkyl moiety of the stationary phase and the non polar part of the molecule plays

Table 7

Equations Relating the Capacity Factor of Norfluoxetine (k') with the Formation Constant (K_f) of the Norfluoxetine-HP-β-CD Inclusion Complex.

buffer/ ACN	$1/k_0'$	K_f/k_0'	$K_f(M^{-1})$	r	n
90/10	0.134 (±0.011)	9.33 (±0.39)	69.63 (±0.014)	0.997	5
80/20	0.2812 (±0.0084)	7.69 (±0.31)	27.63 (±0.011)	0.998	5
70/30	0.4548 (±0.0069)	6.20 (±0.25)	13.630 (±0.0092)	0.998	5
60/40	0.4961 (±0.0062)	4.65 (±0.22)	9.37 (±0.0082)	0.997	5
50/50	0.5428 (±0.0045)	2.86 (±0.16)	5.27 (±0.0060)	0.995	5

r = correlation coefficient, n = number of points.

Table 8

Equations Relating the Capacity Factor of Fluoxetine (k') with the Formation Constant (K_f) of the Fluoxetine-HPβ-CD Inclusion Complex.

Buffer/ ACN	$1/k_0'$	K_f/k_0'	$K_f(M^{-1})$	r	n
90/10	0.0901 (±0.0075)	6.75 (±0.28)	74.92 (±0.01)	0.997	5
80/20	0.2058 (±0.0050)	6.09 (±0.18)	29.58 (±0.01)	0.998	5
70/30	0.3457 (±0.0064)	4.87 (±0.23)	14.09 (±0.01)	0.996	5
60/40	0.4052 (±0.0064)	3.68 (±0.23)	9.08 (±0.01)	0.994	5
50/50	0.4616 (±0.0034)	2.71 (±0.12)	5.87 (±0.01)	0.997	5

r = correlation coefficient, n = number of points.

an important role in determining the retention of the solutes. Since the hydrophobic interactions are affected by various factors, the addition of CD in the mobile phase is expected to cause a change in the retention value of the solutes owing to the formation of inclusion complex. Thus, the decrease in k' values caused by the addition of CDs in mobile phase is based on the formation of an inclusion complex, resulting in a weakening of the hydrophobic

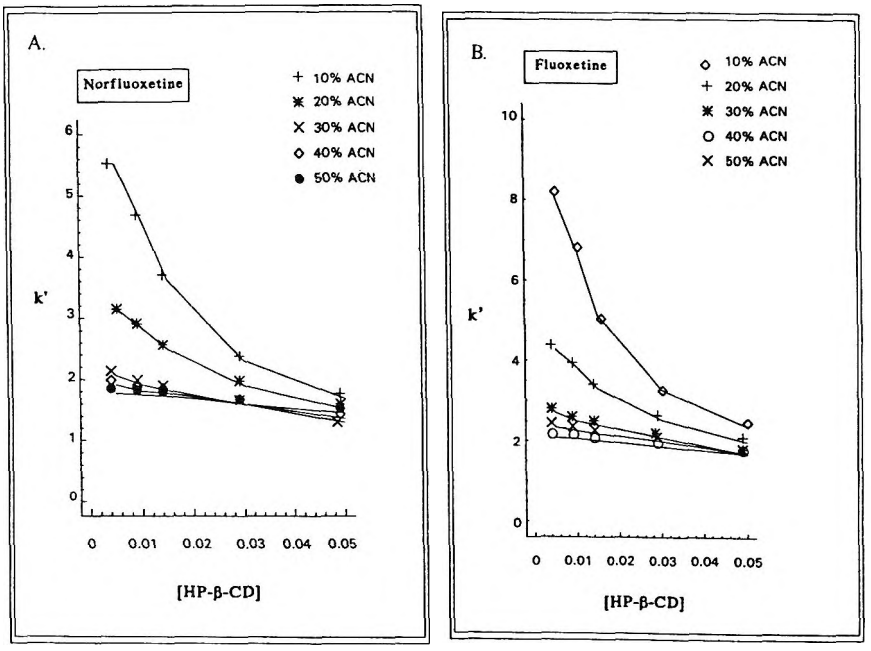


Figure 4. Regression analysis plot of equation 2 (A) and 3 (B) for NR and FL.

interactions between solutes and the stationary phases. Figure 3 describes the decrease of k' values of FL and NR respectively, by increasing the HP-β-CD concentration and using different mobile phase ratios (TEAA 2% w/v; pH: 5.0; flow rate 0.8mL/min).

In the selection of an organic solvent, e.g. acetonitrile, in a reversed-phase system, retention, resolution and the binding constant of inclusion complexes of the solutes (K_f) are dependent on the type of the organic solvent and its content in the mobile phase. Tables 7 and 8 present the equations which permit the calculation of the K_f values of FL and NR in each mobile phase; it has been shown[13] that the capacity factor k' is related to the equilibrium concentration of CD in the mobile phase [CD]m, as follows :

$$\frac{1}{k'} = \frac{1}{k'_o} + \frac{K_f}{k'_o}[CD]_m \qquad (1)$$

where ko' is the capacity factor of the guest molecule in the absence of CD.

Table 9

Equations Relating the Formation Constants of Norfluoxetine and Fluoxetine with the Organic Modifier Concentration

A. $K_f = e^{(a + b \cdot \%ACN)}$ (2)

Compound	a	b	r	n
NR	4.68 (±0.19)	-0.0623 (±0.0058)	0.987	5
FL	4.75 (±0.21)	-0.0627 (±0.0064)	0.984	5

a : intercept, b = slope, r = correlation coefficient, n = number of points.

B. $\log K_f = a + b \cdot \%ACN$ (3)

Compound	a	b	r	n
NR	2.034 (±0.084)	-0.0271 (±0.0025)	0.987	5
FL	2.061 (±0.092)	-0.0272 (±0.0028)	0.984	5

a : intercept, b = slope, r = correlation coefficient, n = number of points.

It is useful to investigate a possible relationship between the K_f value of each drug and organic modifier concentration (%ACN). An exponential model (Table 9A) is proposed in order to describe the dependence of K_f on %ACN and figure 4A present regression analysis plots representing the best fitting on the data. As shown in Table 9B, equation 2 can be converted to the corresponding linear equation 3 (fig.4B).

The K_f value of FL and NR in pure aqueous phase (K_{fw}) can easily be determined by extrapolation, as the intercept of equation 3, i.e., norfluoxetine : 108.1 (±1.2) and Fluoxetine: 115.1 (±1.2).

It is known that very often in RPLC the use of pure aqueous mobile phase leads to a decrease of sensitivity and/or resolution due to tailing and peak broadening. Thus, the concept of determining, indirectly, the K_f values in pure

aqueous phase, allows their comparison with the K_f values determined by using other techniques, on which the use of binary aqueous/organic systems is prohibitive.

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POSSIBILITIES OF PROPOFOL ANALYSIS IN VARIOUS BLOOD COMPONENTS BY MEANS OF HPLC

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ABSTRACT

Propofol (2,6 disopropylphenol) is a short-acting hypnotic agent, effective for the maintenance of anaesthesia when given intravenously as repeated bolus injections or as a continuous infusion. The present paper deals with the analysis of this drug in different parts of the blood. The results obtained by means of HPLC show that propofol not only variously bonds with plasma or solid blood elements, but also penetrates into the interior of blood cells.

The investigations carried out with whole blood, plasma, non-washed or washed cells and washed and lysed cells suggest a different behaviour of thymol (used in chromatographic analysis as internal standard) in relation to propofol.

INTRODUCTION

Drugs in the organism are transported by blood. The leading role in this transport is played by plasma proteins, mainly albumins.[1] It is essential for drugs to cause the least possible change in the organism. Therefore, drugs transported by blood should not bond irreversibly with plasma proteins and should not damage or block morphotic blood elements.

Among numerous hypnotic agents applied in anaesthesiology, physicians are more and more interested in intravenous, short-acting pharmaceutical specifics, allowing for easy sleep guidance.[2] One of the drugs of this kind is Propofol (Diprivan, 2,6-diisopropylphenol).[3,4] It is used not only as a sleeping-draught, but also as a drug to maintain anaesthesia and as a sedative.[3,4,5]

Due to differences in metabolism and renal elimination, individual people show various sensitivities to medicines.[6] Pharmacokinetic developments supported by modern analysis make it possible to optimize the individual medicine dose by measuring the therapeutic agent concentration in blood.[7,8] In the case of propofol, the estimation of its concentration in blood helps to find its minimal dose for individual patients which will be sufficient for sleep maintenance.[9]

So far, two chromatographic procedures have been described in the literature which allow to analyse propofol concetration in blood: extraction[10] and precipitation.[11] The comparison of the data obtained from the application these two methods[12] shows that the calculated values of propofol concentration depend on the amount of the solid blood element - hematocrite.

Questions appear at this point as to whether propofol bonds with morphotic blood elements and whether it penetrates into their interior. Due to the acidic character of the propofol molecule, the problem is very important because the penetration of this substance into blood cells could damage them and handicap their functions.

EXPERIMENTAL

Equipment

A Gilson solvent delivery system, composed of two high pressure pumps Model 305 and 306, manometric module Model 805 and dynamic mixer Model 811C was used. The UV-visible variable wavelength detector was a Model 308 from MIM (Budapest, Hungary).

Chromatographic separations were carried out with a 250 x 4 mm column packed with laboratory-made reversed-phase octadecyl silane sorbent.[12]

The samples were injected into the column by an injection valve Model 7125 from Rheodyne (Cotati, CA, USA).

Reagents

The mixture composed of 67% acetonitrile (gradient grade for chromatography, LiChrosolv series from Merck) and 33% double-distilled water (pH 4.0) was used as a mobile phase. The mentioned pH was achieved by the addition of a proper amount of acetic acid (ca. 0.3-0.5 mL, depending on the water's pH).

Propofol was obtained from ICI-Pharma (Goteborg, Sweden).

Except chemicals mentioned above, the others came from the Polish Factory of Chemical Reagents - POCh (Gliwice. Poland) and were of analytical grade.

The blood for preparation of standards and the blood for analysis was collected in tubes containing sodium citrate and stored at 4 °C for not longer than 5 days.

A stock solution of thymol (used as an internal standard) in methanol (1 mg/mL) was diluted with methanol to the appropriate concentration.

Tetraethylammonium hydroxide (TEAM, 25 % in ethanol) was diluted with ethanol in the volume ratio 3 : 37.

The sorbent for chromatographic separation was obtained by the chemical modification[13,14] of a laboratory-made controlled porosity glass.[15,16] The properties of this material have been described elsewhere.

Methods

Sample preparation

To 2 mL of blood containing propofol (15 μg/mL) and internal standard solution (thymol dissolved in methanol, 20 μL) (samples A - see Table 1 in the Discussion), phosphate buffer (1 mL 0.1 M NaH_2PO_4) and cyclohexane (5 mL) were added. A vessel containing all the components was vigorously shaken for 15 min at 200 rpm. After centrifugation (1200 x g for 5 min) to separate the phases, an aliquot of the cyclohexane layer (ca. 5 mL) was transferred to a clean tube to which TEAH solution (50 μL) was added. The solvent was evaporated to dryness by means of a stream of nitrogen. The residue was redissolved in mobile phase and injected into the chromatographic column.

The same procedure was followed when analysing propofol in plasma obtained from 2 mL blood samples containing the same amounts of propofol and thymol (samples B - see Table 1 in the Discussion). The only difference was the separation of plasma from solid blood elements (by centrifugation) at the beginning of the described procedure.

In order to establish the presence of propofol bonded with solid blood elements, four types of samples were investigated:

i - solid blood elements obtained only by means of blood centrifugation (samples C - see Table 1 in the Discussion);

ii - solid blood elements (obtained after centrifugation of blood) which were next washed four times with 0.9 % NaCl solution (isotonic, isoosmotic solution) to remove the residue of plasma remaining among the solid elements (samples D - see Table 1 in the Discussion);

iii - solid blood elements in the lysed form prepared by their four-time washing with 0.9 % NaCl solution and mixing with three volumes of doubly-distilled water to cause the lysis (samples E - see Table 1 in the Discussion), and

iv - the contents of morphotic blood elements (samples F - see Table 1 in the Discussion) prepared in the following way: solid blood elements obtained as above were first carefully washed four times with 0.9 % NaCl solution, mixed with three volumes of doubly - distilled water and left for 30 min to lyse the cells. Next the suspension was centrifuged to separate the cell wall and the obtained supernatant was subjected to further stages of sample preparation.

In all these cases, the samples of solid blood elements (i-iv) (or C,D,E,F - see Table 1 in the Discussion) were obtained from 2 mL blood samples containing the same amounts of propofol and thymol. Subsequent steps of the preparation procedure were the same as described for whole blood and plasma samples.

Calibration standards

The samples for the calibration curves were prepared from the solution containing 20 mg of propofol in 100 mL of acetonitrile. The solution was appropriately diluted with blood in order to obtain the concentrations of 0.5; 1.0; 2.0; 4.0; 7.0; 10.0; 15.0 and 20.0 μg/mL. Employing these solutions and following the described procedures, six calibration curves (for blood, plasma and four types of the described solid blood element samples) were plotted. To construct these curves, the ratios of the peak heights for propofol (h_P) and internal standard - thymol - (h_T) were used

RESULTS AND DISCUSSION

As already mentioned in the introduction, there are two sample preparation procedures that can be used in the analysis of propofol in blood by means of liquid chromatography.[10-12] The first one[11] requires the precipitation of a part of the proteins contained in the examined blood before injecting the sample into the chromatographic column. According to the second procedure[10]
propofol is extracted from blood into the cyclohexane phase and, after the evaporation of the volatile solvent, the dried residue is disolved in mobile phase and chromatographed. The comparison of both methods[12] suggests the bonding of propofol with morphotic elements of blood, and it also shows that the extraction method gives more precise concentration values.

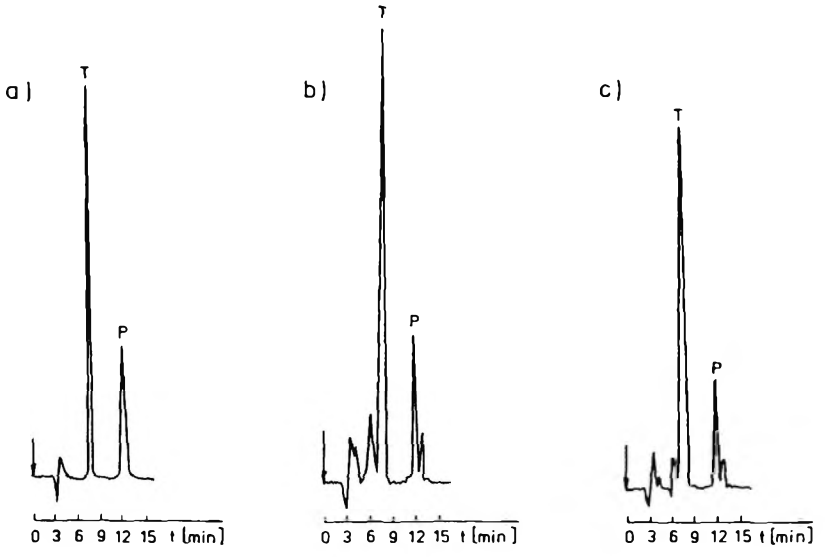


Figure 1. Chromatograms of: a) propofol-thymol mixture (peaks P and T, respectively) dissolved in acetonitrile; b) extract from blood containing propofol (15 μg/mL); c) extract from plasma separated from blood containing propofol (15 μg/ml); Conditions and marks - see Experimental and Fig. 1a.

As the observed data can result from different bonding of propofol with various blood elements, the presence of this anaesthetic agent in two parts of blood (plasma and solid blood elements) was established at the beginning.

In order to find the retention time of propofol and thymol (used as an internal standard) and to show the resolution of the applied chromatographic system, the separation of both substances diluted in acetonitrile was carried out (see Fig. 1a). As appears from the figure, the separation is complete.

In Fig. 1b, the chromatogram of the extract from blood containing 15 μg of propofol per 1 mL is shown. However, there are some substances in the prepared sample which were extracted from blood together with propofol and thymol, but their peaks are sufficiently separated from peaks representing the analyte and internal standard. This chromatogram confirms the results reported by Plummer.[10]

Fig. 1c illustrates the chromatogram of the extracts from plasma, whereas Fig. 2a shows the chromatogram of the extract from non-washed solid blood elements. These separations are similar to that presented in Fig. 1b, but

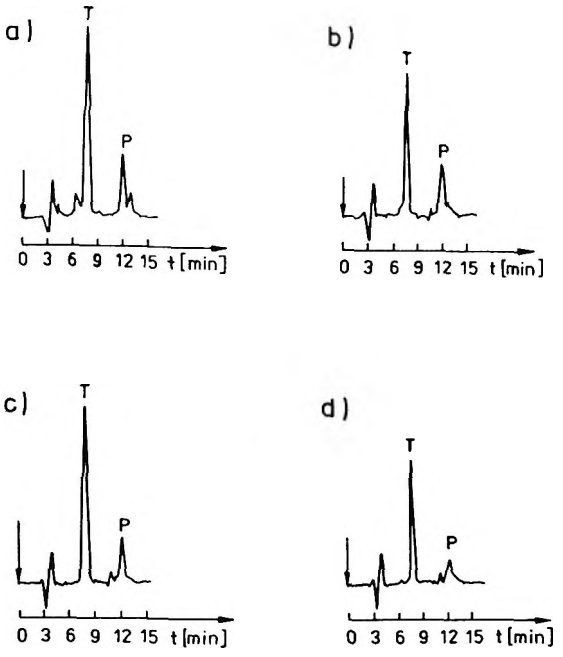


Figure 2. Chromatograms of: a) extract from non-washed solid blood element phase separated from blood containing propofol (15 μg/mL); b) extract from washed (4 times) solid blood element phase obtained by blood centrifugation. Concentration of propofol in initial blood, the same as before; c) extract from washed and lysed solid blood elements separated from blood containing propofol (15 μg/mL); d) extract from the content of solid blood elements separated from blood containing propofol (15 μg/mL); Conditions and marks - see Experimental and Fig. 1a.

distinctly lower heights of propofol and thymol peaks (peaks P and T, respectively) confirm lower amounts of both compounds in these two parts of centrifugally separated blood in relation to the whole blood (see Table 1). Besides, amounts of propofol and thymol contained in the solid blood element phase (before washing) are lower in comparison to those bonded with plasma.

The chromatograms of the extracts from washed or washed and lysed morphotic blood elements are presented in Figs. 2b and 2c, respectively. They look clearer and are very similar to those presented above. On the one hand, they suggest that the peaks unidentified before are probably connected with plasma components; on the other hand, they prove the bonding of propofol and

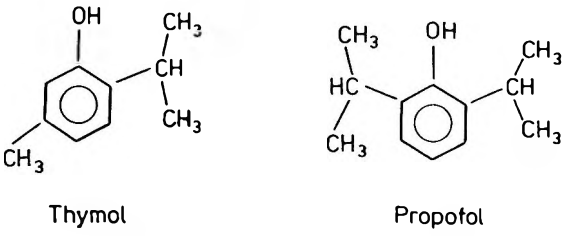
Table 1

Heights of Thymol and/or Propofol Peaks and their Ratios Corresponding to Investigated Samples (Average Values from 7 Separate Measurements)

Symbol of Investigated Sample	Type of Investigated Sample	Thymol Peak Height (h_T) (mm)	Propofol (h_P) (mm)	h_P/h_T
A	Blood containing propofol (15μg/mL)	199.0	64.0	0.3216
B	Plasma obtained by centrifugation of sample A	152.0	46.0	0.3026
C	(i) Solid elements of blood obtained by centrifugation of sample A	68.0	23.0	0.3382
D	(ii) Solid elements of blood (Sample C) washed to remove plasma residues	56.0	19.0	0.3392
E	(iii) Washed solid elements of blood (see D) after lysis	69.0	17.0	0.2463
F	(iv) The content of solid blood elements	47.0	0.9	0.1915

(I), (ii), (iii) and (iv) - see Experimental section.

thymol with solid blood elements. The heights of both propofol peaks in these chromatograms are similar (see Table 1), but they are lower in relation to P peak heights for initial blood, plasma or non-washed solid elements of blood.



Thymol Propofol

Figure 3. Chemical structures of thymol and propofol.

The chromatogram in Fig. 2d shows the penetration of some amount of propofol into the interior of blood cells. This is a very small amount, nevertheless, disadvantageous from a medical point of view. It cannot be excluded that only old or ailing blood cells (whose cell walls are not compact) are penetrated by propofol.

According to the presented results, propofol variously bonds with different components of blood. This conclusion explains the differences discussed in Ref. 12, between the propofol concentration values estimated by means of the precipitation and extraction methods.

In a typical procedure of inducing anaestesia intravenously with propofol, the drug is administrated in the amount of about 2.5 mg/kg of patient body weight. Assuming the average patient body weight of 70 kg and his blood volume of 5 liters, the concentration of propofol in blood should be about 35 μg/ml. As results from radioanalytical and chromatographic data,[17,18] its maximum concentration (about 2 - 3 min after administration) reaches the level of 5-10 μg/mL. The difference between the theoretically calculated and analytically determined values is known from literature[19] and results from propofol absorption in the adiposal tissue and other organs. Because the amount of propofol penetrating blood cells is very small and the applied uv-vis detector is not sensitive enough, blood containing 15 μg/mL of propofol was used for these experiments. The presented experiments were carried out outside the human organism. It is probable that, in natural conditions, the behaviour of propofol is a little different, but such investigation is much more difficult.

Table 1 collects the average values of peak heights and peak height ratios (n = 7) for propofol and thymol extracted from the examined samples. As appears from these data, the ratio of propofol peak height to thymol peak height is similar for first four extracts from blood preparations (blood, plasma, non-washed solid blood elements and washed solid blood elements).

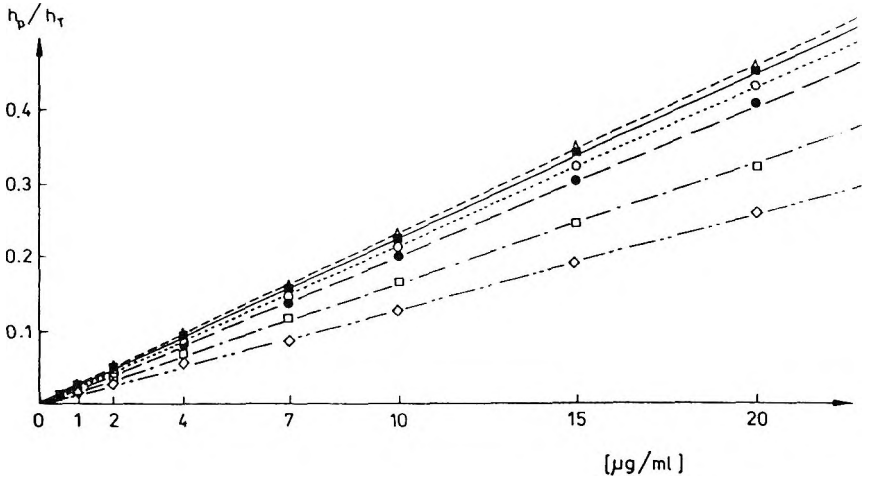


Figure 4. Calibration curves corresponding to investigated blood preparations. Height ratio of propofol peak (h_P) to thymol peak (h_T) vs. propofol conc'n in initial blood. Dotted line with open rings - sample A (see Table 1). Dashed line with black rings - sample B (see Table 1). Solid line with triangles - sample C (see Table 1). Dashed line with black squares - sample D (see Table 1). Dash-single dotted line with white squares - sample E (see Table 1). Dash-double dotted line with white rhombs - sample F (see Table 1).

For the samples E and F (washed and lysed solid blood elements (iii) and/or content of solid blood elements (iv)) the discussed value is significantly lower and decreases respectively. Considering the values presented in can be concluded that:

1. In relation to thymol, propofol is better bonded with solid blood elements then with plasma components - compare h_P/h_T ratio for:

a) plasma (samples B from Table 1) and non-washed solid blood element (samples C), or

b) plasma (samples B) and washed solid blood elements (samples D), or

c) non-washed solid blood elements containing the residual amount of plasma among them (samples C) and washed solid blood elements (samples D).

The h_P/h_T for blood (samples A which are the mixture of plasma and morphotic blood elements) reaches intermediate value.

2. Thymol penetrates better into the interior of blood cells - compare the h_P/h_T ratio for samples D (washed solid blood elements) and for samples F (the content of solid bood elements). The comparison of this value for samples D with E or E with F confirms the same.

The last conclusion can easily be explained. The molecules of thymol was proposed by Plummer as an internal stardard in this analysis due to its similar structure and character with propofol (see Fig. 3). But thymol has a little smaller diameter and is more polar than propofol molecules. In consequence, thymol molecules penerate more easily into the cell interior (over the cell wall) than propofol molecules do. Taking into account the similarity of the h_P/h_T ratio for blood plasma and solid blood elements, it can be assumed that the concentration of propofol in these parts of blood can be estimated approximately on the basis of one calibration curve constructed using one of the mentioned mediums as solvent for calibration solutions. A precise propofol analysis in these parts of blood, and especially its analysis in lysed cells or in the contents of cells require separate calibration curves in all cases. This conclusion is confirmed by the calibration plots presented in Fig. 4.

CONCLUSIONS

1) Propofol is bonded both with plasma proteins and solid blood elements. It penetrates also into the blood cell interior.

2) A precise analysis of propofol in various blood components (and especially in the content of blood cells) requires separate calibration curves constructed for the component in which this drug is investigated.

3) The concentration ratio of propofol and thymol (used as the internal standard) is similar for whole blood, plasma and solid blood elements, but different in the case of the content of blood cells.

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STRONG CATION AND ANION EXCHANGE MEMBRANES AND BEADS FOR PROTEIN ISOLATION FROM WHEY

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ABSTRACT

Protein sorption from raw rennet whey at pH 3.0 onto S-type (strong acid), and at pH 7.2 onto Q-type (strong base), ion exchange beads and membranes was measured. The data were used to analyze protein binding capacities, percentage recoveries, production rates, and design parameters for a commercial-scale process. Performance of S-type beads was superior to Q-type beads, but Q-type and S-type membranes performed comparably, except that the S-type membranes lost capacity during repeated cycling without cleaning, while the Q-type membranes did not. Performance of the S-type membranes was increased by microfiltration of the whey before loading, but this was partially offset by the lower protein content of the microfiltered whey. Increasing the flow rate through the cartridge increased productivity, but the percentage protein recovery decreased. This

was attributed to the pore size of the membrane, which may have been too large for protein to reach the pore wall by diffusion before it passed through the membrane. A smaller pore size may increase the capture of protein and the binding capacity, but at the expense of plugging if the pores are too small.

INTRODUCTION

Commercial ion exchange processes for whey protein separation utilize stirred tanks containing porous beads onto which an ion exchange ligand is immobilized.[1-4] First, whey is stirred with regenerated-cellulose ion exchange beads in a large stainless steel tank, where the proteins adsorb onto the beads. Second, deproteinized whey is drained from the tank. Third, the beads are washed with water to leach out entrained fat, lactose, and ash. Fourth, whey proteins are desorbed from the beads using either an acidic or basic water solution, or a strong salt solution (1 M NaCl). The bead size is chosen to balance slow liquid percolation rates for small beads against slow intra-bead diffusion rates for large beads. Increasing the rate for adsorption of the proteins to the beads is of great practical importance because it maximizes the throughput of the process.

Ion exchange membranes are a new technology, designed to overcome the limitations encountered in conventional commercial processes using beads.[5,6] For beads, the rate of adsorption is controlled by external diffusion of protein to the bead surface (film diffusion), or internal diffusion of protein (intra-bead diffusion) to the adsorption site. Beads can be made smaller to increase diffusion rates, but this increases resistance to flow. For ion exchange membranes, whey flows by convection through the micron-sized pores of the membrane. Therefore, protein is transported into the membrane structure by convection, which is faster than intra-bead diffusion. Flow limitations are negligible if the membranes are made thin. Consequently, properly designed and operated ion exchange membrane separations may outdo bead-based separations in the isolation of proteins from whey and other biological fluids.

The feasibility of using ion exchange membranes for isolation of proteins from microfiltered whey has been demonstrated previously using membranes with a pore size of either 1.2 μm or 50-300 μm.[6] Microfiltration was necessary to prevent plugging of the smaller pore size membrane. Microfiltration decreased the protein content of the whey by 30%, and involved an extra processing step.

There has not been a comparison of the isolation of proteins from whey by ion exchange membranes and ion exchange beads. The requisite fundamental sorption properties of proteins in raw (not microfiltered or demineralized) whey with ion exchange membranes and beads are absent from the literature. A few data points are available for isolation of proteins from raw whey at pH 3.0 using S-type beads,[1,2] but Q-type beads were not evaluated, nor were membranes. The performance of S-type ion exchange membranes for isolation of proteins from microfiltered whey at pH 3.0 was evaluated,[6] but raw whey was not used, nor were beads or Q-type membranes.

In this work, new experimental data for protein sorption from raw whey with S-type and Q-type ion exchange membranes were collected along with similar data for ion exchange beads. These data were used to analyze the protein binding capacities, percentage recoveries, production rates, and design parameters for membranes and beads in a commercial-scale process. The primary focus was on characterization of the newer membrane technology rather than the conventional bead technology. Consequently, fewer experiments were performed with beads.

MATERIALS AND METHODS

Separated (partially defatted by centrifugation) Cheddar cheese whey at pH 6.2, obtained from Associated Milk Producers, Inc. (Arena, WI), was adjusted to either pH 3.0 using 0.375 M HCl, or to pH 7.2 using 1 M NaOH, and stored at 4°C. S Sepharose Fast Flow beads (S-1264, 45-165 μm wet-diameter cross-linked 6% agarose beads, $4x10^6$ Da exclusion limit, Sigma Chemical Co., St. Louis, MO) were repeatedly washed with 0.1 M citric acid/sodium citrate pH 3.0, followed by vacuum filtration to remove residual buffer.

Q Sepharose Fast Flow beads (Q-1126, Sigma) were washed with deionized water, vacuum filtered, washed with 1 M NaCl, vacuum filtered, washed with deionized water, and again vacuum filtered. The wet vacuum-filtered beads had a packed volume of 1.35 mL/g. The initial bulk concentration of water in the vacuum-filtered beads was determined by oven drying at 70°C to be 0.84 g/g for the Q-type beads, and 0.82 g/g for the S-type beads. In 50 mL centrifuge tubes, whey solution and beads were equilibrated with agitation for 24 h at 4°C, and then centrifuged to remove the beads.

The supernatant solutions were analyzed for Kjeldahl total nitrogen (TN) and non-protein nitrogen (NPN) using prior methods.[6] Supernatant pH did not change after equilibration.

Two commercially-available membrane cartridges (Productiv S model PSC10-SP, and Productiv QM model PSC10-QM, BPS Separations, Ltd., Spennymoor, County Durham, U.K.) were evaluated. The protein recovery cycle consisted of equilibration, loading, washing, and elution. Equilibration consisted of pumping 100 mL elution buffer (E buffer), followed by 100 mL loading/washing buffer (L/W buffer) through the cartridge. During loading, whey solution was pumped through the cartridge. Then, L/W buffer was pumped through the unit until the absorbance returned to baseline.

The cycle was completed by pumping E buffer through the unit until baseline absorbance was attained, followed by 0.2 M NaOH until baseline absorbance was reestablished. The cartridge was then disconnected and cleaned. The L/W buffer was 50 mM citric acid/sodium citrate pH 3.0 for the PSC10-SP, and deionized water for the PSC10-QM. For both cartridges, the E buffer was 1 M NaCl in L/W buffer. All buffers were vacuum degassed just prior to use. The flow rate was 10 mL/min, equal to one bed volume (BV) per min.

Cleaning followed a standard procedure,[6] wherein the sealed cartridge containing 0.2 M NaOH was submerged in a 60°C water bath for 1 h. It was then removed from the water bath, reconnected, and backflushed with deionized water, 0.2 M HCl, and deionized water, all in sequence.

An orange color accumulated on the Q-type adsorbents after contacting with whey, increasing in intensity with increasing extent of contact. This was attributed to norbixin, an anatto-derived pigment added during cheesemaking (American Cheese Color, code 41001, Sanofi Bio-Industries, Waukesha, WI). This divalent anion probably bound to the positively-charged adsorbent. It could be removed with acetone, but its concentration in milk (7 μM) was negligible compared to the charge density of the beads (0.18-0.25 M), and probably also of the membranes.

RESULTS

Protein Recovery from Whey by Ion Exchange Beads

Whey protein adsorption to ion exchange beads was determined by measuring the decrease in protein content in the whey solution in contact with the beads. Equating the amount of protein removed from the whey to that adsorbed by the ion exchange beads yields:

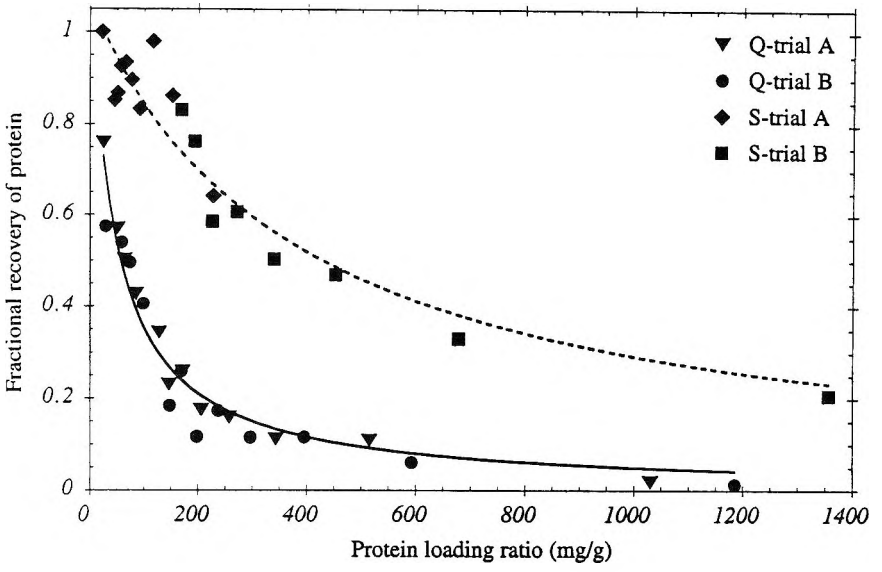

Figure 1. Fraction of protein removed ($y = q_1 m/wc_0$) from whey at pH 3.0 by S-type beads, and from whey at pH 7.2 by Q-type beads, versus the ratio of the mass of protein initially in the whey to the mass of beads ($x = wc_0/m$).

$$w\,c_0 - (w + c_w m)\,c_1 = q_1 m \qquad (1)$$

where w is the mass of whey (45 g), c_0 is the initial bulk concentration of protein in the whey (mg/g), c_W is the initial bulk concentration of water in the beads (g/g), m is the mass of the wet beads (g), c_1 is the bulk concentration of protein after equilibration (mg/g), and q_1 is the average concentration of protein in an equilibrated bead (mg/g).

As shown in Figure 1, as the protein loading ratio wc_0/m increased, the fraction of protein removed from the whey $q_1 m/wc_0$ steadily decreased. The data were fit by least-squares regression to $y = a/(b + x)$, where $y = q_1 m/wc_0$, and $x = wc_0/m$. Using this equation, the fitted parameters carry a specific meaning. At very large protein loading ratios, the maximum capacity of the beads is simply equal to a. The capacity of the beads is half the maximum value when the protein loading ratio is equal to b. For S-type beads, $a = 400 \pm 50$ mg/g, and $b = 380 \pm 60$ mg/g. For Q-type beads, $a = 52 \pm 4$ mg/g, and b

= 45 ± 8 mg/g. The capacity of the beads is the product of x and y. Thus, as the protein loading ratio wc_o/m increases (more whey, fewer beads), the recovery of protein decreases, and the bead capacity increases.

The capacity and protein recovery for S-type beads was always greater than for Q-type beads under the conditions studied. For example, at wc_o/m = 150 mg/g, q_1 = 110 mg/g and $q_1 m/wc_o$ = 0.75 for S-type beads, compared to q_1 = 40 mg/g and $q_1 m/wc_o$ = 0.27 for Q-type beads. In other words, the capacity (or, equivalently, the fractional recovery) is 275% greater for the S-type beads than for the Q-type beads under these conditions.

Recovery and Binding Capacity of Protein from Whey by Ion Exchange Membranes

Whey protein adsorption to ion exchange membranes was analyzed in a similar manner. Equating the amount of protein removed from the whey to that adsorbed by the ion exchange cartridge yields

$$wc_o - ec_e = Q_1 v \qquad (2)$$

where e is the volume of the effluent (mL), which includes the whey loaded and the wash buffer, c_e is the concentration of protein in the effluent (mg/mL), Q_1 is the average concentration of protein in the cartridge (mg/mL), and v is the volume of the membranes in the cartridge (mL). The membrane volume was defined as the total bed volume (10 mL).

As shown in Figure 2, as the protein loading ratio $x = wc_o/v$ increased, the fraction of protein removed from the whey $y = Q_1 v/wc_o$ steadily decreased. The recovery data were fit by least-squares regression to $y = a/(b + x)$. For the PSC10-SP cartridge, a = 26±7 mg/mL, and b = 24±9 mg/mL. For the PSC10-QM cartridge, a = 30±20 mg/mL, and b = 60±40 mg/mL. Under the conditions studied, the capacity and protein recovery for the PSC10-SP cartridge did not differ significantly from the PSC10-QM cartridge.

As with ion exchange beads, the capacity of the cartridge is the product of x and y. Thus, as the protein loading ratio wc_o/v increases (more whey), the recovery of protein decreases, and the cartridge capacity increases.

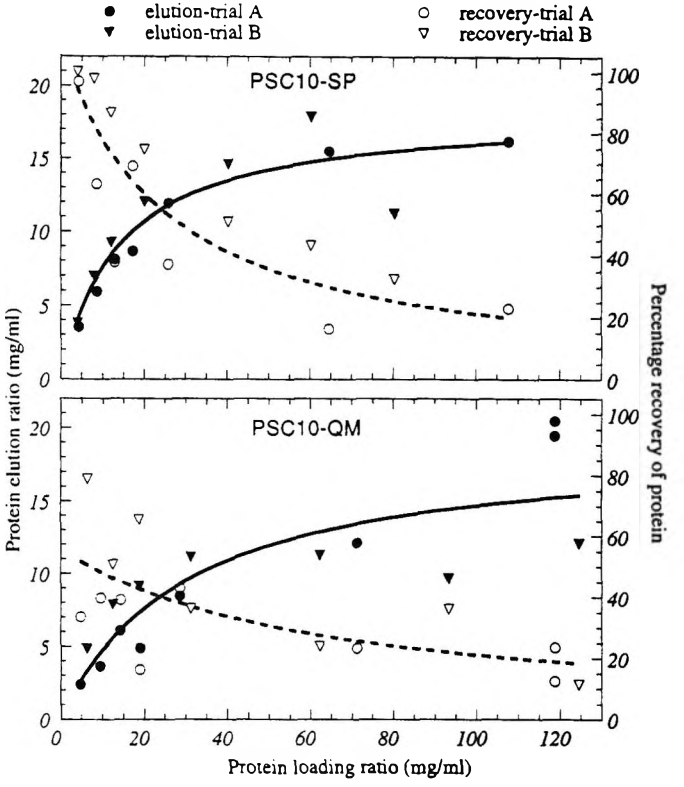

Figure 2 . Ratio of the amount of protein recovered in the elution peak to the volume of the membrane cartridge (left ordinate = pc_p/v), and percentage of protein removed from the whey (right ordinate = $100xQ_1v/wc_o$) by the PSC10-SP at pH 3.0 (top), and by the PSC10-QM at pH 7.2 (bottom), versus the ratio of the amount of protein initially in the whey to the volume of membrane ($x = wc_0/v$). By definition, p is the volume of the elution peak (mL), and c_p is the concentration of protein in the elution peak (mg/mL).

Elution Peak Composition vs. Protein Loading Ratio for Ion Exchange Membranes

The amount of protein in the elution peak was determined after loading from 10 to 250 mL of whey solution into the ion exchange membrane cartridges with cleaning between cycles (Figure 2). The total amount of protein in the elution peak generally increased with increased loading of protein for

Table 1

Percentage of Protein Bound and Recovered. Whey (50 ml, pH 7.2) was Loaded at Different Flow Rates into a PSC10-QM Cartridge. The Wash Buffer was D.I. Water, and the Elution Buffer was 1 M NaCl.

Flow Rate (mL/min)	Protein Bound (%)	Protein Recovered (%)
1.0	53.2±0.7*	50±10
2.0	52±9	40±20
5.0	33	39
10.0	33±7	31±9
18.5±0.2	20±10	28±5

* mean±S.D., n=2

both the PSC10-SP and PSC10-QM. The data were fit to $z = ax/(b + x)$. The parameters in this equation have the same conceptual meaning as before, but this form of the equation is for capacity rather than fractional recovery. For the PSC10-SP cartridge, a = 18±1 mg/mL, and b = 14±3 mg/mL. For the PSC10-QM cartridge, a = 19±3 mg/mL, and b = 30±10 mg/mL. The maximum amount of protein eluted was not significantly different between the two cartridges.

The fraction of protein bound to the membrane that eluted (pc_p/Q_1v) can be computed from the fitted parameters, and is equal to $z \div xy$. It was not significantly different from 100%, indicating complete recovery of protein within the deviation of the fitted parameters. However, only the first portion of the elution peak was collected in order to avoid dilution of the peak due to tailing. A small amount of the protein desorbed from the membrane was lost in the portion of the elution peak which was not collected. Therefore, strictly speaking, not all the protein which bound to the cartridges was present in the elution peak fraction.

Effect of Flow Rate on Protein Recovery from the PSC10-QM Cartridge

In order to determine if the results depended on flow rate, the loading, washing, and eluting cycle, with cleaning between cycles, was repeated at five different flow rates using a fixed loading volume (Table 1). The percentage of protein loaded which bound decreased significantly as the flow rate increased, as did the percentage of protein loaded which was present in the elution peak.

Stability of Cartridge Performance

In a commercial process, the ion-exchange cartridge is repeatedly cycled through the sequential steps of loading, washing and eluting in a semi-continuous manner without cleaning between cycles. To simulate this, ten cycles were repeated under identical conditions to measure any decreases in membrane performance.

For the PSC10-SP membrane cartridge, there was a significant decrease in the amount of protein recovered in the elution peak during repeated cycling without cleaning (Table 2). The mass of protein recovered per cycle decreased by an average of 5-6% per cycle. The elution peak volume was 48.8±0.6 mL. For this protein loading ratio ($wc_o/v = 20$), 106 mg of protein eluted in a single cycle followed by cleaning as calculated from Figure 2. This falls within the range of the early cycling data. The protein production rate of the semi-continuous process was 40 mg protein per h per mL of membrane volume.

For the PSC10-QM cartridge, there was not a significant change in the amount of protein recovered in the elution peak during repeated cycling without cleaning (Table 3). The amount of protein recovered per cycle was 90±10 mg (mean±S.D.) in an elution peak volume of 49.1±0.8 mL. This compares closely to 97 mg of protein eluted for this protein loading ($wc_o/v = 31$) in a single cycle followed by cleaning as calculated from Figure 2. The protein production rate of the semi-continuous process was 40 mg protein per h per mL membrane volume.

For minerals, the amounts recovered for the PSC10-SP cartridge were: K = 2.4±0.2 mg, Ca = 3.3±0.3, Mg = 0.45±0.05, P = 0.23±0.04, and S = 0.9±0.2. For the PSC10-QM cartridge the amounts were: K = 0.44±0.06 mg, Ca = 2.28±0.08, Mg = 0.25±0.01, P = 6.2±0.2, and S = 1.98±0.07. There was a significant decrease in Ca and S with cycling of the PSC10-SP cartridge, paralleling decreases in protein recovery, but there were no decreases in K, Mg, or P. There was no decrease in the amount of minerals recovered with cycling of the PSC10-QM cartridge.

Because whey proteins bind Ca and contain S,[7] these minerals can bind along with the protein or as individual ions. Cations (K, Ca, Mg) bound more to the PSC10-SP cartridge compared to the PSC10-QM cartridge. Anions (S as SO_4 and P as PO_4) bound more to the PSC10-QM cartridge. The minerals contents of the elution peaks were much lower than that of the whey on a concentration basis, and relative to the protein content. Therefore, protein was preferentially adsorbed compared to minerals.

Table 2

Contents of Elution Peaks for the PSC10-SP Cartridge. Each Cycle Consisted of 50 mL of Whey, pH 3.0 (Load), 15 mL of 50 mM Sodium Citrate, pH 3.0 (Wash), 50 mL of 50 mM Sodium Citrate 1 M NaCl, pH 3.0 (Elute), and 15 mL Of 50 mM Sodium Citrate, pH 3.0 (Wash). A Flow Rate of 10 mL/Min was Used.

Cycle	Protein (mg)	K (mg)	Ca (mg)	Mg (mg)	P (mg)	S (mg)
1	126	2.1	3.7	0.5	0.1	1.3
2	115	2.5	3.6	0.5	0.2	1.1
3	112	2.8	3.6	0.5	0.3	1.1
4	101	2.4	3.4	0.5	0.2	0.9
5	97	2.6	3.4	0.5	0.3	0.9
6	90	2.5	3.2	0.4	0.3	0.9
7	84	2.3	3.1	0.4	0.2	0.8
8	30	2.2	2.9	0.4	0.3	0.7
9	78	2.2	3.0	0.4	0.2	0.8
10	70	2.2	2.8	0.4	0.3	0.7
whey*	201	46.3	15.7	2.4	19.0	3.5

* composition of the feed solution

DISCUSSION

The capacity and protein recovery of S-type beads was superior to Q-type beads. This difference may have resulted from the pH of the whey solution in each experiment. At pH 3, all the proteins in whey have a positive net charge, and are able to bind to S-type beads.[4] However, at pH 7.2 some of the proteins in whey, such as the immunoglobulins, have a net positive charge and cannot bind to Q-type beads. At pH > 9, all the proteins in whey have a negative net charge.

Using an elevated pH may increase the performance of the Q-type beads, but it also may promote undesirable alkaline protein denaturation. Protein recovery was comparable for the PSC10-SP and the PSC10-QM for a single cycle followed by cleaning. However, the PSC10-SP lost capacity rapidly during repeated cycling without cleaning, while the PSC10-QM did not.

Table 3

Contents of Elution Peaks for the PSC10-QM Cartridge. Each Cycle Consisted of 50 mL of Whey, pH 7.2 (Load), 15 mL of Water (Wash), 50 mL of 1 M NaCl (Elute), and 15 mL of Water (Wash). A Flow Rate of 10 mL/Min Was Used.

Cycle	Protein (mg)	K (mg)	Ca (mg)	Mg (mg)	P (mg)	S (mg)
1	110	0.5	2.3	0.2	6.5	1.9
2	94	0.4	2.2	0.2	6.3	2.0
3	97	0.5	2.3	0.3	6.5	2.0
4	83	0.4	2.4	0.3	6.3	2.0
5	85	0.4	2.4	0.3	6.4	2.1
6	98	0.5	2.4	0.3	6.3	2.0
7	96	0.4	2.3	0.3	6.3	2.0
8	83	0.4	2.2	0.3	6.1	2.0
9	60	0.3	2.2	0.2	5.9	1.9
10	95	0.5	2.2	0.2	5.9	1.9
whey*	312	49.7	14.0	2.1	20.8	3.7

* composition of the feed solution

The recovery data reported here form a technical basis for comparing beads and membrane cartridges for isolation of proteins from whey. The volume of the membrane cartridge, or the mass of the beads, required to process a commercial-scale quantity of whey were calculated and compared.

For comparison purposes, the whey throughput (T) of each membrane cartridge and of each tank was set equal to the commercial-scale value (220 L/min). The protein content of the whey (c_o) was set at 6 mg/mL.

The commercial process utilizes two stirred tanks of approximately 40,000 L each filled with ion-exchange beads to recover protein from 550,000 L/d of whey.[3,4] Each tank has a cycle time (t_C) of three hours. The superficial residence time in each membrane cartridge (t_R) was fixed at the experimental value (1 min).

Table 4

Comparison of Beads to Membrane Cartridges. Values for X were Calculated from the Fractional Protein Recovery and Figures 1 and 2. Other Values Were: c_0 = 6 mg/mL, T_r = 1 min, and T = 220 L/min.

	Protein Recovered from Whey											
	40%				60%				80%			
Adsorbent	x mg/g	t_c min	w m^3	m,v kg.L	x mg/g	t_c min	w m^3	m,v kg, L	x mg/g	t_c min	w m^3	m,v kg, L
S-Beads	620	180	40	390	290	180	40	840	120	180	40	2000
Q-Beads	85	180	40	2800	42	180	40	5800	20	180	40	12000
PSC10-SP	41	15	3.3	480	19	11	2.5	770	8.5	9.4	2.1	1500
PSC10-SP*	140	41	9.1	270	66	24	5.2	330	29	15	3.3	470
PSC10-QM	15	11	2.3	920	NA**	NA	NA	NA	NA	NA	NA	NA

* 0.45 μm microfiltered whey, c_0 = 4.2 mg/ml

** target protein recovery cannot be attained

The cycle time for the membrane cartridge is the sum of the time required to load whey, wash away contaminants, elute bound protein using salt, and rinse out salt. By definition, the bed volumes (BV) of whey loaded (= w/v) is equal to the protein loading ratio divided by the whey concentration (x/c_0). In data reported here, 8 BV were required to wash, elute, and rinse the cartridge after loading. Therefore, the cycle time for each cartridge is $t_C = t_R(x/c_0 + 8)$. The product of t_C and T is the volume of whey loaded per cycle *i.e.* $w = t_C T$. The volume of each cartridge is $v = wc_0/x$. The mass of beads per tank is $m = wc_0/x$. The known parameters are: c_0, t_R, T, and the fractional protein recovery ($q_i m/wc_0$ or $Q_i v/wc_0$).

Performance data for S-type beads, Q-type beads, the PSC10-SP cartridge, and the PSC10-QM cartridge were used for comparison (Table 4). Six to seven times more adsorbent mass is required for Q-type beads than for S-type beads to achieve either 40, 60 or 80% protein recovery. Two times more adsorbent volume would be required for a scaled-up PSC10-QM cartridge than for a scaled-up PSC10-SP cartridge to achieve 40% protein recovery. It was not possible to achieve 60% or 80% recovery for the PSC10-QM cartridge under the conditions studied.

The extrapolated maximum capacity of S-type beads was 15 times greater than the capacity of the PSC10-SP membrane cartridge. However, the time for

loading, washing, and regeneration of the beads in a stirred-tank adsorption process was 3 h, compared to 9 min for the membrane cartridge. Consequently, the membrane process would require a similar amount of adsorbent compared to the tank process. At 60% and 80% recovery, the S-type membrane process requires less adsorbent than the S-bead process, but the opposite is true at 40% recovery. At all recoveries, the membrane process occupies less space than the tanks process because both 40,000 L tanks could be eliminated.

Protein recovery from microfiltered whey using a PSC10-SP cartridge was included in the comparison using prior results.[6] Compared to unfiltered whey, the membrane volume required to process microfiltered whey is 40-70% smaller, but the process throughput is 30% smaller because of the lower protein content of the whey (c_o = 4.2 mg/mL).

If the residence time (t_R) could be decreased in the membrane process, then the required membrane volume would decrease proportionately, and the capital cost of the membrane process would decrease. However, increasing the flow rate through the cartridge decreased the percentage protein recovery (Table 1), although it did increase productivity.

Recovery may have decreased because the pore size of the PSC10 cartridge was too large to capture the protein. In order for protein to diffuse to the wall of the pore and bind before it passes through the membrane, t_R must greatly exceed the time scale for boundary-layer mass transfer to the pore wall (t_{BLMT}). As an order-of-magnitude approximation,[8] $t_{BLMT} = (d_p)^2/4D$, where d_p is the membrane pore size (50-300 μm), and D is the diffusion coefficient of the whey protein *e.g.* $D = 6.7 \times 10^{-7}$ cm^2/s for bovine serum albumin. For these experiments, t_R was 1 min, and t_{BLMT} was 0.2-6 min. Consequently, the percentage protein recovery may have decreased as t_R decreased, as shown in Table 1, because the membrane pore size was too large.

A smaller membrane pore size would be desirable to eliminate boundary-layer mass transfer effects. It would increase the internal surface area of the membrane, increasing capacity. However, prior microfiltration of the whey would be needed to prevent plugging if the pore size was too small (< 5 μm). Conceivably, a pore size of 5-50 μm would provide a more suitable balance between capacity and plugging than did the PSC10 cartridges.

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PURIFICATION OF FISH OIL ETHYL ESTERS BY HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY USING NON-AQUEOUS SOLVENT SYSTEMS

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ABSTRACT

High-speed countercurrent chromatography (HSCCC) was applied to purification of fish oil ethyl esters (FOEE). The CCC separations were performed using non-aqueous two-phase solvent systems in two steps: The first purification was carried out with a hexane-acetonitrile system where 30 mL of crude FOEE was cleaned in 140 min yielding 23.1 mL of refined FOEE. The product was colorless and free of saturated fatty acid esters. The second and final purification was performed with a hexane-dichloromethane-acetonitrile (5:1:4, v/v/v) where 1 g of the refined FOEE was resolved into three fractions, ethyl hexadecenoate, ethyl octadecenoate and the third composed of

56.4% of ethyl docosahexaenoate and 39.3% of ethyl eicosapentenoate. The separation was completed in 145 min. However, polyunsaturated fatty acids are not resolved with this solvent system due to their low partition coefficients.

INTRODUCTION

Since the last decade, high-speed countercurrent chromatography (HSCCC) has been extensively applied to separation and purification of natural products mostly using organic/aqueous two-phase solvent systems.[1,2]

This paper describes purification of a gram quantity of fish oil ethyl esters (FOEE) which contains two major components, ethyl docosahexaenoate (EDHA) and ethyl eicosapentenoate (EEPA). It is claimed that these esters possess the medicinal property of preventing cardiovascular diseases and exhibit higher antioxidant activities than the free acid form.[3,4] Using two different non-aqueous two-phase solvent systems, these major components were separated as a mixture from minor components.

Recently, an analytical-scale separation of polyunsaturated fatty acids by HSCCC has been reported using organic-aqueous two-phase solvent systems. Application of the method to a gram quantity, however, produced a mixture of 4 components.[5] On the other hand, the HSCCC separation of polyunsaturated fatty acid ethyl esters has not been reported.

EXPERIMENTAL

Apparatus

HSCCC experiments were performed using a coil planet centrifuge equipped with a multilayer coil separation column that was designed and fabricated at the Beijing Institute of New Technology Application, Beijing, P. R. China. The multilayer coil was prepared by winding a 1.6 mm ID PTFE (polytetrafluoroethylene) tube coaxially onto the column holder hub. The total column capacity measured 230 mL.

The HSCCC centrifuge was rotated at 800 rpm with an 8 cm revolution radius. The system was equipped with an FMI pump (Zhejiang Instrument Factory, Hangzhou, P. R. China), a fraction collector and a sample injection valve.

Reagents

Hexane, acetonitrile and dichloromethane were of an analytical grade and purchased from Shanghai Chemical Factory, Shanghai, China. A standard sample mixture for fatty acid ethyl esters was a gift from the Second Oceanography Institute, the Ocean Bureau of P.R. China, Hangzhou, China.

Materials

The fish oil ethyl esters (FOEE), a yellow oily liquid, was purchased from Puto Medicine factory, Zhousan, Zhejiang, P. R. China. This material was used for HSCCC refinement.

HSCCC Refinement of Crude FOEE

The HSCCC experiment was performed with a non-aqueous two-phase solvent system composed of hexane-acetonitrile. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use. In each refinement the multilayer coil column was first entirely filled with the upper stationary phase. Then the lower mobile phase containing 30 mL of crude FOEE was pumped into the inlet of the column at a flow rate of 3.0 mL/min, while the apparatus was rotated at 800 rpm. After 220 mL of mobile phase was pumped and relatively polar impurities were eluted from the column, the HSCCC centrifuge was stopped and the column contents were collected by pressure with nitrogen. The contents were evaporated to dryness to yield the refined FOEE which was further subjected to separation as described below.

Separation of the Refined FOEE

Final purification was performed with a non-aqueous solvent system composed of hexane-dichloromethane-acetonitrile (5:1:4, v/v/v). The solvent mixture was equilibrated and separated as described earlier. In each separation, the multilayer coil was first entirely filled with the upper stationary phase, followed by injection of sample solution (1 mL of the refined FOEE in 10 mL of the mobile phase) through the injection valve. Then the mobile phase was pumped into the inlet of the column at a flow rate of 2.0 mL/min, while the apparatus was rotated at 800 rpm. The effluent from the outlet of the column was collected into test tubes using a fraction collector. Each fraction was subjected to GC analysis for fatty acid ethyl esters.

GC Analysis of Fatty Acid Ethyl Esters

A Shimadzu GC-7AG gas chromatography equipped with a flame ionization detector (FID) was used for GC analysis of fatty acid ethyl esters. GC separations were performed on a Bpl capillary column (50 m X 0.22 mm ID) (SEG Company). The temperature was programmed from 150°C to 280°C at the rate of 3°C/min increment, while the temperature at the injector and the detector was kept constant at 280°C. The carrier gas was nitrogen.

RESULTS AND DISCUSSION

Refinement of Crude FOEE

GC analysis of the FOEE showed that it contained 1.1% of ethyl myristate, 1.7% of ethyl hexadecenoate, 1.8% of ethyl palmitate, 5.5% of ethyl octadecenoate, 0.9% of ethyl octadecanoate, 28.8% of ethyl eicosapentenoate (EEPA) and 41.0% of ethyl docosahexaenoate (EDHA) (Fig. 1 A). Using HSCCC 30 mL of the FOEE was refined with the non-aqueous solvent system of hexane-acetonitrile to produce 23 mL of colorless refined FOEE which contained 51.6% of EDHA, 36.0% of EEPA, 6.7% of ethyl octadecenoate and 2.0% of ethyl hexadecenoate (Fig. 1 B).

We found that refined FOEE was colorless and free of saturated fatty acid esters. It appears that unsaturated fatty acid esters are more hydrophobic and therefore have higher partition coefficients than the saturated fatty acid esters.

Separation of Refined FOEE

Table 1 shows the separation of 1 g of the refined FOEE by HSCCC with the hexane-dichloromethane-acetonitrile (5:1:4, v/v/v) system. Three components I, II and III were obtained. Component III (corresponding to peak C16:1 in GC) and component II (corresponding to peak C18:1 in GC) were ethyl hexadecenoate and ethyl octadecenoate, respectively (see Fig. 1 A and B).

Component I (corresponding to peak C20:5 and C22:6 in GC in Fig. 1 A and C) was a mixture of EDHA and EEPA. Fractions containing component I was collected and dried in vacuum to yield 910 mg of mixture containing 56.4% of EDHA and 39.3% of EEPA (Fig. 1 C).

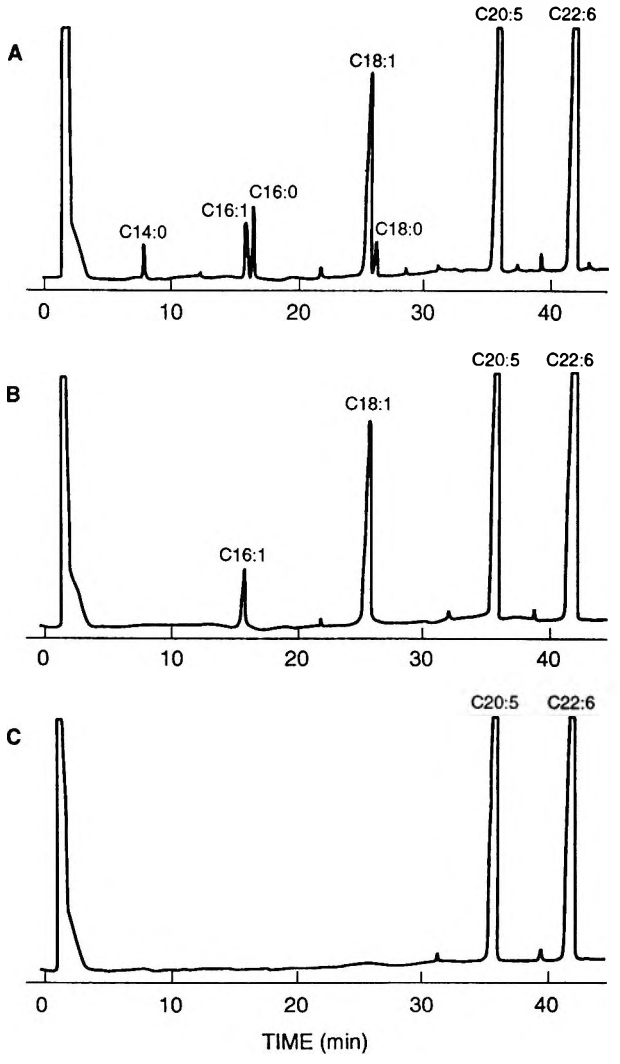

Figure 1. GC analysis of three samples. A: Fish oil ethyl ester (FOEE); B: the refined FOEE obtained from HSCCC refinement of crude FOEE; C: component I obtained from HSCCC separation of the refined FOEE. C14:0 (ethyl myristate), C16:1 (ethyl hexadecenoate), C18:1 (ethyl octadecenoate), C18:0 (ethyl octadecanoate), C20:5 (ethyl eicosapentenoate) (EEPA), C22:6 (ethyl docosahexaenoate) (EDHA).

Table 1

GC Analysis of HSCCC Fractions Obtained from the Refined FOEE

Fraction No.[1]	Retention Time (min)	Detection[2]	Component[3]
1	35	-	
3	40	-	
5	45	-	
7	50	+	I
9	55	++	I
11	60	++++	I
13	65	+++++	I
15	70	++++++	I
17	75	+++++	I
19	80	++++	I
21	85	+++	I
23	90	++	I
25	95	+	I
27	100	-	
29	105	+	II
31	110	+	II
33	115	+	II
35	120	+	II
37	125	-	
39	130	+	III
41	135	+	III
43	140	+	III
45	145	-	

[1] Fraction volume: 5 mL/min.

[2] GC analysis: 0.5 μL of each fraction was injected. -: no fatty acid ester. +: small amounts of fatty acid esters. ++ to ++++++: larger amounts of fatty acid esters.

[3] Component I: a mixture of ethyl docosahexaenoate (EDHA) and ethyl eicosapentenoate (EEPA); Component II: ethyl octadecenoate; Component III: ethyl hexadecenoate.

The overall results of the present studies indicate that HSCCC can be used for separation and purification of fatty acid ethyl esters with non-aqueous solvent systems. The method may play an important role in the field of oil chemistry in future.

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SIMULTANEOUS HPLC DETERMINATION OF VIGABATRIN AND GABAPENTIN IN SERUM WITH AUTOMATED PRE-INJECTION DERIVATIZATION

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ABSTRACT

A simple and partially automated method for the HPLC determination of the new anticonvulsants vigabatrin and gabapentin in serum samples from epileptic patients is described. The first step in sample pre-treatment is the precipitation of the serum proteins using an excess of acetonitrile to which two internal standards have been added. After centrifugation the supernatant fraction of the samples was placed into an autosampler of an HPLC apparatus which can be programmed with different automatic procedures for pre-injection derivatizations.

The amino acids vigabatrin and gabapentin are derivatized by reactions with ortho-phthaldialdehyde (OPA) and mercaptoethanol. The OPA derivatives are separated on a reversed phase column and measured by fluorimetry. Detection of the gabapentin derivatives requires careful removal of oxygen from the mobile phase by degassing continuously with helium. In this respect the fluorescence signal of vigabatrin derivatives is

less sensitive to a loss in intensity by the back diffusion of oxygen into the mobile phase after degassing with helium for only a short time.

INTRODUCTION

Vigabatrin (4-aminohex-5-enoic acid, VIGA) is a γ-vinyl derivative of the γ-aminobutyric acid (GABA) and shows its anticonvulsant effect by inhibition of the GABA degrading enzyme GABA transaminase. Thereby a high specificity is combined with a low toxicity.[1]

Gabapentin (1-(aminomethyl) cyclohexane acetic acid, PENTI) was initially developed as a structural analogue to the inhibitory neurotransmitter GABA. Examination has, however, shown that it neither inhibits the uptake of GABA nor does it influence the enzymatic degradation of GABA. An explanation for the anticonvulsant activity of gabapentin is at present still the subject of research.[1] Advantages of therapeutic drug monitoring for the two new anticonvulsant drugs VIGA and PENTI remain still speculative. At least documentation of patient compliance seems to be important in cases refractory to drug therapy. Furthermore the knowledge of serum concentrations may be necessary for studies concerning side effects and efficacy of the drugs.

The first studies for the analysis of VIGA by HPLC methods were published about 10 years ago.[2-5] Grove et al.[2] carried out protein precipitation in serum and urine samples using trichloroacetic acid and HPLC separation on an ion exchange column in an amino acid analyser. Post column derivatization with ortho-phthaldialdehyde (OPA) was used for fluorimetric detection. Smithers et al.[4] likewise precipitated the proteins from serum and urine samples using an excess of acetonitrile. In order to increase the selectivity of the assay copper chloride was added which should form complexes with the endogenous amino acids. Thus their reaction with dansyl chloride was prevented during the ensuing derivatization. The dansyl derivatives of VIGA and the internal standard (γ-phenyl GABA) were extracted with ethylacetate. After evaporating the solvent off the residues were resolved in mobile phase and injected into a reversed phase column. The separated derivatives were measured by fluorimetric detection.

Chen et al. describe the HPLC separation of the enantiomers of VIGA using UV detection for the diastereomeric derivatives with *tert.*-butyloxy-L-leucine N-hydroxysuccinimide ester.[3] Later on they published an HPLC analysis of VIGA and a primary degradation product in tablets.[5] In this case they carried out a separation on a Partisil® SCX column using UV-detection at

a wavelength of 210 nm. Two further papers on the determination of VIGA in plasma samples using HPLC with fluorimetric detection of OPA derivatives were published in 1991 and 1993. Thereby in both methods the proteins of the plasma samples were precipitated by adding methanol.[6,7]

At least three papers dealing with the HPLC analysis of PENTI have been published. In these papers the same derivatization reagent (2,4,6-trinitrobenzene-sulphonic acid, TNBSA) and the internal standard 1-(aminomethyl) cycloheptane acetic acid (Gö-3609) were used. In the basic study from 1985 of Hengy and Kölle[8] protein precipitation of the plasma samples was carried out using perchloric acid prior to derivatization. The TNBSA products were purified in two liquid liquid extraction steps.

Fraser and MacNeil[9] precipitated serum proteins with acetone and purified the derivatives with acetic acid precipitation. In the third paper by Lensmeyer et al.[10] PENTI and the internal standard were extracted from the serum by a solid phase extraction on a C_{18} cartridge. Derivatization was performed in the methanolic eluate and the reaction products were purified by a further solid phase extraction with a hydrophobic filter membrane (Empore disk® C_{18}).

In the following paper an HPLC method for the simultaneous determination of Vigabatrin and Gabapentin is described. A simple sample pretreatment in which proteins were precipitated with acetonitrile was followed by the automated derivatization of the analytes with the reagent OPA / mercaptoethanol. The reaction products were finally separated on a reversed phase column and measured by fluorimetric detection.

MATERIALS and METHODS

Apparatus and Reagents

As HPLC system for automated derivatization the 1090 LC with a workstation and a 1046A programmable fluorescence detector from Hewlett-Packard, Waldbronn (Germany), was used. Gradient separations were carried out on a BANsil C_{18} column (5 µm, 250 x 4 mm) from ASMT, Enger (Germany).

Acetonitrile, water, and methanol were of HPLC gradient quality from Riedel-DeHaen, Hannover (Germany). All other reagents used were of analytical reagent grade from Merck, Darmstadt (Germany).

Vigabatrin and the internal standard (ISTD) substance γ-phenyl GABA were obtained from Marion Merrel Dow Research Institute, Cincinnati, Ohio (USA), and Gabapentin and the ISTD Gö-3609 were obtained from Parke Davis, Freiburg (Germany).

The ISTD solution consisted of 100 mg γ-phenyl GABA and 10 mg Gö-3609 dissolved in 500 mL acetonitrile and 500 mLwater.

The borate buffer was made with 15.5 mg boric acid dissolved in 500 mL water and adjusted to pH 9.5 with a concentrated sodium hydroxide solution in water.

The OPA reagent mixture consisted of 100 mg *ortho*-phtaldialdehyde, 9 mL methanol, 1 mL borate buffer, and 100 µL mercapto-ethanol.

Calibration and Control Samples

For the production of calibration samples stock solutions were made up with 20 mg VIGA and 10 mg PENTI in 100 mL of a mixture of acetonitrile/water (1:1, v/v). With this solvent mixture the analytes and also the ISTD substances can be dissolved easily, whereas acetonitrile or water alone are not equally suitable as solvents for the compounds mentioned. 50 µL portions of the stock solution were added to 500 µL blank serum in 10 mL centrifuge tubes with screw caps. The tubes were closed and frozen at -18°C.

As no controls for the routine determination of VIGA or PENTI are commercially available serum samples from patients with Sabril® (Viga-batrin) or Neurontin® (Gabapentin) medication were pooled. From the pooled sera 500 µL portions were frozen and analysed following the method described in order to check the run-to-run precision of the results.

Extraction and Derivatization

500 µL ISTD solution and 1000 µL acetonitrile were added to 500 µL of patient serum in 10 mL centrifuge tubes (as well as to the calibration and control samples). The tubes were shaken for 5 min in a vortex apparatus. The mixture was centrifuged for 15 min.

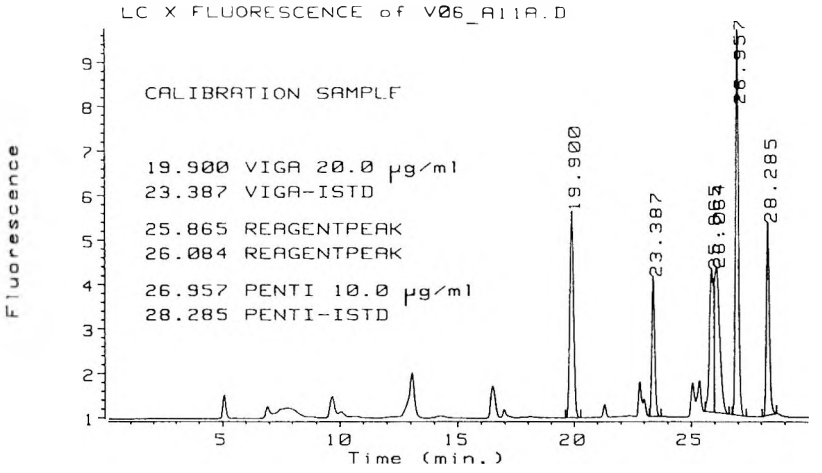

Figure 1. Chromatogram of a calibration sample with 20 µg/mL VIGA and 10 µg/mL PENTI in blank serum

The clear supernatant fraction was transferred to the derivatization procedure in a special "automated pre-column sample preparation system"[14] which consists of the 1090 LC autosampler and a derivatization program of the HP workstation. The sample extract and the reagents for the OPA derivatization are pipetted up from the different vials by the autosampler and transferred into the injector loop.

After mixing the collected portions by a forward/backward movement of the injector syringe and a delay time for completion of the OPA reaction, the derivatization products are injected onto the analytical column. The derivatization steps are as follows: draw 0 µL from vial 0 (containing acetonitrile/water for flushing the outside of the injection needle), draw 3 µL from vial 1 (containing borate buffer as described above), draw 3 µL from vial 2 (containing the OPA reagent mixture as described above), draw 0 µL from vial 0, draw 6 µL from a sample vial, draw 0 µL from vial 0, draw 3 µL from vial 2, draw 3 µL from vial 1, draw 0 µL from vial 0, draw 2 µL of air, mix 20 cycles with a 10 µL volume of the syringe, wait 1 min, inject.

Parameters of Separation and Detection

Chromatography of the derivatization products was carried out by a gradient elution at 40°C to obtain a baseline separation of the four analytes from endogenous compounds and reagent interferences (see figures 1-4).

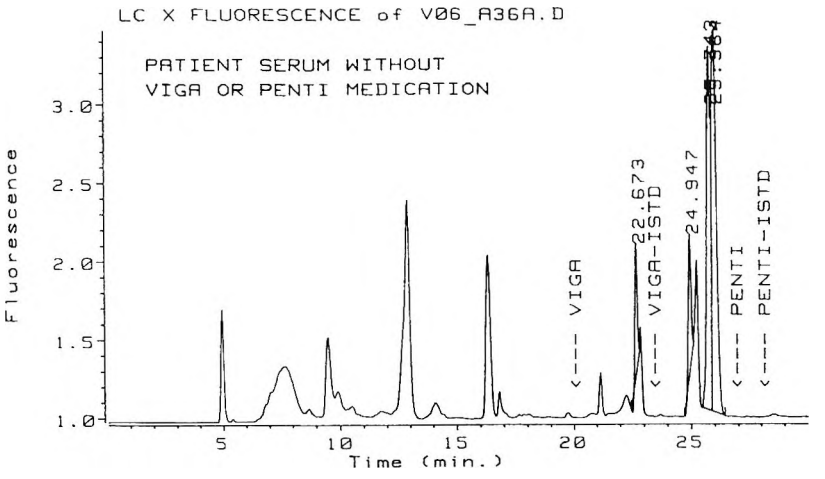

Figure 2. Chromatogram of a serum from an epileptic patient without Sabril® or Neurontin® medication.

Solvent A for the gradient mixture was 0.1% phosphoric acid (pH 2), acetonitrile, and methanol (8:1:1, v/v/v) and solvent B was acetonitrile and methanol (1:1, v/v). The flow rate was 1.0 mL/min. The gradient profile was as follows: 10% B from min 0 to min 1, from 10% to 70% B from min 1 to min 26, 70% B from min 26 to min 29, and back to 10% B from min 29 to min 29.1, stop run at min 30. Delay time before the next injection was 3 min.

The detection parameters of the fluorescence detector were as follows: Excitation = 235 nm, emission = 435 nm, Pmtgain = 8, lamp frequency 55 Hz, response time = 1000 msec.

RESULTS and DISCUSSION

Extraction Conditions and Sample Amount

As described earlier[11,12] it was observed that a one to one ratio of acetonitrile and the aqueous serum sample was not sufficient to yield a complete deproteinization. With an ISTD solution containing equal amounts of acetonitrile and water and a two-fold volume of acetonitrile added to the serum

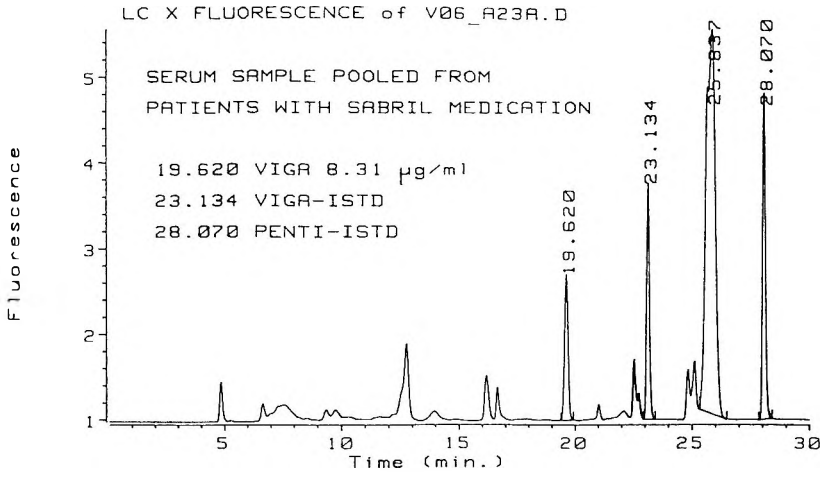

Figure 3. Chromatogram of a patient serum with a VIGA concentration of 8.3 µg/mL.

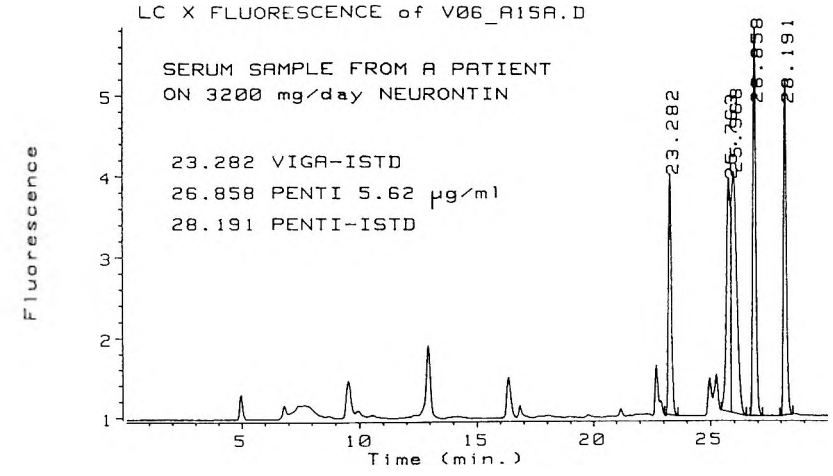

Figure 4. Chromatogram of a patient serum with a PENTI concentration of 5.6 µg/mL.

sample the final amounts of acetonitrile (1250 µL) and the aqueous phase (750 µL) are in a ratio of 5 to 3. With this minimum excess of acetonitrile a complete precipitation of the proteins can be achieved.[13]

The amounts of the samples, of the ISTD solution and of the precipitation reagent, as given above, were chosen to result in a better handling and a better precision in pipetting the volumes. If smaller sample amounts were obtained from the clinic (e.g. paediatric samples) a reduction of the volumes in the pre-treatment of the patient samples causes no problems if ratios of sample amount and ISTD are the same as with the calibration samples.

From a mixture of 100 µL serum, 100 µL ISTD solution, and 200 µL acetonitrile more than enough of the supernatant fraction is obtained for the derivatization procedure in which only 6 µL of the sample extract are needed for one chromatographic separation. Therefore, if necessary, the volumes in the sample pre-treatment step can be reduced even more to an extent which is limited by the precision of the manual pipetting and the handling of the smaller volumes.

Chromatographic Conditions

The main problem in the HPLC analysis of drugs which are amino acids consists in separating the analytes and the internal standards from endogenous amino acids which are always present in the serum samples and which also react with OPA. In order to optimise this separation we developed a gradient with a phosphoric acid/acetonitrile/methanol mixture to separate VIGA, PENTI and the two internal standard substances from the other serum constituents (see figures 1-4).

As can be seen from figures 1-4 the gradient separation delivers peaks of VIGA, PENTI, and the two ISTDs at positions in the chromatograms which are not overlapped by the peaks of derivatives of the endogenous substances in the serum (especially figure 2).

Linearity of Detection

In order to check on the linearity of the fluorimetric detection, different amounts of VIGA and PENTI were dissolved in water/acetonitrile (1:1) in a range much higher than normally expected in patient samples and measured as described above. For VIGA, linearity was measured in a range from 0 to 300 µg/mL with a coefficient of correlation (CC) of 0.99988 and a standard error of estimate (SEE) of 1.9094. The slope was 1.006 and the intercept -.519.

For PENTI, linearity was measured in a range from 0 to 300 µg/mL with a CC of 0.99985, a SEE of 1.3271, a slope of 0.9947, and an intercept of 0.238.

Table 1

Recovery of VIGA and PENTI from Serum Samples after Deproteinization

Vigabatrin						
Conc. [μg/mL]	1.05	2.50	10.0	20.0	40.0	Mean
Recovery [%]	94.34	111.2	100.7	97.59	101.1	100.97
Gabapentin						
Conc. [μg/mL]	1.01	4.90	10.1	19.9	39.8	Mean
Recovery [%]	97.78	100.1	100.2	99.60	99.79	99.47

Recovery of the Extraction Procedure

In order to determine the degree of recovery of the analytes from the serum using the extraction and derivatization methods described above stock solutions containing VIGA and PENTI were added to water as well as to blank serum and analysed (n = 4 for each concentration) in the same way as patient samples.

The results, given in Table 1, show no relevant differences between the results obtained from aqueous or serum samples spiked with VIGA and PENTI. Thus the sample pre-treatment given above yields in a complete recovery of the analytes from serum samples.

Method Precision

Before determining the overall precision of the method described for the analysis of serum samples it must first be checked to what extent the automatic derivatization with the HPLC equipment used for the analysis of VIGA and PENTI contributes to imprecision.

Therefore the derivatization program was carried out twelve times by the autosampler with the same solution of suitable concentrations of the analytes in acetonitrile and water and the deviations of the results were statistically checked.

As seen from the results given in Table 2 the degree to which the apparatus contributes to the imprecision of the automatic derivatization of VIGA and

Table 2

Precision of the HPLC Apparatus for the Automatic OPA Derivatization

	n	Max.	Min	Mean	SD	CV%
VIGA	12	20.00	19.24	19.77	0.2053	1.039
PENTI	12	10.10	10.00	10.07	0.0325	0.323

(SD = standard deviation, CV% = coefficient of variation)

PENTI with OPA is between 1.039% and 0.323% determined as coefficient of variation, if the influence of serum extraction by protein precipitation and the effect of the sample background in the chromatogram due to the serum matrix are omitted.

In order to measure the overall precision of the method described pooled samples from the sera of patients on treatment with Sabril® or Neurontin® were analysed. In each case different concentrations were measured several times on the same day to establish the "within-run" precision.

Likewise the values of pooled samples measured as internal controls over a longer period of time were evaluated in order to establish the "run-to-run" precision. The results in Table 3 show coefficients of variation far below 5% which show that the method described is well suited for routine determinations in clinical laboratory analysis.

Limit of Determination

Normally the range of the determination is related to the "therapeutic range" of the drugs to be analysed. Whereas the detection limit of the method described for the determination of VIGA and PENTI in serum samples might be well below 0.1 μg/mL, a limit of determination was settled with 1.0 μg/mL for Vigabatrin and with 0.5 μg/mL for Gabapentin. These limits meet the practical requirements of the routine analyses of both anticonvulsants.

As shown in table 4 the results of the coefficients of variation of small peaks, even with concentrations just below the determination limits, are tolerable for routine analyses of patient samples.

Table 3

Within-Run and Run-to-Run Precision for VIGA and PENTI in Serum Samples (conc. μg/mL)

	VIGA "within-run"	PENTI "within-run"
N	20	20
Minimum	8.11	3.65
Maximum	8.53	3.80
Mean value	8.370	3.762
standard deviation	0.0913	0.0307
C.V. (%)	1.091	0.816
	VIGA "run-to-run"	**PENTI "run-to-run"**
N	22	22
Minimum	7.82	3.83
Maximum	8.84	3.99
Mean value	8.425	3.925
standard deviation	0.2097	0.0450
C.V. (%)	2.489	1.147

Lower concentrations than those calculated in table 4 are hardly of clinical significance for routine analysis, but are possibly of scientific interest. Then it is recommended that in sample pre-treatment a higher effect of concentrating the analytes must be achieved as for example is described by Lensmeyer et al.[10] for the SPE extraction of PENTI from serum samples.

Special Requirements of the OPA Derivatization of PENTI

On developing the analytical procedure with automated OPA derivatization as described above we started with the development of a routine determination of VIGA in patient sera. In the beginning the mobile phase was degassed with helium only intermittently during refill of the solvent bottles in the HPLC apparatus. With this handling an unexpected but steady decrease of the PENTI and PENTI-ISTD concentrations was observed.

After some experiments it became evident that rediffusion of oxygen from the air resulted in a steadily decreasing intensity of the fluorescence signal in the case of both substances, whereas the OPA derivatives of VIGA and VIGA-ISTD were shown to be not sensitive against the oxygen concentration in the mobile

Table 4

Within-run Precision for low Concentrations of VIGA and PENTI in Serum (conc. μg/ml)

	VIGA "within-run"	PENTI "within-run"
N	10	10
Mean value	0.873	0.387
standard deviation	0.0356	0.0071
C.V. (%)	4.082	1.837

phase. With degassing the solvent reservoirs continuously with a very low helium gas flow we overcame this problem. This gives us the possibility to carry out routine determinations of VIGA and PENTI in the same analytical series. In addition we have the option to calculate the PENTI levels as determined using the VIGA-ISTD. This could be of importance in the near future as we were informed that the PENTI-ISTD will not be produced any longer by the manufacturer.

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USE OF SUPERCRITICAL FLUID EXTRACTION-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY IN THE DETERMINATION OF POLYNUCLEAR AROMATIC HYDROCARBONS FROM SMOKED AND BROILED FISH

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ABSTRACT

Polynuclear aromatic hydrocarbons (PAHs) from smoked and broiled fish were isolated by supercritical fluid extraction (SFE), and quantitatively determined by reversed phase liquid chromatographic (RP-HPLC) methods. The SFE-method was tested first with inert matrix. As expected, the extraction of PAHs from quartz sand gave good recoveries ranging from 80 to 100 % with R.S.D. of 3-11 %. The extraction of fish material showed that methanol had to be used as a modifier, because larger PAH molecules were weakly extracted with pure CO_2. The use of methanol resulted in, however, that lipophilic compounds were co-eluting in greater amounts, interfering the analysis of PAHs by HPLC - UV. That is why the extracts were purified using solid phase extraction (SPE) prior to

chromatographic determination. The extraction of one sample was completed in 20 minutes, while several hours are consumed in the conventional solvent extraction methods. The sensitivity of the method used is in ppb-range, which corresponds well with the concentration of PAHs in smoked fish products.

INTRODUCTION

The polynuclear aromatic hydrocarbons (PAHs) are formed in the inefficient combustion of organic matter. They contaminate foods through direct deposition from the atmosphere as environmental contaminants, or they are produced during cooking, baking or smoking of foods.

Some of the PAHs, such as benzo(a)pyrene, benzo(b)fluoranthene and dibenzo(a,h)anthracene, have been shown to have carcinogenic or mutagenic activity, the most effective being those with 5 or 6 fused rings. Fortunately, the most abundant PAHs in foods are smaller compounds thus being less harmful.[1-3]

Supercritical fluid extraction (SFE) has shown great potential in offering shorter extraction times with higher recoveries and low consumption of organic solvents comparing with traditional liquid extraction methods. The most commonly used fluid is carbon dioxide, CO_2, mainly because of its mild supercritical conditions (t_c= 31.1 °C, p_c= 7.38 MPa).[4]

The extraction and analysis of PAH-compounds have been the most popular application of supercritical fluid techniques in the field of environmental analysis.[5-10] SFE-methods have been developed for extracting PAHs from samples such as soil, marine sediments, aqueous media, and air samples.[9-13] One of the most difficult points in optimizing SFE of PAHs has shown to be the effect of matrix.[9,14]

As a matrix in SFE, fish tissue is rarely used, thus almost unknown. Nam et al. have published some papers dealing with the SFE of PAHs from spiked fish tissues[15] and pesticide residues from river fish.[16] Lee et al. have developed a method for a rapid determination of PCBs from fresh fish;[17] and a few papers about SFE of fish lipids have been published.[18-20]

In this study, we have developed a fast SFE-method for isolating PAHs from fish tissue. PAHs extracted were quantitatively determined by using liquid chromatographic methods. Samples compared were two distinct Baltic Herring (*Clupea harengus*) products: smoked and broiled fish samples.

MATERIALS AND METHODS

Preparation of Samples

Samples of fresh, smoked and broiled Baltic Herring products were purchased from local markets in January, 1995. The bones of broiled fish, the bones and the skin of the smoked fish were removed, and the edible parts of fish were homogenized and lyophilized. The fresh Baltic Herring fillets were homogenized as such. Prior to lyophilization, some fresh Baltic Herring samples were spiked with the synthetic mixture of PAH standard.

Standards

PAH-standards used were naphthalene (N) (E. Merck, Darmstadt, Germany); fluoranthene (F), pyrene (Py), (Aldrich-Europe, Beerse, Belgium); phenanthrene (Phen), anthracene (An), perylene (Per), benzo[a]pyrene (BaP), benzo[a]anthracene (BaA), fluorene (Fl) and chrysene (Ch) (Sigma Chemical Co., St. Louis, MO, USA). Standard stock solutions (1 mg/mL) were prepared in HPLC-grade trichloromethane (E. Merck, Darmstadt, Germany).

SFE-Procedure

Freeze-dried fish sample (1.000 g) and quartz sand (1.00 g) were mixed thoroughly and filled tightly into the extraction cartridge (volume 2.5 mL). In the extraction, ISCO SFX 220 SFE-equipment (ISCO Inc. Lincoln, NE, USA) with dual syringe pumps and restrictor heating device was used. The other pump was in the connection with a CO_2 -cylinder containing helium head pressure (99.998 %; OY AGA AB, Helsinki, Finland) and the other one with a reservoir containing HPLC-grade methanol (Lab-Scan Ltd., Dublin, Ireland). Before extraction, the cartridge filled was held about 10 min. at the extraction temperature (70 °C).

The other extraction conditions were as follows: pressure 350 atm (1 atm = 0.101325 MPa), fluid volume (measured as liquid at the head of the pump) 20 mL, and the fluid flow rate controlled by a piece of fused silica capillary (30 cm x 50 µm i.d., Polymicro Technologies Inc., Phoenix, AZ, USA) 1.5 - 1.7 mL/min. The analytes were collected by inserting the outlet of the restrictor into a tube containing 3 mL of hexane/dichloromethane (3:1). The collection

solvent was held at 5-10 °C in order to diminish restrictor plugging during the extraction.[21] The SFE-extracts were evaporated to dryness under nitrogen and diluted into 1 mL of HPLC-grade hexane (E. Merck, Darmstadt, Germany).

Cleanup of Extracts

The extracts were purified by scaling down the method described by Perfetti et al.[22] Instead of using commercial solid phase extraction (SPE) cartridges, the simple cleanup columns were prepared by packing 1.0 g alumina (Aluminum oxide 90, E. Merck, Darmstadt, Germany) and 0.8 g Silica Gel 60 (E. Merck, Darmstadt, Germany) into separate Pasteur pipettes. Alumina column was placed above silica column, and PAHs were eluted through the columns with 2 x 1.5 mL hexane/dichloromethane (3:1). The eluate was evaporated under nitrogen to about 1 mL after adding 2 mL of acetonitrile into tube. The eluate was further purified by elution through octadecyl SPE-cartridge (Bond Elut C18, 500 mg) with 2 mL acetonitrile. The sample was not further concentrated to avoid loss of more volatile PAHs with steam of acetonitrile. Instead, 1 µg naphthalene was added into the eluate in order to standardize the sample volume. Naphthalene was chosen, because it was not found in the fish samples.

Chromatographic determination

The purified extracts were analyzed by using a high-pressure binary gradient LC-system LC-6A with SCL-6A system controller unit and SPD-6AV spectrophotometric detector at 254 nm. (Shimadzu Co, Kyoto, Japan). The separation of PAHs was achieved with LiChrospher 100 RP-18 column (125 x 4 mm, 5 µm, E. Merck, Darmstadt, Germany) in 24 minutes when stepwise gradient from 50 % to 97.5 % acetonitrile in water and flow rate of 0.8 mL/min was used. In the extracts, PAHs were identified by using co-injection. The quantitative results were calculated by applying external standardization, the compounds quantified are listed in materials section.

RESULTS AND DISCUSSION

The HPLC-chromatograms of the synthetic mixture of PAH standards and PAHs from different Baltic Herring samples are shown in Fig. 1. The chromatogram A shows the resolution of the standard mixture. The detection limit with UV-detection used varied between 2 and 20 ng/mL, for anthracene

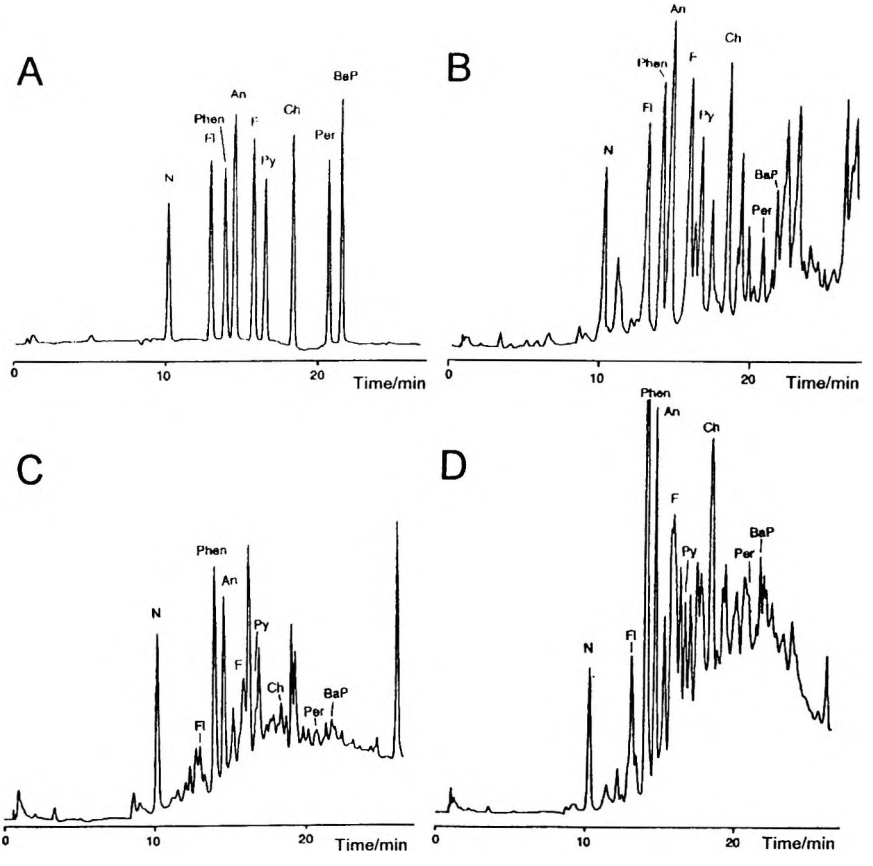

Figure 1. Separation of PAHs by HPLC and UV detection at 254 nm. A: mixture of PAH standards; B: fresh Baltic Herring sample spiked with standards; C: smoked Baltic Herring; D: broiled Baltic Herring. Abbreviations: see Materials section. HPLC-conditions: see Methods section

and pyrene, respectively. Fresh Baltic Herring samples were chosen for spiking matrix, because the amount of PAHs was below the UV-detection limit. Typically, concentrations of individual PAHs in Baltic Herring are less than 5 ng/g eatable part of the fish in dry weight basis.[23]

Fig. 1B. is a chromatogram of the spiked Baltic Herring sample, and Fig. 1C. and Fig. 1D. represent chromatograms of smoked and broiled fish samples, respectively.

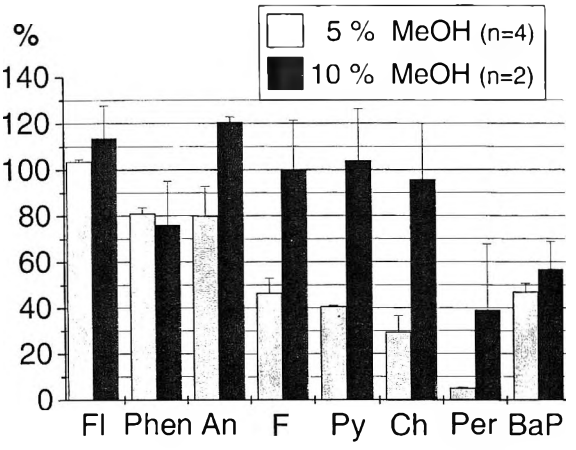

Figure 2. Effect of modifier addition to the recoveries of the individual PAHs spiked into fish tissue. Abbreviations: see Materials section.

As reported in the literature,[9-10] the extraction of spiked PAHs from inert quartz sand matrix was successful, e.g. at 250 atm and 55 °C recoveries varied from 80 to 100 % with R.S.D. of 3-11 %. The optimum conditions for extraction of PAHs from dried fish were studied by varying pressure, temperature and modifier concentration. SFE of fish material showed that the PAHs containing more than three rings were poorly isolated from tissue with neat CO_2. Our findings agreed with that of Nam et al.[15] They increased the solubility of PAHs by adding toluene directly into the sample in the extraction cell.

In our method, methanol, as a dynamic modifier, was added through another pump. All the PAHs studied were more soluble to the binary fluid than to the neat CO_2. Depending on the PAH-compound, the highest recovery was obtained with adding 5 or 10 % of methanol, as can be seen in Fig. 2. They reached recovery of 75-100 %, except perylene and bentzo(a)pyrene.

One explanation for this enhancement of extraction efficiency by the use of modifier may be the occupation of the active sites of fish matrix with modifier.[5,11] This may be the case in the study of Nam et al..[15] The other possible reason may be the co-elution of PAHs with lipids, which has been the problem in solvent extraction.[22] This might be true in our study, because the increasing of the polarity of the fluid by the modifier addition resulted in the extraction of reasonable amount of lipids.

The quantification of the PAHs in the extracts of herring products was sometimes difficult, because lipophilic compounds interfered the analysis of PAHs by HPLC-UV at 254 nm. Attempts were made to select such optimum extraction conditions (p and T), that the amount of lipids in the SFE-extracts decreased without affecting on the recovery of PAHs. The decreasing of the temperature or pressure lowered the solubility of fat to the fluid, but at the same time, recoveries of PAHs were reduced. A quite often used method for decreasing the co-extraction of lipids is to add aluminum oxide in the extraction cell.[17,24-26] In our study, co-extraction of lipids was only slightly reduced by mixing 1.0 g of aluminum oxide to 0.6 g sample prior to the extraction. Therefore, we ignored the use of alumina in the extraction cartridge, and instead, solid phase purification methods described in the methods section were used prior to the quantitative determination by HPLC-UV.

Samples of two distinct Baltic Herring (*Clupea harengus*) products showed great difference in PAH content and distribution of individual compounds as shown in Fig. 3. The amount of PAHs in freeze-dried, fresh samples was too low to be shown in the same scale. The values in the Fig. 3. are multiplied by response factors. The total PAH contents quantified in this work was about 180 ng/g and 1300 ng/g, in smoked and broiled fish samples, respectively. These concentrations are calculated to be corresponding with the edible part of the undried product. The amount of PAHs in smoked fish is in good agreement with published results,[2-3,27-28] although the correlation is difficult due to the differences in fish species,[27] isolation and chromatographic methods,[2-3,27-28] and difference between compounds quantified.[28] In both fish products studied the proportion of fluoranthene was the highest of the PAHs, i.e. about 220 ng/g (40 %) and 1.1 mg/g (30 %) on dry weight basis in smoked and broiled fish, respectively. Fortunately, the amounts of more harmful compounds, e.g. the concentrations of B(a)P were found to be smaller, i.e. about 40 ng/g in smoked and 400 ng/g broiled fish samples on dry weight basis. The PAH-content in edible parts of smoked fish depends obviously on the concentrations of these compounds in the wood smoke and the manufacturing method. The very high concentrations found in broiled fish sample has to be referred with the manufacturing method and consumption manners of this product in Finland. The daily intake of PAHs might increase by eating broiled fish, because according to national habit, the product is eaten as a whole, including the burned skin. In contrast, smoked fish is eaten without skin.

The sensitivity of the SFE-method with HPLC-separation and UV-detection is in ppb-range, which corresponds well with the concentration of PAHs in smoked Baltic Herring samples. More sensitivity is needed, if the

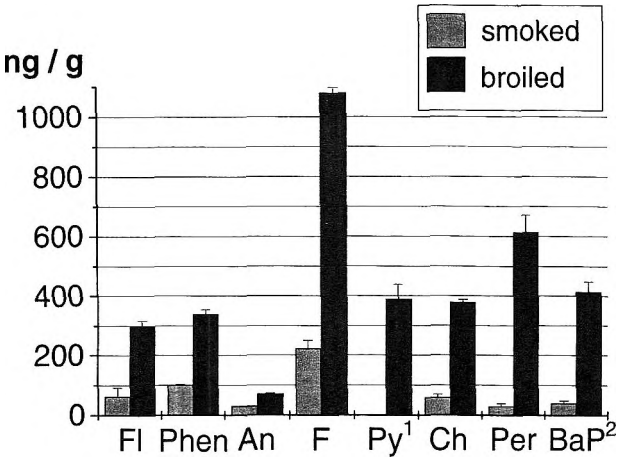

Figure 3. Concentrations of individual PAHs in smoked and broiled Baltic Herring samples (ng/g dry weight). Values are corrected with response factors. Abbreviations: see Materials section. [1] Not quantified from smoked fish. [2] n=2.

PAHs are determined in trace levels, like in fresh fish samples. It can be achieved by increasing the sample size, or increasing the sensitivity or selectivity of the chromatographic determination.

CONCLUSION

The extraction of one sample was completed in 20 minutes, while several hours were consumed in the conventional solvent extraction method. Despite the subsequent purification steps the savings in time and solvent consumption are remarkable. In the future, we consider the increasing of the sensitivity and specificity of the method by fluorescence or mass detection. At the same time the cleanup of the sample could be simplified, and the whole sample preparation procedure minimized.

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LIQUID-SOLID CHROMATOGRAPHY WITH COMPUTER-SELECTED MOBILE PHASES ON THE BASIS OF SNYDER THEORY

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ABSTRACT

The thin layer chromatographic (TLC) retention, R_M, and separation, α, on silica of esters of the natural (2R,3R)-tartaric acid, RO_2C CH(OH) CH(OH) CO_2 R, and related 1,3-dioxolanes were studied. Twenty mobile phases were used, being selected by the previously elaborated computer program LSChrom based on the Snyder parameters strength, ε, localization, m, and polarity, P'.

The available values of ε made it possible to derive conclusions about the adsorption pattern of the samples and to calculate their energy of adsorption, S. The relationships $R_M(m, P')$ and $R_M(S, E_s)$ were quantitatively studied where E_s is the steric constant of group R.

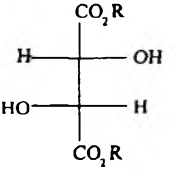
The conclusions summarize the application of LSChrom to TLC of 66 compounds with 161 mobile phases.

INTRODUCTION

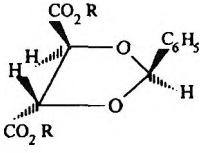
Liquid-solid chromatography (LSC) or, simply, LC such as HPLC, TLC and low or medium pressure LC is of great importance in separation and isolation of organic compounds. The stationary phases are limited, namely silica, modified silicas and alumina. Thus, the variety of mobile phases enables, in principle, the separation of the numerous organic compounds. The mobile phases have to possess an adsorptivity similar to that of the samples (solutes). They are pure solvents or, in the majority of the cases, mixtures of two and more solvents. The choice of the mobile phase for a given separation is usually done by the trial-and-error approach or by optimization approaches in separate cases (for instance, see ref. 1-4).

Recently, we have developed a computer program LSChrom[5] calculating the parameters strength, ε, localization, m, and polarity P' (see below) on the basis of Snyder theory.[6-8] This program simplifies the choice of mobile phases in LSC as shown by its application to TLC on silica or alumina of 54 compounds of different classes mainly diastereoisomers with 141 mobile phases composed of two to six solvents.[9-15] TLC, rather than HPLC, has been used because TLC enables an easy work with multicomponent mobile phases.

Trying to outline the utility of LSChrom, this paper reports its application to TLC of two other classes of compounds, namely the esters of (2R,3R)-2,3-dihydroxybutanic acid **1-6** and the related (4R,5R)-2-phenyl-4,5-dialkoxycarbonyl-1,3-dioxolanes **7-12**. The natural (2R,3R)-tartaric acid and its esters **1-6** are important building blocks for the synthesis of chiral compounds. The presence of the ester groups with an increasing effective volume of group R as in our previous studies[12,13] enabled us to elucidate the role of steric effects and adsorption energy in the cases studied.



1-6



7-12

R = CH_3, CH_2CH_3, $CH_2CH_2CH_3$, $CH(CH_3)_2$, $CH_2CH_2CH_2CH_3$ and $CH(CH_3)CH_2CH_3$

THEORY

The TLC retention, R_M, HPLC retention factor, k', and separation factor, α, are related by the well-known equations:

$$R_M \equiv \log k' = \log\left(\frac{1}{R_F} - 1\right) \quad (1)$$

$$\log\alpha = \log\frac{k'_m}{k'_n} = R_{M_m} - R_{M_n} \quad (2)$$

where R_F is the directly measured parameter in TLC and the subscripts m and n correspond to the number of the first and second compound of the solute pair, respectively. Snyder theory defines the following dimensionless parameters which characterize the mobile phase:

strength, ε, measuring the dimensionless Gibbs energy (ΔG° / $RT\ln 10$) of adsorption of the mobile phase per unit area of the adsorbent surface; the greater the ε value, the weaker is the sample retention.

localization, m, measuring the capability of the mobile phase for interaction *via* the available functional group/s of the composing solvent/s with specific adsorbent sites; it determines, to a great extent, the selectivity of the mobile phase as measured by α of solute pairs; the theory predicts a linear relationship between $\log\alpha$ and *m* as best separation is expected with either minimum or maximum *m* being established in many but not all cases.[8-13]

polarity, *P'*, measuring the total interaction ot the mobile phase with the sample; it tunes the mobile phase strength; a greater P' value means a better dissolution of the sample in the mobile phase and, therefore, a weaker retention.

The adsorptivity of a group *i* in the solute molecule is measured by the dimensionless Gibbs energy of adsorption, Q°_i, with pentane as a mobile phase having $\varepsilon = 0$. If the mobile phase has ε greater than zero, then the net energy of adsorption, Q_i, is reduced by the energy loss for desorption of mobile phase molecule/s from the adsorbent surface:

$$Q_i = Q^\circ_i - \varepsilon a_i \quad (3)$$

where a_i is the relative effective area under adsorption of group i.

To a first approximation, the dimensionless Gibbs energy of adsorption, *S*, of a solute molecule containing more than one group i when $\varepsilon > 0$ is given by

$$S = \sum_i Q_i \qquad (4)$$

This approximation is correct in discussion of the retention of compounds of a given class on same adsorbent.

EXPERIMENTAL

The synthesis of compounds **1-12** studied and their spectral characteristics will be published elsewhere.[16] Compounds **1-6** show intramolecular hydrogen bonds of the type *OH···OH*, while compounds **7-12** are free from intramolecular hydrogen bonding.

TLC was performed on silica $60GF_{254}$ (Merck, Germany) using the procedure given in ref. 17. The solvents used were of analytical-reagent grade. The R_M values were arithmetic means of two to three measurements showing a reproducibility of ± 0.02.

The computer program[5] used was LSChrom Ver.2 for Windows. The strength graph given in ref. 18 is an alternative to this program but, with a limitation in the precision of the reading within the graph and a limitation in the variety of the mobile phases.

RESULTS AND DISCUSSION

The mobile phases used in TLC on silica are shown in Table 1 together with their computer calculated[5] values of ε, m and P'. Mobile phases 1-10, with $\varepsilon = 0.348$, were used for the acyclic compounds **1-6** and mobile phases *11-20*, with $\varepsilon = 0.288$, were applied to the cyclic compounds **7-12**. Independently of the constant value of ε, the mobile phases within any of these two cases are not equivalent because of the variation in their values of m and P'. The total variation is $0.10 \leq m \leq 0.91$ and $0.25 \leq P' \leq 2.92$.

Table 2 shows the data for the experimental R_M values and the statistical quantities average R_M, $\bar{R}_M$, and standard deviation *(S.D.)*. Figure 1 represents the relationship between S.D. and $\bar{R}_M$.

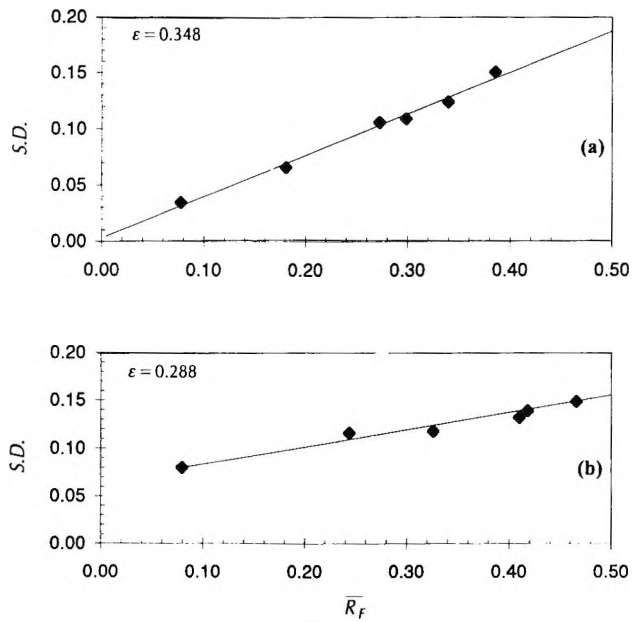


Figure 1. Relationship between *S.D.* and $\overline{R_F}$ values obtained for a given compound and all mobile phases used with equal calculated values of ε. The values of *S.D.* and $\overline{R_F}$ are from Table 2. The slope, intercept and correlation coefficient are (a) 0.37, 0.003, 0.995 and (b) 0.18, 0.065, 0.933.

Table 3 summarizes the values of the retention, R_M, and separation, α, of the compounds obtained by eqns. 1 and 2 from the experimental R_F values. The average values of log α for any solute pair with all mobile phases used are also included. The values of log α are usually positive, i.e. the first compound of the solute pair is better retained than the second compound.

The expected linear relationship between log α and *m* was not clearly seen. The variation of the mobile phases showed that the mobile phases 1,3,4,7,10,15 and 17 separate all solute pairs studied as the absolute values of log α are in the range 0.05-0.47. Mobile phase 6, composed of hexane and isopropanol, leads to best separation (log α = 0.68) of solute pair 1-2.

Table 4 shows data according to Snyder[6] for the adsorption properties of groups *i* participating in the compounds studied. The values of Q_i obtained by eqn.3 refer to the conditions used (ε = 0.348 or ε = 0.288). These data enabled

Table 1

Mobile Phases Used in TLC and their Computer-Calculated[5] Values of Strength, ε, Localization, *m*, and Polarity, *P'*

No.	Mobile Phase	%	ε	*m*	*P'*
	For TLC of Compounds ***1-6***				
1	Hexane-ethyl acetate	70.00 : 30.00	0.348	0.59	1.39
2	Hexane-diethyl ether	48.90 : 51.10	0.348	0.65	1.48
3	Hexane-diisopropyl ether	1.60 : 98.40	0.348	0.10	2.36
4	Hexane-dioxane	81.50 : 18.50	0.348	--	0.97
5	Hexane-acetone	84.90 : 15.10	0.348	0.92	0.86
6	Hexane-isopropanol	96.00 : 4.00	0.348	--	0.25
7	Toluene-ethyl acetate	86.50 : 13.50	0.348	0.45	2.67
8	Toluene-diethyl ether	80.00 : 20.00	0.348	0.48	2.48
9	Toluene-acetone	94.30 : 5.70	0.348	0.53	2.55
10	Toluene-isopropanol	98.15 : 1.85	0.348	--	2.43
	For TLC of compounds ***7-12***				
11	Hexane-ethyl acetate	88.00 : 12.00	0.288	0.58	0.62
12	Hexane-diethyl ether	79.30 : 20.70	0.288	0.63	0.66
13	Hexane-dioxane	91.60 : 8.40	0.288	--	0.50
14	Hexane-acetone	95.30 : 4.70	0.288	0.88	0.34
15	Toluene-methylenechloride	26.50 : 73.50	0.288	0.10	2.92
16	Toluene-ethyl acetate	95.00 : 5.00	0.288	0.20	2.50
17	Toluene-diethyl ether	92.30 : 7.70	0.288	0.20	2.43
18	Toluene-acetone	97.60 : 2.40	0.288	0.24	2.47
19	Toluene-isopropanol	99.28 : 0.72	0.288	--	2.41
20	Toluene-acetonitrile	96.50 : 3.50	0.288	0.20	2.52

the calculation of the energy of adsorption, S, of the compounds studied by eqn. 4. The values obtained, together with the data about the steric constant, E_s,[19] of group R are given in Table 5.

Three-dimensional representations of the retention, R_M, as a function of the mobile phase parameters *m* and *P'* are shown in Figure 2 and Figure 3. This relationship concerns the TLC of a given compound with all mobile phases used.

Figure 4 illustrates another 3-dimensional function, namely R_M of compounds studied with a given mobile phase vs. the solute parameters E_s and *S*.

Table 2

Experimental Values of R_F and Average Values of R_F, $\bar{R}_F$ and Standard Deviation (S.D.) for a Given Compound and All Mobile Phases Used for its TLC

Solute No.	R	R_F of compounds 1-6 for indicated mobile phase 1	2	3	4	5	6	7	8	9	10	$\bar{R}_F$	S.D.
1	CH_3	0.13	0.12	0.12	0.06	0.05	0.03	0.06	0.10	0.07	0.04	0.08	0.03
2	C_2H_5	0.30	0.25	0.27	0.14	0.12	0.13	0.16	0.20	0.14	0.10	0.18	0.07
3	C_3H_7-n	0.43	0.38	0.45	0.26	0.17	0.19	0.25	0.27	0.21	0.12	0.27	0.11
4	C_3H_7-iso	0.47	0.37	0.49	0.32	0.21	0.20	0.27	0.29	0.23	0.14	0.30	0.11
5	C_4H_9-n	0.52	0.39	0.58	0.34	0.23	0.23	0.32	0.36	0.27	0.16	0.34	0.12
6	C_4H_9-sec	0.56	0.48	0.69	0.43	0.25	0.23	0.37	0.38	0.29	0.18	0.39	0.15
		R_F of compounds 7-12 for indicated mobile phase 11	12	13	14	15	16	17	18	19	20	$\bar{R}_F$	S.D.
7	CH_3	0.14	0.17	0.14	0.08	0.41	0.34	0.40	0.33	0.14	0.29	0.24	0.12
8	C_2H_5	0.23	0.30	0.24	0.13	0.47	0.45	0.47	0.40	0.19	0.38	0.33	0.12
9	C_3H_7-n	0.32	0.40	0.32	0.20	0.54	0.54	0.67	0.47	0.25	0.47	0.42	0.14
10	C_3H_7-iso	0.33	0.42	0.34	0.19	0.52	0.53	0.64	0.44	0.23	0.46	0.41	0.13
11	C_4H_9-n	0.39	0.47	0.36	0.22	0.58	0.59	0.74	0.49	0.28	0.54	0.47	0.15
12	C_4H_9-n	0.39	0.49	0.39	0.24	0.61	0.73	0.76	0.51	0.30	0.50	0.49	0.16

Computer-aided Choice of the Mobile Phases

As adopted in our previous studies,[9-15] we started TLC of compounds **1-6** with the experimentally found mobile phase 1 giving R_F values in the favorable range $0.13 \leq R_F \leq 0.56$. This mobile phase is composed of hexane and ethyl acetate in a ratio 70:30 and is characterized by strength, $\varepsilon = 0.348$ calculated by LSChrom. We were interested to keep ε constant. Using LSChrom, we were able to calculate the ratios in which the solvents of mobile phases 2- 10 have to be taken ensuring $\varepsilon = 0.348$. Such a procedure was also applied to compounds **7-12** and thus mobile phases 11-20 were selected having $\varepsilon = 0.288$.

Using mobile phases of equal ε, R_F of all compounds vary in the region 0.03-0.76, i.e., there is no case when the compounds remain at the start line or move with the front line.

Moreover, the R_F values for any compound and all mobile phases used vary in a limited range owing to the variation in m and P', the possible difference between the calculated and experimental values of ε and the presence of complicating factors in TLC as demixing of the solvents composing the

Table 3

Values of the Retention, R_M, and Separation, α, Established for Acyclic Compounds 1-6 and Cyclic Compounds 7-12

Solute No.	R	1	2	3	4	5	6	7	8	9	10	Average
		R_M of compounds 1-6 for indicated mobile phase										
1	CH_3	0.83	0.87	0.87	1.19	1.28	1.51	1.19	0.95	1.12	1.38	
2	C_2H_5	0.37	0.48	0.43	0.79	0.87	0.83	0.72	0.60	0.79	0.95	
3	C_3H_7-n	0.12	0.21	0.09	0.45	0.69	0.63	0.48	0.43	0.58	0.87	
4	C_3H_7-iso	0.05	0.23	0.02	0.33	0.58	0.60	0.43	0.39	0.52	0.79	
5	C_4H_9-n	-0.03	0.19	-0.14	0.29	0.52	0.52	0.33	0.25	0.43	0.72	
6	C_4H_9-sec	-0.10	0.03	-0.35	0.12	0.48	0.52	0.23	0.21	0.39	0.66	
		Log α for indicated mobile phase										
1-2		0.46	0.39	0.43	0.41	0.41	0.68	0.47	0.35	0.34	0.43	**0.44**
2-3		0.25	0.26	0.34	0.33	0.18	0.20	0.24	0.17	0.21	0.09	**0.23**
3-4		0.07	-0.02	0.07	0.13	0.11	0.03	0.05	0.04	0.05	0.08	**0.06**
4-5		0.09	0.04	0.16	0.04	0.05	0.08	0.10	0.14	0.09	0.07	**0.09**
5-6		0.07	0.16	0.21	0.17	0.05	0.00	0.10	0.04	0.04	0.06	**0.09**
		R_M of compounds 7-12 for indicated mobile phase										
		11	12	13	14	15	16	17	18	19	20	
7	CH_3	0.79	0.69	0.79	1.06	0.16	0.29	0.18	0.31	0.79	0.39	
8	C_2H_5	0.52	0.37	0.50	0.83	0.05	0.09	0.05	0.18	0.63	0.21	
9	C_3H_7-n	0.33	0.18	0.33	0.60	-0.07	-0.07	-0.31	0.05	0.48	0.05	
10	C_3H_7-iso	0.31	0.14	0.29	0.63	-0.03	-0.05	-0.25	0.10	0.52	0.07	
11	C_4H_9-n	0.19	0.05	0.25	0.55	-0.14	-0.16	-0.45	0.02	0.41	-0.07	
12	C_4H_9-sec	0.19	0.02	0.19	0.50	-0.19	-0.43	-0.50	-0.02	0.37	0.00	
		Log α for indicated mobile phase										
7-8		0.26	0.32	0.29	0.24	0.11	0.20	0.12	0.13	0.16	0.18	**0.20**
8-9		0.20	0.19	0.17	0.22	0.12	0.16	0.36	0.12	0.15	0.16	**0.19**
9-10		0.02	0.04	0.04	-0.03	-0.03	-0.02	-0.06	-0.05	-0.05	-0.02	**-0.02**
10-11		0.11	0.09	0.04	0.08	0.11	0.11	0.20	0.09	0.11	0.14	**0.11**
11-12		0.00	0.03	0.06	0.05	0.05	0.27	0.05	0.03	0.04	-0.07	**0.05**

The values of R_M and log α are calculated from the experimental R_F values of Table 2 by eqns. 1 and 2, respectively.

mobile phase.[6] For instance, compound **8** and mobile phases 11-20 having calculated value of ε equal to 0.288 and different values of m and P' give R_F in the interval 0.13-0.47. To receive a measure for the similarity of the mobile phases owing to their equal ε, we calculated the values of average $\bar{R}_F'$ R_F' and standard deviation *(S.D.)* for any compound and all mobile phases used for its TLC. A well seen linear relationship between *S.D.* and $\bar{R}_F$ reflecting the structure of the compounds was established (see Figure 1). Thus, the similarity

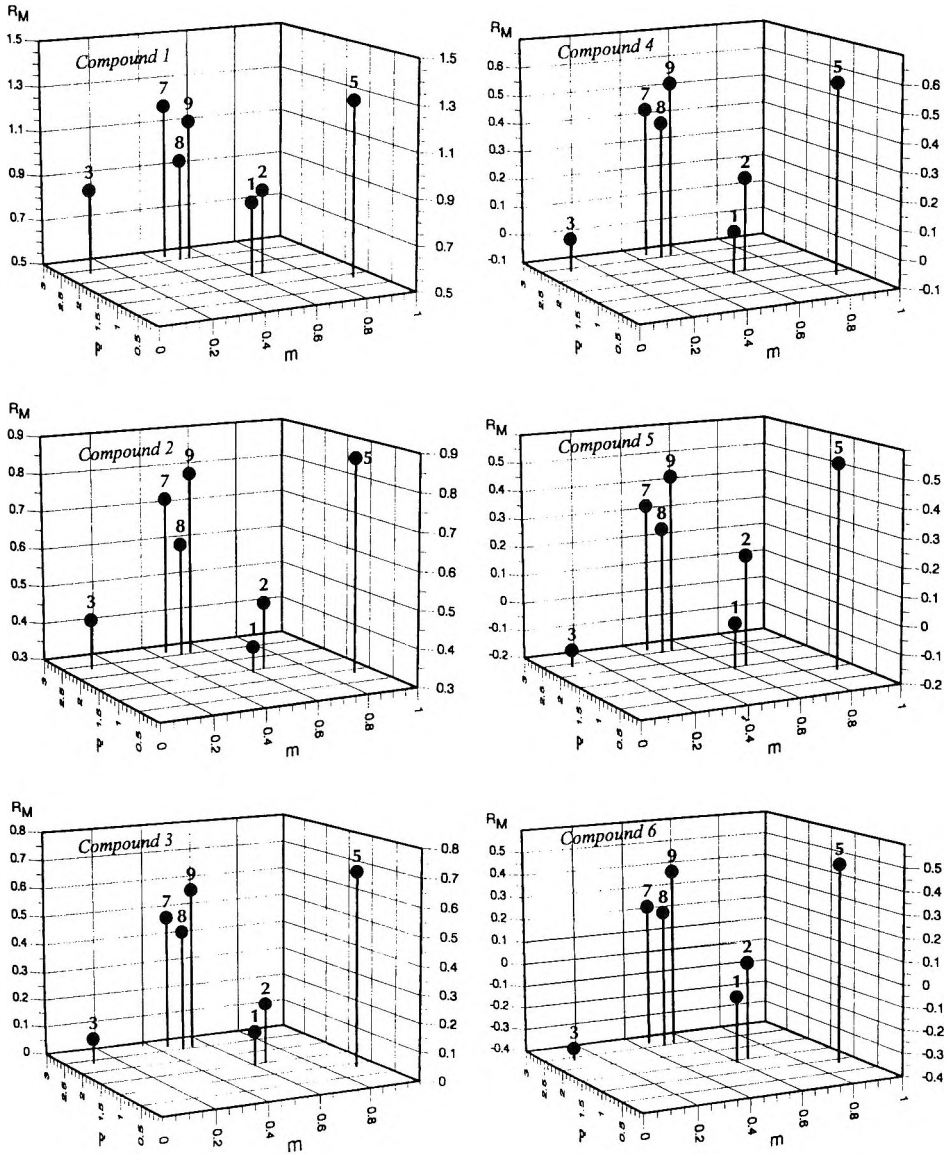


Figure 2. Three-dimensional representations of the retention, R_M , of any compound from **1-6** obtained with all mobile phases used as a function of the mobile phase parameters m and P'. The values of R_M are taken from Table 3. The number near any point specifies the mobile phase. The data for mobile phases 4, 6 and 10 are not included because of the absence of values for m.

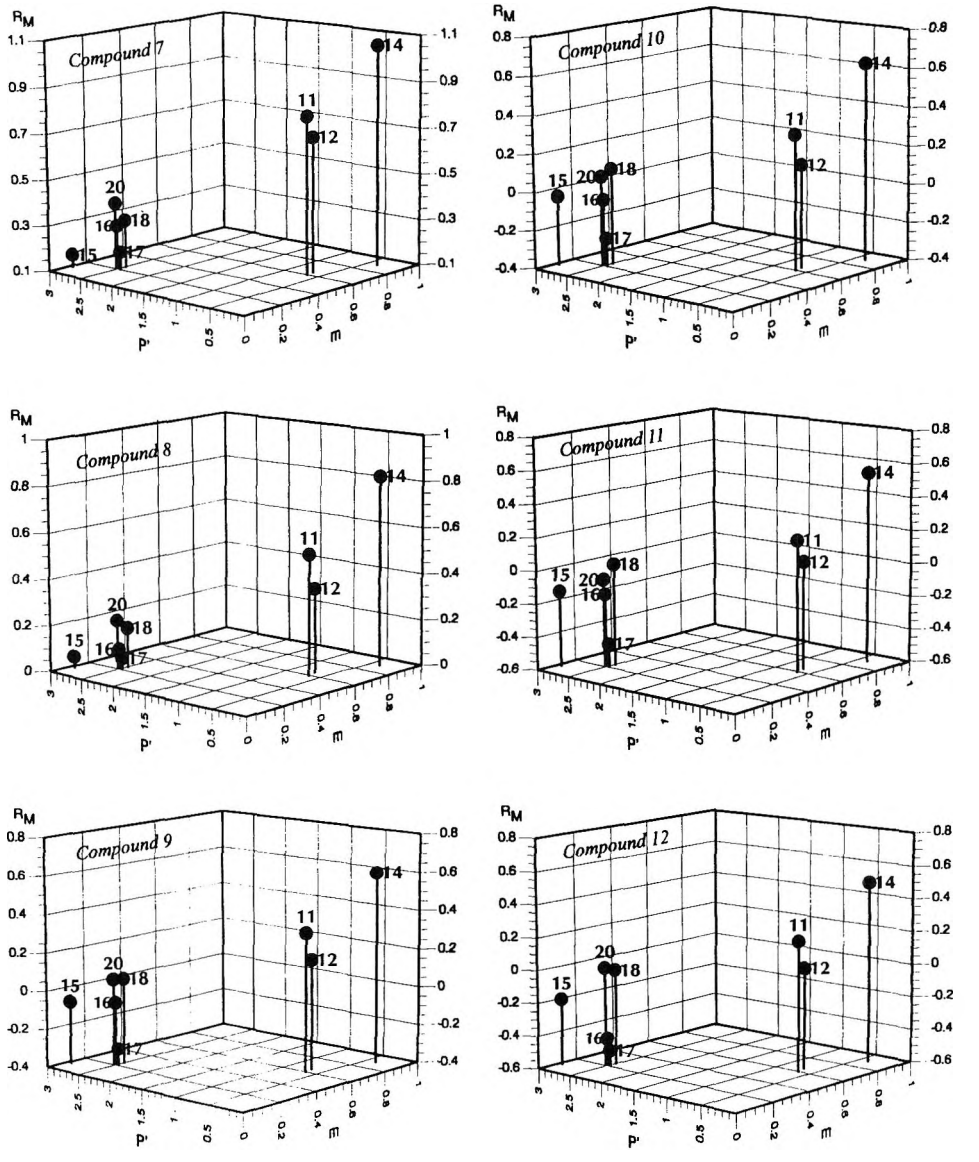


Figure 3. Three-dimensional representations of the retention, R_M, of any compound from **7-12** obtained with all mobile phases used as a function of the mobile phase parameters m and P'. The values of R_M are taken from Table 3. The number near any point specifies the mobile phase. The data for mobile phases 13 and 19 are not included because of the absence of values for m.

Table 4

Data According to Snyder[6] for the Adsorption Properties of Groups *i* Participating in the Compounds Studied

Group *i*	$Q°_i$	a_i	$Q_i = Q°_i - \varepsilon a_i$ ε=0.348	ε=0.288
C_6H_5	1.50	6.00	-0.59	-0.23
OH	5.60	8.50	2.64	3.15
O	3.61	9.00	0.48	1.02
CO_2CH_3	5.27	10.50	1.62	2.25
$CO_2C_2H_5$	5.22	11.40	1.25	1.94
$CO_2C_3H_7$-n	5.17	12.30	0.89	1.63
$CO_2C_3H_7$-iso	5.29	13.00	0.77	1.55
$CO_2C_4H_9$-n	5.12	13.20	0.53	1.32
$CO_2C_4H_9$-sec	5.24	13.90	0.40	1.24

The data for $Q°_i$ and a_i are taken from ref. 6, p. 200 and p. 264. The values of Q_i refer to the cases studied when the mobile phase has ε=0.348 or 0.288.

Table 5

Data for Steric Constant, E_s, of Group R and Energy of Adsorption, *S*, of the Compounds Studied

Group R	E_s	Cpd.	$S = \frac{2Q_{OH}}{2Q_{COOR}}$ + for ε=0.348	Cpd.	$S = \frac{2Q_O}{2Q_{COOR}}$ + for ε=0.288
CH_3	0.00	**1**	8.52	**7**	6.53
C_2H_5	0.07	**2**	7.79	**8**	5.91
C_3H_7-n	0.36	**3**	7.06	**9**	5.29
C_3H_7-iso	0.47	**4**	6.82	**10**	5.13
C_4H_9-n	0.39	**5**	6.34	**11**	4.67
C_4H_9-sec	1.13	**6**	6.09	**12**	4.51

The data for steric constant, E_s, are taken from ref. 19, p. 298. The values of *S* are obtained on the basis of the data of Table 4 and eqn. 4.

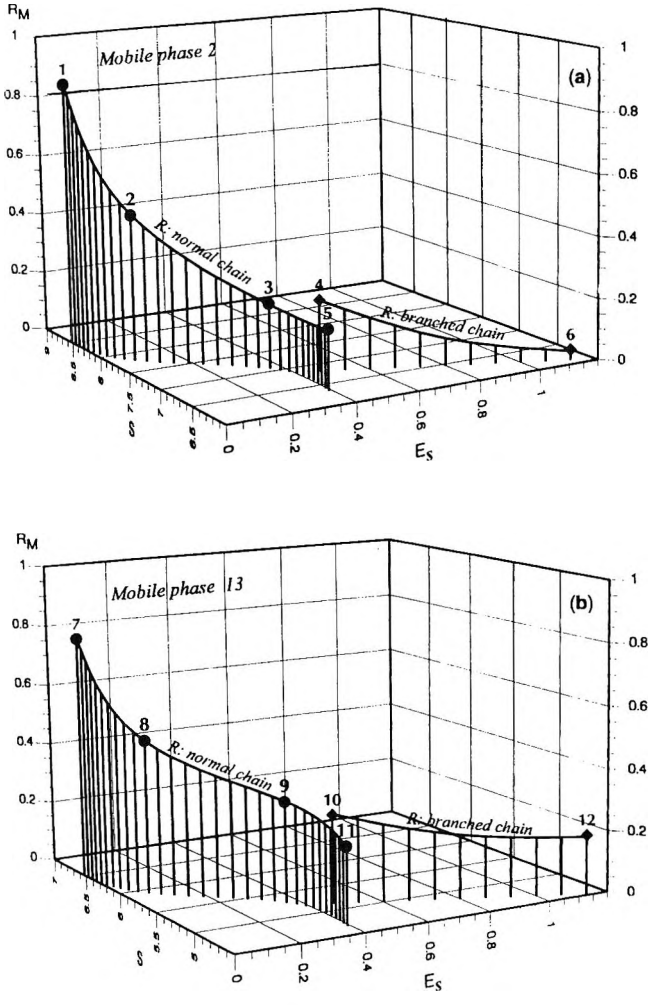


Figure 4. Three-dimensional representations of the retention R_M of (a) compounds **1-6** and (b) compounds **7-12** with the indicated mobile phase as a function of the solute parameters E_s and S. The values of R_M are taken from Table 3. The data for E_s and S are from Table 5. The number near any point denotes the compound.

of the mobile phases of equal calculated values of ε is expressed by *S.D.* at the corresponding R_F.

Expected Adsorption Patterns and Calculation of Solute Adsorption Energy, S

The adsorption pattern is determined by the adsorbing group/s of a solute molecule. The available values of ε for mobile phases used enabled us to deduce the adsorption patterns in the cases studied. As seen from Table 4, all groups *i* participating in the compounds studied, except the phenyl group, show $Q_i > 0$, i.e., the adsorption pattern of compounds **1-6** includes the two hydroxyl groups and the two ester groups and the adsorption of compounds **7-12** occurs *via* the two etherial oxygen atoms and the two ester groups. Then, the energy of adsorption, *S*, of any compound was calculated on the basis of eqn. 4 and the corresponding values of Q_i for the adsorbing groups. Being with greater values of Q_i, the hydroxyl groups in the case of compounds **1-6** and the ester groups in the case of compounds **7-12** have a greater contribution to S, than the remaining groups in solute molecules. *S* varied in the range 6.09-8.52 for the acyclic compounds **1-6** and in the range 4.51-6.53 for the cyclic compounds **7-12**. The greater adsorption energy of the first compounds explains the use of mobile phases with a greater value of ε for their TLC (see Table 1).

Retention, R_M, as a Function of Mobile-Phase Localization, m, and Polarity, P'

This relationship has not been quantitatively studied so far. The data of ref. 6, p. 277, are not sufficient to calculate the localization of all compounds studied, but it is apparently significant. The same is valid for the localizing solvents[8] such as ethyl acetate, diethyl ether, diisopropyl ether, dioxane, acetone, isopropanol and acetonitrile participating in the mobile phases used. Thus, the difference in the localization of the solute and mobile phase is expected to change the solute retention in comparison with the case when such a phenomenon is absent. The expected influence of P' on retention is given under Theory.

Figure 2 and Figure 3 represent R_M *vs.* m and P' in optimum R_M scales which permits a better visualization of some particularities. Thus, the retention of any compound from **1-6** is always greater with mobile phases 5 and 7-9 than that with mobile phases 1-3. This steady retention pattern is probably due to the greater contribution of the hydroxyl groups to adsorption in comparison with the ester groups as mentioned above. The influence of m and P' on R_M is not straightforward, which can be attributed to various specific interactions between the mobile phases and the hydrogen bonded solutes, i.e., the so-called secondary effects.

Within compounds **7-12**, the retention pattern is not so steady, especially with mobile phases 15, 16 and 18 revealing, probably, the increased role of the varying ester groups in the adsorption. A tendency for a stronger retention with mobile phases of smaller values of P' (as it is expected) and greater values of m is seen.

Retention, R_M, as a Function of Solute Steric Constant, E_s, and Energy of Adsorption, S

Steric constant, E_s, is one of the measures for the size of a group. Energy of adsorption, S, depends both on electronic and steric effects under LSC.[6]

The compounds within any of the two classes studied differ only in the size of group R, which varies from methyl to *sec.*-butyl. According to Table 5, this variation leads to an increase of the steric constant, E_s, of group R, which should hinder the retention and a decrease of energy of adsorption, S, which should reduce the retention. As seen from Figure 4, the compounds with a normal and a branched chain of group R form two independent series. The retention gradually decreases as it is expected in these two series and also in all remaining cases. This decrease is greatest for solute pairs 1-2 and 7-8 with a change of group R from CH_3 to C_2H_5. It determines the best separation of the two solute pairs as seen from the average values of log α of 0.44 and 0.20, respectively.

CONCLUSIONS

Our previous studies[9-15] and the present study report the application of the computer program LSChrom based on Snyder theory to TLC of 66 compounds of different classes, mainly diastereoisomers, with 161 mobile phases composed of two to six solvents. These studies lead to the following conclusions which show unequivocally the utility of the practical application of Snyder theory in LSC.

1. Similar in strength and different in selectivity mobile phases are easily selected by LSChrom. This approach makes it possible to avoid the unfavorable cases when the compounds remain at the start line or move with the front.

2. The separation, α, of a solute pair at a constant ε depends, in many cases, linearly on localization, m, of the mobile phase, being maximum at either minimum m or maximum m. In the cases when this linearity is masked by other effects, the best mobile phases giving maximum value of α are experimentally specified from various mobile phases with constant or increasing ε.

3. The computer calculated values of ε, m and P' enable to elucidate the adsorption pattern of the solutes and to study quantitatively various relationships such as $R_{A/}(m,P')$ and $R_{A/}(E_S,S)$. This is important for configurational determinations within diastereoisomers and for a better understanding of the separation process.

ACKNOWLEDGMENT

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HPLC METHOD FOR THE DETERMINATION OF EDTA IN AN OPHTHALMIC CLEANSER

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ABSTRACT

A reversed-phase high-performance liquid chromatographic (HPLC) procedure using ultra-violet (UV) detection for the analysis of edetate disodium (EDTA) via complexation with iron, in an ophthalmic cleanser, is reported. The method is selective, accurate, and reproducible. The peak area versus EDTA concentration is linear over the range of 50-150% of its label claim of 0.30 mg/mL, with a detection limit of 200 ng/mL. The mean absolute recovery of EDTA, using the described method, is 102.7 ± 0.4% (mean ± SD, n = 10).

[†]Current Address: Fujisawa USA, Inc., Pharmaceutical Sciences, 2045 N. Cornell Ave., Melrose Park, IL 60160-1002.

A stress study with heat, acid, base and UV radiation indicates that the method is stability-indicating with no interference from excipients or degradation products.

INTRODUCTION

An HPLC method for the determination of EDTA in a sterile ophthalmic cleanser that contains no dyes or perfumes, is reported. This cleanser is ideal for conditions that require daily eyelid hygeine and can also help in removing accumulated oily debris.

EDTA is a chelating agent that forms stable water soluble complexes with metal ions. EDTA is used commercially as a water softener, antioxidant, antibacterial, and anticoagulant.[1] Several methods exist for the determination of EDTA, including polarography,[2] amperometry,[3] catayltic-fluorometry,[4] spectrophotometry,[5] and potentiometry.[6,7] HPLC methods have been developed for EDTA, based on absorbance ratioing[8] and complexation with either iron or copper.[9-13]

To our knowledge, only two HPLC methods exist for the determination of EDTA in ophthalmic products.[9,13] Neither method examined the effect of stressing the ophthalmic product with heat, acid, base, or UV radiation. The degradation resulting from stressed samples could pose a selectivity problem. Also, the method described by Hall and Takahasi[9] is based on the method described by Bauer et al.,[13] whereby EDTA was complexed with copper.

Yamaguchi et al.[12] reported that the $Fe(EDTA)^-$ complex exhibited greater stability than the $Cu(EDTA)^{2-}$ complex. Consequently, iron complexation was selected for this current work.

The method described herein for the quantitation of EDTA is stability-indicating and satisfies the USP XXII guidelines under Assay Category I.[14] According to the USP XXII guidelines, analytical methods for the quantitation of major components of bulk drug substance or preservatives in finished pharmaceutical products fall under Assay Category I.[14] Data elements required for Assay Category I include precision, accuracy, selectivity, range, linearity, and ruggedness.

EXPERIMENTAL

Chemicals and Reagents

The ophthalmic cleanser was formulated at IOLAB Corporation (Claremont, CA, USA), which was subsequently acquired by CIBA Vision Ophthalmics (Duluth, CA). Edetate disodium was a USP reference standard. Ferric chloride hexahydrate and tetrabutylammonium hydrogen sulfate were purchased from Aldrich (Milwaukee, WI, USA). The water was deionized and then distilled.

All reagents were used without further purification.

Apparatus

The chromatographic system consisted of a Waters Model 600E system controller and pump, a WISP 712 autosampler, and a 486 variable-wavelength UV detector set at 254 nm (Waters Associates, Milford, MA, USA). A stainless-steel Adsorbosphere HS C_{18} column (4.6 x 150 mm, 3 μm, Alltech Associates, Inc., Deerfield, IL, USA) was maintained at ambient temperature.

Mobile Phase

The mobile phase simply consisted of 50 mM tetrabutyl ammonium hydrogen sulfate in water. The flow rate was 1.0 mL/minute with a typical operating pressure of *ca.* 130 bar.

Sample Preparation

An EDTA Stock solution was prepared at 1.50 mg/mL in water. A Standard solution was prepared by diluting the Stock solution 1:125 (v/v) with 0.1 mM ferric chloride hexahydrate prepared in water.

The Test solution was prepared by diluting the ophthalmic cleanser (label claim 0.30 mg/mL EDTA) 1:25 (v/v) with 0.1 mM ferric chloride hexahydrate prepared in water.

System Suitability

The system suitability results are calculated according to Chromatography <621> of the USP XXII from typical chromatograms.[14] The instrument precision, as determined by six successive injections of an EDTA Standard solution, should provide a relative standard deviation (RSD) not greater than 1.0%. The column efficiency should be greater than 4500 theoretical plates. The tailing factor should not exceed 2.0 at 5% peak height.

The Test solution (ophthalmic cleanser with sample work-up) is used to verify that the method meets all suitability limits, with exception of the instrument precision.

Stress Study

The selectivity of the method was studied through the analysis of stressed Test and Placebo (Test solution without EDTA) solutions. The stressed samples were subjected to heat, acidic, basic, and UV light environments.

Three mL aliquots of the Test and Placebo solutions were sealed in transparent containers and exposed to a UV radiation source (200-400 nm, 35 $mWatt/cm^2$) for 40 hours. Other 3.0 mL aliquots were adjusted to either pH 2 with concentrated HCl or pH 12 with 50% NaOH and sealed in glass containers with equal headspace and stored at 85°C for 40 hours.

Data Acquisition

The peak area of EDTA was measured using a PE Nelson 900 series interface and down-loaded to a PE Nelson Turbochrom 3 workstation (Perkin-Elmer Corporation, Cupertino, CA, USA). The chromatographic data was automatically processed for peak area, followed by an unweighted linear regression analysis.

Calculations

The response factor, **RF**, of the Fe-EDTA complex is determined by:

$$RF = \frac{W_S \times F}{V \times PA_S} \qquad (1)$$

where $\mathbf{W_s}$ is the weight (mg) of the edetate disodium standard used, **F** is the purity factor (mg/mg) of edetate disodium in the standard, **V** is the dilution volume, and $\mathbf{PA_S}$ is the peak area of Fe-EDTA complex for the Standard solution. Edetate disodium content of the Test sample, $\mathbf{C_T}$, is:

$$C_T(\text{mg/mL}) = RF \times PA_T \qquad (2)$$

where $\mathbf{PA_T}$ is the peak area of Fe-EDTA complex for the Test solution.

RESULTS AND DISCUSSION

Chromatography

Typical chromatograms obtained from a 20 μL injection of a Standard, Test and Placebo solution are illustrated in Figure 1 (a-c). The retention time of the Fe-EDTA complex is 5.2 minutes, with an overall chromatographic run time of 20 minutes.

System Suitability

The column efficiency for EDTA was 5723 theoretical plates. The tailing factor of EDTA was 1.2. The instrument precision, determined by 6 replicate injections of the Standard solution, exhibited a RSD of 0.5%.

Precision and Accuracy

The precision (RSD) and accuracy (relative error, RE) was determined by analyzing EDTA standards ranging from 50-150% (0.15 - 0.44 mg/mL), in replicates of six (Table 1).

Linearity

A linear response in peak area for EDTA over the range of 50-150% its label claim was observed. The correlation coefficients were 0.999 or better (n=6).

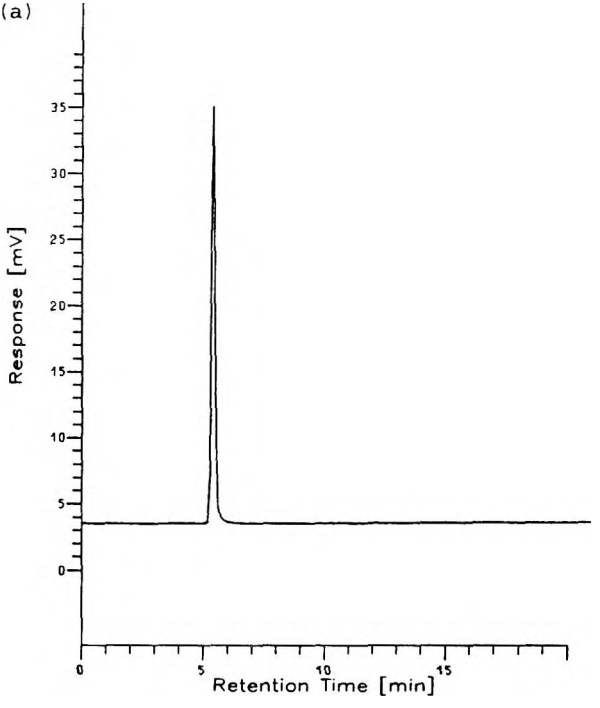
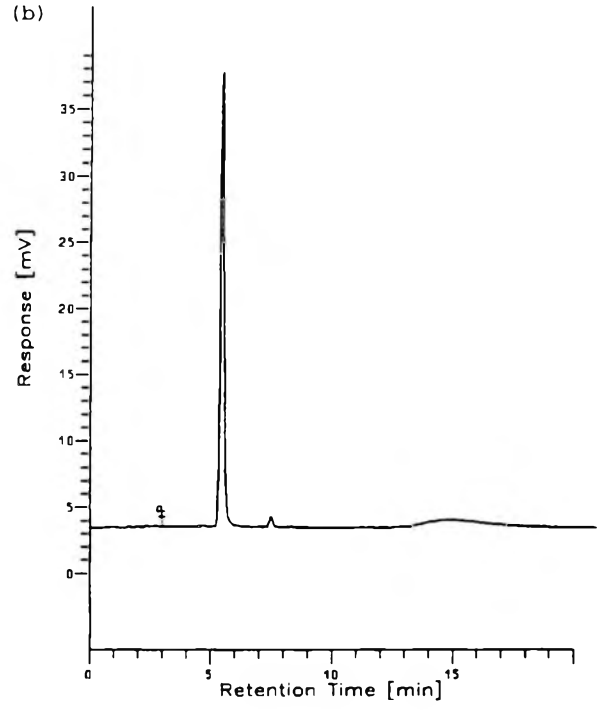
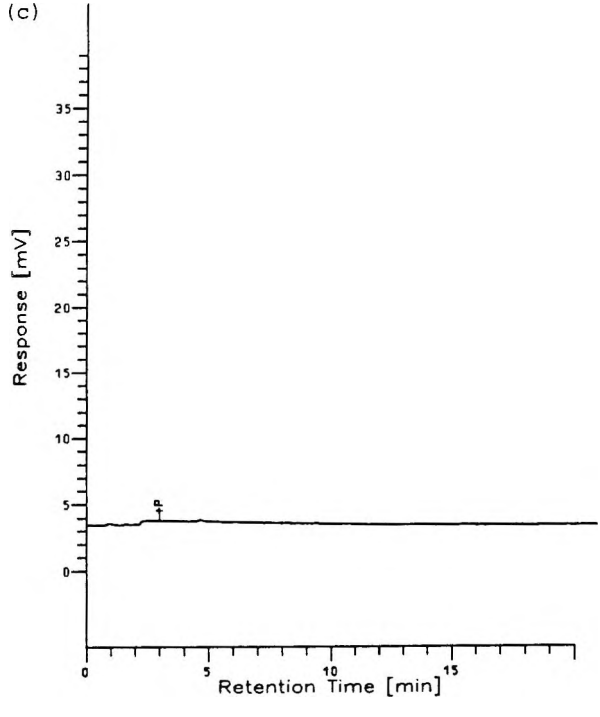


Figure 1. Typical chromatograms of (a) a Standard solution, (b) a Test solution (ophthalmic cleanser containing EDTA). (c) Typical chromatogram of a Placebo (ophthalmic cleanser not containing EDTA).


(b)
Response [mV]
35
30
25
20
15
10
5
0
P
0
5
10
15
Retention Time [min]


(c)
Response [mV]
35
30
25
20
15
10
5
0
P
0
5
10
15
Retention Time [min]

Selectivity

The ophthalmic cleanser was stressed with heat, acid, base, and UV radiation. The heat-stressed ophthalmic cleanser at 40°C for 40 hours did not result in any degradation. The acid-stressed samples were adjusted to pH 2 with concentrated HCl and heated at 85°C for 40 hours. EDTA degradation of 8% was observed for the acid-stressed samples under the described conditions.

The base-stressed samples were adjusted to pH 12 with 50% NaOH and heated at 85°C for 40 hours. EDTA degradation of 2% was observed for the base-stressed samples under the described conditions. Ultraviolet light-stressed samples were placed in the path of a UV lamp at 40 mWatt/cm^2 for 40 hours. EDTA degradation of 32% was observed for the UV stressed samples under the described conditions.

Despite the observed degradation, no interfering peaks at the retention time of the Fe-EDTA complex were observed in any of the stressed samples.

Table 1

Precision and Accuracy of EDTA in Ophthalmic Cleanser

Nominal Conc. (mg/mL)	n	Mean Found Conc. (mg/mL)	%RSD	%RE
0.152	6	0.157	0.3	3.2
0.223	6	0.230	0.4	2.9
0.294	6	0.302	0.5	2.8
0.364	6	0.372	0.2	2.2
0.436	6	0.448	0.2	2.6

CONCLUSION

The precision of the method is below 0.5%, while the accuracy is within 0.8%. The method is rapid and requires minimal sample pretreatment, resulting in *ca.* 100 samples being analyzed daily.

The described assay for the analysis of EDTA is selective, sensitive, and robust. Furthermore, the method is stability-indicating with no interference

from degradation products or excipients under the described stress conditions. Consequently, it is anticipated that this method could be used for the routine analysis of EDTA in ophthalmic preparations.

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ANNOUNCEMENT

BASIC PRINCIPLES OF HPLC AND HPLC SYSTEM TROUBLESHOOTING

A Two-Day In-House Training Course

The course, which is offered for presentation at corporate laboratories, is aimed at chemists, engineers and technicians who use, or plan to use, high performance liquid chromatography in their work. The training covers HPLC fundamentals and method development, as well as systematic diagnosis and solution of HPLC hardware module and system problems.

The following topics are covered in depth:

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- Modes of HPLC Separation
- Developing and Controlling Resolution
- Mobile Phase Selection and Optimization
- Ion-Pairing Principles
- Gradient Elution Techniques
- Calibration and Quantitation
- Logical HPLC System Troubleshooting

The instructor, Dr. Jack Cazes, is Editor-in-Chief of the Journal of Liquid Chromatography & Related Techniques, of Instrumentation Science & Technology, and of the Chromatographic Science Book Series. He has been intimately involved with liquid chromatography for more than 30 years; he pioneered the development of modern HPLC technology. Dr. Cazes was Professor-in-Charge of the ACS Short Course and the ACS Audio Course on Gel Permeation Chromatography for many years.

Details of this course may be obtained from Dr. Jack Cazes, P. O. Box 2180, Cherry Hill, NJ 08034-0162, USA. Tel: (609) 424-3505; FAX: (609) 751-8724

J. LIQ. CHROM. & REL. TECHNOL., 19(9), 1511–1521 (1996)

LIQUID CHROMATOGRAPHY CALENDAR

1996

MAY 7 - 9: VIIth International Symposium on Luminescence Spectrometry in Biomedical Analysis - Detection Techniques and Applications in Chromatography and Capillary Electrophoresis, Monte Carlo, Monaco. Contact: Prof. Willy R. G. Baeyens, University of Ghent, Pharmaceutical Institute, Harelbekestraat 72, B-9000 Ghent, Belgium.

JUNE 16 - 21: "HPLC '96: Twentieth International Symposium on High Performance Liquid Chromatography," San Francisco Marriott Hotel, San Francisco, California. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

JULY 1 - 3: International Symposium on Polymer Analysis and Characterization, Keble College, Oxford University, U.K. Contact: Prof. J. V. Dawkins, Dept. of Chemistry, Loughborough University of Technology, Loughborough, Leicestershire, LE11 3TU, U.K.

JULY 14 - 18: 5th World Congress of Chemical Engineering, Marriott Hotel, San Diego, California. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

JULY 27 - 31: 37th Annual Meeting of the Aqmerican Society of Pharmacognosy, University of California, Santa Cruz, California. Contact: Dr. Roy Okuda, Chem Dept, San Jose State University, One Washington Square, San Jose, CA 95192-0101, USA. Tel: (408) 924-5000; FAX: (408) 924-4945.

AUGUST 7 - 9: 28th Canadian High Polymer Forum, Sarnia, Ontario, Canada. Contact: Kar Lok, BASF Corp, 11501 Steele Creek Rd, Charlotte, NC, 28273, USA. Tel: (704) 587-8240; FAX: (704) 587-8115.

AUGUST 9 - 14: 31st Intersociety Energy Conversion Engineering Conference (co-sponsored with IEEE), Omni Shoreham Hotel, Washington, DC. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

AUGUST 11 - 15: 26th ACS Northeast Regional Meeting, Western Conn State Univ, Danbury, CT. Contact: A. Alder, 11 Long Ridge Rd, Redding, CT 06896, USA; (203) 938-2920; Email: reglmtgs@acs.org.

AUGUST 11 - 16: 3rd International Hydrocolloids Conference, Sydney, Australia. Contact: Gail Hawke, P. O. Box N-399, Grosvenor Place, Sydney, NSW 2000, Australia. Tel: 61 02 241 3388; FAX: 61 02 241 5282.

AUGUST 11 - 16: ICORS '96: 15th International Conference on Raman Spectroscopy, Pittsburg, Pennsylvania. Contact: Sanford Asher, Chem Dept, University of Pittsburgh, PA 15260, USA. Tel: (412) 624-8570; FAX: (412) 624-0588.

AUGUST 12 - 16: 11th Internationa Congress on Thermal Analysis & Calorimetry, Philadelphia. Contact: The Complete Conference, 1540 River Pk Dr, Sacramento, CA 95815, USA. Tel: (916) 922-7032; FAX: (916) 922-7379.

AUGUST 17 - 20: 31st National Heat Transfer Conference, Westin Galleria, Houston, Texas. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

AUGUST 18 - 23: 17th International Conference on Magnetic Resonance in Biological Systems, Keystone, Colorado, USA. Contact: Conference Office, 1201 Don Diego Ave, Santa Fe, NM 87505, USA. Tel: (505) 989-4735; FAX: (505) 989-1073.

AUGUST 25 - 29: 212th ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; Email: natlmtgs@acs,org.

AUGUST 25 - 30: International Symposium on Metal Hydrogen Systems: Fundamentals and Applications, Les Diablerets, Switzerland. Contact: MH-96, Inst of Physics, Univ of Fribourg, Perolles, CH-1700 Fribourg, Switzerland. Tel: 41 37 299 113; FAX: 41 37 299 772.

AUGUST 25 - 30: 12th International Congress on Chemical & Process Engineering, Praha, Czech Republic. Contact: Organizing Committe, CHISA'96, P. O. Box 857, 111 21 Praha, Czech Republic. Tel: 42 2 353287; FAX: 42 2 3116138.

SEPTEMBER 1 - 4: 4th Inetrnational Symposium on Preparative & Industrial Chromatography & Related Techniques, Basel, Switzerland.

Contact: Secretariat Prep'96, Messeplatz 25, CH-4021 Basel, Switzerland. Tel: 41 61 686 28 28; FAX: 41 61 686 21 85.

SEPTEMBER 1 - 6: IUPAC Chemrawn IX, Seoul, Korea. Contact: IUPAC Chemrawn IX, Secretariat, Tongwon B/D 6th Floor, 128-27 Tangjudong, Chongro-ku, Seoul 110-071, Korea. FAX: 82 2 739-6187.

SEPTEMBER 1 - 6: 11th International Symposium on Organosilicon Chemistry, Monpellier, France. Contact: R. Corriu, University of Montpellier II, Place Eugene Batallon, cc007, 34095 Montpellier cedex 05, France. Tel: 67 14 38 01.

SEPTEMBER 1 - 6: 11th Symposium on Quantitative Structure-Activity Relationships: Computer-Assisted Lead Finding and Optimization," Lausanne, Switzerland. Contact: Dr. Han van de Waterbeemd, F. Hoffmann-La Roche Ltd., Dept PRPC 65/314, CH-4002 Basle, Switzerland.

SEPTEMBER 4 - 9: International "Thermophiles '96" Symposium: Biology, Ecology & Biotechnology of Thermophilic Organisms, Athens, Georgia. Contact: J. Wiegel, University of Georgia, Microbiology Department, 527 Biol Sci Bldg, Athens, GA 30602-2605, USA. Tel: 706) 542-2674; fax: (706) 542-2651.

SPETEMBER 7: Field-Flow Fractionation Workshop VIII, Ferrara, Italy. Contact: F. Dondi, Dept of Chem, University of Ferrara, via L. Borsari 46, I-44100 Ferrara, Italy. Tel: 39 532 291154; fax: 39 532 240709.

SEPTEMBER 9 - 11: Sixth International Symposium on Field-Flow Fractionation, Ferrara, Italy. Contact: F. Dondi, Dept of Chem, University of Ferrara, via L. Borsari 46, I-44100 Ferrara, Italy. Tel: 39 532 291154; fax: 39 532 240709.

SEPTEMBER 9 - 11: 110th AOAC International Annual Meeting & Expo, Hyatt, Orlando, Florida. Contact: AOAC International, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301, USA. Tel: (703) 522-3032; FAX: (703) 522-5468.

SEPTEMBER 9 - 12: Saftey in Ammonia Plants & Related Facilities, Westin at Copley Place, Boston, Massachusetts. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

SEPTEMBER 10 - 12: International Thermophiles Workshop: Keys to Molecular Evolution & the Origin of Life, Athens, Georgia.

Contact: J. Wiegel, University of Georgia, Microbiology Department, 527 Biol Sci Bldg, Athens, GA 30602-2605, USA. Tel: 706) 542-2674; fax: (706) 542-2651.

SEPTEMBER 11 - 13: Corn Refiners Assn Scientific Conference, Bloomindale, Illinois. Contact: Corn Refiners Assn, 1701 Pennsylvania Ave, NW, Washington, DC 20036, USA. Tel: (202) 331-1634; FAX: (202) 331-2054.

SEPTEMBER 11 - 13: 2nd Workshop on Biosensors & Biol Techniques in Environmental Analysis, Lund, Sweden. Contact: M. Frei-Hausler, IAEAC Secretariat, Postfach 46, CH-4123 Allschwill 2, Switzerland.

SEPTEMBER 15 - 20: 21st International Symposium on Chromatography, Stuttgart, Germany. Contact: Dr. L. Kiessling, Geselleschaft Deutscher Chemiker, Postfach 900440, D-60444 Frankfurt/Main, Germany.

SEPTEMBER 16 - 18: 3rd Inetrnational Conference on Applications of Magnetic Resonance in Food Science, Nantes, France. Contact: Laboratoire de RMN-RC, 2 rue de la Houssiniere, 44072 Nantes cedex 03, France. Tel: 33 40 74 98 06; FAX: 33 40 37 31 69.

SEPTEMBER 16 - 19: International Ion Chromatography Symposium 1996, University of Reading, Reading, UK. Contact: J. R. Strimaitis, Century International, P. O. Box 493, Medfield, MA 02052, USA. Tel: (508) 359-8777; FAX: (508) 359-8778.

SEPTEMBER 19 - 21: 19th Annual Gulf Coast Chemistry Conference, Pensacola, Florida. Contact: J. Gurst, Chem Dept, University of West Florida, Pensacola, FL 32514, USA. Tel: (904) 474-2744; FAX: 904) 474-2621.

SEPTEMBER 20 - 24: 12th Asilomar Conference on Mass Spectrometry, Pacific Grove, California. Contact: ASMS, 1201 Don Diego Ave, Santa Fe, NM 87505, USA. Tel: (505) 989-4517; FAX: (505) 989-1073.

SEPTEMBER 28 - OCTOBER 5: Federation of Analytical Chem & Spectroscopy Societies (FACSS), Kansas City. 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 17 - 19: 52nd Southwest Regional ACS Meeting, Houston, Texas. Contact: J. W. Hightower, Dept. Chem. Eng., Rice University, Houston, TX 77251, USA.

OCTOBER 24 - 26: 52nd Southwestern Regional Meeting, ACS, Houston, Texas. Contact: J. W. Hightower, Chem Eng Dept, Rice Univ, Houston, TX 77251, USA.

OCTOBER 27 - 31: American Assn of Pharmaceutical Scientists Annual Meeting & Expo, Seattle, Washington. Contact: AAPS, 1650 King St, Alexandria, VA 22314-2747, USA. Tel: (703) 548-3000; FAX: (703) 684-7349.

OCTOBER 29 - 30: ASTM Symposium on Pesticide Formulations & Application Systems, New Orleans, Louisiana. Contact: G. R. Goss, Oil-Dri Corp, 777 Forest Edge Dr, Vrenon Hills, IL 60061, USA. Tel: (708) 634-3090; FAX: (708) 634-4595.

OCTOBER 29 - 31: Cphl Pharmaceutical Ingredients Worldwide '96 Conference, Milan, Italy. Contact: Miller Freeman BV, Industrieweg 54, P. O. Box 200, 3600 AE Maarssen, The Netherlands. Tel: 31 3465 73777; FAX: 31 3465 73811.

NOVEMBER 6 - 8: 31st Midwestern Regional Meeting, ACS, Sioux Falls, South Dakota. Contact: J. Rice, Chem Dept, S. Dakota State Univ, Shepard Hall Box 2202, Brookings, SD 57007-2202, USA.

NOVEMBER 6 - 9: 24th Biennial International Conference on Application of Accelerators in Research & Industry, Denton, Texas. Contact: J. L. Duggan or B. Stippec, University of North Texas, Physics Department, Denton, TX 76203, USA. Tel: (817) 565-3252; FAX: (817) 565-2227.

NOVEMBER 9 - 12: 48th Southeast Regional ACS Meeting, Hyatt Regency Hotel, Greenville, South Carolina. Contact: H. C. Ramsey, BASF Corp., P. O. Drawer 3025, Anderson, SC 29624-3025, USA.

NOVEMBER 10 - 13: 4th North American Research Conference on Organic Coatings Science & Technology, Hilton Head, South Carolina. Contact: A. V. Patsis, SUNY, New Paltz, NY 12561, USA. Tel: (914) 255-0757; FAX: (914) 255-0978.

NOVEMBER 10 - 14: 10th International Forum on Electrolysis in the Chemical Industry, Clearwater Beach, Florida. Contact: P. Kluczynski, Electrosynthesis Co, 72 Ward Road, Lancaster, NY 14086, USA. Tel: (716) 684-0513; FAX: (716) 684-0511.

NOVEMBER 10 - 15: AIChE Annual Meeting, Palmer House, Chicago, Illinois. Contact: AIChE, 345 E. 47th Street, New York, NY 10017, USA.

NOVEMBER 12 - 14: Plastics Fair, Charlotte, North Carolina. Contact: Becky Lerew, Plastics Fair, 7500 Old Oak Blvd, Cleveland, OH 44130, USA. Tel: (216) 826-2844; FAX: (216) 826-2801.

NOVEMBER 13 - 15: 13th Montreux Symposium on Liquid Chromatography-Mass Spectrometry (LC/MS; SFC/MS; CE/MS; MS/MS), Maison des Congres, Montreux, Switzerland. Contact: M. Frei-Hausler, Postfach 46, CH-4123 Allschwill 2, Switzerland. Tel: 41-61-4812789; FAX: 41-61-4820805.

NOVEMBER 18 - 20: 3rd International Conference on Chemistry in Industry, Bahrain, Saudi Arabia. Contact: Chem in Industry Conf, P. O. Box 1723, Dhahran 31311, Saudi Arabia. Tel: 966 3 867 4409; FAX: 966 3 876 2812.

NOVEMBER 17 - 22: 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; FAX: (302) 738-5275.

NOVEMBER 24 - 27: Industrial Research for the 21st Century, 1996 Biennial Meeting, Red Lion Resort, Santa Barbara, California. Contact: R. Ikeda, DuPont Central Research Dept., P. O. Box 80356, Wilmington, DE 19880-0356, USA. Tel: (302) 695-4382; FAX: (302) 695-8207; Email: ikeda@esvax.dnet.dupont.com.

1997

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

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

MAY 5 - 9: 151st ACS Rubber Div Spring Technical Meeting, Anaheim, California. Contact: L. Blazeff, P. O. Box 499, Akron, OH 44309-0499, USA. Tel: (216) 972-7814; FAX: (216) 972-5269.

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. Tel: (814) 865-1200; FAX: (814) 865-3030; Email: kaj@ecl.psu.edu.

MAY 27 - 30: ACS 29th Central Regional Meeting, Midland, Michigan. Contact: S. A. Snow, Dow Corning Corp., CO42A1, Midland, MI 48686-0994, USA. Tel: (517) 496-6491; FAX: (517) 496-6824; Email: usdcc6fc@bmmail.com.

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; Email: reglmtgs@acs.org.

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 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; Email: reglmtgs@acs.org.

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; FAX: (313) 577-2099; Email: jhr@che.wayne.edu.

SEPTEMBER 7 - 11: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; (202) 872-6128; Email: natlmtgs@acs.org.

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 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.ord.

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; Email: miscmtgs@acs.org.

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; FAX: (302) 738-5275.

1998

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

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

JUNE 13 - 19: 26th ACS National Medical Chemistry Symposium, Virginia Commonwealth Univ/Omni Richmond Hotel, Richmond, Virginia. Contact: D. J. Abraham, Virginia Commonwealth Univ, Dept of Med Chem, P. O. Box 581, Richmond, VA 23298, USA. Tel: (804) 828-8483; FAX: (804) 828-7436.

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

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

NOVEMBER 4 - 7: 50th ACS Southwest Regional Meeting, Resw 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 21 - 25: 217th ACS National Meeting, Anaheim, Calif. 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 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 26 - 30: 219th ACS National Meeting, Las Vegas, Nevada. 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 20 - 24: 220th 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.

2001

APRIL 1 - 5: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 19 - 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. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

SEPTEMBER 8 - 12: 224th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2003

MARCH 23 - 27: 225th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

SEPTEMBER 7 - 11: 226th ACS National Meeting, New York City. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2004

MARCH 28 - APRIL 1: 227th ACS National Meeting, Anaheim, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

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

Book:

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

Chapter in a Book:

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

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