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formerly Journal of Liquid Chromatography

VOLUME 19 NUMBER 16 1996

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES

September 1996

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Identification Statement. *Journal of Liquid Chromatography & Related Technologies* (ISSN: 1082-6076) is published semimonthly except monthly in May, August, October, and December for the institutional rate of $1,595.00 and the individual rate of $797.50 by Marcel Dekker, Inc., P.O. Box 5005, Monticello, NY 12701-5185. Second Class postage paid at Monticello, NY. POSTMASTER: Send address changes to *Journal of Liquid Chromatography & Related Technologies*, P.O. Box 5005, Monticello, NY 12701-5185.

Volume	Issues	Institutional Rate	Individual Professionals' and Student Rate	Foreign Postage		
				Surface	Airmail to Europe	Airmail to Asia
19	20	$1,595.00	$797.50	$70.00	$110.00	$130.00

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CODEN: JLCTFC 19(16) i-iv, 2559-2722 (1996)
ISSN: 1082-6076

Printed in the U.S.A.

JOURNAL OF LIQUID CHROMATOGRAPHY
& RELATED TECHNOLOGIES

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Contributions to this journal are published free of charge.

Effective with Volume 6, Number 11, this journal is printed on acid-free paper.

J. LIQ. CHROM. & REL. TECHNOL., 19(16), 2559–2578 (1996)

SOLID-PHASE EXTRACTION STUDY AND PHOTODIODE ARRAY RP-HPLC ANALYSIS OF XANTHINE DERIVATIVES IN HUMAN BIOLOGICAL FLUIDS

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ABSTRACT

An automated reverse phase high performance liquid chromatography (HPLC) - photodiode array method using a multi linear gradient elution is described for the simultaneous analysis of nine xanthines: xanthine, 7-methylxanthine, 3-methylxanthine, 1-methylxanthine, isocaffeine, theobromine, paraxanthine, theophylline and caffeine. The separation method development was based on mobile-phase optimisation and off-line solid-phase extraction (SPE) from human biological fluids: blood serum and urine. Eluent consisted of 0.05 M CH_3COONH_4 and methanol (90:10 v/v) changing to (70:30 v/v) over a period of 20 min. Identification of xanthines was achieved by photodiode - array detector and quantitation was performed at 270 nm. Isocaffeine was used as internal standard at a concentration of 3.06 ng/μL. High extraction recoveries were achieved from Merck RP-18 cartridges using 1% hydrochloric acid as eluent, requiring small volumes, 40 μL of blood serum and 100 μL of urine.

The separation of xanthines was achieved on octylsilica, using a Silasorb C_8, 10μm, 250x4.6 mm i.d. analytical column thermostated at 32 ^{0}C and proved to be highly selective, sensitive, reproducible, accurate and rapid regarding the nine compounds. Detection limits ranged from 2 to 3 ng for 20 μL injected volume while linearity holds up to 20 ng/μL for each compound.

INTRODUCTION

The term xanthine derivatives (methylxanthines), in the case of the present study, refers to mono - di and tri-methylated derivatives of xanthine.

Caffeine (1,3,7-trimethylxanthine, 1,3,7 TMX) and its dimethylated metabolites: theophylline (1,3-dimethylxanthine, 1,3 DMX) and theobromine (3,7-dimethylxanthine, 3,7 DMX) three of the most well known compounds that belong to the group of methylxanthines, are ubiquitous in human biological fluids, due to the dietary intake of coffee, tea and cola drinks. Additionally, theobromine is present due to chocolate consumption.[1]

Theophylline is a mild diuretic agent, a moderate myocardial and central nervous stimulant and a powerful bronchodilator. Caffeine is utilised in the therapy of neonatal apnoea.[2,3]

The methylxanthines are extensively metabolised by the hepatic microsomal mixed function oxidase (cytochrome P_{450}) system so that less than 10% of theophylline and less than 2% of caffeine are excreted unchanged in the urine.

The presence of the pharmacologically active dietary methylxanthines may influence patient compliance with theophylline therapy used in the treatment of asthma and chronic obstructive pulmonary disease. They may also alterate the evaluation and interpretation results of theophylline therapeutic drug monitoring.[1]

Patients on theophylline medication for obstructive airway diseases and consuming caffeine containing beverages excrete the whole spectrum of metabolites in the urine.

It is advantageous to be able to measure the individual metabolites and assess their contribution to the total xanthine level.

Figure 1 presents the interrelationships of methylxanthines and their metabolic products expected to occur in serum.[1]

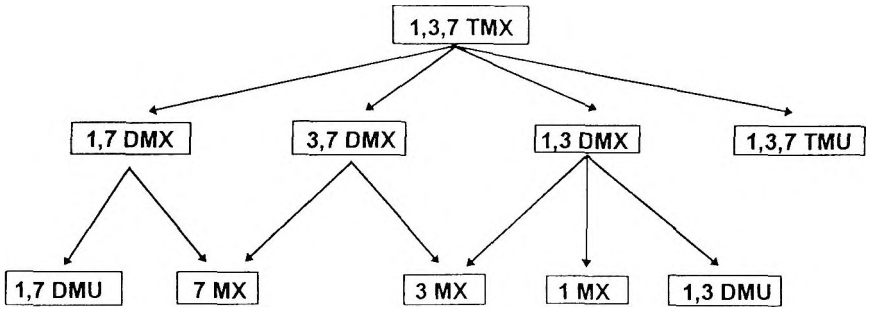

Figure 1. The Interrelationships of the xanthine derivatives and their metabolic products expected to occur in plasma. (U=uric acid).

A large variety of techniques and analytical procedures for determining xanthine derivatives in biological fluids have been reported: Radioimmunoassay (RIA), fluorescence polarisation immunoassay (FPIA), enzyme immunoassay (EIA), capillary electrophoresis and several chromatographic methods with emphasis on HPLC.[4-6]

A number of HPLC methods, reverse phase and normal, have been reported for the determination of methylxanthines in biological fluids. Many of the previously reported assays failed to separate other xanthine derivatives from theophylline and, thus, have substantially overestimated theophylline levels leading to clinical problems. A few have addressed this problem by using isocratic or gradient elution, ion-exchange columns or ion-pairing reagents which improve separation but lead to rapid deterioration of column efficiency.[7-14] High performance liquid chromatographic methods that enable separation and identification of all possible metabolites are required for caffeine and theophylline analysis.

Several procedures for the preparation of biological samples have been reported. Extraction with organic solvent followed by evaporation and reconstitution of the residue and dilution of serum, with or without, eliminating proteins are generally accepted methods.[3,15]

Solid-phase extraction (SPE) recently substituted the classic liquid-liquid extraction, as being rapid, solvent consuming, which leads to reduction in pollution and offering a wide sorbent selection. Therefore, SPE is widely applied to sample preparation especially for samples of biological interest. Most of the assays developed to measure xanthine derivatives and metabolites in biological samples have required a large volume of sample, tedious sample preparation and long chromatogram run times. Furthermore, many of these assays exhibited poor resolution of the compounds of interest.[16]

This prompted us to develop a method that would require small sample volumes, a relatively short and simple sample preparation and a shorter chromatogram run time, as well as lower limits of detection.

The present method deals with the simultaneous determination of xanthine derivatives in human biological fluids, blood serum and urine, in less than 20 minutes time, after solid-phase extraction. It requires only a small sample volume, 40 μL of serum and 100 μL of urine, making it a valuable tool for clinical paediatric research.

It is sensitive and rapid providing high selectivity, satisfactory reproducibility and high accuracy.

EXPERIMENTAL

Instrumentation

A Shimadzu (Kyoto, Japan), quaternary low pressure gradient system was used for chromatographic analysis of xanthine derivatives. The solvent lines were mixed in a FCV-9AL mixer and an LC-9A pump was used to deliver the mobile phase to the analytical column which was thermostated in a CTO-6A oven.

Sample injection was performed by a SIL-9A autosampler and detection was achieved by a SPDM-6A photodiode array detector.

Chromatograms were stored on the hard disk of a Function 386 PC and printed on a SEIKOSHA SP-1900 printer.

Degassing of solvents was achieved by continuous helium sparking in the solvent flasks through a DGU-2A degassing unit.

The analytical column was a Silasorb C_8, 10μm, 250x4.6 mm i.d., purchased by Rigas Labs, Thessaloniki, Greece.

A glass vacuum-filtration apparatus obtained from Alltech Associates, was employed for the filtration of the buffer solution, using 0.2 μm membrane filters obtained from Schleicher and Schuell (Dassel, Germany).

A Glass-col, Terre Haute 47802 small vortexer and a Hermle centrifuge, model Z 230 (B. Hermle, Gosheim, Germany) were employed for the pre-treatment of biological samples.

The SPE study was performed on a Vac-Elut vacuum manifold column processor purchased from Analytichem International, a division of Varian (Harbor City, USA).

All evaporations were performed with a 9-port Reacti-Vap evaporator (Pierce, Rocford, IL, USA).

UV spectra for selecting the working wavelength of detection were taken using a Varian DMS 100S UV/VIS double-beam spectrophotometer.

All computations were achieved using a VIP 312 computer.

Materials and Reagents

All xanthine derivatives were purchased by Sigma (St. Louis, MO, USA).

HPLC gradient grade methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). Ammonium acetate p.a. was also from Merck. Bis de-ionised water was used throughout analysis. Solid phase extraction cartridges C_{18} were from Merck.

The mobile phase was vacuum filtered before use through 0.2 μm membrane filters.

Standard Solutions

Stock solutions of xanthine derivatives were prepared in methanol and stored refrigerated at 4 ^{0}C. These solutions were found to be stable for at least one month.

Working methanolic solutions were prepared from stocks at concentrations 0.25, 0.5, 1.0, 2.0, 3.0, 5.0, 8.0, 10.0, 15.0 and 20.0 ng/μL. Methanolic solution of internal standard was added at a concentration of 3.06 ng/μL.

Chromatography

Peak areas of the xanthine derivatives were measured and the ratio to internal standard was compared for each compound to that of the calibration standards. Maintaining the analytical column at 32 °C ensures reproducible separations.

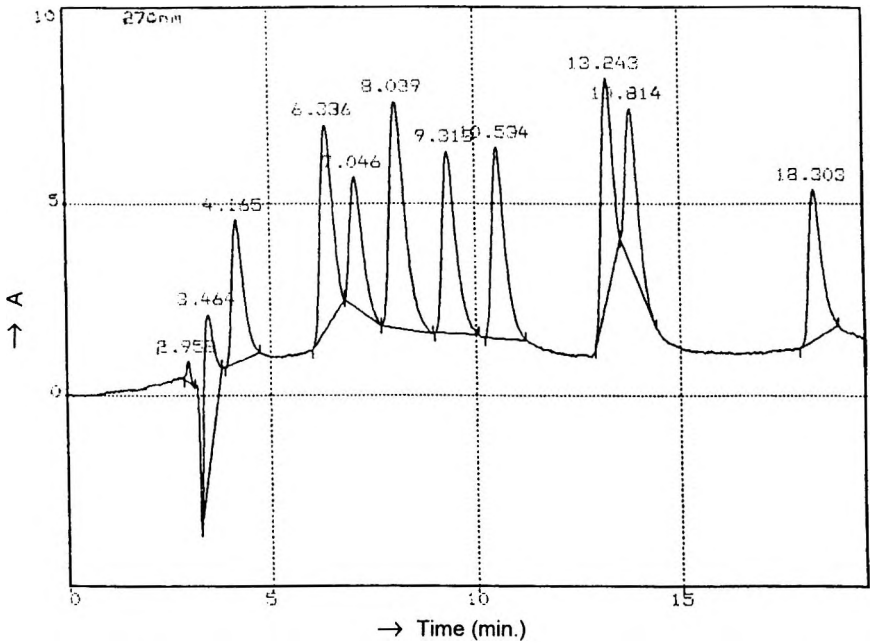

Figure 2. High performance Liquid Chromatogram of Xanthine Derivatives.
Peaks: (4.165) = XA, (6.336) = 7MX, (7.046) = 3MX, (8.039) = 1MX, (9.318) = IC, (10.534) = TB, (13.243) = PX, (13.243) = TP and (18.303) = CA. At a concentration of 3.0 ppm.

RESULTS AND DISCUSSION

Optimisation of the Chromatographic System

The chromatographic system for the separation of the nine xanthine derivatives was chosen among others, regarding the gradient time and eluent consistence, in order to result in optimum separation with high selectivity and detection effectiveness in as shorter analysis time as possible.

A variety of mobile phases was tested in order to find out the optimum chromatographic system for the analysis of xanthine derivatives. The mobile phases, were in principle, binary mixtures of an aqueous solution of ammonium acetate with methanol in several ratios. The final mobile phase is chosen in terms of peak shape, column efficiency and chromatographic analysis time,

selectivity and resolution. An increase in organic solvent percentage yielded a deterioration of chromatogram shape. The optimum conditions are reported in Table 1. Figure 2, shows the chromatogram obtained during the separation of the xanthine derivatives by means of the chromatographic system developed at the present study.

The R_s values for the nine compounds: xanthine (XA), 7-methylxanthine (7MX), 3-methylxanthine (3MX), 1-methylxanthine (1MX), isocaffeine (1,3,9-trimethylxanthine, IC), theobromine (3,7-dimethylxanthine, TB), paraxanthine (1,7- dimethylxanthine, PX), theophylline (1,3- dimethylxanthine, TP) and caffeine (1,3,7- trimethylxanthine, CA) are: 3.11, 1.22, 1.31, 1.37, 1.31, 4.24, 1.16 and 7.02 respectively per couple of compounds.

Performance Characteristics of the Proposed Method

The system described here was used for the simultaneous determination of nine xanthine derivatives: XA, 7MX, 3MX, 1MX, IC, TB, PX, TP and CA.

Optimised chromatographic conditions were set and the following analytical characteristics were evaluated:

- Precision and accuracy.
- Analysis time.
- Calibration data.
- Selection of wavelength.
- Solid-phase extraction and
- Real sample analysis.

Precision and Accuracy

Method validation regarding reproducibility was achieved by replicate injections of standard solutions at low and high concentration levels where peak areas were measured in comparison to the peak area of the internal standard.

Statistical evaluation revealed relative standard deviations at different values for eight injections. Results are shown in Table 2.

Long term stability study was conducted during routine operation of the system over a period of eight consecutive days. Results are illustrated in Table 3.

Table 1

Optimum Conditions for the Chromatographic Separation of Xanathine Derivatives

Operation	Parameter	Value
Separation	CH_3COONH_4 (A)	0.05 M
	MeOH (B)	
	pH	7.0
	Flow rate	1 mL/min
	Inlet pressure observed	160 Kg/cm^2
Gradient System	A:B 90-10 (v/v)	0 min
	A:B 80-20 (v/v)	8 min
	A:B 70-30 (v/v)	15 min
	A:B 70-30 (v/v)	20 min
Detection	Photodiode Array	270 nm
	Sensitivity	0.002aufs
	Temperature	32°±0.1° C

Table 2

Within-Day Precision and Accuracy for the Analysis of Xanthine Dervatives (n=8)

Compound	Added (ng)	Found (ng)	SD	RSD(%)
XA	21.6	20.8	0.3	1.44
	40.8	39.9	0.8	2.00
	60.0	58.2	1.3	2.23
	100.8	105.6	3.9	3.69
7MX	20.4	21.2	0.7	3.30
	40.8	39.3	1.5	3.82
	62.2	61.9	2.3	3.72
	102.0	106.6	8.2	7.69
3MX	20.4	21.8	0.9	4.13
	40.8	39.9	0.7	1.74
	61.2	59.8	4.7	7.86
	102.0	101.2	5.6	5.53

(continued)

Table 2 (continued)

Within-Day Precision and Accuracy for the Analysis of Xanthine Dervatives (n=8)

Compound	Added (ng)	Found (ng)	SD	RSD (%)
1MX	20.4	20.7	1.4	6.76
	40.8	40.7	0.8	1.96
	61.2	58.6	4.6	7.85
	102.0	99.9	7.4	7.41
TB	20.0	21.3	1.5	7.04
	40.0	38.7	2.9	7.49
	60.0	57.8	4.7	8.13
	100.0	98.2	5.9	6.01
PX	20.0	21.1	0.6	2.84
	40.0	38.9	2.7	6.94
	60.0	62.3	5.0	8.03
	100.0	101.9	4.7	4.79
TP	20.4	19.2	1.9	9.90
	40.8	41.5	2.2	5.30
	61.2	61.5	4.4	7.15
	102.0	102.5	7.3	7.12
CA	20.2	21.1	1.2	5.69
	40.4	41.4	0.8	1.93
	60.6	61.3	1.9	3.10
	101.0	102.2	4.9	4.79

Working Range and Detectability

For the simultaneous analysis of the nine xanthine derivatives, the term working range is more proper than linear range since column saturation that takes place at high concentrations leads to poor peak shapes.

The upper limit regarding the higher concentration of injected solution assumes the co-existing concentration of other compounds at a similar level. Therefore, higher concentrations could also be injected in case of other compounds' absence. Upper limit reported is 20 ng/μL.

Table 3

Day-to-Day Precision and Accuracy for the Analysis of Xanthine Derivatives over a Period of 8 Consecutive Days.

Compound	Added (ng)	Found (ng)	SD	RSD (%)
XA	21.6	22.4	1.9	8.48
	40.8	39.4	1.0	2.54
	60.0	59.4	2.2	3.70
	100.8	99.2	5.4	5.44
7 MX	20.4	21.6	0.8	3.70
	40.8	40.4	0.5	1.24
	61.2	61.3	0.2	0.33
	102.0	101.8	4.9	4.81
3MX	20.4	21.1	0.2	0.95
	40.8	40.6	2.3	5.67
	61.2	60.6	3.8	6.27
	102.0	103.7	2.3	2.22
1MX	20.4	20.8	0.6	2.89
	40.8	39.8	1.0	2.51
	61.2	60.4	1.3	2.15
	102.0	101.7	5.5	5.41
TB	20.0	22.3	1.8	7.89
	40.0	38.7	1.7	4.39
TB	60.0	61.8	4.9	7.93
	100.0	95.9	4.7	4.90
PX	20.0	21.2	1.1	5.19
	40.0	40.1	0.9	2.24
	60.0	59.8	1.4	2.34
	100.0	100.3	2.0	1.99
TP	20.4	22.1	0.8	3.62
	40.8	40.8	2.1	5.15
	61.2	62.4	4.2	6.73
	102.0	102.7	2.4	2.34

(continued)

Table 3 (continued)

Day-to-Day Precision and Accuracy for the Analysis of Xanthine Derivatives over a Period of 8 Consecutive Days.

Compound	Added (ng)	Found (ng)	SD	RSD (%)
CA	20.2	20.9	0.7	3.35
	40.4	39.0	1.1	2.82
	60.6	57.5	4.2	7.31
	101.0	101.3	5.8	5.73

Detection limits were calculated as a three-fold signal - to - noise ratio at the baseline (S/N = 3) and found to be 2.0 ng for xanthine, 7-methylxanthine and 1-methylxanthine, while 3.0 ng for the rest of the compounds, for 20μL injected sample volume.

Analysis Time

The analysis time in the proposed method is determined by the retention time of the most strongly retained compound in the chromatographic separation, as analytical column is rapidly equilibrated after the last step of gradient system.

As it is shown in Figure 2 the sample analysis time is less than 19 min., as caffeine, the last eluted compound has a retention time of 18.303 min.

Calibration Data

Calibration of the method was performed by injection of mixed standard of xanthine derivatives covering the entire working range. Ten concentrations were used in the range 0.25 - 20 ng/μL.

The sensitivity setting of the photodiode array detector was adjusted to give almost full-scale deflection for the highest standard concentration. Each sample was injected five times.

Linear correlation between absolute injected amount or concentration and peak area ratio was obtained for all xanthine derivatives using isocaffeine as internal standard at a concentration of 3.06 ng/μL. Isocaffeine was selected for two reasons: it is absent in biological fluids as it isn't a caffeine metabolite and it appears in the middle of the chromatographic analysis time. The results of the statistical treatment of calibration data for xanthine derivatives are summarised in Table 4.

Table 4

Calibration Data for Simultaneous Determination of Xanthine Derivatives*

Parameter	Value	XA	7MX	3MX	1MX	TB	PX	TP	CA
Concentration range	mg/L	0.5-20	0.5-20	0.5-20	0.5-20	0.5-20	0.5-20	0.5-20	0.5-20
Slope	AIU.ng^{-1}	0.01566	0.02286	0.01743	0.03478	0.04017	0.02809	0.02214	0.02597
Intercept		-0.16856	-0.25512	-0.21823	-0.41312	-0.76511	-0.36104	-0.32832	-0.38750
Correlation coefficient		0.98806	0.99536	0.99426	0.99341	0.99102	0.99550	0.99454	0.99398
Detection limit	ng	2	2	3	2	3	3	3	3
Retention time	min.	4.165	6.336	7.046	9.315	10.534	13.243	13.814	18.303

*Peak area ratio measurement with 3.06ng/μL Isocaffeine as internal standard RT = 8.039 min.

Table 5

Recovery of Xanthine Derivatives after SPE using C_{18} Cartridges with Isocaffeine as a Chromatographic Standard

Compound	Recovery (%)
XA	108.3
7MX	97.2
3MX	101.9
1MX	92.9
TB	91.5
PX	96.5
TP	100.6
CA	94.2

Selection of Wavelength

The analytical wavelength 270 nm was chosen for quantitation since it represents a maximum absorbance as results from xanthine compounds' spectra. Quantitation at this wavelength enhances sensitivity for all compounds.

Solid Phase Extraction of Xanthine Derivatives

Several different solid-phase extraction cartridges provided by different manufacturers were tested for the optimisation of xanthine derivatives' isolation and recovery in human blood serum and urine.

Xanthine derivatives were subsequently analysed by HPLC after separation on a C_8 column with isocaffeine as internal standard in case of blood serum and without internal standard in case of urine.

Sample preparation time was approximately one hour including sample evaporation to dryness step. Taking in account that ten samples were simultaneously treated, single analysis time is reduced to six minutes per sample. Extraction efficiency was calculated by extracting standard solutions at six different concentration levels, i.e. 1.0, 2.0, 3.0, 4.0, 5.0, 7.0 ng/μL, of xanthine derivatives.

Recovery of compounds was calculated by comparing peak area ratios against internal standards with those obtained for unextracted methanolic solutions. Results obtained are presented in Table 5.

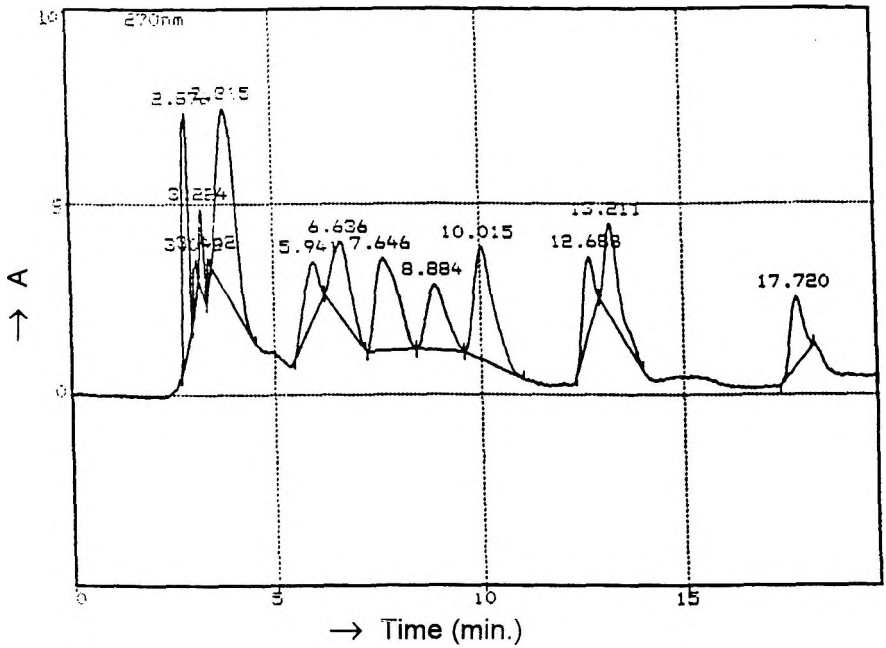

Figure 3. High Performance Liquid Chromatogram of Xanthine Derivatives Extracted from Blood Serum. Peaks: (3.815)=XA, (5.941)=7MX, (6.636)=3MX, (7.646)=1MX, (8.884)=IC, (10.015)=TB, (12.688)=PX, (13.211)=TP and (17.720)=CA.

100 µL of standard solution were applied to the SPE cartridge, which was conditioned by flushing 3 mL MeOH and 3 mL H_2O prior to the addition of sample. After applying the sample, cartridge was dried by sucking air. Xanthine derivatives were eluted using 3 mL 1 % HCI. The sample was subsequently evaporated to dryness under gentle nitrogen steam in a 45 °C water bath and diluted to 100 µL internal standard solution (3.06 ng/µL).

A washing step with water resulted in losses of xanthine derivatives and poor recoveries were noticed. Thus this step was omitted in the present study.

Real Sample Analysis

a. Human Blood Serum

Aliquots of 40 µL of human blood serum were treated with 80 µL of acetonitrile to precipitate proteins in order to release bound xanthine derivatives.

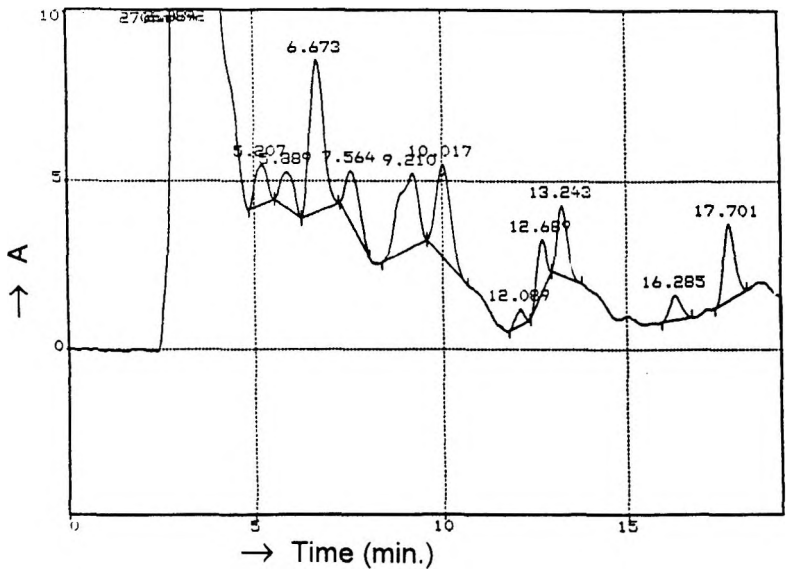

Figure 4. High Performance Liquid Chromatogram of Xanthine Derivatives Extracted from Urine. Peaks: (5.841)=7MX, (7.564)=1MX, (10.017)=TB, (12.689)=PX, (13.243)=TP and (17.701)=CA.

After 2 min. vortex mixing, 100 μL of mixed standard solution were added to the sample at concentrations: 1.0, 2.0, 3.0, 4.0 and 5.0 ng/μL.

The sample was subsequently centrifuged at 3500 rpm for 15 min and the supernatant was transferred to a clean eppendorf tube, where 30 μL of methanol were added. Finally the sample was slowly applied to the SPE cartridge and procedure followed the steps described under solid-phase extraction paragraph.

b. Urine

A similar sample preparation method was followed for urine samples using 100 μL urine sample and 200 μL of acetonitrile for deproteinization. The rest of the procedure is described above. Reconstitution to volume was performed with 100 μl of methanol, as no internal standard was used in case of urine analysis.

The serum and urine samples were pooled samples free from xanthine derivatives and collected from five healthy volunteers. No interference from endogenous compounds from sample matrix was observed in case of blood serum as shown in Figure 3.

Table 6

Calibration Curves for the Analysis of Xanthine Derivatives in Spiked Blood Serum Samples using Isocaffeine (3.06 ng/μL) as Internal Standard

Analyte	Calibration Curve Equation $Y^* = a + bx$	Correlation Coefficient
XA	Y = -0.45033 + 0.02969X	0.99985
7MX	Y = 0.06142 + 0.00611X	0.99507
3MX	Y = 0.00465 + 0.00967X	0.99985
1MX	Y = -0.10697 + 0.01718X	0.99992
TB	Y = 0.06450 + 0.01195X	0.99953
PX	Y = -0.0041 + 0.00582X	0.99195
TP	Y = -0.15740 + 0.01182X	0.99878
CA	Y = -0.09367 + 0.01026X	0.99976

Y* = Peak area ratio of analyte to internal standard. X=ng of the analyte.

Table 7

Calibration Curves for the Analsis of Xanthine Derivatives in Spiked Urine Samples

Analyte	Calibration Curve Equation $Y^* = a + bx$	Correlation Coefficient
7MX	Y = -23846.3 + 623.3X	0.99807
1MX	Y = 742.8 + 556.6X	0.97658
TB	Y = -12.8 + 892.1X	0.98681
PX	Y = -5811.2 + 410.7X	0.99252
TP	Y = -536.0 + 548.0X	0.99008
CA	Y = 13144.2 + 478.8X	0.98469

Y* = Peak area, X=ng of the analyte.

While some urine endogenous compounds interfere with some determined xanthine derivatives. Thus xanthine and 3-methylxanthine could not be analysed in urine samples and isocaffeine could not be used as internal standard as shown in Figure 4.

Table 8

Recovery of Xanthine Derivatives from Human Blood Serum after SPE on C_{18} Cartridges using Isocaffeine as Internal Standard.*

Compound	Added (ng)	Found (ng)	SD	RSD (%)
XA	19.6	19.4 ± 0.5	2.58	99.0
	58.8	59.6 ± 2.1	3.52	101.4
	100.0	100.9 ± 1.2	1.19	100.9
7MX	20.4	19.5 ± 0.3	1.54	95.6
	61.2	65.6 ± 2.5	3.81	107.2
	102.0	94.4 ± 1.8	1.91	92.5
3MX	21.2	21.1 ± 0.8	3.79	99.5
	63.6	63.1 ± 1.5	2.38	99.2
	106.0	107 ± 4.5	4.20	101.0
1MX	19.6	19.9 ± 1.1	5.53	101.5
	57.9	57.9 ± 2.3	3.97	98.5
	98.3	98.3 ± 6.7	6.82	100.3
TB	19.5	19.5 ± 1.6	8.21	96.5
	59.7	59.7 ± 1.5	2.51	98.5
	100.2	100.2 ± 4.1	4.09	99.2
PX	21.2	21.3 ± 0.7	3.29	100.5
	63.6	65.2 ± 1.8	2.76	102.5
	106.0	106.9 ± 6.1	5.71	100.8
TP	20.0	21.2 ± 0.4	1.89	106.0
	60.0	57.8 ± 3.0	5.19	96.3
	100.0	102.1 ±5.1	5.00	102.1
CA	20.4	20.2 ± 0.4	1.98	99.0
	61.2	63.3 ± 2.5	3.95	103.3
	102.0	103.6 ± 2.5	2.41	101.6

* Mean value of six measurements

Table 9

Recovery of Xanthine Derivatives from Human Urine Samples after SPE on C_{18} Cartridges*

Compound	Added (ng)	Found (ng)	SD	RSD (%)
7 MX	40.8	38.4 ± 1.8	4.69	94.1
	61.2	60.4 ± 5.2	8.61	98.7
	102.0	102.5 ± 4.1	4.00	100.5
1MX	39.2	41.6 ± 2.1	5.03	106.1
	58.8	56.9 ± 3.3	5.80	96.8
	98.0	103.7 ± 5.6	5.40	105.8
TB	40.4	41.1 ± 0.8	1.95	101.7
	60.6	58.6 ± 4.1	7.00	96.7
	101.0	98.6 ± 1.7	1.72	97.6
PX	42.4	43.0 ± 0.6	1.40	101.4
	63.6	60.7 ± 2.5	4.12	95.4
	106.0	100.6 ± 1.7	1.68	95.2
TP	40.0	40.2 ± 1.3	3.23	100.5
	60.0	58.6 ± 0.7	1.19	97.7
	100.0	101.7 ± 4.4	4.32	101.7
CA	40.8	38.9 ± 0.8	2.06	95.3
	61.2	63.0 ± 4.0	6.35	102.8
	102.0	98.3 ± 5.5	5.60	96.4

* Mean value of six measurements.

Calibration curves for the analysis of xanthine derivatives in blood serum and urine samples are presented in Tables 6 and 7, respectively. The precision and accuracy studies of solid phase extraction of xanthine derivatives from biological samples were conducted by spiking drug free blood serum and urine samples with known concentrations of the compounds and then by comparing obtained results with those as calculated from regression equations. Results are given in Table 8 for serum samples and Table 9 for urine samples. Each value represents the mean of six measurements carried out.

CONCLUSIONS

Nine xanthine derivatives: xanthine, 7-methylxanthine, 3-methylxanthine, 1-methylxanthine, isocaffeine, theobromine, paraxanthine, theophylline and caffeine were separated and analysed by means of HPLC with photodiode array detection.

Isocaffeine as being absent in biological fluids was selected as internal standard.

The binary gradient eluent system used provides good separation, high selectivity, resolution within a minimum analysis time of approximately 18 min. Detection limits are within 3 ng range for 20 μL injected sample volume.

Day-to-day precision was tested over 8 consecutive days and repeatability (within day run) proved to be very satisfactory (RSD < 10 %).

Solid phase extraction was used for the isolation of the analytes from biological fluids. Only a small volume of the sample is required (40 μL in case of serum and 100 μL in case of urine) making the method a valuable tool for clinical paediatric research.

ACKNOWLEDGEMENTS

The authors of this paper wish to thank N. Asteriadis S. A. for providing the Shimadzu HPLC system.

Also we are grateful to Merck Hellas (A. Maltabe) for the supply of the solid phase extraction cartridges.

REFERENCES

1. T. Leakey, J. Chromatogr., **507**, 199-220 (1990).

2. T. Vree, L. Riemens, P. Koopman-Kinenais, J. Chromatogr., **428**, 311-319 (1988).

3. Y. H. Park, C. Goshorn, O. Hihsvark, J. Chromatogr., **343**, 359-367 (1985).

4. P. Parra, A. Linon, S. Ferre, T. Grix, F. Jane, J. Chromatogr., **570**, 185-190 (1991).

5. Z. K. Shihabi, M. E. Hirsdale, A. M. Bleyer, J. Chromatogr., B. Biomed. Appl., **669(1)**, 163-169 (1995).

6. M. C. Gennaro, C. Abrigo, P. Biglino, The Analyst, **117**, 1071-1073 (1992).

7. A. Wahlländer, E. Rehner, B. Karlaganis, J. Chromatogr., **338**, 369-375 (1985).

8. R. Hartley, J. R. Cookman, I. Smith, J. Chromatogr., **306**, 191-203 (1984).

9. J. Blanchard, S. Harvey, W. Morgan, J. Chromatogr. Sci. **28**, 303-306 (1990).

10. E. Naline, C. P. Palette-Pays, J. Moreau, C. Adreyer, M. Pays, Chromatogr., **604**, 203-211 (1992).

11. J. Lauff, J. Chromatogr., **417**, 99-109 (1987).

12. N. Grgurihovich, J. Chromatogr., **380**, 431-436 (1986).

13. K. Muir, M. Kunitany, S. Riegelman, J. Chromatogr., **231**, 73-82 (1982).

14. N. Scott, J. Chakaborty, V. Marks, J. Chromatogr., **375**, 321-329 (1986).

15. M. Homma, K. Oka, N. Takahashi, Anal. Chem., **61**, 784-787 (1989).

16. M. B. Kester, C. L. Saccar, M. Rocci Jr, H. Mansmann Jr, J. Chromatogr., **380**, 99-108 (1986).

Received March 12, 1996
Accepted April 3, 1996
Manuscript 4108

J. LIQ. CHROM. & REL. TECHNOL., 19(16), 2579–2592 (1996)

EVALUATION OF REVERSED PHASE COLUMNS FOR THE ANALYSIS OF DIPHENYLMETHANE DERIVATIVES

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ABSTRACT

Four reversed phases were tested in RPLC of newly synthesized diphenylmethane derivatives. Non-deactivated Kromasil C-8 and Beckman C-18 and deactivated Supelco LC-8-DB and Supelco LC-18-DB reversed phases were evaluated. Column supplier, deactivation and the presence of amine modifier (triethylamine, TEA) were used as variables and capacity factors (k) and asymmetry factors (As) were measured. Two different mobile phase systems were used: Acetonitrile: ammonium acetate (0.1 M) and methanol: potassium dihydrogen phosphate (0.02 M). According to the results of this comparative study, non-deactivated Kromasil C-8 and Beckman C-18 were found to be the most suitable phases for our applications e.g., synthesis purity and lipophilicity studies concerning antihistamine-like compounds. For most of the compounds involved in this study, the use of these phases requires TEA as an amine modifier. Addition of the amine also made it possible to use the deactivated phases, e.g., Supelco C-8 and C-18 columns. The RPLC method was used to evaluate the lipophilicity of some DPPE derivatives, compared to known antihistaminergics.

INTRODUCTION

Recently, a para-diphenylmethane compound, N,N-diethyl-2-[4(phenyl-methyl)phenoxy] ethanamine HCl (DPPE), has been proven to be a suitable ligand in binding to the anti-estrogenic binding site (AEBS), as well as to intracellular histamine (H_{ic} receptor.[1,2] DPPE has shown greater potency in inhibition of Con A-stimulated DNA synthesis than pyrilamine (an H_1-receptor antagonist), cimetidine (an H_2-receptor antagonist) and verapamil (a calcium channel blocking drug), thus being a potential drug candidate.[3] We have started synthezising DPPE and its analogues in order to develop more and more useful ligands for AEBS and H_{ic}-receptor binding studies. The aim of this study was to develop high performance liquid chromatographic (HPLC) methods for synthesis, product purity and lipophilicity assessment studies for these new compounds. Recently, the anticancer effects of DPPE itself have been studied in a clinical trial, where the compound was analyzed from clinical samples by means of HPLC using ultraviolet light (UV) detection.[4] The selection of a suitable stationary phase for RPLC of these basic and rather lipophilic compounds is crucially important. Deactivated reversed phase packings should be excellent for strongly basic analyses.[5-7] Therefore, they could be used without an amine modifier, which permits their possible application in LC-MS studies in the thermospray (TSP) mode.[8,9] However, also non-deactivated packings were included to this study, one reason being their economical price. Nonetheless, when using non-deactivated columns, it seemed likely that it would be necessary to include an amine modifier for the present compounds, because of their basic nature and amine structure.[10,11]

The retention (as measured by the capacity factors) and peak shapes (asymmetry factors) of DPPE derivatives were studied using conventional and deactivated C_8 and C_{18} reversed phase columns. The following parameters were studied:

1) effect of column supplier, stationary phase chain length and deactivation;

2) the nature of the organic solvent

3) effect of the addition of the amine modifier (triethylamine).

When the column with optimal characteristics had been determined, we also examined

4) effect of pH of the mobile phase and

5) influence of the percentage of organic solvent.

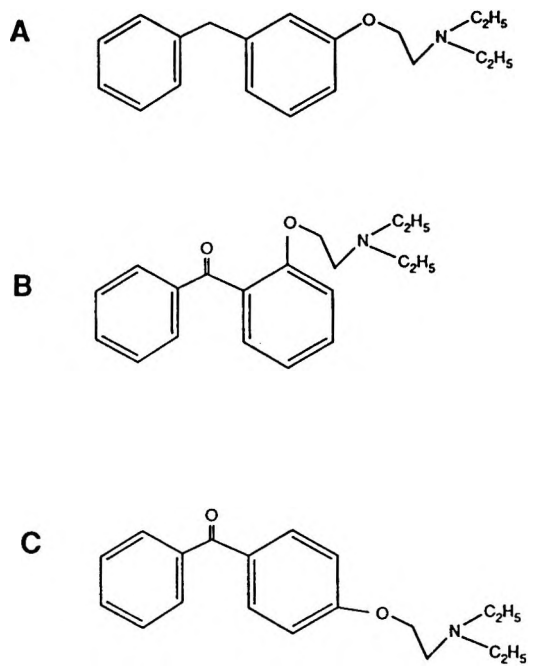

Figure 1. Structures of A) DPPE, B) 2-DBPE and C) 4-DBPE.

Only DPPE derivatives were included in these further studies. Finally, the most suitable system was applied to lipophilicity studies of DPPE derivatives and antihistamines (H_1-receptor antagonists), the latter compounds being structurally and pharmacologically closely related to DPPE.

MATERIALS AND METHODS

Chemicals

The syntheses of N,N-diethyl-2-[4-(phenylmethyl)phenoxy] ethanamine HCl (DPPE) and its derivatives (Fig. 1) have been characterized by spectral data (EI-MS, ^{1}H and ^{13}C NMR), but also studied by chromatography [HPLC-UV, HPLC-MS (TSP)[12]].

The following analytical grade chemicals were used: Astemizole, clemastine, carbinoxamine, cinnarizine, cyclizine, diphenhydramine,

pheniramine, phenyltoloxamine and pyrilamine (Sigma, St. Louis, MO, USA), ammonium acetate (Merck), potassium dihydrogen phosphate (Merck), concentrated acetic acid (Merck), sodium nitroprusside (Merck) and triethylamine (Fluka).

HPLC-grade solvents, methanol and acetonitrile, were purchased from Labscan. Before being used for HPLC, the mobile phases were filtered through a 0.45 μm filter, and degassed with helium.

Liquid Chromatographic System

High performance liquid chromatography was performed with a system consisting of a Beckman programmable solvent module 116, a Beckman variable wavelength UV-detector 166 (set at 240 nm), System Gold data module (Beckman Instruments, San Ramon, CA), Marathon autosampler (Sparks, The Netherlands) equipped with column thermostat and a Rheodyne 7080-080 loop (20 μl) injector (Rheodyne, Cotati, CA). The flow rate was 1.0 mL/min.

Columns

The following columns were used: Supelco LC-8-DB and LC-18-DB, 5 μm, 150x4.6 mm, from Supelco Inc (Bellefonte, PA, USA), Kromasil C-8, 5 μm, 150x4.6 mm from Eka Nobel, Surte, Sweden) and Beckman C-18, 5 μm, 250x4.6 mm from Beckman Instruments (San Ramon, CA, USA).

Mobile Phases

Two main types of mobile phases were used, under isocratic conditions:

1) acetonitrile: 0.1 M ammonium acetate buffer (65:35, v/v) with pH 6.4, and

2) methanol: 0.02 M potassium dihydrogen phosphate buffer (80:20, v/v) with pH 4.5.

Both mobile phase combinations were used, both with and without triethylamine (20 mM), when evaluating the columns. Later, when a suitable column was chosen for further studies, the percentages of organic solvent were varied for both mobile phase types. Also, the pH scale was tested between pH 3.0 and 7.0 for the most applicable mobile phase composition. For LC-MS studies, the mobile phase was acetonitrile: 0.1 M ammonium acetate buffer (65:35, v/v) with apparent pH adjusted to 6.5.

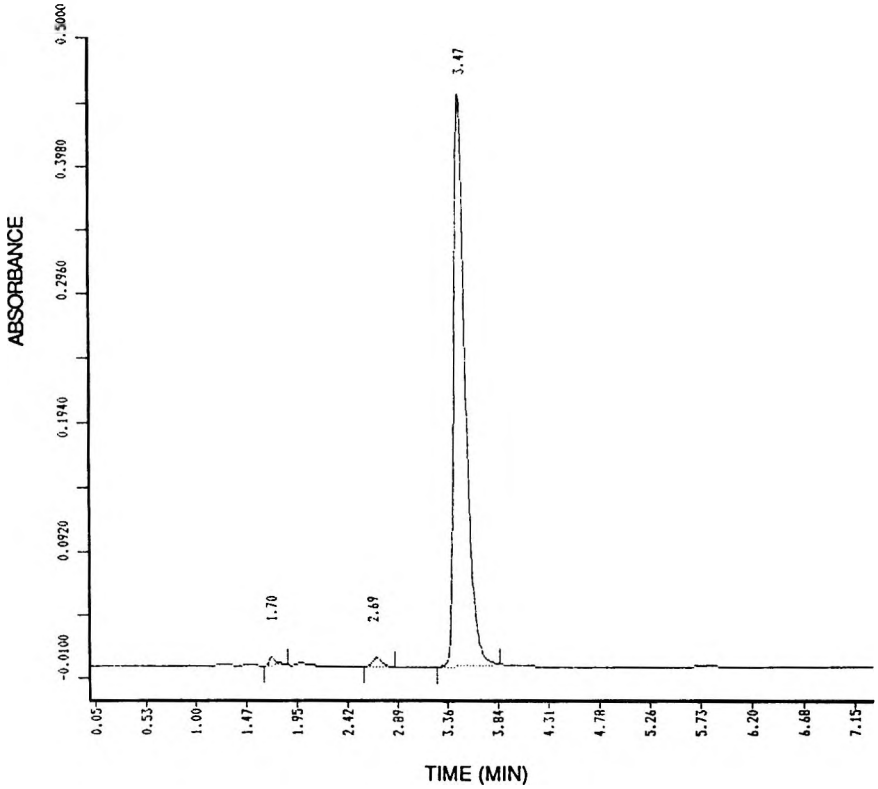

Figure 2. A chromatogram of 2-DBPE. Column: Kromasil C-8, 150x4.6 mm, 5 μm particle size, Mobile phase: Acetonitrile: 0.1 M ammonium acetate with 20 mM TEA, pH 6.5 (50:50), UV detection at 240 nm.

Solutions

Stock solutions (1 mg/mL) of both diphenylmethane derivatives and reference compounds (H_1-antihistamines) were prepared by dissolving them in methanol and filtering the solutions through a Millex-filter disc (0.22 μm, Millipore, Bedford, MA, USA).

Stock solutions were kept at -20 °C in the dark. Standard solutions (100 μg/mL) were prepared as methanolic dilutions. Aliquots of 20 μL of standard solutions were injected onto the column.

Table 1

Capacity Factors and Asymmetry Factors for Non-Deactivated Columns using Acetonitrile/Ammonium Acetate Buffer and Methanol/Phosphate Buffer

Column	DPPE				2-DBPE				4-DBPE			
	with TEA		without TEA		with TEA		without TEA		with TEA		without TEA	
	k	As	k	As	k	As	k	As	k	As	k	As
Acetonitrile : 0.1M Ammonium Acetate Buffer (65:35)												
Beckman C-18	2.34	1.8	3.88	5.8	1.35	1.0	2.11	5.8	1.33	2.0	1.91	5.5
Kromasil C-8	1.29	1.0	1.64	3.0	0.81	1.0	0.96	1.3	0.85	1.0	1.02	1.7
Methanol : 0.02M Potassium Dihydrogen Phosphate Buffer (80:20)												
Beckman C-18	1.07	2.0	3.23	4.5	0.65	2.0	1.80	9.0	0.60	2.0	1.56	6.0
Kromasil C-8	0.77	1.0	1.11	2.3	0.48	1.0	0.72	1.5	0.48	1.0	0.81	1.8

Table 2

Capacity Factors and Asymmetry Factors for Deactivated Columns using Acetonitrile/Ammonium Acetate Buffer and Methanol/Phosphate Buffer

Column	DPPE				2-DBPE				4-DBPE			
	with TEA		without TEA		with TEA		without TEA		with TEA		without TEA	
	k	As	k	As	k	As	k	As	k	As	k	As
Acetonitrile : 0.1M Ammonium Acetate Buffer (65:35)												
Supelco C-8	2.16	1.4	2.18	2.5	1.66	1.6	1.48	1.8	1.67	1.4	1.52	2.7
Supelco C-18	2.00	1.0	3.45	4.3	1.23	1.0	2.05	3.2	1.21	1.3	1.80	2.6
Methanol : 0.02M Potassium Dihydrogen Phosphate Buffer (80:20)												
Supelco C-8	0.94	2.3	2.58	2.7	0.76	2.3	3.83	2.3	0.79	2.3	3.65	3.0
Supelco C-18	1.56	2.3	3.80	6.2	0.98	1.5	2.17	3.9	0.89	1.0	2.00	3.7

Calculations

The following parameters were used for the column evaluation. The capacity factor (k) was calculated using the relationship $(t_r-t_o)/t_o$, where t_r was the retention time of the compound and t_o that of a nonretained compound, measured by using sodium nitroprusside. The peak asymmetry factor was measured at 10 % of the peak height using the ratio of the widths of the rear and front sides of the peak.[13,14]

RESULTS AND DISCUSSION

Non-Deactivated RP Columns and the Effect of the Amine Modifier

Three diphenylmethane derivatives: DPPE, 2-DBPE, 4-DBPE, were first chromatographed with conventional C_8 and C_{18} reversed phases. These compounds were studied with and without triethylamine (TEA), using two types of mobile phases: one containing acetonitrile and acetate buffer and the other mobile phase containing methanol and phosphate buffer. Experimental data showing capacity factors and asymmetry factors when using non-deactivated C_8 and C_{18} phases for RPLC of diphenylmethane derivatives are shown in Table 1.

It is evident that triethylamine is needed with every non-deactivated reversed phase. The situation is the same with both types of mobile phases. Optimal chromatographic conditions were obtained with the Beckman C_{18} column, apparently due to its length (250 mm) and capacity (octadecyl chains). It seems that this column is most suitable for RPLC-UV studies of synthetic products of new diphenylmethane derivatives and related compounds. For lipophilicity studies a C_8 phase with a 15 cm column (Kromasil) should be optimum, as can be seen in the chromatogram of 2-DPPE (Fig. 2), and the portion of organic solvent could be reduced to obtain suitable retention behavior as was found later.

Deactivated RP Phases and the Effect of Amine Modifier

The significance of column deactivation was one of the most crucial points in this investigation. Recently, this parameter has been studied in the RPLC of α-tocopherol analogues.[15] In our studies, the results indicate that column deactivation is a significant factor in RPLC of diphenylmethane derivatives. However, the addition of an amine modifier, in this case triethylamine, has greater effects on capacity factors and peak symmetry of diphenylmethanes than column deactivation (Table 2). When Supelco LC-8-DB with acetonitrile:0.1 M acetate buffer (65:35) was used for DPPE, one obtained k and As values of 2.18 and 2.5 without TEA.

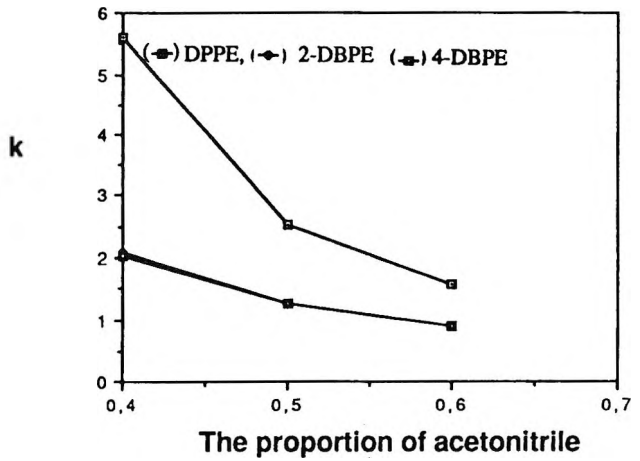

Figure 3. Effect of the proportion of acetonitrile on the capacity factors of DPPE derivatives. Conditions as in Fig. 2 with the exception that pH was 6.0.

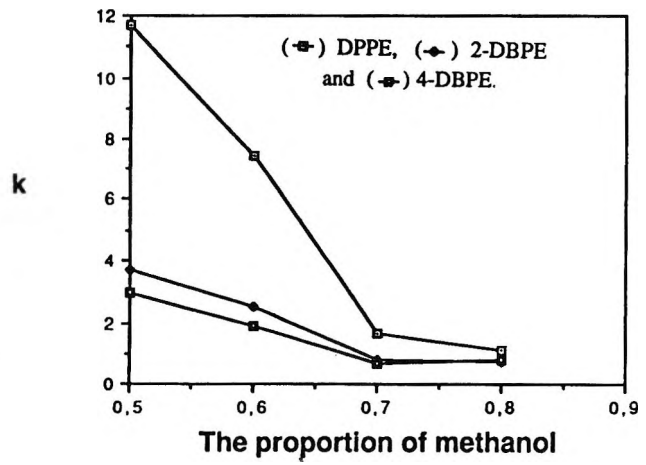

Figure 4. Effect of the proportion of methanol on the capacity factors of DPPE derivatives. Conditions as in Fig. 2. with the exception that mobile phase was MeOH: KH_2PO_4 (0.02M), pH4.5.

When 20 mM TEA had been added to the buffer of the mobile phase, the corresponding values were 2.16 and 1.4, respectively. TEA affects both retention times and peak shapes. When DPPE is chromatographed without TEA, first with a nondeactivated column, Kromasil C-8 (Table 1) and subsequently with a deactivated column, Supelco LC-8-DB (Table 2), the differences in capacity factors and asymmetry values are not so dramatic as is seen with the addition of TEA. Thus, for RPLC of diphenylmethanes, the use of an amine modifier is preferable also in the case of deactivated columns.

For LC-MS studies performed in the TSP mode, a deactivated Supelco LC-8-DB was chosen. This was due to the fact that TEA is not suitable for the solvent system used in TSP, and therefore the Supelco column was the best choice. The non-deactivated RP columns tested were not able separate the sample components sufficiently well to obtain TSP spectra.

Effect of the Organic Solvent

As described before, two main types of mobile phases were used initially, one based on acetonitrile and ammonium acetate buffer (65:35), one consisting of methanol and phosphate buffer (80:20). These combinations were tested with almost all the columns involved, with and without triethylamine.

For further method development with Kromasil C-8, both acetonitrile/acetate buffer and methanol/phosphate buffer combinations were examined in more detail: The percentage of organic solvent was varied. The proportions of acetonitrile (Fig. 3) and methanol (Fig 4) were varied when DPPE, 2-DBPE and 4-DBPE were chromatographed in the presence of 20 mM TEA at pH 6.5 (Acn:NH_4Ac 0.1 M) or pH 4.5 (MeOH:KH_2PO_4 0.02 M). The aim was to use the methanolic mobile phases principally for lipophilicity investigations, but it was found that column back pressures were too high (2.9 kpsi) for routine analyses. This was the case with every suitable proportion of methanol. Thus, the only reasonable alternative for this column and purpose was to use acetonitrile as the organic solvent, even though methanol is generally preferred for log k determinations by RPLC.[10,16]

Effect of the pH of the Mobile Phase and the Column Temperature

After the selection of the mobile phase composition, e.g., Acn: NH_4Ac 0.1 M with 20 mM TEA (50:50), the pH scale was tested with this solution running through the Kromasil C-8 column. The effect of the pH was studied also for the mobile phase consisting of MeOH: KH_2PO_4 0.1 M with 20 mM TEA (60:40), although this composition was not suitable for permanent use because of its pressure inducing effects as described above.

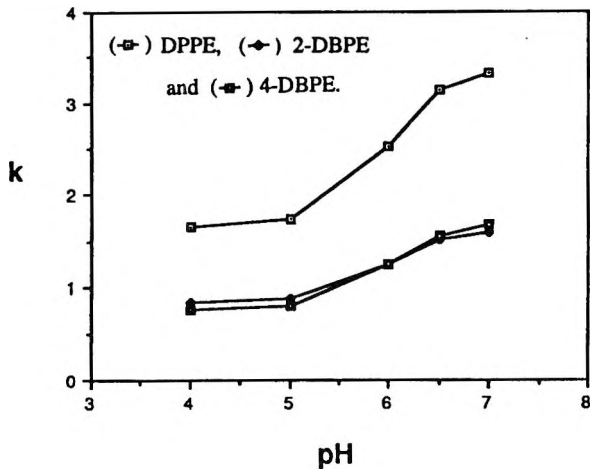

Figure 5. Effect of pH of the mobile phase on capacity factors of DPPE derivatives. Conditions as in Fig. 2.

It is worth noting that, should other types column be used, i.e., which do not exhibit such pressure rises as seen here (for instance a shorter or not so tightly packed column, e.g. C-18 phase), the methanolic mobile phase may be very useful.

The capacity factors of DPPE derivatives clearly depend on the pH of the mobile phase of the acetonitrile/acetate buffer (Fig. 5). pH is one of the most important factors for modification if one is trying optimize the mobile phase of RPLC for lipophilicity studies of new molecules in drug design.[16] The effect of temperature was not remarkable. The change of ambient temperature to 40 °C had little effect on the retention parameters of these compounds (Table 3). Therefore, we continued to use an ambient temperature (25 °C), because, when working at this relatively high pH (6.5), it should prolong column lifetime compared with working at elevated temperatures.[17]

Applications

Purity studies for synthetic DPPE compounds were performed with HPLC with both variable wavelength detection and with diode array detection (unpublished results). Identification and purity studies with HPLC-MS (thermospray) method for DPPE and its derivatives have been described elsewhere.[12]

Table 3

The Effect of Temperature on the Capacity Factors and Asymmetry Factors of DPPE Derivatives*

Compound	40 °C	25 °C
DPPE	k = 2.62 As = 1.7	k = 2.53 As = 2.3
2-DBPE	k = 1.30 As = 1.3	k = 1.26 As = 1.0
4-DBPE	k = 1.33 As = 2.0	k = 1.25 As = 1.0

* Conditions as in Figure 2.

Table 4

Log k Values Obtained by RPLC and Log P Values Obtained from the Literature* for Nine Antihistamines**

Compound	Log k	Log P
Astemizole	0.488	4.10
Clemastine	0.750	5.05
Cinnarizine	1.437	6.14
Cyclizine	0.481	3.97
Diphenhydramine	0.136	3.36
Pheniramine	-0.102	2.02
Phenyltoloxamine	0.519	3.90
Carbinoxamine	0.046	2.17
Pyrilamine	0.136	2.77

* Reference 18. ** Chromatographic conditions as in Figure 2.

The special application described here is the evaluation of lipophilicity of DPPE and related compounds. One of the main areas of interest for our project is to produce lipophilicity data to characterize the physicochemical properties of new compounds and also to use this data in QSAR and CoMFA studies with respect to drug design.

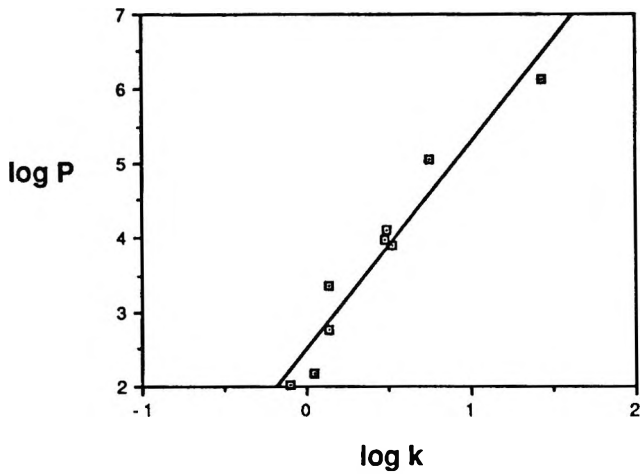

Figure 6. Correlation of log k and log P values of antihistamines. Log k values were obtained at the same liquid chromatographic conditions as in Fig. 2.

Table 5

Log P Values Calculated According to Equation (1) and Log P Values Obtained by Shake-Flask Method for DPPE and its Derivatives

Compound	Log k	Log P (calc.)	Log P (obs.)
DPPE	0.503	4.07	4.55
2-DBPE	0.195	2.97	3.51
4-DBPE	0.211	3.02	3.71

HPLC is especially suitable for this kind of work since it is much more compatible and faster for lipophilicity determinations than conventional methods (e.g. shake flask method), and also provides more data about the molecular properties of the drug than can be obtained with simple lipophilicity determination.[10] We selected the Kromasil C-8, 5 μm, (150x4.6 mm) to be the RPLC column for our lipophilicity studies of the new diphenylmethane derivatives and also for further method development in this field. It is a tightly packed, and a well end-capped column, though it is not actually deactivated. It is not expensive, and its lifetime seems to be quite long. Initially, we used Acn: 0.1 M NH_4Ac buffer with 20 mM TEA (50:50), pH 6.5 as the mobile phase selected in this study. The peaks produced by DPPE compounds are not very sharp in these conditions, but they are symmetric and, importantly, their

retention is optimal for generating their log k values as well as log k values of a set of structurally related antihistamines (Table 4). When subjecting the log k values obtained here and log P values from the literature[18] to linear regression (Fig. 6), an equation:

$$y=2,4602 + 2,8338x \ (R=0.97) \qquad (1)$$

was derived. According to this equation, log P values for DPPE and its derivatives can be calculated. These values are in accordance with those determined with the shake-flask method (Table 5).

According to this investigation, non-deactivated columns may be worthy of evaluation; they may be the columns of choice. For instance, non-deactivated columns, Beckman C-18, 250 mm of length and Kromasil C-8, 150 mm of length were suitable for purity studies and lipophilicity investigations, respectively. They were better than deactivated columns, especially when one considers also the economics of their use, as in the case of Kromasil C-8.

REFERENCES

1. L. J. Brandes, M. W. Hermonat, Biochem. Biophys. Res. Commun., **123**, 724-728 (1984).

2. L. J. Brandes, W. A. Beecroft, G. R. Hogg, Biochem. Biophys. Res. Commun., **179**, 1297-1304 (1991).

3. L. J. Brandes, F. J. LaBella, Biochem. Biophys. Res. Commun., **182**, 786-793 (1992).

4. L. J. Brandes, K. J. Simons, S. P. Bracken, R. C. Warrington, J. Clin. Oncol., **12**, 1281 -1290 (1994).

5. R. J. M. Vervoort, F. A. Maris, H. Hindricks, J. Chromatogr., **623**, 207-220 (1992).

6. T. L. Ascah, B. Freibush, J. Chromatogr., **506**, 357-369 (1990).

7. J. Coene, M. Ghijs, E. Van Den Eeckhout, W. Van Den Bossche, P. Sandra, J. Chromatogr., **553**, 285-297 (1991).

8. M. M. Bursey, C. E. Parker, R. W. Smith, S. J. Gaskell, Anal. Chem., **57**, 2597-2599 (1985).

9. A. J. Alexander, P. Kebarle, Anal. Chem., **58**, 471-478 (1986).

10. T. Braumann, J. Chromatogr., **373**, 191-225 (1986).

11. J. S. Kiel, S. L. Morgan, R. K. Abramson, J. Chromatogr., **320**, 313-323 (1985).

12. A. Martinsen, S. Hyvönen, A. Poso, J. Gynther, J. Chromatogr., submitted for publication (1996).

13. L. R. Snyder, J. J. Kirkland (Eds.), **Introduction to Modern Liquid Chromatography**, Wiley, New York, 1979.

14. J. P. Foley, J. G. Dorsey, Anal. Chem., **55**, 730-737 (1983).

15. J. Verne-Mismer, M. Lamard, J. Wagner, J. Chromatogr., **645**, 251-258 (1993).

16. N. El Tayar, H. Van De Waterbeemd, B. Testa, J. Chromatogr., **320**, 305-312 (1985).

17. L. R. Snyder, **Practical HPLC Method Development,** Wiley, New York, (1988).

18. C. Hansch, (Ed.), **Comprehensive Medicinal Chemistry**, Vol. 6, "Drug Compendium," Pergamon Press, London, 1990.

Received March 18, 1996
Accepted April 8, 1996
Manuscript 4120

J. LIQ. CHROM. & REL. TECHNOL., 19(16), 2593-2602 (1996)

STUDIES ON NEUROSTEROIDS. IV. QUANTITATIVE DETERMINATION OF PREGNENOLONE IN RAT BRAINS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The quantitative determination of pregnenolone in rat brain was done using reverse phase high performance liquid chromatography with fluorescence detection and the internal standard method. The desired fraction was obtained from a rat brain with the combined use of a Bond Elut C8 cartridge for the solid-phase extraction and a lipophilic gel (piperidino-hydroxypropyl Sephadex LH-20) for the ion-exchange chromatography. The fraction was derivatized with 1-anthroyl cyanide followed by purification with 2 successive silica gel columns to remove the excess or decomposed reagent. Separation of the compounds was performed on a J'sphere ODS-L80 column and the calibration graph was linear from 10 to 60 ng/tube (*ca.* 1 g tissue of brain). The method was applied to the determination of pregnenolone in rat brains of Wistar and Sprague-Dawley strains, most of which showed much lower amounts than that previously reported.

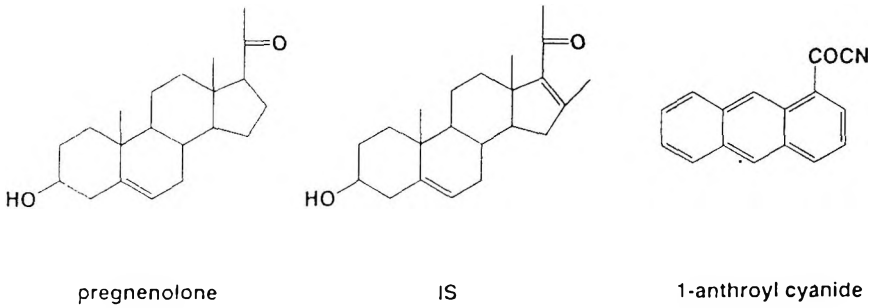

Figure 1. Structures of pregnenolone, IS, and 1-anthroyl cyanide

INTRODUCTION

Since the discovery of dehydroepiandrosterone in rat brains, several 17- and 20-oxosteroids, called "neurosteroids", have been elucidated in mammalian brains,[1] in which pregnenolone is one of the main steroids. Significant interest has thus been focused on their biological properties in this organ.[1] The determination of neurosteroids [65±15 ng/g tissue of rat brain; mean ± standard deviation (S.D.)] has usually been done by gas chromatography-mass spectrometry or radioimmunoassay;[1,2] however, these methods have some problems regarding their simplicity and versatility. High performance liquid chromatography (HPLC) shows promise as a convenient determination method, but highly sensitive derivatization is necessary because neurosteroids are not very responsive to the commonly used detectors.

In a previous paper of this series, we clarified the existence of dehydroepiandrosterone and pregnenolone in rat brains by HPLC using fluorescence (FL) detection.[3] This paper deals with the quantitative determination of pregnenolone in rat brains using HPLC with FL detection and the internal standard (IS) method (Fig. 1).

EXPERIMENTAL

Materials

Oxosteroids were kindly supplied by Teikoku Hormone Mfg. (Tokyo, Japan). 3β-Hydroxy-16-methylpregna-5,16-dien-20-one was prepared in this laboratory and used as an IS.[4] 1-Anthroyl cyanide was purchased from Wako Pure Chemical Ind. (Osaka, Japan).

Bond Elut-C18 and -C8 cartridges, 500 mg (Varian Sample Preparation Products, Harbor City, CA, U.S.A.) were successively washed with hexane, $CHCl_3$, MeOH, and H_2O prior to use. Piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) was prepared using the method reported by Goto et al.[5]

Apparatus

HPLC was carried out using a JASCO TRI ROTAR chromatograph equipped with a Hitachi F-1000 FL detector (λ_{ex} 370 nm, λ_{em} 470 nm)(Hitachi, Tokyo). Reverse phase column (J'sphere ODS-L80, 4 μm, 15 cm x 0.46 cm i.d.) (YMC, Kyoto, Japan) was used under ambient conditions at a flow rate of 1 mL/min.

Procedure for Determination of Pregnenolone in a Rat Brain

Adult male Wistar and Sprague-Dawley rats (7-8 weeks old: 190-215 g) were kept for 2 days at 25 °C under a 12hr/12hr lighting schedule (8:00-20:00). The rat was decapitated and half (*ca.* 1 g tissue) of the entire brain was homogenized in isotonic saline (2 mL) under ice-cooling. MeOH (2 mL) and IS (50 ng) in EtOH (50 μL) were added to the homogenate and centrifuged at 2,800 rpm for 15 min. The precipitate was again suspended in MeOH (2 mL) and centrifuged as described above. Both supernatants were combined and centrifuged again at 2,800 rpm for 30 min. The supernatant was diluted with H_2O (45 mL) and the whole solution was applied to a Bond Elut C8 cartridge, washed with H_2O (6 mL) and eluted with 90% EtOH (5 mL), which was applied to a PHP-LH-20 column (0.6 cm i.d.). The eluate with 90% EtOH was concentrated *in vacuo* to a volume of 1 mL, which was diluted with H_2O (4 mL) and applied to a Bond Elut C18 cartridge. After washing with H_2O (6 mL), the desired compounds were eluted with 80% MeOH (5 mL) which was then evaporated and dried *in vacuo*. The residue was next used for the derivatization reaction. 1-Anthroyl cyanide (500 μg) in MeCN (0.1 mL) containing 0.24% quinuclidine in MeCN (0.05 mL) was added to the residue and kept at 60 °C for 30 min, 2 drops of MeOH was added to decompose the excess reagents and then evaporated under an N_2 gas stream. The residue from the derivatization mixture was applied to 2 successive silica gel columns (each 3 x 0.6 cm i.d.) as described below. After washing with hexane (3 mL) and hexane-AcOEt (20:1, 6 mL), the eluate with hexane-AcOEt (20:1, 20 mL) was evaporated in vacuo and the residue was applied to the next silica gel column.

After washing with hexane (3 mL) and hexane-acetone (60:1, 9 mL), the desired compounds were eluted with hexane-acetone (60:1, 13 mL). After evaporation of the solvent, the residue was dissolved in EtOH and an aliquot of which was applied to HPLC using MeCN-H_2O (15:2) as the mobile phase.

Absolute Recovery Rate Before Derivatization Reaction

A solution of pregnenolone (30 ng) in EtOH (300 μL) was added to the rat brain (*ca.* 1 g tissue) homogenate, deproteinized, treated with Bond Elut C8 cartridge, PHP-LH-20 column and Bond Elut C18 cartridge as described above. A solution of IS (50 ng) in EtOH (50 μL) was addded to the residue and evaporated *in vacuo*. The residue was derivatized with 1-anthroyl cyanide as described above and the recovery rate was calculated using the peak height ratio method.

The above described solutions of pregnenolone (30 ng) and IS (50 ng) were mixed, evaporated, derivatized with 1-anthroyl cyanide and treated with 2 successive silica gel columns followed by HPLC analysis as previously described and the peak height ratio of which was taken as 100%.

Calibration Graph

In the standard addition method, solutions of pregnenolone (10, 20, and 30 ng) in EtOH (100, 200, and 300 μL) and IS (50 ng) in EtOH (50 μL) were added to the rat brain (*ca.* 1 g tissue) homogenate, and each of the resulting solutions was assayed using the proposed method. In the IS method, solutions of pregnenolone (10, 20, 40, and 60 ng) in EtOH (100, 200, 400, and 600 μL) and IS (50 ng) in EtOH (50 μL) were mixed and each of the resulting solutions was applied to a Bond Elut C8 cartridge using the proposed method.

Method Validation

The solutions of pregnenolone (10 and 30 ng) in EtOH (100 and 300 μL) and IS (50 ng) in EtOH (50 μL) were added to the rat brain (*ca.* 1 g tissue) homogenate, and each of the resulting solutions was assayed using the proposed method.

RESULTS AND DISCUSSION

Cleanup of Pregnenolone in Rat Brain

Neurosteroids are not very responsive to the commonly used HPLC detectors; however, fluorometric derivatization shows promise as a reliable determination method. In a previous paper, we used 4-(*N,N*-dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole (DBDH) and

1-anthroyl cyanide as fluorogenic labeling reagents for the separation and characterization of pregnenolone and dehydroepiandrosterone in rat brains.[3] In the preliminary experiment, 1-anthroyl cyanide is superior to DBDH in the derivatization procedure, because the latter reagent produced many decomposed compounds. These data prompted us to use 1-anthroyl cyanide as the derivatization reagent.

The entire brains of adult male Wistar rats were homogenized in isotonic saline and deproteinized with methanol. The supernatant was applied to solid-phase extraction, ion-exchange chromatography, solid-phase extraction and then derivatization with 1-anthroyl cyanide (Fig. 2). The reaction mixture was purified with two successive silica gel columns, applied to HPLC and a typical chromatogram is shown in Fig. 3.

No interference from the endogenous substances in the rat brain was detected at the retention time of IS. To confirm the purity of the peak, the eluate from the corresponding peak was collected, reapplied to HPLC using MeOH-H_2O (9:1) as the mobile phase and showed a single peak, in which any potential interference with the other oxosteroids was clearly minimized as shown in Fig. 4.

Absolute Recovery Rate and Calibration Graph

The absolute recovery rate before the derivatization reaction at 30 ng/tube was examined according to the cleanup procedure. Reasonable net recovery rates (65.0 ± 6.1%, n=5) were obtained throughout the pretreatment (four steps). The calibration graph constructed using the standard addition (added 10-30 ng) and peak height ratio methods showed good linearity ($y=0.040x+0.66$, $r=0.998$). Next the usual IS method was examined as follows. The solutions of pregnenolone at four levels (10 - 60 ng) and IS were mixed and each of the resulting solutions was applied to a Bond Elut C8 cartridge followed by the proposed method. The constructed calibration graph also showed good linearity ($y=0.039x+0.043$, $r=0.996$, 10-60 ng/tube), which had almost the same slope as that obtained using the standard addition method. It was used as the calibration graph in the following experiments.

Method Validation

After spiking of the standard sample (10 and 30 ng) to the rat brain homogenate, the concentrations of pregnenolone were determined using the proposed method. The obtained data are summarized in Table 1. Satisfactory recovery rates were obtained with a relative S.D. (R.S.D.) of less than 13%.

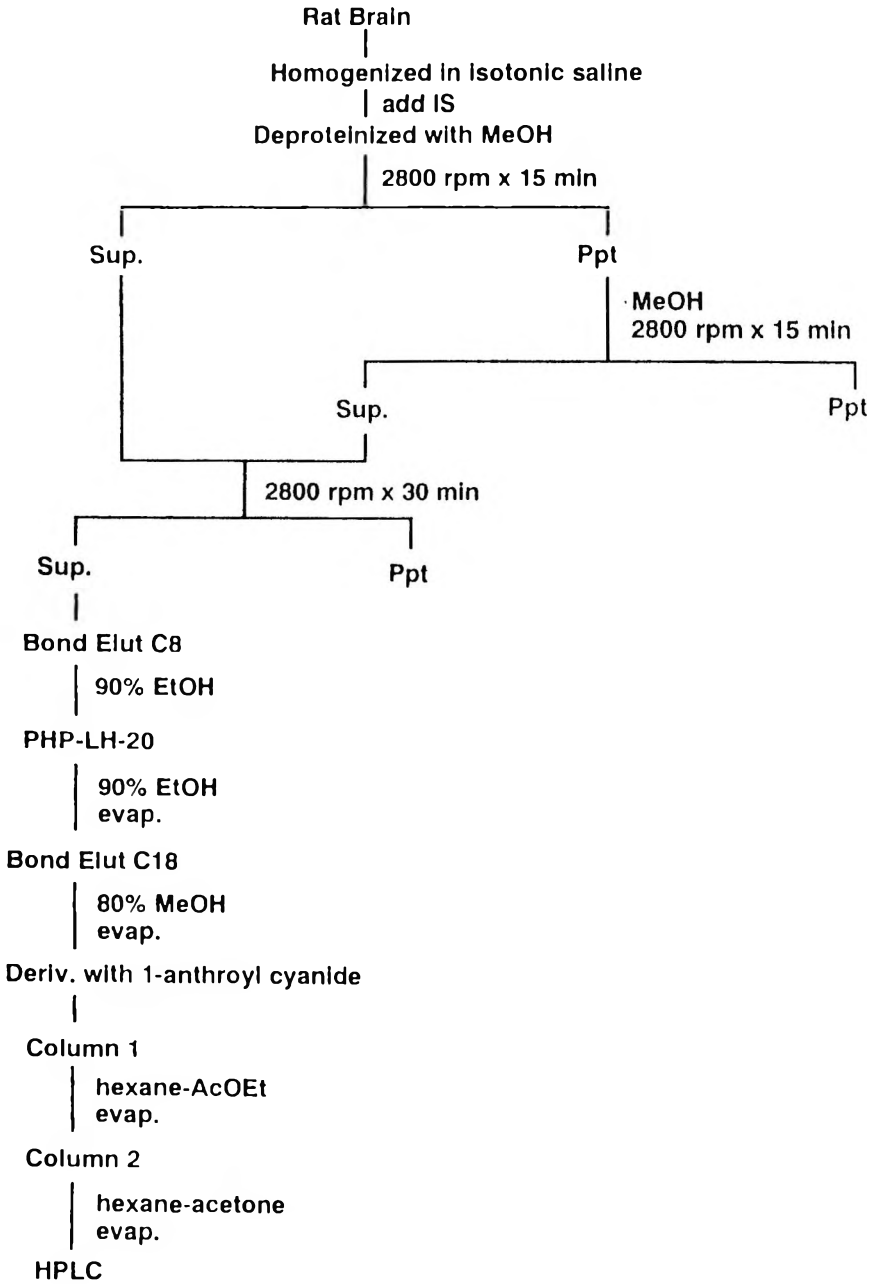

Figure 2. Procedure for determination of pregnenolone in rat brain

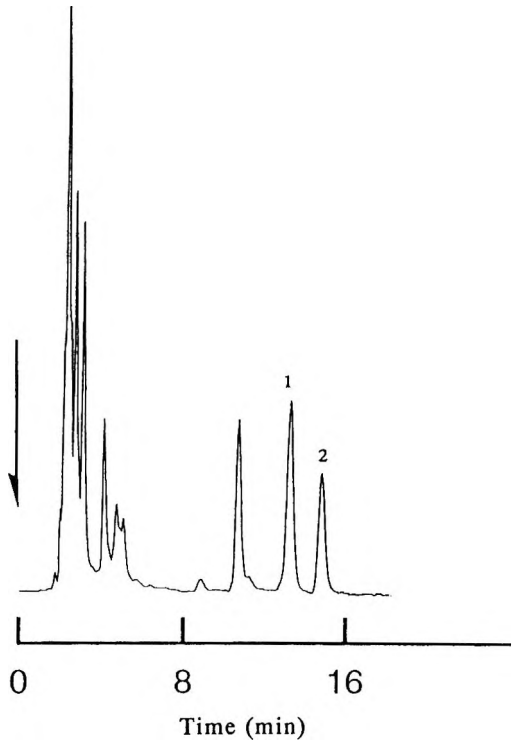

Figure 3. Chromatogram of pregnenolone derivatized with 1-anthroyl cyanide in rat brain. 1. pregnenolone; 2. IS. Conditions: mobile phase, MeCN-H_2O (15:2).

Application of the Method

The quantity of pregnenolone in rat brains of Sprague-Dawley strain is reported as 65 ± 15 ng/g tissue.[2] Brain neurosteroids undergo large circadian variations, with the largest values around the time of lights off under a 12hr/12hr light regimen.[1]

The measurement of pregnenolone was made for the whole brain of young adult male rats and the obtained data are shown in Table 2. Only one rat in five of Wistar (Entry 1) or Sprague-Dawley (Entry 3) strain contained more than 10 ng of pregnenolone/g tissue and circadian variations have not been found as shown in Entries 1 and 2. Also, the other brains contained less than 1 ng of pregnenolone (ng/g tissue) or the steroid could not be detected under these conditions.

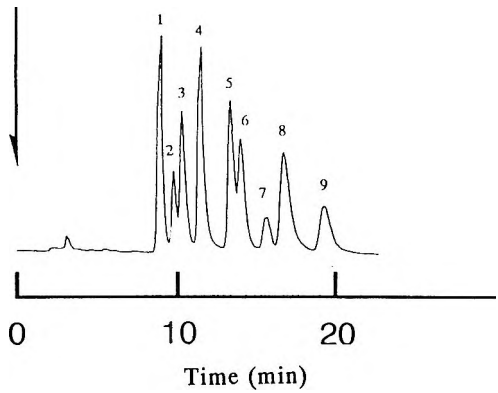

Figure 4. Separation of oxosteroids derivatized with 1-anthroyl cyanide. 1. 3α-hydroxy-5β-androstan-17-one, 3β-hydroxy-5β-androstan-17-one; 2. 3α-hydroxy-5α-androstan-17-one; 3. dehydroepiandrosterone; 4. 3β-hydroxy-5α-androstan-17-one; 5. 3α-hydroxy-5β-pregnan-20-one; 6. 3α-hydroxy-5α-pregnan-20-one; 7. 3β-hydroxy-5β-pregnan-20-one; 8. pregnenolone; 9. 3β-hydroxy-5α-pregnan-20-one; Conditions: mobile phase, MeOH-H_2O (9:1).

Table 1

Accuracy and Precision of Pregnenolone in Rat Brain

Pregnenolone (ng/Tube)			Difference[c]
Added	Found	Expected	(%)
0	16.0[a]		
10	26.0 ± 1.6 (6.2)[b]	26.0	0.0
0	21.0[a]		
30	53.1 ± 6.7 (12.6)[b]	51.0	7.0

[a] mean (n=3), *ca.* 1g tissue.
[b] n=5, mean ± S.D. (R.S.D., %).
[c] % mean difference = 100x (mean of net found - added)/added.

Table 2

Pregnenolone in Rat Brain

Entry	Concentration (ng/g Tissue)					
1[a]	10.5	<1	<1	nd[c]	nd	(Wistar strain)
2[b]	13.1	5.5	<1	<1	<1	(Wistar strain)
3[a]	22.2	<1	nd	nd	nd	(Sprague-Dawley strain)

[a] after 1 hr of light exposure
[b] after 10 hr of light exposure
[c] nd = not detectable

CONCLUSIONS

The quantitative determination of pregnenolone in rat brain was done by reverse phase HPLC with FL detection using 1-anthroyl cyanide as the derivatization reagent and 3 β-hydroxy-16-methylpregna-5,16-dien-20-one was used as the IS. The method was applied to the determination of this steroid in the rat brains of Wistar and Sprague-Dawley strains, most of which showed the much lower amounts than that previously reported. No circadian variations have been found. As the reasons for this difference are not clear at this time, we are now investigating this discrepancy and the significance of this steroid in the brain will be reported in the future.

ACKNOWLEDGEMENTS

Part of this work was supported by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan. The authors thank Teikoku Hormone Mfg. for providing the oxosteroids.

REFERENCES

1. P. Robel, Y. Akwa, C. Corpéchot, Z.-Yi Hu, I. Jung-Testas, K. Kabbadj, C. Le Goascogne, R. Morfin, C. Vourc'h, J. Young, E.-Emile Baulieu, **Neurosteroids: Biosynthesis and Function of Pregnenolone and Dehydroepiandrosterone in the Brain. Brain Endocrinology**, Second edition, ed. by M. Motta, Raven Press, Ltd., New York, 1991, pp. 105-132.

2. C. Mathur, V. V. K. Prasad, V. S. Raju, M. Welch, S. Lieberman, Proc. Natl. Acad. Sci. U.S.A., **90**, 85-88 (1993).

3. K. Shimada, T. Nakagi, M. Yoshizawa, T. Ueda, Anal. Sci., **11**, 445-447 (1995).

4. R. Mickova, K. Syhora, Collect. Czech. Chem. Commun., **29**, 2513-2521 (1964).

5. J. Goto, M. Hasegawa, H. Kato, T. Nambara, Clin. Chim. Acta, **87**, 141-147 (1978).

Received March 8,1996
Accepted March 20, 1996
Manuscript 4101

J. LIQ. CHROM. & REL. TECHNOL., 19(16), 2603–2614 (1996)

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF TEFLUBENZURON AND DIFLUBENZURON IN FISH TISSUES OF FARMED FISH FOR RESIDUE STUDIES

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ABSTRACT

A method for the determination of teflubenzuron and Diflubenzuron in fish tissues by HPLC is presented. The samples were extracted with acetone - tetrahydrofuran. Employing traditional liquid-liquid extraction and using a Si and C_8 solid phase extraction column, a clean extract was obtained. The lower limit of quantification for teflubenzuron was 20 ng/g (mL) for muscle, liver and plasma and for diflubenzuron in muscle and liver, the limit of quantification was 25 ng/g.

INTRODUCTION

Infestation with sea lice, *Lepeophtheirus salmonis* and *Caligus elongatus*, is a growing problem in fish farming. The lice damages the skin, causing unthriftiness, and in severe cases the lice may even result in death of the fish. The parasite may also transmit microbial pathogens. Farmed fish are usually more commonly and more heavily infested compared to wild fish[1,2,3]

A range of methods (chemical, physical and biological) has been introduced for controlling sea lice. Treatment with chemotherapeutics has included the use of dichlorvos, trichlorfon, azamethiphos, carbaryl, ivermectin, pyrethrum and hydrogen peroxide.[2] These drugs are administered by bathing or dipping, except for ivermectin which is applied orally (in feed).[2]

The pesticides teflubenzuron (TFB) from Cyanamid and diflubenzuron (DFB) from Solvay Duphar which can be given orally (in feed) to salmon, are two of the newest drugs used in the treatment of sea lice in salmon.

Although the pharmacokinetics of TFB and DFB in fish is unknown, their elimination from fish tissue is assumed to be dependent on water temperature. Drug levels in different tissues are therefore, to some extent, unpredictable. In order to establish safe withdrawal periods to protect the consumer, a sensitive assay for detecting TFB and DFB in tissues is needed.

This paper describes simple and rapid extraction and clean-up procedures for the HPLC determination of TFB in fish plasma and tissues and for the determination of DFB in muscle and liver. The methods are suitable for pharmacokinetic studies and residue analysis of these compounds in Atlantic salmon and rainbow trout. The method is reliable and sensitive and requires only small quantities of chemical reagents. There is no interference from the tissue matrix in the chromatographic analysis.

MATERIALS AND METHODS

Materials and Reagents

Samples of muscle, liver, and plasma from salmon and rainbow trout were used. All chemicals and solvents were of analytical or HPLC grade. Teflubenzuron (Cyanamid), was donated by Skretting (Stavanger, Norway) and diflubenzuron (Solvay Duphar), was donated by Ewos (Skårer, Norway). Stock solutions (1mg/mL) of TFB and DFB were prepared by dissolving the compound in tetrahydrofuran.

Working standards were prepared by dilution with acetonitrile-water (1 : 1) and dichloromethane - hexane (60 : 40). The solutions were stored in the refrigerator. Extraction columns Bond Elut (3cc/500mg, 1cc/100mg) SI and C_8 (1cc/100 mg) were purchased from Varian (Harbor City, CA, USA). Empty reservoirs of 125 mL (Analytichem) were combined with the SI columns.

Chromatographic Conditions

The analyses were performed on a Perkin-Elmer HPLC system, consisting of a Series 410 Bio solvent delivery system, an ISS 100 sampling system (with hexane as flushing liquid) equipped with a Lauda RMT6 cooler (10 °C) from Messgeräte Werk Lauda (Lauda Königshafen, Germany), and a LC 235C diode array detector (Perkin-Elmer, Norwalk, CT, USA). The detector was operated at 250 nm (fixed wavelength). The integration was carried out using the software programme Turbochrom 4.0 (Perkin-Elmer), which was operated on a Brick personal computer connected to a BJ-330 printer (Canon).

The analytical column (stainless steel, 25 cm x 4.6 mm. ID) and guard column (stainless steel , 2 cm x 4.6 mm. ID), were packed with 5 µm particles of Supelcosil LC-ABZ+Plus (Supelco, Bellefonte, PA, USA). The guard column was connected with an A.318 precolumn filter with an A-102X frits (Upchurch Scientific, USA).

For TFB, the mobile phase was a mixture of dichloromethane- hexane (60: 40). The flow rate was 0.8 mL/min for 6 min, followed by 1.5 mL/min for 7 min (5 min for plasma). For DFB, the mobile phase was a mixture of hexane-diclorometha ne (60 : 40). The flow rate was 0.9 mL/min. for 6 min., followed by 1.5 mL/min. for 8 min.

Sample Pretreatment

Muscle and liver

The tissue sample, (3 g) was weighed into a 50 mL centrifuge tube with a screw cap (NUNC, Roskilde, Denmark). Volumes of 300 µl water (or standard dissolved in acetonitrile-water) and 9 mL acetone-tetrahydrofurane (6 : 4) were added. The mixture was homogenized for approx. 6 sec. in an Ultra-Turrax TP 18/2 (Janke & Kunkel KG, Ika Werk, Staufen, F. R. G.), and then centrifuged for 3 min. (5000 rpm). 6.15 mL of the supernatant (corresponding to 1.5 g) were transferred into a glass-stoppered centrifuge tube. Volumes of 2 mL CH_2Cl_2 and 3 mL hexane were added and mixed for approx. 5 sec .

After centrifugation for 3 min (3000 rpm), the upper layer (organic layer) was transferred to another glass-stoppered tube (the water layer was discarded), mixed with 3 mL hexane, and centrifuged after 5 min (3 min., 3000 rpm). The supernatant was then transferred into another glass-stoppered tube and evaporated to dryness under a stream of nitrogen using a Reacti-Therm heating module at 35 °C, and a Reacti-Vap evaporating unit (Pierce, Rockford, IL, USA). The dry residue was dissolved in 6 mL hexane, and loaded onto a conditioned SI column (500mg).

Clean-up on SPE-column

The SI column was conditioned with 2.5 mL hexane, and 2 mL hexane was added to the column. The sample extract was loaded onto the columm without being suctioned through. The glass-stoppered tube was rinsed with 3 x 1 mL hexane and the empty reservoir washed with 2 x 1.5 mL hexane, which was also loaded onto the column. The column was washed with 3 x 2.5 mL hexane, 2 x 2.5 mL hexane-diethyl ether (95 : 5) and 2.5 mL hexane-diethyl ether (90 : 10). The column was then eluted with 2 x 2.5mL hexane-diethyl ether (60 : 40), with full vacuum, the collected eluate being evaporated to dryness. The dry residue was dissolved in 300 µl CH_3OH, and 1.5 mL water then added. The mixture was loaded into a conditioned C_8 column (with 1 mL CH_3OH and 2 x 1 mL H_2O) and slowly suctioned through (0.2 mL/ min.). The glass-stoppered tube was rinsed with 0.5 mL H_2O - CH_3CN (70 : 30), which also was loaded onto the column . The column was washed with 0.5 mL H_2O-CH_3CN (70 : 30), and 2 x 0.5 mL H_2O-CH_3CN (55 : 45). The column was suctioned to dryness for 5 sec. at a vacuum of -5 in. Hg. and then eluted with 2 x 200µl CH_3CN with full vacuum using a VacMaster system (International Sorbent Technology). The collected eluate was evaporated to dryness. The dry residue was dissolved in 0.5 mL CH_2Cl_2 - hexane (60 : 40). After centrifugation (2 min. 3000 rpm.), aliquots of 20µl were injected into the HPLC at intervals of 16 min. for the determination of TFB and DFB.

Plasma

The pretreatment of plasma samples was as follows: To 500µl plasma were added 200 µl water (or standard dissolving in acetonitrile-water) and 6 mL acetone-tetrahydrofurane (6 : 4). The mixture was shaken for 10 sec. Volumes of 2 mL CH_2Cl_2 and 3 mL hexane were added, mixed for 5 sec. and then centrifuged for 3 min. (3000 rpm.). The upper organic layer was transferred to another glass-stoppered centrifuge tube, mixed with 3 mL hexane, and centrifuged after 5 min. The supernatant was transferred into another glass-stoppered tube and then evaporated to dryness.

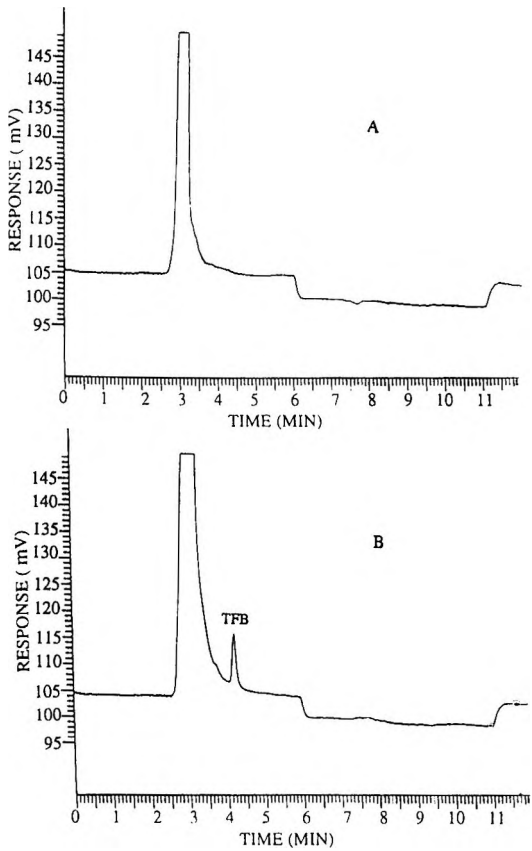

Figure 1. Chromatograms of extracts from fish plasma. A: drug-free plasma, B: plasma spiked with teflubenzuron (200 ng/ mL).

The dry residue was dissolved in 200 µl dichloromethane, and mixed with 3 mL hexane, and loaded (without being suctioned through) onto a conditioned 100 mg SI column (conditioned with 1 mL hexane and 0.6 mL hexane added to the column). The glass tube was rinsed with 3 x 1 mL hexane and the empty reservoir washed with 2 x 1 mL hexane, which also was loaded onto the column. The column was washed with 1mL hexane, 1 mL hexane-diethyl ether (95 : 5) and 0.5 mL hexane-diethyl ether (90 : 10). The column (with full vacuum) was eluted with 2 x 1 mL hexane - diethylether (60 : 40). The collected eluate was evaporated to dryness and the dry residue dissolved in 300 µl CH_3OH and 1.5 mL H_2O then added. Further preparation procedures were as for muscle and liver. Aliquots of 40 µl were injected into the HPLC at intervals of 12 min. for the determination of TFB.

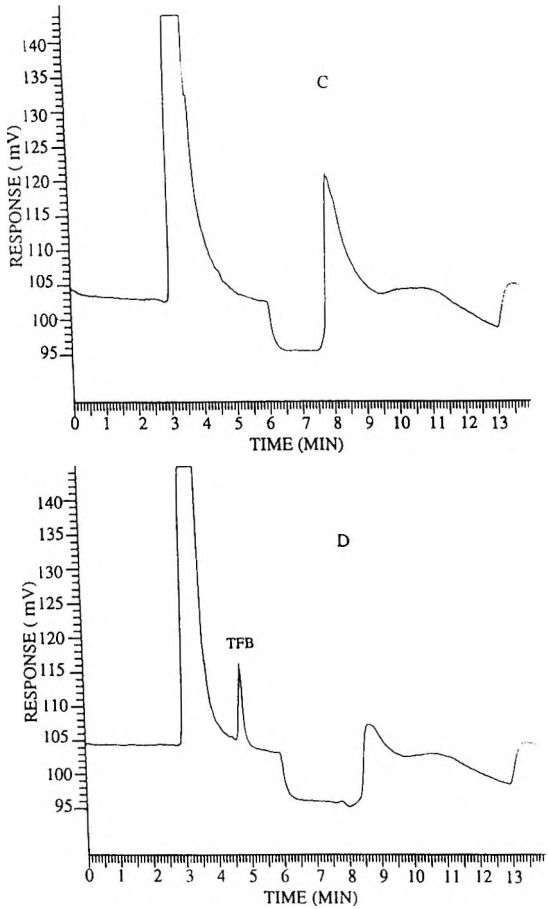

Figure 2. Chromatograms of extracts from fish muscle. C: drug-free muscle, D: muscle spiked with teflubenzuron (200 ng/g).

Calibration Curves and Recovery Studies

The calibration curves for TFB and DFB were obtained by spiking plasma, muscle and liver tissue samples with standard solutions. These were prepared by dilution with acetonitrile - water to yield 20, 30, 50, 100, 200, 500, 1000 and 2000 ng TFB/mL for plasma, and 20, 25, 30, 50, 100, 200, 400 and 500 ng/g both for muscle and liver. For DFB, the standard solutions were prepared to yield 25, 30, 50, 100, 200, 300, and 500 ng/g for muscle and liver.

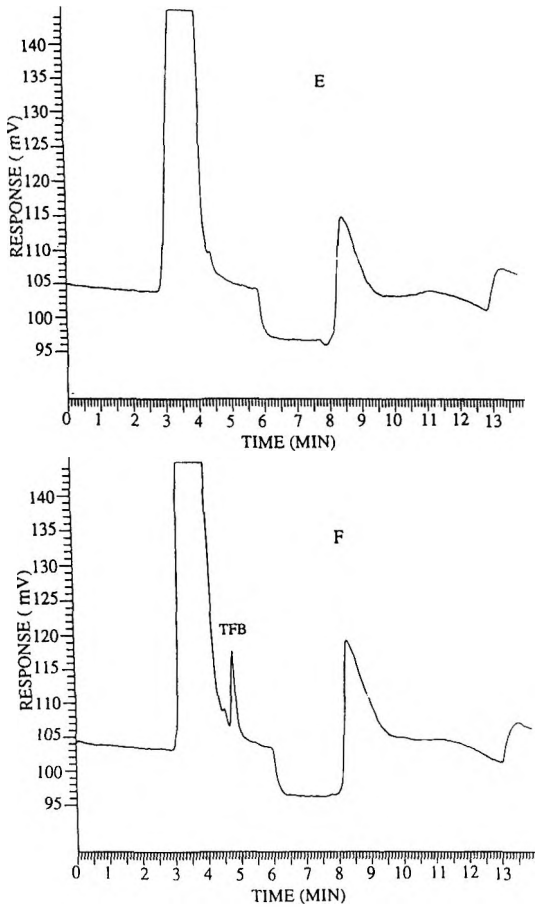

Figure 3. Chromatograms of extracts from fish liver. E: drug-free liver, F: liver spiked with teflubenzuron (200 ng/g).

Duplicate samples were used. The recovery rates were determined by comparing results of analysis of the spiked plasma, muscle and liver samples with those of standard solution (prepared by dilution with dichloromethane - hexane). The linearity of the standard curves for TFB in plasma, muscle and liver and for DFB in muscle and liver was tested using peak-height measurements.

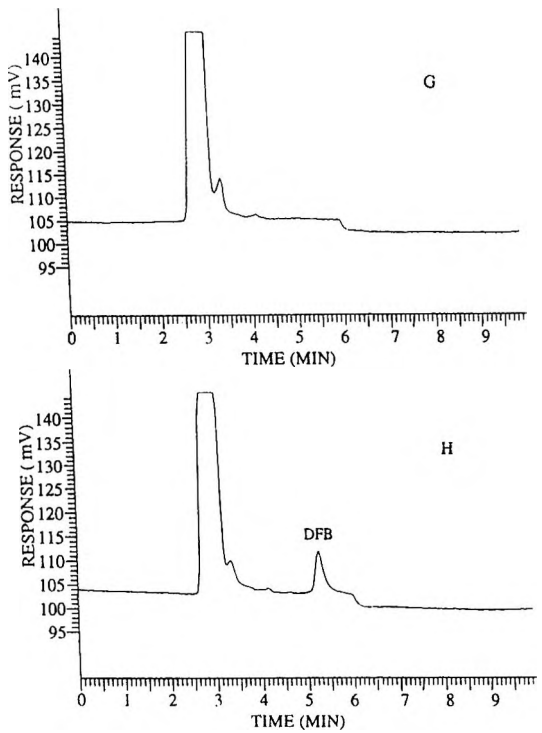

Figure 4. Chromatograms of extracts from fish muscle. G: drug-free muscle, H: muscle spiked with diflubenzuron (200 ng/g).

RESULTS AND DISCUSSION

Chromatograms of clean plasma, muscle and liver, and spiked samples are shown in Figures 1, 2, 3, 4, and 5. The standard curves were linear in the investigated areas; 20 - 2000 ng/mL for TFB in plasma and 20 - 500 ng/g for TFB in muscle and liver, and 25 - 500 ng/g for DFB in muscle and liver.

The linearity of the standard curves for TFB was 0.9995 for plasma and liver and 0.9997 for muscle, the corresponding figures for DFB being 0.9998 for muscle and 0.9996 for liver, when using the external standard method of calculation.

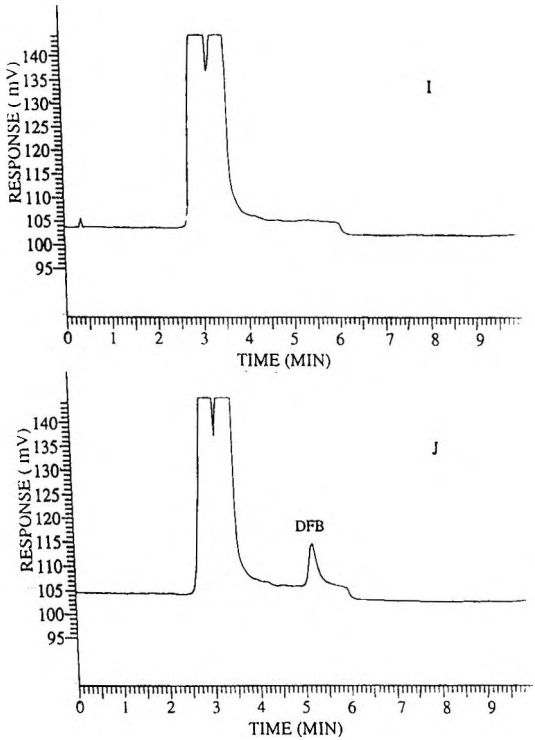

Figure 5. Chromatograms of extracts from fish liver. I: drug-free liver, J: liver spiked with diflubenzuron (200 ng/g).

The precision and recovery for TFB from plasma, and for TFB and DFB from muscle and liver were also calculated and are shown in Table 1. The extraction procedures were validated, and showed good recovery of TFB and DFB. The recovery of TFB was 96% for muscle, and varied from 89 to 90%, and 92 to 96 %, for plasma and liver, respectively. The recovery of DFB varied from 81 to 83% and 83 to 84% for muscle and liver, respectively. The precision of these recovery studies varied from 3.0 to 3.1, 0.8 to 1.5, and 0.5 to 1.6% for TFB in plasma, muscle and liver, respectively. For DFB, the precision varied from 1.0 to 1.9 and 1.2 to 1.5% in muscle and liver, respectively. For TFB, the limit of quantification was 20 ng/g (mL) and the limit of detection 15 ng/g (mL) for plasma, muscle and liver. The limit of quantification for DFB was 25 ng/g and the limit of detection 17 ng/g for muscle and liver. No interference was seen during analysis when calibrating the curves, nor when performing recovery studies.

Table 1

Tissue	No. of samples	Amount in spiked samples (μg/mL-g)	Recovery (%) TFB Mean	TFB S.D.	DFB Mean	DFB S.D.
Plasma	8	0.05	89	3.0		
500μL	8	1.00	90	3.1		
Muscle	8	0.05	96	1.5		
(3g)	8	0.40	96	0.8		
Liver	8	0.05	92	1.6		
(3g)	8	0.40	96	0.5		
Muscle	8	0.05			83	1.0
(3g)	8	0.30			81	1.9
Liver	8	0.05			84	1.2
(3g)	8	0.30			83	1.5

S.D. = standard deviation.

The described method for determination of TFB in plasma can also be used for detection of DFB in plasma, but the procedure has not yet been evaluated. The method was tested under practical conditions by analysing about 60 samples from different rainbow trout, no interfering peaks being observed.

The retention and elution properties of TFB and DFB and the tissue matrix, were studied on bonded-phase extraction columns of polar and non-polar character. Both TFB and DFB are fat-soluble, and residues of fat in the extract strongly influenced the recovery of the two components. It was possible to remove the fat from the tissue extracts by liquid-liquid extractions with hexane and acetonitrile, but this procedure was tedious and, in addition, the recovery was variable.

However, experiments with SI columns to remove the fat, showed that TFB and DFB were strongly retained on the SI column under non-polar conditions. A hexane solution of the tissue homogenate was applied onto the column. Due to the high fat content, TFB and DFB dissolved well in that solvent, and impurities were removed by thorough washing with hexane, and 5 and 10% diethylether in hexane. The compounds were eluted with 40% diethylether in hexane. Also the capacity of the SI-sorbent material had to be

tested. On columns containing only 100 mg sorbent material, the recovery of TFB and DFB from muscle and liver homogenates was variable. On increasing the amount of SI material in the columns to 0.5g, the analyses of spiked tissue showed good reproducibility.

Further purification studies of fish tissue before the HPLC analysis was carried out on less polar extraction columns, and acceptable recovery of TFB and DFB was obtained on C_8 using water-acetonitrile (70 : 30 and 55 : 45).

When a new analytical column (ABZ+Plus) was taken into use, we observed poor peak height and asymetric peak shape. We have found that this can be avoided by removing the organic solvents and conditioning the columns twice as follows:

Time (min.)	H_2O %	CH_2CN %	CH_3OH %	Flow (mL/min.)
20	90	10		1.0
10	50	50		1.0
20	40		60	1.2
15	10	90		1.2
5	15		85	1.0
30	15		85	1.5
5			100	1.5
20			100	1.5

After this procedure, the HPLC system was flushed with dichloromethane for 15 min (1.5 mL/min), prior to the mobile phase. We assume that the combination and the variation of the viscosity and flow, influence column particles in such a way that the result was a good peak shape and good separation. The mobile phase was a mixture of dichloromethane-hexane (85 : 15 for TFB and 60 : 40 for DFB). The flow rate was 0.8 mL/min. for 6 min., followed by 1.5 mL/min. for 7 min. After use, the analytical column was washed for 25 min (1 mL/min) with a mixture of dichloromethane - methanol (80 : 20), followed by dichloromethane for 15 min (1.5 mL/min) and stored in hexane - dichloromethane (90 : 10) for a maximum period of one day. If the column is stored for more than one day, it should be flushed with 100 to 200 mL methanol. New conditioning is not necessary.

An experienced technician can carry out sample clean-up of about 18 samples per day. The assay shows good precision when using the external standard method. The method is robust and sufficiently sensitive, with good recovery.

The method is efficient for quantification of residues of the drugs TFB and DFB. The quantification is linear over a wide concentration range. Only small amounts of solvents are required.

ACKNOWLEDGEMENTS

We are grateful to the Norwegian Research Council for financial support.

REFERENCES

1. T. A. Schram, "*Lepeophtherius Salmonis*, Copepoda, Caligidae, Developmental Stages, Morphology", Norsk Veterinærtidsskrift, **105**, 12. (1993).

2. M. J. Costello, " Review of Methods to Control Sea Lice (Caligidae: Crustacea) Infestations on Salmon (*Salmo Salar*) Farms", Commission of the European Communities contract (No. AQ. 2. 502).

3. T. Håstein, T. Bergsjo, " The Salmon Lice *Lepeophtherius Salmonis* as the Cause of Disease in Farmed Salmonids," Riv. It. Piscic. Ittiop), **A. 11,** 3-4 (1976).

Received November 19, 1995
Accepted February 20, 1996
Manuscript 4038

J. LIQ. CHROM. & REL. TECHNOL., 19(16), 2615–2621 (1996)

DETERMINATION OF ORGANIC ACIDS IN APPLE JUICE BY CAPILLARY LIQUID CHROMATOGRAPHY

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ABSTRACT

The organic acids quinic, malic, shikimic and citric were separated on a packed reverse phase fused-silica capillary column using 0.01 M K_2HPO_4/H_3PO_4 at pH 2.7 as eluent at a flow rate of 2 μL/min, and determined with UV detection. Conventional liquid chromatographic equipment was adapted for such purposes.

The organic acids were able to be separated with detection limits of 2.4, 2.1, 0.04 and 2.9 ng, respectively. Application of the proposed method to the quantification of organic acids in apple juice is reported.

INTRODUCTION

Carboxylic acids are one of the most frequently assayed types of substances among those found in fruits on account of the significant role they play in maintaining quality and nutritional value. In fact, they have a direct influence on the sensory properties of fruits and their juices, and could be added to juices to prevent sedimentation or darkening, or even to flavour them. At the same time, some organic acids are regarded as indicators of ripeness, bacterial activity, or may be used to reveal potential adulteration.[1]

In this study, from among the different analytical techniques available (titrimetric, photometric, enzymatic, electroanalytic and chromatographic) for the determination of organic acids in foods, high performance liquid chromatography was preferred, due to its rapidity, sensitivity and specificity and, since this method entails uncomplicated sample pre-treatment. As in gas chromatography, the actual trend in liquid chromatographic is to reduce the internal diameter of the chromatographic columns to below 1 mm. The most important advantages of microcolumn separations may be summarised as: increased mass sensitivity, higher separation efficiency, improved speed of analysis, less sample volume, and less solvent consumption.[2-4] The purpose of this work was to separate and determinate the principal organic acids in apple juice by capillary liquid chromatography (CLC).

EXPERIMENTAL

Reagents

Analytical standard-grade quinic, malic, shikimic and citric acids were obtained from Sigma (St Louis, MO, USA), and were used without further purification. High purity water was obtained through a Millipore Milli-Q system (Milford, MA, USA). The mobile phases used were buffered solutions containing K_2HPO_4 and adjusted to different pH values with H_3PO_4. All solutions used were filtered across a 0.22 μm Millex membrane to remove any impurities.

Apparatus and Conditions

The micro-LC equipment consisted of a Kontron 422 pump (Kontron Instruments, Milan, Italy) connected to a T-flow-split system wich enabled the mobile phase (0.05 mL/min) to be distributed between the by-pass and the micro-LC column (2 μL/min) connected to a zero-dead volume Valco injector equipped with a 60 nL internal loop (Valco, Houston, TX, USA). Detection was performed by means of Kontron model 430 UV-Vis detector with a 90 nL, 20 mm, flow cell.

Column effluents were monitored at 206 nm and were recorded and integrated with a Data System 450 software from Kontron Instruments. Analyses were carried out in the isocratic mode by using 0.01 M K_2HPO_4/H_3PO_4 at pH 2.7 as eluent at room temperature (20 ± 2 °C). The microcolumn used was a Spherisorb ODS-2 (300 x 0.32 mm i.d., 5 μm) (LC Packings, Amsterdam, The Netherlands).

Apple Juice Samples

The apples were harvested close to optimal processing maturity, and the juice was produced without cold storage previous to sampling. Single-variety juices were extracted from typical varieties employed for apple juice manufacture. Two different technological treatments were employed in order to stabilise the apple juice obtained: ceramic (Gamma Alumina) crossflow microfiltration (MF) with 0.2 μm pore size (Millipore, Milford, MA, USA), and ultrafiltration (UF) by means of a zirconium oxide membrane with 50K of MWCO (TECHSEP Carbosep, Lyon, France). The filtration conditions were as follows: superficial velocity 5 m/s, pressure 3.5 bar, temperature 30 °C for MF and 30 °C and 50 °C for UF. The juices were frozen (-20 °C) until their analytical determination and chromatographic analysis was carried out by direct injection of the sample.

RESULTS AND DISCUSSION

We studied the effect of the elution conditions (pH, ionic strength, organic modifier and flow-rate) on the resolution of the acids concerned, by varying one parameter at a time, while keeping all the others constant. Taking into account the pKs of the different organic acids assayed, the previous studies carried out by our group,[5-7] and the minimum pH recommended for the capillary chromatographic column operation, we assayed the pH ranging between 2.5 and 2.9. For pHs close to 2.9 a good resolution does not exist among the malic and shikimic acids, whereas for low pHs, the elution time of the citric acid is too high. In consequence, a pH of 2.7 was selected. The effect of the ionic strength of the eluent on the retention time of carboxylic acids seems to be slight, but it can be observed, that when the ionic strength decreases (below 10^{-3}M), the width of the peaks increases and the chromatograms lose resolution. In consequence, an ionic strength of 10^{-2}M was fixed. Taking into account the low lipophility of carboxylic acids, the acetonitrile organic modifier percentages were varied between 0.25% and 0.75%. In fact, an improvement in the performance in presence of the organic modifier is not observed. On the contrary, starting from 0.5% of organic modifier, the shikimic and malic acids are partly overlapped. The flow rate, selected on the basis of the resolution, time of analysis and pressure drop across column, was 2 μL/min. Figure 1 shows the chromatogram obtained under these optimised conditions.

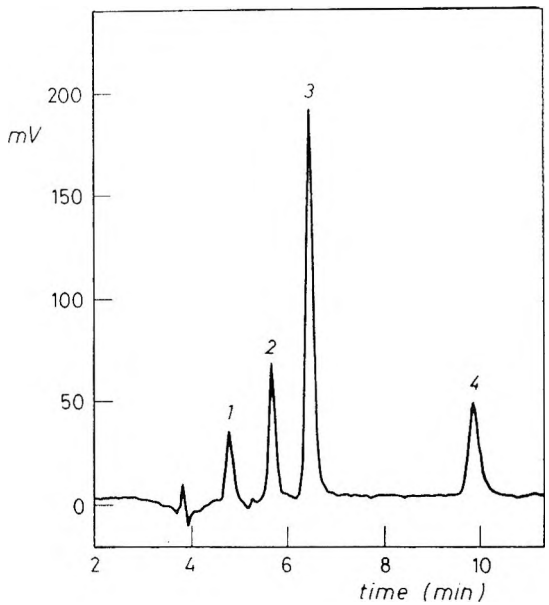

Figure 1. Chromatogram of a standard organic acid mixture. Column: Spherisorb ODS-2, 300x0.32 mm i.d., 5 μm. Mobile phase: K_2HPO_4/H_3PO_4 0.01 M; pH 2.7; flow rate 2 μL/min. 1= quinic acid; 2= malic acid; 3= shikimic acid; 4= citric acid.

The quantification of the organic acids was achieved by using the external standard method. Calibration plots were generated by repeated injections of 60 nL of standard solutions of organic acids of different concentrations, and the resulting plots were stored in the data module. A good correlation of the standards and corresponding peak areas ($r=0.999$) over the range between the detection limits and 120 ng for quinic and citric acids, 600 ng for malic acid and 6 ng for shikimic acid, were established. A volume of 60 nL of the apple juice was then injected and the amount of organic acids was obtained directly from the data module. The data module calibration was checked regularly with standard solutions.

Recovery studies were performed for an apple juice. Each organic acid was spiked at three different concentrations in triplicate on different days. Typical recoveries ranging from 91-105% for organic acids at all spiking levels were obtained. The coefficients of variation were generally less than 5%. These results indicate that the method has an adequate degree of accuracy for the analysis of these solutes. The limits of detection for the described method were 2.4, 2.1, 0.04 and 2.9 ng per injection (signal-to-noise ratio = 3) for quinic, malic, shikimic and citric acids, respectively.

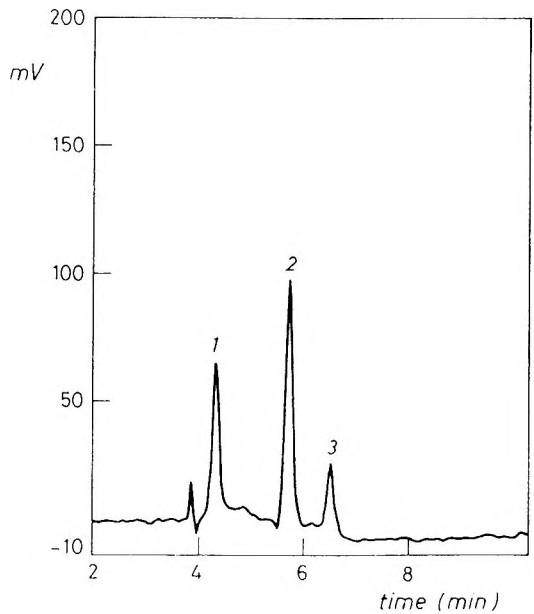

Figure 2. Typical chromatogram of organic acids in apple juice. Column and Chromatographic conditions as in Figure 1. 1= fructose; 2= malic acid; 3= shikimic acid.

A typical chromatogram for an apple juice obtained according to the technologies described above and using the operating conditions specified, is shown in Figure 2. As can be seen, only malic and shikimic acids are present in these apple juices. We could not determine the presence of citric acid as it was found in small concentrations and was only detected when malic acid occurred at greater concentrations, thereby confirming that changes in both acids follow a similar pattern.[6]

As has been reported,[8] the quinic acid content decreases during the ripening of the fruit. This decrease may be the result of the synthesis of chlorogenic acid. As these juices were obtained from ripe apples, their concentrations were below the detection limits of the method.

Table 1 shows the changes in malic and shikimic acids when the apple juice was clarified by membrane technology. As can be seen, the contents of organic acids in the apple juices processed decrease with the technological treatment employed in their stabilisation. This fact has also been reported in the clarification of other products such as wine and grape juice by microfiltration and ultrafiltration.[9-11]

Table 1

Variation of Organic Acid Contents (mg/L) in Apple Juice According to the Type of Membrane Used for its Stabilization.

Acid	Amount in Raw Apple Juice	Amount in MC Apple Juice	Amount in UC (30 °C) Apple Juice	Amount in UC (50 °C) Apple Juice
Malic	7108.6	4421.8		
Shikimic	14.7	5.7		
Malic	5446.3		3318.9	4155.3
Shikimic	23.2		13.0	17.4

MC, microfiltrate; UC, ultrafiltrate

The temperature effect could have a very important influence on the recovery level of the organic acids during membrane treatment. The higher recovery of malic and shikimic acids was monitored in UF at higher temperatures, wich could be explained on the basis of a greater solubilizing effect.

CONCLUSIONS

Reverse phase HPLC with microcolumns provides a rapid, simple and economic alternative for the separation and determination of organic acids. The proposed method is particularly suitable for determining quinic, malic, shikimic and citric acids in apple juice, but it can also be applied to other sample types.

ACKNOWLEDGEMENTS

This work was supported financially by the Comisión Interministerial de Ciencia y Tecnología (Project ALI92-1027-C03)

REFERENCES

1. D. Blanco Gomis, "HPLC Analysis of Organic Acids", in **Food Analysis by HPLC**, L.M.L. Nollet, ed., Marcel Dekker, Inc., New York, 1992, pp. 371-385.

2. M. V. Novotny, D. Ishii, **Microcolumn Separations**, Elsevier, 1985.

3. J. W. Jorgenson, J. de Wit, "Detection in Microcolumn Liquid Chromatography," in **Detectors for Capillary Chromatography**, H.H. Hill, D.G. McMinn, eds., John Wiley & Sons, New York, 1992. pp. 395-421.

4. J. P. Chervet, R. E. J. van Soest, J. P. Salzmann, LC-GC, **5,** 33-38 (1992).

5. D. Blanco, M. J. Morán, M. D. Gutiérrez, A. Sanz-Medel, Chromatographia, **24**, 347-350 (1987).

6. D. Blanco, M. J. Morán, M. D. Gutiérrez, J. J. Mangas, Chromatographia, **25**, 1054-1058 (1988).

7. D. Blanco, M. J. Morán, M. D. Gutiérrez, J. J. Mangas, J. Liq. Chromatogr., **14**, 2707-2719 (1991).

8. D. Blanco, M. J. Morán, M. D. Gutiérrez, J. Moreno, E Dapena, J. J. Mangas, Z. Lebensm. Unters Forsch., **194**, 33-37 (1992).

9. M. Serrano, S. B. Sachs, P. Ribereau-Gayon, Conn. Vigne Vin, **18**, 135-154 (1984).

10. C. Peri, Bull. O.I.V., **60**, 789-800 (1987).

11. C. A. Sims, R. P. Bates, R. P. Johnson, Am. J. Enol. Vitic., **39**, 11-17 (1988).

Received March 1, 1996
Accepted March 19, 1996
Manuscript 4102

J. LIQ. CHROM. & REL. TECHNOL., 19(16), 2623–2633 (1996)

ZOPICLONE AND ZOLPIDEM QUANTIFICATION IN HUMAN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH PHOTO-DIODE-ARRAY DETECTION

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ABSTRACT

A rapid and selective high performance liquid chromatography method for the simultaneous quantification of zopiclone and zolpidem has been developed. Signals are monitored by a photodiode-array detector with a main 305 nm wavelength and a bandwidth of 10 nm. After one step liquid phase extraction, samples of 100 µL are injected into a 5 µm ODS-2 column (30 cm x 4.6 mm I.D.). Drugs are eluted with a mobile phase containing potassium dihydrogen phosphate buffer, 0.01 M, methanol and tetrahydrofuran (30: 65: 5, V/V/V). Retention times of zopiclone and zolpidem are, respectively, 4.5 min and 5.5 min. The procedure has the necessary sensitivity and precision for pharmacokinetic studies (within day coefficients of variation < 7.1% and between-day coefficients of variation < 12.1 %). The linearity and the rapidity of this method are especially attractive for toxicological quantification in emergency toxicology.

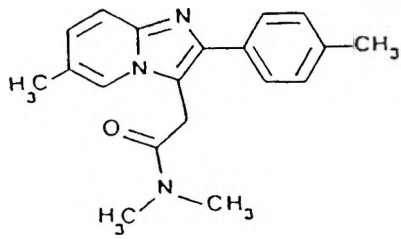

Figure 1. Structure of zolpidem.

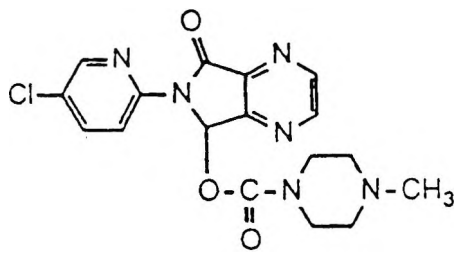

Figure 2. Structure of zopliclone.

INTRODUCTION

Zopiclone and zolpidem are two hypnotics which are widely prescribed, given their rapid onset of action and short elimination half-life. Zolpidem (Fig. 1) is an imidazopyridine agonist of the $GABA_A$ ω1 receptors,[1] whereas zopiclone (Fig. 2) is a cyclopyrolone which presents equal affinity with ω1, ω2 and ω3 receptors.[2]

As benzodiazepines, both compounds present anticonvulsant, myorelaxant, anxiolytic and sedative properties[3,4] but they may produce less dependence than benzodiazepines.[1,2] Nowadays, they are often involved in voluntary intoxications[5,6,7] and experience reveals that they could induce serious side effects.[8,9] Several analytical methods have been proposed for the determination of zopiclone or zolpidem: liquid chromatography (LC) methods using ultraviolet (UV) detection,[7,10,11] fluorimetric detection,[12-17] or diode array detection (DAD),[18] and gas chromatography (GC) methods equipped with nitrogen-phosphorus detection,[19-21] or electron capture detection for zopiclone.[22]

This paper presents a method for the rapid detection and quantification of both zopiclone and zolpidem, suitable for toxicological or pharmacokinetic studies.

MATERIALS

Apparatus

The liquid chromatographic system consisted of a constant flow-rate pump (model 110 A, Beckman Instruments, Germany), a sample injector (Rheodyne, Berkeley, CA, USA), a 5 µm particle-size C_{18} Spherisorb ODS-2 column, 30 cm x 4.6 mm I.D., (Interchrom, Montluçon, France) connected to a multiwavelength photodiode-array detector (DAD) (Waters Chromatography, Milford, USA) with a main sample wavelength of 305 nm and a bandwidth of 10 nm. Zopiclone and zolpidem spectra were previously stored in the library. The identification of each product was based on the comparison of its retention time and unknown spectrum to the different spectra stored in the library. The software calculated a fit value (degree of similarity), a degree of purity ranging from 0 to 1000, and finally identified the compound being as one of those stored in the library. The time window and the fit threshold were set at ±0.02 min and 900/1000, respectively.

Reagents

The various compounds used were kindly provided by manufacturers: zopiclone base and zolpidem hemitartrate from Rhône Poulenc Rorer (Vitry sur Seine, France). Ethanol RP Normapur, dichloromethane RP Normapur, ethyl acetate RP Normapur, orthophosphoric acid RP Normapur and tetrahydrofuran were purchased from Prolabo (Paris, France), and hexane Pestipur grade from SDS (Valdonne Peypin, France). They were of high grade of purity. Methanol, triethylamine and acetonitrile were HPLC grade, potassium dihydrogenphosphate was analytical grade (Merck, Darmstadt, Germany). Solutions at 100 mg/L of zopiclone and zolpidem were prepared monthly in acetonitrile and stored at -20 °C. Appropriate ethanolic dilutions were made each day to obtain solutions at 1 mg/L.

METHOD

Procedure

The mobile phase was a mixture of potassium dihydrogen phosphate buffer, 0.01M, methanol and tetrahydrofuran (30:65:5, V/V/V). At first, the buffer was adjusted to pH 2.6 with orthophosphoric acid, then 0.1% of triethylamine was added to obtain symmetric peaks.

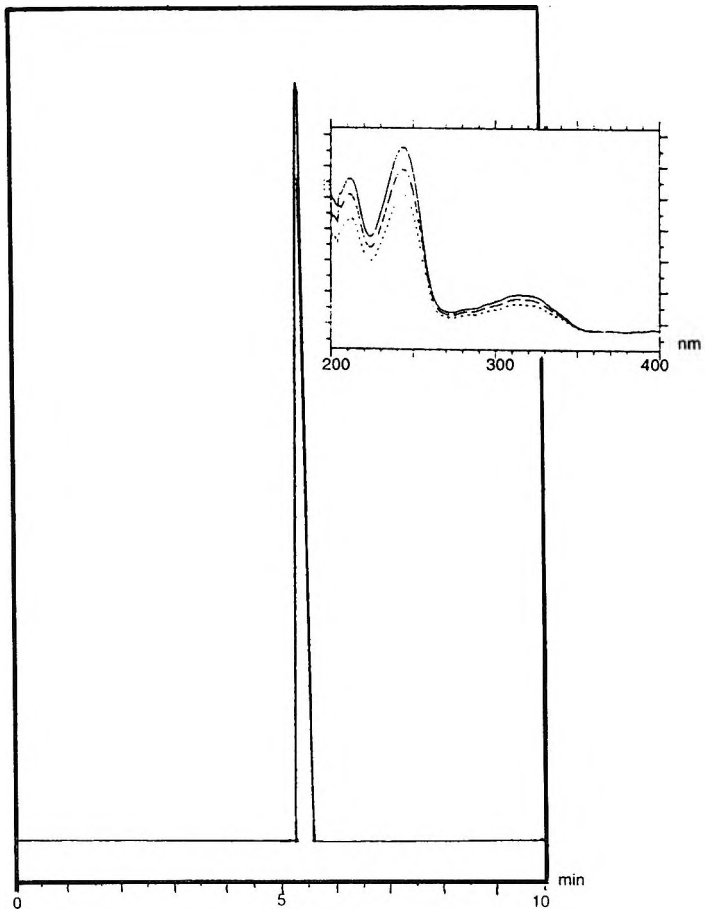

Figure 3. Chromatogram and UV spectrum of a sample containing 100 ng/mL of zolpidem.

The mobile phase was filtered through a 0.45 μm Millipore filter (Millipore, Bedford, MA, USA) and degassed in an ultrasonic cuve (Prolabo, Paris, France). The chromatographic separation was performed at ambient temperature, flow rate 0.8 mL/min.

Two mL of plasma, 2 mL of carbonate buffer, pH 9.2, and 4 mL of hexane/dichloromethane (4 : 3, V/V) were mixed on a horizontal mechanical agitator in a 15 mL centrifuge tube. After a period of centrifugation (10 min, 3000 rpm), the upper organic phase was transferred into a Reacti-Vial and evaporated to dryness under a stream of air.

Table 1

Within-Day Precision of Zopliclone and Zolpidem (n=10)

Theoretical Concentration ng/mL	Zolpidem Mean (± SD) ng/mL	CV (%)	Zopiclone Mean (± SD) ng/mL	CV (%)
50	53.77 ± 2.13	3.9	49.37 ± 3.21	6.5
100	103.39 ± 3.8	3.6	98.19 ± 3.81	3.9
200	198.99 ± 3.70	1.9	192.75 ± 13.70	7.1

Linear regression line of zolpidem: y = 0.968x + 5.537 (r = 0.998)
Linear regression line of zopliclone: y = 0.945x + 3.526 (r = 0.994)

Table 2

Between-Day Precision of Zopiclone and Zolpidem (n=10)

Theoretical Concentration ng/mL	Zolpidem Mean (± SD) ng/mL	CV (%)	Zopiclone Mean (± SD) ng/mL	CV (%)
50	47.43 ± 5.76	12.1	49.39 ± 5.47	11.0
100	99.57 ± 7.77	8.8	97.99 ± 4.71	4.8
200	195.16 ± 15.24	7.8	193.06 ± 19.03	9.8

Linear regression line of zolpidem: y = 0.981x - 0.393 (r = 0.986)
Linear regression line of zopliclone: y = 0.932x + 1.337 (r = 0.994)

The calibration was obtained by programming, daily, the diode-array detector after the injection of a sample containing 100 ng/mL of zopiclone and 100 ng/mL of zolpidem. Then, the concentration of each sample was automatically calculated by the system.

RESULTS

LC chromatograms and UV spectra obtained from the analysis of blank plasmas revealed no interference with analyses. LC chromatograms and UV spectra of a sample containing 100 ng/mL of zolpidem and a sample contai

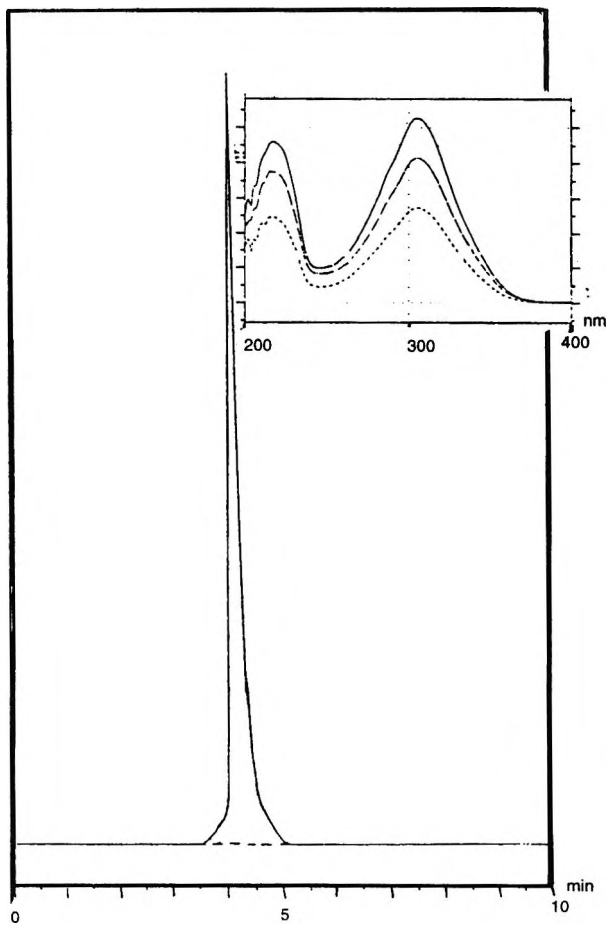

Figure 4. Chromatogram and UV spectrum of a sample containing 444 ng/mL of zopiclone.ning 444 ng/L of zopiclone are displayed in Figs. 3 and 4.

The retention times (RT) of zopiclone and zolpidem are, respectively, 4.5 min and 5.5 min. The intra-assay precision of the method was investigated by analysing 10 replicate samples of plasma containing 50, 100 and 200 ng/mL of zopiclone and zolpidem. Results are given in Table 1.

Between-day precision was calculated by the analysis of 10 replicate samples of zopiclone and zolpidem on different days. Results are given in Table 2.

Table 3

Accuracy of the Method

Theoretical Concentration ng/mL	Zolpidem Mean (± SD) ng/mL	Zolpidem CV (%)	Zopiclone Mean (± SD) ng/mL	Zopiclone CV (%)
50	54.10 ± 2.29	4.2	43.81 ± 3.53	4.2
100	103.87 ± 3.21	3.1	96.24 ± 5.58	5.8
200	204.66 ± 7.84	3.8	208.32 ± 10.1	4.8

Table 4

Overall Recovery of Zolpidem and Zopiclone Added to Blank Plasma

	Zolpidem	Zopiclone
n	10	10
Mean	88.92%	85.90%
CV	8.9%	8.9%
Sd	7.98	7.64

The accuracy was assessed by analysing five replicate samples of three concentrations of zopiclone and zolpidem on different days. Results are given in Table 3. The recovery (Table 4) was evaluated by comparing concentrations after the injection of series of samples of both compounds with, and without, extraction.

This method was linear over the ranges 20-1300 ng/mL for zolpidem and 20-1500 ng/mL for zopiclone. The limits of linearity were evaluated graphically after spiking increasing concentrations of zopiclone and zolpidem.

The instrumental limits of detection were determined by spiking extracts with decreasing concentrations of zopiclone and zolpidem until a response equivalent to three times the background was obtained. The limit of detection is 20 ng/mL for both compounds.

The examination of the blank plasma chromatogram revealed no interference with biological blood constituents. Table 5 gives retention times of psychoactive drugs which could be associated during treatment or intoxications.

Table 5

Interferences: Retention Times (min) of Several Tested Compounds

DCI	RT (min)
Acepromazine	>20
Alprazolam	>20
Amisulpiride	>20
Amitriptyline	>20
Bromazepam	5.67
Clobazam	6.33
Clotiazepam	9.31
Cyamemazine	>20
Desipramine	>20
Diazepam	10.34
Flunitrazepam	6.42
Haloperidol	>20
Imipramine	>20
Levomepromazine	>20
Lorazepam	5.73
Lormetazepam	8.41
Midazolam	10.63
Niaprazine	5.39
Nitrazepam	6.58
Nortriptyline	3.47
Prazepam	17.30

DISCUSSION

Isocratic elution was chosen for the described experiment since it provided a good separation of both compounds and required a short analysis time (RT < 6 min). We optimized the mobile phase proposed by Tracqui[18] by the addition of a small amount of triethylamine (0.1%), so we obtained shorter retention times and a symmetrical peak of zopiclone allowing accurate peak area and drug concentration calculations. As Boniface,[10] we have checked that the addition of higher percentages of triethylamine did not improve the zopiclone peak shape.

Advantages of the proposed method consisted in the simplicity of the procedure (one step liquid extraction), the rapidity of the analysis (RT <6 min), and the high specificity, which afforded a great safety for the identification of each compound: retention time and UV spectra were both taken into account for the identification.

The precision of the method was, at its best, around the median values and we observed that coefficients of variation often slightly increased for very low and high concentrations.

Moreover, the limit of detection (20 ng/mL) of both compounds was sufficient for pharmacokinetic studies, since the ranges of plasma concentrations of zopiclone and zolpidem are, respectively, 20-80 ng/mL[23] and 50-220 ng/mL[24] after orally therapeutic doses. The linearities of the method (20-1300 ng/mL for zolpidem and 20-1500 ng/mL for zopiclone) were consistent with the quantification of each compound after an acute poisoning episode.

Kennel[20] compared several organic solvents and different pH's for zopiclone extraction and obtained low and inconsistent recoveries except with chloroform at pH 6.6 (recovery: 77%). A reduction of the hexane proportion and the addition of dichloromethane at pH 9.2 were shown to be preferable in terms of recovery and reproductibility. Hexane/dichloromethane (4:3, V/V) was chosen as the extracting solvent, since it was little prone to emulsion formation and allowed good recovery (> 85%).

CONCLUSION

The method we suggest presents three major advantages: satisfactory precision, rapidity of the analysis and safety for the simultaneous identification of two common hypnotics. It is particularly suitable for toxicological emergency quantification or pharmacokinetic studies.

REFERENCES

1. P. Attali, J. Benavides, G. Perrault, La Lettre du Pharmacologue, **6**, 69-74 (1992).

2. J. M. Stutzman, C. Delahaye, H. Allain, Therapie, **48**, 33-42 (1993).

3. H. Allain, Ch. Delahaye, La Lettre du Pharmacologue, **3**, 35-68 (1992).

4. G. Perrault, E. Morel, D. J. Sanger, B. Zivkovic, Europ. J. Pharmacol., **187**, 487-494 (1990).

5. Z. Ahmad, M. Herpath, P. Eben, B. M. J., **302**, 292-294 (1991).

6. Y. Regouby, G. Delomez, A. Tisserant, Therapie, **44**, 379-380 (1989).

7. G. Debailleul, F. Abi Khalil, J. Anal. Toxicol., **15**, 35-37 (1991).

8. L. M. Iruela, V. Ibanez-Rojo, E. Baca, Lancet, **342**, 1495-1496 (1993).

9. L. M. Iruela, V. Ibanez-Rojo, E. Baca, Lancet, **342**, 443-444 (1993).

10. P. J. Boniface, I. C. Martin, S. L. Nolan, S. To Tan, J. Chromatogr., **584**, 199-206 (1992).

11. M. J. Royer-Morrot, M. Rambourg, I. Jacob, J. Chromatogr., **581**, 297-299 (1992).

12. L. G. Miller, B. W. Leduc, D. J. Greenblatt, J. Chromatogr., **380**, 211-215 (1986).

13. A. Le Liboux, A. Frydman, J. Gaillot, J. Chromatogr., **417**, 151-158 (1987).

14. A. Asberg, A. Rye Alertsen, O. E. Skaug, Nor. Pharm. Acta, **48**, 41-44 (1986).

15. C. Stanley, P. Mitchell, C. M. Kaye, Analyst, **110**, 83-84 (1985).

16. P. Guinebault, C. Dubruc, P. Hermann, J. P. Thenot, J. Chromatogr., **383**, 206-211 (1986).

17. V. Ascalone, L. Flaminio, P. Guinebault, J. P. Thenot, L. Morselli, J. Chromatogr., **581**, 237-246 (1992).

18. A. Tracqui, P. Kintz, P. Mangin, J. Chromatogr., **616**, 95-103 (1993).

19. D. Debruyne, J. Lacotte, B. Hurault De Ligny, M. Moulin, J. Pharm. Sci., **80**, 71-74 (1991).

20. S. Kennel, P. Kintz, A. Tracqui, P. Mangin, A. A. Lugnier, J. Chromatogr., **527**, 169-173 (1990).

21. Y. Gaillard, J. P. Gay-Monchamp, M. Ollagnier, J. Chromatogr., **619**, 310-314 (1993).

22. Y. Gaillard, J. P. Gay-Monchamp, M. Ollagnier, J. Chromatogr., **622**, 197-208 (1993).

23. Imovane (Zopiclone), Technical documentation, Synthelabo Labs., Paris 1988.

24. Stilnox (Zolpidem), Technical documentation, Synthelabo Labs., Paris 1988.

Received October 3, 1995
Accepted March 23, 1996
Manuscript 3973

J. LIQ. CHROM. & REL. TECHNOL., 19(16), 2635–2648 (1996)

OPTICAL RESOLUTION OF RACEMIC α-AMINO ACIDS ON A DYNAMIC CHIRAL STATIONARY PHASE DERIVED FROM (S)-LEUCINOL BY LIGAND EXCHANGE CHROMATOGRAPHY

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ABSTRACT

A dynamic chiral stationary phase (CSP **7**) for resolving racemic α-amino acids have been prepared by hydrophobically loading (S)-N,N-carboxymethyl dodecyl leucinol monosodium salt onto a commercial reverse phase octadecyl silica gel column. CSP **7** was successfully employed in resolving various racemic α-amino acids. The chromatographic results for resolving various racemic α-amino acids on CSP **7** have been found to be generally better as expected from the chiral recognition model proposed than those on the previously reported dynamic CSP (**2**), (R)-N,N-carboxymethyl dodecyl alaninol monosodium salt which is hydrophobically bound onto a commercial reverse phase octadecyl silica gel column. Especially, CSP **7** seems to be more attractive than CSP **2** in that CSP **7** shows reasonably good resolving ability for the broad range of racemic α-amino acids at the high content of organic modifier (20 % CH_3CN) in the aqueous mobile phase while CSP **2** shows very poor resolving ability.

INTRODUCTION

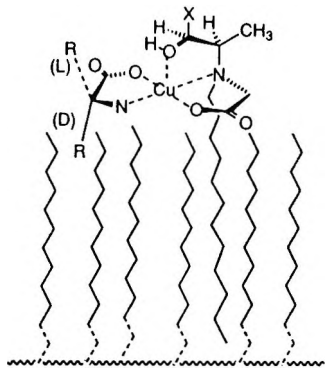
Chiral ligand exchange chromatography has proven to be a very useful means for separating enantiomers of racemic α-amino acids as shown by Davankov and other workers.[1-5] For example, Cu (II) complexes of optically active α-amino acids and their derivatives have been successfully employed in resolving various racemic α-amino acids as chiral mobile phase additives[6,7] or chiral stationary phases (CSPs) after binding covalently[8,9] or hydrophobically[10-12] to solid column support.

In this area, our efforts have been focused on the use of Cu (II) complexes of optically active aminoalcohol derivatives hydrophobically bound to octadecyl-silica gel as dynamic CSPs in resolving various racemic α-amino acids. For example, two dynamic CSPs (CSP **1** and **2**) based on Cu (II) complexes of (1S,2R)-norephedrine derivative and (R)-alaninol derivative hydrophobically adsorbed on octadecyl-silica gel have been developed and used in resolving various racemic α-amino acids.[13-15]

Based on the chromatographic resolution trends of showing higher enantioselectivity on CSP **2** than on CSP **1** for the two enantiomers of α-amino acids having a simple hydrophobic α-alkyl substituent and the chiral recognition model concerning the formation of the energetically different two diastereomeric ternary complexes shown in Figure 1, we have concluded that the phenyl functionality at the first chiral center of CSP **1** is not essential in the chiral recognition and simply disturbs the axial coordination by the hydroxy group of the fixed ligand (chiral selector) in the square planar coordination sphere of the ternary complex.[15]

In the chiral recognition model shown in Figure 1, the α-alkyl substituent of the (D)-enantiomers is intercalated between the octadecyl chains of silica gel while that of the (L)-enantiomers is directed into the bulk of mobile phase and, in consequence, the (D)-enantiomers of α-amino acids having a simple α-alkyl substituent are retained longer than the (L)-enantiomers because of the greater lipophilic interaction between the α-alkyl substituent of the (D)-enantiomers and the octadecyl chains of silica gel. However, the retention mode of the (L)-enantiomers is not precisely explained in that model.

In this study, we propose a retention mode of the (L)-enantiomers, which also utilizes the lipophilic interaction between the α-alkyl substituent of the (L)-enantiomers and the octadecyl chains of silica gel. Based on the chiral recognition modes proposed for the retention of (D)- and (L)-enantiomers, we rationalize that a dynamic CSP derived from (S)-leucinol derivative may show greater enantioselectivity for the two enantiomers of racemic α-amino acids than CSP **1** or **2**.



CSP **1**, X = Phenyl
CSP **2**, X = H

Figure 1. The proposed structure of the diastereomeric ternary complex formed (a) from the fixed ligand of CSP **1**, (D)- or (L)-amino acid and Cu(II) or (b) from the fixed ligand of CSP **2**, (D)- or (L)-amino acid and Cu(II).

EXPERIMENTAL

Instrumentation

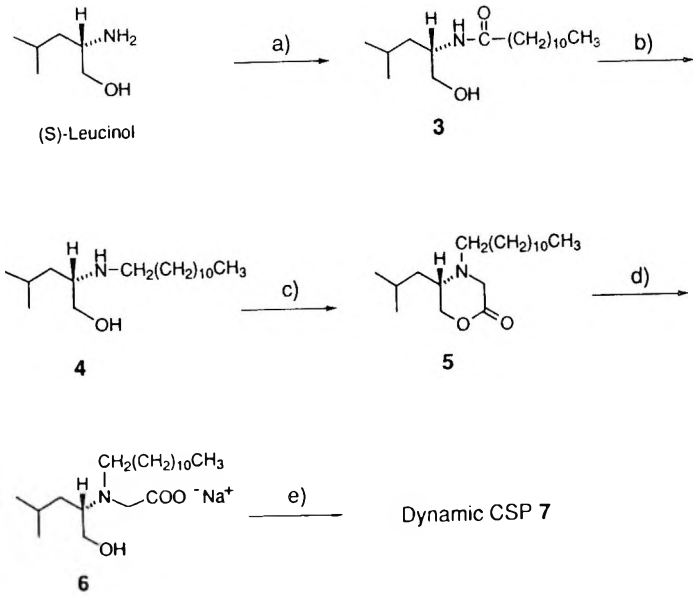
Melting point determination was performed by using a Rigaku Thermal Analyzer TAS 100. ^{1}H NMR spectra were recorded on a Varian Gemini 300 or a Varian EM-360A spectrometer using tetramethylsilane as an internal standard. IR spectra were recorded on a Mattson Galaxy 2000 FT-IR spectrometer. Mass data (EI) were obtained on a VG Trio 2000 GC/MS system.

Chromatographic resolution data were collected on an HPLC system consisting of Waters Model 510 pump, Waters Model U6k Universal Chromatographic Injector, Waters Model 441 Absorbance Detector with 254 nm UV filter and Waters Model 740 Data Module Recorder.

Preparation of Dynamic CSP 7 from (S)-Leucinol

Dynamic CSP 7 was prepared by the procedure shown in Scheme 1. The detailed synthetic procedures are as follows.

(S)-N-Lauroylleucinol 3: To a stirred solution of (S)-leucinol (3.0 g, 25.1 mmole) and triethylamine (5 mL, 36 mmole) in 50 mL of dry methylene chloride was added a solution of lauroyl chloride (3.8 mL, 25.1 mmole) in 10 mL of dry methylene chloride under nitrogen at room temperature.



Scheme 1. (a) lauroyl chloride, triethylamine, methylene chloride, room temperature, 40 min., 98 %. (b) $LiAlH_4$, THF, 48 hr., 97 %. (c) ethylbromoacetate, triethylamine, methylene chloride, room temperature, 48 hr., 42.5 %. (d) 1 N aq. NaOH, MeOH, room temperature, 6 hr. 99 %. (e) Hydrophobic loading onto a commercial reverse phase C_{18} column.

The reaction mixture was stirred at room temperature under nitrogen for 30 min and then washed successively with 0.5 N HCl, 0.5 N NaOH, and water. The organic solution was dried over anhydrous $MgSO_4$ and filtered, and the solvent was removed under reduced pressure. The residue was crystallized from the mixed solvent of methylene chloride and hexane at 0 °C to afford **3** as a white crystalline solid (7.35g, 98.0%). m.p. : 42.0-43.5 °C, ^{1}H NMR ($CDCl_3$) δ 0.87(t, 3H), 0.93(dd, 6H), 1.20-1.38(m, 18H), 1.58-1.67(m,3H), 2.19(t, 2H), 2.58(broad s, 1H), 3.52(dd, 1H), 3.67(dd,1H), 4.02-4.06(m, 1H), 5.60(broad s, 1H),. IR(KBr) cm^{-1} 3304, 2955, 2918, 2851, 1642, 1547.

(S)-N-Dodecyl leucinol 4: A solution of $LiAlH_4$ (2.28 g, 60 mmole) in 50 mL of dry tetrahydrofuran was added to a stirred solution of **3** (5 g, 16.7 mmole) in 15 mL of dry tetrahydrofuran through dropping funnel over 30 min at 0 °C. The whole mixture was refluxed for 2 days. The reaction mixture was cooled to 0 °C and then quenched by adding water. The whole mixture was passed through the bed of celite and then tetrahydrofuran was removed under reduced

pressure. The aqueous solution was extracted with methylene chloride. The methylene chloride solution was dried over anhydrous $MgSO_4$, and filtered and then methylene chloride was removed under reduced pressure. The residue was crystallized from the mixed solvent of methylene chloride and hexane to afford **4** as a white crystalline solid (4.65 g, 97.0 %). m.p. : 70.5-72.0 °C, ^{1}H NMR ($CDCl_3$) δ 0.87(t, 3H), 0.90(d, 6H), 1.19-1.35(m, 20H), 1.40-1.47(m, 2H), 1.57-1.66(m,1H), 2.03(broad s, 2H), 2.49-2.69(m, 3H), 3.21(dd, 1H), 3.60(dd, 1H) IR(KBr) cm^{-1} 3297, 3104, 2959, 2920, 2851, 1469.

(S)-4-Dodecyl-5-isobutyl-2,3,5,6-tetrahydro-4H-1,4-oxazin-2-one 5: To a stirred solution of **4** (4.50 g, 15.8 mmole) in 30 mL of dry benzene was added a solution of ethyl bromoacetate (1.94 mL, 17.3 mmole) in 10 mL of dry benzene. The reaction mixture was refluxed for 36 hr and then cooled to room temperature and concentrated. The white oily residue was purified by column chromatography on silica gel (ethylacetate:hexane:dichloromethane = 1:10:1, v/v/v) to give **5** as a colorless oil (2.18 g, 42.5 %). ^{1}H NMR ($CDCl_3$) 0.86(t, 3H), 0.92(dd, 6H), 1.21-1.32(m, 20H), 1.36-1.44(m, 2H), 1.57-1.66(m, 1H), 2.38-2.57(m, 2H), 3.33(d, 1H), 3.48(d, 1H), 4.12(dd, 1H), 4.39(dd, 1H). IR(KBr) cm^{-1} 2957, 2926, 2855, 1751, 1468. MS(EI) m/e : 325(M^+).

(S)-N,N-Carboxymethyl dodecyl leucinol monosodium salt 6 and hydrophobic loading onto a commercial reverse phase octadecyl-silica gel column (preparation of CSP 7): NaOH solution (1 M inH_2O, 6.20 mL) was added dropwise to a stirred solution of **5** (2.00 g, 6.15 mmole) in 30 mL CH_3OH at room temperature. After being stirred for 5 hr at room temperature, the solvent was evaporated under reduced pressure and the residue was dried under high vacuum for 10 hr to afford oily product (2.24 g, 99%). [IR(KBr) cm^{-1} 3296, 2955, 2926, 2855, 1678, 1595. MS(EI) m/e : 365(M^+)]. Hydrophobic loading of **6** onto a commercial reverse phase octadecyl-silica gel column (Waters μ-Bondapak™ C_{18}, 3.9 x 300mm) to afford dynamic CSP **7** was performed by eluting a solution of **6** (2.0 g) in 30 mL of methanol/water (1:2, v/v) through the column (flow rate : 0.5 mL/min) followed by washing with 150 mL of methanol/water (1:2, v/v, flow rate: 0.3 mL/min). The loaded amount of **6** was not able to be determined. However, the used amount of **6** (2.2 g) was assumed to be large enough to be fully loaded because the bleeding of the excess of **6** from the column was detected by the UV monitor.

Chromatography

Preparation of mobile phase: Mobile phase was prepared by dissolving specified amount of $CuSO_4$ in deionized water or deionized water containing acetonitrile or methanol as an organic modifier.

Chromatography: To resolve racemic α-amino acids on dynamic CSP **7**, a mobile phase was eluted through the column until the baseline (UV monitor, 254 nm) became stable to equilibrate the column and then, an aqueous solution containing a racemic α-amino acid was injected. Flow rate was 0.8 mL/min. Dynamic CSP **7** used in this study was found to be equally effective for the chiral separation of racemic α-amino acids for at least three months.

RESULTS AND DISCUSSION

The chiral recognition model shown in Figure 1 indicates that the lipophilic interaction between the α-alkyl substituent of the (D)-enantiomers and the octadecyl chains of silica gel plays an important role in retaining (D)-enantiomers. However, the chiral recognition mode retaining the (L)-enantiomers does not utilize the lipophilic interaction. Under the conditions of aqueous mobile phase, it seems to be unreasonable to leave out the possibility of the lipophilic interaction between the α-alkyl substituent of the (L)-enantiomers and the octadecyl chains of silica gel. Consequently, we tried to figure out from the study of chemical models any possible retention mode of the (L)-enantiomers, which utilizes the lipophilic interaction between the α-alkyl substituent of the (L)-enantiomers and the octadecyl chains of silica gel.

One possible retention mode of the (L)-enantiomers utilizing the lipophilic interaction between the α-alkyl substituent of the (L)-enantiomers and the octadecyl chains of silica gel with the trans (N,N)-configuration, which is known to be more stable than the cis (N,N)-configuration,[10] is observed from the molecular model study by reconstructing the ternary complex after switching the bonding positions of the N-carboxymethyl unit and the N-alkyl chain of the fixed ligand (chiral selector). (Note that the inversion of configuration at the nitrogen center is fast even at room temperature). Switching the bonding positions of the N-carboxymethyl unit and the N-alkyl chain of the fixed ligand reverses the direction of the N-carboxymethyl unit and, in consequence, the reconstructed ternary complex with the trans (N,N)-configuration allows the α-alkyl substituent of the (L)-enantiomer intercalate between the octadecyl chains of silica gel (the interested leaders are encouraged to construct the ternary complex using molecular models). The two chiral recognition modes for retaining (D)- and (L)-enantiomers selectively are compared in Figure 2. As shown in Figure 2, the two chiral recognition modes are quite similar except the orientation of the methyl group and the hydrogen at the chiral center of the fixed chiral selector of CSP **2**. The methyl group at the chiral center of the fixed chiral selector is positioned at the site of complex square planar ring in the retention mode of the (L)-enantiomer (Figure 2b) and, therefore, disturbs the complex formation. However, in the retention mode of the (D)-enantiomer (Figure 2a) the hydrogen at the chiral center of the fixed chiral selector is positioned at the site of complex square planar ring.

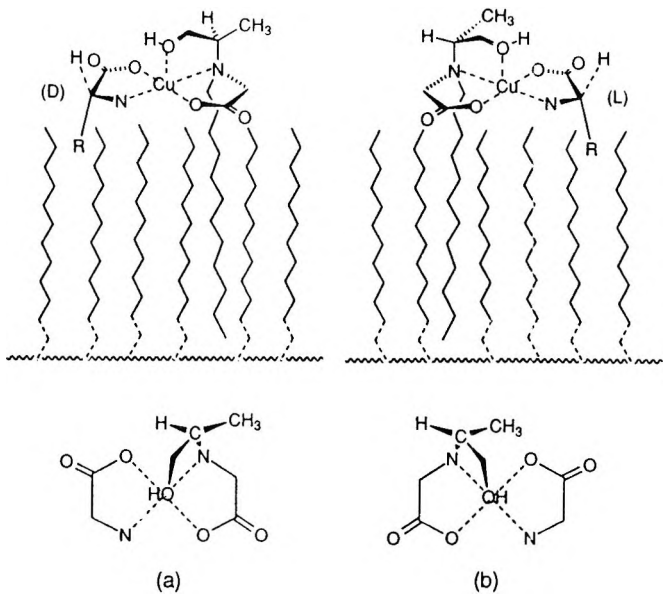


Figure 2. Top: the proposed structures of the ternary complex formed from the fixed ligand [(R)-chiral selector)] of CSP **2**, (a) (D)- and (b) (L)-amino acid and Cu(II). Bottom: schematic representation of the top ternary complexes viewed from above. The ternary complex (a) formed from the fixed ligand, (D)-amino acid and Cu(II) is more stable than the ternary complex (b).

Consequently, the complex shown in Figure 2a should be more stable than that shown in Figure 2b and the (D)-enantiomer should be retained longer. This is consistent with the observed elution orders on CSP **2** for the resolution of α-amino acids which have a simple a-alkyl substituent.[15]

From the chiral recognition model shown in Figure 2, the larger group than the methyl group at the chiral center of the fixed chiral selector, is expected to enhance the stability difference between the two diastereomeric complexes because the larger group may disturb more strongly, the formation of the less stable complex with the less retained enantiomer.

Based on this rationale, we planned to prepare new dynamic CSP **7** starting from (S)-leucinol. The large isobutyl group at the chiral center of CSP **7** instead of the methyl group at the chiral center of CSP **2** might improve the resolving ability of dynamic CSP **7** compared to that of CSP **2**.

Table 1

Resolution of α-amino Acids on (S)-leucinol Derivative 7 Adsorbed on an Octadecyl Silica Gel Column with the Variation of Organic Modifier Content in the Aqueous Mobile Phase at the Constant Cu(II) Concentration (2.5×10^{-4} M).[a]

	20% CH_3CN in Water		10% CH_3CN in Water		100% Water		10% MeOH in Water		20% MeOH in Water	
A[b]	k'[c]	α[d]	k'[c]	α[d]	k'[c]	α[d]	k'[c]	α[d]	k'[c]	α[d]
ala	3.94	1.26	5.29	1.33	8.77	1.72	7.15	1.55	6.60	1.45
	4.95		7.06		15.11		11.09		9.55	
val	4.81	3.30	8.08	3.50	14.69	6.27	10.57	5.06	9.32	4.22
	15.87		28.27		92.07		53.45		39.33	
leu	12.09	2.37	18.18	3.23			22.93	5.16	18.39	4.14
	28.67		58.86				118.43		76.22	
pro	6.73	1.54	9.61	1.92	18.22	3.62	14.30	2.47	13.48	1.99
	10.39		18.46		66.00		35.33		26.78	
met	8.95	1.96	13.60	2.50			19.68	3.31	15.05	2.93
	17.50		34.02				65.11		44.13	
phe	18.55	2.44	35.35	3.19					33.23	6.45
	45.27		112.84						214.25	
pgl	11.35	3.48	16.07	4.69			17.67	7.94	14.13	6.74
	39.47		75.38				140.22		95.20	
asp	10.81	1.27	11.99	1.54			18.30	1.79	16.39	1.59
	13.76		18.46				32.74		26.11	
his	12.67	1.68	14.41	1.71			14.95	1.54	11.55	1.73
	21.33		24.69				23.09		19.94	
gln			5.48	1.25	10.84	1.86	7.94	1.50	6.81	1.33
			6.85		10.19		11.89		9.07	
glu	21.96	1.51	23.93	2.01			35.16	2.47	32.31	2.16
	33.10		48.09				86.93		69.57	
ser	2.12	1.64	3.51	1.36	6.89	1.53	5.34	1.49	4.79	1.46
	3.47		4.76		10.56		7.94		6.99	
thr	2.33	1.53	3.92	1.35	8.26	1.73	7.73	1.46	5.05	1.54
	3.57		5.29		14.28		11.25		7.77	
arg					7.06	2.15	4.87	1.85	4.93	1.42
					15.20		9.02		7.01	

[a]: See text for the chromatographic conditions. For blanks, chromatography was not performed or data was not able to be collected because of the illegibility of chromatogam. In every case, (L)-enantiomer is retained longer than (D)-enantiomer except histidine. In the case of histidine, (D)-enantiomer is retained longer than the (L)-enantiomer. [b]: Full name of amino acids as following. ala:alanine, val:valine, leu:lecuine, pro:proline, met:methionine, phe:phenylalanine, pgl:phyenylglycine, asp:aspartic acid, his:histidine, gln:glutamine, glu:glutamic acid, ser:serine, thr:threonine, arg:arginine.HCl. [c]: Capacity factors for the first and second eluted enantiomer. [d]: Separation factor.

Chiral selector **6** was prepared starting from (S)-leucinol and then hydrophobically loaded onto a commercial octadecyl-silica gel column to afford dynamic CSP **7** as shown in Scheme 1. Dynamic CSP **7**, thus prepared, was very effective in resolving various racemic α-amino acids. Table 1 summarizes the resolution of various racemic α-amino acids on dynamic CSP 7 with varying organic modifier content in the aqueous mobile phase at constant Cu(II) concentration (2.5×10^{-4} M). For blanks in Table 1, data were not able to be collected because of the long retention times or the illegibility of chromatogram.

As shown in Table 1, most of the tested α-amino acids were resolved with reasonable or good separation factors. As expected from the proposed chiral recognition model shown in Figure 2, the separation factors on CSP **7** are found to be comparable to or better than those on dynamic CSP **2**.[15] Table 1 also shows that an increase in the content of the organic modifier in the aqueous mobile phase diminishes the retention of the two enantiomers as denoted by the capacity factors (k'). However, the retention of the more retained enantiomers is diminished more significantly than that of the less retained enantiomers. In consequence, the separation factors decrease as the content of the organic modifier in the aqueous mobile phase increases. The use of acetonitrile as an organic modifier decreases the retention of the more retained enantiomers more significantly than the use of methanol and, consequently, the separation factors decrease more rapidly when acetonitrile is used as an organic modifier. All of these are exactly consistent with those observed on dynamic CSP **2**.[15] These trends on dynamic CSP **2** were previously explained by the chiral recognition model shown in Figure 1.

According to the chiral recognition model shown in Figure 1, the lipophilic interaction between the α-alkyl substituent of the more retained enantiomer and the octadecyl chains of silica gel is expected to decrease as the polarity of the mobile phase decreases by increasing the content of organic modifier in the mobile phase. The reduction in the lipophilic interaction is justly more significant with less polar organic modifier (acetonitrile in this case), whereas the retention of the less retained enantiomers is not notably affected by the organic modifier in the aqueous mobile phase. In consequence, the separation factors decrease as the organic modifier content in the mobile phase increases and this is more significant with the use of less polar organic modifier.

However, the newly proposed chiral recognition model shown in Figure 2 utilizes the lipophilic interaction between both of (D)- and (L)-enantiomers and the octadecyl chains of silica gel. In consequence, the use of organic modifier in the aqueous mobile phase diminishes the retention of the less retained enantiomers as well as the retention of the more retained enantiomers. In this event, the more significant diminution in the retention of the more retained enantiomers than the less retained enantiomers by the use of organic modifier in

the aqueous mobile phase, may be rationalized on the basis that the ternary complex shown in Figure 2a is more stable and tighter than the ternary complex shown in Figure 2b.

In the previous study concerning the resolution of α-amino acids on CSP **2**, we already proposed that the reduction in the lipophilic interaction between the α-alkyl substituent of amino acids and the octadecyl chains of silica gel might be greater with the use of less polar organic modifier in the aqueous mobile and with tighter complexes.[15] Therefore, the retention of the more retained enantiomers is more significantly diminished than the retention of the less retained enantiomers, as the organic modifier content in the aqueous mobile phase increases and, in consequence, the separation factors decrease.

The same rationale can be applied for explaining the resolution trends of racemic α-amino acids on CSP **7** and the chiral recognition model, which utilizes the lipophilic interaction between the octadecyl chains of silica gel and both of (D)- and (L)-enantiomers, for resolving racemic α-amino acids on CSP **7** is shown in Figure 3.

As shown in Table 1, (L)-enantiomers are always retained longer on CSP **7** than the (D)-enantiomers, except histidine. In resolving histidine on CSP **7**, (D)-enantiomer is retained longer than the (L)-enantiomer. The elution orders on CSP **7** shown in Table 1, are opposite to those on CSP **2**.[15] However, the opposite elution orders on CSP **2** and **7** should be considered to be the same in the sense of chiral recognition because the absolute configuration of CSP **2** is opposite to that of CSP **7**. The exception in the elution order of the two enantiomers of histidine on CSP **7** might be explained by the exactly same rationale applied for the elution order on CSP **2**.[15]

It is quite interesting to note that dynamic CSP **7** shows reasonably good resolving ability for the two enantiomers of racemic α-amino acids even at the high content of organic modifier (20 % CH_3CN) in the aqueous mobile phase while dynamic CSP **2** shows very poor resolving ability under the same conditions of mobile phase.[15] Even though the resolutions on CSP **2** or **7** are very excellent with very polar aqueous mobile phase (for example 100 % water), the limitations in the use of CSP **2** or **7** as an analytical purpose with very polar aqueous mobile phase are the long retention times of the two enantiomers of relatively hydrophobic amino acids and consequently resolutions are limited to relatively less hydrophobic several amino acids.

To diminish the retention times of the two enantiomers (and consequently to extend the use of CSP **2** and **7** to the broad range of racemic α-amino acids), the polarity of the mobile phase should be decreased by increasing the content of organic modifier in the aqueous mobile phase.

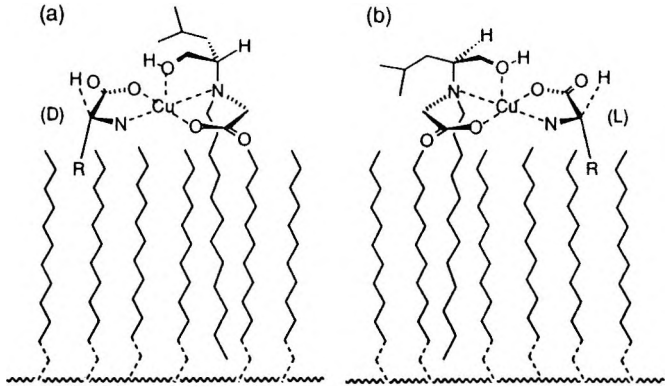


Figure 3. The proposed structures of the ternary complex formed from the fixed ligand [(S)-chiral selector)] of CSP **7**, (a) (D)- and (b) (L)-amino acid and Cu(II). The ternary complex (b) formed from the fixed ligand, (L)-amino acid and Cu(II) is more stable than the ternary complex (a).

In this context, CSP **7** is much more attractive than CSP **2** because CSP **7** shows reasonably good resolving ability for the broad range of racemic α-amino acids even at the high content of organic modifier (20 % CH_3CN) in the aqueous mobile phase, whereas CSP **2** shows very poor resolving ability.[15]

The discrepancy between the resolution behaviors of CSP **2** and CSP **7**, at high content of organic modifier (20 % CH_3CN) in the aqueous mobile phase, may be rationalized by considering that the ternary complex shown in Figure 3b, formed from the fixed ligand of CSP **7**, is less stable. Consequently, it is less compact than that shown in Figure 2a, formed from the fixed ligand of CSP **2**, because the large α-alkyl substituent (such as the isobutyl group in the fixed ligand of CSP **7**) somewhat disturbs the complex formation. As described above, the less compact complex might be less significantly influenced by the polarity of the aqueous mobile phase than the more compact complex.[15] Consequently, the retention time of the more retained enantiomer on CSP **7** decreases more slowly than the one on CSP **2** as the content of the organic modifier in the aqueous mobile phase increases and the resolving ability of CSP **7** is, to some extent, maintained; this is true even at the high content of organic modifier (20 % CH_3CN) in the aqueous mobile phase while that of CSP **2** is almost lost.[15]

The effect of the variation of the Cu(II) concentration in the mobile phase of constant composition [acetonitrile-water (20:80, v/v)] on the resolution trends for resolving racemic amino acids on CSP **7**, is summarized in Table 2.

Table 2

Resolution of α-amino Acids on (S)-leucinol Derivative 7 Adsorbed on an Octadecyl Silica Gel Column with the Variation of Cu(II) Concentration in Acetonitrile-Water (20:80 v/v)[a]

	5.0×10^{-4} M		2.5×10^{-4} M		2.0×10^{-4} M	
A[b]	k'[c]	α[d]	k'[c]	α[d]	k'[c]	α[d]
ala	2.96	1.24	3.94	1.26		
	3.67		4.95			
val	4.16	3.17	4.81	3.30	8.15	3.29
	13.17		15.87		26.82	
leu	8.66	2.45	12.09	2.37	21.15	2.34
	21.23		28.67		49.52	
pro	4.88	1.58	6.73	1.54	10.10	1.51
	7.68		10.39		15.22	
met	6.10	2.03	8.95	1.96	15.23	1.98
	12.36		17.50		30.75	
phe	14.38	2.53	18.66	2.44	35.98	2.47
	36.42		45.27		88.83	
pgl	7.96	3.60	11.35	3.48	19.48	3.38
	28.66		39.47		65.76	
asp	9.77	1.33	10.81	1.27	20.14	1.29
	13.00		13.76		25.99	
his	11.82	1.72	12.67	1.68	19.51	1.61
	20.31		21.33		31.44	
glu	19.80	1.53	21.96	1.51	37.63	1.44
	30.24		33.10		54.29	
ser			2.12	1.64	3.05	1.49
			3.47		4.54	
thr			2.33	1.53	3.81	1.27
			3.57		4.85	

As shown in Table 2, the retention of the two enantiomers decrease appreciably as the Cu(II) concentration increases. However, the enantioselectivity denoted by the separation factors, α, does not show any noticeable tendency. All of these observations are consistent with those on CSP **2**.[15] The appreciable diminution in the retention of the two enantiomers at the high concentration of Cu(II) in the mobile phase, may be explained by the fact, that the increase of the Cu(II) concentration in the mobile phase enhances the formation of the mobile binary complex from Cu(II) and α-amino acids, as described previously to explain the resolution trends on CSP **2**.[15]

In summary, in this study, we proposed an improved chiral recognition model for resolving racemic α-amino acids on CSP **2**, which utilizes the lipophilic interaction between the octadecyl chains of silica gel and both of (D)- and (L)-enantiomers. Based on the chiral recognition model proposed, we designed a new dynamic CSP (**7**) which is expected to show greater enantioselectivity for the two enantiomers of a-amino acids than CSP **2**. The designed CSP was prepared by tentatively loading (S)-N,N-carboxymethyl dodecylleucinol monosodium salt derived from (S)-leucinol onto a commercial reverse phase octadecyl silica gel column and, used in resolving various racemic α-amino acids. As expected, the resolving ability of CSP **7** for the two enantiomers of various racemic α-amino acids, was comparable to or greater than that of CSP **2**. Especially, CSP **7** seems to be more attractive as an analytical purpose than CSP **2** in that, CSP **7** shows reasonably good resolving ability for the broad range of racemic α-amino acids at the high content of organic modifier (20 % CH_3CN) in the aqueous mobile phase, while, CSP **2** shows very poor resolving ability.

ACKNOWLEDGEMENTS

This work was supported by the grants from the Basic Science Research Program, Ministry of Education, Korea (BSRI-95-3410) and from the Organic Chemistry Research Center sponsored by the Korea Science and Engineering Foundation.

REFERENCES

1. V. A. Davankov, in **Chiral Separations by HPLC : Applications to Pharmaceutical Compounds**, A. M. Krstulovic (Editor),Ellis Horwood, Chichester, 1989, Ch. 15, p. 446.

2. V. A. Davankov, J. D. Navratil, H. F. Walton, **Ligand Exchange Chromatography**, CRC Press, Boca Raton, Florica, 1988.

3. S. Lam, in **Chiral Liquid Chromatography**, W. J. Lough (Editor), Blackie, Glasgow and London, 1989, Ch. 5, p. 83.

4. V. A. Davankov, A. A. Kurganov, A. S. Bochkov, in **Advances in Chromatography**, J. C. Giddings (Editor), Vol. 22, Marcel Dekker, New York, 1983, Ch. 3, p. 71.

5. V. A. Davankov, in **Advances in Chromatography**, Vol. 18, J. C. Giddings (Editor), Marcel Dekker, New York, 1980, Ch. 4, p.189.

6. P. E. Hare, E. Gil-Av, Science, **204**, 1226-1228 (1979).

7. J. Wagner, E. Wolf, B. Heintzelmann, C. Gaget, J. Chromatogr., **392**, 211-244 (1987).

8. D. Charmot, R. Audebut, C. Quivoron, J. Liq. Chromatogr., **8**, 1753-1767 (1985).

9. G. Gubitz, J. Liq. Chromatogr., **9,** 519-535 (1986).

10. V. A. Davankov, A. S. Bochkov, A. A. Kurganov, P. Roumeliotis, K. K. Unger, Chromatographia, **13,** 677-685(1980).

11. V. A. Davankov, A. S. Bochkov, Yu. P. Belov, J. Chromatogr., **218**, 547-557 (1981).

12. H. Kiniwa, Y. Baba, T. Ishida, H. Katoh, J. Chromatogr., **461**, 397-405 (1989).

13. M. H. Hyun, N.-E. Lim, S.-N. Choi, Bull. Kor. Chem. Soc., **12,** 594-596 (1991).

14. M. H. Hyun, J.-J. Ryoo, N.-E. Lim, J. Liq. Chromatogr., **16**, 3249-3261 (1993) .

15. M. H. Hyun, D. H. Yang, H. J. Kim, J.-J. Ryoo, J. Chromatogr. A, **684**, 189-200 (1994).

Received February 1, 1996
Accepted February 23, 1996
Manuscript 4079

J. LIQ. CHROM. & REL. TECHNOL., 19(16), 2649–2657 (1996)

DETERMINATION OF SCOPARONE IN RAT PLASMA BY LIQUID CHROMATOGRAPHY AND ITS APPLICATION TO PHARMACOKINETICS

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ABSTRACT

A simple liquid chromatographic method was developed to study the pharmacokinetics of scoparone in the rat plasma. After addition of an internal standard (ferulic acid), plasma was deproteinized by acetonitrile for sample clean-up. The drugs were separated on a reverse phase column and detected by UV detection at a wavelength 340 nm. Acetonitrile-water (30:70, v/v, pH 2.5 adjusted by orthophosphoric acid) was used as a mobile phase. It was applied to the pharmacokinetic study of scoparone in rats after a dose of 5 mg kg^{-1} intravenous

administration. A biphasic phenomenon with a rapid distribution followed by a slower elimination phase was observed from the plasma concentration-time curve.

INTRODUCTION

Artemisia scoparia Waldst. et Kitaib. is used as a folk medicine in Taiwan for the treatment of hepatitis. Scoparone (Figure 1) was isolated from the leaf and stem of *Artemisia scoparia*.[1] It was recently reported that scoparone possesses vasodilator and hypotensive actions,[2-4] immune suppressive activities,[4-5] free radical scavenging properties,[6] anti-anginal effects on the heart[3] and antiatherogenic effect in hyperlipidaemic diabetic rabbits.[7] Recently, scoparone was also used as a research substrate for the differentiation of cytochrome P-450 activities.[8-10] Although, the action mechanism of cardiovascular effects of scoparone have been reported, the determination of scoparone from plasma and its pharmacokinetic properties have not been studied. In this work, we developed a liquid chromatographic (LC) method with UV detection to determine the concentration of scoparone in rat plasma and its related pharmacokinetic profile.

MATERIALS AND METHODS

Chemicals and Reagents

Scoparone (6,7-dimethoxycoumarin) and ferulic acid (internal standard)[11] were purchased from Aldrich (Milwaukee, WI, USA) and Sigma (St. Louis, MO, USA), respectively. Acetonitrile and orthophosphoric acid (85%) were obtained from E. Merck (Darmstadt, Germany). Triple deionized water (Millipore Corp., Bedford, MA, USA) was used for all preparations.

Apparatus and Chromatography

The LC system consisted of an autosampler (SIC model 23, Tokyo, Japan), a variable wavelength UV-VIS detector (Soma, Tokyo, Japan) and a chromatographic pump (BAS, PM-80, West Lafayette, IN, USA). Separation was achieved on a reverse phase Nucleosil $5C_{18}$ column (250 x 4 mm, particle size 5 μm, Macherey - Nagel, Duren, Germany). The mobile phase was acetonitrile - water (30 : 70, v/v; pH 2.5 adjusted by orthophosphoric acid), and the flow rate was 1.0 mL min^{-1}. Scoparone was monitored at a

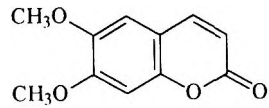

Figure 1. Chemical structure of scoparone.

wavelength of 340 nm throughout the experiments. The system was operated at room temperature (25 °C).

Animals

Male Sprague-Dawley rats (250-300 g) were obtained from the Laboratory Animal Center at the National Yang-Ming University. These animals were specifically pathogen free and kept in our own environmentally controlled quarters (temperature maintained at 24 ± 1 °C and with 12:12 light-dark cycle), for at least 1 week before use. Standard laboratory food and water was available continuously, except when food was withdrawn 18 hours prior to experimentation.

Blood Sampling

Rats were anesthetized with intraperitoneal pentobarbital 50 mg kg^{-1}. Only one-quarter (12.5 mg kg^{-1}, i.p.) of the dose of pentobarbital was administered during the experimental period when required. Blood samples (0.3 mL) were collected via cardiac puncture,[9,10] at interval of 5, 10, 15, 20, 30, 45, 60, 90 and 120 min after intravenous administration of scoparone (5 mg kg^{-1}). Data from six individual rats were used to construct pharmacokinetic profiles by plotting concentration of scoparone in plasma versus time. Sigmaplot for Windows (version 1.01), (Jandel Sci. Corte Madera, CA, USA) was used to plot standard curves and to perform least squares regression analysis on the calibration data.

Treatment of Plasma Samples

Plasma samples were prepared as previously described.[11-12] Each collected blood sample was transferred to a heparinized microcentrifuged at 8000 g for 3 min (Eppendorf Model 5402). The resulting plasma (0.1 mL) was then mixed with 0.2 mL of acetonitrile containing ferulic acid (1 μg mL^{-1}) as internal standard.

The denatured protein precipitate was separated by centrifugation at 8000 g for 3 min, and a 20 μL aliquot of the supernatant was directly injected onto the LC. The same sample handling process was used for recovery and precision determination.

Recovery

Recovery has been defined as a measure of the efficiency of the extraction of the analyte from the sample matrix. In the experiment, recovery of scoparone was determined at low, moderate and high concentrations (0.1, 0.5 and 5 μg mL^{-1}, respectively) from the rat plasma. Two groups of samples were used to assess extraction recovery (i.e. test and control groups). The samples in the test group was spiked with scoparone in rat plasma to yield final concentrations of 0.1, 0.5 and 5 μg mL^{-1}; whereas the samples in the control group were spiked with scoparone after the extraction.

The extraction recovery was calculated as the ratio of the measured concentration of the test samples over the measured concentration of the control samples at the low, moderate and high concentrations. Quadruplicate assays have been performed at the same concentrations.

Precision

Precision over the entire working dose range was determined by replicate analyses of plasma samples (n=4) spiked with three different concentrations (0.1, 0.5, or 5 μg mL^{-1}) of scoparone. To determine intra-assay variance, quadruplicate assays were carried out on the same samples at different times during the day. Inter-assay variance was determined by assaying the spiked samples in quadruplicate on days one, two, four, and six, after spiking. Coefficients of variation (C.V.s) were calculated from these values.

Pharmacokinetic Analysis

A calibration curve was constructed based on the analysis by HPLC of various concentrations of scoparone spiked in rat plasma. The concentrations of scoparone in rat plasma after i.v. administration, was determined from the peak area by using the equation for linear regression from the calibration curve. All data were subsequently processed by the computer program PCNONLIN (SCI Software Inc., Lexington, KY).

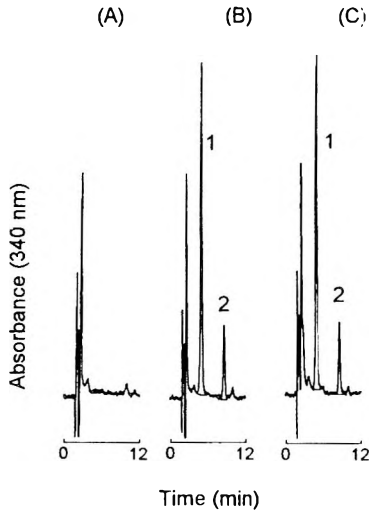

Figure 2. Chromatograms of scoparone in rat plasma: (A) blank plasma. (B) spike scoparone (0.5 μg mL^{-1}) and ferulic acid (internal standard). (C) plasma sample 30 min after a 5 mg kg^{-1} i.v. administration of scoparone (0.46 μg mL^{-1}). 1: ferulic acid; 2: scoparone.

RESULTS AND DISCUSSION

Under the conditions described above, the retention times of scoparone and ferulic acid (internal standard) were found to be 8.52 and 5.04 min, respectively (Figure 2). The main characteristic spectral data obtained in the mobile phase were shown as an absorption maxima at 340 nm for scoparone and at 322 nm for internal standard (ferulic acid).[13]

The recoveries of scoparone from rat plasma were found to be 96.38, 94.63, and 97.49 % for the concentrations 0.1, 0.5, and 5 μg mL^{-1}, respectively. The reproducibility of the method was also defined by examining both intra- and inter-assay variabilities. The intra-assay CVs for scoparone at concentrations of 0.1, 0.5, and 5 μg mL^{-1} were 7.25, 5.51, and 3.61%, respectively, and the inter-assay CVs for scoparone at the same concentrations were 8.63, 5.88, and 3.83%, respectively.

To determine the linearity and the detection limit of the HPLC method, rat plasma samples spiked with six different concentrations of scoparone (0.1-5 μg

mL^{-1}) were analyzed. The peak area ratios (scoparone to ferulic acid) were linearly related to the concentration of drug and the equation for the regression line for scoparone was found to be $y = 0.544x - 0.005$ (r^2=0.999). The lower practical limit of quantification was 0.1 μg mL^{-1}. Under the procedure described above, the detection limit for scoparone, at a signal-to-noise ratio of 4, was 0.05 μg mL^{-1} in rat plasma.

Figure 2(A) shows the chromatogram of blank rat plasma. No discernible peaks were observed within the time frame in which scoparone and ferulic acid were detected. Figure 2(B) shows the chromatogram of rat plasma spiked with scoparone (0.5 μg mL^{-1}) and internal standard. Figure 2(C) shows the chromatogram of scoparone (0.46 μg mL^{-1}) sample obtained 30 min after i.v. administration of scoparone (5 mg kg^{-1}) to a rat.

The data, from the dose, fit best into a two-compartment open model by the computer program PCNONLIN. The following equation applies into a two-compartment pharmacokinetic model:

$$C = Ae^{-\alpha t} + Be^{-\beta t} \quad (1)$$

In the equation 1, A and B are the concentration (C) intercepts for fast and slow disposition phases, respectively, and I and J are disposition rate constants for fast and slow disposition phases, respectively. The K_{12} and K_{21} are micro rate constants between the central and peripheral compartments, and K_{10} as the elimination rate constant. The distribution half-life ($t_{1/2,\alpha}$) and elimination half-life ($t_{1/2,\beta}$) of scoparone, as shown in the initial phase and terminal phase of the plasma concentration-time curve, was determined by the equation of $0.693/\alpha$ and $0.693/\beta$, respectively. Analysis of data after i.v. injection of scoparone at 5 mg kg^{-1} yields equations 2 (and Figure 3), respectively:

$$C = 5.04e^{-0.18t} + 1.26e^{-0.028t} \quad (2)$$

The pharmacokinetic parameters, as derived from these data and calculated by PCNONLIN program, are shown in Table 1.

A statistical nonlinear regression program was accessed through the JANA and PCNONLIN programs for the kinetic analysis. The pharmacokinetic models (one vs. two compartment) were compared according to Akaike's information criterion (AIC)[14] and Schwartz criterion (SC)[15] and, with minimum AIC and SC values, were regarded as the best representation of the plasma concentration time course data. A two-compartment open model with elimination from the central compartment, was proposed and validated

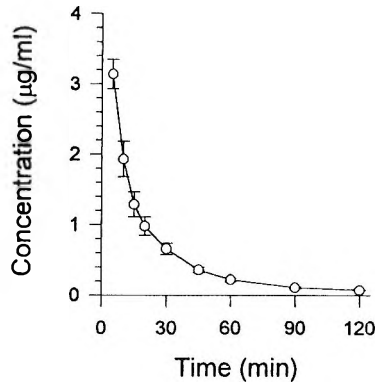

Figure 3. Plasma concentration-time curve after *i.v.* administration of scoparone in rats at dose of 5 mg kg^{-1}.

Table 1

Pharmacokinetic Parameters of Scoparone in Rats After 5 mg kg^{1}, i.v. Administration

Parameters	Estimate
A, μg mL^{-1}	5.04 ± 0.53
B, μg mL^{-1}	1.26 ± 0.18
α, min^{-1}	0.18 ± 0.029
β, min^{-1}	0.028 ± 0.003
K_{10}, min^{-1}	0.085 ± 0.011
K_{12}, min^{-1}	0.063 ± 0.013
K_{21}, min^{-1}	0.058 ± 0.010
Vol, L kg^{-1}	0.85 ± 0.086
$t_{1/2\alpha}$, min	4.68 ± 0.91
$t_{1/2\beta}$, min	27.18 ± 3.19
Cl, L kg^{-1} min^{-1}	0.067 ± 0.005
AUC, μg min mL^{-1}	77.17 ± 6.34
AUMC, μg min^2 mL^{-1}	1991 ± 265
MRT, min	25.55 ± 2.30

Data are expressed as mean ± SEM. Cl: clearance.
See text for other abbreviations.

through the program, to explain the apparent biphasic disposition of scoparone in rat plasma after i.v. administration. The noncompartmental method for calculating disposition parameters of scoparone are based on the theory of statistical moments.[16] The area under the concentration curve of a plot of the product of concentration and time versus time, from zero time to infinity, is often referred to as the area under the moment curve, AUMC.[16]

The ratio of AUMC to AUC for scoparone is a measure of its mean residence time (MRT).[17] MRT, calculated after i.v. administration, is the statistical moment analogy to drug elimination half-life. After administration of scoparone (5 mg kg^{-1}, i.v.), MRT and $t_{1/2,\beta}$ were 25.55 and 27.18 min, respectively (Table 1). Like half-life, MRT is a function of both distribution and elimination. Whereas, half-life tell us the time required to eliminate 50% of the dose, MRT_{iv} tells us the time required to eliminate 63.2% of the dose.

In conclusion, the present method allows a high selectivity and reliability. The relative simplicity permits its use for pharmacokinetic studies. Analysis of data after i.v. injection of scoparone at 5 mg kg^{-1} yields a two-compartment pharmacokinetic model.

ACKNOWLEDGEMENTS

This study was supported by research grant NSC-86 from the National Science Council, Taiwan.

REFERENCES

1. K. S. Jamwal, M. L. Sharma, N. Chandhoke, B. J. Ghatak, Indian J. Med. Res., **60,** 763-771 (1972).

2. M. L. Sharma, Indian J. Med. Res., **87,** 387-394 (1988).

3. J. Yamahara, G. Kobayashi, H. Matsuda, T. Katayama, H. Fujimura, Chem. Pharm. Bull., **37,** 1297-1299 (1989).

4. H. C. Huang, Y. L. Huang, J. H. Chang, C. C. Chen, Y. T. Lee, Eur. J. Pharmacol., **217,** 143-148 (1992).

5. H. C. Huang, S. H. Chu, P. D. Lee Chao, Eur. J. Pharmacol., **198,** 211-213 (1991).

6. H. C. Huang, C. R. Lee, Y. I. Weng, M. C. Lee, Y. T. Lee, Eur. J. Pharmacol., **218,** 123-128 (1992).

7. Y. L., Chen, H. C. Huang, Y. I. Weng, Y. J. Yu, Y. T. Lee, Cardiovasc. Res., **28,** 1679-1685 (1994).

8. D. Muller-Enoch, A. Greischel, Arzneim. Forsch., **38,** 1520-1522 (1988).

9. Y. Horsmans, J. P. Desager, C. Harvengt, Life Sci., **53,** PL421-426 (1993).

10. R. F. Witkamp, S. M. Nijmeijer, W. C. Mennes, A. W. Rozema, J. Noordhoek, A. S. van Miert, Xenobiotica, **23,** 401-410 (1993).

11. T. H. Tsai, C. J. Chou, F. C. Cheng, C. F. Chen, J. Chromatogr. B., **655,** 41-45 (1994).

12. T. H. Tsai, C. J. Chou, C. F. Chen, J. Pharm. Sci., **83,** 1307-1309 (1994).

13. T. H. Tsai, C. F. Chen, Int. J. Pharm., **80,** 75-79 (1992).

14. K. Yamoaka, T. Nakagawa, T. Uno, J. Pharmacokinet. Biopharm., **6,** 165-175 (1978).

15. G. Schwartz, Ann. Stat., **6,** 461-464 (1978).

16. M. Gibaldi, D. Perrier, **Pharmacokinetics**, Marcel Dekker, Inc., New York 1982, pp. 409-417.

17. L. Z. Benet, R. L. Galeazzi, J. Pharm. Sci., **68,** 1071-1074 (1979).

Received February 26, 1996
Accepted March 18, 1996
Manuscript 4091

J. LIQ. CHROM. & REL. TECHNOL., 19(16), 2659–2667 (1996)

DETERMINATION OF MITOMYCIN C IN RABBIT OCULAR TISSUE AFTER TOPICAL ADMINISTRATION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A high-performance liquid chromatographic method is described for the determination of mitomycin C (MMC) in rabbit ocular tissues. The optimial conditions were 20 mM sodium phosphate buffer, pH 7.0 - methanol (70:30) in isocratic elution (flow rate 1mL/min). The detection limit of MMC of this method is 0.02 μg/g for conjunctive and sciera or 0.02 μg/mL for aqueous humor. The effect of pH value and temperature on the stability of MMC was investigated. Acetonitrile was used for extracting MMC from conjunctiva and sclera, and ethyl acetate for aqueous humor. Recoveries ranged from 49% to 92% over a wide range of concentrations (0.1-400 μg/g). The half-life of MMC, after cellulose sponge administration, is 0.58 hours for the conjunctiva and 0.45 hours for the sclera. The peak concentration of MMC in aqueous humor is 1 hour.

INTRODUCTION

Mitomycin C (MMC), an antibiotic-antimetabolite, is regarded as the prototype bioreductive drug in clinical use due to its requirement for activation of cytotoxic metabolites by reductive enzymes. It is known to have a potential antiproliferative effect on cultured rabbit subconjunctival fibroblasts.[1] Glaucoma filtration surgery may fail as a result of fibroblast proliferation at the sclerostomy site, with subsequent obstruction of aqueous humor drainage.[2,3] Antimetabolites with antifibroblastic proliferative activity have been used to modify the normal wound healing response and increase surgical success ratest.[4,5] MMC has gained popularity as the adjunctive drug of choice. The intraoperative cellulose sponge administration of this agent has been successful in improving the outcome of glaucoma filtration surgery in the eyes, with a poor surgical prognosis.[6]

Suresh[7] and Kawase[8] described the method of measuring MMC in aqueous and vitreous humour using normal phase HPLC. Reversed phase HPLC methods to measure MMC concentration in tumour tissue have been previously reported.[9-11] To clarify the pharmacokinetics of MMC in the eye, we developed the present HPLC methods for determining MMC concentration in ocular tissues after cellulose sponge administration, and studied the stability of MMC at different pH values. We also investigated the concentration changes of MMC after a single application in rabbit ocular tissue under different time intervals.

MATERIALS

Reagents

Methanol, acetonitrile were HPLC reagent grade. Ethyl acetate, sodium dihydrogen phosphate, sodium hydrogen phosphate and sodium chloride were analytical reagent grade. All of the above chemicals were purchased from Shanghai Reagent Factory (China). Mitomycin C was obtained from Kywa, Hakko Kegyo (Tokyo, Japan). Water was deionized and double-distilled in a quartz glass still. All other chemicals were of analytical reagent grade.

Apparatus

The chromatographic system consisted of a Shimadzu (Japan) model LC-6A liquid chromatograph, including an SCL-6A system controller, an LC-6A pump, an SIL-6A autosampling injector, a CTO-6A column oven, an SPD-6A UV-VIS spectrophotometric detector and a C-R3A data processing

unit. The analytical column was Zorbax ODS, 250 x 4.0mm, 5 μm (DuPont Instruments, USA). An ultrasonicator (Shanghai, China) was used for extracting the sample and degassing the mobile phase. The extracts of MMC were evaporated in a vacuum desiccator (Shanghai, China) at 40 °C.

METHODS

Chromatographic Conditions

Separations were carried out on a 250 x 4.0mm Zorbax ODS reversed-phase column (DuPont Instruments). The mobile phase consisted of 20 mM sodium phosphate buffer (pH7.0)-methanol (70:30). Elution was performed isocratically at a flow rate of 1 mL/min; the column was maintained at a constant temperature of 40 °C.

The mobile phase components were filtered before using (0.45μm filter, Waters Millipore, USA) and desgassed with an ultrasonicator. The UV-VIS detector was used for monitoring MMC operating at wavelength 360 nm. The standard solution of 0.2 μg/mL of MMC was prepared with sodium phosphate buffer, pH 7.0.

Treatment of Rabbits

Healthy, pigmented rabbits, weighing between 2.0 and 3.0 Kg, were anesthetized with an intramuscular injection of 5% pentobartal sodium 0.44mL/Kg before surgery and incising the conjunctiva 5mm posterior to where the limbus was performed. A 0.4 mg/mL solution of Mitomycin C was prepared by dissolving 2 mg Mitomycin C powder in 5mL of 0.9% sodium chloride solution. Rabbits received Mitomycin C in each eye via cellulose sponge administration. A sponge, mearsuring 3 x 3 x 3 mm, was saturated with Mitomycin C solution (0.4 mg/mL). Sponges were applied directly between the conjunctiva and the sclera in the operated eyes. After thoroughly rinsing with 0.9% sodium chloride, the rabbits were killed at 0.5-, 1-, 2-, 3-hour intervals after drug administration. Then, 100 to 200 μL of aqueous humour were drained via a 26-gauge needle, and excised a section of conjunctiva measuring 15 x 15mm and a section of sclera 15 x 10mm; each section weighed 150 to 250 mg.

Extraction of MMC from Ocular Tissues

For the conjunctiva and sclera, 5mL of cold acetonitrile was added to the 200mg specimen and homogenized. For the aqueous humour, 4 mL of ethyl

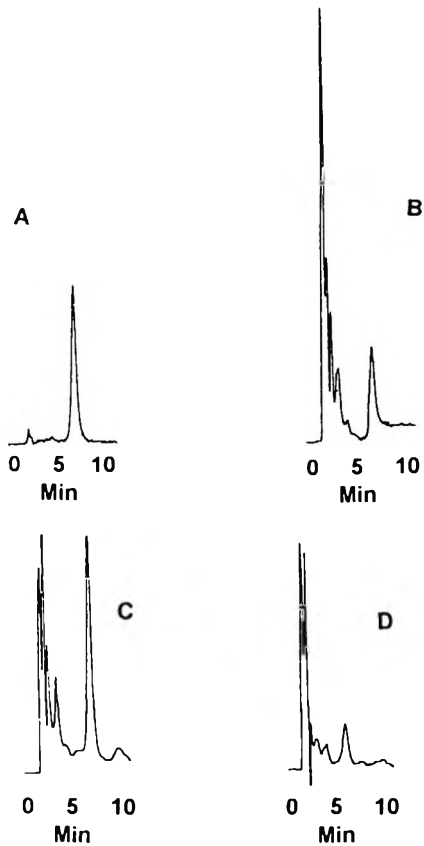

Figure 1. Typical chromatograms (A) 10 μL of MMC standard at a concentration of 0.2 μg/mL; (B) conjunctiva extract, (C) sclera extract and (D) aqueous humour extract after administration.

acetate was added to 0.2 mL of the aqueous humour sample and stirred. Then the samples were ultrasonicated for 30min. After centrifugation at 3000rpm for 10 min at 4 °C, the organic phase was separated and evaporated completely at 40 °C in the vaccum desiccator. After dissolving the residue in 0.4 mL mobile phase, sonication was performed. The filtration was carried out before injecting the sample into the chromatograph. In control extraction, ocular tissues were collected from non-drug administration rabbits and MMC (0.1-400 μg/g) was added to 200 mg conjunctiva and sclera or 0.2 mL aqueous humour. The late extraction was as described above.

Table 1

Recovery of Mitomycin C from Rabbit Ocular Tissues (Mean±SD)

Amount/g Tissue (μg)	Extraction Efficiency (%)		
	Conjunctiva	Sclera	Aq. Humour
400	68 ± 4	59 ± 9	49 ± 12
200	72 ± 8	75 ± 6	68 ± 9
100	74 ± 7	71 ± 13	64 ± 11
50	83 ± 6	81 ± 4	80 ± 7
10	86 ± 9	83 ± 5	81 ± 6
1	92 ± 7	91 ± 9	89 ± 8
0.5	81 ± 10	79 ± 12	85 ± 3
0.1	65 ± 11	64 ± 2	54 ± 5

n = 5 for each concentration.

Table 2

Effect of Evaporation Temperature on the Stability of MMC Extract from Rabbit Ocular Tissue

Temperature (°C)	Recovery, % (Mean ± SD)		
	Conjunctiva	Sclera	Aq. Humour
30	92 ± 11	91 ± 13	88 ± 12
40	92 ± 7	91 ± 9	89 ± 12
50	87 ± 10	80 ± 6	78 ± 9
60	81 ± 8	74 ± 8	62 ± 7
70	76 ± 12	70 ± 5	49 ± 10
80	58 ± 9	52 ± 10	51 ± 13

1.0 μg MMC was added to each tissue per gram and extracted as described in the METHODS section. n = 4.

Stability of MMC

A solution of 0.2 μg/mL of MMC was prepared with 20 mM sodium phosphate of various pH values, and kept it in the dark for various durations. The amounts of chromatography detection were compared with each other and an optimial pH value of solution was determined.

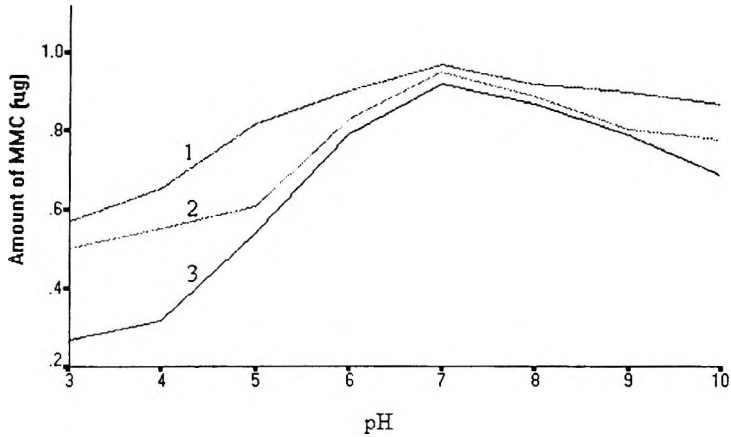

Figure 2. Degradation curve of MMC. 1 μg of MMC was dissolved in 5.0 mL 20 mmol sodium phosphate buffer, at various pH values, and kept at 20 °C in the dark for (1) 24 hrs; (2) 72 hrs; (3) 168 hrs.

RESULTS

The chromtograms of MMC standard and extracts of ocular tissues are shown in Figure 1. The retention time of MMC in this condition was 5.6 min. The detection limit of MMC for this method is 0.02 μg/g tissue or 0.02 μg/mL aqueous humour. The efficiency of extraction in each tissue is listed in Table 1.

The effect of different pH values on Mitomycin C stability is shown in Figure 2. When the pH value is below 6 or above 8, MMC is unstable. The effect of evaporation temperature on stability of MMC extract from rabbit ocular tissues is shown in Table 2. The best appropriate evaporating temperature was at 40 °C, at which there was a better recovery and a shorter evaporation time. The mean concentration of MMC in ocular tissues at different time intervals after cellulose sponge administration is shown in Table 3. The halflives of Mitomycin C were 0.58 hours in conjunctiva, 0.45 hours in sclera, respectively. The peak concentration of MMC in aqueous humor was reached at 1 hour.

DISCUSSION

Andrews[12] obtained different hydrolytic products from MMC with hydrochoric acid. Pan[13] reported that MMC and its metabolites reduction was catalyzed by NADPH-cytochrome P-450 reductase. Neither author, however, investigated systematically, the effect of pH value on the stability of MMC.

Table 3

Mitomycin C Concentration Changes of Ocular Tissue after Administration (Mean ± SD)

Hours	n	Conjunctiva (μg/g)	n	Sclera (μg/g)	n	Aq. Humour (μg/mL)
0	6	1.3859 ± 0.6208	4	2.4686 ± 0.8375	4	0.0330 ± 0.0050
0.5	6	0.7614 ± 0.1743	5	1.1518 ± 0.3604	4	0.0943 ± 0.0312
1	7	0.3691 ± 0.2635	6	0.5082 ± 0.3287	6	0.1214 ± 0.0413
2	4	0.1429 ± 0.0933	4	0.1632 ± 0.1136	5	0.0867 ± 0.0256
3	4	0.0895 ± 0.0796	4	0.1074 ± 0.0871	4	0.0743 ± 0.0154

This experiment studied the degradation of MMC in solution at various pH values, ranging from 3.0-10.0, at different time intervals (Figure2). When the pH was at 7.0, the amounts of MMC loss were 3%, 5% and 8% in 24, 72 and 168 hours, respectively; but when the pH value was at 3.0, they were 43%, 50% and 73%, and 13%, 22% and 31% at a pH value of 10.0. The results indicated that MMC is unstable in acidic and basic conditions, especially in acid.

Cummings et al.[9] chose the mobile phase at pH 5.8, improving the resolution between MMC and its metabolites. Our study showed that MMC was degraded, obviously, at pH 5.8. This fact points out that MMC was also degraded during analysis in Jeffrey's method. Thus, we chose sodium phosphate buffer, pH 7.0-methanol (70:30) as mobile phase to reduce degradation of MMC during the analysis.

The evaporation temperature of organic extract can also influence the stability of MMC. When the temperature is above 50 °C, the degradation becomes remarkably obvious in our experiments (Table 2). Thus, we determined the evaporation temperature at 40 °C in which the drying time was shorter and degradation of MMC was minimized.

The nature of the extraction solvents were compared between acetonitrile and chlorform/2-propanal/ethyl actate. Our studies showed that the former was more advantageous for extracting MMC from conjunctiva and sclera. For aqueous humour, ethyl acetate was used for extraction solvent. The efficiency of liquid-liquid extraction is shown in Table I. Across a wide range of concentrations (0.1-400 μg/g), the extraction efficiency of MMC remained high. The average recovery was 77.6 ± 9.4%, 75.4 ± 10.4% and 71.4 ± 14.8%, for conjunctiva, sclera and aqueous humour, respectively. These data gave a satisfactoy extraction efficiency of MMC from ocular tissues and was superior to Cummings' report.[9] The present study determined the pharmacokinetics of

conjunctiva, sclera and aqueous humour MMC after postoperative topical administration in rabbit eyes. The half-lives were calculated to be 0.58 hours for the conjunctiva and 0.45 hours for the sclera. The peak concentration of MMC in aqueous humor was achieved 1 hour after cellulose sponge administration.

The initial concentration of sclera MMC is higher than conjunctiva, but the half-life of MMC in conjunctiva is longer than sclera. The clearance times of MMC are 3 and 6 hours for sclera and conjunctiva, respectively. These results indicate that the absorption of MMC of sclera is more rapid than conjunctiva and the clearance is also rapid.

REFERENCES

1. T. Yamamoto, J. Varani, H. K. Soong, P. R. Lichter, "Effects of 5-Fluorouracil and Mitomycin C on Cultured Rabbit Subconjunctival Fibroblasts," Ophthalmology, **97**, 1204-10 (1990).

2. G. L. Skuta, R. K. H. Parrish, "Wound Healing in Glaucoma Filtering Surgery," Surv. Ophthalmol., **32**, 149-70 (1987).

3. M. M. Tahery, D. A. Lee, "Review: Pharmacologic Control of Wound Healing in Glaucoma Filtration Surgery," J. Ocul. Pharmacol., **5**, 155-79 (1989).

4. Y. Kitazawa, K. Kawase, H. Matsushita, M. Minobe, "Trabeculectomy with Mitomycin. A Comparative Study with Fluorouracil," Arch. Ophthalmol., **109**, 1693-8 (1991).

5. J. H. Rabowsky, J. M. Ruderman, "Low-Dose 5-Fluorouracil and Glaucoma Filtration Surgery," Ophthalmic. Surg., **20**, 347-9 (1989).

6. G. L. Skuta, C. C. Beeson, E. J. Higginbotham, "Intraoperative Mitomycin versus Postoperative 5-Fluorouracil in High Risk Glaucoma Filtering Surgery," Ophthalmology, **99**, 438-44 (1992).

7. S. C. Srivastava, U. Hornemann, "High-Pressure Liquid Chromatography of the Antibiotics Mitomycin A, B and C and of polar Mitomycin C Conversion Products," J. Chromatogr., **161**, 393-395 (1978).

8. K. Kawase, H. Matsusluta, T. Yamamota, Y. Kitazawa, "Mitomycin Concentration in Rabbit and Human Ocular Tissues after Topical Administration," Ophthalmology, **99**, 203-7 (1992).

9. J. Cummings, L. Chirrey, N. Willmott, G. W. Halbert, J. F. Smyth, "Determination of Mitomycin C, 2,7-Diaminomitose, 1,2-cis-and 1,2-trans-1-hydroxy-2,7-Diaminomitosene in Tumour Tissue by High-Performance Liquid Chromatography," J. Chromatogr., **612**, 105-13 (1993)

10. D. Seigel, N. W. Gibson, P. C. Preusch, D. Ross, "Metabolism of Mitomycin C by DC-Diaphorase: Role in Mitomycin C-induced DNA Damage and Cytotoxicity in Human Colon Carcinoma Cells," Cancer Res., **50**, 7483 (1990).

11. P. R. Hoban, M. I. Walta, C. N. Robson, J. Godden, I. J. Stratford, P. Workman, A. L. Harris, I. D. Hickson, "Decreased NADPH:Cytochome P-450 Reductase Activity and Impaired Drug Activation in a Mammalian Cell Line Resistant to Mitomycin C under Aerobic but not Hypoxic Condition," Cancer Res., **50**, 4692 (1990).

12. P. A. Andrews, Su- Shu Pan, N. R. Bachur, "Liquid Chromatographic and Mass Spectral Analysis of Mitosane and Mitosene Derivatives of Mitomycin C," J. Chromatogr., **262**, 231-247 (1983).

13. Su-Shu Pan, P. A. Andrews, C. J. Glover, N. R. Bachur, "Reductive Activation of Mitomycin C and Mitomycin C Metabolites Catalyzed by NADPH-Cytochrome P-450 Reductase and Xanthine Oxidase," J. Biol. Chem., **259**, 959 (1984).

Received June 12, 1995
Accepted August 1, 1995
Manuscript 5129

J. LIQ. CHROM. & REL. TECHNOL., 19(16), 2669–2679 (1996)

HIGH SENSITIVITY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ELECTROSPRAY TANDEM MASS SPECTROMETRY DETERMINATION OF TERFENADINE IN HUMAN PLASMA

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ABSTRACT

A highly sensitive and selective HPLC-MS-MS method was developed for the determination of terfenadine in human plasma. The analytes, terfenadine and terfenadine related compound A (internal standard), were extracted from human plasma by methyl t-butyl ether/methylene chloride/n-butyl chloride (3:2:1). The organic solvent is evaporated to dryness and reconstituted in 100 µL of acetonitrile/0.02 M ammonium acetate buffer pH 3.5 (50:50). Chromatographic separation is achieved on a TSK gel ODS-80$_{TS}$ column with a mobile phase composed of acetonitrile/1% formic acid/0.01 M ammonium acetate pH 4.0 (85:13:2). The analytes were detected by HPLC in conjunction with electrospray tandem mass spectrometry. The assay was linear in the concentration ranges of 0.2 to 50 ng/mL. The analysis of pooled quality controls demonstrated excellent precision with relative standard deviations being less than 5.2%. The method is accurate with all intraday and over-all mean values being less than 5.3% from theoretical.

INTRODUCTION

Terfenadine (α-[4-(1,1-dimethylethyl)phenyl]-4-(hydroxydiphenylmethyl) -1-piperidinebutanol) is an H_1 antihistaminic drug. This drug has been used in treatment of allergic rhinitis and a variety of skin allergies[1] without any anticholinergic, antiserotoninergic and anti-adrenergic effects.[1-3] Terfenadine undergoes extensive biotransformation on its first pass through the liver, to form mostly the acid metabolite. Other metabolites such as azacyclonol, the 'alcohol' metabolite and a 'ketone' metabolite[4] were also found in urine. Because of the high first pass effect, very low concentrations of terfenadine are found in the blood plasma.[5] Few analytical methods for the determination of terfenadine and its metabolites have been reported.[4,6-9] However, these methods lack the sensitivity (> 2 ng/mL) and the selectivity.

This paper presents a highly sensitive and selective high performance liquid chromatography (HPLC) electrospray (ESP) tandem mass spectrometry (HPLC- MS-MS) method for the quantitation of terfenadine in human plasma, which has an analysis time of less than 4 min. The short analysis time was due to the highly selective MS-MS detector.

EXPERIMENTAL

Materials

Terfenadine and internal standard (terfenadine related compound A) were obtained from USP (Rockville, MD, USA). Heparinized human plasma was purchased from Rockland (Gilbertsville, PA, USA). Acetonitrile, methylene chloride, methyl t-butyl ether and n-butyl chloride (HPLC-grade) were obtained from Burdick & Jackson (Muskegon, MI, USA). Acetic acid and hydrochloric acid (GR grade) were obtained from EM Science (Gibbstown, NJ, USA). Ammonium acetate (HPLC grade) was obtained from Fisher (Fairlawn, NJ, USA). Formic acid (ACS reagent, 98.7%) was obtained from Sigma (St. Louis, MO, USA). Deionized water was processed through a Milli-Q water purification system (Millipore, Bedford, MA, USA)

Chromatographic System

HPLC-MS-MS was performed on a VG Quattro I triple quadrupole (Fisons, Manchester, UK) mass spectrometer interfaced via a electrospray (ESP) probe to a Hewlett-Packard 1090 L HPLC system with a 25-μL sample loop. The source temperature was set at 200°C. The cone voltage and the collision energy were at 35 V and 30 eV, respectively. The bath gas (nitrogen) and ESP

nebulizing gas (nitrogen) were 450 l/hr and 15 l/hr, respectively. The dwell time was 0.2 s. The mass spectrometer was set to detect 472.2/436 and 470.2/203.2 parent/daughter ions for terfenadine and ISTD, respectively, in the positive ion mode. The analytical column was a TSK gel ODS-80_{TS} (150 mm x 2.0 mm I.D., 5-μm particle size, Tosho, Japan). The mobile phase was acetonitrile/1% formic acid/0.01 M ammonium acetate pH 4.0 (85:13:2) at a flow-rate of 0.2 mL/min. Data were collected and processed using IBM PC compatible computer and MassLynx (Version 2.1) software.

Preparation of Standard Solutions

A stock solution of terfenadine (100 μg/mL) was prepared by dissolving 2.5 μg of terfenadine in 25 mL of methanol. A stock solution of internal standard (100 mg/mL) was prepared by dissolving 2.50 mg of terfenadine related compound A (ISTD) in 25-mL of methanol. Standard solutions of terfenadine (2-500 ng/mL) and internal standard (250 ng/mL) were prepared by diluting the stock solutions with methanol/water (25:75).

Quality Control Samples

Pooled quality control samples (QC) (4, 20 and 40 ng/mL) were prepared to determine the precision and accuracy of the method, and to evaluate the stability of samples. A quality control pool at a concentration above the curve range (80 ng/mL, over-curve control) was also prepared to evaluate precision and accuracy when analyzed using partial volume.

Sample Preparation

Calibration standards were prepared by adding 50 μL of the appropriate terfenadine standard solutions (2-500 ng/mL) to 0.5 mL of blank human plasma. Calibration standards and quality controls were processed by adding 0.5 mL of plasma, 50 μL of internal standard solution (250 ng/mL) and 5 mL of methyl t-butyl ether/methylene chloride/n-butyl chloride (3:2:1) into labeled 16x125 screw-cap tubes. The tubes were capped and mixed on a vortex mixer for 1 min and centrifuged at 2500 rpm for 10 min. The aqueous layer was frozen in an acetone/dry ice bath and the organic layer was transferred to a clean tube. The plasma layer was extracted with 5 mL of methyl t-butyl ether/methylene chloride/n-butyl chloride (3:2:1) for a second time. The organic layers were pooled in a clean tube and evaporated to dryness under nitrogen at 40°C. Reconstituted in 100 μL of acetonitrile/0.02 M ammonium acetate pH 3.5 (50:50). Aliquots of 10 μL were injected onto the HPLC system.

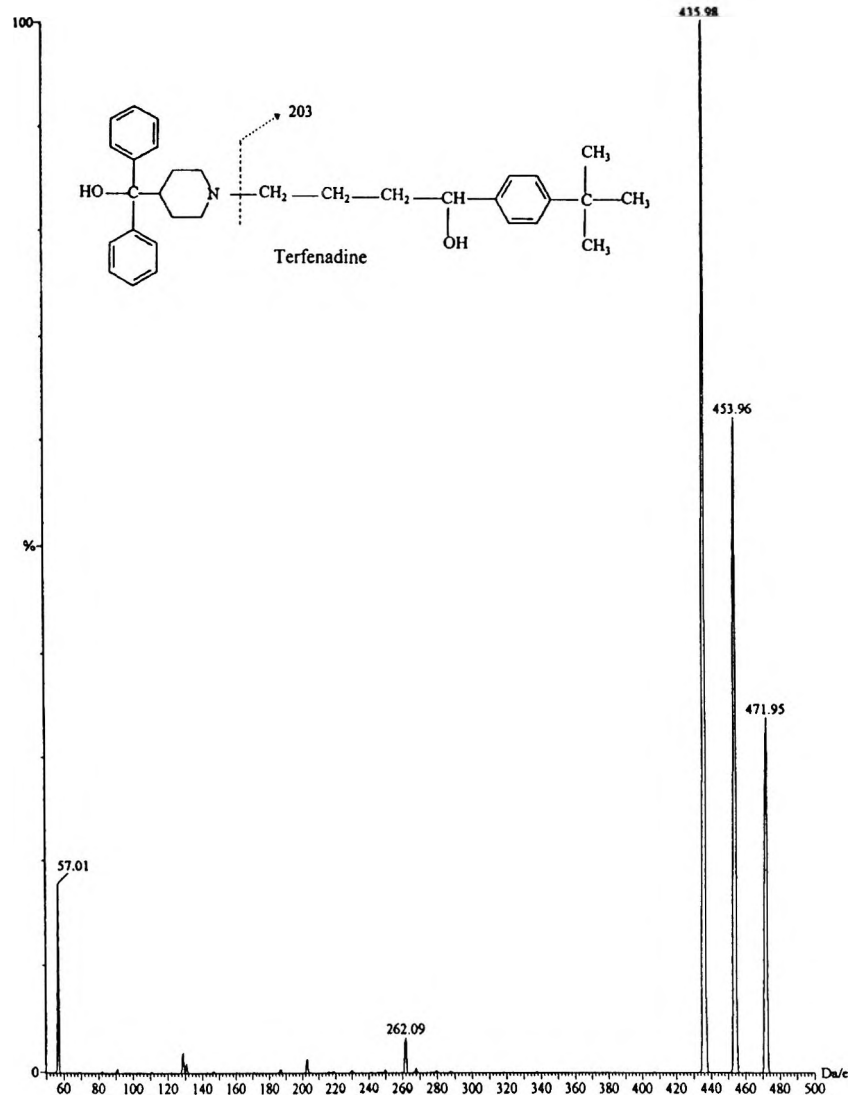

Figure 1. Molecular Structure and Mass Spectrum of Terfenadine.

Validation

Duplicate calibration curves (0.2, 0.5, 1.0, 5.0, 10, 25, and 50 ng/mL) were analyzed on each of three days. Triplicate quality control samples at each concentration (4.0, 20.0, and 40 ng/mL) were analyzed with each calibration

Table 1

Precision and Accuracy of Terfenadine Standards

Calibration Standard Concentration (ng/mL)	Calculated Concentration (Mean±S.D., n=6) (ng/mL)	R.S.D. (%)	Deviation (%)
0.2	0.2±0.011[a]	5.5	0.0
0.5	0.48±0.018[a]	3.8	-4.0
1.0	0.96±0.024	2.5	-4.0
5.0	4.59±0.166	3.6	-8.2
10	9.99±0.502	5.0	-0.1
25	26.9±1.06	3.9	7.6
50	53.1±1.77	3.3	6.2

[a] n = 5.

Table 2

Precision and Accuracy of Terfenadine Quality Controls

Control Concentration (ng/mL)	Calculated (Overall mean±S.D., n=18) (ng/mL)	R.S.D. (%)	Deviation (%)
4.00	4.20±0.220	5.2	5.0
20.0	20.2±0.99	4.9	1.0
40.0	40.0±1.64	4.1	0.0

curve. The calibration curves were obtained by weighted (1/concentration x concentration) least-squares linear regression analysis of the peak area ratios of terfenadine/ISTD vs. the concentration of terfenadine. The equations of the calibration curves were used to calculate the concentration of terfenadine in the controls from their peak area ratios

RESULTS AND DISCUSSION

The molecular structures and mass spectra of terfenadine and terfenadine related compound A (internal standard) are shown in Figures 1 and 2.

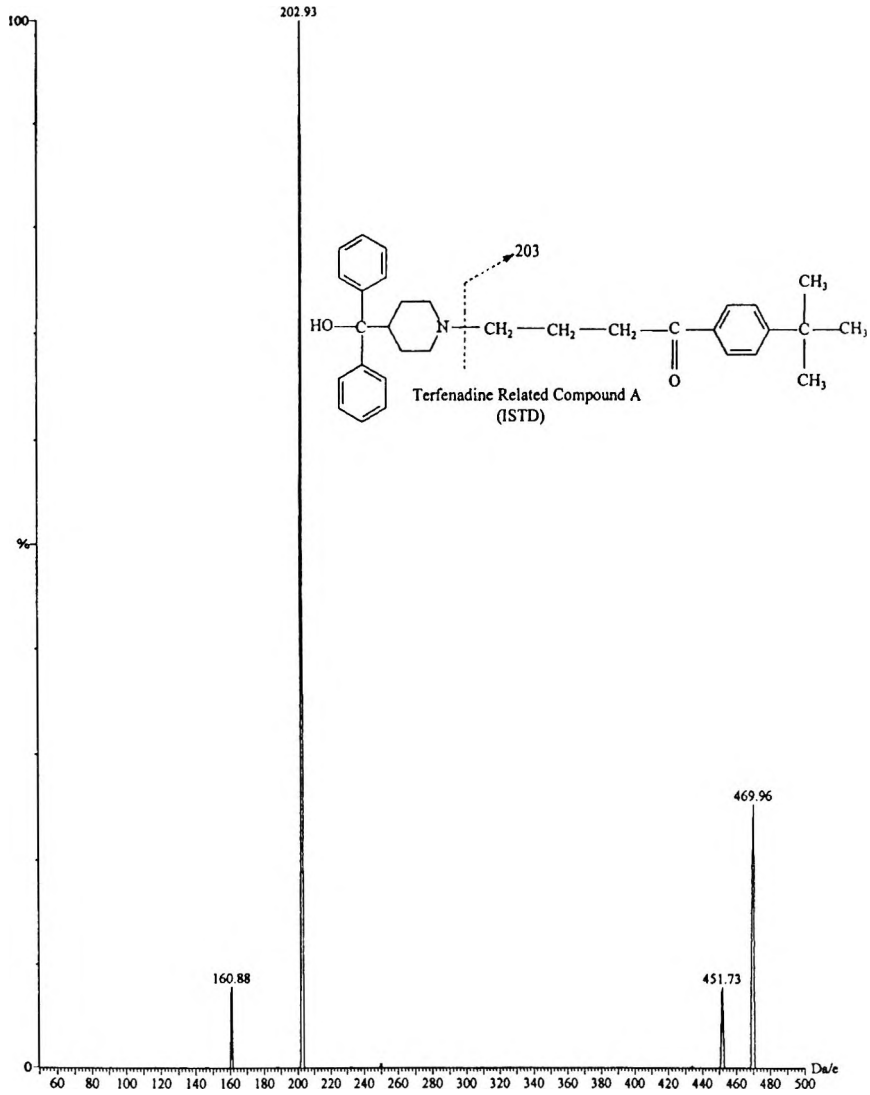

Figure 2. Molecular Structure and Mass Spectrum of Terfenadine Related Compound A (ISTD).

Representative chromatograms are shown in Figure 3 through 5. The mean retention times of terfenadine and the internal standard were 2.5 and 2.8 minutes, respectively. Blank human plasma from 17 pools was tested for endogenous interferences. There were no endogenous interferences found in the

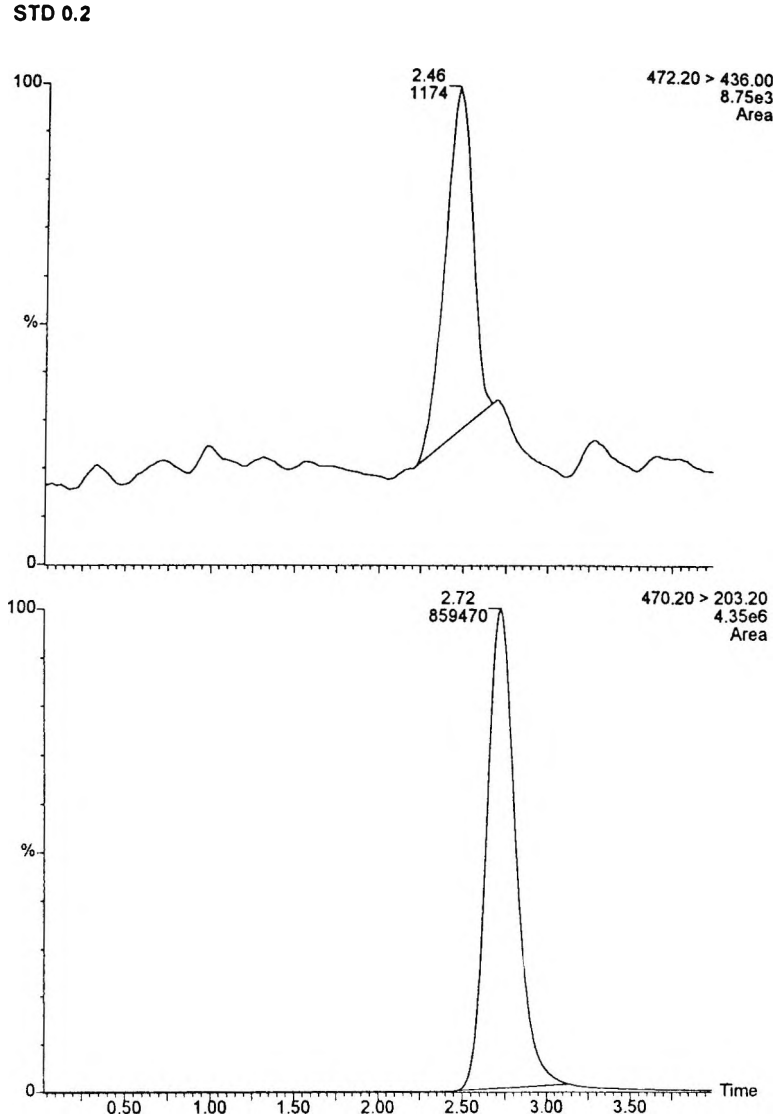

Figure 3. Chromatogram of a 0.20-ng/mL Calibration Standard.

terfenadine and ISTD regions for all the lots tested (Figure 5). Precision and accuracy of terfenadine standards are contained in Table 1. The standards show low values in deviation (<8.2%) and relative standard deviation (<5.5%). Calibration curves for terfenadine in human plasma were linear over the

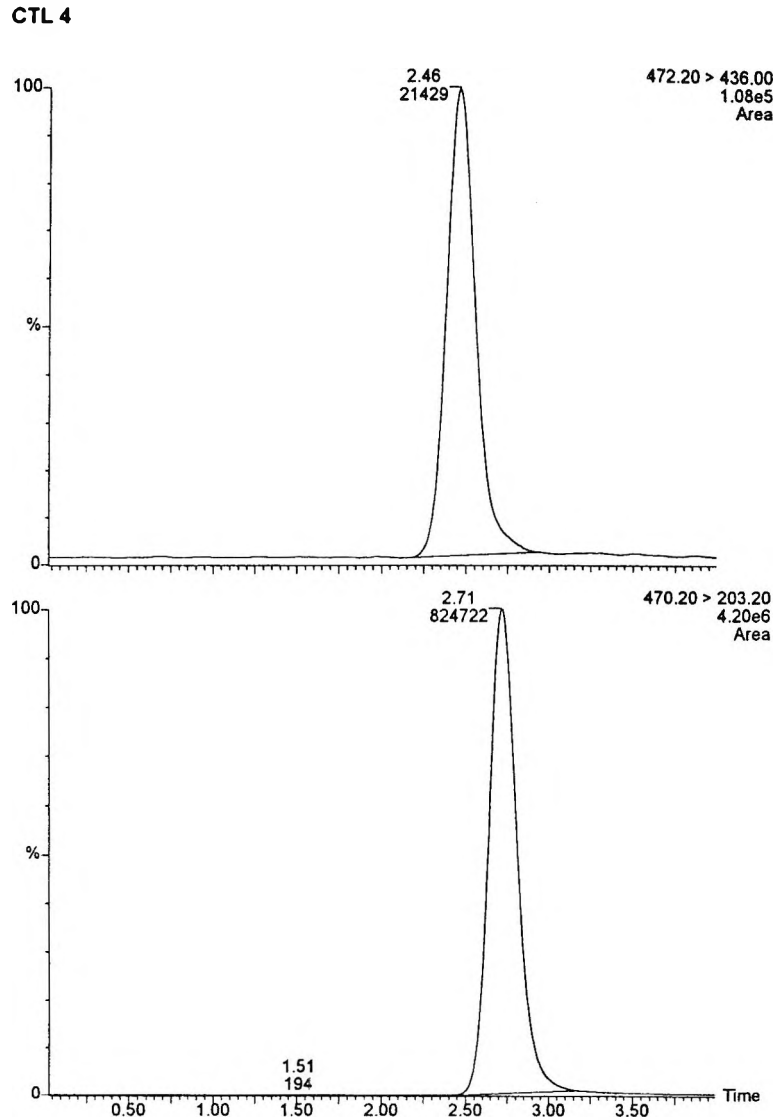

Figure 4. Chromatogram of an 4.00-ng/mL Quality Control Sample.

concentration range of 0.2 to 50 ng/mL. The correlation coefficients were greater than 0.9922 for all curves. Data from the spiked quality control samples are shown in Table 2. The within-day precision of the method, as measured by the relative standard deviation (RSD) of the daily mean (n=6), was less than

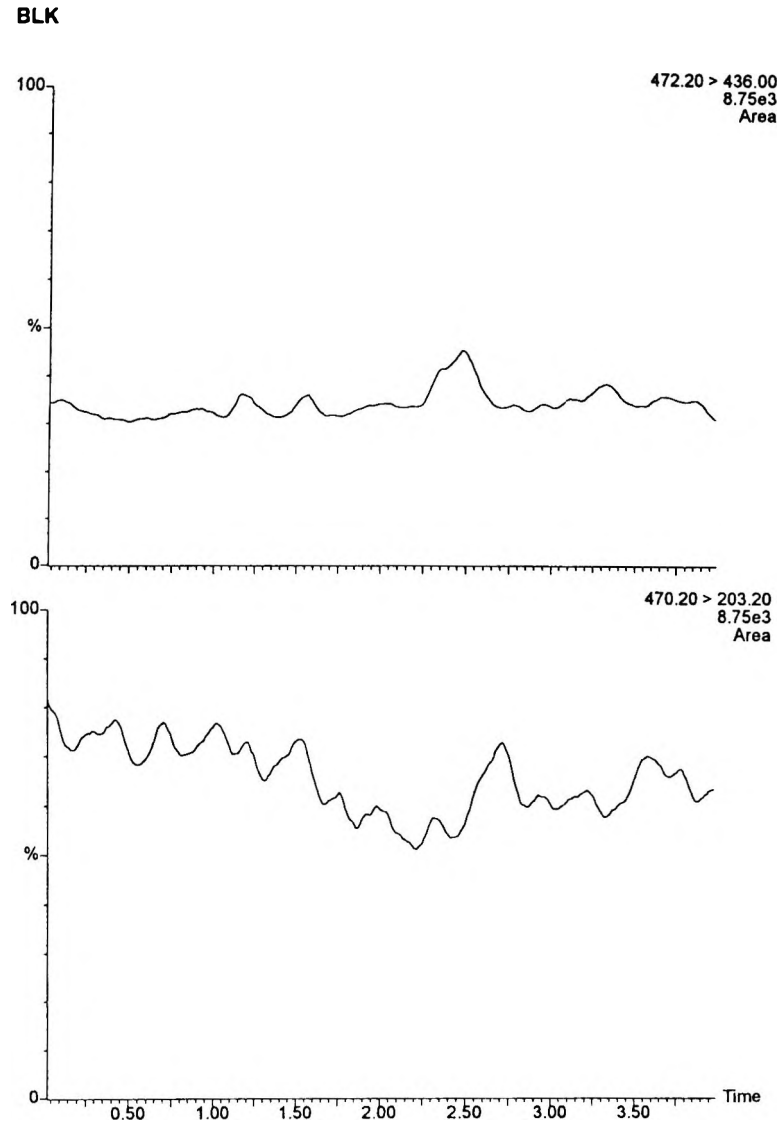

Figure 5. Chromatogram of Blank Human Plasma.

6.7% at the three control concentrations in human plasma. The overall precision was 5.2%, 4.9% and 4.1% (n = 18) for the 4.00-, 20.0- and 40.0-ng/mL terfenadine quality controls, respectively. The accuracy of the method was determined by comparing the means of the measured concentrations with the

nominal (theoretical) concentrations of terfenadine in plasma controls. All of the daily mean (n = 6) and overall mean (n = 18) values for the QC samples were within 5.3% of their expected values.

The lower limit of quantitation (LLOQ) was set at 0.20 ng/mL of terfenadine in human plasma. Five replicates of the lowest standard (0.2 ng/mL) were analyzed to evaluate the LLOQ. At the LLOQ, the RSD (n = 5) of the measured concentrations was 11.5%, and the deviation of the mean of the measured concentrations from their nominal value was 10.0%. Extraction recoveries were determined by comparing the peak areas of extracted QC samples with the peak areas of spiked extracted blanks at the same nominal concentrations. The mean recoveries for terfenadine and the internal standard were 125%, and 99.5%, respectively. Stock and working solutions of terfenadine in methanol and methanol/water (25:75) were stable for at least 2 months when stored at approximately 4°C. Stability was tested by subjecting the QC samples to three freeze/thaw cycles, and storage for 24 hours at room temperature. The thawing and refreezing of QC samples and the storage of QC samples at room temperature had little effect on the precision or accuracy of the results. The mean (n = 3) value was within 11.0% of the expected values.

Process stability was tested by storing the extracted samples in validation day 1 at room temperature overnight before analysis. The storage of extracted samples at room temperature had little effect on the accuracy and precision of the results.

The method presented here for the determination of terfenadine in human plasma shows acceptable linearity, precision, and accuracy down to a concentration of 0.2 ng/mL. The method is simple with relative short analysis time which can result in high sample analysis throughput.

REFERENCES

1. D. McTavish, K. L. Goa, M. Forrill, Drugs, **39**, 552-574 (1990).

2. J. K. Woodward, N. L. Munro, Arzneim.-Forsch., **32**, 1154-1156 (1982).

3. K. V. Mann, K. J. Tietze, Clin. Pharm., **6**, 331-344 (1989).

4. T.-M. Chen, K. Y. Chan, J. E. Coutant, R. A. Okerholm, J. Pharm. & Biomed. Anal., **9**, 929-933 (1991).

5. R. A. Okerholm, D. L. Weiner, R. H. Hook, B. J. Walker, G. A. Dusebout, G. J. Wright, Biopharm. Drug Dispos. **2**, 185-190 (1981).

6. S. Surapaneni, S. K. W. Khalil, J. Liq. Chromatogr., **17**, 2419-2428 (1994).

7. J. E. Coutant, P. A. Westmark, P. A. Nardella, S. M. Walter, R. A. Okerholm, J. Chromatogr., **570**, 139-148 (1991).

8. K. Y. Chan, R. C. George, T.-M. Chen, R. A. Okerholm, J. Chromatogr., **571**, 291-297 (1991).

9. A. Terhechte, G. Blaschke, J. Chromatogr. A, **694**, 219-225 (1995).

Received March 21, 1996
Accepted April 10, 1996
Manuscript 4122

J. LIQ. CHROM. & REL. TECHNOL., 19(16), 2681–2690 (1996)

RAPID AND SENSITIVE DETERMINATION OF CARBARYL, CARBOFURAN AND FENOBUCARB BY LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

A rapid and sensitive procedure is described for the determination of the N-methylcarbamates pesticides, carbaryl, carbofuran and fenobucarb, by liquid chromatography. The pesticides are previously hydrolyzed, in a separate step, to their phenolic derivatives, which, after the separation in a high speed column in a time lower than 2.5 min, are detected in a high sensitivity amperometric cell at a potential of +0.6 V. The method presents detection limits around 0.7 ppb and has been satisfactorily applied to the analysis of these compounds in spiked river water samples.

INTRODUCTION

Carbaryl, 1-naphtyl methylcarbamate, carbofuran, 2,3-dihydro-2,2-dimethylbenzo-furan-7-yl methylcarbamate, and fenobucarb, 2-sec-butylphenyl methylcarbamate, are pesticides of the N-methylcarbamates family, which have a wide range of applications and whose popularity has increased in the time, due i.e., to their short life. A number of papers appear in the bibliography,

concerning their determination in a variety of matrices, by high performance liquid chromatography. The separation between pesticides is generally carried out by reverse phase chromatography and with acetonitrile-water or methanol-water mobile phases. The detection is, in most cases, by UV spectrometry or by fluorimetry, and only in some instances are electrochemical methods used.[1-6] However, these can offer some advantages as greater sensitivity and selectivity without the need of various-step derivatization procedures as in fluorescence. Also, there are references about the coupling of HPLC systems with other techniques such as flow injection analysis, gas chromatography or mass spectrometry. Many of these methods make use of derivatization with different reagents after an alkaline hydrolysis which produces the phenolic derivatives of pesticides.

The hydrolysis reaction is also a good approach to carry out the electrochemical detection since phenols are molecules more easily oxidizable than carbamates, whose direct oxidation implies frequently to apply very high potentials (near +1.3 V). Kissinger et al[7] suggested this possibility, for the first time for carbofuran without going deeply into it, and, later, Olek et al[1] have studied the possibility of measuring several N-methylcarbamates after a pre-column hydrolysis technique. A potassium hydroxide solution in methanol is used to perform the hydrolysis at a temperature of 90 °C, and the procedure, which presents detection limits between 0.01 and 0.04 μg/mL (0.5 to 2 ng injected in 50 μL), is examined for the determination of carbamates in vegetables after liquid partitioning, solvent evaporation and purification on Florisil column steps. A subsequent paper by Krause et al.[2] describes a procedure in which carbamates are separated on a C_8 column using a gradient acetonitrile-water mobile phase and hydrolyzed in-line by post column addition of sodium hydroxide at 100 °C, and detected with a coulometric electrochemical detector. However, the separation takes near 25 min. and the method requires the removal of oxygen from the mobile phase solvents and sodium hydroxide solution. In this way detection limits of around 0.015 μg/mL (0.3 ng in an injected volume of 20 μl) are achieved.

In this paper, we describe a method to analyze three N-methylcarbamates, carbaryl, carbofuran and fenobucarb, in water, by HPLC. The latter is analyzed for the first time by LC with electrochemical detection. The pesticides are hydrolyzed in a prior separate step but the reaction is carried out by the simple addition of 0.02 M sodium hydroxide, at room temperature, according to the results recently reported about the hydrolysis of carbaryl.[8] The separation is effected on a high-speed column using an isocratic mobile phase similar to that used by Olek, and in a time shorter than 2.5 min., and the electrochemical detection is carried out with a high sensitivity amperometric cell. Thus, a simple and rapid procedure is proposed which presents lower detection limits than those mentioned for the other described procedures.

MATERIALS

Apparatus

The chromatographic equipment is composed of a 420 two piston HPLC Pump from Kontron Instruments, a 7125 Rheodyne sample injector equipped with a 20 μL loop, a Pecosphere 3x3 CR C_{18} column (3.3 cm x 0.46 cm, 3μm), a Coulochem II electrochemical detector equipped with a ESA model 5021 conditioning cell and a ESA model 5011 dual analytical cell protected by ESA filters containing 0.2 μm porous graphite filter elements. The high sensitivity analytical cell contains, in series, two porous graphite working electrodes, together with associated reference and counter electrodes.

The working electrodes are a large surface area coulometric electrode and a high efficiency amperometric electrode, more than seven times as efficient as conventional amperometric electrodes (70% vs. 5-10% efficiency). The conditioning cell contains a single porous graphite coulometric electrode.

The acquisition and treatment of data is controlled from an Olivetti 386 PC equipped with the PC Integration Pack software package from Kontron Instruments.

Reagents

Carbofuran, carbaryl and fenobucarb were obtained from Sigma Chemical Co. and used without further purification. Stock solutions of each pesticide in Milli Q grade water (2.5×10^{-4} M) were prepared by weighing. More diluted standards were prepared by suitable dilution. All other chemicals used were of HPLC grade.

HPLC Operating Parameters

The mobile phase was glacial acetic acid/water/acetonitrile, 0.5/49.5/50 made 0.01 M in sodium perchlorate. It was filtered through a 0.45 μm nylon membrane filter and degassed in ultrasonic bath before being used. The flow rate was adjusted to 1.0 mL/min and the system was equilibrated for at least 10 min. prior to injection of the prepared sample or standard.

The conditioning cell was set at 0 V and the detectors 1 and 2 of the analytical cell were set at +0.1 and +0.6 V respectively. The selected sensitivity in the PC Integration Pack was 500 nA full scale (1V).

METHODS

General Procedure for the Determination of Carbofuran, Carbaryl and Fenobucarb

To a 50 mL calibrated flask were added a suitable volume of the pesticide solution and 2 mL of 0.5 M NaOH solution. The solution was diluted with water to a volume near 40 mL and, after shaking for a few seconds, allowed to stand during 10 min. Then 1 mL of glacial acetic acid was added and the solution diluted to the mark with water. Samples were filtered through 0.45 μm nylon filter membranes and degassified in ultrasonic bath before their injection (20 μL) in the chromatographic system. Two chomatograms per sample were obtained and the mean of their peak heights was used as analytical signal.

Procedure for the Determination of Carbofuran, Carbaryl and Fenobucarb in River Water

Aliquots of 30 mL of river water, spiked with different amounts of the pesticides, were treated according to the general procedure and the obtained concentrations were calculated with the aid of the corresponding calibration plots.

RESULTS AND DISCUSSION

The alkaline hydrolysis of the pesticides carbofuran, carbaryl and fenobucarb gives rise to their phenolic derivatives, 2,3-dihydro-2,2-dimethyl-7-benzofuranol, 1-naphthol and 2-sec-butylphenol, respectively. These compounds are more easily oxidizable than the parents N-methylcarbamates and this can be made at a potential value lower than +1.0 V.

Recently,[8] it has been reported that quantitative hydrolysis of carbaryl can be obtained in only 30 s using sodium hydroxide solutions with concentrations $\geq$ 0.02 M. Also, the complete hydrolysis of carbofuran and fenobucarb is obtained in this medium in a short time and has been used as a previous step in their voltammetric determination.[9,10] If the hydrolyzed solutions are subsequently acidified they are stable at least during three hours.

At first, the hydrodynamic curves of the pesticides have been obtained to select the appropriate potential values to detect them in the used system. The mobile phase used was a solution 0.01 M of $NaClO_4$ in acetic

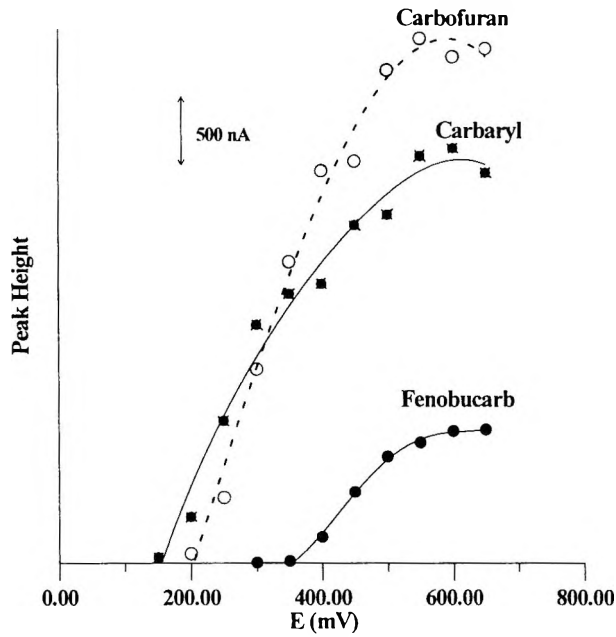

Figure 1. Hydrodynamic curves of carbofuran, carbaryl and fenobucarb in the porous graphite electrode of the coulometric detector.

acid:water:acetonitrile, 0.5:59.5:40, and the potential in the detector 1 was set at +0.1 V whereas the potential in the detector 2 is varied, taking different values between +0.15 and +0.75 V. In Figure 1 a plot of the results obtained is shown. According to these results, and in order to obtain the highest signal, we have selected to set the potential in the detector 2 at +0.6 V. The detector 1 was maintained at +0.1 V.

The influence of acetonitrile proportion in the mobile phase was studied varying it between 40% and 55% (v/v, acetonitrile/water), and the results can be found in Table 1. In all cases the resolution between the carbofuran and carbaryl peaks is satisfactory (Rs greater than 1), but we have selected a 50% proportion of acetonitrile due to the resolution value ($Rs_{1,2}$ greater than 1.5) and the capacity factor values (K' between 1 and 5) obtained. In these conditions the obtaining of the chromatogram takes only 2.5 min. The influence of the pH value of the mobile phase (between pH 2 and pH 5.5 with acetic acid or acetate buffer) on the resolution and capacity factor values, has also been studied. No differences have been observed but we have decided to prepare the mobile phase with acetic acid to avoid undesirable effects in base to changes in the pH values, with real samples, and variations in the oxidation potentials or in the

Table 1

Influence of the Acetonitrile Proportion in the Mobile Phase on the Retention of Pesticides Carbofuran, Carbaryl and Fenobucarb

	Carbofuran		Carbaryl			Febobucarb	
% AcCN (v/v)	t_R(min)	K′	t_R(min)	K′	$Rs_{1,2}$	t_R(min)	K′
40	1.35	2.97	1.86	4.17	3.64	4.31	11.68
45	1.17	2.08	1.51	2.96	2.29	3.02	6.95
50	1.00	1.63	1.21	2.18	1.58	2.17	4.71
55	0.86	1.32	1.00	1.70	1.06	1.64	3.43

detector response. The conductivity of mobile phase was increased with sodium perchlorate. Also, it is well known that the perchlorate are the less-adsorbable on the electrode ions, between the usually used. As result the selected mobile phase is that mentioned in the description of the chromatographic parameters: a solution 0.01 M of $NaClO_4$ in glacial acetic acid/water/acetonitrile 0.5/49.5/50.

In the study of the influence of the mobile phase flow rate we have found that the capacity factors only decrease slightly when the flow rate increases, and N (number of theoretical plates) changes in a similar way.

According to these results and the usefulness of obtaining the chromatogram in a short time we have selected a 1 mL/min flow rate. Different sensitivities have been assayed for the obtaining of the chromatograms and we have set it in 500 nA due to the good signal/noise ratio obtained. Figure 2 shows a chromatogram corresponding to a standard which is 3×10^{-8} M in the three analytes, obtained in the mentioned conditions

Determination Of Carbaryl, Carbofuran And Fenobucarb

Under the optimum experimental chromatographic conditions, a linear relationship between peak height or peak area and concentration of the pesticide was found for the three compounds in the range examined (4×10^{-9} M - 7×10^{-8} M for carbaryl and carbofuran and 4×10^{-9} M - 1×10^{-7} M for fenobucarb), showing the regression equation of Table 2. Due to the greater sensitivity (greater slope) and reproducibility (lower RSD) obtained by using peak height, this parameter has been selected as analytical signal to determine these analytes.

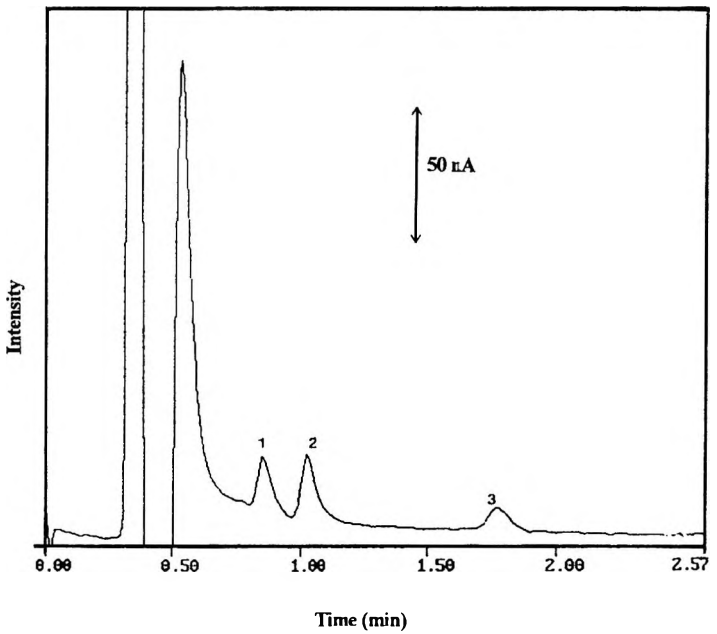

Figure 2. Chromatogram of a 3 x 10^{-8} M solution of carbofuran (1), carbaryl (2) and fenobucarb (3). Mobile phase: acetic acid/water/acetonitrile (0.5/49.5/50) 0.01 M in $NaClO_4$.

Table 2

Regression Parameters for the Pesticide Chromatographic Peaks

Compound	Signal	Slope	Intercept	Correlation Coefficient (r)	RSD (%) p=0.05, n=11
Carbofuran	Height	1.68×10^9	-7.82	0.9992	2.40
	Area	11.8×10^7	-0.37	0.9992	7.90
Carbaryl	Height	1.81×10^9	-2.85	0.9982	1.62
	Area	13.3×10^7	-0.12	0.9980	3.57
Fenobucarb	Height	6.16×10^8	+0.86	0.9997	2.95
	Area	6.65×10^7	+0.06	0.9930	5.88

Table 3

Detection Limit Values Calculated from the Chromatographic Peak Heights

Compound:	Carbofuran	Carbaryl	Fenobucarb
Method	**(M x 10^9)**	**(M x 10^9)**	**M x 10^9)**
3σ	1.00 (4.43)*	0.98 (3.94)	2.00 (8.29)
Winefordner and Long	2.11 (9.34)	3.04 (12.2)	1.60 (6.63)
Clayton ($\alpha = \beta = 0.05$)	2.60 (11.5)	3.76 (15.1)	2.14 (8.87)

* The quantities in brackets are the detection limit values in pg.

Table 4

Determination of Carbofuran, Carbaryl and Fenobucarb in Spiked River Water

Sample:	Concentration	1,2,3	4,5,6	7, 8, 9	10, 11, 12
Compound	**M X 10^8**				
Carbofuran	Added	3.0	5.0	4.0	6.0
	Found	2.86±0.09	4.75±0.03	3.82±0.06	5.76±0.16
Carbaryl	Added	3.0	5.0	4.0	5.0
	Found	2.96±0.22	4.76±0.32	3.91±0.23	4.83±0.26
Fenobucarb	Added	3.0	5.0	5.0	6.0
	Found	2.79±0.01	4.66±0.26	4.91±0.19	5.68±0.27

The detection limits have been calculated by means of different procedures. Hence, the Winefordner and Long[11] and the Clayton[12] methods have been applied as well as that based on the reproducibility of the analyte response at very low concentration.[13] The obtained values are in Table 3, and we can observe that those calculated by the Clayton method with specified assurance probabilities are slightly greater. The proposed procedure has been applied to river water samples, spiked with these compounds and the results are in Table 4.

CONCLUSIONS

In the analysis of N-methylcarbamates, methods of high performance liquid chromatography with electrochemical detection present advantages with respect to the UV or flourimetric detection, as high sensitivity, good reproducibility and easy chemical derivatization procedures. Hence, in the proposed method in which a coulometric detector is used, detection limits near 1 x 10^{-9} M are achieved, and only a previous step of alkaline hydrolysis is needed. This step is, however, simple and quick and does not require any non aqueous medium. Because of these characteristics, this procedure is recommended for controlling residues of these kinds of pesticides in natural waters.

ACKNOWLEDGEMENTS

The authors are grateful to the Central Nuclear de Almaraz (Project CNA93-07) and the CICYT of Spain (Project AL195-1538) for their financial support

REFERENCES

1. M. Olek, F. Blanchard, G. Sudraud, J. Chromatogr., **325**, 239 (1985).

2. R. T. Krause, J. Chromatogr., **442**, 333 (1988).

3. W. J. Mayer, M. K. Greengerg, J. Chromatogr., **211**, 135 (1981).

4. M. B. Thomas, P. E. Sturrock, J. Chromatogr., **357**, 318 (1986).

5. S. Kaway, K. Goto, K. Kano, T. Kubota, J. Chromatogr., **442**, 451 (1988).

6. B. S. Seidel, W. Faubel, H. J. Ache, Kernforschungazent Karlsruhe KfK 5182, pp58, Berlin (1993).

7. P. T. Kissinger, K. Bratin, W. P.King, J. R. Rice, in **Pesticide Analytical Methodology**, J. Harvey Jr., G. Zweig eds, ACS Symposium Series n° 136, Washington, DC, pp 57-88 (1980).

8. K. D. Khalaf, A. Morales-Rubio, M. de la Guardia, Anal. Chim. Acta, **280**, 231 (1993).

9. A. Guiberteau, T. Galeano Díaz, F. Salinas, J .M. Ortiz, Anal. Chim. Acta, **305**, 219 (1995).

10. A. Guiberteau, T. Galeano Diaz, F. Salinas, J. M. Ortiz, J. M. Kauffmann, submitted to Electroanalysis.

11. J. D. Winefordner, G. L. Long, Anal. Chem., **55**, 712A (1983).

12. C. A. Clayton, J. W. Hines, P. D. Elkins, Anal. Chem., **59**, 2506 (1987).

13. J. A. Glaser, D. L. Foerst, G. D. Mckee, S. A. Quave, W. L. Budde, Environmental Science and Technology, **15**, 1427 (1981).

Received January 8, 1996
Accepted March 23, 1996
Manuscript 4071

J. LIQ. CHROM. & REL. TECHNOL., 19(16), 2691–2698 (1996)

ANALYSIS OF ANTINEOPLASTIC POLYSACCHARIDES FROM *MYCOBACTERIUM BOVIS* BCG VACCINE BY HIGH PERFORMANCE ANION EXCHANGE CHROMATOGRAPHY WITH PULSED AMPEROMETRIC DETECTION

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ABSTRACT

Aqueous extracts of *Mycobacterium bovis* BCG vaccine, Tice® substrain, showed potent antineoplastic activity against a murine S180 sarcoma cell line in vivo. Following Sephadex LH-20 chromatography, one fraction (PS1A) was found to have antineoplastic activity, and contains complex polysaccharides. PS1A was further separated into four subfractions by Sephadex G-75 column chromatography. An HPLC method has been established for the analyses of polysaccharides in PS1A and its subfractions. Using a Dionex DX500 HPLC system, separation was achieved on a CarboPac PA1 anion exchange column by gradient elution with 0.1 M NaOH and 0.9 M NaOH/1 M NaOAc. Pulsed amperometry with three potential waveforms (E1=0.1 v, E2=0.7v and E3=-0.1v) was used for detection.

The HPLC chromatogram of PS1A showed three major peaks with retention times of 8.5, 15.5 and 19.25 min., respectively, and these three peaks have been identified in the subfractions of PS1A.

INTRODUCTION

Attenuated *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) has been used as a tuberculosis vaccine for more than 70 years. In 1990, it was approved by the U.S. Food and Drug Administration as an immunotherapeutic agent for the treatment of superficial bladder cancer.[1] A boiling water extract of BCG, termed PS1, showed antineoplastic activity in the murine S180 sarcoma model in vivo.[2] The antineoplastic components of PS1 have been separated by chromatography using both Sephadex LH-20 and Sephadex G-75 columns, and have been shown to be high molecular weight glycans.[3] In order to develop a therapeutic agent from PS1, its production, formulation and metabolism need to be studied. However, before doing any of these functions, it is imperative to develop a rapid and accurate method for the analysis of these antineoplastic polysaccharides.

High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) has been developed recently for the analysis of carbohydrates.[4] At high pH, normally 11-13, the hydroxyl groups of carbohydrates dissociate and become negatively charged, allowing separation by anion exchange chromatography. Pulsed amperometry is more sensitive and selective than either ultraviolet spectrophotometry or refractometry, and can detect picomolar quantities of monosaccharides. HPAEC-PAD has been used for the analysis of monosaccharides, oligosaccharides and polysaccharides. For example, polysaccharides with molecular weights up to 9 kDa have been analyzed using this method.[5] We report here the separation of antineoplastic polysaccharides from BCG using this technique.

MATERIALS AND METHODS

Sodium hydroxide solution (50% w/w), phenol (white loose crystals), sulfuric acid, and acetic acid (glacial) were all purchased from Fisher Scientific (Itasca, IL) and sodium acetate trihydrate from Fluka Chemika (Ronkonkoma, NY). Antineoplastic polysaccharide samples were prepared from freeze-dried ampoules of *Mycobacterium bovis* BCG vaccine, Tice® substrain (lot 105 x 2), previously prepared at this Institute and stored at -20°C. Deionized, distilled water was used for the preparation of HPLC eluents.

Fractionation of PS1

The preparation and fractionation of PS1 have been described previously.[3] Briefly, a solution of PS1, a boiling water extract of BCG, was applied to a Sephadex LH-20 column (Pharmacia Fine Chemicals, Piscataway, NJ), the column eluted with distilled water, and the eluent assayed for carbohydrates by a phenol/sulfuric acid method.[6] The antineoplastic activity of the resultant fractions was determined with a quantitative murine S180 sarcoma assay previously described.[7]

The active fraction, termed PS1A, was applied to a Sephadex G-75 column, and the resultant fractions were also assayed by the phenol/sulfuric acid method and the S180 sarcoma animal model. Animal test data are not reported here. Identified fractions had antineoplastic activity, although to varying degrees.

Analysis of PS1A and its Subfractions with HPAEC-PAD

The HPAEC-PAD method was developed on a Dionex (Sunnyvale, CA) DX500 HPLC system which includes a GP40 quaternary gradient pump, a CarboPac PA1 anion exchange column (4x250 mm), and an ED40 electrochemical detector (gold working electrode). The system was interfaced with a Peaknet chromatography work station. All eluents were degassed, and the flow rate was 1 mL/min.

Samples of PS1A and its subfractions (mg/mL) were dissolved in deionized, distilled water, centrifuged at 10,000 rpm in a microcentrifuge (Fisher Scientific, model 59A), and the supernatant used for the subsequent HPLC analyses.

RESULTS AND DISCUSSION

PS1 was separated into three fractions, designated PS1A, B and C, with the Sephadex LH-20 column. The antineoplastic fraction determined by S180 sarcoma assay, PS1A, was further separated into four fractions with the Sephadex G-75 column, designated PS1A1 through 4.

Separation and detection conditions of the antineoplastic polysaccharides were studied. It was found that the gradient elution program (Table 1) with solvents (a) 0.1 M NaOH and (b) 0.9 M NaOH/1 M NaOAc readily separated polysaccharide components. The waveform of the electrochemical detector for the analysis of polysaccharides is shown in Table 2.

Table 1

GP40 Gradient Program A: 0.1M NaOH; B: 0.1M NaOH/0.9 M NaOAc

Time (min.)	Flow (mL/min)	%A	%B
Init.	1	90	10
3	1	90	10
20	1	30	70
25	1	30	70
30	1	90	10

Table 2

ED40 Detector Waveform

Time (s)	Potential (v)	Integration
0.00	0.1	
0.20	0.1	begin
0.40	0.1	end
0.41	0.7	
0.60	0.7	
0.61	-0.1	
1.0	-0.1	

Using the established procedure, PS1A and its subfractions were analyzed. Figure 1 shows the HPLC chromatogram of PS1A, with three major peaks at retention times of 8.5, 15.5 and 19.25 min., respectively.

Figure 2 is the HPLC chromatogram of PS1A1, which showed a major peak with a retention time of 15.5 min. PS1A1 was characterized as a glucan with a dominant α-1,6 linkage.[3] Figure 3 is the HPLC chromatogram of PS1A2, which showed two peaks with retention times of 15.6 (PS1A2.1) and 19.25 min. (PS1A2.2). Although the molecular weights of PS1A1 and PS1A2.1 were different, 70 kDa and 20 kDa, respectively,[3] they behaved similarly on the anion exchange column. Accordingly, the retention times of PS1A1 and PS1A2.1 were similar, and these two components overlapped on the HPLC chromatogram of PS1A. On the other hand, PS1A2.1 and PS1A2.2

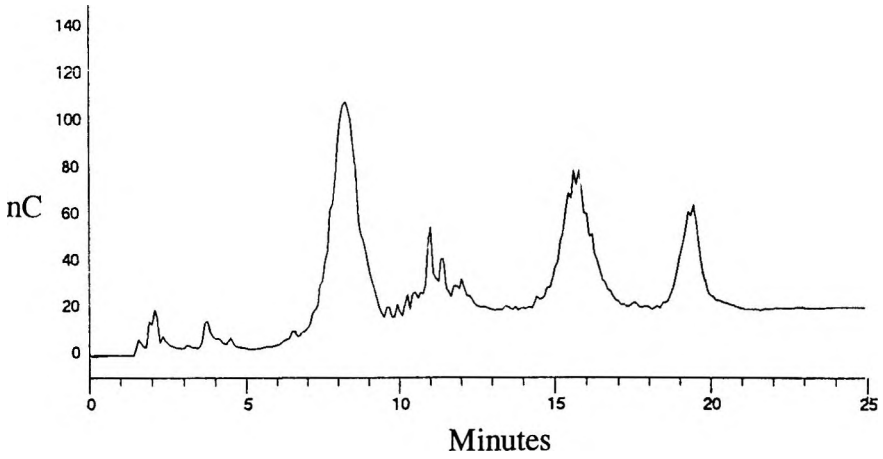

Figure 1. The HPLC chromatogram of PS1A. Conditions: CarboPack PA1 column; gradient elution with 0.1 M NaOH and 0.1 M NaOH/0.9 M NaOAc; flow rate, 1 mL/min.; detector, ED 40 electrochemical detector, pulsed amperometric.

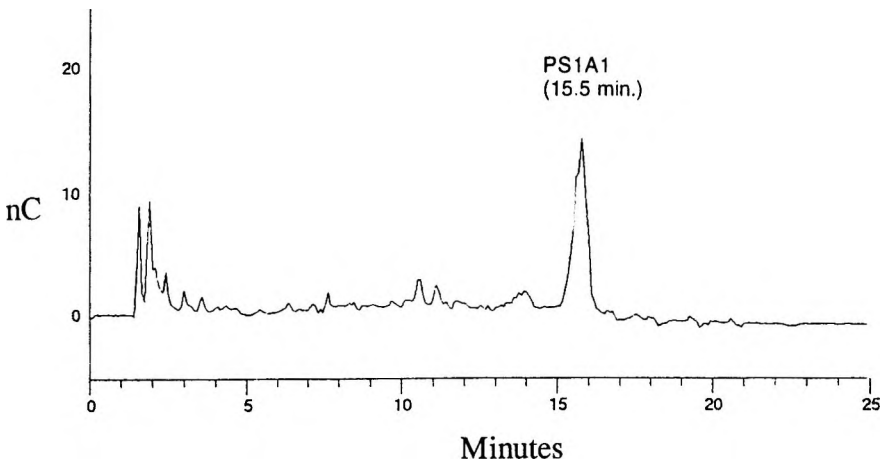

Figure 2. The HPLC chromatogram of PS1A1. Conditions: same as Figure 1.

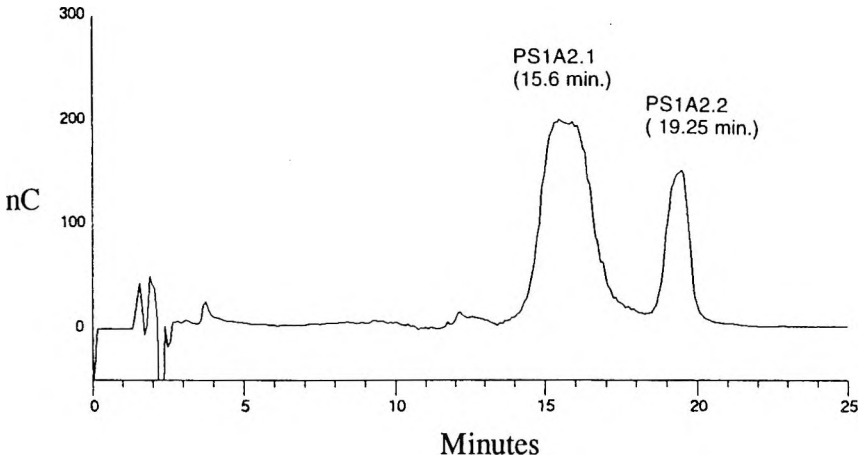

Figure 3. The HPLC chromatogram of PS1A2. Conditions: same as Figure 1.

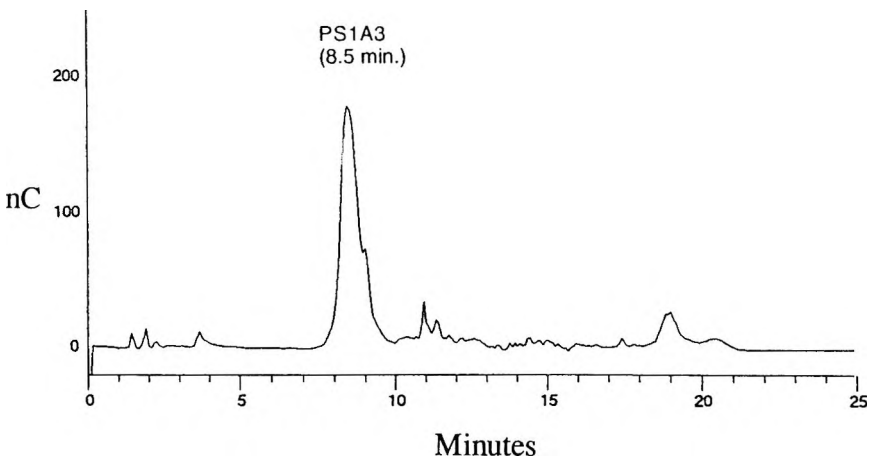

Figure 4. The HPLC chromatogram of PS1A3. Conditions: same as Figure 1.

had similar molecular weights, but behaved differently on the ion exchange column, and were easily separated. It is known that PS1A2.1 and PS1A2.2 are chemically different: the former is a arabinomannan, the latter is an arabinogalactan (unpublished results).

Figure 4 is the HPLC chromatogram of PS1A3, demonstrating one major peak with a retention time of 8.5 min. Monosaccharide compositional analysis showed that PS1A3 contained glucose, arabinose, galactose and an unidentified sugar (unpublished data).

CONCLUSION

An HPAEC-PAD method has been developed on a Dionex DX500 HPLC system for the analysis of antineoplastic polysaccharides from BCG.

PS1 sub-fractions, PS1A and PS1A1-4, were analyzed by this method, and major peaks corresponding to these subfractions were identified on their HPLC chromatograms.

ACKNOWLEDGEMENT

We thank Ms. Afeng Li for the preparation of PS1 and its subfractions.

REFERENCES

1. S. L. Nightingale, J. Am. Med. Assoc. **264**, 677 (1990).

2. Y. Lou, M. E. Klegerman, A. Muhammad, X. Dai, M. J. Groves, Anticancer Res., **14**, 1469-1476 (1994).

3. R. Wang, M. E. Klegerman, I. Marsden, M. Sinnott, M. J. Groves, Biochem. J., **311**, 867-872 (1995).

4. M. R. Hardy, R. R. Townsend, Proc. Natl. Acad. Sci. USA, **85**, 3289-3293 (1988).

5. A. T. Hotchkiss, Jr., K. B. Hicks, Anal. Biochem., **184**, 200-206 (1990).

6. M. Dubois, K. Gilles, J. K. Hamilton, P. A. Rebers, F. Smith, Anal Chem., **28**, 350-356 (1956).

7. M. E. Klegerman, L. Ujjainwala, P. Zeunert, Cancer Lett., **56**, 137-145 (1991).

Received February 18, 1996
Accepted March 18, 1996
Manuscript 4084

J. LIQ. CHROM. & REL. TECHNOL., 19(16), 2699-2708 (1996)

CHIRAL HPLC SEPARATIONS FOR PROCESS DEVELOPMENT OF S-(+)-ISOBUTYL GABA, A POTENTIAL ANTI-EPILEPTIC AGENT

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ABSTRACT

In order to develop an economically-viable route to make S-(+)-isobutyl GABA ((S)3-aminomethyl-5-methylhexanoic acid), many intermediates required chiral analyses to determine their enantiomeric purities. Multiple chiral stationary phases were employed for this purpose. Chiralcel OD-H and Chiralpak AD columns resolved more than 80 % of the tested intermediates.

INTRODUCTION

S-(+)-Isobutyl GABA, shown in Figure 1, is presently under active development as a potential anti-epileptic agent at Parke Davis. Earlier routes to produce it were extremely costly. Our goal was to find a cost-competitive route to produce the compound. Several synthetic routes were explored and reported.[1] Chiral methods were required for (a) screening of chiral agents for chemical resolution, (b) screening products from enzymatic resolutions, (c) purity determination of the resolving chiral agent, and (d) purity determination of the product. Rapid and direct chiral methodology was vitally needed.

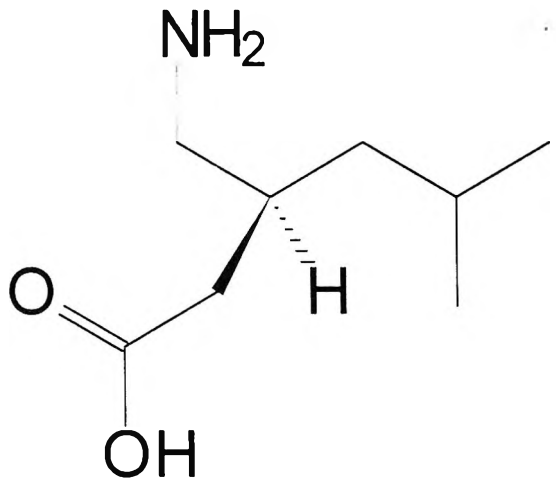


Figure 1. Structure of (S)-(+)-isobutyl GABA.

Chiral HPLC methods for process development of other potential drugs using cellulose carbamate-based and crown ether stationary phases were described previously.[2,3] In this work, we have extended the use of other polysaccharide based stationary phases for chiral separation of various intermediates encountered during the process development to produce (S)-(+)-isobutyl GABA.

EXPERIMENTAL

Apparatus

The liquid chromatographic system consisted of a Hitachi L-6200 intelligent pump, a Micromeritics 728 autosampler and a Valco injector with a 20 μL loop, a Hitachi L-4000H variable wavelength UV detector and a Waters 410 differential refractometer in series, and a Hitachi D-2500 Chromato-Integrator.

The chiral columns are Chiralcels OD, OD-H, OJ, Chiralpak AD, and Crownpak CR(+). They are all 250 x 4.6mm I.D. except the Crownpak column which is 150 x 4.0 mm I.D. They were purchased from Chiral Technologies, Inc., Exton, PA.

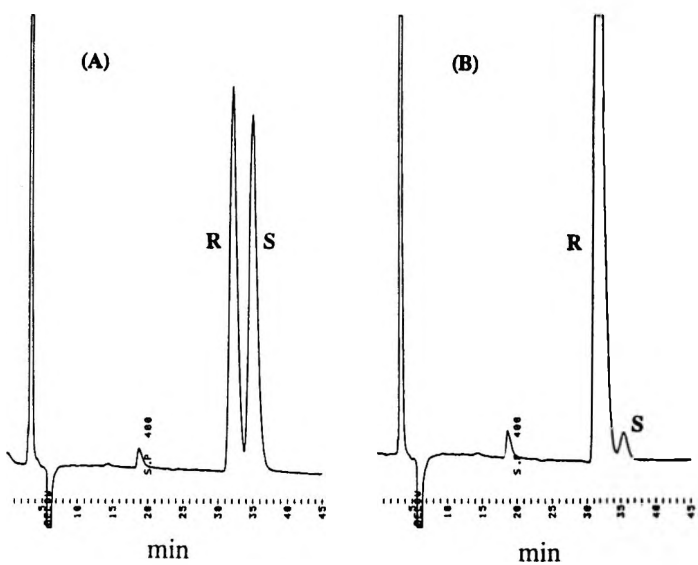


Figure 2. Separation of (A) a racemic mixture and (B) an enriched sample of 3-carbamoylmethyl-5-methylhexanoic acid, compound **13** using the conditions given in Table 1.

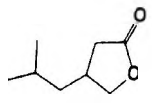
Chemicals

Hexane and isopropanol (HPLC grades) were obtained from EM Science, Gibbstown, NJ. Formic acid (88%) was purchased from J. T. Baker Inc., Phillipsburg, NJ. (S)-(+)Mandelic acid, DL-mandelic acid, (R)-(+)-1-phenylethylamine, (±)-1-phenylethylamine, (R)(+)-1-(4-chlorophenyl)-ethylamine and (S)-(-)-1-(4-chlorophenyl)-ethylamine were purchased from Zeeland Chemical, Zeeland, MI. All other compounds of interest were synthesized by the Chemical Development Department, Parke-Davis Pharmaceutical Research Division, Holland, MI.

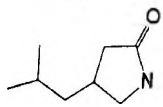
Chromatographic Conditions

The preparation of mobile phase depended upon the type of column used. For Chiralcel OD, OD-H, OJ and Chiralpak AD columns, the mobile phase was either hexane/isopropanol or hexane/isopropanol with a small amount of formic acid added. For the Crown ether column, the mobile phase was aqueous perchloric acid or aqueous perchloric acid/methanol. The detection was RI unless otherwise stated. The amount of sample injected was 50 to 100 μg. The flow rate was varied.

Compounds Studied



$(C_8H_{14}O_2)$
1



$(C_8H_{14}NO)$
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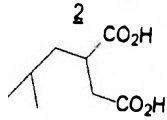

$(C_8H_{14}O_4)$
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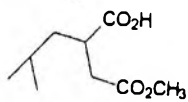

$(C_9H_{16}O_4)$
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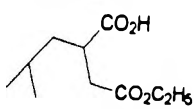

$(C_{10}H_{18}O_4)$
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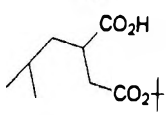

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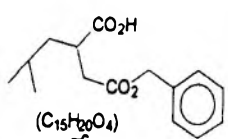

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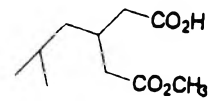

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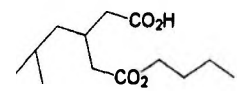

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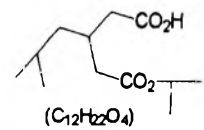

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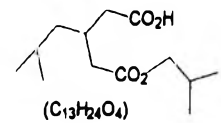

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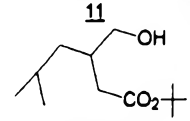

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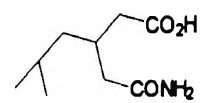
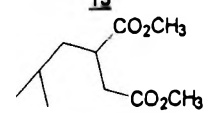


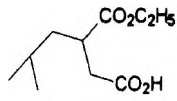
$(C_9H_{17}NO_3)$
13[c]



$(C_{10}H_{18}O_4)$
14

(continued)

Compounds Studied (continued)



($C_{10}H_{18}O_4$)
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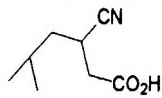

($C_8H_{13}NO_2$)
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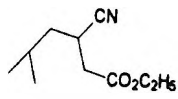

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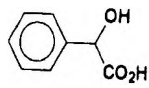
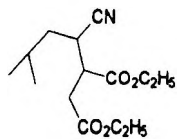


($C_8H_8O_3$)
18[c]



($C_{14}H_{23}NO_4$)
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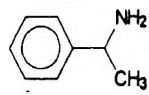
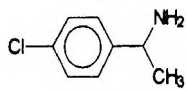
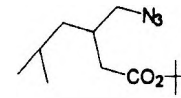


($C_8H_{11}N$)
20[c]



($C_8H_{10}ClN$)
21[c]



($C_{12}H_{23}N_3O_2$)
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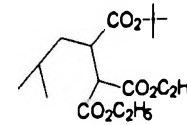

($C_{17}H_{30}O_6$)
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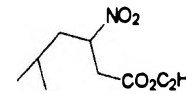
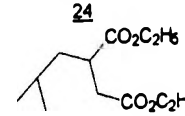
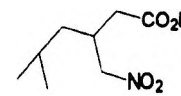


($C_8H_{17}NO_4$)
24



($C_{12}H_{22}O_4$)
25



($C_8H_{15}NO_4$)
26[c]

Table 1

Enantiomeric Separations for Various Compounds Encountered During Process Development of (S)-(+)-Isobutyl GABA[c,e]

Compound	Type of Column	Mobile Phase[a]	Flow[b]	k'_1[d]	α[d]	R_s[d]
1	Chiralpak AD	hexane/IPA (99/1)	1.0	4.30	1.13	2.10
2	Chiralpak AD	hexane/IPA (97/3)	1.0	4.11	1.17	1.64
3	Chiralcel OD-H	hexane/IPA/HCO_2H (95/5/0.1)	1.0	1.33	1.46	3.38
4	Chiralcel OD-H	hexane/IPA/HCO_2H (98.5/1.5/0.1)	1.0	3.16	1.38	4.92
5	Chiralcel OD-H	hexane/IPA/HCO_2H (99/1/0.1)	0.5	3.69	1.06	1.22
6	Chiralpak AD	hexane/IPA/HCO_2H (97/3/0.1)	1.0	1.03	2.28	5.02
7	Chiralcel OD	hexane/IPA/HCO_2H (80/20/0.1)	0.5	0.24	2.27	2.35
8	Chiralcel OD-H	hexane/IPA/HCO_2H (98.5/1.5/0.1)	1.0	4.86	1.09	1.27
9	Chiralpak AD	hexane/IPA/HCO_2H (100/1/0.1)	1.0	4.17	1.11	1.16
10	Chiralcel OJ	hexane/IPA/HCO_2H (100/1/0.1)	0.5	2.47	1.09	0.92
11	Chiralcel OD-H	hexane/IPA/HCO_2H (98/2/0.1)	0.3	1.13	1.10	1.98
12	Chiralcel OD-H	hexane/IPA/HCO_2H (100/0.5/0.1)	0.5	3.21	1.09	1.62

(continued)

Table 1 (continued)

Compound	Type of Column	Mobile Phase[a]	Flow[b]	k'$_1$[d]	α[d]	R_s[d]
13	Chiralcel OD-H	hexane/IPA/HCO_2H (96/4/0.1)	1.0	10.14	1.11	1.49
14	Chiralcel OD-H	hexane/IPA/HCO_2H (98.5/1.5/0.1)	1.0	0.76	2.54	7.79
15	Chiralcel OD-H	hexane/IPA/HCO_2H (95/5/0.1)	0.5	0.53	1.74	5.68
16	Chiralcel OD-H	hexane/IPA/HCO_2H (98/2/0.1)	1.0	5.39	1.07	1.17
17	Chiralcel OD-H	hexane/IPA (98/2)	0.5	1.15	1.13	1.47
18	Chiralcel OD-H	hexane/IPA/HCO_2H (95/5/0.1)	1.0	3.80	1.22	1.96
19	Chiralcel OD-H	hexane/IPA (98/2)	0.5	1.14	1.13	1.64
20	Crownpak CR(+)	aq. $HClO_4$ (pH 1.5)	1.0	7.15	1.51	2.96
21	Crownpak CR(+)	CH_3OH/aq. $HClO_4$ (10/90; pH 1.5)	1.2	13.98	1.53	3.22
22	Chiralpak AD	hexane/IPA (100/1)	0.5	0.60	1.28	1.19
23	Chiralpak AD	hexane/IPA (98/2)	0.6	0.71	1.86	7.23
24	Chiralcel OD-H	hexane/IPA/HCO_2H (100/0.5/0.1)	1.0	1.73	1.10	1.35
25	Chiralcel OD-H	hexane/IPA/HCO_2H (95/5/0.1)	0.5	0.27	2.21	5.10

(continued)

Table 1 (continued)

Compound	Type of Column	Mobile Phase[a]	Flow[b]	k'_1[d]	α[d]	R_s[d]
26	Chiralcel OD-H	hexane/IPA/HCO_2H (80/20/0.1)	1.0	1.53	1.34	2.53

[a] The ratio is volume ratio; IPA = isopropanol.
[b] Flow rate is in mL/min.
[c] Detection is UV @ 214 nm for compounds **13**, **20**, **21** and **26**; 220 nm for compound **7**; 225 nm for compound **6**; 235 nm for compound **18**.
[d] $k`_1$ is the capacity factor of the first eluted enantiomer; α is the stereoselectivity; R_s is the resolution factor.
[e] Structures of the compounds studied are as given above.

RESULTS AND DISCUSSION

The chiral recognition mechanism for the polysaccharide derivative based stationary phases is not clear. Their abilities to achieve the discrimination of enantiomers appears to depend on the conformation of the main chain and the structure of the substituents.[4] Numerous examples have demonstrated the applications of this type of column for chiral resolution.[5] We have found some of these columns very useful for our chiral analytical work.

Table 1 gives k'_1, α, and R_s along with the conditions for the compounds resolved. We believe these are the first direct chiral HPLC separations for these compounds reported, with the exception of mandelic acid and 1-phenylethylamine.[5]

Mobile phase consisted of hexane/isopropanol for most non-carboxylic acid containing compounds. For carboxylic acid-containing compounds, the addition of a small amount of formic acid in the mobile phase was required to facilitate elution. Optimization of the resolution was carried out by decreasing the amount of isopropanol in the mobile phase, changes in flow rate or combinations of both. As expected, greater enantiomeric resolution is achieved when one of the functional groups is adjacent to the chiral center;[6] see compounds **4** and **8**. Under the same conditions, compound **4** has greater α (1.38) and R_s (4.92) than compound **8** (α = 1.09, R_s = 1.27) although compound **4** has a shorter retention time.

3-Carbamoylmethyl-5-methylhexanoic acid, compound **13**, was incorporated into one potential process to produce (S)-(+)isobutyl GABA. We also used Chiralcel OJ and AD columns for the attempted enantiomeric determination of this compound. However, the best separation obtained using

these two columns gave only partial resolution. The enantiomeric separations of a racemic mixture and an enriched sample for this compound are given in Figures 2(A) and 2(B), respectively.

CONCLUSIONS

Direct chiral HPLC technique has become an increasingly important tool in new drug development. It has become an indispensible analytical technique for process development of a chiral drug. We have demonstrated this using a combination of chiral stationary phases for process development of S-(+)-isobutyl GABA. In particular, the use of Chiralcel OD-H and Chiralpak AD columns enabled us to resolve more than 80 % of the tested intermediates.

ACKNOWLEDGMENTS

We gratefully thank Drs. Donald E. Butler and William T. Suggs for their helpful comments. We would also like to thank the organic chemistry colleagues at Parke Davis for their cooperation throughout this work, and Ms. Sandy Dokter for the typing of this manuscript.

REFERENCES

1. M. S. Hoekstra, "Chemical Development of CI-1008, a Pure Enantiomeric Anticonvulsant," presented at the Symposium on Synthetic Organic Chemistry held at Hope College, Holland, Michigan, June 9, 1995; U. S. Patent sreial # 08/458,950; 6-2-95.

2. S. Lin, "Direct Liquid Chromatographic Separation of 2,3-Dihydro-2-ethylbenzofuran-2-carboxylic Acid Enantiomers using a Cellulose Carbamate-Based Chiral Stationary Phase Column," J. Liquid Chromatogr., **18**, 2611-2619 (1995).

3. S. Lin, N. J. Maddox, "Direct Chromatographic Separation of 2-(2-Amino-1,3-thiazol-4-yl)methylglycine and its Methyl Ester Enantiomers using a Chiral Crown Ether Column," J. Liquid Chromatogr., **18**, 1947-1955 (1995).

4. K. Oguni, H. Oda, A. Ichida, "Development of Chiral Stationary Phases Consisting of Polysaccharide Derivatives," J. Chromatogr., A, **694**, 91-100 (1995).

5. **Application Guide for Chiral Column Selection**, Daicel Co., 2nd edition, 1995.

6. M. Hilton, D. W. Armstrong, "Evaluation of the Enantiomeric Separation od Dipeptides using a Chiral Crown Ether Column," J. Liquid Chromatogr., **14**, 3673-3683 (1991).

Received March 11, 1996
Accepted April 3, 1996
Manuscript 4109

J. LIQ. CHROM. & REL. TECHNOL., 19(16), 2709 (1996)

corrected 22 Jan. 97/AP

ERRATUM

Z. Radisavljevic, et al., "Determination of Intracellular and Extracellular Nitrite and Nitrate by Anion Chromatography." [J. Liq. Chrom. & Rel. Technol., **19**(7), 1061-1079 (1996).]

In the **REFERENCES** section (pp. 1077-1079), several references were inadvertently omitted. They are as follows:

18. C. Stein, H. G. Classen, and G. Schwedt, Clin. Chim. Acta, **175**, 167-174 (1988).

19. H. J. Boermans, Am. J. Vet. Res., **51**, 491-495 (1990)

20. J. R. Thayer, R. C. Huffaker, Anal. Biochem., **102**, 110-119 (1980).

21. H. H. Schmidt, T. D. Warner, M. Nakane, U. Forstermann, F. Murad, Mol Pharmacology, **41**, 615-624 (1992).

22. R. L. Gamelli, M. George, D. J. Dries, Z. Radisavljevic, J. Trauma, **37**, 155 (1994).

23. Z. Radisavljevic, Am. J. Med. Sci., **302**, 284-286 (1991).

24. Z. Radisavljevic, Am. J. Hypertens., **8**, 761-767 (1995).

25. R. S. Balaban, Am. J. Physiol., **258**, C377-389 (1990).

26. G. Mautino, N. Paul-Eugene, P. Chanez, A. M. Vignola, J. P. Kolb, J. Bousquet, B. Duglas, J. Leukoc. Biol., **56**, 15-20 (1994).

27. B. Bartholomew, Food Chem. Toxicol., **22**, 541 (1984).

J. LIQ. CHROM. & REL. TECHNOL., 19(16), 2711 (1996)

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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:

- Introduction to HPLC Theory
- 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 founder and Editor-in-Chief of the Journal of Liquid Chromatography & Related Technologies, Editor of Instrumentation Science & Technology, and Series Editor of the Chromatographic Science Book Series. He has been intimately involved with liquid chromatography for more than 35 years; he pioneered the development of modern HPLC technology. Dr. Cazes was Professor-in-Charge of the ACS Short Course and the ACS Audio Course on Gel Permeation Chromatography for many years.

Details 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; E-Mail: jcazes@voicenet.com.

J. LIQ. CHROM. & REL. TECHNOL., 19(16), 2713–2721 (1996)

LIQUID CHROMATOGRAPHY CALENDAR

1996

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.

SEPTEMBER 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 International 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 17 - 20: 10th International Symposium on Cap|illary Electrophoresis, Prague, Czech Republic. Contact: Dr. B. Gas, Dept of Physical Chem, Charles University, Albertov 2030, CZ-128 40 Prague 2, Czech Republic.

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.

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 East 47th Street, New York, NY 10017-2395, USA.

NOVEMBER 11 - 20: 2nd Latin-American Conference on Biomedical, Biopharmaceutical and Industrial Applications of Capillary Electrophoresis, Santiago, Chile. Dr. E. Guerrero, Servicio Medico Legal, Avenida de la Paz 1012, Santiago, Chile.

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.

DECEMBER 17 - 20: First International Symposium on Capillary Electrophoresis for Asia-Pacific, Hong Kong. Contact: Dr. S. F. Y. Li, Dept of Chemistry, National University of Singapore, Kent Ridge Crescent, Singapore 119260, Republic of Singapore.

1997

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

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

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

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

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

MAY 27 - 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.

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

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

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

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

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

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

1998

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

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

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

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

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

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

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 7 - 12: PittCon '99, Orlando, Florida. Contact: PittCon '99, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA. Tel: (800) 825-3221; FAX: (412) 825-3224.

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

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

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

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; Email: natlmtgs@acs.org.

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.

The **Journal of Liquid Chromatography & Related Technologies** will publish, at no charge, announcements of interest to scientists in every issue of the journal. To be listed in the Liquid Chromatography Calendar, we will need to know:

a) Name of the meeting or symposium,
b) Sponsoring organization,
c) When and where it will be held, and
d) Whom to contact for additional details.

Incomplete information will not be published. You are invited to send announcements to **Dr. Jack Cazes, Editor, Journal of Liquid Chromatography & Related Technologies, P. O. Box 2180, Cherry Hill, NJ 08034-0162, USA.**

INSTRUCTIONS TO AUTHORS

The ***Journal of Liquid Chromatography & Related Technologies*** is published in the English language for the rapid communication of research results in liquid chromatography and its related sciences and technologies.

Directions for Submission

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

Submission of a manuscript on diskette, in a suitable format, will significantly expedite its publication.

Manuscripts and computer diskettes should be mailed to the Editor:

Dr. Jack Cazes
Journal of Liquid Chromatography & Related Technologies
P. O. Box 970210
Coconut Creek, FL 33097

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Due to the short production time for papers in this journal, it is essential to order reprints immediately upon receiving notification of acceptance of the manuscript. A reprint order form will be sent to the author with the letter of acceptance for the manuscript. Reprints are available in quantities of 100 and multiples thereof. Twenty (20) free reprints will be included with orders of 100 or more reprints.

Format of the Manuscript

NOTE: Failure to adhere to the following guidelines will delay publication of a manuscript.

1. The preferred dimensions of the printed area of a page are
   6" (15.2 cm) width by 8.5" (21.6 cm) height.
   Use Times Roman 12 point font, if possible.

The general organization of the manuscript should be:

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

2. **Title & Authors**: The entire title should be in bold-face capital letters and centered within the width of the printed area, located 2 inches (5.1 cm) from the top of the page. This should be followed by 2 lines of space, then by the names and addresses of the authors, also centered, in the following manner:

A SEMI-AUTOMATIC TECHNIQUE FOR THE SEPARATION AND DETERMINATION OF BARIUM AND STRONTIUM IN WATER BY ION EXCHANGE CHROMATOGRAPHY AND ATOMIC EMISSION SPECTROMETRY

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

3. **Abstract**: The heading **ABSTRACT** should be typed boldface, capitalized and centered, 2 lines below the addresses. This should be followed by a single-spaced, concise abstract. Allow 2 lines of space below the abstract before beginning the text of the manuscript.

4. **Text Discussion**: Whenever possible, the text discussion should be divided into major sections such as

INTRODUCTION
MATERIALS
METHODS
RESULTS
DISCUSSION
ACKNOWLEDGMENTS

These **major headings** should be separated from the text by two lines of space above and one line of space below. Each major heading should be typed boldface, in capital letters, centered.

Secondary headings, if any, should be placed flush with the left margin, and have the first letter of main words capitalized. Leave two lines of space above and one line of space below secondary headings.

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

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

7. **References** should be numbered consecutively and placed in a separate section at the end of the manuscript. They should be typed single-spaced, with one line space between each reference. Each reference should contain names of all authors (with initials of their first and middle names); do not use *et al.* for a list of authors. Abbreviations of journal titles will follow the American Chemical Society's Chemical Abstracts List of Periodicals. The word **REFERENCES**, in boldface type, should be capitalized and centered above the reference list.

Following are acceptable reference formats:

Journal:

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

Book:

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

Chapter in a Book:

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

8. Each page of manuscript should be numbered lightly, with a light blue pencil, at the bottom of the page.

9. Only standard symbols and nomenclature, approved by the International Union of Pure and Applied Chemistry (IUPAC) should be used. **Hand-drawn characters are not acceptable.**

10. Material that cannot be typed, such as Greek symbols, script letters and structural formulae, should be drawn carefully with dark black India ink. Do not use any other color ink.

Additional Typing Instructions

1. The manuscript must be prepared on **good quality white bond paper**, measuring approximately 8½ x 11 inches (21.6 cm x 27.9 cm). International paper, size A4 is also acceptable. The typing area of the first page, including the title and authors, should be 6" (15.2 cm) wide by 8.5" (21.6 cm) height.

2. All text should be **typed single-spaced**.

3. It is essential to use **dark black** typewriter or printer ribbon so **that clean, clear, solid characters** are produced. Characters produced with a dot/matrix printer are not acceptable, even if they are "near letter quality" or "letter quality." Erasure marks, smudges, hand-drawn corrections and creases are not acceptable.

4. **Tables** should be typed as part of the text, but in such a way as to separate them from the text by a 2-line space above and below the table. Tables should be inserted in the text as close to the point of reference as possible. **A table may not be longer than one page**. If a table is larger than one page, it should be divided into more than one table. The word **Table** (followed by an Arabic number) should precede the table and should be centered above the table. The title of the table should have the first letters of all main words in capitals. Table titles should be typed single line spaced, across the full width of the table.

5. **Figures** (drawings, graphs, etc.) should be professionally drawn in black India ink on separate sheets of white paper, and should be placed at the end of the text. They should not be inserted into the body of the text. They should not be reduced to a small size. Preferred size for figures is from 5 inches x 7 inches (12.7 cm x 17.8 cm) to 8½ inches by 11 inches (21.6 cm x 27.9 cm). Photographs should be professionally prepared, black and white, *glossy* prints. A typewriter or lettering set should be used for all labels on the figures or photographs; they may not be hand drawn.

Captions for figures should be typed single-spaced on a separate sheet of white paper, along the full width of the type page, and should be preceded with the

word **Figure** and an Arabic numeral. All figures and lettering must be of a size that will remain legible after a 20% reduction from the original size. Figure numbers, name of senior author and an arrow indicating "top" should be written in light blue pencil on the back of the figure. Indicate the approximate placement for each figure in the text with a note written with a light blue pencil in the margin of the manuscript page.

6. The reference list should be typed single-spaced. A single line space should be inserted after each reference. The format for references should be as given above.

Manuscripts which require correction of English usage will be returned to the author for major revision.

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ISBN: 0—8247—9382—X

This book is printed on acid-free paper.

Marcel Dekker, Inc.
270 Madison Avenue
New York, NY 10016
(212) 696-9000

Hutgasse 4, Postfach 812
CH-4001 Basel, Switzerland
Tel. 061-261-8482

J. LIQ. CHROM. & REL. TECHNOL., 19(16), (1996)

Contents Continued

JOURNAL OF LIQUID CHROMATOGRAPHY
& RELATED TECHNOLOGIES

Volume 19, Number 16, 1996

CONTENTS

(continued on inside back cover)

MARCEL DEKKER, INC. New York, Basel, Hong Kong
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