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THE EFFECTS OF PORE DIAMETER AND LIGAND CHAIN LENGTH ON FAST LIQUID CHROMATOGRAPHY OF PROTEINS AND PEPTIDES

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ABSTRACT

Fast flow separations of proteins and peptides were successfully carried out on microbore columns containing reversed phase supports of three pore diameters. Increasing flowrates from 0.25 to 3 ml/min (1.2 mm/s - 14.4 mm/s) did not show any adverse effects on the separations when SynChropak RPP-1000 and RPP-4000, which have 1000Å and 4000Å pore diameters, were used. At 3 ml/min, the 1000Å and 4000Å pore diameters yielded narrower peaks for proteins than the 300Å support, whereas the latter exhibited better resolution for peptides. Analyses in less than five minutes were achieved.

A series of ligand chains (C-4, C-8 and C-18) showed few differences in retention or resolution for either protein or peptide standards. Longterm stability of the 300Å and 4000Å supports was in excess of 25,000 column volumes when run at 3 ml/min with 0.1% trifluoroacetic acid.

INTRODUCTION

A common assumption in HPLC is that increasing the flowrate or linear velocity will concommitantly decrease resolution by increasing peak widths. This is based on the van Deemter/Knox equation: $h = Av^{1/3} + B/v + Cv$. For proteins and peptides, the C or mass transfer term is primarily responsible for band spreading because the diffusion coefficients are so small, making $v = ud_p/D_m$ large (1). In these equations, h is the reduced plate height, v is the reduced linear velocity, u is the linear velocity, d_p is the particle diameter and D_m is the diffusion coefficient of the solute in the mobile phase. In size exclusion chromatography, the adverse effects of high linear velocity on peak width increase with the molecular weight of the solute, as would be expected (2). To the contrary, in reversed phase chromatography, general improvement in peptide resolution with increased flowrate has been observed (3). Denaturing and renaturing of proteins during reversed phase chromatography has also been seen to be flowrate dependent (5).

High flowrates have been used successfully to achieve fast separations by high performance liquid chromatography. Fast analyses have been demonstrated with 1-5 μm nonporous supports (4, 6-8), perfusive supports (9, 10) and large pore supports (11, 12). Short columns were essential to many of these applications so that rapid analysis times could be achieved without excessive back pressures.

The use of microbore columns to cut solvent consumption and to increase sensitivity and recovery has seen increasing popularity, particularly for the analysis of submicrogram samples (13-17). It was recently demonstrated that proteins and peptides could be separated in five minutes or less on microbore reversed phase columns run at 3 ml/min

(12). That study suggested that supports with 1000Å or 4000Å average pore diameters may be optimal for the technique. This report describes a study of the effects of pore diameter on the resolution of protein and peptide mixtures by reversed phase chromatography in microbore columns at high linear velocities. The effects of ligand chain length on resolution under these conditions are also examined. Because the linear velocities are considerably higher than those normally used for 2.1 mm I.D. columns, the stability of the columns was also tested.

EXPERIMENTAL

Chemicals

Ribonuclease A (MW 13,700), cytochrome c (MW 12,500), lysozyme (MW 14,400), transferrin (MW 81,000) and bovine serum albumin (BSA) (MW 68,000) were purchased from Sigma Chemical Company (St. Louis, MO). The decapeptide standard was from Synthetic Peptides Incorporated (Edmonton, Canada). Acetonitrile was from WAKO Pure Chemical Industries, Ltd. (Osaka, Japan). Methanol and isopropanol were from Baxter Scientific Products (McGaw Park, IL). Trifluoroacetic acid (TFA) was from Pierce Chemical Co. (Rockford, IL) or WAKO Pure Chemical Industries, Ltd. HPLC-grade water was from Baxter Scientific Products or WAKO Pure Chemical Industries, Ltd.

Methods

Gilson Model 303 or 305 pumps were used. The mixer was equipped with a 1.5 ml chamber. The acetonitrile experiments employed a Gilson Model 116 detector with a 12 µl cell. The alcohol experiments used an ISCO Model V^4 detector with a 3.5 µl cell. The systems were

controlled and data collected with a Gilson Model 715 data system. Rheodyne Model 7125 injectors were used.

SynChropak silica-based reversed phase columns (50 x 2.1mm) were used (Lafayette, IN). SynChropak RP-4, RP-8 and RP-P columns contained 6.5 µm particles with 300Å pores and C-4, C-8 and C-18 ligands. SynChropak RP4-1000, RP8-1000 and RPP-1000 were 7 µm with 1000Å pores and the same ligands. SynChropak RP4-4000, RP8-4000 and RPP-4000 were 10 µm with 4000Å pores and the same ligands. The C-18 and C-8 bonded phases were monomeric and the C-4 was polymeric.

Methods were as described previously for the acetonitrile experiments (12). The 35 min linear gradient was programmed to begin after the 3.5 ml volume of the mixer and tubing had passed. The alcohol gradients went from 0.1% TFA in water to 0.05% TFA in methanol or isopropanol and were not adjusted for the dead volume. The methanol gradient went from 10% to 100% in five minutes and the isopropanol gradient went from 0 - 15% in five minutes.

RESULTS AND DISCUSSION

Effect of Pore Diameter on Protein Resolution

It was previously shown that excellent resolution of five standard proteins could be maintained on microbore columns of 2.1 mm I.D. at flowrates of 3 ml/min (12). The system employed a gradient delay of 3.5 ml and 35 min gradients at varied flowrates from 0.25 ml/min (1.2 mm/s) to 4.5 ml/min (21.6 mm/s). Resolution by reversed phase chromatography was compared for octadecylsilyl (C-18) supports with 300Å, 1000Å and 4000Å pore diameters (12). The delay time differed by 12- to 18-fold,

depending on the flowrate. On the 4000Å support, the resolution of the mixture was better at 3 ml/min than at 0.25 ml/min. The gradient conditions were the same for each pore diameter, although the design of the experiment resulted in a different gradient volume for each flowrate. The gradient time was kept constant for these experiments; therefore, the slope of the gradient in %B/min was constant but the volume or (gradient time x flowrate) increased with flowrate.

Figure 1 compares the resolution of proteins on supports with three different pore diameters under identical conditions at a flowrate of 3 ml/min (14 mm/s linear velocity). Under these conditions, resolution on the 300Å support was definitely inferior to that of the 1000Å and 4000Å packings despite the larger particle diameter of the latter. As seen in the graphs in Figures 2a and 2b, resolution was similar on all three columns at 0.25 ml/min, a standard flowrate for 2.1 mm I.D. columns. As the flowrate was increased, resolution remained constant on the 300Å support but increased on the larger pore materials. This is more dramatic for the protein pair of lysozyme and transferrin than lysozyme and cytochrome c. Transferrin has a molecular weight of 81,000 Daltons whereas the other two proteins are smaller than 20,000 D.

Figure 2c illustrates that the resolution of transferrin and BSA did not change significantly with flowrate on any pore. It was observed that the peak width for BSA increased with flowrate on the 300Å support whereas it decreased slightly on the 1000Å and 4000Å supports, as shown in Figure 3. Despite this fact, the resolution on the 4000Å support was slightly less because the retention of BSA was 3-6% lower than on the other two supports. BSA also exhibited some heterogeneity under these conditions, as seen in Figure 1, giving higher and more variable

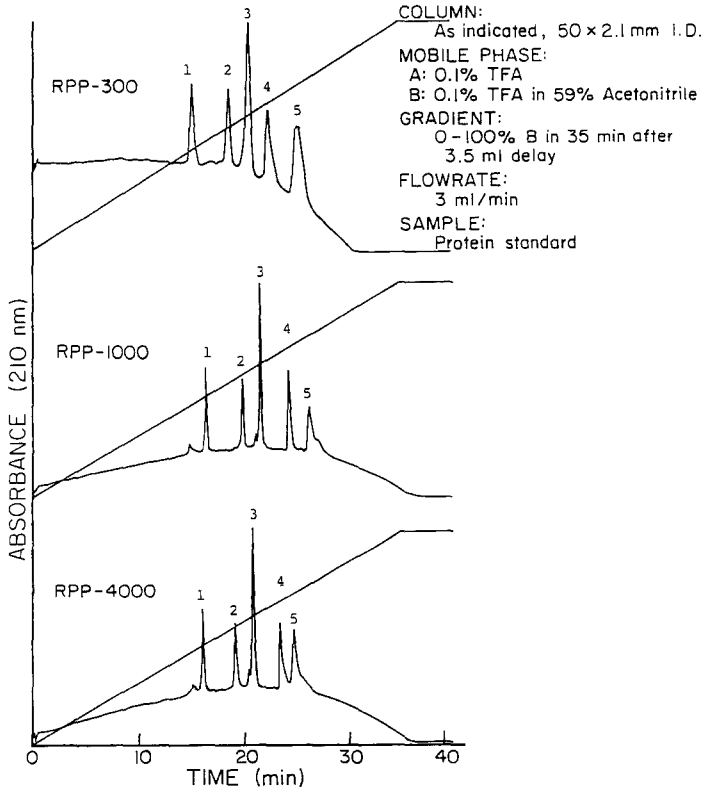
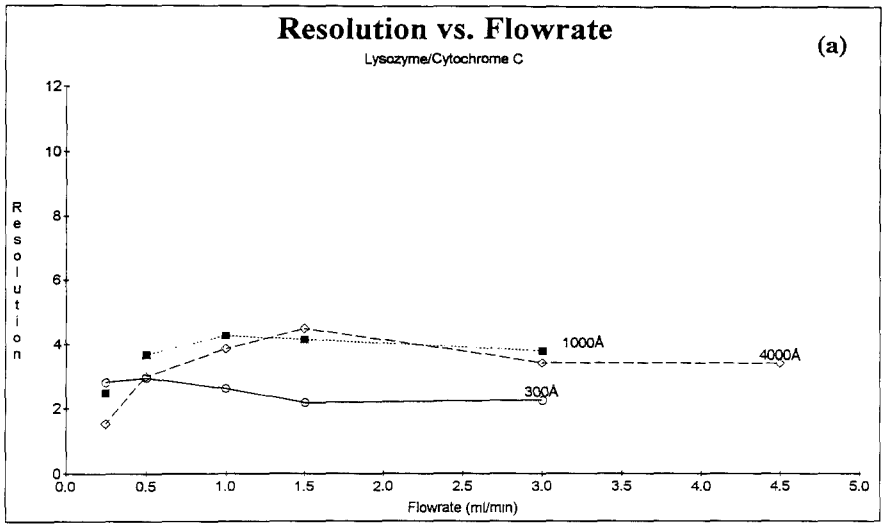


FIGURE 1. The effect of pore diameter on resolution of proteins. Columns: SynChropak RPP, RPP-1000 and RPP-4000, 50 x 2.1mm I.D. Flowrate:
3 ml/min. Gradient: 35 min gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in 59% acetonitrile (delay volume is 3.5 ml). Sample:
1. ribonuclease A, 2. cytochrome c, 3. lysozyme, 4. transferrin and 5. bovine serum albumin. 1.2 µg of cytochrome c and 2.4 µg of each of the other proteins were injected.



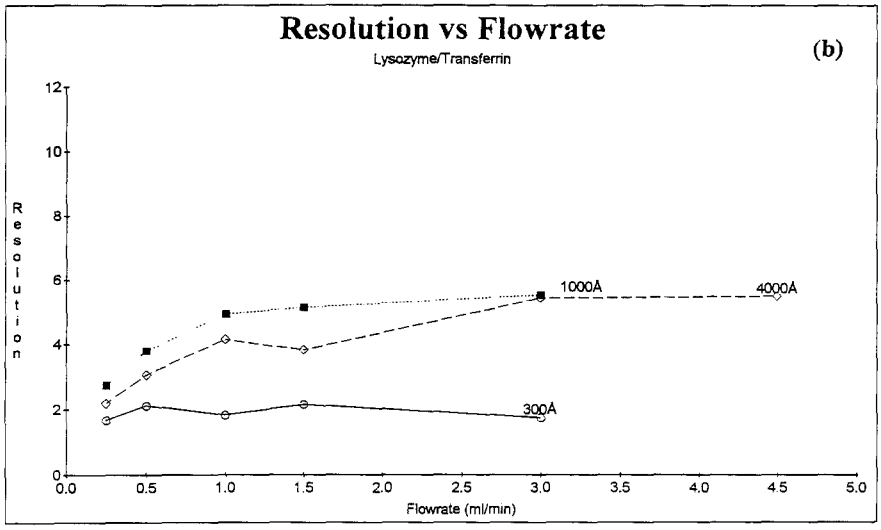


FIGURE 2. The effect of flowrate (linear velocity) on resolution of protein pairs: a. lysozyme/cytochrome c; b. lysozyme/transferrin; c. transferrin/BSA. Conditions as in Figure 1.

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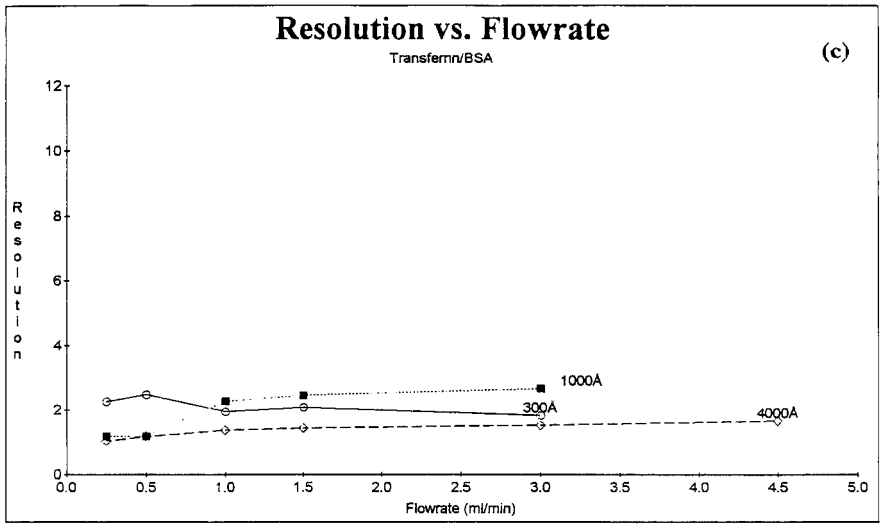


FIGURE 2 (Continued)

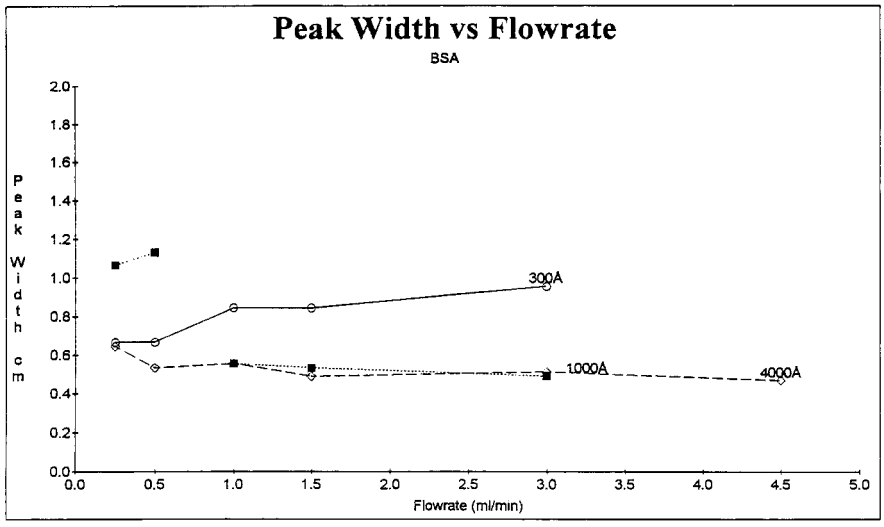


FIGURE 3. The effect of flowrate (linear velocity) on peak width for BSA. Conditions as in Figure 1.

peak widths. The lower retention on the 4000Å support is due to its lower surface area and larger particle diameter.

To verify these observations with another set of columns and an alternative gradient and mobile phase, a five minute methanol gradient at 3 ml/min was used. The peak widths of cytochrome c were the same on both 300Å and 4000Å supports, but the lysozyme and bovine serum albumin peaks were narrower on the 4000Å. Better resolution was again achieved on the 4000Å support than the 300Å despite the larger particle diameter of the former, as seen in Figure 4. Under these gradient conditions, some of the BSA peaks were totally resolved from the primary one so BSA did not look as broad.

Effect of Pore Diameter on Peptide Resolution

To test whether pore diameter would influence the effects of linear velocity on the resolution of small peptides, a decapeptide mixture was run in a parallel study. It can be seen in Figure 5 that the resolution of peptides was better on the 300Å and 1000Å supports, which are 7 µm, than on the 4000Å, which is 10µm. The graphs in Figure 6 confirm that at all flowrates the 300Å and 1000Å supports are superior to the 4000Å in terms of resolution of peptides.

Effect of Ligand Chain

The preceding studies utilized reversed phase columns with octadecyl (C-18) ligands. To investigate whether a shorter ligand chain might influence resolution on microbore columns run with high linear velocities, 300Å and 4000Å silica supports with C-4, C-8 and C-18 ligands were compared at 3 ml/min. Five minute gradients with methanol or isopropanol were implemented. There were few differences in selectivity,

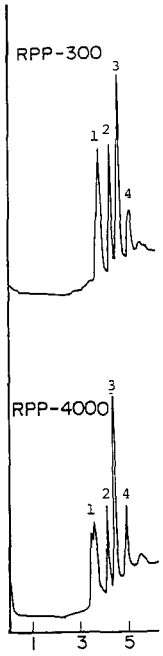


FIGURE 4. The effect of pore diameter on protein resolution. Columns: SynChropak RPP and RPP-4000, 50 x 2.1mm I.D. Flowrate: 3 ml/min. Gradient: 5 min gradient from 0.1% trifluoroacetic acid in 10% methanol to 0.1% trifluoroacetic acid in 100% methanol. Sample: 1. ribonuclease A (75 µg),
2. cytochrome c (15 µg), 3. lysozyme (10 µg) and 4. bovine serum albumin
(40 µg).

resolution or retention among the chain lengths. For peptides, the C-8 and C-18 chains gave slightly better resolution. The two larger proteins gave somewhat higher band widths on the C-18 columns, confirming the general theory that short chain ligands are better for protein analysis. Generally, differences caused by ligand chain lengths were indistinguishable with these proteins and peptides. The more notable

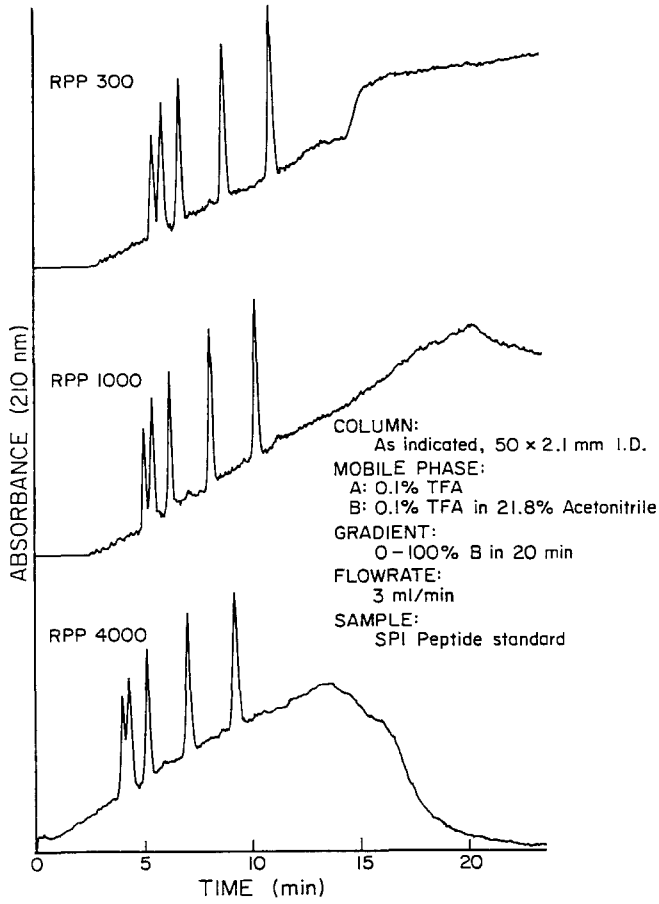
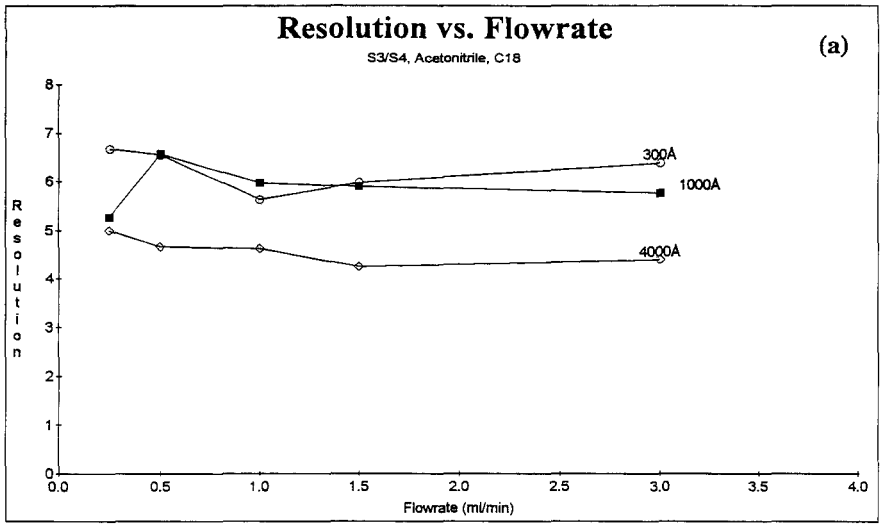


FIGURE 5. The effect of pore diameter on peptide resolution. Columns: SynChropak RPP, RPP-1000 and RPP-4000, 50 x 2.1mm I.D. Flowrate: 3 ml/min. Gradient: 20 min gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in 21.8% acetonitrile. Sample: 2 µg SPI peptide standard.



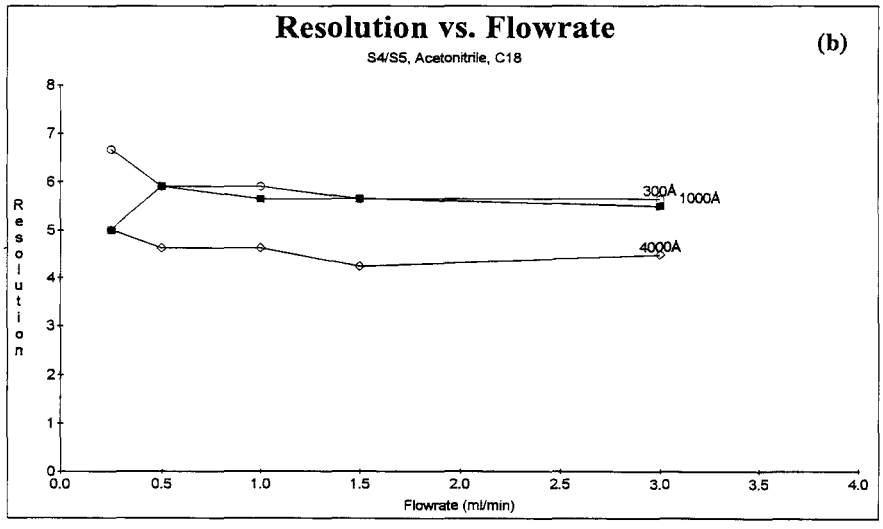


FIGURE 6. The effect of flowrate (linear velocity) on resolution of peptide pairs. Columns: SynChropak RPP, RPP-1000 and RPP-4000, 50 x 2.1mm I.D. Flowrate: 3 ml/min. Gradient: 20 min gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in 21.8% acetonitrile. Sample: 2 µg SPI peptide standard.

differences were between the pore diameters. For all ligands, 4000Å gave superior resolution of proteins while 300Å was best for peptides.

Stability

To assess whether the porous supports used in this study could tolerate the harsh conditions of high linear velocities with trifluoroacetic acid in the mobile phase, a stability study was carried out using 0.1% trifluoroacetic acid at 3 ml/min on C-18 300Å and 4000Å columns. After each liter of throughput, the columns were tested with peptide standards. On both columns, retention times decreased no more than 5 - 8% from the initial run and peak widths remained relatively constant after 4 liters of solvent. This indicates that at least 25,000 column volumes or 250 runs of 5 min duration at 3 ml/min were not deleterious.

CONCLUSIONS

Microbore columns containing 1000Å or 4000Å reversed phase supports were effectively used at linear velocities of 15 - 20 mm/s to analyze proteins rapidly. At all flowrates except the slowest, the resolution of protein pairs not including BSA was better on 1000Å and 4000Å reversed phase columns than on 300Å. Resolution involving BSA was not as high on the 4000Å support because of its shorter retention and its heterogeneity.

Peptide resolution was best on the 300Å support under all conditions. The decapeptides would have total access to the surface of each of the 300Å, 1000Å and 4000Å supports. The lower resolution of the peptides on the 4000Å supports was partially due to the lower retention caused by the significantly lower surface area of the 4000Å support. The fact that the improvement of resolution with flowrate was observed for

proteins but not peptides would suggest that the band spreading was caused by the relative sizes of the solute and the pores. The pore diameter effects were confirmed when alternative chain lengths were used. Negligible differences on protein and peptide retention were seen between various ligands under the very short analysis conditions of this comparative study (3 ml/min, 5 min gradient).

The 1000Å and 4000Å silicas used in this study have some bimodal characteristics while the 300Å has a more uniform pore structure (18). The silica with an average pore diameter of 1000Å has sets of pores which are between 500Å and 3000Å (18). The 4000Å average pore diameter silica additionally contains some larger pores up to 5000Å (18). Mass transfer of proteins was facilitated by the presence of the large pores (> 500Å) which were absent in the 300Å supports. Some improvement of resolution with increased flowrate could be due to decreased contact time with the hydrophobic bonded phase, because contact of proteins with ligand chains sometimes results in unfolding or denaturation.

Both 300Å and 4000Å non-endcapped reversed phase supports held up well to the high flowrates and acidic conditions used in this study. Little degradation was seen after the equivalent of 250 gradient runs.

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PIRKLE-CONCEPT CHIRAL STATIONARY PHASES FOR THE HPLC SEPARATION OF PHARMACEUTICAL RACEMATES

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ABSTRACT

The enantioselectivity of various Pirkle-concept chiral stationary phases (CSPs) for the direct HPLC separation of chiral drugs is explored and discussed. Seven different CSPs are surveyed for their utility to separate enantiomeric pairs belonging to the following major pharmaceutical classes: cardiovascular medicines (antihypertensives, antiarrhythmics, antianginals, diuretics), adrenergic drugs (vasopressors), antiinflammatory and analgesic compounds, topical anesthetics, antihistaminics, and antimalarial therapeutics.

INTRODUCTION

The clinical, research and regulatory significance of chiral drugs continues to spur new developments into stereoselective methods of analysis. The majority of therapeutics administered today are both synthetic and racemic, despite well-documented differences in the pharmacodynamics and pharmacokinetics of the individual isomers.

One isomer can sometimes be many times more physiologically active and/or toxic than the other (1-3). Although the trend is to develop effective new therapeutic agents in optically pure form in order to fully exploit nature's "handedness", undoubtedly many drugs will continue to be developed as racemates for economic, technical or other reasons. Nevertheless, new regulatory guidelines (4) will make it necessary for the drug industry to evaluate the enantiomeric purity and toxicity/activity of any new drug introduction, including the development of "racemic switches" (5). Such driving forces necessitate that fast, sensitive and reproducible chromatographic methods be developed for the direct separation and quantitation of optical antipodes.

Enantiomeric separation by high performance liquid chromatography (HPLC) has taken various approaches over the last 25 years, with the most recent progress focused on the design and manufacture of totally synthetic chiral stationary phases, or CSPs (6-14). Such a wide variety of HPLC CSPs led Wainer to propose a classification scheme for choosing the most appropriate phase based on the structure of the solutes and the type of CSP, i.e., based on the chiral recognition mechanisms involved (15). The "three-point interactive rule" of chiral recognition first proposed by Dagliesh for paper chromatographic separations of amino acids (16) was later extended to HPLC and verified by Baczuk et al (17). Pirkle and his coworkers, however, began the first rational approach to the design of CSPs for HPLC using various optically active π-acids and π-bases and proved their wide utility (18-22). Resolution is achieved through a variety of diastereomeric interactions, including hydrogen bonding, dipole stacking, charge transfer, steric hinderance, etc . The enantiomer which forms the most energetically stable complex with the CSP is retained on the column longest. The "reciprocality

concept" put forth by Pirkle (20,22) allowed several generations of CSPs to evolve by analogy, including the many phases grafted by Oi and his group (23-32).

In this paper the utility of various CSPs of the type developed by Oi and coworkers are evaluated for their utility in resolving enantiomers of some important pharmaceutical compounds. Representative compounds of various drug classes, widely different in chemical structure, are surveyed and column enantioselectivity determined. General aspects of method development are also discussed.

EXPERIMENTAL

Chemicals

Analytical grade standards of all pharmaceuticals surveyed were obtained from Sigma (St. Louis, MO), Aldrich (Milwaukee, WI), or Fluka (Ronkonkoma, NY) chemical companies, depending on availability. Solutions for HPLC analysis were prepared in the range of 1 to 4 mg/ml by dissolving in hexane/ethanol (in various proportions, with ca 0.1% trifluoroacetic acid added) and sonicated as necessary. 99%+ reagent grade ammonium acetate and spectrophotometric grade trifluoroacetic acid were purchased from Aldrich. HPLC-grade solvents were obtained from either Fisher Scientific or Aldrich. All chemicals were used without further purification or filtration.

Instrumentation

The Hewlett Packard 1050 HPLC system (Palo Alto, CA) consisted of a dual piston pump and solenoid valve proportioning system, multiple wavelength diode array-based detector with 8 μl flow cell, and a Rheodyne 7125 injector (Cotati, CA) equipped with a 20μl loop for full or partial injections from 2 to 20 μl. The optimal

detection wavelength for each compound was first determined by scanning the absorption spectrum from 200 to 400 nm using a Hewlett Packard 8452A diode array spectrophotometer. Data acquisition, storage and analysis were performed by the Hewlett Packard 1050 Chemstation software.

Chromatography

Table 1 lists the seven "brush" or Pirkle-concept CSPs used in the survey. All are commercially available as Chirex brand from Phenomenex, Inc. (Torrance, CA). Each CSP consists of an optically pure amino acid or carboxylic acid covalently bound to γ-aminopropyl silinized silica gel (5 μm particle size) and derivatized via an amide or urea linkage with a π-electron group. All columns were slurry-packed using a conventional technique into steel columns of standard 250 x 4.6 mm i.d. analytical dimensions and used without guard columns.

A variety of isocratic mobile phase systems were explored for their ability to enhance enantioseparation and control retention time. Two basic eluent compositions, a reversed phase system consisting of

TABLE 1

Chiral Stationary Phases

Amino Acid	π-Electron Group	Linkage	Designation
(R)-phenylglycine	N-3,5-dinitrobenzoic acid	amide	3001
(R)-naphthylglycine	N-3,5-dinitrobenzoic acid	amide	3005
(S)-valine	(R)-1-(α-naphthyl)ethylamine	urea	3014
(S)-proline	(S)-1-(α-naphthyl)ethylamine	urea	3017
(S)-proline	(R)-1-(α-naphthyl)ethylamine	urea	3018
(S)-tert-leucine	(R)-1-(α-naphthyl)ethylamine	urea	3020
(S)-indoline-2-carboxylic acid	(R)-1-(α-naphthyl)ethylamine	urea	3022

ammonium acetate in methanol or methanol/water, and a normal phase system containing hexane/ethanol/trifluoroacetic acid (with or without 1,2-dichloroethane), were used throughout. In all cases, retention times were primarily controlled by varying the concentration of alcohol. Similar eluents have been used with related Pirkle phases with good results (28,33), and these provided guidelines for the current study. Trifluoroacetic acid (TFA) was added to normal phase systems to improve peak shape. Premixed at a ratio of 1 part TFA to 20 parts ethanol, the final concentration of TFA in the mobile phase varied from 0.1-1.5%, and depended on the concentration of ethanol necessary to eluent all components while still preserving enantioseparation. Flow rates varied from 0.7 to 1.0 ml/min. Observed column pressures ranged from 600 to 1000 psi. All analytical runs were performed at ambient temperature.

RESULTS AND DISCUSSION

Optimizing the Separation

Ethanol concentration appeared to play a larger role than column selectivity in controlling retention time. The greatest determinant of enantioselectivity, on the other hand, was the CSP itself, rather than any manipulation of the mobile phase composition. Stereoselectivity of the CSP for the chiral solutes could quickly be evaluated by testing any given sample under conditions which eluted the peak or peaks within the first 5 to 15 minutes. If the required stereoselectivity, i.e., spatial complimentarity and stereospecific interaction, was present, separation would be observed within this time frame. Further optimization of the separation would then be pursued. Additional changes in mobile phase composition (within the parameters chosen) did little to bring about separation if selectivity was not already apparent.

TABLE 2
Separation Factors for Racemic Drugs

Compound Name	Clinical or Therapeutic Use	UV abs Max (nm)	Column Enantioselectivity Separation Factor Alpha* Chiral Stationary Phase						
			3001	3005	3014	3017	3018	3020	3022
Acebutolol	Antihypertensive	244	1	1	1	P D	1	1	1.09 D
Atropine	Anticolinergic	258			1			1	1
Bendroflumethiazide	Diuretic	272	1.11 D	1.07 F	1.14 F	P B	1.09 D	1.17 F	P F
Bepridil	Antianginal	250	1		1.22 R	1	1.16 D	1.09 L	P J
Brompheniramine	Antihistaminic	264			1.30 B			1.15 G	P D
Captopril	Antihypertensive	242	1	1					
Carbinoxamine	Antihistaminic	262			1.15 C			1.10 G	P D
Chloramphenicol	Antibacterial	272			1			1	1
Chloroquine	Antimalarial	346	1	1	1.22 B	P A	1.20 A	1.21 B	1.13 A
Chlorthalidone	Diuretic	240			P J			1.08 K	1
Clemastine	Antihistaminic	236	1	1	2.86 D	1	1.26 B	3.04 D	1.35 F
Clenbuterol	Bronchodilator	248	1	1	1	1	1.15 B	P L	1.27 B
Colchicine	Gout suppressant	352			1			1	1
Cyclopentolate	Mydriatic	260			1.13 J			1.10 G	1.07 D
Dichloroisoproterenol	Bronchodilator	272			1.06 L			1	1.13 J
Diltiazem	Antianginal	248	1	1	1	1	1	1	1
Diperodon	Anesthetic, local	242			1.20 G			1.09 L	P G
Disopyramide	Antiarrhythmic	262	1	1	1	1	P G	1	1
Doxylamine	Bronchodilator	262			P D			1.07 G	
Ephedrine	Bronchodilator	258			1			1	P D
Epinephrine	Bronchodilator	282			1			1.07 D	1.10 D
Glafenine	Analgesic	346	1	1	P D	1	1	P D G	1
Hydroxy-phenyl-5-phenyl-hydantoin	Anticonvulsant	238			1			1.11	1
Hydroxyphenylethylamine	Vasopressor	258			1			1	
Hydroxyzine	Antihistaminic	238	1		1	1	P F	1	1
Indapamide	Antihypertensive	248	1	1	1	1	P F	1	1.08 F
Indoprofen	Analgesic, NSAID	284	1	1.08 U					

Isoetharine	Bronchodilator	282			P F				1.21 D	1.08 D
Isoproterenol	Bronchodilator	282			1.08 E				1.21 D	1.18 D
Isoxsuprine	Vasodilator	276			1.40 D				1.37 D	P G
Labetolol 1	Antihypertensive	308			P D				1.08 G	1.08 D
Labetolol 2	Antihypertensive	308			1				1.22 G	1.11 D
Mephenesin	Skeletal muscle relaxant	272	1	1	1	1	1		1	1
Metanephrine	Vasopressor	280			1				1	1.10 D
Metaproterenol	Bronchodilator	278			1.10 E				1.24 D	1.13 D
Methocarbamol	Skeletal muscle relaxant	274			1.09 L				1.09 G	1
Methotrimeprazine	Analgesic	254	1		1	1	1		1	1
Methoxamine	Vasopressor	290			1.10 K				1.11 G	1.11 D
Methoxyverapamil	Antiarrhythmic	278			P L					1.15 D
Metoprolol	Antihypertensive	276			P M				1	1.08 G
Midodrine	Adrenergic stimulant	292	1.09 D	1	1.09 F	1	1		1.10 F	1
Nadolol	Antihypertensive	270	1	1	1	1	P F		1	P F
Nefopam	Analgesic	268	1	1	P R	1	1.14 B		P G	1.10 F
Nicotine	Antismoking therapeutic	260			1.10 B				P G	P D
Normetanephrine	Vasopressor	280							1	P D
Octopamine	Vasopressor	276			1				1	P D
Oxybutynin Chloride	Anticolinergic	258			1				1	1
Oxyphencyclimine	Anticolinergic	244	1	1	1	1.50 A	1		1	1
Phenylbutazone	Antiinflammatory	246	1	1	1	1	1		1	1
Phenylethanolamine	Vasoconstrictor, topical	224			1					1
Prilocaine	Anesthetic, local	242			1.13 J				1.17 G	1.14 G
Primaquine	Antimalarial	268	1		1.18 D	1	1		1.16 F	1
Primidone	Anticonvulsant	240	1	1	1	1	1		1	1
Proglumide	Anticolinergic	242	1.27 D	1.45 U	1.17 R		1		1	1
Promethazine	Antihistaminic	254			1.12 J				1.12 G	1.08 K
Propranolol	Antiarrhythmic	292			1.11 H				1.08 K	1.10 K
Quinacrine	Antimalarial	282	1	1	P D	1.38 A	P A		1.10 D	1.33 A
Sulpiride	Antidepressant	242			1				1	1
Suprofen	Analgesic, NSAID	288	1	P U						
Suxibuzone	Antiinflammatory	246	1	1	1	1	1		1	1
Synephrine	Vasopressor	276			1				1	1.12 D
Turbutaline	Bronchodilator	278			1.11 J				1.28 D	1.24 D
Terfenadine (Seldane)	Antihistaminic	260	1		1	1	1		P L	P F
Tetrahydrozoline	Adrenergic stimulant	242	1		1.11 D	1.28 B	1.07 B		1.07 D	1.12 B
Thioridazine	Antipsychotic	264	1		P R		1		P L	1.10 F
Timolol	Antihypertensive	298	1	1	1	1	1		1	1

(continued)

TABLE 2 (continued)
Separation Factors for Racemic Drugs

Compound Name	Clinical or Therapeutic Use	UV abs Max (nm)	Column Enantioselectivity Separation Factor Alpha* Chiral Stationary Phase						
			3001	3005	3014	3017	3018	3020	3022
Tropicamide	Anticolinergic	254	1	1	P G	1	1	1	1.10 D
Verapamil	Antiarrhythmic	280			1.10 N			1.05 L	1.11 G
Warfarin	Anticoagulant	282			1			1	1.03 P

* Alpha (α) = K2/K1; P = Partially separated; Blank denotes no separation attempted. Mobile phase conditions are indicated by letter codes following alpha values for all complete or partial separations and are explained at the bottom of the table.

Normal Phase Solvent Systems:

	Hexane (%)	1,2-Dichloro-ethane (%)	Ethanol-TFA† (%)
A	40	35	25
B	50	35	15
C	40	50	10
D	55	35	10
E	43	50	7
F	58	35	7
G	60	35	5
H	70	25	5
I	73	20	7
J	75	20	5
K	76	20	4
L	77	20	3
M	82	15	3
N	87	10	3
O	92	5	3
P	88	10	2
Q	78	20	2
R	62	35	3

Reversed Phase Solvent Systems:

	Ammonium Acetate (M)	Methanol (%)	Water (%)
S	0.005	100	0
T	0.01	100	0
U	0.025	100	0
V	0.03	100	0
W	0.05	100	0

† Note: Ethanol and TFA (trifluoroacetic acid) were pre-mixed 20:1.

An initial survey of most compounds was performed at 25, 15, or 10% ethanol, and 35 or 20% 1,2-dichloroethane, with hexane making up the remainder. Some of the more polar test compounds could be sufficiently retained only by dropping the concentration of ethanol down to 1-3%. Phases used with this system were 3001, 3014, 3017, 3018, 3020 and 3022. Phase 3005 appeared well-suited to the analysis of carboxylic acids and amides and was evaluated under both normal and reversed phase conditions. In normal phase mode, various combinations of hexane and ethanol (premixed with TFA) were tried with some success. Reversed phase systems consisting of 25mM ammonium acetate in methanol/water mixtures also proved effective.

The capacity of these low-molecular weight CSPs for separating and purifying a wide variety of enantiomers has been amply demonstrated (23-32). The current survey significantly expands the known applications of these phases. The selection of compounds was designed to be representative of the diverse classes of pharmaceuticals which are either being developed enantiomerically pure, or converted in a "racemic switch", because of reduced side effects and/or enhanced potency.

Table 2 summarizes the efforts to separate and optimize the resolution of 70 racemic pharmaceuticals of wide structural variety using seven different CSPs. At least 24 clinical/therapeutic drug categories are represented. Not all compounds were tested on all CSPs. Percentages of successfully resolved enantiomers (Table 3) ranged from 10% to 45% per CSP. Enantiomer peaks were considered resolved if baseline or valley between the pair dropped to less than 25% of the height of the first peak. When partially and fully resolved enantiomers are combined together, the percentages ranged

from 10% to 65%. Successful resolution of all compounds on all columns was 60%, and the total of successful and partial separations achieved was 80%; only 20% of the selected compounds showed no stereospecific interaction with the tested CSPs.

Separations of Cardiovascular Drugs

The β-adrenergic blocking agents labetolol and nadolol both contain multiple stereogenic centers. Phase 3020 successfully resolved all four isomers of labetolol (Figure 1), a compound which has been administered for hypertension as both a racemate and partially purified, but was later shown to cause liver toxicity (5).

TABLE 3

Separation Utility of Seven Pirkle-Concept CSPs

	3001	3005	3014	3017	3018	3020	3022	ALL
S	3	4	24	3	7	29	29	46
P	0	1	11	3	5	6	13	10
T	31	24	66	26	28	64	65	70
%S	10	17	36	12	25	45	45	60
%S+P	10	21	53	23	43	55	65	80

S = Separated enantiomeric pair (baseline or valley height between the pair is less than 25% of the height of peak one).

P = Partial separation of the enantiomeric pair (valley height is greater than 25% of the height of peak one).

T = Total number of compounds tested.

Nadolol, containing three chiral centers, was partially resolved into three peaks on phases 3018 and 3022. The best result was obtained on the latter phase and is shown in Figure 2. Superior enantioresolution of methoxyverapamil, one compound in the widening class of calcium channel modulators, was achieved in under 7 minutes on phase 3022 (Figure 3). Six of the seven test columns showed at least partial stereoselectivity for the diuretic bendroflumethazide (Table 2). Peak resolution obtained on phase 3001 is shown in Figure 4.

Separations of Antiinflammatory and Analgesic Compounds

Initial chiral HPLC methods for the analysis of non-steroidal antiinflammatory drugs (NSAIDs) required prior derivatization. Derivatization with chiral reagents is prone to error (34) and achiral derivatization (35-36), while providing detection benefits, can complicate the chromatography. Unpublished data from Oi and coworkers, and, more recently, unpublished data from Jamali's group, have shown the wide utility of phase 3005 for the direct resolution of NSAID enantiomers. In Figure 5 the separation of indoprofen enantiomers using this phase is presented. Figure 6 shows the enantioseparation of nefopam, another useful analgesic drug, on phase 3018.

Separations of Adrenergic Compounds

The separations of two adrenergic-stimulating amino alcohols, synephrine and tetrahydrozoline, are shown in Figures 7 and 8, respectively. Relatively few chiral chromatography systems have shown the capacity for direct enantioresolution of these compounds (37) Because their pharmacological effects reside primarily in the (-)-

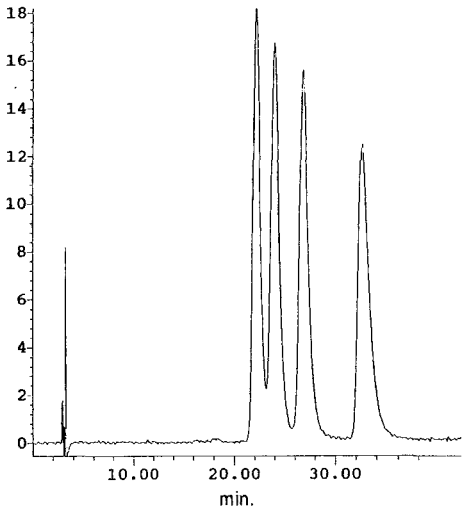

FIGURE 1. Chromatographic separation of racemic Labetolol (β-adrenergic receptor blocker) on Chirex phase 3020. The mobile phase was hexane/1,2 dichloro-ethane/ethanol-trifluoroacetic acid (60:35:5, with ethanol-TFA premixed 20:1) at a flow rate of 1.0 ml/min. UV detection was used at 308 nm. Assignments of the four isomers were not known.

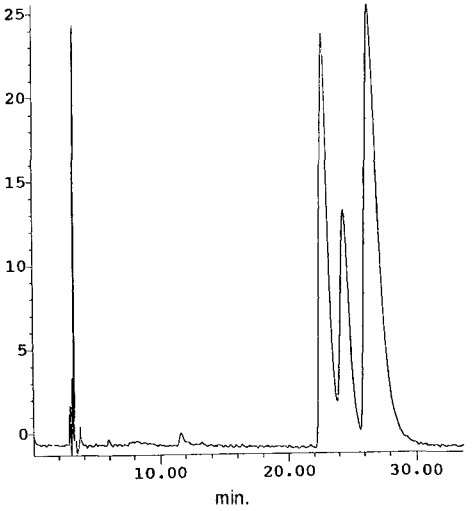

FIGURE 2. Chromatographic separation (partial) of racemic Nadolol (β-adrenergic receptor blocker) on Chirex phase 3022. The mobile phase was hexane/1,2-dichloroethane/ethanol-trifluoroacetic acid (58:35:7, with ethanol-TFA premixed 20:1) at a flow rate of 1.0 ml/min. UV detection was used at 270 nm.

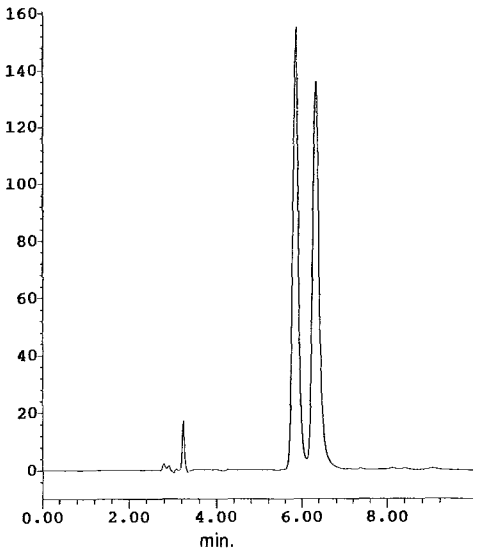

FIGURE 3. Chromatographic separation of racemic Methoxyverapamil (calcium channel modulator) on Chirex phase 3022. The mobile phase was hexane /1,2-dichloroethane/ethanol-trifluoroacetic acid (55:35:10, with ethanol-TFA premixed 20:1) at a flow rate of 1.0 ml/min. UV detection was used at 278 nm. $\alpha = 1.15$

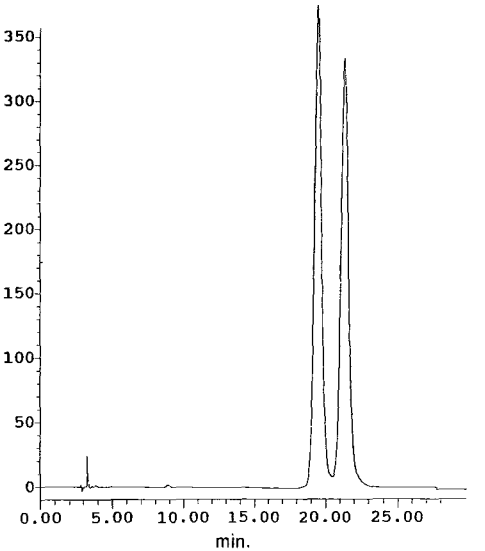

FIGURE 4. Chromatographic separation of racemic Bendroflumethazide (diuretic) on Chirex phase 3001. The mobile phase was hexane/1,2-dichloroethane/ ethanol-trifluoroacetic acid (55:35:10, with ethanol-TFA premixed 20:1) at a flow rate of 1.0 ml/min. UV detection was used at 272 nm. $\alpha = 1.11$

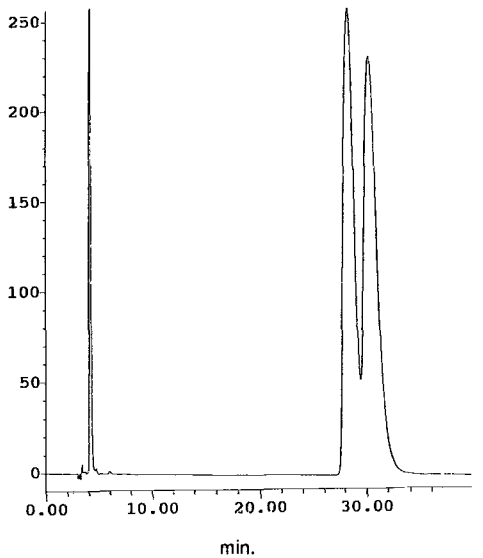

FIGURE 5. Chromatographic separation of racemic Indoprofen (NSAID) on Chirex phase 3005. The mobile phase was 0.025 M ammonium acetate in 100% methanol at a flow rate of 1.0 ml/min. UV detection was used at 284 nm. $\alpha = 1.08$

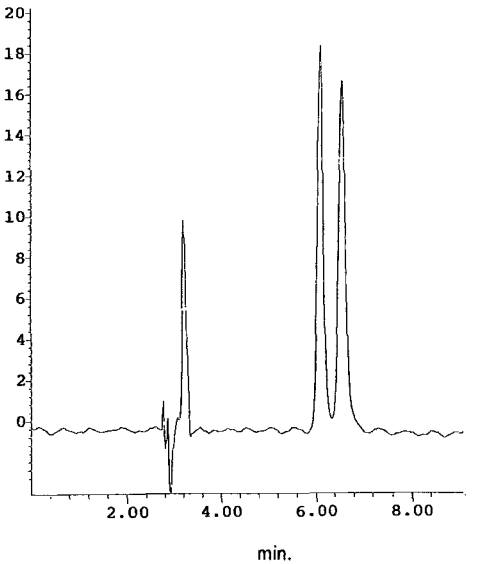

FIGURE 6. Chromatographic separation of racemic Nefopam (analgesic) on Chirex phase 3018. The mobile phase was hexane/ 1,2-dichloroethane/ethanol-trifluoroacetic acid (50:35:15, with ethanol-TFA premixed 20:1) at a flow rate of 1.0 ml/min. UV detection was used at 268 nm. $\alpha = 1.14$

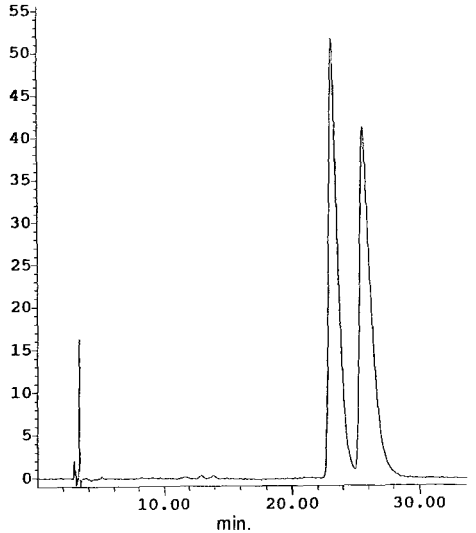

FIGURE 7. Chromatographic separation of racemic Synephrine (vasopressor) on Chirex phase 3022. The mobile phase was hexane/1,2-dichloroethane/ethanol-trifluoroacetic acid (55:35:10, with ethanol-TFA premixed 20:1) at a flow rate of 1.0 ml/min. UV detection was used at 276 nm. $\alpha = 1.12$

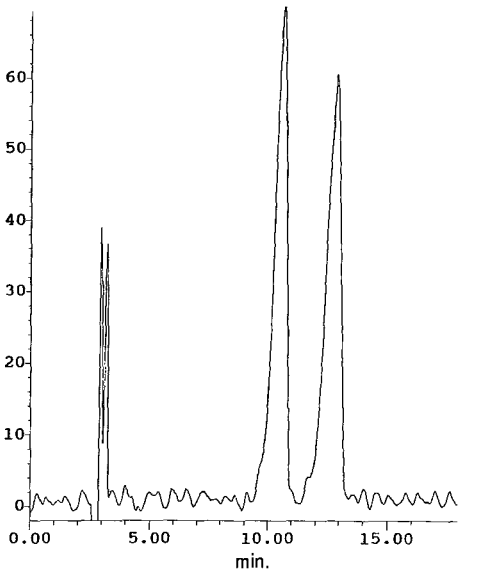

FIGURE 8. Chromatographic separation of racemic Tetrahydrozoline (β-adrenergic stimulant) on Chirex phase 3017. The mobile phase was hexane/1,2dichloro-ethane/ethanol-trifluoroacetic acid (50:35:15, with ethanol-TFA premixed 20:1) at a flow rate of 1.0 ml/min. UV detection was used at 242 nm. $\alpha = 1.28$

(R)-enantiomers, the unique stereoselectivity of these columns will be of benefit. Note the successful resolution of tetrahydrozoline on several of the test CSPs (Table 2).

Separations of Antihistaminic Drugs

Of the six antihistaminic drugs tested in this survey, four were well-resolved on several columns and two were partially resolved (Table 2). Separations of two compounds are shown in Figures 9 and 10. The differences in pharmacological activity and disposition of the pheniramine derivatives, chlor- and brompheniramine, are well known (38), but attempts to resolve and study the enantiomers by chiral chromatography have met with limited success (39). These CSPs now extend the current analytical capabilities.

Separations of Antimalarial Therapeutics

The enantiospecific differences between antipodes of the antimalarial drugs is another area of active investigation (40). Three compounds were chosen for testing in the current study: chloroquine, primaquine and quinacrine. The excellent separations of all three compounds obtained on several CSPs should prove to be valuable analytical tools for investigating their different pharmacodynamic and pharmacokinetics properties.

Separations of Topical Anesthetics

Two topical anesthetics were selected for the current survey. Both appeared to have similar stereoselectivity on three of the columns, but no resolution on the other four. Optimum results for the chiral separations of diperodon and prilocaine are shown in Figures 13 and 14.

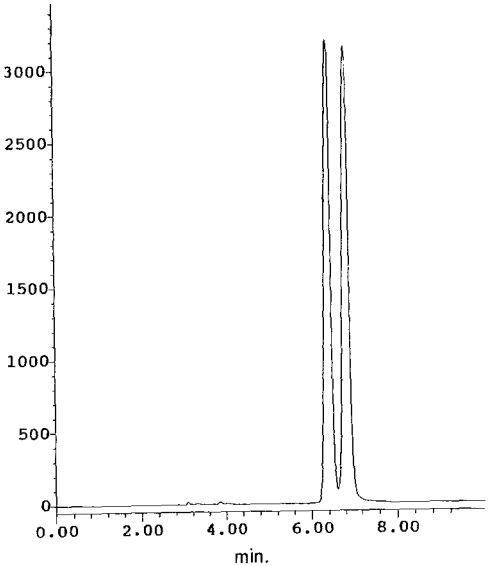

FIGURE 9. Chromatographic separation of racemic Promethazine (antihistaminic) on Chirex phase 3020. The mobile phase was hexane/1,2-dichloroethane/ethanol-trifluoroacetic acid (60:35:5, with ethanol-TFA premixed 20:1) at a flow rate of 1.0 ml/min. UV detection was used at 254 nm. $\alpha = 1.12$

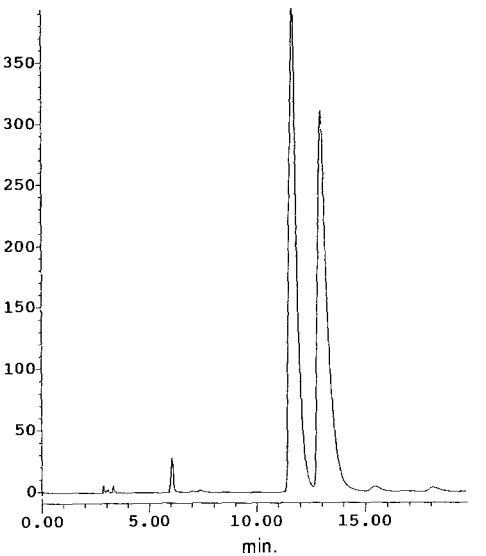

FIGURE 10. Chromatographic separation of racemic Brompheniramine (antihistaminic) on Chirex phase 3020. The mobile phase was hexane/1,2-dichloroethane/ethanol-trifluoroacetic acid (60:35:5, with ethanol-TFA premixed 20:1) at a flow rate of 1.0 ml/min. UV detection was used at 264 nm. $\alpha = 1.15$

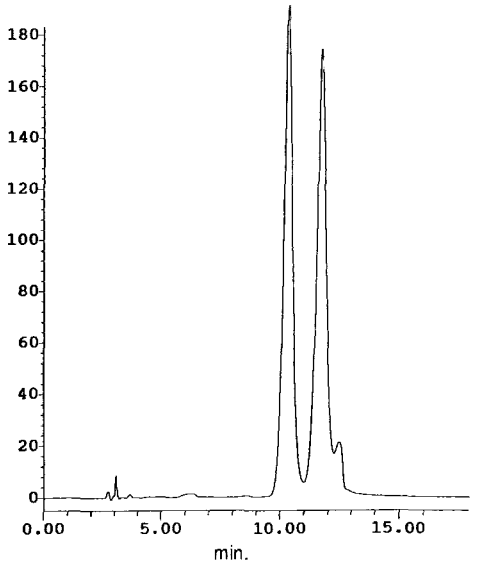

FIGURE 11. Chromatographic separation of racemic Primaquine (antimalarial) on Chirex phase 3014. The mobile phase was hexane/1,2-dichloroethane/ethanol-trifluoroacetic acid (55:35:10, with ethanol-TFA premixed 20:1) at a flow rate of 1.0 ml/min. UV detection was used at 268 nm. $\alpha = 1.18$

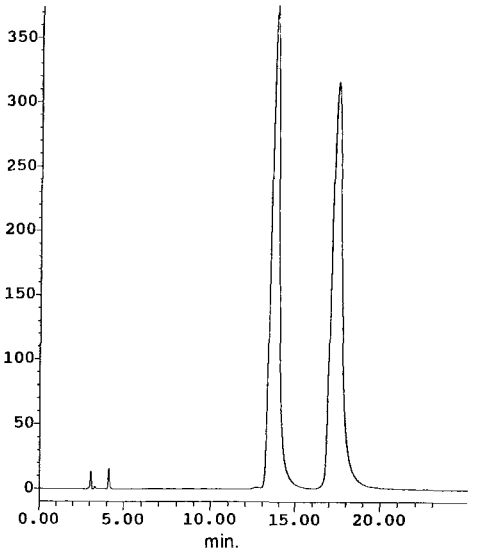

FIGURE 12. Chromatographic separation of racemic Quinacrine (antimalarial) on Chirex phase 3022. The mobile phase was hexane/1,2-dichloroethane/ethanol-trifluoroacetic acid (40:35:25, with ethanol-TFA premixed 20:1) at a flow rate of 1.0 ml/min. UV detection was used at 282 nm. $\alpha = 1.33$

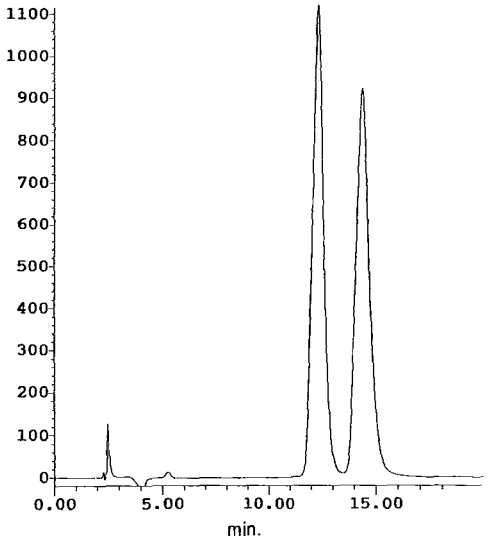

FIGURE 13. Chromatographic separation of racemic Diperodon (local anesthetic) on Chirex phase 3014. The mobile phase was hexane/1,2-dichloroethane/ ethanol-trifluoroacetic acid (60:35:5, with ethanol-TFA premixed 20:1) at a flow rate of 0.7 ml/min. UV detection was used at 242 nm. $\alpha = 1.20$

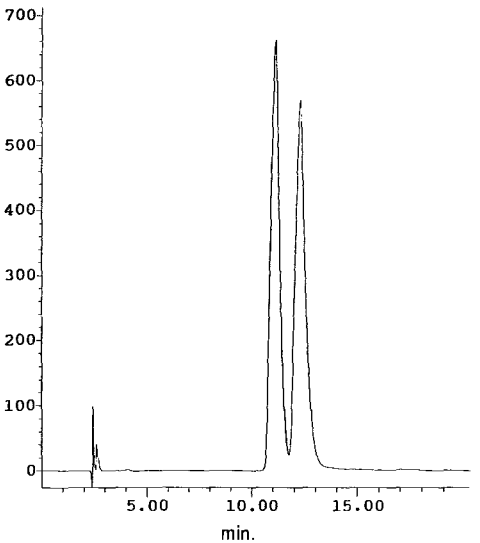

FIGURE 14. Chromatographic separation of racemic Prilocaine (local anesthetic) on Chirex phase 3014. The mobile phase was hexane/1,2-dichloroethane/ ethanol-trifluoroacetic acid (75:20:5, with ethanol-TFA premixed 20:1) at a flow rate of 0.7 ml/min. UV detection was used at 242 nm. $\alpha = 1.13$

CONCLUSION

The large number of possible interactions (41-42) provided by the multiple chemical functionalities contained in each CSP is what gives these types of columns their broad applicability. Compared with other, naturally-derived CSPs, these low-molecular weight phases demonstrate considerably higher efficiencies, are robust and long-lasting, and more suitable for quantitative work (7,9). Some columns in the current study have been actively used for more than one year without any perceptible degradation in performance (unpublished data). Reversed phase separations are possible using several phases in this class, but separations performed under normal phase conditions often afford greater enantioselectivity (14,43).

Although chiral separations are known to be highly compound-specific with respect to any given CSP (6-7,9,13,42,44), the resolution of a wide variety of racemic pharmaceuticals was clearly demonstrated in the current study using a select number of Pirkle-concept phases. An easy approach to rapid method development was presented. High-efficiency packings and exceptional CSP selectivities combined to produce baseline separations for the majority of test compounds. The application of these phases to the areas of chiral drug synthesis, analysis in biological fluids and enantiomeric purity determinations will make these CSPs an excellent choice for chiral separations and aid in the development of superior therapeutics.

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SEPARATION OF THE STEREOISOMERS OF HEXAMETHYL-PROPYLENEAMINE OXIME (HM-PAO) BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The Resolvosil BSA-7, Chiracel OD, and Chiralpak AD columns were examined to determine whether one might be suitable for the separation of enatiomers of the ligand hexamethyl-propyleneamine oxime (HM-PAO) and/or its technetium-99m complex. Using hexane/IPA as eluent, partial separation (R_S = 0.96) of the d- and l-ligand enantiomers was achieved on the OD column, but as the meso-form of the ligand was eluted midway between the d- and l-enantiomers. There was excellent separation of the technetium-99m complexes of d- and l-HM-PAO on the OD column, and partial separation (R_S = 0.90) of the meso- and d-complexes. Only partial resolution of the Tc-99m complexes of d,l- and meso-diastereoisomers was achieved on the AD column, but this column provided baseline resolution of all three stereoisomeric forms of the ligand. Thus, the OD column could be used for HPLC analysis of Tc-99m complexes of HM-PAO stereoisomers, while the AD column is suitable for analysis of the ligand stereoisomers.

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INTRODUCTION

While the influence of stereochemistry on the pharmacological action of drugs is well known [1,2], there have been relatively few examples of stereochemical effects of biodistribution properties of the technetium-99m complexes employed in diagnostic nuclear medicine [3,4]. One notable example in recent years is the technetium complex of hexamethyl-propyleneamine oxime (HM-PAO). This ligand, shown in figure 1, has two chiral centers, giving rise meso-, d-, and l-isomers. Synthesis of the ligand in a 2-step process gives an equal mixture of the meso- and d,l-diastereoisomer [5]. This mixture was labeled with technetium-99m to give, in high yield, a lipophilic Tc(V) complex with zero net charge [6], which is, presumably, a mixture of the three stereoisomers shown in figure 1. When administered intravenously to laboratory animals [7] and man [7,8], this

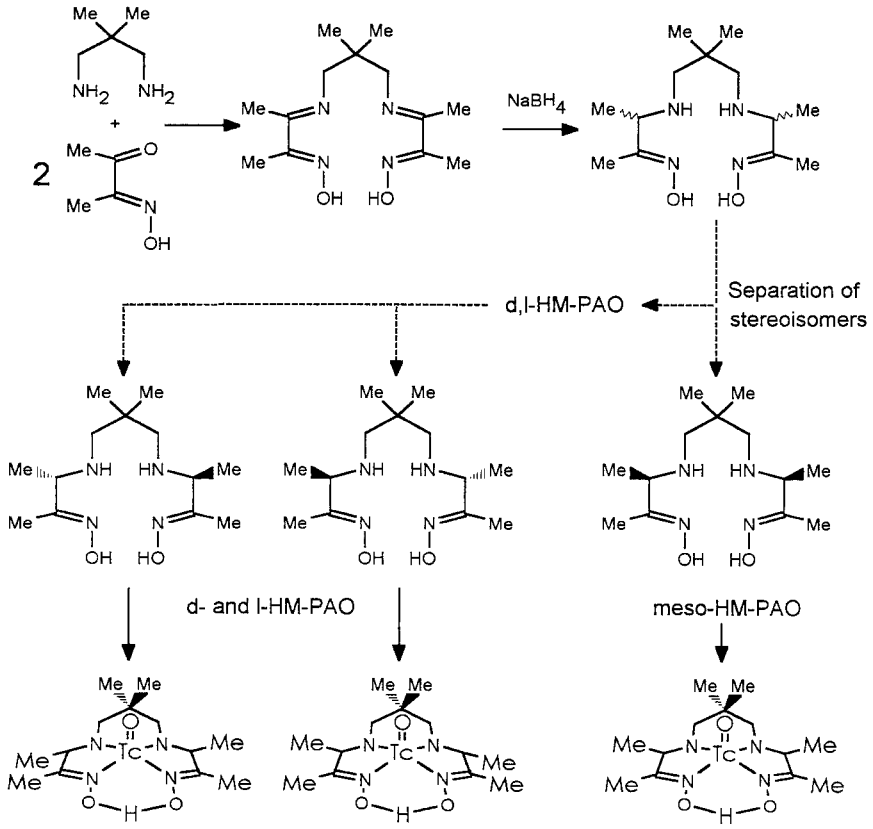

Figure 1. The synthesis of HM-PAO, and the structures of the stereoisomers of the ligand and its technetium complex

technetium complex displayed rapid uptake into the brain, with slow washout from this organ.

Separation of the ligand into the meso- and d,l-diastereoisomers was achieved by repeated recrystallization [9]. Analysis of the stereoisomeric purity of the separated diastereoisomers has been described using normal phase HPLC [5], and quantitation of the signals associated with minor differences in the ^{1}H nmr [10] and IR [11] spectra of meso- and d,l-HM-PAO. The technetium complexes from the two diastereoisomeric forms of the ligand displayed quite different in vivo properties in rats; the complexes had similar brain uptake, but the meso-form displayed far faster cerebral washout than the d,l-complex [12]. The Tc-99m complex of d,l-HM-PAO was also shown to possess these desirable properties in man [13], and has since become a the basis of a commercially-available pharmaceutical product; Ceretec™. The d,l-diastereoisomer complex has also been used extensively for *in vitro* Tc-99m labeling of leukocytes and platelets [14]; on re-injection, these labeled cells are used for imaging of sites of infection and thrombi, respectively. Recently, evidence has been presented which indicates that the cell labeling properties of the d- and l-enantiomer complexes are not identical [15]. Similarly, the brain uptake/retention properties of these two complexes may also be different [16,17]. Hence, it is important to the interpretation of data in studies involving Tc-99m HM-PAO to know the proportion of each of the stereoisomers.

As we needed to prepare d,l-HM-PAO for use by our colleagues in cerebral extraction studies [18], we found it necessary to develop reliable methods for the determination of stereochemcial purity. We now describe some new HPLC methods for the analysis of HM-PAO.

MATERIALS AND METHODS

HPLC-grade solvents were filtered and degassed prior to use. The HM-PAO (meso- and d,l-diastereoisomer mixture) was synthesized by the method described previously [5], with minor modifications. Meso-and d,l-HM-PAO were separated by repeated crystallization from acetonitrile as described previously [5,19]. The individual d- and l-enantiomers were separated by crystallization as their (-)-tartrate and (+)-tartrate salts (respectively) directly from the meso/d,l-mixture (eliminating the need for the initial isolation of the d,l-diastereoisomer, described previously [17]) as follows:

d-HM-PAO D-(-)-tartrate: D-(-)-tartaric acid (3.3 g, 22 mmol) was dissolved in hot ethanol (40 mL) and was added to a suspension of HM-PAO (meso/d,l-mixture, 6 g, 22 mmol) in ethanol (50 mL). The resulting mixture was heated to brief boiling, insoluble material was removed by filtration and washed with ethanol (10 mL). The yellowish solution was allowed to stand at room temperature for one week. The white crystalline solid which formed was isolated by filtration and washed with ethanol (3 x 25 mL) to give 6.23 g of a mixed isomer HM-PAO tartrate salt. The melting point of this product was 167-169 °C. The product was recrystallized a total of three times from ethanol, and the purity of product was monitored by melting point and ^{1}H NMR (D_2O) after each recrystallization. The purified product (0.41g) was obtained as short needles, mp 168-169.5 °C (lit. 17 167.5-168 °C). ^{1}H NMR (D_2O) δ 1.108 [s, 6H, C(CH_3)$_2$], 1.432 and

1.458 (s, 12H, $CHC\underline{H}_3$), 1.858 [s, 6H, $C=N(OH)C\underline{H}_3$], 3.000 (q, 4H, $C\underline{H}_2NH$), 3.945 (q, 2H, $C\underline{H}CH_3$), 4.297 (s,2H, tartaric C<u>H</u>OH). ^{13}C NMR (D_2O) 12.02 [$C(\underline{C}H_3)_2$], 16.28 [$\underline{C}(CH_3)_2$], 22.56 ($CH\underline{C}H_3$), 33.79 [$C=N(OH)\underline{C}H_3$], 55.25 ($\underline{C}H_2NH$), 59.05 ($\underline{C}HCH_3$), 74.31 and 74.48 (tartaric <u>C</u>HOH), 155.34 [$\underline{C}=N(OH)CH_3$], 178.78 (tartaric <u>C</u>OOH). d-HM-PAO D-(-)-tartrate was dissolved in water to a concentration of 2.5 g/100 mL to measure its specific rotation. $[\alpha]_D^{25} = -26.42^o$ (lit. 17 -27.67°).

l-HM-PAO was obtained in a similar manner from HM-PAO (meso/d,l-mixture, 3.0 g, 11 mmol) and L-(+)-tartaric acid (1.7 g, 11 mmol). After a total of four crystallizations from ethanol l-HM-PAO L-(+)-tartarate (0.36 g) was obtained, mp 170-172 °C (lit. 17173-175 °C). ^1H NMR (D_2O) δ 1.095 [s, 6H, $C(C\underline{H}_3)_2$], 1.419 and 1.445 (s, 12H, $CHC\underline{H}_3$), 1.850 [s, 6H, $C=N(OH)C\underline{H}_3$], 2.986 (q, 4H, $C\underline{H}_2NH$), 3.927 (q, 2H, $C\underline{H}CH_3$), 4.278 (s,2H, tartaric C<u>H</u>OH). ^{13}C NMR (D_2O) 12.88 [$C(\underline{C}H_3)_2$], 17.21 [$\underline{C}(CH_3)_2$], 23.85 ($CH\underline{C}H_3$), 34.66 [$C=N(OH)\underline{C}H_3$], 56.18 ($\underline{C}H_2NH$), 59.93 ($\underline{C}HCH_3$), 75.47 (tartaric <u>C</u>HOH), 156.38 [$\underline{C}=N(OH)CH_3$], 179.80 (tartaric <u>C</u>OOH). l-HM-PAO D-(+)-tartrate was dissolved in water in a concentration of 2.5 g/100 mL to measure its specific rotation. $[\alpha]_D^{25} = 27.06^o$ (lit. 17 28.08°).

HPLC analyses of the ligand, and the preparation of Tc-99m complexes, were conducted using the free base form of the ligand. Tartrate salts were converted to the free base form by treatment of aqueous solutions of the ligand with excess solid sodium carbonate, followed by extraction of the free base into diethyl ether and evaporation of the organic solvent.

The Tc-99m complexes of HM-PAO stereoisomers were prepared by methods similar to those described previously [5,12,17,18]. The radiochemical purities (RCPs) of the complexes were determined by HPLC analysis using a reversed-phase system [18]. For certain studies, the complexes were purified by a solid-phase extraction procedure reported previously [18,20]. By this procedure, Tc-99m HM-PAO complexes were obtained in ethanolic solution; suitable for use in the normal phase HPLC studies.

Two HPLC systems were used:

System 1. Two Rainin Rabbit HPX pumps, controlled by a personal computer operating Gilson 712 software, fitted with a Kratos UV detector set at 230 nm.

System 2. A Spectra-Physics Model SP8700 HPLC system equipped with an ISCO V^4 UV/visible detector and a radiometric detector connected to a Spectra-Physics Model SP4270 integrator/recorder.

Three analytical chiral HPLC columns, Chiracel OD and Chiralpak AD (5 μm, 150 x 4 mm, Chiral Technologies, Inc.) and Resolvosil BSA-7 (7 μm, 150 x 4 mm, Alltech Associates, Inc.) were examined for their potential to separate all three stereoisomers of the ligand and/or their complexes. In all cases, column integrity was checked regularly using the standard compounds and chromatography conditions recommended by the suppliers.

RESULTS and DISCUSSION

There are to date very few reported examples of the resolution of Tc-99m enantiomeric complexes. Verbruggen et al resolved the two enatiomeric technetium complexes of the achiral ligand termed MAG_3 by formation of a pair of diastereoisomers by esterification with a chiral alcohol [21]. Recently, the rhenium analog of the brain perfusion radiopharmaceutical Tc-99m L,L-ECD [22] was resolved from the Re complexes of other ECD stereoisomers by HPLC using a cyclodextrin-based column [23]. We have previously reported the resolution of the enantiomeric complexes of a Tc-99m PnAO-nitroimidazole complex using the Chiracel OD column [24]. This column was selected for evaluation with HM-PAO as it has proved successful in the resolution of a wide variety of drug enantiomers [25]. Tc-99m HM-PAO stereoisomers were also studied on the Chiralpak AD and Resolvosil BSA-7 columns.

Chiracel OD column

An initial study involving d,l-HM-PAO and the Chiracel OD column was conducted by Daicel Chemical Industries Ltd at our request; it was reported that the d- and l-enantiomers could be resolved using this system [26]. Our initial studies with this column were conducted at ambient temperature using hexane/IPA (97:3) as eluent. Typical chromatograms are shown in figure 2a. The d,l-mixture is resolved into two peaks, with the l-enatiomer eluting first. The meso-isomer has a retention time midway between the d- and l-isomers. The separation of d- and l-isomers deteriorated on raising the proporation of IPA in the eluent. However, separation was improved by raising the column temperature to 40 ^{o}C and by the addition of 0.1% diethylamine to IPA; as shown in figure 2b [26]. While this HPLC system does not provide baseline separation (R_S for d,l-HM-PAO = 0.96), it might be suitable for a quantitative assay of isomer purity of the d- and l-enantiomers. Following personal communication of our results to colleagues, this HPLC system has been employed for the preparative separation of d- and l-enatiomers from the d,l-mixture, resulting in the comparison of the Tc-99m enantiomer complexes in an animal model [16]. It should be noted that we found there was some variation in the separation of d,l-HM-PAO with the batch number of the column obtained from the supplier[#] .The Chiralcel OD column was also examined with respect to the separation of the Tc-99m complexes of HM-PAO. As shown in figure 3, this system provided excellent separation of the d,l-diastereoisomer complexes. However, baseline separation of Tc-99m meso-HM-PAO from Tc-99m l-HM-PAO was not achieved. These data indicate that the Chiracel OD system can be used to separate d- and l-HM-PAO (ligand and complex) from a d,l-mixture, but will not fully resolve HM-PAO (ligand and complex) as a mixture of the diastereoisomers.

We have recently shown that certain Tc-99m propyleneamine oxime (PnAO) complexes can undergo inversion of the TcO core. Thus, if the ligand is unsymmetical about the central carbon atom, Tc-complexation results in the formation of an enantiomeric pair of complexes, which can interconvert rapidly in the presense of water

[#] These studies were conducted over a five year period. It was found that the separation of the ligand enantiomers deteriorated with columns purchased after 1991. After sharing our findings with Chiral Technologies Inc, it appears that differences may well be related to changes in column manufacture.

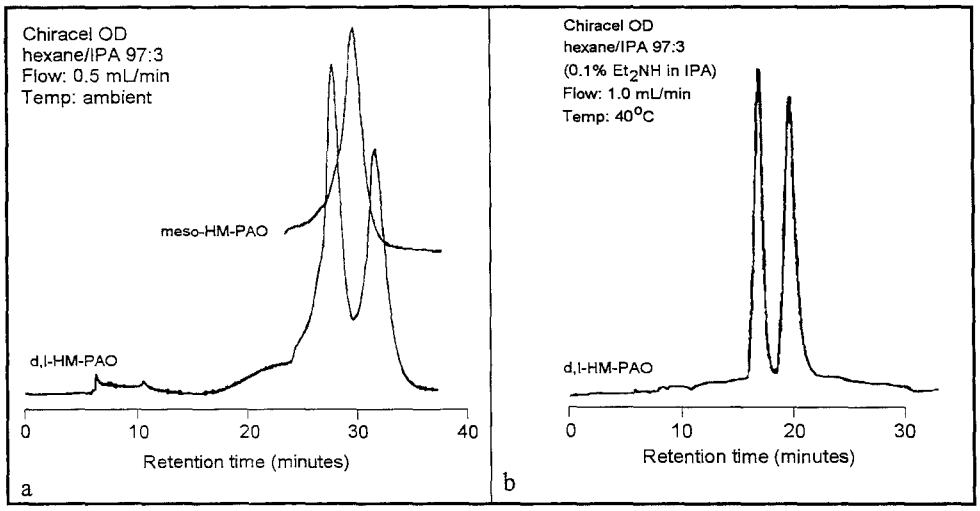

Figure 2. HPLC Chromatograms of meso- and d,l-HM-PAO (ligand) on the Chiracel OD column.

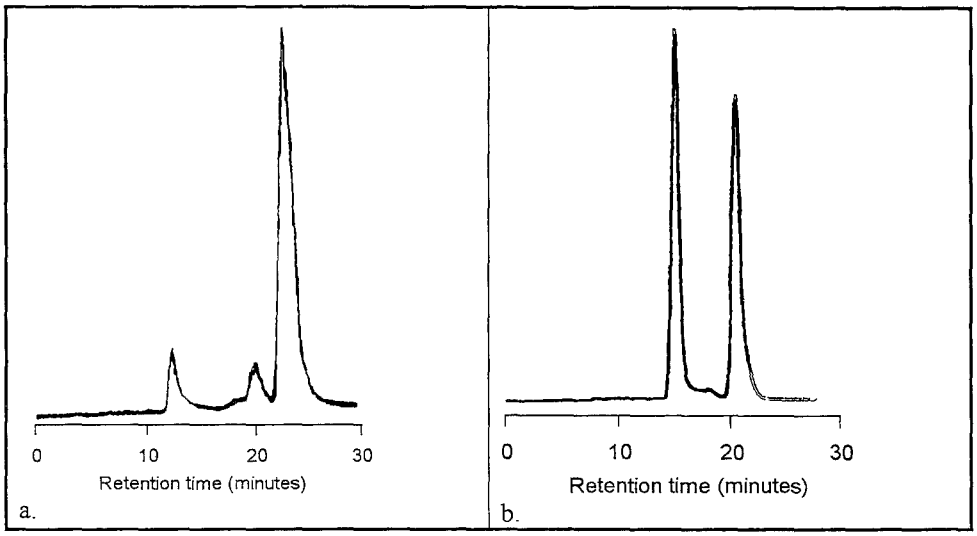

Figure 3. Separation of the Tc-99m complexes of HM-PAO on the Chiracel OD column

a. Chromatogram of the Tc-99m complexes from a meso-/d,l-mixture of HM-PAO (meso-form > 50%)

b. Chromatogram of Tc-99m d,l-HM-PAO

[Chiracel OD column hexane/IPA 85/15. Flow 1mL/min, temperature ambient]

[27]. In the case of the Tc-99m complex of d- or l-HM-PAO, a process in which the net result is inversion of the TcO core will give a product identical to the starting material. Thus, isolated fractions thought to be the complexes of either d- or l-HM-PAO should not convert to another HM-PAO complex over time unless racemization of the chiral center is involved. Water (to ~15%) was added to isolated HPLC fractions of Tc-99m d- and l-HM-PAO (from Chiracel OD HPLC (hexane/IPA 75:25) of Tc-99m d,l-HM-PAO). The solutions were allowed to stand at ambient temperature, and samples were reanalyzed over a period over several hours. While some peak broadening was observed (presumably due to the presense of water in the injectate), no additional peaks were formed.

Meso-HM-PAO can, in theory, form two technetium complexes, in which the two methyl substituents (adjacent to the nitrogen atoms) are both either syn- or anti- with respect to the Tc-oxygen atom (figure 4). The X-ray crystal structure of Tc-99 meso-HM-PAO [6] suggests that only one complex is formed (the syn-isomer), and conventional HPLC analyses of the Tc-99m complex have also indicated that only one complex is formed [28]. At all hexane/IPA solvent ratios employed in this study, Tc-99m meso-HM-PAO was observed as a single peak. Water (to ~15%) was added to an isolated HPLC fraction of Tc-99m meso-HM-PAO (from Chiracel OD HPLC (hexane/IPA 75:25)). As was the case with isolated d- and l-complexes, re-analysis showed that some peak broadening occured, but no additional peaks were formed. These data provide further evidence that only one of the two possible geometrical isomers of meso-HM-PAO Tc-complexes is formed. By comparison, another achiral linear tetradentate ligand, meso-ECD, gave two rhenium(V)O complexes (resolved on a cyclodextrin chiral column) [23]; which are, presumably, the syn- and anti-forms of the complex (figure 4).

In order to assign individual peaks as d- and l- , samples of these enantiomers were obtained by crystallization of the tartrate salts of the crude meso/d,l-stereoisomer mixture obtained on ligand synthesis. HPLC chromatograms of the isolated d- and l-enantiomers of HM-PAO (free base form) are shown in figure 5, and the chromatograms of the complexes obtained from these ligands are shown in figure 6. The elution order of the ligand enantiomers on the OD column was l- then d-. The chromatogram of the isolated d-HM-PAO ligand enantiomer shows a single peak, while that for the l-HM-PAO ligand enantiomer indicates that there is either meso- or d-isomer present as an impurity. For the complexes, the l-enantiomer again has a shorter elution time than the d-enantiomer. However, because the complexes have better separation than the ligands on this HPLC system, the isomer impurity obtained on recrystallization of HM-PAO L-(+)-tartrate salt can be seen to be the meso-form in the analysis of Tc-99m l-HM-PAO. In addition, the d-complex also displays some meso-complex as an impurity. From the chromatograms of the complexes, it was determined that the % meso-ligand remaining in the d- and l-samples are 17.8 and 12.5%, respectively.

Table 1 displays the dependence of peak separation of the technetium complexes on solvent composition (hexane/IPA ratio). Baseline separation of d- and l-complexes was achieved with all eluents with % hexane $\geq$ 25%. Good separation (R_S = 0.90) of meso- and d-complexes was obtained using 85% hexane.

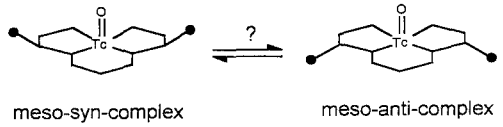

Figure 4. Schematic representation of the syn- and anti-forms of the Tc-complexes of meso-ligands, e.g. HM-PAO and ECD

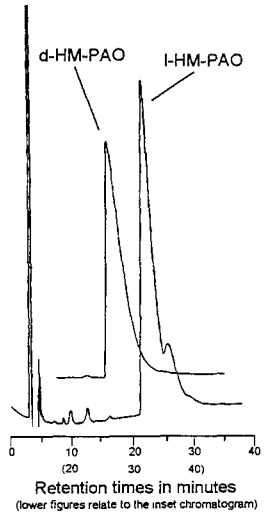

Figure 5. Analysis of isolated d- and l-enantiomers of HM-PAO (ligand) on the OD column.

Chiralpak AD column

Figure 7 displays a series of chromatograms obtained with samples of HM-PAO (ligand) on the Chiralpak AD column, eluted at room temperature with hexane/ethanol (containing 0.1% diethylamine). This system provides excellent separation of all three stereoisomers. In table 2 are listed the peak retention times and R_S values of d-, l-, and meso- peaks at several different hexane/ethanol ratios. By comparison, the Tc-99m complexes of HM-PAO were not resolved on this system. With isocratic elution and % EtOH >10%, the Tc-99m stereoisomer complexes co-eluted. At 10% EtOH, the meso-complex was observed as a shoulder to the d,l-peak. The complexes were not eluted using 5% EtOH, but using a shallow gradient elution profile (5 --> 10% of EtOH), the meso-peak could be separated ($R_S < 1$) from the d,l-peak.

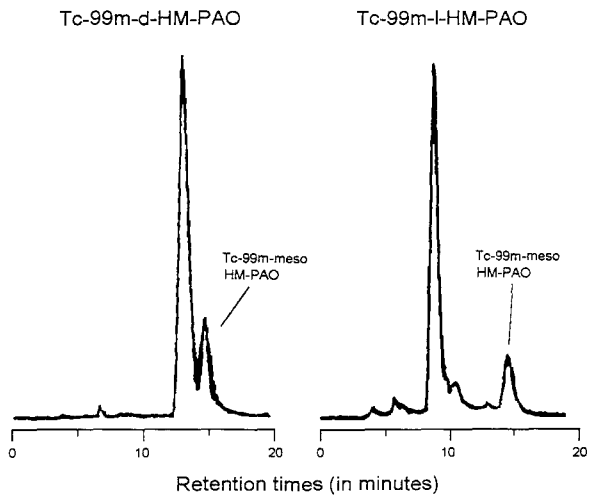

Figure 6. HPLC chromatograms of Tc-99m complexes from isolated d- and l-HM-PAO

Table 1. Separation of the Tc-99m complexes of meso-, d,- and l-HM-PAO on the Chiracel OD column at several solvent (hexane/IPA) ratios.

% hexane	R_S		% hexane	R_S	
	l and d	d and meso		l and d	d and meso
0	0.87	0.32	80	3.20	0.85
25	1.02	0.26	85	3.27	0.90
50	1.16	0.32	90	2.91	0.61
75	2.64	0.69			

Comparison of these results with those obtained on the OD column might appear to be somewhat surprising given the similarity between the OD and AD columns. Both columns have a silica gel base, to which is attached a polysaccharide based on a 1,4-glucose repeating unit derivatized (2-, 3- and 6-positions) with a 3,5-dimethylphenyl carbamate moiety. The fundamental difference is that the OD column is based on cellulose (β-1,4 linkage) and the AD system is based on amylose (α-1,4 linkage). This difference gives rise to very different three dimentional structures, and hence different resolving characteristics.

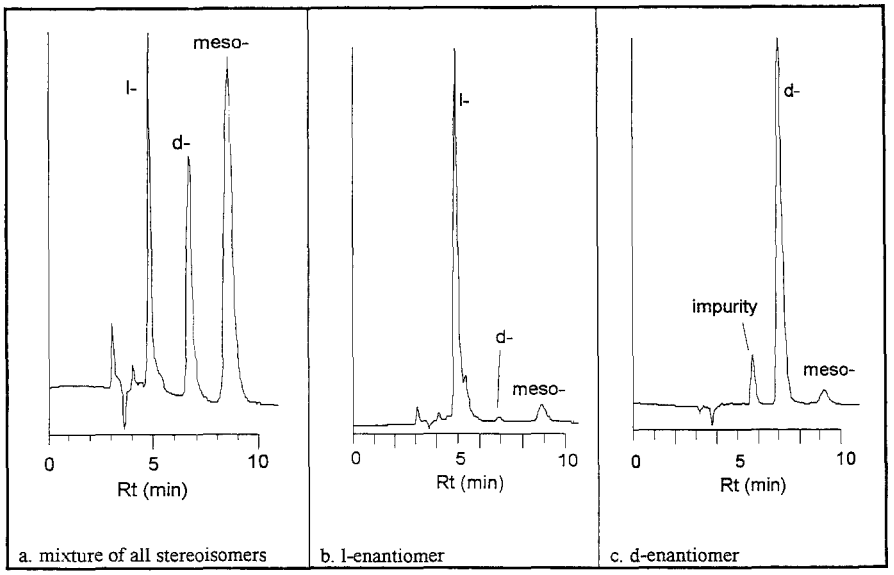

Figure 7. HPLC chromatograms of HM-PAO on the Chiralpak AD column.

Table 2. Separation of meso-, d,- and l-HM-PAO on the Chiralpak AD column at several solvent (hexane/EtOH) ratios.

% hexane	R_t (min)			R_S	
	l-	d-	meso-	l and d	d and meso
65	4.4	5.6	6.8	1.2	1.1
75	4.8	6.8	8.8	1.9	1.9
70	5.6	8.8	11.1	2.2	1.4
85	6.8	11.0	14.8	2.9	1.7

Chromatograms b and c in figure 7 show the results of ligand analyses on the AD column of two samples described in the previous section; l- and d-HM-PAO obtained by recystallization of the tartrate salts of the orginal meso/d,l-mixture. There is reasonably good agreement between the results of analyses of these samples as their Tc-99m complexes on the OD column, and as the free ligand on the AD column (these results are shown in table 3). For any given sample, the analytical results for the free ligand on the OD column and the complexes (formed from this ligand mixture) on AD column could only be similar provided that the avidity of the meso- and d,l-stereoisomers of HM-PAO for technetium are almost identical. The data given in table 3 would indicate that the HM-PAO stereoisomers have similar affinities for technetium.

Table 3. Comparison of the results of analyses of purified samples of d- and l-HM-PAO; analysis as free ligand on the Chiralpak AD column and as Tc-99m complexes on the Chiracel OD column.

sample/column	%		
	l-	d-	meso-
purified d- on OD	< 1	82.2	17.8
purified d- on AD[1]	6.1	74.3	19.6
purified d- on AD[2]	< 1	79.2	20.8
purified l- on OD	87.5	< 1	12.5
purified l- on AD[1]	87.3	1.8	10.9
purified l- on AD[2]	86.1	1.9	12.0

Key 1 - % l- includes the main peak and shoulder peak
2 - % l- includes the main peak but not the shoulder peak

In the chromatograms of HM-PAO ligand on the AD column, a shoulder to the main l-peak was observed (see figure 7). This peak is clearly seen in the samples of "purified" l-enantiomer, and, to a lesser extent, in samples which are mixtures of the stereoisomers. In the sample of "purified" d-enantiomer (figure 7c), the peak which appears to be l-HM-PAO has a R_t which corresponds to the shoulder peak rather than the main l-peak. impurity shown as l- in figure 7c . On labelling this sample with Tc-99m, it is clear that this impurity does not appear form Tc-99m l-HM-PAO complex (table 3). At present, the identity of this shoulder peak is not known. One possibility is that it is an oxime isomer. In HM-PAO, the oximes prefer to adopt the E-configuration, although E,Z- and Z,Z-isomers have been observed [29]. When isolated, Z-oxime HM-PAO isomers only give low yields of Tc-99m HM-PAO complexes [29].

An HPLC system based upon the AD column provides a convenient method for the determination of stereoisomeric purity of samples of HM-PAO. This system was used to analyze the products from several tartrate salt recrystallizations of HM-PAO. Table 4 displays the results of one of these studies, showing that one stereoisomer can be isolated with high enantiomeric purity after just two recrystallizations starting from the reaction product.

Resolvosil BSA-7 column

Due to the sensitivity of this column to ethanol, the samples of Tc-99m d,l-HM-PAO used with this column were not subjected to the solid phase purification procedure, described above. While methods of HM-PAO complex stabilization are known [30], aqueous solutions of Tc-99m d,l-HM-PAO formed from the the commercially-available kit degrade rapidly to form one or more so-called secondary complexes [5,29,31-34]. A steady increase in the proportion of these secondary complexes was observed (as a broad

Table 4. Results of the analysis of one series of tartrate salt recrystallization samples on the Chiralpak AD column.

	% of each HM-PAO stereoisomer		
	d-	l-	meso-
1st crystallization	72.2	9.0	18.8
2nd crystallization	90.1	4.3	5.6
3rd crystallization	92.4	6.7	1.0

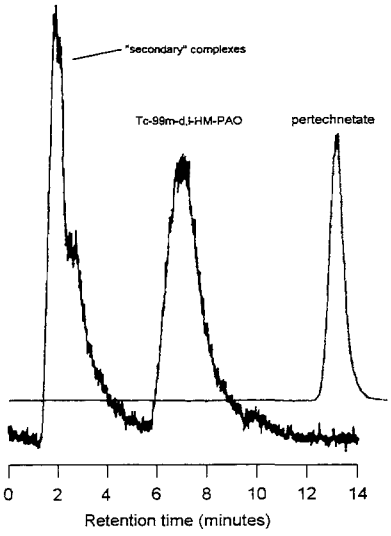

Figure 8. HPLC chromatogram of Tc-99m d,l-HM-PAO (4 hours post preparation) on the Resolvosil BSA system. A chromatogram of Tc-99m pertechnetate, obtained on the same system, is overlayed.

peak close to the void volume) on analysis of the aqueous solution of Tc-99m d,l-HM-PAO on the Resolvosil BSA-7 system over a period of several hours. However, this system fails to resolve the d- and l- enantiomer complexes, which appear as a broad peak at $R_t \sim 7.5$ minutes. Another major degradation product of Tc-99m d,l-HM-PAO prepared from the commercially-available kit is Tc-99m pertechnetate [31]. On the reversed-phase HPLC systems generally used for RCP determination of Tc-99m d,l-HM-PAO, pertechnetate appears in the void volume, and is indistinguishable from the secondary complexes. On the Resolvosil BSA-7 system, Tc-99m pertechnetate appears as

a sharp peak with greater retention than Tc-99m d,l-HM-PAO (figure 8). Therefore, while this HPLC system does not resolve the complexes of the d- and l-enantiomers, it has some potential for the rapid determination of radiochemical purity in those situations when it is desirable to distinguish between pertechnetate and the secondary complexes.

CONCLUSIONS

Three chiral HPLC columns were examined to determine their potential for the separation and analysis of HM-PAO stereoisomers. Tc-99m d,l-HM-PAO appeared as a single broad peak on the Resolvosil BSA-7 column. However, this peak was well separated from the radioactive impurities, Tc-99m pertechnetate and the so-called secondary complexes of Tc-99m d,l-HM-PAO. Therefore, while the Resolvosil BSA-7 column failed to separate the complex stereoisomers, it might be useful for routine analysis of the complex. The Chiracel OD column, using hexane/IPA as eluent, did separate the d- and l-HM-PAO ligand enantiomers. However, as separation was poor, and meso-HM-PAO was eluted midway between the enantiomers. The Tc-99m complexes of the d- and l-enantiomers are well separated on the Chiracel OD column, although there is only modest separation of the complexes from the meso- and d-ligands. Baseline separation of all three stereoisomers of the ligand was achieved on the Chiralpak AD column. This system does appear to be suitable for the routine analysis of the isomeric purity of HM-PAO samples. In addition, as semi-preparative versions of this column are commercially-available, it should be possible to isolate small quantities of HM-PAO enantiomers directly from the reaction mixture or from samples in which the proportion of one stereoisomer is increased through tartrate-salt recrystallization.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC PROFILING OF CANNABIS PRODUCTS

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ABSTRACT

An HPLC method with photodiode array detection (DAD) is described for the qualitative and quantitative determination of neutral and acidic cannabinoids in Cannabis sativa L. The complex chromatographic pattern can be used for the classification of Cannabis chemotypes, the monitoring of the psychotropic potency and the comparison of Cannabis products of different origin.

INTRODUCTION

Psychotropic products of the chemotype I (drug type) (1,2) and chemotype II (intermediate type) of Cannabis sativa L. - like herbal Cannabis (marijuana), Cannabis resin (hashish) and extracts of Cannabis resin (hashish oil) - are the most abused illicit drugs of the world. On the other hand, the chemotype III (fiber/industrial type) becomes in Europe

more and more important as a renewable, fast growing fiber producing plant. It is an economically and ecologically interesting alternative source for the production of natural fibers, which can be used for example as an inexpensive, rugged raw material in the paper, car or building industry.

For the last ten years our laboratory has analyzed several hundred samples of Cannabis sativa L. of different origin as part of botanical, phytochemical and forensic research projects, using an isocratic HPLC method (1-3). Due to the lack of suitable standards this method did not allow to measure the main neutral *and* acidic cannabinoids by direct quantitation. The same holds true for other published HPLC methods (4-8). This paper describes the first HPLC method with photodiode array detection (DAD) for the aquisition of full cannabinoid profiles.

MATERIALS AND METHODS

Instrumentation

All HPLC analyses were performed on a Hewlett-Packard (HP, Waldbronn, Germany) HPLC system consisting of a 1090M liquid chromatograph, a 1090L autosampler, a 1040M photodiode array detector, a Vectra 486/33N computer with HPLC Chemstation Rev. A.02.00 software and a Desk Jet 550C printer.

Chromatographic Conditions

The HPLC separation of Cannabis extracts was performed at 40°C oven temperature on a 200 x 2.0 mm i.d. column with a 20 x 2.0 mm i.d. precolumn, packed with Spherisorb ODS-1, 3 µm (Stagroma, Wallisellen,

Switzerland). Solvent A was water, containing 8.64 g/L orthophosphoric acid (85%), solvent B was acetonitrile. The gradient profile was as follows: 0-38 min, 47-60% B, linear; 38-48 min, 60-70% B, linear; 48-50 min, 70-47% B, linear; 50-60 min, 47% B, isocratic. The flow rate was 200 µL/min. The solvents were filtered under vacuum through a 0.45-µm nylon membrane filter and degassed by sonication prior to use and by a constant flow of helium during use. After use the column was washed with acetonitrile. Precolumn and column have been replaced after 50 and 150 runs, respectively. The quantitation of THCA-B, CBD, CBG and THC was performed at 210 nm, CBDA, CBN and THCA-A at 224 nm. The peak identity was ascertained by on-line scanning of UV spectra from 192 to 350 nm at a sampling rate of 3.125 spectra/sec.

Chemicals and Reagents

(-)-Δ^9-(*trans*)-tetrahydrocannabinolic acid A (THCA-A) was isolated in our laboratory (9). (-)-Δ^9-(*trans*)-tetrahydrocannabinol (THC) and cannabichromene (CBC) were donated by the UN Narcotics Laboratory (Vienna, Austria). Cannabidiol (CBD), cannabigerol (CBG) and cannabinol (CBN) were obtained by the Swiss Federal Office of Public Health (Berne, Switzerland). (-)-Δ^9-(*trans*)-tetrahydrocannabinolic acid B (THCA-B), cannabidiolic acid (CBDA), (-)-Δ^9-(*trans*)-tetrahydrocannabivarin (THV), (-)-Δ^9-(*trans*)-tetrahydrocannabivarinic acid (THVA), cannabiripsol (CBR), cannabielsoin (CBE) and cannabicyclol (CBL) were kindly supplied by the Research Institute of Pharmaceutical Sciences, University of Mississippi (Oxford, MS, USA). Chemicals and reagents were of HPLC or analytical grade, purchased from Merck (Basel, Switzerland).

Cannabis Samples

Cannabis of chemotype I and II was collected from own Cannabis plantations, authorized by the Swiss Federal Office of Public Health. Chemotype III was collected from plantations of the Swiss Federal Office of Agriculture. Hashish and hashish oil have been confiscated by police and customs authorities.

Sample Preparation

100 mg dried (40°C, 24h), pulverized herbal Cannabis (Cannabis, marijuana), 50 mg Cannabis resin (hashish) or 50 mg Cannabis oil (hashish oil) was extracted with 1.0 mL methanol-chloroform (9:1, v/v) by sonication during 15 min. 100 µL of the filtered extract was diluted with 300 µL of methanol and aliquots of 1 µL were used for HPLC.

Quantitation

HPLC quantitation was performed by the external standard method, measuring the peak areas of the cannabinoids at their maxima of 210 or 224 nm, respectively. The calibration graphs (linear regression analysis) were obtained by triple analysis of different injection volumes (0.5 - 5 µL) of standard mixtures containing 10, 100 and 1000 ng/µL of each cannabinoid.

RESULTS AND DISCUSSION

The *sample preparation* is much faster and simpler than the previous method used routinely in our laboratory (3). As isolated

cannabinoid acids, like THCA-A and CBDA, the dominant biogenic cannabinoid acid of Cannabis drug type and fiber/industrial type, respectively, are now available as standards, the evaporation and thermal decarboxylation of the Cannabis extracts can be avoided and direct quantitation is possible. Previously, these steps were necessary for the indirect quantitation of THCA-A, THCA-B and CBDA by measuring the amount of the corresponding neutral cannabinoids (THC, CBD) before and after decarboxylation of the acids at high temperature. The efficiency of the extraction was checked by duplicate extraction of two samples and showed a *recovery* of 97.6 to 99.4% for the main cannabinoids.

The *chromatographic system* is based on acetonitrile-water-phosphoric acid, a solvent with low UV cut-off and allowing the detection down to 192 nm, and 3-µm spherical reversed phase material in a low-diameter column, allowing to reduce the flow rate to 200 µL/min. A sharp symmetrical peak-shape and sufficient separation of the cannabinoids from the complex plant matrix can be achieved (see Fig. 1 and 2). The large polarity range of the cannabinoids requires the use of a fine tuned solvent gradient program to limit the run to 60 min for a full cannabinoid profile. Co-extracted lipophilic plant constituents necessitate the replacement of the column and precolumn after 150 and 50 runs, respectively, because of deteriorating peak-shapes and -resolution (especially between CBC and THCA-A).

Peak identification was achieved by standards and their characteristic DAD-UV spectra (see Fig. 3) with a library match of > 990 within a retention time window of ± 0.2 min. Retention time windows were adjusted every 10th run with a standard mixture, if necessary. An indication of peak homogeneity was given by peak purity check demonstrated by an up-slope, apex and down-slope peak spectra match of 990 or more. The *limit of detection*, measured with standard mixtures,

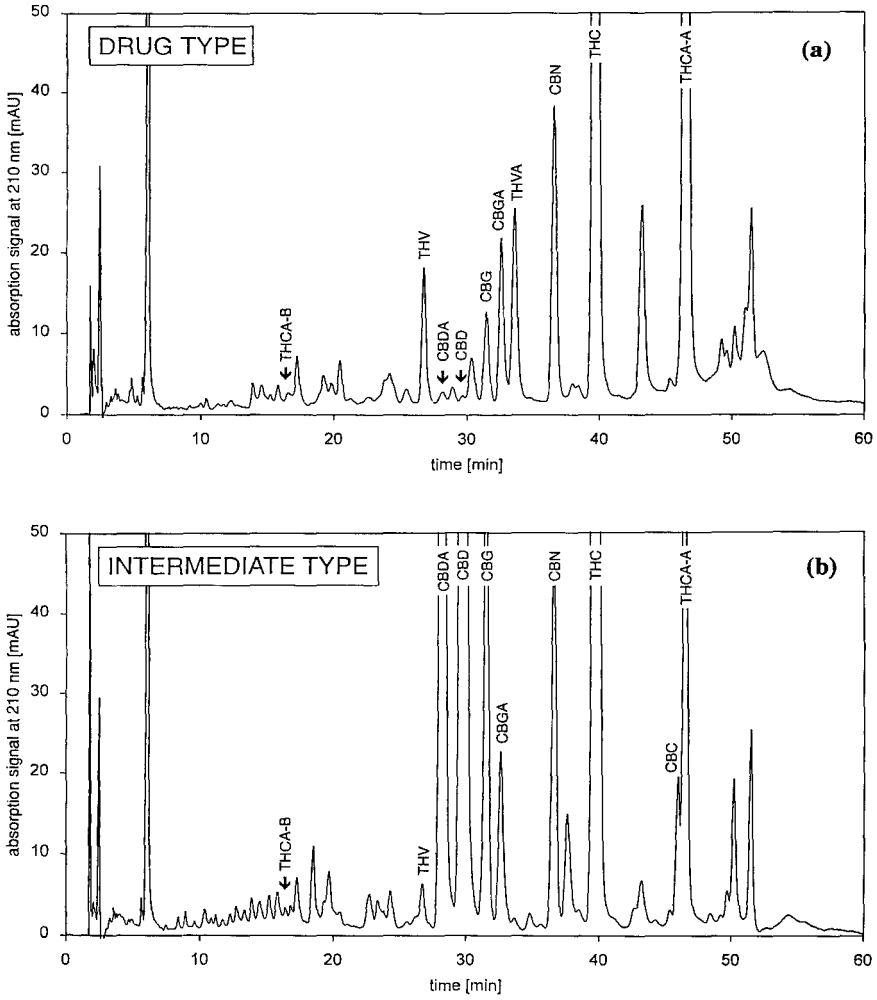

FIGURE 1. HPLC profiles of chemotypes of Cannabis sativa L.: (A) Chemotype I (drug type); (B) chemotype II (intermediate type); (C) chemotype III (fiber/industrial type).

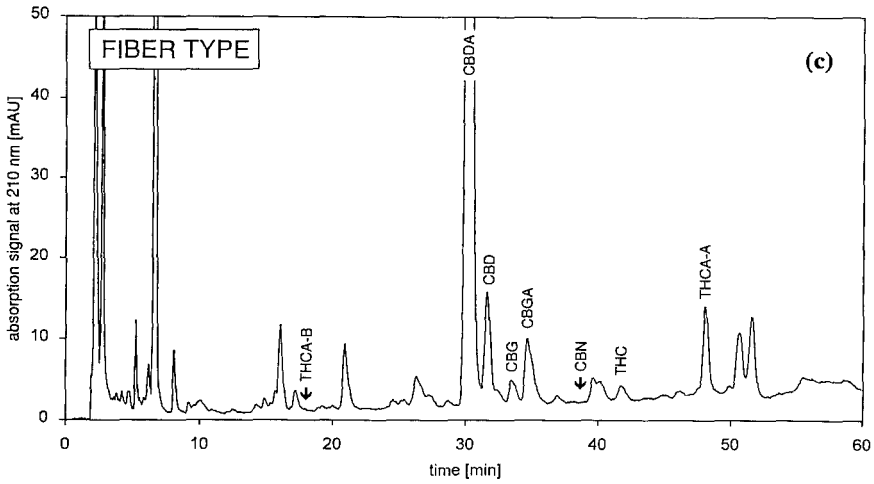

FIGURE 1 (Continued)

was 1 ng cannabinoid on the chromatographic system with a signal to noise ratio of 3 or higher. The *limit of quantitation* was about 25 ng cannabinoid per 1 µL extract, corresponding to 0.1% cannabinoids per drug sample and allowing to acquire full UV spectra. The *linearity* was determined in the range of 5 to 5000 ng for each cannabinoid. A linear relationship was found between the peak area and the concentration of THC, CBG, CBD, CBN, THCA-A, THCA-B and CBDA. The correlation coefficient r of these cannabinoids was > 0.9999. The inter-day precision of the calibration was checked by analyzing two replicates of solutions containing 100 and 1000 ng/µL cannabinoid standards at five different days within one month. The relative standard deviations were between 3.0 and 5.9% at the low (100 ng/µL) concentration level and between 1.3 and 4.3% at the high (1000 ng/µL) concentration level. The calibration solutions are stable over five weeks if stored at -20°C. The over-all

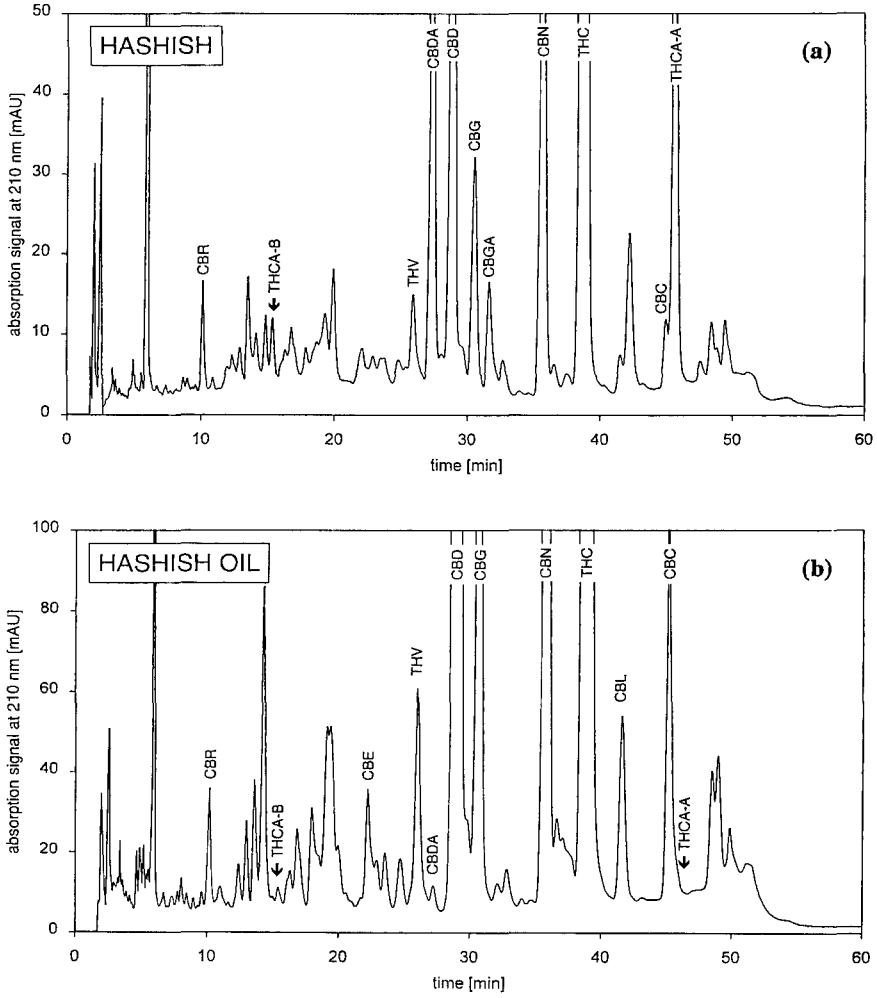

FIGURE 2. HPLC profiles of other Cannabis products: (A) Cannabis resin (hashish); (B) Cannabis resin extract (hashish oil).

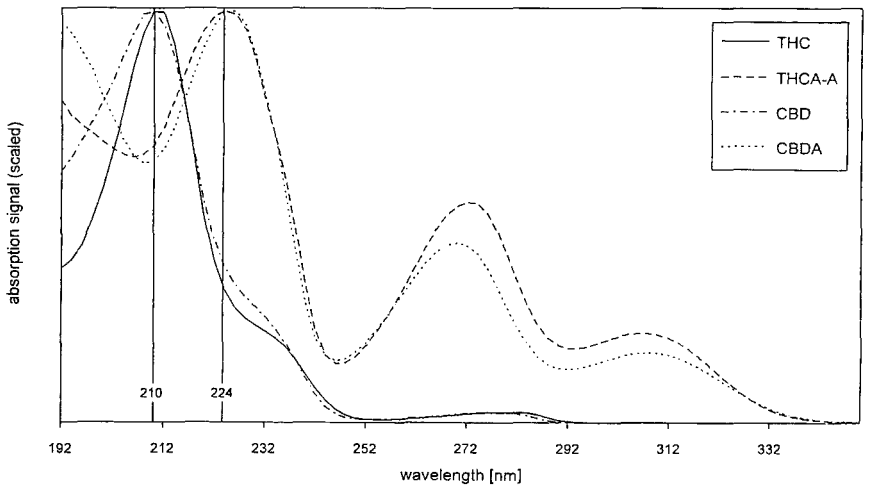

FIGURE 3. DAD-UV spectra of neutral and acidic cannabinoids.

precision of the method was determined by analyzing two replicates of five Cannabis extracts within one day and on five different days within two months. The relative standard deviations for the intra-day and inter-day variation were 2.3 to 5.8% and 2.7 and 5.3%, respectively.

As can be seen in Figures 1 and 2, the HPLC profiles of extracts of Cannabis products show up to 40 different peaks. With standards and DAD-UV spectra, 13 major or minor cannabinoids could be identified as CBR, THCA-B, THV, CBDA, CBD, CBG, CBGA, THVA, CBN, THC, CBL, CBC, and THCA-A. The quantitation included the key cannabinoids THC, THCA-A, CBD, and CBDA as well as THCA-B, CBG and CBN. The resulting characteristic chromatographic pattern makes the forensically and pharmacologically important differentiation of chemotype I-III of herbal Cannabis feasible. Chemotype I, the so called drug type, is characterized by the dominant key cannabinoids THC and THCA-A. The THC and THCA-A concentrations varied from 0.1 to 2.5% and from 0.1 to 8.2%,

respectively. Chemotype II, the intermediate type, shows high concentrations of THC/THCA-A as well as CBD/CBDA. Both chemotypes are psychoactive. A typical specimen of this type contained 4.31% (± 0.12%) CBDA, 2.30% (± 0.09%) CBD, 0.17% (± 0.01%) CBG, 0.33% (± 0.01%) CBN, 1.19% (± 0.06%) THC and 1.95% (± 0.10%) THCA-A. CBD and CBDA are the major cannabinoids of the psychoinactive chemotype III, the fiber or industrial type, whereas the THC/THCA-A content is < 0.5%. Only traces of THCA-B could be detected in all analyzed samples. Chemotype IV, the propyl isomer/C3 type (10, 11), can be differentiated by the dominant key cannabinoids (-)-Δ^9-(*trans*)-tetrahydrocannabivarin (THV) and its corresponding acid (-)-Δ^9-(*trans*)-tetrahydrocannabivarinic acid (THVA). This least frequent chemotype, originating mainly from South Africa (11, 12), contains also remarkable amounts of THC/THCA-A (THV/THVA : THC/THCA-A > 1) and exhibits psychoactivity. Low concentrations of THV/THVA can be detected in most Cannabis samples of chemotype I and II. Figure 2 demonstrates that HPLC profiles of the resin (hashish) and the resin extract (hashish oil) are usually more complex than those of herbal Cannabis (marijuana). Resin extracts which are produced by solvent extraction or more often by direct distillation of the resin using high temperature resulting in a reduction of the acids and decomposition of unstable cannabinoids.

CONCLUSION

The described HPLC-DAD method can be used for classifying Cannabis chemotypes, for monitoring the psychotropic potency of Cannabis products by quantitation of THC/THCA, for checking the identity of Cannabis specimens of different origin by comparing subtle differences

in their chromatographic pattern and within other applications, where the information of a full cannabinoid profile is useful. It completes our earlier published GC/MS procedure, which was developed for profiling mainly the non-cannabinoid constituents of Cannabis products and used for the determination of the geographical origin of confiscated Cannabis samples (13, 14).

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF NONAMIDATED AND GLYCINE- AND TAURINE-AMIDATED BILE ACID 3-GLUCOSIDES

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ABSTRACT

The high-performance liquid chromatographic separation of the 3-glucosides of nonamidated lithocholic, chenodeoxycholic, ursodeoxycholic, deoxycholic and cholic acids, and their double conjugate forms with glycine and taurine has been carried out on a C18 reversed-phase column. Satisfactory separation not only of each of the three groups of the nonamidated and amidated bile acid 3-glucosides but also of the individual compounds in the same group was attained by employing acetonitrile-0.3% potassium phosphate buffer (pH, 7.0) as mobile phase. The retention data reported here provide an insight into structural elucidation of these biologically important bile acid 3-glucosides.

INTRODUCTION

Recently, the glycosidic conjugates of bile acids are of substantiated interest in biosynthesis, metabolism, and physiological significance in connection with hepatobiliary diseases. At present, three types of glycosidic conjugation are known in bile acid metabolism in humans: glucuronidation (1,2), *N*-acetylglucos aminidation (3,4), and glucosidation (5-8). Of these glycosidic conjugates, bile acid glucosides, novel conjugates, have recently been shown to be formed in human liver microsomes by a glucosyltransferase and are preferentially excreted in human urine from patients with hepatobiliary diseases.

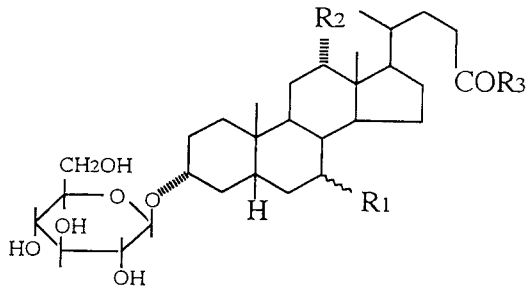
High-performance liquid chromatography (HPLC) with a reversed-phase column seems to be a most reliable method for analysis of such polar, nonvolatile and thermolabile compounds. In fact, a direct HPLC analysis of glycine-, taurine-, sulfate-, glucuronide- and *N*-acetylglucosaminide-conjugated bile acids without prior deconjugation has been successfully applied (9-11).

As part of our synthetic program on potential bile acid metabolites, we have recently synthesized a series of nonamidated and glycine- and taurine-amidated bile acid 3-glucosides as authentic specimens (Fig. 1) (12). In this paper, we clarify the retention behaviors of the bile acid 3-glucosides on a reversed-phase HPLC and compare with those of analogous bile acid 3-glucuronides and 3-*N*-acetylglucosaminides reported previously (10,11).

EXPERIMENTAL

Samples and Reagents

The 3-glucosides of nonamidated and glycine- and taurine-amidated bile acids were synthesized in these



	R1	R2	R3	
1	H	H	OH	(LCA 3-Glc.)
2	α-OH	H	OH	(CDCA 3-Glc.)
3	β-OH	H	OH	(UDCA 3-Glc.)
4	H	α-OH	OH	(DCA 3-Glc.)
5	α-OH	α-OH	OH	(CA 3-Glc.)
6	H	H	$NHCH_2COOH$	(Glyco-LCA 3-Glc.)
7	α-OH	H	$NHCH_2COOH$	(Glyco-CDCA 3-Glc.)
8	β-OH	H	$NHCH_2COOH$	(Glyco-UDCA 3-Glc.)
9	H	α-OH	$NHCH_2COOH$	(Glyco-DCA 3-Glc.)
10	α-OH	α-OH	$NHCH_2COOH$	(Glyco-CA 3-Glc.)
11	H	H	$NHCH_2CH_2SO_3H$	(Tauro-LCA 3-Glc.)
12	α-OH	H	$NHCH_2CH_2SO_3H$	(Tauro-CDCA 3-Glc.)
13	β-OH	H	$NHCH_2CH_2SO_3H$	(Tauro-UDCA 3-Glc.)
14	H	α-OH	$NHCH_2CH_2SO_3H$	(Tauro-DCA 3-Glc.)
15	α-OH	α-OH	$NHCH_2CH_2SO_3H$	(Tauro-CA 3-Glc.)

FIGURE 1. Structures of bile acid 3-glucosides.

laboratories by the methods recently reported (12). All the chemicals used were of analytical reagent grade. Solvents used were of HPLC grade and were degassed by sonication prior to use.

Apparatus

The HPLC apparatus used was a Hitachi L-6000 chromatograph (Hitachi,. Tokyo, Japan) equipped with a Shimadzu SPD-2A ultraviolet detector (Shimadzu, Kyoto, Japan); the wavelength selected for all measurements was 205 nm. A Cosmosil 5C18 column (5 μm, 150 mm X 4.6 mm I.D.) (Nacarai Tesque Inc., Kyoto, Japan) was used under ambient conditions. Acetonitrile-0.3% potassium phosphate buffer mixture (pH, 3.5~7.5; ratio from 25:75 to 35:65, v/v) was used as eluent at a flow rate of 1.0 ml/min.

RESULTS AND DISCUSSION

The chemical structures of bile acid 3-glucosides examined in this study are shown in Fig. 1. These 3-glucosides include the derivatives of the nonamidated (1~5) and glycine- (6~10) and taurine- (11~15) amidated forms of five prominent naturally occurring bile acids [*i.e.*, lithocholic acid (LCA), chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA), deoxycholic acid (DCA), and cholic acid (CA)] and differ from one another in the number, position and configuration of hydroxyl groups at positions C-3, C-7 and/or C-12 in the 5β-steroid nucleus.

Recently, Goto *et al.* have reported the HPLC behaviors of bile acid 3-glucuronides (10) and 3-*N*-acetylglucosaminides (11) on a C18 reversed-phase column using acetonitrile-potassium phosphate buffer mixture as the mobile phase and found that the capacity factor (k') of those conjugates are extensively dependent on both the structure of the substrates and the acidity of the mobile

phase. On the basis of these findings, the effect of pH of mobile phase on the k' values of each of the three groups of nonamidated and glycine- and taurine-amidated bile acid 3-glucosides were initially examined. In order to facilitate a comparison of their mobilities with those of the analogous glucuronide and *N*-acetylglucosaminide conjugates (10,11), acetonitrile-0.3% potassium phosphate buffer (pH, 3.5~7.5) was used as the eluent system. The result is expressed graphically in Fig. 2. As expected, the k' values (relative to tauro-DCA) of each compound respond to the structural differences in the 5β-steroid nucleus and C-17 side chain, and separation efficiency and analysis time of individual members of compounds are markedly influenced by pH of the mobile phase.

Among the three groups of bile acid 3-glucosides, the nonamidated forms were the most sensitive to the pH of mobile phase and then followed by glycine conjugates, and their k' values increased with decreasing pH from *ca.* 6~7. In neutral or slightly alkaline condition (pH, 7.0~7.5), each of these two groups of compounds exhibited a well-shaped peak with a relatively small k' value and short retention time. On the contrary, the k' values for the taurine conjugates are scarcely affected throughout the whole pH range (3.5~7.5) examined. The phenomenon is compatible with those observed with analogous bile acid 3-glucuronides (10) and 3-*N*-acetylglucosaminides (11), and can be explained in terms of the difference in the degree of dissociation in the three types of side chain (9). Based on the above data, acetonitrile-0.3% potassium phosphate buffer of pH=7.0 was chosen as suitable mobile phase.

Five nonamidated bile acid 3-glucosides differing in the number, position and configuration of hydroxyl groups in the aglycone moiety were well resolved on Cosmosil 5C$_{18}$ column using the appropriate eluent system, and the

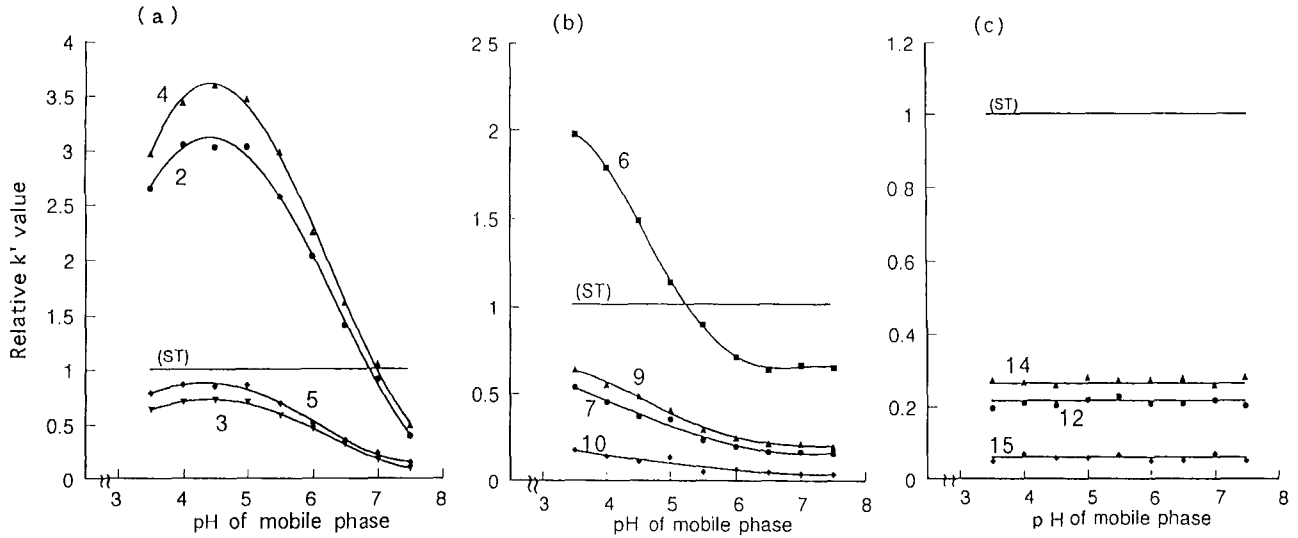


FIGURE 2. Effect of pH of mobile phase on relative k' values of(a) nonamidated and (b) glycine- and (c) taurine-amidated bile acid 3-glucosides. St (standard) = taurodeoxycholic acid. HPLC conditions: column, Cosmosil 5C18; mobile phase, acetonitrile- 0.3% potassium phosphate buffer (pH, 3.5~7.5),(30:70~35:65, v/v); flow rate, 1.0 ml/min; detection, UV at 205 nm. The numbering corresponds to that in Fig. 1.

TABLE 1. Relative k' values of nonamidated and amidated bile acid 3-glucosides on reversed-phase HPLC.[1]

Nonamidated		Glycine-amidated		Taurine-amidated	
1	2.94	6	2.37	11	3.50
2	0.84	7	0.86	12	1.26
3	0.16	8	0.16	13	0.23
4	1.00	9	1.10	14	1.52
5	0.25	10	0.29	15	0.38

[1] k' Values are expressed relative to that of DCA 3-Glc. (4); mobile phase, acetonitrile-0.3% potassium phopsphate buffer (pH, 7.0; 25:75, v/v). The numbering corresponds to that in Fig.1.

following order of increasing mobility was observed: UDCA 3-Glc. (3)< CA 3-Glc. (5)< CDCA 3-Glc. (2)< DCA 3-Glc. (4)< LCA 3-Glc. (1). The same elution order was also observed on the corresponding glycine and taurine double conjugates. The order of mobility in each group usually corresponds to the number of hydroxyl groups in the substrates and is consistent well with those observed for the corresponding bile acid 3-glucuronides (10) and 3-N-acetylglucosaminides (11). However, the 3-glucosidic conjugates of UDCA having a β-hydroxyl group at position C-7 had decidedly the earliest mobility in each group, probably due to a decrease of the hydrophobic interaction between the β-face of the steroid nucleus and the surface of the stationary phase.

Table 1 shows the k' values [relative to DCA 3-Glc. (4)] for the 15 bile acid 3-glucosides determined on a

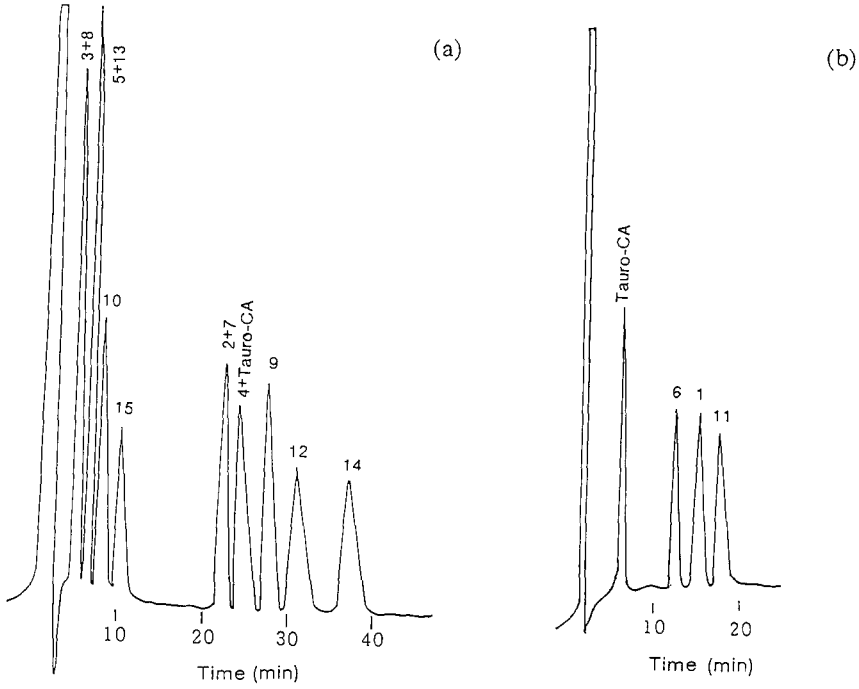


FIGURE 3. HPLC separation of a mixture of bile acid 3-glucosides. Conditions: column, Cosmosil 5C18; mobile phase, acetonitrile-0.3% potassium phosphate buffer (pH, 7.0) (a) 23:77 (v/v) and (b) 28:72 (v/v). Peak identification and the numbering as in Fig. 1.

Cosmosil 5C18 column under identical HPLC conditions [acetonitrile-0.3% potassium phosphate buffer (pH, 7.0) (25:75, v/v)]. Typical HPLC chromatograms for the separation of a mixture of these compounds are also illustrated in Fig. 3. It is not practical to analyze simultaneously the 3-glucosidic conjugates of monohydroxylated LCA (1, 6 and 11) and other di- and

trihydroxylated bile acids in short analysis time under an isocratic condition , because of higher lipophilicity of the formers compared with the latters. In addition, the taurine conjugates in each group of bile acid 3-glucosides were found to be always eluted more slowly than the corresponding nonamidated and glycine-amidated analogs. Although two pairs of nonamidated and glycine-amidated bile acid 3-glucosides [*i.e.*, CDCA 3-Glc. (2) *vs* . glyco-CDCA 3-Glc. (7) and UDCA 3-Glc. (3) *vs* . glyco-UDCA 3-Glc. (8)] overlapped with the eluent system, they were resolved by decreasing the pH of mobile phase.

The present retention data reported here may be helpful for characterizing the structures of these biologically important bile acid 3-glucosides, and the method depends on the ability to determine simultaneously nonamidated and glycine- and taurine-amidated bile acids without prior group separation and deconjugation.

NOTES

The following trivial names and abbreviations are used in this paper:

Lithocholic acid (LCA) = 3 α -Hydroxy-5 β -cholanoic acid

Chenodeoxycholic acid (CDCA) = 3 α ,7 α -Dihydroxy-5 β -cholanoic acid

Ursodeoxycholic acid (UDCA) = 3 α ,7 β -Dihydroxy-5 β -cholanoic acid

Deoxycholic acid (DCA) = 3 α ,12 α -Dihydroxy-5 β -cholanoic acid

Cholic acid (CA) = 3 α ,7 α ,12 α -Trihydroxy-5 β -cholanoic acid

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STUDIES ON NEUROSTEROIDS. I. RETENTION BEHAVIOR OF DERIVATIZED 17-OXOSTEROIDS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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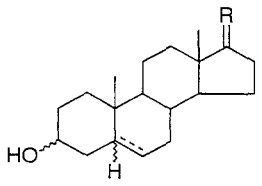
ABSTRACT

The retention behavior of five 17-oxosteroids (dehydroepiandrosterone, epiandrosterone, androsterone, 5β-androsterone, and 5β-androstane-3β ol-17-one) derivatized with 5-dimethylamino-1-naphthalenesulfonic hydrazide, 4-(*N,N*-dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole or *p*-nitrophenylhydrazine are examined using reversed-phase high-performance liquid chromatography. Inclusion chromatography using cyclodextrin as a mobile phase additive is also used for this purpose and found effective in separating the isomeric derivatized 17-oxosteroids.

INTRODUCTION

Since the discovery of dehydroepiandrosterone (Ia) in rat brain, several 17- and 20-oxosteroids, called "neurosteroids", have been elucidated in mammalian brain [1]. In the last few years, major progress has been made towards the elucidation of the molecular mechanism of action of steroid hormones in the brain and anterior pituitary. The determination of neurosteroids (< 70 ng/g of brain) has been usually done by gas chromatography-mass spectrometry or radioimmunoassay, but these methods have some problems in their simplicity and versatility [2]. High-performance liquid chromatography (HPLC) is promising for the establishment as a convenient determination method, but highly sensitive derivatization is necessary because neurosteroids are not very responsive to the commonly used detectors. In order to establish a reliable determination method, fluorometric derivatization is a promising technique and is necessary to clarify the retention behavior of the derivatized neurosteroids and their isomers.

In this paper, the retention behavior of five 17-oxosteroids [Ia, epiandrosterone (IIa), androsterone (IIIa), 5β - androsterone (IVa), and 5β -androstane-3β -ol-17-one (Va)] derivatized with fluorogenic reagent, 5-dimethylamino-1-naphthalenesulfonic hydrazide (DNSNHNH$_2$) or 4- (*N, N* - dimethylaminosulfonyl) - 7 - hydrazino- 2, 1, 3 -benzoxadiazole (DBDH), is examined using reversed-phase HPLC (Fig. 1). Inclusion chromatography using cyclodextrin (CD) as a mobile phase additive is also used for this purpose [3], and the *p*-nitrophenylhydrazones (NP) of these steroids are used to clarify the inclusion behavior during this chromatography.



I : 3β-OH, Δ5
II : 3β-OH, 5α-H
III : 3α-OH, 5α-H
IV : 3α-OH, 5β-H
V : 3β-OH, 5β-H

a : R = O

b : R = NNHO2S— —NMe2

c : R = NNH— —SO2NMe2

d : R = NNH— —NO2

Figure 1. Structures of 17-oxosteroids and their derivatives
Ia: dehydroepiandrosterone IIa: epiandrosterone
IIIa: androsterone IVa: 5β-androsterone
Va: 5β-androstane-3β-ol-17-one

MATERIALS AND METHODS

Materials

α-, β-, and γ-CDs were kindly supplied by Nihon Shokuhin Kako (Tokyo, Japan). Heptakis - (2,6 - di - O - methyl)-β-CD (Me-β-CD; 10.5 methyl residues/mol) was prepared and donated by Kao (Tokyo). 17-Oxosteroids were kindly donated by Teikoku Hormone Mfg. (Tokyo). $DNSNHNH_2$, DBDH, and *p*-nitrophenylhydrazine (NPH) were purchased from Tokyo Kasei Kogyo (Tokyo).

Derivatization Procedure

The derivatization of the 17-oxosteroids with $DNSNHNH_2$ and DBDH has been done using previously described procedures [4,5] to give the corresponding hydrazones (DNS- and DBD-17-oxosteroids), respectively. The derivatization with NPH has been done in the usual way using HCl as the catalyst.

Apparatus

HPLC was carried out using a JASCO TRI ROTAR chromatograph equipped with a UVIDEC-100-II UV (JASCO, Tokyo) or Hitachi F-1000 fluorescence (FL)(Hitachi, Tokyo) detector. A TSKgel ODS 80 TM (5 μm) column (15 cm x 0.46 cm i.d.)(TOSOH, Tokyo) was used at ambient temperature at a flow rate of 1 ml/min, and the void volume was measured with $NaNO_3$ (UV 210 nm) or MeOH (λ ex 280 nm, λ em 320 nm). The pH of the mobile phase containing KH_2PO_4 was adjusted with H_3PO_4.

RESULTS AND DISCUSSION

Retention Behavior of DNS-17-oxosteroids

$DNSNHNH_2$ is widely used as the fluorometric derivatization reagent for carbonyl compounds and several methods for the determination of 17-oxosteroids in biological fluids using this reagent have been reported [4]. The separation of five DNS-17-oxosteroids (I-Vb) *via* reversed-phase HPLC using MeOH or MeCN as an organic modifier was examined. The use of the ion suppressor [0.25% KH_2PO_4 (pH 3.0)] was effective in giving a symmetrical and single peak, but the complete separation has not been done as shown in Fig. 2a, b. These data prompted us to try inclusion chromatography using CD as the mobile phase additive for

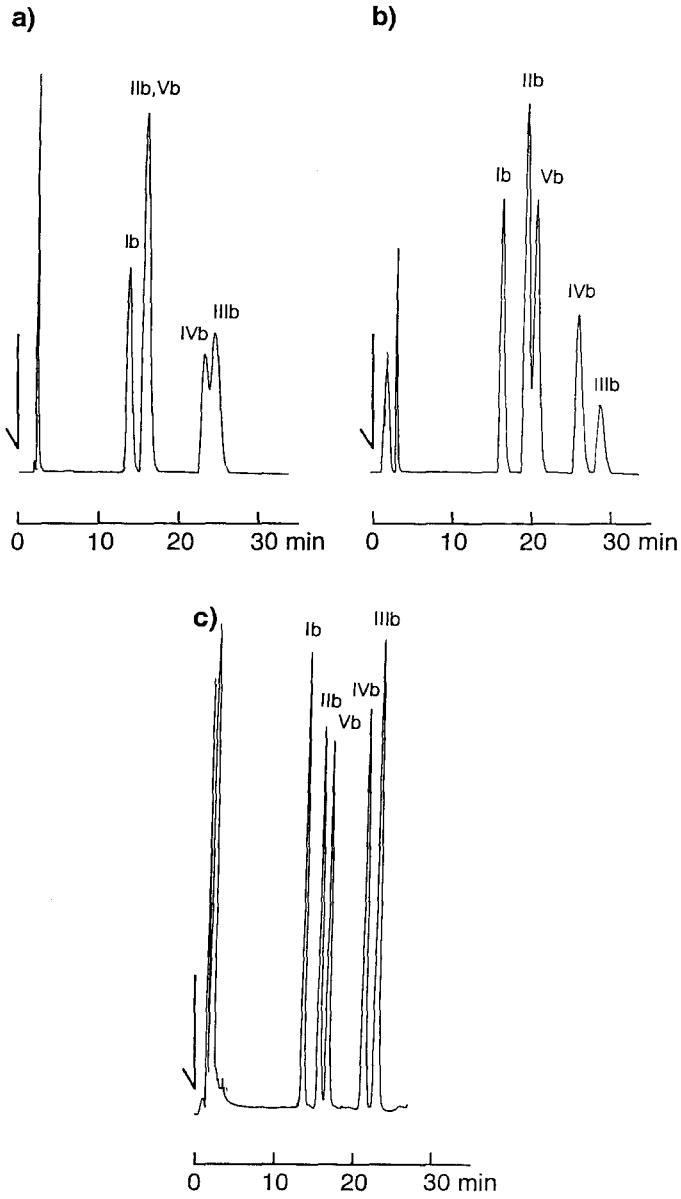


Figure 2. Separation of DNS-17-oxosteroids
Conditions: mobile phase, a) MeOH-0.25% KH_2PO_4 (pH 3.0)(3:1) b) MeCN-0.25% KH_2PO_4 (pH 3.0)(3:2) c) MeCN-0.25% KH_2PO_4 (pH 3.0)(3:2) containing Me-β-CD (2 mM); t_0, a) 1.8 min b) 1.5 min c) 1.6 min; detection, FL (λ ex 340 nm, λ em 525 nm).

Table 1. Effect of CD on the Rk' value of DNS-17-oxosteroids

		Rk' [a]			
		γ - CD		Me-β-CD	
	k' [b]	2 mM	5 mM	2 mM	5 mM
Ib	6.7	0.81	0.69	0.77	0.73
IIb	7.7	0.77	0.63	0.76	0.68
IIIb	12.8	0.77	0.66	0.76	0.71
IVb	12.1	0.75	0.63	0.78	0.75
Vb	8.0	0.63	0.44	0.78	0.75

Conditions: mobile phase, MeOH-0.25% KH_2PO_4 (pH 3.0) (3 : 1) containing CD as indicated. a) The k ' value obtained without CD was taken as 1.0 . b) The k ' value obtained without CD. t_0 1.8 min.

the separation of these derivatives. The effect of γ - and Me-β-CD on the relative capacity factor (Rk') of these derivatives is shown in Table 1. The former host compound is more effective than the latter one and Rk' of Vb (3β, 5β-isomer) was most decreased by the addition of γ - CD in the mobile phase. These data are compatible with the previously obtained data on cardiac steroids [6], that is, γ - CD is remarkably more effective than the other CDs in decreasing the k' values of compounds having an A/B cis ring junction and 3β-hydroxy group. The complete separation of the five derivatives was done by the addition of Me-β -CD and the chromatogram is shown in Fig. 2c.

Retention Behavior of DBD-17-oxosteroids

Recently, DBDH has been developed as a fluorogenic labeling reagent for aldehydes and ketones [5]. The resultant hydrazones were separated on a reversed-phase column and fluorometrically detected at sub-pmol levels. Furthermore, the hydrazones were more

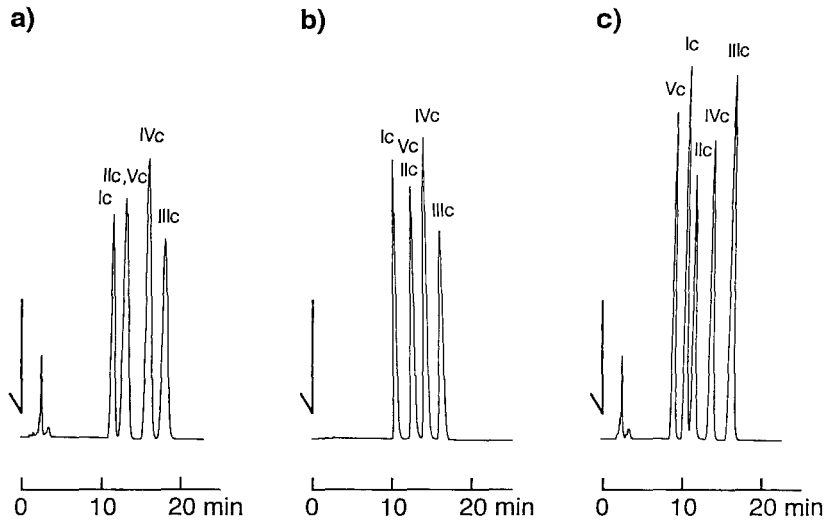


Figure 3. Separation of DBD-17-oxosteroids
Conditions: mobile phase, a) MeOH-H_2O (7:2)
b) MeCN-H_2O (2:1) c) MeOH-H_2O (7:2) containing γ -CD (2 mM); t_0, a) 1.7 min b) 1.5 min c) 1.7 min; detection, FL (λ ex 450 nm, λ em 550 nm).

sensitively detected by peroxyoxalate chemiluminescence than by fluorescence [5]. These data prompted us to use DBDH as a derivatization reagent for the 17-oxosteroids and the obtained hydrazones (I-Vc) were subjected to separation on reversed-phase HPLC. The use of MeOH or MeCN as an organic modifier each gave a symmetrical and single peak but did not give satisfactory separation of the five DBD-17-oxosteroids as shown in Fig. 3a, b. The addition of γ -CD in the mobile phase produced a sharp decrease in the Rk' of Vc, which is compatible with that of the DNS derivative (Table 2), and the complete separation of five derivatives was then obtained as shown in the chromatogram (Fig. 3c).

Table 2. Effect of CD on the Rk' value of DBD-17-oxosteroids

		Rk' [a]		
		γ - CD [b]	Me-β-CD	
	k' [c]	2 mM	2 mM	5 mM
Ic	4.0	0.82	0.82	0.71
IIc	4.7	0.78	0.80	0.67
IIIc	6.4	0.80	0.78	0.70
IVc	5.7	0.77	0.81	0.69
Vc	4.5	0.64	0.80	0.68

Conditions: mobile phase, MeOH-H_2O (4: 1) containing CD as indicated. a) The k ' value obtained without CD was taken as 1.0 . b) Due to its solubility, the experiment with 5 mM has not been done. c) The k ' value obtained without CD. t_0 1.7 min.

Retention behavior of NP-17-oxosteroids

The above described retention behavior of DNS- and DBD-17-oxosteroids in inclusion chromatography showed that γ - CD is more effective than Me-β -CD for decreasing the Rk ' values of these derivatives. In spite of the derivatization residue (DNS and DBD) at the 17-position, the Rk ' values of derivatized V having a 3β , 5β - configuration sharply decreased with the addition of γ -CD in the mobile phase. These data prompted us to examine the retention behavior of PN-17-oxosteroids (I-Vd) using inclusion chromatography. If the inclusion selectively occurs at the introduced fluorophore at the 17-position, the retention behavior of the derivatives having the NP residue, which is smaller than DNS or DBD residue, should be more affected by the addition of Me- β -CD having an inner diameter smaller than γ -CD. But γ -CD is more effective than Me-β -CD as in the case of the DNS- and DBD-derivatives (Table 3). The complete separation of these

Table 3. Effect of CD on the Rk' value of NP-17-oxosteroids

		Rk' [a]		
		γ - CD [b]	Me-β-CD	
	k' [c]	2 mM	2 mM	5 mM
Id	8.6	0.66	0.91	0.74
IId	10.1	0.61	0.87	0.67
IIId	12.7	0.65	0.98	0.76
IVd	11.0	0.65	0.99	0.74
Vd	9.9	0.52	0.86	0.65

Conditions: mobile phase, MeOH-H_2O (4: 1) containing CD as indicated. a) The k ' value obtained without CD was taken as 1.0 . b) Due to its solubility, the experiment with 5 mM has not been done. c) The k ' value obtained without CD. to 1.8 min.

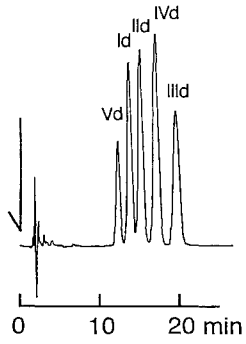


Figure 4. Separation of NP-17-oxosteroids
Conditions: mobile phase, MeOH-H_2O (4:1) containing γ -CD (2 mM); t_0 2.0 min; detection, UV (254 nm).

derivatives were also done by the addition of γ-CD in the mobile phase (Fig. 4).

Conclusions

In order to establish the determination method for neurosteroids, the chromatographic behavior of derivatized 17-oxosteroids has been examined with reversed-phase HPLC including inclusion chromatography. The addition of a host compound in the mobile phase is effective in separating these isomers, and five derivatized 17-oxosteroids were clearly separated by this method. In spite of the introduced derivatization residue (DNS, DBD, and NP) at the 17-position, γ-CD is more effective than Me-β-CD in decreasing the k' value of these compounds. The compound having the 3β-, 5β-configuration is most affected in its retention behavior by the addition of γ-CD. These data indicate that inclusion with a steroid moiety rather than the introduced derivatization residue may play an important role in this chromatography procedure.

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DIRECT INJECTION ANALYSIS OF 6β-HYDROXYCORTISOL AND CORTISOL IN URINE BY HPLC-UV WITH ON-LINE ISRP PRECOLUMN

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URINARY STEROIDS DIRECT INJECTION

The simultaneous measurement of 6β-hydroxycortisol (6β-OHF) and cortisol (FF) is interesting for the evaluation of enzyme induction in man. An on-line HPLC-UV analysis of urinary steroids is described. In a first step, the biological sample was injected onto an ISRP (Internal Surface Reversed Phase) precolumn with water for the elimination of proteins and indesirable products and for the concentration of hydrophobic molecules. In a second step, a simple gradient of acetonitrile (ACN) in water, by a backflush procedure, eluted the retained analytes which are analysed by conventional RP-HPLC coupled with UV detection.

INTRODUCTION

The importance of measuring 6β-hydroxycortisol as an index for studying microsomal enzyme induction of cytochrome P-450 3A (CYP 3A) is well recognised to explore the action of some drugs or foreign chemicals compounds (1-3).

Many HPLC procedures for the determination of steroids in biological fluids have been described last years. With regard to 6β-OHF analysis some techniques have been developped using either normal phase (3-8) or reversed phase (9-14). Very often, these methods need an important treatment of the sample, with liquid-liquid extraction (3, 5, 9, 12) or adsorption on silica cartridges (4, 8, 11), joined alkalin and, sometimes, acid washings.

We have developped an alternative liquid chromatography which suppress the step of extraction and the employment of internal standard. This technique use a backflush system with a ten-port valve and ISRP precolumn, concept designed for the time by Hagestam and Pinkerton (15) and reported for some applications as quantification of drugs (16-18). The recovery of analytes is 100%.

MATERIAL AND METHODS

Steroid Standards

Cortisol, cortisone, corticosterone, 11-deoxycortisol, desoxycorticosterone, prednisone and prednisolone were obtained from Sigma Chemicals. 6β-hydroxycortisol was purchased from Steraloids (distributed in France by Touzard et Matignon).

Methanolic stock solutions of each steroid at 1.000 g/l were stored at 4°C. Daily, fresh dilutions were prepared in mobile phase.

Solvents

Methanol and acetonitrile were purchased from Carlo Erba (HPLC quality).

Chromatography

Initial conditions were leaded by the method developped in our laboratory for the analysis of cortisol and cortisone in saliva (19).

The chromatographic equipment is a fully automatised system purchased from Spectra Physics (now Thermo Instrument Products) with :

- quaternary pump model P-4000, with solvent degazer (porous membranes)
- sample preparator autoinjector AS-3000, thermostat equipped
- detector focus 2000 scanning from 190 to 800 nm
- interface SN 4000.

Data were collected and evaluated with a Spectra Physics PC 1000 software on a Getek 486 computer.

Mobile phase was delivered at a ten-port valve (select-sil 99T) with manual or electropneumatic command (figure 1).

In position 1, the sample was flushing with water into a ISRP cartridge (Ultrabiosep C_{18}, particules of 10 µ, SFCC), in an optimal time of 5 minutes. In position 2, with a backflush system, all the retained molecules were dissolved in mobile phase (ACN / water), separated into an analytical column Ultrabase C_{18} (250 X 4.6 mm, 5 µ, SFCC) and analysed with a scan mode from 235 to 254 nm.

With isocratic mode, some assays were done, concentration of ACN varying between 20 and 40 %, in order to verify the comportment of all the steroids (figure 2).

Because of interferences in urine injection in the first part of chromatograms, we have choosed to work with gradient mode. To retain more 6β-OHF we have begun the chromatographic conditions

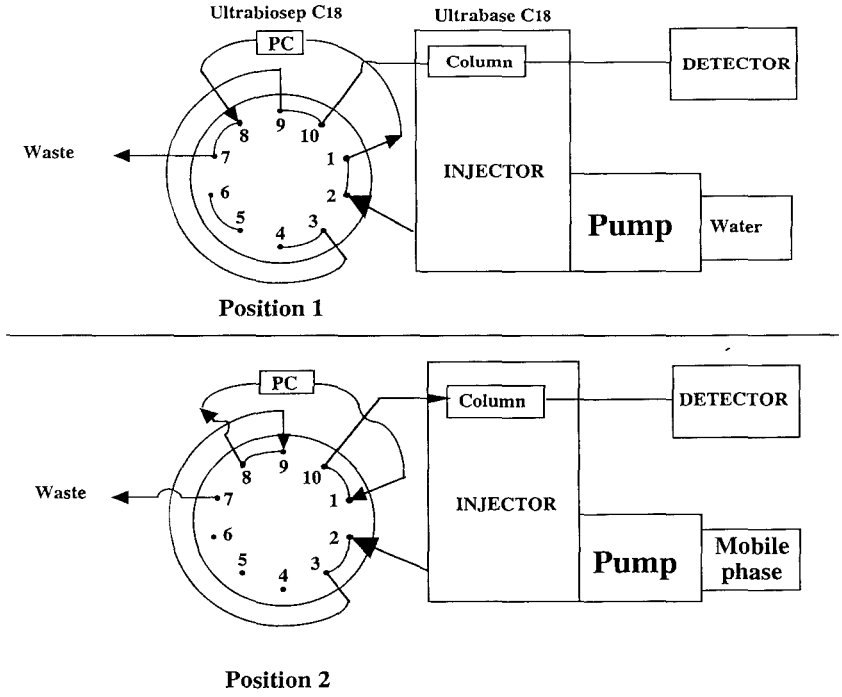

FIGURE 1 :
valve switching system for on-line urine injection.

by 20 % of ACN in water. After 6β-OHF elution, this percentage was rapidly increased to 40 for the quick elution of the other steroids. Some different assays concerning the changement of concentration indicated the optimal time at 15 min. After 25 min., we reconditioned the column to the initial percentage of 20 for 5 min. At least, we came back to position 1 of the valve to sweep precolumn in water for 5 min.

The scheme of final chromatographic conditions are given in figure 3 and table 1.

The system was ready for a new injection.

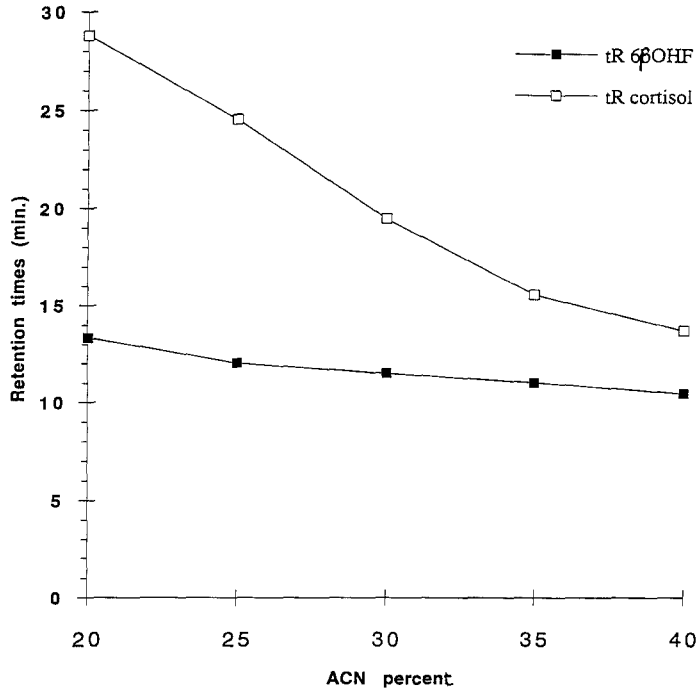

FIGURE 2 :
progression of retention times of 6β-hydroxycortisol and cortisol, according to volumic concentrations of solvent.

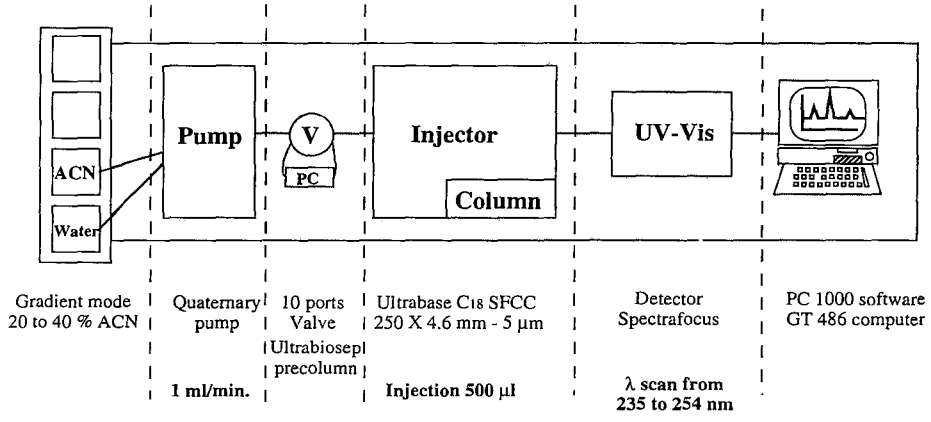

FIGURE 3 :
Chromatographic conditions for an on-line analysis of urinary steroids (LC/UV).

TABLE 1 :
Resume of the Valve Position during the chromatogram Time.

Time	ACN percent (1 ml/min.)	Valve position
0	0	1
5	0	1
5.01	20	2
15	20	2
15.01	40	2
25	40	2
25.01	20	2
30	20	2
30.01	0	1

The urine samples collected from 78 subjets were frozen and, after thawing, centrifuged at 3000 rpm and filtred into 0.22 μ membranes (Millex GS). A pool of urines served as control, in addition to standard solutions (100 and 200 μg/l), systematically injected after four biological samples.

RESULTS

The control of a standard solution with the seven steroids gave the following retention times (figure 4):

6β-OHF :	13.26 ± 0.04	(k' = 2.63)
Prednisolone :	22.58 ± 0.03	(k' = 11.94)
Cortisol :	22.77 ± 0.03	(k' = 12.13)
Cortisone :	23.11 ± 0.04	(k' = 12.47)
Prednisolone :	25.38 ± 0.04	(k' = 14.74)
Corticosterone	25.65 ± 0.05	(k' = 15.01)
11-deoxycortisol :	26.58 ± 0.05	(k' = 15.94)
Deoxycorticosterone :	27.46 ± 0.05	(k' = 16.82)

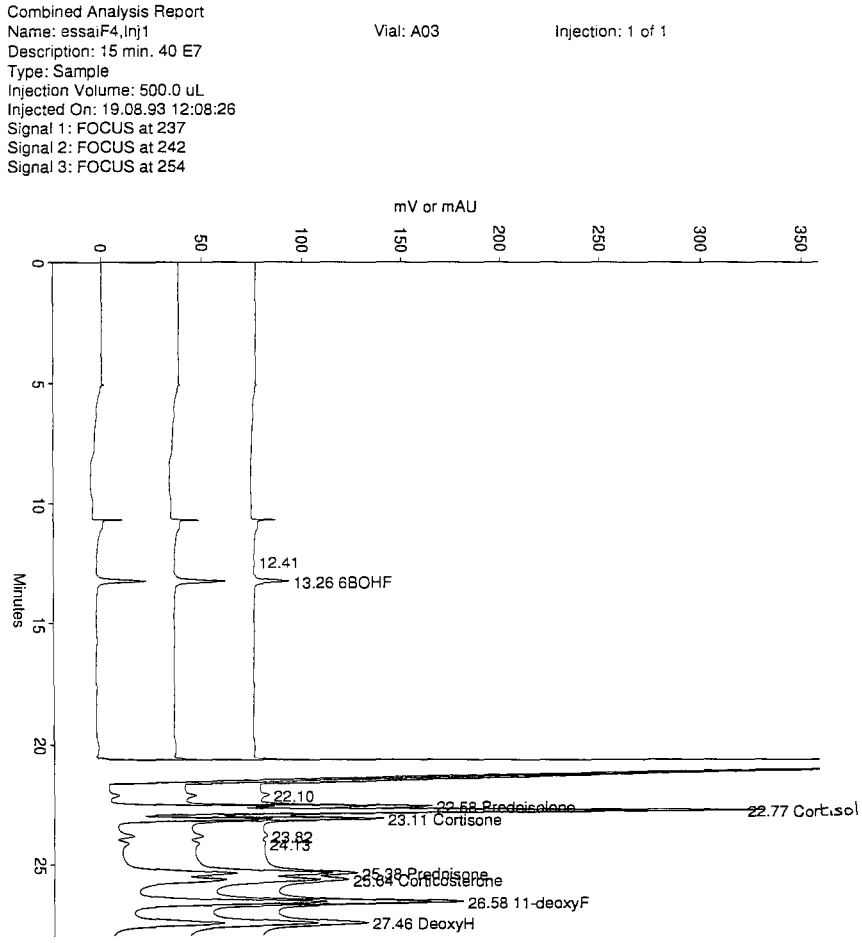

FIGURE 4 :
Chromatogram of a standard solution containing the seven steroids, with detection at 237, 242 and 254 nm.

Recovery

This technique with on-line direct injection has permitted the absolute recovery of steroid compounds.

Linearity and Precision

The response of the detector is linear for concentrations of metabolites from 0 to 5.000 mg/l. All the correlation coefficients of standard curves were equal to 0.999.

The coefficients of variation (CV) intra-assay for 6β-OHF and cortisol were included between 0.81 and 3.30 %, the best values founded at 242 nm and the worse ones at 254 nm. The CV inter-assays were always lower than 4 % for standard solutions and than 6 % for the reference urine thawed every day and treated with the other biological samples.

Detection Limit

The detection limits for 6β-OHF and cortisol were respectively 4.00 µg/l and 3.00 µg/l, that is to say for an injection of 500 µl 2.00 ng and 1.50 ng injected. The quantification limit was 10.00 µg/l for both steroids.

DISCUSSION

With this technique, we have quantified 76 urines out of 78, without any calculation (with only double injection). For the two other samplings, which were very loaded and one of them jaundiced, an interference peak at 13.56 min. obstructed the 6β-OHF one, and did't permit an accurate quantification (figure 5). We thank it was

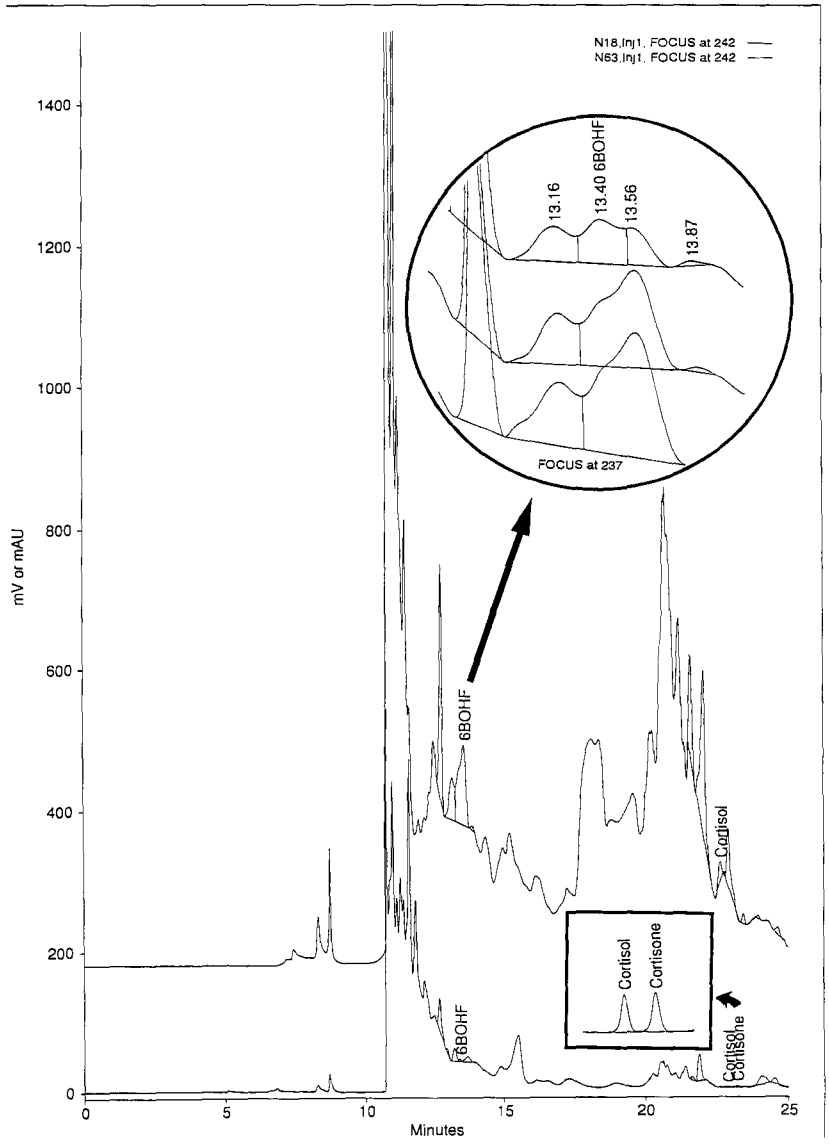

FIGURE 5 :
Chromatograms of urine samples, with direct injection on the column switching technique (LC/UV)

(a) normal urine

(b) loaded jaundiced urine with difficulties to quantity 6β-OHF, because of interference peak at 13.56 minutes.

better to extract selectively the steroid molecule than to give an approximate value with the sophistical software. For cortisol and cortisone determination, no problem occurred, all the integrations were accurated.

This technique has been evaluated by a similar method with liquid-liquid extraction procedure and the same chromatographic system.

One part of the filtred urine was extracted with 5 parts of ethylacetate, then, after centrifugation (10 min. ca 1330 g), the organic phase was washed with one part of NaOH solution 0.1N and one part of water. After removing aqueous phase, the eluate was reduced to dryness under a stream of nitrogen and the extract was reconstituted in 200 µl of mobile phase. The recoveries for 6β-OHF and cortisol were respectively 95.6 % and 92.3 % (n = 10). Chromatographic conditions were the same as described method, except isocratic mode with 30 % of ACN and the absence of the valve with precolumn.

We determined the linear regression between the two methods for 6β-OHF and cortisol with 76 or 78 urinary samples (table 2). The good correlation coefficients (r) and the test of Student applied on the slope (t) showed the quality of the regression.

The two samples with interference peak were easy to detect on the chromatograms. Also, we suggest than, with our simple technique without long and expansive treatment of the biological complex mixtures like urine or plasma, we can quantify some of important steroids and establish directly the ratio of 6β-OHF and cortisol for enzyme induction studies, with the same injection for the almost totality of the samples. If a problem occurs for the quantification of 6β-OHF, we advise the treatment of the biological fluid and the control with the extract product, relieved of interference peak. For studies about cortisol and cortisone, in urine or in saliva, the same simplified technique with isocratic mode (30 % ACN) can be used with success.

The steroids levels for the 76 urines tested (healthy male adults) were :

TABLE 2
Linear regression between HPLC-UV with on-line urine direct injection and HPLC-UV with liquid-liquid extraction of steroids by ethyl acetate

Steroid	Linear regression	n	Correlation (r)	Student (probability)
6β-OHF	Y = 1.006 X	78	0.9223	42.86 (0.000)
	Y = 0.992 X	76	0.9805	83.90 (0.000)
Cortisol	Y = 1.009 X	78	0.9945	155.15 (0.000)

mean and SD of 6β-OHF : 165 ± 30 µg / 24 H
FF : 47 ± 29 µg / 24 H
with a ratio of 5.4.

Our results are in agree with other authors who used similar techniques (20) or immunoenzymology (21).

CONCLUSION

With an automatised HPLC system and Scan detector, we can inject twice 20 samples a day. After short-time sample preparation, the technician is free for an other work. In addition, there is no manipulation of toxic solvents as ethylacetate, methylene chloride or ether. In this study, we have showed that is possible to work with ISRP concept for solvent volumic concentration about 40 %, adverse to others papers which limit this percentage to 20 (22, 23).

In conclusion, this method is simple, sensitive and reliable, and permits the simultaneous determination of 6β-OHF and cortisol for toxicological application.

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ANALYSIS OF METRIBUZIN AND ASSOCIATED METABOLITES IN SOIL AND WATER SAMPLES BY SOLID PHASE EXTRACTION AND REVERSED PHASE THIN LAYER CHROMATOGRAPHY

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ABSTRACT

A method to analyze metribuzin, 4-amino-6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5(4H)-one, and its major metabolites, deaminated metribuzin, DA, 6-(1,1-dimethylethyl)-3-(methylthio) -1,2,4-triazin-5(4H)-one, diketometribuzin, DK, 4-amino-6-(1,1-dimethylethyl)-1,2,4-triazin-3,5(2H,4H) -dione and deaminated diketometribuzin, DADK, 6-(1,1-dimethylethyl)-1,2,4-triazin-3,5(2H,4H)-dione in soil and water samples by reversed-phase thin layer chromatography (RPTLC) is described. Soil samples were extracted with MeOH:0.01 *M* $CaCl_2 \cdot 2H_2O$ (4:1) and the extracts filtered to remove the sediment. The extract then was diluted with deionized water and eluted through a C18, solid phase extraction (SPE) cartridge. Water samples (150-mL) were eluted directly through C18, SPE cartridges. Both soil and water extracts were chromatographed on pre-coated RPTLC plates. The concentration of metribuzin was determined by UV densitometry at 290 nm. Recoveries of metribuzin from soil samples fortified at 2 mg kg^{-1} ranged from 73-86%. Recoveries from water samples fortified at 10 and 100 $\mu g\ L^{-1}$ ranged from 85 to 92%. The detection limit of the method for metribuzin is 30 nanograms.

INTRODUCTION

Metribuzin is triazine herbicide that is used extensively in Louisiana and states in the mid-south for weed control in sugarcane and has been detected in groundwater (1). The triazines are the most widely applied herbicide class with an estimated market size of 1,425 million dollars (2). Although the triazines exhibit a low degree of toxicity to humans and animals (3), the carcinogenic potential of these compounds is still unclear. Metribuzin has been identified as an endocrine disrupter (4). The USEPA has evaluated the carcinogenic potential of metribuzin and has placed it in group D, indicating inadequate human and animal evidence of carcinogenicity. Recently, a comprehensive search was made of the STORET water quality data base, which is maintained by the Office of Water, U.S. Environmental Protection Agency (1). In this search, metribuzin was detected in 343 of 3,208 groundwater samples.

The concerns for water quality have led to increased pesticide monitoring programs throughout the U.S. The cost of these programs has often been prohibitive, thereby limiting the number of samples. In attempt to address these issues several analytical techniques have been investigated that retain the sensitivity of traditional methods such as gas chromatography (GC) and high pressure liquid chromatography (HPLC), while decreasing the cost and sample analysis time. One technique that has received considerable attention is enzyme immunoassay. This technique is rapid and cost effective (5). The principal disadvantage is its lack of specificity, due to cross reactions (6). The technique however, is well suited for screening large numbers of samples. Positive samples later can be confirmed by another technique, such as GC or HPLC.

In recent years, thin layer chromatography (TLC) has also received increased attention as a quantitative analysis tool (7). This emphasis has arisen partly from the significant advances that have been made in TLC stationary phases. Analytical TLC plates now are available in a wide range of normal and reversed phase sorbents, in both the standard and high performance (HPTLC) mode. Also, the availability of low cost scanning densitometers has increased the

utility of TLC as a quantitative tool. Although it is unlikely that TLC will replace GC and HPLC as the method of choice for pesticide analysis, numerous situations exist where TLC would provide a viable, rapid, low cost alternative to these methods. TLC may be particularly useful for sample screening. Potential advantages of TLC include: 1) high sensitivity, 2) minimum sample preparation time, and 3) low cost.

Several authors have reported TLC systems for the analysis of metribuzin and other triazines. Plant extracts containing metribuzin and its metabolites, DA, DK and DADK were analyzed by TLC on silica gel plates with a mobile phase of chloroform/dioxane (95:5 v/v) (8). In a related study, metribuzin present in benzene extracts of plant tissue was analyzed by TLC on silica gel G plates using chloroform/dioxane (9:1 v/v) and on cellulose F plates using benzene as the mobile phase (9). Water extracts of plant tissue containing metribuzin were analyzed on silica gel G using *n*-butanol:ethanol:water (40:11:19 v/v/v) and chloroform/dioxane (9:1 v/v) (9).

Water samples containing atrazine and simazine were extracted with chloroform and chromatographed on silica gel G using a mobile phase of chloroform/acetone (9:1 v/v). Recoveries from samples fortified at a concentration of 10 ppb were 86% for atrazine and 83% for simazine (10). Water samples fortified with triazine and chlorophenoxy herbicides at a concentration of 10 ppb were analyzed by C18, solid phase extraction (SPE) and TLC. Atrazine and simazine were analyzed on silica gel GF plates with a mobile phase of chloroform/acetone (9:1 v/v). Silvex, 2,4-D and 2,4,5-T were determined on silica gel GF plates with a mobile phase of hexane/glacial acetic acid/diethyl ether (72:30:18 v/v/v). Recoveries ranged from 70-88% for the triazines and 93-100% for the chlorophenoxy herbicides (11). More recently, soil samples containing atrazine and its metabolites, hydroxyatrazine, deethylatrazine and depropylatrazine were extracted with MeOH and analyzed by reversed phase, C18, high performance thin layer chromatography (RP-HPTLC). Recoveries for soils fortified at a concentration of 2 ppm ranged from 87-97% (12). This paper describes

a simple and direct method for the analysis of soil and water samples containing metribuzin and its metabolites DA, DK and DADK using SPE and RPTLC.

MATERIALS

Soils

The soils investigated included surface horizons (A or Ap) from a Commerce silty clay (fine-silty, mixed, nonacid, thermic Aeric Fluvaquent), and an Ocholocknee sandy loam (coarse-loamy, siliceous, acid, thermic Typic Udifluvent) from East Baton Rouge parish, Louisiana, an Evesboro loamy sand (coarse-loamy, siliceous, mesic Aquic Hapludult) from Sussex county, Delaware and a Conover loam soil (loamy, mixed, mesic Udollic Ochraqualf) from Ingham county, Michigan. The horizon designation Ap indicates that the soil has been cultivated and the designation A indicates that the soil has not been cultivated. The moisture content at 0.33b is taken to be an estimate of the field capacity moisture content of the soil in question. Surface horizons The Ochlocknee, Evesboro, Conover and Commerce soils were used in recovery experiments and varied in clay content from 12 to 26% and in organic matter content from 0.9 to 1.8%. Selected characteristics of these soils are presented in Table 1.

Herbicides

The herbicide used was metribuzin [93-94% pure], 4-amino-6- (1,1-dimethyl ethyl)-3- (methylthio)-1,2,4-triazin-5(4H)-one. In addition, three of its major metabolites were studied, deaminated metribuzin [99.6%, DA], 6-(1,1-dimethyl ethyl) -3-(methylthio)-1,2,4-triazin-5(4H)-one, diketometribuzin [98.7%, DK], 4-amino- 6-(1,1-dimethylethyl) -1,2,4-triazin-3,5(2H,4H)-dione and deaminated diketo metribuzin, [84.8%, DADK], 6-(1,1-dimethylethyl)-1,2,4-triazin-3,5 (2H,4H)-dione (Figure 1). Parent metribuzin and metabolites were obtained from the Miles Corporation, Kansas City, MO. Standard solutions of metribuzin were prepared in HPLC grade MeOH at concentrations of 0.01, 0.1 and 1.0 $\mu g\ \mu L^{-1}$.

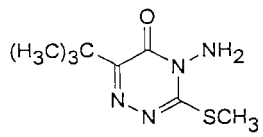
TABLE 1

Selected Soil Properties for the Evesboro, Ocholocknee, Conover and Commerce Soils.

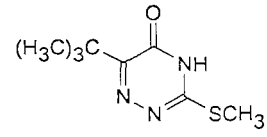
Soil Series Horizon	pH[§]	OM	Particle Size Sand	Silt	Clay	0.33 Bar
		----------%----------				
Evesboro						
Ap	5.3	0.94	71	16	13	9.6
Ocholocknee						
A	4.9	1.09	67	21	12	15.0
Conover						
Ap	6.6	1.79	60	20	20	20.6
Commerce						
Ap	5.2	1.52	41	33	26	29.0

§ - **pH** = pH of 1:1 soil/deionized water suspension, **OM** = soil organic matter content (13), **Particle Size** (14), **0.33 Bar** = water content (g/100 g dry soil) at 0.33 bars pressure.

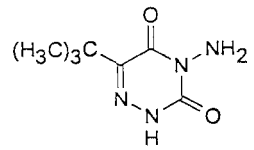
Standards for the metribuzin metabolites DA, DK and DADK were also prepared in HPLC grade MeOH at concentrations of 0.1 and 1.0 $\mu g\ \mu L^{-1}$. All solvents used for TLC were analytical grade and deionized water ($\geq$ 18 mohm-cm) was obtained with a nanopure deionization system (Barnstead/ Thermolyne Corp., Dubuque, IA).



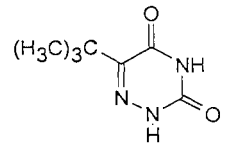
Metribuzin



DA



DK



DADK

FIGURE 1. Chemical structures for metribuzin, DA, DK and DADK.

METHODS

Water Extraction Procedures

Two techniques were investigated to extract water samples. In the first method, water samples (500 mL) were extracted in a 1-L separatory funnel with 3 x 25-mL dichloromethane. The organic fractions were combined and then dried through anhydrous sodium sulfate. The sodium sulfate was rinsed with a final 25-mL of dichloromethane and the dried organic fraction was then evaporated under a gentle air stream. The residue was reconstituted in 100 µL of HPLC grade MeOH for analysis. In the second method, water samples (150 mL) were filtered through C18, SPE cartridges (SEP-PAK®, Waters Associates, Milford. MA). Prior to filtration, the cartridges were activated with 2-mL of HPLC grade MeOH and 5-mL deionized water. After filtration, the columns were rinsed with 2-mL of HPLC grade MeOH to elute the sorbed metribuzin. The samples were dried under a gentle air stream and then re-constituted in 100-µL MeOH.

Soil Extraction Procedures

Soil (50 g) was transferred to 250-mL erlenmeyer flasks and extracted with 150-mL MeOH:0.01*M* $CaCl_2 \cdot 2H_2O$ (4:1) on a rotary shaker (200 RPM) for 18 hours. The suspension was then filtered through Whatman #4 filter paper. The soil was washed with an additional 50 mL of MeOH:0.01*M* $CaCl_2 \cdot 2H_2O$ and the filtrates were combined. At this stage two techniques were employed. In the first method the extracts were evaporated to dryness under vacuum at 60° C, re-dissolved in MeOH, transferred to 20-mL vials and redried under a gentle air stream. Prior to analysis, samples were reconstituted in 200 µL MeOH. In the second method 5-mL of the extract was diluted with 145-mL of deionized water and filtered through an activated C18, SPE cartridge. Metribuzin and any associated metabolites were eluted from the cartridge with 2-mL MeOH. The extract was dried under an air stream and reconstituted in 100 µL MeOH.

Thin Layer Chromatography

RPTLC was performed on reversed-phase, hydrocarbon impregnated uniplates, (10 x 20 cm, 250 micron thickness, Analtech Inc., Newark, DE). Standards and sample extracts were drawn into microcapillary pipettes (1, 2 and 4 µL) and applied with a Nanomat III (Camag Inc., Greenville, NC). A standard curve, which varied in concentration depending on the samples being analyzed, was included on each plate. The optimum mobile phase was determined to be MeOH:H_2O (45:55 v/v). Spotted plates were equilibrated in a vertical chamber containing the mobile phase for 0.5-h prior to development. Plates were developed for a distance of 10 cm, dried and scanned with a variable wavelength Shimadzu CS9000U Dual Wavelength Flying Spot Scanner. Standard curves were analyzed by linear regression analysis (MSTATC, East Lansing, MI).

Herbicide Recovery

To evaluate the extraction efficiency of the proposed method, 1-L of deionized water and 1-L of tap water was spiked with metribuzin at rates of 10

and 100 μg L^{-1}. Samples were analyzed as outlined above. The extraction efficiency for soil was evaluated by adding 50-g of the Evesboro, Ochlocknee Conover and Commerce soils to 250-mL erlenmeyer flasks, moistening the soil to field capacity (10, 15, 20 and 29%, respectively) and amended them with metribuzin at a rate of 2 mg kg^{-1} soil. Samples were aged for 24-hours and extracted and analyzed as outlined above.

Response to Other Herbicides

The performance of the method with other herbicides was also evaluated. Compounds evaluated included; atrazine, (6-chloro-N-ethyl-N'- (1-methylethyl) -1,3, 5-triazine-2,4-diamine), cyanazine, 2-[[4-chloro-6-(ethylamino)-1,3,5-triazin-2-yl]amino]-2-methylpropanenitrile, and alachlor, 2-chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)acetamide. These compounds were spotted along with the metribuzin at a concentration of 2 μg to determine if they could be resolved in the proposed system.

RESULTS AND DISCUSSION

A series of experiments was conducted to determine the optimum wavelength for analysis of metribuzin and it metabolites. The UV-absorbtion spectrum of metribuzin, DA, DK and DADK was constructed by spotting 2 μg of each compound on a RPTLC plate and scanning the spots from 200 to 370 nm (Figure 2). The maximas for metribuzin, DA, DK and DADK were found to be 290, 235, 254 and 254 nm, respectively. Further analyses of each compound was performed at their respective maxima.

Several TLC systems were investigated to perform these separations. The first stationary phase investigated was a RP-HPTLC, C18, bonded phase plate. These plates proved adequate for the parent metribuzin, but not for the separation of metribuzin, DA, DK and DADK together. Various mobile phases composi-

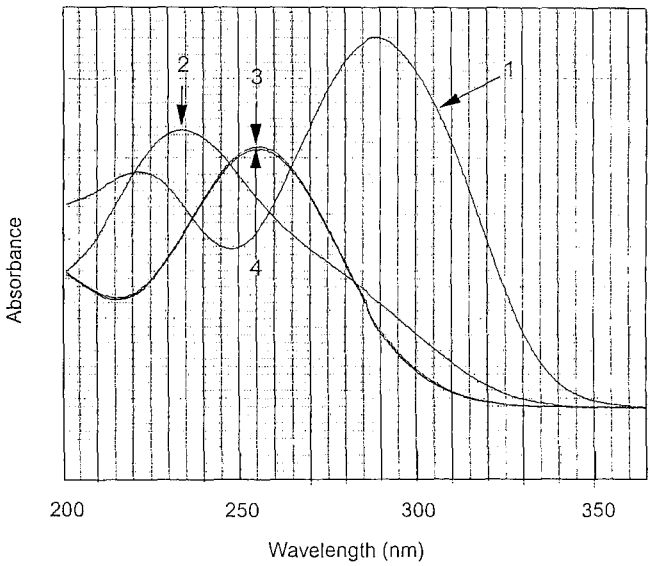


FIGURE 2. Absorbance spectrum for metribuzin (1), DA (2), DADK (3) and DK (4).

tions were investigated including; MeOH:H_2O (from 85:15 to 60:40 v/v), Acetonitrile:H_2O, MeOH:acetic acid, MeOH:tetrahydrafuran:H_2O, etc. The most promising approach appeared to be with MeOH:H_2O (60:40 v/v), however this amount of water resulted in a very slow development and still yielded an unsatisfactory separation (Figure 3a). The stationary phase was changed to a RPTLC, hydrocarbon impregnated plate. This plate allows for a greater percentage of H_2O in the mobile phase, up to 100%, without significant increases in development time. Several mobile phase combinations were evaluated starting at MeOH:H_2O (50:50 v/v) (Figure 3b). After several experiments, a mobile phase consisting of MeOH:H_2O (45:55 v/v) was found to yield the optimum separation (Figure 3c). It should be noted that water percentages greater than 55% resulted in a decreased resolution of the four compounds.

Although slight variation in the absolute retention R_f (Table 2) was observed among experimental systems, relative trends in R_f values were consistent.

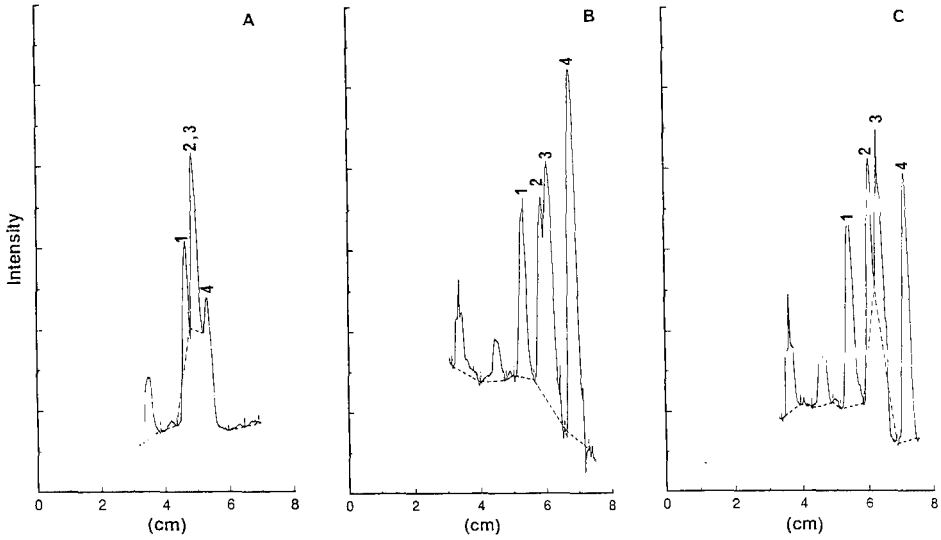


FIGURE 3. Densitometer chromatograms of A) metribuzin (1), DA (2), DADK (3) and DK (4) on a HPTLC, C18 plate, MeOH:H20 (60:40 v/v), B) metribuzin (1), DK (2), DADK (3)and DA (4) on a RPTLC plate, MeOH:H20 (50:50 v/v), C) metribuzin (1), DK (2), DADK (3)and DA (4) on a RPTLC plate, MeOH:H20 (45:55 v/v).

The observed variation is most likely related to the combined effects of intramolecular attraction between the co-chromatographed compounds and the presence of interfering compounds in the water and soil extracts. All plates were thoroughly dried before development making it unlikely that residual MeOH is causing the observed effect. It is conceivable that residual H_2O was present in the soil and water extracts, although this was not directly observed. If this was the case then the observed R_f values would tend to be lower. Despite the observed variations, all of the compounds were well resolved in all systems.

Standard curves for parent metribuzin and its metabolites DA, DK and DADK were linear or curvilinear, depending on the range of concentrations investigated (Figure 4). Although the curves could be described by quadratic or cubic regression equations, these equations generally resulted in a poor fit at the

TABLE 2

Retention Factors (R_f) for Metribuzin, DA, DK and DADK on RPTLC Plates in Various Systems.

Compound	System[§]	R_f
Metribuzin	soil	0.51
	water	0.51
	cochromatography	0.52
	standard curve	0.58
DA	soil	0.79
	water	0.78
	cochromatography	0.82
	standard curve	0.83
DK	soil	0.63
	water	0.62
	cochromatography	0.63
	standard curve	0.74
DADK	soil	0.67
	water	0.67
	cochromatography	0.68
	standard curve	0.78

§ - **soil** = fortified soil samples, **water** = fortified water samples, **co-chromatography** = co-chromatographed compounds, **standard curve** =compounds chromatographed alone.

lower concentration ranges. A better fit was generally obtained if the curve was broken into two ranges, 100 to 800 ng and 1000 to 3000, 4000 or 5000 ng (Table 3). The detection limit of this method was 30 ng for spotted standards.

Recoveries of parent metribuzin from fortified water samples were highly dependent on the method employed. Water samples fortified at 10 and 100 $\mu g\ L^{-1}$ that were extracted with methylene chloride resulted in recoveries of 43 and 70%,

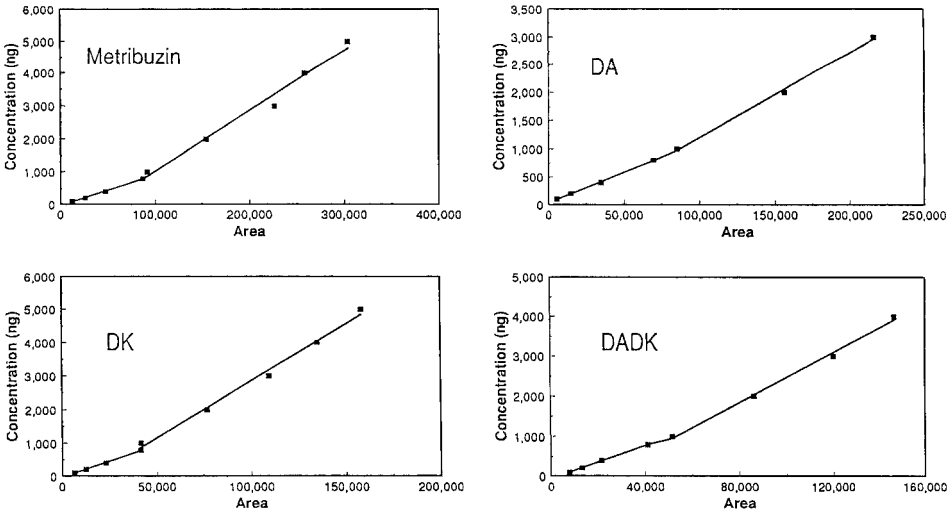


FIGURE 4. Standard curves for metribuzin, DA, DK and DADK as described by linear equations.

TABLE 3

Regression Data for Metribuzin, DA, DK and DADK.

Compound	Conc. Range	Linear r^2
	ng applied	
Metribuzin	(100-800)	0.997***
	(1000-5000	0.981***
DA	(100-800)	0.998***
	(1000-3000)	0.997***
DK	(100-800)	0.998***
	(1000-5000)	0.991***
DADK	(100-800)	0.999***
	(1000-5000)	0.996***

*** significant at P(0.001)

respectively. Recoveries of parent metribuzin from water samples analyzed by SPE techniques were significantly improved. For deionized water and tap water samples fortified at 10 $\mu g\ L^{-1}$ the recoveries were 85 and 89%. For deionized water and tap water samples fortified at 100 ug L^{-1} the recoveries were 92 and 91%, respectively. In all cases, the RPTLC system yielded a well resolved chromatogram, free of interferences (Figure 5a). In addition to an increase in recovery, the SPE technique also resulted in significant decreases in analysis time and solvent use. The method also avoided the use of dichloromethane, a recognized carcinogen.

Recoveries of parent metribuzin from fortified soil samples were dependent on the method employed. Only the Evesboro and Conover soils were evaluated by the evaporation method. Recoveries of metribuzin by this method were 48 and 50% for the Evesboro and Conover soils, respectively. It is possible that evaporating the sample to dryness was causing the observed low recoveries. A similar result was reported for the analysis of metribuzin in soils (15). In this method, soil samples were extracted by Soxhlet in 80% MeOH and the solvent reduced by evaporation, but not taken to dryness. The remaining solvent was then extracted with benzene in a separatory funnel. Recoveries were reduced by 20% if the samples were taken to dryness. An alternate method employing SPE was investigated. This method does not include an evaporation step, but rather relies on SPE for concentration and cleanup of the sample. The recoveries by this method were 76, 73, 76 and 87% for the Evesboro, Ochlocknee, Conover and Commerce soils, respectively. For all soils, the RPTLC system resulted in well resolved chromatograms that were free of interferences (Figure 5b). Solid phase extraction not only significantly increased the recoveries, but also decreased the analysis time.

The performance of the RPTLC system was also evaluated for atrazine, alachlor and cyanazine, in combination with metribuzin. All four compounds were well resolved when co-chromatographed (Figure 5c). The R_f values for alachlor, atrazine and cyanazine were 0.26, 0.39 and 0.53, respectively.

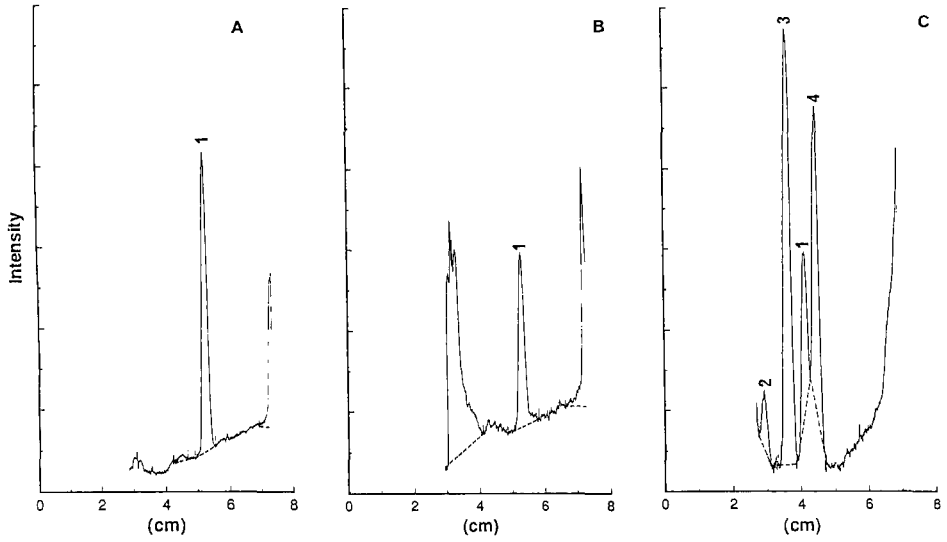


FIGURE 5. Densitometer chromatograms of A) metribuzin (1), in a tap water sample, B) metribuzin (1), in a fortified soil sample, and C) metribuzin (1), alachlor (2), atrazine (3) and cyanazine (4).

The described RPTLC method for metribuzin analysis of soil and water samples is a simple, rapid and cost effective alternative to other available procedures. The extraction procedures yielded recoveries for soils ranging from 73 to 87%, and for water samples ranging from 85 to 92%. The limit of detection of the method was 30 ng. The procedure may be particularly valuable for rapid screening of soil and water samples prior to confirmatory analysis.

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DETERMINATION OF PENTACHLOROPHENOL AND CYMIAZOLE IN WATER AND HONEY BY C-18 SOLID PHASE EXTRACTION AND QUANTITATIVE HPTLC

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ABSTRACT

Pentachlorophenol and cymiazole were extracted from water and honey using C-18 solid phase extraction, column eluates were chromatographed on a high performance preadsorbent silica gel plate containing a fluorescent phosphor, and the pesticides were quantified by densitometric scanning of fluorescence quenching. Recoveries from water at concentrations of 0.25-5 ppm ranged from 97.7 to 100% for PCP and 89.5-94.9 for cymiazole. Recoveries from honey at 10 and 50 ppm ranged from 94.0-96.1% for PCP and 91.9-93.7 for cymiazole.

INTRODUCTION

In previous papers, methods based on solid phase extraction (SPE) and quantitative silica gel TLC were reported for the determination of chlorophenoxy acid and triazine herbicides (1), organochlorine insecticides (2), organophosphorus insecticides (3), and carbamate insecticides

(4) in environmental water samples. Pentachlorophenol (PCP) is a widely used fungicide and molluscicide that is employed as a wood preservative in beehives, while cymiazole is an acaricide that is applied to the control of a honeybee parasitic mite among other applications. Because of the possibility of contamination of honey in apiculture as well as drinking and environmental waters through other uses of these pesticides, methods are needed for residue analysis. Previously published determinations of PCP in water involved conventional solvent extraction combined GC/MS (5), while PCP was determined in honey by C-18 SPE and GC/MS (6). The determination of the acaricide cymiazole in honey was reported using hexane extraction and HPLC (7). Methods have not been published for TLC analysis of water for these pesticides, nor for the solid phase extraction of cymiazole. This paper extends the SPE/quantitative TLC methodology to the determination of PCP and cymiazole in water and honey, using C-18 SPE columns for extraction, preadsorbent high performance silica gel plates for separation, and densitometric scanning of quenched zones for quantification.

EXPERIMENTAL

Standards

Standard PCP was obtained from the EPA Pesticide and Industrial Chemicals Repository (Las Vegas, NV) and standard cymiazole was supplied by Ciba-Geigy AG, Muenchwilen, Switzerland. Stock standard solutions were prepared with

concentrations of 5.00 mg/ml and were diluted 1:50 to prepare TLC standards with concentrations of 100 ng/ul. Toluene was the solvent used for PCP solutions and acetonitrile for cymiazole.

Thin Layer Chromatography

TLC was carried out on 10 x 10 cm Whatman laned, high performance preadsorbent silica gel plates with fluorescent indicator (catalog no. 4806-711) that were precleaned by development with methylene chloride-methanol (1:1) before use. Standards and samples from the SPE column were applied to the preadsorbent using a 10 ul Drummond digital microdispenser, and plates were developed in a Camag twin-trough chamber as described earlier (4) with toluene-methanol (9:1) for PCP or hexane-acetone-methanol-glacial acetic acid (35:10:5:0.1) for cymiazole. Pesticide zones were detected, after drying the mobile phase with warm air from a hair drier for 5 min, by fluorescence quenching under 254 nm UV light in a viewing box. Zone areas were measured by scanning with a Shimadzu CS-930 densitometer in the single beam, reflectance mode at 215 nm for PCP or 265 nm for cymiazole. Percent recovery was calculated by comparing the areas of samples with standards representing 100% recovery.

Analysis of Samples

Samples were analyzed by use of Supelco 6 ml solid phase extraction tubes containing 1 g C-18 sorbent (catalog no. 5-7055) and a J.T. Baker glass manifold no. 7018-00 that was

generally operated as described earlier (4). The flow rate of sample through the columns was 8-10 ml/min, and columns were dried by drawing vacuum for 10-15 min before elution of the pesticides into a graduated vial. For determination of PCP, water samples were initially adjusted to pH 2 with concentrated HCl and honey samples were dissolved in acidified water (10 g of honey per 100 ml of water containing 0.4 ml of 4 M HCl), the sample container and column reservoir were rinsed with pH 2 water, the column was washed with two column volumes of water after passing the sample and before drying, and PCP was eluted with two 3 ml portions of methanol using gentle pressure from a rubber bulb. For determination of cymiazole, water was initially adjusted to pH 9 with 1 M NaOH and honey was dissolved in pH 9 water (10 g per 100 ml), the sample container and column reservoir were rinsed with pH 9 water, the column was washed with 2 column volumes of pH 9 water after passing the sample and before drying, and cymiazole was eluted with two 3 ml portions of hexane. Eluates were evaporated just to dryness under nitrogen in a water bath at 40° C and reconstituted in 2.00 ml of the eluting solvent. Vacuum drying did not remove all of the water from the column, and 0.1 to 0.3 ml of water was eluted with the organic solvent and remained in the vial after drying under nitrogen. This water did not interfere with subsequent TLC analysis of the reconstituted samples. Two 2.00 ul aliquots of sample (representing 500 ng of pesticide if recovery is 100%) and two 5.00 ul aliquot of the TLC standard (containing 500 ng of pesticide) were spotted on each plate, and the average sample

and standard areas were compared to determine percent recovery.

Initial SPE recovery studies were carried out by analyzing deionized water spiked with the pesticides, and then the method was extended to spiked river water and honey samples that were preanalyzed and found not to contain either pesticide. Water was fortified at concentration levels of 5.00, 1.00, 0.50, and 0.25 ug/ml (ppm) by adding 100 ul of stock pesticide solution (500 ug) to 100, 500, 1000, and 2000 ml, respectively. Honey was fortified at 50.0 and 10.0 ppm by spiking 10.0 and 50.0 g samples with 100 ul of pesticide stock solution.

RESULTS AND DISCUSSION

PCP and cymiazole formed compact bands with R_f values of 0.52 and 0.64 in their respective mobile phases described above. The sensitivity of detection of each pesticide was ca. 200 ng by fluorescence quenching under 254 nm UV light. Calibration curves relating zone area and weight spotted between 300 and 500 ng typically had linear regression coefficients of ca. 0.98, which permitted the reliable determination of recovery based on area comparison between samples and a single standard within this linear range spotted on each plate.

Samples containing PCP were adjusted to pH 2 so that the acid was unionized and would be retained by the C-18 column. Samples to be analyzed for cymiazole were alkalinized to pH

9 because its solubility in aqueous solutions is reduced and extraction into hexane is quantitative at this pH (7).

Recovery tests for the pesticides from spiked deionized water were carried out in duplicate at various concentrations, and percent recoveries of PCP were 99.5 and 98.5 at 5 ppm, 98.3 and 98.8 at 1 ppm, 97.7 and 100.0 at 0.5 ppm, and 98.0 and 99.5 at 0.25 ppm. Percent recoveries of cymiazole from deionized water were 93.8 and 94.4 at 5 ppm, 91.5 and 92.8 at 1 ppm, 89.5 and 91.6 at 0.5 ppm, and 90.9 and 92.6 at 0.25 ppm. Water from a local river was spiked at 0.5 ppm, and the recoveries for duplicate analyses by the SPE/TLC method were 98.4 and 99.2 for PCP and 93.3 and 94.9 for cymiazole. There were no zones detected by fluorescence quenching in the chromatograms of these samples that interfered with the scanning of the analyte zones. Spiked honey samples were analyzed in duplicate and respective recoveries were 95.4 and 96.1% for PCP and 91.9 and 92.4% for cymiazole at 50.0 ppm, and 94.0 and 95.5% for PCP and 92.1 and 93.7% for cymiazole at 10.0 ppm. Again, no interfering zones were present in sample chromatograms.

The recovery and repeatability data presented above for spiked samples demonstrate that the SPE/HPTLC approach can be successfully applied for routine analyses of PCP and cymiazole in water and honey samples at the concentration levels specified. Recoveries of both compounds from water were at least 89% for all concentrations tested. Recoveries of PCP from honey were in the same range as reported earlier by Sep-Pak SPE and GC/MS (6). Recoveries of cymiazole from honey were

somewhat lower than by conventional solvent extraction and HPLC (7) but were acceptable (>90%) considering the advantages of SPE. The SPE/HPTLC method combines the convenience and low solvent consumption of SPE with the simplicity and high sample throughput of preadsorbent quantitative HPTLC. It will be applicable to any water or honey samples not containing co-extractable impurities that interfere with the chromatography, detection, or scanning of the pesticide analytes. The limits of determination can be lowered by passing more sample through the SPE column, reconstituting the column eluate with a smaller volume of solvent and/or spotting a larger aliquot of sample, or scanning zones containing weights of analyte close to the 300 ng lower end of the calibration curve.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE QUANTITATIVE DETERMINATION OF PHENIRAMINE IN PLASMA

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ABSTRACT

A simple, sensitive and reproducible high performance liquid chromatographic (HPLC) method for the determination of pheniramine in plasma has been developed and validated. The assay is performed after single extraction of pheniramine and amitriptyline (internal standard) from alkalinized plasma into ether. The drug and the internal standard were eluted from a μ-Bondapak C_{18} column at 40°C with a mobile phase consisting of methanol: water (62:38%, v/v) adjusted with phosphoric acid to an apparent pH 3.5 at a flow rate of 1.2 ml/min. The effluent was monitored with an ultraviolet detector set at 262 nm. Standard curves for the analyte in plasma were linear ($r>0.999$) in the range of 20-400 ng/ml and the minimum detectable concentration in plasma is 10 ng/ml. The within-day coefficient of variation (CV) ranged from 3.57% to 6.51% at three different concentrations. The between-day CVs varied from 5.03% to 7.84%. The absolute recoveries of pheniramine ranged from 94% to 96.9% and the relative recoveries ranged from 92% to 109.3% at three different concentrations. Stability tests showed that pheniramine is stable for at least 3 weeks in plasma after freez-

ing. The method is applied for the determination of the pharmacokinetic parameters of pheniramine after administration of a 75-mg tablet (Avil-retard) to six beagle dogs.

INTRODUCTION

Pheniramine maleate is an H_1-receptor antagonist commonly used for hypersensitivity reactions and pruritus of varying origin. It is most indicated in cases of allergic conditions such as hay fever, urticaria, conjunctivitis, and eczema of nervous origin[1,2]. Screening of the literature revealed most of the analytical methods have been developed for the determination of pheniramine in pharmaceutical dosage forms[3-10], and only few in biological fluids[11-13]. These include, spectrophotometric[3], gas-liquid chromatography (GLC)[4,5,11,12], and high-performance liquid chromatography (HPLC)[6-10,13]. The spectrophotometric method is generally unsuitable for pharmacokinetic and bioavailability studies because the potential interference of other compounds that may co-exist during the extraction procedure. The reported GLC and HPLC methods, possess adequate resolution for identifying pheniramine, however, it requires a relatively large sample, time consuming and involving tedious extraction and derivatization steps.

In this report a simple, rapid, sensitive, accurate and reproducible HPLC assay for the determina-

tion of pheniramine in plasma is described. The proposed method is also applied for the determination of the pharmacokinetic parameters of pheniramine after the administration of a single oral dose of 75 mg of pheniramine maleate tablet (Avil^{R}-retard) to six beagle dogs.

MATERIALS AND METHODS

Instruments

The following apparatus from Waters Associates, Milford, MA, U.S.A., was used. A model 6000A solvent delivery pump, model 481 variable wavelength detector, model 730 M recorder integrator data module, column heater, and model U6K universal injector. Chromatographic separation was performed using a u-Bondapak C_{18} steel column (300 mm length x 3.9 mm i.d., 10 μm particles).

Materials

All the solvents used were of HPLC grade. All other chemicals and reagents were of spectroquality or analytical grade. Pheniramine maleate was kindly supplied by Hoechst AG, Frankfurt Main, Germany and amitriptyline (internal standard) was purchased from Winlab Limited, Maidenhead, Berkshire, U.K.

Standard Solutions

Pheniramine maleate (equivalent to 10 mg pheniramine base) was dissolved in 100 ml HPLC water. This stock solution was diluted 100-fold in water to give the working standard solution (1 ug/ml). The working internal standard solution (10 ug/ml) was prepared by diluting the stock solution 1 mg/ml 100-fold in HPLC water.

Chromatographic Conditions

The mobile phase consisted of methanol:water (62:38% v/v) adjusted with phosphoric acid to an apparent pH 3.5. It was degassed daily by passing it through a 0.45-um membrane filter (Millipore, Bedford, MA, USA). The mobile phase was pumped isocratically at a flow rate of 1.2 ml/min, and at 40°C. The chart speed was 0.3 cm/min., and the effluent was monitored using UV detection at 262 nm and attenuation at 0.005 AUFS.

Procedure

To a screw-capped glass centrifuge tube (10 ml), 1 ml plasma, 30 ul of the internal standard (10 ug/ml), and 500 ul of 1 M sodium carbonate were added. The mixture was shaken on a vortex mixer for 30 sec. Five milliliters of diethylether was added for extraction and the mixture was shaken on a vortex mixer for 2 min.,

and centrifuged for 10 min., at 4,000 rpm. Following centrifugation, the organic layer was transferred into another glass centrifuge tube and evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 250 ul of the mobile phase, vortexed for 30 sec., and transferred to a disposable polypropylene microcentrifuge tube (1.5 ml) and centrifuged for 5 min., at 12,000 rpm, in a microcentrifuge to ensure that no particulate matter would be injected into the column. An aliquot was then injected directly into the loop injector.

Animal Studies

Six healthy female beagle dogs weighing between 6.5 and 11.5 kg were used. Pheniramine maleate 75 mg tablet (Avil^{R}-retard, Hoechst AG, Germany) was administered by gastric intubation. The dogs were fasted for 24 h before drug administration and continued fasting until 4 h post dose but allowed free access to water. Venous blood samples (5 ml) were taken from the femoral vein into heparinized tubes before drug administration and at 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0, and 24.0 h after the drug was given. The plasma was then separated after centrifugation and stored frozen at -20°C pending analysis.

RESULTS AND DISCUSSION

The composition and pH of the mobile phase were varied to achieve the optimum chromatographic conditions. A mobile phase consisting of methanol:water (62:38%, v/v) adjusted with phosphoric acid to an apparent pH 3.5 gave optimum resolution of pheniramine and amitriptyline (I.S.) and no interference from other components in plasma was observed.

The volume of methanol in the mobile phase drastically affected the resolution and retention time of both pheniramine and the internal standard. For example, changing the methanol ratio from 62% to 70% resulted in decrease in retention times of both drugs and interference with the endogenous plasma constituents. Decreasing the percentage of methanol to 50% resulted in increase in retention of the internal standard and loss of resolution.

The effect of the pH of the mobile phase was also studied. At an apparent pH above 4.5 the sensitivity decreased dramatically (10 times). However, at an apparent pH 3, the pheniramine peak was not resolved from the endogenous acidic components in plasma. Using an apparent pH 3.5 resulted in sharpening the peak of pheniramine and no interference from other components in the plasma was observed. The optimum flow rate of 1.2 ml/min., resulted in retention times of 4.5 and 6.1 min., for pheniramine and amitriptyline, respectively.

TABLE 1

Retention Times of Some Tested Drugs

Drug	Retention time (min)*
Metoclopramide	3.0
Pheniramine	4.5
Chlorpheniramine	4.5
Mebeverine	4.7
Diltiazem	4.8
Amitriptyline	6.1
Ketoprofen	6.6
Phenylbutazone	8.0
Diazepam	8.4
Ibuprofen	9.2
Flurbiprofen	12.0
Itraconazole	12.0

* From injection into the column.

Several drugs were tested as internal standard such as chlorpheniramine, metoclopramide, diltiazem, mebeverine and other compounds (Table 1). Most of the drugs tested either interfere with pheniramine (chlorpheniramine), coelute with the endogenous plasma peaks (metoclopramide), or produce low recovery (mebeverine) under the alkaline extraction. Amitriptyline peak was sharp, symmetrical, well resolved from endogenous components in plasma and reproducible. Further, amitriptyline recovery was excellent (97.4%). Therefore, amitriptyline was selected as the internal standard. Table 1 lists the retention times of the tested drugs.

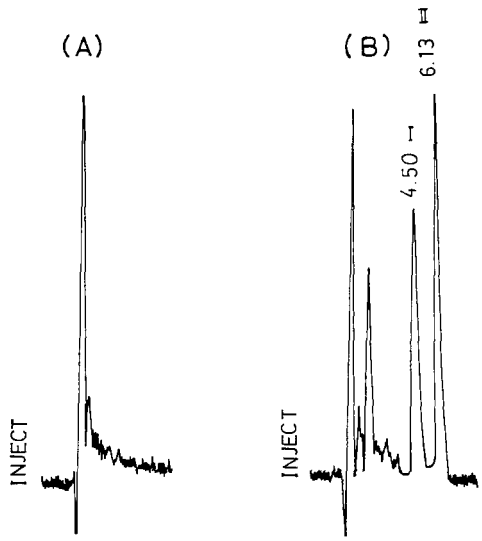

Figure 1: Chromatograms of a dog plasma samples collected before (a) and 4.0 hr after administration of 75 mg Avil^{R}-retard tablet (b).

Key: I; Pheniramine (Conc. 81.0 ng/ml).
II; Amitriptyline (internal standard).

Figure 1 shows chromatograms from a dog plasma samples collected before and 4.0 h after administration of 75 mg oral dose of pheniramine (Avil^{R}-retard).

Quantification

The quantification of the chromatogram was performed using peak-height ratios of the drug to the internal standard. For each assay a six-point calibration curve was prepared by spiking drug-free plasma samples

(1 ml) with 0, 20, 40, 80, 120, 200, and 400 ng pheniramine. Calibration samples were processed identically and simultaneously as described. The concentrations and peak-height ratios were linearly related over this range. Each point on the calibration curve was based on eight determinations. Least squares linear regression analysis of the data resulted in the following equation:

$$Y = 0.0054 + 0.01\ X,\ r = 0.999$$

Standard curves were constructed over an eight-week period to determine the variability of the slopes and intercepts. The results showed small day-to-day variability in the slopes and intercepts. The coefficient of variation for the slopes was 4.34% which indicates a high stability and precision for the assay.

Precision

The within-day precision (random analytical variation) was evaluated by replicate analysis of pooled plasma samples containing pheniramine at three different concentrations. All specimens used to study precision and bias were interspersed with clinical specimens during analysis. The within-day precision showed a coefficient of variation (CV) of 3.57 to 6.51% (Table 2). In addition, the assay was accurate even at plasma concentration as low as 30 ng/ml (bias=2.33%).

TABLE 2

Within-day and Between-day Precision of Pheniramine in Human Plasma.

Within-day*			Between-day**		
Added Conc. (ng/ml)	Measured Conc. (ng/ml)	Bias %	Added Conc. (ng/ml)	Measured Conc. (ng/ml)	Bias %
30			30		
Mean	30.7	2.33	Mean	29.6	-1.33
S.D.	2.0		S.D.	2.32	
CV%	6.51		CV%	7.84	
100			100		
Mean	97.0	-3.0	Mean	98.7	-1.30
S.D.	4.91		S.D.	6.54	
CV%	5.10		CV%	6.63	
300			300		
Mean	291.8	-2.73	Mean	295.3	-1.57
S.D.	10.42		S.D.	14.84	
CV%	3.57		CV%	5.03	

* Mean values represent six different plasma samples for each concentration.

** Between-day reproducibility was determined from 6 different runs over a 4-week period at the three concentrations. The concentration of each run was determined from a single calibration curve run on the first day of the study.

The between-day variation (total analytical variation) was similarly evaluated on several days up to 4 weeks. The between-day CVs varied from 5.03 to 7.84% (Table 2).

Accuracy

The absolute and relative analytical recovery from plasma for pheniramine at three different concentrations were measured in the following way. The drug and internal standard were added to drug-free plasma to achieve the concentrations shown in Table 3. These plasma were then analyzed by the developed method. Following extraction, evaporation, and reconstitution, a carefully measured fixed volume of the supernatent was then injected and the peak-height was measured. Absolute recovery was calculated by comparing these peak heights with the peak heights obtained by the direct injection of the same fixed volume of the pure aqueous drug standards. As shown in Table 3 absolute recoveries of pheniramine ranged from 94 to 96.9%.

The relative recovery of the drug was calculated by comparing the concentrations obtained from the drug-supplemented plasma with the actual added concentrations. The relative recovery ranged from 92 to 109.3% (Table 3).

TABLE 3

Absolute and Relative Recovery of Pheniramine from Human Plasma*.

Conc. (ng/ml)	Mean Peak Heights (Cm)		Absolute Recovery %	Relative Recovery %	Range Relative Recovery
	Aqueous	Plasma	Mean±SD	Mean±SD	
30	1.52	1.43	94.0±1.2	102.4±6.7	92.0-109.3
100	3.10	2.95	96.9±4.4	97.0±4.9	92.0-106.0
300	8.22	7.92	96.4±1.6	97.3±3.5	93.3-102.3
I.S.	3.70	3.60	97.4±5.3		

* Six replicate analyses of each concentration.

Stability

Stability studies of plasma samples spiked with pheniramine (30, 100 and 300 ng/ml) were performed over a 3-week period (Table 4). Plasma samples were stored in a freezer at -20°C until the analysis. The results demonstrate that pheniramine can be stored frozen in plasma for at least 3-weeks without appreciable degradation.

Limit of Detection

The limit of quantification for this method was attained with plasma samples containing 10.0 ng/ml of pheniramine. It was defined as the concentration in plasma that resulted in a detectable peak of approximately three times the noise level.

TABLE 4

Effect of Storage at -20°C on Pheniramine Stability in Human Plasma.

	Added Conc. (ng/ml)		
	30	100	300
Days		% Recovered	
0	100.0	98.1	96.3
4	110.0	105.0	107.7
8	93.3	91.0	93.7
12	103.3	108.0	100.0
15	95.0	96.2	96.7
21	86.7	94.0	96.3
Mean	98.1	98.7	98.5
S.D.	8.2	6.5	5.0
CV%	8.4	6.6	5.1

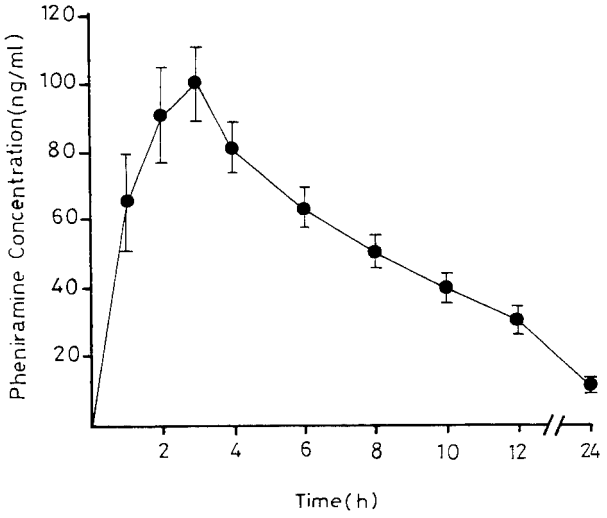

Figure 2: Mean (±SD) plasma concentration-time profiles of pheniramine following administration of a 75-mg tablet (Avil-retard) to six beagle dogs.

Clinical Application

Figure 2 shows the mean plasma concentration-time profile of pheniramine after administration of a 75-mg tablet (Avil^{R}-retard) to six beagle dogs. The calculated pharmacokinetic parameters (mean±SD) were the area under the plasma concentration-time curve (AUC_{o--oo}) (1023.53±109.6 ng.h/ml), peak plasma concentration (C_{max}) (104.2±9.7 ng/ml), peak time (T_{max}) (2.83±0.41 h), elimination rate constant (K_{el}) (0.11±0.02 h^{-1}) and elimination half-life ($t_{½}$) (6.47±1.14 h).

Conclusion

The HPLC method developed in this study has the sensitivity, accuracy, reproducibility and stability which makes it versatile and valuable in many applications, specifically in pharmacokinetic studies and bioavailability-bioequivalency studies of pheniramine pharmaceutical products.

ACKNOWLEDGEMENT

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ISOLATION AND CHARACTERIZATION OF (PHEOPHYTIN)MERCURY(II)

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ABSTRACT

Mercury-substituted chlorophylls, i.e., (pheophytin *a*)mercury(II) and (pheophytin *b*)mercury(II), were separated by reversed-phase HPLC with a C_{18}-bonded silica column and a mobile phase of acetone-methanol. Their spectroscopic and chromatographic characteristics strongly suggest that both (pheophytin *a*)mercury(II) and (pheophytin *b*)mercury(II) form 1 : 1 (metal ion : chlorin ring) complexes.

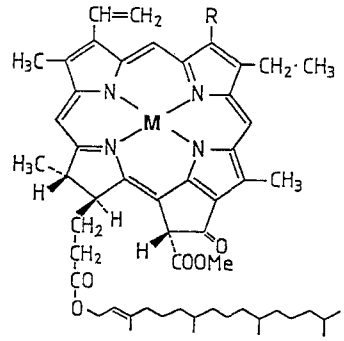
INTRODUCTION

Metallochlorophylls, in which magnesium of chlorophylls is substituted by other metal ions, are attractive in view of photochemical applications since they have intense visible light absorption bands. Among them, mercury-substituted chlorophylls should have interesting photochemical properties because the large diameter of mercury(II) ion

causes distinct electronic interaction between the central metal ion and the chlorin ring. Although several isolation methods by means of HPLC have been studied for iron- (1,2), zinc- (3,4) nickel- (5), copper- (6) and manganese-substituted chlorophylls (7), isolation of mercury-substituted chlorophylls has not been reported. The difficulty in the isolation of mercury-substituted chlorophylls is considered due to their very labile properties such as demetallation (8). In this short communication, a simple and rapid isolation method of (pheophytin *a*)mercury(II) [(pheo-*a*)Hg] and (pheophytin *b*)mercury(II) [(pheo-*b*)Hg] using reversed-phase HPLC is reported. Furthermore, it is indicated that both [(pheo-*a*)Hg] and [(pheo-*b*)Hg] form 1 : 1 (metal ion : chlorin ring) complexes by comparing the spectroscopic and chromatographic characteristics with those of zinc-substituted chlorophylls, i.e., (pheophytin *a*)zinc(II) [(pheo-*a*)Zn] and (pheophytin *b*)zinc(II) [(pheo-*b*)Zn], which are typical 1 : 1 complexes with no axial ligand. The structure of these complexes is shown in Figure 1.

EXPERIMENTAL

Chlorophylls were extracted from spinach and purified by a literature method (9), followed by their pheophytinization with dilute hydrochloric acid. Pheophytin *a* [(pheo-*a*)H_2] and *b* [(pheo-*b*)H_2] were seperated by reversed-phase HPLC with a mobile phase of acetone-acetonitrile (1 : 1, v / v). Crude (pheo-*a*)Hg and (pheo-*b*)Hg were prepared by stirring a mixture of (pheo-*a*)H_2 or (pheo-*b*)H_2 dissolved in acetone and a slight excess of anhydrous mercury(II) acetate dissolved in ethanol for 1 h. Zinc complexes, (pheo-*a*)Zn and (pheo-*b*)Zn, were prepared by reaction of zinc acetate with (pheo-*a*)H_2 or (pheo-*b*)H_2 (3). The HPLC apparatus was a



M	R	Compd.
Hg	CH_3	(pheo-a)Hg
Hg	CHO	(pheo-b)Hg
Zn	CH_3	(Pheo-a)Zn
Zn	CHO	(Pheo-b)Zn
2H	CH_3	(pheo-a)H_2
2H	CHO	(pheo-b)H_2

FIGURE 1. Structure of (pheo-*a*)Hg, (pheo-*b*)Hg, (pheo-*a*)Zn, (pheo-*b*)Zn, pheophytin *a* [(pheo-*a*)H2] and pheophytin *b* [(pheo-*b*)H2].

Hitachi 638-80 equipped with an Inertsil ODS-2 column (GL Science). The contour chromatograms were obtained by using a Waters 991J photodiode array detecter. The column temperature was kept at 25 ℃ and the mobile phase flow rate was 1.0 ml / min. Helium gas was bubbled through a reservoir containing the solvent of mobile phase. FTIR spectra were recorded on JEOL JIR-100 spectrophotometer by the polyethylene film method. All operations were carried out in the dark and in argon atmosphere.

RESULTS AND DISCUSSION

HPLC conditions

The separation was carried out just after the crude products were obtained in order to avoid their degradation. Metallochlorophylls have the amphipatic nature due to a lipophilic phytyl group and a relatively hydrophilic matallochlorin. Therefore, a reversed phase C_{18}-bonded silica gel (ODS) column was used. Binary components such as acetone-acetonitrile, acetone-methanol, etc, were examined as the mobile phase. As a result, acetone-methanol was the most suitable for the separation. Figure 2 shows the contour chromatogram for the crude (pheo-*a*)Hg. In this chromatogram, several unreacted or decomposed metal-free species, i.e., pheophorbide *a* (pheophytin derivative which has no phytyl group), (pheo-*a*)H_2, and pheophytin *a'* [(pheo-*a'*)H_2] are detected. Pheophytin *a'* is a C-10 epimer of (pheo-*a*)H_2 (10). Chlorophylls and their derivatives have two strong absorption bands. The exceedingly intense band near 400 nm is assigned to a Soret band and the band near 650 nm to a Q band (11). These bands are shifted upon the replacement of two protons in pheophytin by the metal ion(1-6). From this tendency, the species 2 and 3 in Figure 2 are considered to be mercury complexes. This is supported by the fact that these species have no fluorescence. The presence of two mercury complexes obtained from (pheo-*a*)H_2 is not surprising because chlorophyll *a* and its derivatives have a C-10 epimer, i.e., chlorophyll *a'*, which is formed by epimerization in the polar solution (10,12,13). In fact, the reversed-phase chromatograms for other metallochlorophylls show two species, *a* and *a'* (1,5,6). The retention time of the species *a'* is larger than that of *a* (1,5,6) and the species *a'* has the same spectral

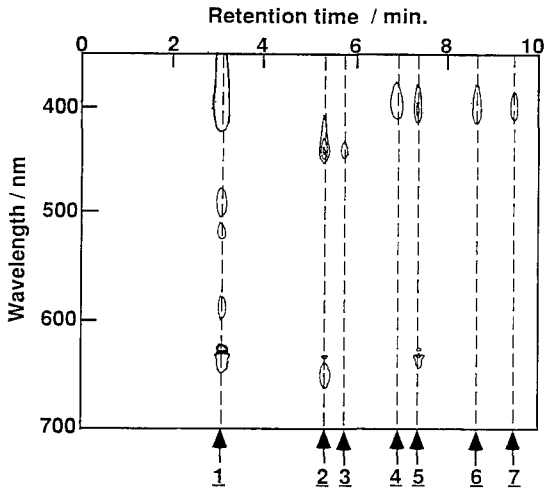


FIGURE 2. Contour chromatogram for crude (pheo-*a*)Hg.
Mobile phase : acetone/methanol (60/40).
1 = pheophorbide *a*, **2** = (pheo-*a*)Hg, **3** = (pheo-*a'*)Hg, **4**, **5** = unknown metal-free species, **6** = (pheo-*a*)H_2, **7** = (pheo-*a'*)H_2.

characteristics as the species *a* (1,6,10,12). This is in good agreement with the present case. Hence, it is considered that the species 2 is (pheo-*a*)Hg and 3 is (pheo-*a'*)Hg. Figure 3 shows the contour chromatogram for the crude (pheo-*b*)Hg. Based on the same reason as that mentioned above, the species 2 and 3 in this chromatogram are considered to be (pheo-*b*)Hg and (pheo-*b'*)Hg, respectively. The retention time of (pheo-*a*)Hg is larger than that of (pheo-*b*)Hg. This elution order agrees with that for other metallochlorophylls in the reversed-phase HPLC system (1,3-6), consistent with the general tendency that the aldehyde group (-CHO) in (pheo-*b*)Hg has larger polarity than the methyl group (-CH_3) in (pheo-*a*)Hg.

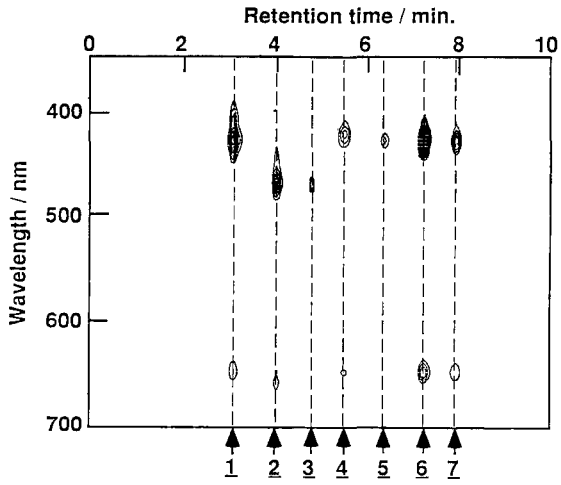


FIGURE 3. Contour chromatogram for crude (pheo-*b*)Hg.
Mobile phase : acetone/methanol (60/40).
1 = pheophorbide *b*, **2** = (pheo-*b*)Hg, **3** = (pheo-*b*')Hg, **4**, **5** = unknown metal-free species, **6** = (pheo-*b*)H_2, **7** = (pheo-*b*')H_2.

Identification of (pheo-*a*)Hg and (pheo-*b*)Hg

In the above discussion, it is presumed that (pheo-*a*)Hg and (pheo-*b*)Hg are 1 : 1 (metal ion : chlorin ring) complexes with no axial ligand, similar to other metallochlorophylls, e.g., (pheophytin)zinc complexes. For the mercuryporphyrins, of which the skeletal structure is closely related to that of mercury-substituted chlorophylls, 2 : 1 and 3 : 2 ("double sandwitch type") complexes, which have acetato-mercury bonds, are known, in addition to 1 : 1 complexes (14,15). It is suggested from their spectroscopic and chromatographic characterization that both (pheo-*a*)Hg and (pheo-*b*)Hg are 1 : 1 complexes as follows. Figure 4 shows the FTIR spectrum of (pheo-*a*)Hg, together with those of (pheo-*a*)Zn and (pheo-

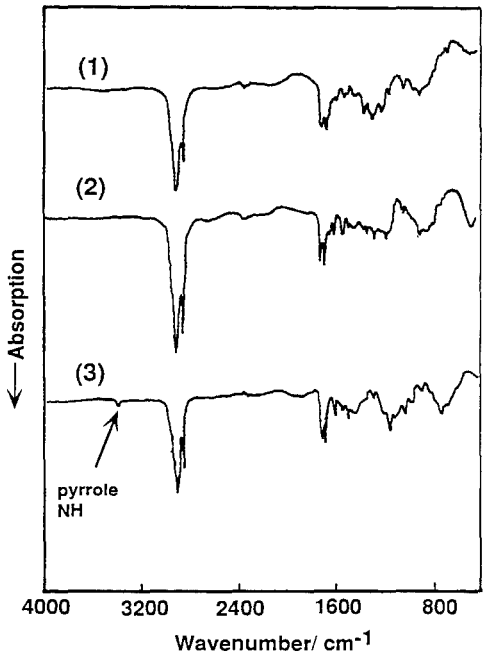


FIGURE 4. FTIR spectra of **(1)** (pheo-*a*)Hg, **(2)** (pheo-*a*)Zn and **(3)** (pheo-*a*)H_2.

a)H_2. The spectral pattern of (pheo-*a*)Hg is very similar to that of (pheo-*a*)Zn, the typical 1 : 1 complexes with no axial ligands. The incorporation of the mercury ion is apparent from the absence of the pyrrole N-H stretching absorption, while it is detected at ca. 3400 cm^{-1} in the spectrum of (pheo-*a*)H_2. More important information is obtained from the CO stretching region. Figure 5 shows the CO stretching region of the spectrum for (pheo-*a*)Hg, together with those of (pheo-*a*)Zn and (pheo-*a*)H_2. The spectral pattern of the CO stretching region is very similar to that of (pheo-*a*)Zn, in the manner of the numbering in Figure. 5. If (pheo-

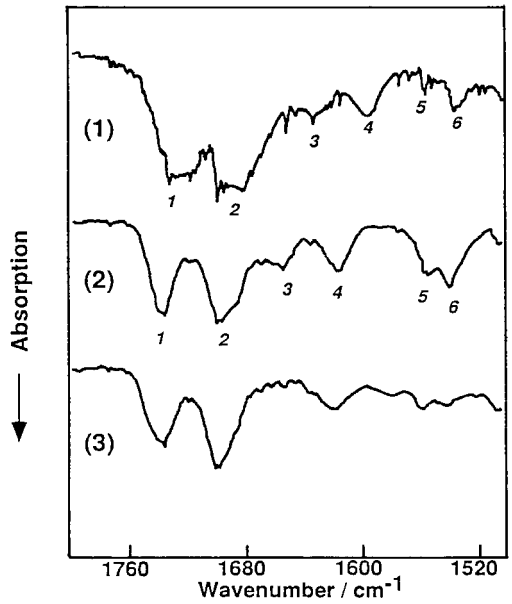


FIGURE 5. FTIR spectra in carbonyl stretching region for **(1)** (pheo-*a*)Hg, **(2)** (pheo-*a*)Zn and **(3)** (pheo-*a*)H_2.

a)Hg is 2 : 1 or 3 : 2 complex, a strong band of the mercury bound acetate should be observed at ca. 1560 - 1580 cm^{-1}, referred to mercury porphyrins (14,15). However, such absorption is not detected here, suggesting that (pheo-*a*)Hg is 1 : 1 complex. In the same way, (pheo-*b*)Hg is also considered to be 1 : 1 complex.

For the reversed-phase HPLC of metallochlorophylls using acetone-methanol as a mobile phase, the dependence of the capacity factor (k') on the mixing ratio of acetone-methanol is sensitive to the coordination geometry (5,7). In the case of 1 : 1 complex with no axial ligands, the k' value decreases in proportion to the increasing concentration of acetone

TABLE 1

Absorption Maxima of the Complexes

Complex	λ max of Soret band /nm	Q band /nm
(pheo-*a*)Hg	449[a]	677[a]
(pheo-*b*)Hg	472[a]	664[a]
(pheo-*a*)Zn	425[a]	652[a]
	427[b]	655[b]
(pheo-*b*)Zn	452[a]	637[a]
	455[b]	640[b]

[a] Solvent : acetone/methanol (60/40)
[b] Taken from ref. 3, where the solvent is acetone/methanol (25/75).

(5,7). The relationship between the k' value and the mixing ratio of acetone-methanol was examined for (pheo-*a*)Hg, (pheo-*b*)Hg, and the corresponding zinc complexes. When the acetone concentration increased from 20 to 80 %, the log k' decreased linearly from 0.31 to -0.38, -0.20 to -0.93, 0.59 to -0.33, and 0.41 to -0.44 for (pheo-*a*)Hg, (pheo-*b*)Hg, (pheo-*a*)Zn, and (pheo-*b*)Zn, respectively. Thus, the tendency of the k' value for the mercury complexes is very similar to that for the zinc complexes. This finding is consistent with the 1 : 1 structure of the mercury complexes.

Table 1 summarizes the wavelengths at the absorption maxima of (pheo-*a*)Hg and (pheo-*b*)Hg, together with those of the corresponding zinc complexes. The spectral pattern reflects the π electron symmetry of the macrocycles and the symmetry is strongly affected by the coordination

geometry (16). The patterns of (pheo-*a*)Hg and (pheo-*b*)Hg were very similar to those of corresponding zinc complexes, indicating these mercury complexes are 1 : 1 complexes with no axial ligand. It is noteworthy that the peak wavelengths of (pheo-*a*)Hg and (pheo-*b*)Hg are longer than those of any other corresponding metallochlorophylls. Hence, (pheo-*a*)Hg and (pheo-*b*)Hg could be used as the photosensitizers around 450 - 470 nm, if increase their stabilities.

In conclusion, labile complexes (pheo-*a*)Hg and (pheo-*b*)Hg are effectively isolated by reversed phase HPLC with a C18-bonded silica column. Their spectroscopic and chromatographic characterization indicate that (pheo-*a*)Hg and (pheo-*b*)Hg are 1 : 1 (metal ion : chlorin ring) complexes with no axial ligand.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR DETERMINATION OF ATENOLOL FROM HUMAN PLASMA AND URINE: SIMULTANEOUS FLUORESCENCE AND ULTRAVIOLET DETECTION

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ABSTRACT

A rapid, reliable analytical method was required to study the disposition of atenolol following oral administration in subjects in various stages of pregnancy. Available methods showed wide variability due to matrix interference. A simple HPLC method is reported for the determination of atenolol in human blood and urine. Atenolol and the internal standard, albuterol, were isolated using solid phase extraction and separated isocratically on a C-18 analytical column with a mobile phase consisting of a mixture of an aqueous solution of mono-basic ammonium phosphate and N,N-dimethyloctylamine, and acetonitrile (93:7 v/v). Atenolol and the I.S. were monitored in the effluent using both fluorescence (228/310 nm, excitation/emission) and ultraviolet (224 nm) detection. Within-run and between-run precision showed a c.v. <5% for concentration of 50-400 ng/ml with an error <5%. Recovery following solid phase extraction ranged from 72.8% at 50 ng/ml to 95.5% at 500 ng/ml. The method is linear over a range of 50-

750 ng/ml. The assay has been applied for quantification of atenolol plasma levels for the determination of pharmacokinetic parameters following oral dosing.

INTRODUCTION

Atenolol, a selective ß-1 adrenergic blocking agent, is currently used for the treatment of hypertension, angina pectoris and certain types of arrhythmias (1-3). Several methods have been previously reported for the determination of atenolol in biological fluids. These include gas-chromatographic methods requiring derivatization (4-5), and high performance liquid chromatographic (HPLC) techniques using either solid phase or liquid extraction with ultraviolet or fluorescence detection (6-8). Since the UV maximum for atenolol occurs at 224 nm, it is often difficult to obtain HPLC chromatograms free from interference because of substances having high UV absorbance at similar wavelengths in the matrix. Atenolol shows high native fluorescence at excitation/emission wavelengths of 228/310 nm, respectively. Although HPLC chromatograms using fluorescence detection are generally free from interference relative to UV detection, there has been a lack of a suitable internal standard for analysis of atenolol. Moreover, methods of analysis of atenolol in biological specimens using fluorescence detection from several previously reported methods show wide variability due to interfering substances.

This paper describes a HPLC method for determining atenolol in human plasma and urine using albuterol as an internal standard. The sample is prepared by solid phase extraction for sample cleanup and the reconstituted sample is injected into an HPLC and separated on a C_{18} reversed-phase analytical column. The effluent is monitored with a fluorescence detector at an excitation/emission wavelength of 228/310 nm, respectively. Before the conditions of the method were optimized, the UV chromatogram showed evidence of interfering substances that were co-eluting with atenolol and the internal standard. To avoid any quenching or enhancement of the fluorescence of atenolol or albuterol due to co-eluting endogenous subtances that could affect the accuracy and reproducibility of the method, conditions were modified until complete resolution of all peaks was accomplished by monitoring the effluent using UV detection at 224 nm. The method described gives excellent chromatographic separation and is rapid, sensitive and reproduceable, with a sensitivity limit of 50 ng/ml.

EXPERIMENTAL

Materials and Reagents

Atenolol, albuterol (internal standard), and human serum were purchased from Sigma Chemical Co. (St. Louis, MO). Ammonium phosphate monobasic and *N,N*-

dimethyloctylamine (DMOA) were purchased from Aldrich Chemical Co. (Milwaukee, WI). HPLC grade acetonitrile and methanol were obtained from Fisher Scientific (Pittsburgh, PA). Octadecyl (C-18) solid phase extraction columns (100 mg) were obtained from Baxter Healthcare Co. (Muskegon, MI).

Instrumentation

The HPLC system consisted of the following components: Perkin Elmer, Series 410 LC solvent delivery pump, LC 90 UV spectrophotometric detector, LS-4 fluorescence spectrophotometic detector, LCI-100 laboratory computing integrator (for fluorescence detector), Hitachi AS-4000 auto sampler, and 2000-D computing integrator (for UV detector). Atenolol was separated on a C-18 reverse phase, 250 x 4.6 mm I.D., 5 μ analytical column, preceeded by a C-18 guard column, both Adsorbosphere C-18, Alltech Associates, Inc, (Deerfield, IL). Samples were eluted isocratically at a mobile phase flow rate of 1.5 ml/min. The effluent was monitored using a variable wavelength UV detector at an analytical wavelenth of 224 nm placed in series with a fluorescent detector with an excitation/emission wavelenth of 228 nm and 310 nm, respectively.

Mobile Phase

The mobile phase consisted of a mixture of an aqueous solution containing 25 mM mono-basic ammonium

phosphate and 1 mM *N,N*-dimethyloctylamine (DMOA) with acetonitrile, 93:7 v/v, adjusted to pH 3.0 with phosphoric acid 85%. The mobile phase was filtered through a Nylon 66 membrane filter (Sartolon, Sartorius, Germany) and deaerated with helium gas.

Preparation of Standard Solutions:

Accurately weighed atenolol was dissolved in an appropriate volume of distilled water to obtain a stock solution containing 1 mg/ml. This solution was further diluted to obtain working standard solutions with concentrations of 1 and 10 μg/ml. Pooled normal human serum was spiked with appropriate volumes of these standards to achieve standard solutions containing 50, 100, 200, 250, 500 and 750 ng/ml, that were used to obtain a standard curve. A standard curve of atenolol in urine was prepared in a similar manner. The internal standard solution was prepared by dissolving an appropriate amount of albuterol in water to give a concentration of 1 mg/ml. This solution was further diluted to obtain an internal standard solution with a concentration of 5 μg/ml.

Sample Preparation

To 500 μl of spiked serum or patient serum sample was added 50 μl of internal standard solution. The mixture was vortexed for 30 seconds then loaded on a preconditioned 100 mg, C-18 solid-phase extraction

column. Extraction columns were preconditioned by passing through one column volume of methanol followed by two column volumes of water. Serum samples containing the internal standard were passed through the solid phase columns and the loaded columns washed with 3 x 0.2 ml of water. Atenolol and the internal standard were eluted from the column using 2 x 0.5 ml of methanol. The combined methanol extracts were evaporated to dryness under a stream of dry, filtered air at 40-45° C. The residue was reconstituted with 150 µl of water, vortexed for 30 seconds, then centrifuged at 14,000 g for 4 minutes. The supernatant was transferred into injection vials and 50 µl was injected into the HPLC using an autoinjector.

Spiked standards and patient serum specimens were treated similarly. However, urine specimens required dilution in which 10 µl of urine was diluted to 500 µl with distilled water. Internal standard solution was added and the identical procedure as previously described was followed.

RESULTS AND DISCUSSION:

Both UV absorbance and fluorometric methods have been widely used for the detection of atenolol following HPLC separation (6,7). UV spectra reported by Verghese *et al.* (7) and confirmed in our laboratory indicate that the molar absorbance of atenolol is

sufficiently intense for detection at concentrations as low as 15-25 ng/ml from small sample volumes (500 μl). Atenolol also displays high intrinsic fluorescence. Albuterol proved to be an ideal internal standard for this assay since it was found to migrate closely to atenolol, showed a UV maximum at 224 nm and a fluorescence excitation/emission at 228/310 nm, respectively, and their peaks could be completely resolved under the conditions of the method. Samples were detected by passing the HPLC effluent serially through a variable wavelength UV detector at 224 nm followed by a fluoresence detector at excitation/emission wavelengths of 228 nm and 310 nm, respectively, as shown in Fig 1. Typical HPLC chromatographs of atenolol and the internal standard, albuterol, in plasma using UV absorbance and fluorescence detection are shown in Fig 2. Chromatographs using UV detection indicated the presence of at least two potentially interfering peaks, one that eluted in close proximity to atenolol and the other close to the internal standard. Although endogenous interfering substances in human plasma may show only weak or complete absence of fluorescence, co-elution of peaks may cause quenching or enhancement of fluorescence resulting in variability in accuracy and reproducibility of the analytical method. Modifications in mobile phase composition and pH, and sample cleanup

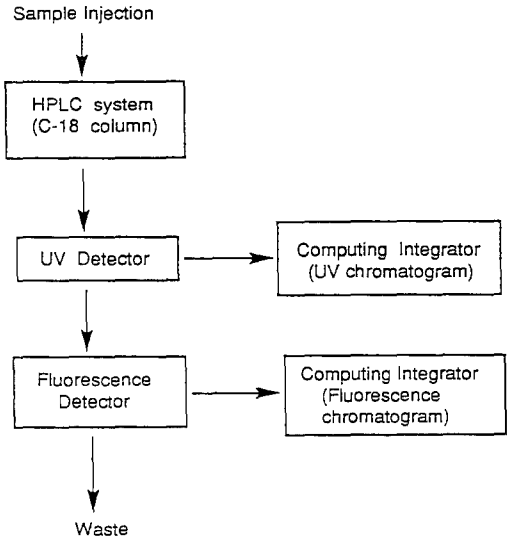

Figure 1. Schematic of the HPLC system.

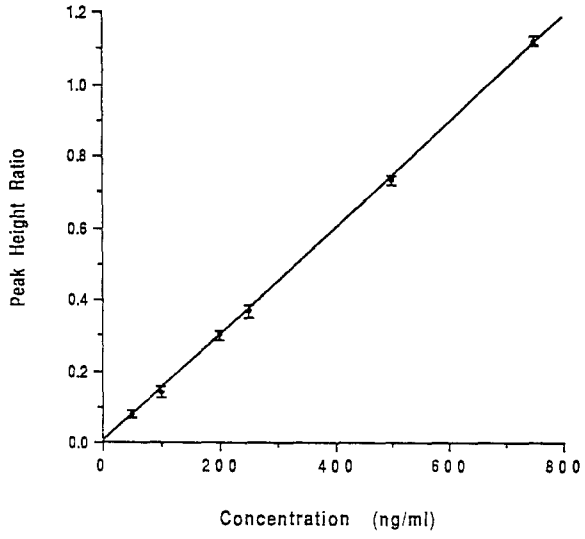

Figure 2. Standard curve of peak height ratio of atenolol to internal standard versus atenolol concentration (shown as means ± s.d. of five determinations at each concentration).

procedures, were made until optimal conditions were achieved whereby all UV and fluoresence peaks could be completely resolved. The retention times for the internal standard and atenolol using UV detection were 7.1 and 10.4 minutes, and for fluorescence detection, 7.3 and 10.6 minutes, respectively. Since the UV and fluorescence detectors were coupled in series, the fluorescence peaks lagged their respective UV peaks by approximately 0.2 minutes. The assay cycle period was approximately 30 minutes.

All results reported herein have used fluorescence detection since this method was relatively free from interference compared to UV detection. Depending on the excitation/emission wavelengths used, 228/310, 228/606 or 278/606, the limit of detection for the method was 5 ng/ml, 10 ng/ml or 25 ng/ml, respectively. However, when the assay conditions were optimized to achieve separation of all essential peaks, the results using UV detection were comparable to fluorescence. Thus, the method is equally applicable to quantitate atenolol using either fluorescence or UV detection.

Variability of the method was determined by assaying six known serum concentrations on five separate days. Results of this study are shown in Table I. A standard curve plot of peak height ratio of atenolol to internal standard versus atenolol concentration is

TABLE I. Standard curve and day-to-day variability of Atenolol assay in serum at six concentrations determined on five consecutive days.

Atenolol Concentration ng/ml	Peak Height Ratio Mean ± S.D.
50	0.076 ± 0.010
100	0.142 ± 0.016
200	0.299 ± 0.014
250	0.368 ± 0.018
500	0.731 ± 0.013
750	1.117 ± 0.013

Standard Curve (five consecutive days)
Slope = 1.48×10^{-3}
y-Intercept = -1.98×10^{-3}
Correlation coefficient = 0.9995

shown in Figure 3. The standard curve shows that the method is linear over a concentration range of 50 - 750 ng/ml, with a mean slope of 1.48×10^{-3}, a *y*-intercept of -1.98×10^{-3}, and a mean correlation coefficient of 0.9995.

Within-run and between-run precision and accuracy were determined at four different concentrations. Within-run precision was determined at 20, 50, 150 and 400 ng/ml, each sample being assayed six times within the same run. The results shown in Table II demonstrate that the method is precise with a coefficient of variation of less than 5 percent for concentrations of 50 - 400 ng/ml with a percent error of less than 5 percent. Although the accuracy of the method was

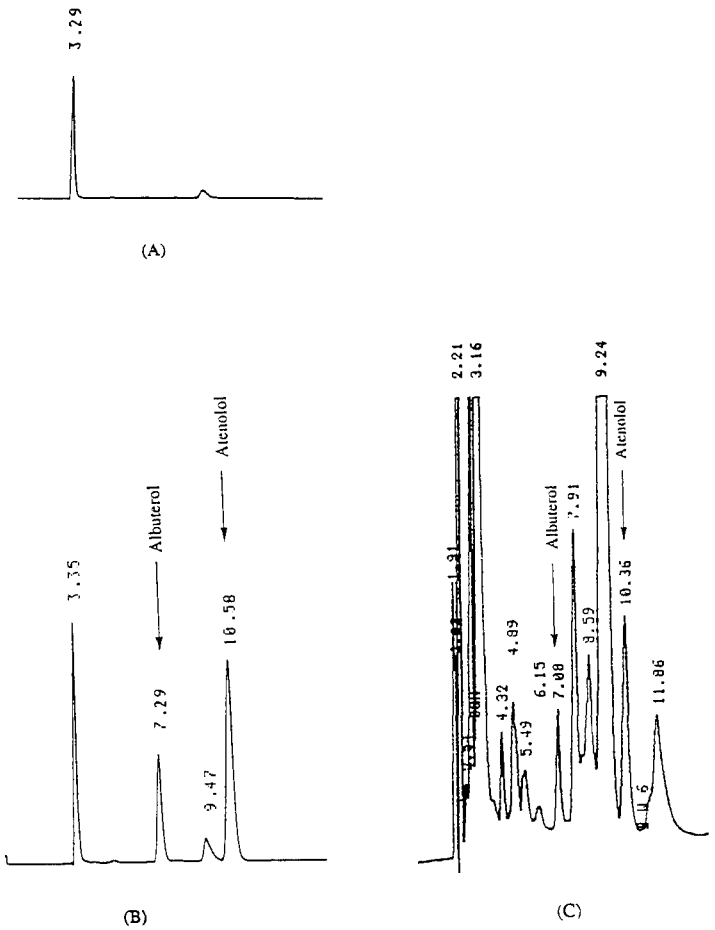

Figure 3. Typical HPLC chromatograms using fluorescence detection showing (A) human plasma blank; (B) human plasma containing atenolol, 22.3 ng and I.S., 22.3 ng; (C) HPLC chromatogram using UV detection obtained from the same injection shown in (B) above, indicating clear separation of atenolol and I.S. from interfering peaks from the matrix.

TABLE II. Within-run precision and accuracy for determination of atenolol in serum.

Atenolol Concentration, ng/ml Actual	Measured* ± S.D.	C.V.%	% Error
20	19.6 ± 3.0	15.3%	2.0%
50	52.3 ± 2.2	4.1%	4.6%
150	154.7 ± 3.7	2.4%	3.1%
400	389.1 ± 6.8	1.7%	2.7%

*Mean of 6 assays

excellent for the 20 ng/ml sample, the assay variability was wide, with a coefficient of variation of 15.3%. Between-run precision was determined by repeated assays of four spiked serum samples containing 150, 250, 400 and 700 ng/ml of atenolol on six separate days. The results of this study are presented in Table III. The coefficient of variation for all samples tested ranged from 0.82 - 6.45%, with a percent error of 0.72 - 4.50%.

Recovery (extraction efficiency) following solid phase extraction was determined on serum specimens spiked with atenolol at four known concentrations (50, 100, 250 and 500 ng/ml) and corresponding concentrations in water. The percent recovery was determined by comparing the peak height of the extracted plasma samples to the peak height for the non-extracted aqueous solution of equal concentration. Table IV

TABLE III. Between-run precision and accuracy for determination of atenolol in serum.

Atenolol Concentration, ng/ml Actual	Measured* ± S.D.	C.V.%	% Error
150	147.5 ± 9.5	6.5%	1.7%
250	251.8 ± 2.1	0.8%	0.7%
400	382.0 ± 15.3	4.0%	4.5%
700	706.2 ± 10.1	1.4%	0.9%

*Mean of 6 assays

TABLE IV. Recovery of atenolol and internal standard from spiked serum samples following C_{18} solid-phase extraction.

Plasma Concentration (ng/ml)	Mean Recovery* ± S.D. % Atenolol	I.S.
50	72.8 ± 14.5	
100	77.8 ± 10.9	
250	88.1 ± 2.6	
500	95.5 ± 3.1	
I.S., 250 ng		92.3 ± 5.1

shows a mean recovery ranging from 72.8 ± 14.5% for the 50 ng/ml sample to 95.5 ± 3.1% for the 500 ng/ml sample. The mean extraction efficiency for the internal standard was 92.3 ± 5.1%.

The stability of atenolol in plasma was determined since samples are frequently stored frozen for a period of time prior to analysis. It has been recommended

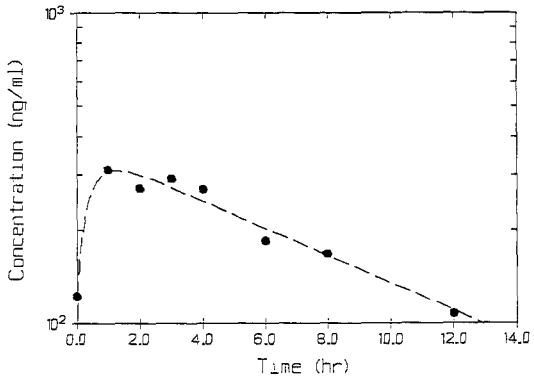

Figure 4. Plasma concentration-time profile of atenolol following administration of a 50 mg oral dose to a subject in the third trimester of pregnancy. The data is fitted a one-compartment oral absorption model. Fitted parameters are k_{el} = 0.10 hr^{-1}; k_a = 2.20 hr^{-1}; AUC = 3.55 $\mu g \cdot hr/ml$, V_d = 34.9 L.

that samples should not be stored for a long period prior to analysis because of instability (6). Samples containing known concentrations of atenolol were maintained at ambient temperature (23°), 4° and -20° C for a period of seven days. The samples were all analyzed within the same run. No significant trend in sample concentration change were seen following storage at the different temperatures over a seven day period.

The method was applied to an investigation of changes in the pharmacokinetic profile of atenolol in women throughout pregnancy. The pharmacokinetics of atenolol was studied in 14 patients in their first,

second and third trimesters of pregnancy. A typical plasma concentration-time curve over the 12 hour dosing interval following chronic oral dosing of 50 mg twice a day is shown in Figure 4. The data were fitted to a one-compartment model with extravascular dosing. All model-defined parameters (k_{el}, k_a, V_d, AUC) were obtained and the total clearance was calculated.

The analytical method described is reliable and sensitive, and is applicable for the determination of atenolol in blood or urine.

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RELATIONSHIP STUDY BETWEEN REVERSED PHASE HPLC RETENTION AND OCTANOL/WATER PARTITION AMONG AMPHOTERIC COMPOUNDS

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ABSTRACT

The retention of eight non-congeneric amphoteric compounds was followed in a reversed phase (RP HPLC) system (C_{18}/methanol-water vol. 50:50) in the pH range 4 - 9. The chromatographic behavior of the amphoterics is explained by means of their species distribution diagram (pH profile of the protonation macro- and microspecies) in the same pH interval.

Maximum retention was observed at the isoelectric point of the compounds even in cases when the zwitterionic species was in a great excess over the neutral (uncharged) one. This finding reveals, that the retention at the isoelectric point (ie.p.) must be generated by the retention of the less polar, neutral form. The great similarity of pH dependent partition (octanol-water) and retention (C_{18}/methanol-water) pattern provides further proof to the concept, that in case of amphoteric compounds the neutral species is transferred into the octanol phase during octanol/water partition.

Linear correlation analysis showed that $logP_{oct/w}$ and $logk'_{C18}$ are analogue lipophilicity parameters i.e.: the physico-chemical content of $logk'_{C18}$ corresponds to that of the octanol/water true partition coefficient.

INTRODUCTION

The biological, therapeutical importance of amphoteria relies on the amphoteric character of the human body proteins and polypeptides, further, on the relatively large number of amphoteric drug compounds. The unambiguous mathematical definition of lipohilicity and protolytic dissociation for amphoterics is a much more complicated task than in case of monofunctional acids or bases, where the true partition coefficient (logP, the concentration rate of the neutral, uncharged species in the organic and the aqueous layer) can be obtained by Eqns. 1 and 2[1]:

$$\text{acids:} \quad logP = logP_{app} + \log (1 + 10^{pH-pKa}) \qquad (1)$$

$$\text{bases:} \quad logP = logP_{app} + \log (1 + 10^{pKa-pH}) \qquad (2)$$

Due to the similarity of their physico-chemical content, a very close relationship exists between the RP HPLC retention (t_R ; logk') and $logP_{oct/w}$, which was recognized earlier and could be utilized in a rather broad spectrum. This correlation proved particularly significant in RP HPLC systems where C_{18} as stationary phase was applied[2]. For this, numerous of examples could be referred from the circle of non-dissociating compounds[3], acids[4] and bases[5].

Relationship between $logP_{app}$ and logP was derived[6] also for amphoteric compounds including the stepwise dissociation constants (pK_{a1}, pK_{a2}):

$$\log P = \log P_{app} + \log (1 + 10^{pH-pKa1} + 10^{pKa2-pH}) \quad (3)$$

Eqn.3 is valid only for "ordinary" amphoterics (in general: $\Delta pK_a \rangle 4$), where beside the cation and anion forms the uncharged (but none of the the zwitterionic) species is present in the equilibrium mixture.

A more complicated relationship must be valid between logP and $\log P_{app}$ if $\Delta pK_a \ll 4$, i.e. overlapping protonation exists thus the solution of such compounds contains also the zwitterionic form ("zwitterionic amphoterics").

The relative concentration of protonation microspecies at a given pH value can be expressed by the protonation microconstants[7] ($k_1^{\pm}$ $k_2^{\pm}$ k_1^o k_2^o in Figure 1). The latters may be calculated if pK_{a1} and pK_{a2} macroconstants are known and one of the microconstants is experimentally available.[7,8] On this basis the diagram of pH dependent distribution of microspecies for a given amphoteric can be prepared (microspeciation). This diagram may supply valuable information about the actual protonation state and lipophilicity of the compounds. Takács-Novák et al.[9,10,11] basing on certain experimental data suggest, that only the neutral species partitions between the octanol and aqueous phase. The authors derived a relationship between logP and $\log P_{app}$ for zwitterionic amphoterics[10]:

$$\log P = \log P_{app} + \log (1 + 1/ k_1^o [H^+] + k_2^o / k_2^{\pm} + k_2^o [H^+]) \quad (4)$$

It seems probable, that the values of microconstants and data of species distribution as well as the true partition coefficients may provide valuable ideas to define the mechanism of effect for amphoteric drug compounds.

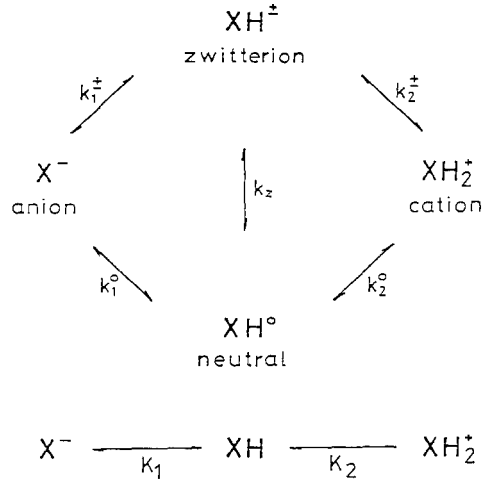


FIGURE 1 Protonation scheme of amphoterics

In the course of the present work the pH profile of reversed phase (RP HPLC) retention was followed in pH range 4 - 9. Since the retention on C_{18} stationary phase showed an evident analogy with octanol/water partition, it seemed reasonable anticipation, that the pH dependent RP HPLC retention should allow some new insight to the equilibrium partitioning state of different amphoteric compounds.

The C_{18} retention, although with some reserves,[2,13] is accepted as a lipophilicity parameter, moreover, in some cases, it proved suitable in QSAR research[12,14]. The RP_{C18} HPLC provided particularly good correlations with the octanol/water partition, when the mobile phase, beside water (aqueous buffer solution) contained methanol as organic modifier.[15,16] The correlation between $logk'_{C18}$ and $logP_{oct/w}$ was also close in case of ionizable compounds[17] although certain authors[18,19] suggested the use of correction. Our results unanimously reveal the

predominant contribution of the neutral (less polar) species in developing the final retention of the amphoteric solute. This behavior can be met regardless of that the uncharged form is the major or the minor component of the equilibrium solution.

EXPERIMENTAL

Model substances

Pyridoxine (HCl), Morphine ($HCl.3H_2O$), Nitrazepam, Sulfadimidine were a quality of Hungarian Pharmacopoeia.[20] Perfloxacin, Norfloxacin were synthesized at Chinoin Pharmaceutical Works (Budapest), Niflumic acid was also generously supplied by its manufacturer, Gedeon Richter Chemical Works (Budapest). All these substances were used without further purification. 11-amino undecanoic acid (11-AA) 99% (Aldrich).

Materials

Buffer solutions (for Chromatography) in pH range 3 - 8 were prepared by mixing the proper volumes of 0.067 M aqueous solutions of potassium dihydrogenphosphate and disodium hydrogenphosphate (KH_2PO_4, $Na_2HPO_4.2H_2O$ anal. grade, Reanal, Budapest). The pH of these solutions was tested by pH-metry. As mobile phase the 50:50 vol. mixture of the buffer solutions (20°C) and methanol (20°C) was used. After mixing of the solutions a final pH control at the ready for use methanolic mobile phase was performed.

For pH measurement combined glass electrode (Radiometer GK2320 C) and a reference pH meter (Radiometer PHM93) were used. The solutions were thermostatted at 20°C and stirred by magnetic stirrer.

TABLE 1

The pH shifting effect of methanol in aqueous buffer solutions

pH (± 0.02)		Δ (shifting)
aqueous buffer solution	after methanolic dilution (1 :1)	
3.12	4.03	0.91
5.08	6.10	1.02
6.09	7.27	1.18
7.47	8.74	1.27
8.40	9.24	0.87

Electrode calibration was performed using standard buffer solutions (Aldrich, pH 2 - 11). The accepted pH values are the average of three subsequent readings, the standard deviation was less than ±0.02 pH unit.

Table 1 shows the pH values of the aqueous and the corresponding methanolic buffer solutions.

Methanol, HPLC grade (Chemolab,Budapest).

Chromatography

The HPLC apparatus was comprised in Waters (Millipore,USA) Model 501 solvent delivery system, Labor MIM (Budapest,Hungary) Model QE 308 variable wavelength UV photometer as detector, Yokogava (Tokyo,Japan) Type 3051 recorder. For the detection of 11-AA refractometry was applied (Waters Differential Refractometer, Model R 401). The packing was filled into steel columns 250 x 4.6 mm. I.D.) the adsorbent, Hypersil 5 ODS was purchased from Bioseparation Technique Ltd., (Budapest, Hungary) in a particle size 5 μm.

As eluent the 50:50 mixture of methanol-aqueous buffer solutions was used. The eluents, after pH control, were filtered and degassed prior to chromatography. The flow rate was 1.0ml/min. A Model 7125 sampling valve (Rheodyne, Berkley, USA) was applied.

The column temperature was controlled by recirculating water through an isolated stainless jacket from thermostat (Ultrathermostate, MLW Type U_2C, Freital, Germany).

The model substances were solved in the eluent. The chromatograms were recorded and the retention data were collected by a Hewlett-Packard integrator Model 3396 Ser.2.

Each retention data was calculated as an average of three parallel runs. The mobile phase hold up time was signalled by the solvent peak of methanol.

Determination of the octanol/water pH-partition profile

The traditional shake-flask method was used for logP measurements. The apparent partition coefficients ($logP_{app}$) were determined in wide pH range at 7 different pH values including the ie. point pH. The experimental details and the obtained $logP_{app}$ values were published elsewhere.[9,10] Here only the pH-partition profile of the molecules is presented in Figure 3.

Determination of the protonation macro- and microconstants

The protonation macroconstants were determined by standard methods (potentiometry or UV spectroscopy) at $25 \pm 0.1^{\circ}C$, $I = 0.2M$ ionic strength. Combined pH-metry and UV spectroscopy was applied to determine the protonation microconstants.[8,11] The pH-dependent relative concentrations (%) of microspecies were calculated using

protonation microconstants. The distribution diagrams are shown in Figure 4.

RESULTS, DISCUSSION

In Table 2, the RP HPLC retention values of the model compounds and also the pK values as well as the isoelectric points are included. Figure 2 shows the structural formulas of the compounds.

In assigning the pH interval to be studied (4 - 9) the stability of the chromatographic column and the reproducibility (comparability) of the results were considered. This limitation occasionally diminished the possibilities to compare the RP HPLC retention and octanol/water partitioning behavior in a wider pH interval. The pH profile of octanol/water partition is shown by Figure 3, while pH dependence of species distribution (macro- and microspeciation) can be seen on Figure 4.

As the species distribution in the equilibrium mixtures at the ie.p. concerns, the model compounds may be divided into three groups. 11-AA is a typical zwitterionic amphoteric that is at the pH of ie.p. practically only the zwitterionic species exists; on the contrary, nitrazepam and sulfadimidine are true ordinary amphoterics. In their solution, beside the cation and the anion form, as third component the neutral (uncharged) species is present. The other five (compds No. 2-6 in Table 2) substances represent a transition: in their solution at the pH interval, near to the ie.p., the zwitterionic and neutral species co-exist in commensurable amount. The relative concentration of the neutral form increases from niflumic acid (compd. No. 2) through the morphine (compd. No. 6).

TABLE 2

RP-HPLC retention times (min) of model substances

N°	substance	pK_{a1} pK_{a2} (ie.p.)	pH of the eluent				
			4.03	6.10	7.27	8.74	9.24
1.	11-AA	10.74 4.56 (7.65)	5.40	4.65	4.80	5.10	5.55
2.	niflumic acid	4.44 2.26 (3.35)	57.10	29.70	17.10	13.80	12.90
3.	pyridoxine	9.16 5.05 (7.10)	3.50	5.47	6.86	5.67	5.53
4.	norfloxacin	8.51 6.22 (7.37)	3.62	5.24	8.90	7.60	5.62
5.	pefloxacin	7.80 6.02 (6.91)	4.03	13.31	33.00	14.80	8.42
6.	morphine	9.54 8.34 (8.94)	2.77	2.95	4.93	8.60	10.70
7.	nitrazepam	10.66 2.94 (6.80)	12.60	12.20	12.30	12.50	12.50
8.	sulfadimidine	7.38 2.36 (4.87)	3.35	3.75	3.52	3.39	3.48

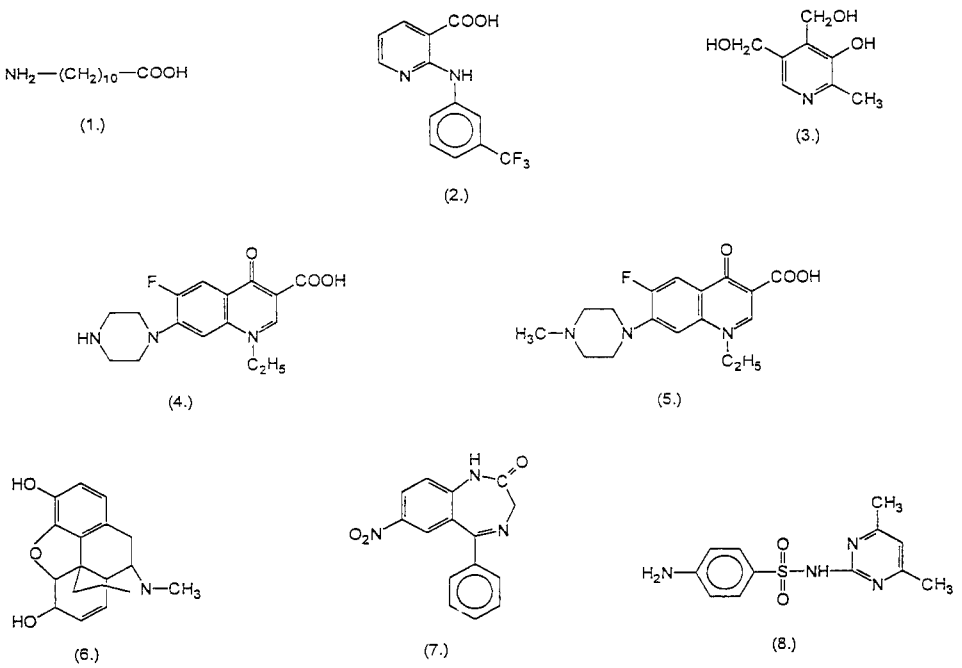


FIGURE 2 Chemical structure of the model compounds

Figure 5 shows the pH profile of RP HPLC retention of model compounds expressed by logk' values. The shape of curves are very characteristic.

In case of **11-AA** mild retention depression my be observed in the region of ie.p. This is a behaviour which must be typical of pure zwitter-ionic amphoterics where the charged poles are isolated.

The retention of **niflumic acid** shows a definite increase, approaching to the ie.p. though, the relative concentration of the zwitterion at this region is higher than 90%. This fact unambiguously indicates the adsorbance of the neutral form since from the two

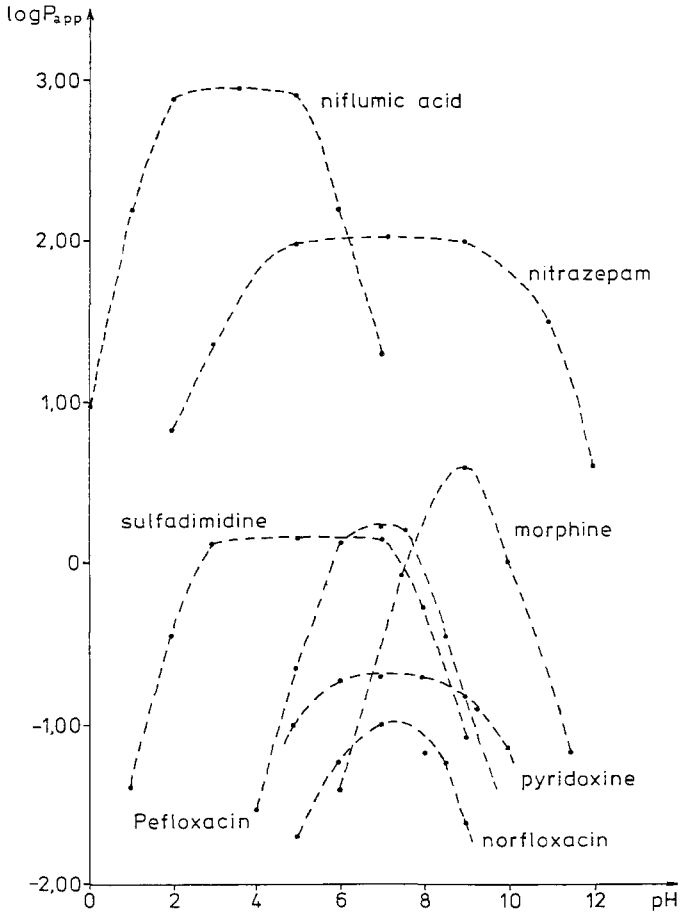
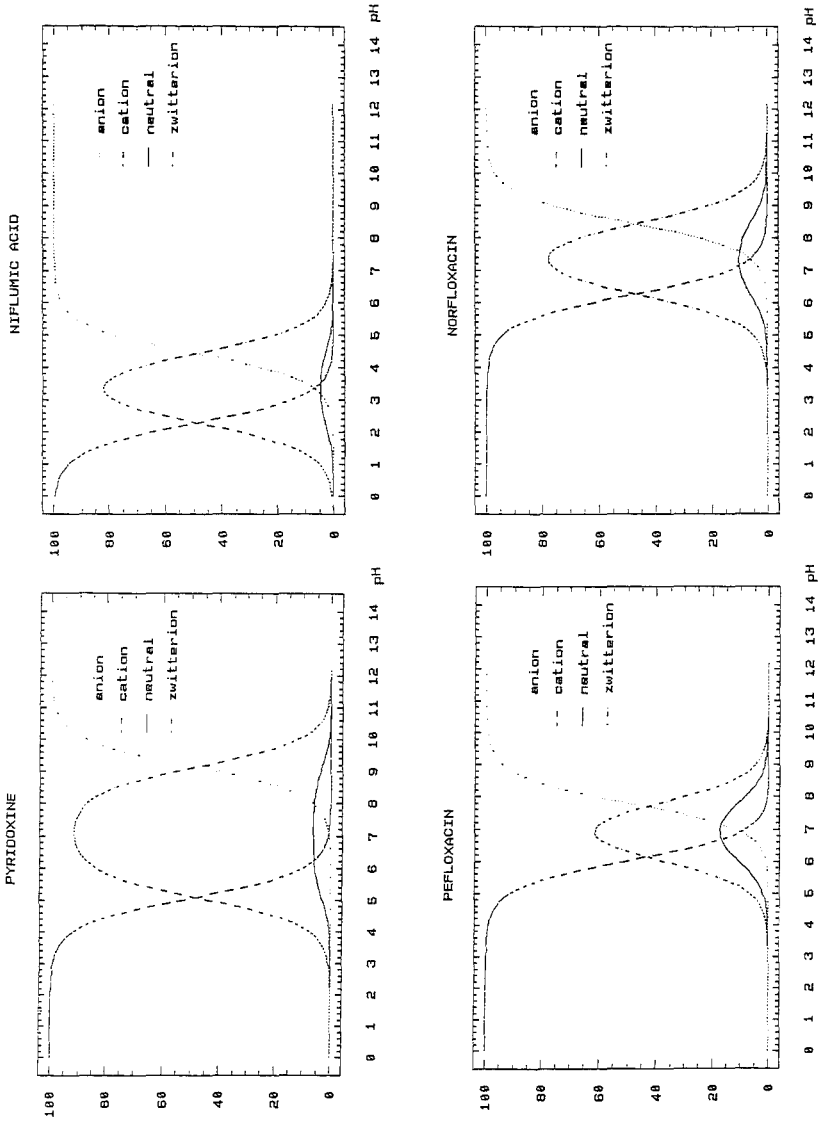


FIGURE 3 pH profile of octanol/water partition


NIFLUMIC ACID
PYRIDOXINE
NORFLOXACIN
PEFLOXACIN
anion
cation
neutral
zwitterion
pH
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
0 20 40 60 80 100

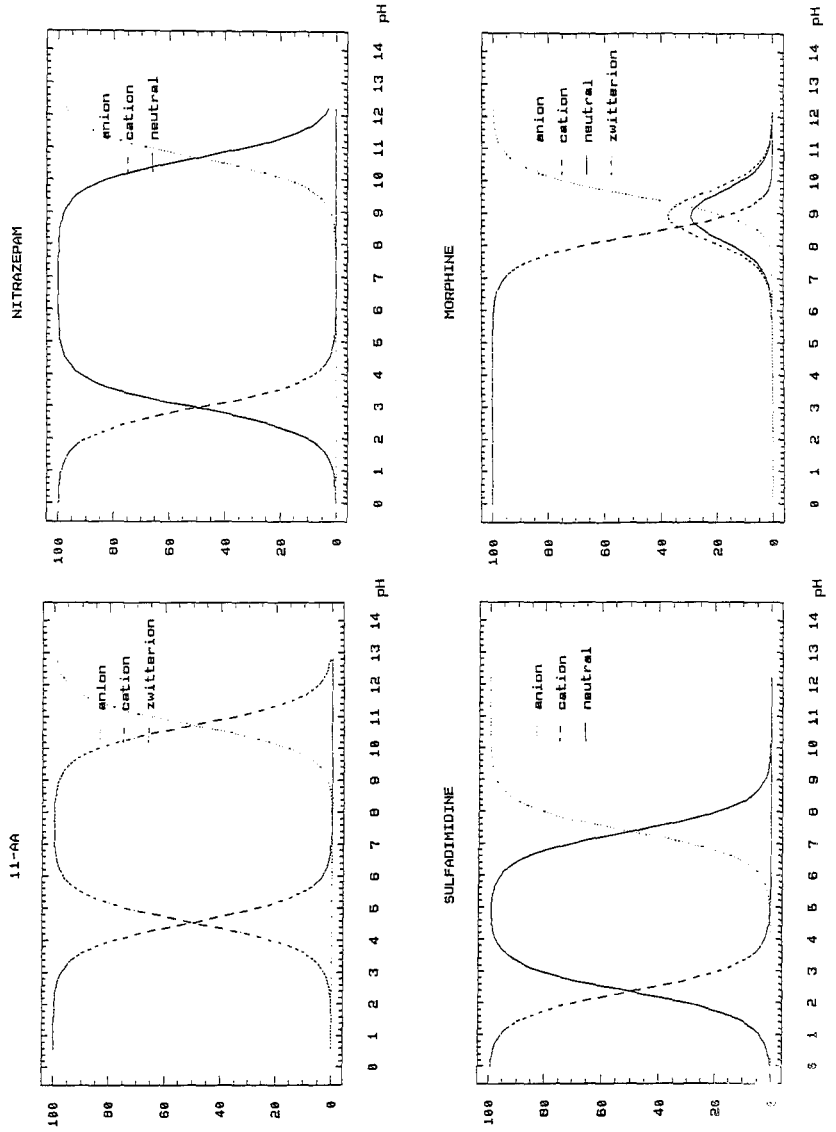


FIGURE 4 Species distribution diagram of the model substances

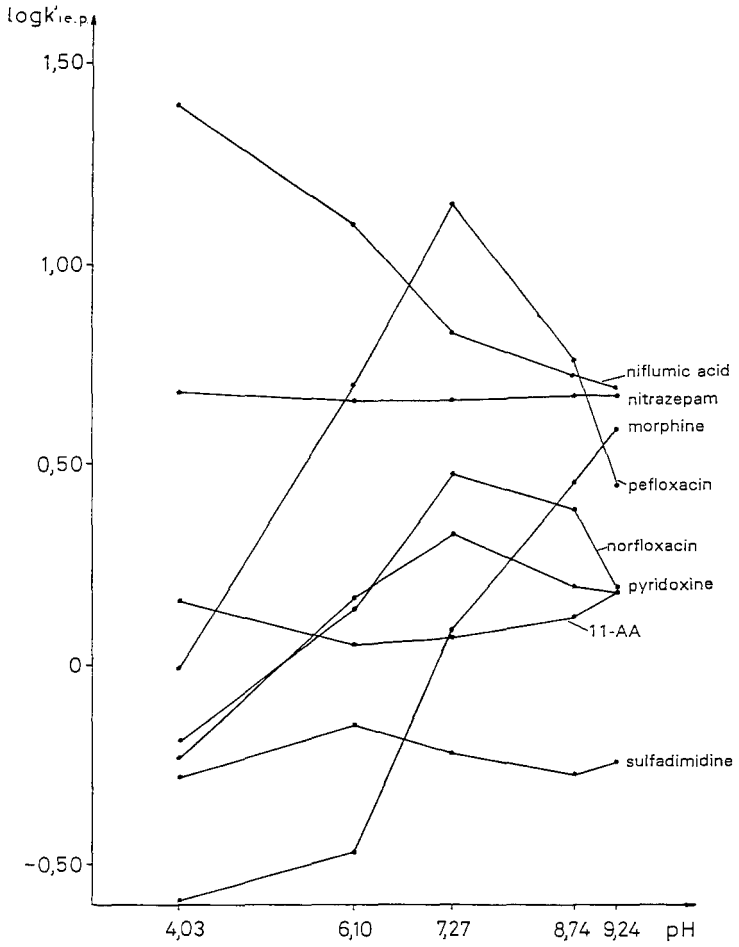


FIGURE 5 pH profile of RP-HPLC retention

protonation isomers $XH^{\pm}$ and XH^{o}, the neutral form is the less polar i.e. more lipophilic. The very steep rise of the retention by pH depression may be a consequence of the great difference in acid-base strength (ie.: lipophilicity) of the niflumic acid cation and anion species (7-ie.p. = 3.65). The plausibility of this explanation is supported by the species distribution diagram (Figure 4). Near to the ie.p. the amount (contribution to the retention) of the more polar anionic form rapidly decreases followed by the appearance of the cationic form. Quite reasonable to assume, that a maximum retention could be achieved at the ie.p. (pH 3.35). In addition, the ion pairing between niflumic acid and phosphate ions[11] as a weak retention modifying factor must not be excluded.

Norfloxacin and **pefloxacin** show a definite retention maximum at the ie.p. region, evidently caused by the adsorbance of the neutral form existing in gradually increasing concentration. The significantly higher retentions in the alkaline region (cf. retentions at pH 4.03 and 9.24) may arise from differences in the neutral form concentrations:

pH	z %	n %	a %	c %
4.03	0.73	0.21	-	99.06
9.24	2.72	0.77	96.51	-

z: zwitterionic, n: neutral, a: anionic c: cationic form

or may be indicators of different lipophilicity of cationic and anionic species. However, this latter assumption is less probable since the acid--base strength of pefloxacin's carboxyl and amine groups is rather equal (7-ie.p. = 0.09).

The retentions of **morphine** are increasing towards the alkaline zone and must decrease above pH ⟩ 9.24 (which interval not tested).

The correspondent species distribution diagram clearly shows, that the retention increase is related to the adsorbance of the neutral from.

Pyridoxine has a definite maximum curve. The maximal retention appears at the ie.p., indicating again the adsorbance of the neutral form.

The retentions of **nitrazepam** are located along a straight line; by means of the species distribution diagram (Figure 4) it can be seen that through the pH interval (4.03 - 9.24) studied the uncharged is the practically existing form. The monoions appearing at the peripheral alkaline and acidic pHs, due to their almost equal and small polarity (7 - ie.p. = 0.20) have hardly influence on the overall retention; the retention curve describes an almost straight line. The **sulfadimidine** curve runs similar to that of nitrazepam, though, the low retention values may reduce the reliability of evaluation.

The results above unambiguously confirm the conception about the dominant contribution of the neutral species adsorbance to the retention of amphoterics. This effect seems to work quite independently from the actual ratio of the zwitterionic and uncharged form, appearing not only by the ordinary amphoteric compounds but also by those representing a transition between the pure ordinary and zwitterionic amphoterics. This experience, in addition to the similar feature of lipophilicity pH profile and retention pH profile (cf. Figures 3 and 5) proves by a series of non-congeneric compounds, that partitioning properties of C_{18}/methanol-water and octanol/water systems are very similar.

By linear regression analysis between the true partition coefficients (data in Table 3) and $\log k'_{ie.p.}$ values a correlation coefficient r = 0.768 has been found including all molecules. If the two fluoroquinolones (compds. 4 and 5) had been omitted from the analysis the r value

TABLE 3

The true partition coefficients (logP) and the capacity factors ($logk'_{ie.p.}$) of model compounds

substance	logP	$logk'_{ie.p.}$ [c]
niflumic acid	4.43[a]	1.379
pyridoxine	0.33[a]	0.326
norfloxacin	-0.02[a]	0.484
pefloxacin	1.07[a]	1.146
morphine	1.22[a]	0.587
nitrazepam	1.96[b]	0.670
sulfadimidine	0.19[b]	-0.152

a.) logP value calculated by Eqn. 4
b.) logP value calcutated by Eqn. 3
c.) logk' value calculated from retention times (Table 2); t_o = 2.20 (min).

has improved to 0.948, which can be considered a significant linear relationship. (The outlier behavior of fluoroquinolones points out to some specific interactions and requires further investigations.)
Based on the above findings, $logk'_{ie.p.}$ retention and logP (true partition coefficient) as physico-chemical content concerns, should be regarded analogue lipophilicity parameters.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 18(4), 827-829 (1995)

THE BOOK CORNER

CAPILLARY ZONE ELECTROPHORESIS, F. Foret, L. Krivankova and P. Bocek
VCH Weinheim, Germany, 1993, xiv + 346 pages DM 228.00. ISBN: 3-527-30019-8

Capillary zone electrophoresis (CZE) is a powerful analytical technique which recently gained popularity among other separation modalities. It had proven its efficiency in separation and analysis of small ions and molecules and also macromolecules such as proteins, nucleic acid, viruses and cells among others. The book consists of 10 chapters which ends with a list of references upto 1992.

Topics covered include:

- Fundamental concepts and theoretical principles
- Phenomena accompanying electrophoresis
- Practice of capillary electrophoresis
- Instrumentation - principles, components and how to operate it
- Applications

The book is well illustrated as it contains 201 figures and 32 tables. The chapters are clearly presented in a concise format.

The book is recommended for graduate students, analytical chemists in both pharmaceutical and biotechnology industries as well as academic professionals.

Reviewed by
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GAS CHROMATOGRAPHIC ENVIRONMENTAL ANALYSIS - PRINCIPLES, TECHNIQUES, INSTRUMENTATION, by Fabrizio Bruner, VCH Publishers, Weinheim, Germany, 1993, xii + 233 pp., DM 98.00; ISBN: 3-527-28042-1

This book is a welcome addition to the scientific literature, as it is one of the few books available on the applications of gas chromatography to environmental analysis specifically.

The volume consists of five chapters with good illustrations and figures. Each chapter ends with a list of references up to 1992.

The author discusses, in Chapters 1 and 2, the general principles and instrumentation required for gas chromatographic environmental analysis. Chapter 3 is dedicated to mass spectrometry and its usefulness and significance in environmental organic analysis. While Chapter 4 is devoted to the chromatographic analysis of volatile air and water pollutants such as hydrocarbons, sulfur gases, halocarbons, among others. Finally, Chapter 5 deals with sample preparation and analysis of organic micropollutants from complex matrices.

Dr. Bruner discusses, in detail, the techniques required for sample preparation, since he states that "it is useless to exploit even the most sophisticated apparatus when the injected sample does not represent the original one."

This book is highly recommended for analytical chemists in both industrial, academic contexts who are involved in research, in environmental analysis and also graduate students. It is also an excellent reference for governmental research centres, chemical companies.

Reviewed by
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DIODE ARRAY DETECTION IN HPLC, edited by L. Huber & S. A. George, Chromatographic Science Series, J. Cazes Editor, Volume 62, Marcel Dekker, Inc. New York, Basel, Hongkong, 1993, vii + 400 pp., $150.00; ISBN: 042474947-4

This book represents Volume 62 in the Chromatographic Science Series published by Marcel Dekker. The book is well presented and discusses the principles and techniques of diode array detection and its application in high performance liquid chromatography. The advantages of diode array detection over conventional absorbance detectors are also well presented. Diode array detection offers high selectivity and sensitivity as it is coupled by modern software which can evaluate and process a large amount of data, provided in a very short time. The most significant improvements made in optics of the diode array detector is also discussed.

The book is written by eight contributors, who are experienced in this technique. It also is well illustrated, as it contains 239 figures, 22 tables, and each chapter ends with references; however, the most recent references are cited in 1990.

The volume consists of 14 chapters, classified into four parts, with the following topics:

I. Theory and Design:

* Historical Developments

* Modern Developments

II. Advantages of Diode Array Detectors to Chromatographers:

* Diode Array Detection Advantages for the Chromatographer

* Spectral Matching and Peak Purity

* Chemometrics and Photodiode Array Detection

III. Applications of Diode Array Detectors:

* The Use of Diode Array Detectors in the Pharmaceutical Industry

* Clinical Applications

* Toxicological Applications

* Applications in the Analysis of Amino Acids, Peptides and Proteins

* Food and Beverages

* Environmental Applications

* Chemical, Petrochemical, and Polymer Applications

IV. Guidelines on how to optimize Sensitivity, Selectivity, Automation:

* Optimization of Diode Array Detection, and

* Using Diode Array Detectors for Automated Routine Analysis.

This book is highly recommended to analytical, pharmaceutical and environmental chemists and also for biochemists, and graduate students.

Reviewed by
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JOURNAL OF LIQUID CHROMATOGRAPHY, 18(4), 831-836 (1995)

LIQUID CHROMATOGRAPHY CALENDAR

1995

FEBRUARY 13 - 15: PrepTech '95, Sheraton Meadowlands Hotel, East Rutherford, New Jersey. Contact: Dr. Brian Howard, ISC Technical Conferences, Inc., 30 Controls Drive, Shelton, CT 06484-0559, USA.

MARCH 6 - 10: PittCon'95: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, New Orleans, Louisiana. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

APRIL 2 - 7: 209th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

MAY 21: Techniques for Polymer Analysis and Characterization, a short course, Sanibel Island, Florida. Contact: ISPAC Registration, 815 Don Gaspar, Santa Fe, NM 87501, USA.

MAY 22 - 24: 8th International Symposium on Polymer Analysis and Characterization, Sanibel Island, Florida. Contact: ISPAC Registration, 815 Don Gaspar, Santa Fe, NM 87501, USA.

APRIL 25 - 28: Biochemische Analytik '95, Leipzig. Contact: Prof. Dr. H. feldmann, Inst. fur Physiologische Chemie der Universitat, Goethestrasse 33, D-80336 Munchen, Germany.

MAY 23: Miniaturization in Liquid Chromatography versus Capillary Electrophoresis, Pharmaceutical Institute, University of Ghent, Ghent, Belgium. Contact: Dr. W. R. G. Baeyens, Univ of Ghent, Pharmaceutical Inst, Harelbekestraat 72, B-9000 Ghent, Belgium.

MAY 28 - JUNE 2: HPLC'95, 19th International Symposium on Column Liquid Chromatography, Convention Center, Innsbruck, Austria. Contact: HPLC'95 Secretariat, Tyrol Congress, Marktgraben 2, A-6020 Innsbruck, Austria.

MAY 31 - JUNE 2: 27th Central Regional Meeting, ACS, Akron Section. Contact: J. Visintainer, Goodyear Research, D415A, 142 Goodyear Blvd, Akron, OH 44236, USA.

JUNE 6 - 8: 28th Great Lakes Regional ACS Meeting, LaCrosse-Winona Section. Contact: M. Collins, Chem. Dept., Viterbo College, La Crosse, WI 54601, USA.

JUNE 11 - 14: 1995 International Symposium and Exhibit on Preparative Chromatography, Washington, DC. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

JUNE 13 - 16: Capillary Electrophoresis, Routine Method for the Quality Control of Drugs: Practical Approach (in English); L'Electrophorese Capillaire, Methode de Routine pour le Controle de Qualite des Medicaments: Approche Pratique (in French), Monpellier, France. Contact: Prof. H. Fabre, Lab. de Chimie Analytique, Inst. Europeen des Sciences Pharmaceutiques Industrielles de Montpellier, Ave. Charles Flahault, 34060 Montpellier Cedex 1, France.

JUNE 14 - 16: 50th Northwest/12th Rocky Mountain Regional Meeting, ACS, Park City, Utah. Contact: J. Boerio-Goates, Chem Dept, 139C-ESC, Brigham Young Univ, Provo, UT 84602, USA.

JULY 9 - 15: SAC'95, The University of Hull, UK, sponsored by the Analytical Division, The Royal Society of Chemistry. Contact: The Royal Society of Chemistry, Burlington House, Picadilly, London W1V 0BN, UK.

JULY 7 - 8: FFF Workshop, University of Utah, Salt Lake City, UT. Contact: Ms. Julie Westwood, FFF Research Center, Dept. of Chem., University of Utah, Salt Lake City, UT 84112, USA.

JULY 10 - 12: FFF'95, Fifth International Symposium on Field-Flow Fractionation, Park City, Utah. Contact: Ms. Julie Westwood, FFF Researcvh Center, Dept. of Chem. Univ. of Utah, Salt Lake City, UT 84112, USA.

AUGUST 20 - 25: 210th ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 4 - 7: 13th International Symposium on Biomedical Applications of Chromatography and Electrophoresis and International Symposium on the Applications of HPLC in Enzyme Chemistry, Prague, Czech Republic. Contact: Prof. Z. Deyl, Institute of Physiology, Videnska 1083, CZ-14220 Prague 4, Czech Republic.

SEPTEMBER 12 - 15: 5th International Symposium on Drug Analysis, Leuven, Belgium. Contact: Prof. J. Hoogmartens, Inst. of Pharmaceutical Sciences, Van Evenstraat 4, B-3000 Leuven, Belgium.

OCTOBER 18 - 21: 31st Western Regional Meeting, ACS, San Diego, Calif. Contact: S Blackburn, General Dynamics, P. O. Box 179094, San Diego, CA 92177-2094, USA.

OCTOBER 22 - 25: 25th Northeastern Regional Meeting, ACS, Rochester, New York. Contact: T. Smith, Xerox Corp, Webster Res Center, M/S 0128-28E, 800 Phillips Rd, Webster, NY 14580, USA.

NOVEMBER 1 - 3: 30th Midwestern Regional ACS Meeting, Joplin, Missouri. Contact: J. H. Adams, 1519 Washington Dr., Miami, OK 74354-3854, USA.

NOVEMBER 1 - 4: 31st Western Regional ACS Meeting, San Diego, California. Contact: T. Lobl, Tanabe Research Labs, 4450 Town Center Ct., San Diego, CA 92121, USA.

NOVEMBER 5 - 7: 30th Midwestern Regional Meeting, ACS, Joplin, Missouri. Contact: J. H. Adams, 1519 Washington Dr, Miami, OK 74354, USA.

NOVEMBER 29 - DECEMBER 1: Joint 51st Southwestern/47th Southeastern Regional Meeting, ACS, Peabody Hotel, Memphis, Tenn. Contact: P.K. Bridson, Chem Dept, Memphis State Univ, Memphis, TN 38152, USA.

DECEMBER 17 - 22: 1995 International Chemical Congress of Pacific Basin Societies, Honolulu, Hawaii. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1996

FEBRUARY 26 - MARCH 1: PittCon'96: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, Chicago, Illinois. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

MARCH 24 - 29: 211th ACS National Meeting, New Orleans, LA. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

MARCH 31 - APRIL 4: 7th International Symposium on Supercritical Fluid Chromatography and Extraction, Indianapolis, Indiana. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

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

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

AUGUST 18 - 23: 212th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

OCTOBER 16 - 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.

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

1997

APRIL 6 - 11: 213th ACS National Meeting, San Antonio, Texas. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 7 - 12: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1998

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