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VOLUME 20 NUMBER 15 1997

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES

September 1997

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Identification Statement. *Journal of Liquid Chromatography & Related Technologies* (ISSN: 1082-6076) is published semimonthly except monthly in May, August, October, and December for the institutional rate of $1,750.00 and the individual rate of $875.00 by Marcel Dekker, Inc., P.O. Box 5005, Monticello, NY 12701-5185. Periodicals postage paid at Monticello, NY. POSTMASTER: Send address changes to *Journal of Liquid Chromatography & Related Technologies*, P.O. Box 5005, Monticello, NY 12701-5185.

Volume	Issues	Institutional Rate	Individual Professionals' and Student Rate	Foreign Postage		
				Surface	Airmail to Europe	Airmail to Asia
20	20	$1,750.00	$875.00	$75.00	$110.00	$130.00

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CODEN: JLCTFC 20(15) i–iv, 2313–2508 (1997)
ISSN: 1082-6076

Printed in the U.S.A.

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

J. LIQ. CHROM. & REL. TECHNOL., 20(15), 2313–2325 (1997)

CHROMATOGRAPHIC PROPERTIES OF MIXED CHEMICALLY BONDED PHASES WITH ALKYLAMIDE AND AMINOPROPYL LIGANDS

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ABSTRACT

A series of mixed chemically bonded phases, which contained alkylamide and aminopropyl ligands, were prepared by subsequent reaction of the silica-based aminopropyl phase with suitable alkanoyl chlorides. Physicochemical properties of these phases were studied by using various techniques such as elemental analysis, ^{29}Si and ^{13}C solid-state NMR and adsorption.

In addition, suitable chromatographic measurements were carried out in order to study the effect of the length of the terminal alkyl chain on the solute's retention under reversed phase conditions.

INTRODUCTION

In spite of the development of many novel packing materials for reversed-phase high performance liquid chromatography (RP HPLC), e.g., polymeric and carbonaceous packings,[1-4] chemically bonded phases (CBP) have been increasingly used.[5] This is due to their interesting physicochemical properties and relatively high stability under reversed-phase conditions.[6] At these conditions the retention and selectivity depend strongly on the specific and nonspecific interactions of the analyte with the components of the mobile and stationary phases. It should be noted, that molecular interactions in the stationary phase are more complex in comparison to those in the mobile phase because they are influenced by many factors such as: the type and structure of the bonded ligands, their coverage density as well as the surface properties and porosity of siliceous supports.[2,6-14] The situation becomes even more complex for chemically bonded ligands, which possess some specific functionalities, e.g., internal and/or external $-NO_2$, $-NH_2$, -OH, -CN, $-C_6H_5$, -NHCO- groups.[2,6,11,15-18]

In order to improve chromatographic separations of polar substances, new types of chemically bonded phases, which contain the specific internal groups were synthesized.[5] Among them, alkylamide phases attracted special attention because of their utility for separation of various basic compounds.[19-27] Amide groups are also present in the silica-based phases synthesized by immobilization of proteins, e.g., bovine serum albumin (BSA).[28,29] The BSA phases were used for normal-phase, as well as reversed-phase separations, including chiral separations. Retention mechanism on these phases is complex and not fully explained yet. Another example of the specific phase was reported by Feibush and Santasania,[30] who studied the shielded hydrophobic phase (SHP), which contained ion-exchange groups in the hydrophobic zone. This phase has been shown to be useful for direct injection analysis of drugs in biological fluids.

In the current work, a series of mixed chemically bonded phases, which in addition to residual surface silanols containing two different ligands, alkylamide and aminopropyl, was studied. The amide ligands were obtained by the secondary reaction of aminopropyl ligands, created in the first step of the silica modification, with various alkanoyl chlorides. Thus, the resulting phases contained terminal alkyl chains of different lengths, e.g., $-CH_3$, $-C_6H_{13}$, $-C_{12}H_{25}$, $-C_{16}H_{33}$, and $-C_{18}H_{37}$. Surface properties of these packing materials were characterized by various physicochemical methods such as elemental analysis, ^{29}Si, and ^{13}C solid-state NMR and adsorption. Their chromatographic properties were investigated using two different test mixtures: homologous series of alkylbenzenes and alkylanilines.

EXPERIMENTAL

Materials

Chemically bonded phases were prepared by using 5 μm LiChrospher Select B silica from E. Merck, Darmstadt, Germany. Its BET specific surface area and total pore volume were equal to 570 m^2/g and 0.96 cm^3/g, respectively. The average pore width was about 5.8 nm. The concentration of accessible surface silanols was estimated to be equal to 5.18 $\mu mol/m^2$.

The following surface modification reagents were used: γ-aminopropyldimethyl-metoxysilane from Huls (Bristol, PA, USA), acetylchloride from Fluka (Buchs, Switzerland), hexanoyl chloride, lauroyl chloride, palmitoyl chloride, and stearyol chloride from Aldrich (Milwaukee, WI, USA). Specially prepared dry morfoline[31] (Riedel de Haën, Seelze, Germany) was used as an activator.

The remaining chemicals: methanol, toluene, and n-hexane, all analytical grade purity, were purchased from Aldrich. The deionized water was purified in laboratory using a Millipore (El Paso, TX, USA) Milli-Q reagent water system.

Synthesis of Chemically Bonded Phases

Mixed alkylamide phases were synthesized in a sealed glass reactor by a two-step process.[19-26] Prior to the synthesis, the silica gel (SG, about 15 g) was dried at 180°C under vacuum (10^{-3} Pa) in a glass reactor without contact with the environment for 12 h.[32] Then, it was reacted with 20 mL of monofunctional aminopropylsilane under environmentally isolated conditions at 110°C ± 5°C for 5 hrs. The product, aminopropyldimethylsilyl phase (SG-NH_2), was filtered, washed with dry toluene, methanol, and n-hexane, and finally dried by flowing nitrogen. In each second modification step about 3 g of the SG-NH_2 phase was placed in the glass reactor and heated at 120°C under vacuum (10^{-3} Pa) for 12 h, and then allowed to react with suitable alkanoyl chloride in the presence of dry morpholine at 50, 60, 80 and 100°C ± 5°C, respectively. The resulting material was filtered, washed with dry toluene, methanol, and n-hexane, and then dried at room temperature.The following alkanoyl chlorides were used: acetylchloride (SG-AP_1 - code of the resulting phase), hexanoyl chloride (SG-AP_2), lauroyl chloride (SG-AP_3), palmitoyl chloride (SG-AP_4), and stearoyl chloride (SG-AP_5). Note that the synthesized

Table 1

Physicochemical Properties of Mixed Alkylamide Phases

Phase	Terminal Alkyl Chain	P_C (%)	P_N (%)	α_a(μmol/m^2)[a]
SG-NH_2	----	6.93	1.58	2.33
SG-AP_1	-CH_3	8.32	1.68	1.38
SG-AP_2	-C_5H_{11}	9.93	1.57	0.98
SG-AP_3	-$C_{11}H_{23}$	15.24	1.62	1.45
SG-AP_4	-$C_{15}H_{31}$	18.33	1.65	1.55
SG-AP_5	-$C_{17}H_{35}$	19.75	1.62	1.59

[a] Concentration of aminopropyl groups in the case of the SG-NH_2 phase or alkylamide groups in the case of the SG-AP phases; the concentration of residual aminopropyl groups in the SG-AP phases is equal to 2.33 - α_a.

alkylamide phases possessed different terminal alkyl chains: -CH_3, -C_5H_{11}, -$C_{11}H_{23}$, -$C_{15}H_{31}$, -$C_{17}H_{35}$, respectively. Physicochemical properties of the resulting phases are summarized in Table 1, whereas the scheme of the modification process is shown in Figure 1.

Column Packing

About 1.2 g of a given phase was added gradually to 35 mL of 2-propanol. The slurry was sonicated in an ultrasonic bath for 5 min and packed in stainless steel columns (60 x 4.6 mm I.D.) using a Haskel (Burbank, CA, USA) model DST-162-52 air driven fluid pump. Prior to packing, the system was pressurized to about 6500 psi. Methanol was used as the carrier solvent.

Physicochemical Measurements

The specific surface area, total pore volume, and average pore width of the LiChrospher Select B silica were determined form low temperature nitrogen adsorption data measured using a Model 1800 Sorptomatic instrument (Carlo Erba, Milan, Italy). The concentration of surface silanols was determined using the method proposed by Nondek and Vyskocil[33] based on the GC determination

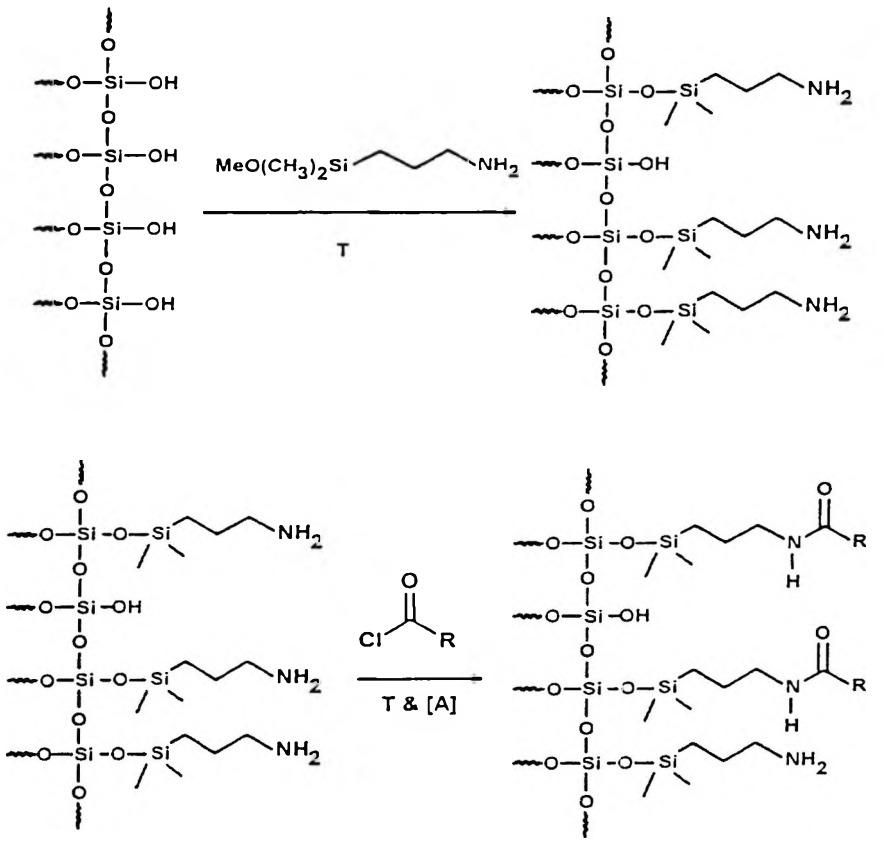

Figure 1. Two-step modification of the silica surface; T denotes heating, [A] denotes activator, and R = $-CH_3$, $-C_5H_{11}$, $-C_{11}H_{23}$, $-C_{15}H_{31}$, $-C_{17}H_{35}$, respectively.

of methane formed during the reaction between dimethylzinc- tetrahydrofurane complex and silanol groups. The surface coverage of bonded ligands was calculated from the carbon (P_C) and nitrogen (P_N) loadings[21] determined by using a Perkin Elmer (Norwalk, CT) Model 240 CHN elemental analyzer.

Solid state NMR measurements, before and after chemical modification, were performed using a Bruker (Rheinstatten, Germany) Model MSL 200 spectrometr. Magic-angle-spinning (MAS) was used at a spinning rate of 4 KHz. ^{29}Si cross-polarization (CP) MAS NMR spectra were recorded with a pulse repetition time of 2 s.

In the case of ^{13}C CP/MAS NMR spectra, a contact time of 12 ms was applied. All NMR spectra were externally referenced to liquid tetramethylsilane. The chemical shifts (L) are given in ppm.

Chromatographic Measurements

HPLC measurements were carried out using a Hewlett Packard (Waldbronn, Germany) Model HP-1050 liquid chromatograph system with a UV detector and a Rheodyne (Berkeley, CA) Model 7125 sampling valve fitted with 20 μL loop. The retention data were recorded and processed using a Hewlett Packard Vectra QS/16S computer with ChemStation. All chromatographic measurements were performed at room temperature using a flow rate of 0.5 cm^3/min. Methanol-water (35 - 65 % v/v) mixture was employed as the mobile phase. The capacity factor (k') values were calculated by utilizing the void volume determined by means of D_2O.

RESULTS AND DISCUSSION

The ratio of aminopropyl bonded ligands (see Table 1) to the surface silanols (about 5.18 μmol/m^2 for the silica studied) indicate that only about 45% of accessible silanols were reacted during the first step of chemical modification (see Figure 1). The second step of modification involved the reaction of suitable alkanoyl chlorides with aminopropyl bonded ligands (see Figure 1) and led to formation of alkylamide ligands. Note, that during the second modification step the concentration of residual surface silanols (about 55%) did not change. The values of the surface coverage, summarized in Table 1, for all alkylamide phases studied indicate that about 60% of aminopropyl ligands, i.e., only about 28% of the initial concentration of surface silanols, were converted to amide ligands. The SG-AP_2 phase was an exception because the conversion was about 40% only. Thus, on average, each alkylamide phase possessed about 55% of residual silanols, 17% of residual aminopropyl ligands, and 28% of alkylamide ligands. It appears that the length of the terminal alkyl chain did not influence the final composition of mixed alkylamide phases.

Shown in Figure 2 are the ^{29}Si CP/MAS NMR spectra for the bare (a) and modified (b) silicas. The spectrum b was recorded for the SG-AP_5 phase. A comparison of both spectra shows that the chemical modification of the silica caused a substantial reduction of the number of geminal (Q_2, δ = -91 ppm) and free (Q_3, δ = -100 ppm) surface silanols. Simultaneously, the number of siloxane groups (Q_4, δ = -108 ppm) increased.

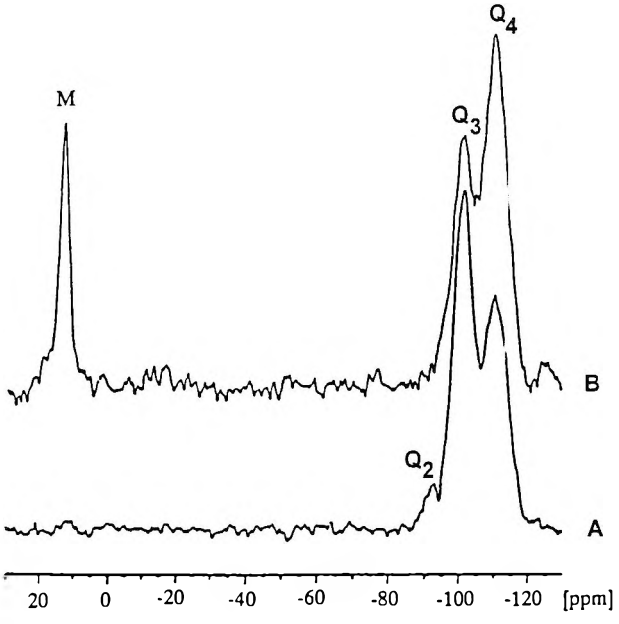

Figure 2. ^{29}Si CP/MAS NMR spectra for LiChrospher Select B silica before (A) and after (B) chemical modification.

In addition, the spectrum b contains the peak M (δ = +14.4 ppm) that corresponds to chemically bonded monofunctional silyl ligands.[12,18] That peak clearly demonstrates the formation of a covalent bonding during attachment of monosilane. An additional confirmation of this bonding was obtained by recording the ^{13}C CP/MAS NMR spectra for the SG-NH_2 and SG-AP_1 phases, which are shown in Figure 3.

As can be seen on both spectra the peak A (δ = -2.5 ppm) corresponds to the formation of the -$Si(CH_3)_2$- bond. The peaks localized in the range of chemical shifts between δ = +10 ppm to δ = +60 ppm correspond to the remaining -CH_2 groups. An additional peak 4 (δ = +174 ppm) is observed on the spectrum for the SG-AP_1 phase and can be related to the carbon atom present in amide group.

Chromatographic properties of the alkylamide phases listed in Table 1 were evaluated on the basis of the RP HPLC data measured for alkylbenzenes and alkylanilines. An exemplary chromatogram is shown in Figure 4 for alkylanilines separated under reversed phase conditions on the SG-AP_5 phase.

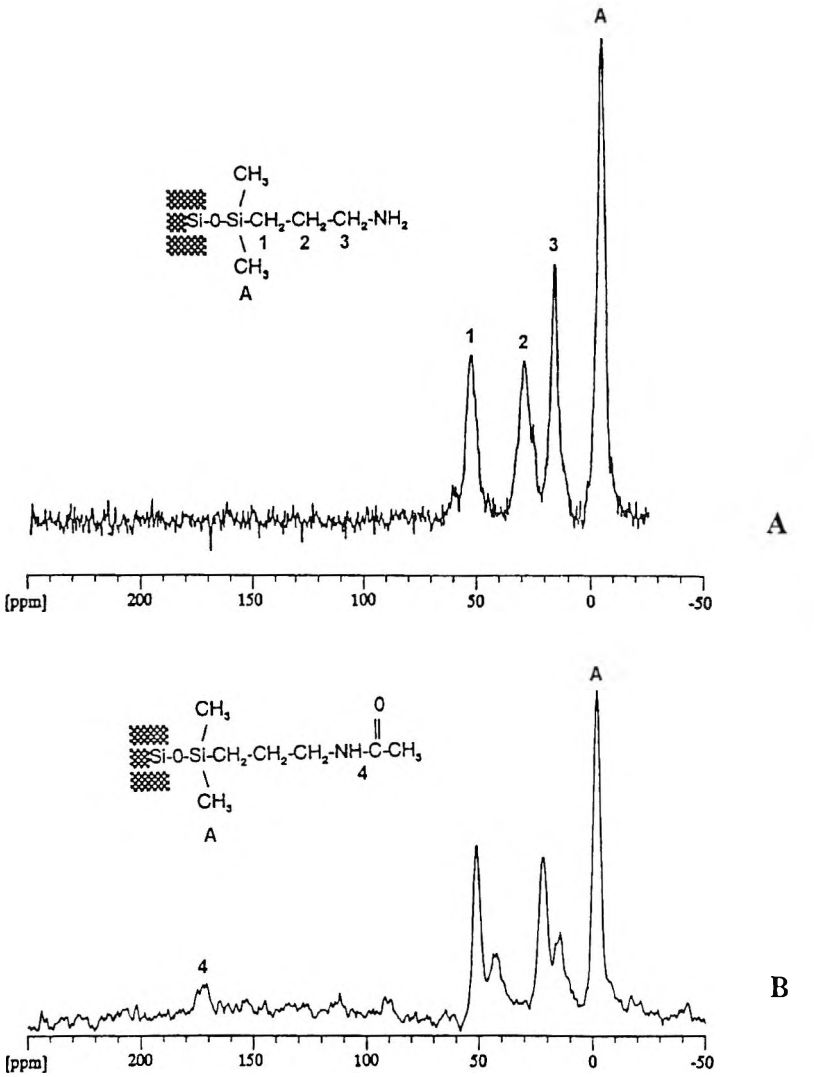

Figure 3. ^{13}C CP/MAS NMR spectra for monomeric SG-NH_2 (A) and SG-AP_1 (B) bonded phases.

Although retention times for alkylanilines are relatively long, the peak symmetry is very good. Also, as can be seen in Figure 5, the retention data measured under reversed phase conditions for homologous compounds on alkylamide phases show normal behavior, i.e., the plots of the logarithm of the capacity factor (log k') on the number of carbon atoms (n_C) in the alkyl chain of

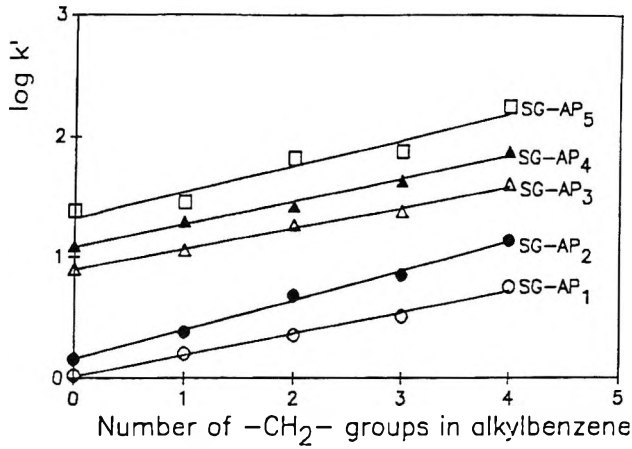

Figure 4. Exemplary chromatographic separation of alkylanilines (s - solvent peak, 1 - aniline, 2 - methylaniline, 3 - dimethylaniline, 4 - diethylaniline) on the SG-AP_1 phase; conditions as in Figure 5.

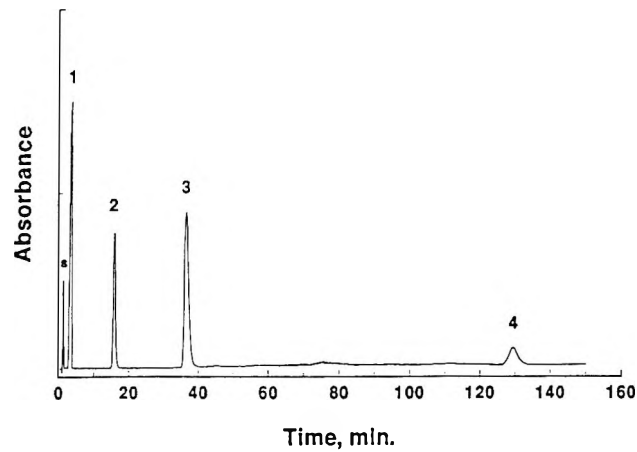

Figure 5. Dependence of log k' *vs.* the number of methylene groups in alkylbenzenes chromatographed using 35% v/v methanol in water and 1 mL/min flow rate.

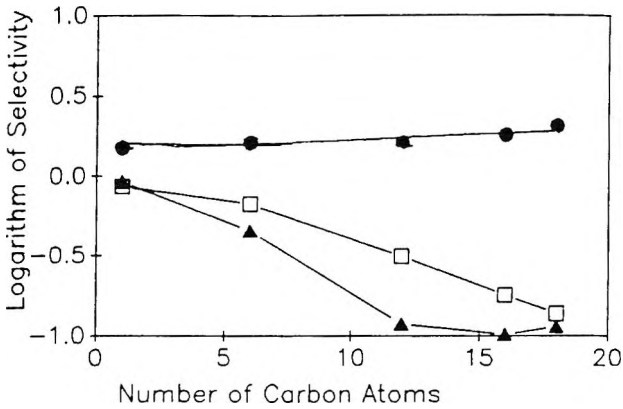

Figure 6. Logarithm of the selectivity *vs.* the number of carbon atoms in the terminal alkyl chain of the alkylamide phases studied; filled circles refer to the methylene selectivity, whereas open squares and filled triangles refer to the hydroxyl and amine selectivities, respectively.

the solute are linear. The slope of each linear plot shown in Figure 5 is equal to the logarithm of the methylene selectivity, which is proportional to the free energy of transfer of the methylene group between the mobile and stationary phases. This quantity is useful to estimate the hydrophobicity of chemically bonded phases because its value increases with increasing contribution of hydrophobic interactions in the stationary phase. Hence, the logarithm of the methylene selectivity increases with increasing number of carbon atoms in the terminal alkyl chain of the bonded ligands.

As can be seen from Figure 6 (see the solid line with filled circles), the methylene selectivity data for the alkylamide phases studied satisfy this condition. Simultaneously, the contribution arising from specific interactions should decrease when the length of the terminal alkyl chain increases.

Shown in Figure 6, plots for the hydroxyl and amine selectivities fulfill this condition too; their values decrease when the length of the terminal alkyl chain increases. The nonspecific and specific selectivity data presented in Figure 6 demonstrate that the length of the terminal alkyl chain in the bonded alkylamide phases plays an important role in controlling the hydrophobicity of these phases as well as their chromatographic performance.

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Received January 11, 1997
Accepted February 8, 1997
Manuscript 4346

J. LIQ. CHROM. & REL. TECHNOL., 20(15), 2327–2355 (1997)

CHEMOMETRIC ACCOUNT FOR RETENTION OF LYSOSOMOTROPIC ALKANOLAMINES IN PLANAR CHROMATOGRAPHY SYSTEMS

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ABSTRACT

Planar chromatography retention data (in form of thermodynamically meaningful R_M coefficients) for twelve model alkanolamines, indicating lysosomotropic activity, were determined in eighty chromatographic systems using eight different silica gel layers with varied chemically bonded stationary phases. In performed thin layer chromatography experiments, aqueous binary mobile phases containing methanol or acetonitrile as organic modifier, in five different proportions, were applied. Additionaly, nine quantum chemical descriptors were calculated for test solutes by a semi-empirical molecular

modelling procedure (AM1) to describe their structural properties. Chemometric technique by the multivariate method of principal components analysis (PCA) was appplied to the all completed chromatographic retention and computational chemistry data for identification of the most selective and useful planar chromatography systems as well as molecular interactions of solutes which affect their retention phenomena in the particular separation mode. The statistically significant relationship was revealed between quantitative measure of antimicrobial activity of alkanolamines and values of calculated scores of principal components.

INTRODUCTION

Some alkanolamines, preferably in the uncharged form, indicate lysosomotropic activity since *in vivo* penetrate the native lysosomal and cytoplasmic membranes leading to inhibition of choline transport,[1] norepinephrine uptake,[2] phosphostranferase activity,[3] or modification and enrichmment of membrane phospholipids polar head groups.[4] Such feature allows use of alkanolamines as the protectors of hematopoietic precursor cells against some cytotoxic compounds[5] or, like N-ethyl-3-carbazole carboxaldehyde phenylhydrazones[6] and 4-aza-5∝-sitostanes,[7,8] as the specific antimicrobial agents.[9,10] Moreover, the structural factors govering the membrane transport process of alkanolamines are still not rationalized[11] and unable to identify such compounds useful for the property oriented *in vivo* chemotherapy. However, the increasing application of advanced chemometric methods employing a large set of physical and chemical parameters in structure-biological property studies[12,13] gives quite real possibility to establish more reliable models of such defined activity of alkanolamines. For instance, by chemometric evaluation of chromatographic retention data, it was possible to recognize[14-20] the continuity of changes in alkanolamines hydrophobicity and its relation with Wiener topological index, expressing ability of these solutes for the non-specific molecular interactions.

A chemometric technique by the method of principal components analysis (PCA) is the multivariate statistical procedure which uses the well-defined linear transformation

$$y = \boldsymbol{W} * x$$

changing vectorial description of the stationary stochastic process $x = R^K$ into the vector $y = R^K$ by applying the intermediate matrix $\boldsymbol{W} \varepsilon R^K * N$, where K

$<< N$, in such manner that the output surface with reduced dimensions still retain all the principal informations referring to the considered process. Accurate statistical theory implies that PCA transformation creates the set of K orthogonal vectors which have great impact into variance of input data. The great amount of information included in the mutually correlated input data is changed during PCA transformation into the set of statistically independent minor components K arranged according their validity.[21] PCA transformation can be referred to as the form of the disadvantage (loss) compression of input data, which is known in communications theory as the Karhunen-Loeve transformation.[22]

PCA as most frequently employed in chemical applications is the special case of factorial methods of data analysis[23] which, in chromatography, have been used in such areas, distinguished and reviewed recently by Kaliszan,[24,25] as elucidation of molecular mechanism of separation in chromatographic systems, prediction of retention based on the structural descriptors of solutes, evaluation of the separation properties of stationary phase materials and mobile phase composition, optimization of separation conditions in multivariable chromatographic systems, prediction of biological activities, and pharmacological classification of xenobiotics based on their retention in diversified chromatographic systems.

Especially, in case of planar chromatography, the different factorial methods of multivariate analysis were introduced to classify the strength of mobile phases in RPTLC systems for separation of triazine derivatives,[26] characterize suitability of the impregnating agents for TLC determination of lipophilicity of phytotoxic 2-nitro-4-cyanophenyl esters,[27] evaluate effects of support matrix[28] or organic modifier[29] on the TLC estimation of hydrophobicity parameters of peptides or commercial anticancer drugs, respectively.

Application of PCA to TLC data made it possible to establish the procedure for precise identification of near 450 drugs and their metabolites[30,31] or optimization of TLC separation of flavonoids.[32] Use of the PCA procedure to data matrix, founded on the TLC retention values and topological molecular descriptors, enabled parametrization of molecular activity of amino acids[33] and alkanolamines.[14,16,17,20]

In the present studies, it was shown that systematic information extracted by performed PCA analysis of retention behaviour of alkanolamines in diverse planar chromatography systems not only lead to the explanation of molecular mechanisms of their selective separation but also has direct relevance to the potential pharmacological properties of such solutes.

EXPERIMENTAL

Materials and Methods

HPTLC measurements were performed on methanolic solutions (*ca.* 10 mg/mL) of the following twelve test alkanolamines with chemical structures shown in Figure 1:

> 2-aminoethanol (coded as MEA), bis(2-hydroxyethyl)amine (DEA), tris(hydroxymethyl)aminomethane (APD) (POCh, Gliwice, Poland), bis(2-hydroxypropyl)amine (DIP), N-(2-hydroxyethyl)ethylenediamine (NAE), (Merck-Schuchardt, Hohenbrunn, Germany), N,N'-bis(2-hydroxyethyl)-methylamine (MeDEA), N,N'-bis(2-hydroxyethyl)-*n*-butylamine (BuDEA), N,N'-bis(2-hydroxyethyl)-*tert*-butylamine (tBuDEA), N,N'-bis(2-hydroxyethyl)-aniline (PhDEA), N,N'-bis(2-hydroxyethyl)-p-toluidine (pTDEA), tris(2-hydroxyethyl)amine (TEA) (Fluka, Buchs, Switzerland) and N,N'-bis(2-hydroxyethyl)-o-toluidine (oTDEA) (Bayer, Leverkusen, Germany).

HPTLC experiments was performed with 10 x 10 cm, glass-backed HPTLC plates, precoated with silanized silica, coded as C2 (Merck, Darmstadt, Germany; #5746); bonded octylsiloxane silica, coded as C8 (Merck; #13725); bonded octadecylsiloxane silica, coded as C18 (Baker, Phillipsburg, NJ, USA, #7012-01); wettable (partially silanized) bonded octadecylsiloxane silica, coded as WC18 (Macherey-Nagel, Düren, Germany; #811 075); bonded diphenyl silica, coded as DPH (Whatman, Maidston, England; #44814-820); bonded cyanopropylsiloxane silica, coded as CN (Merck, #16464); bonded aminopropylsiloxane silica, coded as NH2 (Merck, #12572); and bonded propyldiol silica, coded as DIOL (Merck, # 12668).

The plates were used as received. Mixtures of deionized water containing HPLC grade methanol or acetonitrile (Merck) in the proportions 20:80, 40:60, 60:40, 80:20 and 90:10 (v/v) were applied as mobile phases. Before use, the appropriate mixture of solvents was left to stand 1h at room (21°C) temperature.

Samples of solutes (0.5 μL) were spotted individually with a type 701 Hamilton (Reno, USA) 10 μL microsyringe. The plates were developed horizontally in DS-II sandwich type, all-Teflon tanks (Chromdes, Lublin, Poland)[34] until the solvent front reached a point 0.8 cm from the upper edge of the plate.

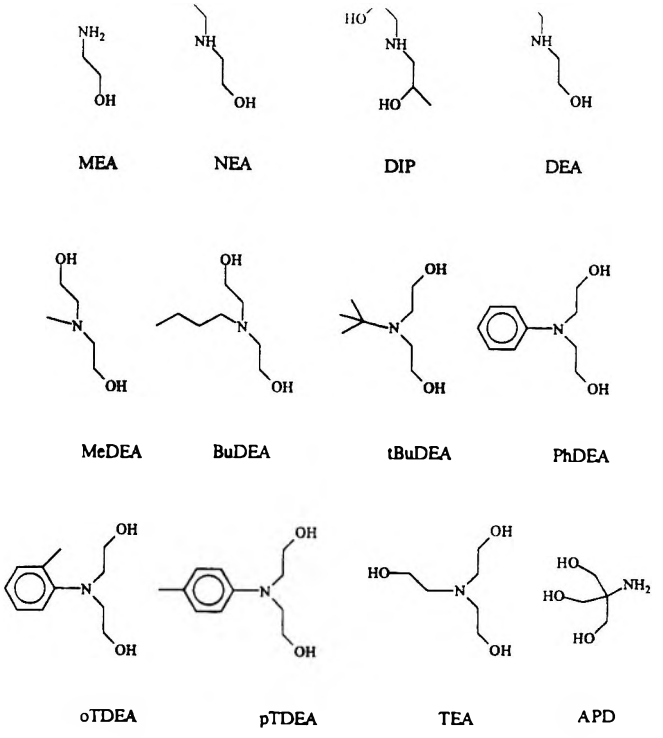

Figure 1. Structure of alkanolamines studied. For abbreviation see Table 1.

Spots of the separated alkanolamines were visualized with ninhydrin (Merck) solution (0.4 g in *n*-butanol-acetic acid (d = 1.04), 100:1 (*v/v*)) and heating the plate at 80°C for 60 min. The chromatograms on the TLC plates were densitometrically evaluated with the 16 (4-bit) grayscale scanner ScanJet 3p (Hewlett-Packard, Warsaw, Poland) connected to IBM PC/486-DX2 microcomputer and working under Quantiscan v.2.0 (Biosoft, Cambridge, U.K.) software.[35]

The retention data in planar chromatography systems (#38, 39, and 40 in Table 7) were obtained in measurements performed on Whatman 1 cellulose layers with n-butanol + acetic acid + water (77:6:17 v/v) in system 38, pyridine + ethyl acetate + water + acetic acid (42:25:25:8) in system 39, or n-butanol saturated with 0.1% ammonia solution in system 40 as mobile phase. Spots visualization was identical as in TLC experiments.

Table 1

Structural Parameters of Model Alkanolamines Derived From Molecular Calculations

No.	Parameter	Unit	Code	Alkanolamine*					
				MEA	**NAE**	**DIP**	**DEA**	**MeDEA**	**BuDEA**
1	Molecular Weight	a.m.u.	M.W	61.08	104.15	133.19	105.14	119.16	161.24
2	Total Energy	kcal/mol	TE	-20306.9	-32575.9	-42062.9	-34878.8	-38459.5	-49238.0
3	Binding Energy	kcal/mol	BE	-937.7	-1652.6	-2157.1	-1596.5	-1865.5	-2708.8
4	Electronic Energy	kcal/mol	EE	-62018.9	-132660.7	-196274.7	-134710.4	-166961.5	-262843.8
5	Heat of Formation	kcal/mol	HE	-58.6	-58.3	-118.1	-107.1	-101.1	-119.6
6	Energy of HOMO	eV	HOMO	-9.8453	-9.4624	-9.1483	-9.5388	-9.3220	-9.2232
7	Energy of LUMO	eV	LUMO	3.1720	2.7349	3.0755	2.6272	2.5151	2.4658
8	Maximum Charge	electrons	MAX	0.211	0.1493	0.1994	0.2017	0.2012	0.2008
9	Minumum Charge	electrons	MIN	-0.3490	-0.3465	-0.3344	-0.3293	-0.3305	-0.3353
10	Dipole Moment	debye	D	1.310	2.650	1.129	2.413	2.660	2.420

Table 1 (continued)

Structural Parameters of Model Alkanolamines Derived From Molecular Calculations

No.	Parameter	Unit	Code	Alkanolamine*					
				tBuDEA	PhDEA	pTDEA	oTDEA	TEA	APD
1	Molecular Weight	a.m.u.	M.W.	161.24	181.23	195.26	195.26	149.19	121.14
2	Total Energy	kcal/mol	TE	-49228.4	-53821.1	-57434.2	-57415.5	-49444.6	-42260.7
3	Binding Energy	kcal/mol	BE	-2699.1	-2770.7	-3072.2	-3053.4	-2249.2	-1688.8
4	Electronic Energy	kcal/mol	EE	-275136.9	-293915.1	-338942.9	-328753.3	-235332.3	-184140.0
5	Heat of Formation	kcal/mol	HE	-109.9	-48.2	-74.5	-55.8	-150.7	-140.5
6	Energy of HOMO	eV	HOMO	-9.0876	-8.0646	-9.1318	-7.9409	-9.3556	-10.1574
7	Energy of LUMO	eV	LUMO	2.3916	0.6804	0.4272	0.7089	2.3072	2.9622
8	Maximum Charge	electrons	MAX	0.2005	0.2056	0.2000	0.2057	0.2023	0.1854
9	Minumum Charge	electrons	MIN	-0.3350	-0.3328	-0.3358	-0.3346	-0.3336	-0.3397
10	Dipole Moment	debye	D	1.327	1.082	2.023	1.244	2.258	2.392

* Abbreviations: MEA = 2-aminoethanol; NEA = N-(2-hydroxyethyl)ethylenediamine; DIP = bis(2-hydroxypropyl)amine; DEA = bis(2-hydroxyethyl)amine; MeDEA = N,N'-bis(2-hydroxyethyl)methylamine; BuDEA = N,N'-bis(2-hydroxyethyl)-n-butylamine; tBuDEA = N,N'-bis(2-hydroxyethyl)-tert-butylamine; PhDEA = N,N'-bis(2-hydroxyethyl)aniline; o-TDEA = N,N'-bis(2-hydroxyethyl)-ortho-toluidine; pTDEA = N,N'-bis(2-hydroxyethyl)-para-toluidine; TEA = tris(2-hydroxyethyl)amine; APD = tris(hydroxymethyl)aminomethane.

Thermodynamically correct values of R_M coefficients for test solutes were calculated from triplicate measurements of R_F parameter using the Bate-Smith and Westall formula:[36]

$$R_M = \log [(1/R_F) - 1] = \log k' \quad (1)$$

Structural Calculations

For each test solute, the nine structural parameters (*i.e.* total energy, binding energy, electronic energy, heat of formation, energy of HOMO, energy of LUMO, maximum charge, minimum charge and dipole moment) were calculated with HyperChem 4.5 (Hypercube, Inc., Waterloo, Ontario, Canada) molecular modeling software installed on an IBM-compatible 486/DX2-66 MHz personal computer. These calculations were performed on the *in vacuo* optimized geometry of molecular structure of test solutes (as presented in Fig. 1), after employing a semi-empirical technique AM1 with restricted Hartree-Fock (RHF) mode. The Polak-Ribiere conjugate gradient procedure was used for optimization. The values of dipole moments for analytes were calculated using the molecular mechanics (MM+) method.

Chemometric Calculations

Retention data registered in the eighty planar chromatograpy systems (see Tables 2-9) as well as structural data for the test solutes (compare Table 1) were subjected to the multivariate statistical procedure by principal components analysis (PCA).[21,23] The previously reported retention data for test solutes in twenty six HPTLC[14,17] and three paper chromatography[37] systems were included in these calculations.

All calculations were performed with Statistica 4.3 (StatSoft, Inc., Tulsa, OK, USA) software implemented on an IBM-compatible 486/DX2-66 MHz personal computer.

RESULTS AND DISCUSSION

PCA of Structural Data

In Table 1, the values of structural descriptors calculated by the molecular

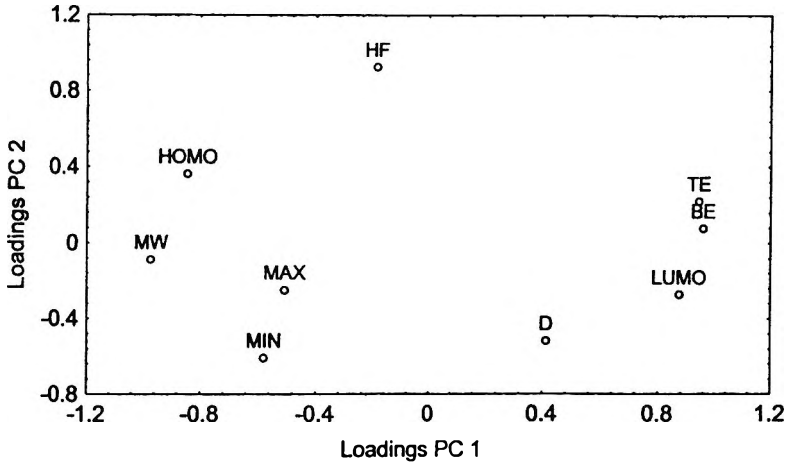

Figure 2. Two-dimensional plot of loadings input to calculate first two principal components (PC1 and PC2) due to the structural parameters from PCA of molecular data in Table 1. For abbreviation on the plane see Table 1.

modelling package, for a set of model alkanolamines, were presented. It can be assumed[24,38] that differences in molecular size or relative ability of individual solutes to undergo nonspecific, dispersive intermolecular interactions have been reflected by the molecular descriptors such as molecular weight MW; total energy TE; binding energy BE; electronic energy EE; and heat of formation HE. The ability of solutes to undergo charge transfer and/or hydrogen bonding interactions has been expressed by the two respective structural parameters: energy of highest occupied molecular orbital (HOMO) and the energy of lowest unoccupied molecular orbital (LUMO). The ability of solutes for electrostatic interactions as the dipole-dipole and dipole-induced dipole type have been quantified, respectively, by maximum/minimum excess charge on individual atoms the solute molecule (MAX and MIN, respectively and dipole moment D.

For assessment of molecular mechanism of observed retention of alkanolamines, the 12 x 10 matrix determined for 12 solutes and 10 structural descriptors was subjected to PCA. In Fig. 2, the loadings of calculated two principal components are presented. In this case, the summarized accounts of both PC1 and PC2 for the total input data variance was near 78 %. The PC1 explained 59.5 % of variance as well as PC2 only 18.5 %.

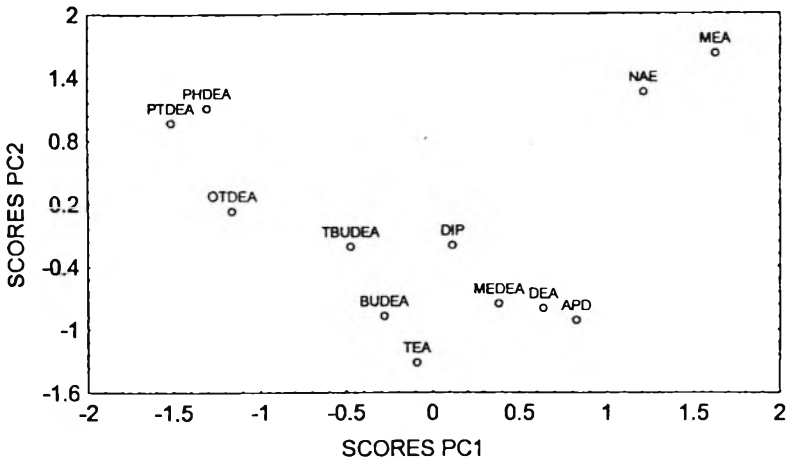

Figure 3. Two-dimensional plot of scores input to calculate first two principal components (PC1 and PC2) due to the individual solutes from PCA of molecular data in Table 1, from PCA of retention data of alkanolamines in Tables 2-9. For abbreviation on the plane see Table 1.

The PC1 is determined mostly by structural parameters associated with descriptors of molecular size of solutes, *i.e.* total energy TE, binding energy BE, electronic energy EE and slightly with such electronic descriptors as energy of LUMO and dipole moment D. It can be assumed that PC1 can be physicochemically interpreted as measure of solutes' ability to undergo the weak non-specific van der Waals interactions (dipole-dipole, dipole-induced dipole and dispersion type) supported by the more specific hydrogen bond formation. The sequence of molecular descriptors on the PC1 axis indicate that dispersion forces prevailing on the sum of both type of dipole-dipole interactions. Contribution of hetero- and homo-intermolecular hydrogen bond formation into potential associative interactions of alkanolamines must lead to a change of their dipole moment as suggest the close proximity of their respective decriptors TE and LUMO on the loading plane in Fig. 2. Similarly, PC2 is loaded mostly by structural parameters related to other descriptors of solute molecular size, such as the heat of formation HF and electronic descriptor as the energy of HOMO, which suggests that PC2 can be related to the quantitative measure of the second characteristic interaction pattern during associations of alkanolamines, *i.e.* specific electron pair donor-electron pair acceptor (charge transfer) interactions supported by the long distance dispersion interactions.

The ability of formation of such charge transfer complexes is dependent on the vector sum of the dipole moments of donor and acceptor molecules[38] as confirmed by proximity of the respective molecular descriptors (HOMO with MIN/MAX) on the loading plane in Fig. 2. The existence of *n*-*n*, *n*-π and π-π type of intermolecular interactions can be predicted by the presence of electron rich substituents in the alkanolamines moiety, in the stationary phase structures and in the molecules of organic modifiers of aqueous binary mobile phase.

Preliminary conclusions drawn from Fig. 2 on the possible interactions pattern of alkanolamines have been confirmed by the scores plot presented in Fig. 3. Alkanolamines containing a phenyl-like substituent connected to the tertiary amine group, *i.e.* PhDEA, oTDEA and pTDEA (see Fig. 1), giving ability for the charge transfer associations, have most extreme values of PC2, and they are alkanolamines which posses the lowest values of PC1. It is well known that the polarizability for π-electron systems in phenyl substituent is high, which favors the molecular orientation and possibility for dispersion interactions, so the PCA classified properly the mentioned solutes in the same cluster.

The PC1 and PC2 are both loaded very high by solutes MEA and NAE (see Fig. 1), containing the lone pairs of the *n*-electrons, as well as the primary hydroxyl and amine substituents which are both the strongest proton donor group. For seven alkanolamines in the central part of the scores plot (Fig. 3), the relative increase of contribution of all the three van der Waals interactions (orientation, inductive along with dispersive) during the chromatographic process can be predicted.

This conclusion is supported by the highest values of calculated dipole moments (see Table 1) and polarizability[18] for these solutes. For bis(2-hydroxypropyl)amine (DIP), located in the centre of the scores plane, it can be postulated that this molecule is possessed of very limited contribution of charge transfer interactions or hydrogen bond formation during any possible association process. This solute can be probably considered as the standard compound possesing molecular ability to participate exclusively in the non disturbed van der Waals interactions. One can also observe that PC1 is near parabolic function of the molecular mass of examined solutes.

Thus revealed here, PCs divides excellently the two more chemical, in nature, intermolecular interactions patterns which dominate for alkanolamines association and their close relations with the more physical type of van der Waals interactions.

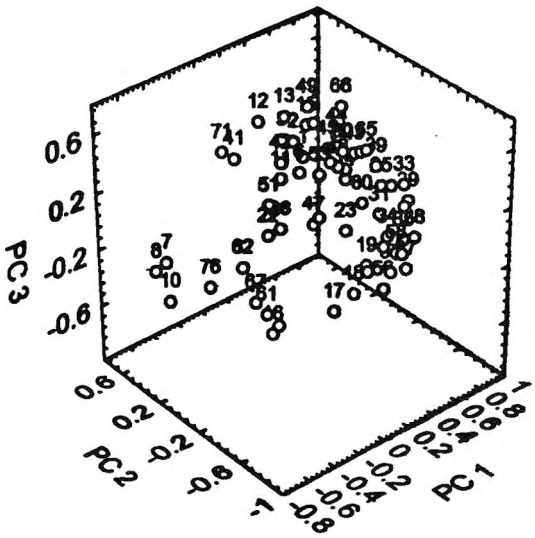

Figure 4. Spatial plot of loadings input to calculate first three principal components (PC1, PC2 and PC3) due to the individual planar chromatography systems from PCA of retention data of alkanolamines in Tables 2-9. The numbers on the plane refer to the code of chromatographic systems in Tables 2-9.

PCA of Retention Data

The values of R_M coefficients for twelve test alkanolamines were measured on the eight diversified hydrocarbonaceous silica gel stationary phases using ten different aqueous binary mobile phases (compare Tables 2-9). The retention data of solutes in the three paper chromatography[37] systems (see Table 7) were also included in these considerations. The purpose of PCA was to find the structure established in such a retention data matrix containing 12 x 80 elements. Assuming that the relative errror of measured R_M coefficients was independent of the employed chromatographic conditions (mainly in reversed-phase mode), a PCA transformation gives the matrix values with a constant variance.

With the use of PCA, a few hidden variables, such as principal components (PCs), can be calculated which explain almost all the observed variance of the retention data set. It appears that the calculated first three

principal components, together, explained nearly 59% of the total variance of the initial data matrix, the former accounting for 32.5 %, the second 15.5 %, and third only 11.0 %.

In Fig. 4 the loading inputs to calculated first, second and third principal component, PC1, PC2, PC3 respectively, due to the individual planar chromatography system, are shown. The structure of two-dimensional loading plot in Fig. 5 can be related to the planar chromatographic systems design, *i.e.* composition of binary mobile phase and type of stationary phase. It appears that the main PC1 can be associated with the water fraction in the binary mobile phase as well as changing of the organic modifier from methanol to acetonitrile. This means that PC1 explains the variations in the retention data of alkanolamines caused by mobile phase strenght and solvent strenght selectivity. The second PC2 can be asssociated with type of stationary phase, especially increasing polar character of such phase, and its selectivity. The PC3 is highly loaded (see Fig.4) by retention data registered on the cyano- and aminopropylsiloxane bonded silica layers, and can be related with contribution of the strong, specific interactions of solutes with the stationary phase. Thus, in accordance with the dynamic nature of any chromatographic process, the PCA revealed the three fundamental factors for the systematic variance of registered retention data of alkanolamines as the mobile phase strenght, stationary phase polarity and functionalities in solute molecule.

In Fig. 5, close to the origin of both PC1 and PC2 scales (point with coordinates 0,0), retention data referring to the planar chromatography system number 61 are located. Data for this system (see Table 6) can be considered as the hydrophobicity parameters R_{Mw} of solutes have since been calculated as the water extrapolated (acetonitrile free mobile phase) R_M values on the octadecylsiloxane bonded silica HPTLC plates. Thus the (0,0) point in the loadings plane (Fig. 5) can be regarded as the indicator of diminished contribution of the polar (silanophilic) interactions of stationary phase with solutes during chromatographic retention. The fact that octylsiloxane and diphenylsilica layers, used in systems 16 and 30, respectively, indicate greater bonded phase surface area and bonding density than octadecylsiloxane silica,[39-41] imply further reduction of silanophilic interactions and cause the location of both mentioned TLC systems at the same place in Fig. 5.

The PCA differentiated very well between reduced and enhanced potency to the silanophilic as well as proton donor interactions of the cyano- and aminopropylsiloxane bonded phases[39] by grouping them in the opposite sides of Fig. 5, in the right- and left-upper corner, respectively. Conversely, the other pair of polar stationary phases, *i.e.* propyldiol and cyanopropylsiloxane bonded silica, have been located in the one cluster (at the upper-right corner) indicating

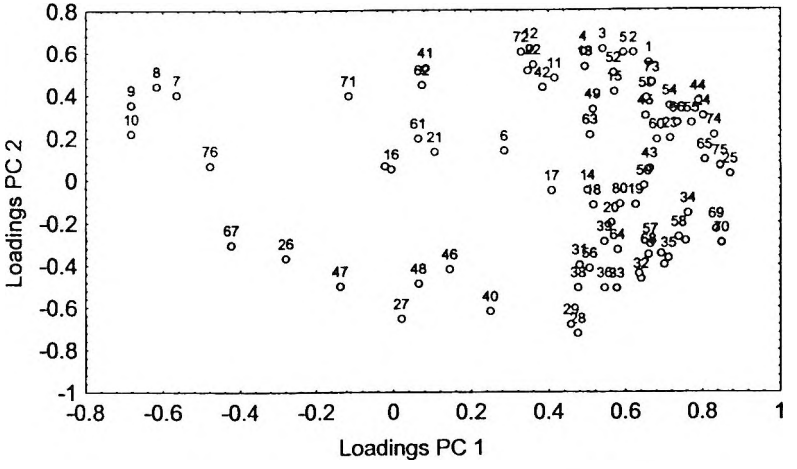

Figure 5. Two-dimensional plot of loadings input to calculate first two principal components (PC1 and PC2) due to the individual planar chromatography systems from PCA of retention data of alkanolamines in Tables 2-9. The numbers on the plane refer to the code of chromatographic systems in Tables 2-9.

their similar strong solvatation by the mobile phase components in a surface phase which diminishes the effect of the presence of polar substituents in these chemically bonded phases on the overall sorbent selectivity. Poole *et al.*[40,41] reported an analogous phenomenon on the results of inspection of retention properties of solid-phase extraction cartridges filled with the mentioned polar silica packings. The enhanced and comparable access of considerable fraction of the original pore volumes of the cyano- and propyldiol- bonded silica by small molecules has been observed[39,40] in contrast to the octadecylsiloxane bonded silica in which the pores are near completly blocked after chemical modification.[23,39] Thus, the chromatographic systems with the octadecylsiloxane phases comprising different ability for solvatation by sorption of organic modifier from mobile phase[42] were grouped in a separate place (the lower-right corner) in Fig. 5.

The position of the paper chromatography systems (numberd as 38, 39, 40, see also Table 7) in Fig. 5, close to the TLC systems (numbered as 31, 66, 28, 58), with alkyl or diphenyl bonded silica and employing rather high concentration of organic modifier in mobile phase, reflects similar predominant significance of the solutes' solvatation in the bulk mobile phase on their

Table 2

Retention Data (log k') for Alkanolamines Determined on Propyldiol Silica Layers (DIOL) with Aqueous-Methanol or Aqueous-Acetonitrile Mobile Phases

No.	Solute	Methanol Concentration (% v/v) 20	40	60	80	90	Acetonitrile Concentration (% v/v) 20	40	60	80	90
1	MEA	-1.269	-0.846	-0.804	-0.823	0.901	-1.680	-1.366	-1.024	-0.217	-0.485
2	NAE	0.583	-0.294	-1.000	-1.074	-0.876	-1.122	0.010	0.734	0.590	0.498
3	DIP	-0.967	-0.925	-0.849	-0.861	-0.944	-1.063	0.745	1.957	-0.112	-0.298
4	DEA	-1.372	-0.837	-0.467	0.001	-0.876	1.800	1.500	1.121	-0.578	0.471
5	MeDEA	1.292	1.270	1.118	1.115	0.984	1.295	1.355	1.446	1.374	1.369
6	BuDEA	1.561	1.425	1.272	1.238	1.090	1.555	1.596	1.528	1.325	1.343
7	tBuDEA	1.761	1.635	1.555	1.423	1.264	1.050	0.826	0.960	1.150	1.382
8	PhDEA	-0.858	-0.803	-0.808	-0.838	-0.901	-1.056	-1.066	-1-163	-0.676	-0.587
9	oTDEA	-0.820	-0.807	-0.761	-0.823	-0.990	0.990	-0.251	-1.390	-0.625	-0.556
10	pTDEA	-0.998	-0.762	-0.753	-0.700	-0.886	-0.580	-0.620	-0.650	-0.694	-0.457
11	TEA	0.927	0.846	-0.761	0.603	0.444	0.863	0.873	0.838	1.021	1.121
12	APD	-1.045	-0.903	-0.864	-0.879	-0.955	-0.711	-1.283	-1.000	-0.500	0.498
TLC System No.:		1	2	3	4	5	41	42	43	44	45

Table 3

Retention Data (log k') for Alkanolamines Determined on Aminopropylsiloxane Silica Layer (NH) with Aqueous-Methanol or Aqueous-Acetonitrile Mobile Phases

No.	Solute	Methanol Concentration (% v/v)					Acetonitrile Concentration (% v/v)				
		20	40	60	80	90	20	40	60	80	90
1	MEA	-0.620	-0.590	-0.544	-0.500	-0.471	1.065	0.920	0.010	0.159	0.400
2	NAE	-0.520	-0.752	-0.798	-0.738	-0.513	-0.380	-1.331	-1.100	-0.147	1.043
3	DIP	-1.137	-1.068	-0.873	-0.895	0.093	-1.060	-1.129	-1.550	-1.195	-0.779
4	DEA	-1.430	-1.348	-0.992	-0.779	-0.781	-1.153	-1.409	-0.470	0.944	-0.244
5	MeDEA	-1.065	-0.475	0.031	0.220	0.137	-1.454	-1.206	-0.661	0.864	-0.125
6	BuDEA	-0.824	-0.937	0.178	-0.410	-0.894	-0.820	-2.230	-1.387	1.037	-0.850
7	tBuDEA	-1.032	-0.522	-0.256	-0.679	-0.979	-1.347	-1.973	-1.710	1.737	1,742
8	PhDEA	-1.078	1.071	1.687	1.841	1.874	-1.324	-1.438	-1.765	-0.528	-1.200
9	oTDEA	-0.983	1.043	1.742	1.921	1.916	-1.312	-1.121	-1.796	-1.428	-1.615
10	pTDEA	0.811	1.116	1.773	1.930	1.950	-0.846	-1.048	-1.060	-1.297	-1.070
11	TEA	0.137	0.146	-0.700	-0.794	-1.161	-1.195	-1.928	-1.638	1.391	-0.021
12	APD	-1.200	-0.888	-0.535	-0.656	-0.830	-1.001	-1.196	-0.840	-0.329	-0.400
TLC System No.:		6	7	8	9	10	46	47	48	49	50

Table 4

Retention Data (log k') for Alkanolamines Determined on Cyanopropylsiloxane Silica Layer (CN) with Aqueous-Methanol or Aqueous-Acetonitrile Mobile Phases

No.	Solute	Methanol Concentration (% v/v) 20	40	60	80	90	Acetonitrile Concentration (% v/v) 20	40	60	80	90
1	MEA	1.114	1.160	1.188	1.192	1.198	0.640	0.714	0.677	0.578	0.518
2	NAE	1.301	0.925	0.904	1.034	1.089	-1.253	0.205	1.369	1.414	1.366
3	DIP	1.138	-1.019	-0.904	-1.177	-0.687	1.737	1.778	2.053	1.200	0.778
4	DEA	-0.378	-0.535	-0.606	-0.808	-1.031	-0.546	-0.670	-0.807	-0.912	-1.063
5	MeDEA	1.106	1.252	1.232	1.085	-.910	1.275	1.450	1.409	1.167	1.076
6	BuDEA	1.146	1.396	1.449	1.226	1.104	1.588	1.520	1.577	1.211	0.936
7	tBuDEA	1.325	1.682	1.692	1.475	1.292	1.836	1.885	1.926	1.544	1.161
8	PhDEA	0.423	0.202	-0.097	-0.478	-0.811	0.317	-0.041	-0.457	-0.808	-1.088
9	oTDEA	0.921	0.417	-0.386	-0.449	-0.746	0.471	0.041	-0.356	-0.533	-1.101
10	pTDEA	0.662	0.873	0.773	0.693	0.536	0.430	0.072	-0.450	-0.694	-1.143
11	TEA	0.816	0.874	0.773	0.693	0.536	0.840	0.852	0.955	0.790	0.597
12	APD	-1.130	-1.151	-1.075	1.900	-1.604	-0.671	-1.620	-2.230	-1.973	-1.146
TLC System No.:		11	12	13	14	15	51	52	53	54	55

Table 5

Retention Data (log k') for Alkanolamines Determined on Diphenylsilica Layer (DPH) with Aqueous-Methanol or Aqueous-Acetonitrile Mobile Phases

No.	Solute	Methanol Concentration (% v/v)					Acetonitrile Concentration (% v/v)				
		20	40	60	80	90	20	40	60	80	90
1	MEA	-0.459	-0.600	-0.984	-1.075	-1.068	1.115	0.842	0.930	0.491	0.518
2	NAE	0.738	0.895	0.952	0.976	0.697	0.968	0.803	1.072	1.089	1.366
3	DIP	1.266	1.338	0.984	0.593	0.496	1.394	1.704	1.737	1.444	-0.778
4	DEA	-0.260	-0.529	-0.876	-1.090	-1.054	-0.693	-0.994	-1.916	-2.459	-1.063
5	MeDEA	0.738	0.719	0.831	0.720	0.132	0.713	0.779	0.784	0.812	1.076
6	BuDEA	0.940	1.010	0.565	0.078	-0.583	0.968	0.928	0.864	0.882	0.936
7	tBuDEA	1.004	1.035	0.925	1.430	1.595	0.817	1.099	1.195	1.153	1.161
8	PhDEA	0.489	0.363	-0.230	-0.715	-0.893	0.193	-0.125	-0.693	-1.444	-1.088
9	oTDEA	1.844	0.301	-0.117	-0.612	-0.888	0.396	-0.011	-0.629	-1.625	-1.101
10	pTDEA	0.782	0.339	-0.113	-0.596	-0.883	0.358	-0.027	-0.623	-1.374	-1.143
11	TEA	0.107	0.157	-0.010	-0.074	-0.633	0.200	0.573	0.528	-0.609	-1.200
12	APD	1.397	1.418	1.460	1.430	1.348	1.635	1.576	1.510	1.625	-1.146
TLC System No.:		16	17	18	19	20	56	57	58	59	60

Table 6

Retention Data (log k') for Alkanolamines Determined on Wetted Silica Layer (WC18) with Aqueous-Methanol or Aqueous-Acetonitrile Mobile Phases

No.	Solute	Methanol Concentration (% v/v)					Acetonitrile Concentration (% v/v)					
		20	40	60	80	90	0	20	40	60	80	90
1	MEA	-0.509	-0.371	-0.376	-0.230	0.514	-0.652	-0.862	0.157	0.153	0.984	0.838
2	NAE	0.703	0.546	0.437	0.781	1.114	0.099	0.389	0.674	0.771	0.852	1.161
3	DIP	1.305	0.843	0.211	-0.144	1.183	2.049	1.680	1.200	1.525	1.062	-0.635
4	DEA	0.088	-0.253	-0.483	-0.847	-0.744	-0.427	0.441	0.325	-0.061	-0.241	0.057
5	MeDEA	0.317	0.369	0.325	0.736	0.888	0.084	0.497	0.424	0.051	0.978	1.438
6	BuDEA	0.804	0.561	0.398	0.772	0.893	0.509	0.730	0.438	0.061	0.706	0.928
7	tBuDEA	0.898	0.775	0.451	-.785	1.039	0.532	0.914	0.895	0.102	1.121	1.508
8	PhDEA	0.481	0.098	-0.301	-0.603	-0.862	0.356	0.376	-0.010	-0.694	-1.205	-1.048
9	oTDEA	0.733	0.320	-0.146	-0.512	-0.782	0.560	0.648	0.115	-0.578	-0.928	-0.928
10	pTDEA	0.873	0.420	-0.062	-0.431	-0.723	0.594	0.711	0.178	-0.491	-0.785	-0.744
11	TEA	0.565	0.344	0.178	0.431	0.833	-0.054	0.364	0.233	0.020	0.807	1.331
12	APD	0.868	-0.522	0.146	-0.246	-0.231	-0.254	-0.505	-0.349	-0.164	-0.984	-0.785
TLC System No.:		21	22	23	24	25	61	62	63	64	65	66

Table 7

Retention Data (log k') for Alkanolamines Determined on Octadecylsiloxane Silica Layer (C18) with Aqueous-Methanol or Aqueous-Acetonitrile Mobile Phases and in Paper Chromatography Systems

No.	Solute	Methanol Conc'n (% v/v)				Acetonitrile Conc'n (% v/v)				Paper Chromatography		
		40	60	80	90	40	60	80	90	A*	B**	C#
1	MEA	1.541	1.534	1.453	1.358	0.788	0.547	1.518	1.457	0.630	-0.070	0.327
2	NAE	1.621	1.512	1.450	1.431	0.624	1.056	1.423	1.638	0.825	0.052	0.176
3	DIP	1.504	1.116	0.790	1.171	1.002	1.204	1.416	1.539	0.231	-0.477	-0.410
4	DEA	2.685	1.417	0.029	1.472	0.718	-1.003	-1.924	-0.851	0.575	-0.213	0.052
5	MeDEA	1.721	1.072	0.555	1.504	1.123	1.204	1.367	1.327	0.477	-0.212	-0.288
6	BuDEA	1.748	1.064	0.459	1.490	0.405	0.845	1.306	1.225	-0.035	-0.525	-0.865
7	tBuDEA	-1.740	-0.954	-0.126	1.735	-0.501	0.103	1.618	1.638	0.176	-0.432	-0.825
8	PhDEA	0.673	0.080	-0.525	1.215	0.689	-0.380	-1.437	-1.457	-0.908	-1.995	-0.688
9	oTDEA	1.777	0.780	-0.171	1.967	2.594	-0.335	-1.924	-1.225	0.001	-0.630	-0.908
10	pTDEA	1.173	0.343	-0.323	1.542	0.483	-0.320	-1.119	-1.005	-0.005	-0.650	-0.930
11	TEA	1.453	0.733	0.151	1.741	0.449	1.101	1.253	1.220	0.550	-0.213	0.087
12	APD	1.324	1.435	1.540	1.867	1.287	1.518	1.732	1.766	0.658	-0.454	-0.035
TLC System No.:		26	27	28	29	67	68	69	70	38	39	40

Table 8

Retention Data (log k') for Alkanolamines Determined on Octylsiloxane Silica Layer (C8) with Aqueous-Methanol or Aqueous-Acetonitrile Mobile Phases

No.	Solute	Methanol Concentration (% v/v)				Acetonitrile Concentration (% v/v)				
		40	60	80	90	20	40	60	80	90
1	MEA	1.358	1.442	1.525	1.706	0.146	0.898	1.369	1.690	1.499
2	NAE	1.431	1.751	1.751	1.789	-0.066	-0.370	0.097	1.462	1.256
3	DIP	1.171	1.930	1.930	1.282	-2.000	-1.500	-0.129	1.195	1.905
4	DEA	1.142	1.802	1.802	1.841	0.845	-0.538	-0.611	-0.771	-0.201
5	MeDEA	1.504	1.317	1.317	0.769	0.880	1.269	1.697	1.905	1.578
6	BuDEA	1.490	1.440	1.440	1.100	0.810	1.252	1 662	1.987	1.721
7	tBuDEA	1.735	1.262	1.262	1.162	1.290	1.681	2.083	1.955	1.727
8	PhDEA	1.215	0.405	-0.387	-0.781	0.760	0.236	-0.311	-0.812	-0.784
9	oTDEA	1.967	1.580	-0.205	-0.535	0.582	0.183	-0.238	-0.622	-0.601
10	pTDEA	1.542	0.681	-0.185	-0.628	0.498	0.156	-0.203	-0.532	-0.702
11	TEA	1.741	1.418	1.085	0.739	0.579	1.217	1.469	1.591	1.557
12	APD	1.867	1.805	1.751	1.730	0.794	-0.506	-0.574	-0.664	-0.188
TLC System No.:		30	31	32	33	71	72	73	74	75

Table 9

Retention Data (log k') for Alkanolamines Determined on Silanized Silica Layer (C2) with Aqueous-Methanol or Aqueous-Acetonitrile Mobile Phases

No.	Solute	Methanol Concentration (% v/v)				Acetonitrile Concentration (% v/v)				
		40	60	80	90	20	40	60	80	90
1	MEA	1.271	1.415	1.510	-0.082	0.696	0.769	0.720	1.076	1.602
2	NAE	1.952	0.950	-0.070	1.119	-0.677	1.209	1.562	1.613	-1.603
3	DIP	1.634	1.229	0.792	0.670	0.943	1.442	1.540	1.740	1.729
4	DEA	-0.783	-0.873	-0.886	-1.369	0.502	0.036	-0.923	-1.301	-0.194
5	MeDEA	1.212	0.903	0.586	0.343	1.510	1.450	1.351	1.362	1.580
6	BuDEA	1.291	0.877	0.438	0.135	1.187	0.087	-0.567	-0.978	1.507
7	tBuDEA	1.594	1.061	0.492	0.220	-0.005	1.012	1.462	1.513	1.908
8	PhDEA	-0.053	-0.349	-0.670	-1.015	1.610	-0.040	-0.567	-1.431	-1.187
9	oTDEA	0.653	0.010	-0.602	-0.984	1.801	0.163	-0.549	-1.613	-0.909
10	pTDEA	0.131	-0.185	-0.586	-0.874	1.709	0.041	-0.501	-0.581	-0.615
11	TEA	1.043	0.610	0.176	-0.010	1.196	1.106	1.061	1.301	1.421
12	APD	1.251	1.573	1.915	1.826	1.738	1.623	1.549	1.919	1.987
TLC System No.:		34	35	36	37	76	77	78	79	80

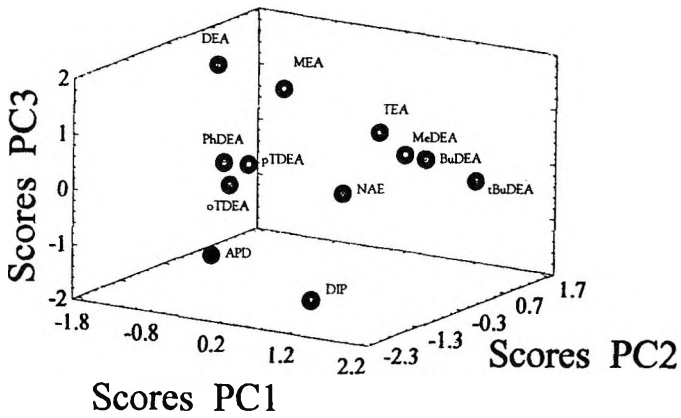

Figure 6. Spatial plot of scores input to calculate first three principal components (PC1, PC2 and PC3) due to the individual solutes from PCA of retention data of alkanolamines in Tables 2-9. For abbreviation on the plane see Table 1.

retention. Moreover, in mentioned TLC systems the transport of solute molecules from the bulk mobile phase to the non-polar chemically bonded stationary phase is less favored by competitive sorption of molecules of organic modifiers from binary mobile phase in the surface phase.[17,20,23,42]

To explain which original variables have high impact on the above-calculated PCs, their respective scores have been investigated. Fig. 6 gives a calculated scores plot, which shows the contribution of the twelve solutes to the original Pcs. Here, it is seen that solutes such as MeDEA, BuDEA, tBuDEA and NAE, DIP, TEA (see Table 1 for codes) are most extreme on the mobile phase strenght axis PC1. The tBuDEA and BuDEA load very high on the both first and second PC as shown in Fig. 7.

The solutes containing phenyl substituent in their structures, *i.e.* PhDEA, oTDEA and pTDEA, load the second PC2, explaining that their retention is driven, in fact, by presence or lack of hydrophobic interactions with stationary phase. The PC3 is loaded by such polar solutes as MEA, DEA and TEA which contain exposed hydroxy group(s), giving possibility for the specific interactions, *i.e,*. hydrogen bond formation, with the polar stationary phase, *e.g.*, cyano- and aminopropylsiloxane or propyldiol bonded silica. On results of PCA, one can assume method development for optimizing chromatographic

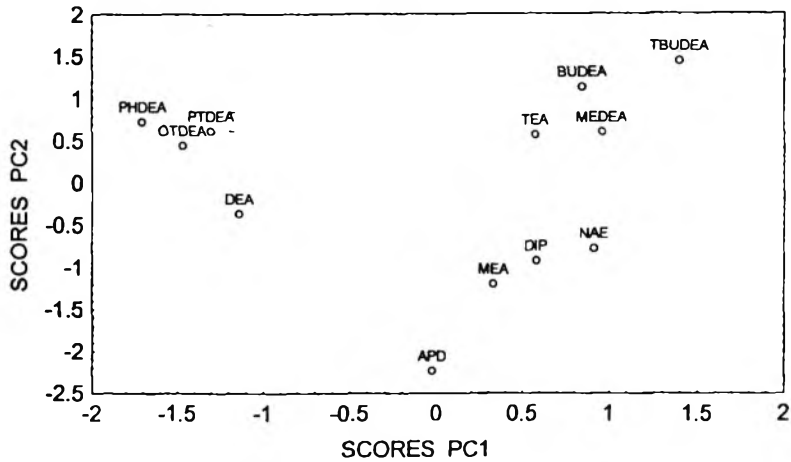

Figure 7. Two-dimensional plot of scores input to calculate first two principal components (PC1 and PC2) due to the individual solutes from PCA of retention data of alkanolamines in Tables 2-9. For abbreviation on the plane see Table 1.

separation of the three groups of alkanolamines in which the retention process can be regulated, primarily, by molecular interactions in the mobile phase (for the N-alkyl substituted solutes loading PC1), or by interactions with the stationary phase (for the N-phenyl substituted solutes loading PC2), as well as by modification (derivatization) of solute structure (for solutes loading PC3). However, the values of explained variance of experimental retention data by each calculated PC, indicates the relatively low and comparable contribution of the stationary phase polarity and its ability to hydrogen bond formation to the overall retention of solutes in the particular planar chromatography system employing aqueous-methanol or aqueous-acetonitrile binary mobile phase.

A similar conclusion was drawn for retention on HPLC columns with diversified bonded functional groups by Snyder *et al*[43] on the basis of gradient elution theory as well as Park *et al.*[44] and Abraham *et al.*[45] using linear solvation energy relationships. Similarly, on the basis of thermodynamically formulated theory of chromatographic processes[46] the dominating role of intermolecular association between solute and more polar components of bulk mobile phase during retention of alkanolamines have been previously indicated[15,19,20] after inspection of the limited retention data registered in adsorption TLC systems.

Prediction of Alkanolamines Bioactivity

Systematic information revealed by chemometric analysis with PCA of retention data, determined in various planar chromatography systems, can be useful to clarify the observed lysosomotropic activity of alkanolamines. The statistically significant relationship has been calculated by a multiregression method between the logarithm of minimal bactericidal concentration (log *MBC*)[9] of alkanolamines and calculated principal components scores presented in Fig. 6 :

$$\log MBC = -0.465\ (+/-\ 0.100) * PC1 + 0.438\ (+/-\ 0.100) * PC2 - 0.209\ (+/-\ 0.100) * PC3 - 1.635\ (+/-\ 0.105) \quad (2)$$

$n = 12;\ R = 0.932;\ F_{(3,8)} = 15.021;\ SE = 0.364;\ p < 0.001$

The moderate level of antimicrobial activity against *Pseudomonas pseudoalcaligenes* observed for BuDEA and DEA,[9] which were significantly related with scores calculated here for PC1 and PC2, respectively, can be explained by their ability to hydrogen bond formation for the former compound and electrostatic interactions for the second compound, both accompanied with dispersive interactions, during penetration of bacterial membrane. Thus, the established above model equation (2) indicates that some modification of structural features of alkanolamines, especially in kind of substituents connected to the tertiary amine group, which causes any disturbances in their subtle balanced ability to specific molecular interactions leads to the sharp changes of lysosomotropic and antimicrobial activity of such compounds. Similar effects have been observed for the highly molecular bactericidal agents 4-aza-5α-sitostanes in which extended lipophilicity leads to increase of their antimicrobial activity.[7,8]

CONCLUSION

Principal Components Analysis provides a useful interpretation of the retention characteristics of the planar chromatography systems used for alkanolamines separation, under reversed-phase conditions, in terms of optimization of their selectivity, and the structural features of solutes that affect retention in particular chromatographic systems. The polar bonded silica layers, containing cyanopropylsiloxane or propyldiol functionalities, give near comparable separation properties of ionizable alkanolamines as the standard, commercially available octadecylsiloxane phase. The performed PCA select the three planar chromatography systems in which the pure hydrophobic interactions dominate the retention process of alkanolamines. In spite of the

limited set of solutes used in these studies, the conclusions drawn on the PCA of retention data of alkanolamines were useful for the correct prediction of their antimicrobial activity.

ACKNOWLEDGMENTS

These studies were supported by an internal grant AM-503-11-137-17/1996 from the Medical University of *Lódz* (*Lódz*, Poland) offered to G. B. Also, one of the authors (H. Y. A.-E.) wishes to thank the Administration of King Faisal Specialist Hospital and Research Centre for its support to the Bioanalytical and Drug Development research program.

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Received February 28, 1997
Accepted March 30, 1997
Manuscript 4389

J. LIQ. CHROM. & REL. TECHNOL., 20(15), 2357-2366 (1997)

SIMULTANEOUS DETERMINATION OF ZINC(II) AND IRON(III) IN HUMAN SERUM BY LIQUID CHROMATOGRAPHY USING POST-COLUMN DERIVATIZATION WITH 4-(2-PYRIDYLAZO)-RESORCINOL

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ABSTRACT

A liquid chromatographic method for the determination of sub-μg/mL levels of zinc(II) and iron(III) in human serum has been studied by use of a post-column derivatization with 4-(2-pyridylazo)-resorcinol (PAR) as a chelating agent. Human serum samples are pretreated with hydrochloric acid and trichloroacetic acid for deproteination, and then sodium acetate solution is added for adjusting the pH of the aqueous sample to 3.5. The sample is injected onto a 10 μm bonded phase, strong-acid ion exchange column, and eluted with 0.340 mol/L tartrate buffer (pH 3.75) at a flow rate of 1.0 mL/min. The eluate is mixed with 10^{-4} mol/L PAR solution as the post-column reactant at the same flow-rate of the elution of the metal ions at 30°C, and the absorbance of the mixed solution is monitored at 515 nm (Zn) and 715 nm (Fe). The human serum samples can be analyzed with good precision by the proposed method.

INTRODUCTION

Many analytical methods for the determination of metals in biological samples have been reported, using spectrophotometry,[1,2] electrochemistry,[3,4] and chromatography.[5] In clinical analysis of human serum, iron and zinc as well as copper are important elements to be determined for the evaluation of human bloods and bodies.[6] Normal concentration levels of zinc and iron in serum are in the range of 1.0-2.0 μg/mL.[7] Deficits of both metals in blood are known to cause diseases of oxygen transport and other biological process. Liquid chromatography (LC) has an advantage of enabling multi-element determinations and automatic applications. Until now, very few papers have been reported on the LC determination of zinc(II) in human serum for routine analysis because of lack of suitable chelating agents.[8,9] The purpose of this study is to develop a simple and rapid LC method for the quantitation of zinc and iron in serum samples. The proposed method is based on the separation of the two metal ions on a strong cation-exchange column with tartrate buffer, followed by post-column derivatization of the metal species with 4-(2-pyridylazo)resorcinol (PAR) as a chelating agent. The absorbance of the resultant solution is monitored spectrophotometrically at 515 nm (zinc) and 715 nm (iron). The present paper describes the post-column LC method for the routine analysis of human serum samples.

EXPERIMENTAL

Apparatus and Reagents

A Tosoh (Tokyo, Japan) CCPD liquid chromatograph equipped with a Rheodyne 7125 sample injection valve (100-μL) and a Shimadzu (Kyoto, Japan) SPD-AS UV-VIS spectrophometer fitted with a 8-μL flow cell was used. The derivatization of the eluate with PAR was carried out using a Shimadzu LC-9A delivery pump and a 1/16 x 0.4 x 1000 mm reaction coil at 30°C. A TSKgel IEC (Na^+ form) (Tosoh, Tokyo, Japan) column (150 x 4.6 mm i.d.; particle size 10 μm) was used. The flow rate was 1.0 mL/min. Data was processed with a Shimadzu model CR-5A integrator/recorder. A Toa Denpa (Saitama, Japan) HM-30s pH meter was used for pH measurements.

PAR was of analytical reagent grade from Wako Junyaku Chemicals (Tokyo, Japan). Metal ion standards of a desired concentration were prepared by dilution of stock 1000 μg/mL standard solutions for atomic absorption spectrometry. Serum samples (Wako control sera I, II) were purchased from

Wako Junyaku Chemicals. Deionized water used was prepared from a Millipore Milli-Q water purification system. The mobile phase (0.340 mol/L tartrate buffer) was prepared by dissolving 9.98 g of tartaric acid and 23.7 g of sodium tartrate in 500 mL of deionized water. The derivatization solution (10^{-4} mol/L PAR) was prepared by dissolving 5.3 mg of PAR, 7.35 mL of 17 mol/L acetic acid and 25 mL of 15 mol/L ammonia in 250 mL of deionized water. The other reagent grade chemicals were purchased from Wako Junyaku Chemicals.

LC Separation and Determination of Metal Ions

A slightly acidic aqueous solution (ca. pH 4) containing metal ions of interest, was injected onto a strong-acid ion exchange (10 μm, particle size) column and eluted with the mobile phase at 1.0 mL/min flow rate. The eluate, after LC separation, was mixed with the PAR solution and flowed to the reaction coil at 1.0 mL/min flow rate for derivatization. The absorbance of the resultant solution was monitored spectrophotometrically at 515 nm for all metal inos, except for the detection of iron(II) at 715 nm.

The concentration of the metal ion was determined by measuring the peak height on the chromatogram. In the serum analysis, the aqueous sample was injected onto the analytical column after the pretreatment procedure was carried out as described below.

Sample Preparation for Serum Analysis

Sample solution (500 μL) was placed in a 50-mL stoppered Teflon centrifuge tube and 1.0 mol/L hydrochloric acid (250 μL) added. The solution was heated in a steam bath at about 80°C for 2 min. After cooling to room temperature, the solidified mixture was stirred with 20 (w/v) % trichloroacetic acid (250 μL) and centrifuged at 2000 g for 5 min. The supernate solution was filtered with a 0.45-μm Millipore filter, and the serum concentration was decreased to 1/2 compared to that of the original serum.

The filtered solution (200 μL) was treated with 1.2 mol/L sodium acetate (200 μL) in a 5-mL Teflon test tube. Just prior to injections, hydroxylamine hydrochloride (about 5 mg) was added to the resultant solution (400 μL) for the reduction of iron(III) to iron(II).

Preparation of Calibration Curves for Serum Analysis

The calibration curves were constructed by adding known amounts of metal standards (10 μg/mL) to the serum sample I solution (500 μL) that was treated with the procedues described above. By plotting the peak heights against the metal concentrations, the slopes of the calibration curves for the determination of zinc(II) and iron(III) in serum samples were obtained. The intercepts of the curves were obtained from the reagent blank test.

RESULTS AND DISCUSSION

Post-Column LC Using PAR for Several Metal Ions

The post-column LC methods have been studied for the determination of traces of some heavy metal ions by use of citrate buffer as the mobile phase and PAR as the derivatization agent.[10] We have attempted to develop a convenient and useful post-column LC method for serum analysis. Figure1 shows a typical chromatogram obtained for direct injection of a mixture containing 1.0 mg/mL of each of the metal ions according to the procedure described above. The elution peaks appeared at retention times of 1.80 min for copper(II), 3.75 min for zinc(II), 4.45 min for nickel(II), 7.75 min for cobalt(II), 14.00 min for iron(II, III), and 30.20 min for manganese(II).

No difference in retention time was observed between iron(II) and iron(III) under the experimental conditions. The zinc(II) peak partially coeluted with the nickel(II) one when they were separated with 0.340 mol/L tartrate buffer of any pH values lower than 3.45 (1:1 molar ratio of tartaric acid to tartrate).

For the purpose of this study, however, there is no problem of peak separation between zinc(II) and nickel(II) because of a negligible presence of nickel(II) in human serum. The effect of pH of mobile phase on retention times of the six metal ions was investigated over the range of pH 3.45-4.53 under the constant concentration (0.340 mol/L) of tartrate buffers used.

Data shown in Fig. 2 indicate that the retention times gradually decreased with increasing pH of mobile phase. Indistinguishable peaks were observed among the five metal ions except manganese(II) when a 0.340 mol/L sodium tartrate buffer (pH 6.9) was used as the mobile phase.

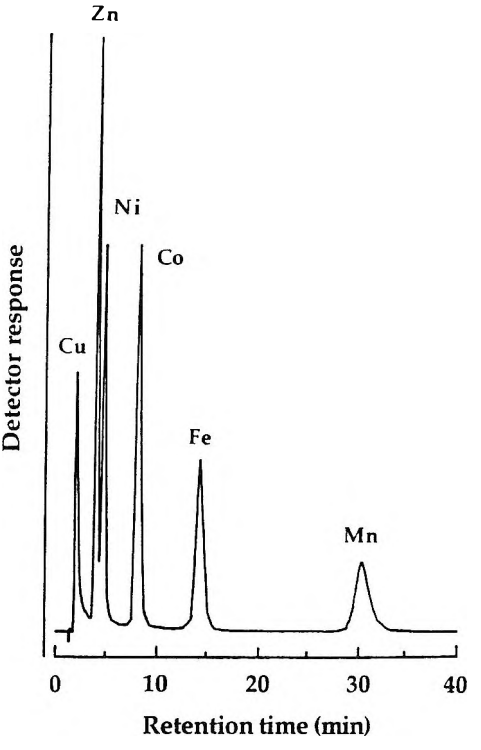

Figure 1. A typical chromatogram of some metal ions by post-column derivatization after LC separation. Concentration of metal ions:1.0 μg/mL each; mobile phase:0.340 mol/l tartrate buffer (pH3.75); derivatization solution:10^{-4} mol/l PAR solution; wavelength: 515 nm; flow rate: 1.0 mL/min; other LC conditions as in text.

The effect of concentration of tartrate buffer on the retention times was also investigated over the concentration of 0.280-0.370 mol/l at a constant pH of 3.75. It can be seen from Figure3 that the retention times gradually decreased with increasing concentration of tartrate.

The above results are understood in terms of the complex formation of metal tartrates onto the cation exchange column. We have selected a 0.340 mol/L tartrate buffer (pH 3.75) as being appropriate for the mobile phase.

The other experimental variables such as the concentrations of PAR and ammonia buffer, flow rates and column temperature were investigated, and the usual conditions were selected as described in the experimental section because

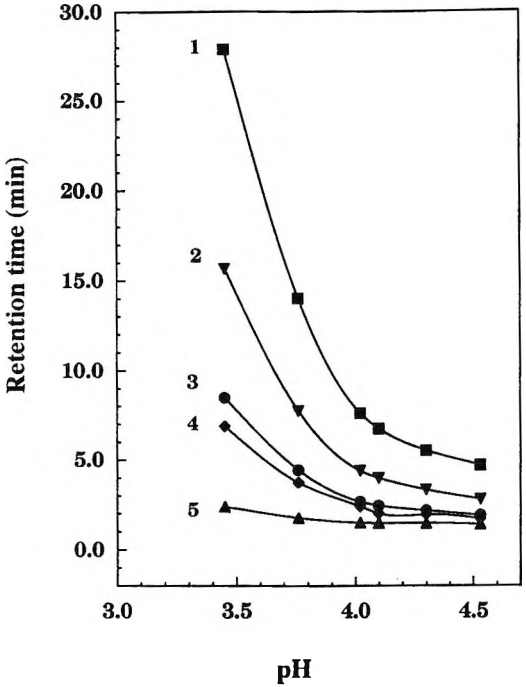

Figure 2. Effect of pH (mobile phase) on retention times. Total concentration of tartrate: 0.340 mol/L 1: Fe;2: Co; 3: Ni:; 4: Zn; 5:Cu; other conditions as in Fig.1.

they gave no serious effect on the peak heights of zinc(II) and iron(III). The iron(III) peak was relatively small and less producible in height compared with the others, and therefore, we have decided to get higher responses due to the iron(II) complex by reducing the iron(III) to iron(II) with the addition of hydroxylamine hydrochloride to samples just before injections.

Calibration Curves and Detection Limits

Calibration curves of peak height vs. metal concentration were constructed according to the procedure of the experimental section. Except for copper(II), peak heights were linearly proportional to metal concentration up to 2.0 μg/mL at correlation coefficients of above 0.998. The calibration curve for copper(II) was evaluated using the quadratic equation against the metal concentration ranging 0.1-1.0 mg/mL. It appears that the copper(II) species had an adsorption nature on the analytical column under the present conditions.

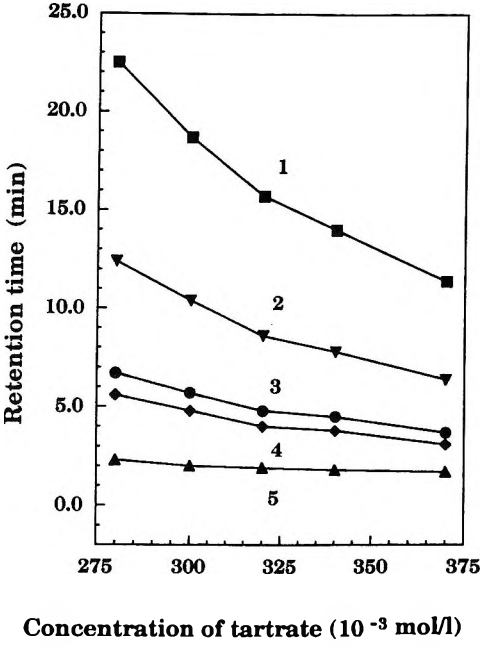

Figure 3. Effect of tartrate concentration on retention times. The pH of mobile phase: 3.75; other conditions as in Figs.1 and 2.

Accordingly, the determination of copper in serum by the proposed LC method was routinely unusable. The iron(II) complex at 715 nm had an absorbance of seven times higher than the corresponding iron(III) one at 515 nm. The detection limit for iron(II) at 715 nm attained to 0.01 μg/mL. The others at 515 nm were 0.01 μg/mL for zinc(II), 0.04 μg/mL for nickel(II) and cobalt(II), and 0.10 μg/mL for manganese(II).

Application to Serum Analysis

Based on the above results, the proposed LC method enables the five metal ions except copper(II) to be determined at least in the sub-μg/mL concentration levels. Control human sera (Wako I, II) were selected as real samples to evaluate the present post-column LC method. Taking into account the matrix effect of the serum samples, we have constructed the calibration curves for the determination of zinc(II) and iron(III) by applying the standard

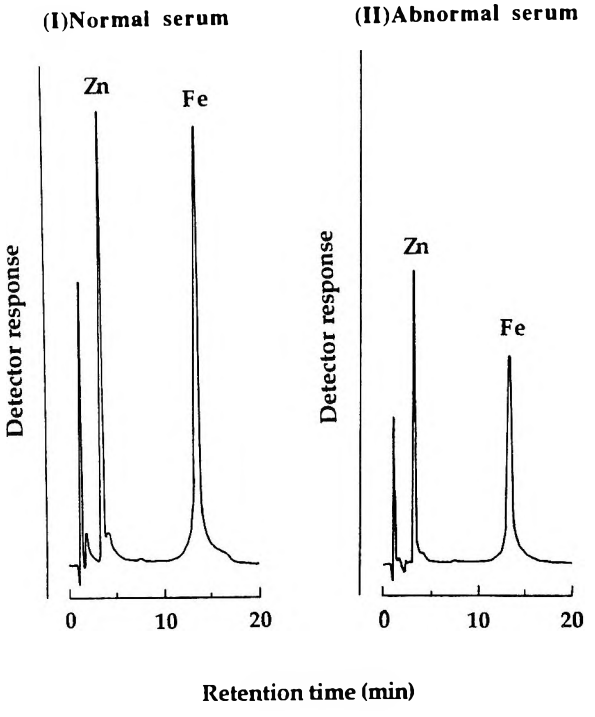

Figure 4. Determination of zinc and iron in control sera by the proposed LC method. (I) control serum I; (II) control serum II; the detections were carried out at 515 nm for zinc(II) and at 715 nm for iron(III).

addition method of analysis: known amounts of the two metal standards were added to serum sample solutions (Control serum I). The following equations were obtained by using the slopes obtained from the above experiments and the intercepts of the blank test: Y (peak height, arbitrary unit) = 53.2X (metal concentration, μg/mL) + 1 for zinc(II) and Y = 34.8X + 4 for iron(II), respectively. Fig. 4 shows the chromatograms obtained for Control sera I and II. The analytical results calculated from the peak heights, summarized in Table 1, were in good agreement with the recommended values obtained by spectrophotometry using bathophenanthroline (iron) and sodium 2-(5-bromo-2-pyridylazo)-5-(N-propyl-N-sulfopropylamino)-phenol (zinc). Therefore, the post-column LC method can successfully be applied to the simultaneous determination of zinc and iron in human serum.

Table 1

Analytical Results for Analyses of Control Sera (Wako)[a]

Sample	Metal	Concentration Found (μg/mL)	Certified Value (μg/mL)
Serum I	Zn	1.42 ± 0.02	1.34 ± 0.17
(Normal)	Fe	1.76 ± 0.07	1.80 ± 0.18
Serum II	Zn	0.84 ± 0.02	0.82 ± 0.14
(Abnormal)	Fe	0.67 ± 0.03	0.62 ± 0.07

[a] Five replicate determinations were made.

CONCLUSION

The present report presented a very useful LC method for the simultaneous determination of zinc and iron in serum. Since the method is rapid and accurate, it is recommended that this technique be used for routine analysis of biological samples as well as environmental ones.

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Received February 23, 1997
Accepted March 30, 1997
Manuscript 4372

J. LIQ. CHROM. & REL. TECHNOL., 20(15), 2367–2376 (1997)

DETERMINATION OF VANADIUM, COBALT, NICKEL, AND IRON IN BROMOPEROXIDASES FROM *PSEUDOMONAS PUTIDA* AND *CORALLINA PILULIFERA* BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH SPECTROPHOTOMETRIC DETECTION

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ABSTRACT

A highly sensitive, selective, and simple method for the determination of vanadium, cobalt, nickel, and iron has been proposed for the analysis of the enzymes. The method is based on the preliminary chelation of the metal ions with 2-(5-bromo-2-pyridylazo)-5-(diethylamino)phenol at pH 4.5 and the subsequent separation by reversed-phase high performance liquid chromatography using a trimethyl-bonded phase column and an aqueous acetonitrile containing tetrabutylammonium bromide, and spectrophotometric detection at 595 nm. The detection limits, defined as three times standard deviation of a blank signal, are 2.2 pg of vanadium, 0.72 pg of cobalt, 16.5 pg of nickel and 98.8 pg of iron in 100 mm^3-injection, respectively.

Vanadium, cobalt, nickel, and iron in the enzymes can be successfully determined without preseparation and preconcentration.

The proposed method is evaluated by comparing the analytical results with those obtained by inductively coupled plasma (ICP) atomic emission spectrometry and ICP mass spectrometry.

INTRODUCTION

Biological halogenation catalyzed by haloperoxidases has environmental, agricultural and pharmacological significance through their roles in the biosynthesis of volatile halogenated compounds,[1] antibiotics, and hormones.[2] So far, the enzymes catalyzing the halogenation have been considered heme iron-containing peroxidases.[3-5] However, a class of haloperoxidases discovered in marine algae[6,7] and fungi[8] has contained vanadium which has been identified by ESR.[9,10] Moreover, Itoh et al. found bromoperoxidase from *Pseudomonas putida* which has been activated by the incubation with cobalt ion.[11]

Thus, because some metal ions such as iron, vanadium, and cobalt function as the active site, the detection and the determination of the trace metals are of importance for clarifying the mechanism of the enzymatic reactions, and further investigation of metal enzymes will depend on the availability of a highly sensitive method for the metal determination.

Recently, reversed-phase high performance liquid chromatography (RP-HPLC) with spectrophotometric detection has been accepted as a powerful method for trace metal determinations.[12] One of the most convenient HPLC methods for metals is the pre-column derivatization method which is based on the preliminary chelation of the metal ions with a suitable chromogenic reagent such as azo dye compounds and the subsequent separation by HPLC and spectrophotometric detection.

2-(5-bromo-2-pyridylazo)-5-(diethylamino) phenol (5-Br-PADAP), among many azo dyes, is excellent in sensitivity for detection of metal ions, which has been employed for the simultaneous determination of vanadium, cobalt, nickel, and iron in high-purity silicon.[13] In this report, the HPLC method using 5-Br-PADAP is applied to the enzyme analysis.

EXPERIMENTAL

Reagents

2 x 10^{-3} M 2-(5-bromo-2-pyridylazo)-5-(diethylamino)phenol (5-Br-PADAP) solution was prepared as follows: a 0.175-g portion of 5-Br-PADAP (Dojindo Laboratories, Kumamoto, Japan) was dissolved into a 25-g portion of poly(oxyethylene) 4-nonylphenyl ether with 10 oxyethylene units (PONPE-10) (Tokyo Chemical Industry, Tokyo, Japan) with warming on a water bath and stirring; after the solution was diluted with about 150 cm^3 of water with warming and gently stirring, the resultant solution was transferred into a 250-cm^3 calibrated flask and made up to the mark with water. The surfactant, PONPE-10 was employed for solubilization of 5-Br-PADAP and its chelates. Standard solutions of metal ions were 100 mg dm^{-3} or 1000 mg dm^{-3} solution for atomic absorption spectrometry (Kanto Chemical, Tokyo, Japan) and were accurately diluted by keeping 0.2 M acid concentration.

A pH 4.5 acetate buffer solution was prepared by dissolving sodium hydroxide in 2 mol dm^{-3} acetic acid while measuring pH of the solution. The mobile phase was 46 or 48 %w/w aqueous acetonitrile solution containing 2 x 10^{-3} or 3 x 10^{-3} mol kg^{-1} of tetrabutylammonium bromide (TBA), 5 x 10^{-3} mol kg^{-1} of sodium acetate, and 10^{-4} mol kg^{-1} of ethylenediamine-tetraacetic acid (EDTA). EDTA acts as a masking agent for the metal contaminant from the HPLC apparatus. Distilled, deionized water was purified with a Millipore Milli-Q system.

Apparatus

The chromatographic system consisted of a Shimadzu LC6A pump, a Rheodyne 7125 injector with a 100-mm^3 sample loop and a Shimadzu SPD-6AV variable-wavelength spectrophotometric detector with a 10 mm flow-through cell. A Cosmosil 5TMS column (trimethyl-bonded phase column, 4.6 mm x 250 mm, Nacali Tesque, Kyoto, Japan) was used.

For measurement of the absorption spectra of the eluted chelates, a Shimadzu SPD-M10AVP photodiode-array detector was used, together with its associated software package. Sample weighing was performed with a Zartorius L420S electronic balance (readability: 1 mg).

Recommended Procedure

A sample solution (1-g portion) was accurately weighed into a 30-cm^3 screw capped Teflon bottle on an electronic balance. To the solution, 1 cm^3 of 2 x 10^{-3} M 5-Br-PADAP solution and 2 cm^3 of pH 4.5 acetate buffer solution were added. The whole mixture was diluted to 10 g with water on the balance and the weight of the resultant solution (W) was accurately weighed. The whole solution was tightly closed and heated on a boiling-water bath for 30 min and then cooled. Before and after heating, the weight of the solution (W) did not vary. The test solution, thus obtained, was injected with a 100-mm^3 loop injector onto the column. The rate of the mobile phase was 1.0 cm^3 min^{-1}. Chromatograms were obtained by measuring the absorbance at 595 nm.

The peak height (in mV and absorbance unit) of the chelates detected on the chromatograms was normalized, with dilution factor (W/10) calculated from the accurate weigh (W) of the test solution, i.e. (mV or absorbance unit) x W/10. The normalized values were used for determining the concentration of the metal ions.

Purification of Enzyme

Bromoperoxidase from *Corallina pilulifera* was purified from the crude extract with ammonium sulfate fractionation and the subsequent column chromatography, using a DEAE-Sepharose CL, a Sepharose 6B, and a Cellulofine GC-700m according to a previous report.[14] Bromoperoxidase, from *Pseudomonas putida* was also purified by the method previously reported.[11] Both solutions containing enzyme, were dialyzed in a cellulose-tubing for 2 days against several changes of water; the dialysis-inside solutions were used as samples to be analyzed and the outside solution containing no enzyme, was used as a blank.

Preparation of Enzyme Sample Solution

4-cm^3 of the inside solutions containing an enzyme and 4-cm^3 of the outside solution (as dialysis blank) obtained through the dialysis, were respectively heated with 4 cm^3 of concentrated nitric acid in a Teflon bottle on a hot-plate, nearly to dryness and then, each residue, with one drop of concentrated nitric acid, was diluted with water to 5-g on the electronic balance with accurate weighing. One-g portion of each solution, which was accurately weighed, was analyzed by the recommended procedure.

RESULTS AND DISCUSSION

Derivatization of Metal Ions with 5-Br-PADAP

The complexation of vanadium(V), cobalt(II), nickel(II), and iron(III) with 5-Br-PADAP was quantitative at pH 4.5 with acetate buffer solution.[15] The cobalt(II) chelate is easily oxidised to the cobalt(III) chelate which gives a net charge of +1 on the chelate. The test solution should be heated on a boiling-water bath to reduce the time of the derivatization since the color development of vanadium(V) and nickel(II) chelates with 5-Br-PADAP is slow; the heating time was fixed at 30 min.

Separation and Detection of 5-Br-PADAP Chelates

Among vanadium(V), cobalt(II), nickel(II), iron(III), cadmium(II), copper(II), zinc(II), manganese(II), and aluminum(III), four chelates, vanadium(V), cobalt(II) (as cobalt(III) chelate), nickel(II), and iron(III) (as iron(II) chelate as described below) chelates, gave well resolve peaks using an aqueous acetonitrile mobile phase independent of the addition of TBA. Here it was found that, even though the iron(III) chelate was injected onto the column, an eluted-iron chelate was the iron(II) chelate although it is not clear why iron(III) is reduced to iron(II). Because the spectrum of the eluted iron chelate, observed using a photo diode-array detector, agreed with that of iron(II) chelate, which has two maxima at 558 and 748 nm, measured with a conventional spectrophotometer; while iron(III) chelate has one maximum at 595 nm. The chelates of metal ions, such as copper(II) and zinc(II), decomposed on the column during elution and gave no peaks on the chromatogram; the chelates such as cadmium(II), manganese(II), and aluminum(III) chelates do not form at given condition, pH 4.5. As a result, these foreign metal ions did not interfere with the detection of vanadium(V), cobalt(II), nickel(II), and iron(III).

When TBA was absent in an acetonitrile-water mobile phase, the chelates eluted in the order of vanadium(V), nickel(II), iron(II), and cobalt(III) chelates and especially cationic cobalt(III) chelate, gave rise to slow elution. Since it took about 60 min to elute the cobalt(III) chelate, the chelate gave a broadened peak and poor detection. On the other hand, as TBA was added to the mobile phase, TBA constrained the cobalt(III) chelate to be eluted before the nickel(II) chelate and ensured highly sensitive for cobalt.[13] In the present method, the

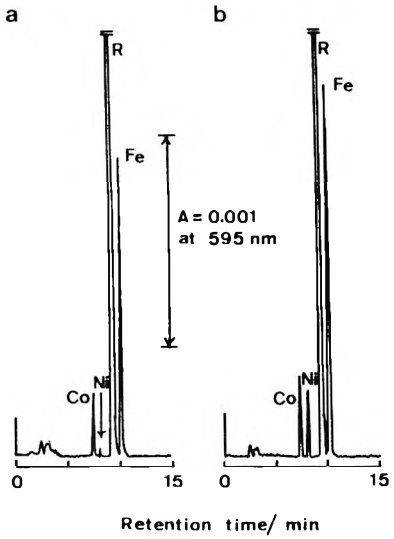

Figure 1. Analysis of bromoperoxidas from *Pseudomonas putida*. Mobile phase: 48 %w/w aqueous acetonitrile containing $3x10^{-3}$ mol kg^{-1} TBA;a: blank (outside solution); b: sample (inside solution containing enzyme);R: free 5-Br-PADAP.

cobalt(III) chelate could be eluted within 8 min and it took only 12 min per one run. TBA is adsorbed to the reversed phase(RP) stationary support based on the ion exchange and hydrophobic interaction mechanisms; from those results, the ion exchange adsorption of the cationic cobalt(III) chelate is prevented by preferential ion exchange of TBA cation with silanol proton and the chelate is excluded from the polar stationary phase, the surface of which is mostly covered by TBA cation.[16] Since the retention time of the cobalt(III) chelates decreased with increasing the concentration of TBA[13] and TAB was dynamically retained onto the RP stationary phase,[16] the concentration of TBA in the mobile phase should be kept at constant to preserve the uniform retention of TAB on the stationary support.

The retention time of all chelates also decreased with increasing acetonitrile concentration. The eluting conditions were optimized in view of the compromise between peak resolution and total elution time. The separation of each chelate from other chelates was optimized by varying the concentration of acetonitrile and the retention time of the cobalt(III) chelate was adjusted by changing TBA concentration.

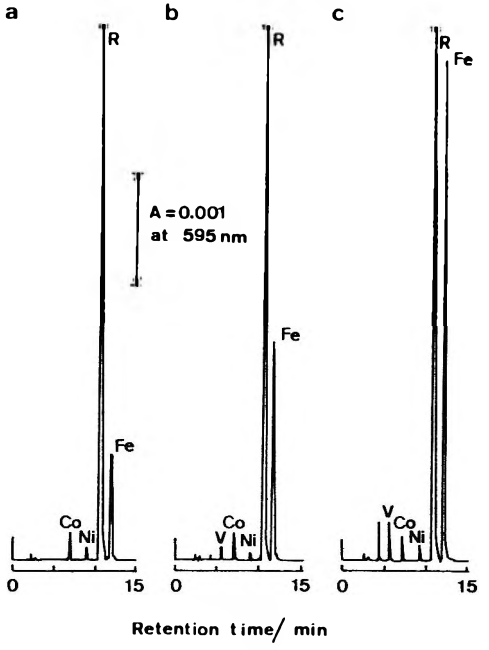

Figure 2. Analysis of bromoperoxidases from *Corallina pilulifera*. Mobile phase: 46 %w/w aqueous acetonitrile containing $3x10^{-3}$ mol kg^{-1} TBA;a: blank (outside solution); b: sample 1 (inside solution 1 containing enzyme);c: sample 2 (inside solution 2 containing enzyme); R: free 5-Br-PADAP.

The concentrations of acetonitrile and TBA in a mobile phase were chosen in the range from 46 to 48 %w/w acetonitrile and the range from 2 x 10^{-3} to 3 x 10^{-3} mol kg^{-1} TBA, respectively. Addition of TBA to the mobile phase should serve to establish simultaneous and rapid HPLC determination for these chelates.

When a 100-mm^3 aliquot of a test solution was injected onto the column with 46 %w/w or 48 %w/w acetonitrile mobile phase containing 3 x 10^{-3} mol kg^{-1} of TBA, the peak height calibration curves (0.005 absorbance unit full scale) of vanadium(V), cobalt(II), nickel(II), and iron(III) linear up to 300 pg of vanadium(V), 160 pg of cobalt(II), 2400 pg of nickel(II), and 1500 pg of iron(III) in 100 mm^3-injection, respectively. When 80 pg of vanadium(V), 40 pg of cobalt(II), 240 pg of nickel(II), and 1000 pg of iron(III) were injected and determined, the relative standard deviations for three determinations of vanadium(V), cobalt(II), nickel(II), and iron(III) were 2.8 %, 1.1 %, 4.3 % and 0.8 %, respectively.

Table 1

Determination of Vanadium and Iron in *Corallina pilulifera*

Sample No.		Concentration of Metal Ions, ng/cm^3-Dialyzed Solution (mol/mol/enzyme) This Method	ICP-AES*	ICP-MS**
1	V	4.5 ± 0.5 (0.42)	3.0 (0.28)	3.1 (0.29)
	Fe	97 ± 17 (8.3)	101 (8.7)	64 (5.5)
2	V	12.0 ± 0.8 (0.53)	11.0 (0.49)	13.0 (0.58)
	Fe	320 ± 4 (12.9)	266 (10.8)	198 (8.0)

* inductively coupled plasma atomic emission spectrometry.
** inductively coupled plasma mass spectrometry.

The detection limits, defined as the concentration corresponding to 3 times the standard deviation of the reagent blank, were 2.2 pg of vanadium(V), 0.72 pg of cobalt(II), 16.5 pg of nickel(II), and 98.8 pg of iron(III) in a 100-mm^3 injection, respectively, when elution conditions were fixed at 46 %w/w acetonitrile and 2 x1 0^{-3} mol kg^{-1} TBA.

Analysis of Enzyme

The present method was applied to the determination of vanadium, cobalt, nickel, and iron in the bromoperoxidases from *Pseudomonas putida* and *Corallina pilulifera*. A 1-g portion of the solution treated with nitric acid was analyzed by the recommended method.

Figure 1 shows typical chromatograms obtained in the analysis of *Pseudomonas putida* by the proposed method. As shown in Fig. 1-a, even if no metal ion was added to the test solution as a blank, the peaks corresponding to cobalt(III), nickel(II), and iron(II) chelates were caused on the chromatogram. This is mainly attributed to the contaminant from the reagents

used. The chromatogram was used as the blank chromatogram. In 1 cm^3 of the dialyzed solution, 1.3 ng of cobalt, 54.6 ng of nickel and 6.7 ng of iron were found, respectively, and vanadium was not detected. From these results, we would reveal a novel class of bacterial bromoperoxidase.[11]

Figure 2 was obtained from analyses of the blank solution(a) and of the bromoperoxidases (b: sample 1, c: sample 2) from *Corallina pilulifera*. Table 1 gives the results of determination of vanadium and iron in the bromoperoxidases from *Corallina pilulifera*. For comparison, the data obtained with inductively coupled plasma (ICP) atomic emission spectrometry and ICP mass spectrometry (MS) are also listed. Vanadium and iron in the enzymes can be successfully determined by the proposed method.

The proposed method is one of the most sensitive methods for vanadium and cobalt; the detection limits of both metal ions are comparable with or lower than those reported for ICP-MS which were 23 - 30 pg cm^{-3} for vanadium and 0.49 - 10 pg cm^{-3} for cobalt , respectively.[17,18] The method here provides a selective and sensitive determination of vanadium, cobalt, nickel, and iron, and is applicable to the analysis of various enzymes.

ACKNOWLEDGMENT

The authors thank Mr. K. Ohashi and Ms. R. Ueda of Seiko Instruments Inc., for the determination of metal ions with ICP-AES and ICP-MS.

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Received November 9, 1996
Accepted November 30, 1996
Manuscript 4323

J. LIQ. CHROM. & REL. TECHNOL., 20(15), 2377–2387 (1997)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH PEROXYOXALATE CHEMILUMINESCENCE DETECTION OF SYNTHETIC PEPTIDE, EBIRATIDE

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ABSTRACT

Ebiratide [L-methionyl-L-glutamyl-L-histidyl-L-phenyl alanyl-D-lysyl- N-(8-aminooctyl)- L- phenylalanin -amide-S,S-dioxide] is one of the synthetic analogues of ACTH4-9 for Alzheimer disease. High performance liquid chromatography with peroxyoxalate chemiluminescence detection (HPLC-PO-CL) has been examined for the analysis of this compound. The fluorogenic reaction product of ebiratide with DBD-F [4-(N,N-dimethylaminosulphonyl)-7-fluoro-2,1,3-benzoxadiazole] was detected by the chemiluminescence generated with bis(2,4,6-trichlorophenyl)oxalate (TCPO) or bis[4-nitro-2-(3,6,9-trioxadecyloxycarbonyl)phenyl]oxalate (TDPO) in the presence of hydrogen peroxide. TDPO gave about five times higher CL intensity than TCPO using 100mM hydrogen peroxide. The

detection limit of DBD derivative of ebiratide was 25 fmol on column with TDPO. The HPLC-PO-CL was applied to the determination of ebiratide extracted from rat plasma by the solid phase extraction with Bond Elute C18. The method gave a good linearity over the range from 0.25 to 10 pmol on column with the recovery of 85% for the spiked rat plasma and was able to estimate the time course of ebiratide at a sub-pmol/mL level.

INTRODUCTION

A specific and sensitive determination method for trace amount of drug substance is important in the development of pharmaceutical products. Such a method is also important for the assay of clinical samples. For example, decrease in the amount of blood needed for biochemical examination will give great advantages for patients.

Ebiratide is produced by Hoechst AG as a therapeutic drug relating to the brain functional learning and memory.[1] This compound was injected up to 600μg/day in clinical studies.[2] Its concentration in the blood was expected to be at a low level; consequently, it has been necessary to develop a specific and sensitive determination method.

We have reported HPLC-fluorescence detection of ebiratide using precolumn derivatization with DBD-F,[3,4] and achieved the detection limit of 250 fmol on the column.[5] This paper describes the application of HPLC-PO-CL method to the determination of a fluorescent DBD-ebiratide with the combination of a solid phase extraction (sorbent extraction) for the rat plasma after administration (i.v.).

EXPERIMENTAL

Chemicals

Ebiratide was synthesized by Hoechst AG (Frankfurt, Germany). DBD-F and imidazole (fluorometric grade) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). TCPO, TDPO, hydrogen peroxide (30%), and nitric acid were purchased from Wako Pure Chemical Industries (Tokyo, Japan). Acetonitrile, methanol, and distilled water for HPLC grade, were obtained from Kanto Chemicals Industries (Tokyo, Japan). Other chemicals were of analytical reagent grade.

Standard Solutions

Standard solutions to validate the method were prepared as follows; appropriate amounts of ebiatide were dissolved in water to obtain a 100 μM solution. The solution was diluted with 0.1 M borate buffer solution (pH 9.0) to obtain 10 pM to 10 μM of ebiratide stock solutions. Appropriate amounts of stock solutions were added to 400 μL of plasma to obtain spiked plasma samples of 0.5 to 50 ng/mL. Control solutions were prepared with 400 μL of water in the same manner.

Extraction Procedure of Ebiratide from Plasma

A rat plasma sample (400 μL) was diluted with 800 μL of water and applied to a cartridge of Bond Elute C18 column containing 100 mg of ODS (Varian Associates, Inc., Harbor City, CA, USA), which was activated in advance by a successive washing with each 2 mL of methanol and water. After the matrix was washed out with 1 mL of water, ebiratide was eluted and collected into a polypropylene reaction tube (2 mL, ASSIST, Tokyo, Japan) containing 1 mL of 0.005M HCl (in 30% aqueous methanol). The eluent was dried by a vacuum centrifuge freeze dryer (Tokyo Rika Co. Ltd., Tokyo, Japan), and the resultant residue was redissolved in 100 μL of 0.1M borate buffer solution (pH 9.0) for fluorescence derivatization.

Fluorescence Derivatization of Ebiratide with DBD-F

The sample solution and 100 μL of DBD-F acetonitrile solution were mixed in the reaction tube and kept standing at 50°C for 30 min. The resultant mixture was used for HPLC-PO-CL analysis.[5]

HPLC-PO-CL Apparatus

The system (Fig.1) consists of a Hewlett Packard Model 1090L HPLC system with a Vydac protein & peptide column 5C18 (150 x 4.6 mm I.D., The Separations Group, Hesperia, CA, USA), for analytical separation with an injection amount of 100 μL and a flow rate of 1.0 mL/min, a JASCO BIP-100 pump (Japan Spectroscopic Co. Ltd., Japan) for the delivery of chemiluminescence reagent through an Inertsil column ODS (150 x 4.6 mm

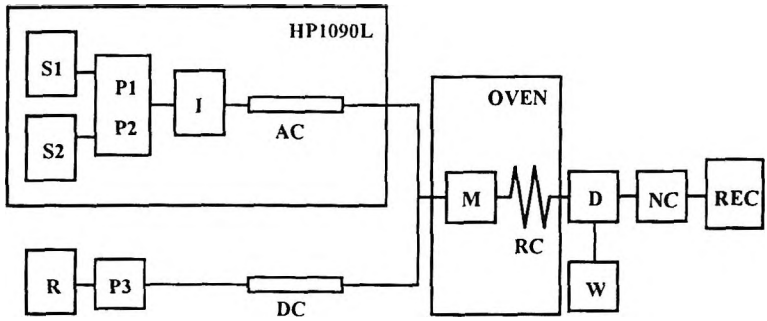

Figure 1. Schematic diagram of HPLC-PO-CL system. S1:50mM Imidazole nitrate (pH6.0):organic solvent*(3:2v/v), S2: 50mM Imidazole nitrate (pH6.0); organic solvent*(2:3v/v),; R, PO-CL reagent consisting of oxalate/hydrogen peroxide; P1, P2 and P3, pumps; I, injector; AC, analytical column; DC, dummy column; M, tee mixer; RC, reaction coil; OVEN was maintained at 30°C; D, detector; NC, noise cleaner; REC, recorder; W, waste. *organic solvent is a mixture of acetonitrile and methanol (2:1v/v)

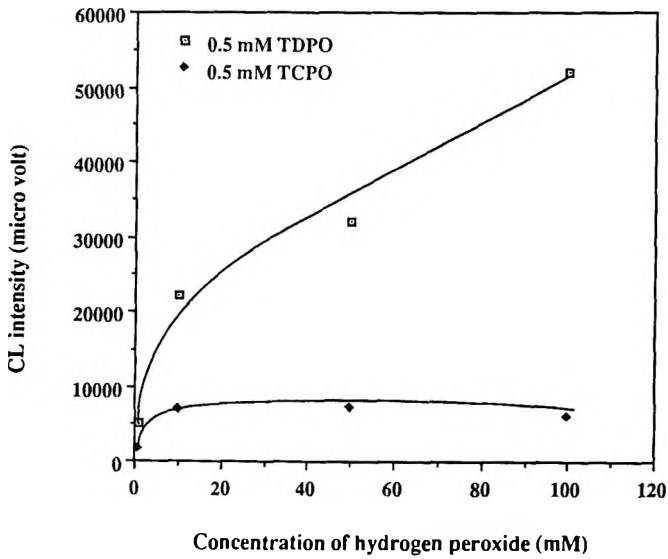

Figure 2. Effect of the hydrogen peroxide concentration on chemiluminescence. CL intensity was obtained with injected 2.5 pmol of ebiratide using 0.5 mM oxalate.

Table 1

Chemiluminescence Signal and Noise from Ebiratide in TDPO or TCPO System*

Oxalate	H_2O_2[mM] [mM]	Signal [micro volt]	Noise [micro volt]	Signal/ noise
	100	52140	202	258
TDPO	50	32140	165	195
system	10	22164	43	237
	1	4942	35	258
	100	5908	41	144
TCPO	50	7161	63	116
system	10	6893	40	171
	1	1734	44	39

*Each system consists of 2.5 mM TDPO or TCPO and Hydrogen peroxide. DBD-Ebirate was injected pmol as ebiratide on column. HPLC conditions: a flow rate of 1.0 mL/min using a mixture of solvent S1 and S2 (50:50). The flow rate of PO-CL reagent was maintained at 1.5 mL/min.

I.D., GL Science, Tokyo, Japan) for pressure control with a flow rate of 1.5 mL/min. A JASCO 825CL chemiluminescence detector (Japan Spectroscopic Co. ltd.) and a Type 3066 Pen recorder (Yokogawa Hokushin Electric, Tokyo, Japan) with a noise cleaner UNI-1(UNION, Tokyo, Japan).

RESULTS AND DISCUSSION

Chemiluminescence Intensity with TCPO and TDPO

Using 0.5 mM TCPO or TDPO and 1,10,50, and 100 mM hydrogen peroxide, the effect of the concentration of hydrogen peroxide on the PO-CL intensities of DBD-ebiratide derivative was studied. As shown in Fig.2 and Table 1, both the CL intensities of DBD-ebiratide and the noise in a TDPO system increased with the concentration.

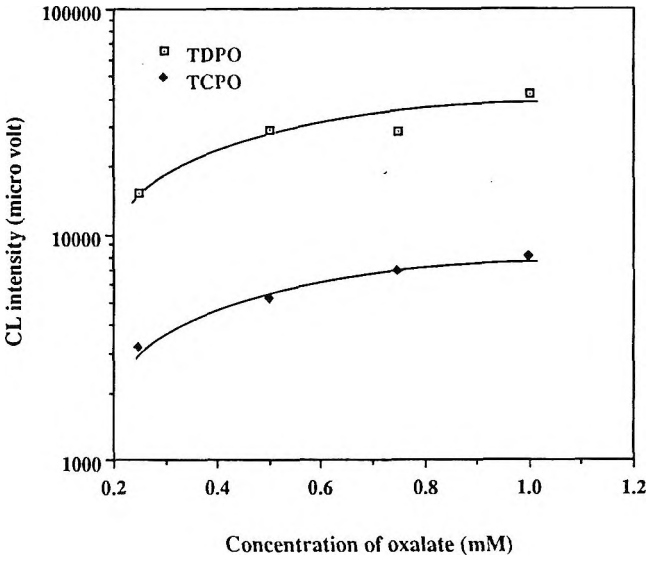

Figure 3. Effect of the oxalate concentration on chemiluminescence intensity. CL intensity was obtained with injected 2.5 pmol of ebiratide using 100 mM hydrogen peroxide.

On the other hand the noise level in a TCPO system was almost constant from 1 to 100 mM investigated. Figure 3 shows the effect of the concentration of oxalate on chemiluminescence intensity. DBD-ebiratide in a TDPO system gave about 5 times higher CL intensity than that in a TCPO system over the range of 0.25 to 1.0 mM of oxalates with 100 mM hydrogen peroxide.

HPLC-PO-CL detection is based on the reaction of aryloxalate with hydrogen peroxide, followed by the generation of dioxetanedione transferring its energy to fluorescent compounds to yield emission. Although both TCPO and TDPO might generate dioxetanedione sufficiently to excite the fluorescent compound injected, and thus was expected to give the same CL intensity, there were differences in both their signal and back ground noise of chemiluminescence.

The results showed that chemiluminescence generated in a TCPO system decreased with the high concentration of hydrogen peroxide, while in a TDPO system such a depression of chemiliminescence was not observed. This phenomenon was reported by Orlovic et al.[6] on the chemiluminescence from 9, 10-diphenylanthracene using TCPO, hydrogen peroxide and imidazole.

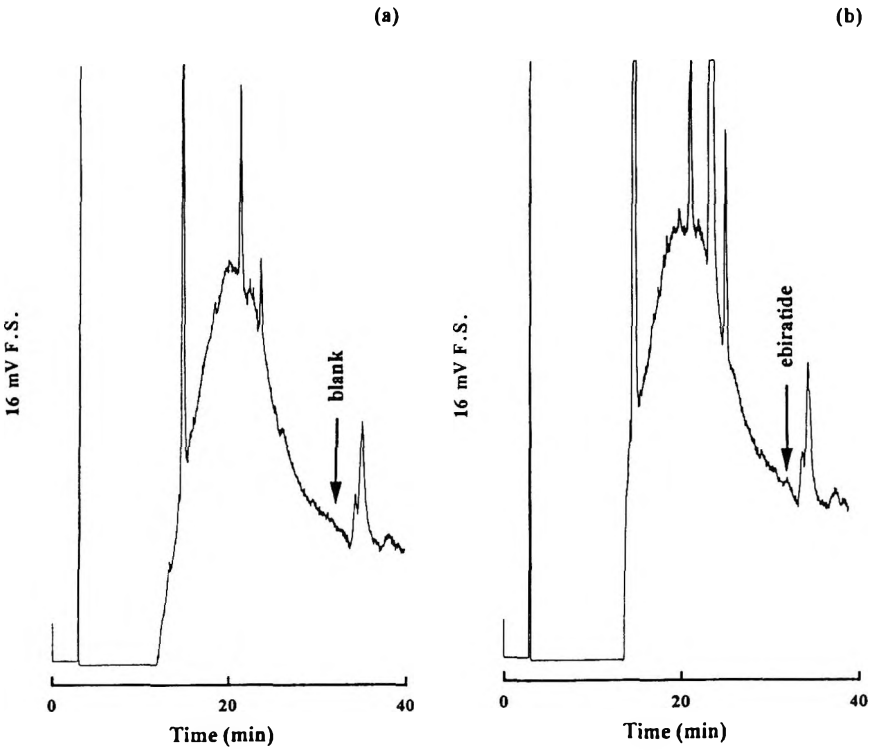

Figure 4. HPLC-PO-CL chromatogram of ebiratide-DBD. (a) Blank, and (b) ebiratide 25 fmol. HPLC conditions: Linear gradient at a flow rate of 1.0 mL/min using a mobile phase starting with a 100:0 mixture of solvent S1 and S2 changing to the mixing ratio to 55:45 and 30:70 in 15 and 60 min respectively, followed by 15 min initialisation with S1. The flow rate of PO-CL reagent was maintained at 1.2 mL/min.

It was reported that the PO-CL reaction with DFPO gave the reaction products of its hydroxyperoxy oxalate ester, half ester, and phenol.[7] TCPO was also considered to form such components, especially 2,4,6-trichlorophenol, which decreases PO-CL intensity by easily getting the energy of dioxetanedione. The results suggested that the differences in CL intensities contributed to the decomposed products from those oxalates. In practical use of HPLC-PO-CL a high intensity of chemiluminescence was required to obtain a high sensitivity.

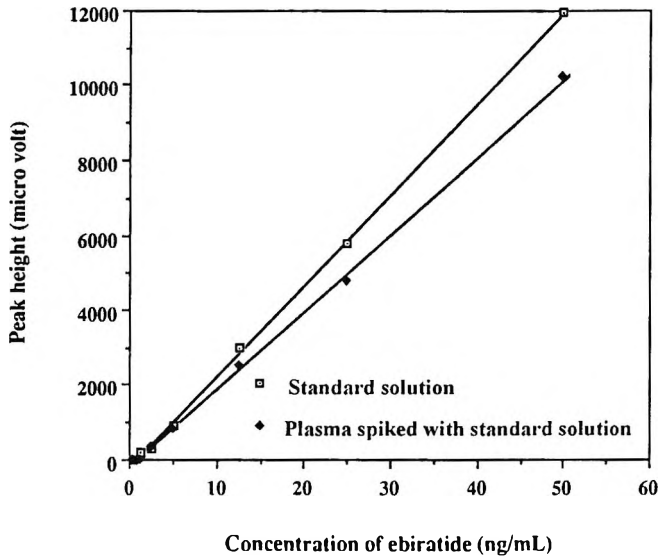

Figure 5. Absolute recovery study of ebiratide.

Therefore, the PO-CL reagent for ebiraitde was finally decided to consist of 0.5 mM TDPO and 100 mM hydrogen peroxide. TDPO was actually a preferable oxalate for this study to give the high sensitivity with the detection limit of ebiratide of 25 fmol on column (signal-to-noise ratio of 2) as shown in Fig. 4 (a) and 4 (b).

Biomedical Application of HPLC-PO-CL

In order to extract the peptide from plasma sample, sorbent extraction with Bond Elute C18 using 0.005 mol/L HCl in aqueous methanol as an extracting solvent was applied. The peptide is known to be stable around pH 3.[5] The residue obtained by centrifuge freeze drying was redissolved in 100 μL of 0.1 mol/L borate buffer solution for fluorescence derivatization.

The absolute recovery of ebiratide was calculated by the ratio of the slopes of linear regression lines obtained from the standard solution ($y = 242.66x - 198.46$, $r^2 = 0.999$, $n = 12$) and the plasma spiked with standard solutions treated with Bond Elute C18 sorbent extraction ($y = 206.40x - 190.67$, $r^2 =$

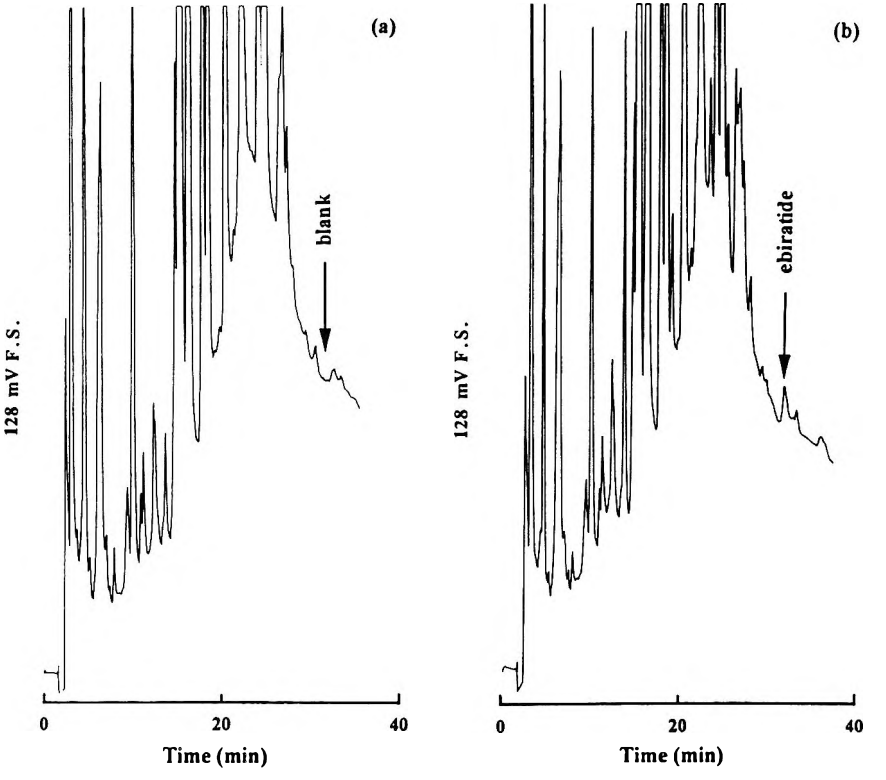

Figure 6. HPLC-PO-CL chromatogram of ebiratide-DBD. (a) Blank sample extracted from control rat plasma. and (b) ebiratide extracted from rat plasma sample (5 min after administration). HPLC conditions: Linear gradient at a flow rate of 1.0 mL/min using a mobile phase starting with a 100:0 mixture of solvent S1 and S2 changing to the mixing ratio to 55:45 and 30:70 in 15 and 60 min respectively, followed by 15 min initialisation with S1. The flow rate of PO-CL reagent was maintained at 1.2 mL/min.

0.999, n = 12) (Fig. 5). This method gave a good linearity over the range from 0.25 to 10 pmol on column with the recovery of 85% for the spiked rat plasma. Ebiratide was administered (i.v.) at the dose of 10 mg/kg to SD rat (body weight of 500 g, n = 3).

The blood was collected at 5, 30, 60, and 120 min after injection and centrifuged at 3000 rpm for 10 min at 5°C to collect plasma. Each sample was derivatized according to the procedure mentioned in the EXPERIMENTAL

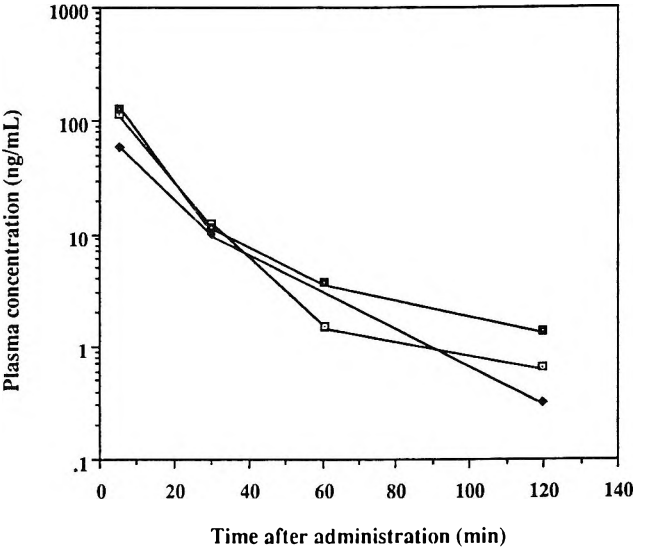

Figure 7. Plasma concentration of ebiratide (10mg/kg, i.v., n=3).

and analyzed by HPLC-PO-CL. As shown in Figs. 6 (a) and 6 (b), DBD-ebiratide was separated from the components of control plasma sample. Figure 7 shows the time course of the mean plasma concentration of ebiratide after injection.

It was found that HPLC-PO-CL, with the combination of sorbent extraction, was able to be applied to the determination of ebiratide in the rat plasma at the concentration of 0.25 pmol on column. On the other hand, the detection by the HPLC-Fluorescence method[5] gave 10 pmol on column under the same HPLC separation conditions as the HPLC-PO-CL method.

CONCLUSIONS

Although radioimmunoassay is known as a highly sensitive analytical method for ebiratide,[8] it requires specific antibodies and special facilities and is restricted in its application. The application of HPLC with fluorescence detection to plasma sample was difficult because of its deficiency in sensitivity.[5] On the other hand, the proposed HPLC-PO-CL is a very specific and sensitive method for the determination of ebiratide with the detection limit of 25 fmol on

column. The sensitivity is 10 times higher than that of HPLC-fluorescence detection.[5] The proposed HPLC-PO-CL method has successfully been applied to the determination of ebiratide in rat plasma with sorbent extraction which would be useful to the other peptide analyses.

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Received December 20, 1996
Accepted January 31, 1997
Manuscript 4363

J. LIQ. CHROM. & REL. TECHNOL., 20(15), 2389–2397 (1997)

SIMULTANEOUS SEPARATION AND DETECTION OF TEN COMMON FAT-SOLUBLE VITAMINS IN MILK

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ABSTRACT

A method for the separation of ten fat-soluble vitamins on a reversed phase C_{18} column with UV detection is described. The choice of a suitable organic solvent in conjunction with an aqueous phase composition is important in achieving a good separation of the ten common fat-soluble vitamins which include different forms of vitamins A, D, K and α-tocopherol (E). Two different mobile phases were developed for the isocratic separation of the vitamins. It was found that the separation of the ten vitamins is very sensitive to a small change in the composition of the mobile phase. The method was applied to determine the vitamins in milk sample, after sample treatment which includes saponification, drying, and reconstitution of the vitamins with the mobile phase.

INTRODUCTION

Most of the vitamin comes to us through the natural and fortified processed foods. Fish oils and egg yolks are rich natural sources of vitamins A and D. Vitamin A exists in three oxidation states. Some have different isomeric forms which are important in biochemical functions. Vitamin D is usually produced in our own tissues. Different forms of vitamin D exist upon thermal activation and non enzymatic process. Vitamin E (atocopherol) is produced by plants that enter our food supply, such as vegetable oils.

On the other hand, the fat-soluble vitamin Ks are obtained from plant, animal, and bacterial sources. The two forms of vitamin K, phylloquinones (K_1) and menaquinones (K_2), provide roughly half each of the amounts absorbed into the human body and stored in the liver. Menadione (K_3) is frequently used as an electron carrier to oxygen. Vitamin K_3 is also a cofactor of cyclic photophosphorylation.

High performance liquid chromatography (HPLC) with UV or fluorescence detection, is a common analytical method widely used in the qualitative and quantitative determination of vitamins in physiological samples and foodstuffs. These methods have been reviewed recently.[1-2] Microcolumn HPLC with multi-channel UV-VIS detection for the determination of vitamins in foodstuffs have been reported.[3-5] Specific methods for analysing individual vitamins are available with fluorimetric detection[6-7] or electrochemical methods.[8-9] Ion exchange chromatographic method for assay of vitamins is available as well.[10] Other methods, such as specific enzyme-linked ligand-sorbent assay of vitamins in human plasma and urine, have recently been reported.[11]

In addition, automated methods for the determination of a particular vitamin in human sera have been described.[12-13] These HPLC methods are good for the determination of individual vitamins or a group of vitamins. It would be more economical and time-saving to simultaneously determine as many vitamins as possible in an analysis.

In this paper, an HPLC method is described for separating ten common fat-soluble vitamins. The method was then applied to measure the vitamins in milk sample. The milk sample requires simple procedural modifications of the sample treatment process, which includes saponification, evaporation, and reconstitution of the vitamins prior to HPLC analysis.

EXPERIMENTAL

Chemicals

All-trans vitamin A (alcohol, aldehyde, acid), 13-cis vitamin A acid, D_2, D_3, E (α-tocopherol) and K_1, K_2, as well as K_3 were purchased from Sigma (MO, USA). HPLCgrade ethanol, acetonitrile, and tetrahydrofuran were obtained from Riedel-de Haen AG (Germany). Methanol and n-hexane were purchased from Mallinckrodt Specialty Chemicals (Kentucky, USA). Isopropanol was purchased from AJAX Chemicals (Australia). Other chemicals were of analytical grade.

Apparatus

Experiments were performed on a modular liquid chromatograph equipped with a Rheodyne 7126 injector fitted with a 20-μL loop. An analytical C_{18} column (particle size 5 μm, 25 x 0.46 cm) (Alltech, CA, U.S.A.) and a model UV-1 variable wavelength spectrophotometer (Rainin) with a 12-μL flow-cell attachment were used. Chromatograms were recorded with a Hewlett-Packard 3396 II integrator. A high precision solvent-delivery system SSI 300LC (Alltech, CA, U.S.A.) was used.

Chromatographic Conditions

The eluent was monitored at 250 nm for UV detection. The flowrate was set at 1.0 mL/min. The isocratic reversed phase HPLC separation of vitamins was carried out using a mobile phase that contains ethanol and methanol (20: 80, v/v). The separation could also be achieved with another mobile phase which is composed of ethanol and water (95: 5,v/v).

Extraction of Vitamin Ds

A milk sample (50 mL) was mixed with 30 mL of ethanolic potassium hydroxide (10: 30, v/v). Ethanolic potassium hydroxide solution was prepared by the previous method.[14] The mixture was saponified at 80°C for 20 min based on a previous procedure.[15] The vitamins from the saponified mixture were 2x extracted with 10 mL of n-hexane. The n-hexane extract was evaporated to dryness. The dried extract was reconstituted with 1.0 mL of the mobile phase. An aliquot (5 μL) was injected onto HPLC for analysis.

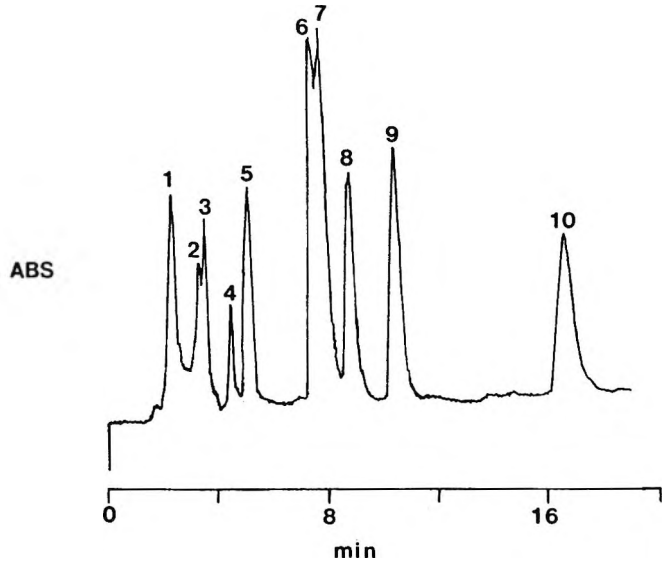

Figure 1. Chromatogram of vitamin standards. See text for experimental details. Peaks: 1, K_3 (20 ng); 2, A cis-acid (50 ng); 3, A all-trans acid (55 ng); 4, A alcohol (50 ng); 5, A aldehyde (35 ng); 6, D (80 ng); 7 D1 (80 ng); 8, E (180 ng); 9, K (70 ng); 10, K (70 ng). Eluent: ethanol and methanol (20: 80, v/v).

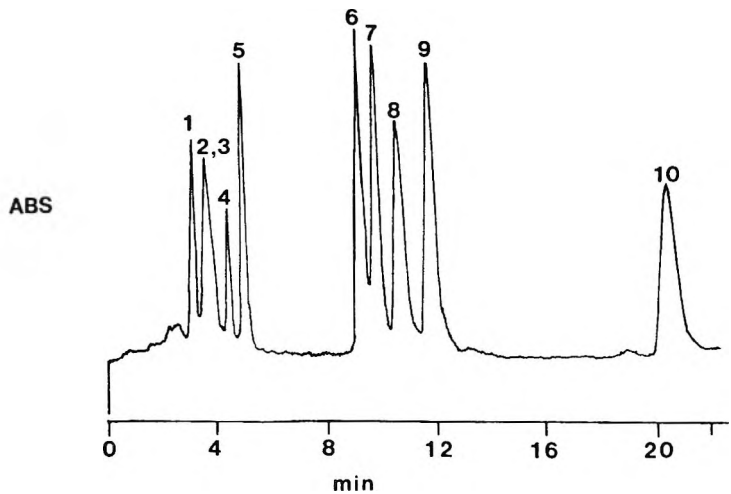

Figure 2. Chromatogram of vitamin standards. See Figure 1 for experimental conditions and labels. Eluent: ethanol and water (95: 5, v/v).

RESULTS AND DISCUSSION

A representative chromatogram for the isocratic separation of the common fat soluble vitamins, with the mobile phase containing ethanol and methanol (20: 80, v/v), is shown in Figure 1. The second mobile phase containing ethanol and water (95: 5, v/v) was also developed for the isocratic separation of the vitamins (Figure 2). The mobile phase composition was studied to improve resolution between peaks. The isomeric separation of the all-trans-, and 13-cis-, retinoic acid, was not achieved with ethanol-water as the mobile phase. On the other hand, the ethanol-methanol solvent system allows the separation of structurally similar vitamin As and a qualitative detection of the two retinoic acids, which are peaks number 2 and 3 in Figures 1 and 2. The resolution between these two vitamin peaks markedly decreased as the methanol concentration of the mobile phase slightly increased. Likewise, the resolution between vitamins D_2 and D_3 also decreased with the elevation of methanol concentration. The two vitamin Ds structurally differ from each other by an extra double bond in vitamin D_2. Hence, the chromatographic behavior of these two compounds are very similar.

The mobile phase which contains ethanol and water (95: 5, v/v) could separate vitamins D_2 and D_3. However, the overall run time takes longer (> 20 min) and coelution of the isomeric vitamins A occurs with this ethanol-water solvent system as the mobile phase. In contrast, the ethanol-methanol solvent system takes about 16 min to complete the elution. Neither the change of the flow rate nor the elution strength of the ethanol-water mobile phase could resolve the isomeric peaks.

The composition of the two different mobile phases described in this study has to be carefully prepared to allow resolution between isomeric vitamins A, as well as vitamin D_2 and D_3. The two mobile phases are complimentary to each other in terms of elution time and the resolution capability. The disadvantage of using acetonitrile instead of methanol in the mobile phase is longer elution time and similar selectivity results.

The determination of fat-soluble vitamins presents detection problems.[16] They show differing UV absorbance maxima. Their absorbance spectra are often affected by interferring compounds that show similar spectral characteristics. The absorbance spectra of the ten fat-soluble vitamins were initially studied using a Waters diode-array UV detector (Model 990, MA, USA) connected to the HPLC system. The results are shown in Figure 3 (A and B).

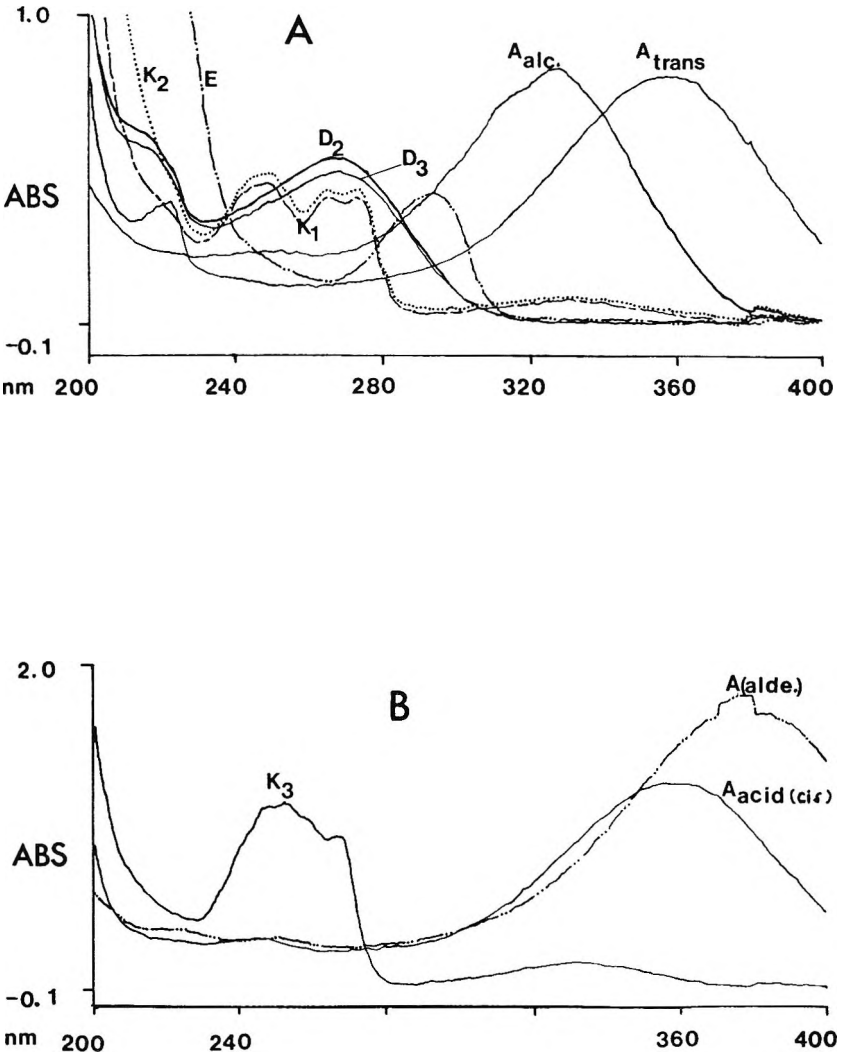

Figure 3. Ultraviolet Absorption Spectra of vitamins. See text for experimental conditions.

The spectra were obtained with the vitamin standards and presented in two Figures for clarity. The diode-array detection provides both the qualitative and quantitative information. The composition of analyses under each peak can be established by comparing the spectra taken through the peak elution. A

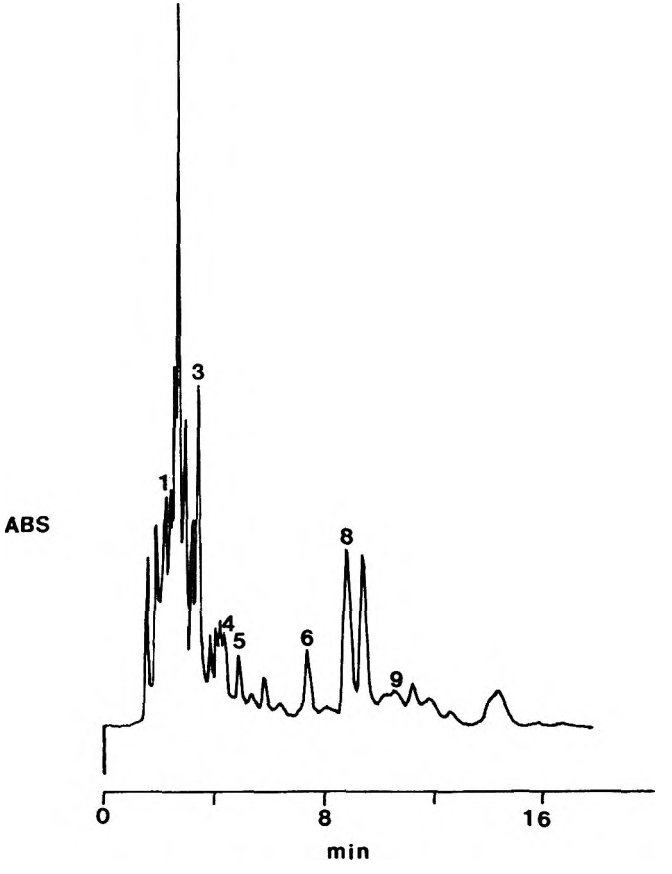

Figure 4. Chromatogram of vitamins from a milk sample. See Figure 1 for experimental conditions and labels.

relative strong absorbance common to the ten vitamins, appears around 250 nm at which no interference from the milk sample was detected. For a more specific vitamin analysis, the spectral data provide useful information for establishing the absorbance maximum under the described experimental conditions.

The application of the method to milk samples was performed. The chromatogram of vitamins from a milk sample is shown in Figure 4. The detection of vitamin Ks was not achieved by the described procedures. Hence, the milk sample was spiked with vitamin K standards prior to the sample treatment. However, vitamin K_3 was not stable enough in the extraction

process. The extraction procedure was also studied using different extraction solvents after saponification. The saponification of milk sample to hydrolyze fats is necessary before extraction. n-Hexane is an effective extraction solvent for vitamins A, D, and E. Although the detection limits of the vitamins are in the range of a few nanograms at three times of the background noise level, a large volume of milk sample is required for the detection of the fat-soluble vitamins. The correlation parameters of linearity of the line graphs are close to unity in the range of nanomolar concentration.

In conclusion, the isocratic separation method for analysing the ten vitamins is simple and efficient. The wavelength can be changed for the detection of a particular vitamin of interest to minimize interferences from matrix in biological and food samples. The method is useful for other applications.

ACKNOWLEDGMENTS

Support for this work was provided, in part, by CUHK (no. 0205-35000). B.Y.G. acknowledges the graduate assistantship from the HKP-China exchange program.

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Received September 10, 1996
Accepted November 15, 1996
Manuscript 4284

J. LIQ. CHROM. & REL. TECHNOL., 20(15), 2399–2414 (1997)

ANALYSIS OF AMINOPHYLLINE IN THIGH CREAM FORMULATIONS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Aminophylline is a 2:1 mixture of theophylline and ethylenediamine which is widely used as a bronchorelaxant for the treatment of asthma. More recently, it has been claimed that formulations of aminophylline in topical creams are useful for the reduction of lower body fat. As part of a study of the efficacy of such formulations, we have developed a chromatographic method for the analysis of aminophylline in cream preparations. The method involves dissolving the creams in an acetone-water solvent, derivatizing the aminophylline components with dansyl chloride and analyzing the resulting mixtures by reversed-phase high performance liquid chromatography (HPLC). Because other components of the creams are separated from the aminophylline components during the HPLC analysis, no extractions of aminophylline from the creams are required. However, vigorous

stirring of the reaction mixture during the dansylation is required to ensure that all of the aminophylline components are derivatized. Several commercial aminophylline-based thigh cream formulations were analyzed by this method. It was found that these formulations varied significantly in the amount of aminophylline present.

INTRODUCTION

Aminophylline (1H-purine-2,6-dione-3,7-dihydro-1,3-dimethyl-1,2-ethane diamine) is a bronchorelaxant used primarily in the treatment of asthma. It consists of two components, theophylline and ethylenediamine, in a 2:1 molar ratio. As a bronchial dilator, aminophylline is commercially available in several dosage forms. These include both single-dose and sustained-release capsules and tablets, solutions and suppositories.

A number of methods have been reported for the quantitative determination of aminophylline in biological and pharmaceutical samples. The majority of these methods, however, measure theophylline content only. Some of the earliest methods were based on extraction of theophylline with organic solvents, precipitation of a theophylline salt, and gravimetric determination of the residue.[1] The official USP method is based on the titration of silver theophyllate with ammonium thiocyanate.[2] Other volumetric methods include the titration of theophylline with alkali to a potentiometric or colorimetric endpoint,[3,4] the titration of ethylenediamine with a strong acid (e.g., HCl),[5] and the complexation of theophylline with mercuric acetate and subsequent titration of excess mercury ions with ammonium thiocyanate.[6] Alternatively, cupric acetate has been used followed by back-titration with EDTA.[7] There is also a nonaqueous titration procedure, utilizing sodium methoxide or acetous perchloric acid as the titrant, which allows for the determination of both components in a single titration procedure.[8,9]

Several spectroscopic methods have been developed in which the UV absorbance of theophylline is measured at 275 nm. Isolation of theophylline from its matrix is achieved in several ways. For pharmaceutical and many biological samples, extraction with an organic solvent is often used.[10,11] For serum samples, extraction with a mixture of ammonium sulfate, chloroform, and hexane followed by back-extraction of theophylline into aqueous borate buffer (pH 9) has been reported.[12] Charcoal extraction has also been used in which theophylline is adsorbed on charcoal and eluted with an organic solvent.[13] In another application, the theophylline present in blood samples was analyzed by oxidation with potassium dichromate in an acidic medium.[14]

The oxidation product was then isolated by steam distillation and its absorbance measured at 257 nm. Finally, spectral subtraction methods were utilized to determine aminophylline content from the UV absorbances of samples containing benzyl alcohol as a preservative, as well as mixtures of aminophylline and phenobarbitol.[15]

Many methods have been developed for the analysis of theophylline in biological samples based on high performance liquid chromatography (HPLC) and gas chromatography (GC).[16] Chromatographic conditions must be developed on a case-by-case basis to prevent interference from other drugs and/or theophylline metabolites. Ethylenediamine may also be analyzed by GC or HPLC although, until recently, its prior derivatization and separate analysis from theophylline was required.

A few years ago, Lau-Cam and Roos developed a chromatographic method which allowed for the simultaneous determination of both theophylline and ethylenediamine in solid and liquid dosage forms, specifically tablets and solutions.[17] The method is based on the HPLC separation of the two components as their dansyl derivatives (i.e., dansyl-theophylline and bis-dansyl-ethylenediamine). It involves extraction of tablets into water or dilution of liquid dosage forms, reaction of the extract with dansyl chloride (5-dimethylamino-1-naphthalenesulfonyl chloride) in an alkaline medium, and analysis of the resulting mixture by reversed-phase HPLC. Separation is achieved on a standard octadecylsilica column using a quaternary mobile phase. Excellent precision, accuracy and recovery were obtained on the analyses of a variety of dosage forms.

Recently, aminophylline has become available as an over-the-counter cream. It has been marketed by the cosmetic industry as an agent for the reduction of lower body fat. For asthma patients, aminophylline functions by dilating the bronchioles.[18] The receptors upon which it acts are also found in fat cells, with an especially high concentration occurring in the femoral regions of women. It has, therefore been hypothesized that when applied topically, these creams are absorbed into the thigh where aminophylline serves to dilate the fat cells and thus facilitate the breakdown of resistant fat storage.[19]

The topic of thigh creams and the debate as to whether or not they actually work has received much media attention within recent years with articles appearing in countless women's magazines and even in a national news publication.[20] There are currently over 50 different aminophylline-based thigh cream preparations on the market. At costs ranging from $10-$30 per bottle, a year's supply of this product can cost the consumer up to $900.

There are other concerns, however, besides cost. Aminophylline is known to occasionally produce serious cardiopulmonary side effects in the dosages administered to asthma patients. Although, it has been reported that when applied topically, no trace of the drug has been found in blood chemistry tests,[21] there have been no studies published to date that actually monitored cardiopulmonary functioning in response to acute and/or chronic use of such creams.

We have recently begun a study to determine the efficacy and cardiopulmonary effects associated with regular use of these creams. Since the actual dosing contained in these creams is not readily available to the consumer, it is conceivable that they may contain far less than the reputed effective dose of 2% aminophylline. Quantifying the percent active ingredient in the cream preparations used in this study is, therefore, necessary.

Despite the extensive list of methods previously developed for the quantitative analysis of aminophylline (or at least its theophylline component), no one to date has done such an analysis on a cream formulation. The newest analytical method, that of Lau-Cam and Roos, was developed specifically for pharmaceuticals in solid and liquid dosage forms. A cream matrix presents a more formidable challenge. The purpose of this study, therefore, is to develop a means of quantitatively analyzing aminophylline in cream dosage forms through modification of Lau-Cam and Roos method.

EXPERIMENTAL

Materials

The thigh cream products were purchased from various commercial sources, as described later. Aminophylline standard was obtained from Sigma Chemical Co. (St Louis, MO). Dansyl chloride was obtained from Lancaster Synthesis, Inc. (Windham, NH). Chromatographic-grade methanol, other solvents and sodium carbonate (used to adjust pH) were obtained from Fisher Scientific, Inc. (Fair Lawn, NJ).

Instrumentation and Conditions

All analyses were performed on an HPLC system consisting of a Perkin-Elmer Series 410 solvent delivery system, a Rheodyne Model 7125 injector (10 μL loop), and a Perkin-Elmer Model LC-135 diode array detector set at

254 nm. Separations were achieved on a Microsorb-MV C-18 column, 15 cm x 4.6 mm i.d., 5 μm particle size (Rainin Instrument Co., Inc., Woburn, MA) Chromatograms were recorded and processed on a Perkin-Elmer Omega data system.

The isocratic mobile phase consisted of 69% methanol, 29% water, 1.5% acetic acid, and 0.5% triethylamine. The flow rate was 1.0 mL/min.

Preparation of Samples

Standard solutions were prepared from a stock solution containing 1 mg/mL aminophylline in water. 1-5 mL of the stock solution were mixed with 10 mL of dansyl chloride solution (5 mg/mL in acetone) and 10 mL of sodium carbonate solution (0.9 mg/mL in 50% aqueous acetone, by volume). The mixtures were allowed to stand at room temperature in the dark for 12 hours, then brought to a volume of 50 mL and analyzed by HPLC under the conditions described above.

Cream sample solutions were prepared by adding 10 mL of 50% aqueous acetone to Erlenmeyer flasks containing an accurately weighed quantity of cream (100-400 mg) as described in Table 10. The suspensions were then stirred for several minutes to dissolve the cream. Dansylation of the resulting solutions was performed in a similar manner as the standards under various conditions of reagent concentration, pH, and temperature as described later.

Further Preparation of Creams by Extraction

Removal of the organic, water-insoluble cream components prior to dansylation was attempted in the following manner: a 10 mL aliquot of cream solution prepared as described above was first transferred to a 50 mL centrifuge tube. 10 mL of chloroform was then added to the tube and the mixture was shaken vigorously for 2 minutes. After centrifuging at 3000 rpm for 5 minutes, the aqueous layer was removed, dansylated as described above, and finally, analyzed by HPLC.

Standard Addition Analysis

A total of 20 mL of a dansylated aminophylline standard (0.1 mg/mL in water) was added to an equal volume of a previously analyzed cream sample and rechromatographed.

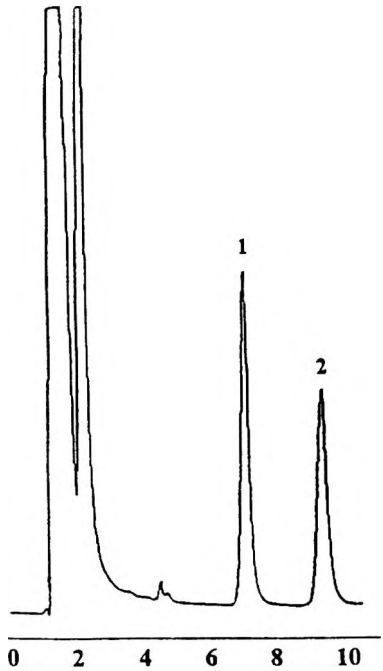

Figure 1. Chromatogram of a standard solution of aminophylline after dansylation. Identified peaks correspond to (1) dansyl-theophylline and (2) bis-dansyl ethylenediamine.

Recovery Experiments

A mass of aminophylline weighing 13 mg was added to 1.30 g of a previously analyzed thigh cream formulation and stirred to form a homogenous mixture. 130 mg of this mixture was then prepared for analysis as outlined above.

RESULTS AND DISCUSSION

Figure 1 shows a typical chromatogram obtained from the analysis of a standard aminophylline solution using the method developed by Lau-Cam and Roos. The retention times for dansyl-theophylline and bis-dansyl-

Table 1

Results of Thigh Cream Analysis Under Initial Derivatization Conditions

Sample No.	Theophylline (mg/g)	Ethylenediamine (mg/g)	Molar Ratio
1	4.97	0.128	13:1
2	3.74	0.132	9:1
3	4.87	0.147	11:1
4	5.51	0.124	15:1
5	5.64	0.143	13:1
6	5.42	0.153	12:1

ethylenediamine were 7.01 min and 9.21 min, respectively. At 254 nm, the theophylline component gave a similar detector response as bis-dansyl-ethylenediamine, corresponding to the 2:1 molar ratio present in aminophylline samples.

All of our preliminary data on the cream samples was obtained using University Medical's Original Thigh Cream (University Medical Products, Newport Beach, CA). Due to the complex nature of its inactive ingredients, we perceived this cream to be the most difficult to analyze, and on this basis, chose it for all of the initial method development. The results shown in Table 1 were obtained when the cream samples were prepared and dansylated in exactly the same manner as the standards (i.e., identical conditions of reagent conc., pH, and temperature).

Figure 2A shows a typical chromatogram obtained from this analysis. In the presence of the other cream components, both theophylline and ethylenediamine exhibited a slight decrease in retention time. Ethylenediamine was most affected. More importantly, bis-dansyl-ethylenediamine exhibited a much lower response than did dansyl-theophylline, resulting in molar ratios of theophylline to ethylenediamine between 5 and 8 times greater than the expected 2:1 molar ratio (see Table 1).

Cream is a very complex matrix. Since the aminophylline in the cream was analyzed in the presence of this matrix, it appeared likely that the anamolous quantitative results were caused by interference from the cream.

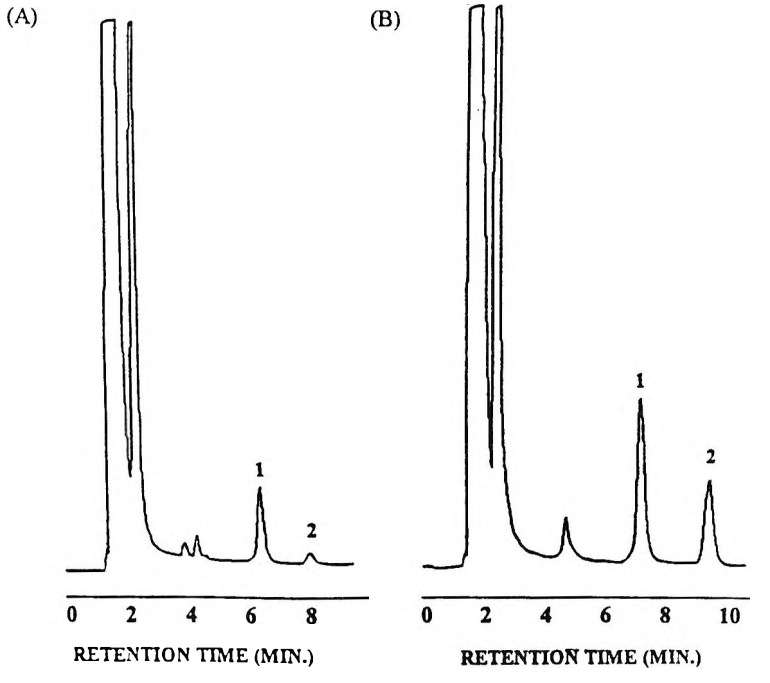

Figure 2. Chromatogram of cream formulation under (A) initial dansylation conditions and (B) optimized dansylation conditions. Peak identities are the same as that described in Figure 1.

Two modes of interference were possible:

1. The cream matrix could be distorting the chromatographic separation and/or relative responses of the two components.

2. The cream components could be inhibiting the dansylation of one or both of the compounds in the aminophylline in an unequal manner.

Investigations were conducted to determine the source of the anamolous results and to minimize them, as discussed below.

Investigation of Possible Chromatographic Interference

As stated above, the possibility existed of cream components interfering with the quantitative data. A standard addition experiment was conducted to

Table 2

Standard Addition Experiment

	Dansyl-theophylline Peak Area	Bis-Dansyl-ethylenediamine Peak Area
Before Addition	52279984	7908328
After Addition	95216896	51262802
Δ Peak Area	42936912	43354476

investigate this possibility in which an aliquot of derivatized standard was added to a previously analyzed sample and then rechromatographed. Table 2 shows the corresponding peak areas obtained. After addition of the derivatized standard, the peak areas increased by a similar amount for each component. The nearly identical increase in response indicates that the cream matrix did not affect the quantitative chromatographic results for either component. Therefore, it can be concluded that incomplete and/or unequal dansylation of the target compounds in the presence of the cream was the source of the anamolous results.

Attempted Extraction of Interfering Cream Components

The second possibility of the cause for the anomolous ratio of theophylline to ethylenediamine was interference of cream components (e.g., various paraffins, herbal extracts and oils) in the dansylation process. To minimize these effects, we attempted to extract the potential interfering compounds into chloroform and away from the reaction mixture prior to dansylation.

Table 3 shows data obtained on four duplicate cream samples. As the number of extractions increased, the molar ratio was observed to decrease, approaching the theoretical value of 2:1.

These results, however, were apparently caused by a decrease in the amount of theophylline detected (see Table 3) rather than an overall improvement in the dansylation. Theophylline was most likely lost by back-extraction into the chloroform layer.

Table 3

Extraction of Cream Samples with Chloroform

Number of Extractions	Theophylline (mg/g)	Ethylenediamine (mg/g)	Molar Ratio
1	3.71	0.35	3.5:1
2	3.13	0.30	3.4:1
3	2.95	0.38	2.6:1
4	1.89	0.37	1.7:1

Table 4

Extraction of Aminophylline Standard with Chloroform

Number of Extractions	Theophylline (mg/g)	Ethylenediamine (mg/g)	Molar Ratio
None	0.0200	0.0034	2.0:1
4	0.0039	0.0034	0.4:1

To confirm the occurrence of this theophylline loss, a 0.025 mg/mL solution of aminophylline was analyzed once with, and once without extraction into chloroform. The data presented in Table 4 shows that after four extractions only one-fifth of the original theophylline content remained in the aqueous layer removed for analysis. This confirmed the occurrence of back-extraction of the theophylline into the organic phase.

Optimization of Reaction Conditions

The demonstrated loss of theophylline during the attempted extraction of cream components prior to dansylation necessitated our reverting to performing the derivatization and analysis in the presence of the cream. Since the apparent problem with the analysis appeared to be interference by cream components in the dansylation process, we attempted to optimize the derivatization by a systematic variation of reaction conditions. Table 5 describes these variations and the resulting analytical data.

Table 5

Optimization of Dansylation Conditions

Condition Varied	Theophylline (mg/g)	Ethylenediamine (mg/g)	Molar Ratio
Reagent Conc. (increased 3-fold)	13.26	0.55	8:1
pH (raised to 3.5)	13.32	0.36	12:1
Temperature (heated to 60°C)	9.87	0.39	8:1
Vigorous Stirring (magnetic stirrer at 300 rpm)	8.92	1.35	2:1

As shown there, each variation produced an increase in the amount of both components detected compared to that obtained in initial experiments (Table 1). However, in most cases, the molar ratios were still higher than the expected 2:1 molar ratio, indicating incomplete dansylation of the target compounds. Only under conditions of vigorous stirring of the reaction mixture was the expected 2:1 molar ratio achieved.

Method Validation

Table 6 shows the optimized derivatization conditions used in the remainder of this study. Figure 2B shows a chromatogram of a cream formulation derivatized under these optimized conditions. It exhibits the expected relative response for the two aminophylline components. System precision data, shown in Table 7, were obtained from successive injections of a single cream solution after derivatization. The relative standard deviation (RSD) of results obtained for the ethylenediamine component is somewhat greater than that obtained for theophylline but still is under 3%. The precision of the entire method was evaluated by derivatizing and analyzing six separate samples of the same cream formulation. The results shown in Table 8 demonstrate good analytical reproducibility.

Table 6

Optimized Reaction Conditions

Dansyl Chloride Concentration	pH	Temperature	Special Conditions
2 mg/mL	2.2	ambient	in the dark stirred virorously

Table 7

System Precision

Injection	Theophylline (mg/g)	Ethylenediamine (mg/g)	Molar Ratio
1	8.69	1.32	2.19:1
2	8.59	1.28	2.24:1
3	8.60	1.36	2.11:1
mean	8.63	1.32	2.18:1
RSD	0.52%	2.47%	2.46%

Table 8

Method Precision

Sample	Theophylline (mg/g)	Ethylenediamine (mg/g)	Molar Ratio
1	8.52	1.32	2.15:1
2	8.88	1.26	2.34:1
3	8.72	1.23	2.36:1
4	9.20	1.30	2.36:1
5	9.10	1.28	2.37:1
6	8.69	1.32	2.19:1
mean	8.85	1.28	2.29:1
RSD	2.9%	2.8%	4.3%

Table 9

Recovery Experiment

Component	Amount Added, mg	Amount Recovered, mg	Percent Recovery	Corrected Recovery
Theophylline	1.07	1.02	95%	97%
Ethylenediamine	0.178	0.157	88%	94%

A recovery experiment was also conducted in which a weighed amount of aminophylline was added to a cream formulation prior to extraction, derivatization and analysis. Subtracting the amounts of each component which were initially found in the unspiked formulation gave the results shown in Table 9. However, the percent recovery determined in this way underestimates the actual recovery of the drug from a single analysis of such a formulation, since these results are based on extraction and analyses of two cream formulation samples, one spiked and one unspiked. Employing a propagation-of-errors approach, a better estimate of recovery from a single extraction and analysis was obtained by calculating a "corrected recovery", which is essentially the square root of the recovery based on the two analyses. The results, also shown in Table 9, indicate that at least 94% of each of the drug components were extracted and analyzed by this method.

Additional Analyses of Commercial Creams

Three commercial creams were analyzed by this method. Results are shown in Table 10. Amounts of creams used for the analysis needed to be varied to obtain similar chromatographic responses. These results indicate that the percent active ingredient (calculated by adding the percentages of the two aminophylline components) varies widely among the commercial formulations.

CONCLUSION

In this study we have shown that the dansylation of both theophylline and ethylenediamine in thigh cream formulations is adversely affected by other components of these creams. This interference, which complicates the analysis of these components by reversed-phase HPLC, has been minimized through optimization of dansylation conditions. With this new method we have demonstrated excellent system and method precision as well as acceptable

Table 10

Aminophylline Content in Several Cream Preparations

	Amount of Cream Used For Analysis (mg)	Theophylline (mg)	Ethylenediamine (mg)	Percent Amino-phylline
University Medical Orig.[a]	260	2.30	0.33	1.00
Thinny Thighs[b]	400	1.05	0.18	0.31
Thigh High[c]	130	2.31	0.39	2.08

[a] University Medical Products, Newport Beach, CA.
[b] Winning Solutions, Inc., Westport, CT.
[c] Faneuil Companies, Scottsdale, AZ.

recovery. It appears that analyzing such complicated matrices requires a careful investigation and optimization of reaction and analytical conditions, especially when derivatization is involved. The analytical results show that there is a wide variation in the percent aminophylline present in various brands of thigh creams. This may have important consequences in conducting clinical studies of the safety and efficacy of these formulations.

ACKNOWLEDGMENTS

This study was supported in part from a Florida Atlantic University Research Initiation Grant. Acknowledgment is also made to the donors of The Petroleum Research Fund, administered by the American Chemical Society, for partial support of this research.

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Received March 2, 1997
Accepted March 31, 1997
Manuscript 4388

J. LIQ. CHROM. & REL. TECHNOL., 20(15), 2415–2426 (1997)

SEPARATION OF HIGH MOLECULAR WEIGHT SAPONINS OF *ARCHIDENDRON ELLIPTICUM* BY HYDROPHILIC INTERACTION CHROMATOGRAPHY

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ABSTRACT

Saponins of high molecular weight (Mr 2,000-2,100) from several species in the plant family Leguminosae were separated at both analytical and preparative scale using wide pore aminopropyl HPLC with acetonitrile-water mixtures, and gradient wide-pore butyl reversed-phase HPLC. The basis for the aminopropyl separation appears to be hydrophilic interaction chromatography, and the pore size of the media appears to play a key role in the separation.

INTRODUCTION

This paper reports the application of hydrophilic interaction chromatography methodology to the successful isolation of the cytotoxic elliptosides A-J from *Archidendron ellipticum* (Bl.) Nielsen (Leguminosae), at

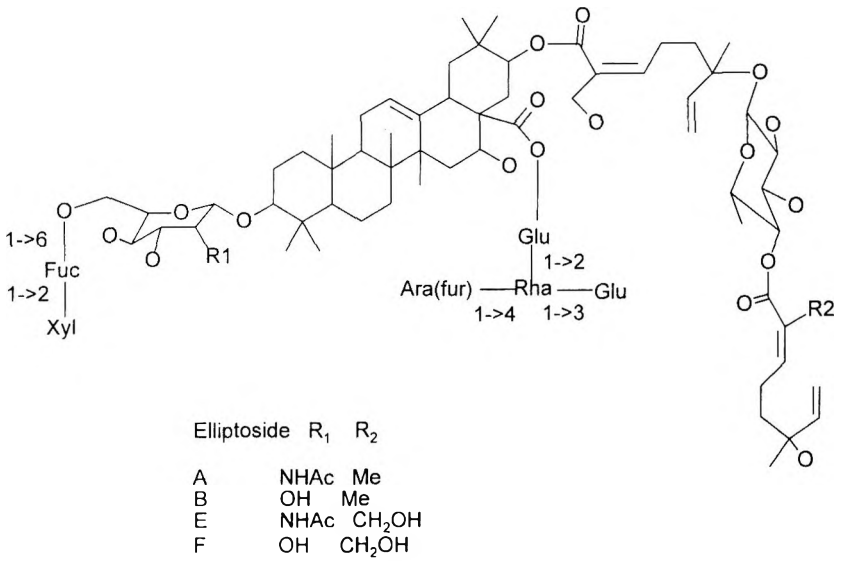

Figure 1 Structures of Elliptosides A,B, E, and F.

both the analytical and preparative scale. These closely related structures are ester glycosides of acacic acid with eight sugar residues and two monoterpene esters (Figure 1). Details of the structure elucidation and biological properties of elliptosides A-J are presented elsewhere.[1] We have found other saponin mixtures from the related trees *Archidendron jiringa* and *Cathormion altissimum* to contain similar cytotoxic saponins and have also separated these on an analytical scale. This technique has also been successfully applied in our laboratory, to the separation of saponins from other non-legume sources (data not shown).

RESULTS

The crude organic extract of *A. ellipticum* was partitioned between $CHCl_3$, MeOH, and water to give a cytotoxic aqueous fraction. This fraction was further permeated through Sephadex LH-20 in MeOH to give an early-eluting (i.e., higher molecular weight) cytotoxic fraction, composed of a complex mixture of saponins, as judged by ^{1}H-NMR. Later fractions had reduced or no cytotoxic activity. A variety of conventional separation methods were applied to the bioactive mixture in an effort to purify the saponins. Separations on silica gel, C_{18}, diol, cyano, PRP-1, and PRP-3 chromatography media were

evaluated by TLC and/or HPLC without obtaining adequate resolution. Numerous countercurrent systems were scouted by partition TLC on silica gel and several trial runs were made on a Sanki CPC apparatus. These methods were not effective, and, in addition, the use of centrifugal countercurrent methods led to significant loss of bioactivity in the recovered fractions.

The use of a C_4 wide-pore separation[2] using a MeOH-H_2O-HOAc gradient partially resolved the saponin mixture; however, NMR and mass spectral analysis made it clear that further separation would be required to obtain pure compounds. Wide-pore aminopropyl media resolved the mixture into six major peaks. Retention times for the peaks increased with increasing content of CH_3CN in the mobile phase, indicating that the separation did not reflect reverse-phase chromatography. When analyzed by C-4 HPLC, each of the peaks also proved to be a mixture. The sequential application of amino and C-4 HPLC, however, permitted complete separation of elliptosides A-H in pure form (Figures 2 and 3). Wide-pore phenyl separation of a third peak from the aminopropyl chromatography yielded two further saponins, elliptosides I and J. The compounds isolated by this method had consistent ^{1}H- and ^{13}C-NMR spectra, as well as a single major pseudomolecular ion by FABMS and, thus, were judged to be pure.

The effect of pore size on the aminopropyl separation was studied in three ways:

1) Chromatography columns having aminopropyl bonded phase chemistry, but different nominal pore size, were used to characterize the Sephadex LH-20 eluate from *A. ellipticum*. As shown in Figure 4, a 60Å column (Rainin Dynamax, 8μ irregular, 4.6 x 250 mm) did not give a useful chromatographic separation, while a column with 120Å pores (Waters Carbohydrate column, 4.6 x 300 mm) gave some resolution, and a wide pore 300Å column (YMC Amino, 4.6 x 250 mm) gave a superior separation of the same material. Further trial separations using 120Å, 5μ spherical aminopropyl and polyamine media (YMC) gave poor resolution, confirming the necessity for wide pore media and eliminating particle size and shape as major factors affecting the separation.

2) The organic and aqueous extracts of several other plants which also exhibited cytotoxicity similar to that from *A. ellipticum* were processed by the same partition and Sephadex LH-20 chromatography steps described above. The earliest Sephadex LH-20 peak was then analyzed using an analytical YMC 300Å 5 μ aminopropyl HPLC column.

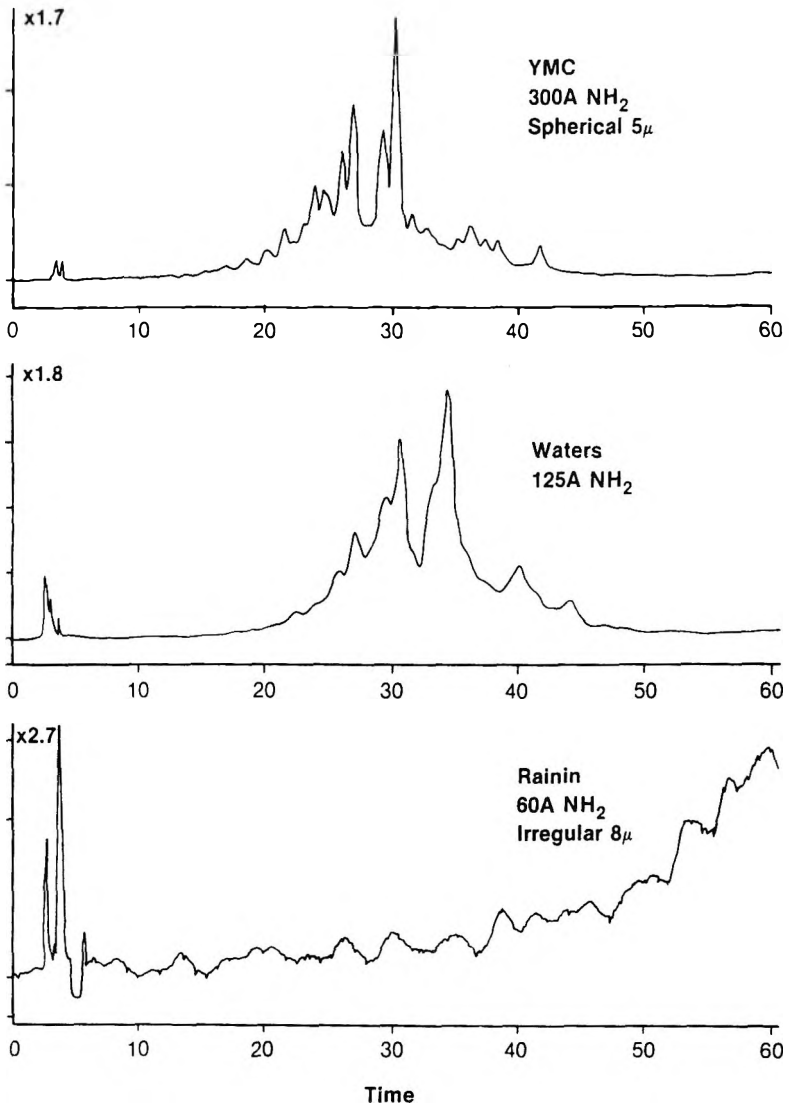

Figure 2. Comparison of the separation of crude *A. ellipticum* saponin mixture on a) YMC 300Å 5 μ spherical particle analytical column; b) Waters 125Å "Carbohydrate" column c) Rainin 60 Å aminopropyl 8 μ irregular particle column. All columns were 250 mm in length, flow 1 mL/min., 80% CH_3CN, detection at 230 nm.

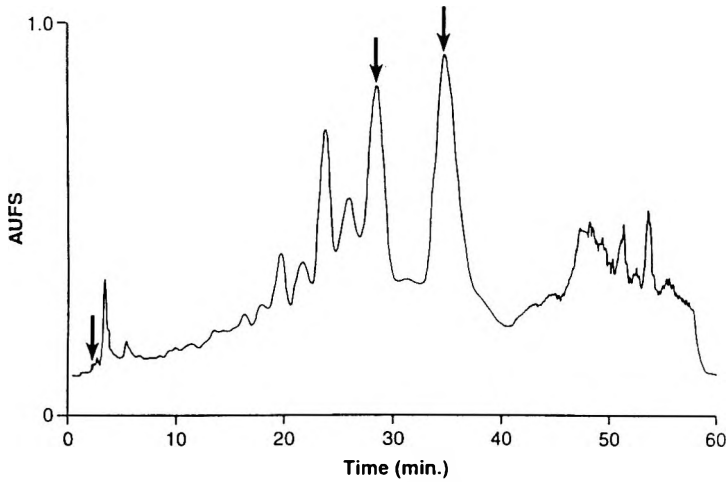

Figure 3. Preparative separation of crude saponin mixture from *A. ellipticum* on YMC 20mm x 250 mm 300 Å 5 μ spherical aminopropyl column, 80% CH_3CN, 15 mL/min, detection 238 nm. Load 100 mg. Arrows indicate elliptoside A-D and E-H containing fractions respectively.

Fractions from the plants *Cathormion altissimum* (Hook.f.) Hutch.& Dandy and *Archidendron jiringa* (Jack) I.Nielsen gave a similar high-resolution separation of saponin peaks (Figure 5). Thus, this method could be applied to the separation of saponin mixtures from several plants in the family Leguminosae.

3) Construction of computer models of the elliptosides showed that the maximum dimensions of the molecules should be no greater than 100Å. While a compound of this size would not usually be considered large enough to demand 300Å pores for efficient mass transfer, the effect of hydration of the saponin may increase the effective size of the molecule.

In addition, the functionalization and hydration of the aminopropyl silica media may decrease the effective pore size of the particle. Alternatively, the saponins might form mixed micelles of a large enough size to require larger pore media, but in that case separation of the distinct components would not appear probable.

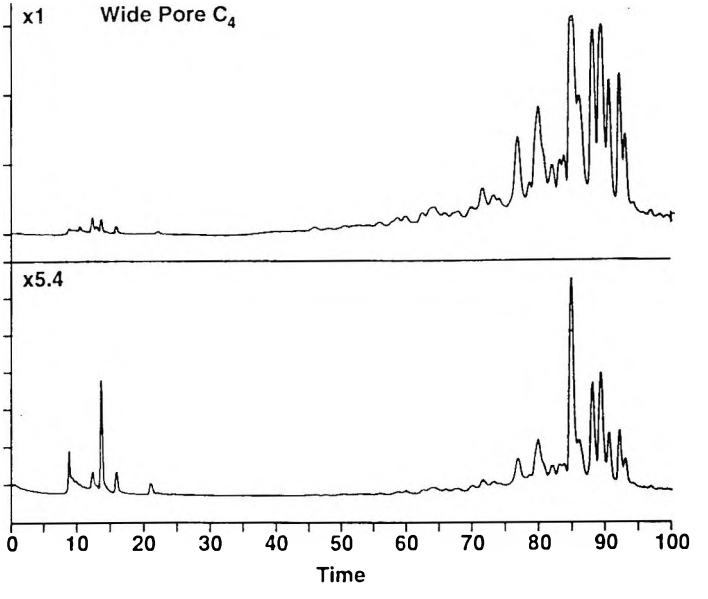

Figure 4. Preparative separation of partially purified elliptosides A-D on Rainin C_4 300 Å 5 μ spherical column, gradient from 50% MeOH (40 mM HOAc) to 100% MeOH (40 mM HOAc). Load 25 mg.

DISCUSSION

The majority of previously characterized saponins have been of molecular weight <1500 daltons (i.e., containing one to four sugar moieties). Saponins containing larger numbers of sugars have generally been difficult to purify due to the complexity of such mixtures. In the present case, monoterpene esters attached to different parts of the molecule further complicated the task. While simple saponins have been successfully purified by conventional chemical techniques such as adsorption and partition chromatography, as well as by countercurrent chromatography,[3] more complex mixtures of high molecular weight saponins have historically been difficult to purify to homogeneity.

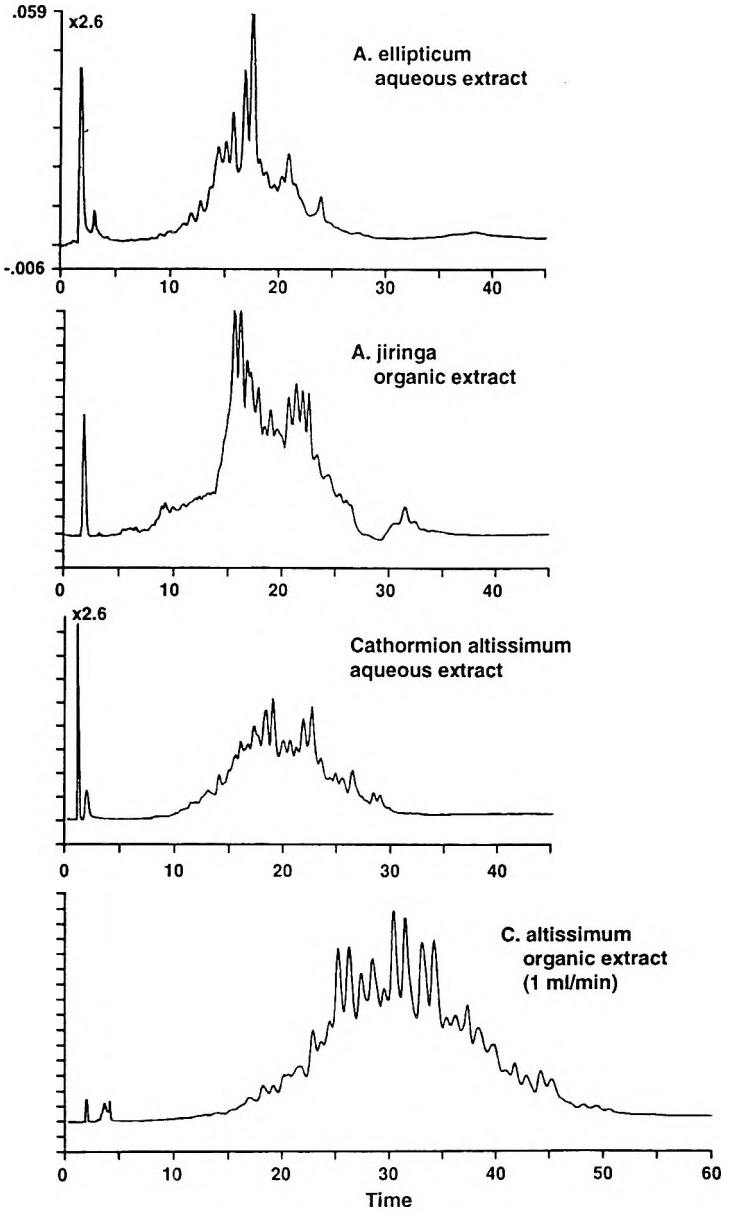

Figure 5. Analytical separation of saponin fractions from various plant samples on aminopropyl bonded phase media. 2 mL/min 80% CH_3CN except for d) 1 mL/min.

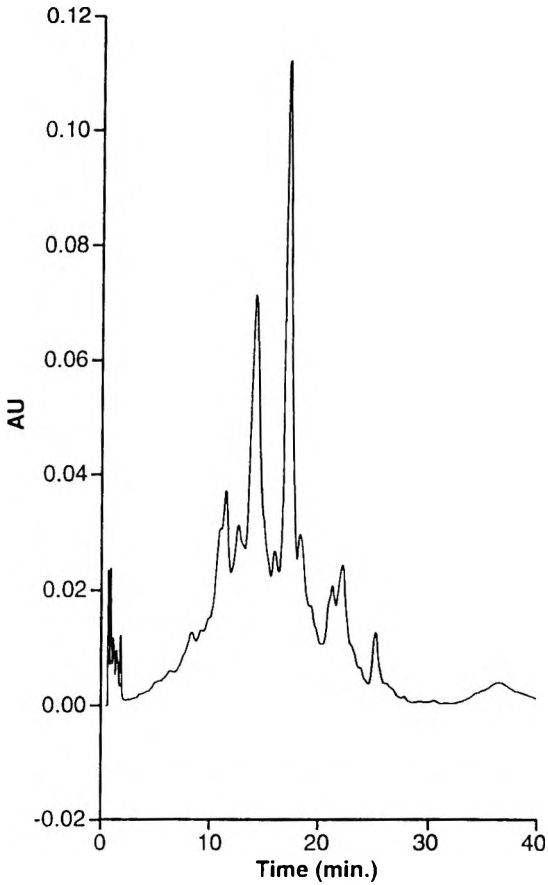

Figure 6. Analytical separation of crude saponin mixture from *A. ellipticum* on YMC 4.6 mm x 250 mm 300 Å 5 μ spherical polymeric amino column, CH_3CN-H_2O gradient, 2 mL/min, detection 230 nm. Load 100 μg.

A recent advance in large saponin purification methodology is the use of wide-pore butyl (C_4) HPLC,[2] where high molecular weight (Mr1,800) saponins from *Quillaja saponaria*, useful as immune adjuvants,[4] were purified from a complex mixture. The use of wide-pore (330Å) HPLC media was not specifically identified as being crucial to this separation. The well-established technique of separating simple sugars and oligosaccharides by the use of so-called "carbohydrate" HPLC columns (aminopropyl phase-bonded silica gel) eluted with mixtures of acetonitrile and water has been well-known since 1975.[5,6] A partition mechanism has been proposed for the separation,[7,8,9]

involving formation of a water-rich layer in the stationary phase. The most polar analytes are proposed to associate preferentially with the water-rich layer. The recent formulation of the concept of hydrophilic interaction chromatography (HILIC[10]) has been made to generalize this method of separation. While the elliptosides contain eight sugars, the basis for their separation on aminopropyl media would appear not to depend on the specific sugar moieties, since elliptosides A and B, which are not separated by this system, have different internal sugars (Glc vs. GlcNAc), while elliptosides A and E, which have identical sugar composition but a different monoterpene, are separated. The most polar saponins are eluted after the less polar saponins, the opposite elution order from that expected in reverse-phase chromatography. HILIC appears to be the basis for this separation.

"Carbohydrate" separation technology or HILIC has rarely been applied to the separation of glycosides of any type, including saponins. The only examples found in the literature were the analytical separation of ginseng saponins on an amino column of unspecified pore size,[11] and separation of neutral steroidal *Dioscorea* saponins.[12]

The aminopropyl column performance rapidly deteriorated after several days of preparative use. Replacement of the guard column returned the system to its initial performance. The use of a vacuum flash chromatography step using CH_3CN-H_2O on flash chromatography grade aminopropyl media prior to HPLC did not appear to improve column lifetime, and resulted in poor mass recoveries.

Short column lifetime is a recognized limitation of aminopropyl HPLC media.[13] Switching to a "polymeric" amino bonded phase column has yielded useful separations with substantially increased column lifetime (Figure 6). The wide pore amino column separation of high molecular weight saponins was successfully applied to the resolution of saponin mixtures from diverse plants in the family Leguminosae. It may also prove useful for separation of complex saponin mixtures from other families.

MATERIALS AND METHODS

General

All solvents were HPLC grade. Gel permeation was carried out using glass columns packed with Sephadex LH-20 (Pharmacia) and eluted with

MeOH, and monitored with a variable wavelength monitor (ISCO V^4) tuned to 230 nm, or detuned to higher wavelengths at higher loadings. HPLC was carried out using a Waters model 600E gradient pumping system equipped with a model 990 diode array detector, or a Waters Delta Prep 3000 with a Waters model 481 variable wavelength detector. Columns used were Rainin Dynamax wide pore C_4 (250 mm, 5μ, 300 Å) and YMC aminopropyl (250 mm, 5μ spherical 300Å), polyamine (PBMN, 250 mm, 5 μ spherical 300Å), and phenyl (250 mm, 5 μ spherical 300Å) columns of various diameters, equipped with guard columns containing the same packing materials. A Waters "Carbohydrate" column (4.6 x 300 mm, 125Å), YMC narrow pore (120 Å) aminopropyl column, and Rainin Dynamax aminopropyl column (4.6 x 250 mm, 60Å, 8 μ irregular) were also used in characterizing the effect of pore size on the separation.

Extracts

Extracts used in the present work were obtained from the NCI natural products repository. The extracts were prepared as described below from plant materials collected by NCI contractors in South America, Africa, and Southeast Asia. Voucher herbarium specimens were deposited by the collectors.

Isolation of Saponins from *Archidendron ellipticum* Leaf Extract

Milled leaves of *Archidendron ellipticum* (824 gms) were percolated with a mixture of CH_2Cl_2-MeOH (1:1, v/v) to extract the organic soluble materials. The solvent was drained and a MeOH rinse was performed on the marc. The combined solvents were evaporated to dryness on a rotary evaporator at 35°C. This yielded 98 g of organic extract (11.8% w/w). 14.4 g of extract were dissolved and partitioned with a mixture of $CHCl_3$ (500 mL), MeOH (450 mL), and H_2O (200 mL). Two further 500 mL portions of $CHCl_3$ were used to wash the aqueous phase. The aqueous phase was evaporated to give 4.03 gms of cytotoxic polar material. This material was chromatographed on a 7 x 38 cm column of Sephadex LH-20 in MeOH, using uv detection at 238 nm to differentiate the eluted fractions. The earliest eluting peak was evaporated to give 949 mg of crude saponin mixture with increased cytotoxicity. This material was separated by wide-pore aminopropyl HPLC (YMC 20 x 250 mm, 5μ spherical, 300Å column) using 80% acetonitrile as eluant, followed by a linear gradient to 72% CH_3CN (see Figure 3). This resolved six major peaks, two of which were then separately chromatographed on wide- pore butyl HPLC (Rainin 21.4 x 250 mm 5μ spherical, 300Å column), using a gradient from 50% MeOH/H_2O, 40 mM HOAc to 100% MeOH, 40 mM HOAc (see Figure

4). In this fashion elliptosdes A-H were isolated in the following amounts: A-16 mg, B-5.1 mg, C -5.9 mg, D-3.4 mg, E -25 mg, F-11.8 mg, G- 14.7 mg, H -10.6 mg.

Isolation Using a Vacuum Flash Chromatography Step

The same sequence of separation was followed as above, however, a vacuum flash chromatography column step using aminopropyl media (YMC 120Å, 40-63μ, irregular) was performed before the aminopropyl HPLC in an effort to increase the HPLC column lifetime. The column was eluted with 70% CH_3CN, 50% CH_3CN and H_2O.

Purification of Aqueous Extract of *Archidendron Ellipticum* Leaves

The marc from the organic extraction above was extracted overnight with distilled water and the aqueous extract lyophilized to give an aqueous extract. Trituration of 314 mg of extract with MeOH yielded a methanol-soluble fraction which was permeated on Sephadex LH-20 in MeOH. The earliest peak from this chromatography (29 mg) was identical in cytotoxic activity, aminopropyl HPLC profile, and NMR spectrum to the corresponding fraction from the organic extract.

ACKNOWLEDGMENTS

Grateful acknowledgement is made to L. Pannell, D. M. Sheely and G. Gray for mass spectral analyses, the New York Botanical Garden, Missouri Botanical Garden, and the University of Illinois at Chicago for the collection of botanical material, A. Monks and D. Scudiero for cytotoxicity measurements, J. H. Cardellina, II and M. R. Boyd for helpful discussions, and T. McCloud for preparation of extracts.

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Received January 27, 1997
Accepted March 27, 1997
Manuscript 4381

J. LIQ. CHROM. & REL. TECHOL., 20(15), 2427-2439 (1997)

DETERMINATION OF BIOGENIC AMINES AND RELATED COMPOUNDS IN THE GANGLIA AND THE AURICLE AND VENTRICLE OF THE HEART OF THE SNAIL HELIX LUCORUM L. BY HPLC WITH AMPEROMETRIC DETECTION

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ABSTRACT

Biogenic amines and related compounds in the suboesophageal ganglia and the auricle and ventricle of the heart of the snail Helix lucorum L. have been identified and quantified using the technique of reversed phase ion-pair HPLC with amperometric detection. HPLC analysis revealed the presence of two catecholamines, Dopamine (DA) and Norepinephrine (NE), one indoleamine, Serotonin (5HT), its immediate precursor 5-Hydroxytryptophan (5HTP) and their metabolites 3,4-Dihydroxy phenylacetic acid (DOPAC) and 5-Hydroxyindole-3-acetic acid

(5HIAA). Quantitatively, DA and 5HT were the most prominent monoamines detected, with the ganglia containing the highest concentrations of both. The ganglia contained also assayable amounts of NE and DOPAC.

INTRODUCTION

Biogenic amines, such as the indoleamine serotonin and the catecholamine dopamine, were found to act as central neurotransmitters in molluscs[1] and, also to be implicated in the control and regulation of the cardiac activity in gastropods and bivalves.[2] However, progress in research in these fields requires accurate data detailing the levels of monoamines and monoamine metabolites and precursors in nervous and non-nervous tissues of these species. In the last decade, high performance liquid chromatography (HPLC) with amperometric detection became one of the most important techniques for the transmitter research,[3-6] since it is a sensitive, precise, rapid, and relatively simple technique for the determination of very small amounts of these endogenous compounds in biological tissues.

The aim of the present study is: 1) to investigate the retention behaviour of catecholamines (DA, E, and NE), indoleamines (5HT), their precursors (DOPA, TRYP, and 5HTP) and their main metabolites (DOPAC, 3MT, HVA, 5HIAA, and 5HTOH), see Figures 1 and 2, in reversed phase liquid chromatography (RPLC), 2) to find optimum conditions for their separation, and 3) to detect the above compounds in the suboesophageal ganglia and in the auricle and the ventricle of the heart of the snail Helix lucorum L.using HPLC with electrochemical detection.

EXPERIMENTAL

Chromatography

The liquid chromatography system consisted of a Shimadzu LC-9A solvent delivery unit with a double-plunger reciprocating pump, a Model 7125 syringe loading sample injector fitted with a 20 μL loop (Rheodyne, Cotati, CA), and a Gilson Electrochemical detector (Model 141). The working electrode was a small disc of glassy carbon (3mm diameter) and the electrode

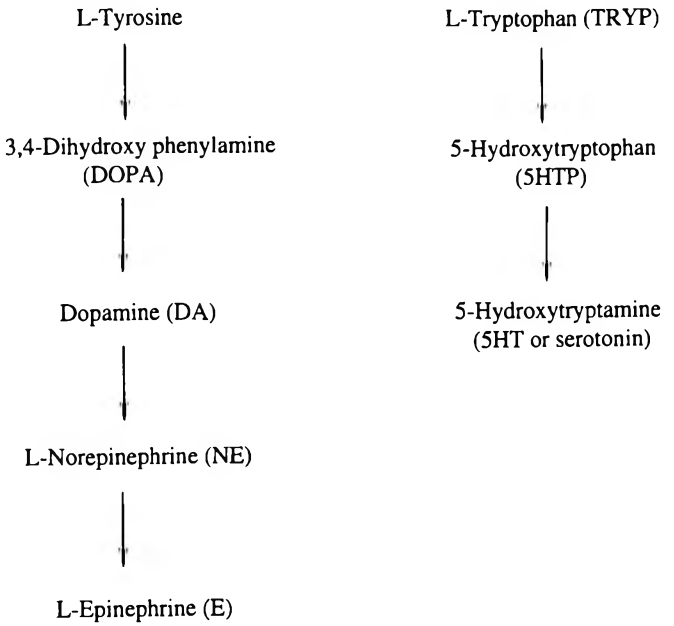

Figure 1. Biosynthetic pathway of the catecholamines and an indoleamine from their precursor aminoacids.

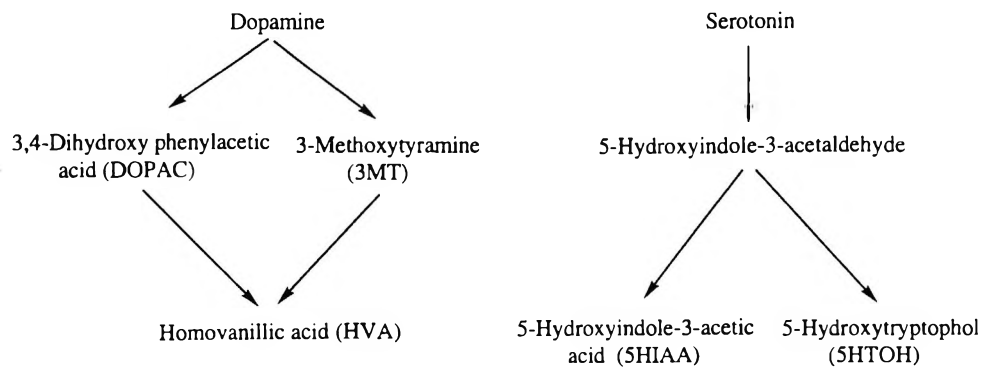

Figure 2. Metabolic pathway of Dopamine and Serotonin.

potential was maintained at 0.65 V vs a Ag/AgCl (3.0 M NaCl) reference electrode. The electrochemical detector was interfaced to a PC 486 IBM compatible computer via a 14-bit AD-DA card. The same computer was also used to carry out all calculations reported in this paper.

The mobile phase was a mixture of an aqueous buffer (plus an ionic modifier) with an organic solvent. The aqueous buffer (pH=3.3) consisted of 0.085 M sodium acetate, 0.09 M tartaric acid, and 1.0 mM Na_2EDTA. Methanol was used as an organic modifier and sodium octyl sulphonate (SOS) as an ion-pairing agent. The methanol and SOS concentration, as well as the chromatografic column, are specified in the Results and Discussion section. The mobile phase was filtered through a mixed esters membrane filter (0.45μm Schleicher & Schuell GmbH, Germany), sonicated and degassed under vacuum. The mobile phase was run at a flow rate 1.0 mL/min and was recycled. All separations were carried out at ambient temperatures.

Chemicals

All chemicals were used, as received, from commercial sources. NE hydrochloride, E and DA hydrochloride were obtained from Aldrich Chemical Co. (Milwaukee, WI). DOPA, 3MT hydrochloride, 5HT creatine sulfate complex, 5HTP, DOPAC, 5HIAA, HVA, TRYP, 5HTOH, Nω-Methylserotonin (M5HT) oxalate, 3,4-Dihydroxybenzylamine (DHBA) hydrobromide, and Epinine (EPI) hydrochloride were available from Sigma Chemicals Co. (St. Louis, MO). All other chemicals were of analytical-reagent grade.

Standards and Sample Preparation

Stock solutions of the analytes and internal standards were prepared in the aqueous buffer of the mobile phase at a concentration of 0.1mg/mL and stored at 4°C. Standard solutions (0.32-0.032μg/mL) were prepared at weekly intervals by an appropriate dilution of the stock solutions using the same buffer as the diluent.

The suboesophageal ganglia as well as the auricles and the ventricles of the hearts were excised out by removing the shells. These tissues after dissection were pooled separately to give samples of 0.01-0.04 g and stored at -70°C until immediately prior to analysis. Then, the samples were thawed and homogenized in the aqueous buffer containing an internal standard, using

a Potter-Elvehjem homogenizer. Then the homogenates were centrifuged at 20,000 r.p.m. for 10 min and, in the end, aliquots of the clear supernatants were either injected immediately onto the chromatographic column or kept at 4°C for up to 48 h.

RESULTS AND DISCUSSION

Retention Behaviour and Identification of Biogenic Amines and Related Compounds

Choice of the reversed-phase column packing material

Reversed phase supports from different suppliers can show large differences in chromatographic behaviour.[7] In order to find a suitable packing material for our study three different columns were tested: Two from Alltech, Adsorbosphere Catecholamine 3μm (100mm x 4.6mm I.D.) and Adsorbosphere HS C18 5μm (250mm x 4.6mm I.D.), and one from MZ-Analysentechnik GMBH, Kromasil C18 5μm (250mm x 4.6mm I.D.). DA, 5HT, and M5HT were chosen as test compounds, since DA and 5HT are the most significant amines detected in the ganglia of snails[1] and M5HT was used as an internal standard in this study.

The Kromasil column exhibited unexpected peak tailing or fronting when methanol was used as an organic modifier and only the introduction of isopropanol, instead of methanol, to the mobile phase improved the overall chromatographic performance of this column. However, the other two columns showed a good chromatographic behaviour in any case. Consequently, both of them could be chosen for further optimization study, taking into account only that the top of the shorter column filled with 3μm particles is more likely to be clogged than the longer one filled with 5μm particles, when we inject the untreated supernatant directly onto the chromatographic column in order to avoid precolumns, a possible source of band broadening. This has led us to choose the longer Adsorbosphere column in this investigation, although it results in chromatograms with excessive retention in comparison with the other Adsorbosphere column. This attempt to find the suitable chromatographic column allowed, also, the positive identification of DA and 5HT in all tissues examined. Chromatograms produced from making injections of samples onto all different chromatographic columns tested almost always showed two peaks which corresponded, in terms of individual column retention time, to DA and 5HT.

Table 1

Retention Times (min) for All Test Biogenic Amines and Related Compounds Using Different Mobile Phase Composition

		Eluent Composition		
Test Solute	**Aqueous Buffer pH = 3.3**	**As Eluent of Previous Col. plus 6% Methanol**	**As Eluent of Previous Col. plus 25mg/L SOS**	**As Eluent of Previous Col. Except for pH = 4.1**
NE	3.5	3.1	3.3	3.4
E	4.5	3.4	3.8	4.0
DOPA	6.3	4.2	4.3	4.1
DA	7.5	4.6	5.7	6.0
5HTP	21.3	9.4	10.2	9.2
3MT	23.5	9.5	13.0	13.5
5HT	24.0	10.5	14.2	15.0
DOPAC	40.2	18.9	18.2	15.7
TRYP	49.6	22.0	24.6	20.1
5HTOH	76.0	31.0	29.8	30.0
5HIAA	98.0	38.4	37.0	34.9
HVA	162.5	60.7	57.5	48.5

Effects of organic modifier

The retention times of solutes in a pure aqueous mobile phase depicted in Table 1 have also been used in solute hydrophobicity estimation, but each RPLC system provides a distinct individual hydrophobicity measure of solutes.[8,9] Generally the more polar the solute, the less it is retained and for solutes of equal molecular size and polarity, the differences in RPLC retention may arise from steric effects.[9]

As an organic solvent, such as methanol, is added to a pure aqueous eluent, the mixture becomes less and less solvophobic. At the same time its surface tension lowers. Thus, the addition of an organic modifier is expected to facilitate the transfer of polar solutes, such as the solutes under examination, from the stationary to the mobile phase.[10] In addition, the introduction of methanol to the mobile phase is accompanied by the extraction of this organic solvent by the alkyl chains of the stationary phase, which has resulted in changes in the nature of the surface of the stationary phase. Thus, the solutes will inevitably interact less with the sorbed organic modifier through dipole-dipole and hydrogen interactions.[11] As a consequence of all of the above

mentioned, the addition of methanol in the mobile phase produces a decrease in retention time for all compounds examined, but no change in the elution order of these compounds. This is depicted in Table 1 for a representative methanol concentration of 6%. Therefore, methanol can be used as a factor for shortening the run time, but not as a means of optimizing separation or identification of solutes, since the effect of methanol on the retention behaviour of solutes is, to a first approximation, independent of each individual solute.

Effects of ionic modifier

The addition of an ion-pairing agent to an aqueous mobile phase containing an organic modifier, plus a buffer to control pH, has been demonstrated to be effective in minimizing the peak tailing attributed to secondary interactions between polar ionogenic compounds and unreacted silanol groups on the silica surface and, more importantly, in controlling the retention of ionic or ionizable compound. Retention in ion-pair RPLC can be described by a dynamic ion-exchange mechanism, i.e., ion-pair formation takes place between the sample ion and hetaeron (the pairing ion) adsorbed to the stationary phase.[12-14]

In practice, the effect observed from the addition of SOS at a concentration of 25 mg/L on the retention of the examined solutes is shown in Table 1. The retention of amines, NE, E, DA, 3MT, and 5HT, and of zwitterionic solutes, DOPA, 5HTP, and TRYP, is enhanced, while that of acidic compounds, DOPAC, 5HIAA, and HVA, and of the neutral one, 5HTOH, is reduced slightly. The observed influence of SOS on the retention of these compounds is in good agreement with the predictions based on both the process governing retention in ion-pair RPLC and the degree of ionization of solutes at a given pH. At the relatively low pH = 3.3 the amines and aminoacids are protonated and thus positively charged, while the acids are essentially nonionized. On the other hand, the stationary phase should be negatively charged because of the adsorbed hydrophobic negatively charged octyl sulfonates and it should interact strongly with the opposite charged solutes causing an increase in their retention. On the contrast, the retention of unionized acids and neutrals should be almost unaffected. The observed slight decrease in the retention of these compounds can be explained by a slight lessening of their interaction with the stationary phase as it becomes more polar through adsorption of the hetaeron.

The knowledge of the effect of the ionic modifier on the retention of the solutes of interest can be used as a means of optimizing the seperation of the solutes and of further confirmation of the identities of the sample components responsible for the peaks having column retention times similar to those of

standard analytes. In other words, if we make injections of standards and samples using mobile phases containing different concentrations of SOS and the retention behaviour of the corresponding standard and sample chromatogram peaks is similar, this is a strong evidence that the same analyte is responsible for these peaks.

Effects of mobile phase pH

In RPLC the retention of ionogenic solutes, such as the compounds examined in the present study, is a strong function of the mobile phase pH with different solutes showing different behaviour both qualitatively and quantitatively.[15] As indicated in Table 1, pH affects retention in the opposite manner for acidic (DOPAC, 5HIAA, and HVA) and basic solutes (NE, E, DA, 3MT, and 5HT). In addition, the retention of the acids is more sensitive to a change in mobile phase pH than the retention of the amines, since as the mobile phase pH increases, the acids are deprotonated to a greater extent, resulting in a decrease in their retention. Conversely, the amines carry essentially the same charge over the narrow pH range examined (3.3-4.1) and their retention increases only slightly.

The retention of the neutral metabolite 5HTOH is not affected by the mobile phase pH, while the retention of the amino acids, DOPA, 5HTP, and TRYP, resembles that of the acids, i.e., their retention increases with increasing mobile phase pH. This is probably due to an initial deprotonation of the protonated amino acids to form the more polar zwitterions.

Thus, pH can be used as a paramater for optimizing separations and identifying the solutes in the samples in the same manner as the addition of SOS in the eluent.

Figure 3 gives typical chromatograms of a standard mixture and a sample from the suboesophageal ganglia as representative for the other tissues investigated. Under the chromatographic conditions of Figure 3, a complete separation of NE, DA, 5HTP, DOPAC, 3MT, 5HT, 5HTOH, M5HT, 5HIAA, and HVA was achieved and peaks corresponding to NE, DA, 5HTP, DOPAC, 5HT, and 5HIAA were identified in the snail ganglia by retention times of standards. Moreover, as systematically described above, changes in the mobile phase composition gave further evidence that these biogenic amines and related compounds were present in all snail tissues examined, except for NE which eluted close to the solvent front peak. For this reason, no clearly detectable response of NE was visible as a separate peak, especially in case where a relatively small peak with no good baseline was obtained.

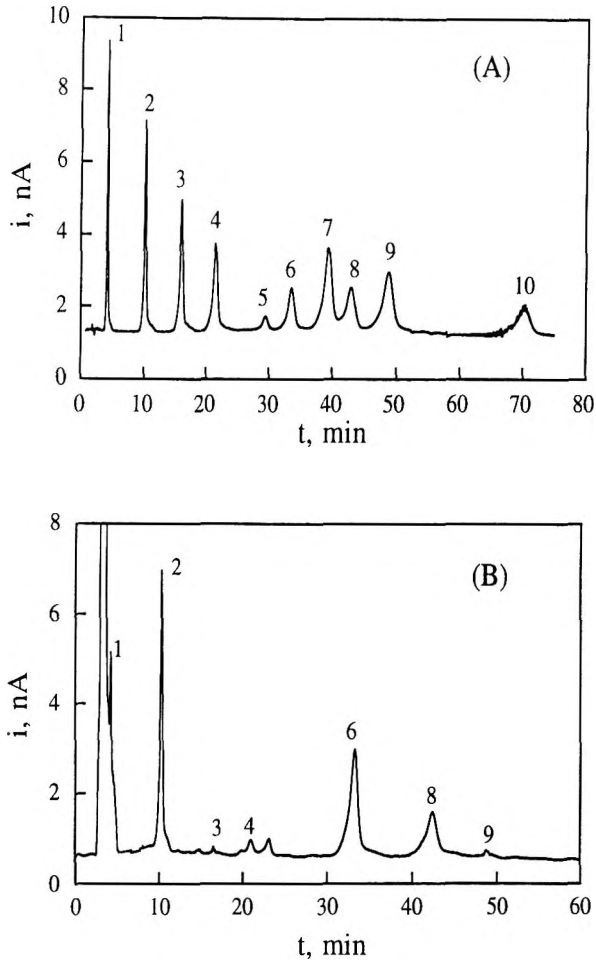

Figure 3. Typical chromatograms of 20 µL of a mixture containing 0.32 µg/mL of the reference and internal standards (A) and of a supernatant of snail ganglia (B); mobile phase aqueous buffer (pH=3.3)-methanol (96:4 v/v) containing SOS (40 mg/L). Peaks: 1=NE, 2=DA, 3=5HTP, 4=DOPAC, 5=3MT, 6=5HT, 7=5HTOH, 8=M5HT (internal standard), 9=5HIAA and 10=HVA. See text for other chromatographic conditions.

It should be noted here, that changes in chromatographic performance that occur with the continuous use of a given column, necessitate the periodic reoptimization of mobile phase parameters (SOS and methanol concentrations, and pH). However, reoptimization of column performance, using our knowledge about the effect of these parameters on retention of solutes of

interest, summarized in Table 1, can generally be accomplished in a few hours. In this investigation the necessary alterations of mobile phase composition ranged between 2-4 % for the methanol content and 20-40 mg/L for the SOS concentration.

Quantification of the Assayable Biogenic Amines and Related Compounds

Internal standardization

The use of internal standards in quantitative analytical procedures is generally considered to be superior to direct calibration. The addition of M5HT to reference solutions and tissue samples in equal concentrations, effectively controlled probable variable volumes injected and with-run variations in column or detector electrode performance. Calculation of sample concentrations was based on the comparison of the peak area ratios (relative to the internal standard, M5HT) for tissue samples to the corresponding peak area ratios for standards, i.e., $\frac{(CA/M5HT)_{sample}}{(CA/M5HT)_{standard}}$. In particular, for the quantification of a catecholamine (CA) present in a sample the following equation was used:

Concn of CA in sample, μg/g wet tissue =

$$\frac{(CA/M5HT)_{sample}}{(CA/M5HT)_{standard}} \times (\text{concn of CA in standard, } \mu g/mL) \times \frac{(\text{volume, mL})_{sample}}{(\text{weight, g})_{wet\ tissue}}$$

It is of interest to note that the use of M5HT increases the total chromatography time and, for this reason, in such assays, it is common to test internal standards, DHBA and EPI, in place of M5HT. However, the first one eluted soon after the solvent front next to DOPA and the other coeluted with DA for a given composition of the mobile phase. Consequently, since the use of M5HT simplifies establishing optimal mobile phase composition, we have chosen M5HT as an internal standard and shortened the run time for each sample using the gap between DA and 5HT for the analysis of the succeeding sample, at least in case where the most prominent biogenic amines, DA and 5HT, were only detected in tissues.

Table 2

Levels of 5HT, DA, NE, and DOPAC in Various Tissues of the Snail Helix Lucorum L.

	Concentration in μg/g Wet Tissue			
Tissue	**5HT**	**DA**	**NE**	**DOPAC**
Gaglia	15.26 ± 0.94	4.14 ± 0.75	0.51 ± 0.07	0.49 ± 0.09
Auricle	6.74 ± 0.85	0.27 ± 0.03	NA*	NA*
Ventricle	4.31 ± 0.56	0.02 ± 0.006	NA*	NA*

Values are means ± SEM (n=5)
* NA = not accurately assayable

Quantitative results

The results of the determination of the levels of biogenic amines and related compounds in different snail nervous and non nervous tissues are shown in Table 2. It is seen that 5HT occurs in greatest quantities with the most in the ganglia, followed by the auricle and the ventricle. As concerns catecholamines, DA is the dominant one in all tissues examined, although its amount in the heart muscle and especially in the ventricles is negligible compared to that in the nervous tissues. Moreover, another catecholamine, NE and the major DA metabolite, DOPAC (see Figure 3B) were found in roughly the same quantities in the ganglia but at about a 10 fold lower concentration than DA, while in heart tissues they could not be accurately measured.

In general, the results of this investigation are consistent with the results of previous studies of the gastropodes,[1,16] although there is a paucity of data detailing the quantification of biogenic amines and related compounds in the molluscan tissues.

It is worth noting that a variation in DA, 5HT, NE, and DOPAC concentrations were observed between the values determined by the analysis of different samples, see Table 2, although the samples analysed did not consist of tissues of individual animals but of two or three pooled tissues of different animals. Such variation is commonly observed in molluscan species[1,16] and is a feature of invertebrates in general.

Future Directions

In the future, it will be of interest to determine whether the biogenic amines and related compounds detected in the snail ganglia and in the heart auricles and ventricles display significant changes in their concentrations when the animals are maintained under different exogenous conditions, such as estivation, hibernation, etc. It is clearly of importance to investigate a possible correlation of differances in the levels of these edogenous compounds with behavioural dormant states of the snails.

Naturally, such studies are stongly dependent on accurate data. In this respect, we believe that the method described in this work takes into account all precautions to obtain optimum conditions and maximum sensitivity and, as a result, enables an accurate determination of catechol- and indoleamines and related compounds in biological tissues.

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Received January 1, 1997
Accepted January 31, 1997
Manuscript 4341

J. LIQ. CHROM. & REL. TECHNOL., 20(15), 2441–2450 (1997)

DIRECT LIQUID CHROMATOGRAPHIC SEPARATION OF 3R-TRANS, 3S-CIS, AND 3R-CIS 1,1-DIMETHYLETHYL (4R-CIS)-6-CYANOMETHYL-2,2-DIMETHYL-1,3-DIOXANE-4-ACETATE ENANTIOMERS ON A CELLULOSE TRIS (3,5-DIMETHYLPHENYL CARBAMATE) CHIRAL COLUMN

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ABSTRACT

Direct liquid chromatographic separation of 3R-trans, 3S-cis and 3R-cis 1,1-dimethylethyl (4R-cis)-6-cyanomethyl-2,2-dimethyl-1,3-dioxane-4-acetate enantiomers on a cellulose tris (3,5-dimethylphenyl carbamate) column is described. The 3R-cis isomer is an intermediate in the synthesis of atorvastatin (a HMG-CoA reductase inhibitor). The detection limit for each of the undesired 3R-trans and 3S-cis isomers was 0.2% by area percent normalization. The separation of the undesired 3S-trans diastereomer was occasionally observed, and was found to be greatly affected by slight differences in mobile phase composition.

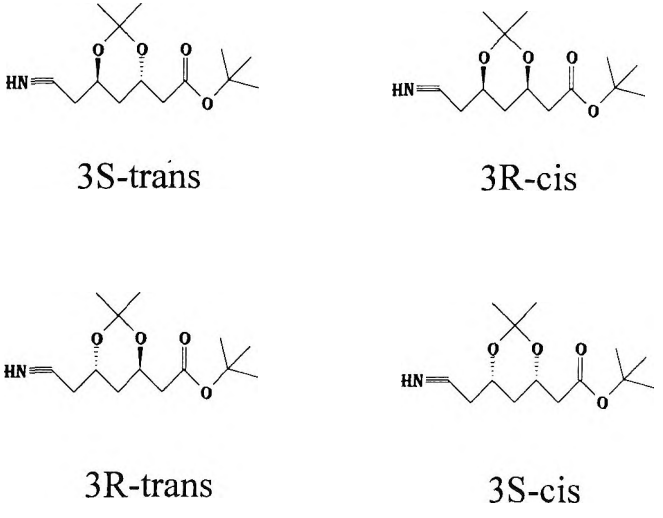

Figure 1. Structures of the isomers.

INTRODUCTION

1,1-Dimethylethyl (4R-cis)-6-cyanomethyl-2,2-dimethyl-1,3-dioxane-4-acetate (3R-cis) is a key intermediate in the convergent synthesis of atorvastatin.[1,2] This ethical pharmaceutical has been shown, in human clinical trials, to significantly reduce serum cholesterol levels by inhibiting the enzyme HMG-CoA reductase.[3,4] In order to control the level of undesired stereoisomers in the bulk pharmaceutical product, a chiral assay for the 3R-cis intermediate was developed. This was done since this intermediate contains all of the chiral centers present in the atorvastatin molecule, and thus, the chiral purity of the final bulk pharmaceutical is determined by the chiral purity of the 3R-cis intermediate.

A cellulose tris (3,5-dimethylphenyl carbamate) chiral column was used to obtain the separation of the undesired 3R-trans, 3S-cis and occasionally the 3S-trans isomers, from the 3R-cis isomer (Figure 1). The effect of eluent composition (hexane: isopropanol ratio, trifluoroacetic acid content), flow rate, and column temperature on the separation of the 3R-trans, 3S-cis, 3R-cis, and 3S-trans isomers was investigated.

EXPERIMENTAL

Apparatus

The HPLC system was composed of the following: a Hitachi L-6200 pump, an Alcott 728 autosampler with a 20μL injector loop, a Hitachi L-4200H detector, a Waters TCM column heater, and a Hitachi D-2500 chromato-integrator. The column used was a Chiracel OD-H, 5 micron, 250 x 4.6mm ID from Chiral Technologies Inc, Exton, PA.

Reagents

HPLC grade hexane(s) and isopropanol were purchased from EM Science, Gibbstown, NJ. All 1,1-dimethylethyl (4R-cis)-6-cyanomethyl-2,2-dimethyl-1,3-dioxane-4-acetate isomers were synthesized in the Chemical Development Department of the Parke-Davis Pharmaceutical Research Division, Holland, MI.

Chromatographic Conditions

The mobile phase used consisted of 97 parts hexane, and 3 parts isopropanol (mixed v/v). The column temperature was 30°C (except for the temperature study). The ultraviolet detection wavelength was 215 nanometers. Flow rate was 0.8 mL/min (except for the flow rate study). The amount of sample injected for the determination of chiral purity was about 200 μg (in a 20μL injection volume).

RESULTS AND DISCUSSION

The hexane/isopropanol mobile phase used in this system resulted in the separation of the 3R-trans, 3S-cis and 3R-cis isomers. Isopropanol content, flow rate, and column temperature were varied to optimize the separation of the enantiomers while maintaining a low detection limit.

Table 1 and Figure 2 show the results of varying the isopropanol content in the mobile phase from 1 to 7% (v/v). The isomers resolution from each other increased with decreased isopropanol content.

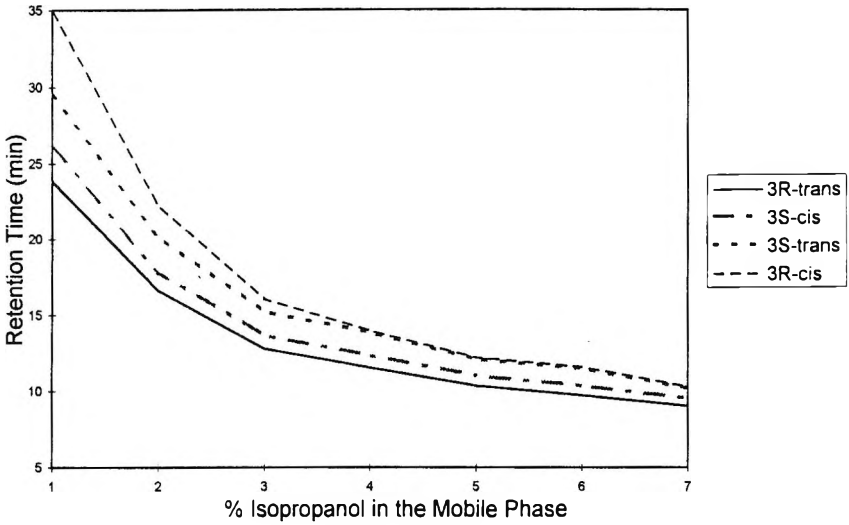

Figure 2. Graph of isopropanol content versus isomer retention time.

Table 1

Resolution Resulting from Alterations in the Hexane/Isopropanol Ratio of the Mobile Phase

Hexane IPA Ratio	Resolution 3R-trans, 3S-cis	Resolution 3S-cis, 3S-trans	Resolution 3S-trans, 3R-cis	Resolution 3S-cis, 3R-trans
99.1	1.89	2.14	2.99	----
98.2	1.44	2.35	1.82	----
97.3	1.46	2.13	0.89	----
96.4	1.51	Coeluted	----	2.34
95.5	1.39	Coeluted	----	1.96
94.6	1.31	Coeluted	----	2.17
93.7	1.33	Coeluted	----	1.39

Although all four isomers were separated when the mobile phase contained 1 and 2 % isopropanol, the peaks were broadened due to longer retention on the column. This resulted in a loss of the desired detection level of 0.2%.

Table 2

Resolution and Retention Times of the Isomers at Variable Flow Rates

	0.6 mL/min	0.8 mL/min	1.0 mL/min
Retention 3R-trans (min)	17.56	12.78	10.56
Retention 3S-cis (min)	18.80	13.67	11.30
Retention 3S-trans (min)	20.99	15.22	12.58
Retention 3R-cis (min)	22.54	16.04	13.26
Resolution 3R-trans, 3S-cis	1.50	1.46	1.33
Resolution 3S-cis, 3S-trans	2.11	2.13	1.86
Resolution 3S-trans, 3R-cis	1.20	0.89	0.80

Table 3

Resolution and Retention Times of the Isomers at Different Column Temperatures

	25°C	30°C	35°C
Retention 3R-trans (min)	14.39	12.78	12.19
Retention 3S-cis (min)	15.36	13.67	13.08
Retention 3S-trans (min)	17.43	15.22	14.32
Retention 3R-cis (min)	19.18	16.04	15.26
Resolution 3R-trans, 3S-cis	1.32	1.46	1.48
Resolution 3S-cis, 3S-trans	2.22	2.13	1.58
Resolution 3S-trans, 3R-cis	1.64	0.89	1.16

The mobile phase flow rate was varied from 0.6 mL/min to 1.0 mL/min. Resolution was slightly increased with a lower flow rate (Table 2). The detection limit remained acceptable at the lower flow rate of 0.6 mL/min.

The column temperature was varied from 25°C to 35°C, and this had some effect on the resolution of the isomers. As temperature was decreased the resolution slightly increased. The results are shown in Table 3. Trifluoroacetic acid was added to the mobile phase at the 0.1% level (v/v). This modifier had no effect on the separation (data not shown).

Table 4

Mobile Phase Aging Experiment Testing the Resolution of the 3S-Trans Diastereomer From the 3R-Cis Intermediate Over Time

Time	0 Days	1 Day	3 Days	8 Days	13 Days
Retention 3R-trans (min)	12.78	12.70	12.72	13.44	13.47
Retention 3S-cis (min)	13.67	13.59	13.63	14.38	14.40
Retention 3S-trans (min)	15.22	15.86	15.16	16.18	16.16
Retention 3R-cis (min)	16.12	15.86	16.00	17.36	17.72
Resolution 3R-trans, 3S-cis	1.46	1.49	1.52	1.42	1.38
Resolution 3S-cis, 3S-trans	2.13	2.08	2.07	2.17	2.17
Resolution 3S-trans, 3R-cis	0.89	0.76	0.90	1.11	1.50

The resolution of 3R-trans, 3S-cis, and 3R-cis from each other on the HPLC system was consistent using the parameters in the chromatographic conditions section. These conditions achieved separation of the above three isomers, while retaining the ability to detect them at low levels. It was observed that the fourth isomer 3S-trans coeluted with the 3R-cis intermediate in most of the above experiments. If some separation occurred, the low detection limit was lost. While investigating the method before validation, mobile phase was used which had been prepared a month earlier. All four isomers were well resolved from each other. When new mobile phase was prepared, the resolution of the 3S-trans isomer from the 3R-cis intermediate was lost. After much investigation, it was determined that as time passed after mobile phase preparation, resolution of the 3S-trans isomer from the 3R-cis intermediate on the HPLC system was improved. The rate of this 'aging' phenomenon varied depending on the brand (JT Baker and Fisher), purity, or age of the hexane used. This observation was investigated to determine if the 3S-trans isomer could be separated from the 3R-cis intermediate consistently.

An experiment was done to show the 'aging' phenomenon. Mobile phase was prepared and the resolution of all four isomers was measured after preparation, and on the following days. The results are shown in Table 4 and Figure 3.

These results show that the changes in mobile phase, with time, were not caused by a decrease in the hexane:isopropanol ratio due to evaporation. If this were the case, retention times would get progressively earlier; however, elution occurred later after 2 weeks.

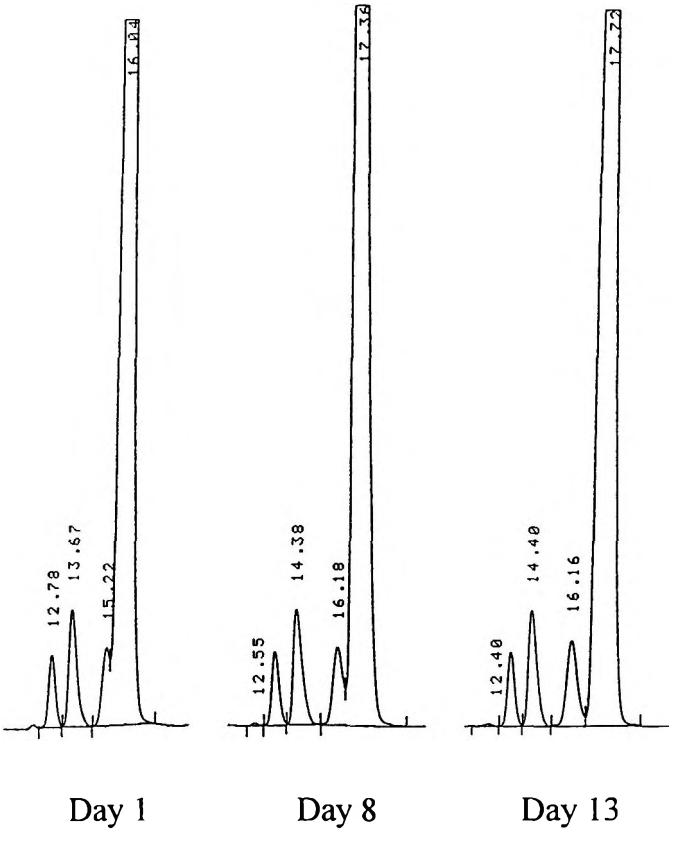

Figure 3. Mobile phase 'aging' experiment.

Water content in the mobile phase was varied (from 0.01-0.9%) and showed no effect in the resolution of 3S-trans and 3R-cis isomers. Mobile phase (which had been 'aged') was tested for peroxide formation,[6] because it has been reported that hydroperoxides may form by the oxidation of hydrocarbons in the presence of dissolved oxygen.[5] No peroxides were detected.

Air was bubbled through both the hexane, before mobile phase preparation, and the mobile phase itself. This was done as an attempt to speed the 'aging' process by exposure to oxygen in the air. The resulting mobile phases did not separate the 3S-trans isomer from the 3R-cis intermediate.

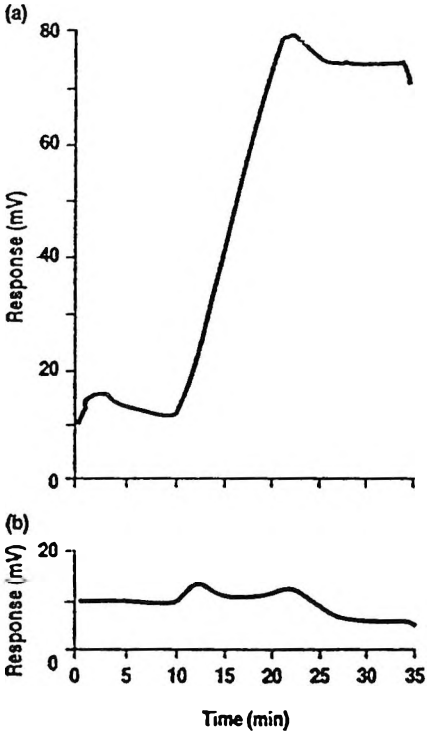

Figure 4. Blank gradient baselines for mobile phases containing (a) year old hexane and (b) fresh hexane.[7]

An experiment was done to test the affects of light exposure on the mobile phase. Mobile phase was prepared, then half was stored in the dark and half in the light. Both were tested at intervals over two weeks time. They 'aged' at the same rate showing that light had no effect on the 'aging' process (data not shown).

Problems with hexane/isopropanol mobile phase over time in normal phase HPLC have been previously reported.[7] Specifically, T. Michnik and D. Federighi of Cell Therapeutics, Inc.,[7] generated two different blank gradient runs using their own method. The first, used one year old hexane in the mobile phase, and the second, three month old hexane. These are shown in Figure 4.[7] The 'age' of hexane can have some effect on normal phase HPLC chromatography; however, the explanation for the change over time is uncertain.

CONCLUSIONS

This method is effective in determining the chiral purity of the 3R-cis intermediate in the synthesis of atorvastatin. It has been routinely used to detect the undesired 3S-cis enantiomer down to the 0.2% level. The 3R-trans isomer is nicely separated, but is not of concern, because it is not detected in the intermediate.

Although the separation of the undesired 3S-trans diastereomer was observed using 'aged' mobile phase, the reason for this is not understood, limiting reproducibility. Another method was, therefore, developed to quantify the 3S-trans isomer.

ACKNOWLEDGMENTS

We gratefully thank Drs. Donald E. Butler, Sechoing Lin, Thomas N. Nanninga, and William T. Suggs for helpful comments and suggestions.

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Received December 22, 1996
Accepted January 31, 1996
Manuscript 4365

J. LIQ. CHROM. & REL. TECHNOL., 20(15), 2451-2459 (1997)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE ASSAY OF TEBUFENOZIDE INSECTICIDE IN SUSPENSION FORMULATIONS

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ABSTRACT

A simple, reliable, and robust liquid chromatographic method is described to separate and quantify tebufenozide insecticide in suspension concentrates. The analyte was extracted with acetonitrile and partitioned with hexane to remove lipoid and other nonpolar additives in the formulation to improve selectivity and specificity. The concentration of the insecticide in the polar phase was adjusted volumetrically and analysed using an RP-8, 10-μm, 200 × 4.6 mm i.d. bonded phase column, with diode-array detection at 236 nm and 50% water/50% acetonitrile-dioxane (4:1, v/v), as the mobile phase. The linear concentration range, limit of detection, and limit of quantification for the insecticide were 5 to 500 ng (in 40-μL injection volume), 1.0 ng and 5.0 ng, respectively. Triplicate analysis of 3 lots of commercial suspension concentrates gave values that were agreeable with those given on the labels. The method, with necessary modifications, could be extended and used routinely as a quality control method in the analysis of different types of tebufenozide formulations.

INTRODUCTION

With the current public concern about the widespread use of broad-spectrum neurotoxic insecticides in forest insect control programs and their perceived risk to human health and the environment,[1] considerable interest has been shown in recent years to develop and test promising new chemicals which are environmentally benign and have a narrow-spectrum of activity. Tebufenozide [N,N'-*t*-butyl-N.-(3,5-dimethylbenzoyl)-N-(4-ethylbenzoyl) hydrazine], also known as MIMIC® or RH-5992, developed and marketed by Rohm and Haas Co. (Spring House, PA, USA), is a hormonal insecticide acting as an insect growth regulator interfering with the molting process of lepidopteran insects.[2] It is a nonsteroidal ecdysone agonist causing premature and incomplete ecdysis, and eventual death of the exposed insects. The material is found to have low mammalian and aquatic toxicities.[3] Because of these desirable properties, it is field tested in Canada to control the insect pest, spruce budworm (*Choristoneura fumiferana* Clemens), a destructive defoliator and killer of spruce and fir forests of the New England states in USA and the Maritime provinces in Canada.

Aerial application of insecticides is an economical method used in forestry to cover large areas of infested forests. In the past few years, different aqueous and oil-based formulations of tebufenozide have been field tested to evaluate their stability, sprayability, target coverage, and biological performance. Few analytical methods have been reported in open literature to quantify the active ingredient (AI) in the formulations.[3] In 1994, Rohm and Haas introduced a low volume suspension concentrate (SC) for forestry use to enhance its deposition characteristics on conifer needles and its biological effectiveness, and, also to improve its dispersion, suspension, and shelf-life. The new formulation consisted of a suspension of finely ground particles (3 to 5 μm) of tebufenozide mixed in a fluid medium containing oils, water, emulsifiers, stickers, surfactants, and microgranular solid matrices. The chemical identity of additives and their composition in the SC are the proprietary information of the company. The formulation was mixed with water at the spray site to form an emulsion which was then sprayed aerially over the budworm-infested forests. Although a high performance liquid chromatographic (HPLC) method has been reported in literature to monitor the tebufenozide content in experimental formulations,[4] the method did not specifically address the analysis of the new SC which contained a host of additives such as oils, emulsifiers, surfactants, solid matrices, etc., which could cause interference in the analysis. This paper describes a reliable, reversed-phase HPLC method to quantify the AI in the SC formulations, and examines its suitability as a quality control method to monitor the AI content in other tebufenozide formulations that will be used routinely in forestry spray operations to control the spruce budworm populations.

EXPERIMENTAL

Materials

HPLC-grade acetonitrile, dioxane, hexane, and methanol were purchased from Caledon Laboratories Ltd. (Georgetown, ON, Canada). Ultrapure water was drawn from a Milli-Q water purification system (Millipore Corp., Bedford, MA). Analytical grade tebufenozide (99.6% purity, m.p. 186-1880C) was kindly donated by Rohm and Haas Co., USA. Among the three lots of commercial SC formulations used in the analysis, one (SC-1) was from the 1994 field study, the second (SC-2) was from the scientist in charge of the Formulation Project at the local CFS research centre, and the third (SC-3) was supplied courtesy of Rohm and Haas Canada Inc. (West Hill, ON). However, all three SC formulations, containing 24.0 g AI/100 mL as per the label claim, were initially manufactured and distributed by the parent Rohm and Haas Co. (Spring House, PA).

Chromatography

The instrument used was a Hewlett-Packard (HP) (Palo Alto, CA) model 1090M HPLC, fitted with an autosampler, variable volume auto-injector, a binary solvent delivery system with He degassing, and two dual-syringe metering pumps giving stable and reproducible flows. The instrument was connected to a UV-visible diode-array detector (DAD) with a wavelength range of 190 to 600 nm. The HPLC system was monitored by a HP-9000/310 computer work station operated by HP-7995 R software. An HP LiChrosorb RP-8, 10-μm, 200 × 4.6 mm i.d. analytical column, preceded by a MOS-Hypersil C-8, 5-μm, 20 × 4.6 mm i.d. guard column, both thermostated at 40°C, were used. The elution was achieved isocratically using a mobile phase consisting of 50% water/50% acetonitrile-dioxane (4:1, v/v) with a flow-rate of 0.8 mL/min. Prior to analysis, the mobile phase was filtered using a 0.20-μm Nylaflo nylon membrane filter (Gelman Sciences, Rexdale, ON) and degassed. The sample and reference wavelengths were set at 236 and 430 nm, respectively, and the injection volume used was 40-μL. The system was equilibrated for 30 min before making an injection.

Stock and Working Solutions

A stock solution of tebufenozide was prepared by weighing 100.0 mg of the analytical material into a volumetric flask, dissolving it in acetonitrile, and adjusting

the volume to 100 mL. The flask was covered with aluminum foil to prevent photolytic loss of the analyte and stored in darkness at 0°C. Five working solutions in the concentration range of 0.125 to 12.5 μg/mL were prepared by serial dilution of the stock solution to calibrate the HPLC. Forty-μL of each working solution was injected into the HPLC six times and the DAD response (mAU) was recorded.

A calibration curve was prepared by plotting the average mAUs against concentration. The detector response was linear in the concentration range of 5 to 500 ng. The slope and intercept of the straight line, calculated using the least-square regression analysis, were 2.026 and 0.602, respectively, with a coefficient of determination of 0.998.

The SC formulations had the tendency to separate into two phases and the solid ingredients in the mixture sedimented at the bottom of the container on prolonged storage. Each container was first inverted several times to break-up the lumps and then agitated vigorously on a mechanical shaker for 2 h. About 1 mL of each formulation was pipetted into a weighing bottle and its exact mass was determined on an analytical balance. The formulation was then transferred, quantitatively, to a 50-mL Teflon centrifuge tube and its volume was adjusted to 30 mL with acetonitrile. The tube was agitated for 1 h for dissolution of the AI and then centrifuged at 6000 rpm for 20 min to coagulate and sediment some of the additives in the formulation.

A 20-mL aliquot of the supernatant was transferred to a 50-mL separatory funnel, 7 mL of hexane was added and shaken for 10 min. The bottom acetonitrile layer, containing the AI and other polar additives, was transferred to a 100-mL volumetric flask. The hexane layer containing the oil and other nonpolar materials was partitioned two more times, with 15 mL of acetonitrile each time, to remove the trace levels of AI in the hexane phase. The acetonitrile layers were added to the initial sample in the volumetric flask and the hexane layer was then discarded. The volume of the pooled acetonitrile was adjusted to 100 mL with further addition of the solvent.

A measured volume of the filtered acetonitrile extract was then taken in a graduated centrifuge tube and its volume adjusted by either concentration under N_2 (Meyer N-Evap®) or dilution with acetonitrile, so that the concentration of AI in the formulation extract was within the concentration range of the calibration curve prepared for the tebufenozide standard. A 40-μL aliquot of each extract was injected several times (n = 6) into the HPLC and the average DAD response was calculated. The concentration of the AI in the extract was computed from the calibration curve and expressed as g AI/100 mL of the SC.

RESULTS AND DISCUSSION

Method Development

The objective of this work was directed towards the development of a simple, rapid, and accurate HPLC method for the routine analysis of tebufenozide in SC formulations which, are nowadays, viewed favourably for forestry use. Using the previous work[4] as a guideline, suitable mobile and stationary phases, detection mode, column temperature, etc. were selected. Quantitative recovery of the analyte from each of the three SCs was obtained initially by repetitive experimentation using different solvents for extraction. Acetonitrile dissolution followed by hexane partition gave good analyte recovery from the formulations. Hexane partition, although somewhat time consuming, helped to remove the lipoid components and other nonpolar additives in the formulation, enhancing optimum analyte resolution and separation with good peak purity.

The use of RP-8 bonded phase column (10-μm, 200 × 4.6 mm i.d.) and the mobile phase consisting of 50% water/50% acetonitrile-dioxane (4:1, v/v), produced good elution pattern of the analyte and its separation from the impurity peaks in the formulation. The necessity of dioxane, its amount and effect on the peak separation were investigated, and 10% v/v of the solvent in the mobile phase was found to be optimum, producing good peak separation. However, slight variations (± 2%) in the composition did not unduly affect the analysis, indicating the ruggedness of the method. The DAD detection mode chosen (sample λ set at 236 nm) responded linearly to the analyte in the concentration range of 5 to 500 ng when 40-μL aliquots of the standard solutions were injected into the HPLC. The selection of column temperature (40°C) and the mobile phase flow rate of 0.8 mL/min gave optimum run time and good peak separation of the analyte. With ambient temperature or increased flow rate, the run time decreased but the peak resolution and peak quality were below marginal.

Method Validation

Figure 1 shows typical chromatograms obtained for the reagent blank and tebufenozide standard, after injecting 40-μL volumes of each into the RP-8 column and eluting with the selected mobile phase. It can be seen that the analyte was eluted and detected, and its peak was well resolved with good baseline separation. The retention time (RT) was 18.2 min. The precision of the method was determined by replicate injection (n = 6) of the same standard solution and measuring the corresponding RTs and peak areas. The relative standard deviations (RSD) in RTs and peak areas from the mean were 1.2 and 1.6%, respectively. This exercise was

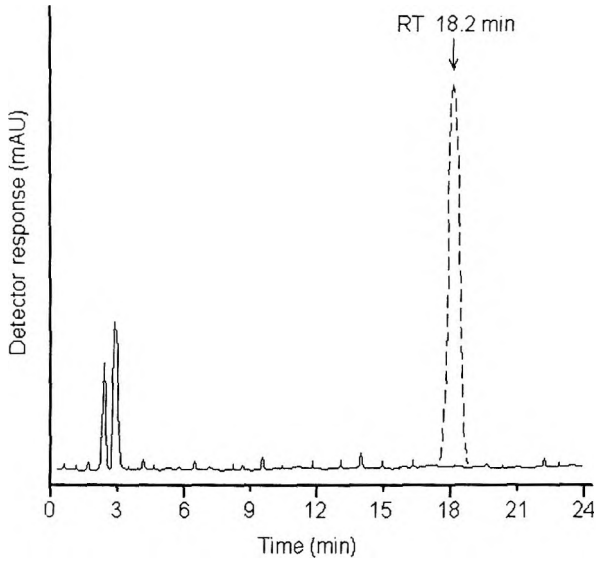

Figure 1. Liquid chromatogram of a blank solution (——) and of a 2 μg/mL tebufenozide standard (- - - -) after a 40-μL injection.

repeated for the other standard solutions and similar results were obtained, indicating the precision of the chromatographic response. Repeat injections on different days indicated a good degree of reproducibility of the results. The linearity of the detector response was confirmed by plotting peak area counts against the analyte concentration range studied (5 to 500 ng) and a linear relationship passing through the origin was obtained with a coefficient of determination of 0.998.

The calibration of the HPLC system reported above was carried out before and during the analysis of SC formulations to check that the instrument was performing within the limits set during validation.

The limit of detection (LOD) was reported in this paper as the concentration which gave a signal to noise ratio of 2:1. The ratio was determined by measuring the peak area of the analyte and dividing it by the absolute value of the largest noise fluctuation from the baseline of the chromatogram of a blank solution.[5] The limit of quantification (LOQ) of the analyte, determined with acceptable precision and accuracy, was expressed arbitrarily as five times the LOD value. The LOD and LOQ values obtained in this study were 1.0 and 5.0 ng, respectively.

Table 1

Tebufenozide Concentrations in Three Lots of Suspension Concentrate (SC) Formulations

Sample Lot	Expected or Label Conc. (% w/v)	Measured Conc. (% w/v) (mean ± SD)	RSD (%)	Abs. Error (%)
SC-1	24.0	22.3 ± 1.6	7.2	7.1
SC-2	24.0	24.2 ± 0.9	3.8	0.8
SC-3	24.0	22.7 ± 1.1	4.8	5.4

Formulation Analysis

The acetonitrile extracts of the three SC formulations after hexane partition, were injected into the HPLC, and the average (n = 6) peak areas were computed. The corresponding concentrations were read from the calibration curve and the %AI in the formulations (w/v) were calculated and compared with the data on the label. The results are recorded in Table 1.

Figures 2 and 3 show typical chromatograms obtained for the acetonitrile extract of SC-1 formulation before and after hexane partition, respectively. Comparison of the sample chromatograms shows noticeable impurity peaks in Figure 2, probably from oil and other nonpolar components present in the extract. Although the analyte peak was somewhat resolved, the baseline separation and peak purity were unsatisfactory. In addition, drift in RT was appreciable from sample to sample (range, 18.0 to 18.8 min). A minor peak near the up-slope and a shoulder at the down-slope of the analyte peak in Figure 2 introduced considerable error in quantification, primarily due to selectivity and specificity in assaying the AI in the formulation. However, the hexane partition, although time consuming, rectified the problem by completely removing the minor peak and the shoulder in the chromatographic trace (Figure 3), as well as the drift in RT.

The AI measured in the three sample lots of SC formulations (Table 1) agreed reasonably well (22.3 to 24.2%, w/v) with the expected or label value (24.0%, w/v) of each sample. The absolute error (deviation of the measured value from the expected or theoretical value in absolute terms of percentage) ranged from 0.8 to 7.1%, whereas the range in RSD was 3.8 to 7.2%. The sample lot, SC-2, received from the local Formulation Project had the lowest RSD (3.8%) and absolute error (0.8%), whereas the field sample (SC-1) had the highest values for both.

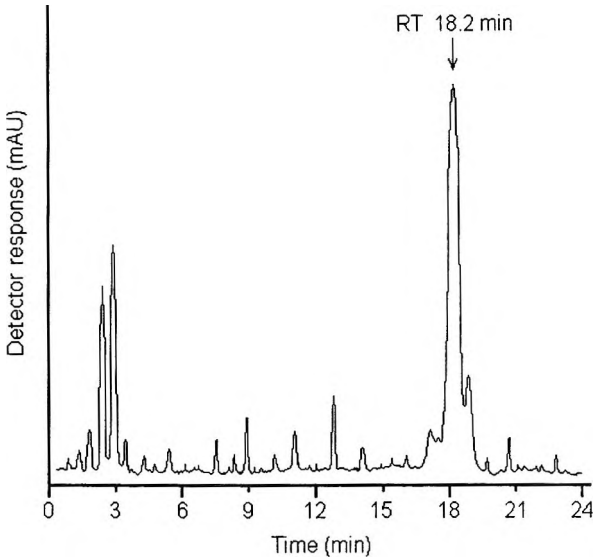

Figure 2. Liquid chromatogram of a suspension concentrate in acetonitrile (40-μL injection); interference from formulation matrices observed.

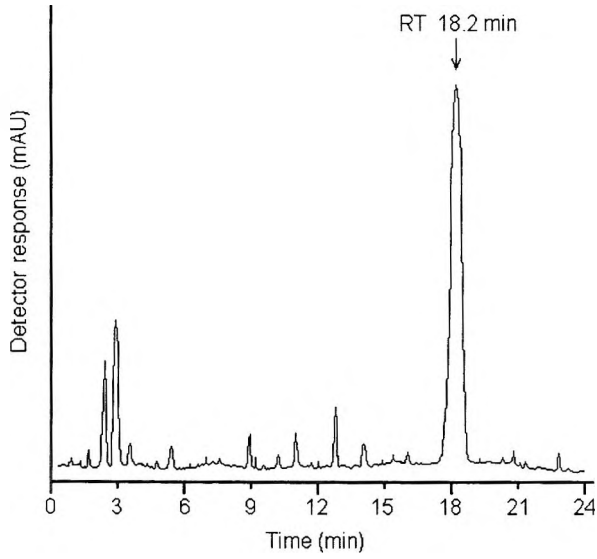

Figure 3. Liquid chromatogram of a suspension concentrate after acetonitrile extraction and hexane partition (40-μL injection).

CONCLUSIONS

The HPLC method described in this paper, provides an easy, accurate, and rugged technique for the extraction and quantification of tebufenozide in commercial SC formulations. This method could be extended easily to routinely analyse the AI in aqueous and emulsifiable concentrates, granular, and wettable powder formulations of tebufenozide, and could become a standard method for quality control operations.

ACKNOWLEDGMENTS

The author expresses sincere thanks to R. Nott for laboratory assistance, L. Sloane for preparation of the manuscript, and Rohm and Haas Company for supplying the suspension concentrate formulations and analytical grade tebufenozide used in this study.

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Received January 12, 1997
Accepted February 14, 1997
Manuscript 4343

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SOLID-PHASE EXTRACTION FOR THE DETERMINATION OF TRICYCLIC ANTIDEPRESSANTS IN SERUM USING A NOVEL POLYMERIC EXTRACTION SORBENT

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ABSTRACT

A fast and easy solid-phase extraction method was developed for the determination of amitriptyline, doxepin and their metabolites (nortriptyline and nordoxepin) in porcine serum matrix by high performance liquid chromatography. The spiked serum sample was pretreated with 2% phosphoric acid followed by a simple and rugged SPE procedure using OasisTM HLB extraction cartridges.

The SPE method requires only one mL of simple solvent throughout the entire SPE process. High and reproducible recoveries were obtained even though the cartridges ran dry. For five replicate analyses, the average recoveries of parent tricyclic antidepressants and their metabolites were all greater than 94%, and the RSDs were all less than 4.0%.

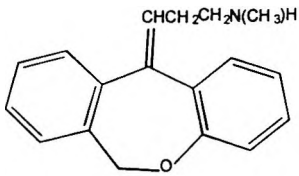
INTRODUCTION

Over the past 40 years, tricyclic antidepressants (TCAs) have been widely used to treat patients suffering from depression. They inhibit the reuptake of the neurotransmitters serotonin and norepinephrine in the central nervous system, and they are among the most widely prescribed drugs in current medicine. The monitoring of their concentrations in biological fluids such as plasma, serum or urine can be important in determining clinical efficacy, predicting side effects, and monitoring compliance.[1-2] The quantification of these drugs is also useful in emergency settings.[3] Moreover, TCAs are also used to treat enuresis, migrainous headaches, and obsessive-compulsive disorders.[4]

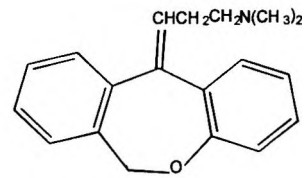
Many analytical methods have been applied to the analysis of TCAs. These include gas chromatography,[5-7] liquid chromatography,[8-18] and immunoassay.[19-21] As shown in Figure 1, the structures of TCAs are very similar; this might exhibit mutual interference in any immunoassay format which lacks antibody specificity. Liquid chromatography is the predominant method used for the measurements of antidepressants. A number of HPLC methods for determining TCAs and their metabolites have been developed and reviewed.[15-16] As pointed out by Wong,[15] the attributes for using HPLC are as follows. First, it is cost-effective due to the low reagent cost for extraction solvent and mobile phase. Second, it allows the possibility of simultaneous assays of multi-drugs and metabolites. Third, the routine use of LC always presents minor technical problems; experience and simple procedure enhance its application. The modes of HPLC include ion-paring, normal phase, and reversed phase chromatography. Among them, the reversed phase chromatography is the dominant method for the analysis of TCAs.

For the sample preparation prior to the HPLC analysis of TCAs in biological fluids, either liquid-liquid extraction[8,10-12,14-17] or solid-phase extraction[9,13,15,16,18] is used. The purpose of the sample preparation is to extract analytes of interest from the sample matrix in a high concentrated form prior to HPLC analysis.

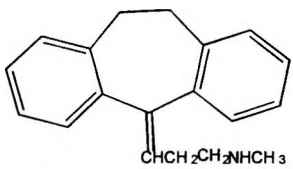
The trends of sample preparation have been shifted from liquid-liquid extraction to solid-phase extraction. In the most recent survey conducted by LC-GC, 40% of respondents use SPE.[22] Reversed phase sorbents, such as C_8 and C_{18}, are the most widely used packing materials. However, a troublesome feature of the reversed phase SPE is its irreproducibility. Also SPE is not easy to use. Many authors have reported that reversed phase SPE cartridges should not run dry before loading sample solution.[23-25] If accidentally the cartridges



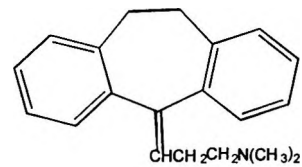
Nordoxepin



Doxepin



Nortriptyline



Amitriptyline

Secondary Amines Tertiary Amines

Figure 1. Structures of tricyclic antidepressants. They have very similar structures with two phenyl rings and one seven member ring-structure. Nordoxepin and nortriptyline are secondary amines; doxepin and amitriptyline are tertiary amines. Nordoxepin is a metabolite of doxepin, nortriptyline is a metabolite of amitriptyline. All of them are basic compounds with pKa values around 10.

run dry, the consequences are low and variable recoveries. This tedious and time consuming process is the major drawback for SPE. Therefore, it is desirable to have an easy SPE procedure which can provide excellent recovery and reproducibility.

In this paper, we report a fast and easy SPE method for high and reproducible recoveries of amitriptyline, doxepin, and their metabolites (nordoxepin and nortriptyline) in porcine serum matrices by an improved

HPLC assay. The HPLC method utilizes a Symmetry® C_{18} reversed phase column and a simple mobile phase. Oasis™ HLB extraction cartridges are used to extract the analytes from the serum matrix. The sorbents retain analytes even when the cartridges run dry.

EXPERIMENTAL

Reagents and Materials

The nordoxepin hydrochloride was from Alltech (Deerfield, IL); doxepin hydrochloride, nortriptyline hydrochloride, and amitriptyline hydrochloride were from Sigma Chemical Company (St. Louis, MO). Acetonitrile, methanol, and phosphoric acid were HPLC grade and were from J. T. Baker (Phillipsburg, NJ). Porcine serum was obtained from Equitech-Bio (Ingram, Texas). 1cc/30 mg OasisTM HLB extraction cartridges were obtained from Waters Corporation (Milford, MA).

Standard Solutions

A stock standard of each compound was prepared by dissolving 1.0 mg of the pure compound in 1.0 mL of water. Working solutions were prepared by diluting these stock standards in 20 mM phosphate buffer, pH 7.0/methanol (80:20).

Standard curves were prepared in 20 mM phosphate buffer, pH 7.0/methanol (80:20) over a concentration range from 0.50 to 10 μg/mL for amitriptyline, and from 0.25 to 5.0 μg/mL for doxepin and nortriptyline. Each standard solution contained 2.5 μg/mL nortriptyline as the internal standard.

Extraction Procedures

Aliquots of freshly thawed drug-free serum were spiked with drug solutions to produce the desired concentrations. Two levels of sample concentrations were prepared. At high level, the concentrations for nortriptyline and doxepin were each at 0.5 μg/mL; for amitriptyline was at 1.0 μg/mL. At low level, the concentrations for nortriptyline and doxepin were each at 0.1 μg/mL; for amitriptyline was at 0.2 μg/mL. Each level contained 0.5 μg/mL of nordoxepin as the internal standard. These spiked serum samples

were then acidified with 20 μL of phosphoric acid. The samples were vortex-mixed for five seconds and loaded onto OasisTM HLB extraction cartridges, which had been activated with 1 mL of methanol followed by 1 mL of water. After loading, the cartridges were washed with 1 mL of 5% methanol solution, which was discarded. The cartridge was eluted with 1 mL of methanol. The eluate was evaporated to dryness in a heating block at 40°C under a gentle stream of nitrogen and reconstituted with 200 μL of 20 mM phosphate buffer, pH 7.0/methanol (80:20).

HPLC Apparatus and Operating Conditions

Isocratic elution was used throughout the entire study. The HPLC system consisted of a Waters 616 LC system, a 717 plus Autosampler, and a 996 Photo Diode Array Detector. The Millennium 2010 Chromatography Manager was used for system control and data acquisition. The column used was a Waters Symmetry® C_{18} (3.9 mm x 150 mm, 5 μm particle size) preceded by a Sentry™ guard column (3.9 mm x 20 mm). The chromatography was carried out at 35°C. The mobile phase was 20 mM potassium phosphate, pH 7/methanol at 30:70 (v/v). The flow rate was 1.0 mL/min. For the concentration determination, 20 (μL each of the sample and the standard solution were injected. The effluent was monitored at 254 nm.

RESULTS AND DISCUSSIONS

Chromatographic Analysis

As shown in Figure 1, TCAs have two phenyl rings and one seven member ring-structure. On the basis of changes in the side chain, they can be divided into secondary (nordoxepin and nortriptyline) and tertiary amines (doxepin and amitriptyline). Nordoxepin is a metabolite of doxepin, nortriptyline is a metabolite of amitriptyline. They are basic compounds with pKa values around 10. They interact with the residual sites of silanol groups present on the silica based reversed phase sorbent, and cause peak tailing in HPLC and low recovery for SPE. To overcome this problem, it has been shown that the adding of competing amines, such as trimethylamine or triethylamine, is necessary in order to obtain good peak shapes for TCAs.[8,12,13,15-16] Good peak shapes are important for good quantitation, especially at low concentration. In this study, we were able to obtain good peak shapes with a very simple mobile phase, 20 mM potassium phosphate, pH 7/methanol at 30:70 (v/v), without adding any competing reagent.

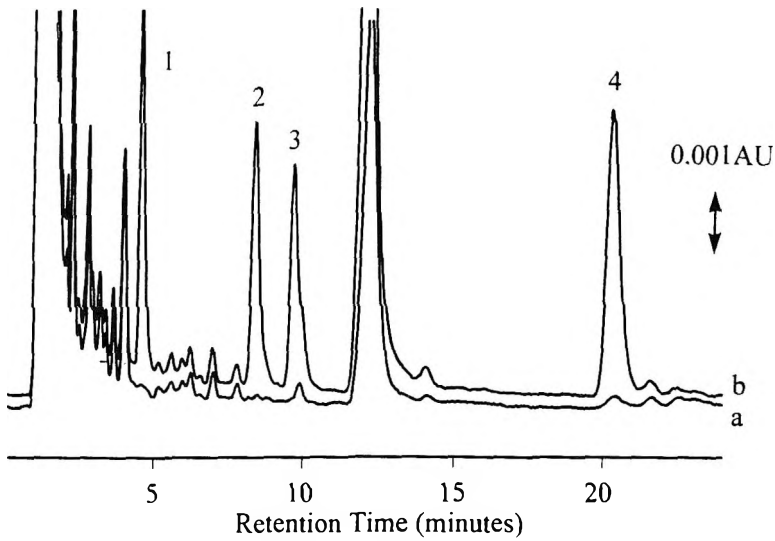


Figure 2. Representative chromatograms of a serum blank (curve a); a serum sample spiked with 1.0 (g/mL of amitriptyline, 0.5 (μ/mL each of nortriptyline and doxepin, and 0.5 (μ/mL of nordoxepin as the internal standard (curve b). Peak 1 is nordoxepin (I.S.), peak 2 is nortriptyline, peak 3 is doxepin, and peak 4 is amitriptyline. The column used was a Symmetry® C_{18} (3.0 mm x 150 mm, 5 mm particle size) preceded by a Sentry™ guard column (3.9 mm x 20 mm). The mobile phase was 20 mM phosphate, pH 7.0/methanol (30:70) at a flow rate of 1.0 mL/min, and the effluent was monitored at 254 nm.

The representative chromatograms of a serum blank and a spiked serum sample are shown in Figure 2. The serum blank is shown in curve a, and the spiked TCAs in the serum matrix, at the concentration of 1.0 μg/mL of amitriptyline and 0.5 μg/mL each of nortriptyline and doxepin, is shown in curve b. The elution sequence is nordoxepin (peak 1), nortriptyline (peak 2), doxepin (peak 3), and amitriptyline (peak 4). Here, the nordoxepin is used as the internal standard. No endogenous interferences were found to interfere with the quantitation of peaks of interest.

Calibration curves were based on peak-area ratio to the internal standard, nordoxepin. Within the concentration range described in the experimental section, linear plots were obtained for doxepin, amitriptyline, and nortriptyline. The correlation coefficients were 0.999476, 0.999888, and 0.999678 for doxepin, amitriptyline, and nortriptyline, respectively.

Table 1

Recovery of TCAs from Spiked Porcine Serum*

	Compound	Nordoxepin (I.S.)	Nortriptyline	Doxepin	Amitriptyline
	Spiked Conc.	0.50	0.50	0.50	1.00
High	(μg/mL)				
Level	Recovery (%)	103	99.7	94.0	102
	RSD (%)	3.5	2.3	1.3	2.5
	Spiked Conc.	0.50	0.10	0.10	0.20
Low	(μg/mL)				
Level	Recovery (%)	97.6	103	102	101
	RSD (%)	3.3	3.4	3.2	3.7

* A simple and rugged SPE method was applied to each analysis. Only one mL of simple solvent was applied to each step of the SPE procedure. Results of five replicate analyses.

Recovery of TCAs

TCAs are basic compounds and have high protein binding capacity in the range of 95%.[26] To eliminate the loss of recovery due to the binding of analytes to the albumin, it is necessary to free the protein bound drugs. In this study, we observed approximately 80% of recoveries when the sample solution was not acidified. With the addition of 20 μL of phosphoric acid into the sample solution, we were able to obtain recoveries greater than 94% for these high protein binding compounds.

The results for the TCAs are summarized in Table 1. At high level, the recoveries were 99.7%, 94.0%, and 102% for nortriptyline, doxepin and amitriptyline, respectively. At low level, the recoveries were 103%, 102%, and 101% for nortriptyline, doxepin, and amitriptyline, respectively. For five replicate analyses, the RSDs were all less than 4.0%. It should be noted that these results were obtained even though the cartridges ran dry before loading the sample onto cartridges. This procedure totally eliminates the time consuming and tedious stopcock manipulation necessary with traditional SPE cartridges, while providing excellent and reproducible results.

The absolute recovery of the internal standard, nordoxepin, was determined by comparing the average peak area from five replicate analyses to the average peak area of the ten standard injections. The mean recoveries were 103% with 3.5% RSD for the high level, and 97.6% with 3.3% RSD for the low level. The ten standard injections had a RSD of 2.5%.

Comparison to Liquid-Liquid Extraction and C_{18} SPE

Extraction of TCAs is traditionally carried out by either liquid-liquid extraction or solid-phase extraction. Liquid-liquid extraction procedures are carried out at high alkaline pH with nonpolar solvents, such as n-hexane or n-heptane. Addition of isoamyl alcohol is a necessary step to prevent formation of an emulsion, which can cause low recovery. The sample solution is shaken and centrifuged. The organic layer is then carefully decanted into another test tube, evaporated to dryness, and reconstituted into a proper solvent for reversed phase HPLC analysis.[8,11] Alternatively, the organic phase is back extracted with diluted acid for subsequent HPLC analysis.[10,12]

For the liquid-liquid extraction method without the back extraction step, Amitai et al.[11] first alkalinized sample solutions to pH 10.5 with 0.1 M of sodium hydroxide. To the sample solutions, they added 8 mL of hexane/isoamyl alcohol (97/3, v/v), shook for 10 minutes, and centrifuged for another 10 minutes. The aqueous layer was frozen by placing the sample solutions in a methanol-dry ice bath. Finally, they decanted the organic phase into other test tubes, evaporated to dryness and reconstituted for HPLC analysis. They reported that the recoveries of parent TCA compounds, such as amitriptyline and doxepin, from plasma were 93% with 2% SD; the recoveries of TCA metabolites, such as nortriptyline and nordoxepin, from plasma were lower at 72% with 3% SD. Similarly, Ghahramani et al.[8] reported that the recoveries of amitriptyline, nortriptyline, and desipramine (used as an internal standard) from plasma at a concentration of 0.05 μg/mL were 90%, 87%, and 76%, respectively; the absolute recoveries, therefore, were 68.4 % and 66.1 % for amitriptyline and nortriptyline, respectively. The within-batch coefficients of variation were reported to be less than 7.4%.

For the liquid-liquid extraction method with the back extraction step, El-Yazigi et al.[12] transferred the organic phase into a tube containing 100 μL of 0.03% phosphoric acid, shook, centrifuged, and finally transferred the acid layer for HPLC analysis. They reported that the recoveries from plasma at a concentration of 0.05 μg/mL were 85.7% and 87.4% for amitriptyline and nortriptyline, respectively; at a concentration of 0.2 μg/mL, the recoveries were 70.4% and 76.5%. No absolute recoveries were reported. The within-batch

coefficients of variation were reported to be less than 9.8%.[12] Similarly, Atta-Politou et al.[10] reported that the recoveries from plasma at a concentration of 0.05 μg/mL were 93.4% with 11.8% SD and 88.7% with 7.7% SD for amitriptyline and nortriptyline, respectively; at a concentration of 0.15 μg/mL, the recoveries were 89.4% with 4.2% SD and 95.7% with 4.2% SD for amitriptyline and nortriptyline, respectively.

An alternative way to extract TCAs is solid-phase extraction method. With SPE, several steps involved in the liquid-liquid extraction are eliminated. These include shaking, centrifuging, and transferring the extracts. Therefore, sources of variability are minimized and the analysis time is also reduced significantly. In addition, SPE requires less solvent and glassware, is less costly in labor and materials, and is easier to automate compared to liquid-liquid extraction. The SPE method requires six simple steps. These steps include preconditioning cartridges, loading the sample solution, washing the cartridge, eluting the analytes, evaporating, and reconstituting into a proper solvent for HPLC analysis.

Bidlingmeyer et al.[18] used Sep-Pak® C_{18} cartridges to clean up the interferences from the serum matrix. They prewashed cartridges with 10 mL of methanol followed by 5 mL of 0.1 N NH_4OH. Then they loaded 1 mL of spiked serum, into which 2 mL of 0.1 N NH_4OH was added, onto the cartridges. These cartridges were flushed with 10 mL of 0.1 N NH_4OH, followed by 10 mL of methanol: water (50:50). The final elution was with 3 mL of 2% butylamine in methanol. The eluate was then evaporated to dryness and redissolved in 100 μL of mobile phase. The entire procedure took about fifteen minutes. The recoveries of amitriptyline and nortriptyline at a concentration of 0.1 μg /mL of serum were 97 % (with RSD of 14%) and 102 % (with RSD of 4%), respectively. These recoveries were reported after adjustment with the internal standard which was protriptyline.

SPE, first introduced in 1978, is now among the most commonly used rapid sample preparation techniques for cleaning up sample matrices and concentrating analytes prior to analysis. The most commonly used sorbents are the porous silica particles surface-bonded with C_{18} or other hydrophobic alkyl groups. The presence of silanols on the surface of packing material complicates the interaction of analytes, especially the basic compounds such as TCAs, with the sorbents. Therefore, solid-phase extraction cartridges that are packed with these sorbents are difficult to work with. It may take a long time to develop and to optimize the methods. In the above method reported by Bidlingmeyer et. al, large volumes of solvent were used, and the addition of a competing agent, butylamine, is necessary for the elution.

Table 2

Comparison of Liquid-Liquid Extraction and Solid-Phase Extracton Using Sep-Pak® C_{18} Or Oasis™ HLB Cartridges

	Percent Recovery (RSD)[a] Amitriptyline	Nortriptyline	Solvents Used	Automation	Reference
Liquid-Liquid Extraction					
Without back extraction	93 (2%)	72 (4%)	>8.0 mL	Difficult	11
	68 (<7%)	66 (<7%)	5.3 mL	"	8
With back extraction	78 (<10%)	82 (<10%)	2.3 mL	"	12
	91 (8%)	93 (6%)	6.4 mL	"	10
Solid-Phase Extraction					
Sep-Pak® C_{18}	97 (14%)	102 (4%)	40.1 mL	Easy	18
Oasis™ HLB	99 (4%)	100 (3%)	4.2 mL	"	

[a] Concentration ranges from 0.05 to 0.20 μg/mL of analyte. All recoveries were reported as absolute recovery, except for results from references 12 and 18.

Once the methods have been optimized, the cartridges must remain wet after conditioning and equilibration, in order to retain analytes from an aqueous sample matrix. These steps make the silica-based SPE sorbents time consuming, tedious, and irreproducible.

In this study, we used Oasis™ HLB cartridges to extract TCAs from serum matrix. The Oasis™ HLB sorbent is a polymeric reversed phase sorbent. This macroporous polymer [poly(divinylbenzene-co-N-vinylpyrrolidone) exhibits both hydrophilic and lipophilic characteristics. The lipophilic monomer is divinylbenzene; it provides the reversed phase properties necessary for analyte retention. The hydrophilic monomer is N-vinyl pyrrolidone; it gives the packing the necessary amount of hydrophilic property to prevent the wettability problems encountered with the traditional silica based reversed phase sorbents. These two unique and distinct characteristics are carefully balanced.

The Oasis™ HLB sorbent contains no silanol group, which simplifies the retention mechanism between sorbents and the analytes. As a result, a simple and more general SPE protocol (see experimental section) can be applied.

With only one mL of simple solvents throughout the entire SPE procedure, we were able to obtain excellent recoveries (all greater than 94%) with good reproducibility (less than 4.0% RSD).

Table 2 summarizes the above methods used for the extraction of TCAs. Clearly, the Oasis™ HLB extraction cartridge has tremendous advantages over the liquid-liquid, and traditional silica based C_{18} SPE methods. This cartridge simplifies the sample preparation procedure to an extraordinary degree. It provides fast and easy method development while giving us high and good reproducible results.

CONCLUSIONS

The assay described here provides a faster and simpler extraction method than the previously published methods, including liquid-liquid and solid-phase extraction. This method requires only one mL of simple solvent in each step of the SPE procedure. Additionally, unlike traditional reversed phase sorbents, we can let these Oasis™ HLB extraction cartridges run dry on a vacuum manifold prior to sample loading. This feature reduces errors caused by the sample preparation, and improves method ruggedness. The unique property of this sorbent makes SPE more efficient, more rugged, and less tedious than conventional SPE. This method is highly precise, easy to perform, and suitable for determining the TCAs in the biological matrices.

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Received January 27, 1997
Accepted March 5, 1997
Manuscript 4385

J. LIQ. CHROM. & REL. TECHNOL., 20(15), 2475–2493 (1997)

DETERMINATION OF AMINO ACIDS IN URINE BY CAPILLARY ELECTROPHORESIS WITH INDIRECT UV DETECTION

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ABSTRACT

This study has described a relatively simple method for determination of 14-18 amino acids (AA's) by CE with indirect UV detection using both myristyltrimethyl-ammonium bromide (MTAB)-coated and uncoated capillaries. The method used either salicylate or benzoate as UV-absorbing additive in carbonate buffer and the non-absorbing AA's were detected indirectly at 230 nm. The optimization of the assay conditions was demonstrated with an MTAB-coated capillary using salicylate-carbonate as separation buffer. Factors that affect indirect signal response and migration behavior of AA's such as buffer concentration, pH, and the relative amount of UV-absorbing additive in buffer have been studied. Comparison of MTAB-coated and uncoatd capillaries on the analysis of AA's

has been made. It has been found that the limits of detection of 18 AA's by the method were in the order of 53.6 to 785 pg injected. The method developed was used in the determination of AA's in urine sample and preliminary results were provided.

INTRODUCTION

Amino acids (AA's) are important biological molecules. Determination of AA's in proteins, peptides, and biological fluids may help us, not only in mapping the structures of these macromolecules, but in understanding their metabolic pathways as well in diagnosing diseases. There are more than 50 hereditary diseases of AA metabolism that have been identified today.[1] Diseases like aminoacidurias (e.g., phenylketonuria, branched-chain ketoaciduria also known as maple syrup urine disease, histidinemia and so forth), marked by high levels of urinary AA's can be diagnosed through screening of AA's in urine specimens.[2]

Determination of AA's have been accomplished by liquid chromatography (LC) and capillary electrophoresis (CE).[3-10] Because most AA's, except the aromatic ones, lack absorptivity of UV and visible radiation that can be used for their sensitive detection, pre- or post-column derivatization reaction of AA's with a fluorophore or a chromophore is often employed. However, the derivatization of AA's in LC and CE methods is limited by many factors, such as the availability of derivatizing agent, the reaction rate, the stability, and the solubility of product, the ability of derivatizing agent to react with both primary and secondary AA's, and the absorption and emission wavelengths of derivatized product in regard to the outputs of the instrument.

CE is a micro-analytical technique with unrivaled separation efficiency that is well suited for the determination of AA's in biological fluids. Detection of AA's in CE can also be done by indirect approaches, similar to those used in LC. For example, indirect fluorescence detection of AA's has been reported.[11,12] To achieve low limits of detection, a laser light that ensures a tightly focused beam is essential for efficient fluorescence detection. Alternatively, indirect UV detection is more practical if the limits of detection is not a major concern, since most commercial CE instruments are equipped with UV detectors.

Indirect UV detection in CE may be characterized by monitoring of the UV absorbance of the separation buffer, where background UV-absorbing additive is displaced by zones of non-UV-absorbing solute ions and resulted in

negative peaks. A few studies on indirect UV detection of AA's by CE have been reported, including the detection of 7 AA's with salicylate,[13] 3 AA's with quinine sulfate,[14] and 18 AA's with either *p*-aminosalicylic acid or 4-(N,N'-dimethylamino)benzoic acid in the presence of α-cyclodextrin.[15]

In this study we have developed a simple CE method for the analysis of 14-18 AA's, using either salicylate or benzoate as UV-absorbing additives in carbonate buffer by MTAB-coated and uncoated capillaries. Factors that affect indirect signal response and migration behavior of AA's, such as buffer concentration, pH, and the relative amount of UV-absorbing additive in buffer have been studied. The potential use of the method in the analysis of urinary AA's has been investigated and preliminary results are presented.

EXPERIMENTAL

Chemicals and Solutions

All AA's used in this study were purchased from Sigma (St. Louis, MO). Myristyltrimethylammonium bromide (MTAB) was obtained from Aldrich (Milwaukee, WI). Sodium salicylate, sodium benzoate, sodium carbonate (anhydrous), sodium hydroxide, and hydrochloric acid were purchased from Fisher Scientific (Pittsburgh, PA).

Aqueous solutions were prepared with deionized water (Barnstead/Thermolyne NANOpure system, Dubuque, IA). Stock standard solutions of AA's were prepared in deionized water at concentrations of 1.0 mg/mL and 0.1 M. Prior to CE analysis, fresh working solutions of various AA's were prepared by serial dilutions from the stock solutions with deionized water. 0.1 M sodium salicylate and sodium benzoate were prepared in deionized water and used as stock solutions of UV-absorbing additives. 10 mM MTAB was prepared in deionized water and used as stock coating solution. The separation buffers were prepared by dissolving the appropriate amount of sodium carbonate and 0.1 M UV-absorbing additive stock solutions, as well as 10 mM MTAB stock solution if the dynamic coating was being used, in deionized water.

The pH values of the buffers were adjusted to the desired values with 0.1 M HCl solution. In this work, all buffers were filtered through 0.45-μm cellulose-acetate-membrane syringe filters (Alltech, Deerfield, IL) before use.

CE Instrument

A Beckman (Fullerton, CA) P/ACE 2050 CE instrument and an IBM PC with System Gold software were used for this study. On-column UV absorption was detected at 230 nm and the capillary temperature was maintained at 25°C. Sample injection was done by pressure at 0.5 psi for 5 s. If an uncoated capillary was used, the cathode was placed in the outlet of the capillary and the anode was placed in the inlet. If MTAB had been added in separation buffer, the polarity of the electrodes had to be reversed because the surfactant dynamically coated the inner surface of the capillary through the formation of a bilayer (with cationic heads facing outwards into the aqueous solution), and reversed the direction of electroosmotic flow. The details of experimental conditions can be found in the figure legends.

Capillary Columns

Open tubular fused-silica capillaries (360 μm O.D., 100 μm I.D.), with polyimide coating on the outer surface, were purchased from Polymicro Technologies (Phoenix, AZ). A new capillary was conditioned by rinsing sequentially with 0.1 M NaOH, deionized water and separation buffer, and the capillary was used as uncoated capillary. An MTAB-coated capillary was prepared by flushing the uncoated capillary with salicylate-carbonate (or benzoate-carbonate) buffer containing 0.15 mM MTAB at 0.5 psi for 3 min. Between runs, the capillary was rinsed for 90 s using the separation buffer.

Urine Sample Pretreatment

Urine samples used in this study were collected from a healthy volunteer. All samples were first filtered through 0.45-μm cellulose-acetate-membrane syringe filters (Alltech), and then diluted four times by deionized water (1 portion of urine to 4 portions of water) prior to the CE analyses.

RESULTS AND DISCUSSION

The signal response of indirect UV detection is affected by many factors, including the relative concentration of UV-absorbing additive in the separation buffer, the charge ratio of UV-absorbing additive to analyte ions, and the pH and concentration of the separation buffer. In this study, we have investigated

Table 1

Properties of the Non-UV Absorbing AA's and UV Additives*

Amino Acids	M.W.	pKa_1(COOH)	$pKa_2(NH_3^+)$	pKa_3(R)	pI
Aspartic acid (Asp)	133.10	1.88	9.60	3.65 (COOH)	2.77
Glutamic acid (Glu)	147.13	2.19	9.67	4.25 (COOH)	3.22
Cysteine (Cys)	121.16	1.96	10.28	8.18 (SH)	5.07
Glycine (Gly)	75.07	2.34	9.60		5.97
Alanine (Ala)	89.09	2.34	9.69		6.00
Valine (Val)	117.15	2.32	9.62		5.96
Leucine (Leu)	131.17	2.36	9.60		5.98
Isoleucine (Ile)	131.17	2.36	9.60		6.02
Serine (Ser)	105.09	2.21	9.15		5.68
Threonine (Thr)	119.12	2.09	9.10		5.60
Asparagine (Asn)	132.12	2.02	8.80		5.41
Glutamine (Gln)	146.15	2.17	9.13		5.65
Proline (Pro)	115.13	1.99	10.60		6.30
Hydroxyproline (OH-Pro)	131.13	1.92	9.73		5.83
Citrullline (Cit)	175.19	NA	NA		NA
Ornithine (Orn)	132.16	NA	NA		NA
Methionine (Met)	149.21	2.28	9.21		5.74
Lysine (Lys)	146.19	2.18	8.95	10.53 (NH_3^+)	9.74
Arginine (Arg)	174.20	2.17	9.04	12.48 (NH_3^+)	10.76
Histidine (His)	155.16	1.82	9.17	6.00 (NH_3^+)	7.59
Uric acid (UA)	168.11			5.40 (NH)	
Salicylic acid (SA)	138.12	2.97		13.40 (OH)	
Benzoic acid (BE)	122.12	4.19			

* See reference 22.

pKa_1, the dissociation constant for the α-COOH group.

pKa_2, the dissociation constant for the α-NH_3^+ group.

pKa_3, the dissociation constant for the R group present in the molecule.

pI, isoelectric point.

NA, not available.

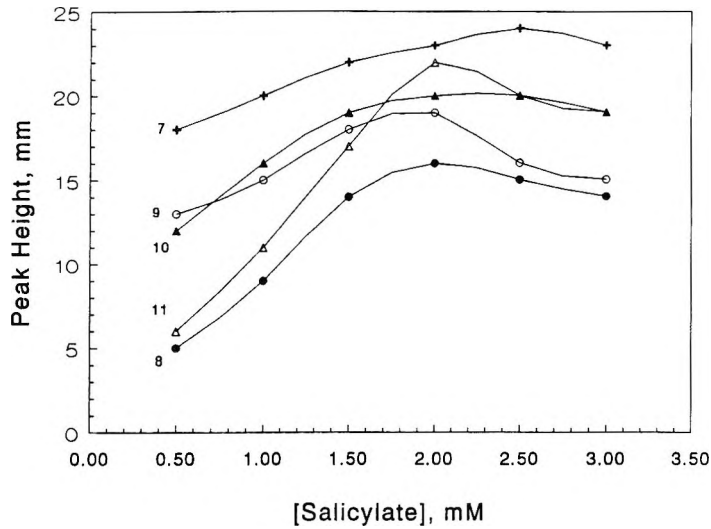

Figure 1. The influence of salicylate concentrations on the indirect UV responses. Experimental conditions: uncoated capillary, 77 cm x 100 μm (i.d.); temperature, 25°C; UV filter, 230 nm; separation potential, 15 kV; separation buffers, 5.0 mM carbonate/0.5-3.0 mM salicylate/0.15 mM MTAB at pH 10.7; injection pressure/time, 0.5 psi/5 s, AA's, 10 μg/mL. The identities of AAs are given in Table 2.

the factors that affect the sensitivity of indirect UV detection and the migration characteristics of selected AA's using an MTAB-coated capillary. The analytical performances of MTAB-coated and uncoated capillaries on the separation of AA's were compared. The method developed was applied to the determination of AA's in urine samples.

Optimization of the Separation Conditions and the Sensitivity of Indirect UV Detection

UV-absorbing additives

The selection of UV-absorbing additive is of the utmost importance in indirect UV detection. According to the previous studies,[16-18] an effective UV-absorbing additive ought to provide a high UV absorbance at a wide range of wavelengths, and a close match of ionic mobility with those of analyte ions. Unmatched ionic mobilities between UV-absorbing additive and analyte ions may result in low detection sensitivity (due to the low displacement ratio of UV additive to analytes), poor peak symmetry, and reduced separation efficiency.

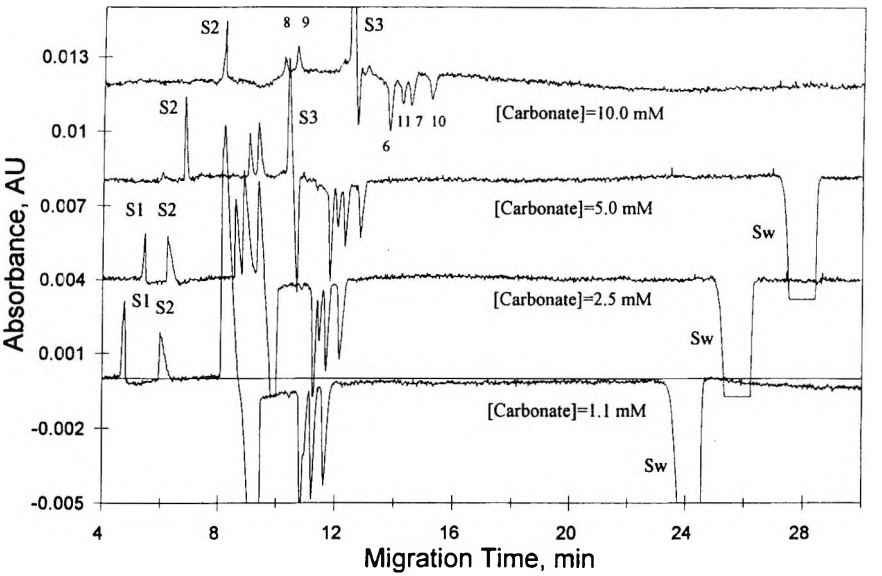

Figure 2. The influence of carbonate concentrations on the indirect UV responses and the migration times of AA's. Separation buffers: 1.1-10 mM carbonate/2.0 mM salicylate/0.15 mM MTAB at pH 10.7. The other experimental conditions were the same as those in Figure 1. Peak identities: S_1, system peak 1 (chloride), S_2, system peak 2 (carbonate/bicarbonate), S_3, system peak 3 (salicylate), S_w, system peak (water), the other peak identities: see Table 2.

In this work, salicylate was used as a UV-absorbing additive in the studies of signal response, separation efficiency, and analytical performances of the MTAB-coated and uncoated capillaries because it has a dissociation constant and a molecular mass compatible to those of AA's being studied (Table 1). Benzoate was also used as UV-absorbing additive for the reason of having compatible mobility and molecular mass to those of AA's. The detection wavelength of both salicylate and benzoate was chosen at 230 nm rather than 200 nm, as interference experienced from urine samples was much less at this wavelength.

Indirect signal response

In indirect UV detection, the concentration ratio of UV additive to co-ions in the separation buffer, determines the sensitivity of the signal response. As shown in Figures 1 and 2, the indirect responses of five AA's increased either with an increase in salicylate concentration (Figure 1), or with a decrease in

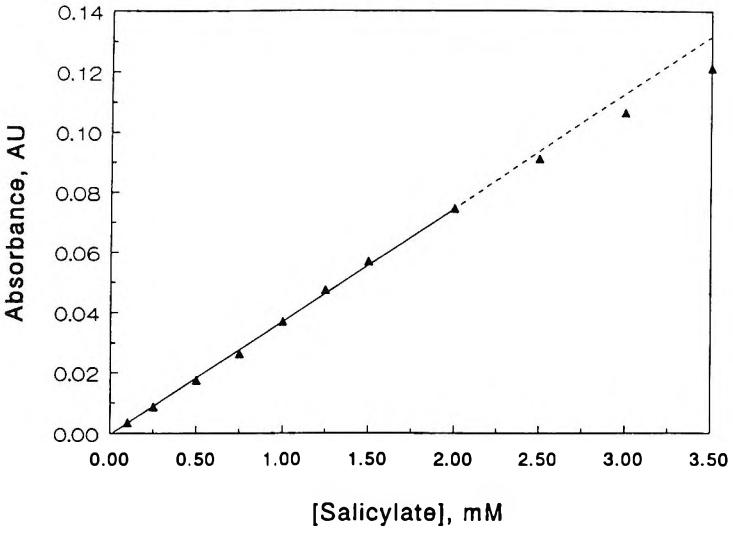

Figure 3. The UV absorbance of salicylate at different concentrations. Experimental conditions: uncoated capillary, 77 cm x 100 μm (i.d.); temperature, 25°C; UV filter, 230 nm; separation buffers, 5 mM carbonate/0.1 mM-3.5 mM salicylate at pH 10.7. The experiment was done by sequentially pushing eleven buffers containing various amounts of salicylate through the separation capillary.

carbonate concentration in separation buffer (Figure 2). These results reveal that the response in indirect UV detection is related to the relative displacement of UV-absorbing additive to co-ions by the analyte ions. Furthermore, if the concentration of the UV-absorbing additive (salicylate) exceeded its upper linear dynamic range of detection (2.0 mM) in CE (Figure 3), the responses of the analyte ions (AA's) either decreased or reached plateaus (Figure 1), because the displacement of the UV-absorbing additives with the analyte ions no longer follows the same relationship.[19] Accordingly, 2.0 mM salicylate was chosen as the concentration of UV-absorbing additive for later work in order to retain a linear detection response with maximum sensitivity of detection.

The pH influences the degrees of dissociation of buffer ions and UV additive, as well as of AA's; therefore, it affects the apparent mobilities of ionic species in CE and the signal responses of indirect detection. As shown in Figure 4, when the pH of buffer solution was equal to or less than 10.3, it not only caused the low-signal responses of AA's but also worsened the separation efficiencies due to the diminishing distinction in apparent mobilities among the AA's. However, at pH values equal to or greater than 10.7, the signal

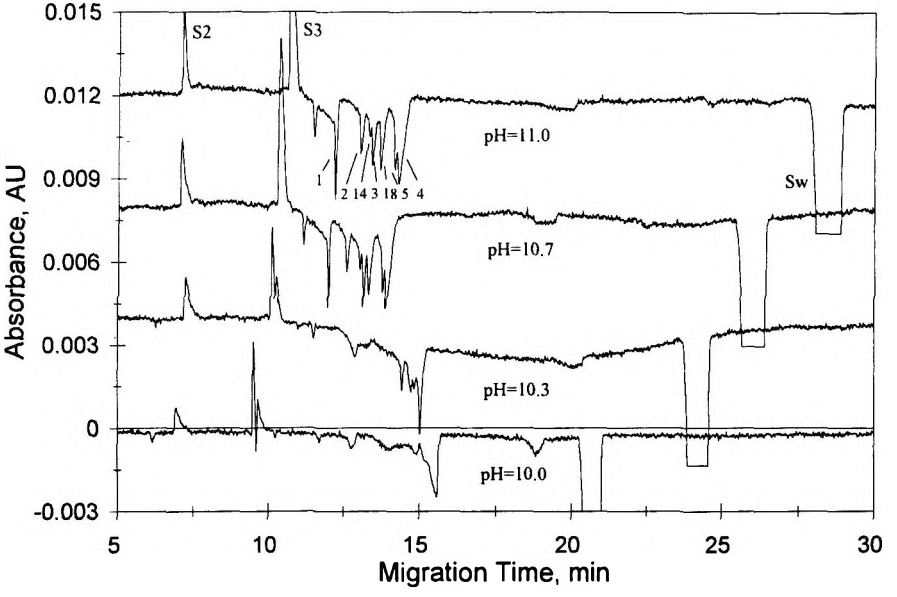

Figure 4. The influence of the buffer pH on the indirect UV responses and the migration times of AA's. Separation buffers: 5.0 mM carbonate/2.0 mM salicylate/0.15 mM MTAB at pHs 10.0-11.0. The other experimental conditions and peak identities were the same as those in Figures 1 and 2.

responses and the separation efficiencies of the AA's had both increased and improved because the zwitterionic AA's were converted to anions at the higher pHs and these UV-transparent anions displaced more UV-absorbing additive anions with more distinctive mobilities.

Migration and separation of AA's

Once a surfactant known as myristyltrimethylammonium bromide (MTAB) was added to the salicylate-carbonate buffer and filled in an uncoated capillary, the positively charged heads of the surfactant molecules dynamically formed ion-pairs with the negatively charged silanol ($Si\text{-}O^-$) groups on the inside wall of the fused silica capillary at pH > 2.[20] If the amount of surfactant exceeded a monolayer coverage of the capillary surface, the hydrophobic chain of the surfactant in buffer would be pushed to the hydrophobic chain on the surface by the polar medium, resulting in a self-assembled molecular bilayer of the surfactant, which has the positive heads facing outwards into the aqueous

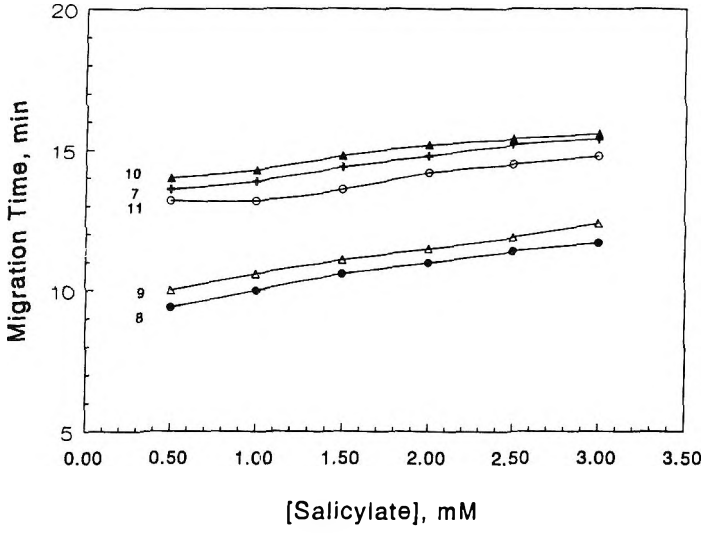

Figure 5. The influence of salicylate concentrations on the migration times of AA's. Experimental conditions were the same as those in Figure 1.

buffer. Because of the dynamic coating of MTAB on the inside surface of the capillary, the surface charge of the uncoated capillary had changed from negative to positive, and the electroosmosis adopts the same flow direction as the electrophoresis of anionic AA's.

Migration and separation of AA's are influenced by the buffer concentration. Experimental results show that the migration time of AA's decreased with decreasing carbonate concentration (Figure 2), and salicylate concentration (Figure 5).

Although higher signal responses were obtained when lower carbonate concentrations and a fixed salicylate concentration were used (Figure 2), the best separation efficiency was achieved when carbonate concentrations were between 2.5 and 5.0 mM and salicylate concentrations were between 1.5 and 2.0 mM.

Based on the studies shown in Figures 1 to 5, the optimum conditions for separation and indirect detection of AA's in MTAB-coated capillary were those in which the salicylate concentration was at 1.5 mM to 2.0 mM; the carbonate concentration, 2.5 mM to 5.0 mM; and the buffer pH, 10.7 to 11.0.

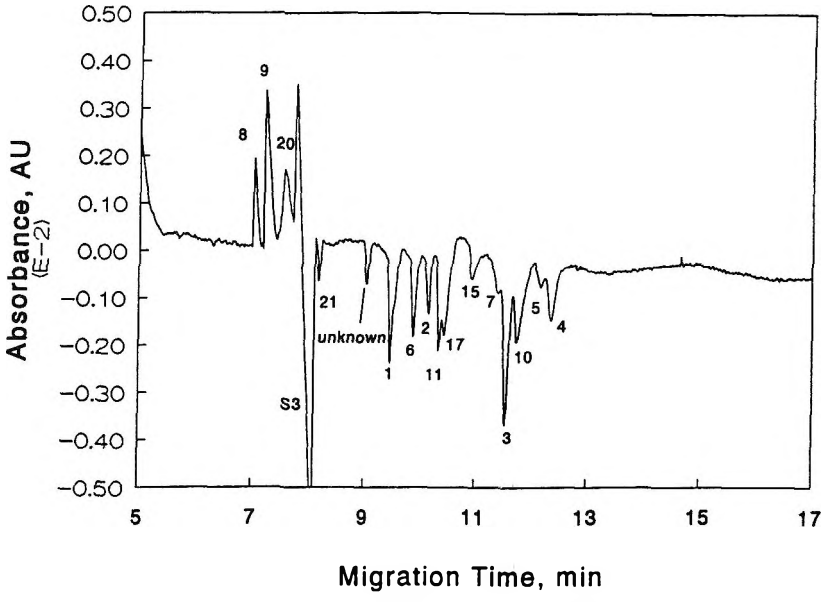

Figure 6. *The electropherogram of AA's using an MTAB-coated capillary capillary* with salicylate as a UV absorbing additive. Separation buffer: 5.0 mM carbonate/2.0 mM salicylate/0.15 mM MTAB at pH 10.7. The other experimental conditions and the peak identities were the same as those in Figures 1 and 2.

Analytical Performance

Under the optimum conditions, separations of a mixture of 14-18 AA's were accomplished in MTAB-coated capillaries using either salicylate (Figure 6) or benzoate (Figure 7) as UV-absorbing additives. The results were compared with those obtained from an uncoated capillary (Figure 8). As slab gel electrophoresis, CE exploits differences in the acid-base behavior of the different AA's, i.e., differences in the sign and magnitude of their net electric charges at a given pH, which are predictable from their pK_a values (Table 1). In other words, the migration orders of AA's in Figures 6 to 8 were governed by the apparent mobilities of AA's in the different systems, which were the vector sums of electrophorectic and electroosmotic mobilities of the individual AA's. In this work, the peak identities in Figures 6 to 8 were assigned by comparisons of electropherograms of the mixture to those of the individual AA's and by stepwise addition of known AA's to the mixture.

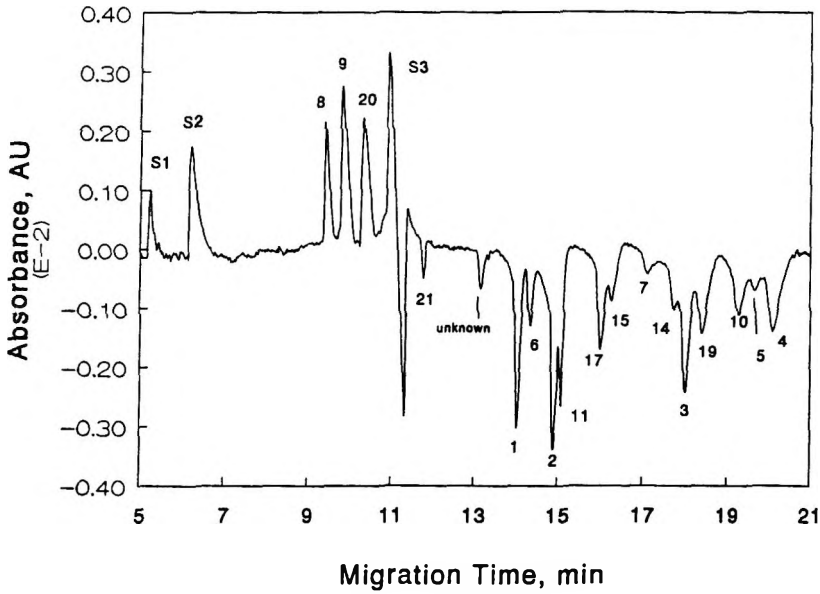

Figure 7. The electropherogram of AA's using an MTAB-coated capillary with benzoate as a UV absorbing additive. Separation buffer: 5.0 mM carbonate/2.0 mM benzoate/0.15 mM MTAB at pH 10.7. The other experimental conditions and the peak identities were the same as those in Figures 1 and 2 except the S_3 peak is the peak of benzoate.

In an MTAB-coated capillary, the electroosmotic flow of the buffer solution had the same direction as the electrophoretic flow of the AA's under the separation conditions employed and moved toward the anode at the capillary outlet. The migration order of AA's depended solely on the electrophoretic mobility of AA's because the contribution of electroosmotic mobility to the apparent mobility of AA's remained constant.

According to the equation:[20] $\mu_{ep} = \frac{Q}{6\pi\eta r}$ electrophoretic mobility (μ_{ep}) is proportional to the charge of the molecule (Q), and inversely proportional to the viscosity of the separation media (η) and the hydrodynamic radius (r) of the molecule. Because the hydrodynamic radius of an AA is related not only to the mass and the shape of the molecule but also to the degree of hydration of the molecule,[21] it is rather difficult to estimate its relative magnitude by comparing its mass with those of AA's in the mixture. However, it is relatively easy to

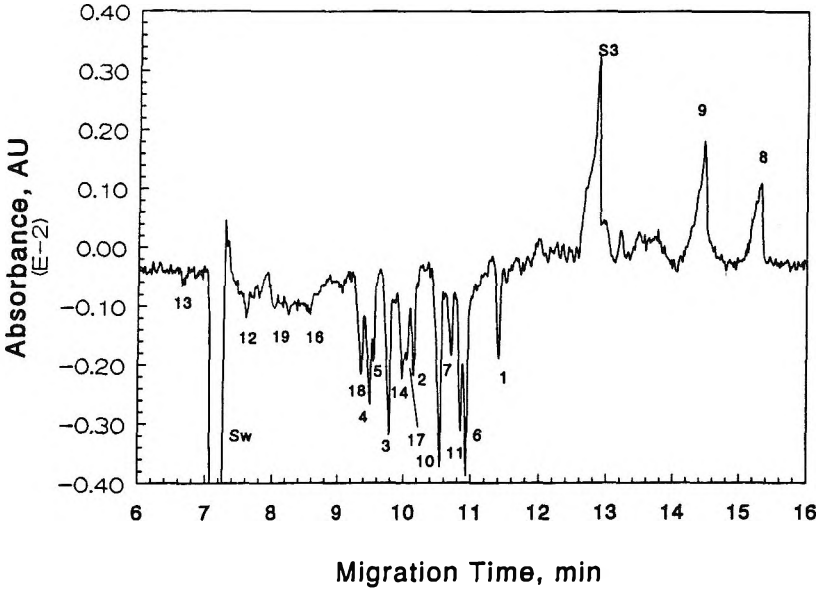

Figure 8. The electropherogram of AA's using an uncoated capillary. Separation buffer: 5.0 mM carbonate/2.0 mM salicylate at pH 10.7. The other experimental conditions and the peak identities were the same as those in Figures 1 and 2.

calculate the net charges of AA's by considering AA's that have a single α-amino group, a single carboxyl group, and an R group that does not ionize as diprotic acids (H_2L^+), and AA's with an ionizable R group as triprotic acids (e.g., H_3L^+ for AA's with negatively charged R groups, and H_3L^{2+} for AA's with positively charged R groups). Quantitatively, the net charges of AA's ($Q_{H_2L^+}$) be calculated either as diprotic AA's:

$$Q_{H_2L^+} = \alpha_{H_2L^+} Z_{H_2L^+} + \alpha_{HL} Z_{HL} + \alpha_{L^-} Z_{L^-} \qquad (1)$$

(where $\alpha_{H_2L^+}$, α_{HL} and α_{L^-} are the fractions in forms of H_2L^+, HL and L^-, and $Z_{H_2L^+}$, Z_{HL} and Z_{L^-} are the charges on these species) or as triprotic AA's with a negatively charged R group:

$$Q_{H_3L^+} = \alpha_{H_3L^+} Z_{H_3L^+} + \alpha_{H_2L} Z_{H_2L} + \alpha_{HL^-} Z_{HL^-} + \alpha_{L^{2-}} Z_{L^{2-}} \qquad (2)$$

(where $\alpha_{H_3L^+}$, α_{H_2L}, α_{HL^-} and $\alpha_{L^{2-}}$ are the fractions in forms of H_3L^+, H_2L, HL^- and L^{2-}, and $Z_{H_3L^+}$, Z_{H_2L}, Z_{HL^-} and $Z_{L^{2-}}$ are the charges on these species) or as triprotic AA's with a positively charged R group:

$$Q_{H_3L^{2+}} = \alpha_{H_3L^{2+}} Z_{H_3L^{2+}} + \alpha_{H_2L^+} Z_{H_2L^+} + \alpha_{HL} Z_{HL} + \alpha_{L^-} Z_{L^-} \qquad (3)$$

(where $\alpha_{H_3L^{2+}}$, $\alpha_{H_2L^+}$, α_{HL} and α_{L^-} are the fractions in forms of H_3L^{2+}, H_2L^+, HL and L^-, and $Z_{H_3L^{2+}}$, $Z_{H_2L^+}$, Z_{HL} and Z_{L^-} are the charges on these species). Since the pI values of AA's were below the pH (10.70) of the separation buffer; therefore, all AA's in Figure 6 carried net negative charges. The order of migration is Asp (the charge-to-mass ratio: -1.447×10^{-2}), Glu* (-1.301×10^{-2}), Cys (-1.422×10^{-2}), salicylate* (-7.256×10^{-3}), urate* (-5.949×10^{-3}), unknown peak, Gly (-1.234×10^{-2}), Ser* (-9.255×10^{-3}), Ala (-1.023×10^{-2}), Gln* (-6.663×10^{-3}), OH-Pro* (-6.889×10^{-3}), His* (-6.261×10^{-3}), Thr (-8.189×10^{-3}), Val (-7.877×10^{-3}), Asn (-7.475×10^{-3}), Ile (-7.063×10^{-3}), Leu (-7.063×10^{-3}). It is noted that the species with an asterisk (*) did not follow the sequence of their charge-to-mass ratios due to the differences in the shape and the degree of hydration. Using an MTAB-coated capillary with salicylate as the UV absorbing additive, 14 AA's could be separated within 13 min. In another experiment benzoate was used to substitute salicylate, 16 AA's could be resolved in less than 21 min with an order of migration (Figure 7) similar to that of Figure 6.

In an uncoated capillary, the electroosmotic flow of buffer solution moving toward the cathode was greater than the electrophoretic flow of the negatively charged AA's moving toward the anode.[20] The migration order of the AA's in an uncoated capillary (Figure 8) was basically a reversal of those in the MTAB-coated capillaries (Figures 6 and 7) because of the combined effect of the two opposite flows. With salicylate as the UV absorbing additive, 18 AA's could be separated in less than 16 min in an uncoated capillary.

It is worth noting that the AA's with pIs close to the buffer pH showed low responses in the indirect detection signal because these AA's carried less negative charges and accordingly they displaced less UV absorbing additives. Table 2 shows the detection limits (defined as signal/noise = 3) of the AA's being studied by both MTAB-coated and uncoated capillaries, and they ranged from 53.6 to 785 pg. For the AA's that have large differences between pIs and buffer pH (e.g., Glu, Asp, etc.), the detection limits were lower than those AA's that have small differences (e.g., Lys, Arg, etc.), due to the greater charge densities on the former ions.

Table 2

The Detection Limits of AA's in Different Separation Conditions[a]

		Uncoated Capillary $N^c = 1.73\times10^5$	MTAB-Coated Capillary $N = 1.67\times10^5$	
Peak No.[b]	**Amino Acid**	**Salicyate**	**Salicyate**	**Benzoate**
		Limit of Detection[d], pg		
1	Gly	119	73.2	65.4
2	Ala	125	146	68.7
3	Val	74.2	62.8	106
4	Leu	137	169	172
5	Ile	687	549	687
6	Ser	61.0	110	196
7	Thr	162	628	458
8	Asp	131	91.6	85.8
9	Glu	94.7	53.6	65.4
10	Asn	58.4	146	275
11	Gln	78.5	137	110
12	Lys	391	439	NA
13	Arg	687	517	NA
14	Met	196	NA	687
15	His	NA	220	275
16	Pro	687	NA	NA
17	OH-Pro	275	110	137
18	Cit	131	NA	162
19	Orn	785	439	NA
20	Cys	NA	157	85.8
21	UA	NA	200	275

[a] The separation conditions were the same with Figures 6-8.

[b] The peak numbers were the same as those used for peak identities in all figures.

[c] The theoretical plate numbers (N, plates/m) of the capillary columns were calculated using valine peaks in Figures 6 and 8.

[d] Limit of detection was defined as the injection volume (57 nL) times the concentration of AA at S/N = 3.

NA, not available.

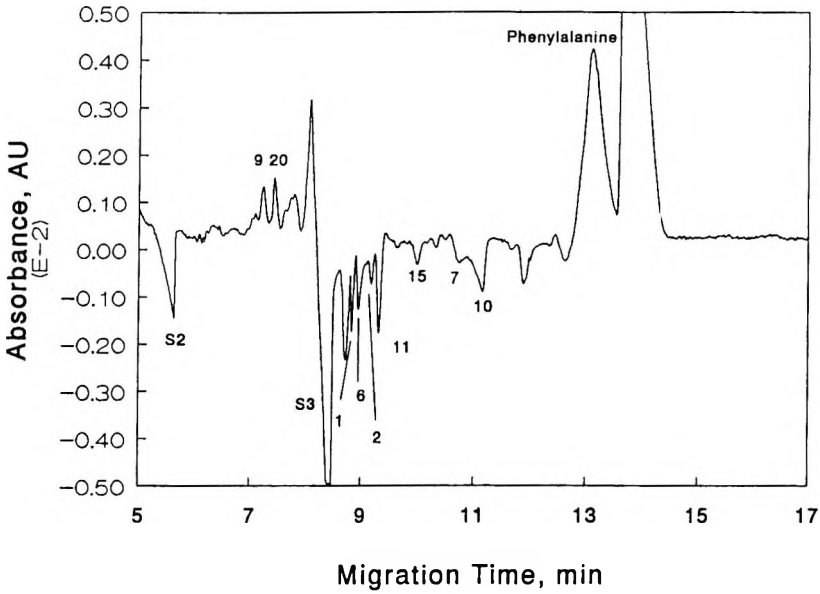

Figure 9. The electropherogram of human urine sample. Experimental conditions were the same as those in Figure 6.

Determination of AA's in Urine Sample

The developed method using MTAB-coated capillary was applied to the analysis of AA's in human urine sample. The sample pretreatment procedure consisted of a filtration step with a 0.45-μm cellulose-acetate-membrane syringe filter and a fourfold dilution with deionized water (1 portion of urine to 4 portions of water). As shown in Figure 9, eight AA's, i.e., Glu, Cys, Gly, Ser, Ala, Gln, Thr, and Asn were detected under the chosen conditions. The peak identities of AA's in the urine sample were verified by sequential addition of known AA's to the test sample. The concentrations of AA's in urine could be found from the linear calibration curves of the AA's ($r \geq 0.996$), which were constructed using various concentrations of the AA's (from 25 to 100 μM), versus the peak heights of the AA's (the calibration curves are not shown). The results are summarized in Table 3, together with the measured values and the normal values cited from the literature. All the values of AA's reported from our preliminary analysis are reconciled with the normal range reported, with the exceptions of cysteine and asparagine in urine.

Table 3

The Levels of Eight Amino Acids in Urine Detected by CE

Amino Acids (Calibration Equation[b] and Regression Coefficient)	Urinary AA (μM) CE Method	Normal Range[a]*
Glu (y = 5.0 + 0.280x, r = 1.0000)	71.4	37-180
Cys (y = 0.5 + 0.172x, r = 0.9984)	319.8	13 - 87
Gly (y = -2.5 + 0.412x, r = 0.9960)	248.8	108 - 2800
Ser (y = -1.0 + 0.336x, r = 0.9983)	193.5	108 - 467
Ala (y = 1.0 + 0.292x, r = 0.9994)	119.9	40 - 533
Gln (y = 1.0 + 0.496x, r = 0.9992)	241.9	93 - 573
Thr (y - 0.228x, r = 0.9991)	98.7	57 - 293
Asn (y = 2.5 + 0.344x, r = 0.9997)	152.6	180 - 467

* See reference 23.

[a] Assuming the average volume of urine for a 70 kg adult is 1500 mL/24 hrs.

[b] In the equations, y is the peak height (mm) and x is the concentration of AA in μM.

CONCLUSIONS

This study has shown a relatively simple method for determination of 14-18 AA's by CE with MTAB-coated and uncoated capillaries. The optimization of the analysis conditions was demonstrated with an MTAB-coated capillary using salicylate as the UV absorbing additive in carbonate buffer. Factors that affect indirect signal response and migration behavior of AA's, such as buffer concentration, pH, and UV absorbing additive were studied. The separations of AA's in both MTAB-coated and uncoated capillaries were compared. The detection limits of 18 AA's found in this method were in the order of 53.6 to 785 pg injected. The method developed has been applied to the determination of AA's in urine sample and preliminary results has been presented.

ACKNOWLEDGMENT

This work was supported by the Graduate Council of Cleveland State University.

REFERENCES

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Received December 12, 1996
Accepted January 30, 1997
Manuscript 4354

J. LIQ. CHROM. & REL. TECHNOL., 20(15), 2495 (1997)

ERRATUM

ESTIMATION OF THE RP-HPLC LIPOPHILICITY PARAMETERS LOG K', AND LOG K_W, A COMPARISON WITH THE HYDROPHOBICITY INDEX φ_0

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Table 4 of this paper [J. Liq. Chrom. & Rel. Technol, 20(11), 1703-1715 (1997)] contained an error. The correct Table 4 follows:

Table 4

Log k' and Log P_{app} for Some Study Hydrazides

Compounds	------- φ_{MeOH} (%)/log k' -------						log k_w	log P_{app}
	25	35	45	55	65	75		
3	-	1.580	1.015	0.514	0.088	-0.273	3.133	3.319
4	-	1.939	1.330	0.716	0.225	-0.343	3.891	4.182
9	0.880	0.541	0.209	-0.086	-0.377	-0.590	1.582	1.552
10	1.239	0.823	0.438	0.086	-0.222	-0.479	2.039	2.073
11	1.109	0.709	0.330	-0.004	-0.290	-0.530	1.867	1.877
12	0.869	0.563	0.250	-0.044	-0.317	-0.663	1.623	1.599

J. LIQ. CHROM. & REL. TECHNOL., 20(15), 2497 (1997)

EDUCATION ANNOUNCEMENT

BASIC PRINCIPLES OF HPLC AND HPLC SYSTEM TROUBLESHOOTING

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- Logical HPLC System Troubleshooting

The instructor, Dr. Jack Cazes, is founder and Editor-in-Chief of the Journal of Liquid Chromatography & Related Technologies, Editor of Instrumentation Science & Technology, and Series Editor of the Chromatographic Science Book Series. He has been intimately involved with liquid chromatography for more than 35 years; he pioneered the development of modern HPLC technology. Dr. Cazes was Professor-in-Charge of the ACS Short Course and the ACS Audio Course on GPC, and has taught at Rutgers University. He is currently Visiting Scholar at Florida Atlantic University.

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

J. LIQ. CHROM. & REL. TECHNOL., 20(15), 2499–2507 (1997)

LIQUID CHROMATOGRAPHY CALENDAR

1997

SEPTEMBER 2 - 5: 12th International Bioanalytical Forum, Univ. of Surrey, Guildford, UK, sponsored by the Chromatographic Society (U.K.). Contact: Dr. E. Reid, 72 The Chase, Guildford GU2 5UL, U.K. Tel/FAX: (0) 1483-565324; Email: D.Stevenson@surrey.ac.uk.

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

SEPTEMBER 8 - 10: 1997 PrepTech Conference, Hyatt Regency Hotel, Orlando International Airport, Florida. Contact: S. Galla, ISC Technical Conferences, Inc., 30 Controls Dr., Box 559, Shelton, CT 06484-0559, USA. Tel: (203) 926-9300; FAX: (203) 926-9722.

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

SEPTEMBER 14 - 19: ACS Int'l Symposium on Systems Approach to Service Life Prediction of Organic Coatings, Breckenridge, Colorado. Contact: J. Martin, NIST, Bldg. 226, Rm B-350, Gaithersburg, MD 20899, USA. Email: jmartin@nist.gov.

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

SEPTEMBER 22 - 25: AIChE Conference on Safety in Ammonia Plants & Related Facilities, Fairmont Hotel, San Francisco, California. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA. Tel: 1-800-242-4363.

SEPTEMBER 30 - OCTOBER 2: 53rd Southwest ACS Regional Meeting, Tulsa, Oklahoma. Contact: F. B. Growcock, Amoco Corp., E&PT, P. O. Box 3385, Tulsa, OK 74012, USA. Tel: (918) 660-4224; Email: fgrowcock@amoco.com.

OCTOBER 5 - 8: Conference on Formulations & Drug Delivery, La Jolla, California, sponsored by the ACS Div. of Biochem. Technol. Contact: ACS Meetings, 1155 16th St, NW, Washington, DC 20036. Tel: (202) 872-6286; FAX: (202) 872- 6013; Email: miscmtgs@acs.org.

OCTOBER 6 - 10: Validation d'une Procedure d'Analyse, Qulification de l'Appareillage; Application a la Chromatographie Liquide, Montpellier, France. (Training course given in French) Contact: Prof. H. Fabre, Tel: (33) 04 67 54 45 20; FAX: (33) 04 67 52 89 15; Email: hfabre@pharma.univ-montpl.fr.

OCTOBER 19 - 22: 49th ACS Southeast Regional Meeting, Roanoke, Virginia. Contact: J. Graybeal, Chem Dept, Virginia Tech, Blacksburg, VA 24061, USA. Tel: (703) 231-8222; Email: reglmtgs@acs.org.

OCTOBER 21 - 23: Sensors Expo: Conference on Exposition of Sensors, Detroit, Michigan. Contact: Expocon Mgmt. Assoc., 3363 Reef Rd. P. O. Box 915, Fairfield, CT 06430-0915, USA. Tel: (203) 256-4700; Email: sensors@expo.com.

OCTOBER 21 - 23: Biotechnica Hannover '97: Int'l. Trade Fair for Biotechnology, Hannover, Germany. Contact: D. Hyland, Hannover Fairs USA, Inc., 103 Carnegie Center, Princeton, NJ 08540, USA.

OCTOBER 21 - 24: 152nd Fall Technical Meeting & Rubber Expo'97, Cleveland, Ohio, sponsored by ACS Div. of Rubber Chem. Contact: ACS Meetings, 1155 16th St, NW, Washington, DC 20036. Tel: (202) 872-6286.

OCTOBER 21 - 25: 33rd ACS Western Regional Meeting, Irvine, California. Contact: L. Stemler, 8340 Luxor St, Downey, CA 90241, USA. Tel: (310) 869-9838; Email: reglmtgs@acs.org.

OCTOBER 25 - 30: 24th Annual Conference of the Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Providence, Rhode Island. Contact: ACS Div. of Anal. Chem., Tel: (301) 846-4797; FAX: (301) 694-6860.

OCTOBER 26 - 29: ISPPP'97 – 17th International Symposium on the Separation of Proteins, Peptides, and Polynucleotides, Boubletree Hotel, Washington, DC. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA. FAX: (301) 898-5596.

OCTOBER 26 - 29: 8th Symposium on Handling of Environmental & Biological Samples in Chromatography and the 26th Scientific Meeting of the Group of Chromatography and Related Techniques of the Spanish Royal Society of Chemistry, Almeria, Spain. Contact: M. Frei-Hausler, IAEAC Secretariat, Postfach 46, CH-4123 Allschwill 2, Switzerland. FAX: 41-61-4820805.

OCTOBER 29 - NOVEMBER 1: 32nd ACS Midwest Regional Meeting, Lake of the Ozarks, Osage Beach, Missouri. Contact: C. Heitsch, Chem Dept, Univ of Missouri-Rolla, Rolla, MO 65401, USA. Tel: (314) 341-4536; FAX: (314) 341-6033.

NOVEMBER 2 - 6: AAPS Annual Meeting & Expo, Boston, Mass. Contact: AAPS, 1650 King Street, Alexandria, VA 22314-2747, USA. Tel: (703) 548-3000.

NOVEMBER 2 - 6: 11th International Forum on Electrolysis in the Chemical Industry, Clearwater Beach, Florida. Contact: P. Klyczynski, Electrosysthesis Co., 72 Ward Rd., Lancaster, NY 14086, USA.

NOVEMBER 6 - 8: 2nd North American Research Conference on Emulsion Polymers/Polymer Colloids, Hilton Head Is., SC. Contact: A. V. Patsis, SUNY New Paltz, Inst. of Mater. Sci., New Paltz, NY 12561, USA.

NOVEMBER 7 - 8: 6th Conference on Current Trends in Computational Chemistry, Jackson, Miss. Contact: J. Leszcynski, Jackson State Univ., Chem. Dept., 1400 J. R. Lynch St., Jackson, MS 39217, USA. Tel: 601) 973-3482; Email: jersy@iris5.jusms.edu.

NOVEMBER 11 - 15: 5th Chemical Congress of North America, Cancun, Mexico. Contact: ACS Meetings, 1155 16th St, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-6286; FAX: (202) 872-6128.

NOVEMBER 16 - 21: AIChE Annual Meeting, Westin Bonaventure/Omni Los Angeles, Los Angeles, California. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA. Tel: 1-800-242-4363.

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

NOVEMBER 20 - 21: New Horizons in Chemistry Symposium, Los Angeles. Contact: J. May, Tel: (213) 740-5962; Email: jessy@methyl.usc.edu.

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FEBRUARY 28 - MARCH 1: 28th Annual Spring Prog. in Polymers: Degradation & Stabilization of Polymers, Hilton Head Is., South Carolina. Contact: A. V. Patsis, SUNY New Paltz, Inst. of Mater. Sci., New Paltz, NY 12561. Email: ims@mhv.net.

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

MARCH 8 - 12: AIChE Spring National Meeting, Sheraton, New Orleans. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA.

MARCH 15 - 18: 14th Rocky Mountain Regional Meeting, Tucson, Arizona. Contact: R. Pott, Univ of Arizona, Tucson, AZ 85721.

MARCH 23 - 25: 5th Meeting on Supercritical Fluids: Materials and Natural Products Processing, Nice, France. Contact: F. Brionne, Secretariat, ISASF, E.N.S.I.C., 1 rue Grandville, B.P. 451, F-54001 Nancy Cedex, France. Email: brionne@ensic.u-nancy.fr.

MARCH 29 - APRIL 2: 215th ACS National Meeting, Dallas, Texas. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036, USA.

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

MAY 20 - 22: 32nd Middle Atlantic ACS Regional Meeting, Wilmington, Delaware. Contact: G. Trainor, DuPont Merck, P. O. Box 80353, Wilmington, DE 19880-0353. Email: reglmtgs@acs.org.

MAY 26 - 29: VIIIth International Symposium on Luminescence Spectrometry in Biomedical and Environmental Analysis: Detection Techniques and Applications in Chromatography and Capillary Electrophoresis, Las Palmas de Gran Canaria (Canary Islands), Spain. Contact: Dr. Jose Juan Dantana Rodriguez, University of Las Palmas of G. C., Chem. Dept., Faculty of Marine Sci., 35017 Las Palmas de G. C., Spain. Tel: 34 (9) 28 45.29.15 / 45.29.00; FAX: 34 (9) 28 45. 29.22; Email: josejuan.santana@quimica.ulpgc.es.

MAY 27 - 29: 30th Central Regional ACS Meeting, Ann Arbor, Michigan. Contact: D. W. Ewing, J. Carroll Univ., Chem. Dept., Cleveland, OH 44118. Tel: (216) 397-4241. Email: ewing@jevaxa.jeu.edu.

JUNE 1 - 3: 31st Great Lakes Regional ACS Meeting, Milwaukee, Wisconsin. Contact: A. Hill, Univ. of Wisc., Chem. Dept., Milwaukee, WI 53201, USA. Tel: (414) 229-4256; Email: reglmtgs@acs.org.

JUNE 2 - 5: Third International Symposium on Hormone and Veterinary Drug Residue Anbalysis, Congres Centre Oud Sint-Jan, Bruges, Belgium. Contact: C. Van Peteghem, Dept. of Food Analysis, University of Ghent, Harelbekestraat 72, B-9000 Ghent, Belgium. Tel: (32) 9/264.81.34.

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

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

JULY 12 - 16: SFC/SFE'98: 8th International Symposium & Exhibit on Supercritical Fluid Chromatography & Extraction, Adams Mark Hotel, St. Louis, Missouri. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA. Tel: (301) 898-3772; FAX: (301) 898-5596; Email: Janetbarr@aol.com.

AUGUST 23 - 28: 216th ACS National Meeting, Boston, Massachusetts. Contact: ACS, 1155 16th Street, NW, Washington, DC 20036, USA.

AUGUST 31 - SEPTEMBER 3: AIChE Conference on Safety in Ammonia Plants & Related Facilities, Charleston Place, Charleston, South Carolina. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA. Tel: 1-800-242-4363.

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

SEPTEMBER 13 - 18: 22nd International Symposium on Chromatography, ISC'98, Rome, Italy. Contact: F. Dondi, Chem. Dept., Universita di Ferrara, Via L. Borsari, 46, I-44100 Ferrara, Italy. Tel: 39 (532) 291154; FAX: 39 (532) 240707; Email: mo5@dns.Unife.it.

SEPTEMBER 24 - 26: XIVth Conference on Analytical Chemistry, sponsored by the Romanian Society of Analytical Chemistry (S.C.A.R.), Piatra Neamt, Romania. Contact: Dr. G. L. Radu, S.C.A.R., Faculty of Chemistry, University of Bucharest, 13 Blvd. Reepublicii, 70346 Bucharest - III, Romania.

NOVEMBER 4 - 7: 50th ACS Southwest Regional Meeting, Res. Triangle Pk, North Carolina. Contact: B. Switzer, Chem Dept, N Carolina State University, Box 8204, Raleigh, NC 27695-8204, USA. Email: switzer@chemdept.chem.ncsu.edu.

NOVEMBER 15 - 20: AIChE Annual Meeting, Fontainbleu/Eden Roc, Miami Beach, Florida. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA.

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MARCH 7 - 12: PittCon '99, Orlando, Florida. Contact: PittCon '99, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA.

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

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

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

2000

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

MARCH 26 - 31: 219th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036.

JUNE 25 - 30: HPLC'2000 – 24th International Symposium on High Performance Liquid Phase Separations, Seattle, Washington. Contact: Barr Enterprises, P.O.B. 279, Walkersville, MD 21793, USA.

AUGUST 20 - 25: 220th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

DECEMBER 14 - 19: 2000 Int'l Chemical Congress of the Pacific Basin Societies, Honolulu, Hawaii. Contact: ACS Meetings, 1155 16th St., NW, Washington, DC 20036, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: pacific@acs.org.

2001

APRIL 1 - 6: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036.

AUGUST 25 - 31: 222nd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2002

APRIL 7 - 12: 223rd ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 8 - 13: 224th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2003

MARCH 23 - 28: 225th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 7 - 12: 226th ACS National Meeting, New York City. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2004

MARCH 28 - APRIL 2: 227th ACS National Meeting, Anaheim, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC.

AUGUST 22 - 27: 228th ACS National Meeting, Philadelphia, Pennsylvania. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128.

2005

MARCH 13 - 18: 229th ACS National Meeting, San Diego, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 28 - SEPTEMBER 2: 230th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2006

MARCH 26 - 31: 231st ACS National Meeting, Atlanta, GA. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

SEPTEMBER 10 - 15: 232nd ACS National Meeting, San Francisco, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2007

MARCH 25 - 30: 233rd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 19 - 24: 234th ACS National Meeting, Boston, Massachusetts. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs @acs.org.

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

Chapter in a Book:

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

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JOURNAL OF LIQUID CHROMATOGRAPHY
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Volume 20, Number 15, 1997

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J. LIQ. CHROM. & REL. TECHNOL., 20(15), (1997)

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