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MOLECULAR MASS CHARACTERIZATION OF POLY(4-VINYLPYRIDINE)

R. MENDICHI, G. AUDISIO, AND A. GIACOMETTI SCHIERONI
Istituto di Chimica delle Macromolecole (CNR)
Via E. Bassini 15
20133 Milan, Italy

ABSTRACT

Poly(4-vinylpyridine) samples, synthesized by anionic and radical initiators, have been characterized by Size Exclusion Chromatography, Light Scattering and Intrinsic Viscosity. The mobile phase used in SEC characterization was 50% methanol and 50% 0.1M $LiNO_3$. Broad molecular weight distribution samples of P4-VP, characterized via Low Angle Laser Light Scattering, have been used in the calibration of the SEC system. The polymers show different molecular weight distribution and high polydispersity. Mark-Houwink constants for P4-VP in the SEC mobile phase have also been obtained.

INTRODUCTION

In this paper we present a molecular mass characterization of Poly(4-vinylpyridine) (P4-VP) polymers synthesized in our laboratories. Several papers present a characterization of P4-VP, by way of intrinsic viscosity (1,3-6), light scattering (3-5), osmometry (3,7), sedimentation (7) and pyrolysis (8). To our knowledge nobody presents the whole molecular weight distribution (MWD) of P4-VP polymers by Size Exclusion Chromatography (SEC or GPC) means.

SEC characterization of P4-VP presents various difficulties with respect to the choice of the mobile phase and the calibration of the system. In order to obtain both the sample's solubility and the columns packing compatibility we used 50% methanol and 50% 0.1M $LiNO_3$ as the mobile phase. To test how good this new solvent was we performed the off-line intrinsic viscosity measure of P4-VP samples which was carried out using both methanol and the SEC mobile phase.

An SEC calibration curve was constructed using three P4-VP broad MWD samples characterized by means of static Low Angle Laser Light Scattering (LALLS). Substantially absolute values of the weight-average molecular weight, M_w, and intrinsic viscosity, $[\eta]$, have furnished a reference for checking the SEC method.

EXPERIMENTAL

Materials

Three P4-VP samples were synthesized using anionic initiators, Butyl Lithium (BuLi), under various conditions. The synthesis procedure is described in reference (1). A further two P4-VP samples were synthesized using radical initiators, Cupric Acetate (CuAc) and Azobisisobutyronitrile (AIBN). The last procedures are described in reference (2).

SEC narrow standards, seven Poly(ethylene oxide) (PEO) and four Poly(ethylene glycol) (PEG) with molecular weights ranging from $4.4 \cdot 10^2$ to $8.5 \cdot 10^5$ g/mole, were obtained from Waters (Milford, MA)

Methods

SEC: The SEC system, Waters, consisted of a 600E pump, a U6K injector, 410 differential refractometer and 490 UV detectors (of 254 nm wavelength). Data reduction was carried out by the Waters 840 Data and Chromatography Control Station. The columns set was composed of two Waters Ultrahydrogel (1000 and 250 Å pore size). The experimental conditions consisted of: a mobile phase of 50% methanol and 50% of 0.1M $LiNO_3$ degassed with helium, room temperature, a 0.6 ml/min flow rate, a sample's concentration of 2 mg/ml and a 50 µl injection volume.

Intrinsic viscosity: The intrinsic viscosity, [η], value was determined using an Ubbelohde capillary viscometer (Schott Gerate) with methanol and the SEC mobile phase as solvents at a temperature of 25 °C. Each solution was filtered through a 0.45 μm filter. Sample concentrations ranging approximately from 0.1 to 0.5 g/dl. [η] value were calculated in the usual manner as reduced viscosity, η_{sp}/c, extrapolated to zero concentration according to Huggins equation. Intrinsic viscosity has been correlated with molecular weight by means of the Mark-Houwink equation.

Light scattering: The theory and the experimental protocol of LALLS Chromatix KMX-6 (LDC/Milton Roy) have been described previously (9,10). LALLS measures were performed at a wavelength of 632.8 nm, room temperature and an angle of scattered light collection θ=4°-5°. The solvent used was methanol, filtered through a 0.2 μm filter. Specific refractive index increment dn/dc=0.205 was calculated as follows. At wavelength λ=436 nm dn/dc=0.267 (5); at λ=546.1 dn/dc=0.224 (4), at λ=632.8 the value was calculated by extrapolation with the inverse of λ^2 (Cauchy Equation). The intensity of scattered light was measured at five concentrations ranging from 1 to 6 mg/ml. Each solution was filtered several times through 0.45 μm filters to obtain dust-free solutions. The weight-average molecular weight, M_w, and the second virial coefficient, A_2, were obtained according to the usual Debye equation

$$\frac{k \cdot c}{R_\theta} = \frac{1}{M_w} + 2 \cdot A_2 \cdot c \qquad (1)$$

where R_θ is the excess Rayleigh factor, k the polymer optical constant, and c the concentration.

Mark-Houwink constants: The value of k and a for the narrow standards, PEO/PEG, in the SEC mobile phase at room temperature was obtained by way of a least-squares linear fit of $\mathrm{Log}([\eta]_i)$ vs. $\mathrm{Log}(M_i)$, where $[\eta]_i$ is the value obtained from off-line measurement and M_i is the vendor value of molecular weight. The value of the constants for P4-VP in methanol was obtained from reference (1), in the SEC mobile phase solvent was calculated by using the calibration method based on broad MWD samples.

RESULTS AND DISCUSSION

Five P4-VP samples, as received from the synthesis without additional fractionation or cleaning, were characterized. Results and conditions of the synthesis are summarized in Table I.

SEC Mobile Phase

There are only a few solvents suitable for use in SEC of P4-VP polymers. The mobile phase is chosen primarily for sample solubility and secondarily for compatibility with the stationary phase of the columns, finally leading unwanted non-size-exclusion secondary effects. The choice of SEC mobile phase for P4-VP polymers presents two alternatives:

- N-N Dimethyl Formamide (DMF).
- Mixture of water and organic solvent (methanol).

DMF is a commonly used solvent in SEC runs. It is compatible with many packing gels and many polar and apolar polymers. However DMF is of little practical use and the results are occasionally difficult to interpret. We chose the second alternative as. Methanol is a good solvent of P4-VP polymers. Unfortunately a 100% of methanol is not compatible with the packing of Ultrahydrogel columns or other columns used on aqueous SEC. We have used 50% of methanol and 50% 0.1M $LiNO_3$ as the mobile phase. $LiNO_3$ salt was added to prevent the aggregation of macromolecules. P4-VP is completely soluble in the mobile phase. Furthermore Ultrahydrogel aqueous columns allow the use of up to 50% of organic solvent by gradual introduction via the gradient method.

The values of intrinsic viscosity, $[\eta]$, and Huggins coefficient, K_H, of P4-VP samples both in methanol and in SEC mobile phase are reported in Table II. Intrinsic viscosity values in SEC mobile phase significantly decrease respect to the value in methanol. The values of K_H for the five samples of P4-VP in methanol range from 0.32 to 0.43. The values are typically of a random-coil polymer in a good solvent. In the SEC mobile phase the values of K_H range from 0.51 to 0.60. Both $[\eta]$ decrease and K_H increase, going from the methanol to the SEC mobile phase solvent, state decreasing in solvent polymer affinity. Of course this fact is the price to pay for the presence of water, a poor solvent of P4-VP polymers, in the SEC mobile phase.

Table I: Polymerization of Poly(4-Vinylpyridine).

Sample		BuLi1	BuLi2	BuLi3	AIBN	CuAc
Solvent		Toluene	Toluene	Toluene	Methanol	Methanol
Monomer	mmol	95	190	470	260	270
Initiator	mmol	0.32	0.32	1.92	1.90	0.55
Init/Monom	$\cdot 10^3$	3.37	1.68	4.08	7.31	2.03
Solvent	ml	50	100	200	153	28
Temperature	°C	35	35	35	60	60
Time	h	16	16	16	4	6
Yield	%	95	82	40	25	20

Table II: Characterization of Poly(4-Vinylpyridine)

	SEC		LALLS		INTRINSIC VISCOSITY					
					SEC mobile phase			Methanol		
	$M_w \cdot 10^{-5}$ g/mole	D	$M_w \cdot 10^{-5}$ g/mole	$A_2 \cdot 10^4$ ml·mole·g^{-2}	[η] dl/g	K_H	$M_v \cdot 10^{-5}$ g/mole	[η] dl/g	K_H	$M_v \cdot 10^{-5}$ g/mole
BuLi1	1.81	9.7	1.88	7.07	0.65	0.51	1.57	0.88	0.43	1.47
BuLi2	1.69	6.8	1.66	7.25	0.59	0.59	1.37	0.84	0.38	1.37
BuLi3	0.67	8.4	0.68	7.91	0.31	0.60	0.58	0.45	0.32	0.55
AIBN	1.32	6.4			0.51	0.57	1.12	0.73	0.38	1.12
CuAc	0.75	11.6			0.35	0.55	0.69	0.52	0.36	0.68

SEC calibration curve

The calculation of molecular weight averages by SEC for any polymer species other than the narrow standards available requires the transformation of molecular weight into that of the specified polymer. The universal calibration (11) has wide applicability. However it requires accurate values of the Mark-Houwink constants for both samples and standards, as well as requiring the absence of non-size-exclusion secondary

effects between the sample and gel. In our case suitable narrow standards were lacking, the Mark-Houwink constants were not known and there where conceivable non-size-exclusion secondary effects. Therefore we preferred a calibration with P4-VP broad MWD samples.

The method used to obtain the true calibration curve was suggested by Chiantore and Hamielec (12). Such method requires the universal calibration curve and two chromatograms of broad MWD samples of known M_w. It starts from a primary narrow standards, PEO/PEG, calibration curve. The primary calibration function is denoted

$$M_s(v) = \phi(v) \qquad (2)$$

given k_s and a_s, values reported in Table III, the universal calibration may be expressed as

$$([\eta] \cdot M)(v) = k_s \phi(v)^{1+a_s} \qquad (3)$$

the true calibration curve for P4-VP polymers can be written as

$$M_p(v) = \alpha \phi^{\beta}(v) \qquad (4)$$

where α and β are constants

$$\alpha = \left(\frac{k_s}{k_p}\right)^{\frac{1}{1+a_p}} \qquad \beta = \frac{(1+a_s)}{(1+a_p)} \qquad (5)$$

Combining the definition of the weight-average molecular weight and the eq.(4) the value M_w of every sample can be expressed as

$$M_{w_i} = \alpha \int_0^{\infty} F_i(v) \phi^{\beta}(v) dv \qquad (6)$$

where $F_i(V)$ is the normalized chromatogram of the sample.

Dividing the two eq.(6) we obtain a new equation where only β is unknown. Using an iterative method we can calculate β. Once β has been obtained α may be calculated using eq.(6). Thus the calibration curve, eq.(4), for P4-VP was defined. Finally from the calculated values α and β using eq.(5) we also obtain Mark-Houwink constants, k_p and a_p, of P4-VP in the SEC mobile-phase at room temperature.

In the calibration we used three BuLi samples. From three samples, in three pairs, we calculated the values α and β that minimize the difference between SEC and LALLS M_w values.

The weight-average molecular weight, M_w, of three BuLi samples was obtained from LALLS measure. Both the M_w value and the second

Table III: Mark-Houwink constants at room temperature

Polymer	Solvent	$k \cdot 10^4$ dl/g	a
P4-VP	Methanol	2.4	0.69
P4-VP	SEC mobile phase	0.935	0.74
PEO, PEG	SEC mobile phase	1.74	0.76

virial coefficient A_2 of P4-VP samples are reported in Table II. The M_w values of the samples range from 0.68 to $1.88 \cdot 10^{-5}$ g/mole and the related MWD are adequately far apart to overlap.

SEC results

Table II summarized the characterization results. Examples of P4-VP MWD are also shown. Figure 1 shows the MWD of P4-VP initiated by Butyl Lithium, BuLi3 sample. Figure 2 shows the MWD of P4-VP initiated by Cupric Acetate, CuAc sample. In divergence with the results of Parravano (1) the molecular weight distributions of each sample are unimodal. Only the MWD of the CuAc sample shows a little bimodal form. As seen from Table II the polydispersity index, $D=M_w/M_n$, ranges from 6.4 to 11.6. However these high values are not extraordinary for anionic and radical polymerization.

The agreement between the molecular weight averages obtained from SEC, LALLS and Intrinsic Viscosity is very good. Of course the agreement between SEC and LALLS M_w values depend principally on the fact that the SEC calibration curve was obtained from LALLS M_w data. Elsewhere we point out the good agreement between M_w values obtained from SEC, M_v values obtained from intrinsic viscosity via the Mark-Houwink equation and k and a value reported in Table III, both in methanol and in the SEC mobile phase. This fact we think confirms the consistency of the SEC characterization method.

Finally the Mark-Houwink constants for P4-VP in the SEC mobile-phase at room temperature, a=0.74 (±0.04) k=0.935 (±0.06)$\cdot 10^{-4}$ dl/g,

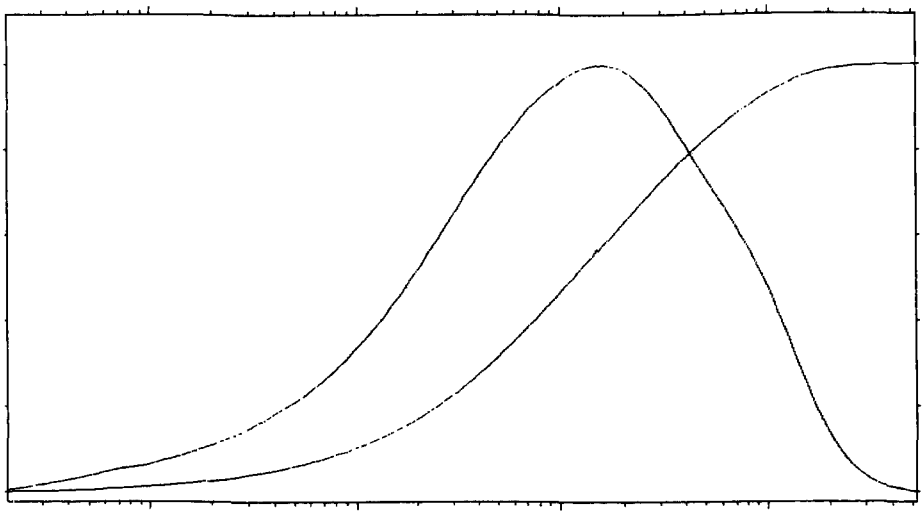

Figure 1: Molecular weight distribution of BuLi3 sample.

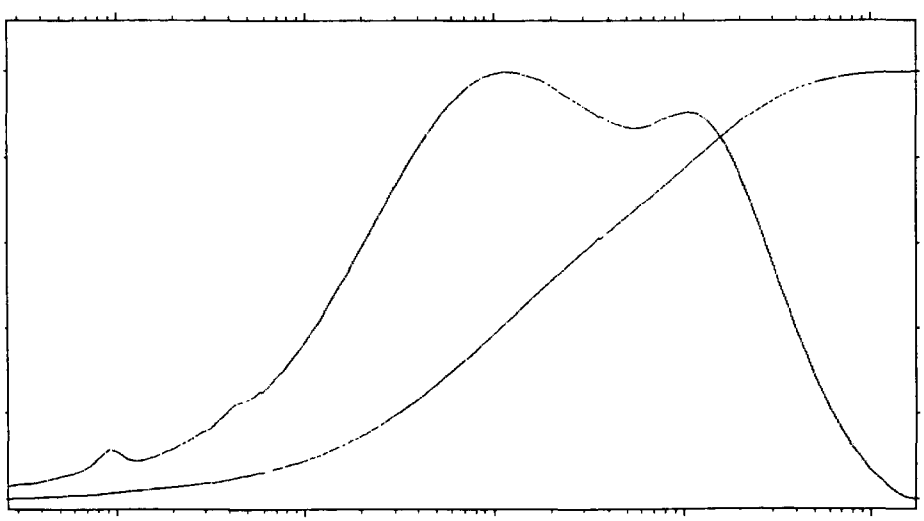

Figure 2: Molecular weight distribution of CuAc sample.

were calculated according to eq.(5). Values precision of k and a is quite low. This low precision depends on the calculation method based on broad MWD samples in opposition to the classical method based on some narrow standards.

CONCLUSION

We have described a SEC method for the characterization of molecular weight distribution of P4-VP polymers. The method uses as a mobile phase a mixture of Methanol and 0.1M $LiNO_3$.

Three dilute solution characterization methods have been utilized to create a coherent and consistent picture of P4-VP molecular mass properties. The agreement between SEC and both off-line viscometry and LALLS data is very good. Thus the intrinsic viscosity and LALLS data confirm the SEC method of characterization of whole MWD of P4-VP. The agreement between the data also confirms the validity of the broad MWD samples calibration method of the SEC system.

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LIQUID CHROMATOGRAPHIC ANALYSIS AND SEPARATION OF POLYPEPTIDE COMPONENTS FROM HONEY BEE VENOMS

GY. SZÓKÁN[1], J. HORVÁTH[1], M. ALMÁS[1], GY. SAFTICS[2], AND A. PALÓCZ[2]
[1]*Department of Organic Chemistry*
Eötvös University
Eötvös, Budapest
[2]*Beekeeping Farm and Products Ltd.*
Tököl, Budapest

Abstract

Reversed-phase HPLC on different columns with acetonitrile-water-trifluoroacetic acid eluent system was used to characterize honey bee venoms and to separate and determine quantitatively its peptide components.

Introduction

Honey bee venom is a rich source of pharmacologically active peptides. It is a complex mixture of biogenic amines and peptides with both pharmacological and immunological activities [1-6]. It contains a wide range of proteins and peptide toxins in big quantity of water (80-85 %), only approximately 0.1μg of dry venom can be isolated from a single bee. Honey bee venom is collected by an electrical milking method or by extraction from bee venom glands or sticks. Its characterisation needs the quantitative determination of the components in it.

The main components of bee venom are the low-molecular weights proteins melittin, apamine and MCD (mast cell degranulating-) peptide and the enzymes

phospholipase A_2 and hyaluronidase beside small peptides, physiologically active amines, sugars, phospholipids, amino acids and pheromones.

For the characterization of this complicated biological matrix many methods have already been described. From chemical point of view the peptide components can be separated by means of electroforetic and chromatographic procedures [1,7-11]. Because of water-soluble biomacromolecules present in venom, gel filtration was at first the most important technique for their purification and separations [12-14]. In order to obtain pure components from the crude venom, a conventional preparative gel chromatographic method based on the work of Habermann and Reiz was used and further developed [1,6]. For its fractionation and analysis different gels have been used as various types of Sephadex and Sepharose [12-13]. In these cases the components elute according to their molecular size. The elution order was as expected, although the components could not be adequately separated. (See Fig. 1)

3 components: melittin, apamine and MCD peptide could be readily separated on column of Heparin-Sepharose CL-6B [15]. IEC gives only a limited separation, too. In principle, the preparative method found by Habermann, would be suitable for the assay of bee venom, but as the separation time is up to 24 hrs , it is impracticable for quality control purposes. Therefore the development of a rapid and efficient method for the qualitative and quantitative characterisation of bee venom was very desirable for both chemists and beekeepers. Unfortunately the conventional gel types lack mechanic stability, witch was a major limitation when operating with high mobile phase velocity In order to achieve the high performance conditions for the separation, suitable rigid supports (as Shodex OH and ToyoSoda TSK SW) were developed for analysis of differents venoms [13].

Recently different high performance liquid chromatographic methods based upon FPLC media have been applied: at first gel filtration with Superose column and ion exchange with MonoS column. The elution profiles from the various separation techniques differed significantly [14]. Exclusion chromatography by Räder on an I-125 protein column gave good results: almost complete separation was possible within 12 min under isocratic conditions [17]. According to the experiences of many authors, separation trials with silica gel [8], amino-phase, ion-exchange resins [16] and reversed phase were unsatisfactory because of partial resolutions. Sometimes they gave good chance for final purification, but they were not enough for fingerprint analysis or quantitative characterisation of bee venom [17].

As RP-HPLC is one of the most powerful technique for separation of peptides and proteins, we examined the possibilities of reversed phase HPLC for the finger-print characterisation and assessment of purity of bee venoms originated from different sources.

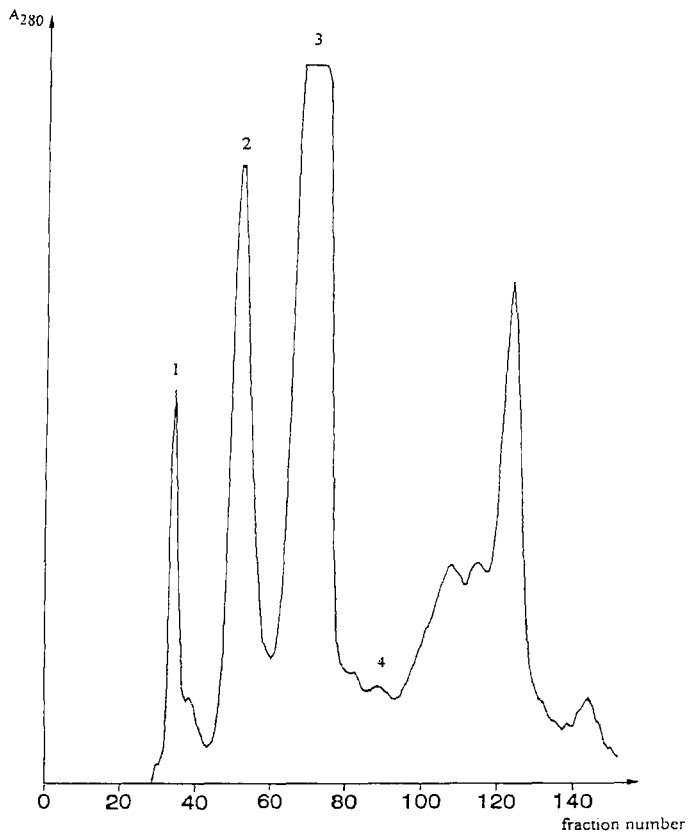


FIGURE 1. Conventional preparative chromatographic pattern of bee venom. Column: Sephadex G-50 (1400x200 mm). Eluent 0.1 M ammonium formiate buffer (pH 4.5). Flow rate: 0.4 ml/min. Fraction volume: 4ml. Identified peaks: hyaluronidase (1), phospholipase A_2 (2), melittin (3), apamine (4).

The structure of main bee venom components:

Ala-Leu-Cys-Asn-Cys-Asn-Arg-Ile-Ile-Ile-Pro-His-Met-Cys-Trp-Lys-Lys-Cys-Gly-Lys-Lys-NH2 Tertiapin

Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln-NH2 Melittin-F

Tyr-Ile-ILe-Asp-Val-Pro-Pro-Arg-Cys-Pro-Pro-Gly-Ser-Lys-Phe-Ile-Lys-Asn-Arg-Cys-Arg-Val-Ile-Val-Pro Secapin

Ala-Gly-Pro-Gln-Histamine Procamine A

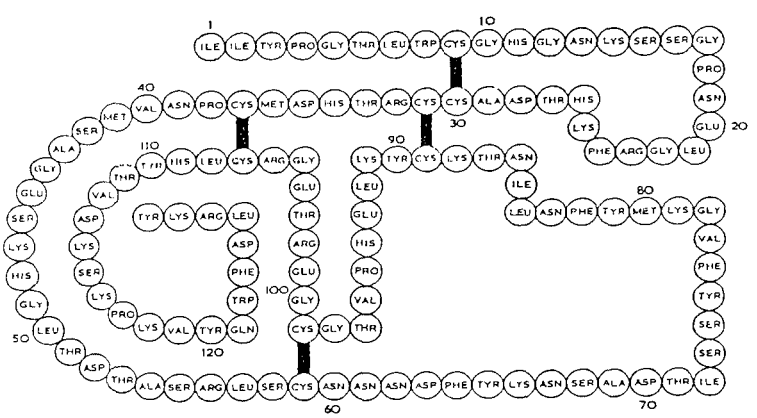
Ala-Gly-Gln-Gly-Histamine Procamine B

Ala-Gly-Pro-Ala-Gln-Histamine Nelson's peptide

H-Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln-NH2 Melittin

Cys-Asn-Cys-Lys-Ala-Pro-Glu-Thr-Ala-Leu-Cys-Ala-Arg-Arg-Cys-Gln-Gln-His-NH2 Apamine

Ile-Lys-Cys-Asn-Cys-Lys-Arg-His-Val-Ile-Lys-Pro-His-Ile-Cys-Arg-Lys-Ile-Cys-Gly-Lys-Asn-NH2 MCD-peptide



Phospholipase A_2

Experimental

Materials

Commercial-grade dried bee venoms were obtained from Hungarian beekeepers (Locations:Tököl, Szolnok, Hungary). Hyaluronidase and melittin were obtained from Fluka (F. R. G.), apamine, MCD peptide and phospholipase A_2 were all obtained from Sigma (St. Louis, MO, U.S.A.). Solvents were supplied from Reanal (Hungary).

Preparation of bee venom

Method A.

After electrical milking and collection the crude venom was dried or lyofilized from water.

Method B.

Extraction from powdered bee glands and sticks

a, with water, then lyofilization

b, with a mixture of n-butanol-pyridine-acetic acid-water (60:20:6:24 v/v), then evaporation, solvent change to water and lyofilization.

Apparatus and methods

Conventional preparative column chromatography

300 mg dissolved bee venom is placed on a Sephadex G-50 column (1400 mm x 20 mm I.D.). An aqueous 0.1 M ammonium formiate buffer of pH 4.5 is used as the eluent. The flow-rate was 0.4 ml/min. Each fraction was 4 ml. The separation is finished after 160 fractions and detection is carried out at 280 nm by Spektronom 203 (see Fig. 1). On the base of chromatographic pattern the fractions were pooled and lyophilized twice from water.

High-Performance Liquid Chromatography

Separations were performed on a KNAUER-HPLC instrument consisting of two pumps (Model 64) with analytical pumphead, a gradient programmer 50B, a variable-wavelength UV monitor fitted to an 8 µl flow-cell, an injection valve with 20 µl sample loop (Knauer GmbH, Bad Homburg, F.R.G.) and a sample injector (Knauer, F.R.G.). Peaks were recorded on a Model OH-314/1 chart recorder (Radelkis, Hungary).

Qualitative analysis

The bee venom was separated by a Knauer chromatograph using four reversed phase columns: Hypersil ODS, 125 mm x 4.6 mm, 6µ particle size, Hypersil WP-300 C18, 125 mm x 4.6 mm, 5µ particle size (Shandon), Delta-Pak C18 300, 300 mm x 3.9 mm, 10µ particle size (Waters) and Econosphere 300 C4, 250 mm x 4.6 mm, 7µ particle size (Alltech). The bee venom solution was prepared by dissolving 5mg of bee venom in 1ml distillated water or 1 ml A eluent. The mobile phases were: A: 0.1% TFA in water and B: 0.1% TFA in acetonitrile:water (80:20). The bee venom was separated by linear gradient 5% B - 80% B at 40 min. The flow-rate was maintained at 1.5 ml/min. The elution was monitored at 220 or 280 nm with recorder response set at 0.32 absorbance units full scale.

Quantitative analysis

The apamine, melittin and phospholipase A_2 standards were used for calibration. The apamine standard solution was prepared by dissolving 0.25 mg of apamine in 1 ml distillated water. The phospholipase A_2 standard solution was prepared by dissolving 1.00 mg of phospholipase A_2 in 1 ml distillated water. The melittin standard solution was prepared by dissolving 4.00 mg of melittin in 1 ml distillated water or eluent A. The mobile phases, flow-rate and monitor were the same as in case of qualitative analysis. For Hypersil WP-300 C18 column the mobile phase was 84% A eluent and 16% B eluent in case of apamine, 60% A and 40% B eluent at 5 min and after 5 min 45% A and 55% B eluent for phospholipase A_2 55 % A and 45% B eluent at 5 min and 45%A and 55% B eluent in case of melittin.

Results and discussion

We found, that RP-HPLC is the most efficient technique for separation of peptides, proteins and other components in bee venom. High resolution and efficiency were achieved using C_4, C_8 or C_{18} chemically bonded phases with acetonitrile-water or acetonitrile-water-methanol eluent mixtures containing ion-suppresser and ion-pairing reagent. Methanol-water eluent is not sufficient one, because methanol is not enough strong modifier, acetonitrile is better. The elution window was very narrow for the optimal resolution and it has different values for the different columns (Table I-II, Fig. 2-3).

The separation of all main components takes place satisfactorily because of their similar chemical structure and behaviour a very similar range of molecular weights. For chromatography of these biopolymers the columns with wide pore diameter (300 Å) gave better patterns, as the conventional ones (100 Å). The resolved components are readily detectable in the low UV-range (200-220 nm) with reproducible results on the base of peptide-amide chromophore units present in the molecules. Furthermore the absorption of these polypeptides is very similar. At higher absorbance wavelengths (280 nm) the proteins could be detected selectively, so the measurement of major melittin content (at 280 nm) can give important information for very rapid characterization of bee venoms. The elution profiles from the various RP-columns differed only very slightly (Table II). Similar pattern was obtained with FPLC on ProRPC (C8) column by Einarsson [14].

All polypeptide components of the bee venom contain more basic amino acid residues, therefore ion paring reagents were required to resolve them by RP-HPLC. Ragnarsson used different alkanesulphonic acids for determination single peptides, as apamine or MCD-peptide [18]. Bennett worked always with trifluoracetic acid as good ion pair forming agents in RP-HPLC of polypeptide hormones [20]. In our case the RP-HPLC was performed with it as especially favourable ion suppressor and ion-pairing reagent in the eluent. By this way the main components of bee venom could be easily analysed with reproducible results. Isocratically it was not possible to separate the all components, but the linear gradient resulted in good separation. By applying a gradient of 5-85% acetonitrile in 0.1% TFA a well-resolved pattern approximate 18-20 different peaks could be detected in honey bee venom when monitoring absorbance at 220 nm (Fig. 2). Optimising the gradient form it is was possible the improve further the resolution, e.g. on the first part of the chromatographic pattern: the minor components

Table I Solvent composition (%) for elution at different RP-columns. (Quantitative determinations).

Column type	Apamine		Melittin	
	A %	B %	A %	B %
Econosphere C4	85	15	50	50
DeltaPak C18	97	3	44	56
Hypersil C18	84	16	45	55

Table II Retention of bee venom components on different RP-columns.

Components	Retention time (t_R min)			
	Column type			
	Econosphere 300 Å C4	DeltaPak 300 Å C18	Hypersil 300 Å C18	Hypersil 100 Å C18
Apamine	8.4	13	3.7	-
MCD-peptide	9,4	15,2	9.4	-
Phospholipase A_2	23.6	25,0	19.3	-
Hyaluronidase	30,4	30,0	30.4	-
Melittin	31.2	32,4	26.6	15.2*

*:Isocratic value

surrounding the apamine could be separated very well (9 further peaks), applying very only soft gradient in the first 10 min of the chromatography (Fig. 4).

According to the chromatographic pattern the linear peptides (as melittin) have longer retention on C_{18} phase, then the cyclic peptides (the components containing one or more disulphide bridges e.g. apamine, MCD-peptide etc.). Even the enzymes with higher M_W (phospholipase A_2 and hyaluronidase) are eluated before melittin because of intermolecular cystine residues indicating that the hydrophobic side chains of the linear peptide (melittin) are more accessible for interaction with the hydrophobic stationary phase, then in the case of a more compact, cyclic compound as MCD-peptide or an enzyme. Here we observed a decreasing peptide retention due to the lower contact area between peptide and stationary phase. If a linear peptide is the

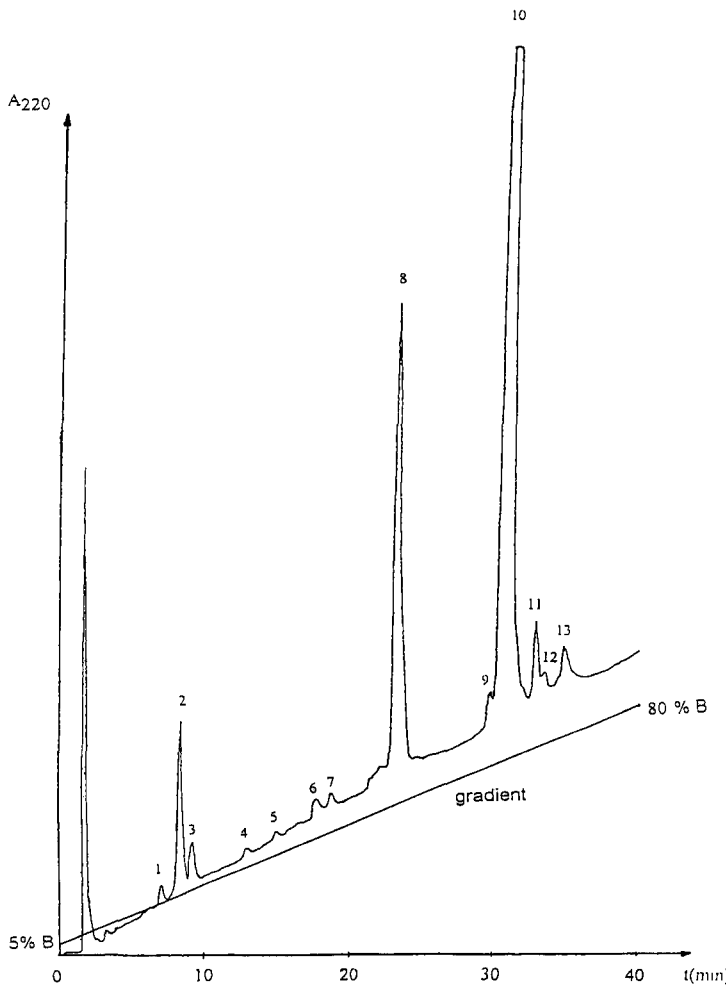


FIGURE 2. Chromatographic pattern of bee venom.
Column: Econosphere 300 A C4 (250x4 mm). Eluent A: 0.1% TFA in water, eluent B: 0.1% TFA in acetonitrile-water (80-20). Linear gradient: 5% B - 80% B at 40 min. Injection volume: 20 µl. Detection 220 nm. Flow rate: 2.0 ml/min. Identified peaks: minor peptides (1, 4-7), apamine (2), MCD-peptide (3), phospholipase A_2 (8), hyaluronidase (9), melittin (10), melittin derivatives (11-13). A=0.32.

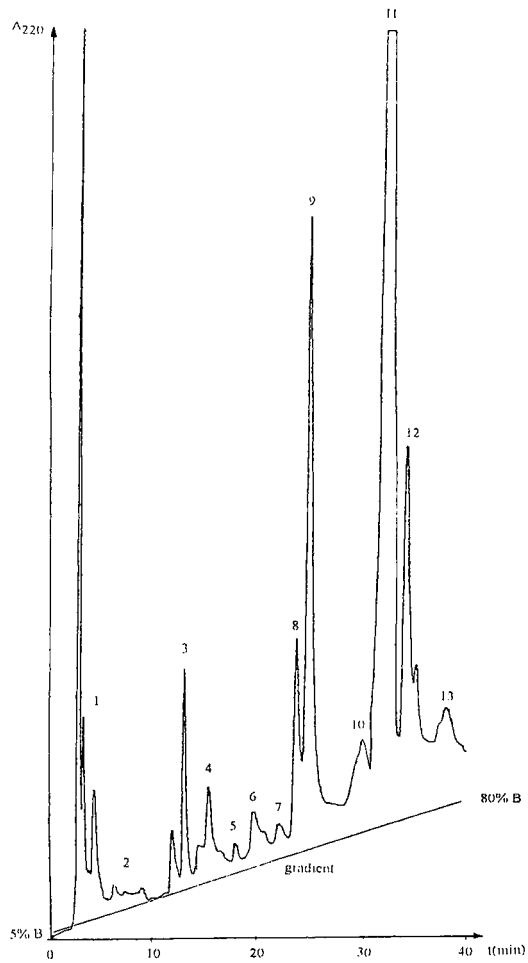


FIGURE 3 Chromatographic pattern of bee venom.
Column: Delta-Pak C18 300 (300x3.9 mm). Eluent A: 0.1% TFA in water, eluent B: 0.1% TFA in acetonitrile-water (80-20). Linear gradient: 5% B - 80% B at 40 min. Injection volume: 20 µl. Detection 220 nm. Flow rate: 2.0 ml/min. Identified peaks: amino acids, biogenic amines (1), procamines (2), apamine (3), MCD-peptide (4), tertiapin (5), melittin-F (6), secapin (7), lysophospholipase (8), phospholipase A_2 (9), hyaluronidase (10), melittin (11), melittin derivatives (12), acid monophosphoesterase (13). A=0.32.

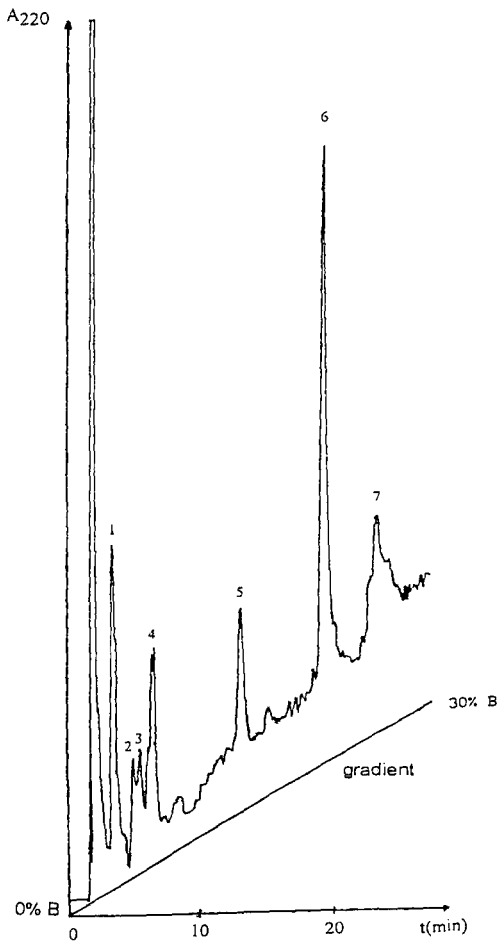


FIGURE 4. Chromatographic pattern of the minor components of bee venom surrounding the apamine.
Column: Delta Pak C18 300 A (300x3.9 mm). Eluent A: 0.1% TFA in water, eluent B: 0.1% TFA in acetonitrile-water (80-20). Linear gradient: 0% B - 30% B at 30 min. Injection volume: 20 µl. Detection 220 nm. Flow rate: 1.5 ml/min. Identified peaks: minor peptides (1-5), apamine (6) MCD-peptide (7). A=0.04.

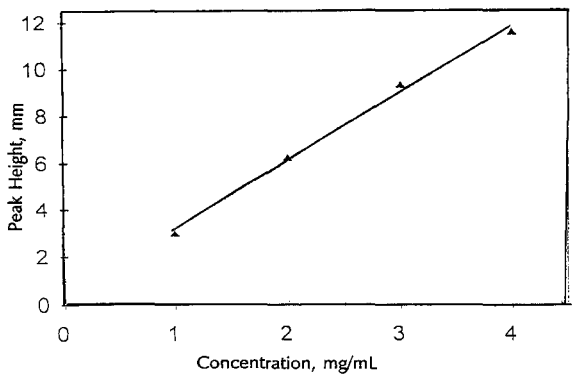


FIGURE 5. Calibration curve of melittin

analyte, there is a larger contact area between the analyte and the retentive site, which results in a strong retention (see retention data of melittin).

On the base of their cyclic structure, the small quantities (below 1%) of tertiapin and secapin could be identified on the chromatographic pattern (prediction: peak 7-tertiapin, peak 8-secapin).

The different procamines are very hydrophilic peptides because of C-terminal histamine and Gln residue, so they are coeluted at the very beginning of the gradient. Amino acids and biogenic amines are together with the eluent peaks in our system.

A more minor, partly degraded form of melittin, called melittin-F locks the first seven residues of the native molecule, and its retention time is much more lower, than that of the parents compound.

The peaks of the chromatographic pattern (Fig. 2-4) are quite well identified, and so different honey bee venoms could be characterised very precisely by this finger-print (and quantitative composition, of course).

Under these conditions a reproducible, about complete separation of bee venom into most of its components was possible within 40 min. Under isocratic condition (see experimental parts) quantitative determination of melittin, apamine, phospholipase A_2 was possible using standards of the corresponding components. The plots of the peak height of the determined component versus the known concentration were used as calibration curves to calculate the quantity of bee venom components (Fig. 5). The calibration graph of standard melittin, apamine and phospholipase A_2 was linear and

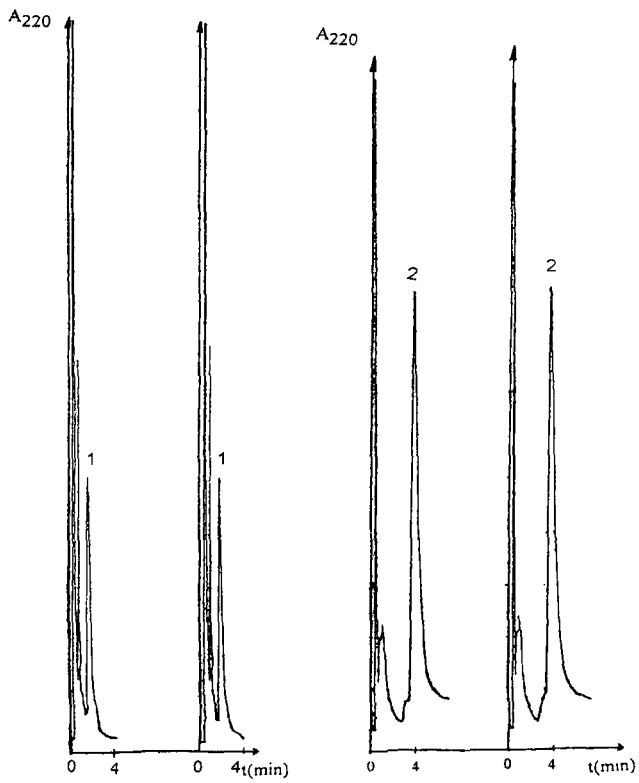


FIGURE 6. Reproducibility of quantitative measurements.
Column: Hypersil WP-300 C18 (125x4.6 mm). Flow rate: 1.5 ml/min. Detection: 220 nm. Eluents: apamine (1): 84% A and 16% B, melittin (2): 45 % A and 55 % B. A=0.32.

Table III Distribution of the components apamine, melittin and phospholipase A_2 in bee venom and in GPC fractions.

Sample	Location or isolation method	Apamine %	Melittin %	Phospholipase A_2 %
bee venom	SERVA	3.2	51.5	-
bee venom	Latvia	4.4	52.0	12.6
bee venom	Tököl	3.4	51.0	-
bee venom	Tököl by extraction	4.3	45.8	-
bee venom	Kengyel	4.5	61.0	13.8
dried bee venom	Szolnok*	3.5	57.1	12.5
lyophilized bee venom	Szolnok	3.4	57.0	12.6
frozen bee venom	Szolnok	3.5	57.0	12.5
bee venom, after 1 year	Szolnok	3.4	56.9	10.4
Melittin	via picrate	-	87.3	-
Melittin	GPC	-	85.8	-
Phospholipase A_2	GPC	-	-	95.5

* Hyaluronidase 2.8%, MCD peptide 1.4% (measured by similar procedure using standards for calibration)

passes through zero. Reproducibility of quantitative measurements was excellent with 0.2% error for apamine and 0.4-0.5% for melittin (Fig. 6). Optimising the method the different peaks do not disturb the quantitative determination of each other, isocratically base line separation could be achieved for the main components (see experimental part).

During measurements the melittin is pure monomer polypeptide, although it is known to exist in a tetrameric form (in concentrated aqueous solution at high ionic strength).

It was found, that in pure batches of bee venom the amount of main component melittin was between 50-60% (see Table III), the minor component, apamine was less,

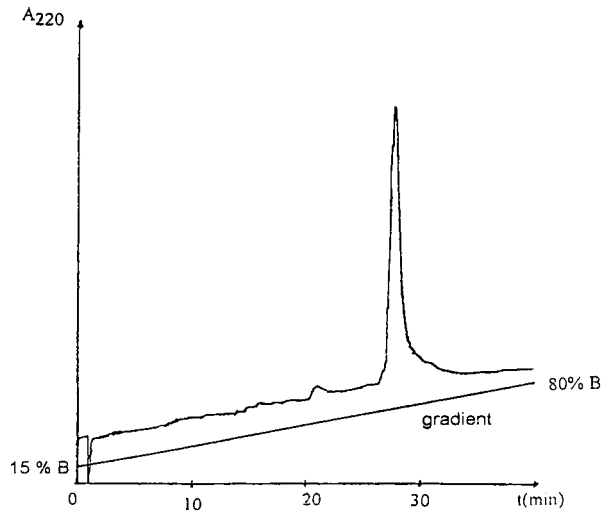


FIGURE 7. Chromatographic pattern of pure melittin(1) from GPC. Column: Hypersil WP-300 A C18 (125x4.6 mm). Eluent A: 0.1% TFA in water, eluent B: 0.1% TFA in acetonitrile-water (80-20). Linear gradient: 15% B - 80% B at 40 min. Injection volume: 20 µl. Detection 220 nm. Flow rate: 1.5 ml/min. A=0.32.

approximate 2-6%. Our RP-HPLC could be used directly to finger-print analyse the bee venom in a single run derived from different sources as well as for competing various manufacturer's materials, too. The system described above was excellent for quantitative determination of bee venom's enzymes e.g. phospholipase A_2, which usually is determined on the base of its biological activity. Comparing the values of composition, the stability of lyophilized and frozen bee venom has not changed: it seems, that the compositions do not depend on the year of production (Table III). The preparative isolation methods of pure components could be controlled very adequately (Fig. 7, Table III). At GPC fractionation the purity of isolated components in the fractions could be checked very precisely (Table III).

If the isolations took place by means of extraction from bee glands and sticks, we found, that n-butanol-pyridine-acetic acid-water (60:20:6:24 v/v) mixture is not only the best one, but the chromatographic pattern of obtained substance is very similar to

that of original bee venom and the ratio of the component in different batches was practically the same. Melittin could be prepared from solution via its picrate salt [7] as pure substance. Its purity checked by HPLC was higher, than 85 %. Applying the optimised gradient profile for separation, in some cases the finger-print analysis showed different melittin peaks (on great quantity) according to N-formil-melittin, melittin-F and desamido-melittin substances.

The RP-columns can be used with displacement chromatographic technique, too. A method elaborated recently by Fellegvári [19] helps chiefly the preparative work in isolation of main components from honey bee venom; e.g. melittin [21].

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OPTIMUM SEPARATION AND COMPOUND CLASS SEPARATION OF THE METABOLITES OF BENZO[a]PYRENE-DNA ADDUCTS WITH REVERSED-PHASE LIQUID CHROMATOGRAPHY

MALI ROZBEH AND ROBERT J. HURTUBISE*
Department of Chemistry
University of Wyoming
Laramie, Wyoming 82071

ABSTRACT

Mobile phases were optimized for the reversed-phase liquid chromatographic separation of a complex mixtures of fourteen metabolites of benzo[a]pyrene (B[a]P). The metabolites constituted groups of isomers that were difficult to separate. The groups of isomers were tetrols, dihydrodiols, diones, and monohydroxyl-benzo[a]pyrenes. The window diagram optimization approach was used to initially optimize the binary mobile phases. Based on the data obtained from the optimum binary mobile phases, a solubility parameter optimization method was employed to obtain an optimum ternary mobile phase. Both the binary and ternary mobile phases were very effective in separating the metabolites. However, complete baseline resolution of the complex mixture of the metabolites was not achieved under the conditions investigated. Nevertheless, it was possible to obtain a separation of all fourteen of the metabolites with some overlap of the chromatographic bands. Also, compound class separation was obtained with the classes separating in the order of tetrols, diones, dihydrodiols, and monohydroxyl-benzo[a]pyrenes.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are not highly reactive chemically, but exert their carcinogenic activity after metabolism by humans, or lower animals, through metabolites which are sufficiently reactive to modify cellular macromolecules such as nucleic acids (DNA, RNA) and proteins. Identification of metabolites from some of the PAHs has been reported, with the metabolites from benzo[a]pyrene (B[a]P) being studied the most extensively (1). Information on metabolic activation of B[a]P has been summarized recently (2-4). Benzo[a]pyrene is a ubiquitous environmental contaminant found in the atmosphere, waterways and oceans, soil, marine life and in the food chain. Since the report of B[a]P as a potent carcinogen, numerous studies on the carcinogenesis of B[a]P have been performed (1-5). In laboratory experiments, the biotransformation products of B[a]P are usually analyzed by means of HPLC (6-14). The separation of some B[a]P metabolites using HPLC was initiated by the work of Selkirk et al. (11). However, the approach they developed did not completely resolve an isomeric mixture of four tetrols, dihydrodiols, diones, and monohydroxylated metabolites. Croy et al. (15) refined the earlier work of Selkirk et al. (11) by chromatographically recycling various isomers. They found that 1-OH-B[a]P and 7-OH-B[a]P co-chromatographed with 3-OH-B[a]P. However, their methodology did not isolate of 2-OH-B[a]P. Elnenaey and Schoor (14) developed a method to separate twelve isomeric monohydroxylated metabolites by using HPLC with fluorescence detection and various methanol-water gradients. Their study showed that several metabolic isomers have almost identical retention times. Wang and O'Laughlin (16) focused on the development of a sensitive method using laser-induced fluorescence detection in conjunction with HPLC for the separation and detection of tetrols that were formed by fish that metabolized B[a]P.

Reported here are HPLC methods for the rapid separation of fourteen B[a]P metabolites into four main compound-class types. The compound-class types are tetrols, dihydrodiols, diones, and monohydroxylated benzo[a]pyrene

compounds. Also, mobile-phase optimization methods for obtaining the optimal binary and ternary mobile phases for the separation of complex mixtures of the metabolites are reported. These methods are based on the work of Cooper and Hurtubise (17,18). They discussed the use of the window-diagram optimization approach and a mobile-phase optimization method for ternary mobile phases based on solubility parameter concepts for the separation of complex hydroxyl aromatic mixtures. The window diagram approach has been applied rather extensively to HPLC (19-24). In addition, Schoenmakers and coworkers (25-27) developed a method for estimating optimum compositions of ternary mobile phases for separating complex mixtures based on solubility parameters. Collectively, most of the metabolites used in this work have not been investigated previously by HPLC. For example, the separation of tetrols from diones, dihydrodiols and monohydroxyl-benzo[a]pyrenes has not been studied in detail. Moreover, little or no HPLC chromatographic data are available for many of the compounds investigated in this work.

EXPERIMENTAL

Apparatus

The liquid-chromatograph used was a Waters unit equipped with a model 6000A pump (Waters Assoc., Milford, MA, U.S.A.), a U6K injector, a dual channel free-standing ultraviolet 440 model detector set at 254 nm, and a Linear 1200 dual channel, 5.0 V recorder (Linear Instruments Co. Concord, CA). A model FIAtron heating block (Oconomowoc, WI, U.S.A.) constant temperature control system was used to keep the temperature of the column at 25 ±0.1° C. The chromatographic column employed was a 5-μm Baker-bond C_{18} (250mm x 4.6mm i.d.) obtained from J.T.Baker (Phillipsburg, NJ, U.S.A.).

Reagents

HPLC grade methanol and water were obtained from J.T.Baker Inc. (Phillipsburg, NJ, U.S.A.). Acetonitrile was HPLC grade and was purchased from

Fisher Scientific (Fair Lawn, NJ, U.S.A.). The organic modifiers and water were prefiltered through a Millipore type HA 0.45 μm filter. The B[a]P metabolites are listed in Table 1 and were obtained from the National Cancer Institute (NCI) repository at Midwest Research Institute (MRI, Kansas City, MO). All chemicals were used without further purification.

Procedures

Solutions of 0.1 mg/mL for an individual metabolite and 0.01 mg/mL for the mixture of standards were prepared in methanol or acetonitrile depending on the mobile phase composition. The retention volumes of each metabolite were determined by injecting 3.0-4.0 μl of the standard solution into the chromatographic system. To assure stability, the solutions were stored under nitrogen gas at -15° C and in the dark. However, under these conditions, 6-hydroxybenzo[a]pyrene was unstable in solution, and it decomposed after four days. Therefore, it was prepared freshly each 4-5 days.

The capacity factors (k') were calculated from the equation, $k'=(\overline{V}_R-\overline{V}_m)/\overline{V}_m$, where $\overline{V}_R$ is the retention volume (mL) and $\overline{V}_m$ is the column void volume (mL). The void volume for the C_{18} column was obtained by injection of a methanol solution of potassium nitrite.

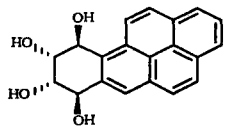
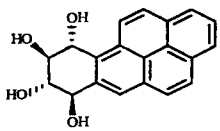
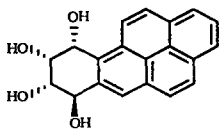
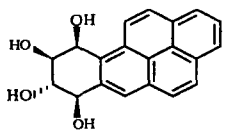
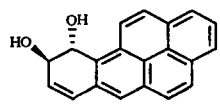
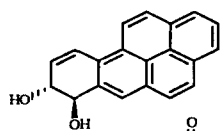
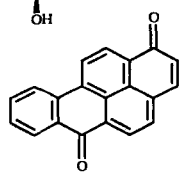
RESULTS AND DISCUSSION

General Considerations

The variable most frequently employed in optimizing liquid-chromatographic separations is the composition of the mobile phase. A number of mobile-phase optimization strategies have been developed over the years to obtain the optimal mobile phases for the separation of mixtures of compounds. In this work, the window diagram approach (23,28,30) and the solubility parameter optimization method developed by Schoenmakers et al. (27) and Drouen et al. (26) were combined to develop a relatively easy way of optimizing the mobile phases for the separation of the B[a]P metabolites. The window-diagram method was employed

TABLE 1

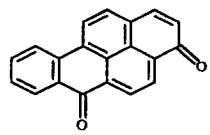
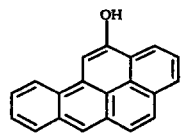
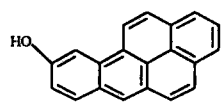
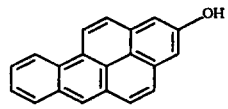
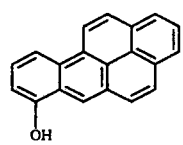
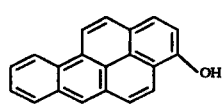
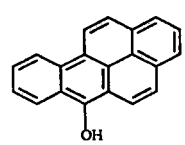
Names and Structures of the Model Compounds

Compound	Structure
1. Benzo[a]pyrene-r-7,t-8,9,c-10-tetrahydrotetrol (Tetrol I-1)	
2. Benzo[a]pyrene-r-7,t-8,c-9,t-10-tetrahydrotetrol (Tetrol II-1)	
3. Bezo[a]pyrene-r-7,t-8,9,10-tetrahydrotetrol (Tetrol I-2)	
4. Benzo[a]pyrene-r-7,t-8,c-9,10-tetrahydrotetrol (Tetrol II-2)	
5. Benzo[a]pyrene-trans-9,10-dihydrodiol	
6. Bezo[a]pyrene-trans-7,8-dihydrodiol	
7. Benzo[a]pyrene-1,6-dione	

(continued)

TABLE 1 (Continued)

Names and Structures of the Model Compounds

Compound	Structure
8. Benzo[a]pyrene-3,6-dione	
9. 12-Hydroxybenzo[a]pyrene (12-OH-B[a]P)	
10. 9 -Hydroxybenzo[a]pyrene (9-OH-B[a]P)	
11. 2-Hydroxybenzo[a]pyrene (2-OH-B[a]P)	
12. 7-Hydroxybenzo[a]pyrene (7-OH-B[a]P)	
13. 3-Hydroxybenzo[a]pyrene (3-OH-B[a]P)	
14. 6-Hydroxybenzo[a]pyrene (6-OH-B[a]P)	

to determine the optimum binary mobile phases. Then, the optimum binary mobile phases were used with the solubility parameter optimization method to acquire the optimum ternary mobile phases. Below brief discussions of the window diagram optimization method and the solubility parameter optimization method are given.

The window diagram method is a graphical method for describing chromatographic retention data. The optimum chromatographic conditions are obtained by maximizing the selectivity of the most difficult to resolve chromatographic peak pairs as a function of various experimental variables (23,28,30). In this work, the separation factor, S, was used as the optimization criterion for the window diagram (29). The separation factor is defined by eqn. 1, where t_i is the retention time of component i, and t_j is the retention time of component j.

$$S = \frac{t_i - t_j}{t_i + t_j} \qquad (1)$$

The advantage of using eqn. 1 is that it not necessary to measure t_o (void time). Schoenmakers et al. (27) and Drouen et al. (26) developed a systematic optimization criterion from their work based on solubility parameter theory. The optimization criterion from their work used in this research was the product of resolution factors, πR_s, which is defined by eqn. 2, where k_i and k_{i+1} are the capacity factors for each pair of adjacent peaks in a chromatogram.

$$\Pi R_S = \Pi_{i=1}^{n-1} \frac{k_{i+1} - k_i}{k_{i+1} + k_i + 2} \qquad (2)$$

The criterion expressed by eqn. 2 gives a relative number designated to select an optimum ternary mobile phase composition, but not necessarily a satisfactory chromatogram. Cooper and Hurtubise (18) reported a detailed comparison between the solubility parameter and window optimization methods for the reversed-phase chromatographic separation of hydroxyl aromatics. They showed that by comparing eqn. 1 and eqn. 2 the only difference between S and

πR_s is that πR_s corresponds to the product of separation factors, or S values, for each adjacent peak pair in a chromatogram. This relationship provides a convenient means of directly comparing the optimization in the window diagram and the solubility parameter optimization method. With both optimization methods, two conditions must be met. First, one must be able to recognize a given solute in different chromatograms. Secondly, one must be able to predict variations of capacity factors with mobile phase composition.

Window Diagram Optimization for the Metabolites of B[a]P

In this work, isomers related to metabolic pathways for B[a]P were selected for separation. Generally, classes of isomers including tetrols, dihydrodiols, diones and monohydroxy-benzo[a]pyrenes can be found in urine, blood sera and lung adenomas samples of cancer patients (5,31). Fourteen different metabolites of B[a]P were investigated with a C_{18} column at 25° C with methanol:water (MeOH:H_2O) and acetonitrile:water (ACN:H_2O) mobile phases.

Initial data for the window diagram optimization procedure were obtained by acquiring k' values for the metabolites with two different isocratic mobile phases which gave a wide range of k' values. Anywhere from 9 to 14 different binary mobile phases were used to construct the window diagrams. The window diagrams were plotted based on separation factors versus mobile phase composition for seven pairs of compounds (eqn. 1). The optimum mobile phase composition for MeOH:H_2O was found to be 81.75:18.25, which gave good resolution of a fourteen component mixture (Figure 1). It is evident from Figure 1 that this binary mobile phase is capable of separating the monohydroxyl isomers, except for the 2-OH-B[a]P and 9-OH-B[a]P and B[a]P-t-9,10-dihydrodiol from the tetrols. Also, 12-OH-B[a]P appears between the two diones. It should be mentioned that a mixture of just the four tetrols can be separated with MeOH:H_2O (55:45). This result is supported by Rojas et al. (8).

In order to examine the selectivity of different mobile phases, other organic modifiers including acetonitrile and tetrahydrofuran were studied. Based on the experimental k' values obtained with different acetonitrile compositions, a window

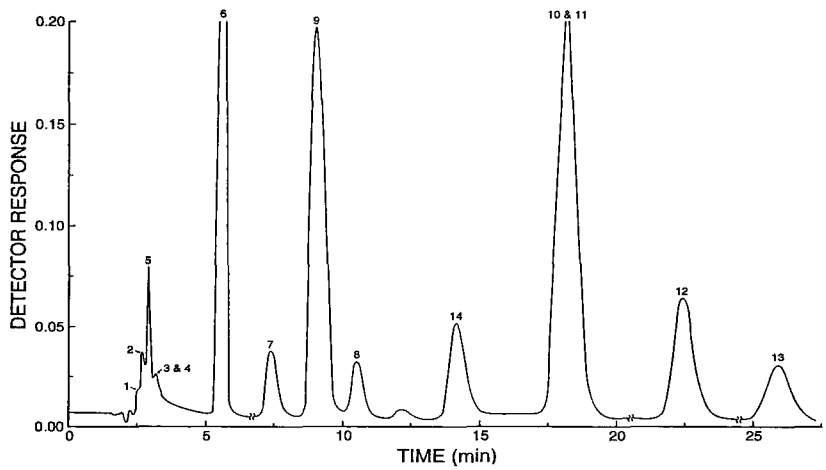


FIGURE 1. Chromatogram of fourteen metabolites of benzo[a]pyrene obtained with the optimum binary mobile phase MeOH:H_2O (81.75:18.25). The names and structures of the compounds are given in Table 1.

diagram was constructed for the ACN:H_2O mobile phase compositions. The optimum mobile phase obtained was ACN:H_2O (65:35). Figure 2 shows chromatogram for the fourteen different metabolites of benzo[a]pyrene separated with ACN:H_2O (65:35). Also, with ACN:H_2O (65:35), the compounds were separated in to four classes, namely, tetrols, dihydrodiols, diones and monohydroxy-B[a]P compounds (Figure 2). Separation of diones and monohydroxy-B[a]P has been confirmed by others using HPLC (10-12,32). The separation order for the monohydroxyl aromatic compounds in the mixture of fourteen metabolites with increasing retention time was in the following order: 12-OH-, 9-OH-, 2-OH-, 7-OH-, 3-OH- and 6-OH-B[a]P (Figure 2). Results for five of the hydroxylated B[a]P (12-OH-, 9-OH-, 2-OH-, 7-OH-, 3-OH-B[a]P), from this investigation, are identical to the results of Elnenaey and Schoor(14). Elnenaey and Schoor (14) used a gradient mobile phase system to separate 12

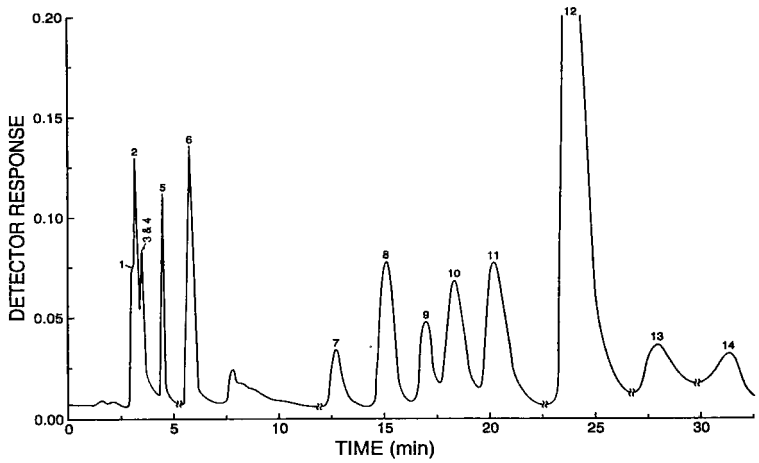


FIGURE 2. Chromatogram of fourteen metabolites of benzo[a]pyrene obtained with the optimum binary mobile phase ACN:H_2O (65:35). The names and structures of the compounds are given in Table 1.

isomeric monohydroxyl-B[a]P and concluded that the 6-OH-B[a]P may co-chromatographed with 9-OH-B[a]P. However, the results from our work indicated that 6-OH B[a]P is unstable, and it has to be used with in 2-3 days of preparation (33). Also, investigation of THF:H_2O (51.5:48.5) which has the same polarity (34) as ACN:H_2O (65:35), indicated several disadvantages of this mobile phase compared to the other binary mobile phases investigated. These include the decomposition of dihydrodiols and 6-OH-B[a]P, and short very retention times for overall separation of the fourteen metabolites.

In general, the optimum mobile phase, ACN:H_2O (65:35), resulted in elution of the complex mixture with the desired resolution between the most difficult to separate pairs, 2-OH-B[a]P and 9-OH-B[a]P. Comparison of Figure 1 and Figure 2 reveals that a much better separation including compound class separation and separation of six monohydroxyl isomers in a complex mixture of fourteen metabolites was achieved using the binary ACN:H_2O (65:35). However,

better baseline resolution was achieved with MeOH:H_2O (81.75:18.25) in the region of monohydroxylated metabolites (Figure 1). Comparison of the respective chromatograms for Figure 1 and Figure 2 shows that ACN:H_2O (65:35) resulted in an increase in the capacity factors (k') for 6-OH-B[a]P and 12-OH-B[a]P. This is most likely due to selective interactions of these two metabolites with acetonitrile in the mobile phase. The increase in the elution times for 6-OH-B[a]P and 12-OH-B[a]P resulted in a better class separation with ACN:H_2O (65:35) compared to MeOH:H_2O (81.75:18.25). Nevertheless, the k' values for the diones increased with ACN:H_2O and caused these compounds to elute near the hydroxyl aromatics (compare Figure 1 and Figure 2). However, ACN:H_2O (65:35) was a better mobile phase than MeOH:H_2O (81.75:18.25) for both compound class separation and for the separation of the fourteen metabolites.

Table 2 shows capacity factors for the fourteen metabolites of B[a]P obtained from the window diagram optimization method for the two different binary mobile phases investigated. The data show that most of these components are eluted in the respective mixtures within a k' range of approximately 1 and 15.61

Solubility Parameter Optimization For the Metabolites of B[a]P

With the binary mobile phases, band broadening was somewhat of a problem suggesting that a ternary mobile phase might provide lower k' values and sharper bands for the metabolites. Also, it was important to improve the separation of some of the pairs of the metabolites and increase resolution between the classes of metabolites such as diones and monohydroxyl-B[a]P metabolites. Therefore, the mobile phase optimization method based on solubility parameters which was developed by Schoenmakers and coworkers (26,27) was applied to the complex mixture of metabolites. Also, because of the results of Cooper and Hurtubise (17,18) for the separation of a mixture of twenty one monohydroxyl aromatic mixtures using a ternary mobile phase from the solubility parameter approach, it was concluded that this approach would be applicable for optimizing the separation of the metabolites on a C_{18} column.

TABLE 2

The k'-Values Obtained for the Metabolites of Benzo[a]pyrene with the Optimum Binary and Ternary Mobile Phases Using the Window Diagram and Solubility Parameter Methods

No.	Compound	k'-values		
		MeOH:ACN :H_2O (17:50:33)	MeOH:H_2O (81.75:18.25)	ACN:H_2O (65:35)
1	Tetrol I-1	0.31	0.53	0.43
2	Tetrol II-1	0.40	0.58	0.50
3	Tetrol I-2	0.47	0.79	0.53
4	Tetrol II-2	0.47	0.82	0.90
5	B[a]P-t-9,10-dihydrodiol	0.74	0.65	0.90
6	B[a]P-t-7,8-dihydrodiol	2.26	1.81	1.76
7	B[a]P-1,6-dione	6.49	2.39	5.51
8	B[a]P-3,6-dione	7.21	4.21	6.08
9	12-OH-B[a]P	9.76	3.56	7.00
10	9-OH-B[a]P	10.0	7.32	7.21
11	2-OH-B[a]P	11.2	7.37	7.64
12	7-OH-B[a]P	13.6	9.23	9.6
13	3-OH-B[a]P	14.8	10.8	10.8
14	6-OH-B[a]P	15.0	5.77	12.4

The binary mobile phase ACN:H_2O (65:35) obtained with the window diagram approach was used to calculate a composition of MeOH:H_2O that had the same polarity as ACN:H_2O. These two binary mobile phases were employed as initial mobile phases to obtain the optimum ternary mobile phase with the solubility parameter approach. After obtaining the initial binary mobile phases the procedure used for optimization of ternary mobile phases was as follows (17,18,34): a) A mobile phase selection diagram was constructed based on ln k' values obtained from ACN:H_2O (65:35) and MeOH:H_2O (83.2:16.8) versus the

composition of the ternary mobile phases of MeOH:ACN:H_2O (27); b) Using eqn. 2, the change in the criterion, πR_s, was calculated from the estimated ln k' values obtained from the mobile-phase selection diagram; c) The experimental values of ln k' were obtained from the first maximum πR_s value; d) The chromatographic data obtained from the first ternary mobile phase was used to construct a new mobile phase selection diagram, and steps c and d were repeated until no further improvement in the separation of metabolites occurred.

Using the solubility parameter approach discussed above, the optimum ternary mobile phase mixture obtained was MeOH:ACN:H_2O (17:50:33). Figure 3 shows the chromatogram obtained with the optimum ternary mobile phase. The chromatogram showed some improvements over the chromatogram obtained with MeOH:H_2O (81.75:18.25) in Figure 1. First, there was no overlap between the tetrols as a group and B[a]P-t-9,10-dihydrodiol. Second, the diones were separated from the hydroxyl aromatics, because of the migration of 12-OH-B[a]P with this optimum ternary (Figure 3). Also, class separation was in order of increasing retention time for tetrols, dihydrodiols, diones and monohydroxyl-B[a]P metabolites, without any overlap of compound classes (Figure 3). In addition, the improved separation of 2-OH-B[a]P and 9-OH-B[a]P was obtained compared to MeOH:H_2O (81.75:18.25) (Figure 1 and Figure 3). Comparison between the chromatograms in Figure 2 and in Figure 3 showed little improvement in the separation in the region of tetrols and dihydrodiols. However, the chromatogram in Figure 3 shows some definite improvements over the binary mobile phases in Figure 1 and Figure 2. The bands are sharper and the compound classes were well separated (Figure 3). Nevertheless, as shown in Figure 3, there was poor separation between 12-OH-B[a]P and 9-OH-B[a]P (Figure 3). In an attempt to further improve the resolution between 12-OH-B[a]P and 9-OH-B[a]P and the other metabolites, the experimental data from Figure 3 were used to obtain another ternary mobile phase using the solubility parameter approach. The composition of that mobile phase was MeOH:ACN:H_2O (11.8:55.8:32.4). However, the resulting chromatogram was clearly poorer than its predecessor, and there was a

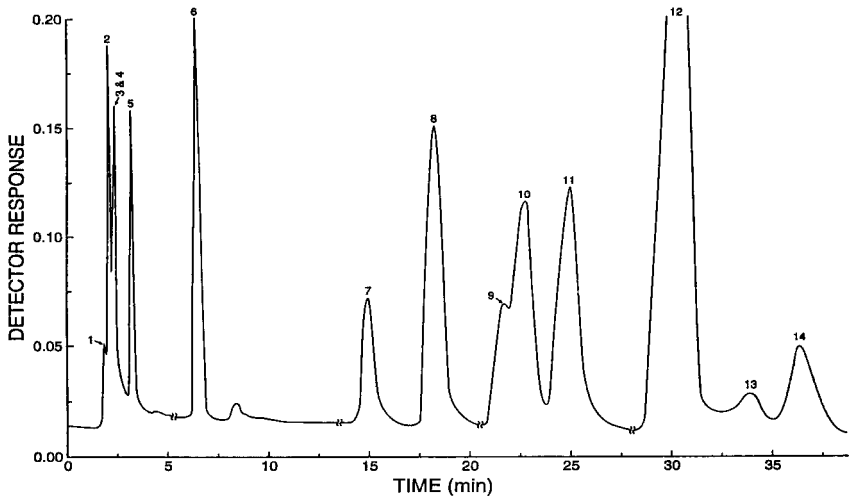


FIGURE 3. Chromatogram of fourteen metabolites of benzo[a]pyrene obtained with the optimum ternary mobile phase MeOH:ACN:H_2O (17:50:33). The names and structures of the compounds are given in Table 1.

decrease in the value of the πR_s criterion. Table 2 shows the k' values obtained for the optimum ternary mobile phase.

CONCLUSIONS

Undoubtedly, the ACN:H_2O (65:35) from the window-diagram optimization method gave a better overall separation for the hydroxyl-B[a]P compounds and also gave good compound-class separation. It was shown that the optimization scheme used for the ternary mobile phase resulted in a somewhat better overall resolution for at least 12 of the compounds and a better class separation for the mixture of metabolites (Figure 3) because each class of compound was separated by a greater range than with ACN:H_2O (65:35). However, ACN:H_2O (65:35) was

essentially as effective as the ternary mobile phase and gave a better separation of 9-OH-B[a]P and 12-OH-B[a]P. A desirable k' value range ($1<k'<15$) was obtained with both optimum binary mobile phases and the ternary MeOH:ACN:H_2O (17:50:33) (Table 2). The most difficult to separate adjacent peak pairs 2-OH-B[a]P and 9-OH-B[a]P, 6-OH-B[a]P and 12-OH-B[a]P and 7-OH-B[a]P and 3-OH-B[a]P can be resolved with both the optimum ACN:H_2O mobile phase and the ternary mobile phase. However, our results showed that complete baseline resolution with one specific optimum mobile phase was not possible for such a complex mixture of isomers under the conditions described.

ACKNOWLEDGEMENTS

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AN EVALUATION OF COLUMN-TO-COLUMN RETENTION VARIABILITY FOR COMPOUNDS RELATED TO PANADIPLON (U-78,875) USING THREE DIFFERENT REVERSE-PHASE HPLC SEPARATION MODES

J. P. SCHOLL
The Upjohn Company
Control Research and Development Laboratories
Kalamazoo, Michigan 49001

ABSTRACT

In order to assess the potential for substituting one brand of column for another in the analysis of multiple analytes, panadiplon (U-78,875) and eight of its related compounds were examined with respect to relative retention and peak shape on six different reversed-phase C8-bonded silica columns in the isocratic and gradient separation modes, and on six different bare silica columns in the dynamically modified silica (DMS) mode. No two columns in any separation mode showed identical retention behavior for all nine analytes. Gradient chromatography demonstrated generally more uniform relative retention for all columns than either the isocratic or the DMS mode, which is largely attributable to a reduction in time available for partitioning as a consequence of increasing solvent strength. DMS may offer improvements over isocratic reversed-phase HPLC in reproducibility for multiple analytes containing either alkyl homology or single, strongly interactive groups. In general, gradient chromatography provides the greatest immunity from column-to-column variability for analysis of multiple analytes.

INTRODUCTION

An issue of prime importance in the development of rugged HPLC methodology is the consistent performance of columns. The establishment of specificity for all compounds of interest entails not only the purity of peaks represented by those compounds, but an examination of multiple lots of HPLC columns and column packing material. During the lifetime of an HPLC method, changes in a manufacturer's column production methodology can have a serious impact on the performance of the method (relative retention, peak shape). This provides a great incentive to develop methods that are less dependent on column variability, as the column is the most significant component of an HPLC system that is not under a developer's control.

There are three commonly used strategies that have been employed to reduce column-to-column variability for multiple analytes. In the case of basic compounds, addition of small amounts of an amine modifier to the mobile phase can improve poor retention time reproducibility and peak shape due to analyte interaction with residual silica silanols[1-4]. Sadek and Carr[4] demonstrated that certain amines are much more efficient at covering residual silanols than others; improvement in analyte retention characteristics is also very analyte-dependent.

A second strategy that could be employed to obtain improved analyte retention behavior is dynamically modified silica (DMS). By adding a long-chain quaternary amine salt to an organo-aqueous mobile phase near neutral pH (see Reference 5), underivatized silica is dynamically coated. This coating is effective at shielding many of the partially ionized silanol sites on the silica surface, producing a hydrophobic layer similar to the bonded-phase chains of alkyl bonded silica. Several workers have investigated the effects of dynamic modifier identity and concentration[5-11], buffer pH[8-9,11], organic modifier identity and concentration[8-11], and stationary phase parameters[5] for DMS. Reference 12 reviews the use of DMS as a general technique for improving the reproducibility of column selectivity in RP-HPLC. It was anticipated that the use of dynamically modified silica

could also improve the fidelity of retention for compounds related to U-78,875.

For gradient HPLC, it is likely that the change in solvent strength during a chromatographic run might be effective in controlling the time of elution, and, indirectly, could mitigate differences in retention caused by differences in column chemistries. Since an increase in solvent strength "forces" the analyte from the column, the net effect might be a diminished dependence on column-specific retention mechanisms. This was the rationale for examining different bonded-phase packings in the gradient elution mode.

The objective of this work was to examine three different reversed-phase HPLC separation modes for impurities and degradation products of a developmental drug candidate (panadiplon, U-78,875) to determine which, if any, is less dependent upon variation of the chromatographic stationary phase. The modes examined were: 1) isocratic; 2) dynamically modified silica (DMS); and 3) gradient.

MATERIALS

Instrumentation

The chromatographic equipment consisted of a Hewlett-Packard 1090L HPLC system, configured with a Waters WAVS® Box to switch 3 columns during one run. An external LDC UVMonitor D detector with cadmium lamp and 229 nm filter was used. Mixtures of U-78,875 and eight of its related compounds (either two or three individual compounds per vial; Figure 1) were prepared in concentrations sufficient to give peak responses of at least 0.1 AU, in either mobile phase (isocratic or DMS) or dimethylformamide (gradient).

Columns

For the isocratic and gradient separation modes, the same C8-bonded, 250 X 4.6 mm i.d columns were compared. These were: Zorbax R_x-C8 (serial

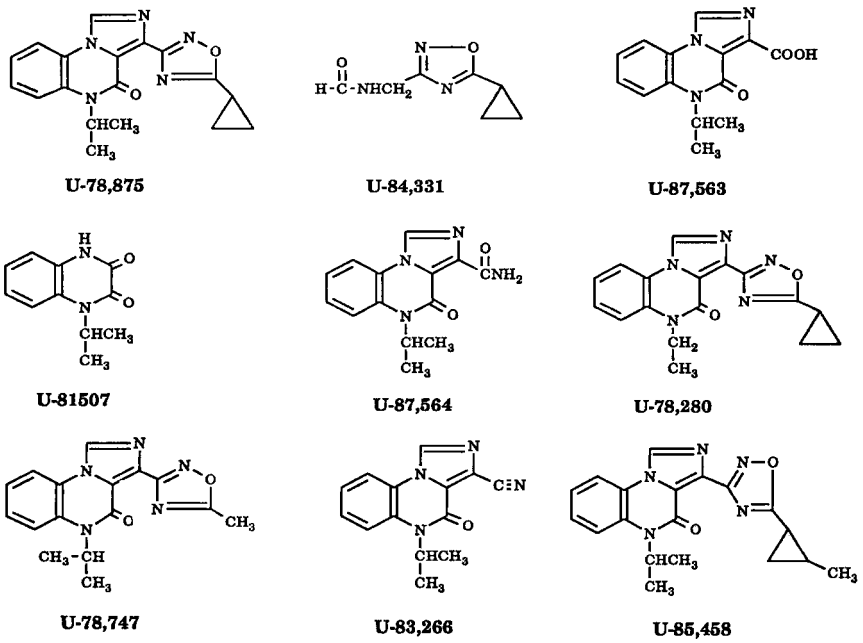

Figure 1. Structures of Panadiplon (U-78,875) and related compounds.

number AU2932); YMC A-203-5 C8 (s/n 4252672); Spherisorb C8 (s/n C2478); Zorbax C8 (s/n L15786); Nucleosil C8 (s/n 051789R-P); and Jones Apex C8 (s/n 4M25325F/38703). For the DMS mode, the columns compared (250 X 4.6 mm i.d.) were: Zorbax SIL (s/n B6898); YMC Silica (s/n 4255489); Spherisorb Silica (s/n 07240KP); Nucleosil Silica (s/n 07240MP); Jones Apex Silica (s/n 4M25300/44152); and Lichrosorb Silica (s/n 070985-C). All columns were packed with 5 micron particle size, silica-based material. Table 1 gives pore size, surface area, and carbon load (where applicable) for the column types used. In all cases, the same batch of mobile phase was used for comparison of six columns under each separation mode, and duplicate injections of compound solutions were made.

TABLE 1
Characteristics of 5 Micron Bare and C8-Bonded Silicas Used in this Work (from Manufacturers' Literature). All Columns were 250 mm long X 4.6 mm i.d.

Column Type	Pore Size (Å)	Surface Area (m^2/g)	Carbon Load (%)
Zorbax Rx-C8	80	180	5.5
Zorbax C8	70	300	12
Zorbax SIL	70	300	--
YMC C8	120	*	10
YMC Silica	120	290	--
Spherisorb C8	80	220	6
Spherisorb Silica	80	220	--
Nucleosil C8	100	350	9
Nucleosil Silica	100	350	--
Jones Apex C8	100	170	7
Jones Apex Silica	100	170	--
Lichrosorb Silica	60	550	--

*Surface Area for Bonded-Phase material not given

METHODS

Isocratic

For comparison of columns in the isocratic mode, the mobile phase was prepared by mixing 350 ml of acetonitrile (Baxter Burdick and Jackson) and 650 ml of DI water, adding 2.0 ml of N,N-dimethyloctylamine (DMOA; Ames Laboratories) and 20 mg of 1,4,8,11-tetraazacyclotetradecane (CYCLAM; Aldrich Chemical). While stirring magnetically, the pH to is adjusted to 7.5 $\pm$ 0.1 with 85% phosphoric acid. The flowrate was 1.0 ml/minute and injection volume 10 µl for the isocratic system.

Dynamically Modified Silica

For the DMS system, the mobile phase consisted of 100 mM KH_2PO_4 (Mallinckrodt), pH 6.5 buffer:Acetonitrile:n-Butanol (Baxter Burdick and

Jackson), 830:140:30, containing 10 mM Dodecyltrimethylammonium Bromide (Aldrich, 99%). A flowrate of 1.0 ml/minute was used with the DMS system, with 20 µl injections.

Gradient

Gradient mobile phase A consisted of 950 ml of water, 50 ml acetonitrile, and 20 mg CYCLAM, with pH adjusted to 7.0 with a dilute (1:20) aqueous solution of phosphoric acid, while mobile phase B consisted of 950 ml of acetonitrile and 50 ml of water. The gradient method injection volume was 20 µl and flowrate 0.8 ml/minute, with the following program:

Time (min.)	%A	%B
0.0	95	5 (isocratic)
4.5	95	5 (begin initial gradient)
11.5	65	35 (begin shallow gradient)
47.5	50	50 (begin final gradient)
59.5	5	95 (isocratic)
64.5	5	95 (begin down ramp)
66.5	95	5 (begin equilibration)
76.5	95	5 (isocratic equilibration)

Data Collection

Void time was deemed to be the first full inflection (maximum or minimum) of the baseline. Peak integration, relative retention, and tailing factor were calculated using the in-house software developed by Upjohn Control; USP definitions were used.

RESULTS AND DISCUSSION

Isocratic Chromatography

Figure 2 shows the retention behavior of these compounds on the six C-8 bonded phase columns investigated, using isocratic conditions. It is apparent that there is little similarity in retention behavior for all nine

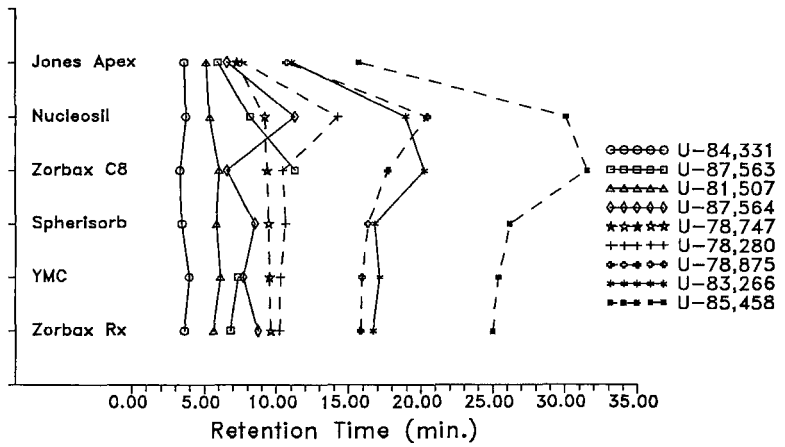

Figure 2. Isocratic retention times for six columns.

compounds for the column set as a whole; only the Zorbax Rx, YMC, and Jones materials maintain the same elution order. There is a total of nine retention order reversals within this set of columns; U-87,563 (a carboxylic acid) did not give a well-formed peak on the Spherisorb column. The variability (percent RSD for retention relative to U-78,875) of retention ranges from 7.5% for U-85,458 (most retained) to 48.4% for U-84,331 (least retained); these values lie in the same approximate range as observed by Hansen et al.[13] for comparison of bonded-phase columns. Peak shape (Table 2) was acceptable for most compounds except those having either strong hydrogen-bonding character (the primary amide U-87,564) or ionic character (carboxylic acid U-87,563, and enolizable amide U-81,507). These three analytes could be expected to chromatograph poorly for some column packings, at the relative high pH (7.5) of this mobile phase, due to a partial ion exchange retention mechanism on unshielded silanols.

Dynamically Modified Silica

Figure 3 shows the relative retention variability and asymmetry for DMS chromatography. The overall precision in relative retention for these

TABLE 2
Asymmetry Expressed as USP Tail Factor for U-78,875 and Eight Related Compounds Using Three Different Reverse-Phase Separation Modes

COMPOUND	Isocratic		DMS		Gradient	
	range	mean	range	mean	range	mean
U-78,875	1.1-1.3	1.2	1.0-1.5	1.3	0.9-1.5	1.2
U-84,331	0.8-2.1	1.6	1.2-1.4	1.3	0.9-1.5	1.3
U-81,507	1.6-6.1	3.3	1.2-1.7	1.4	1.2-4.5	2.3
U-87,563	1.7-4.0*	2.5	1.2-2.8	2.0	0.7-3.8	1.7
U-87,564	1.4-5.0	2.3	0.9-1.4	1.1	1.2-2.0*	1.6
U-78,747	1.2-3.2	1.6	1.1-1.4	1.2	0.9-1.7	1.4
U-78,280	1.1-1.4	1.3	0.9-1.3	1.1	0.9-1.8	1.3
U-83,266	0.8-1.3	1.1	0.9-1.4	1.2	0.8-1.2	1.0
U-85,458	1.0-1.2	1.1	1.0-1.7	1.3	1.0-1.6	1.3

*(n=5) - No data for Spherisorb due to poor peak shape

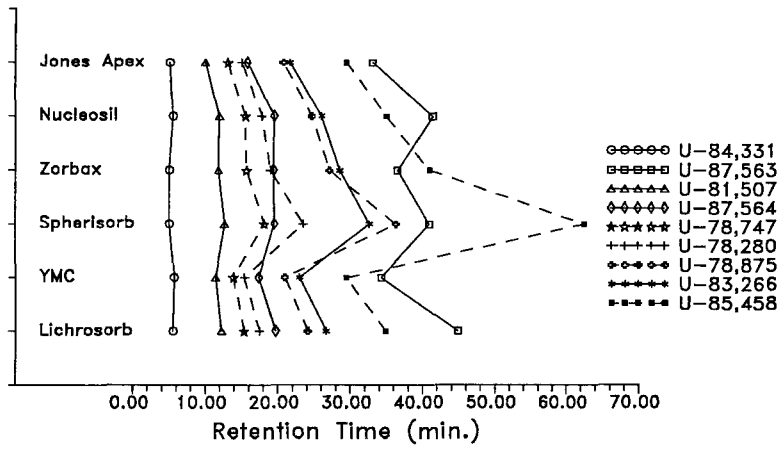

Figure 3. DMS retention times for six columns.

compounds was somewhat better in this separation mode than with bonded-phase chromatography, but there are six retention order reversals: four for the Spherisorb silica; and for both the Zorbax and Spherisorb silicas in the case of the acidic U-87,563.

Hansen et al[13] used DMS to show much improved peak symmetry and a much improved fidelity of relative retention for eleven tricyclic amine drugs. In that study, the percent RSD for retention of ten such compounds (relative to imipramine) was uniformly less than ten percent, across an eleven-column set of silica materials. While all of the compounds of the present work contain nitrogen, none of them are expected to be cationizable, as were the nitrogens of the compounds compared in Reference 13. While it is difficult to rationalize the difference in DMS retention behavior between the tricyclic amine drugs and U-78,875-related compounds, one hypothesis might be that the ionizable nitrogens confer an electron-rich center on the tricyclic compounds that is not present (at least, to the same degree) in U-78,875-related compounds. If these centers allow the analytes to partition in a manner different from U-78,875-related compounds, one might explain

the difference in column-to-column retention variability between Reference 13 and the present work.

The one aspect of chromatography that the DMS separation mode controlled quite well for these compounds was peak shape. All 9 compounds provided peaks with USP tailing factors of at most 2.8 (shown by U-87,563 on Spherisorb silica); likewise, there was no instance of peak shape so poor that a retention time was not assignable, as was the case for the other two separation modes examined. The improvement in peak shape for DMS confirms the observations of Hansen et al[5,12].

Gradient Chromatography

Figure 4 shows that gradient elution, just as the other two elution modes studied, does not prevent retention order reversals; however, it reduces the number of reversals to a minimum of four (without the information of amide U-87,564 retention, which gave no recognizable peak in this mode for the Spherisorb column). It also reduces the variability in retention across all columns to a level below that observed for either of the other two separation modes. U-87,563 was the only compound investigated for which the percentage RSD for retention relative to U-78,875 was greater than 10%. As this compound is a carboxylic acid (expected to be ionized at the pH of the mobile phase), there is the possibility of mixed retention mechanisms (ionic as well as hydrophobic).

Comparison of the Three Separation Modes

The dashed lines in Figures 2-4 show the subset of compounds differing only in aliphatic substitution. These seem to "track" very well across the column set for DMS mode (Figure 3), and the percentages RSD for retention relative to U-78,875 for these compounds (U-78,747, U-78,280, and U-85,458) are all below 10%. This suggests that alkyl homology does little to change the retention mechanism, in this separation mode. There are undoubtedly differences in adsorption capacity of the silicas for long-chain quaternary compound[14], which is a likely determinant of the general

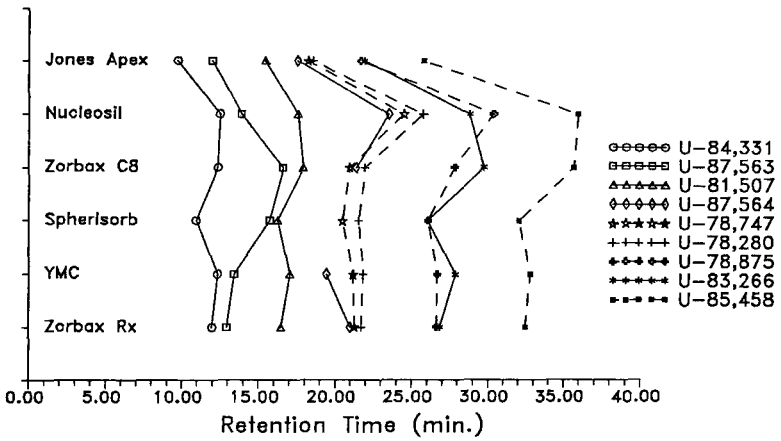

Figure 4. Gradient retention times for six columns.

differences in retention of the alkyl homologues across the column set. The "tracking" of retention for the alkyl homologues across the six columns is not so strong for bonded phase chromatography (Figures 2 and 4) as for DMS (Figure 3), possibly due to differences in the nature of hydrophobic interaction of the alkyl homologues with alkyl chains on the C8-bonded stationary phases. That the alkyl homologues retain more predictably in the DMS mode than in the C8-bonded modes suggests that greater control of hydrophobic aspects of retention may be possible for DMS chromatography than for C8-bonded chromatography. These bonded phases are known to have a variety of carbon loads; overall geometric differences between them are likely.

The better precision of relative retention for gradient chromatography than for the other two separation modes is probably due largely to "compression" of the time available for partitioning as the mobile phase strength is raised. Figure 5 plots the variation in retention relative to U-78,875 on each set of six columns. There is less variation in the case of each compound in the gradient mode than in the DMS mode, while the DMS

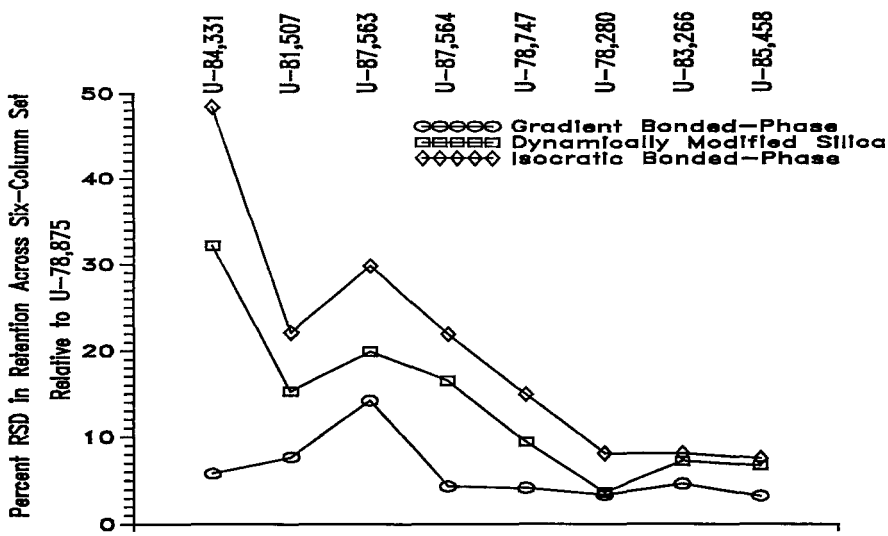

Figure 5. Variability of retention for eight compounds relative to U-78,875 in three different HPLC separation modes.

mode is more uniform in relative retention than the bonded-phase isocratic mode. The reduced variation between gradient and isocratic chromatography should be attributable in part to the influence of increasing elution strength in mitigating bonded-phase packing differences. The variation in DMS isocratic chromatography is intermediate between the bonded-phase isocratic and bonded-phase gradient modes, which suggests that differences in silica are still very important.

There is only a slight general improvement in peak shape for gradient chromatography over that observed isocratically, most likely also due to the "compression" effect of a gradient. While the DMS mobile phase pH was somewhat lower than that of the isocratic mobile phase pH (6.5, rather than 7.5) much better symmetry was observed for the hydrogen-bonding U-87,564 and acidic U-81,507 and U-87,563 in the dynamically modified silica mode than either of the bonded-phase modes.

CONCLUSIONS

An investigation of the elution behavior of U-78,875 and eight related compounds with isocratic and gradient reversed-phase, and dynamically modified silica HPLC has shown that none of these separation modes show identical retention behavior for all compounds investigated on any pair of the six packings investigated for any mode. The spectrum of compounds that one might encounter in an arbitrary impurities or metabolite mixture for any drug substance will probably have a wide range of acidity and/or oxidation state, and will be chemically more heterogeneous than the majority of compounds investigated for DMS in previous studies[5-12]. DMS appears to be most useful for standardization of separations where only alkyl homologues are examined; or for mixtures of compounds exhibiting single, strong retention mechanisms.

Gradient chromatography did not prevent retention order reversals for the present study, but it reduced the overall variability between packings due to its ability to control the time of elution via increasing solvent strength, and may be an improvement over either isocratic reversed phase or DMS, for an arbitrarily chosen compound mixture. If resolution of multiple components is not critical (as in single-analyte assays), it may be possible to replace any of a number of columns for the intended column, if the assay has been designed to be sufficiently rugged. This work suggests that, for resolution of multiple analytes of varying structure, gradient chromatography promises greater immunity from unforeseen changes in HPLC column production methodology than either isocratic reversed-phase or dynamically modified silica chromatography.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ISOLATION, SPECTROSCOPIC CHARACTERIZATION, AND IMMUNOSUPPRESSIVE ACTIVITIES OF TWO RAPAMYCIN DEGRADATION PRODUCTS*

C. PAUL WANG**, KELVIN W. CHAN, ROBERT A. SCHIKSNIS, JOANN SCATINA, AND SAMUEL F. SISENWINE
Division of Drug Metabolism
Wyeth-Ayerst Research
CN 8000
Princeton, New Jersey 08543

ABSTRACT

A high performance liquid chromatographic method has been developed for the isolation of two degradation products of rapamycin which is currently under development as an immunosuppressive agent. Prior to isolation, the drug was incubated at 37°C in rat bile or ammonium acetate (pH 8.0). The isolation was achieved by a Supelco, PLC-18 21.2 x 250 mm, 18 μm column using methanol/ammonium acetate gradient mobile phase. After evaporation of methanol, the remaining eluates were lyophilized. The isolated degradation products were characterized by negative ion fast atom bombardment mass spectrometry (FAB MS) and proton nuclear magnetic resonance spectroscopy (^{1}H NMR). Degradation product A was found to be a macrolide ring-opened hydrolysis product of rapamycin where the C25 ester bond had been hydrolyzed. Degradation product B was determined to be a ring-opened isomer of rapamycin. B had less than 4% of the potency of rapamycin in a thymocyte proliferation assay, while A had minimal activity at concentrations tested.

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**To whom all correspondence should be addressed.

INTRODUCTION

Rapamycin, [3S-[3R*[S*(1R*,3S*,4S*)],6S*,7E,9S*,10S*,12S*,14R*,15E,17E, 19E,21R*,23R*,26S*,27S*,34aR*]]-9,10,12,13,14,21,22,23,24,25,26,27,32,33,34a-Hexadecahydro-9,27-dihydroxy-3-[2-(4-hydroxy-3-methoxycyclohexyl)-1-methylethyl]-10,21-dimethoxy-6,8,12,14,20,26-hexamethyl-23,27-epoxy-3H-pyrido[2,1-c] [1,4]oxaazacyclohentriacontine-1,5,11,28,29 (4H,6H,31H)-pentone, (Figure 1), an antitumor and antifungal agent isolated from the fungus *Streptomyces hygroscopicus*(1,2), has been found to have potent immunosuppressive activity while exhibiting little toxicity in primates(3,4). The drug is currently under clinical trials as an immunosuppressive agent.

Rapamycin is chemically unstable; under basic or acidic conditions, it degrades via β-elimination to form a ring-opened isomer of rapamycin. The drug is also unstable in biological fluids; extensive degradation was observed in rat serum samples even after storage at -20°C for 18 days. Therefore, prior to pursuing the isolation of biliary metabolites of rapamycin in rats, the drug was incubated at 37°C in rat bile. Two major rapamycin degradation products, designated A and B, were observed. To further verify that the degradation process is not induced or catalyzed by endogenous materials and to facilitate the isolation of large quantities of the products, we have also incubated rapamycin in an ammonium acetate solution of a similar pH to bile (pH 8.0).

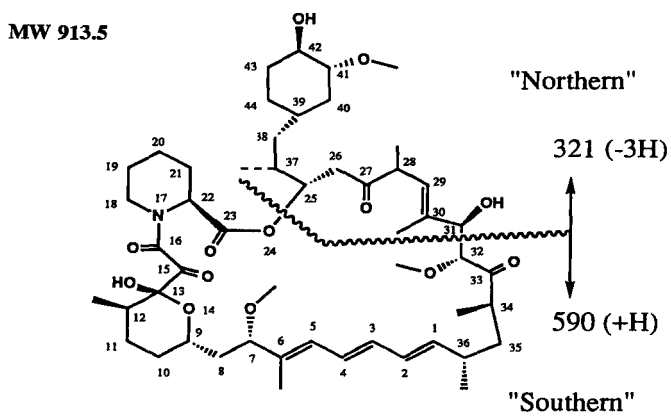

FIGURE 1 Chemical Structure of Rapamycin and its Major Fragmentation Pathway

We hereby report the isolation, structural characterization and immunosuppressive activity of the major degradation products of rapamycin after incubation of rapamycin at 37°C in rat bile or ammonium acetate (pH 8.0).

EXPERIMENTAL

Materials

Rapamycin was obtained from Wyeth-Ayerst Research, Rouses Point, NY. Ammonium acetate (HPLC grade) and ammonium hydroxide were obtained from J.T. Baker (Phillipsburg, NJ). All solvents used in the study were HPLC grade. Control rat bile was obtained in-house (Drug Metabolism Division, Wyeth-Ayerst Research, Princeton, NJ).

Instrumentation

Two HPLC systems were used in the isolation. One consists of a LDC Analytical, Model CM 4000 pump, a Waters WISP Model 710B autosampler (Waters Associate, Milford, MA), and a LDC Analytical SpectroMonitor 5000, photodiode array detector. The second system is composed of a Waters 600E system controller and pump, a Waters 490E programmable multiwavelength detector, a Waters U-6K manual injector and a Hewlett-Packard 3390A integrator. Isolation of degradation products was achieved using Supelcosil LC-18, 5 μm, 4.6 x 250 mm column (Supelco, Bellefonte, PA) and Supelcosil PLC-18, 18 μm, 21.2 x 250 mm column. The nuclear magnetic resonance spectrometers used were Bruker AM-400 (Billerica, MA) and Varian VXR-400 (Palo Alto, CA). A MS 50 mass spectrometer equipped with a FAB ion source operated in the negative ion mode (Kratos Analytical, Ramsey, NJ) was used.

Preliminary isolation of compounds A and B from *in vitro* incubation of rapamycin at 37°C in rat bile

Rapamycin (50 μg in 20 μl methanol) was added to control rat bile per 1.0 ml bile or 500 μg in 40 μl methanol per 1.25 ml bile. The samples were vortexed and incubated at 37°C in a shaking water bath for up to 15 hr.

The samples were then injected directly onto a Supelco LC-18, 25 cm x 4.6 mm, 5 μm column to evaluate degradation. Separation was achieved with a linear gradient from 65% to

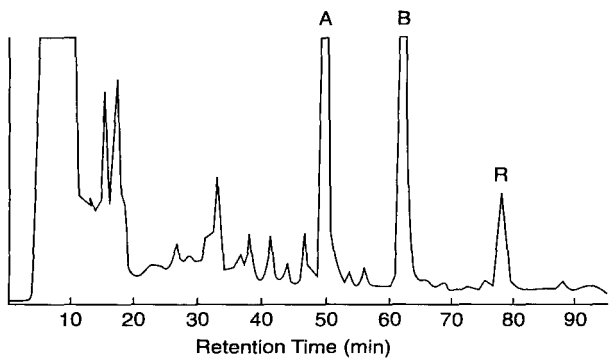

FIGURE 2 HPLC (Supelco LC-18, 25 cm x 4.6 mm, 5 μm) Chromatogram of Rapamycin Incubated with Rat Bile for 15 hr (A: Peak A, B: Peak B, R: Rapamycin)

86% methanol in 0.05M ammonium acetate over 80 min at a flow rate of 0.5 ml/min. Detection was by monitoring UV absorbance at 276 nm. Two major degradation peaks were observed in the chromatogram (Figure 2). The peaks were designated as compound A (retention time 49.2 min.) and compound B (retention time 61.5 min.). The eluates containing compounds A and B were collected separately from multiple injections. The collections were dried in a Savant AS-160 concentrator at room temperature in the manual mode.

Isolation of compounds A and B after incubation of rapamycin at 37°C in rat bile

To isolate sufficient quantities of compounds A and B, 30 tubes that each contained 0.9 mg rapamycin in 50 μl ethanol and 2.0 ml control rat bile were prepared. The tubes were incubated in a 37°C water bath for 13.5 hr and then stored at -80°C until analysis.

Isolation of compounds A and B was performed with a preparative Supelco PLC-18, 25 cm x 21.2 mm, 18 μm column. The mobile phase gradient profile is described in Table 1. Detection was by UV absorbance at 276 nm. The injection volume was 2 ml of the biliary sample. As shown in Figure 3, the retention times of compounds A and B and rapamycin were 31.1, 39.4, and 60.6 min., respectively. The HPLC eluates containing compounds A and B were collected into 20 ml scintillation vials, and the purity of the collected eluates was analyzed by the analytical HPLC system described above. Methanol was then removed from the eluates with the Savant AS-160 at room temperature in the manual mode. The remaining aqueous residues were pooled and then lyophilized. The material was redissolved and purified again as described in this section.

TABLE 1

Gradient System used in the Supelco PLC-18, 250 x 21.2 mm, 18 μm Column for the Isolation of Rapamycin Degradation Products[a]

Time (min.)	Flow rate (ml/min.)	Percent methanol	Percent 0.05M ammonium acetate
0.0	4.0	77	23
45	4.0	86	14
50	4.0	95	5
60	4.0	95	5
65	4.0	77	23

[a]Linear gradient was used between each time point.

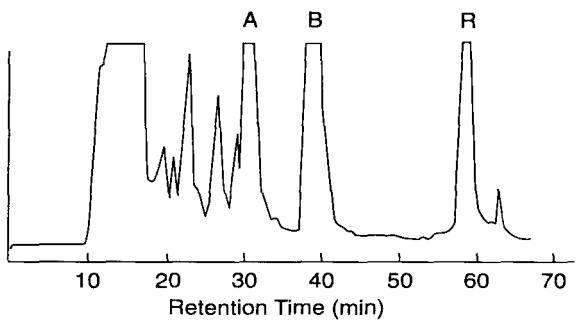

FIGURE 3 HPLC (Supelco PLC-18, 25 cm x 21.2 mm, 18 μm) Chromatogram of Rapamycin Incubated with Rat Bile for 13.5 hr (A: Peak A, B: Peak B, R: Rapamycin)

Isolation of compounds A and B after incubation of rapamycin in 0.1M ammonium acetate (pH 8.0)

Rapamycin (150 mg) was dissolved in 15 ml ethanol, which was added to 300 ml of 0.1M ammonium acetate (pH 8.0 with ammonium hydroxide). The mixture was incubated in a 37°C water bath for 24 hr. The suspension was filtered while it was still warm, and the filtrate was lyophilized to dryness. The dried white powder was redissolved in methanol/water (65/35). Isolation of compounds A and B was performed on the preparative system as described above (Figure 4). After removal of methanol using the Savant concentrator, the aqueous solution was pooled and lyophilized.

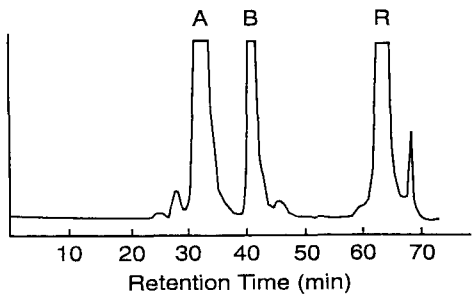

FIGURE 4 HPLC (Supelco PLC-18, 25 cm x 21.2 mm, 18 μm) Chromatogram of Rapamycin Incubated with Ammonium Acetate Solution (pH 8.0) (A: Peak A, B: Peak B, R: Rapamycin)

The thymocyte proliferation assay

The isolated rapamycin degradation products A and B were subjected to an *in vitro* immunosuppressive activity test. This thymocyte proliferation assay was conducted in-house at Wyeth-Ayerst Research, Princeton, NJ.

Spectroscopic characterization of compounds A and B

All FAB mass spectra were obtained in the negative ion mode. The primary beam was 1 mA of 7 kV xenon atoms. Resolution of the instrument was adjusted to 2000 (10% valley). Triethanolamine was employed as the matrix for the samples. Each sample was dissolved in methylene chloride, and an aliquot of the solution was mixed with the matrix on a copper probe tip before insertion into the mass spectrometer for analysis. High resolution measurements of the molecular ion species were carried out by peak matching with reference ions from cesium iodide at a resolution of 10,000. All ^{1}H-NMR spectra were obtained from samples dissolved in alumina neutralized, deuterated chloroform.

RESULTS

Immunosuppressive activity of A and B by thymocyte proliferation assay

When compared to the IC50 of rapamycin, the immunosuppressive activity of B is less than 4% of that of rapamycin. Product A appears to be inactive.

Structural elucidation of compound A

The mass spectra of A isolated from *in vitro* incubation of rapamycin in rat bile or 0.1M NH_4OAc are essentially the same. The exact mass of the molecular ion species at m/z 930 was found to be 930.5595 by high resolution MS. This mass is consistent with an elemental composition of $C_{51}H_{80}NO_{14}$ (theoretical mass = 930.5578) and suggests that the ion at m/z 930 is the $[M-H]^-$ of a product in which one mole of H_2O has been added to rapamycin. The observation of the $[M-H]^-$ suggests that the macrolide ring is opened and the fragment ions at m/z 339 and 590 show that hydrolysis has occurred in the northern portion of rapamycin (Figure 1). Therefore, formation of A is likely due to hydrolysis of the C_{25} ester bond (Figure 5).

In the ^1H-NMR spectra of A, the H-25 resonance, which occurs at 5.15 ppm for rapamycin, is observed at 3.85 ppm. This chemical shift is similar to those observed for H-25 in spectra of hydrolyzed rapamycin analogs containing C25-OH. Upon dissolution of A in $CDCl_3$, no evidence of a C_{25}=C_{26} alkene group is observed. A has been nomenclatured as 1-{[2R-Hydroxy-6S-[22-(4R-hydroxy-3R-methoxy-1S-cyclohexyl)-14R,20-dihydroxy-2S,13R-dimethoxy-3, 9S,11R,15,17R,21R-hexamethyl-12,18-dioxo-docosa-3E,5E,7E,15E-tetraenyl]-3R-methyl-tetrahydro-pyran-2-yl]-oxo-acetyl}-piperidin-2S-carboxylic acid.

Structural elucidation of compound B

The negative ion FAB mass spectra of B isolated after *in vitro* incubation of rapamycin in rat bile or ammonium acetate solution were essentially identical. The mass spectrum of B is very similar to that of rapamycin with the exception of the molecular ion species being detected at m/z 912 instead of 913. The exact mass of this ion determined by high resolution MS was 912.5513, which is consistent with an elemental composition of $C_{51}H_{78}NO_{13}$ (i.e. one hydrogen less than rapamycin, theoretical mass = 912.5473). These data suggest that B is an isomer of rapamycin in its macrolide ring opened form (Figure 6) because the free carboxylic acid in ring opened rapamycin can favor the formation of $[M-H]^-$ and generate the carboxylate anion more easily than rapamycin.

Similarly, the ^1H-NMR spectra of B obtained from rat bile and 0.1M NH_4OAc solution incubations match, indicating that B isolated from the two sources is the same. The ^1H-NMR spectra of all B materials examined by NMR contain the H-25 double-doublet resonance at 6.75 ppm which is diagnostic for the presence of a C_{26}=C_{25} alkene functionality and characteristic of this ring opened form of rapamycin. The nomenclature of B is 23,25-Deepoxy-25,26-didehydro-23-hydroxyrapamycin.

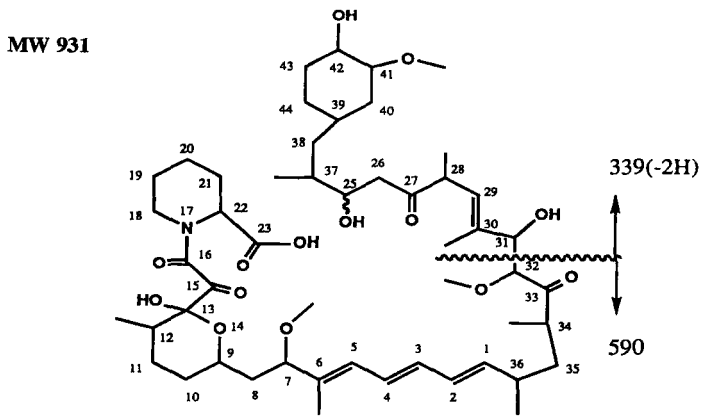

FIGURE 5 Chemical Structure of Degradation Product A and its Major Fragmentation Pathway

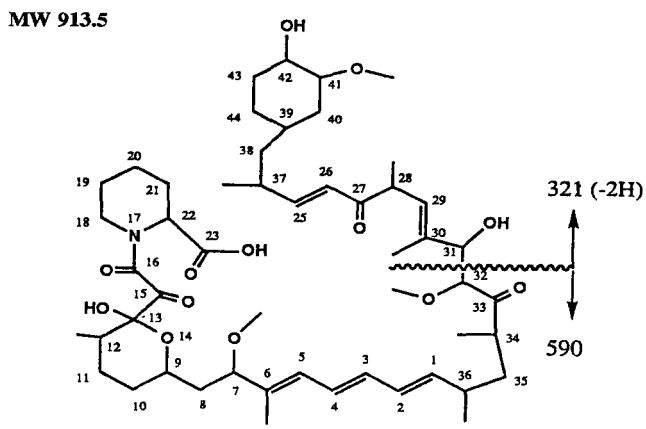

FIGURE 6 Chemical Structure of Degradation Product B and its Major Fragmentation Pathway

DISCUSSION

An analytical and a preparative HPLC procedures for the isolation of rapamycin degradation products A and B have been developed. Using ammonium acetate in the mobile phase is a critical factor to achieve satisfactory resolution and isolation of the degradation products. Since rapamycin degradation products are unstable, the sample handling procedure described earlier is critical for the successful isolation of the degradation products. A and B are generated by *in vitro* incubation of rapamycin with rat bile at 37°C for 13 hr. This degradation process is not necessarily catalyzed by endogenous materials because it also occurs in 0.1M NH_4OAc to form the same products. However, the endogenous compounds appear to affect the degradation of rapamycin since there is a difference in the relative peak areas of A and B when rapamycin is incubated in rat bile and 0.1M NH_4OAc (pH 8.0) (Figures 3 - 4).

The results described in this report show that the degradation products formed in either matrix are the same; they have been identified by HPLC, ^1H-NMR, and MS. Compound B was determined by MS and NMR to be a macrolide ring-opened isomer of rapamycin (Figure 6). Compound A has been characterized by MS and NMR to be a ring-opened hydrolysis product of rapamycin in which the C_{25} ester bond has been hydrolyzed (Figure 5). Both A and B are unstable to air, acid and base so that samples are best stored under nitrogen and kept at -80°C.

The immunosuppressive activities of A and B have been measured by the thymocyte proliferation assay; the potency of the degradation products was found to be less than 4% of that of rapamycin. Therefore, unless they are present *in vivo* at much higher concentrations than is rapamycin, they do not contribute significantly to the immunosuppressive action of rapamycin.

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INVESTIGATION OF RETENTION BEHAVIOR FOR RACEMATE DRUGS ON AVIDIN- AND MODIFIED AVIDIN-COLUMN

Y. ODA*, N. MANO, N. ASAKAWA, Y. YOSHIDA, AND T. SATO
Eisai Co. Ltd.
Tsukuba Research Laboratories
Tokodai 5-1-3
Tsukuba, Ibaraki 300-26, Japan

ABSTRACT

The binding properties of a native avidin were investigated by recording the fluorescence spectra of the native avidin, and by measuring the avidin-drug binding and the hydrophobicty of drugs. Some emission intensities were diminished by mixing avidin and drugs solutions compared with the native avidin solution. Also, the Scatchard plot analysis were performed to evaluate the avidin-drug binding. Then, modified avidin columns were prepared by acylation of amino groups and carboxyl groups to examine the retention and enantioselective properties of drugs. As a result, these functional groups contributed to chiral recognition of the avidin column for some drugs. Based on the results in this study, the avidin column have multiple binding sites for chiral separation.

INTRODUCTION

High-performance liquid chromatography (HPLC) has been usually considered as a tool for the evaluation of drug enantiomers, and many chiral stationary phases (CSPs) have been developed. While proteins are complex and high molecular weight polymers composed of L-amino acids, therefore

some protein- drug interactions can reflect the enantioselectivity of the drug. Protein-conjugated CSPs such as the albumin [1-3], α_1-acid glycoprotein [4], ovomucoid [5] and flavoprotein [6] have been applicable to the enantiomeric separation in a wide range of drugs. These columns are used under reversed-phase chromatographic conditions, which are suitable to analyze drug enantiomers in plasma [7]. We have previously developed an avidin-conjugated column which can allow to separate some drug enantiomers in plasma with direct injection method [8-10]. The retention and enantioselectivity on the avidin column were greatly affected by changing the mobile phase composition [11]. However, a native avidin has been little studied for the binding of drugs. In order to characterize the binding sites of the avidin column, some studies were performed.

EXPERIMENTAL

Apparatus

A chromatograph was set up with a Shimadzu model LC-9A system {two LC-9A pumps, a SIL-6B auto injector, a SCL-6B system controller, a SPD-6A UV monitor (254nm) and a C-R4AX integrating recorder}. Fluorescence spectra were measured with a Hitachi model F-2000 fluorescence spectrophotometer (excitation wave length 280 nm). A Shimadzu model CPR-005 (3000 rpm) as a centrifuge was used.

Materials

Native avidin was purified from chicken egg white according to the previously described method [12]. (±)-Trihexyphenidyl hydrochloride (TP), (±)-Cloperastine hydrochloride (CP) and solvents of HPLC grade were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). (±)-2-Phenylpropionic acid (PP) from Aldrich Chem. Co. (Milwaukee, WI, USA), (±)-Ibuprofen (IP) from Sigma chemical Co. (St. Louis, MO, USA) and (±)-chlormezanone (CM) from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) were used. Dissuccinimidyl suberate, sulfosuccinimidyl acetate and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride were purchased from Pierce (Rockford, IL, USA). All other chemicals were of analytical reagent grade. Centrifree micropartition systems (MPS-3) for ultrafiltration were

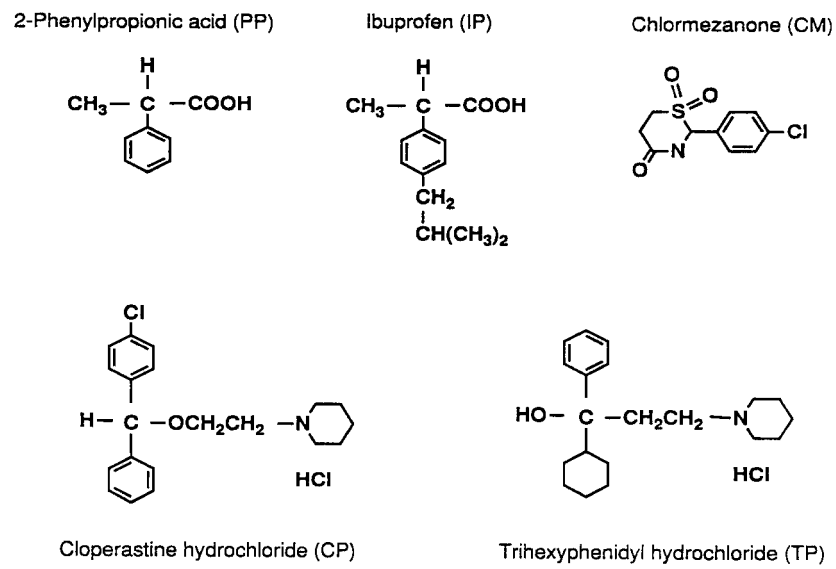

Fig.1 Structures of 2-phenylpropionic acid, ibuprofen, trihexyphenidyl hydrochloride, cloperastine hydrochloride and chlormezanone.

obtained from Amicon Inc. (Beverly, MA, USA). An ODS column used was Inertsil ODS-2 (5 μm, 15cm x 4.6mm i.d.) made by GL sciences Inc. (Tokyo). The structures of PP, IP, CM, CP and TP were shown in Fig.1.

Avidin column preparation

Nucleosil NH_2 (5μm, 2g) and dissuccimidyl suberate (2g) were allowed to react overnight in 30 ml of acetonitorile at room temperature. After the activated silica gel had been washed with 60 ml of acetonitorile and then suspended in 30 ml of 0.1 M sodium hydrogen carbonate aqueous solution with 1g of native avidin, this mixture was stirred for 2h at room temperature . Avidin-conjugated silica gel was packed into 15cm x 4.6mm i.d. stainless-steel column by conventional high-pressure slurry-packing procedure with 2-propanol - water mixture solvent (1/2 = v/v) at packing-pressure ca. 250kg / cm^2.

Chiral separation with avidin column

Samples were prepared by dissolving known amounts (0.08~40 mg / ml) of a drug in water - methanol and then a 25 μl portion of the sample was injected. Mobile phase was 20 mM potassium phosphate buffer - methanol mixed solution, and the flow rate was 1.0 ml / min. All operations were carried out at room temperature.

Measurement of free drug concentration

The binding of a drug to native avidin was determined as follows. A 1 ml portion of 1 μM native avidin or without avidin solution in the 20 mM potassium phosphate buffer (pH 7) was mixed with a 1 ml portion of drug solution in the 20 mM potassium phosphate buffer (pH 7) containing less than 20 % methanol. The drug concentration was varied to make the binding ratio, bound drug concentration / native avidin concentration, to be in a range 0.6 to 3.6. The 1 ml portion of the mixed solution was subjected to the ultrafiltration using MPS-3 at room temperature. After the centrifugation for 10 min, the drug concentration in the filtrate was determined by HPLC using an ODS column. The mobile phase was methanol - water - perchloric acid mixed solution and all operations were performed at ambient temperature with the flow rate of the mobile phase being 1.0 ml /min. The mobile phase composition was selected appropriately.

Fluorescence spectra of avidin and avidin - drug complexes

A native avidin solution (5 μg / ml) was prepared with 20 mM potassium phosphate buffer (pH 7) and a drug was dissolved at 35 ng / ml in 20 mM potassium phosphate buffer (pH 7) / methanol (5 / 1). The avidin - drug complex solution used was the mixture of the same volume of the native avidin solution and a drug solution, respectively. Fluorescence spectrum was measured by scanning 280 - 450 nm as emission wave length.

Measurement of drug hydrophobicity

The hydrophobicity of each drug is expressed as their calculated r-value on ODS column [13]. The mobile phase was the mixture of methanol - 10 mM potassium phosphate buffer (pH 7). The methanol concentration was varied from 25~80 % at an interval of 4 %. The flow rate was at 1.0 ml / min. The r-value was calculated by the least squares method as

$$\log k' = r \log (1 / [\text{methanol}]) + q$$

where k' is the capacity factor of a drug on ODS column.

RESULTS AND DISCUSSIONS

Loading capacity of avidin column and native avidin - drug binding

Loading capacity of the chiral separation column becomes an important consideration in scaling up separation procedures. In general, the chiral specific binding sites on protein-conjugated column are limited, therefore loading capacity is low. In addition, there are some nonspecific interactions between the chiral drugs and protein-conjugated stationary phases, which leads to the low column efficiency. Fig. 2 shows the representative chromatograms on the avidin column obtained by varying injection amounts. IP and CM were not optically resolved, when 20 ~ 30 μg of IP or CM was injected on the avidin column. While PP, CP and TP racemates could be separated up to 0.2 ~ 1 mg injection amounts.

The differences of the protein binding between drug enantiomers exert significant effect on the enantiomeric separation with an immobilized protein column [14]. Therefore, we examined the binding constants (K) and the binding sites (n) on a native avidin molecule. These results are shown in Table 1. The K value of these five drugs was in the following order: IP > CP > TP > CM > PP. The number of binding sites of PP was the minimum (n = 4.2) and that of IP was the maximum (n = 5.6) of these drugs. Although This result seemed to mean that the loading capacity was not so much different for each drug on the avidin column, PP was allowed to inject almost 1mg on the avidin column and 20 μg of IP was over loaded. Of course, although degree of overloading may rather be correlated to the retention, on the other hand, our studies may mean that the avidin column has nonspecific interactions for chiral discrimination with IP and CM, which leads to the low chromatographic efficiency [15].

Next we examined the effect of drug competitions for the binding sites on the native avidin by measuring free drug concentration of a drug in the presence of an other drug in avidin solution (data not shown). All drugs studied compete with the same binding sites on the native avidin ,or bind at different sites which can affect each other by inducing conformational changes in the native avidin, because the displacement phenomena were observed by competitions studies.

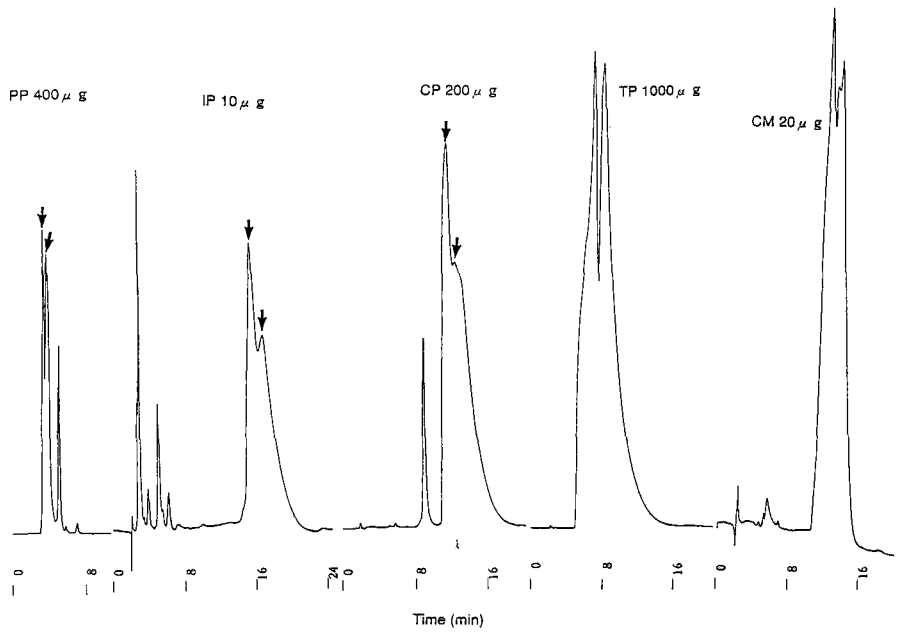

Fig.2 Representative chromatograms on the avidin column.
amounts injected, PP: 400 μg, IP: 10 μg, CP: 200 μg, TP: 1000 μg, CM: 20 μg
HPLC conditions, PP and CM: 20 mM phosphate buffer (pH 7) / methanol = 90 / 10, IP, CP and TP: 20 mM phosphate buffer (pH 7) / methanol = 70 / 30. See text for other conditions.

Table 1

Scatchard plot analysis of avidin - drug binding

	K	n	R	N
PP-avidin	0.55	4.2	-0.94	6
IP-avidin	1.49	5.6	-0.91	6
CP-avidin	1.20	5.1	-0.94	7
TP-avidin	0.93	5.2	-0.97	7
CM-avidin	0.61	5.0	-0.90	6

K: binding constant, n: binding site number

R: correlation coefficient, N: measurement number

See text for the conditions.

Fluorescence spectra of tryptophan residue on avidin

A native avidin molecule has 16 tryptophan residues [16], therefore fluorescence spectrum of the native avidin can be reflected differences of the native avidin conformation [17]. Fig. 3 shows one of the fluorescence spectra of the native avidin, drug and the native avidin-drug complex. The fluorescence intensity of avidin was weakened by the formation of avidin-PP, avidin-CM or avidin-TP complexes, although each drug has no fluorescence. IP and CP had no effects on fluorescence intensity of avidin. Therefore, some drugs can have some interactions with tryptophan residues on the surface of the native avidin molecule. It is known that the avidin has a very strong interaction with biotin which binds to tryptophan residues of the avidin [18] and the stereoselectivity of some drugs disappeared by the formation of avidin-biotin complex [11].

Effect of the pH on avidin column

Since the native avidin has many ionic amino acid residues [16], the properties of avidin column are changed by the pH of mobile phase. Table 2 shows the effect of the mobile phase on the avidin column. The k' values of acidic drugs (PP, IP) increased in the low-pH region, and the opposite results were obtained for basic drugs (CP, TP). However, there are different influences for the tryptophan residues of the native avidin between PP and IP, or CP and TP. While neutral drug, CM, showed the k' values obtained at pH 5 and pH 7 were almost 40% higher than that at pH 6 on the avidin column. The chiral separation of both acidic and basic drugs were not so affected the pH of the mobile phase, but as for neutral drug, CM enantiomers were not separated at pH 6.

Effect of zinc additive on avidin column

It is known that the metal ion such as zinc (II) is often an important structural components in some proteins to organize protein conformation [19]. Therefore, the effect of zinc additive on the avidin column was examined (Table 3). A tris buffer was used as a mobile phase instead of a phosphate buffer, because a phosphate is subject to form a the metal-phosphate chelate. The tris-buffer gave strong retentions to acidic drugs and weak retentions to basic drugs on the avidin column. Enantioselectivity of PP, CM and CP were lost by the change of the phosphate buffer to the tris buffer. But addition of zinc ions in the tris buffer was allowed to recover the enantioselectivity of CM and

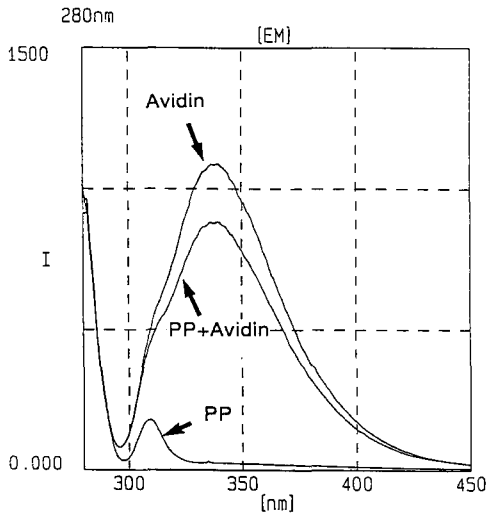

Fig. 3 Emission spectra of native avidin, PP and avidin-PP complex. See text for the measurement conditions.

Table 2

Effect of Mobile Phase PH on Avidin Column

	PP[1]		CM[1]		TP[2]		CP[2]		IP[2]	
	k_1	α	k_1	α	k_1	α	k_1	α	k_1	α
pH 7	0.69	1.13	5.54	1.09	4.77	1.56	9.68	1.13	7.13	1.13
pH 6	2.44	1.22	4.07	1.00	2.35	1.60	4.57	1.15	11.2	1.14
pH 5	5.36	1.10	5.59	1.13	2.05	1.51	3.02	1.14	15.3	1.14

HPLC condition: avidin column (150 mm X 4.6 mm), Flow Rate (1.0 ml / min), UV254nm

Mobile Phase, 1) 20 mM Phosphate Buffer / Methanol = 10 / 90

2) 20 mM Phosphate Buffer / Methanol = 30 / 70

* k_1 means the capacity factor of the first eluted enantiomer.

** α means the separation factor ($\alpha = k_2 / k_1$).

Table 3

Effect of Zinc Additive

	PP[1]		CM[1]		TP[2]		CP[2]		IP[2]	
	k_1	α	k_1	α	k_1	α	k_1	α	k_1	α
0 mM	1.43	1.00	5.69	1.00	2.05	1.48	4.34	1.00	9.95	1.13
5mM $ZnCl_2$	2.51	1.00	5.16	1.17	1.14	1.61	2.20	1.15	10.9	1.00

HPLC condition: avidin column (150 mm X 4.6 mm), Flow Rate (1.0 ml / min), UV254nm

1), Mobile Phase, 50 mM Tris buffer (pH 7) / Methanol = 90 / 10

2), Mobile Phase, 50 mM Tris buffer (pH 7) / Methanol = 70 / 30

* k_1 means the capacity factor of the first eluted enantiomer.

** α means the separation factor ($\alpha = k_2 / k_1$).

CP and to lose that of IP. Thus, adding zinc ions to the mobile phase may be sometimes an useful procedure for chiral separation of drug enantiomers. But these experiments gave the avidin column strong damages, so retention properties had not recovered to initial states with the using phosphate buffer. The tris buffer is a primary amine which is bound to the carboxyl groups of avidin column, and / or to other high affinity sites on the silica matrix. Thus, these results mean that both the retention and enantioselectivity are affected by small residues of tris(hydroxymethyl) aminomethane and metal ions on the avidin column, and then it is difficult to remove completely the trace level of tris buffer and metal ions from the avidin column.

Hydrophobicity of drug

The r-value is well correlated to the hydrophobic properties of the solute such as non-polar surface area and the 1-octanol / water partition coefficient [13]. Therefore, the hydrophobicity of each drug was expressed as r-value given in Table 6. The hydrophobicity order was CP > TP > IP > CM > PP. This order is similar to that of the binding constant value (K) of drug-avidin: IP > CP > TP > CM > PP (Table 1) except IP. The native avidin has a moderate hydrophobicity as it contains many hydrophobic amino acid residues [16]. Lindner et al. reported that a correlation was found between retention

behaviour and hydrophobicity for the cellulase CSP [20]. In our study, the hydrophobic interaction plays an important role in the binding drugs on the native avidin. Then, because the native avidin is a basic protein (pI: 10.5) [21], the avidin column is expected to retain electrostatically acidic drugs (such as IP) much strongly than basic drugs. The elution order from the avidin column was CP > IP > TP > CM > PP at pH 7 of the mobile phase (Table 2). A correlation was found between the retention behaviour and the hydrophobicity of drugs (except IP), therefore the hydrophobic interaction is important for retention of drugs on the avidin colunm, although the electrostatic interaction is also necessary to the enantioselectivity.

Acylation of amino group on avidin column

Acylation of a primary amino group (such as a lysine residue) was performed *in situ* by recycling sulfosuccinimidyl acetate (100 mg) in 20 mM sodium hydrogen carbonate buffer / acetonitrile for 5h [22] at room temperature (amino acylate avidin column). Fig. 4 shows the chromatograms obtained with this amino acylate avidin column. Comparison of the avidin column, the k' values of acidic drugs (PP, IP) markedly decreased and enantioselectivity disappeared. As for basic drugs (CP, TP), the k' values increased and enantioselectivity improved. Table 4 shows the effect of mobile phase pH on the amino acylate avidin column. The retention properties for varying pH were almost the same as those on the avidin column. Both the k' value and enantioselectivity of neutral drug (CM) were not so affected by acylation of amino groups.

Acylation of carboxyl group on avidin column

The avidin column has several carboxyl groups, such as aspartic acid residues and glutamic acid residues [15]. A carboxyl acylate avidin column was obtained by recycling 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (200mg) and methylamine (200mg) in 20 mM potassium dihydrogen phosphate buffer (pH 4.8) for 12 h *in situ* at room temperature [23]. Fig. 5 shows the chromatograms obtained with the carboxyl acylate avidin column. The k' values of acidic drugs increased compared with those on the avidin column, while those of basic drugs were reversed. The enantioselectivity of CP and IP disappeared and that of TP became very weak. Table 5 shows the effect of mobile phase pH on the carboxyl acylate avidin column. The

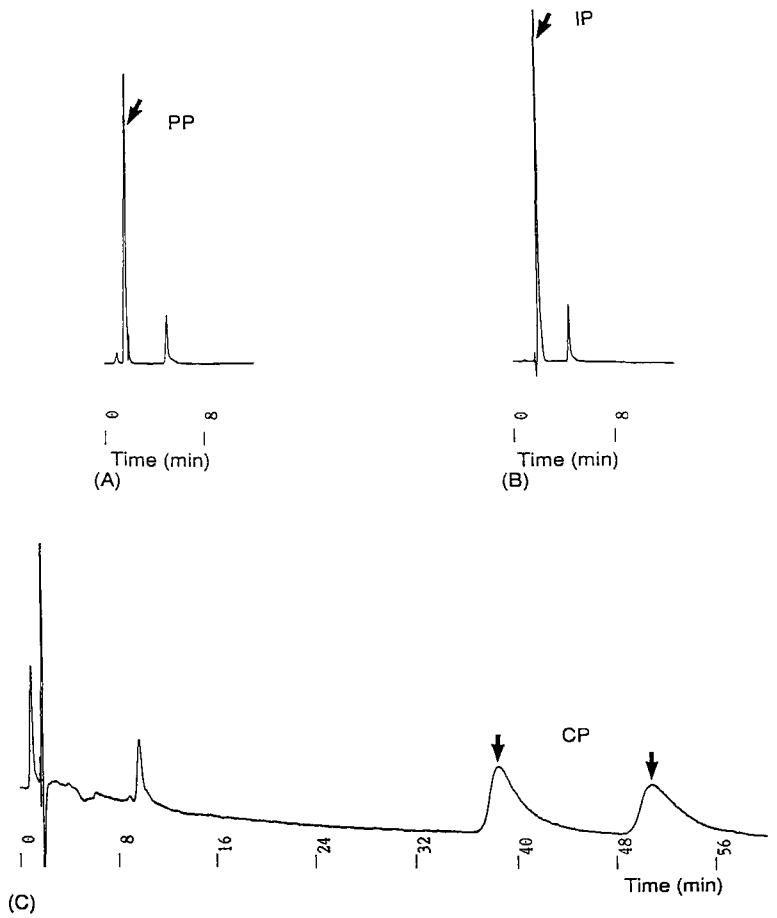

Fig. 4 Representative chromatograms on the amino acylate avidin column. (A) PP, (B) IP, (C) CP, (D) TP and (E) CM
HPLC conditions, (A) and (E): 20 mM phosphate buffer (pH 7) / methanol = 90 / 10, (B), (C) and (D): 20 mM phosphate buffer (pH 7) / methanol = 70 / 30. See text for other conditions.

(continued)

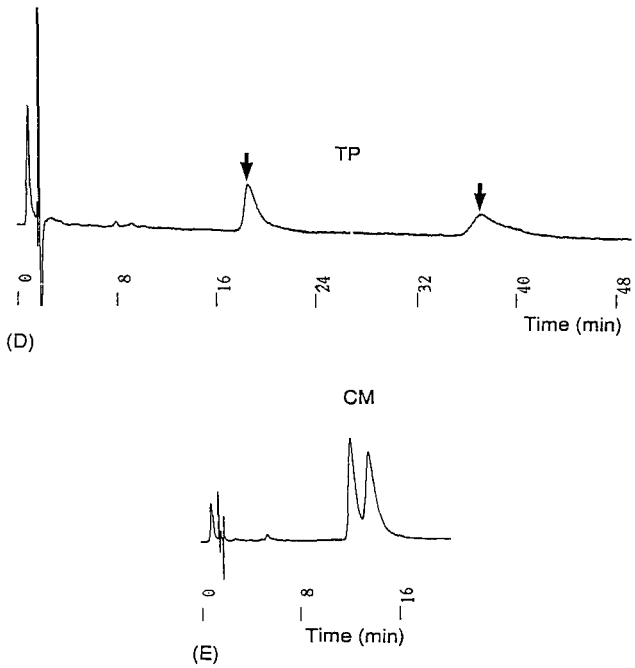

Fig. 4 (Continued)

Table 4

Effect of Mobile Phase PH on Amino Acylate Avidin Column

	PP[1)]		CM[1)]		TP[2)]		CP[2)]		IP[2)]	
	k_1	α	k_1	α	k_1	α	k_1	α	k_1	α
pH 7	0.09	1.00	6.67	1.14	10.6	2.09	23.1	1.33	0.31	1.00
pH 6	0.30	1.00	5.10	1.00	7.58	2.45	18.0	1.45	1.82	1.00
pH 5	1.41	1.00	6.74	1.02	4.07	2.41	7.95	1.44	3.75	1.00

HPLC condition: amino methylate avidin column (150 mm X 4.6 mm)

Flow Rate (1.0 ml / min), UV254nm

Mobile Phase, 1) 20 mM Phosphate Buffer / Methanol = 10 / 90

2) 20 mM Phosphate Buffer / Methanol = 30 / 70

* k_1 means the capacity factor of the first eluted enantiomer.

** α means the separation factor ($\alpha = k_2 / k_1$).

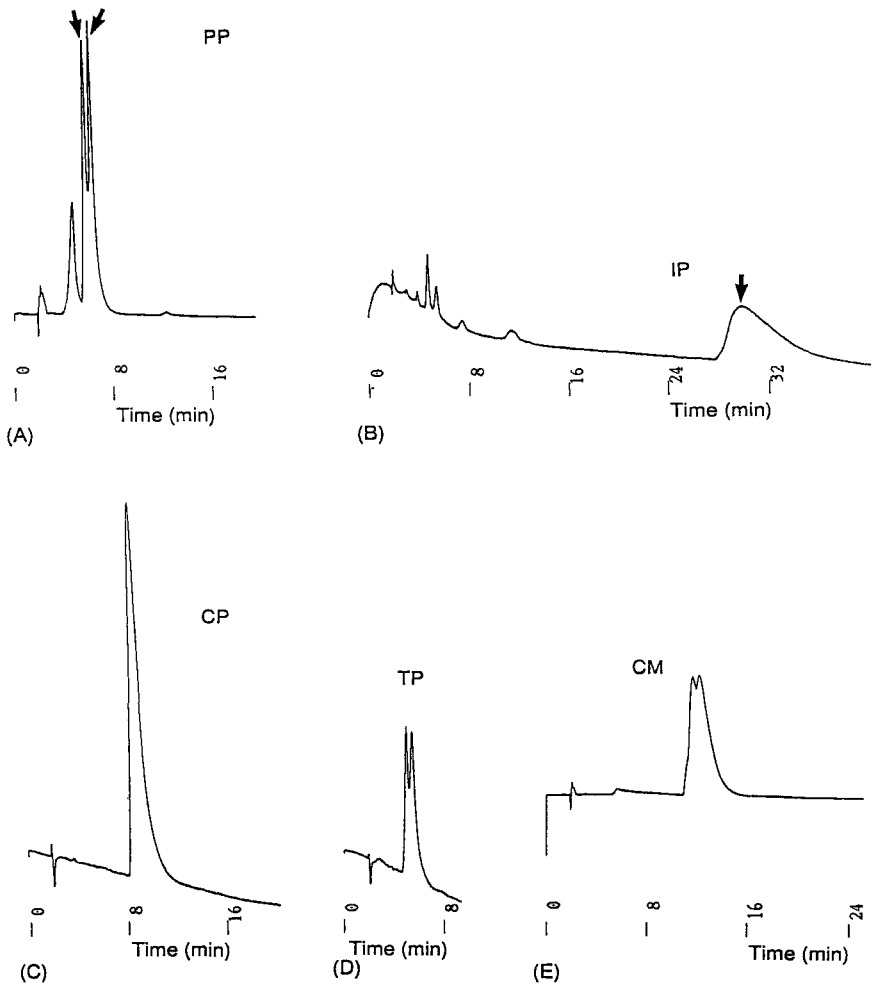

Fig. 5 Representative chromatograms on the carboxyl acylate avidin column. (A) PP, (B) IP, (C) CP, (D) TP and (E) CM
HPLC conditions, (A) and (E): 20 mM phosphate buffer (pH 7) / methanol = 90 / 10, (B), (C) and (D): 20 mM phosphate buffer (pH 7) / methanol = 70 / 30. See text for other conditions.

Table 5

Effect of Mobile Phase PH on Carboxy Acylate Avidin Column

	PP[1]		CM[1]		TP[2]		CP[2]		IP[2]	
	k_1	α	k_1	α	k_1	α	k_1	α	k_1	α
pH 7	2.13	1.13	5.20	1.05	1.61	1.15	3.62	1.00	14.7	1.00
pH 6	5.21	1.11	4.95	1.12	0.40	1.34	1.09	1.00	30.0	1.00
pH 5	8.71	1.10	4.73	1.15	0.06	1.00	0.16	1.00	45.8	1.00

HPLC condition: carboxy methylate avidin column (150 mm X 4.6 mm)

Flow Rate (1.0 ml / min), UV254nm

Mobile Phase, 1) 20 mM Phosphate Buffer / Methanol = 10 / 90

2) 20 mM Phosphate Buffer / Methanol = 30 / 70

* k_1 means the capacity factor of the first eluted enantiomer.

** α means the separation factor ($\alpha = k_2 / k_1$).

Table 6

r-values of drugs on ODS column

	r	q
PP	1.94	3.29
IP	7.48	13.8
CP	8.17	16.2
TP	7.74	15.5
CM	4.12	7.29

$\log k' = r \log (1 / [MeOH]) + q$

tendency of retention for acidic drugs or basic drugs were the similarity that on the avidin column. However, CM enantiomers were separated at pH 6 of mobile phase, which was a different result observed on the avidin column and the acylate amino avidin column.

We modified these functional groups on the avidin column to examine their contribution to the chiral separation. Whereas, the specific modification of

the native avidin may affect a remote binding site by conformational changes, because the native avidin is flexible and its tertiary structure is not fixed. While, the avidin conformation seems to be stabilized (not completely) by immobilization on a silica support with a succinimide linker compared with not immobilized avidin [11]. Therefore, acylations of amino groups or carboxyl groups of the avidin column were performed *in situ* to minimize an alteration of tertiary structure caused by these modifications. Also, Haginaka et al. reported that the blocking of primary amino groups on an ovomucoid (OVM) column gave higher or almost equal enantioselectivities for basic drugs compared with the unmodified OVM column [24, 25]. These results mean that the modifications of amino groups of avidin play a key role for the chiral separation of acidic drugs, and the accessibility of basic drugs to the chiral recognition sites may be hindered by the electrostatic repulsion between drugs and the primary amino groups. While the retention of acidic drugs became strong and that of basic drugs became weak by the acylation of carboxyl groups of the avidin column, so these results were vice versa those obtained with the amino acylate avidin column. There are two possible explanations, the first is that the Coulombic interactions between the charged drugs and the charged avidin molecules exist in the retention mechanism on the avidin column, and the second is that the secondary structure of the avidin is affected by the modifications from ionic groups to uncharged groups on the avidin column despite the fact the avidin is immobilized. As for the chiral separation, the carboxyl groups of the avidin column are necessary to separate IP enantiomers and TP enantiomers, although those do not participate the enantioselectivity of PP.

The k' value and the enantioselectivity of CM were little affected by the acylation of amino and carboxyl groups of the avidin column. However, CM enantiomers were not separated at pH 6 of the mobile phase buffer on the avidin column (Table 2), therefore histidine residues (imidazolium group: pK = 6) of avidin may contribute to the enantioselectivity of CM.

CONCLUSION

Changes in the properties of the mobile phase (pH, metal ion modifier) and acylation of amino and carboxyl groups on avidin column can alter the binding ability for drug enantiomers on the avidin column. The change of the

microenvironment on the avidin column altered the ability to retain and discriminate drug enantiomers [26]. Our results also suggest that the retaining phase contains multiple chiral and non-chiral binding sites on the avidin column. The binding of the drugs on the avidin column may be mainly due to hydrophobic interactions. Then, the avidin column has many different binding sites giving the possibility of enantioselective interactions with a broad range of drugs. Mechanistic studies for chiral recognition on the avidin column are of interest in prediction of the optimum HPLC conditions and the increments of the column efficiency.

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DETERMINATION OF BENZBROMARONE AND BENZARONE IN PHARMACEUTICALS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

A. GUMIENICZEK AND L. PRZYBOROWSKI
Department of Medicinal Chemistry
Medical Academy in Lublin
Chodźki Str. 6
20-093 Lublin, Poland

ABSTRACT

Assay procedure based on high-performance liquid chromatography has been developed for the specific determination of benzbromarone and benzarone in pharmaceutical dosage forms, using a LiChrosorb RP-18 column, a mobile phase acetonitrile-phosphate buffer pH 2,6 (9:1 v/v) and UV detection at 240 nm. Benzbromarone and benzarone have been extracted from tablets with methanol and determined in the range 2-12 µg/ml with fairly good recovery (mean 99,63% and 99,78% for benzbromarone and benzarone, respectively) and reproducibility.

INTRODUCTION

Benzbromarone, 2-ethyl-3-benzofuranyl 4-hydroxy-3,5-dibromophenyl ketone is a widely used uricosuric drug which increases urinary urate excretion.

Its debrominated metabolite - benzarone, 2-ethyl-3-benzofuranyl 4-hydroxyphenyl ketone, is also pharmacologically active. It has been shown to have uricosuric and more powerful fibrinolytic activity and is proposed for treatment of vascular disorders. The following methods have been used for estimation of benzbromarone in pure substance: non-aqueous titration[1,2], voltammetry and controlled potential coulometry[3]. A few analytical procedures, almost exlusively based on gas chromatography-mass spectrometry and high-performance liquid chromatography, have been described for determination of benzbromarone and/or benzarone in biological samples (plasma, urine)[4-11]. However, there are no publications about the analysis of these drugs in their dosage forms which are needed as analytical tools for stability studies of drugs and for their determination in commercial formulations. That is why it was considered to develop assay procedure which would serve as a rapid and reliable method for quality control of benzbromarone and benzarone pharmaceutical formulations.

MATERIALS

Reagents

Benzbromarone and benzarone were obtained from Sanofi-Labaz (France). Desuric® (100 mg of benzbromarone) and Fragivix® (100 mg of benzarone) tablets (Laboratiores Labaz, France) were used. Phosphate buffer: 0,067 M potassium dihydrogen phosphate adjusted to pH 2,6 with phosphoric acid; pH tolerance ± 0,05, acetonitrile and methanol LiChrosolv® for chromatography (E.Merck, Germany) were applied. All other solvents and reagents were of analytical grade.

Apparatus

A liquid chromatograph , type LC-730 (Laboratorni Pristroje Praha, Czechoslovakia) with a variable - wavelength UV detector and 250 x 4 mm steel column packed with LiChrosorb RP-18 (dp = 7 µm) was used. A reciprocating shaker, type 327 (Premed, Poland) was applied.

METHODS

The mobile phase was acetonitrile-phosphate buffer pH 2,6 (9:1, v/v). The flow rate was 1 ml/min. Detection was by UV absorption at 240 nm, detector output range was 0.08 AUFS, recorder chart speed was 0,3 cm/min.

Solutions

Stock solutions (1 mg/ml) of benzbromarone and benzarone were prepared by dissolving appropriate amounts of the substances in methanol. These solutions were stable for at least six months at 4°C. Working dilutions of 0,1 mg/ml of benzbromarone and benzarone were prepared from the stock solutions.

Calibration curve for benzbromarone assay

From the working solution of benzbromarone 0,2, 0,4, 0,6, 0,8, 1,0 and 1,2 ml volumes were pipetted into 10-ml measuring flasks.
Then, 0,2 ml of internal standard solution (0,1 mg/ml of benzarone) was added and made with acetonitrile up to 10,0 ml. 20 µl of each sample was then injected into the column. All measurements were repeated three times for each concentration. The peak heights were measured and the peak heights ratios of analyte to internal standard were then plotted against the res-

pective concentration of benzbromarone, in order to obtain the calibration curve.

Calibration curve for benzarone assay

The calibration for assay of benzarone was prepared in the same way as it is described above for benzbromarone, using benzbromarone as an internal standard (the same solutions and volumes pipetted). The calibration curve was based on the peak heights ratios of benzarone to that of internal standard against the respective concentration of benzarone.

Sample preparation

Tablets of benzbromarone

Tablets of benzbromarone were ground to a fine powder and amounts equivalent to 2-12 mg (after a declaration) of the compound were extracted with methanol in 100-ml volumetric flasks.
Filtered 1,0 ml volumes of the extracts were transfered into 10-ml flasks, 0,2 ml of internal standard solution (0,1 mg/ml) was added and made with acetonitrile up to 10,0 ml. Then, 20 µl of each sample was injected into the column.

Tablets of benzarone

Estimation of benzarone in tablets was developed in the same way as in the case of benzbromarone, using benzbromarone as an internal standard.

RESULTS AND DISCUSSION

A reversed-phase HPLC procedure was developed as a suitable method for the analysis of benzbromarone and benzarone dosage forms.

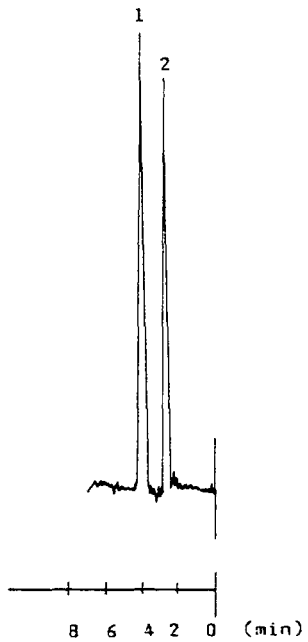

FIGURE 1. Typical chromatogram of benzbromarone (1) and benzarone (2). Peaks correspond to 6 µg/ml of benzbromarone and 4 µg/ml of benzarone.

The chromatographic conditions were adjusted in order to provide a versatile HPLC procedure capable of separating benzbromarone and benzarone and therefore also potentially useful for pharmacokinetic studies. A mixture of acetonitrile-phosphate buffer pH 2,6 (9:1 v/v), at flow rate of 1,0 ml/min was found to be an appropriate mobile phase, allowing for adequate and rapid separation of benzbromarone and benzarone (retention times 4,08 and 2,75 min, respectively). Typical chromatogram of benzbromarone and benzarone under described HPLC conditions is presented in Figure 1.

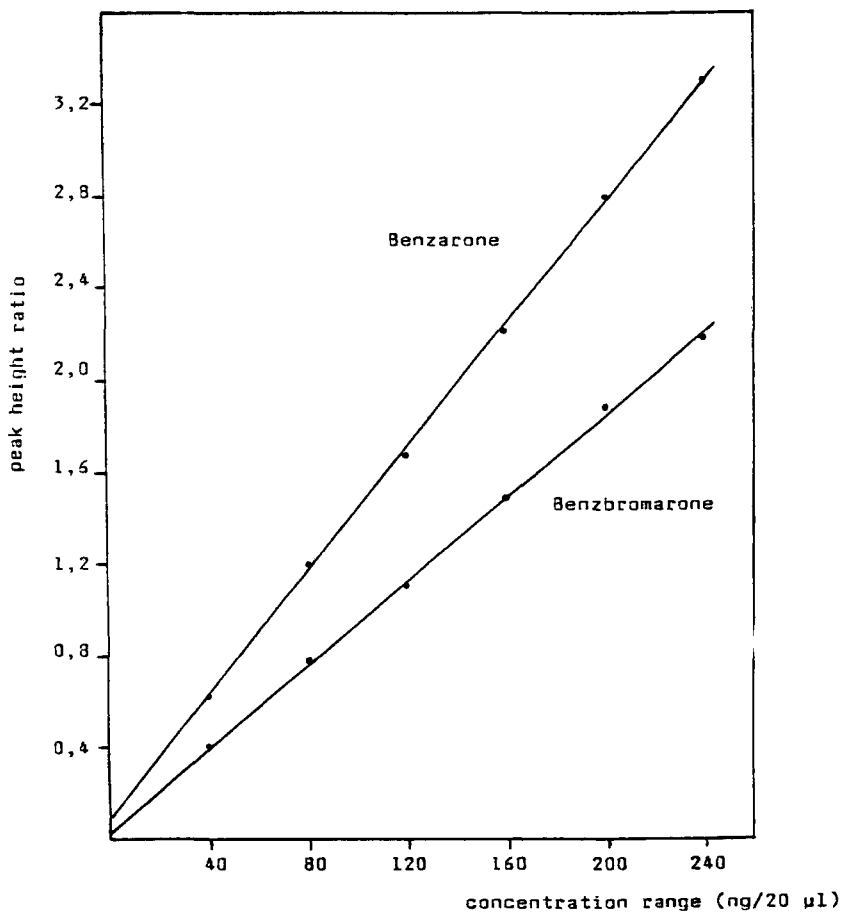

FIGURE 2. Standard curves for benzbromarone and benzarone assay (each point is the mean of three determinations).

TABLE 1

Results of the Determinations of Benzbromarone and Benzarone in Dosage Forms (n = 5, at each concentration)

No.	Amount expected (ng/20 µl)	Amount found (mean ± SD)	Coefficient of variation (%)
	BENZBROMARONE		
1	40	39,45 ± 0,54	1,37
2	80	79,72 ± 0,54	0,68
3	120	119,77 ± 0,54	0,45
4	160	158,92 ± 0,83	0,52
5	200	201,85 ± 0,54	0,27
6	240	238,79 ± 0,54	0,23
	BENZARONE		
1	40	39,67 ± 0,37	0,93
2	80	79,94 ± 0,37	0,46
3	120	120,06 ± 0,30	0,25
4	160	158,99 ± 0,36	0,22
5	200	201,79 ± 0,30	0,15
6	240	238,33 ± 0,30	0,13

SD - standard deviation

For quantitative applications linear calibration curves were obtained over the working concentration range 2-12 µg/ml. For assay of benzbromarone $y = 0,00899x + 0,04933$, $r = 0,9995$, for assay of benzarone $y = 0,01341x + 0,092$, $r = 0,9997$ where y - peak height ratio of benzbromarone or benzarone to that of internal standard and x - concentration of drugs in ng per 20 µl of the mobile phase.

Methanol was chosen as the extraction organic solvent because of solubility properties of benzbromarone and benzarone and a reversed-phase mode of chromatography. Recovery of benzbromarone after extraction from tablets was found to be 99,63% for benzbromarone and 99,78% for benzarone.
The precision of the chromatographic analysis in tablets was determined at six concentrations of both drugs. The coefficients of variation were obtained by repeating the procedure five times for each sample (Table 1).

The described method is simple and fairly reliable for pharmaceutical analysis.

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We wish to thank the company Sanofi-Labaz for supplying benzbromarone and benzarone standards.

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SYNTHESIS AND DETERMINATION OF N-ACETYLOCTOPAMINE BY HPLC WITH ELECTROCHEMICAL DETECTION. BIOASSAY IN *NIPPOSTRONGYLUS BRASILIENSIS* (NEMATODA)

L. ELKIHEL[1], J. M. GROSCLAUDE[2], B. NEMBO[3], C. JACQUOT[2], P. GAYRAL[3], F. GOUDEY-PERRIERE[3], Y. LETOURNEUX[1]*, AND H. BARRETEAU[2]

[1]Laboratoire de Génie Protéique-Physicochimie des Substances Naturelles
Université de La Rochelle
Pôle Sciences
Av. Marillac, 17 000 La Rochelle, France
[2]Laboratoires de Pharmacologie
[3]Biologie et Contrôle des Organismes Parasites
Faculté de Pharmacie
Université de Paris-Sud
92296 Châtenay-Malabry Cedex, France

ABSTRACT

Determination of biogenic amines in invertebrates, by means of high-performance liquid chromatography (HPLC), is of considerable physiological significance. In insects, octopamine is released into the hemolymph in response to stress, and exhibits some myogenic properties in both insects and nematodes. The first part of the present paper deals with the synthesis of N-acetyloctopamine from octopamine and with its characterization. The second part deals, for the first time, with the detection of these biogenic amines in insects and nematodes using the chromatographic system with electrochemical detection. N-acetyloctopamine was detected in a chromatographic system enabling determination in biological

* To whom correspondence should be addressed.

samples. The interest of the used method is in the simultaneous detection of various biogenic amines. Improvements can certainly be made for detection of more specific to invertebrates compounds such as tyramine, octopamine and N-acetylated derivatives.

INTRODUCTION

Biogenic amines thought to act as neurotransmitters or neurohormones have been found in many invertebrates including insects (where noradrenaline is particularly abundant) and nematoda [1]. Two features of amine metabolism in invertebrates compared to the well known metabolic pathways in vertebrates are noteworthy: high levels of octopamine [2-4] and preponderance of the N-acetylation pathway in enzymatic degradation ([2], [5]).

In nematodes, the presence of N-acetyltransferase has been demonstrated recently [5], and consequently, the N-acetylated derivatives of dopamine and serotonin have been characterized and determined in *Nipppostrongyius brasiliensis* [4]. The assay of these derivatives is of considerable interest, at least in insects such as cockroaches [3], as their levels have been found to be influenced by sex, age, experimental housing and feeding conditions [6-10].

Although the N-acetylated derivatives of dopamine and serotonin can now be detected, N-acetyloctopamine, which is not yet commercially available, must be synthesized before being characterized in biological samples.

We currently employ a method of simultaneous detection of a variety of biogenic amines initially described in ref. [11] for vertebrate tissues, adapted for detection of additional compounds in cockroaches [3], and nematodes [1, 4, 12, 13]. Both octopamine and N-acetylated derivatives of dopamine and serotonin can thus be detected in the same assay.

The first part of the present paper deals with the synthesis of N-acetyloctopamine from octopamine, and its characterization. The chromatographic separation system was coupled with electrochemical detection employed in our laboratory for assay of biogenic amines in insects and nematodes. It enabled simultaneous detection of tryptophan (Trp), 5-hydroxytryptophan (5-HTP), 5-hydroxytryptamine (5-HT), 5-hydroxyindol acetic acid (5-HIAA), N-acetylserotonin (N-Ac-5-HT), tyramine (Tyr), dopamine (DA), dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine (3-MT), homovanillic acid (HVA), noradrenalin (NAd), N-acetyldopamine (N-Ac.DA) and octopamine (OA).

EXPERIMENTAL

N-acetyloctopamine synthesis and characterisation :

Low yields and formation of several by-products were obtained by the classical method using direct N-acetylation of 1-(4-hydroxyphenyl)-2-aminoethanol (octopamine) with acetic anhydride and pyridine. In contrast, 1-(4-hydroxyphenyl)-2-(N-acetylamino)ethanol (N-acetyloctopamine) was readily available by selected N-acetylation in high yield (93 %) using octopamine and acetic anhydride in methanol (**Figure 1**).

Melting points were measured on a Mettler FP 52 and are uncorrected. The IR spectra were obtained using a Bomem MB-100. The frequency values are expressed in cm^{-1}. Nuclear magnetic resonance (^1H NMR) spectra were recorded

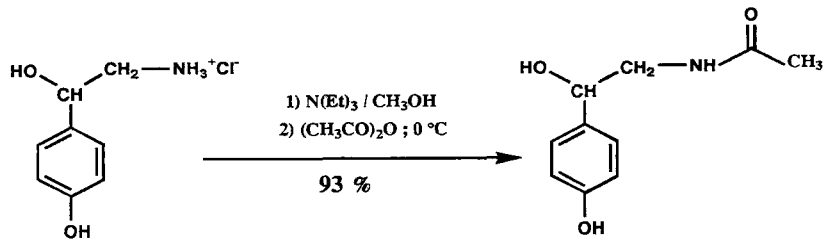

Figure 1 : synthesis of N-acetyloctopamine

on a Jeol 90 spectrometer. The chemical shift values are expressed in δ values (parts per million) relative to tetramethylsilane as an internal standard. High-resolution mass spectra were recorded on HP 5889A quadripolar. UV absorption spectra were recorded on a Uvikon 930 (Kontron) spectrometer in chloroform solution (wavelenghts are in nm). Analytical thin layer chromatography (TLC) was performed on Merck 60F-254 silica gel plates.

1-(4-hydroxyphenyl)-2-(N-acetylamino)ethanol: acetic anhydride (0.089 mL, 0.949 mmol) was added dropwise to a stirred solution of octopamine hydrochloride (0.15 g, 0.709 mmol) and triethylamine (0.11 mL, 0.709 mmol) in methanol (5 mL), at 0°C and under nitrogen atmosphere. After 1h, the mixture was evaporated and the product was crystallized from ethanol-petroleum ether, which gave 0.128 g (yield : 93 %) of pur product as colourless needles ; **Rf** 0.27 (dichloromethane-methanol, 9:1) ; **mp** = 204-206 °C ; **IR** (film) : 3400 (OH alcohol) ; 3400-3010 (NH amide) 1650 (CO amide) ; 1620 (C=C) ; **UV** : λ_{max} : 274 ; **^{1}H NMR** ($CDCl_3$) : δ = 2.02 (s ; 3H ; -CO-C<u>H</u>3) ; δ = 6,57 (m ; 1H ; -N<u>H</u>CO-). ; **MS** : 195 ($M^{\cdot +}$; 1%) ; 177 ($M^{\cdot +}$-18 ; 61%) ; 135 ($M^{\cdot +}$ - CH_2=C=O ; 100%) ; 107 (30%) ; 77 (24%).

Recovery of adult worms :

White rat parasited with the nematoda *Nippostrongylus brasiliensis* were maintained in the laboratory according to a previous protocol [14]. Coprocultures of faeces from infested rats were stored at 25°C and, after 6 to 9 days, third-stage larvae were collected from faecal cultures, rinsed and inoculated into rats by skin puncture (2500 larvae per 250 g female rat). Maturation needs migration to the lungs, then from the trachea to the oesophagus and onwards to the intestine, the larvae become adults. The first eggs appear in the faeces five to six days after infestation.

Rats were killed 7 days after infestation, internal mucus of the small intestine was taken off and placed in 0.15 M NaCl at 37°C for 1 hr. Nematodes were collected after sedimentation, then rinsed three times with physiological saline. Worms were then wiped dry, weighed and immediatly frozen at -20°C until assay.

Sample preparation :

Weighed nematodes were homogenized (1 mg/50 µl) in perchloric medium (0.4 N perchloric acid, containing 0.1 % EDTA-Na_2, 0.1 % sodium metabisulfite and 0.1 % L-cystein), using an Ultraturrax (PolyLabo, Paris, France). Proteins were precipitated with perchloric acid ; after centrifugation (Sorvall superspeed, 4°C, 20 min at 3000 *g*), 20 µl of supernatant were assayed for biogenic amine detection.

Separation and determination of biogenic amines :

Samples were analysed by means of chromatography with electrochemical detection according to [3, 13], with some minor adaptations. The

chromatographic system consisted of a Beckman 112 pump (constant flow rate of 0.9 ml/min); a Rheodyne injection valve with a 20 µl loop ; a reverse-phase column (Ultrasphere ODS, 5 µm, 150 x 4 mm, Beckman) and a Metrohm 641 VA electrochemical detector (sensitivity 1 nA) equipped with a glassy carbon electrode (set to 0.85 V potential versus a KCl/AgCl reference electrode) [1]. This potential was varied between 0.80 to 0.9 V to try and find the best conditions to detect N-acetyloctopamine in our chromatographic system.

The mobile phase was a mixture of phosphate buffer [0.1 M KH_2PO_4, heptane sulphonic acid (5 mM)] and methanol (92.5/7.5, v/v) ; pH was adjusted to 3.8 using 3 M KOH.

Total elution was obtained within 25 min. The system allowed the simultaneous detection of noradrenaline (NAd), 5-hydroxytryptophan (5-HTP), octopamine (OA), 3,4-dihydroxyphenylacetic acid (DOPAC), *N*-acetyldopamine (NADA), dopamine (DA), 5-hydroxyindoleacetic acid (5-HIAA), N-acetylserotonin (N-Ac-5-HT), tyramine (Tyr), homovanillic acid (HVA), tryptophane (Trp), 3-methoxytyramine (3-MT) and serotonin (5-HT), in the order of elution.

Chemicals

All reagents were of analytical grade ; methanol was provided from Merck, while tryptophan, 5-hydroxytryptophan, serotonin, 5-hydroxyindolacetic acid, N-acetylserotonin, noradrenaline, tyramine, octopamine, 3,4-dihydroxyphenylacetic acid, dopamine, 3-methoxytyramine, homovanillic acid and N-acetyldopamine were provided from Sigma Co.

RESULTS

Detection of N-acetyloctopamine :

20 µl of solutions containing 1 to 10 µg / ml of N-acetyloctopamine were injected and chromatographed at different potentials at a given sensitivity. A solution of 200 ng/20µl detected with an electrode potential of 0.85 V, produced a peak height of 18 cm, while no peak was observed at the retention time of octopamine (detection level of octopamine at 0.85 V : 18 ng/ml corresponding to 0.36 ng/20 µl) . This observation confirmed the purity and stability of the synthesized N-Ac.OA, which contains, even dissolved in buffer, less than 0.18% OA.

Retention times of the different amines in the chromatographic system are listed in **Table I**. Retention time of N-Ac.OA alone or after addition to the other substances was 3.80 ± 0.02 min (n = 15). This retention time is rather close to that of NAd (retention time 4.12 ± 0.04 min) which represents a difficulty for determination of this compound in biological samples from invertebrate tissues containing high levels of NAd.

A chromatogram of 20 µl of a standard solution containing tryptophan (8 ng), octopamine (4 ng), tyramine (2 ng), 5-hydroxytryptophan, 5-hydroxytryptamine, 5-hydroxyindol acetic acid, N-acetylserotonin, dopamine, 3-methoxytyramine, homovanillic acid, noradrenaline, N-acetyldopamine (0.4 ng of each) along with 40 ng of N-acetyloctopamine (N-Ac.OA) is shown in **Figure 2** (potential 0.9 V).

Table I : Retention time expressed in minutes for the different substances detected simultaneously in the analytical system.

Substances	Retention time (in min)
N-Ac-OA	3.80 ± 0.02
NAd	4.12 ± 0.04
5-HTP	5.33 ± 0.05
OA	5.53 ± 0.05
DOPAC	7.23 ± 0.06
NADA	7.28 ± 0.06
DA	9.48 ± 0.09
5-HIA	12.02 ± 0.12
N-Ac-5-HT	13.40 ± 0.12
Tyr	14.92 ± 0.13
HVA	16.07 ± 0.14
Trp	18.58 ± 0.19
3-MT	21.10 ± 0.15
5-HT	24.77 ± 0.24

In order to obtain a relationship between peak height and concentration of the solution, different amounts of N-Ac.OA were added to the standard solution . In **Table II**, peaks (expressed in cm) are given for OA and N-Ac.OA (various concentrations) at different potentials. The detector response for these two amines was dependent on the injected amount of the sample and of the elecrode potential (**Figure 3**). For N-Ac.OA as for OA and Tyr, peaks were well separated and a maximum response was observed at 0.9 V. The effect of the electrode potential was evaluated as the percentage fraction of the detector response at 0.9 V.

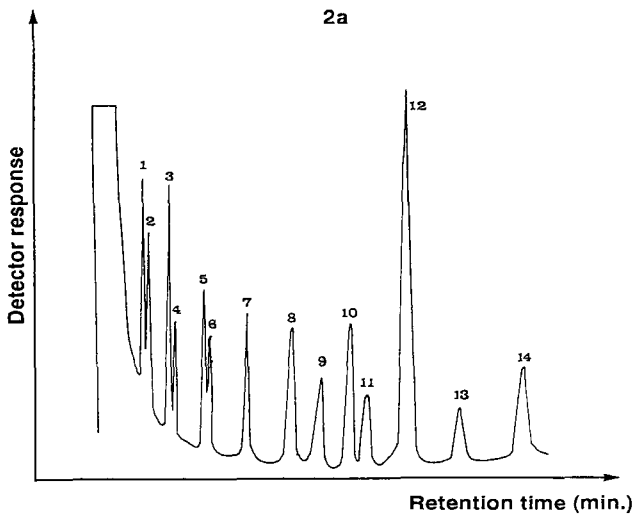

Figure 2 : Chromatogram obtained by injection of 20 µl of the standard solution containing, tryptophan (8 ng), N-acetyloctopamine (40 ng), octopamine (4 ng), tyramine (2 ng) and 0.4 ng of the other compounds (in the order of elution)

1. N-acetyloctopamine ; **2.** noradrenaline ; **3.** 5-hydroxytryptophan ; **4.** octopamine ; **5.** 3,4-dihydroxyphenylacetic acid ; **6.** *N*-acetyldopamine ; **7.** dopamine ; **8.** 5-hydroxyindolacetic acid ; **9.** N-acetylserotonin ; **10.** tyramine; **11.** homovanillic acid ; **12.** tryptophan ; **13.** 3-methoxytyramine ; **14.** serotonin.

2a : injection of 20 µl of a standard solution containing N-acetyloctopamine (40 ng).

2b : injection of 10 µl of standard solution containing N-acetyloctopamine (20 ng) and 10 µl of a solution containing N-acetyloctopamine (100 ng).

(continued)

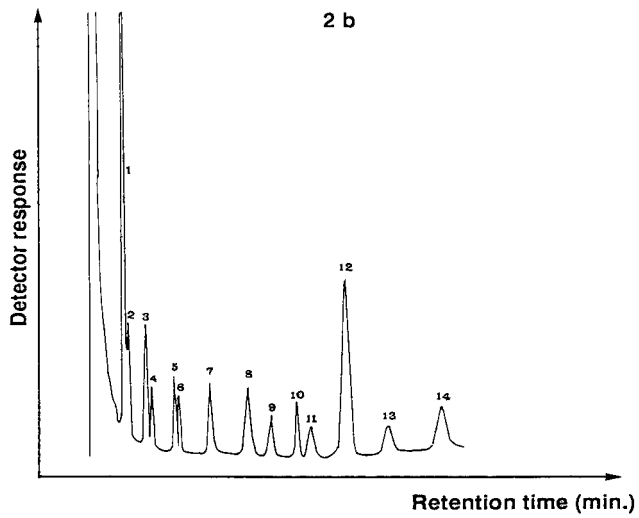

Figure 2 (Continued)

Table II : Peak heights expressed in centimeters after injection of a solution containing 2 µg/ml of octopamine or 1 to 10 µg/ml of N-Ac.OA at different electrode potentials.

	N-Ac.OA			OA
Potential	**1µg/ml**	**2 µg/ml**	**10 µg/ml**	**2 µg/ml**
0.80 V		1.1	6.3	0.1
0.83 V		3.7		0.5
0.85 V		6.4	18	1.3
0.87 V		11		2.8
0.90 V	11.6	28		4

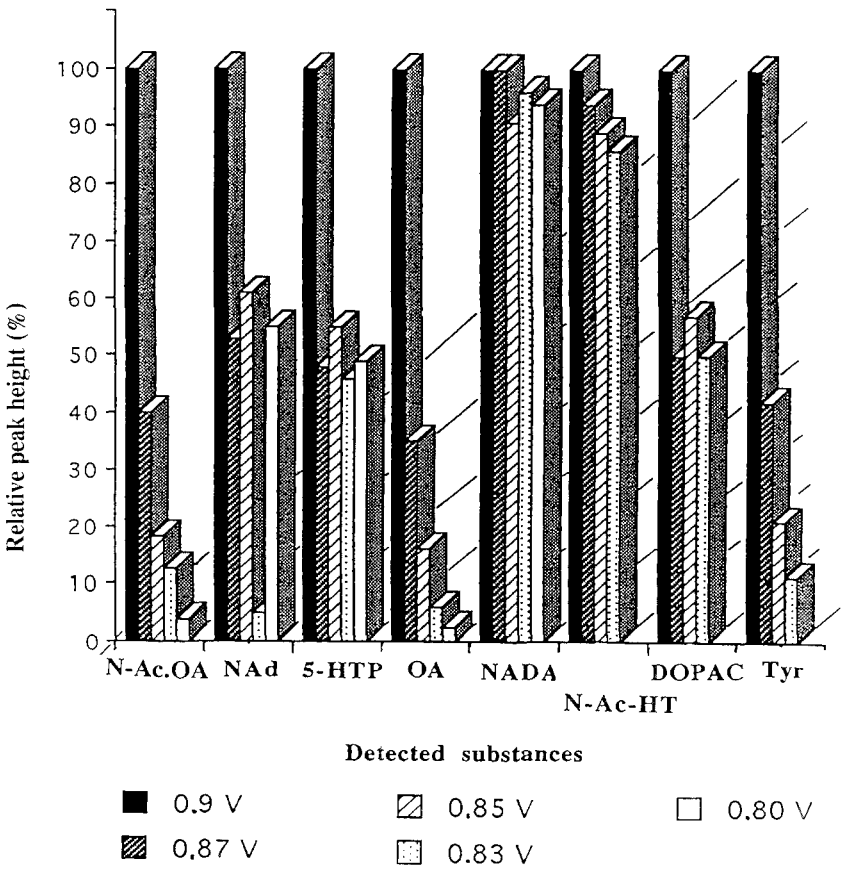

Figure 3 : Percentage response of different substances at different electrode potentials expressed as peak height relative to the peak height of the same substance at 0.9 volts.

Table III : Amine detection levels (ng/ml) of standard solution and correspondent concentrations in biological samples (1 mg of nematode / 50 µl buffer) at 0.90 and 0.83 V.

Reference substances	Standard solution (ng/ml)		Nematode samples (ng/g)	
	0.90 V	0.83 V	0.90 V	0.83 V
NAD	0.44	0.4	22	20
5-HTP	0.29	0.31	14.5	15.5
DOPAC	0.46	0.46	23	23
DA	0.53	0.49	26.5	24.5
5-HIA	0.53	0.58	26.5	29
Trp	4	44	200	2200
5-HT	0.83	0.85	41.5	42.5
N-Ac.OA	30.77	108.11	1538	5.405
OA	9.75	66.66	487.5	3333
NADA	5.88	3.071	294	153.5
N-Ac-5-HT	6.25	3.51	312.5	175.5
Tyr	3.85	16.66	192.5	833

Detection response expressed in ng of amines / ml of injected solution are listed for 0.90 V and 0.83 V in **Table III** which shows the detector responses to typical concentrations in biological samples on the basis of 1 mg of nematode / 50 µl buffer.

Assay in biological samples :

Chromatogram of a nematode sample is illustrated in **Figure 4a** (0.90 V), and detection levels are listed in **Table III**.

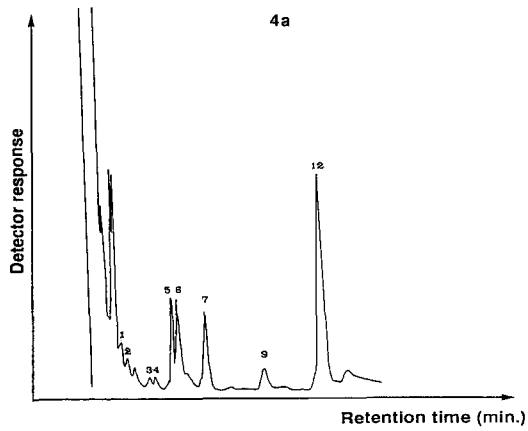

Figure 4 a : Chromatogram of the nematod sample

1. N-acetyloctopamine ; **2.** noradrenaline ; **3.** 5-hydroxytryptophan ; **4.** octopamine ; **5.** 3,4-dihydroxyphenylacetic acid ; **6.** N-acetyldopamine ; **7.** dopamine ; **9.** N-acetylserotonin ; **12.** tryptophan ; the other compounds were not detected in this sample.

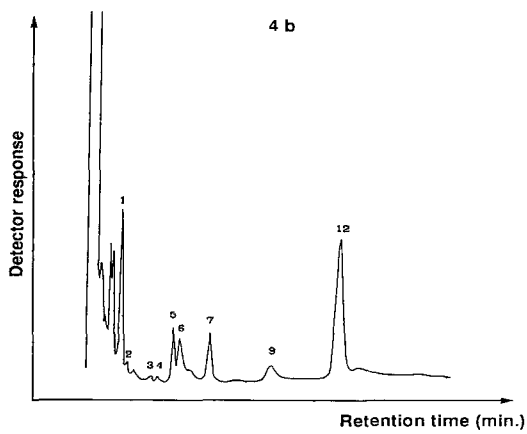

Figure 4b : Chromatogram of 10 µl of nematod sample mixed with the same volume of a solution of N-acetyloctopamine (2 µg/ml, 20 ng).

N-Ac.OA was detected in biological samples by *(i)* comparison of sample peaks with those from a mixture of reference substances with the same retention times as N-Ac.OA at different detection potentials; *(ii)* by spiking samples with a solution of N-Ac.OA (10 µl sample added to 10 µl of a 2 µg/ml solution of N-Ac.OA) (**Figure 4b**). The limit of sensitivity at 0.9 V was around 31 ng/ml of the solution.

DISCUSSION AND CONCLUSION

N-Ac.OA, a non-commercially available amine, was detected for the first time in a chromatographic system enabling quantitative analysis in biological samples. Determination of this amine in invertebrates is important since OA is of considerable physiological significance [2]. In insects, octopamine is released into the hemolymph in response to stress, and it has been shown to have myogenic properties in both insects and nematodes.

The interest of the used method is the simultaneous detection of various biogenic amines. One may remarks that the detection system is more sensitive to amines found in vertebrate nervous system and detection thresholds are lower for compounds such as Trp, 5-HTP, 5-HT, 5-HIAA, DA, DOPAC, 3-MT, HVA. Improvements can certainly be made for detection of compounds more specific to invertebrates such as tyramine, octopamine and N-acetylated derivatives. It should be noted that the levels of NAd and OA must be taken into account as they are often found at high concentration in invertebrates and may mask the N-Ac.OA peak. In such cases, samples will require appropriate dilution.

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INFLUENCE OF THE MICROBIAL STARTER AND THE BREADMAKING STEP ON THE FREE AMINO ACID PROFILES OF WHEAT SOURS, DOUGHS, AND BREADS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

C. COLLAR AND M. A. MARTÍNEZ-ANAYA
Laboratorio de Cereales
Instituto de Agroquímica y Tecnología de Alimentos
Jaime Roig, 11
46010 Valencia, Spain

Abstract

Reversed-Phase High Performance Liquid Chromatography profiles of twenty-two free amino acids from sour doughs (240 dough yield, 35 °C, 20 h, 1.1% ash content), bread doughs (17.5% sour dough addition, 0.5-0.6% ash content) and breads respectively inoculated with *Lactobacillus brevis*, 25a (with and without yeast) and *Lactobacillus plantarum*, L-73 and B-39 (without yeast) are investigated. Interactive influence of both the microbial composition of the starter and the breadmaking steps are observed on the individual amino acid pattern. After proofing, a significant decline is noticed in dicarboxylic acids and amides, Ser, and hydrophobic amino acids as well as in total amino acid content in all samples. The extent in the amino acid assimilation by lactobacilli and mainly by yeast is closely concerned with the strain of lactobacilli. In general, homofermentative lactobacilli induce bigger decreases. In addition, aromatic amino acids noticeably deplete during fermentation of both unsoured doughs and doughs started with *L. plantarum* strains. Predominant amino acids such as Ala and GABA are sharply promoted as a consequence of enzymatic reactions. Baking generally induces a significant increase in main amino acids except in unsoured doughs and doughs started with L-73. The presence of yeast in the microbial composition of the heterofermentative bacterial starters (25a) leads to greater increases in amino acid levels during baking.

Introduction

The positive effects of sour dough addition in breadmaking performance and in extension of bread microbial shelf-life have been extensively supported in the literature for rye (1-2) and wheat (3-7) systems, and related to the production of suitable metabolites by the starting microflora. Sour dough improving action is associated with the equilibrated balance between ethanolic and lactic fermentation (1) and depends on many interactive factors -microbial starter composition (4, 6, 8), sour dough process (9), processing conditions (3, 10-12) and ingredients (8)- that regulate the metabolism of starting yeast and lactic acid bacteria.

In wheat products, sour dough plays a dual role as rising and acidifying agent to promote both aroma and volume of bread (5). Consequently, in addition to acidification degree, active metabolites such as amino acids are of interest since they serve as an important source of bread flavor precursors (13), are involved in metabolism of dough microorganisms (14-17), and improve sour dough functionality (18) and bread dough machinability (19).

During sour dough fermentation of both rye and wheat systems, amino acid metabolism regarding nutritional requirements and involved enzymatic activities, depends mainly on the microbial starter composition (20-23) and on some processing conditions (13, 17, 24). Concerning wheat sours, the extraction rate of flour and the fermentation temperature are reported as the main factors with positive influence on level of total free amino acids (24) and on extent of accumulation of hydrophobic and basic amino acids during fermentation (17). Incorporation of sour doughs to bread doughs affects initial levels of amino acids in doughs depending on the sour dough starter composition (25) since microbial mass of microorganisms constitutes a source of amino acids (23). As well, qualitative and quantitative composition of amino acids of fermented doughs affects intensity and typical bread flavor mainly through the formation of specific Maillard compounds during baking.

The influence of the microbial starter on the individual amino acid pattern has been investigated in rye (13, 26) and wheat (17, 24) sour doughs so as in wheat straight doughs and breads (15, 16, 19, 27, 28); but no systematic research has been carried out on free amino acid profiles of started wheat sours, doughs and breads despite the interest of the dynamics of these metabolites along breadmaking.

In this paper, a previously optimized methodology for the determination of 22 individual amino acids is successfully applied to wheat sours, doughs and breads started with homo and heterofermentative bacterial starters.

Experimental

Reagents and chemicals. Amino acids (AA, Sigma grade), dansylaminoacids, and dansyl chloride were purchased from Sigma. Acetonitrile (ACN, HPLC grade) and water (chromatography-grade) were furnished by Merck; acetone (ultraviolet grade), was from Panreac. Buffers were prepared from analytical-grade chemicals. Solvents were filtered with an Afora filter holder, and samples, with a Swinney filter (Millipore). Millipore HA-grade 0.45-μm filters and Millipore FH-grade 0.22-μm filters were used for aqueous solvent and ACN filtration, respectively.

HPLC equipment. A Hewlett-Packard (Palo Alto, CA) HPLC system, composed of a 1050 pumping system, a rheodyne injector, a 1040 A diode array detector set at 254 nm, a 9000 Pascal Chem Station and an oven set at 30 °C, was used.

Chromatographic conditions. An ODS HS/3 column (3 μm particle size, 8.3 cm length, 4.6 mm i. d.) and a precolumn (3 μm, 3 cm, 4.6 mm) both from Perkin-Elmer (Norwalk, CT) were used as stationary phase. Mobile phase consisted of buffer 12 mM K_2HPO_4, pH 7 (A) and ACN (B). Gradient program was from 10% to 43.6% B in 42 min,

up to 70% B in 3 min, and isocratic elution during 10 min for washing purposes. Flow rate was 1.50 mL/min.

Sample preparation. Three lactobacilli strains (*Lactobacillus brevis*, 25a with (+) and without (-) yeast, and *Lactobacillus plantarum*, L-73 and B-39 without yeast) were used as starters. Composition of microbial starters (propagated cultures: CHR. Hansen's Laboratorium, Denmark A/S), analytical data on flours used (supplier: Federal Center for cereal, potato and lipid research, Detmold, Germany), and conditions for sour dough (FSD) preparation and dough formula are summarized in Table I. Control doughs (without starter) were also prepared for comparative purposes. The German Standard method "Kastengebächversuch" was used for baking test (5). Briefly, doughs were mixed at 26-27 °C (UBD); after dough resting (20 min), scaling (700 g), hand rolling and intermediate proofing (10 min), doughs were panned and proofed (FBD) (60 min, 32°C, 75-80% RH) before baking (B) (240°C, 40 min).

Extraction, purification and chromatographic determination of free AA. Freeze-dried FSD, UBD, FBD (10 g) and B (5 g) were each extracted twice with 20 mL 40% ethanol (v:v) (25). After centrifugation at 23 000 g for 20 min at 1-3°C, supernatants were made up to 50 mL. Aliquots of extracts were deproteinized by ultrafiltration (cut-off 10 kDa, Millipore cartridges). Protein-free extracts were brought to pH 2.0 and applied onto a column packed with 5 mL Dowex 50W-X2 resin (50-100 mesh, H^+ form), previously equilibrated with 0.01 M HCl (16). The ammonia eluates were evaporated to dryness in a rotary evaporator, Dried extracts were dansylated (28) and used for AA determination (15, 27) using the internal standard addition method for AA quantification. A standard mixture of 20 protein AA, γ-aminobutyric acid and ornithine were dissolved in 0.01 N HCl and made up to 200 mL. Norvaline was used as the internal standard (69 mg/200 mL 0.01 HCl). Aliquots of the AA standard mixture and purified sample extracts, containing approximately 50 μg of amino nitrogen were placed in small vials, and the internal stardard (0.1 mL) was

Table I.- Starters and flours used for sour and bread dough preparation.

STARTER COMPOSITION AND FLOUR CHARACTERISTICS		
lactobacilli	**strain**	**cfu/g**
Lactobacillus plantarum	L-73	2.0×10^{10}
Lactobacillus plantarum	B-39	5.0×10^{11}
Lactobacillus brevis	25-a	6.0×10^{11}
yeast	**cells/g**	
Instant Active Dried Yeast (IADY)	2.7×10^{10}	
flour	**sour dough**	**bread dough**
energy of deformation ($\times 10^3$ ergs)	-	233
curve configuration ratio	-	0.92
ash (%, mb)	1.11	0.54
moisture (%)	12.22	14.79
Hagberg Index	-	360
SOUR DOUGH CONDITIONS		
dough yield	240	
fermentation time	20 h	
fermentation temperature	35 °C	
bacterial inoculum	10^7 bacteria/g flour	
yeast inoculum (25-a+)	10^6cells/g flour	
DOUGH FORMULA		
sour dough (%, flour basis)	17.5	
flour (g)	1000	
ascorbic acid (mg)	50	
malt flour (g)	1.8	
water (ml)	620	
vegetable fat (g)	10	
sugar (g)	10	
salt (g)	15	
IADY (g)	20	

respectively added. Samples were evaporated to dryness in a heating-stirring module coupled to an evaporating unit (Pierce, Rockford, IL) and redissolved in 0.5 mL of 0.1 N K_2CO_3 buffer, pH 10.5, followed by addition of 0.2 mL of dansyl chloride (Dns-Cl) solution (0.5 g/25 mL of dry acetone) and 1.0 mL of dry acetone (final pH 9.3- 9.6). The mixture was heated at 100°C for 2 min., then evaporated to dryness, and redissolved in 1.7 mL of initial mobile phase. Aliquots (20μ L) of each sample and standard were injected into the HPLC system.

Statistical evaluation. Two paralell experiments and three replicates per experiment were made. Data of individual amino acid levels in FSD, UBD, FBD and B were statistically analyzed in a Microvax computer (Digital, Northboro, MA) using programs of the BioMeDical statistical Package (BMDP). Univariate analysis (two-ways analysis of variance) was performed to calculate significant interactions (starter x breadmaking step) by applying 7D program (Tukey test). Multivariate data handling procedures (factor and K-means clustering analysis) were conducted in order to classify samples on the basis of the significant grouping individual amino acids, by applying respectively 4M and KM programs.

Results and Discussion

Chromatographic separation and quantification of amino acids

Previously optimized RP-HPLC experimental conditions (28) lead to the complete separation of 23 amino acids (20 protein AAs, GABA, Orn and Nval as internal standard) in a standard mixture and in FSD, UBD, FBD and B samples, in less than 37 min (Figure 1).

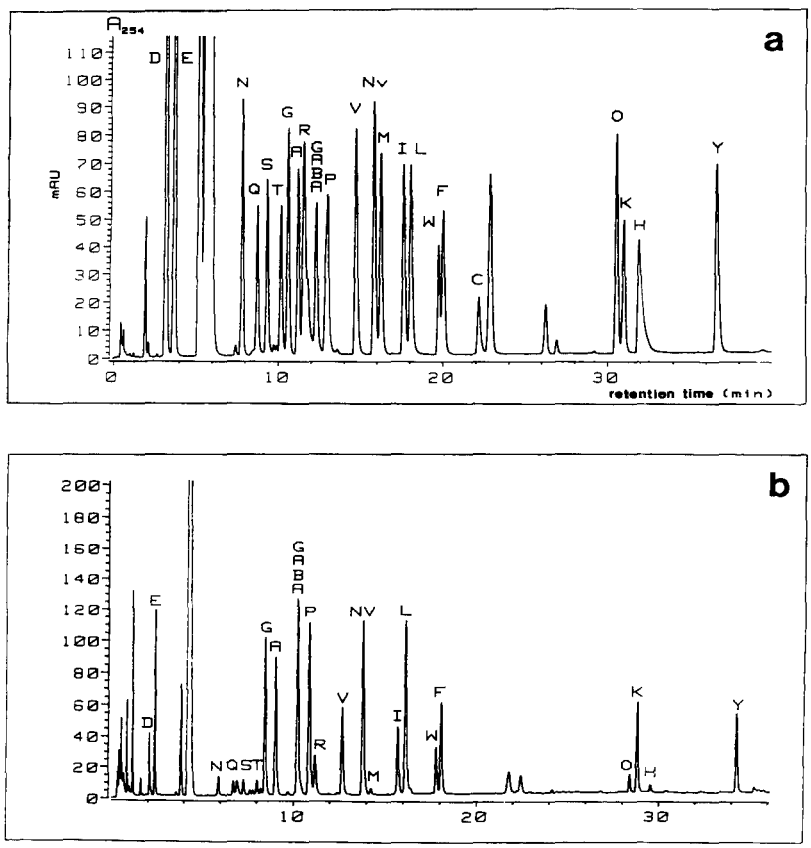


Figure 1.- Separation of dansylamino acids (Dns-AA) of a standard mixture (a) and of fermented sour dough samples started with *Lactobacillus brevis*, 25a+ (b), by reversed-phase high-performance liquid chromatography. Concentration of Dns-Cl [Dns-Cl] and molar ratio [Dns-Cl]/[AA]= 8.5 mM, 4.6. Column: ODS (3 μm particle size, 8.3 cm length, 4.6 mm i.d.). Mobiles phases: A= 12mM K_2HPO_4, pH 7.0; B= ACN. Linear gradient, 10 to 43.6% B over 42 min; flow rate: 1.5 mL/min; injection volume: 20 μL; column temperature: 30 °C. Dns-AAs: (D) Asp, (E) Glu, (N) Asn, (Q) Gln, (S) Ser, (T) Thr, (G) Gly, (A) Ala, (GABA) γ-amino butyric acid, (P) Pro, (R) Arg, (V) Val, (M) Met, (I) Ile, (L) Leu, (W) Trp, (F) Phe, (C) Cys, (O) Orn, (K) Lys, (H) His and (Y) Tyr.

Good resolution and reproducibility are achieved in the separation of individual AAs. Coefficients of variation (CV) for relative retention times in standards and samples range from 0.10 and 1.30%, being less than 1% for most AA. Variability in response factors (f) relative to Nval and in AA quantification are less than 5% except for Thr, Tyr and Arg (<9%). Maxima diode array detector (DAD) responses correspond to Gly (f= 0.5713), Orn (f= 0.6033) and Lys (f=0.6347); minima are for Cys (f= 3.2076), Arg (f=2.6836) and Thr (f= 2.3913). Peak purity checked through specific DAD software and by comparison of UV spectra at different wavelength is higher than 99.5% (match > 995) for all AA except for Cys and Met (match= 900) when present in trace amounts.

Interactive effects of microbial starter and breadmaking step on the free amino acid profile of samples

Data of individual and total amino acid content of sours, doughs and breads are summarized in Table II. Interactive effects between type of starter (B-39, 25a-/+, L-73) and type of sample (FSD, UBD, FBD, B) are observed on most AA. Among the pool of samples, total amino acid content (mg/ 100 g sample, dry basis) ranges from 167.25 mg (B-39-) to 216.19 mg (L-73-) within FSD, from 86.25 mg (control, 25a+) to 125.99 mg (L-73-) within UBD, from 58.93 mg (control) to 107.59 mg (25a-) within FBD, and from 66.26 mg (control) to 123.03 mg (25a+) within B. Prominent AA include Glu, Ala, GABA, and Pro in all samples; and Arg, Ile, Leu, Trp, Phe and Lys in FSD. In an opposite way, Cys and Met are minor AA, sometimes present in trace amounts.

Fermentation and baking steps influence the individual and total AA levels depending on the microbial composition of started sour doughs (Table II). After prooving, in general, a significant ($P < 0.05$) decline is observed in dicarboxylic acids and amides, Ser and hydrophobic AA as well as in total amino acid content in all doughs (Figure 2). As it was

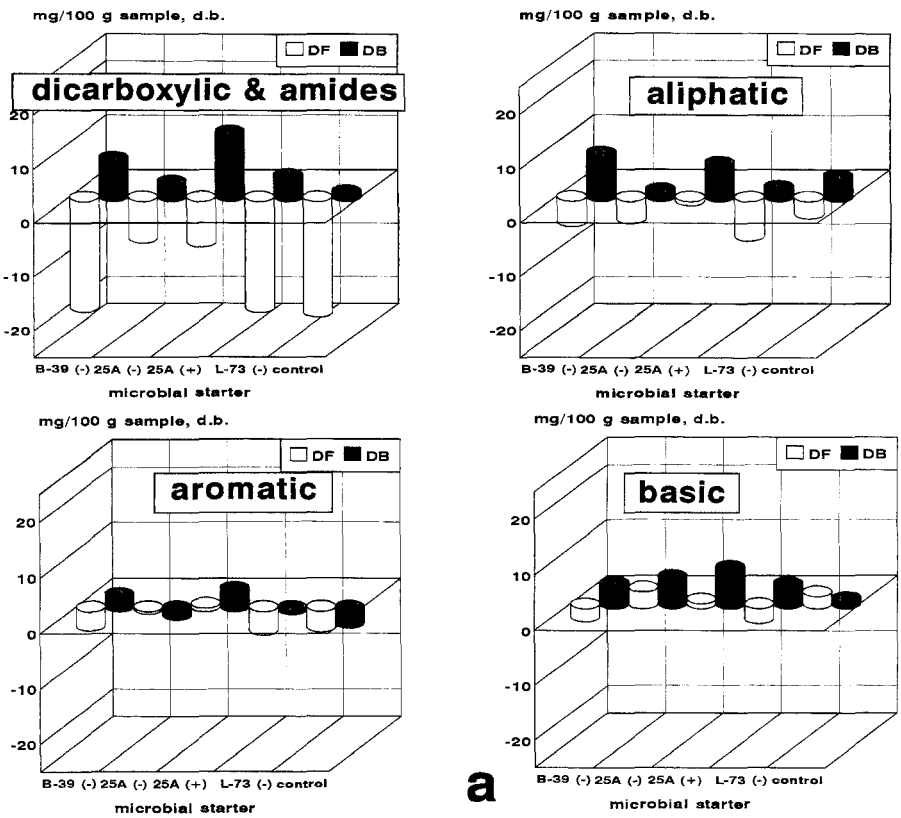


Figure 2.- Changes in main (a), minor (b) and total (c) free amino acid groups during fermentation (DF) and baking (DB).

(continued)

previously reported (16), the extent in the AA assimilation by lactic acid bacteria and mainly by yeast is closely concerned with the strain of lactobacilli in good accordance with their nutritional requirements on AA (14). In general, homofermentative lactobacilli (B-39, L-73) induce bigger decreases during proofing than the heterofermentative lactobacillus 25a (+/-) (Table II). In addition, aromatic AA noticeably decrease during fermentation of control

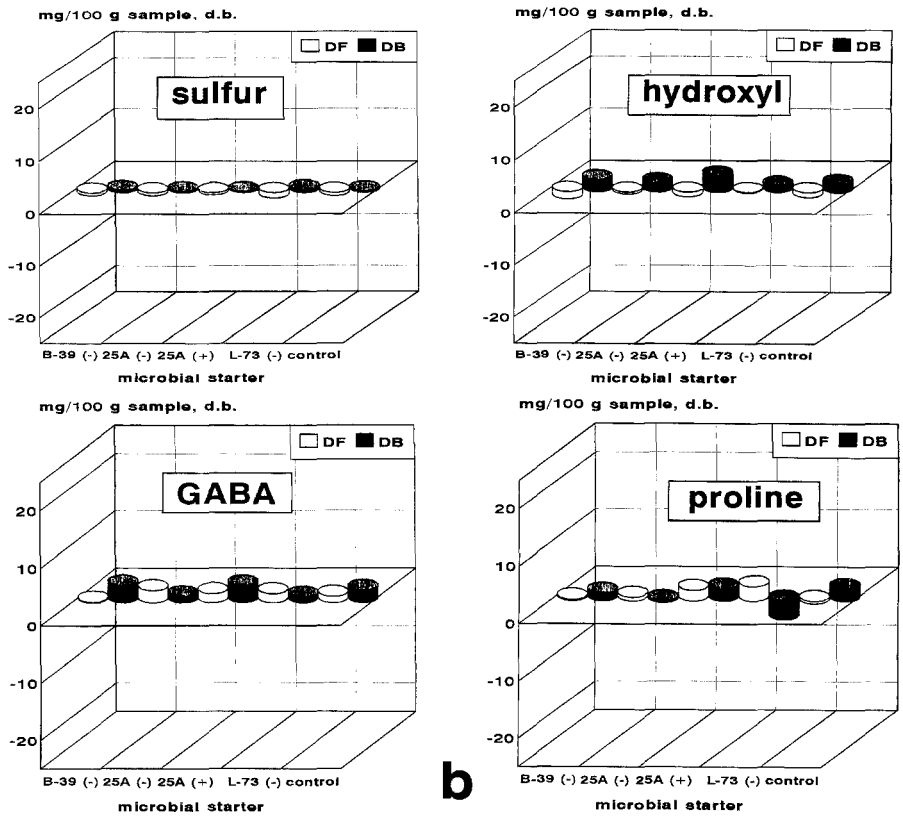
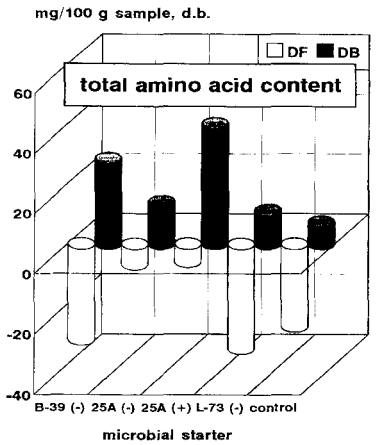


Figure 2 (Continued)

doughs and of doughs started with *L. plantarum* strains (Figure 2). On the other hand, predominant AAs such as Ala and GABA are sharply promoted as a consequence of enzymatic reactions. Protein AAs accumulate by exoproteolysis (13) because lactic acid bacteria no longer metabolize AAs so rapidly. GABA (25a, L-73) increases as a consequence of the effect of glutamate decarboxylase (29) as there is a simultaneous reduction in Glu content. Orn, which is involved in the biosynthetic pathway of Arg (30), is released particularly in 25a started doughs, probably from the microbial mass (25).



C

Figure 2 (Continued)

Baking generally induces a significant ($P < 0.05$) increase in main AAs except in control doughs and in doughs started with L-73 (Figure 2). This observation should be attributed to an hydrolytic thermal degradation and to an intense proteolysis of proteins favoured by the acidic pH (4.43-4.56) of soured fermented doughs during oven spring. The net AA increase masks their utilization in non-enzymatic Maillard reactions. The protein degradation is not so intense in control doughs (pH 5.57) in which the reaction between AAs and sugars during baking to form melanoidins becomes more evidenced. Concerning heterofermentative 25a as starter, the presence of yeast in its microbial composition (25a-, 25a+), leads to the biggest increases in amino acid levels during baking (Figure 2).

Starter composition does not significantly influence the levels of Ser, Thr and Cys in all samples, leading to similar amounts in both soured and control samples (< 4 mg). Within FSD, homofermentative B-39 started samples show the highest amounts of Ala (9.58 mg) and

Table II.- Individual and total amino acid content of unstarted and started sours, doughs and breads.

starter	sample	MEAN CONTENT OF INDIVIDUAL AND TOTAL FREE AMINO ACIDS*																					
		Asp	Glu	Asn	Gln	Ser	Thr	Gly	Ala	GABA	Pro	Arg	Val	Met	Ile	Leu	Trp	Phe	Cys	Orn	Lys	His	Tyr	TAA
Lactobacillus plantarum B-39 (-)	fermented sour dough	5.22	8.22	8.27	4.01	3.73	2.70	4.71	9.58	16.04	6.50	21.68	8.87	1.79	7.03	18.28	8.70	10.84	0.54	0.24	8.71	3.04	7.75	167.25
	unfermented bread dough	6.59	19.81	5.33	4.93	2.32	2.12	2.07	6.37	5.79	11.05	9.35	4.36	0.70	2.80	4.29	7.18	3.07	0.37	0.80	4.11	0.87	3.04	106.26
	fermented[a] bread dough	1.99 **	10.03 **	1.46 **	2.56 **	1.06 **	1.99	2.45	7.89 **	5.95	11.27	8.00	3.20	0.30	0.66 **	0.94 **	6.49	1.15 **	0.23	0.77	3.26 **	0.74	2.16 **	74.50 **
	bread[b]	**4.20 ** **	**14.36 ** **	**2.77 ** **	**2.03 ** **	**2.12 ** **	**3.17**	**3.18**	**10.62 ** **	**8.86 ** **	**12.59 ** **	**11.06**	**4.23**	**0.42**	**2.18 ** **	**3.21 ** **	**6.40**	**2.58 ** **	**0.53 ** **	**1.43 ** **	**3.10**	**1.05**	**3.18 ** **	**102.93 ** **
Lactobacillus brevis 25a (-)	fermented sour dough	6.53	9.60	8.52	3.77	5.71	2.82	5.89	6.83	18.99	8.49	5.58	11.04	2.58	10.61	23.08	9.76	14.40	0.67	9.06	4.37	3.71	3.12	174.59
	unfermented bread dough	7.08	17.30	5.52	6.17	2.45	1.73	2.20	7.06	6.06	12.33	1.93	4.92	1.01	4.07	7.62	7.91	4.86	0.24	2.34	2.69	0.66	2.35	107.59
	fermented[a] bread dough	5.66	15.36 **	3.16 **	4.05 **	1.80 **	2.00	2.68	9.28 **	8.16 **	12.97	2.50	4.72	0.54 **	1.98 **	3.04 **	7.99	3.96	0.12	4.01 **	3.42 **	0.75	2.75 **	100.80
	bread[b]	6.57	17.38 **	4.37 **	2.88 **	2.09 **	3.39	2.90	10.44 **	9.21 **	12.70	8.49 **	4.75	0.38	3.51 **	1.59 **	6.60 **	3.65	0.49 **	3.43	2.76	1.04	2.93 **	114.95 **
Lactobacillus brevis 25a (+)	fermented sour dough	5.43	10.45	5.54	4.18	4.08	2.70	6.45	8.12	20.88	10.42	3.25	13.43	2.93	12.34	26.52	11.19	17.02	0.40	9.81	4.89	2.47	3.27	186.08
	unfermented bread dough	6.14	14.42	4.54	6.64	2.24	1.97	1.83	5.47	4.87	8.11	1.98	4.10	0.82	2.75	5.29	5.65	3.56	0.28	2.70	2.48	0.98	1.55	88.81

	fermented[a] bread dough	4.21	11.80 **	2.77 **	4.38 **	1.49	1.82	2.00	7.97 **	6.43 **	9.94 **	2.64	4.12	0.45 **	1.72 **	2.89 **	5.94	3.38	0.25	3.20	2.88	0.32 **	2.07 **	82.82 **
	bread[b]	8.03 **	18.69 **	5.25 **	3.36 **	2.87 **	3.49 **	2.95 **	10.71 **	9.31 **	12.02 **	7.34 **	5.04	0.43	3.10 **	3.37	7.17 **	4.69 **	0.41	4.22 **	2.94	1.25 **	2.93 **	123.03 **
Lactobacillus plantarum L-73 (-)	fermented sour dough	5.72	7.85	9.11	4.92	1.40	2.67	5.31	8.80	20.62	10.72	25.98	12.15	2.95	9.87	30.33	13.83	17.98	0.53	0.37	9.86	2.85	12.41	216.19
	unfermented bread dough	7.04	18.36	6.76	6.91	1.77	2.08	2.24	7.49	6.76	13.00	10.35	5.48	1.15	3.61	8.13	8.45	5.15	0.34	1.09	4.72	1.36	3.84	125.99
	fermented[a] bread dough	2.17 **	11.52 **	1.80 **	2.81 **	1.33	2.38	3.49 **	10.82 **	8.31 **	15.51 **	8.18 **	3.51 **	0.18 **	0.75 **	0.98 **	8.01	1.96 **	0.27	1.11	4.61	0.90 **	3.14	91.31 **
	bread[b]	3.86	14.57 **	2.28	1.81	1.61	3.26	3.37 **	10.62	9.29 **	12.26 **	13.02 **	4.80	0.51	0.98	1.75	6.44 **	2.59 **	0.47	1.18	3.21	1.16	3.57	102.56 **
CONTROL	unfermented bread dough	7.88	20.25	6.31	5.82	2.13	1.86	1.55	5.74	4.31	9.74	2.68	2.18	0.32	1.37	1.43	6.16	1.04	0.63	0.92	1.81	0.58	1.53	86.25
	fermented[a] bread dough	4.98 **	9.46 **	0.66 **	3.53 **	1.24 **	1.80	2.03 **	3.96 **	5.39	9.31	4.78	2.10	0.23	0.32 **	0.65	4.28 **	0.28 **	0.11 **	0.68	2.34	0.39	0.38 **	58.93 **
	bread[b]	3.49	13.65 **	1.14	1.47 **	1.75	2.88	2.32	5.95 **	7.48 **	11.42 **	5.62	3.37	0.27	0.55	0.94	0.59 **	0.45	0.32	0.82	2.12	0.73	0.85	66.26

(*) mg amino acid/100 g sample, d.b.

TAA= total amino acid content

(**) Indicates a statistically significant change between mean values ($P < 0.05$) for fermentation (a) and baking (b) steps when 2nd order interactions (starter / breadmaking step) are significant.

the lowest in Glu (8.22 mg), Gly (4.71 mg), GABA (16.04 mg), Pro (6.50 mg), Val 8.87 mg), Ile (7.03 mg), Leu (18.28 mg), Trp (8.70 mg) and Phe (10.84 mg). Heterofermentative 25a accounts for greater amounts of Glu (9.60 mg) and Ser (5.71 mg), being hydrophobic AAs promoted in presence of yeast (25a+). Within UBD, inoculated doughs show higher levels of individual and total AAs except in Asp, Glu and Asn, when data are compared with control doughs. In general, homofermentative lactobacilli (L-73, B-39) lead to higher levels of AAs than heterofermentative (25a+). The presence of yeast significantly decreases the amount of AAs. FBD including uninoculated samples do not statistically differ in addition in Gly (2.0-3.5 mg), Met (0.2-0.5 mg) and His (0.3-0.9 mg) contents, but soured samples show higher levels of free AAs than control doughs with the exception of Asp content (4.98 mg). *L. plantarum* strains (L-73, B-39) leads to similar AA levels, and lower than *L. brevis* starters (25a-, 25a+). Differences in AA pattern are minimized in B samples. Inoculated samples account for greater amounts of AAs than control breads, but similar levels of Ser (1.4-2.9 mg), Thr (2.9-3.5 mg), Gly (2.4-3.4 mg), Pro (11.4-12.7 mg), Met (0.3-0.5 mg) and Cys (0.3-0.5 mg) are observed. In addition, soured samples follow the same profile of Ala (10.4-10.7 mg), GABA (8.9-9.3 mg), Val (4.2-5.0 mg), Trp 6.4-7.2 mg), Lys (2.8-3.2 mg), His (1.0-1.3 mg) and Tyr (3.0-3.6 mg). Within homofermentative lactobacilli, B-39 induces higher hydrophobic AA content; and with heterofermentative lactobacillus 25-a, the presence of yeast increases dicarboxylic acids and amides (+13%) as well as Orn contents (+23%). Levels of Asp, Glu, Asn, Gln, Orn, Ile and total AAs are more prominent in breads started with hetero than with homofermentative lactobacilli (Table II).

Classification of samples according to the amino acid pattern

Multivariate data handling procedures (factor and K-means clustering analysis) are applied in order to classify samples according to the effects of microbial starter and type of

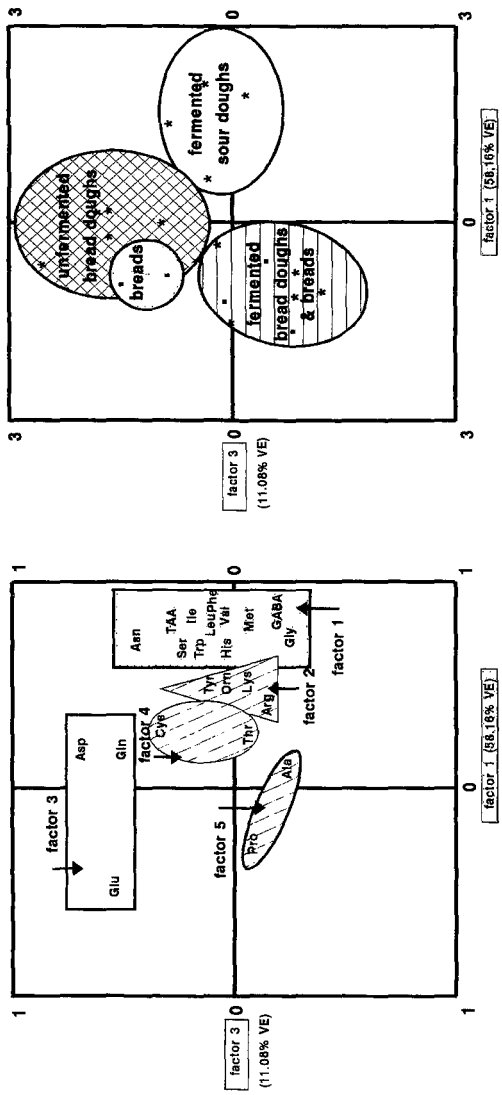


Figure 3.- Distribution of samples (N=19) in the plane defined by factor 1 vs. factor 3 (factor analysis) on the basis of their individual amino acid pattern (N=23).

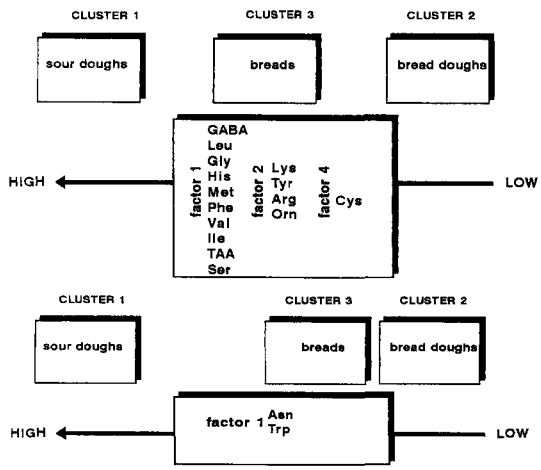


Figure 4.- Trends of individual amino acids of samples distributed in three prefixed clusters (K-means clustering).

sample on the significant grouping individual AAs. When absolute values for analytical variables (Nr= 23) are considered for FSD, UBD, FBD and B samples (Nr= 19), three factors account for 84% of the total cumulative variance (VE) (Figure 3a). Factor 1 (58.16% VE) positively correlates with Val, Phe, Ile, Met, Leu, GABA, TAA, Gly, His, Trp, Asn and Ser (loadings over 0.600); factor 2 (15.16% VE) is closely connected with Arg, Tyr, Lys (positive correlation) and Orn (negative correlation), and factor 3 (11.08%) relates with Asp, Glu and Gln contents. The most suitable classification of samples was obtained after plot of scores of factor 1 vs factor 3 (Figure 3b). Three groups are outlined: (a) FSD (4 cases) showing the higher levels of AAs of factor 1 and medium content of AAs of factor 3, (b) UBD and B (25a) (7 cases) characterized by high levels of AAs of factor 3 and medium contents of AAs of factor 1, and (c) FBD and B (8 cases) defined by low levels of AAs of factor 1 and factor 3. Maximum and minimum values of AAs of factor 1/factor 3

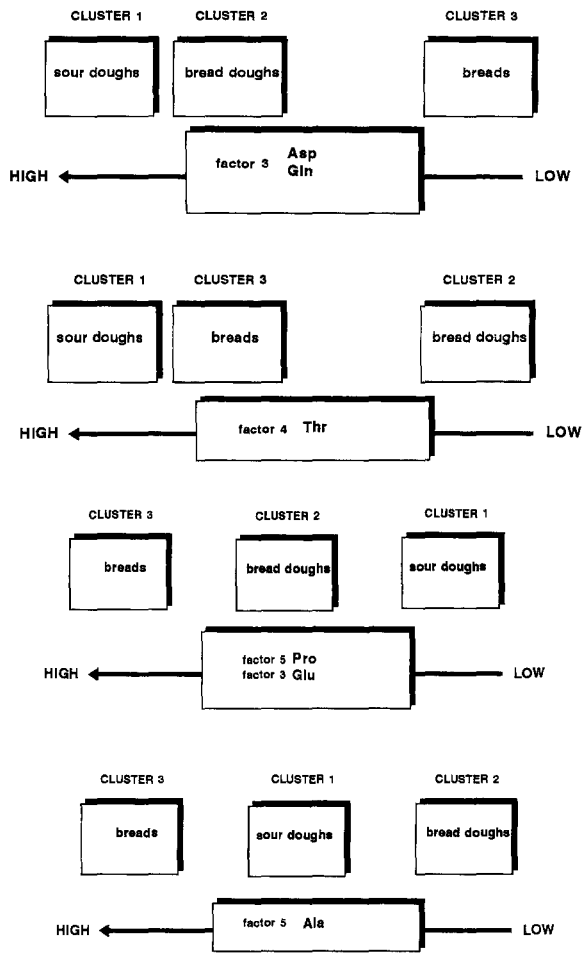


Figure 4 (continued)

correspond respectively to FSD (25a+)/ UBD (control), and B (control)/ FBD (B-39) (Table II).

Sample distribution from factor analysis is in good accordance with AA profiles of samples after K-means clustering analysis of data (Figure 4). In three predefined clusters, the pool of samples is grouped according to samples included in each breadmaking step: FSD (cluster 1), UBD and FBD (cluster 2) and B (cluster 3). FSD account for maximum levels of main AA (factors 1-4), minimum amounts of Pro (factor 5) and Glu (factor 3) and intermediate values for Ala (factor 5). UBD and FBD reach the lower values of main AAs (included in factors 1, 2, 4 and Ala) and intermediate contents of Pro and Glu. B samples characterize by higher amounts of Pro, Glu and Ala, lower in Asp and Gln and intermediate values for main AA. Similarities among samples are also observed: BD and B (Asn, Trp), SD and B (Thr), and SD and BD (Asp, Gln).

Results ponted out that almost all individual AAs play a significant role in characterizing breadmaking samples. Breadmaking step is a separative factor more relevant than the type of starter in the classification of samples.

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Appendix

Key to Abbreviations

RP-HPLC	reversed-phase high performance liquid chromatography
AA	amino acid

FSD	fermented sour dough
UBD	unfermented bread dough
FBD	fermented bread dough
B	bread
Dns-Cl	dansyl chloride
Dns-AA	dansyl amino acid
Asp	aspartic acid
Glu	glutamic acid
Asn	asparagine
Gln	glutamine
Ser	serine
Thr	threonine
Gly	glycine
Ala	alanine
GABA	γ -aminobutyric acid
Pro	proline
Arg	arginine
Val	valine
Nval	norvaline
Met	methionine
Ile	isoleucine
Leu	leucine
Trp	tryptophan
Phe	phenylalanine
Cys	cystine
Orn	ornithine
Lys	lysine
His	histidine
Tyr	tyrosine
A_{254}	absorbance at 254 nm
mAU	absorbance milliunits
Rt	retention time
CV	coefficient of variation
f	response factor
DAD	Diode Array Detector
UV	ultraviolet
TAA	total amino acids
ACN	acetonitrile
VE	variance explained

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ELUCIDATION BY REVERSE PHASE HPLC OF SOME CITRUS FLAVANONES AND THEIR RESPECTIVE DIHYDROCHALCONES: STRUCTURAL STUDY

J. A. DEL RIO, J. CASTILLO, AND O. BENAVENTE-GARCÍA
Department of Plant Biology
Faculty of Biology
University of Murcia
Espinardo, 30071 Murcia, Spain

ABSTRACT

A high-performance liquid chromatographic method for the elucidation of the principal *Citrus* flavanone glycosides in biosynthesis and their respective dihydrochalcones has been developed. A C_{18} reverse phase column and an elution isocratic-gradient system is used with a mixture of water, acetonitrile and acetic acid. We describe the optimization process by studying the influence of the structural characteristics of these compounds on the quantitative chromatographic parameters: k', w and R.

INTRODUCTION

Flavonoids are secondary metabolites widely distributed through the plant kingdom, to which many roles have been attributed [1]. *Citrus* species are of great interest because accumulate high amounts of flavanone glycosides in their fruit and young tissues [2, 3, 4]. *Citrus aurantium* has the widest variety of flavanones of all the citric species of the spanish levante. Among these of great importance from a biosynthetic point of view are: naringin, hesperetin 7-*O*-glucoside, neohesperidin and hesperidin [3, 4].

The key enzyme in flavonoid biosynthesis is chalcone synthase, which catalyses the condensation between the ester CoA of *p*-coumaric acid and 3 molecules of malonylCoA, forming the structure known as chalcone, which makes up the basic skeleton of all known groups of flavonoids [5]. These chalcones are very unstable compounds and until now it has been impossible to isolate and quantify them in the proportions in which they are found during flavonoid biosynthesis in plants.

Some authors consides that chalcones cycle spontaneously as a consequence of a chemical equilibrium

with their corresponding flavanones, an equilibrium displaced towards the flavanones. However, without denying the existence of this displaced equilibrium, other authors have suggested that specific enzymes are responsible for chalcone cyclation, the so-called chalcone-cyclases or chalcone isomerases [6, 7, 8, 9]. Whatever the case, the instability of the chalcone type intermediaries makes it impossible to quantify them in a stationary state.

The selective catalytic hydrogenation of the double bond between the carbons in the α and β positions with the carbonile group of the chalcone makes it possible to obtain the corresponding dihydrochalcones. These are very stable compounds in the biosynthetic conditions of the plant and, by quantifying them, it is possible to indirectly quantify the chalcones in the stationary state of the chalcone structures.

As a previous step to developing this technique on extracts of *Citrus* tissue, the present work attempts to optimize an analytical method using HPLC which would permit a fast and accurate elucidation of the above mentioned flavanones and their respective dihydrochalcones.

MATERIALS AND METHODS

Chemicals

Naringin (N), hesperidin (H), hesperetin 7-*O*-glucoside (H7), neohesperidin (NH), naringin dihydrochalcone (NDC), hesperedin dihydrochalcone (HDC), hesperetin 7-*O*-glucoside dihydrochalcone (H7DC) and neohesperidin dihydrochalcone (NHDC) were obtained from Zoster S.A., Murcia, Spain. Dimethylsulphoxide (DMSO) was used as solvent [10], and the solution was filtered through a 0.45 μm nylon membrane.

Chromatographic Analysis

For the elucidation of flavanones and dihydrochalcones we used a μBondapak C_{18} (250 x 4 mm ID) analytical column with an average particle size of 5 μm. Several solvents were used and these are described in Results. In all the solvent systems the flow rate was 1 ml/min at room temperature.

HPLC analysis was performed using a Beckman liquid chromatograph with a Model 110B solvent-delivery module and a System Gold Module 168 diode array detector (Beckman Instruments, Inc, CA., USA). The absorbance change was monitored at 280 nm.

In order to carry a quantitative study, the elucidation capacity of the different mobile phases used was verified by determining the following parameters [11] for each flavonoid in each of the said phases:

a) Retention time (t_{Ri} = experimental value).

b) Capacity factor $k' = (t_{Ri} - t_o) / t_o$

t_o = mobile phase interstitial volume (void volume)/flow rate.

c) Width of the peak (w).

d) Resolution $R = 1/4(N)^{1/2}((\alpha-1)/\alpha)(k'/(k'+1))$

N = number of theoretical plates.

α = selectivity factor.

RESULTS AND DISCUSSION

Chromatographic Analysis Optimisation. Preparation of Flavonoid Mixture.

The structural study and the optimization of the procedure for analysing the flavanones and their respective dihydrochalcones mentioned in Materials and Methods (Chemicals) was realized by using a mixture of these substances in DMSO. This mixture contained (in 20

ml DMSO): naringin (N), 13 mg; hesperidin (H), 6 mg; hesperetin 7-*O*-glucoside (H7), 3 mg; neohesperidin (NH), 7 mg; naringin dihydrochalcone (NDC), 2 mg; hesperidin dihydrochalcone (HDC), 1 mg; hesperetin 7-*O*-glucoside dihydrochalcone (H7DC), 4 mg and neohesperidin dihydrochalcone (NHDC), 20 mg.

In a first step, HPLC elucidation was optimized by using isocratic systems in which only the proportions of the mobile phase components were varied [10]. Acetic acid (0.5 % v/v) was included in all mobile phases for to improve separation [10, 12, 13].

Mobile Phases with Water and Methanol.

Figure 1, shows how the order and the corresponding ln k' values of these flavonoids are affected by their degree of polarity according to the different substitution in the B ring [10]. The presence of the methoxyl group in position 4' also means that the 3'-hydroxy-4'-methoxy flavonoids (NH and NHDC) are less polar that the respective 4'-hydroxy flavonoids (N and NDC). An increase in the percentage of methanol in the mobile phase (Fig. 1) similarly affects the four structures represented, both flavanones and both

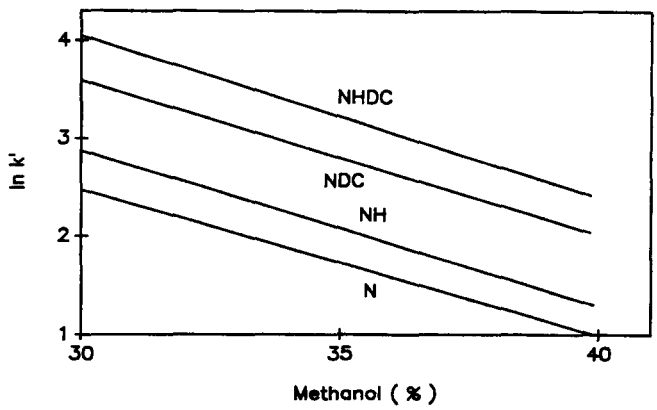


FIGURE 1. Variation of ln k' versus methanol percentage of mobile phases with water-acetic acid-methanol. N: Naringin; NH: Neohesperidin; NDC: Naringin dihydrochalcone; NHDC: Neohesperidin dihydrochalcone.

dihydrochalcones showing similar slopes for decreases in ln k' values. The dihydrochalcones eluted much later than the respective flavanones and so their k' values are higher.

Figure 2 shows variations in ln k' according to variations in the percentage of methanol in the mobile phase for the flavonoids hesperidin, neohesperidin, hesperetin 7-*O*-glucoside and their respective dihydrochalcones. The k' value of glucosylated structures undergoes a greater relative change than that

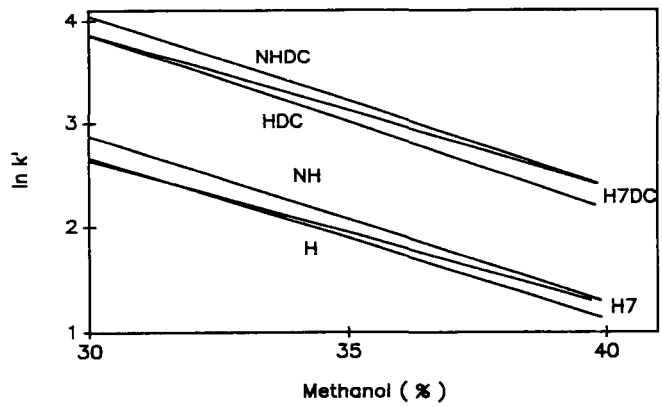


FIGURE 2. Variation of ln k' versus methanol percentage of mobile phases with water-acetic acid-methanol. H: Hesperidin; H7: Hesperetin 7-*O*-glucoside; NH: Neohesperidin; HDC: Hesperidin dihydrichalcone; H7DC: Hesperetin 7-*O*-glucoside dihydrochalcone; NHDC: Neohesperidin dihydrochalcone.

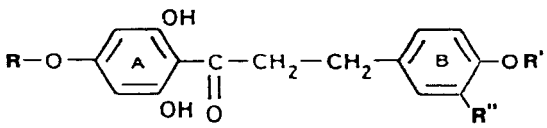
of the rhamnoglucosides; H7DC and H7, show a lower slope for the decrease of their ln k' values; these values are identical to the values of their rutinosides with a 30 and 32 methanol percentage, respectively, and are identical to those of their neohesperidosides with a 40 and 41 methanol percentage, respectively.

Furthermore, it can be seen that the k' values of hesperidin and neohesperidin show a similar decrease as the percentage of methanol in the mobile phase

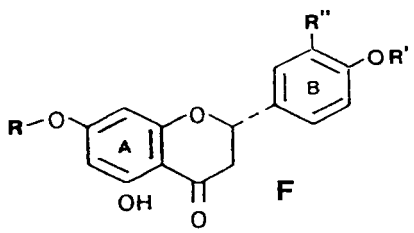
increases. The k' of the rutinosides is always below that of their corresponding neohesperidosides. The presence of 7-*O*-neohesperidoside substitution would affect the stability of the intramolecular hydrogen bond and the neohesperidoside structures would not be planar to rutinosides [14]. There would certainly be a greater hydrophobic interaction between the neohesperidoside molecules and those of the stationary phase of the column, which increase the k' value of these compounds compared with their respective rutinosides [10].

The k' values of the flavanones is always below that of their corresponding dihydrochalcones. This clearly contradicts the experimental data obtained when the solubility of these compounds was analysed. NH shows a solubility (at room temperature) of 0.03 g/l in water, and 1.6-1.7 g/l in water-ethanol (vol 1:1), whereas NHDC shows a solubility of 0.4 g/l in water and 120-130 g/l in water-ethanol (vol 1:1). The cause of this alteration in the k' values of both flavonoid types with respect to the solubility data probably has a structural origin (Fig. 3).

For example, the molecular structure and absolute configuration of neohesperidin dihydrochalcone have been



DC



F

FIGURE 3. Basic structures of flavanones (F) and dihydrochalcones (DC). R: neohesperidosyl, rutinosyl or glucosyl; R': H or methyl; R'': H or hydroxyl.

determined by single-crystal X-ray analysis. The crystal structure consists of two crystallographically independent molecules which differ mainly in the orientation of the isovanillyl B-ring. The molecules are linked together by an intricate arrangement of intermolecular hydrogen bonds formed between the host and solvent water molecules [15].

The presence of this intrincate system of intermolecular hydrogen bonds, which is not found in the flavanones and which results in a voluminous mode of

molecular packing, particulary affects the reverse phase-dihydrochalcone interaction, increasing the k' values of these flavonoids.

The high values obtained for the parameters k' and w (between 7 and 1 min) in the water-methanol phases, which permitted the resolution of all the compounds in the mixture, together with the overlapping effects between glucosides and rhamnoglucosides by the different variations in ln k' according to changes in the percentage of methanol in the mobile phases studied, support the use of mobile phases with water and acetonitrile, as in previous studies [10].

Mobile Phases with Water and Acetonitrile.

Acetic acid (0.5 % v/v) was included in all the mobile phases used. This type of mobile phase with 25 % acetonitrile considerably reduces the retention times of all the flavonoids contained in the standard mixture, which produces a simultaneous overlapping of many and impedes accurate elucidation. When the percentage of acetonitrile in the mobile phase falls below 20 %, flavanones and dihydrochalcones are better resolved, but the other chromatographic parameters are adversely

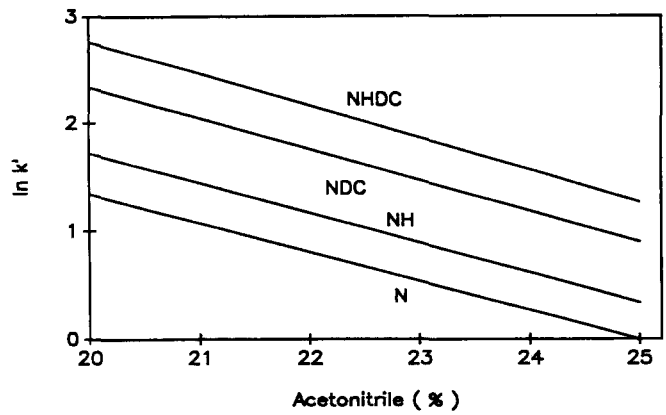


FIGURE 4. Variation of ln k' versus acetonitrile percentage of mobile phases with water-acetic acid-acetonitrile. N: Naringin; NH: Neohesperidin; NDC: Naringin dihydrochalcone; NHDC: Neohesperidin dihydrochalcone.

affected, particularly the w and k' values which increase with respect to those the 20-25 % acetonitrile volume percentage range.

Figure 4 shows how the form of substitution in the B ring when the same type of glycosylation is maintained in the 7 position, does not affect chromatographic resolution, similar to what happens in the water-methanol phases. N, NH, NDC and NHDC show similar slopes for the decrease in ln k' values when the percentage of acetonitrile in the mobile phases increases.

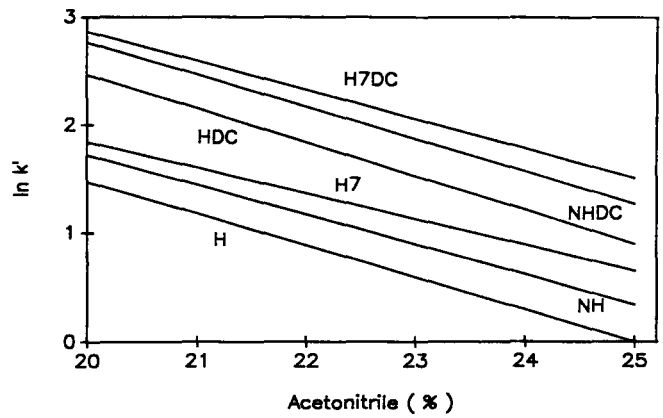


FIGURE 5. Variation of ln k' versus acetonitrile percentage of mobile phases with water-acetic acid-acetonitrile. H: Hesperidin; H7: Hesperetin 7-*O*-glucoside; NH: Neohesperidin; HDC: Hesperidin dihydrichalcone; H7DC: Hesperetin 7-*O*-glucoside dihydrochalcone; NHDC: Neohesperidin dihydrochalcone.

The flavonoids H7 and H7DC show a different behaviour to that described in the water-methanol phases since throughout the range considered (20-25 % acetonitrile), the k' values for both compounds were better than those of their rhamnoglucosylated flavanones and dihydrochalcones, respectively. The glucosides, rutinosides and neohesperidosides show similar slopes for the decrease in their k' values when the percentage of acetonitrile in the mobile phases varies, both for flavanones and dihydrochalcones. This fact served for

TABLE 1

Chromatographic Parameters of the Flavonoid Standard Mixture of Flavanones and Dihydrochalcones (DC) with Mobile Phase: Water-Acetic Acid-Acetonitrile (Gradient System from 20 % to 25 % Acetonitrile percentage, for 7 min).

Flavonoid	k'	α	w	R
Flavanones				
Naringin	3.04	1.10	0.37	1.10
Hesperidin	3.34	1.17	0.40	1.87
Neohesperidin	3.92	1.13	0.40	1.64
Hespt.7-*O*-glu.	4.41	1.26	0.45	3.06
Dihydrochalcones				
NaringinDC.	5.57	1.05	0.40	1.03
HesperidinDC.	5.86	1.15	0.52	2.22
NeohesperidinDC.	6.75	1.12	0.48	2.17
Hespt.7-*O*-gluDC.	7.53	1.08	0.57	1.51

considering the mobile phases containing water and acetonitrile as the most suitable for obtaining the simultaneous resolution of both types of flavonoid compound.

To improve the k' values while maintaining the w values close to those obtained using mobile phases with 25 % acetonitrile (below 0.7 min in all cases) and

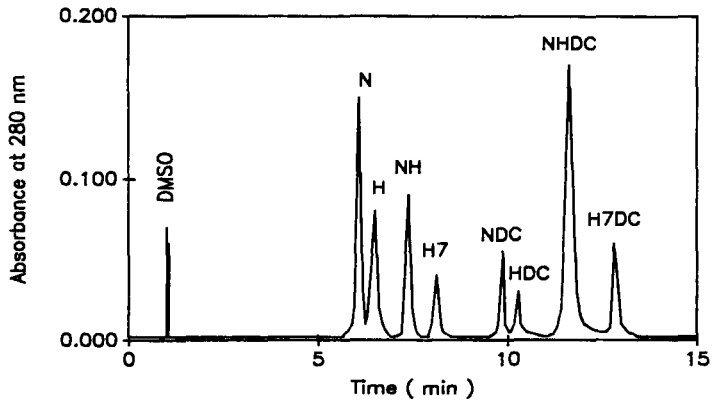


FIGURE 6. Chromatogram of flavanone and dihydrochalcone standard mixture elucidated by means of a gradient-isocratic system with mobile phase: water-acetic acid-acetonitrile. N: Naringin; H: Hesperidin; NH: Neohesperidin; H7: Hesperetin 7-*O*-glucoside; NDC: Naringin dihydrochalcone; HDC: Hesperidin dihydrichalcone; NHDC: neohesperidin dihydrochalcone; H7DC: Hesperetin 7-*O*-glucoside dihydrochalcone.

keeping the values of R similar to those obtained using 20 % (above 1.0 min in all cases), a lineal gradient system was designed which increased the percentage of acetonitrile of the mobile phase from 20 (80:0.5:20 water-acetic acid-acetonitrile) to 25 % (75: 0.5: 25 water-acetic acid-acetonitrile) in a given time, maintaining this composition until all the flavonoids in the mixture were resolved; this gradient change of

acetonitrile percentage shows an optimum time value of 7 min. An analysis of several chromatographic parameters obtained with this system is to be found in Table 1. Figure 6 shows the chromatogram of the flavonoids of the completely elucidated standard mixture.

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A HPLC METHOD FOR THE SEPARATION AND QUANTIFICATION OF THE ENANTIOMERS OF HYDROXYCHLOROQUINE AND ITS THREE MAJOR METABOLITES

YIMIN WEI, GLORIA A. NYGARD, AND SHOUKRY K. W. KHALIL
Department of Pharmaceutical Sciences
North Dakota State University
Fargo, North Dakota 58105

ABSTRACT

A two step HPLC method for analysis of the enantiomers of Hydroxychloroquine (HCQ) and its three major metabolites, Desethylhydroxychloroquine (DHCQ), Desethylchloroquine (DCQ) and Bisdesethylchloroquine (BDCQ), was developed. Fluorescence detection was used at λ_{ex}=230nm and λ_{em}=385nm with a 370nm cut-off filter. This method has higher sensitivity and better resolution of the parent drug and its three metabolites when compared to published methods.

In the first step, a cyano column was used to separate and collect fractions containing HCQ and its three metabolites. The mobile phase was 20% pH 6.0 (0.015M K_2HPO_4) buffer, 30% methanol and 50% acetonitrile at a flow rate of 2 ml/min and 50°C. This method was linear over the concentration ranges of 2-20, 20-300 and 200-2000 ng/ml of blood (r>0.99) and was used for quantitation.

In a second step, chiral separation was performed on a chiral-AGP column using a mobile phase of 94% pH 7.0 (0.05M $NH_4H_2PO_4$, 0.005M dihexylamine) buffer, 5% isopropanol and 1% acetonitrile at a flow rate of 1 ml/min and 35°C. Baseline separation was obtained for the enantiomers of the parent drug and its metabolites.

Resolution of the enantiomers on the chiral column for HCQ, DHCQ, DCQ and BDCQ was 3.2, 3.2, 2.9 and 2.5, respectively. The limit of detection for HCQ and its metabolites was less than 1 ng for each enantiomer.

INTRODUCTION

Hydroxychloroquine (HCQ) is used widely as an antimalarial and in the treatment of rheumatoid arthritis and systemic lupus erythematosus. HCQ has three major metabolites, desethylhydroxychloroquine (DHCQ), desethylchloroquine (DCQ), and bisdesethylchloroquine (BDCQ). Two multistep HPLC methods for the determination of the enantiomers of HCQ and its metabolites have been published [1,2]. In both of these methods, the parent drug and its metabolites were separated and quantitated in the first stage, and then the ratios of the enantiomers were determined in the second stage. The methods require fractional collection of the separate eluting peaks (racemic drug or a metabolite) which are then injected onto another HPLC system for chiral separation of the enantiomers.

The first of these methods [1] uses the quantitative procedure of Tett, et al. [3] as its first stage. With fluorescence detection this method has good sensitivity but exhibits only minimal separation of HCQ from its metabolites. This poor resolution makes it difficult to collect pure fractions of drug or metabolite for use in the second stage of analysis. The chiral separation also exhibited several limitations: baseline separation of the enantiomers was not obtained, special equipment for postcolumn alkalinization was required, and an elution time of nearly one hour was needed to separate each pair of enantiomers. The second method [2] had good resolution

of compounds in both stages of the procedure, but obtained only limited sensitivity with the use of a UV detector. In addition, both methods have the disadvantage of using diethylether in sample preparation.

The method presented in this paper also uses a two-stage procedure, but incorporates several improvements over the other two methods. These include use of a less volatile extraction solvent and choices of chromatographic conditions leading to increased detector sensitivity and excellent resolution of the racemic compounds and enantiomeric pairs. This method is applicable to the study of the pharmacokinetic properties of HCQ and its three major metabolites (DHCQ, DCQ and BDCQ) and for the determination of enantiomer ratios of each of the compounds.

MATERIALS AND METHODS

Instrumentation

The analysis was performed on a Hewlett Packard model 1090 HPLC system fitted with a Hewlett Packard model 1046A fluorescence detector. The effluent was monitored at λ_{ex}=230nm and λ_{em}=385nm through a 370nm cut-off filter. The output was recorded on a Hewlett Packard model 3392A integrator. The column used for achiral assay was a cyanopropyl column (250 x 4.6mm I.D., 5μ) manufactured by Baxter/Burdick & Jackson with a cyano guard column (10 x 3mm I.D., 7μ) manufactured by Applied Biosystems. The column used for the enantiomeric separation was a Chiral-AGP (100 x 4.0mm I.D., 5μ) manufactured by ChromTech AB (distributed by Regis).

Reagents

HCQ-sulfate, R(-)-HCQ, DHCQ, BDCQ and DCQ were obtained from Sanofi/Winthrop Pharmaceuticals. Propranolol-HCL was obtained from Sigma. All organic solvents were HPLC grade. K_2HPO_4, $NH_4H_2PO_4$, H_3PO_4, dihexylamine and NaOH were analytical grade.

Solution Preparation

Drug solutions: Stock solutions containing HCQ, DHCQ, BDCQ and DCQ were prepared at concentrations of 100 μg/ml methanol. Working dilutions to 10, 1 and 0.1 μg/ml methanol were prepared from the stock solutions.

Internal standard (IS) solution: IS stock solution containing propranolol-HCL was prepared at 500 μg/ml methanol.

Blood lysing solution: Blood lysing solution was prepared by diluting 20 μl IS stock in 500 ml deionized distilled water.

Extraction solution: Ethyl acetate and isopropanol were mixed (90:10) for extraction solution.

Quality Control (QC) Samples

Drug free whole blood was spiked with known concentrations of HCQ and its metabolites. The QC samples were stored frozen and used to determine intraday and interday precision of the assay.

HPLC Conditions

Achiral chromatography: Mobile phase consisted of 20% buffer (0.015M K_2HPO_4, pH adjusted to 6.0 with

H_3PO_4), 30% methanol and 50% acetonitrile. Flow rate was 2.0 ml/min at 50°C.

Chiral chromatography: Mobile phase consisted of 94% buffer (0.05M $NH_4H_2PO_4$ containing 0.005M dihexylamine, pH adjusted to 7.0 with 3N NaOH), 5% isopropanol and 1% acetonitrile. Flow rate was 1.0 ml/min at 40°C.

Sample Preparation and Analysis

Whole blood was spiked to prepare standards containing 2-2000 ng each of the drug and metabolites/ml blood. All samples (standards and QCs) were processed by adding 2.0 ml of blood lysing solution to 1.0 ml of whole blood which had been placed in a polypropylene tube. They were then vortexed for 10 seconds, sonicated for 10 minutes and centrifuged at 3000 rpm for 20 minutes. Exactly 2.0 ml of the supernatant was transferred to a second tube where 1.0 ml of 0.1N NaOH and 8.0 ml of extraction solution were added. These mixtures were vortexed for 30 seconds and centrifuged for 15 minutes at 3000 rpm. The organic layer was transferred to a third tube using a disposable polyethylene pipet and evaporated to dryness at 35-40 degrees C under a gentle stream of air. The residue was reconstituted in 250 μl methanol by vortexing for 30 seconds. The reconstituted samples were transferred to plastic HPLC vials and injected (at volumes of 10-100 μl) onto the achiral column.

The peaks of HCQ and its three metabolites eluting from the achiral system were collected separately and evaporated to dryness. The individual dried fractions were then reconstituted in 200 μl of the chiral system mobile phase, and 10-100 μl was injected onto the chiral column to separate the enantiomers.

Absolute recovery of HCQ and the metabolites was estimated by comparing the achiral peak heights of spiked whole blood standards extracted in triplicate to methanol solutions of the compounds.

RESULTS AND DISCUSSION

Achiral Separation

A cyano HPLC column was used to separate and quantitate HCQ and its metabolites. The achiral system gave excellent resolution of the compounds and allowed easy collection of the pure single peaks (Table 1 and Figure 1). The limit of quantitation was less than 2ng/ml of blood for each of the four compounds. The standard curves were calculated using three concentration ranges: 2-20ng/ml, 20-300ng/ml and 200-2000ng/ml. All the correlation coefficients of the standard curves of HCQ, DHCQ, BDCQ and DCQ were greater than 0.99 indicating that this method can be used for a very large range of concentrations. The mean

TABLE 1

Capacity Factors and Retention Times of HCQ and Its Three Major Metabolites on the Achiral HPLC System.

Compound	K[a]	t_r[b] (min)
HCQ	9.1	9.72
DHCQ	13.9	14.34
BDCQ	18.6	18.90
DCQ	24.4	24.52

a. Capacity factors.
b. Retention times.

recoveries of HCQ, DHCQ, BDCQ and DCQ from blood were 75.2%, 86.7%, 47.0%, and 94.8%, respectively.

Quality control blood samples containing HCQ and the metabolites were analyzed in triplicate on three separate days versus freshly spiked standards. Tables 2 and 3 summarize the results of the QC analysis. Intraday and interday variation over a wide range of concentrations (4ng/ml to 1500ng/ml) indicate good reproducibility (CV% range 0.2-14.7%) and accuracy (90.6-115.4%) of the method.

Chiral Separation

A two-stage procedure was chosen because simultaneous separation of all the enantiomers of HCQ

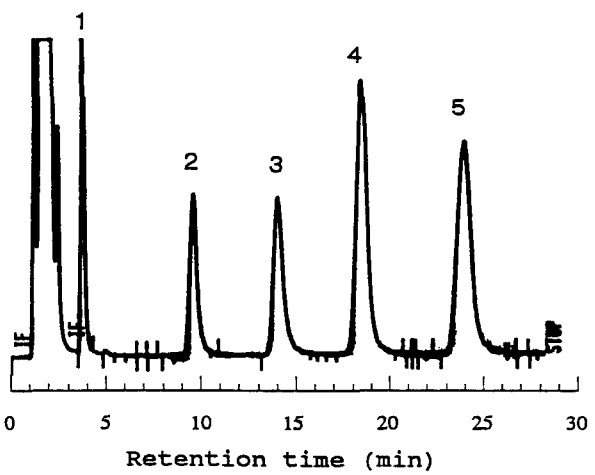

FIGURE 1. Chromatogram of achiral separation of spiked whole blood (50ng/ml). Peaks: 1 = IS, 2 = HCQ, 3 = DHCQ, 4 = BDCQ, 5 = DCQ.

TABLE 2

Accuracy and Reproducibility of Quantitation of HCQ and Its Metabolites--Intraday Quality Control Data (n=3).

	Blood Concentration (ng/ml)			
	Added	Measured	CV%	Accuracy%
HCQ	4.0	4.1	1.9	102.1
	250.0	252.5	3.8	101.0
	1500.0	1392.0	5.3	92.8
DHCQ	4.0	3.6	4.0	90.6
	250.0	255.3	0.2	102.1
	1500.0	1610.9	10.3	107.4
BDCQ	4.0	4.6	10.4	115.4
	250.0	244.3	7.0	97.7
	1500.0	1597.6	9.7	106.5
DCQ	4.0	3.8	6.0	95.4
	250.0	248.4	1.8	99.4
	1500.0	1611.0	10.4	107.4

TABLE 3

Accuracy and Reproducibility of Quantitation of HCQ and Its Metabolites--Interday Quality Control Data (n=3).

	Blood Concentration (ng/ml)			
	Added	Measured	CV%	Accuracy%
HCQ	4.0	4.1	4.9	102.8
	250.0	243.0	7.1	97.2
	1500.0	1377.4	2.9	91.8
DHCQ	4.0	3.7	2.4	92.9
	250.0	248.6	14.2	99.5
	1500.0	1496.8	1.0	99.8
BDCQ	4.0	3.9	14.7	98.7
	250.0	261.6	6.6	104.6
	1500.0	1586.9	8.3	105.8
DCQ	4.0	4.0	3.7	100.0
	250.0	251.6	9.5	100.6
	1500.0	1494.8	3.5	99.7

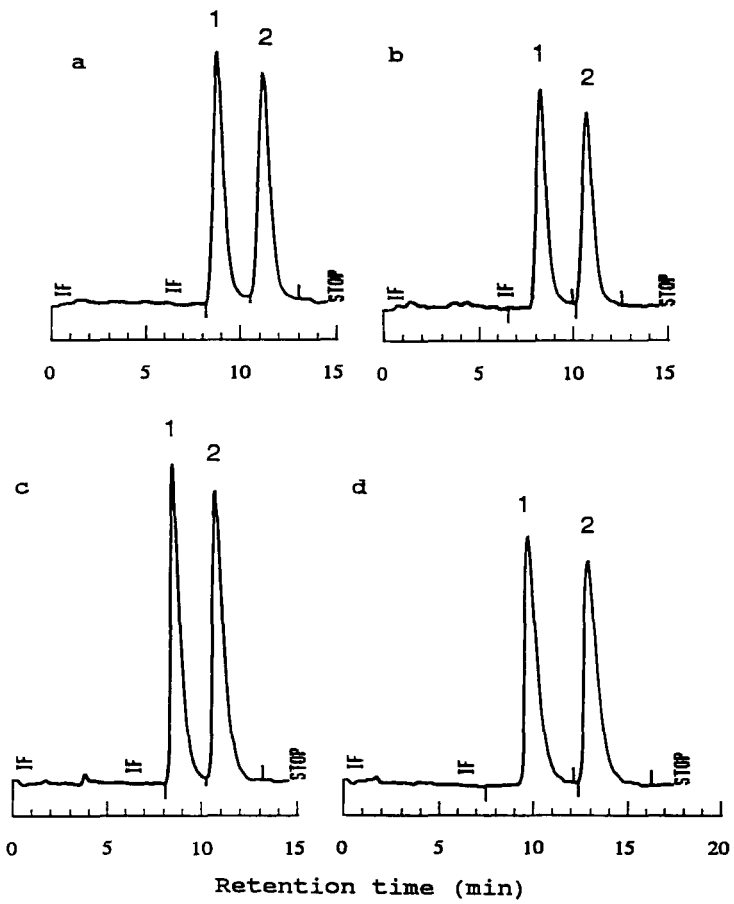

FIGURE 2. Chromatograms of chiral separation. a. HCQ, b. DHCQ, c. BDCQ and d. DCQ. Peak 1 = S(+)enantiomer, Peak 2 = R(-)enantiomer.

TABLE 4

Resolution and Retention Time Data for the Enantiomeric Separation of HCQ and Its Three Metabolites.

Compound	Resolution	t_r(S) (min)	t_r(R) (min)
HCQ	3.2	8.78	11.25
DHCQ	3.2	8.29	10.80
BDCQ	2.9	8.73	11.00
DCQ	2.5	10.04	13.30

t_r(S): S(+) enantiomer retention time
t_r(R): R(-) enantiomer retention time

and its three metabolites on a chiral column is not feasible. The first step involved an achiral separation to resolve and collect the racemates (Figure 1). This was followed by the chiral separation of each pair of drug or metabolite enantiomers (Figure 2). The fractions collected from the achiral column were validated as pure single peaks by being reinjected onto the achiral column. The ratio of each pair of enantiomers was determined by calculating the ratio of their peak areas.

The resolution and retention times of the enantiomer pairs are listed in Table 4. The detection limit for each enantiomer was less than 1 ng. Baseline separation of the compounds and sharp peaks contributed to high sensitivity of the analysis.

The elution order of the enantiomers of HCQ was determined by injecting a pure R(-)-HCQ enantiomer onto the chiral column (Figure 3). The enantiomers of the metabolites were assumed to have the same elution order as HCQ (S, then R) under the conditions of this method [1,2].

The choice of excitation and emission wavelengths depends on the absorbance and fluorescence

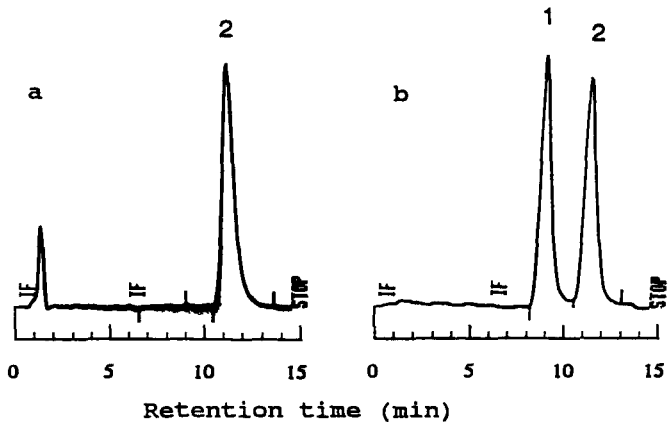

FIGURE 3. Chromatograms on chiral system. a. R(-)-HCQ enantiomer, b. HCQ racemate. Peak 1 = S(+)-HCQ, Peak 2 = R(-)-HCQ.

characteristics of the particular molecular structures, and also depends on the design of the detector. In the current work, the use of a lower excitation wavelength (230nm instead of 320-330nm, as has been commonly used in other work [1,4]) gave increased sensitivity of detection. Compared to the published methods [1,2], the current method has higher sensitivity as well as better resolution of the enantiomers. It is therefore well suited to the analysis of samples in pharmacokinetic and bioavailability studies of HCQ and its metabolites and in studies designed to determine the disposition of the enantiomers.

ACKNOWLEDGEMENT

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INFLUENCE OF KINETIC PROPERTIES OF EXTRACTION SYSTEMS ON THE SEPARATION AND PRECONCENTRATION OF SOME ELEMENTS BY COUNTERCURRENT CHROMATOGRAPHY

P. S. FEDOTOV, T. A. MARYUTINA, V. M. PUKHOVSKAYA, AND B. Ya. SPIVAKOV
Vernadsky Institute of Geochemistry and Analytical Chemistry
Russian Academy of Sciences
Kosygin str. 19
Moscow 117975, Russia

ABSTRACT

The influence of kinetic properties of several extraction systems on the separation of some elements by countercurrent chromatography (CCC) has been studied. The chromatographic behaviour of Eu(III), Fe(III), Ta(V), Nb(V), Hf(IV), Zr(IV) has been investigated in two kinetically different extraction systems on the basis of di-2-ethylhexylphosphoric acid (D2EHPA) and tetraphenylmethylenediphosphine dioxide (DTPMDP). It has been shown that the values of mass transfer coefficients determine the type of elution (isocratic or step), which is necessary for the element separation. The data on batch extraction (mass transfer coefficients and partition coefficients) and parameters of chromatographic peaks (half-widths) can be interrelated by the expression proposed.

INTRODUCTION

Countercurrent chromatography (CCC) is based on retention (without solid support) of either phase (stationary) of a two-phase liquid system in a rotating column under the action of centrifugal forces while the other liquid phase (mobile) is being continuously pumped through [1]. A few devices providing the retention of the stationary phase in the field of mass forces in the absence of solid support have been suggested. The most successful device that gained acceptance is a planetary centrifuge [2]. A column (or a column unit) of a certain configuration rotates around the central axis of the device with the aid of a planetary gear.

So far, CCC has been mainly used for separation and determination of organic and bioorganic substances [3]. The studies of the last five years have shown that the technique can be successfully applied to separation and preconcentration of inorganic ions for their more selective and sensitive determination. The possibility of CCC separation of ortho- and pyrophosphate anions, Cs and Sr, rare earth and other elements in systems on the basis of extraction reagents of different types has been demonstrated [4-7].

The theory of CCC as a chromatographic separation method has being developed for a few last years. Equations for the calculations of partition coefficients, number of theoretical plates, separation factors, peak resolution have been set up on the basis of classical expressions used in extraction chromatography [8,9]. The influence of the flow rate of the mobile phase and the retention factor S_f (ratio of the stationary phase volume V_s to the total column volume V_c) on the separation efficiency and peak resolution has been studied [9-13]. A few procedures for optimization of chromatographic processes have been also proposed [8, 14-16].

The absence of solid support in CCC practically excludes the possibility of adsorbtion. It is assumed that the retention of substances in a separation column can be predicted with the help of partition coefficients. The partition coefficients D obtained under CCC conditions (dynamic) and determined from an elution curve (Eq. 1) are postulated to coincide with the partition coefficients estimated from batch extraction experiments [8].

$$D = \frac{V_r'}{V_s} = \frac{V_r'}{V_m}\frac{V_m}{V_s} = \frac{V_r'}{V_m}\frac{1-S_f}{S_f} = \frac{V_r-V_m}{V_m}\frac{1-S_f}{S_f} \qquad (1)$$

where V_r - retention volume, V_m - mobile phase volume in the column ($V_m = V_c - Vs$), V_r' - corrected retention volume ($V_r' = V_r - V_m$).

Examination of the reported data has shown that this approach is valid for physical distribution of substances between two liquid phases.

The distribution of inorganic compounds is also dependent on the hydrodynamic properties of the system used, partition coefficients of substances to be separated and parameters of the planetary centrifuge operation (rotation and revolution speeds, direction and speed of the mobile phase pumping, sample volume). However, the systems for inorganic separations are very different from those for organic separations as in most cases they contain a complexing reagent (ligand). The complexation process, its rate and the mass transfer rate are the main factors that determine the separation efficiency. The kinetics is of particular importance, because CCC separation can be a non-equilibrium process. Thus, it has been shown for several systems that the batch partition coefficients for inorganic compounds are different from dynamic ones [17-19].

This work is an attempt to correlate kinetic properties of solvent extraction systems and results of separation of inorganic substances by CCC. Kinetic data, which could be used, are very limited. Freiser et al. studied the influence of kinetic factors on the separation efficiency in the centrifugal partition chromatography (CPC) [17, 18]. The kinetics of extraction of lanthanides by bis(2,4,4-trimethylpentyl)phosphinic acid in heptane was investigated and the data obtained were correlated with the results of the CPC separation of

the elements [17]. The studies on the separation efficiency for the distributing species, a palladium complex and an organic solute (3-picoline) for the heptane - water phase pair were also reported [18].

It has become clear from our results as well as from the studies by Freiser et al. that the fundamentals of separation of substances in rotating coil columns need to be specially studied with respect to inorganic compounds.

EXPERIMENTAL

Apparatus

Chromatographic investigations were made on a device consisting of a planetary centrifuge with a vertical column drum fabricated in the Institute of Analytical Instrumentation, St. Petersburg, a peristaltic pump and a fraction collector.

The column of the device rotated around its axis and at the same time revolved around the central axis of the device. The rotation and revolution speeds were equal (ω = 350 r/min). The planetary centrifuge model had the following design parameters: revolution radius R = 140 mm, rotation radius r = 50 mm. The column was made of a teflon tube with an inner diameter of 1.5 mm and a wall thickness of 0.75 mm. The total inner capacity of the column was V_c = 24 ml.

A stirred diffusion cell was used for the investigation of the kinetics of batch extraction. The rotation speed of the motor-driver stirring rod was 150 r/min. The organic phase/aqueous phase interface had an area of 9 cm.

A γ-detector (Tesla, Czechoslovakia) was used for radiometric measurements of Eu and Fe concentrations (radioisotopes ^{154}Eu and ^{55}Fe).

Polychromator ICAP-61 ("Thermo Jarrell Ash Corp.", USA) with first order resolution 0.031 nm was used for ICP-AES determination of Hf, Nb, Ta, and Zr.

Reagents

Tetraphenylmethylenediphosphine dioxide (DTPMDP) was synthesized at the Institute of Organoelement Compounds, Moscow. Di-2-ethylhexylphosphoric acid (D2EHPA) was a high-purity grade (99.9%) reagent. The other chemicals were analytical grade reagents.

Measurement of batch extraction parameters

The equilibrium partition coefficients were determined under batch extraction conditions (using a separatory funnel). The volumes of aqueous and organic phases were equal to 5 ml. The initial concentration of an element under study in the aqueous phase was 5 ppm. Fe(III) in DTPMDP-based system and Eu(III) in both DTPMDP- and D2EHPA-based systems were equilibrated for 5 min. Zr(IV), Hf(IV) in D2EHPA-based systems required 15-min mixing to attain equilibrium. When Fe(III), Ta(V), Nb(V) were extracted in D2EHPA-based systems, test solutions were contacted for 5 hours to attain equilibrium.

When the kinetics of batch extraction in the stirred diffusion cell was investigated, the volumes of organic and aqueous phases were 10 and 30 ml, respectively. The initial concentration of the element in the aqueous phase was 5 ppm. The total volume of the aqueous phase fractions taken for concentration measurements were less than 3 ml.

The mass transfer coefficients were determined with the use of t-dependence of -ln(1-E_t) [20].

$$E_t = \frac{C_o - C_t}{C_o - C^*} \qquad (2)$$

where C_o - initial, C_t - current, C^* - equilibrium concentration of the element in the aqueous phase, mol/l.

It is known that

$$-\ln (1-E_t) = k\,a \qquad (3)$$

where $a = S/V$ (S - phase interface area, cm^2; V - aqueous phase volume, ml; k - mass transfer coefficient, cm/s). The tangent of an angle of t-dependence of -ln(1-E_t) is equal to ka.

Chromatographic procedure

Before the chromatographic experiment was begun, the components of the two-phase liquid system were stirred and brought into equilibrium for mutual saturation of the phases, after which the aqueous phase was used as the mobile phase and the organic one as the stationary phase. The coil column in the stationary mode was filled with the organic phase. After that, while the column rotated, the aqueous phase was fed to its inlet. The mass force field, which arose during rotation, made it possible to retain a constant volume of the stationary phase V_s while the mobile phase was continually pumped through. The pumping speed was 1.0 ml/min. After equilibrium between the mobile and stationary phases had been established, a sample was introduced into the column; the concentration of Eu(III) or Fe(III) in the sample was 200 ppm, the sample volume was 0.1 ml. When the behaviour of Ta(V), Nb(V), Hf(IV), Zr(IV) under the preconcentration conditions was investigated, the sample volume was 10 ml and the concentration of each metal in the sample was 50 ppm.

The volume of the stationary phase retained in the column depends on the extraction system nature, the rotation speed of the column and the pumping speed of the mobile phase. When the influence of the quantity of the stationary phase in the column on the chromatographic peak shape was investigated, a fixed volume of the stationary phase V_s^t (less than V_s allowed by experimental conditions) was introduced with the flow of the mobile phase into the column in the stationary mode. After that, while the column was rotated, the aqueous phase was continuously fed to its inlet.

Table 1

Wavelengths and corresponding analytical ranges of ICP-AES determination of elements

Element	Wavelength, nm	Analytical range, ppm
Hf	282.022	0.010 - 1000
Nb	319.498	0.020 - 500
Ta	240.063	0.020 - 200
Zr	349.621	0.003 - 250

ICP-AES determination of elements

The operation conditions of the determination were the following: forward r.f. power - 1.1 kW; reflected r.f. power - less than 50 W; outer argon flow - 15 l/min, auxiliary argon flow - 0.29 l/min; carrier argon flow - 0.72 l/min; observation height - 16 mm above load coil; nebulizer - of cross-flow type; solution uptake rate - 1.5 ml/min. Operation system "Thermo SPEC" 4.20 on the basis of a personal computer IBM PC-AT 386 was used. The wavelengths and analytical ranges are presented in Table 1.

RESULTS AND DISCUSSION

Study of the kinetic properties of D2EHPA-based systems

D2EHPA is an effective extractant for separation and preconcentration of rare earth and other elements. Kinetic peculiarities of the interaction between D2EHPA and various metal ions have been relatively well studied. Eu(III) is known to be extracted much faster than Fe(III) in systems with D2EHPA [20].

However, for the chromatographic data interpretation additional studies of kinetic properties of the systems used and determination of Eu(III) and Fe(III) mass transfer coefficients are required. These studies were made by use of a stirred diffusion cell. Figure 1 illustrates t-dependences of $-\ln(1-E_t)$ for Eu(III) and Fe(III) extraction for the systems on the basis of D2EHPA. It can be seen that dependences *1* and *2* for Fe(III) extraction (systems containing 0.05 and 0.5 mol/l D2EHPA) and dependence *3* for Eu(III) extraction (system containing 0.5 mol/l D2EHPA) are straight lines, whereas the dependence *4* for Eu(III)

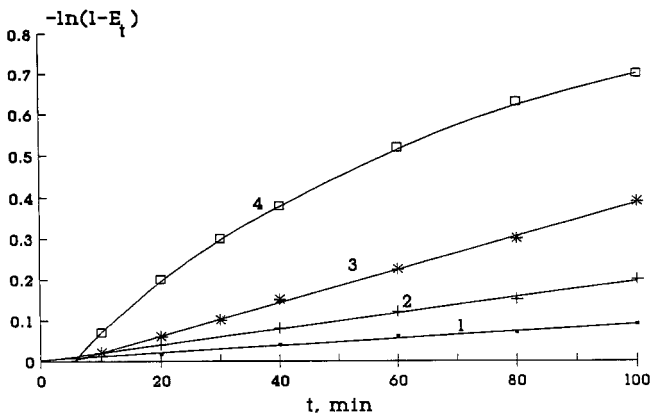

Fig. 1: Time-dependences of -ln(1-E_t) for Eu(III) and Fe(III) extraction in D2EHPA-based systems. *1* - Fe(III), 0.05 mol/l D2EHPA - n-decane - 0.2 HCl; *2* - Fe(III), 0.5 mol/l D2EHPA - n-decane - 3.0 mol/l HCl; *3* - Eu(III), 0.05 mol/l D2EHPA - n-decane - 0.2 mol/l HCl; *4* - Eu(III), 0.5 mol/l D2EHPA - n-decane - 0.5 mol/l HCl.

extraction (system containing 0.5 mol/l D2EHPA) is more complicated. The formation of thin condensed films at the interphase surface in Eu-containing systems with high D2EHPA concentration [20] may explain why the Eu(III) extraction retards with time.

t-Dependences of -ln(1-E_t) for Fe(III) extraction in D2EHPA-based systems are straight lines (Fig. 1) but mass transfer coefficients are much lower than in the case of Eu(III) (Table 2). Besides, the extraction of Fe(III) in the systems with D2EHPA is practically irreversible. For instance, the equilibrium partition coefficient (D_{bat}) of Fe(III) in the 0.05 mol/l D2EHPA - n-decane - 2.0 mol/l HCl system is about 1 in the extraction, whereas in the back-extraction this value is about 10. The mechanisms of the extraction and back-extraction of Fe(III) in the system under investigation are apparently different. It should be noted that the mass transfer coefficients for Eu(III) and Fe(III) extraction are not influenced by changing HCl concentration (from 0.2 to 3.0 mol/l), all other factors being the same.

Ta and Nb belong to elements kinetically more inert than Fe, that is why the investigation of their behaviour under batch extraction and CCC conditions is theoretically important. The 0.5 mol/l D2EHPA - n-decane - 2-3 mol/l HNO_3 systems are more selective to Ta(V), Nb(V), Hf(IV), Zr(IV) in comparison with the 0.5 mol/l D2EHPA - n-decane - HCl systems [21].

The results of the study on Ta(V), Nb(V), Hf(IV), Zr(IV) extraction kinetics in the 0.5 mol/l - n-decane - 2.0 mol/l HNO_3 system are presented in Fig. 2 and Table 2. *t* - Dependences of -ln(1-E_t) are straight lines for all elements, but mass transfer coefficients for Ta(V) and Nb(V) are appreciably lower than for Hf(IV) and Zr(IV).

Table 2

Mass transfer coefficients (k) and batch partition coefficients (D^{bat}) for Eu(III), Fe(III), Ta(V), Nb(V), Hf(IV), Zr(IV) in D2EHPA-based extraction systems

Element	Extraction system	k, cm/s	D^{bat}
Eu(III)	0.05 mol/l D2EHPA-n-decane-0.2 mol/l HCl	2.0×10^{-4}	0.8
	0.5 mol/l D2EHPA-n-decane-0.5 mol/l HCl	6.7×10^{-4}(a) 2.8×10^{-4}(b)	5.2
Fe(III)	0.05 mol/l D2EHPA-n-decane-0.2 mol/l HCl	4.0×10^{-5}	18.6
	0.05 mol/l D2EHPA-n-decane-2.0 mol/l HCl	1.2×10^{-4}(c)	10.9
	0.5 mol/l D2EHPA-n-decane-3.0 mol/l HCl	9.3×10^{-5}	3.1
Ta(V)	0.5 mol/l D2EHPA-n-decane-2.0 mol/l HNO_3	5.7×10^{-5}	12.1
Nb(V)		1.3×10^{-4}	33.8
Hf(V)		6.5×10^{-4}	>100
Zr(V)		7.1×10^{-4}	>100

(a) $t < 30$ min

(b) $t > 60$ min

(c) back-extraction

The chromatographic behaviour of Eu(III), Fe(III), Ta(V), Nb(V), Hf(IV), Zr(IV) in D2EHPA-based systems

The chromatographic behaviour of Eu(III) in the 0.05 mol/l D2EHPA - n-decane - HCl system at different HCl concentrations is shown in Fig. 3. It is seen that the elution of Eu(III) begins in all cases immediately after a volume of mobile phase equal to V_m has passed through the column. The peak width increases with the decrease in HCl concentration. Dynamic (D^{dyn}) and batch (D^{bat}) partition coefficients of Eu(III) in systems under investigation are given in Table 3. It is of interest that at a constant V_s value (12 ml) the shapes of elution curves of Eu(III) are not influenced by the rotation speed (from 350 to 500 r/min) and pumping speed of the mobile phase (from 0.4 to 1.0 ml/min). Therefore, the

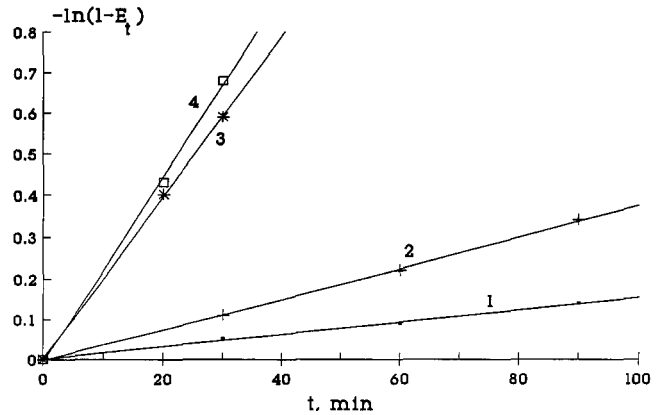

Fig. 2: Time-dependences of -ln(1-E_t) for Ta(V), Nb(V), Hf(IV), Zr(IV) in 0.5 mol/l D2EHPA - n-decane - 2.0 mol/l HNO_3. *1* - Ta(V), *2* - Nb(V), *3* - Hf(IV), *4* - Zr(IV).

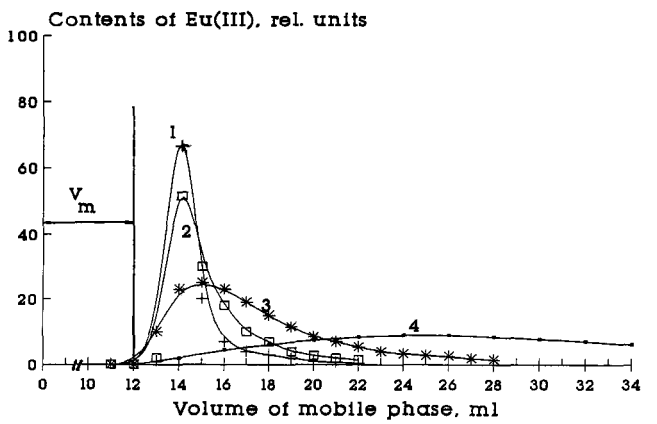

Fig. 3: Chromatographic behaviour of Eu(III) in 0.05 mol/l D2EHPA - n-decane - HCl systems. S_f = 0.5. Mobile phase: *1* - 0.70, *2* - 0.40, *3* - 0.25, *4* - 0.20 mol/l HCl.

Table 3

Batch and dynamic partition coefficients for Eu(III) in the 0.05 mol/l D2EHPA - n-decane - HCl system

C_{HCl}, mol/l	D^{bat}	D^{dyn}
0.70	0.06	0.12
0.40	0.12	0.12
0.25	0.20	0.18
0.20	0.80	1.20

nature of the extraction system, its kinetic properties may have a greater effect on the chromatographic process than the hydrodynamic parameters of the planet centrifuge operation.

D^{dyn} = 1.0 is considered to be the optimal value for obtaining a well-resolved peak of an individual component [8]. A significant broadening of the peak at D^{dyn} = 1.2 does not make it possible to use the chosen extraction system under the chosen experimental conditions for isocratic separation of Eu(III) from other elements.

The isocratic separation of some rare earth elements in the 0.02 mol/l D2EHPA - n-heptane - 0.02 mol/l HCl system was carried out using high-speed CCC [7] (a planetary centrifuge with three identical multilayer coiled columns connected in series; rotation speed 900 r/min, pumping speed 5 ml/min, total column volume 270 ml). Such a device allows one to increase the efficiency of the mass exchange between the mobile and stationary phases. However, a considerable increase in the total column capacity leads to an increase in the time required for the separation (several hours) and to a large volume (about 100 ml) of the mobile phase needed for the elution of each element. Hence, the practical use of these techniques may be very limited.

The equilibrium for Fe(III) extraction in D2EHPA - n-decane - HCl systems is achieved within several hours under the batch extraction conditions. When the chromatographic behaviour of Fe(III) in these systems is examined, it would not be correct to discuss dynamic partition coefficients since under the same conditions a part of Fe(III) is eluted from the column, whereas the other part is extracted into the stationary phase and is not eluted even after the volume of the mobile phase equal to 10 V_m is passed through the column. Consequently, the first part of Fe(III) should have the partition coefficient about 0.1

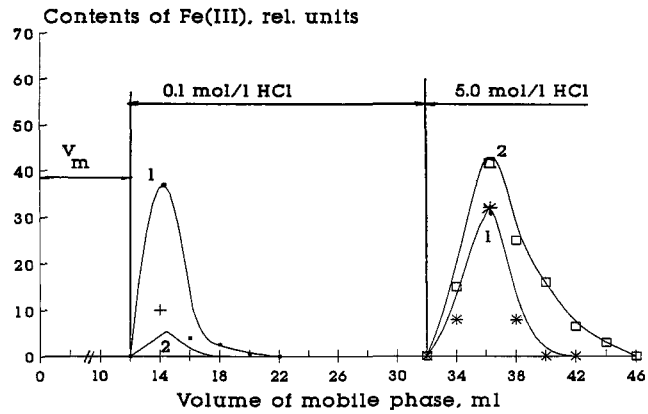

Fig. 4: Chromatographic behaviour of Fe(III) in D2EHPA - n-decane - HCl systems. S_f = 0.5. Step elution. Stationary phase: *1* - 0.05, *2* - 0.5 mol/l D2EHPA.

and the second part of Fe(III) about 10 or higher. About 50% of Fe(III) is eluted in the case of 0.05 mol/l and 5% in the case of 0.5 mol/l D2EHPA. The corresponding values of mass transfer coefficients are 4.0 x 10^{-5} and 9.3 x 10^{-5} cm/s (Table 2). Hence, the less the magnitude of mass tranfer coefficient, the greater the quantity of Fe(III) eluted immediately after the V_m volume is passed through the column. Fe(III) retained by the stationary phase can be eluted only by a highly concentrated HCl solution, since the mechanism of back-extraction of Fe(III) is different from that of extraction.

Similar curves circumscribe the chromatographic behaviour of Ta(V) and Nb(V) in the 0.5 mol/l D2EHPA - n-decane - 2.0 mol/l HNO_3 system (Fig.5). As is seen, a part of Ta(V) (40%) and Nb(V) (35%) is not extracted and eluted after the V_m volume has passed through the column. Zr(IV) and Hf(IV) are extracted quantitatively.

The extraction system used provides relatively high values of the equilibrium partition coefficients for Ta(V) and Nb(V) (Table 2). However, the quantitative extraction is not attained because of a low rate of the mass transfer process. The comparison of the chromatographic behaviour of elements under investigation and their mass transfer coefficients allows us to assume the following. To concentrate species with mass transfer coefficients k < 10^{-4} cm/s under experimental conditions used (V_c = 24 ml), their partition coefficients must be 100 or higher. Otherwise, the species will be partly eluted after the V_m volume is passed through the column.

Therefore, when kinetically inert compounds are concentrated in the stationary phase under CCC conditions, a column with a larger total volume can be used. However, in the separation of any species, a larger column volume results in a longer separation time and broader chromatographic peaks [7].

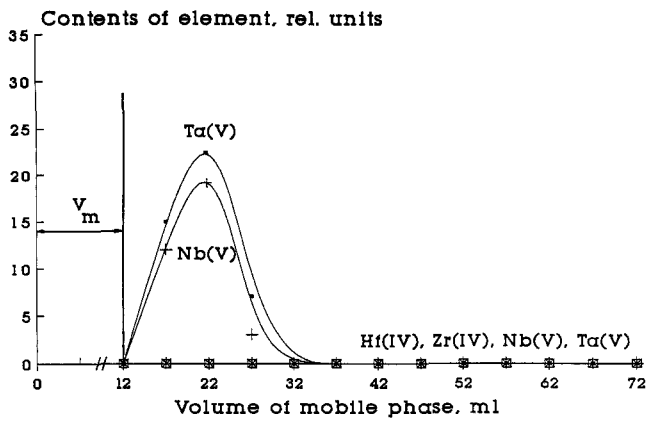

Fig. 5: Chromatographic behaviour of Ta(V) (·), Nb(V) (+), Hf(IV) (o), Zr(IV) (*) in 0.5 mol/l D2EHPA - n-decane - 2.0 mol/l HNO_3 system. $S_f = 0.5$.

Study of the kinetic properties of DTPMDP-based systems

Neutral organophosphorous compounds (NOC) are known to be advantageously distinguished from acidic organophosphorous compounds by kinetic properties. Thus, the equilibrium attainment in an extraction system on the basis of NOC is faster and the mass transfer is more efficient. The values of mass transfer coefficients of Eu(III) and Fe(III) in DTMPDP - chloroform - HCl - NH_4SCN systems are given in Table 4. Both t-dependences are straight lines. NH_4SCN was used as complexing agent, which increased the extraction of Eu(III) and Fe(III) and enabled one to work with low DTPMDP concentrations [22]. The results obtained demonstrate that the mass transfer coefficients of Eu(III) and Fe(III) in the systems on the basis of DTPMDP are significantly higher than those for the systems with D2EHPA.

The chromatographic behaviour of Eu(III) and Fe(III) in DTPMDP-based systems

The chromatographic behaviour of Eu(III) in the 0.005 mol/l DTPMDP - chloroform - 0.5 mol/l NH_4SCN - HCl system at different HCl concentrations is shown in Fig.6. D^{dyn} and D^{bat} of Eu(III) in the systems under investigation are given in Table 5. The values of D^{dyn} are lower than those of D^{bat}. This fact testifies that the elution of Eu(III) is not an equilibrium process. The width of the chromatographic peaks increases with a decrease in HCl concentration. However, in contrast to systems with D2EHPA, Eu(III) was retained in the separation column after a volume of V_m had passed off.

Figure 7 (the origin of the coordinates corresponds to the volume of the mobile phase in the column) shows the curve shape and position as a function of the stationary phase

Table 4

Mass transfer coefficients (k) and batch partition coefficients (D^{bat}) for Eu(III) and Fe(III) in DTPMDP-based extraction systems

Element	Extraction system	k, cm/s	D^{bat}
Eu(III)	0.005 mol/l DTPMDP - $CHCl_3$ - 0.4 mol/l HCl - 0.5 mol/l NH_4SCN	1.8×10^{-3}	3.8
Fe(III)	0.003 mol/l DTPMDP - $CHCl_3$ - 1.0 mol/l HCl - 0.5 mol/l NH_4SCN	8.6×10^{-4}	2.7

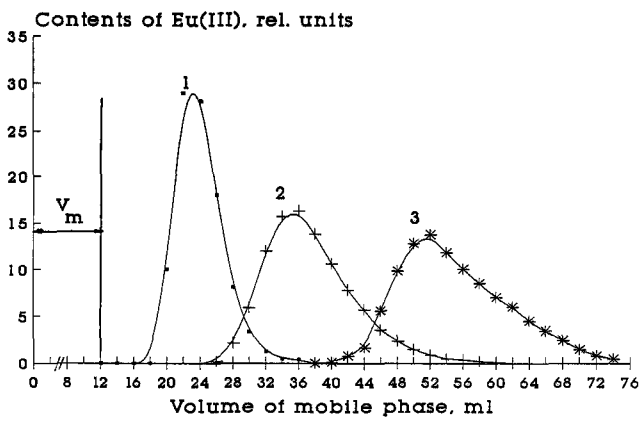

Fig.6: Chromatographic behaviour of Eu(III) in the 0.005 mol/l DTPMDP - chloroform - HCl - 0.5 mol/l NH_4SCN system. $S_f = 0.5$. HCl concentration in the mobile phase: *1* - 1.0, *2* - 0.6, *3* - 0.4 mol/l.

Table 5

Batch and dynamic partition coefficients for Eu(III) in the 0.005 mol/l DTPMDP - chloroform - HCl - 0.5 mol/l NH_4SCN system

C_{HCl}, mol/l	D^{bat}	D^{dyn}
1.0	1.8	0.9
0.6	2.7	1.8
0.4	3.8	3.0

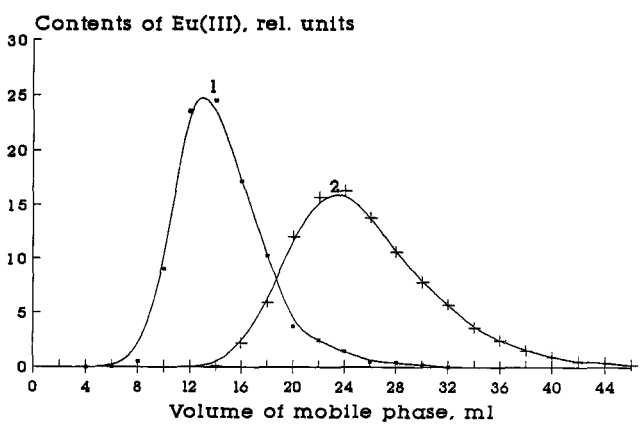

Fig. 7: Influence of the stationary phase volume on the shape of the elution curves of Eu(III) in the 0.005 mol/l DTPMDP - chloroform - 0.6 mol/l HCl - 0.5 mol/l NH_4SCN system. *1* - $S_f = 0.25$ ($V_s = 6$ ml), *2* - $S_f = 0.5$ (V_s=12 ml).

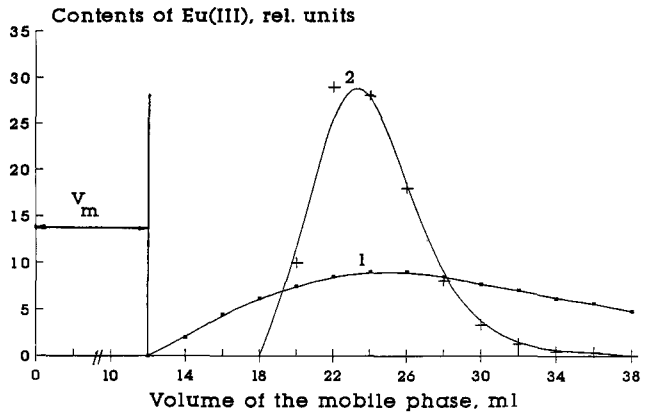

Fig. 8: Influence of the mass transfer coefficient on the Eu(III) peak shape. *1* - 0.05 mol/l D2EHPA - n-decane - 0.20 mol/l HCl system. S_f=0.5. D^{dyn}=1.2. k=2.0 x 10^{-4} cm/s. *2* - 0.005 mol/l DTPMDP - chloroform - 1.0 mol/l HCl - 0.5 mol/l NH_4SCN system. S_f = 0.5. D^{dyn} = 0.9. k = 1.8 x 10^{-3} cm/s.

volume in the column. The chromatographic peak shifts to the left and narrows if the extractant quantity is twice lower (V_s^f =6 ml, peak *1*), all the other factors being the same. The values of D^{dyn} calculated from the chromatograms are the same and equal to 1.8.

Figure 8 illustrates once more the importance of the contribution from chemical kinetics to the separation process. Two peaks are quite different due to the kinetic peculiarities of the extraction systems used, although the corresponding values of dynamic partition coefficients are close (1.2 and 0.9).

Hence, the mass transfer coefficients determine the type of elution, which is necessary for the element separation under given experimental conditions. The systems providing high values of k (about 10^{-3} cm/s) are suitable for isocratic separation as well as for separation by step elution, whereas kinetically more inert systems (k is about 10^{-4} cm/s) require only step elution.

It should be noted that the solvent extraction and chromatographic characteristics for the substances to be separated can be linked by empirical expression 4 [19].

$$\frac{\dfrac{D_{(1)}^{bat} V_{s(1)}}{W_{1/2(1)}}}{\dfrac{D_{(2)}^{bat} V_{s(2)}}{W_{1/2(2)}}} \approx \frac{k_{(1)}}{k_{(2)}}, \qquad (4)$$

where $W_{1/2}$ - half-width of chromatographic peak, indices (1) and (2) relate to two different extraction systems.

Thus, having the batch extraction data (partition coefficients and mass transfer coefficients) for two systems and the results of a chromatographic experiment for one of them, one can estimate $W_{1/2}$ of a chromatographic peak obtained by use of the second system. However, Eq. 4 is valid for partition coefficient values in the range of 1 - 10 and for reversible processes of extraction and back-extraction. The hydrodynamic conditions of both experiments should be identical. Theoretical estimation of $W_{1/2}$ allows one to avoid a time-consuming experimental selection of chromatographic conditions. Thus, if the estimated value is relatively high when $D_{(2)}^{bat}=1$, elution of the component of interest begins with the V_m volume and the chosen system is unsuitable under given conditions for isocratic separation from other components. An acceptable magnitude of $W_{1/2}$ can be achieved by changing the extraction system, choosing concentrations of its constituents, changing V_s volume.

CONCLUSION

The systems for CCC inorganic separations in most cases contain a complexing reagent (ligand). Because of the necessity of taking into account metal-ligand interaction and distribution of the complexes formed, the fundamentals of inorganic separations should be very different from organic ones. The contribution from some chemical kinetics factors to the separation of inorganic species by CCC has been estimated.

It has been shown that the kinetic properties of extraction systems on the basis of complexing reagents have a greater influence on the separation (preconcentration) process than hydrodynamic parameters of the planet centrifuge operation (rotation and pumping speeds). It has been demonstrated that the systems providing high values of mass transfer coefficients (about 10^{-3} cm/s) are suitable for isocratic separation as well as for separation by step elution. When complex formation and mass transfer rates are lower (k is about 10^{-4} cm/s), only step elution is required. If the elements under investigation are kinetically very inert (k is about 10^{-5} cm/s), some difficulties arise even when these elements are being concentrated. High partition coefficients (about 10^2) are necessary for the quantitative extraction of such elements into the stationary phase.

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SEPARATION OF THE 2- AND 6-NITRO-3-ACETAMIDO-4-CHLOROBENZOIC ACID PRECURSORS OF A POTENT HYDROXY-ANTHRANILIC ACID OXYGENASE INHIBITOR BY pH-ZONE-REFINING-COUNTERCURRENT CHROMATOGRAPHY

Y. MA[1], Y. ITO[1], D. S. TOROK[2], AND H. ZIFFER[3]

[1]Laboratory of Biophysical Chemistry
National Heart, Lung, and Blood Institute
National Institutes of Health
Bethesda, Maryland 20892
[2]Laboratory of Clinical Science
National Institute of Mental Health
National Institutes of Health
Bethesda, Maryland 20892
[3]Laboratory of Chemical Physics
National Institute of Diabetes and Digestive and Kidney Diseases
National Institutes of Health
Bethesda, Maryland 20892

ABSTRACT

Multigram quantities of 2- and 6-nitro-3-acetamido-4-chlorobenzoic acids were separated by pH-zone-refining CCC. A two-phase solvent system composed of methyl tertiary-butyl ether, acetonitrile and water was employed using trifluoroacetic acid as a displacer acid in the organic mobile phase and ammonia as a retainer base in the aqueous stationary phase.

INTRODUCTION

Quinolinic acid, a metabolite of tryptophan in the kynurenic pathway, is present in high levels in the brain and blood of patients and primates with a broad spectrum of inflammatory neurological diseases (1). The level of quinolinic acid present was correlated with the severity of the neurological distress and prompted the suggestion that the neurological disturbances resulted from high levels of quinolinic acid. One approach to reducing the high levels of quinolinic acid was to employ inhibitors of enzymes responsible for the conversion of tryptophan to quinolinic acid, Saito et al. (2) found that 6-chlorotryptophan and its metabolite, 4-chloro-3-hydroxy-anthranilic acid were potent inhibitors of 3-hydroxy-anthranilic acid oxygenase. For animal studies large quantities of 4-chloro-3-hydroxy-anthranilic acid had to be prepared. We employed a reaction sequence developed by Glibin et al. (3) where a critical step in their sequence involves nitration of 3-acetamido-4-chlorobenzoic acid followed by separation of the isomeric mononitro-derivatives, 2- and 6-nitro-3-acetamido-4-chlorobenzoic acid (Fig. 1). These two isomers were difficult to separate by repeated fractional crystallization of their barium salts. Our

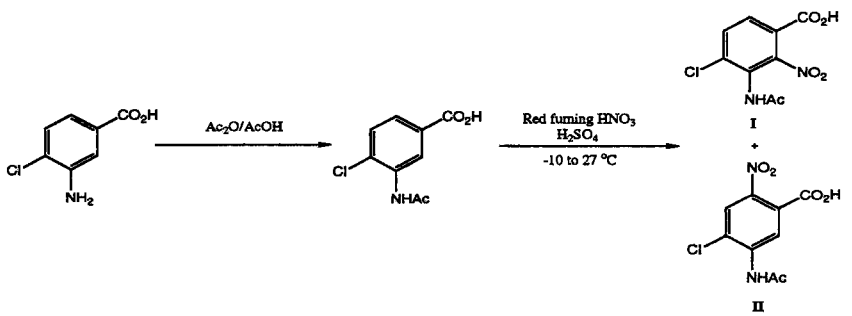

FIGURE 1. Synthesis and structures of isomers I and II: 2- and 6-nitro-3-acetamido-4-chlorobenzonic acids.

attempts to find a solvent system for separating these isomers by column or flash chromatography were unsuccessful.

A recently developed preparative method, pH-zone-refining CCC (4-8), yields highly concentrated rectangular peaks of analytes comparative to those observed in displacement chromatography (9). The method has been applied to ionizable compounds, either acids or bases, which are eluted in the order of their pK_a's and hydrophobicities. In this paper, we describe the use of pH-zone-refining CCC (in a displacement mode) for the separation of multigram quantities of 2- and 6-nitro-3-acetamido-4-chlorobenzoic acid. Each analyte was characterized by ^{1}H and ^{13}C NMR.

EXPERIMENTAL

Synthesis of 3-Acetamido-4-Chloro-2-Nitrobenzoic Acid (I) and 3-Acetamido-4-Chloro-6-Nitrobenzoic Acid (II)

To a hot (90°C) solution of 3-amino-4-chlorobenzoic acid (200 g, 1.17 mol) in glacial acetic acid (1000 ml) was added acetic anhydride (541 g, 500 ml, 5.30 mol) over 5 min. The solution was kept at 90°C for 30 min and cooled to 4°C. The resulting solids were filtered and washed with cold ether (2 X 1000 ml) to afford 3-acetamido-4-chlorobenzoic acid (212 g, 85 %, mp 206-208°C).

To a stirred solution of 3-acetamido-4-chlorobenzoic acid (200 g, 0.936 mol) at -10°C (ice/MeOH) was added a solution of red fuming nitric acid (787 g, 500 ml, 12.5 mol) in sulfuric acid (460 g, 250 ml, 4.96 mol) over 3 hr. The reaction was stirred at -10°C for 30 min then slowly warmed to RT and poured over ice (1000 g). The resulting yellow solid was filtered and washed with cold water (2 X 750 ml) to afford a mixture of nitro isomers I and II (Fig. 1).

CCC Apparatus

A commercial model (Ito Multilayer Coil Separator/Extractor, Potomac, MD, USA) of the high-speed CCC centrifuge was used throughout the present

studies. The basic design of the apparatus was reported elsewhere (4).

The separation column was prepared in our laboratory by winding a single piece of 1.6 mm ID 160 m long PTFE (polytetrafluoroethylene) tubing around the column holder hub forming 16 coiled layers with 325ml capacity.

The revolution speed was regulated with a speed controller (Bodine Electric Company, North Chicago, IL, USA). An optimum speed of 600 - 800 rpm was used in the present studies.

Reagents for CCC Separations

Methyl tertiary-butyl ether (HPLC grade) and ammonium hydroxide (reagent grade) were purchased from Fisher Scientific Company, Fair Lawn, NJ, USA. Acetonitrile (HPLC grade) and trifluoroacetic acid (reagent grade) were purchased from Baxter Healthcare Corporation, Muskegon, MI, USA.

Preparation of Solvent Phases and Sample Solutions

The solvent pairs were prepared as follows: Methyl tertiary-butyl ether, acetonitrile and distilled water (4:1:5, v/v/v) were thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated. The upper organic phase was

acidified with trifluoroacetic acid (0.32%) (pH 1.74) while aqueous ammonia was added to the lower aqueous phase (0.8%, pH 11.2).

The sample solution was prepared by dissolving a crude synthetic mixture of the isomers in 100 ml of a phase mixture consisting of equal volumes of each phase. The pH of the sample solution was adjusted to about 8.7 with aqueous ammonia.

Separation Procedures

The displacement mode of pH-zone-refining CCC was performed as follows: the column was first entirely filled with the aqueous phase containing 0.8% aqueous ammonia (retainer base) followed by sample injection through the sample port. Then, the acidified organic phase containing 0.32% trifluoroacetic acid (displacer) was pumped into the inlet of the column in the tail to head elution mode while the column was rotated at 600 rpm which was later raised to 800 rpm after 10 fractions had been collected. The above manipulation of the column rotation was to minimize the carryover of the stationary phase. The effluent from the outlet of the column was continuously monitored with a uv detector (Uvicord S, LKB Instruments, Bromma/Stockholm, Sweden) at 206 nm and collected at two minute intervals (6.6ml/tube) with a fraction collector (Ultrorac, LKB Instruments).

In this elution mode, the two phases establish a reverse pressure gradient through the column where the pressure at the column inlet often plunges into a negative range causing suction of an extra volume of solvent from the reservoir through the one-way check valves of the metering pump. In order to prevent this complication, a piece of PTFE tubing (0.4mm ID x 3m long) was placed at the outlet of the detector to maintain the column pressure above an atmospheric level. This device also prevented formation of gas bubbles inside the flow cell of the uv detector, thus reducing the noise level in the recording of the elution curve.

After the desired peaks were eluted, the apparatus was stopped and the column contents were collected into a graduated cylinder by connecting the inlet of the column to a nitrogen line at 80 psi. The retention of the stationary phase relative to the total column capacity was computed from the volume of the stationary phase collected from the column.

Analysis of CCC Fractions

The pH value of each fraction was manually determined with a portable pH meter (Accumet Portable Laboratory, Fisher Scientific Company, Pittsburgh, PA, USA).

CCC fractions were analyzed by reversed phase high performance liquid chromatography (HPLC) with a Shimadzu HPLC consisting of a Model LC-6A pump, a manual injector kit, a Model SDP-6A detector, and a Model C-R5A recording data processor (Shimadzu Corporation, Kyoto, Japan) and a Mos-hypersil-1 RPC-8 column, 5 μm, 250 x 4.6 mm (Keystone Scientific Co., Bellefonte, PA, USA). The mobile phase, composed of 0.1M aqueous NH_4OAc and methanol at a volume ratio 4:1, was isocratically eluted at a flow rate of 0.9 ml/min, and the effluent was monitored at 280nm.

Two structural isomers, 3-acetamido-4-chloro-2-nitrobenzoic acid (I) and 3-acetamido-4-chloro-6-nitrobenzoic acid (II) were identified by ^1H and ^{13}C-NMR ($MeOH-d_4$).

I: ^1H-NMR($MeOH-d_4$): 2.11(s,3H), 7.88(d,J=8.14 Hz,1H), 8.03(d,J=8.14Hz,1H), 9.13(bs,1H); ^{13}C-NMR($DMSO-d_6$): 22.5, 123.8, 128.9, 130.6, 132.0, 138.5, 149.4, 163.8, 169.6.

II: ^1H-NMR($MeOH-d_4$): 2.29(s,3H), 8.14(s,1H), 8.84(s,1H), 9.12(bs,1H); ^{13}C-NMR($DMSO-d_6$): 23.9, 123.6, 125.5, 126.4, 127.2, 139.5,143.2, 165.4, 169.8.

RESULTS AND DISCUSSION

Figure 2 shows the chromatogram obtained from the separation of 15 grams of crude nitro-isomers with the

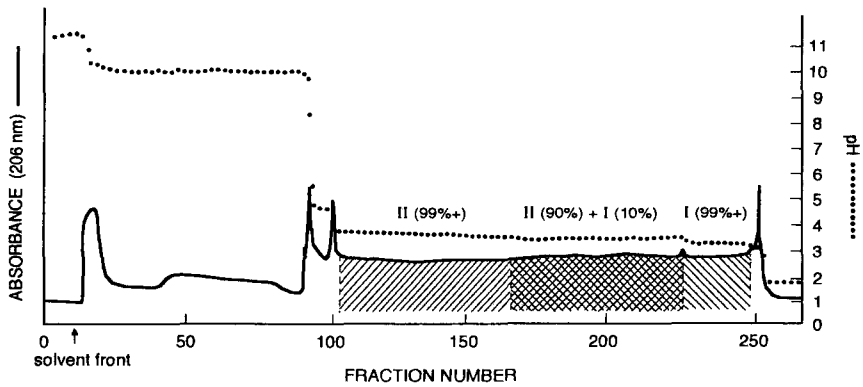

FIGURE 2. Chromatogram of isomeric mononitro-derivatives of 2- and 6-nitro-3-acetamido-4-chlorobenzoic acid by displacement mode of pH-zone-refining CCC. Experimental conditions were as follows: Apparatus: High-speed CCC centrifuge equipped with a multilayer coil of 1.6 mm ID and 325 ml capacity; Solvent system: Methyl-tertiary-butyl ether/acetonitrile/water (4:1:5); Stationary phase: Lower phase (0.8% aqueous ammonia); Mobile phase: Upper phase (0.32% trifluoroacetic acid); Flow rate: 3.3ml/min; Sample: Nitration product of 3-acetamido-4-chlorobenzoic acid (15 grams) dissolved in 100ml in equal volumes of the upper and lower phases; Revolution: 800rpm (600 rpm until 66ml of mobile phase was eluted).

displacement mode of pH-zone-refining CCC. The two isomeric mononitro-derivatives, II and I (see Fig. 1), were eluted in successive rectangular peaks associated with sharp impurity peaks at their boundaries. The separation required slightly over 8 hours. The pH measurement of the fractions revealed that the pH curve (dotted line) formed a characteristic downward staircase pattern where each plateau of the analyte corresponds to the distinct pH zone.

The first peak represents elution of isomer II and the second peak, that of isomer I. HPLC and NMR analyses of peak fractions showed that the early portion of the first peak (fractions 103-165, 5.4 g) and the entire portion of the second peak (fractions 229-254, 2.2 g) were essentially pure, while fractions at the later portion of the first peak (fractions 166-225, 5.3 g) were contaminated with about 10% of isomer I as indicated in the chromatogram. If desired, this fraction may be rechromatographed under the identical condition to improve the yield of pure fractions.

The overall results of our studies demonstrate that pH-zone-refining CCC can be efficiently used for purification of multigram quantities of structural isomers from a crude reaction mixture in 8 hours.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION AND DETERMINATION OF SALICYLALDOXIME AND β-RESORCYLALDOXIME IN AQUEOUS SOLUTIONS AND IN HUMAN URINE

J.-P. HIMBERG[1,2], M. KOSKINEN[1], M.-L. RIEKKOLA[2], AND H. ELO[1]*

[1]*Division of Chemistry*
Department of Pharmacy
University of Helsinki
P.O. Box 15 (Fabianinkatu 35)
FIN-00014 University of Helsinki
Helsinki, Finland

[2]*Division of Analytical Chemistry*
Department of Chemistry
University of Helsinki
P.O. Box 6 (Vuorikatu 20)
FIN-00014 University of Helsinki
Helsinki, Finland

ABSTRACT

A high-performance liquid chromatographic method for the separation and quantitation of salicylaldoxime (2-hydroxybenzaldoxime, SAO) and β-resorcylaldoxime (2,4-dihydroxybenzaldoxime, RES) is reported. Analytical methods for these compounds are needed for studies on the reactions of their antineoplastic copper(II) chelates with biomolecules and for studies on the metabolism of the chelates. A reversed-phase column (Supelcosil LC-18, 150 x 4,6 mm, 5 μm mean particle size) was used as the stationary phase and a mixture

*To whom all correspondence should be addressed.

of tetrahydrofurane and water (volume ratio 55:45) as the mobile phase. UV absorption at 294 nm was used for detection. The retention times of RES and SAO were 4.5 min and 5.3 min, respectively, with a flow rate of 0.5 ml/min. For quantitation of SAO and RES in spiked human urine, the samples were pretreated by extracting with diethyl ether, evaporating the ether phase and dissolving the residue in the eluent. The components of the urine matrix present after this treatment did not interfere with the separation of RES and SAO. In the case of aqueous solutions as well spiked urine samples, the limit of detection of SAO was 0.2 μg/ml and that of RES 0.1 μg/ml.

INTRODUCTION

The copper(II) chelates of salicylaldoxime and its 4-hydroxy analog β-resorcylaldoxime, i.e. *trans*-bis(salicylaldoximato)copper(II) ($CuSAO_2$) and *trans*-bis(β-resorcylaldoximato)copper(II) ($CuRES_2$), as well as some analogs of them have remarkable antiproliferative activity against various tumour cell lines in cell culture [1-2] (see Fig. 1 for the structures of the compounds). $CuSAO_2$ has also been shown to have potent and in some cases even curative antitumour activity against Ehrlich ascites carcinoma in mice [1,3]. The compounds also have interesting and potentially important immunopotentiating properties *in vivo* [1,2,4] as well as *in vitro* [5]. In contrast to the chelates, the free ligands or copper(II) ions are essentially inactive against tumour cells at least *in vitro*, being devoid of antiproliferative activity in the concentration range in which $CuSAO_2$ and $CuRES_2$ totally block tumour cell proliferation [1].

Several lines of reasoning strongly suggest that $CuSAO_2$ and $CuRES_2$ are rapidly metabolized *in vivo* and possibly also *in vitro*. First, intraperitoneal administration of a chelate to rats gives rise to a visually observable green colour in the urine of the animals [4], suggesting that the copper has been liberated or that the chelate has been metabolized so that it becomes soluble in water and gets a new colour. ($CuSAO_2$ and $CuRES_2$ are almost insoluble in water and their colour is different from that observed in the urine of the treated animals.)

Second, after intraperitoneal administration of a suspension of $CuSAO_2$ or $CuRES_2$ to rats, the chelate rapidly disappears from the peritoneal cavity, in spite of its extremely low aqueous solubility. Simultaneously, the pancreata of the animals become hard and get a dark green or almost black colour becoming very easily detectable visually [4,6]. When such pancreata are put in an aqueous fixing solution, the solution is almost immediately coloured green. The colour of the exudate is different from that of either one of the chelates, suggesting again either the liberation of a metabolized form of the chelate, or the liberation of copper ions from the chelate [and possible formation of new complexes with some endogenous ligand(s)] [4,6].

Third, $CuSAO_2$ and $CuRES_2$ as well as many potent analogs of them are known to very rapidly react in the test tube with glutathione and cysteine, two common thiols present in living cells [7]. This reaction may well take place also *in vivo* and in cultured cells, and may even be involved in the mechanism of action of the chelates since the ability of glutathione and cysteine to rapidly destroy $CuSAO_2$, $CuRES_2$ and various antiproliferative and non-antiproliferative analogs is distinctly correlated with the antiproliferative activity of the compounds [7].

In order to be able to study the reactions of $CuSAO_2$ and $CuRES_2$ with thiols in more detail (e.g. to study the possible liberation of the ligands from the chelates), reliable methods are needed for the analysis of their ligand parts, salicylaldoxime (SAO) and β-resorcylaldoxime (RES) (see Fig. 1 for structural formulas of the oximes). More importantly, such methods are needed for the study of the metabolism of the chelates. Obviously, development of methods for the quantitation of the free ligands in urine is especially warranted, since the ligands or their metabolites may well be excreted in the urine. A literature study, however, does not reveal any methods for the analysis of SAO or RES. Therefore, we developed an HPLC method for the separation and quantitation of SAO and RES in aqueous solutions and in human urine.

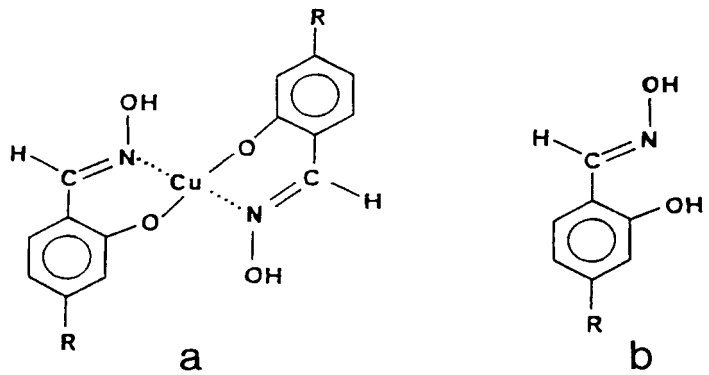

FIGURE 1. (a) The structures of $CuSAO_2$ (R = H) and $CuRES_2$ (R = OH). The systematic names of the compounds are *trans*-bis(2-hydroxybenzaldoximato)-copper(II) and *trans*-bis(2,4-dihydroxybenzaldoximato)copper(II), respectively. (b) The structures of the free ligands, SAO (R = H) and RES (R = OH). The systematic names of the ligands are 2-hydroxybenzaldoxime and 2,4-dihydroxybenzaldoxime, respectively.

EXPERIMENTAL

Chemicals

Analytical grade SAO was obtained from E.Merck (Darmstadt, Germany). RES was synthesized from 2,4-dihydroxybenzaldehyde and hydroxylamine according to standard methods. Details of the synthesis will be published elsewhere [8]. HPLC grade tetrahydrofurane (THF) was obtained from Rathburn (Walkerburn, Scotland). Water was purified by using a Millipore Alpha Q water purification system. Acetic acid was from E. Merck (Titrisol).

Urine Sample Preparation

Human urine was obtained from a 23-year-old male volunteer. Fresh samples were used daily. Urine was spiked with the aldoximes by adding an

appropriate volume of an aqueous solution (1 mg/ml) of the aldoxime to a 5-ml measuring flask and by filling with urine.

Pretreatment of Urine Samples for Chromatography

Urine samples of 1 ml volume were acidified by adding 200 µl of 0.2 M aqueous acetic acid, followed by mixing with a vortex-type shaker. The sample was then extracted once with 5 ml of diethyl ether, vortexing the tube for 1 min with the maximum effect of the mixer. The ether phase was transferred to another test tube and evaporated to dryness in a water bath (ca. 32°C) with the aid of a nitrogen stream. The residue was redissolved in 1 ml of the eluent used in the chromatography. This solution was injected into the HPLC system.

Chromatographic Apparatus and Conditions

All chromatographic measurements were carried out using a high-performance liquid chromatographic system consisting of two LC-10AD liquid chromatograph solvent delivery systems and an SPD-M6A photodiode-array UV-VIS detector (Shimadzu Corporation, Kyoto, Japan). A Hyundai Super 386N Plus computer equipped with a 120 B hard disk and 4 MB extended memory was used for data acquisition and processing in this system, employing Shimadzu LC workstation [Class-LC10 version 1]. The disk operating system was MS-DOS version 5.0. Injection was done using a Shimadzu SIL-6B auto injector, controlled by a SCL-6B system controller.

A Supelcosil LC-18 column (catalog no. 5-8230, 150 x 4.6 mm I.D., 5µm mean particle size), obtained from Supelco, Inc., Supelco Park, Bellefonte, PA, U.S.A., was employed. A LiChroCART 4-4 (Cat. 50957) precolumn containing LiChrospher 100 RP-18 packing material (particle size 5 µm) was used and was obtained from E. Merck.

Column dead volume determination was performed with the aid of 0.03 M aqueous $NaNO_3$.

Chromatographic separations were carried out at room temperature using an isocratic system with a mixture of THF and water (55:45 volume ratio) as the mobile phase. A constant flow rate of 0.5 ml/min was used. Injection volume was 20 µl. Detection was based on UV absorption at 294 nm, measured with the diode array detector. For quantitation of SAO, peak heights gave optimal results and were thus used throughout the study, while for RES, peak areas gave optimal results and were used.

RESULTS AND DISCUSSION

SAO was found to have intense absorption maxima at ca. 214, 258 and 295 nm, and RES at ca. 218 and 277 nm, and UV detection was used throughout the study. At first, attempts were done to develop methods of analysis for aqueous solutions of SAO by using methanol, ethanol or acetonitrile as such or as mixtures with water as the eluent. In these experiments, the same column was employed as in the final method developed, and the flow rate was varied between 0.5 and 1.0 ml/min. In the case of methanol-water mixtures, experiments were also performed using various column temperatures (25 - 50°C). In these studies, no satisfactory methods were found, too rapid elution and/or excessive tailing and peak broadening being the problems.

THF-water mixtures were, however, found to be suitable as the mobile phase. When THF-water mixtures were employed as the eluent, the best results were obtained with a THF/water volume ratio 55:45. With higher THF concentrations, RES and SAO eluted too rapidly considering analysis from biological matrices that may contain large amounts of various hydrophilic

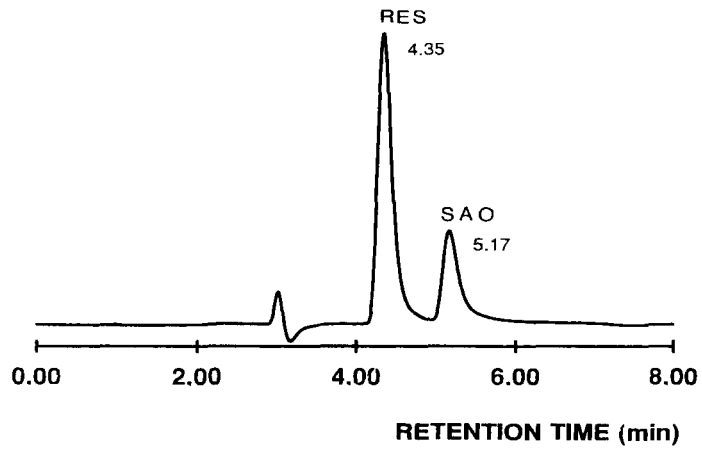

FIGURE 2. Chromatogram of an aqueous solution of SAO (12 μg/ml) and RES (8 μg/ml) (separation conditions in Experimental).

substances. With higher water contents, peak broadening caused serious problems.

With the method developed, we could also separate SAO and RES from each other (see Fig. 2), the 2,4-dihydroxylated congener RES eluting before the monohydroxylated SAO, just as would be expected. In the case of the analysis of aqueous solutions, any wavelength between ca. 200 and 300 nm could be used for detection of the analytes.

The method developed was applied also to the separation of the two congeners as well as to the quantitation of each congener in spiked human urine samples. Thus, after the pretreatment of the samples by acidification, extraction with ether, evaporation of the ether phase and redissolution of the residue, they were injected into the HPLC system. Good separation of the two aldoximes was again obtained. In the case of the pretreated urine samples, the wavelength used

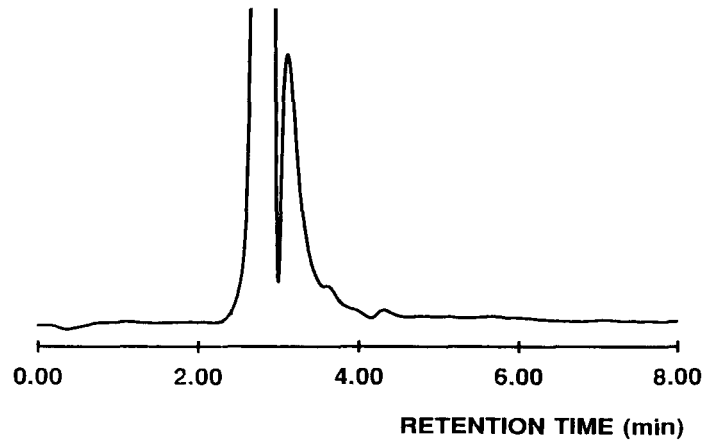

FIGURE 3. Chromatogram of a blank urine sample after the pretreatment procedure (separation conditions in Experimental).

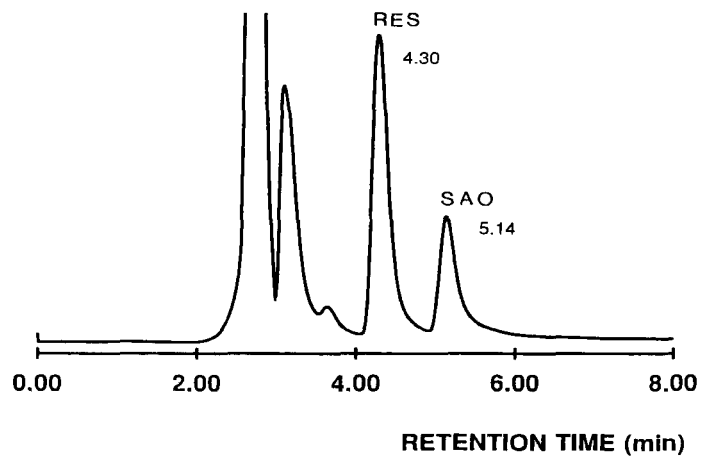

FIGURE 4. Chromatogram of urine sample spiked with SAO (20 μg/ml) and RES (10 μg/ml) and pretreated according to the method described in Experimental (separation conditions in Experimental).

TABLE 1

Recoveries of RES and SAO from spiked human urine samples.

Intra-day

Compound	Amount spiked (μg/ml)	n	Amount recovered (μg/ml) Mean	S.D.	C.V. (%)	Recovery (%)
RES	10	6	8.4	0.7	7.7	84.2
	50	6	44.8	4.5	10.1	89.6
	100	6	86.7	6.9	8.0	86.7
SAO	10	6	8.6	0.4	5.1	86.4
	50	6	42.9	2.4	5.5	85.8
	100	6	82.7	8.7	10.6	82.7

Inter-day

Compound	Amount spiked (μg/ml)	n	Amount recovered (μg/ml) Mean	S.D.	C.V. (%)	Recovery (%)
RES	10	3	8.7	0.6	7.0	87.3
	50	3	45.1	3.1	6.8	90.2
	100	3	88.2	8.8	10.0	88.2
SAO	10	3	8.7	0.7	8.5	82.0
	50	3	41.9	5.7	13.6	83.8
	100	3	84.6	8.7	10.3	84.6

for detection was found to be more critical. When the wavelength was 294 nm, absorption by the components of the pretreated urine matrix was minimal in the region of the chromatogram where RES and SAO were eluted (see Figs. 3 and 4). If detection was performed at the highest absorption maxima of SAO or RES, the matrix components of urine were not completely separated from SAO and RES, thus preventing the analysis of small concentrations of the aldoximes.

The method developed makes possible the quantitation and detection of clearly lower concentrations of RES, as compared to SAO, since the UV absorption of RES is more intense than that of SAO and also since the peak of RES is sharper than that of the slower-eluting SAO. In the case of aqueous solutions as well spiked urine, the limit of detection of SAO is 0.2 μg/ml and that of RES 0.1 μg/ml.

SAO and RES were quantitated separately using the same method as was used for the separation of the congeners. For the aqueous solutions of SAO, the method was linear in the range between 5 and 500 μg/ml ($r = 0.998$), and for those of RES between 1 and 500 μg/ml ($r = 0.999$). For the urine samples spiked with SAO, the method was linear in the range between 5 and 250 μg/ml ($r = 0.995$), and for those of RES between 1 and 100 μg/ml ($r = 0.998$). The method can, however, be used for the quantitation of higher concentrations up to ca. 1 mg/ml, if calibration is performed using several standard solutions with different concentrations. Such high concentrations, however, probably cannot exist in real urine samples.

In the case of the spiked urine samples, the mean recoveries of SAO and RES were approximately 82 - 90% (see Table 1 for details). The intra-day and inter-day repeatabilities of the method developed were acceptable (Table 1).

We found that, when dissolved in water, SAO has a strong tendency to be adsorbed on glass surfaces, necessitating the preparation of new standard solutions daily. A similar phenomenon was not observed with RES.

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DETERMINATION OF AMRINONE IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET DETECTION

REETA BANSAL, A. T. LOURIDAS, R. D. GOTTESMAN, AND J. V. ARANDA
Centre for Perinatal and Developmental Pharmacology Research
Departments of Pediatrics and Pharmacology
McGill University—Lady Davis Institute for Medical Research
3755 Côte Ste-Catherine Road
Montreal, Quebec, Canada H3T 1E2

ABSTRACT

A specific, rapid and sensitive high pressure liquid chromatographic method is described for the determination of amrinone in human plasma. The method involves the use of a commercially available 4 μm particle size reverse phase Waters Nova pak Cyano Column, guard pak Cyano cartridges and a UV/VIS detector. The extraction was based on liquid - liquid single step method. Extraction is carried out with ethyl acetate on a 100 μl plasma sample. The residue is reconstituted with 100 μl of the solution 50:50 (Acetonitrile and 3.2 pH phosphate buffer) and 20 μl of the final solution is injected into the column. Elution is carried out at ambient temperature using acetonitrile and 3.2 pH phosphate buffer (70:30 by volume) as mobile phase at a flow rate of 2.0 ml/min at 1200 PSI. Detection is at 210 nm. Mean retention time ($\pm$ SEM) was 4.18 $\pm$ 0.26 minutes. Separation requires 5 minutes and the sensitivity limit is 0.25 μg/ml - 10 μg/ml. This technique was used in 19 newborn and young infants receiving amrinone. Their plasma concentrations of amrinone were 1.48 $\pm$ 2.13 mg/L during a constant infusion of amrinone 5 to 10 micrograms/kg/minute indicating applicability of this technique in therapeutic drug monitoring.

INTRODUCTION

Amrinone is a synthetic bipyridine derivative (5-amino-3,4'-bipyridin-6-(IH)-one) with potent cardiac inotropic and pulmonary/systemic vasodilatory effects. This prototype of a new class of nonglycoside, noncatecholamine cardiotonic agents is currently used in the treatment of heart failure.

Patients with congestive heart failure (CHF) have a high incidence of sudden death that may be due to either an acute exacerbation of pump function or arrhythmias (1,2). Maskin et al (3) reported that in patients with severe heart failure, the prevalence of complex ventricular arrhythmias is 92% and that of ventricular tachycardia is 71%. Drug aggravation of arrhythmias has occurred with many cardioactive drugs (4). Amrinone is also metabolized by the liver via acetylation and glucuronidation (5). Both amrinone and it's major metabolite (the physiologically inactive n-acetyl derivative) are excreted by the kidneys (5). Amrinone and its metabolites may, because of delayed acetylation or renal elimination, accumulate in some patients. This problem is of great relevance in the newborn and young infant wherein drug elimination and metabolism are also deficient (9). Amrinone is used for cardiovascular support in pediatric patients, thus therapeutic drug monitoring of amrinone concentrations would be beneficial.

A direct relationship between the plasma concentration of amrinone and its pharmacodynamic effect has been suggested (6), thus measurement of plasma amrinone may be useful in the clinical setting. One of the reported methods for detection of amrinone by HPLC was based on a more time consuming extraction procedure (7). Another method does not report the recovery (8). We, therefore, developed a microassay using HPLC and ultraviolet-detection method.

MATERIALS AND METHODS

Patients and Adult Volunteers

Plasma samples (0.2 ml/sample) were obtained from 19 babies ages 3 days to 4 years. All babies received amrinone for cardiovascular support with a loading dose of 2 mg/kg for 10 minutes intravenously followed by a constant intravenous infusion of 5 to 10 microgram/kg/min. Venous blood samples were collected in a heparanized tube during this continuous intravenous infusion for amrinone assay. In addition, drug free venous blood (1-2 ml) was collected from healthy human volunteers (n=5) receiving no medications. Blood was collected into plastic tubes containing lithium heparin and centrifuged for 10 min. at 3000 rpm. Plasma was separated and stored at -80°C until the time of analysis.

Sample Preparations and Extraction Procedures

Extraction was performed in a silanized tube. Ethylacetate (1000 μl) was added to patients' plasma samples or spiked plasma standards (100 μl) containing amrinone in a silanized tube. The mixture was vortexed for 30 seconds. All samples were centrifuged at 2000 rpm for 5 minutes. The organic phase was transferred into a glass tube and the solvent was evaporated under nitrogen at 30°C for about 10 minutes. The residue was reconstituted in 100 μl of the (50:50 V/V) Acetonitrile and phosphate buffer at pH 3.2 and 20 μl was injected into a column.

ASSAY OF AMRINONE

Reagents and Solutions

All chemicals used were of analytical grade unless otherwise stated. Sodium dihydrogen orthophosphate, sodium phosphate monobasic, orthophosphoric acid, sodium hydroxide, ammonium hydroxide (analytical grade), ethyl acetate and acetonitrile (HPLC grade) were obtained from BDH (Pooled, U.K.). Sigmacote (silanising reagent) and amrinone were purchased from Sigma Chemical Company (U.S.A.). Only HPLC-grade de-ionized water was used. All glassware used during the sample preparation was silanized with Sigmacote, rinsed with toluene-methanol and several times with methanol and de-ionized water.

Apparatus

The instruments included a PH-M-82 Standard pH meter, IEC Centra-8R Centrifuge, and Concentrator - Jouan RC 1010. A Waters HPLC System consisting of a 510 HPLC pump, model Waters 715 Ultra WISP autosampler, and 994 UV/VIS detector. Peak height measurements of the drug were integrated on a Waters model 820 integrator plotter. A 4 μm, 8 mm x 100 mm Waters Nova Pak reverse phase cyano column and guard-pak cyano cartridges (Waters, Milford, MA, USA) were used.

Chromatographic Conditions

For the analysis of amrinone, the mobile phase was phosphate buffer 5 mM, pH 3.2 (30%), acetonitrile (70%) (70:30 by volume). The mobile phase was filtered before use with a 0.45 μm

polyvinylidene difluoride membrane filter (Millipore) and degassed under suction. The column was operated at ambient temperature at the flow rate of 2.0 ml/minute and the column effluent was measured at the sensitivity of 1.0 or 0.05 absorbance units (AUFS). Wavelength was 210 nm and the run time was < 10 minutes. The resolution of the chromatographic system was verified daily by injection of 20 μl of a solution containing amrinone. Amrinone has a UV maximum absorption with a retention time of 4.18 ± 0.26 minutes (Figures 1 and 2).

Standard Solutions

Stock solution of amrinone (100 μg/ml) was made from powdered form after dissolution with 1 M hydrochloric acid and addition of water pH adjusted to 3-4. The stock solution of amrinone was diluted with plasma to prepare six calibration standards (0.25, 0.5, 1.0, 2.0, 5.0, and 10.0 μg/ml). All stock solutions were stored at room temperature and were protected from prolonged exposure to light.

Preparation of Validation Samples

Plasma samples (100 μl) were prepared in triplicate at each of five concentrations. One set of triplicate samples was assayed upon preparation. The other two were stored in a laboratory freezer for validation at a later date. The validation samples were analyzed under single-blind conditions after one week.

Recovery, Precision, Accuracy and Reproducibility

Extraction recovery was estimated by comparison of the peak heights of an extracted and an unextracted sample containing the same amount of the compound. Typical chromatograms of extracted and unextracted samples are shown in Figures 1 and 2. A chromatogram from a baby's blood sample is shown in Figure 3.

Five samples for each concentration ranging from 0.25 to 10 μg/ml were studied under the same chromatographic conditions in order to check the recovery, reproducibility and precision of the method. The average recovery for unextracted and extracted blood plasma samples was found to be $98 \pm 2\%$ and $88.9 \pm 3\%$, respectively. Intra-assay precision was determined by analysis of spiked samples containing

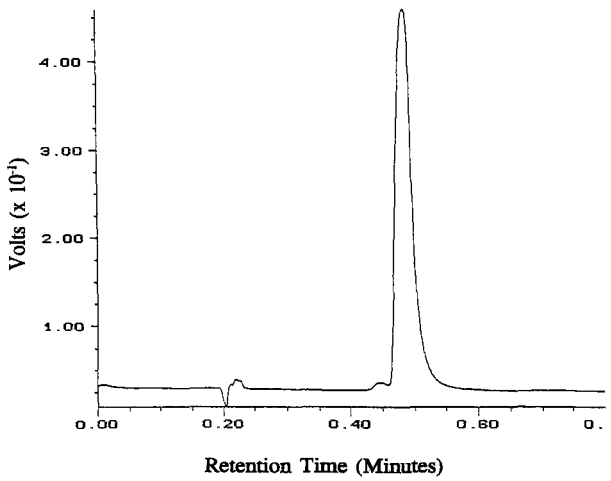

FIGURE 1: Chromatogram of amrinone from unextracted samples.

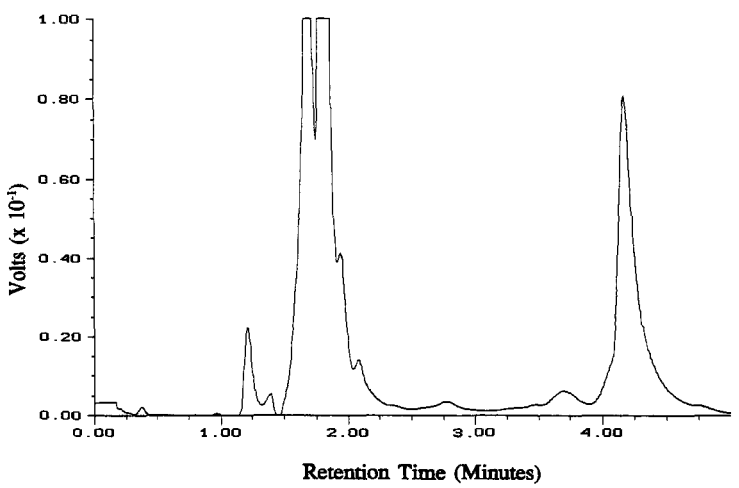

FIGURE 2: Chromatogram of amrinone from extracted plasma samples. Mean retention time (± SEM) is 4.18 ± 0.26 minutes.

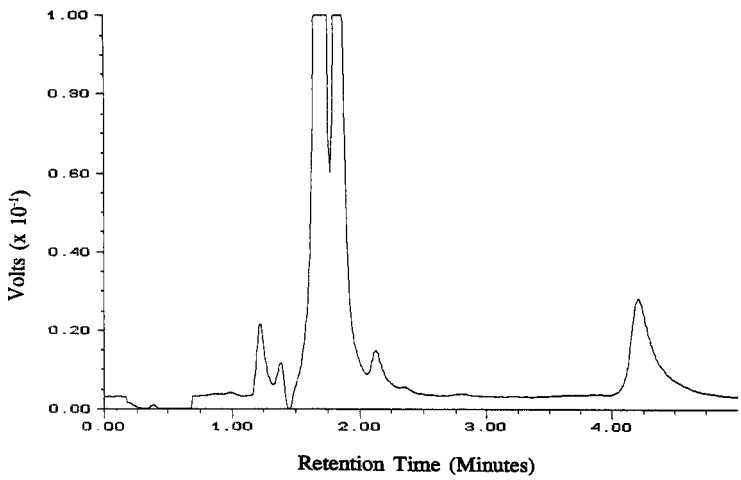

FIGURE 3: Chromatogram of amrinone from a newborn baby receiving a loading dose of 2 mg/kg via intravenous bolus followed by constant infusion of 5 μg/kg/hr.

amrinone at five different concentrations (Table 1). Inter assay precision was determined by analysis of spiked samples on 5 consecutive days. Accuracy and reproducibility was calculated as the percentage difference between amount of drug added to drug-free plasma and amount of drug measured. The intra-assay coefficient of variation was $< 5\%$ and a day-to-day coefficient of variation was $< 10\%$ in the therapeutic range of amrinone.

Calculations

Standard curves and concentrations of amrinone were calculated from peak heights. Calibration curves were constructed after the addition of known concentrations of amrinone to plasma sample by linear regression analysis of peak height versus concentrations.

Statistical Analysis

When peak heights of plasma containing 0.25, 0.50, 1.0, 2.5, 5.0, 8.0 and 10.0 μg/ml of amrinone were plotted against concentration, the resulting line had a slope of 0.0012, a y-intercept of -

0.003, x-intercept of -0.038, a standard error of 0.009 and a correlation coefficient of 0.996. Correlation coefficient squared is 0.993. The standard error is < 0.0011 at 95%, confidence interval 0.090 to 0.096. The two tailed p value is < 0.001. The slope is very significantly different than zero.

RESULTS AND DISCUSSION

The present paper describes an HPLC method for the determination of amrinone in 100 μl of blood plasma. Liquid-liquid extraction on reversed phase has emerged as a fast and simple technique for recovery of amrinone. The present work shows that this technique may be used for the assay of amrinone. Previous methods for amrinone was based on a more time-consuming extraction procedure (9). In the method described here, the Nova pak Cyano columns were found to be most suitable as compared to the other methods, giving higher recoveries and requiring smaller volumes of elution solvent.

Using the extraction method described above, endogenous plasma components did not interfere with amrinone at plasma concentrations ranging from 0.25 μg/ml to 10 μg/ml. When the absolute peak height of amrinone were plotted against the concentration, the relationship was linear and passed through the origin. In the investigated concentration range, the regression line was linear ($y = 0.999$) with an intercept on the y-axis close to the origin (0.0032). Results of analysis of spiked serum for the determination of precision and accuracy of the method are given in Table 1.

Recovery of amrinone, assessed by the injection of known amounts onto the chromatographic column averaged 88.9 $\pm$ 3% (n=6) for amrinone. The inter and intra-assay precisions of the method for amrinone were $< 5.0\%$ and $< 10\%$ respectively. The precision of the method was evaluated in a blind study in the concentration range of 0.25 μg to 10.0 μg amrinone per ml. The experimentally determined concentrations were similar to the actual concentrations.

The applicability of the method for amrinone was demonstrated by the analysis of plasma samples from a patient after an intravenous dose of 2 mg amrinone per kg body weight. The concentration of amrinone in patient samples were 1.5, 1.0, 0.9, 0.75, 0.45 μg/ml after 1,2,3,4,5 minutes.

The precision and accuracy obtained with the present method make it suitable for blood plasma level measurements in patients receiving usual clinical doses of amrinone. In 19 newborn and young infants receiving amrinone with a loading dose of 2 mg/kg followed by a constant infusion of 5 to 10 micrograms/kg/minute, the mean plasma concentrations during steady state was 1.48 $\pm$ 2.13 mg/L.

TABLE 1

Reproducibility, Precision, and Accuracy of the Method

Amount Spiked (μg/ml)	Amount found * (μg/ml)	Confidence Interval (95%)	Standard Error of Mean	Coefficient of variation (CV) (%)
0.25	0.26 $\pm$ 1.80	0.25-0.28	0.054	4.69
0.50	0.51 $\pm$ 0.79	0.48-0.54	0.102	4.48
1.00	1.02 $\pm$ 0.58	0.99-1.05	0.112	2.50
2.50	2.45 $\pm$ 2.49	2.41-2.50	0.168	1.52
5.00	4.97 $\pm$ 1.61	4.93-5.02	0.160	0.723

* Each value is expressed as Mean $\pm$ SD; (n=5)
Volume was 100 μl

Amrinone is given together with other drugs, as was the case for the patients included in this study. In such cases, the specificity for this amrinone assay was tested by using those medications that can possibly be administered to patients who are also receiving amrinone. Fentanyl, furosemide, morphine, calcium chloride, dobutamine, dopamine, midozalam, ampicillin sodium, gentamicin, clafoxan, diazepam, phenytoin, pavulon, gentamicin, vitamin K did not interfere with the measurement of amrinone. The method described is precise, sensitive and selective for amrinone and is also rapid and simple to perform.

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UTILIZATION OF HPLC-ELISA TO ASSESS SERUM LEVELS OF THYMOSIN α_1 FOLLOWING SUBCUTANEOUS ADMINISTRATION TO HUMAN SUBJECTS

P. H. NAYLOR, C. W. NAYLOR, D. SASAKI, AND M. G. MUTCHNICK
Department of Internal Medicine
Division of Gastroenterology
Wayne State University School of Medicine
Detroit, Michigan 48201

ABSTRACT

Thymosin α_1 ($T\alpha_1$) is a 28 aa peptide which is prepared synthetically for clinical use in settings where an immune modulating peptide is predicted to have efficacy. With the increase in clinical studies utilizing $T\alpha_1$ as a single agent or in combination with other therapeutic agents the need to accurately assess the pharmacokinetics of serum levels of the authentic $T\alpha_1$ in serum has become clear. Utilizing radioimmunoassays, previous studies have demonstrated elevated levels of thymosin like material in serum for 3-6 hours following administration. This study reports the development of an extraction-analysis procedure which combines HPLC specificity with ELISA sensitivity to measure the levels of authentic $T\alpha_1$ following subcutaneous administration. The precision, specificity and sensitivity data for the procedure is presented as well as data confirming the ability of the procedure to assess levels in human subjects following subcutaneous administration.

INTRODUCTION

Thymosin α_1 ($T\alpha_1$) is a 28 amino acid peptide currently undergoing evaluation in clinical trials where modulation of immune response may be a

critical factor in the control or clearance of disease (1-6). $T\alpha_1$ is administered subcutaneously, generally as two or three injections over the period of a week. The in vivo events which result in positive effects such as the clearance of the hepatitis B virus, an enhancement of response to vaccination or improvement in the survival time of cancer patients receiving various therapies are believed to center around $T\alpha_1$'s capacity to enhance immune responses. The specific mechanism(s) of action, however, has not been defined. In vitro studies suggest that an important mechanism may be the induction of cytokine production or an increase in cytokine receptors (7,8). Initially isolated from the thymus, $T\alpha_1$ has also been identified in high levels in spleen tissue, is secreted by lymphocytes and is present in serum (9-14). As with many cytokines and protein hormones, lower but detectable levels are also found in essentially all tissues and cells (13-15).

Using a radioimmunoassay based on a rabbit antibody which is specific for $T\alpha_1$ and has been shown to recognize epitopes contained in the N-terminal and C-terminal regions, elevated serum levels of thymosin like material (TLM) have been detected after subcutaneous (sc) administration (16). The subjects in the published study were part of a Phase II clinical trial of thymosin as a follow-up to irradiation in lung cancer patients. In all subjects, pharmacological levels of TLM were measured between 1 and 6 hrs. Similar results have been obtained in individuals who were HIV positive and in elderly individuals. (unpublished results of Naylor et al).

The studies presented here, describe the development of an HPLC-ELISA method to determine the levels of authentic 28 aa $T\alpha_1$ in serum following sc administration. The ELISA is required for detection since even following injection of 1.6 mg of $T\alpha_1$, serum levels are not high enough to be measured with the uv detector of the HPLC unit. The specificity, sensitivity, linearity, precision and accuracy of the method is defined. The utility of the method is demonstrated using serum from an individual receiving a sc injection of $T\alpha_1$.

MATERIALS AND METHODS

Sep-Pak Extraction Procedure

Prior to HPLC analysis, the Tα_1 in samples was extracted using a Waters C18 Sep-Pak. The sep-pak contains C18 ubondapak reverse phase resin and was prepared by sequentially washing with Methanol, HPLC grade water and the buffer of choice (Buffer A = 20 mM Potassium Phosphate, PH 6.0). The sample (typically 2 mls of serum) was loaded onto the column and vacuum applied using a Waters Sep-Pak Manifold. The effluent was collected and in the initial studies the removal of thymosin α_1 verified by ELISA. After two washes with buffer A (2 ml ea), 2 mls of Buffer B(50% acetonitrile,50 % Buffer A) was drawn through the sep-pak and collected. A final wash using 50% acetonitrile was also collected in the initial studies. All collected samples were evaporated using a Savant Speed-Vac and resuspended into 500 ul of HPLC quality water.

HPLC procedure

The HPLC procedure utilized a Perken-Elmer Series 4 Liquid Chromatograph with an ISS-100 autoinjector, LC235 uv Detector and a programmable ISCO fraction collector. The flow rate was 1 ml/minute, the gradient was 10-50% Buffer B in Buffer A (see above), and the uv detector set for 214 nm. Injection volumes were typically 100 ul.

ELISA procedure

The ELISA procedure was a modification of that previously published by Weller et al (11, 14). In brief, rabbit antibody specific for Tα_1 (1/2000 dilution in PBS-Tween-Azide buffer(PTA)) was incubated overnight at 4°C with equal volumes of standard or sample. Tα_1 coated ELISA plates were prepared by incubating Tα_1 in PBS overnight at 4°C in the wells of Immunolon 4 96 well plates. After washing the ELISA plates, an aliquot (50 ul) of the overnight incubation mixture was added and the plates incubated for 40 minutes at 37°C. The

free antibody (ie that which was left over following the overnight establishment of the equilibrium) was captured by the $T\alpha_1$ on the plate. The antibody bound to the $T\alpha_1$ on the plate was detected by sequential additions, (following washes with PTA) of biotinylated-rabbit antibody(Vector Antibodies Inc), avidin-biotinylated alkaline phosphatase (Vector Antibodies Inc) and p-nitrophenyl-phosphate substrate.(Sigma). The optical density at 405 nm was determined and the amount of $T\alpha_1$ in the sample determined by comparison to a curve generated using know standard concentrations of $T\alpha_1$. The curves were typically log-linear with correlation coefficients of at least 0.95 and slopes of at least -.5. Standard deviations for the standards of less that 2-10% were typically obtained.Sample variations were 10-20%. Trimming of the means of serial dilutions yielded the 5-10% errors in the reported data.

RESULTS

The HPLC was used as the criterion for evaluating the extraction procedure. The retention time of $T\alpha_1$ in the HPLC was highly reproducible (0.2% error). The system was also linear with respect to the size of the peak area as compared to varying concentrations of known $T\alpha_1$ (R value = 0.999) and recoveries(99.4 =/- 9.1%. (n= 9)(Table 1)). The reproducibility of the system with respect to injection of the same sample was 5.7% for the Peak Area and 3.4% for the peak height. (n=6)

The extraction procedure resulted in recoveries of $T\alpha_1$ from serum samples spiked with known concentrations of $T\alpha_1$ of 95% based on HPLC Area and 91% when based on HPLC peak height (n= 6). The standard deviation between samples was 6.6 % based on peak area and 9.9 % based on peak height. There was no detectable $T\alpha_1$ in the non-retained sample, or the washes. The serum from a normal individual was subjected to the procedure in parallel to the sample which was spiked with synthetic Tal. Due to the lack of sensitivity of the UV detector, no $T\alpha_1$ was

TABLE I
Accuracy of HPLC Method by Percent Recovery

Concentration $T\alpha_1$ in ng/ml	Peak Area	Calculated Peak Area*	%Recovery**
0.0310	2013313	64945580	87.7
0.0310	2006401	64722612	87.4
0.0310	2099191	67715838	91.4
0.0625	4759637	76154192	102.8
0.0625	4704228	75267648	101.6
0.0625	4637822	74205152	100.2
0.1250	10085500	80684000	108.9
0.1250	10210900	81687200	110.3
0.1250	10137690	81101520	109.5

Average Recovery: 99.4 +/- 9.1

* Peak area/concentration
** % Recovery = Calculated Peak Area/Average Peak Area per mg(74053749)

detected in normal serum, thus the HPLC could be used to define recoveries in the spiked samples. Figure 1 shows that the $T\alpha_1$ in normal serum could be assessed by ELISA and that consistent with the HPLC results,spiked serum had higher levels than normal serum. There were no additional peaks as assessed by HPLC in the spiked serum and when TLM was assessed by ELISA there were no additional peaks in either the normal or spiked sample.

The specificity of the HPLC retention time for $T\alpha_1$ was assessed by comparing retention time of $T\alpha_1$ with the 14 aa N-terminal and 14 aa C-terminal peptide (Figure 2). The retention time for $T\alpha_1$ was 14.0, for N-14 $T\alpha_1$ it was 11.4 and for C-14 $T\alpha_1$ was 9.1. The procedure clearly differentiates between the three peptides.

Serum from an individual injected with 1.6 mg of $T\alpha_1$ was obtained prior to injection and at 0.5,1,2,3,4,6, and 24 hrs. TLM at levels significantly above baseline was present between 0.5 and 4 hrs (Figure 2). A decline was clearly evident at 6 hrs and levels were at baseline by 24 hrs. Samples were then subjected to the Sep-Pak,HPLC, ELISA procedure to determine whether the TLM was present as the authentic 28 aa peptide. As indicated in Figure 3, the only detectible immunoreactivity had a retention time identical to $T\alpha_1$.

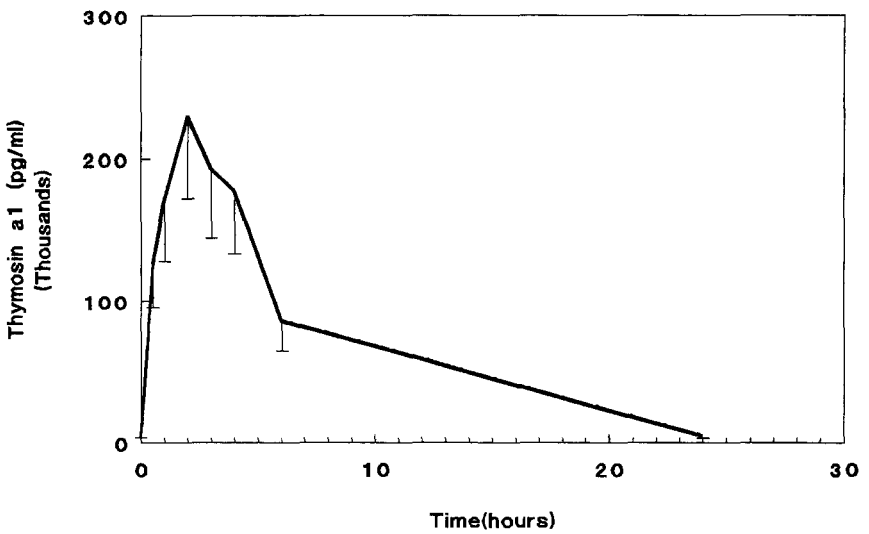

FIGURE 1

Serum samples with and without added Tal were subjected to the Sep-Pak-HPLC procedure and the presence of $T\alpha_1$ assessed by ELISA. Identical single immunoreactive peaks were identified in both samples. The values are expressed as pg/ml of resuspended extracted samples to demonstrate the lack of background in the HPLC-ELISA system.

DISCUSSION

Since $T\alpha_1$ has only recently entered Phase 3 trials, there is little published data regarding the circulating levels following administration to man. These studies are unique since for the first time the levels of the authentic 28 aa peptide have been measured. This is in contrast to the only previous study where an RIA was used to measure TLM in the serum (16). The HPLC-ELISA system thus confirms that following sc injection, the authentic 28 amino-acid $T\alpha_1$ is

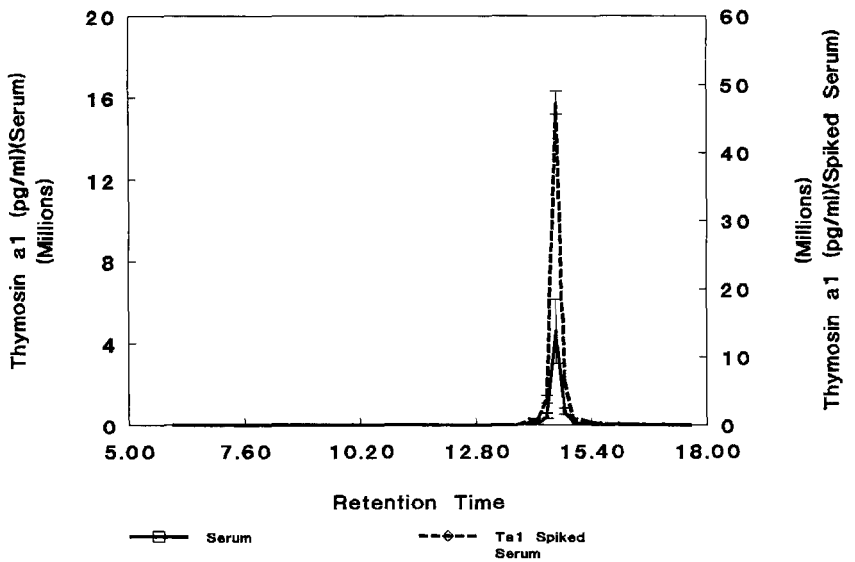

FIGURE 2

Serum was drawn at the times indicated after a 1.6 mg subcutaneous injection of $T\alpha_1$ to an individual with autoimmune hepatitis. The levels of immunoreactive $T\alpha_1$ were assessed by ELISA. The error bars represent the standard error of the mean of 8-10 values.

present in serum as defined by the retention time of the immunoreactive material. Recoveries for the procedure are at least 90%, resulting in a high degree of confidence in the observation. The maximum achievable levels approached 200 ng/ml compared to basal levels of 6 ng/ml. These results are similar to those reported using the RIA to detect TLM in serum from lung cancer patients undergoing $T\alpha_1$ treatment. In these studies, peak levels 10-50 times greater than baseline were reported. As here, the peak levels of $T\alpha_1$ (presumably now validated as authentic by this report) were 3-6 hrs post sc injection. The kinetics are similar to those reported for other proteins, such as interferon-α,

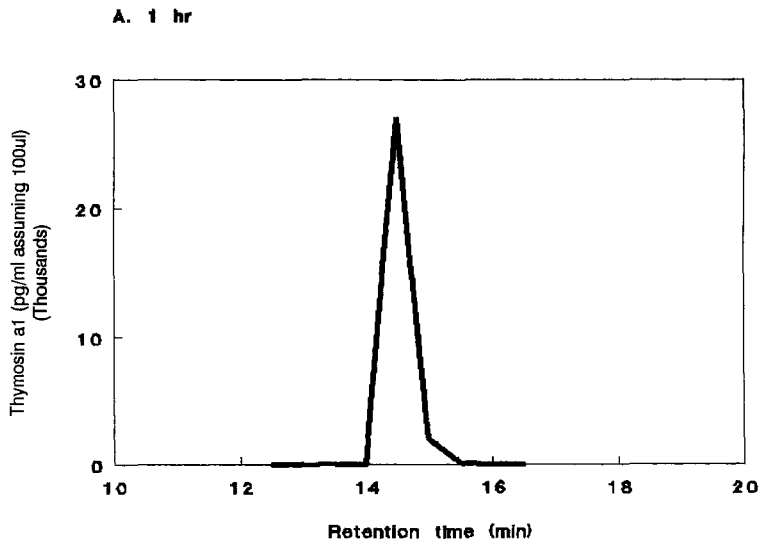

FIGURE 3

Serum at various times post injection of $T\alpha_1$ (1.6 mg,sc as in figure 2) was subjected to the Sep-Pak-HPLC-ELISA procedure. Data from two representative time points where pharmacological levels were detected (at 1 and 3 hrs post injection) are shown. In all samples only a single immunoreactive peak at the retention time of authentic $T\alpha_1$ was detected. The results are expressed as pg/ml of resuspended sample to demonstrate the relationship between sample and non-specific background.

where clearance is generally via filtration through the renal glomeruli followed by proximal tubular reabsorption with subsequent proteolytic degradation by lysosomal enzymes (17). The liver typically has little role unless the proteins are highly glycosylated. The presence of only the authentic peptide at all time points is consistent with this route of clearance.

Thus this study reports a methodology for assessing the levels of authentic $T\alpha_1$ in serum following administration to humans. Although levels approaching 0.2

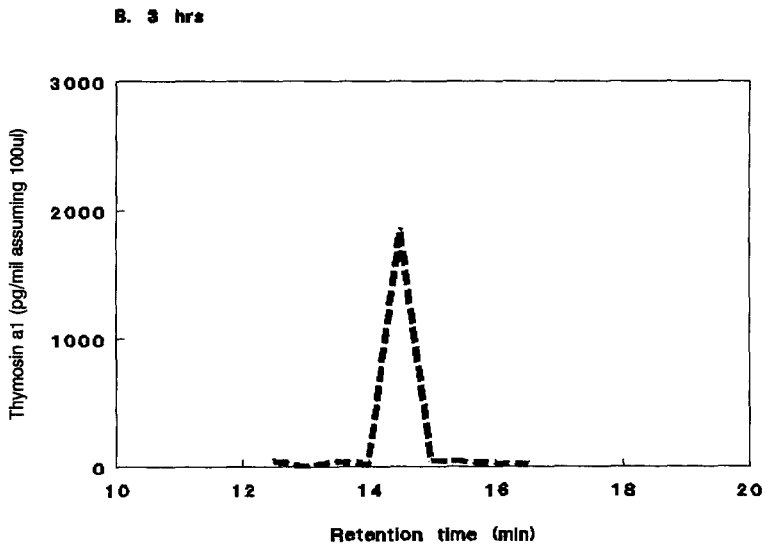

FIGURE 3 (Continued)

ug/ml were achieved, these levels are not adequate for measurement by the uv where minimal detectable levels of thymosin a1 were 1 ug/ml. The sensitivity of the HPLC-ELISA method also allows for the determination of the kinetics in normal individuals since sub bioactive doses (10-100 fold lower) could be used and detected. An additional utility of this methodology is that it detects authentic $T\alpha_1$ in serum from normal individuals. Consistent with our previous study where a sizing HPLC column was used, only a single peak with retention time identical to that of $T\alpha_1$ was detected (13). This methodology is predicted to be especially important in subsequent studies where Ta1 levels in individuals following long term $T\alpha_1$ administration are evaluated. Based on the previous study in lung cancer patients, it is predicted that long term treatment will result in an increase in baseline $T\alpha_1$ levels. This HPLC-ELISA method will allow the determination of whether these levels are due to authentic $T\alpha_1$ or a TLM

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A RAPID HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR DETERMINATION OF HOMOCYSTEINE IN PORCINE TISSUE

P. B. YOUNG[1], A. M. MOLLOY[2], J. M. SCOTT[2], AND D. G. KENNEDY[1]
[1]*Department of Biochemistry*
Veterinary Sciences Division
Department of Agriculture for Northern Ireland
Stormont, Belfast BT4 3SD
Northern Ireland, United Kingdom
[2]*Trinity College*
Dublin, Ireland, United Kingdom

ABSTRACT

A simple yet sensitive method for determination of total L-homocysteine (HC) in tissues is described. The assay involves incubation of a tissue homogenate in the presence of a reducing agent to liberate homocysteine from protein followed by derivatization of thiols with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F). Homocysteine is then quantified using fluoresence detection following high performance liquid chromatography on a 5 μM reverse phase column.

The method was applied to heart tissue taken from normal pigs and the results compared to values obtained from pigs intermittently exposed to nitrous oxide (N_2O). It was found that N_2O treatment resulted in a reduction in methionine synthase activity (EC 2.1.1.13) and concomitantly in a massive accumulation of homocysteine in both tissue and plasma.

INTRODUCTION

In recent years there has been an increase in interest in the sulphur containing amino acid homocysteine (HC) which has been implicated in several pathological conditions, most notably coronary artery disease (1,2).

Homocysteine, formed from S-adenosylhomocysteine, can be metabolized to cystathionine, by condensation with serine, or can be remethylated to methionine. This latter reaction is catalyzed by two enzymes the most widely distributed of which is methionine synthase. The anaesthetic gas N_2O has been demonstrated to be a potent inhibitor of methionine synthase giving rise to an accumulation of HC in plasma (3,4).

It has been reported that, in the presence of copper, homocysteine can exhibit prooxidative activity and that this may result in oxidative modification of low density lipoprotein (LDL) (5,6). It is possible that this may contribute to the onset of coronary artery disease. There is also some evidence suggesting that LDL modification may occur not in plasma but in the arterial intima (7).

A method has previously been described for the measurement of HC in mouse and rat tissues (8) based on the measurement of S-adenosylhomocysteine formed by condensing endogenous HC with adenosine, the reaction being catalysed by S-adenosyl-homocysteine hydrolase (AdoHc hydrolase). However, this method is laborious, time consuming and commercially available AdoHc hydrolase is expensive.

Recent advances in the production of fluorogenic thiol derivatives have been utilised in the measurement of HC in plasma (9,10). In this article we detail a rapid method for determining the HC content of tissues after derivatizing with the thiol specific compound ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F), described by Toyo`oka and Imai (9), followed by HPLC with fluoresence detection. This method is sensitive and robust, yet given its simplicity it is suitable

for the analysis of large numbers of samples. The method was used in this study to determine the HC content of heart tissue taken from normal pigs and from pigs intermittently exposed to N_2O.

MATERIALS AND METHODS

Reagents

Methanol (HPLC grade) was obtained from Rhone-Poulenc Ltd (Manchester, UK). D,L-Homocysteine and tri-n-butylphosphine were obtained from Aldrich Chemical Co. Ltd (Gillingham, UK). SBD-F was obtained from Wako (Dusseldorf, Germany). All other reagents were obtained from BDH Ltd (Poole, UK).

Standard solutions

A stock solution of 10 mmol/L D,L-homocysteine was prepared in 0.1 mol/L hydrochloric acid and stored at 4°C for 1 week. This was diluted to give a final concentration of 5 μmol/L in water prior to use.

HPLC instrumentation

The liquid chromatography system consisted of Hewlett Packard HP 1090 liquid chromatograph, a Merck Hitachi F1050 fluoresence detector (EX 385, EM 515) and a Hewlett Packard HP 3392A integrator. The analysis was performed using a LiChrospher 100 RP 18, 25 x 0.4 cm I.D., 5 μM particle size (E. Merck, Darmstadt, Germany) at ambient temperature. A chart speed of 0.3 cm/min was used.

Chromatographic conditions

The mobile phase gradient used was as described by Araki and Sako (10). Briefly, solvent A consisted of 0.1 mol/L acetate buffer (pH 4.0) containing 2 % methanol (v/v) and solvent B of 0.1 mol/L phosphate buffer (pH 6.0) containing 5 % methanol (v/v).

The buffers were filtered through a type HV filter 0.45 μM (Millipore, Bedford, MA, U.S.A.), mixed with methanol and degassed prior to use. A linear gradient from solvent A to solvent B over 20 minutes (0-100%) at a flow rate of 1 ml/min was used.

Animal study

Sixteen 5 week old Landrace pigs were randomly divided into 2 equal groups. Both groups received the same commercial diet. Group 1 was maintained under a polythene canopy in an atmosphere of 15 % (v/v) N_2O for 4 days. The canopy was then raised and the pigs maintained in air for 3 days. This procedure was then repeated. Group 2 was maintained in air for the entire duration of the experiment. All animals were bled and killed after 4 weeks and tissues immediately frozen in, and stored under, liquid nitrogen.

Homocysteine analysis

Two grams of chopped heart were homogenized with 8 ml of 50 mmol/L phosphate buffer (pH 7.4) containing 10 mmol/L EDTA. This homogenate was then derivatized with SBD-F using a similar procedure to that employed for plasma by Araki and Sako (10). In short, 50 μL of 10 % (v/v) tri-n-butylphosphine in dimethylformamide was added to 250 μL of the homogenate and the mixture incubated at 4°C for 30 minutes to release protein-bound homocysteine. This was followed by the addition of 250 μL of chilled 10 % (w/v) trichloroacetic acid

containing 1 mmol/L EDTA under vigorous vortexing, followed by centrifugation at 1500g for 10 minutes at 4°C. An aliquot (100 μL) of the supernatent was then mixed with 200 μL of 2.5 mol/L borate buffer (pH 9.5) containing 4 mmol/L EDTA and 100 μL of SBD-F (1.0 mg/ml) in 2.5 mol/L borate buffer (pH 9.5). The mixture was then incubated for 60 minutes at 60°C. A 5 μL aliquot was then used for HPLC analysis. Each specimen was homogenized and analysed in duplicate and results expressed as nmol homocysteine /gram tissue (wet wt.).

Plasma was analysed by the same method by substituting 250 μL of plasma for 250 μL of tissue homogenate.

Methionine synthase analysis

Total methionine synthase activity was measured radio-enzymatically as described by Keating *et al.* (11).

Statistical analyses

Statistical analyses were carried out using Student's two-tailed t test with equal or unequal variance as appropriate. Differences between means were considered significant at $p < 0.05$. All values cited in the text are means ± SEM.

RESULTS

HPLC analysis

Figure 1 shows the HPLC chromatograms of: (a) homocysteine standard (5 μmol/L), (b) control pig heart extract and (c) the same control pig heart tissue fortified with 20 nmol homocysteine /gram tissue.

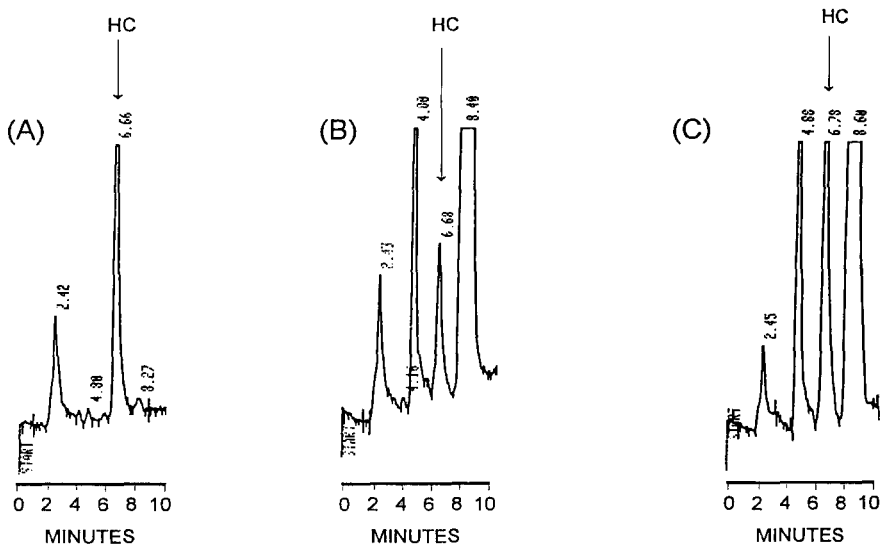

FIGURE 1. Chromatograms of: (A) homocysteine standard (5 μmol/L), (B) a normal pig heart homogenate and (C) the same pig heart homogenate fortified with 20 nmol homocysteine / gram tissue.

The reproducibility of the assay was determined by analysing one sample 10 times within one day and by analysing the same sample over 6 different days. The values obtained within day were 8.9 ± 0.2 nmol/g (n=10, CV 8.5 %) and between day were 9.1 ± 0.4 nmol/g (n=6, CV 8.9 %).

Six different heart samples were fortified with HC at each of two levels (10 nmol/g and 20 nmol/g). The mean recovery of HC from samples fortified at 10 nmol/g was 104.1 ± 3.3 %, and from samples fortified at 20 nmol/g was 106.3 ± 3.8 %.

The linearity of the assay was assessed over a concentration range equivalent to 0-250 nmol/g. The equation of the line of best fit was y =

1.063x - 0.259 (correlation coefficient = 0.9993). The limit of detection, defined as 3 times the signal to noise ratio, was less than 1.25 nmol/g tissue.

Results obtained by analysis of heart tissue from control and N_2O treated pigs for methionine synthase activity and tissue homocysteine content and for plasma homocysteine concentration are summarised in Table 1. All results are the mean of duplicate analysis.

Total methionine synthase activity was significantly lower in the heart of N_2O treated animals than in heart tissue from air controls (38 % of control value). This was reflected in a significantly higher homocysteine content in both tissue and plasma in the N_20 treated group.

DISCUSSION

The present report describes a method for the determination of HC in tissue. The method is a modification of the assay of Araki and Sako (10) who described the determination of HC in plasma by HPLC separation of SBD adducts. The present method proved to be reproducible and reliable and gave good recovery of HC from pig heart homogenate when added at both 10 nmol/g and 20 nmol/g (104.1 ± 3.3 % and 106.3 ± 3.8 %, respectively). A previously reported method (8) produced similar figures in terms of reproducibility and recovery. However detection in that method was based on determining the radioactivity in HPLC fractions, making it tedious and restricting the batch size. With the current method up to 20 tissue specimens can be homogenized and derivatized in duplicate in one working day and with the use of an autosampler the HPLC can be carried out overnight.

Ueland *et al.* (8) demonstrated that the HC content of murine tissue isolated and frozen *post mortem* did not differ significantly from the HC

Table 1. Tissue methionine synthase activity and homocysteine content of plasma and heart tissue from pigs treated with N_2O and corresponding controls.

TREATMENT GROUP	METHIONINE SYNTHASE nmol/g/hr	PLASMA HOMOCYSTEINE µmol/L	TISSUE HOMOCYSTEINE nmol/g
GROUP 1 N2O	49.8 * ± 5.2	496.0 * ± 3.3	88.0 * ± 6.7
GROUP 2 AIR	132.9 ± 12.5	26.3 ± 3.3	9.6 ± 0.7

Values are means ± SEM of eight N_2O-treated pigs and eight air controls. Asterisks indicate significant differences from air control group (* $p < 0.0005$) as assessed by Student's t test.

content of tissue frozen *in vivo*. However, in this study it was found that the HC content of heart tissue increased significantly if the tissue was allowed to stand at room temperature for as little as 10 minutes *post mortem* before being frozen in liquid nitrogen (data not shown).

In this study we have also shown that treatment of pigs with N_2O brings about a significant reduction in methionine synthase activity in heart tissue (activity fell to 38 % of control animal values). This was accompanied by an accumulation of HC in plasma (496 ± 28.9 µmol/L), a concentration which is very much higher than the 58 µmol/L observed by Van der Westhuyzen *et al.* in N_2O treated fruit bats (4). We have also demonstrated that HC is present in measurable amounts in normal porcine heart tissue (9.6 ± 0.7 nmol/g) and that exposure of the animal to N_2O brings about an accumulation of HC in tissue (88.0 ± 6.7 nmol/g). This assay is currently being applied in a study examining the effects of hyperhomocysteinaemia on the pathogenesis of coronary artery disease in pigs.

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A HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR HISTAMINE IN PLASMA USING SOLID PHASE EXTRACTION AND FLUORESCAMINE DERIVATIZATION

DENISE R. LOWE[1], CLARK MARCH[2], JOHN E. JAMES[3], AND H. THOMAS KARNES[4]

[1]*Scientist, Pharmacokinetics*
Whitby Research
2801 Reserve Street
Richmond, Virginia 23261-7426
[2]*Virginia Commonwealth University*
Medical College of Virginia
Richmond, Virginia 23298-0533
[3]*Alcon Laboratories, Inc.*
6201 South Freeway
Fort Worth, Texas 76134
[4]*Associate Professor, Virginia Commonwealth University*
Medical College of Virginia
Richmond, Virginia 23298-0533

ABSTRACT

A reversed phase HPLC method for the determination of histamine in plasma was developed using solid phase extraction with fluorescamine derivatization. Betazole was used as an internal standard. Histamine and the internal standard were extracted from plasma using a cation exchange solid phase extraction cartridge, derivatized pre-column and the derivatized extracts were separated on a C_8 column. The solid phase extraction provided recoveries of 85-90%. The method was found to be linear through the range of 1-10 ng/ml and the histamine derivative was stable at 4°C for 18 hours. The method was found to be accurate to within 7% bias and precise to within 18% CV. Selectivity could not be rigorously assessed because of endogenous histamine, although comparison to RIA results suggested good selectivity. The limit

* Author to which correspondence should be addressed

of detection was calculated to be 13 pg on column, although the current method was limited by the small proportion of sample injected on column. The method is sufficient for measuring histamine levels expected during allergic response.

INTRODUCTION

The low baseline concentration of histamine in plasma requires highly sensitive analytical methodology for accurate measurement. Bioassay,[1,2] radioimmunoassay,[3,4] , enzyme isotope[5] and gas chromatography[6,7,8] have been utilized for quantitation of histamine. These methods have lacked specificity and/or sensitivity and most have been utilized for measuring histamine in tissue extracts or in urine. High performance liquid chromatography (HPLC) methods have been reported for determination of histamine in urine, animal tissues or body fluids.[9,10,11,12,13] Bettero et. al. reported a procedure for fluorescamine derivatization and detection of histamine in tears without prior sample preparation and suggested that this could be applied to plasma.[14] A previous study has shown, however, that many derivatization techniques lack specificity and that biological samples require a clean-up procedure prior to derivatization/HPLC analysis.[7] A method employing post column derivatization with o-phthaldehyde for analysis of histamine in plasma and brain microdialysates has been reported.[15,16] This work was based on previous work in which o-phthaldehyde was used as a precolumn derivatization reagent for analysis of histamine in plasma, urine and rat brain.[17,18] The current method utilizes ion-exchange sorbant extraction combined with fluorescamine derivatization and HPLC analysis for analysis of histamine in plasma. Advantages of this method over previous systems include obviation of ion pairing agents in the mobile phase, enhanced selectivity and automation capability of the solid phase extraction, obviation of multiple pumps and post column reactors and the use of a commercially available bonded phase column.

MATERIALS

Chemicals and Reagents

Imidazole, and histamine diphosphate (lot # 39208) were purchased from Sigma Chemical Company (Sigma Chemical Co. P.O. Box 14508 St. Louis MO 63178). Acetonitrile (ACN, B&J HPLC grade), nitric acid and hydrochloric acid (HCl), were purchased from Baxter (Baxter 8855 McGraw Rd., Columbia MD 21045) sodium borate was purchased from Baker Chemical Company (Baker Reagent Grade lot # 39208) betazole reference standard was obtained from the United States Pharmacopeia (USP Ordering Department Twinbrook Parkway, Rockville Maryland 20852). CBA (Carboxylic Acid) cartridges were obtained from Analytachem International, Harbor City, CA.

Apparatus

A Gilson pump (model #382) and manometric module (model #802-B) (Thomson Instrument Co., 9001 Braddock Rd., Suite 300, Springfield Va. 221151) was used for mobile phase delivery. A Rheodyne injector (Rheodyne Inc. P.O. Box 996 Cotata CA 94928) was used with a 50 μl injection loop. The analytical column was a Phenomenex Ultramex C-8 column 250 mm x 4.6 mm, 5μm (lot # pp/7116D) with a gaurd column packed with Pelligaurd LC-8 packing (lot # 1106 Supelco, Supelco Park, Bellefonte PA 16823-0048). Fluorescence detection was accomplished with the McPherson FL-750 Spectrofluorescence detector equipped with a high sensitivity attachment and a 150W short arc lamp power supply (model #750-03) to power a mercury xenon source (McPherson 53 Main St. Action Mass 72). The detector was set at λex 366nm and λem 440nm cutoff, gain 45. Chromatographic signals were acquired and processed using an HP integrator (model# 3396A, Hewlett Packard Corp. P.O. Box 1000, Avondale PA 16823-0048). An AASP prep station (Varian Instrument Group, Walnut Creek Division, 2700 Mitchell Drive, Walnut Creek, CA. 94598) was used for processing solid phase extractions cartridges.

METHODS

Extraction and Derivatization Procedure

Spiked Plasma samples were prepared in concentrations of 10, 7.5, 5.0, 2.5, and 1 ng/ml by dilution of an aqueous 100 ng/ml stock solution with normal plasma. Controls were prepared in concentrations of 8, 4, and 1.5 ng/ml in plasma. Solid phase extraction was performed using the CBA cartridges. Each cartridge was conditioned with 1.0 ml of MeOH, followed by 2 ml of 0.01 M phosphate buffer pH = 7. Five hundred microliters of spiked plasma with 100 μl of a 1.0 mg/ml solution of the betazole internal standard added, were diluted with 2 ml of ice cold phosphate buffer and were passed through the cartridge at a rate of 0.2-0.3 ml/min. The cartridges were allowed to dry for 30 seconds and were subsequently rinsed with 1.0 ml of hexane. The samples were then eluted with 1.0 mL of 40/60 0.1 M HCl/MeOH. Samples were dried under nitrogen at 60°C and reconstituted in 100 μl of sodium borate buffer (0.2 M pH = 9). The reconstituted sample was combined with 50 μl of 20 μg/ml fluorescamine, in acetonitrile and vortexed. Samples were refrigerated at 4°C until injection.

Chromatographic Conditions

HPLC conditions for the fluorescamine derivative of histamine were optimized by a series of experiments which evaluated solvent strength, ionic strength and pH. The optimal HPLC conditions were found to be a mobile phase of 20:80 ACN: Imidazole buffer (0.50 M pH = 7). The injection volume was 50 μl and the flow rate was 1.0 ml/min.

RESULTS

Stability of the Fluorescamine Derivative

At room temperature the sample maintained at least 90 % or better of its initial concentration for the first hour, followed by a decline to 20 % of the initial signal within 2 hours. Refrigeration at 4°C was found to increase the stability defined as 90% retention of original concentration to 18 hours. Sodium metabisulfite (100 μl of 0.1M) and benzoic acid (50 μl of 0.1M) were added as preservatives without any significant effect.

Selectivity and Recovery

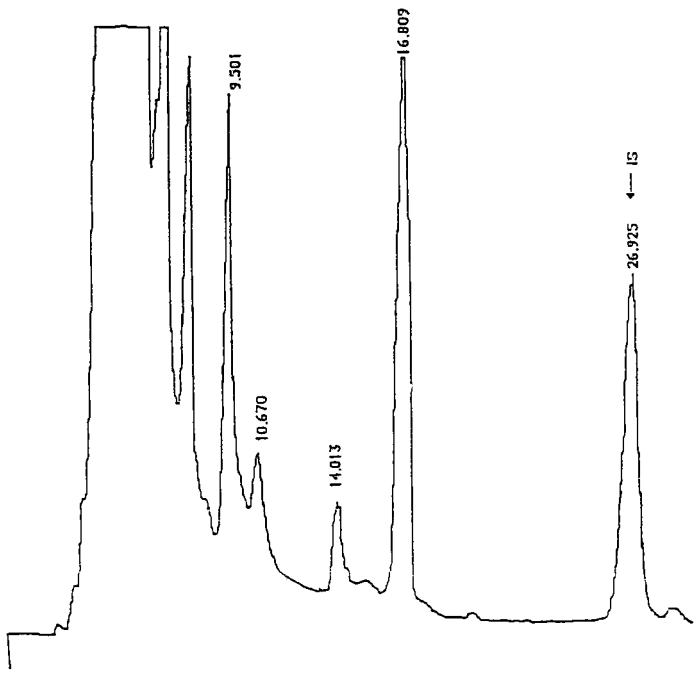
The absolute recovery of the extraction method was determined by comparing the mean area counts (n = 5) for unextracted samples in water to the mean area counts for samples extracted from plasma. Recoveries of 86.9, 89.5% and 87.2 were obtained for concentrations of 10, 7.5 and 5 ng/ml, respectively. Selectivity was evaluated by examining normal plasma extracted, derivatized and injected prior to each run. Low level peaks were observed in some of these samples. Analysis of these plasmas by RIA for histamine demonstrated reasonable correlation between the baseline peaks and histamine concentration. This suggested that these peaks were due to endogenous histamine. Chromatograms of normal plasma and plasma spiked with 4.0 μg/ml of histamine are shown in Figures 1 and 2, respectively.

Linearity and Detectability

A plot of concentration vs. response produced a straight line fitting the form $y = mx + b$, where the slope of the line (m) is the analytical sensitivity. All data were subjected to weighted linear regression with weighting factor of 1/concentration. The mean slope of three weighted calibration curves was 0.1838. A plot of mean peak height ratios (n = 3) versus spiked concentration produced a correlation coefficient of 0.989 and a log-log fit of this data produced a slope of 0.9246. This data indicates acceptable conformance to the linear model. Linearity was tested over the calibration range of 1.0 - 10 ng/ml which corresponds to the concentration range observed in patients exhibiting an allergic response. The limit of detection for the method was determined to be 13 pg on column and was calculated based on 3 times the standard deviation of the noise. The limit of quantitation based on k = 10 is 0.15 ng/ml in a plasma sample or 50 pg on column. Because of the variability at the low end of concentration, however (CV = 11.97 %, error = 17.09% at 1.5 ng/ml), the limit of quantitation was established at 1.0 ng/ml.

Accuracy and Precision

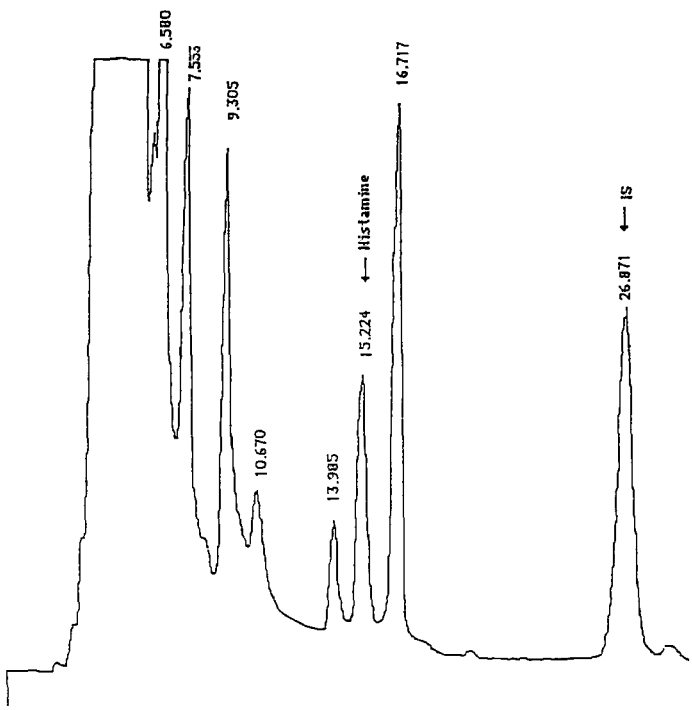
Accuracy was assessed by calculation of the percent bias of mean control results (n = 5) from their respective spiked concentrations. Controls were prepared in plasma



1. Chromatogram of normal plasma subjected to the procedure described.

Table 1. Accuracy and Precision

Concentration (ng/ml)	Concentration (ng/ml)	Percent CV		Percent Bias
		Within Run (n = 9)	Between Run (n = 5)	
8	8.3	16.6	4.0	4.0
4	4.3	17.7	4.3	7.0
1.5	1.6	12.0	16.4	5.6



2. Chromatogram of plasma spiked with 4.0 ng/ml of histamine and subjected to the procedure described.

samples that were shown to have no detectable levels of histamine and assayed independent of the standard curve on a between run basis. The method demonstrated acceptable accuracy with bias measured at all concentrations less than 20% (Table 1). Precision was assessed on both a within run (n = 9) and between run basis (n = 5). All coeffieients of variation were less than 18% showing acceptable precision. Recovery plots of predicted versus measured concentrations demonstrated no significant nonzero intercept, showing that the procedure of spiking normal plasma with undetectable levels of histamine was acceptable.

DISCUSSION

The described method has been shown to be accurate and precise to within acceptable limits for quantitation of histamine through the range of 1.0 - 10.0

ng/ml in plasma. This range is sufficient for determination of histamine levels expected from allergic responses but not sufficient for measurement of baseline levels of histamine. The method is not limited by detectability of the histamine derivative but by precision of the overall process below 1.0 ng/ml. If technical problems can be overcome, this approach may provide for analysis of histamine in plasma at the subnomogram per milliliter concentration levels observed for endogenous baseline histamine.

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COMPARATIVE STUDY OF THE RETENTION BEHAVIOR OF LIPIDIC PEPTIDES ON RP-18 AND SUPELCOSIL™ LC-ABZ STATIONARY PHASES

D. KORAKAS[1] AND K. VALKÓ[2]*

Department of Pharmaceutical Chemistry
School of Pharmacy
University of London
29-39 Brunswick Square
London WC1N 1AC, United Kingdom

ABSTRACT

A series of lipidic amino acids and peptides were investigated by systematic change of the mobile phase composition using traditional octadecylsilica stationary phase and the newly developed Supelcosil™ LC-ABZ column. The mobile phases contained various concentrations of methanol and acetonitrile combined with 0.1% trifluoroacetic acid (TFA). The log k' values were plotted as a function of organic phase concentration in the mobile phase. In some cases parabolic curves were obtained on the traditional octadecyl silica stationary phase due to the presence of the free silanol groups, while on the Supelcosil™ LC-ABZ column straight lines were obtained. Much lower concentration of organic phase was needed for getting similar retention times on the Supelcosil™ LC-

[1]On leave from University of Ioannina, Department of Chemistry, Section of Organic and Biochemistry, 451 10 Ioannina, Greece
[2]Present address: Department of Physical Sciences, Langley Court, Beckenham, BR3 3BS United Kingdom

ABZ column, so no retention was observed at higher organic phase concentrations where the silanol effect could have been observed. The slope and the intercept values of the linear section of the log k′ vs organic phase concentration lines were calculated and the chromatographic hydrophobicity index (φ_0) was also expressed for each compound. By the help of the log k′ vs organic phase concentration plots the optimum conditions for the separation of diastereomeric isomers were predicted.

INTRODUCTION

Lipidic amino acids and peptides were suggested as possible conjugates to pharmacologically active compounds by Gibbons et. al. [1] and Sakarellos et. al. [2]. They are expected to possess a degree of membrane-like character which is sufficient to facilitate the passage of poorly absorbed drugs across biological membranes to reach their active sites. Quantitative structure activity relationships were revealed for a series of lipidic amino acid conjugates of ß-lactame antibiotics [3] which showed the importance of the lipidic side chain in the in vivo action. The antigenic role of single residues and the role of the lipidic side chain to facilitate the formation of the ß-folding have been also investigated for the lipidic peptides studied [4]. The compounds investigated are might serve as possible conjugates to pharmacologically active molecules. The 10 peptide (compound 5) was found to be active on Torpedo acetylcholine receptor. Its modification (compound 6 and 7) might results in more active compounds.

As the synthesis of lipidic amino acid derivatives always results in diastereomeric mixtures, the separation of the isomers is of great importance. Very probably the biological activity of the isomers is different. This is to be measured after preparative HPLC separation of the isomers.

As the lipidic amino acids and peptides have amphophilic character (cationic and anionic plus long hydrophobic side chain) they can be regarded as "difficult" compounds in the chromatography. High performance liquid chromatography can be very useful not only for the final purification of compounds and analytical quality control but also for the separation of diastereomeric isomers which always occur during the synthesis. The Supelcosil™ LC-ABZ column was designed to be suitable for analyses of acidic, basic, zwitterionic, and neutral compounds, without the use of silanol-suppressing mobile phase conditions and additives. As the compounds are soluble in aqueous solutions and they are zwitterions the newly developed Supelcosil™ LC-ABZ column seemed to be a promising stationary phase. For revealing the similarities and dissimilarities between the traditional octadecylsilica phases and the new one the retention of the compounds expressed by log k' values were measured as a function of methanol, acetonitrile, acetonitrile TFA concentration.

EXPERIMENTAL

The compounds were synthesized as it was described by Gibbons et. al. [1] and Sakarellos et. al. [2]. They were chromatographically pure. The chemical structure of the compounds can be seen in Table 1. Under some HPLC conditions, the D, L diastereomers of compound 7 were separated and denoted as 7a and 7b for the first and the later eluting peaks, respectively.

The HPLC measurements were carried out on a Gilson HPLC system consisting of Model 303 pumps, Model 115 variable wavelength UV detector and Rheodyne injector (Anachem, Luton, U. K.). Samples were dissolved 1 mg/ml concentration in water or aqueous methanol and 20 μl was

TABLE 1.

No.	Structure of the Compound
1.	Boc-NH-CH(CH_2)$_{13}CH_3$-CONH(CH_2)$_{13}CH_3$
2.	HCl-H_2N-CH(CH_2)$_{13}CH_3$-CONH(CH_2)$_{13}CH_3$
3.	HCl-H_2N-CH(CH_2)$_3CH_3$-CONHCH(CH_2)$_{13}CH_3$-$COOCH_3$
4.	Boc-NH-CH(CH_2)$_3CH_3$-CONHCH(CH_2)$_{13}CH_3$-$COOCH_3$
5.	Trp-dAsn-Pro-Ala-Asp-Tyr-Gly-Gly-Ile-Lys
6.	Trp-Asn-Pro-Ala-dAsp-Tyr-Gly-Gly-Ile-Lys
7	Trp-Asn-Pro-Gly-Asp-Tyr-Gly-Gly-Ile CO-NH-(D,L)CH(CH_2)$_{10}CH_3$-COOH

injected onto the column. Detection was carried out at 210 nm (0.1 AUFS). Spherisorb ODS 5 μm (4.6 x 50 mm) column (PhaseSep, Deeside, U. K.) and Supelcosil^{TM} LC-ABZ (4.6 x 150 mm) (Supelco Inc., Bellefonte, PA, U. S. A.) columns were used. The mobile phases were various concentration of methanol - water (mobile phase A: MeOH ranging 95 - 35 %), acetonitrile - water (Mobile phase B: AcN ranging 95 - 15%), acetonitrile, 0.1% TFA - water mixtures (mobile phase C: AcN ranging 95 - 15%). The flow rate was always 1 ml/min.

The retention time measurements were repeated three times consecutively and the average was taken into account in the calculations of the capacity ratio (k'). The first solvent peak was regarded to be the dead time.

RESULTS AND DISCUSSION

The log k' values of all of the 8 compounds were calculated at various concentration of methanol,

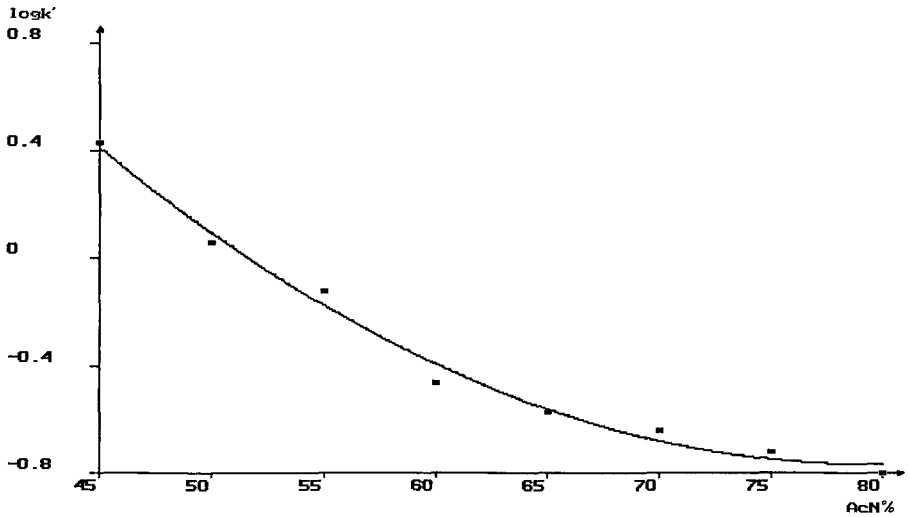

FIGURE 1.The plot of the log k′ vs acetonitrile concentration obtained on Spherisorb ODS column for compound 7. The mobile phase also contained 0.1% TFA.

acetonitrile, acetonitrile and 0.1% TFA mixtures. The obtained log k′ values were plotted as a function of the organic phase concentration and the slope and the intercept values of the straight lines were calculated. For some derivatives (compound 7) parabolic curves were obtained on Spherisorb ODS stationary phase as it can be seen in Figure 1. This finding is due to the residual silanol groups on the stationary phase [5]. In those cases only the linear section of the curve at lower organic phase concentration was considered in calculation of the slope (S) and intercept (log k'_0) straight values to achieve correlation coefficients always higher than 0.99. The slope (S) and the intercept (log k'_0) values are listed in Table 2 and 3 on the Spherisorb and

TABLE 2.

The slope (S) and the intercept ($\log k'_0$) values of the straight line obtained on Sperisorb ODS column by changing the methanol, acetonitrile (with and without 0.1% TFA) concentration.

No.	$\log k'_{0MEOH}$	S_{MeOH}	$\log k'_{0ACN}$	S_{ACN}	$\log k'_{0ACNTFA}$	S_{ACNRFA}
1.	3.0056	-0.0402	1.2029	-0.0181	0.9898	-0.0190
2.	2.2519	-0.0368	0.9183	-0.0138	1.3440	-0.0261
3.	2.1203	-0.0336	1.0999	-0.0167	1.1013	-0.0215
4.	1.2716	-0.0188	1.0489	-0.0160	1.0867	-0.0211
5.	2.6764	-0.0500	0.7850	-0.0115	0.3014	-0.0118
6.	2.9925	-0.0569	0.8457	-0.0129	0.4254	-0.0138
7a	2.4550	-0.0447	1.9078	-0.0279	2.6389	-0.0504
7b	2.3720	-0.0431	1.9078	-0.0279	2.4741	-0.0435

TABLE 3.

The slope (S) and the intercept ($\log k'_0$) values of the straight line obtained on Supelcosil™ LC-ABZ column by changing the methanol, acetonitrile (with and without 0.1% TFA) concentration.

No.	$\log k'_{0MEOH}$	S_{MeOH}	$\log k'_{0ACN}$	S_{ACN}	$\log k'_{0ACNTFA}$	S_{ACNRFA}
1.	0.8635	-0.0170	0.7706	-0.0144	1.3116	-0.0271
2.	0.8890	-0.0182	1.0591	-0.0184	1.2332	-0.0258
3.	0.9639	-0.0203	0.6847	-0.0116	1.4809	-0.0293
4.	0.8691	-0.0175	0.7638	-0.0133	1.0173	-0.0238
5.	3.0629	-0.0636	1.2290	-0.0180	2.0698	-0.1052
6.	2.6267	-0.0522	1.4029	-0.0195	2.0190	-0.0982
7a	3.3949	-0.0491	2.1530	-0.0237	4.1595	-0.1045
7b	3.5253	-0.0496	2.1012	-0.2148	4.3605	-0.1025

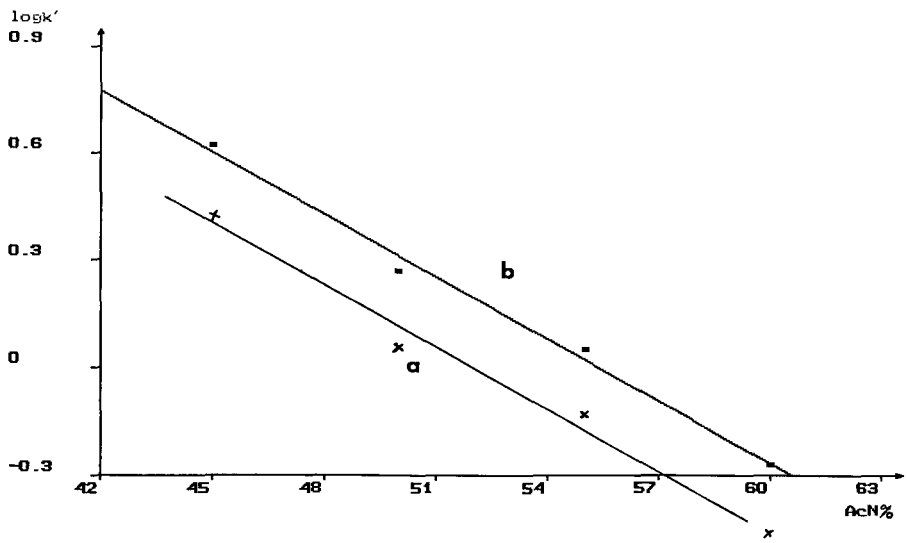

FIGURE 2. The "linear" portion of the plot of the log k' vs acetonitrile concentration obtained on Spherisorb ODS column for compounds 7. The mobile phase contained 0.1% TFA.

Supelcosil column, respectively. Figure 2 shows the linear section of log k' vs acetonitrile concentration plots for compound 7a and 7b on Spherisorb ODS column. It can be seen that the largest difference in the log k' values could be obtained at higher acetonitrile concentration (around the minimum area of the parabolic curve). Figure 3 shows the same plot obtained on Supelcosil^{TM} LC-ABZ column. The opposite trend could be observed. The separation of the diastereomeric isomers takes place at lower acetonitrile concentration, the difference between the log k' values are the largest. It is also noticeable, that much lower acetonitrile concentration can be used with Supelcosil^{TM} LC ABZ column

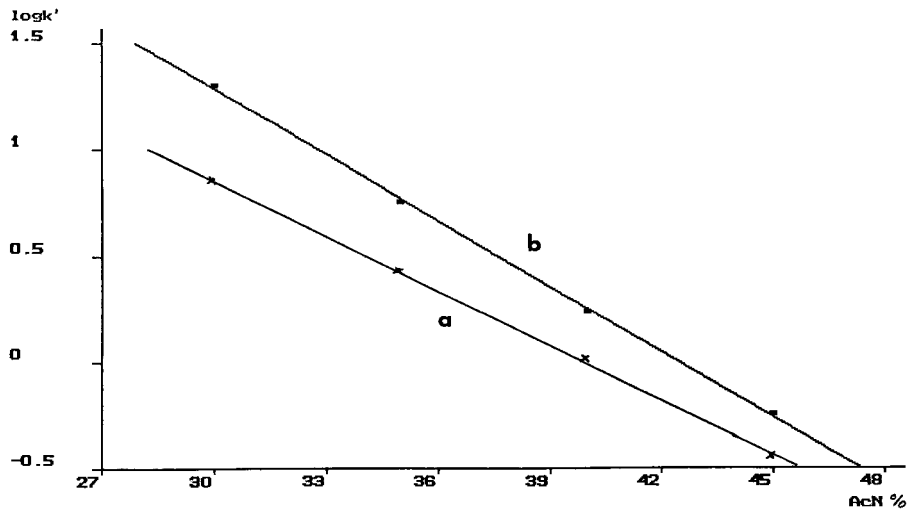

FIGURE 3.The plot of the log k′ vs acetonitrile concentration obtained on Supelcosil^{TM} LC-ABZ column for compound 7.

to obtain the same log k′ region. This means that the stationary phase itself is less hydrophobic and retain less the compounds. It is also very interesting that the slope of the log k′ vs AcN concentration plots are much higher on Supelcosil^{TM} LC-ABZ column than on the Spherisorb ODS for compounds 5 - 7 containing longer peptide residues.

Compound 5 and 6 differs from each other only with the optical isomers of two amino acids. They could be separated only on the Supelcosil^{TM} LC-ABZ column. The separation factor values (α) as a function of the acetonitrile concentration curves on Spherisorb ODS and Suplecosil^{TM} LC-ABZ column are shown in Figure 4 and 5, respectively. Again slightly better (never base line)

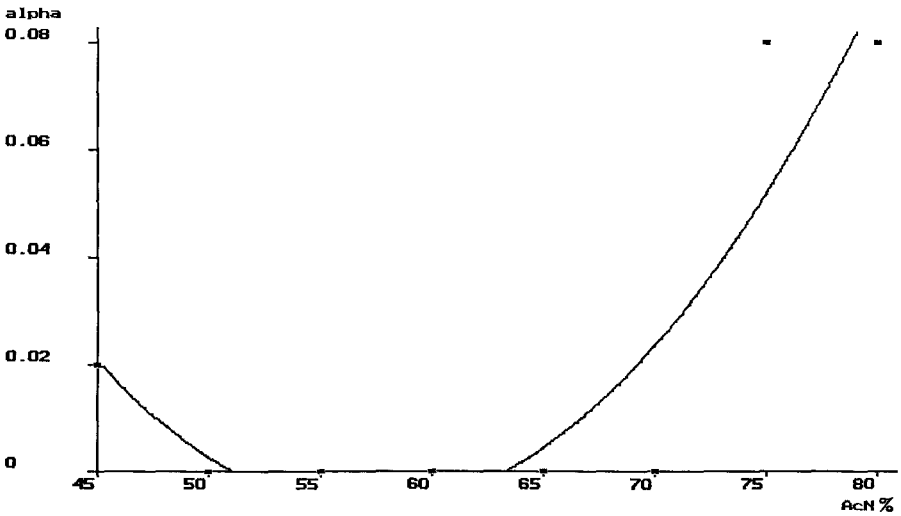

FIGURE 4. The plot of the separation factor (α) vs acetonitrile concentration obtained on Spherisorb ODS column for the racemic mixture of compounds 5 and 6. The mobile phase contained 0.1% TFA.

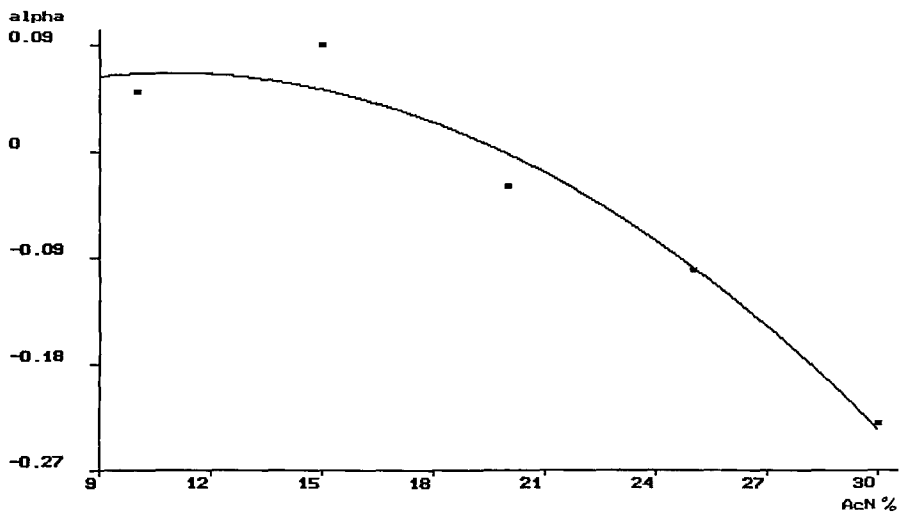

FIGURE 5. The plot of the separation factor (α) vs acetonitrile concentration obtained on Supelcosil™ LC-ABZ column obtained for the racemic mixture of compound 5 and 6. The mobile phase contained 0.1% TFA.

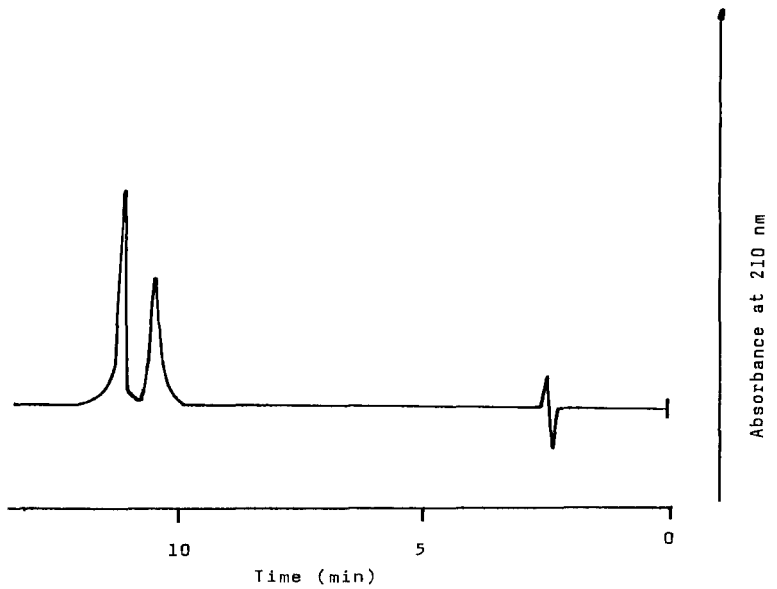

FIGURE 6. The chromatogram obtained by injecting the diastereomeric mixture of compound 5 and 6 on Supelcosil™ LC-ABZ column. The mobile phase 15% acetonitrile, 0.1% TFA; the flow rate was 1.00 ml/min; detection was carried out at 210 nm. 20 μl was injected from the solution containing 0.5 mg/ml of each compound.

separation could be observed at higher acetonitrile concentration on the Spherisorb column. Beautiful base line separation of the two isomers on the Supelcosil™ LC-ABZ column could be achieved as it is shown in Figure 6.

In order to reveal the mechanism of the solute stationary phase interactions on the two columns the correlation of the slope and intercept values obtained on the two columns with various mobile phase mixtures (A, B and C) was also investigated. The correlation

TABLE 4.

The correlation coefficients of the slope (S) and the intercept ($\log k'_0$) values obtained on Sperisorb ODS and Supelcosil™ LC-ABZ column in the three mobile phase systems (A: MeOH - water; B: AcN - water, C: AcN, 0.1% TFA - water).

	Spherisorb ODS	Supelcosil™ LC ABZ
MeOH - Water	0.881	0.935
AcN - Water	0.999	0.939
AcN 0.1%TFA - Water	0.991	0.779

coefficients can be seen in Table 4. As it was observed earlier [6], the high correlation coefficient between the slope and intercept values shows higher similarity of the retention mechanism of the compounds. As it can be seen in Table 4, the correlation between the slope and the intercept values was high in almost every conditions. The compounds behaved as structurally unrelated in the Spherisorb ODS column with methanol and water as mobile phase, and on Suplecosil™ LC-ABZ column with acetonitrile, TFA and water mobile phase. It also can be seen that the extrapolated log k' to the zero organic phase concentrations are different obtained from the various mobile phase systems. The reason for that can be that mobile phase A and B did not contain buffer, so the actual pH could change by changing the organic modifier concentration, and the log k' vs organic phase plots could deviate from linearity around the 0% in a great extent. The mobile phase system C, which contained 0.1% TFA can be regarded as more defined one, as the compounds are in protonated form at every mobile phase composition.

TABLE 5.

The chromatographic hydrophobicity index values (φ_0) for the compounds investigated obtained in acetonitrile, 0.1% TFA and water mixtures.

Compound No.	Spherisorb ODS	Supelcosil^{TM} LC-ABZ
1.	52.2	48.4
2.	51.5	47.7
3.	51.2	50.5
4.	51.4	42.8
5.	25.9	19.7
6.	30.8	20.6
7a	52.4	39.8
7b	56.8	42.5

For the characterization of the hydrophobicity of the compounds investigated we calculated the chromatographic hydrophobicity index as it was suggested recently [7]. The hydrophobicity index is ranging from 1 - 100 meaning the acetonitrile concentration by which the compounds have the retention time twice as much as the dead time. So the higher is the value the more hydrophobic the compound is. The calculated hydrophobicity index values obtained in the fully protonated system (acetonitrile, TFA, water) and in acetonitrile - water mixtures are listed in Table 5. The hydrophobicity index of the two columns show good correlation (the correlation coefficient is 0.92), although the compounds behaved slightly more hydrophilic on the Supelcosil^{TM} LC-ABZ column than on the Spherisorb ODS.

CONCLUSIONS

In conclusion the effect of the free silanol groups could be eliminated on the specially treated reversed phase column. The log k' vs organic phase concentration plots were straight lines, while parabolic curves were obtained on the traditional reversed phase stationary phase. The separation of diastereomeric isomers could be carried out only on the Supelcosil™ LC-ABZ stationary phase at lower organic phase concentration. Poor separation of the isomers could be achieved on the traditional reversed-phase stationary phase as well utilizing the silanol effect at higher organic phase concentrations.

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4. I. Papadouli, S. Potamianos, I. Hadjidakis, E. Bairaktari, V. Tsikaris, C. Sakarellos, M. T. Cung, M. Marrand and S. J. Tzartos, Biochem. J., 269 239-243 (1990)

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6. K. Valkó, J. Liq. Chromatogr. 10 1663-1686 (1987)

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JOURNAL OF LIQUID CHROMATOGRAPHY, 17(16), 3585-3591 (1994)

LIQUID CHROMATOGRAPHY CALENDAR

1994

SEPTEMBER 4 - 9: 4th European Rheology Conference, Seville, Spain. Contact: C. Gallegos, Dept. de Ingenieria Quimica, Universidad de Seville, 41071 Seville, Spain.

SEPTEMBER 5 - 7: 25th International Symposium on Essential Oils, Grasse, France. Contact: Assoc. des Ingenieurs et Techniques de la Parfumerie (A.I.T.P.), 48 ave. Riou-Blanquet, 06130 Grasse, France.

SEPTEMBER 8 - 10: Surfaces in Biomaterials 1994, Scottsdale, Arizona. Contact: Surfaces in Biomaterials Foundation, c/o Ardel Management, P. O. Box 26111, Minneapolis, Minn, USA.

SEPTEMBER 12 - 15: 3rd International Conference on Separations in Biotechnology, University of Reading, U.K. Contact: SCI, Conference Office, 14/15 Belgrave Square, London SW1X 8PS, U.K.

SEPTEMBER 25 - 28: Future of Spectroscopy: From Astronomy to Biology, Ste.-Adele, Que., Canada. Contact: Doris Ruest, Conference Mgr., NRC Canada, Ottawa, Ont., Canada K1A 0R6.

SEPTEMBER 27 - 29: CCPC International Conference on Chemical Process Automation, Houston, Texas. Contact: AIChE Express Service Center, 345 East 47th Street, New York, NY 10017, USA.

OCTOBER 3 - 4: Course on Capillary Electrophoresis, Budapest, Hungary. Contact: Dr. F. Kilar, Central Research Laboratory, Medical School, University of Pecs, Szigeti ut 12, H-7643 Pecs, Hungary.

OCTOBER 5 - 7: 9th Inetrnational Symposium on Capillary Electrophoresis, Budapest, Hungary. Contact: Dr. F. Kilar, Central Research Laboratory, Medical School, University of Pecs, Szigeti ut 12, H-7643 Pecs, Hungary.

OCTOBER 9 - 14: 186th Meeting of the Electrochemical Society. Contact: Electrochem. Soc., 10 S. Main St., Pennington, NJ 08534-2896, USA.

OCTOBER 16 - 19: 46th Southeastern Regional Meeting, ACS, Birmingham, Alabama. Contact: L. Kispert, Chem Dept, Univ of Alabama, Box 870336, Tuscaloosa, AL 35115, USA.

OCTOBER 17 - 19: 3rd Int'l Symposium on Supercritical Fluids, Strasbourg, France. Contact: Int'l Soc for the Advancement of Supercritical Fluids, 1 rue Grandville, B. P. 451, F-54001 Nancy Cedex France.

OCTOBER 18 - 22: 30th ACS Western Regional Meeting, Sacramento, California. Contact: S. Shoemaker, Calif. Inst of Food & Agricultural Res., Univ. of Calif-Davis, 258 Cruss Hall, Davis, CA 95616-8598, USA.

OCTOBER 23 - 27: Annual Meeting of the American Association of Cereal Chemists, Nashville, Tennessee. Contact: J. M. Schimml, AACC, 3340 Pilot Knob Road, St. Paul, MN 55121-2097, USA.

OCTOBER 24 - 26: 2nd Internat'l Conference on Chemistry in Industry, Bahrain, Saudi Arabia. Contact: SAICSC-ACS, P. O. Box 1723, Dhahrain 31311, saudi Arabia.

NOVEMBER 2 - 5: 29th Midwestern Regional Meeting, ACS, Kansas City, Kansas. Contact: M. Wickham-St. Germain, Midwest Res. Inst, 425 Volker Blvd, Kansas City, MO 64110, USA.

NOVEMBER 3: Anachem Symposium, Dearborn, Michigan. Contact: Paul Beckwith, Program Chairman, Detroit Edison Co., 6100 W. warren, Detroit, MI 48210, USA.

NOVEMBER 7 - 10: 13th Int'l Conference on the Application opf Accelerators in Research & Industry, Denton, Texas. Contact: J. L. Duggan, Univ. of N. Texas, Dept. of Physics, P. O. Box 5368, Denton, TX 76203, USA.

NOVEMBER 10 - 11: 17th Int'l Conference on Chemistry, Biosciences, Pharmacy & Environmental Pollution, New Delhi, India. Contact: V. M. Bhatnagar, Alena Chem of Canada, P. O. Box 1779, Cornwall, Ont., Canada K6H 5V7.

NOVEMBER 11 - 12: 3rd Conference on Current Trends in Computational Chemistry, Jackson, Mississippi. Contact: J. Leszczynski, Jackson State University, Dept. of Chem., 1400 J. R. Lynch St., Jackson, MS 39217, USA.

NOVEMBER 13 - 16: 50th Southwest Regional Meeting, ACS, Fort Worth, Texas. Contact: H. C. Kelly, Texas Christian Univ, Chem Dept, Ft. Worth, TX 76129, USA.

NOVEMBER 13 - 18: Annual Meeting of the A.I.Ch.E., San Francisco, California. Contact: AIChE Express Service Center, 345 East 47th St., New York, NY 10017, USA.

NOVEMBER 14 - 17: 33rd Eastern Analytical Symposium, Somerset, New Jersey. Contact: Sheri Gold, EAS, P. O. Box 633, Montchanin, DE 19710, USA.

NOVEMBER 16 - 18: Envirosoft'94, 5th Int'l Conference on Development and Application of Computer Techniques to Environmental Studies, San Francisco, California. Contact: Computational Mechanics, Inc., 25 Bridge St., Billerica, MA 01821, USA.

DECEMBER 4 - 6: IBEX'94, International Biotechnology Expo & Scientific Conference, Moscone Center, San Francisco, California. Contact: Cartlidge & Associates, Inc., 1070 Sixth Avenue, Suite 307, Belmont, CA 94002, USA.

1995

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

1996

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

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

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

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

OCTOBER 16 - 19: 52nd Southwest Regional ACS Meeting, Houston, Texas. Contact: J. W. Hightower, Dept. Chem. Eng., Rice University, Houston, TX 77251, USA.

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

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

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

1997

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

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

1998

MARCH 29 - APRIL 3: 215th ACS National Meeting, St. Louis, Missouri. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 23 - 28: 216th ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1999

MARCH 21 - 26: 217th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 22 - 27: 218th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2000

MARCH 26 - 31: 219th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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

2001

APRIL 1 - 6: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 19 - 24: 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.

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Volkswriter 3, 4	Volkswriter Deluxe 2.2
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F. D. Pierce and H. R. Brown
Utah Biomedical Test Laboratory
520 Wakara Way
Salt Lake City, Utah 84108

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Following are acceptable reference formats:

Journal:

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

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

2. C. T. Mant, R. S. Hodges, "HPLC of Peptides," in HPLC of Biological Macromolecules, K. M.

Gooding, F. E. Regnier, eds., Marcel Dekker, Inc., New York, 1990, pp. 301-332.

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

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

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