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CENTRIFUGAL PARTITION CHROMATOGRAPHY: STABILITY OF VARIOUS BIPHASIC SYSTEMS AND PERTINENCE OF THE "STOKE'S MODEL" TO DESCRIBE THE INFLUENCE OF THE CENTRIFUGAL FIELD UPON THE EFFICIENCY

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ABSTRACT

The stability of various biphasic systems has been determined using a High Performance Centrifugal Partition Chromatograph, and its relationship with various physical parameters has been estimated. The stability seems to be related to $\frac{\gamma}{\Delta\rho}$, the ratio of the interfacial tension to that of the density difference between the two phases, and which is a parameter accounting for both the "Stoke's radius" of the mobile phase, *i.e.* the radius of droplets of mobile phase which will have the same average linear velocity, $\bar{u}$, in the channels than the experimentally determined value, and the "fragility" of these droplets. The average linear velocity of the mobile phase is not dependent upon the acceleration field in the range studied, which means that, using the "Stoke's model" (droplets, even if they do not exist), the higher the acceleration field, the smaller the droplets. Consequently, there is a linear relationship between the efficiency and the rotational speed, provided all other parameters are kept constant.

INTRODUCTION

Stability of the stationary phase is of first importance in Centrifugal Partition Chromatography, where it determines the utility of a biphasic system to

achieve a chromatographic purification. We have shown recently[1] that, by plotting the ratio of the minimum volume of mobile phase to the total volume of the column, we can obtain, for a given flow rate, V_m^{min}/V_C , versus F/S, where F is the flow rate and S the section of the channel where the chromatographic process occurs, leads to a straight line, the slope of which allows comparison of the stability of the stationary phase between instruments and biphasic systems. This slope gives the average linear velocity of the mobile phase, $\bar{u}$, in a channel, which must not be confused with the average velocity in the entire instrument which includes ducts and connections, where there is no stationary phase.

We have determined $\bar{u}$ for various biphasic systems, using the High Performance Centrifugal Partition Chromatograph (HPCPC) already described[2], and have attempted to correlate these data with the physical data, *i.e.* the density difference $\Delta\rho$, the interfacial tension, γ, between the two phases, and the viscosities, η_{UP} and η_{LP}, of the upper and the lower phases for these biphasic systems. We found that the "Bond number"[3,4], B, which is a dimensionless number describing the relative importance of gravitational to surface tension forces, does not account for the CPC stability of the solvent systems, but it corresponds to the Ito classification of the so called "hydrophobic and hydrophilic systems"[5], in countercurrent chromatography.

A better CPC classification for the solvent system stability in centrifugal partition chromatography has been presented using the parameter $\frac{\gamma}{\Delta\rho}$, which accounts for both the Bond number and the "Stoke's radius" of the mobile phase, *i.e.* the radius of droplets of mobile phase which will have the experimentally determined linear velocity $\bar{u}$.

Higher values of $\frac{\gamma}{\Delta\rho}$ correspond to biphasic systems with small Bond number and large droplets, resulting in a hydrodynamic system with fast and stable flow of the mobile phase into the stationary phase, while lower $\frac{\gamma}{\Delta\rho}$ corresponds to biphasic systems with a large Bond number and small droplets, yielding a hydrodynamic system with slow and fragile droplets which are easily broken during their course through the channels, thus favoring emulsification and destabilization of the stationary phase.

In the course of these studies, we discovered that, for a given biphasic system, $\bar{u}$ was not dependent upon the rotational speed in the range ordinarily

used by chromatographers (*c.a.* $\omega >$ 500 rpm). This means that, if we choose a very simple "Stoke's model" for the hydrodynamic behavior of the biphasic system in the HPCPC, *i.e.* one for which the mobile phase can be described as droplets with a "Stoke's radius", a, defined by $a = \sqrt{\frac{9\ \eta_{SP}\ \bar{u}}{2\ \Delta\rho\ g}}$, where η_{SP} is the viscosity of the stationary phase, and g the acceleration field, then there is a very simple relationship between that radius and the acceleration field, which is : $a^2\ g$ = Constant, for a given biphasic system.

As the interface between the two phases, and thus the efficiency of the chromatographic process, is directly related to the mutual dispersion of the phases, *i.e.* the number of droplets and their diameter, we will show that, if all other parameters are kept constant (particularly the flow rate and the ratio of the mobile phase in the CPC column), then the efficiency of the chromatographic process is linearly related to the rotational speed, *i.e.* to the square root of the acceleration field.

EXPERIMENTAL SECTION

Apparatus. A Series 1000 HPCPC (Sanki Laboratories, Mount Laurel, NJ, USA) was used[2]. It is a bench top CPC (30 x 45 x 45 cm, ≈ 60 Kg); the column is a stacked circular partition disk rotor which contains 2136 channels with a total internal volume of 240 ml. The column is connected to the injector and the detector through two high pressure rotary seals containing a drilled sapphire rod passing through two toroidal seals similar to those used with HPLC pump pistons. The partition disks are engraved with 1.5 x 0.28 x 0.21 cm channels connected in series by 1.5 x 0.1 x 0.1 cm ducts. A 4-port valve included in the Series 1000 allows the HPCPC to be operated in either the descending or ascending mode. The HPCPC was connected to an HPLC System Gold (Beckman, San Ramon, CA, USA), including a solvent delivery pump Model 126, a diode array detector Model 168 with a semi-prep scale flow cell, and a manual sample injector. A few turns of a 1/16" steel tubing loop, immersed in a warm water bath (≈40°C), was connected between the outlet of the HPCPC and the inlet of the detector, acting as a noise suppressor. Kinematic viscosities were measured at 23°C using Cannon-Fenske routine viscometers (Touzard & Matignon, Vitry/Seine, France). Interfacial tensions were measured at 23°C using the spinning drop method, with a Site 04 apparatus (Krüss, Palaiseau, France). Densities were determined by

weighing a 25 ml flask of each phase (average of 3 to 4 measurements). Solvent polarity has been estimated using the Reichardt's dye (Aldrich, catalogue number 27,244-2), following the procedure described by S. J. Gluck *et al.*[6]

Chemicals. The following solvents came from Prolabo (Paris, France) : dimethylsulfoxide, tetrahydrofuran, n-butanol, chloroform, methanol, n-propanol, heptane, acetone. The sec-butanol, methyl isobutyl ketone, and n-octanol came from Aldrich (Milwaukee, Wisconsin, USA). Water was de-ionized.

Procedure. Stability of the biphasic systems was estimated by varying the flow rate from 0 to 10 ml/min, or less if the backpressure in the HPCPC was reaching 6 MPa (≈0.86 kPSI), and measuring the volume V_m^{min} previously defined; the rotational speed was generally 1000 or 1200 rpm, and some experiments were performed at different rotational speeds, to evaluate the influence of this parameter upon the stability of the stationary phase. All the systems were evaluated in ascending and descending mode except the system octanol / water, for which the ascending mode was not explored, because our pump was unable to pump octanol at high flow rates.

The experiment related to the efficiency was performed with 4-hydroxybenzoic acid as a marker of the void volume and diethyl phthalate as the analyte, in the descending mode using the heptane / methanol biphasic system.

RESULTS AND DISCUSSION

Twelve biphasic systems have been studied, which represent a wide range of physical and chemical properties (see Table I for abbreviations). EtOAc/Water and $CHCl_3$/Water are not very useful for purification purposes, but they have a large interfacial tension; Oct/Water is used for partition coefficient determination, and its upper phase is rather viscous; HEP/MeOH is widely used as a non polar system; WDT2, 4 and 5 are a new class of medium polarity biphasic systems we introduced recently[7], containing water, dimethylsulfoxide and tetrahydrofuran, and which show very good solvating properties; MIBK/AcO/W and $CHCl_3$/MeOH/PrOH/W are medium polarity systems too, while the three butanol-containing systems are polar systems, widely used for purification of polar compounds, such as peptides.

Table I : Abbreviations used in this paper.

Abbreviations	Solvents and their volume ratio		
EtOAc/Water	Ethyl acetate Water, 50/50		
$CHCl_3$/Water	Chloroform Water, 50/50		
n-BuOH/Water	n-Butanol Water, 50/50		
sec-BuOH/Water	sec-Butanol Water, 50/50		
HEP/MeOH	Heptane Methanol, 50/50		
Oct/Water	Octanol Water, 50/50		
	Water	Dimethylsulfoxide	Tetrahydrofuran
WDT2 (see ref. 7)	11.7	26.3	62
WDT4	21.5	21.2	57.3
WDT5	24.5	16.2	59.3
BAW	n-Butanol Acetic acid Water, 40/10/50		
MIBK/AcO/W	Methylisobutyl ketone Acetone Water, 25/50/25		
$CHCl_3$/MeOH/PrOH/W	Chloroform Methanol n-Propanol Water, 29/38.7/6.5/25.8		

Table II groups the physical data for the biphasic systems, and their phase polarities, estimated through the Reichardt's E_TN[6]. The density differences are in the range 0.07 to 0.14 g/cm^3 for all the systems except those containing $CHCl_3$, and the interfacial tension is generally small (< 3 dyne/cm) except for three systems which show much higher values. Viscosities are in the range 0.5 to 4 cP, except for the upper phase of Oct/Water system, for which it is 7.1 cP.

Stability of the systems. As we recently reported[1], the stability of a biphasic system can be estimated by the slope of the line which links the minimum volume of mobile phase we can obtain for a given flow rate to that flow rate, or, in order to compare both phase systems and instruments, by the slope of the following equation :

$$\frac{V_m^{min}}{V_c} = \frac{(1-d)}{\bar{u}} \frac{F}{S} + d \qquad [1]$$

where V_m^{min}, V_C , $\bar{u}$, F and S have been already defined, and d is the ratio of the volume of the ducts and connections to that of the column, where there is no stationary phase.

Table II : Physical and Chemical data of the Biphasic Systems

System[a]	$\Delta\rho$	γ	η cP		E_TN[b]	
	g/cm^3	Dyne/cm	Upper	Lower	Upper	Lower
EtOAc/Water	0.097	13.2	0.47	1.10	0.50	**1**
$CHCl_3$/Water	0.478	32.8	0.98	0.55	not determined	
HEP/MeOH	0.073	1.16	0.41	0.57	**0.23**	0.73
WDT2	0.102	0.59	0.72	2.37	0.45	0.55
WDT4	0.130	2.15	0.62	2.88	0.45	0.64
WDT5	0.115	1.26	0.72	2.60	0.48	0.67
MIBK/AcO/W	0.084	0.25	0.70	1.42	0.72	0.68
$CHCl_3$/MeOH/PrOH/W	0.213	0.42	1.78	0.97	0.69	0.57
Oct/Water	0.137	8.5[c]	7.10	0.80	not determined	
n-BuOH/Water	0.144	2.91	3.05	1.33	0.65	0.89
BAW	0.101	1.21	2.89	1.51	not determined	
sec-BuOH/Water	0.093	0.53	3.64	2.02	0.73	**1**

$\Delta\rho$ = density difference; γ = interfacial tension; η = viscosity

(a) The solvent systems are roughly sorted according to the Ito Classification in hydrophobic, intermediate and hydrophilic groups.

(b) E_TN is the normalized Reichardt index, *i.e.* non-polar liquids have an E_TN close to 0 and polar liquids have an E_TN close to 1; bold numbers means the polarity is approximative, due to the insolubility of the Reichard's dye in the corresponding phase

(c) from Handbook of Chemistry and Physics

Regression analysis for the twelve systems, in the descending and ascending modes (except for the Oct/Water system in the ascending mode) is shown in Table III, for various rotational speeds of the HPCPC column; the calculated linear velocity, $\bar{u}$, of the mobile phase in the channels, is shown too.

Three significant observations emerge from these results :

1 • We were very surprised to find that the slope of the regression line is not dependent upon the rotational speed of the HPCPC column, in the range ordinarily used by chromatographers (*c.a.* 500 to 2000 rpm). We tested the lower limit of this non-dependence with the system WDT4, in the descending mode, and found that it was still true at 400 rpm, while the stationary phase was no longer stable at 200 rpm. We did not check how the lower limit was system

dependent, since, as we will show below, it is always better for the chromatographer to work at higher rotational speeds.

2 • Even if we can describe a system as more stable or less stable, they are all stable enough to allow for chromatographic runs; for example we can calculate from the data of Table III that the retention of the stationary phase ($1-V_m^{min}/V_C$) will be 54% for F = 5 ml/min for the system $CHCl_3$/MeOH/PrOH/W in the descending mode, and 41% for the same flow rate, for the system WDT2 in the ascending mode. For the system BAW, the retention of the stationary phase will be 57% and 40% for F = 10 ml/min in the descending and ascending mode, respectively. We will define as "very stable" the systems where the retention of the stationary phase is > 60% for F = 10 ml/min, as "less stable" those where the retention is < 50% for the same flow rate, and as "stable" the intermediates systems; dotted lines on Table III separate these categories.

3 • In a previous paper[1] we wrote that d, the ratio of the volume of ducts and connections to that of the column, should be a constant for the HPCPC since they are geometrically defined, but we see in Table III that d varies from 14% (WDT4 in the descending mode) to 33% ($CHCl_3$/MeOH/PrOH/W in the descending mode) with an average of 22% for the descending mode, and 21% in the ascending mode. Using the dimensions given by the manufacturer (see experimental), we find that the ducts represent a total volume of ≈ 32 ml; adding 5 ml for the connections between disks and for the two rotary seals leads to a minimum value of d ≈ 15%, which is approximately the minimal value we find for the system WDT4 in the ascending mode. Higher values for d must be understood as a consequence of the geometry of the channels and ducts, and the way the mobile phase is injected in the channels, *i.e.* the nature of the passage of a continuous mobile phase, in the ducts, to a discontinuous mobile phase, in the channels, and of the inverse phenomenon. We must then define a "dynamic duct", which is the part of the column where there is no stationary phase, and thus no chromatographic process. The best way to measure the dynamic ducts for a CPC column and a biphasic system is to calculate the

Table III : Data for the linear regression analysis of the relationship : $\frac{V_m^{min}}{V_c} = \frac{(1-d)}{\bar{u}} \frac{F}{S} + d$

Descending Mode : Lower Phase as Mobile Phase

System[a] (rot. speed , rpm)	$10^3 \frac{1-d}{\bar{u}}$ (min/cm) (10^3 Std. error)	10 d (10 Std. error)	r^2 (nb of exp.)	$\bar{u}$ (cm/sec)	
$CHCl_3$/Water (700)	0.46 (0.05)	2.02 (0.05)	0.988 (3)	28.8	
Oct/Water (1000)	0.52 (0.06)	2.23 (0.06)	0.988 (3)	24.8	very
EtOAc/Water (1200)	0.64 (0.04)	2.65 (0.05)	0.984 (6)	19.0	stable
n-BuOH/Water (800, 1200)	0.96 (0.08)	2.13 (0.09)	0.96 (8)	13.6	
WDT5 (800, 1200, 1400)	1.1 (0.06)	2.26 (0.07)	0.96 (13)	11.7	
WDT4 (400, 1200, 1400)	1.1 (0.05)	2.28 (0.06)	0.99 (6)	11.9	stable
HEP/MeOH (1200)	1.3 (0.09)	2.3 (0.07)	0.986 (5)	9.6	
BAW (1200)	1.3 (0.1)	2.1 (0.1)	0.985 (4)	9.8	
WDT2 (1000, 1400)	2.5 (0.2)	2.6 (0.1)	0.97 (9)	4.9	
sec-BuOH/Water (1200)	2.7 (0.2)	1.6 (0.1)	0.97 (10)	5.1	less
MIBK/AcO/W (1000)	2.9 (0.4)	2.5 (0.5)	0.96 (4)	4.3	stable
$CHCl_3$/MeOH/PrOH/W (700)	3.16 (0.09)	1.9 (0.1)	0.997 (5)	4.3	

Ascending Mode : Upper Phase as Mobile Phase

System[a] (rot. speed , rpm)	$10^3 \frac{1-d}{\bar{u}}$ (min/cm) (10^3 Std. error)	10 d (10 Std. error)	r^2 (nb of exp.)	$\bar{u}$ (cm/sec)	
$CHCl_3$/Water (700)	0.46 (0.01)	2.29 (0.01)	0.998 (3)	28.1	very
EtOAc/Water (1200)	1 (0.05)	1.48 (0.05)	0.990 (6)	14.2	stable
n-BuOH/Water (800, 1200)	1.2 (0.05)	1.54 (0.05)	0.990 (7)	11.8	
HEP/MeOH (1200)	1.5 (0.2)	1.9 (0.2)	0.988 (3)	9.0	
WDT5 (800, 1400)	1.8 (0.1)	1.8 (0.1)	0.97 (8)	7.53	stable
WDT4 (1200)	2.0 (0.3)	1.4 (0.4)	0.97 (3)	7.15	
sec-BuOH/Water (1200)	2.3 (0.1)	2.8 (0.2)	0.97 (8)	5.3	
BAW (1200)	2.5 (0.2)	1.8 (0.2)	0.986 (5)	5.5	less
$CHCl_3$/MeOH/PrOH/W (700)	2.6 (0.7)	3.3 (0.7)	0.81 ! (5)	4.3	stable
MIBK/AcO/W (1000)	2.8 (0.1)	2.9 (0.2)	0.988 (7)	4.3	
WDT2 (1400)	5.0 (0.6)	1.7 (0.5)	0.95 (5)	2.8	
Octanol/Water	deficient pumping				

(a) Systems are sorted in order of decreasing stabilities, and the dotted lines make the separations between very stable, stable and less stable systems (see text).

V_C = 240 ml; S = 0.059 cm^2; F from 1 to 10 ml/min.

slope and intercept of equation [1] by regression analysis. From our results, we find a dynamic duct of ≈ 22% for most of the biphasic systems, the worst being 33% for the system $CHCl_3$/MeOH/PrOH/W in the ascending mode. 22% instead of 15% corresponds to a layer of pure mobile phase of ≈ 8 μl in each channel, which is ≈ 88 μl.

Correlation between the stability and the physical parameters. The exact nature of the flow of mobile phase into the stationary phase in the channels of the HPCPC column is still a matter of controversy, and a direct visualization of that flow will be very useful, as it has been already done for the Ito CCC apparatus[8]

As is usual in other fields (*e.g.* sedimentation of proteins in a centrifuge), we will adopt a very simple model to describe the hydrodynamic of the flow in a channel. We will imagine the mobile phase in a channel as droplets with an average radius, "a", corresponding to Stoke's Law. For the average linear velocity $\bar{u}$, we experimentally found :

$$\bar{u} = \frac{2\, a^2\, \Delta\rho\, g}{9\, \eta_{SP}} \qquad [2]$$

where $g = \omega^2 R$, R being the average radius of the HPCPC centrifuge, and ω the rotational speed, $\Delta\rho$ the density difference between the two phases and η_{SP} the viscosity of the stationary phase, which can be the upper or the lower one, depending upon the chosen mode.

The independence, for a given biphasic system and a given mode, of the average linear velocity of the mobile phase, $\bar{u}$, toward the acceleration field, g, leads to the simple relationship :

$$a^2\, g = A \qquad [3]$$

A being a constant for a specific system and mode; this means that the higher the acceleration field, the smaller will be the radius of the droplets of mobile phase in a channel. Comparing various biphasic systems, A will characterize the dispersion of the mobile phase in the stationary phase, since a smaller value of A means that, for a given acceleration field, the continuous mobile phase coming from the duct will break into many smaller droplets, while a larger value of A means it will break into few larger droplets. "A" may be called the dispersion term; its dimension is $L^3\, T^{-2}$. From [2] and [3], we get :

$$A = \frac{9}{2}\frac{\bar{u}\,\eta_{SP}}{\Delta\rho} \qquad [4]$$

As the dimension of A is the same as that of the ratio $\frac{\gamma}{\Delta\rho}$ (*i.e.* L^3T^{-2}) where γ is the interfacial tension, we can then write the following equation :

$$A = B\frac{\gamma}{\Delta\rho} \qquad [5]$$

where B is a dimensionless number. From [3] and [5] it becomes :

$$B = \frac{a^2\, g\, \Delta\rho}{\gamma} \qquad [6]$$

Equation [6] can be used to compare B to the numerous dimensionless numbers we can find in the literature, and thus find out if it has already been described : B was defined in 1928 by W.N. Bond *et al.*[3], and is called the Bond number[4]. It characterizes the relative importance of gravitational to surface-tension forces, and accounts for the fragility of a droplet. "A droplet of liquid in motion through another liquid differs in its behavior from a solid sphere in that it may (a) be deformed, (b) have a circulation set up within itself by the shearing effect of the relative motion of the two fluids. These effects upset the stability of the drop, causing it to oscillate about the spherical shape and eventually to burst into fragments or, at least, into smaller drops"[4]. From [4] and [5] we get :

$$B = \frac{9}{2}\frac{\bar{u}\,\eta_{SP}}{\gamma} \qquad [7]$$

Like A, B is not dependent upon the acceleration field. Table IV gives the values of A and B calculated with equation [4] and [7], for the twelve solvent systems, in the descending and ascending modes; systems are sorted in order of decreasing stability in the descending mode. Figure 1 shows the twelve systems with the Bond number corresponding to the descending mode, and sorted in order of increasing Bond number. From Table IV and Figure 1, we can conclude that B does not account for the stability of the systems in centrifugal partition chromatography, but it corresponds to the Ito classification of the so-called "hydrophobic and hydrophilic systems"[5], which accounts for the hydrodynamic behavior of solvents systems in the Ito CCC apparatus. From Table IV, it seems that the stabilities of the solvent systems are related to both

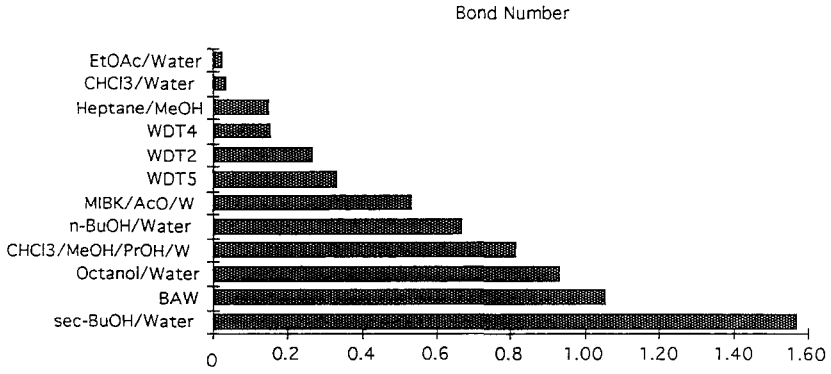

Figure 1 The twelve systems and their Bond numbers, corresponding to the descending mode.

Table IV : The dispersion term, A, and the Bond number, B.

	Descending mode		Ascending mode	
System	A	B	A	B
CHCl3/Water	2.66	0.04	1.46	0.02
Octanol/Water	57.87	0.93	•	•
EtOAc/Water	4.14	0.03	7.28	0.05
n-BuOH/Water	13.54	0.67	4.70	0.23
WDT5	3.67	0.33	7.62	0.69
WDT4	2.56	0.15	7.15	0.43
Heptane/MeOH	2.42	0.15	3.18	0.20
BAW	12.64	1.06	3.69	0.31
WDT2	1.58	0.27	2.90	0.50
sec-BuOH/Water	9.04	1.57	5.14	0.90
MIBK/AcO/W	1.61	0.53	3.27	1.08
CHCl3/MeOH/PrOH/W	1.61	0.82	0.88	0.45

The systems are sorted according to their decreasing stability in the descending mode.
A in cm^3sec^{-2}, B dimensionless

the dispersion of the mobile phase (A) and to the fragility of the droplets (B) : higher values of A (large droplets) combined with lower values of B (less fragility) result in very stable systems (*e.g.* $CHCl_3$/Water, EtOAc/Water); lower values of A (small droplets) combined with higher values of B (more fragility) yield less stable systems (*e.g.* MIBK/AcO/W, $CHCl_3$/MeOH/PrOH/W).

Table V shows the twelve systems sorted according to $\frac{A}{B} = \frac{\gamma}{\Delta\rho}$, a parameter which accounts both for the dispersion of the mobile phase and for the fragility of the droplets; Figure 2 shows the correlation between this parameter and the average linear velocities of the mobile phases in the channels. It can be seen from these data that $\frac{\gamma}{\Delta\rho}$ varies in the same way as the average linear velocities we experimentally determined.

This correlation could be interpreted as follows :

1. $\frac{\gamma}{\Delta\rho}$ small = A small and B large

   Small and unstable droplets moving slowly, and easily broken into smaller ones, leading to some possible emulsification, dragging the stationary phase out of the column. These systems can be used at low flow rate, in order to minimize emulsification and keep a sufficient amount of stationary phase in the column.

2. $\frac{\gamma}{\Delta\rho}$ large = A large and B small

   Large and very stable droplets moving fast; if so, rapid mass transfer between mobile and stationary phase is not favored for these systems, which should display poor chromatographic efficiencies.

3. $\frac{\gamma}{\Delta\rho}$ medium = A and B medium

   This is the common case, and is the best suited for chromatographic applications; the droplets are small enough to allow for a reasonable rate of mass transfer between the two phases, and fast enough to keep a large amount of stationary phase in the column. Some emulsified layer may be present in the channels, like described by D. Armstrong *et al.*[9], but this has no negative repercussion upon the stability of the stationary phase.

Efficiency and Rotational Speed. The improvement of the resolution between peaks by increasing the rotational speed of the CPC column has been recognized since the early beginnings of CPC[10], even if the reason for this phenomenon was never systematically explored. Using our very simple model

Table V : Comparison of the parameter $\frac{\gamma}{\Delta\rho}$ and the experimental average linear velocities of the mobile phase, in descending and ascending mode.

System	$\frac{\gamma}{\Delta\rho}$	ū DM	ū AM
EtOAc/Water	136.51	19.0	14.2
CHCl3/Water	68.62	28.8	28.1
Octanol/Water	62.04	24.8	
n-BuOH/Water	20.21	14.2	11.3
WDT4	16.61	11.9	7.2
Heptane/MeOH	15.96	9.6	9.0
BAW	11.97	9.8	5.5
WDT5	10.97	13.1	7.5
WDT2	5.84	4.9	2.8
sec-BuOH/Water	5.74	5.1	5.3
MIBK/AcO/W	3.02	4.3	4.3
CHCl3/MeOH/PrOH/W	1.97	4.3	4.3

ū in cm/sec, $\frac{\gamma}{\Delta\rho}$ in $cm^3\ sec^{-2}$

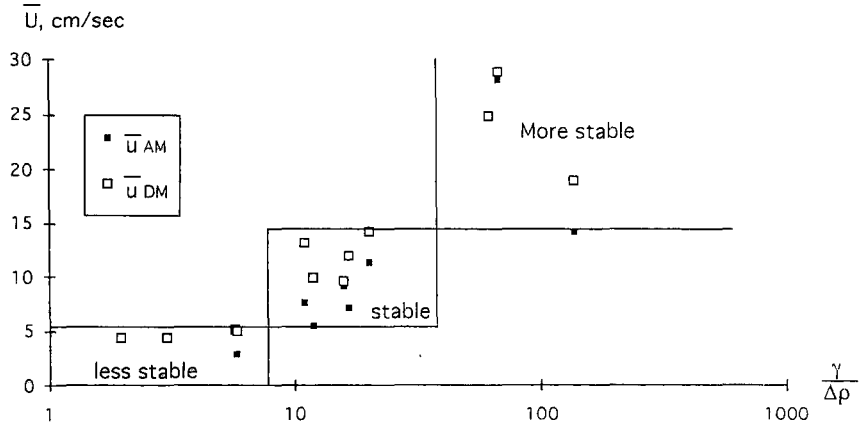

Figure 2 Correlation between the average linear velocity, ū, of the mobile phase in the channels of the HPCPC, and the parameter $\frac{\gamma}{\Delta\rho}$.

$\bar{u}_{AM}$ = average linear velocity in the ascending mode
$\bar{u}_{DM}$ = average linear velocity in the descending mode

to describe the hydrodynamics of the flow in a channel, *i.e.* the "Stoke's model", with droplets simulating spheres with a radius, "a", corresponding to Stoke's law, for the average linear velocity ū, we can express the interfacial area between the two phases as a function of the acceleration field as follows :

from equation [1], the volume of mobile phase in the channel (not in "dynamic" ducts and connections) is :

$$\mathcal{V}_m^{min} = V_m^{min} - V_{ducts} = \frac{(1-d)}{\bar{u}} \frac{F}{S} V_c \qquad [8]$$

Since the droplets have a surface $s = 4 \pi a^2$, a volume $v = 4/3 \pi a^3$, then the number of droplets in the channels is $n = \mathcal{V}_m^{min} / v$, and the interfacial area between the two phases in the channels is :

$$I = n s = \mathcal{V}_m^{min} \frac{3}{a} \qquad [9]$$

From [3], [4], [8], and [9], we obtain :

$$I = (1-d) V_c \frac{F}{S} \frac{27}{2} \frac{\eta_{SP}}{\Delta\rho} A^{-3/2} g^{1/2} \qquad [10]$$

where all terms are constant for a given system and a mode, except for F and g.

For a given biphasic system and a given flow rate, then the efficiency should be directly related to the interfacial area :

$$N \approx I \approx g^{1/2} \qquad [11]$$

where N is the number of theoretical plates for a given peak.

We have tested equation [11] with the system n-Heptane/Methanol in the descending mode, the rotational speed being varied between 700 and 2000 rpm. The flow rate was kept constant and equal to 7 ml/min, and the volume of mobile phase, V_m, was 120 ± 4 ml, which is 27 ml more than the calculated V_m^{min}, in order to keep this volume as constant as possible throughout the experiments, as it has been shown that this parameter has a strong influence upon the efficiency[1]. V_m was determined using 4-hydroxybenzoic acid (non retained solute), and diethyl phthalate was the analyte, with a partition coefficient of 0.27 ± 0.01. The number of plates has been estimated by using both the width at half height and at the base of the

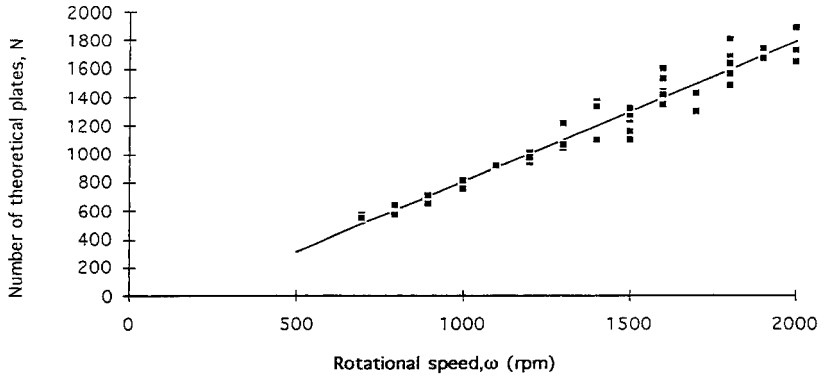

Figure 3 Relationship between N, the number of theoretical plates, and ω, the rotational speed of the HPCPC column, all other parameters being kept constant.

peak, and runs where the two values were not in close agreement were rejected. Runs where V_m was not constant were also rejected too. 59 out of 77 injections were taken into consideration.

Figure 3 shows the results of this investigation; as predicted by equations [10] and [11], based on the "Stoke's model", N is proportional to $g^{1/2}$, *i.e.* to the rotational speed ω, in the range ordinarily used by the chromatographer (500 to 2000 rpm).

Regression analysis leads to the equation :

$$N = 0.98\ (0.03)\ \omega - 175\ (40)$$

$$n = 59 \qquad r^2 = 0.95 \qquad s = 87 \qquad \mathcal{F} = 1126$$

where n is the number of experiments, r the correlation coefficient, s the standard deviation, and $\mathcal{F}$ the Fisher's test parameter (ω in rpm).

We conclude from these results that, even if it has not been proven that the mobile phase flows as droplets in the stationary phase, in the channels of the HPCPC column, we can use the simple "Stoke's model" to account for the dependence of the efficiency upon the rotational speed. The upper limit of this relationship should be reached when N approximates the number of physical plates, *i.e.* the number of channels, 2136 with our instrument. Figure 4 allows the

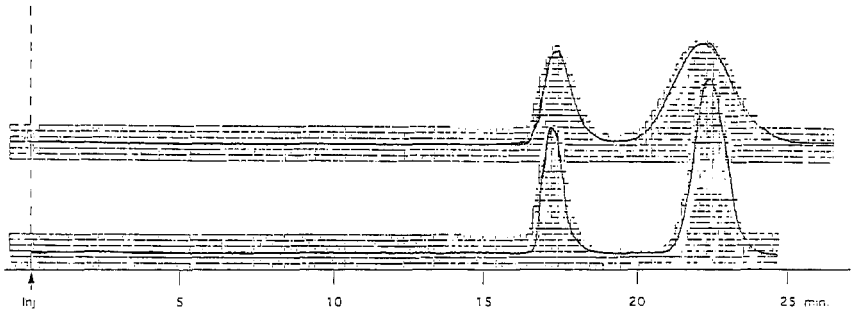

Figure 4 Comparison of HPCPC chromatograms obtained at 700 and 2000 rpm, all other parameters being kept constant.
Solvent system : Heptane / Methanol, descending mode
Unweighted but identical quantities injected
Flow rate : 7 ml/min; $\frac{Vm}{Vc} = 0.5$ Upper : 700 rpm; lower : 2000 rpm

comparison of two chromatograms obtained at 700 and 2000 rpm, all other parameters being kept constant.

CONCLUSION

The "Stoke's model", which is very simple and simulates the mobile phase to droplets with an average radius calculated by using the Stoke's law and the average linear velocity deduced from the regression analysis of the dependence of the volume of the mobile phase in the CPC column with its flow rate, gave us a simple parameter ($\frac{\gamma}{\Delta\rho}$) to estimate the stability of a biphasic system in centrifugal partition chromatography, and is fully compatible with the evolution of the efficiency of the CPC column with the acceleration field. The best results will be obtained for higher rotational speed, whatever the system is, not because the retention of the stationary phase is higher, but because the dispersion of the mobile phase in the stationary phase becomes better.

The back pressure, which is a non-chromatographic parameter, and mainly due to hydrostatic pressure ($\Delta P \approx \Delta\rho\ g)^9$ will be the major obstacle to achieve the best performance of a CPC apparatus. Instruments must be able to

work at high pressure, in order to use them with any biphasic system at higher rotational speeds to obtain as many theoretical plates as the column can yield.

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FRACTIONATION OF [^{14}C]METOLACHLOR METABOLITES BY CENTRIFUGAL PARTITION CHROMATOGRAPHY

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ABSTRACT

Metolachlor (2-chloro-N-(2'-ethyl-6'-methylphenyl)-N-(2-methoxy-1-methylethyl)acetamide) is one of the most widely used and important herbicides throughout the world. In order to understand the biodegradation pathways for this compound it is essential to develop large scale separation and purification procedures for its metabolites. However, analyzing crude plant extracts for these compounds poses several problems. In particular, the complex matrix and low levels of many metabolites requires a preparative separation and detection procedure that is both sensitive and robust. The use of [^{14}C] labeled compounds and scintillation counting satisfies the sensitivity requirement but creates an additional problem when using preparative LC. Irreversible adsorption to the stationary phase frequently occurs when analyzing "real-world" biological samples or extracts thereof. This is particularly undesirable if the matrix contains radioactive components. This problem can be avoided by using a technique with a liquid stationary phase such as centrifugal partition chromatography (CPC). CPC is used for the preparative fractionation of [^{14}C]metolachlor metabolites contained in crude corn plant extract. Also, a rapid analytical HPLC method is developed for the separation of standard non-radiolabeled metolachlor metabolites on a dimethylphenyl-derivatized β-cyclodextrin stationary phase.

INTRODUCTION

Metolachlor (2-chloro-N-(2'-ethyl-6'-methylphenyl)-N-(2-methoxy-1-methylethyl)acetamide) is one of the α-chloroacetamide herbicides that is used extensively in the cultivation of corn and soybeans as a pre-emergence control for most annual grasses and many broadleaf weeds. It is one of the most widely employed herbicides and frequently it is applied in combination with atrazine (1). Residues of metolachlor and its metabolites may occur in crops as a result of labeled uses and in environmental water as a result of run-off, spills or improper application(s). The most common methods used to measure metolachlor residues in water are gas chromatography and immunoassays (2-4). The detection of metolachlor in aqueous media has been reported in the range of 0.1 to 0.9 ppb by enzyme-linked immunosorbent assay (ELISA) (4).

Metabolism studies of specific α-chloroacetamide herbicides have been reported previously (5-8). An investigation of the metabolic fate of [^{14}C]propachlor (2-chloro-N-isopropylacetanilide) in the leaves of corn, sorghum, sugarcane, and barley was conducted by thin-layer chromatography (TLC). Evidence was found that one of the pathways for metabolism of α-chloroacetamide herbicides in higher plants involved glutathione conjugation (5). Recently, the initial metabolism of [^{14}C]acetochlor (2-chloro-N-(ethoxymethyl)-N-(2'-ethyl-6'-methylphenyl)acetamide) in corn and soybean seedlings was studied by high-performance liquid chromatography (HPLC) combined with structural assignments by fast atom bombardment mass spectrometry (FAB-MS) (6). At least eight

extractable products from the metabolism of metolachlor by the soil fungus *Chaetomium globosum* were resolved by gas-liquid chromatographic analysis (GLC) (7). Several key metolachlor metabolites in corn have been identified and a possible metabolic pathway has been proposed (8,9). Two-dimensional TLC and ionic exchange chromatography (IEC) have been used to fractionate and characterize metolachlor metabolites contained in crude extracts from corn (10).

All of the aforementioned studies made effective use of a variety of analytical methodologies. However, as is often the case when working with complex extracts of biological materials; larger, pure or partially pure sample fractions are often needed for more extensive multiple analyses. Preparative HPLC is often useful in this respect but irreversible retention is often a problem with biological samples. Complete sample recovery is often desirable, particularly when working with radioactively labeled compounds. Incomplete recovery in HPLC converts costly stationary phase into radioactive solid waste.

Centrifugal partition chromatography (CPC), a form of countercurrent chromatography (CCC), has been shown to be particularly useful for preparative separations (11-13). For example, it was determined that approximately 880 mg of D,L-tryptophan methyl ester can be separated into enantiomerically pure fractions with one injection (13). We have employed CPC to fractionate petroleum catalytic cracker feeds and asphalts in amounts between 1 and 10 grams per injection (14). Since a liquid rather than a solid stationary phase is used, strongly retained solutes can be completely recovered by flushing the system. The ability

to use dual-mode operation (*i.e.*, both normal-phase and reversed-phase separations in a single run) makes CPC well suited for the fractionation of samples that contain compounds with a wide range of polarities. In addition, separation efficiencies improve with increased flow rate (15). This trend is opposite to that observed for other chromatographic techniques. To our knowledge, there have been no reports on the use of CPC for the separation of radioactive pesticide metabolites.

In this work we evaluate the utility of CPC in the fractionation and characterization of [^{14}C]metolachlor metabolites contained in crude extract of mature corn plants. Both UV detection and liquid scintillation counting detection were used. Also, we demonstrate a rapid analytical HPLC method for the separation and characterization of standard non-radiolabeled metolachlor metabolites on a dimethylphenyl-derivatized β-cyclodextrin stationary phase.

EXPERIMENTAL

Materials

The following materials were obtained from CIBA-GEIGY Corporation (Greensboro, NC): ground mature corn plants (control material), standards of non-radiolabeled metolachlor metabolites (C-25702, CGA-37735, CGA-50720, CGA-13656, CGA-37913, CGA-40919, CGA-41507, CGA-46127, CGA-40172, CGA-51202, CGA-46576, CGA-110186, and CGA-118243), and [^{14}C]radioactive crude corn plant extract. The structures of all metabolites are given in Figure 1. A

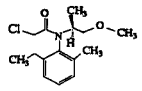
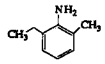
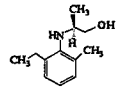
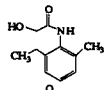
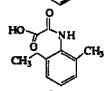
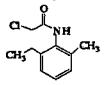
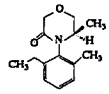
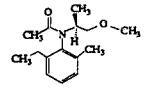
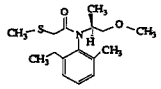
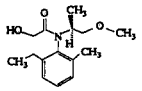
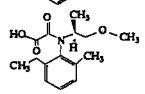
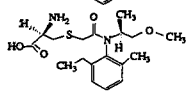
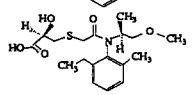
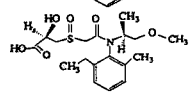
Structure	Abbrev
	Metolachlor
	C-25702
	CGA-37913
	CGA-37735
	CGA-50720
	CGA-13656
	CGA-40919
	CGA-41507
	CGA-46127
	CGA-40172
	CGA-51202
	CGA-46576
	CGA-110186
	CGA-118243

Figure 1. Structure of Metolachlor and its metabolites

biodegradable liquid scintillation cocktail, Ecolume, was purchased from ICN Biomedicals Inc. (Irvine, CA). HPLC grade 1-butanol, methanol, hexane, chloroform, triethylamine, trifluoroacetic acid, acetic acid and 7 ml scintillation vials were purchased from Fisher (Fairlawn, NJ). HPLC grade dichloroacetic acid was purchased from Aldrich (Milwaukee, WI). All water used was distilled and passed through a Barnstead D8922 cartridge to trap organics and filtered through a 0.45 μm Alltech nylon 66 membrane to remove particles.

Centrifugal Partition Chromatography

The CPC apparatus, Model CPC-NMF, equipped with 6 partition cartridges Type 250W (resulting in a total volume of 120 ml) from Sanki Laboratories Inc. (Mount Laurel, NJ). was used. Up to 12 cartridges (with 240 ml total volume) can be used in this instrument as has been described previously (11). A Rheodyne Model 7010 valve with 1000 μl sample loop was used to introduce the sample and a Rheodyne Model 7000 valve was used for mode selection when changing between descending and ascending modes. A Shimadzu LC-6A pump and a Shimadzu SPD-6A spectrophotometric UV detector were used with the CPC. The Recorder Company Series 4500 strip chart recorder, was used to record the CPC chromatogram. After elution, samples were collected with an Isco Cygnet fraction collector (Lincoln, NE).

Corn plants grown in a greenhouse were treated with [^{14}C]doped metolachlor herbicide. The corn was harvested when mature. The mature corn stalks were

mixed with methanol/water (8:2) on a shaker for approximately 30 minutes. The filtrate from three extractions were combined and concentrated using a rotary evaporator. The CPC experiments were performed using a two-phase ternary solvent system: 1-butanol/acetic acid/water. This solvent system was made by mixing 1860ml 1-butanol, 280ml acetic acid, and 1860ml water by volume. This forms a two phase mixture, each saturated with the other. The two phases (one butanol rich and one water rich) can then be separated from one another and used in the CPC. Fractionation was optimized on a nonradioactive corn extract control using UV (254 nm) detection. A 500 μl aliquot the [^{14}C]radioactive crude extract was injected directly into the CPC in the descending mode (*i.e.*, predominantly aqueous mobile phase, switching to the ascending mode (*i.e.*, predominantly butanol mobile phase) after two hours. Fractions were collected and 5 ml of scintillation cocktail was added to a 1 ml portion of each fraction which had been pipetted into a scintillation vial. A Beckman LS 7500 system, Beckman Instruments, Inc., Fullerton, CA was used to record the β-decay events. Blank solutions consisted of 5 ml scintillator cocktail and 1 ml water saturated with l-butanol. All fractions and blanks were counted for 5 minutes.

High Performance Liquid Chromatography

An HPLC method was developed for the separation of metolachlor metabolite standards (C-25702, CGA-37735, CGA-50720, CGA-13656, CGA-37913, CGA-40919, CGA-41507, CGA-46127, CGA-40172, CGA-51202, CGA-

46576, CGA-110186, and CGA-118243). A system controller, Shimadzu SCL-6B, two Shimadzu LC-6B pumps, and a Shimadzu SPD-2AM detector were used in the gradient mode. The metabolite standards were separated using a CYCLOBOND I-DMP column obtained from Advanced Separation Technologies (Whippany, NJ). The elution program contained three steps. First, a mobile phase of 30:70, methanol:buffer (by volume) was employed for 10 minutes. This was followed by a linear gradient from 30:70 to 50:50 methanol:buffer in 10 minutes. An isocratic elution with 50:50, methanol:buffer was continued for 20 minutes. The buffer consisted of 0.1% triethylammonium acetate (pH=7.1), the flow rate was 1 ml/min and UV detection was at 254 nm.

Results and Discussion

Correct choice of the solvent system plays a significant role in the optimization of CPC separations (16). In this study, three solvent systems were evaluated: hexane/water, chloroform/water, and 1-butanol/water systems. Although a third component, methanol, was added to the hexane/water and chloroform/water systems to adjust the partitioning of the sample between the stationary phase and the mobile phase, no desirable fractionations were obtained with these solvents. The best results were obtained with the 1-butanol/water solvent system. This was optimized by adding various ternary components such as: 2-propanol, triethylamine, dichloroacetic acid, trifluoroacetic acid, and acetic acid. The best CPC fractionation of the corn plant sample was

obtained with a 1-butanol/acetic acid/water (1860ml/280ml/1860ml) solvent system (see Experimental).

As can be seen in Figure 1, two of the metolachlor metabolites are weak bases, *i.e.*, a primary amine (C-25702) and an amino alcohol (CGA-37913). All the rest of the compounds are amides. However, three of the amide compounds also contain carboxylic acid functional groups (*i.e.*, CGA-118243, CGA-110186, and CGA-51202) while one compound contains an amino acid moiety (CGA-46576). Using UV detection (Figure 2A, top) the CPC fractionation of the crude corn plant extract was optimized so that the total peak area in the descending mode was roughly equivalent to that in the ascending mode. It appears that there are at least four distinct peaks in the descending mode (at 15, 19, 30 and 45 minutes). The first peak at 15 min also represents the dead time (t_o) for this separation. It is highly likely that each peak is composed of several closely or co-eluting compounds. This is particularly apparent in the ascending mode (Figure 2A) where many of the peaks are so close together (after the initial one at 117 min) that they are often difficult to distinguish one from another.

Using exactly the same chromatographic conditions that were used to generate Figure 2A, crude corn plant extract previously treated with [^{14}C]metolachlor was fractionated (see Experimental). The resulting radiochromatogram shown in Figure 2B. It is apparent from this figure that over two thirds of the radioactive compounds are eluted in the ascending mode. When the radiochromatogram is directly compared to the above UV chromatogram, it is

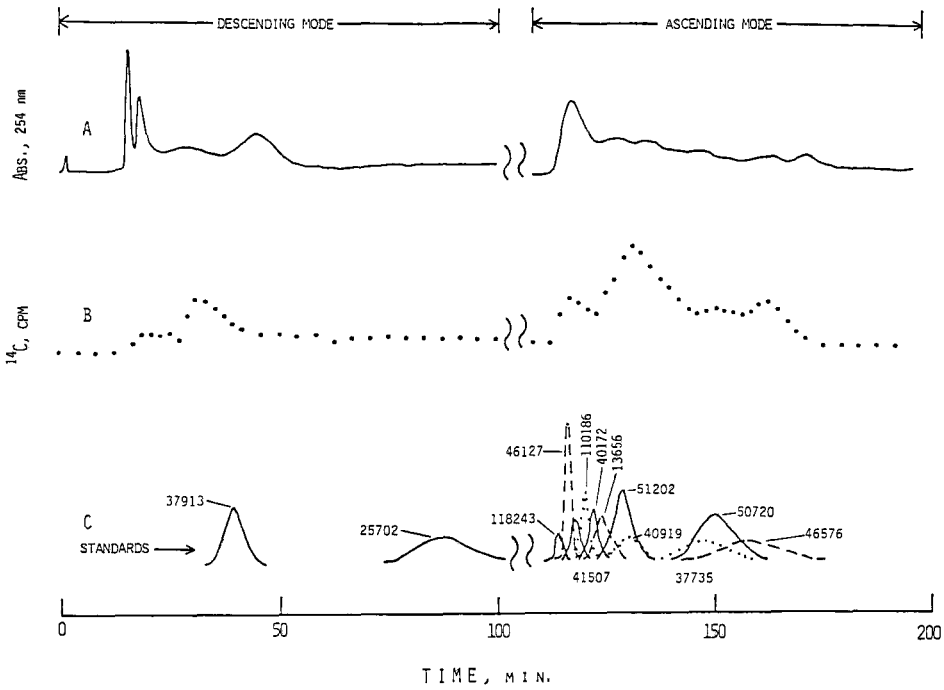


Figure 2. CPC fractionations of [^{14}C]metolachlor metabolites contained in crude corn plant extract using (A) UV detection and (B) liquid scintillation counting detection. Chromatogram (C) is a composite showing the retention of 13 metolachlor metabolite standards (structures given in Fig. 1). Identical conditions were used for all these CPC separations (see Experimental for details).

apparent the relative magnitudes of similarly retained peaks are very different. Also peaks sometimes appear in one chromatogram but not the other. Even though essentially the same material was fractionated in both cases, this is not an unexpected result given the very different selectivities of the detection methods. Clearly there are a number of UV absorbing pigments and compounds that elute in the descending mode (*i.e.*, which has a mobile phase predominantly of water

plus acetic acid) that are completely unassociated with [^{14}C]metolachlor or its degradation products. It seems that there may be a higher correlation between the UV peaks and those of the radiochromatogram in the ascending mode (Figures 2A and 2B). However, it appears that the radiochromatogram, like the UV chromatogram is made up of a large number of overlapping peaks.

Figure 2C shows the elution order of 13 known metolachlor metabolites when separated by CPC using conditions identical to those employed for the corn plant extract. Only the two amine compounds are eluted in the descending mode. Compound 25702 has no corresponding peak in either the UV chromatogram or the radiochromatogram. Interestingly, a major portion of both the UV absorbance and radioactivity in the descending mode chromatogram does not correspond to any of the tested metolachlor degradation products.

Eleven of the thirteen metolachlor metabolite standards elute in the ascending mode. The retention times of the standards correspond to many of the peaks seen in the radiochromatogram and UV chromatogram. The largest peak in the radiochromatogram has a maximum at about 130 minutes. It is broad, and has shoulders indicating that it includes several compounds. Two metolachlor metabolites (CGA-51202 and CGA-40919) elute at about this time. Both compounds result from the dehalogenation of metolachlor (Figure 1), which seems to be an important early step in its biodegradation. Clearly in the ascending mode there are similarities between the retention of the metabolite standards (Figure 2C) and the various maxima in the radiochromatogram (Figure 2B). This was not the case in the descending mode.

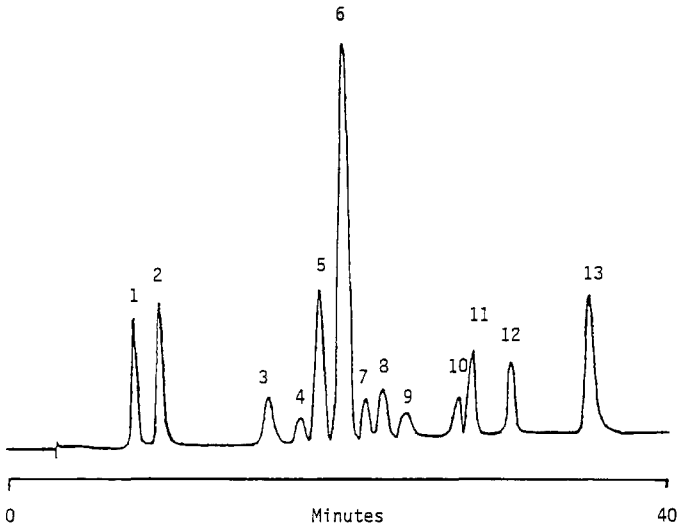


Figure 3. HPLC chromatogram of non-radiolabeled metolachlor metabolite standards on the dimethylphenyl-β-cyclodextrin stationary phase. A background subtraction approach was used along with gradient elution (see Experimental for details). The peaks are as follows: 1. CGA-37735, 2. CGA-50720, 3. CGA-13656, 4. CGA-51202, 5. C-25702, 6. CGA-37913, 7. CGA-118243, 8. CGA-40919, 9. CGA-46576, 10.CGA-40172, 11. CGA-110186, 12. CGA-41507, and 13. CGA-46127.

Gradient HPLC Separation of Metabolite Standards

Currently there are few effective analytical methods for chromatographic separation of the 13 metolachlor metabolites shown in Figure 1. It was found that these metabolite standards could be separated by HPLC on a dimethylphenyl-β-cyclodextrin (DMP-β-CD) stationary phase in the reversed phase mode using gradient elution. The results are shown in Figure 3. Interestingly, the elution order was found to be different than reported on a conventional C_{18} stationary

phase (*i.e.* CGA-50720 < CGA-37735 < CGA-37913 < CGA-51202 < CGA-118243 < CGA-46576 < C-25702 < CGA-13656 < CGA-40919 < CGA-40172 < CGA-110186 < CGA-41507 < CGA-46127) (10). This selectivity difference occurs because the DMP-β-CD stationary phase interacts with solutes via a combination of hydrophobic, hydrogen-bonding and π-π interactions. In the reversed phase mode, the hydrophobic character of DMP-β-CD stationary phase is known to be the result of the dimethylphenyl substituents as well as the interior of the cyclodextrin cavity. pH plays an important role in controlling both the retention and selectivity of the reversed phase separation on the DMP-β-CD stationary phase, just it does with a C_{18} column. It should be noted that the separation of metabolite standards on a DMP-β-CD phase is complete in 40 min. This is approximately twice as fast as the analogous separation on a C_{18} column (10). When running a gradient a broad "solvent-hump" appears over which the peaks are superimposed. This is eliminated with a baseline subtraction's method that is available with most HPLC instruments.

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HPLC CHIRAL OPTIMIZATION OF A UNIQUE β-AMINO ACID AND ITS ESTER

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ABSTRACT

Enantiomeric optimization of a unique ß-amino acid (ß-amino-3-pyridylpropionic acid) and its ethyl ester was achieved with a Daicel Crownpak CR (+) column requiring no derivatization. Using this column, the pH of the mobile phase was adjusted with perchloric acid and studied to determine the optimum pH. A plot of the capacity factor as a function of pH for the amines showed the critical pH required for chiral recognition. From this data, the retention of the amines shift from a hydrophobic non-enantioselective retention mechanism (pH > 2) to complex formation between the crown ether and the amines causing enantioselectivity (pH ≤ 2). At pH 1, lowering the column temperature from ambient to sub-ambient temperatures caused increased retention and enantiomeric separation of the amines. At pH 1 and a column temperature of ~7°C, concurrent enantiomeric baseline resolution (R_s > 1.5) of the ß-amino acid and ester was achieved. The addition of methanol under these conditions caused a decrease in retention and enantiomeric separation of the amines, especially for the ß-amino acid enantiomers.

INTRODUCTION

Currently, the development of chiral drugs has received more attention due to the Food & Drug Administration's (FDA) policy statement for the development of new chiral drugs [1]. Essentially, the policy allows the drug manufacturer to market the chiral drug as either a racemate or a single enantiomer. Interpretation of the policy will depend largely on individual FDA reviewers considering particular cases. However, it is clear from the policy that development of a racemate will require rigorous pharmacological, toxicological, and pharmacokinetic justification for FDA approval. This has prompted many drug manufacturers to develop single enantiomers or avoid asymmetric drugs altogether. While the latter is not always desirable, the former is becoming more feasible due to enantioselective syntheses and chromatographic techniques able to resolve enantiomers on the analytical, as well as the preparative scale. Thus, development of single enantiomers is becoming more prevalent in the drug industry.

Development of single enantiomers of unique ß-amino acids and their esters is on-going at Searle. These compounds are key intermediates in the synthesis of potent anti-platelet/anti-coagulant drugs. Several papers have been published on the enantiomeric separation of amino acids. The most utilized techniques have been pre-column derivatization with a chiral reagent followed by high-performance liquid chromatography

(HPLC) analysis [2-8], chiral ligand-exchange chromatography [9-13], and more recently, direct analysis by HPLC using chiral crown ethers as the stationary phase [14-22]. For an efficient enantiomeric separation, the latter's distinct advantage is no derivatization or special mobile phase requirements. This paper describes the utilization of a chiral crown ether stationary phase made by Daicel (Crownpak CR (+)) for the enantiomeric separation of a unique ß-amino acid and its ester. The effect of pH, temperature, and organic mobile phase modifier was investigated.

MATERIALS AND METHODS

Reagents and materials

ß-Amino-3-pyridylpropionic acid and ethyl ß-amino-3-pyridylpropionic ester were synthesized by the Discovery Department of Searle Research and Development. Both compounds were synthesized as hydrochloride salts. Mobile phase and diluting solvent constituents were obtained from the following vendors: Millipore Milli-Q water; methanol, Burdick & Jackson (Muskegon, MI, USA); perchloric acid, 70% A.C.S. reagent, Aldrich (Milwaukee, WI, USA).

Apparatus

The chromatographic system used for the development of the method consisted of the following: a Varian Model 2010

HPLC pump (Walnut Creek, CA, USA), a Varian Model 9090 autosampler, and a Kratos Model 783 UV detector (Foster City, CA, USA). The column was a Daicel Crownpak CR (+) (150 mm x 4 mm I.D., 5 μm particle size) obtained from Regis Chemical Company (Morton Grove, IL, USA) and cooled using a Jones Model 7950 temperature controller (Lakewood, CO, USA). The temperature of the column was measured with a Barnant 90 Model 600 type K thermocouple obtained from Barnant Company (Barrington, IL, USA). Chromatographic measurements (i.e., retention times, peak areas, etc.) were made with an in-house chromatographic data management system.

Chromatographic conditions

The mobile phase was prepared by adjusting the pH of the Milli-Q water to 1 using 70% perchloric acid and degassed for 5 min before use. Other pertinent HPLC parameters were as follows: column, Daicel Crownpak CR (+), 5 μm particle size; flow rate, 0.5 mL/min; injection volume, 10 μL; column temperature, ~7°C; detection, UV at 210 nm; total run time, 20 min.

Sample preparation

The ß-amino acid and ester samples were prepared by dissolving the compounds in Milli-Q water. Concentrations of approximately 1 mg/mL were used.

RESULTS AND DISCUSSION

Structural features

The absolute configuration of the ß-amino acid and its ethyl ester is shown in Figure 1. The synthesis of these compounds was presented elsewhere [23]. The structures show that these amines are chromophoric and therefore UV active. In addition, they have a free amine adjacent to the chiral center. These combined structural features make them ideal candidates for HPLC enantiomeric separation on a chiral crown ether column using UV detection.

Effect of pH

For chiral recognition to be achieved with the Daicel Crownpak CR (+) column, an acidic mobile phase was required for complex formation between the crown ether and the ammonium ion derived from the amine function. Perchloric acid was used to adjust the pH of the mobile phase owing to

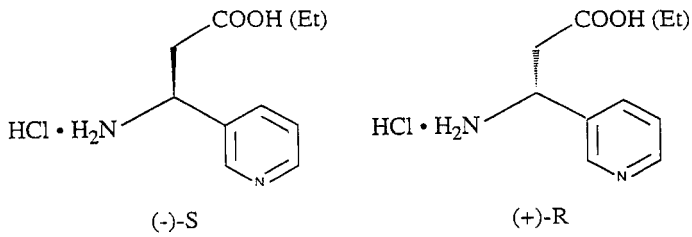

Figure 1. The absolute configurations of (+)-R-, (-)-S-ß-amino-3-pyridylpropionic acid and its ester.

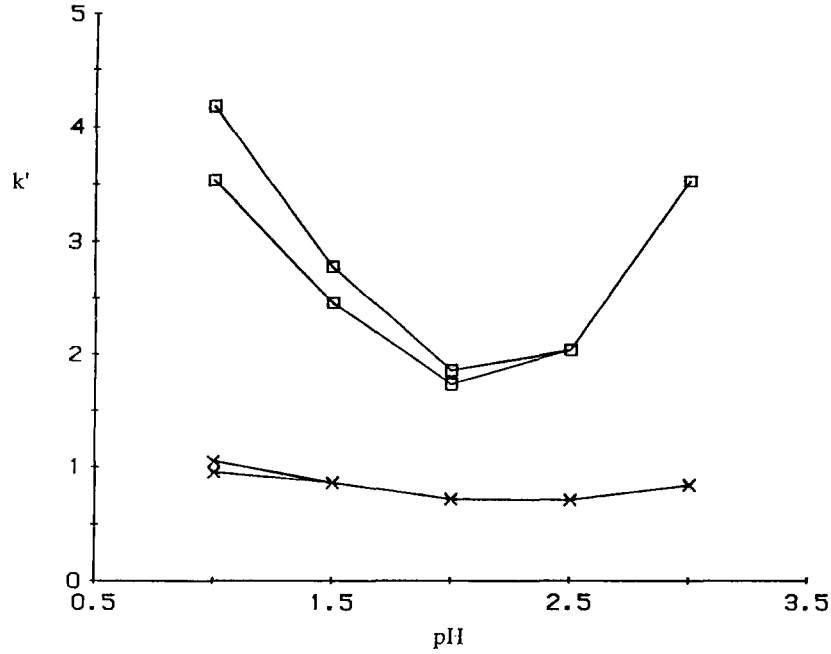

Figure 2. Capacity factor (k') as a function of pH for ß-amino acid (x) and its ester (□). The longest retained enantiomer being (-)-S.

its low UV-absorption. A plot of the capacity factor as a function of pH for the amines is shown in Figure 2. As the pH is decreased, retention of the amines decreased initially, with no evidence of chiral recognition. However, at pH 2, the ester enantiomers begin to resolve whereas the acid enantiomers do not. Below pH 2, the ester enantiomers are fully resolved. Only at pH 1 do the acid enantiomers show chiral recognition. Plotting the resolution (R_s) as a function of pH for these amines (refer to Figure 3) shows that at pH 1.5 the ester

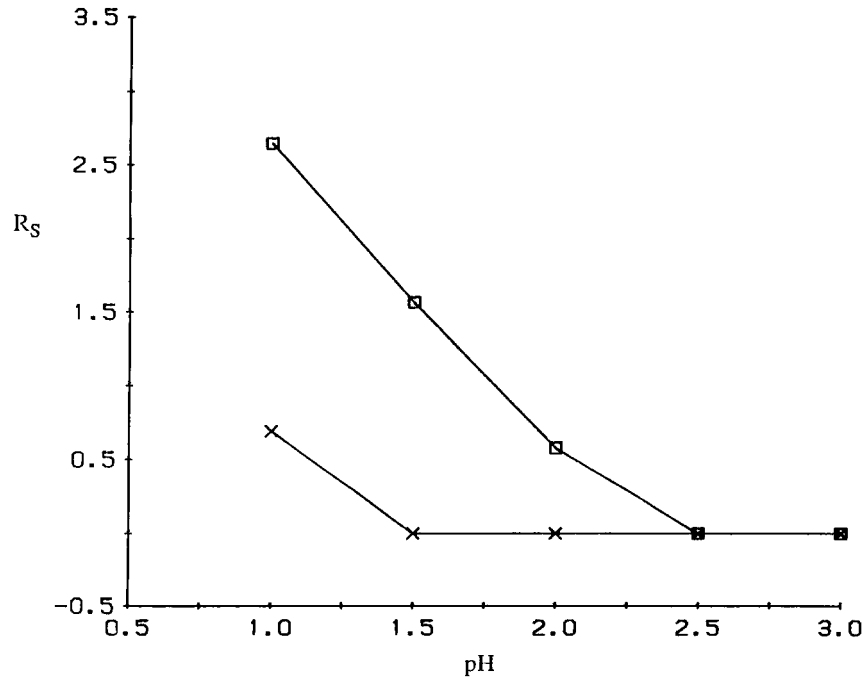

Figure 3. Resolution (R_s) as a function of pH for ß-amino acid and its ester. Symbols and enantiomeric elution same as in Figure 2.

enantiomers are baseline resolved, whereas the acid enantiomers show only partial resolution at pH 1.

This pH study illustrates the mechanism of the enantioselective retention of these amines. Shinbo et al. [20] reported that on a crown ether column, the amine is distributed between the mobile phase and stationary phase predominately by the following two reactions:

$$A^+ + X^- \rightleftharpoons [A^+X^-]_{sp} \quad (1)$$

$$A^+ + C_{sp} + X^- \rightleftharpoons [(AC)^+X^-]_{sp} \quad (2)$$

where A^+ is the protonated amine, X^- is the anion (perchlorate) in the mobile phase, C_{sp} is the crown ether, and $(AC)^+$ is the complex between the crown ether and the amine. The sp subscript indicates the stationary phase. Reaction (1) is non-enantioselective and contributes to the overall retention of the amines mainly through hydrophobic interactions. Reaction (2) determines the retention of the amines due to enantioselective recognition. Figure 2 illustrates these competing reactions. At pH 3, both the acid and ester show no enantiomeric resolution. The retention is determined mainly by reaction (1). Lowering the pH to 2.5 results in a significant decrease in retention for the ester and a slight decrease for the acid; however, there is still no chiral recognition. At pH 2, reaction (2) has begun to dominate over reaction (1), leading to chiral recognition for the ester enantiomers. The acid enantiomers are still unresolved at this pH. Continued lowering of the pH to 1.5 increases the chiral recognition of the ester enantiomers demonstrating that reaction (2) is clearly dominating. Finally at pH 1, the acid enantiomers are partially resolved. Since the manufacturer of the crown ether column does not recommend going below pH 1 due to column instability, the maximum separation allowed by pH was achieved at pH 1.

Effect of temperature

A pH of 1 provided adequate enantiomeric resolution of the ester enantiomers, but only partial resolution of the acid

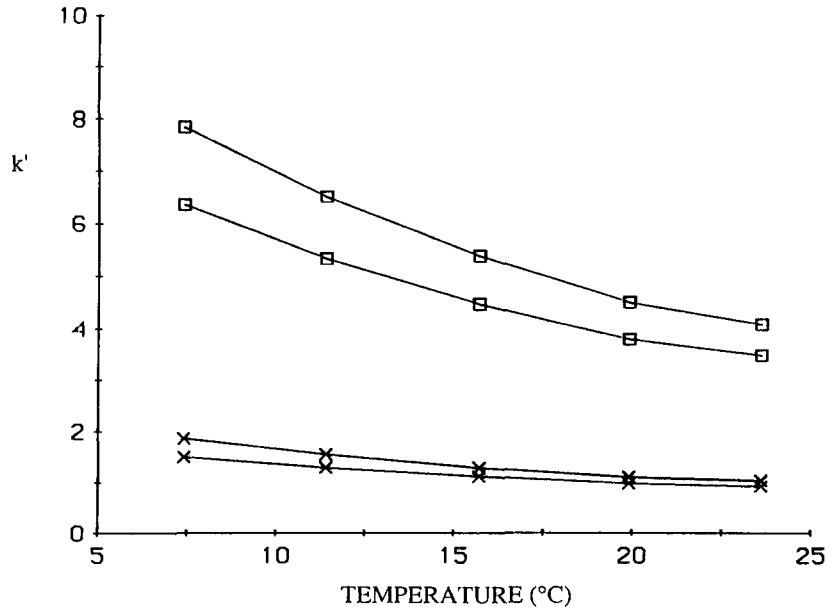

Figure 4. Capacity factor (k') as a function of temperature for ß-amino acid and its ester. Symbols and enantiomeric elution same as in Figure 2.

enantiomers. It had been reported [14,17,20,21] that lowering the temperature of a crown ether column leads to increased enantioselectivity. A plot of the capacity factor as a function of the temperature for the amines is shown in Figure 4. As the temperature decreased, the enantiomeric resolution increased for both the acid and ester enantiomers. Figure 5 is a plot of R_s as a function of temperature for the amines. Lowering the temperature dramatically affected the enantiomeric resolution of the acid. Resolution of the acid enantiomers more than doubled when going from ambient (R_s = 0.8) to approximately

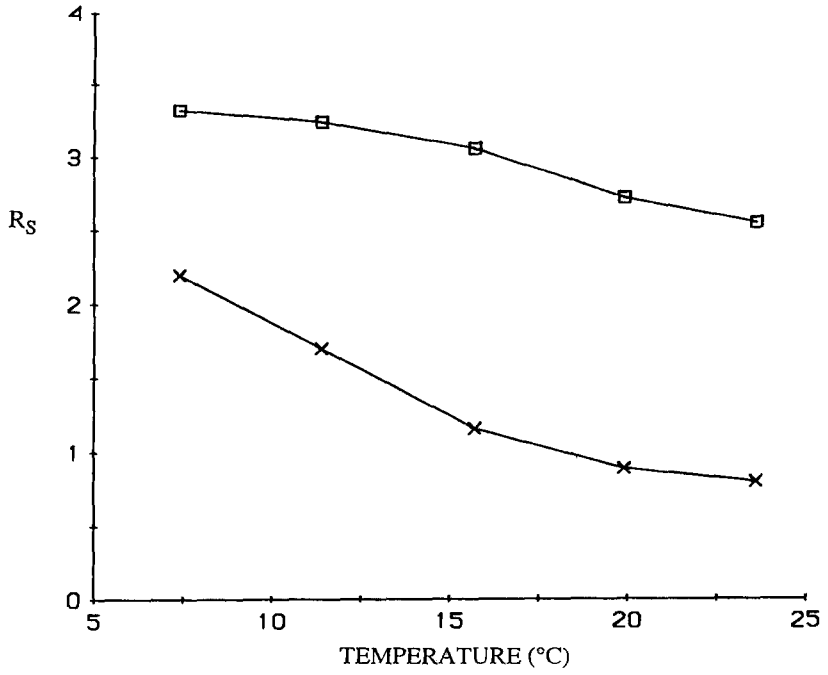

Figure 5. Resolution (R_s) as a function of temperature for ß-amino acid and its ester. Symbols and enantiomeric elution same as in Figure 2.

7°C (R_s = 2.2). The enantiomeric resolution increased slightly for the ester. Optimum HPLC enantiomeric resolution for the acid and ester enantiomers is illustrated in Figure 6 at pH 1 and a column temperature of ~7°C. The elution order of the enantiomers was confirmed by injection of the single enantiomers. The (+)-R-enantiomers eluted first for both the ß-amino acid and ester. This was consistent with the manufacturer's published report regarding elution order on the Crownpak CR (+) column [24]. Assuming a complex

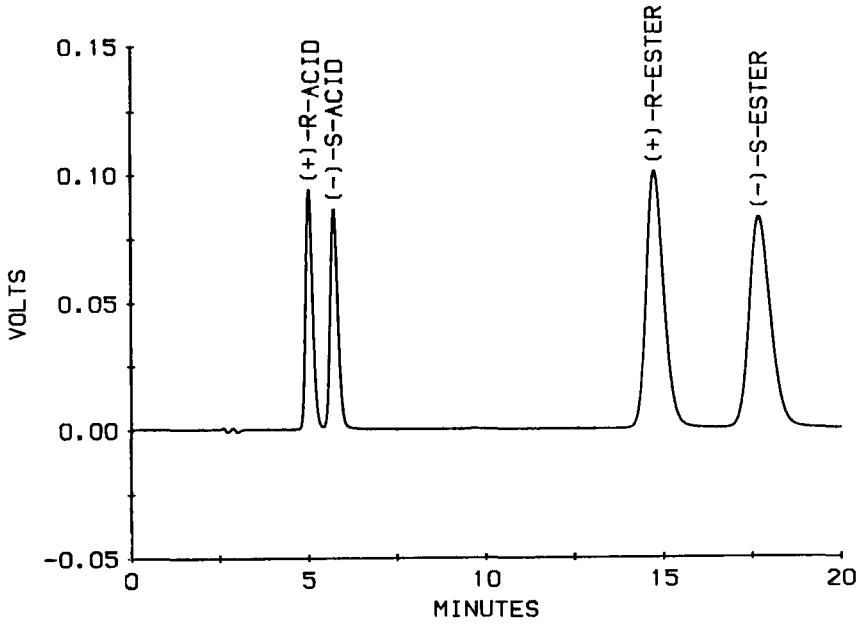

Figure 6. Optimum HPLC chiral separation of ß-amino acid and its ester. See Materials and Methods section for chromatographic conditions.

TABLE 1

Free-Energy Differences for the ß-Amino Acid and its Ester at Various Temperatures

	Δ(ΔG°), cal/mol	
Temperature, °C	Acid	Ester
23.6	-67	-93
19.9	-71	-101
15.7	-80	-109
11.4	-103	-112
7.4	-115	-115

stoichiometry of 1:1 between the crown ether and the amines, the free-energy difference can be calculated according to the following general equation [18]:

$$\Delta(\Delta G^\circ) = -RT\ln\alpha \quad (3)$$

where α is the selectivity coefficient. The free-energy differences for the ß-amino acid and ester at various temperatures are given in Table 1. The temperature dependence of $\Delta(\Delta G^\circ)$ indicates that the stability of the complex with the (-)-S-enantiomer depends to a large extent on the enthalpy term, whereas the less stable complex (i.e., (+)-R- enantiomer) is more determined by the entropy term [25]. In addition, when going from ambient to ~7°C, the acid undergoes a larger absolute change in its free-energy differences than the ester. This indicates that the stability of the crown ether-amine complex for the acid enantiomers is more dependent on the enthalpy term than the ester enantiomers. Thus, sub-ambient temperatures were required for baseline resolution of the acid enantiomers.

Effect of organic modifier

The effect of organic modifier (i.e., methanol) was also investigated. A plot of the capacity factor as a function of percent methanol for the amines is shown in Figure 7. As the percent methanol increased, a significant decrease in retention for the ester enantiomers and a slight decrease for

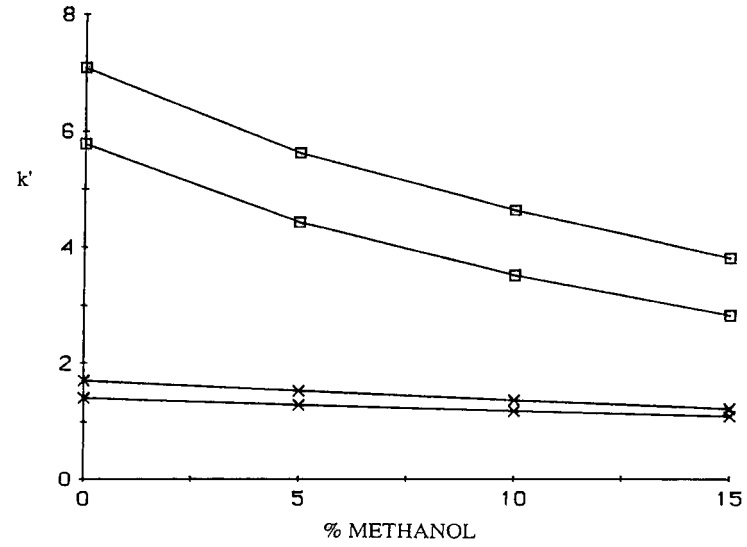

Figure 7. Capacity factor (k') as a function of percent methanol for ß-amino acid and its ester. Symbols and enantiomeric elution same as in Figure 2.

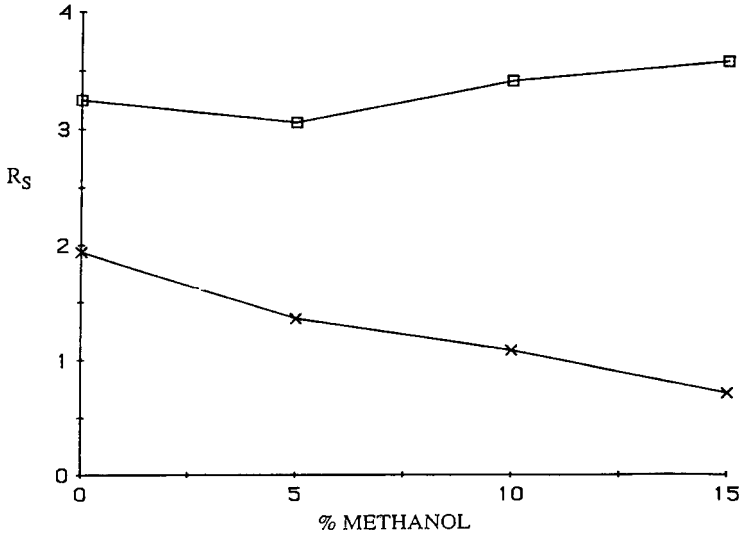

Figure 8. Resolution (R_s) as a function of percent methanol for ß-amino acid and its ester. Symbols and enantiomeric elution same as in Figure 2.

the acid enantiomers was obtained. Although resolution slightly increased for the ester enantiomers, a large loss in resolution for the acid enantiomers was observed as shown in Figure 8. Therefore, methanol was not added to the final method.

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AN INVESTIGATION CONCERNING THE STABILITY OF A STERICALLY PROTECTED CYANOPROPYL MODIFIED SILICA SUBSTRATE

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ABSTRACT

The hydrolytic stability of a sterically protected cyanopropyl modified silica phase, used in reversed-phase mode, has been studied. This Zorbax SB-CN stationary phase shows superior hydrolytic stability in comparison with conventional CN stationary phases against intensive flushing with aggressive eluents of high and low pH, plain and also with the addition of an organic modifier. In addition to chromatography and elemental analysis, solid-state NMR was also used to characterize the changes in the properties of these phases. Moreover, the effect of sample volume and nature of the sample solvent on overloading of the SB-CN columns was studied. Volume overloading was already observed, when 10 μl of the sample solved in pure organic modifier was injected on the column. On the other hand, if the eluent was used as the sample solvent, no significant loss of column performance could be observed, at least up to 50 μl of sample injections.

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INTRODUCTION

The most wide-spread form of reversed-phase high-performance liquid chromatography (RP-HPLC) employs stationary phases containing relatively long alkyl chains (e.g. C_8 and C_{18}). However, for some applications the use of less hydrophobic packings that do not require strong RP-eluents can be profitable. Reversed-phase separations with cyano-bonded (CN) phases potentially may have a large number of application areas, e.g., RP-HPLC analysis of proteins and peptides, have been reported [1]. One of the reasons why CN-bonded phases have not found general use up till now is the poor reproducibility of the synthesis of the stationary phases and the subsequent problems with their (in)stabilities. These latter problems are often due to a rapid loss of ligands from the silica support, especially when working with low pH mobile phases [2,3].

Recently, a new way to protect the siloxane bonds connecting the ligands to the silica surface from hydrolysis was reported [3]. The development of these sterically protected silica-based stationary phases have made it possible to obtain reproducible stationary phases with improved stability against ligand hydrolysis. For these sterically protected phases the siloxane bonds between the ligand and the silica support are sterically protected from hydrolysis by bulky side groups present on the ligand chain. Monofunctional silanes containing two isopropyl side groups instead of the conventional methyl groups were found to produce very stable packings [3].

An additional advantage of cyano-bonded silica columns is that they can be used both in the normal and the reversed-phase mode. In the normal-phase mode, the cyano column acts essentially as a deactivated silica surface resulting in the interaction of analytes with the residual silanols [4]. However, the selectivity is markedly different from that of silica. If the polarity of the solvent

in the normal phase mode is increased, then the effect of the silanols is suppressed leaving the cyano groups as the principal adsorption sites [5]. In the reversed-phase mode, the cyano column acts like a short chain alkyl silica due to the hydrophobic spacer and the bulky side groups [6]. The primary hydrophobic interaction of the analyte with the bonded phase is through the isopropyl side groups whose concentration determines strongly the hydrophobicity of the surface. The residual silanols also play a key role in the overall retention properties of these phases.

The hydrolytic stability of the sterically protected cyanopropyl modified silica, used in the reversed-phase mode, is the issue of this study. The aging effect of several eluents on the process of stationary phase deterioration was studied. Four buffering eluents of high and low pH, totally aqueous and with the addition of methanol were recirculated continuously in a closed system through the columns. Before and after these aging experiments, chromatographic tests, elemental analysis and solid-state NMR were used to characterize changes in the properties of the phases. Finally, the stabilities of these sterically protected stationary phases were compared with those of conventional CN stationary phases. It is emphasized here that by eluent recirculation further deterioration of the column may be suppressed by saturation of the eluents with ligands and/or silica material. The results from these aging experiments should, therefore, be considered as the minimal change in the properties of the stationary phase that may occur during the laboratory use of these phases in HPLC practice.

Many workers have observed that the strength of the sample solvent relative to that of the RP-HPLC mobile phase may have considerable effects on the peak shapes and peak heights of the solutes [7-12]. Peak broadening, peak distortion and multiplication of peaks has been reported when the sample solution was significantly stronger than that of the mobile phase [9-11]. Similar effects

were observed during the stability study of the sterically protected stationary phase investigated here. Therefore, the effect of sample volume and the nature of the sample solvent on the possible overloading of these columns were also studied to determine the optimal conditions for the chromatographic characterization before and after the aging experiments.

EXPERIMENTAL

Chemicals and materials

Five identical sterically protected cyanopropyl modified silica columns, Zorbax SB-CN (150 mm x 4.6 mm I.D.; Rockland Technologies, Inc., Newport, DE, USA) were investigated in this study. One column was used as a reference column, while the other four columns were subjected to artificial aging procedures. The artificial aging experiments consisted of a continuous exposure of the columns separately to four different aggressive eluents. These eluents are listed in Table I. For economic and also practical reasons, these eluents were recirculated continuously in a closed system at a flow rate of 0.5 ml/min for a

Table I. Eluent compositions used for the aging experiments.

Aging exper. no.	Buffering eluent	pH	Volume fraction of methanol in eluent
1	0.05 M Phosphate	2.2	0
2	0.05 M Phosphate	2.2	0.5
3	0.05 M Phosphate	8.0	0
4	0.05 M Phosphate	8.0	0.5

period of ten days (240 hours). Hence, the columns were flushed with about 7200 column volumes of a specific eluent. As was already mentioned, possible saturation of the eluent with dissolved silica and/or ligands, may reduce the aging effect of these experiments compared to realistic laboratory practice. After finishing a typical series of aging experiments, the columns were carefully rinsed sequentially with water, water-methanol mixtures and methanol. In this way, deposition of the buffering salts in the columns was prevented. In the next step, the columns were subjected to chromatographic RP-HPLC test procedures with three test mixtures at suitable eluent compositions. Finally, the contents of the columns were used for NMR experiments and elemental analysis.

Three mixtures of test components were used for the chromatographic characterization of the stationary phases. The test components were all of reference grade. Test mixture 1 consisted of benzene, methylbenzene, ethylbenzene, n-propylbenzene and n-butylbenzene (Aldrich Chemie, Germany). Test mixture 2 consisted of methyl-p-hydroxybenzoate, ethyl-p-hydroxybenzoate, n-propyl-p-hydroxybenzoate and n-butyl-p-hydroxybenzoate (Sigma Chemical Corporation, St. Louis, MO, USA). Test mixture 3 consisted of 2-n-pentylpyridine, 2-n-hexylpyridine, 2-n-heptylpyridine and 2-n-octylpyridine (Merck, Darmstadt, Germany). All test components were dissolved in the eluent. The injection volume ranged from 2 to 50 μl and the injected amount of sample was in all cases about $6*10^{-7}$ g.

For the aging experiments and chromatographic characterizations, solvents and chemicals of analytical reagent grade (Merck) were used. For the preparation of the buffers, deionized water was used. The water was prepared by a Milli-Q water purification system (Millipore, Bedford, MA, USA) prior to use. All eluents were freshly prepared, filtered over 0.22 μm membrane filters (Millipore) and degassed in an ultrasonic bath prior to use.

INSTRUMENTATION AND PROCEDURES

Chromatography

The chromatographic experiments were performed with a model PU 4100 liquid chromatograph pump (Unicam, Cambridge, UK). Furthermore, a Marathon autosampler equipped with a 20 μl sample loop (Spark Holland, Emmen, The Netherlands) and a model PU 4110 UV/VIS detector (Unicam) operated at 254 nm were used. A Nelson Model 760 interface and a Nelson 3000 data system (Nelson Analytical, Cupertino, CA, USA) was applied for data acquisition and subsequent data handling.

For the aging experiments, an instrument was used with an eight-headed metering pump (Metering Pumps, London, UK). Each of these pumps can be purged separately with a specific eluent. The pumping system was provided with a home-made pulse damping system, which includes a pulse damper (Waters, Millipore), a 3 m x 0.1 mm I.D. capillary and a permanent 0.2 μm filter coupled in series. This system ensured a constant flow through the columns during the aging experiments.

Elemental Analysis

The carbon, hydrogen and nitrogen content of the original and the aged stationary phases was obtained with a Perkin Elmer Analyzer, Model 240 (Perkin Elmer Corp., Norwalk, CT, U.S.A.). Tungsten oxide was added to the packings as a catalyst for the analysis. The carbon and hydrogen contents (P_C and P_H, respectively) and the ligand surface density (α_L) of the stationary phases are given in Table II. The repeatability of these analyses was 0.3%.

From the values of the carbon content of the bulk phase and the specific surface area of the substrate, the average ligand density was calculated with an equation derived by Berendsen [13]:

Table II. A summary of carbon and hydrogen contents (P_C and P_H) and ligand surface densities (α_L) of the aged columns 1-4 and the reference column. The repeatability of the analysis is 0.3%.

packing	P_C % (w/w)	P_H % (w/w)	α_L (μmol.m^{-2})
1	4.35	0.91	2.15
2	4.06	0.83	2.0
3	3.93	0.84	1.93
4	3.88	0.82	1.91
Reference	4.29	0.82	2.12

$$\alpha_L = \frac{P_C}{S_{BET}\,(M_C - P_C\,(M_L - 1))} \qquad (1)$$

where

α_L = ligand surface density (mol.m^{-2})

P_C = the amount of carbon (g/g)

S_{BET} = specific BET surface area of the substrate (m^2.g^{-1})

M_C = the amount of carbon per mol bonded silane (g.mol^{-1})

M_L = molecular weight of the silane molecule (g.mol^{-1})

Solid state ^{29}Si NMR measurements

The solid state ^{29}Si NMR spectra were obtained on a Bruker MSL-400 Fourier Transform NMR spectrometer at 79.49 MHz. Aluminium oxide rotors (7 mm O.D.) of the standard Bruker double bearing type were filled with representative samples of ca. 250 mg of packing and were spun at about 2.5 kHz.

^{29}Si cross polarization magic angle spinning (CP MAS) NMR spectra of all stationary phases were obtained with a cross polarization contact time of 6 ms. The pulse interval was 1 s. Typically 2000 FIDs (Free induction decay) with an acquisition time of 10 ms were accumulated in 1K datapoints and zero-filled to 8K prior to Fourier transformation. A line broadening of 20 Hz was used prior to zero-filling and Fourier transformation. The spectral width for all spectra was 20 kHz.

RESULTS AND DISCUSSION

Results of the aging process

Elemental Analysis

The carbon and hydrogen contents of the reference and of the aged packings 1 - 4 are summarized in Table II. The loss of carbon observed for all aged packings was relatively low and did not exceed 10%. Aging experiment 1 did not result in a loss of the bonded phase, while a slight loss of bonded phase was observed for the other aging experiments in the order 4>3>2 of column number.

Chromatography

The effect of long-term exposure of the SB-CN columns to aggressive eluents on their chromatographic properties is illustrated in the Figures 1, 2 and 3. These figures depict the chromatograms of the test mixtures 1, 2 and 3, respectively, eluted on the columns before and after the aging experiments. The chromatogram indicated as "reference" represents the reference column, while the other chromatograms represent the aged columns; chromatograms 1 - 4. The numbers on the right side of the chromatograms correspond to the number of the aging experiment as listed in Table I. In addition to that a number of

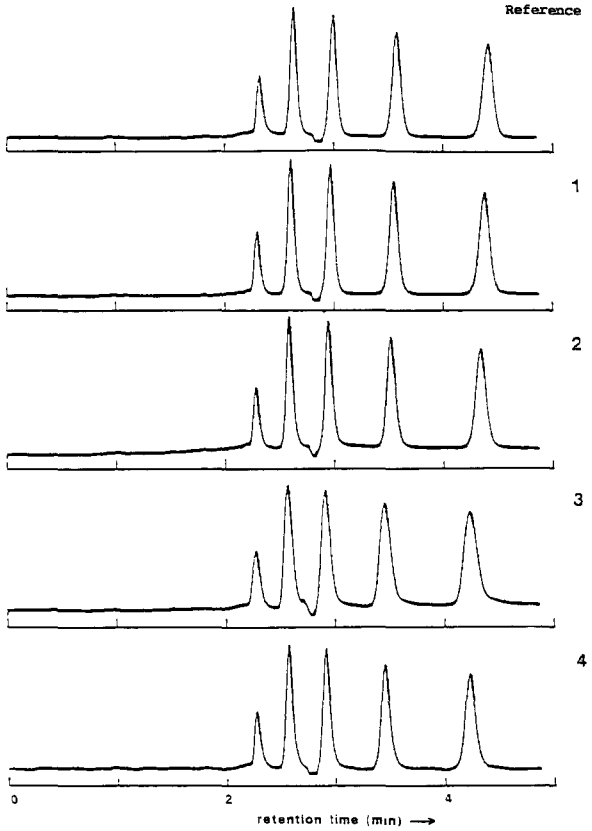

Figure 1.
Chromatograms of the n-alkylbenzenes test mixture eluted on the Zorbax SB-CN stationary phases before (chromatogram indicated as "reference") and after the aging experiments, chromatograms 1-4. The numbers on the right side of the chromatograms correspond to the number of the aging experiment as outlined in Table I.
Chromatographic test conditions: mobile phase: methanol-water (60/40, v/v); flow rate: 1.0 ml/min; detection: UV at 254 nm, 0.01 AUFS; sample volume: 20 μl; sample solvent: methanol-water (60/40, v/v); injected amount: $6*10^{-7}$ g.

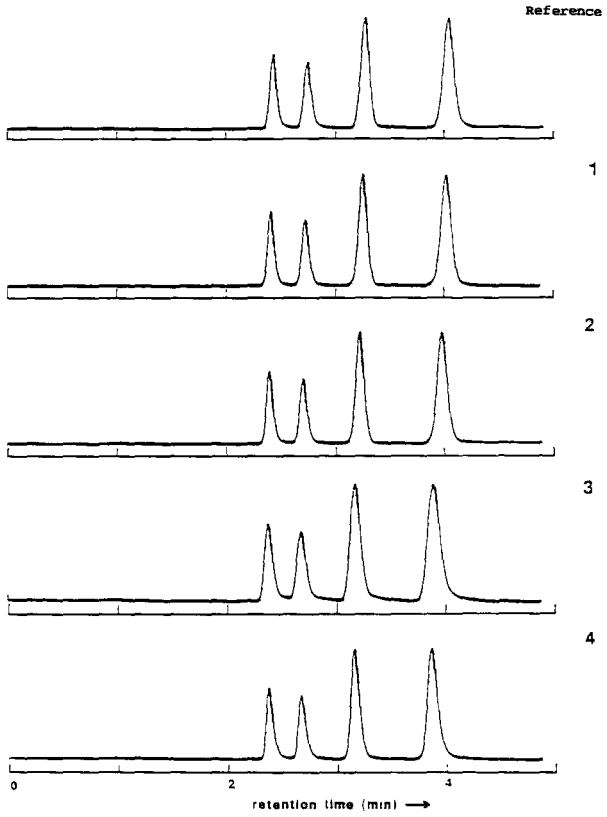

Figure 2.
Chromatograms of the n-alkyl esters of p-hydroxybenzoic acid test mixture eluted on the Zorbax SB-CN stationary phase before (chromatogram indicated as "reference") and after the aging experiments, chromatograms 1-4. The numbers on the right side of the chromatograms correspond to the numbers of the aging experiments as outlined in Table I.
Chromatographic test conditions: mobile phase: methanol-water (55/45, v/v); flow rate: 1.0 ml/min; detection: UV at 254 nm, 0.01 AUFS; sample volume: 20 μl; sample solvent: methanol-water (55/45, v/v); injected amount: $6*10^{-7}$ g.

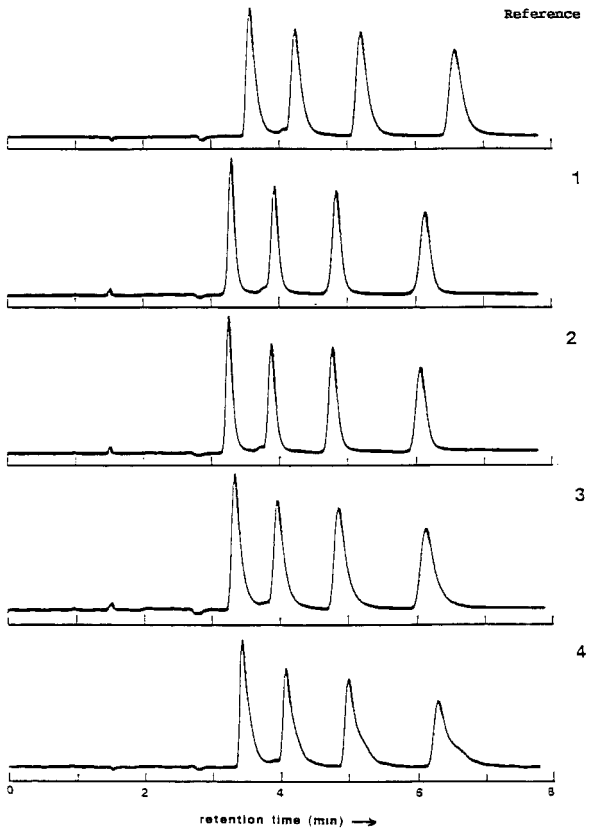

Figure 3.
Chromatograms of the n-alkylpyridines test mixture eluted on the Zorbax SB-CN stationary phase before (chromatogram indicated as "reference") and after the aging experiments, chromatograms 1-4. The numbers on the right side of the chromatograms correspond to the numbers of the aging experiments as outlined in Table I. Chromatographic test conditions as in Figure 1.

Table III. Summary of the capacity factors (k') of n-heptylbenzene, selectivity (α) between ethylbenzene and n-heptylbenzene, plate numbers (N) and asymmetry factors (A_S) both determined for n-heptylbenzene. The chromatographic measurements were performed on aged columns 1-4 and on the reference column. The plate numbers were calculated taking into account the asymmetry factor measured at 10% of the total peak height [14].

column	k'	α	N	A_S
1	5.8	5.34	6800	1.0
2	5.8	5.36	6420	0.97
3	5.7	5.09	3930	1.14
4	5.8	5.0	5520	0.99
Reference	5.6	5.54	5910	1.0

chromatographic data calculated from the chromatograms for ethyl- and heptylbenzene are presented in Table III as a example.

The chromatographic characteristics of the columns did not change much after flushing the columns with the aggressive eluents applied in this study. The retention times of the n-alkylbenzenes and the n-alkyl esters of p-hydroxybenzoic acid homologous series, as well as the selectivity between ethylbenzene and n-heptylbenzene dropped somewhat on the columns 3 and 4 after the aging with alkaline eluents of pH=8. Also a decrease in efficiency was observed for column number 3. These observations of a slight loss of ligands made by chromatography and elemental analysis correspond to the somewhat increased peak asymmetries measured for the n-alkylpyridines for the aging experiments 3 and 4. These latter substances being very sensitive to silanophilic interactions of the stationary phase will respond to the slightly increased amount of silanols due to the loss of ligands. The observed increased asymmetry factors may also be explained by a slight

dissolution of the silica substrate and consequently of a partial collapse of the column bed structure. However, generally spoken, it can be concluded that the aging effect of aggressive eluents on the column performance is low, even if plain aqueous buffers are used as the eluents.

Solid state ^{29}Si NMR measurements

To confirm the observations made by chromatography and by elemental analysis also the reference phase as well as the aged phases were analyzed by NMR spectroscopy. ^{29}Si CP MAS NMR spectra of the reference packing indicated as "reference" and of the aged stationary phases 1-4 are shown in Figure 4 The numbers on the right side of the spectra correspond to the number of the aging experiment, as listed in Table I. Here, too, no significant loss of ligands (signal at 12 ppm) was observed. These observations are in good agreement with the results of the chromatography and with elemental analysis.

The results described above, correspond to those already reported, on the stability of this type of stationary phase [3]. In [3] the decrease in retention on the sterically protected diisopropyl-3-cyanopropyl stationary phase (Zorbax SB-CN) was compared with the decrease for the corresponding conventional dimethyl-3-cyanopropyl phase (Zorbax CN). The stationary phases were subjected to degradation experiments with alternating isocratic runs, followed by gradient elution under water/acetonitrile/TFA conditions. These data demonstrated that the isopropyl protecting groups are very effective in stabilizing the bonded phases and protecting those from hydrolytic deterioration. No measurable loss of the diisopropyl-3-cyanopropyl phase was observed by elemental analysis measurements of carbon, hydrogen and nitrogen content after a 4-day test period. These observations by Kirkland et al. are in good agreement with our results. The test conditions applied in the previous work of Kirkland differ strongly from those used in our study and were performed over a 4-day period instead of a 10-day period in our work. However, as in [3] the SB-CN phases kept their original

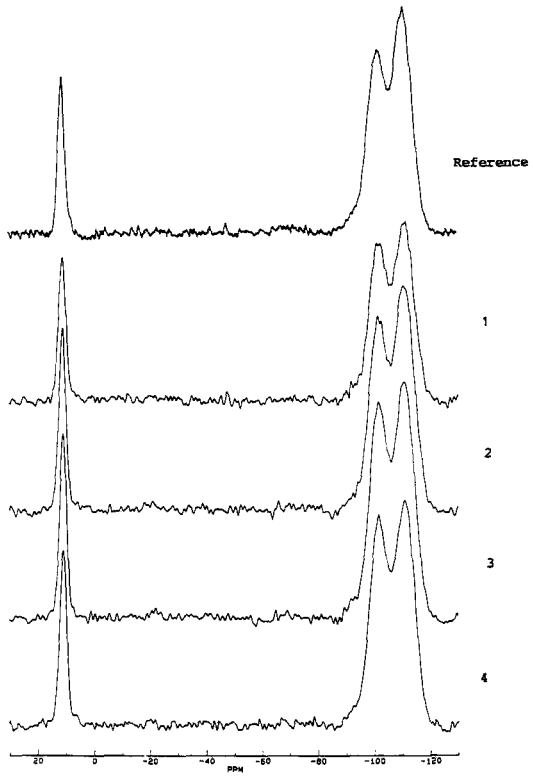

Figure 4.
^{29}Si CP-MAS NMR spectra of the Zorbax SB-CN stationary phases before (spectrum indicated as "reference") and after the aging experiments, spectra 1-4. The numbers on the right side of the chromatograms correspond to the numbers of the aging experiments as outlined in Table I.
Measurement conditions: N_S: 2000; contact-time: 6 ms; pulse interval time: 1 s; acquisition time: 10 ms; line broadening: 20 Hz.

chromatographic behaviour to a large extent, even when the phases were used under aggressive eluent conditions.

Sample injection studies

In order to determine the optimal chromatographic test conditions some SB-CN columns were used for a number of different injection experiments prior to the aging experiments. Test mixture 1, consisting of benzene, methylbenzene, ethylbenzene, n-propylbenzene and n-butylbenzene, was used for these tests. This mixture was dissolved in two different sample solvents consisting of pure methanol or the eluent. By varying the concentration of the different test solutions the absolute injected amount of n-alkylbenzenes on the columns was kept constant at 6.10^{-7} g. In this way, volume overloading could be studied independently of mass overloading. The applied injection volumes ranged from 2 to 50 μl. Plate numbers and asymmetry factors were calculated from the chromatograms.

In Figures 5 and 6 the chromatograms of the n-alkylbenzenes dissolved in pure methanol and in the eluent, respectively, are presented. The numbers on the right side of the chromatograms correspond to the applied injection volume, in μl, of a specific sample solution. From these figures it can be seen, that volume overloading can be already observed, when 10 μl of the sample dissolved in methanol is injected on the column. On the other hand, if eluent was used as the sample solvent, no significant loss of column performance could be observed, even when 50 μl of the sample was injected. A summary of column plate numbers and asymmetry factors, calculated for n-butylbenzene, is presented in Table IV. These data clearly show that a strong decrease in plate number, as well as an increase in asymmetry factor, is already caused by the injection a few μl of test mixture 1 dissolved in methanol. Opposite to that, the column performance is hardly influenced by injection of relatively high volumes of this test mixture dissolved in the eluent. To avoid any interference by this overloading effect during our further study of the Zorbax SB-CN columns, all chromatographic tests

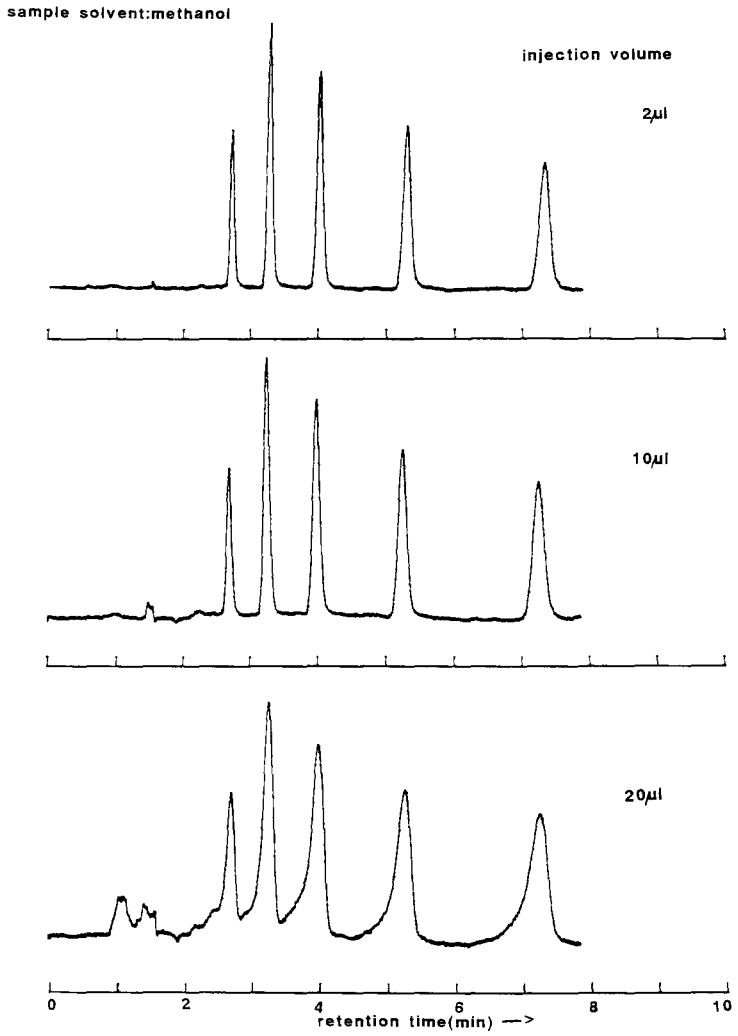

Figure 5.
Chromatograms of the n-alkylbenzenes, dissolved in pure methanol, eluted on the Zorbax SB-CN stationary phase. The numbers on the right side of the chromatograms correspond to the applied injection volume of the solution in μl. Chromatographic test conditions: mobile phase: methanol-water (55/45, v/v); flow rate: 1.0 ml/min; detection: UV at 254 nm, 0.01 AUFS; sample volume: 2, 10 or 20 μl; sample solvent: methanol; injected amount: $6*10^{-7}$ g.

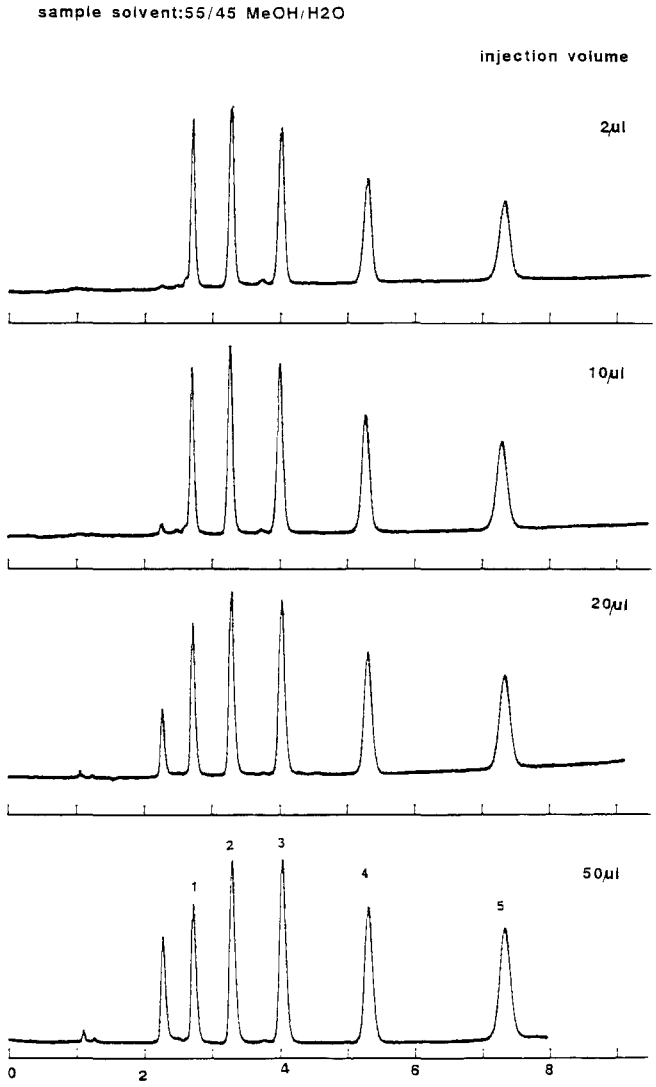

Figure 6.
Chromatograms of the n-alkylbenzenes, dissolved in eluent, eluted on the Zorbax SB-CN stationary phase. The numbers on the right side of the chromatograms correspond to the applied injection volume (in μl) of the solution.
Chromatographic test conditions: eluent: methanol-water 55/45 v/v; flow rate: 1.0 ml/min; detection UV at 254 nm, 0.01 AUFS; sample volumes 2, 10, 20 or 50 μl; sample solvent: methanol-water 55/45 v/v; injected amount of sample $6*10^{-7}$ gr.

Table IV. A summary of plate numbers (N) and peak asymmetry factors (A_S) both calculated for n-butyl benzene. The plate number was calculated taking into account the asymmetry factor measured at 10% of the total peak height [14].

injection volume (μl)	sample solvent: methanol		sample solvent: eluent	
	N	A_S	N	A_S
2	8700	1.05	8300	0.95
10	6800	1.14	8800	0.97
20	2400	0.44	9200	0.95
50	-	-	8600	0.99

in this study were continued with 20 μl sample injections, using the eluent as the sample solvent.

CONCLUSIONS

The Zorbax SB-CN stationary phases investigated in the present study show high stability against intensive flushing with aggressive eluents used in the aging experiments. Only the packings aged with alkaline eluents exhibit a slight loss in retention and selectivity, accompanied by a decrease in column efficiency for the packing aged with plain buffer of pH 8. As a consequence for these packings aged with alkaline eluents, a slight loss of bonded phase is observed by elemental analysis.

In this study it is also shown that the sterically protected cyanopropyl modified silica can be applied very well as a reversed-phase stationary phase.

As also observed for other types of reversed phase packings the volume loadability of the Zorbax SB-CN columns is limited, when pure organic modifier

is used as the sample solvent. In these cases a strong decrease in plate number, as well as an increase in asymmetry factor, was observed after the injection of only a few μl of sample. However, this problem can easily be overcome by dissolving the sample in the eluent. Little change in the performance of these columns was observed in the injection range from 2 to 50 μl, when the sample was dissolved in the eluent.

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COMPARISON OF VARIOUS SORBENTS FOR THE ENRICHMENT OF SAMPLES OF ALIPHATIC AMINES USING SOLID-PHASE EXTRACTION PRIOR TO THE DETERMINATION BY HPLC WITH FLUORIMETRIC DETECTION

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ABSTRACT

Possibilities of the determination of lower aliphatic amines by high performance liquid chromatography (HPLC) with fluorimetric detection after derivatization with o-phthaldialdehyde (OPA) and sample enrichment using solid-phase extraction were investigated. Various materials including octadecyl silica and strong and weak cation exchangers with organic matrices were compared as the sorbents for enrichment of aqueous samples of amines. The best results were achieved using the weak cation exchanger Spheron C 1000 as the sorbent and methanolic perchloric acid as the desorption liquid in the enrichment step. The recovery, linearity and detection limits of the method were determined. The concentration limits of determination of the amines are in the range of nmole.l^{-1}, with the enrichment factor of 240 in the preconcentration step.

INTRODUCTION

Aliphatic amines are organic bases often found in environmental and biological samples. As lower primary and secondary amines can form cancerogenic nitrosamines in presence of nitrites or other nitrosation agents (1), the need for a sensitive analytical method for their determination is obvious.

High performance liquid chromatography (HPLC) is well suited for the analysis of aliphatic amines, but its applications in trace analysis of these compounds are connected with problems originating from the low sensitivity of the detection using standard HPLC methods. Because the aliphatic amines do not absorb significantly in the UV region, it is not possible to use UV detection in their analysis. Fortunately, there is a plethora of derivatization agents making it possible to prepare fluorescent derivatives of aliphatic amines, such as N,N-dimethyl-5-aminonaphthalene-1-sulphochloride (dansylchloride) (2,3,4), N,N-dibutyl-5-aminonaphthalene-1-sulphochloride (bansylchloride) (5), 4-phenylspiro[furan-2-(3H),1'-phthalan]-3,3'-dione (fluorescamine) (2), 1,2-naphtoylbenzimidazole-6-sulphochloride (1,2-NBI-6-SO_2Cl) (6), acridone and acridine derivatives (5), 9-fluorenylmethylformate (9-FMOC) (7), 9-fluorenylmethyl chloroformate (9-FMOC-Cl) (8,9), 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-F and NBD-Cl) (10,11,12), o-carboxybenzoic acid (OPT) (11), 1,2-naphthoquinone-4-sulphonate (13), 3-(2-furoyl)-quinoline-2-carbaldehyde (FQCA) (14), o-phthaldialdehyde (OPA) (2,10,11,15,16) and other. These derivatives make it not only possible to use sensitive fluorimetric detection, but also to increase the selectivity of determination and to suppress the effects of possible interfering compounds present in real samples. Pre-column derivatization often improves

chromatographic behaviour of the amines in common HPLC systems (enhances the selectivity of separation, decreases peak tailing, etc.).

Even with sensitive fluorimetric detection, sample enrichment is often necessary to achieve low detection limits required in trace analysis. Preconcentration of various compounds in aqueous samples is usually accomplished by liquid extraction or by adsorption of sample compounds on a suitable sorbent packed in a small column or in a cartridge. The latter technique, so-called "liquid-solid extraction" (LSE) or "solid-phase extraction" (SPE), has become increasingly popular because it avoids using harmful solvents and tedious manual operations usually connected with liquid-liquid extraction procedures.

Nonpolar sorbents, either organic polymers or alkyl modified silicas, are used most frequently for the enrichment of organic compounds, where the affinity of the nonpolar parts of organic molecules to the nonpolar (usually hydrocarboneous) surface of the adsorbent is the driving force of adsorption, much like in reversed-phase HPLC systems (17,18,19,20). However, compounds with small polar molecules are often only slightly retained by nonpolar sorbents. Ionized or ionizable compounds can be pre-concentrated on the columns or cartridges packed with ion exchangers. For example, phenoxyacid herbicides and other carboxylic acids in water can be enriched on anion exchangers (21,22).

Styrene-divinylbenzene cation exchange resins have been used both for off-line (23,24) and on-line (25) preconcentration of amines in aqueous samples prior to their chromatographic analysis. In addition to ion exchangers, unmodified silica was also used for enrichment of amines in aqueous samples (26) and in the air (27), followed by in-situ derivatization and HPLC analysis.

The main objective of the present work was to compare octadecyl silica and various cation exchangers with hydrophilic matrices as potential sorbents for sample enrichment of aliphatic amines in aqueous samples prior to the derivatization and separation by HPLC with fluorimetric detection.

MATERIALS AND METHODS

Apparatus

HPLC was performed using either an HP 1090M liquid chromatograph equiped with an HP 1046A fluorimetric detector and an HP 9000/310 data station (Hewlett-Packard, Avondale, PA, U.S.A.) or a liquid chromatograph comprised of an HP 1046A fluorimetric detector and an HPP 4001 high-pressure pump, an LCI 30 manual sample injector with a 20 µl sample loop, a CI 100 integrator and a TZ 4221 line recorder (all from Laboratory Instrument Works, Prague, Czech Republic). A glass cartridge column, 150x3 mm I.D., packed with Separon SGX C18, 7 µm, (Tessek, Prague) was used in both instruments.

A Specord M 400 UV-VIS spectrophotometer (Carl Zeiss, Jena, Germany) was used to analyze the fractions of the eluate in the determination of breakthrough volumes by frontal analysis and in the recovery tests. A Dorcus vacuum manifold (Tessek, Prague) was used for off-line solid-phase extraction experiments.

Chemicals

Methanol and ethanol, both UV spectroscopic grade, sodium hydroxide, sodium tetraborate (decahydrate) and

sodium hydrogencarbonate were obtained from Lachema, Brno, Czech Republic; o-phthaldialdehyde (OPA), 2-mercaptoethanol, n-ethylamine, n-propylamine, n-butylamine, n-pentylamine, n-hexylamine and n-heptylamine were all from Fluka, Buchs, Switzerland; perchloric acid was from Carlo Erba, Milano, Italy. A Mili-Q apparatus (Millipore, Bedford, MA, U.S.A.) was employed to pretreat water used for preparation of all the solutions and of the mobile phase. All the other chemicals were used as obtained, without further purification.

Sorbents

Separon SGX C18 (60 μm), Separon HEMA-BIO CM (60 μm) and Separon HEMA-BIO SB (60 μm) were obtained from Tessek, Prague, Czech Republic, as the Silica-cart and HEMA-cart systems consisting each of a pre-packed 1 ml polypropylene cartridge (medical grade Tatren PD 140) packed with the individual sorbent.

Spheron C 1000, Spheron SB 1000, both 25-40 μm, were purchased as bulk materials from Lachema, Brno and were packed in the laboratory into the empty polypropylene cartridges of the same size as the packed Silica-cart and Hema-cart cartridges.

Separon SGX C18 (60 μm) is octadecyl silica of the same type as the packing used in reversed-phase HPLC, with larger particle size.

Both Separon HEMA and Spheron cation exchangers are based on the hydrophilic organic matrix, a macroporous co-polymer of 2-hydroxyethyl methacrylate and ethylene dimethacrylate. The Separon HEMA-BIO matrix was subject to additional chemical treatment, increasing the content of the hydroxyl groups in the matrix and making its

surface more hydrophilic than that of the untreated Spheron matrix.

Spheron C 1000 and Separon HEMA-BIO CM are both weak cation exchangers containing carboxylic groups, with cation-exchange capacities of 2 ± 0,25 mmole.g^{-1}.

Spheron SB 1000 and Separon HEMA-BIO SB are strong cation exchangers with sulphobutylic exchange groups and cation-exchange capacities of approximately 1.5 mmole.g^{-1}.

Derivatization Procedure (2)

Solution A: 50 mg of o-phthaldialdehyde dissolved in 5 ml of ethanol

Solution B: 22.5 µl of 2-mercaptoethanol dissolved in 5 ml of ethanol

Solution C: borate buffer, pH=10.5

The OPA derivatization reagent was prepared by mixing 1.5 ml of A with 1.5 ml of B and 90 ml of C. The mixed reagent was kept in a dark bottle in the refrigerator and was stabile for 2 days. After this period, fresh reagent was prepared. The derivatization was performed in a 10 ml volumetric flask by adding 3 ml of the OPA reagent to the solution of amine(s). The mixture was kept at ambient temperature for 5 minutes and then diluted to final volume with methanol.

Determination of the breakthrough curves and of the recovery in the sorption and desorption procedures.

All ion exchange cartridges were washed with water, 5 ml of 0.1 M perchloric acid and again with water to neutral pH before the use to make sure that the cation exchangers are in the H^+ form.

Separon SGX C 18 was washed with 5 ml of methanol and with water to remove possible organic impurities from the cartridge.

The breakthrough curves were determined by frontal analysis in which the stock solution containing 10^{-3} mole.l^{-1} n-butylamine in water was passed through the cartridge fixed in the Dorcus vacuum manifold. The flow rate was kept between 5-10 ml.min^{-1} during the sorption procedure, fractions of the effluent were collected and the amine in the fractions was derivatized with o-phthaldialdehyde as described above.

The concentrations of n-butylamine were measured using a Specord M 400 UV-VIS spectrophotometer at the wavelength of 335 nm, corresponding to the absorption maximum of the OPA derivative. The determination was based on the calibration curve constructed for the concentration range 3.10^{-6} to 2.10^{-4} mole.l^{-1} of n-butylamine in water. The calibration curve was linear with correlation coefficient of 0.9998. The experimental absorbance values were corrected for the absorbance of the blank solution containing only the derivatization reagent in water.

Methanol acidified to pH=3 with perchloric acid was used for desorption of n-butylamine from the Separon SGX C18 Silica-cart cartridge while 0.1 - 1.0 M perchloric acid in water or in aqueous methanol served as the desorption liquid from the cation exchange cartridges.

A Separon SGX C18 Silica-cart cartridge was used to test the possibilities of in-situ derivatization during the sample enrichment step. The cartridge was pre-conditioned by washing with 5 ml of the mixture of the solutions A and B (OPA with mercaptoethanol). The aqueous sample of n-butylamine was brought to pH=10.5 necessary for the derivatization reaction by addition of the borate buffer immediately before the enrichment.

Each fraction of the effluent was divided into two portions. The first was analyzed spectrophotometrically without any further treatment while the other was mixed with the OPA reagent before the analysis, as described above. Pure ethanol was used for desorption and the fractions of the desorbate were collected and analyzed in the same way as the effluent in the sorption step.

HPLC Procedure

The method was first tested for the analysis of aqueous samples containing 10^{-9} – 10^{-8} mole.l^{-1} n-butylamine, using the liquid chromatograph built from the individual parts, including an HP 1046A fluorimetric detector. 500 ml volume of each sample was passed through a cartridge packed with Spheron C 1000 weak cation exchanger in the Dorcus vacuum manifold. After the enrichment step, the amine was desorbed from the cartridge by elution with 3 ml 1 M perchloric acid in 50% methanol; the desorbate was neutralized with 1 M sodium hydroxide and derivatized as described above.

Two artificial samples containing 10^{-7} mole.l^{-1} and 10^{-9} mole.l^{-1}, respectively, of each ethyl-, n-propyl-, n-butyl-, n-pentyl-, n-hexyl- and n-heptylamine in water were prepared. The first sample was derivatized without any pre-treatment and the second sample was subject to the same enrichment procedure as the samples containing only n-butylamine. Both samples were analyzed by HPLC using the HP 1090M liquid chromatograph with the HP 1046A fluorimetric detector.

The chromatographic conditions in the two instrumental setups were identical: isocratic elution with methanol-water 70:30 as the mobile phase at 0.5 ml.min^{-1}. The excitation and the emision wavelengths were

set to yield maximum sensitivity of detection: λ_{ex}=223 nm and λ_{em}=435 nm. Sample volume of 20 µl was injected into the liquid chromatograph in each experiment.

RESULTS AND DISCUSSION

Tests of the Sorbents for Solid-Phase Extraction

1. Octadecyl silica

The retention volume of n-butylamine was evaluated from the inflexion point on the breakthrough curve (plot of the concentration of n-butylamine in the effluent vs. time elapsed from the start of the frontal analysis) and the breakthrough volume was determined from the intersection point of the tangent (at the inflexion point) with the baseline.

The breakthrough curve of 10^{-3} M n-butylamine in water on a Separon SGX C18 cartridge is shown in Fig.1a. The profile of the breakthrough curve is atypical, with the breakthrough volume at 25 ml followed by a slow continuous increase of the concentration of n-butylamine up to 120 ml, where a steep increase in concentration occurs. It is not clear if this behavior can be attributed to the presence of the residual silanol groups in the sorbent or to another effect.

Methanol brought with $HClO_4$ to pH=3 was used for the elution of n-butylamine sorbed on the cartridge and 98% of the sorbed compound was eluted in 6 ml of the eluate (desorption curve 1 in Fig. 2). The desorption with acidified methanol would be suitable for the recovery of n-butylamine, but the shape of the breakthrough curve indicates that only very low sample volumes can be

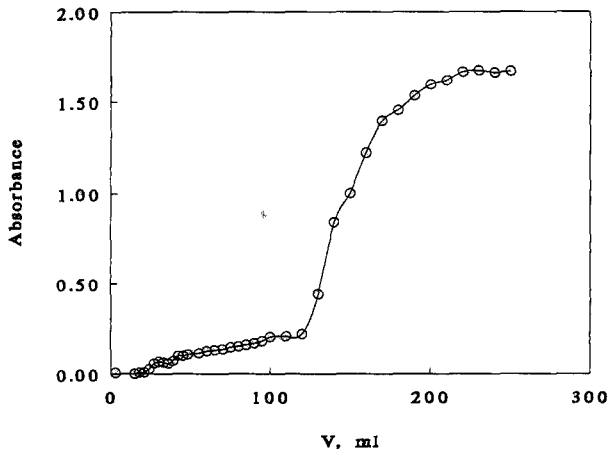

FIGURE 1a

Breakthrough curve of 10^{-3} M n-butylamine in water on a Separon SGX C18 cartridge.
V - volume of the effluent; Absorbance measured in fractions after derivatization with OPA at 335 nm.

employed in this system, resulting in inacceptably low enrichment factors, which precludes efficient use of this sorbent for the enrichment of aqueous samples of alkylamines.

The octadecyl silica material was tested as possible support for in-situ derivatization during the solid-phase extraction step. For this purpose, the Separon SGX C18 cartridge was first conditioned with the OPA reagent, as described in Experimental and the aqueous solution of n-butylamine was buffered to pH=10.5 before the sorption. Although the high pH is not compatible with continuous use of silica based columns, it can be applied in the work with a cartridge intended for a single use.

To investigate the process of in-situ derivatization in detail, the fractions of the effluent

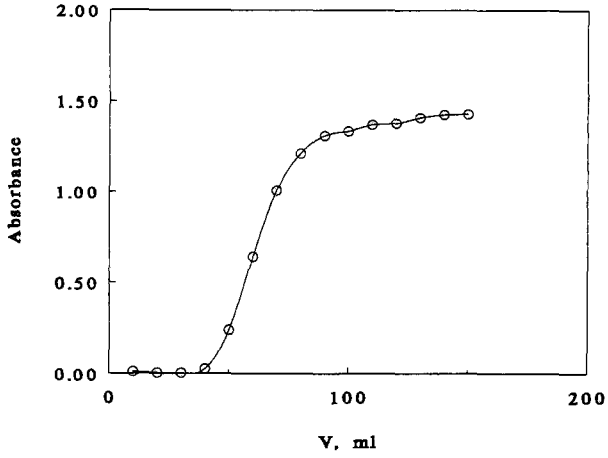

FIGURE 1b

Breakthrough curve of 10^{-3} M n-butylamine in water (buffered to pH 10.5) on a Separon SGX C18 cartridge conditioned with the OPA reagent.
V - volume of the effluent; Absorbance measured in fractions after derivatization at 335 nm.

from the cartridge during the sorption and desorption processes were divided into two portions, the first of which was measured photometrically without any treatment and the other was subject to the derivatization procedure with OPA prior to the photometric determination (see Experimental). Untreated portions of the fractions of the effluent collected during the sorption process did not show significant absorbance. However, the absorbance increased in the portions subject to the derivatization procedure and the resulting breakthrough curve had almost symmetrical profile and the breakthrough volume of 44 ml (Fig. 1b).

Pure ethanol was used as the desorption liquid for n-butylamine retained on the cartridge preconditioned

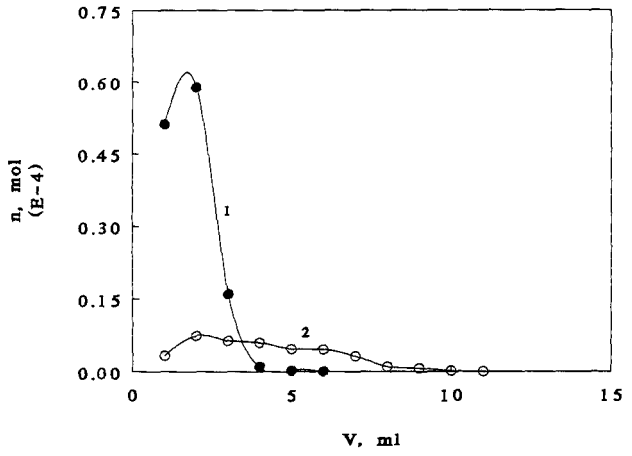

FIGURE 2

Desorption curves of n-butylamine after sorption on a Separon SGX C18 cartridge. 1. Desorption with methanol acidified with $HClO_4$ to pH=3 from unconditioned cartridge; 2. Desorption with ethanol from the cartridge conditioned with the OPA reagent;
V - volume of the desorbate; n - mass of the amine in fractions.

with OPA. No significant absorbance was found in the untreated fractions of the desorbate, in contrast to the portions subject to the derivatization procedure, where the desorption curve shown in Fig. 2 (curve 2) was obtained. This desorption curve was shallow and only approximately 70% of the derivative was recovered in the desorption step.

These experiments indicate that the pre-conditioning of the C18 cartridge with the OPA reagent results in an improved profile of the breakthrough curve with respect to the unconditioned cartridge, possibly because the reagent blocks the unreacted silanol groups in the sorbent, but that real in-situ derivatization of

n-butylamine hardly occurs under the conditions employed.

2. Cation exchangers

The main driving force of the sorption of alkylamines on cation exchangers in the H^+ form are ion-exchange interactions between the protonized form of the amine and the exchange groups of the exchanger. Strong cation exchangers posses $-SO_3^-$ groups, which are dissociated over the wide range of pH values from 1 to 14. On the other hand, carboxylic groups of weak cation exchangers are dissociated only in alkaline solutions and do not show ion-exchange properties in acidic solutions.

In addition to the ion-exchange mechanism, interactions with the matrix of the cation exchanger may contribute to the sorption of amines. Ion-exchangers based on styrene-divinylbenzene copolymers often show strong hydrophobic interactions with various organic compounds. In the alkylamine series, these interactions can be expected to increase with increasing length of the alkyl in the amine, so that the sorption capacities, breakthrough volume and recovery can be significantly influenced by the structure of the amine.

These effects are likely to be of minor importance for the sorption of amines on polymeric ion-exchangers with hydrophilic matrices, such as Spheron or Separon HEMA-BIO materials based on ethyleneglycol-methacrylate copolymers. The two types of matrices differ in additional hydrophilization introducing hydroxyl groups into the structure of the HEMA-BIO matrix.

The experimental breakthrough curves of 10^{-3} M n-butylamine on the cartridges packed with the cation exchangers tested and with unconditioned Separon SGX C18

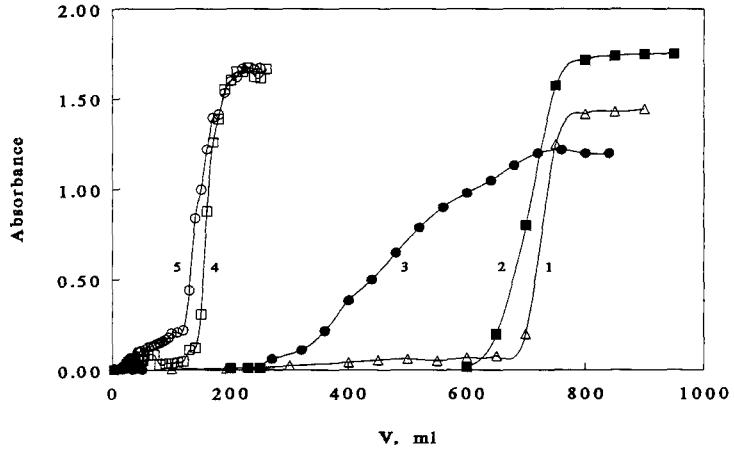

FIGURE 3

Breakthrough curves of 10^{-3} M n-butylamine in water on cartridges packed with Spheron C 1000 (1), Spheron SB 1000 (2), Separon HEMA-BIO SB (3), Separon HEMA-BIO CM (4) and Separon SGX C18 (5).
V - volume of the effluent; Absorbance measured in fractions after derivatization at 335 nm.

are compared in Fig.3. The exchangers with the hydrophilized Separon HEMA-BIO matrix provide significantly lower breakthrough volumes than the Spheron materials. The profile of the breakthrough curve on the weak cation exchanger HEMA-BIO CM is similar to that observed for the octadecyl silica SGX C18 material, possibly because of simultaneous effects of the sorption on the carboxylic ion-exchange groups and on the lipophilic hydroxy groups in the matrix of the exchanger. This may be also the reason for the gradual slope of the breakthrough curve observed on the strong cation exchanger Separon HEMA-BIO SB, where the first breakthrough is observed at 270 ml, but the equilibrium is not achieved before 700 ml of the effluent have

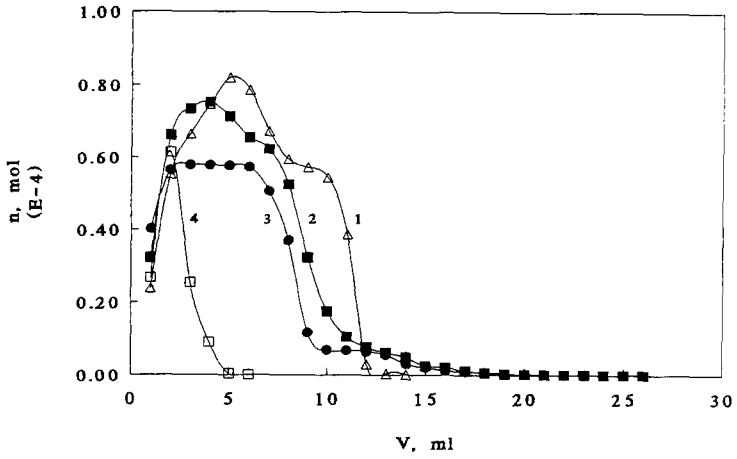

FIGURE 4

Desorption curves of n-butylamine from cartridges packed with Spheron C 1000 (1), Spheron SB 1000 (2), Separon HEMA-BIO SB (3) and Separon HEMA-BIO CM (4) with 0.1 M $HClO_4$ in water as the desorption liquid.
V - volume of the desorbate; n - mass of the amine in fractions.

passed through the cartridge. The Spheron cation exchangers with the matrix not subject to additional hydrophilization showed much sharper breakthrough curves at significantly higher breakthrough volumes (640 ml for Spheron SB and 730 ml for Spheron C 1000). These results indicate that the materials with untreated matrix are more suitable for the enrichment of n-butylamine than hydrophilized HEMA-BIO cation exchangers.

Recoveries from ion exchangers saturated with n-butylamine in the sorption step to the full ion exchange capacities were compared in another set of experiments. Fig. 4 shows desorption curves of n-butylamine obtained with 0.1 M perchloric acid in

water as the desorption liquid. Strong cation exchangers show tailing desorption curves, which means that larger volumes of the desorption liquid should be used in comparison to weak cation exchangers. This indicates that carboxylic cation exchangers are more suitable for the enrichment of amines than cation exchangers with sulfonic ion-exchange groups. The elution from the Separon HEMA-BIO CM cartridge can be accomplished in 5 ml of the desorption liquid whereas 14 ml are necessary for the Spheron C 1000 cartridge. However, because of much higher breakthrough volume (730 ml) on the latter cation exchanger, the maximum enrichment factor for this material is higher than for other sorbents studied and the recovery of 92% is satisfactory and better than with the Separon HEMA-BIO CM and Spheron SB 1000 cation exchangers (Table 1). After comparison of the breakthrough volumes, retention volumes, enrichment factors and recoveries under comparable conditions, Spheron C 1000 was selected as the sorbent most suitable for the enrichment of aliphatic amines.

Composition of the Desorption Liquid

To achieve the elution of an ionized solute from an ion-exchanger it is necessary to employ mobile phase containing ions that compete with the solute ions for the ion exchange functional groups. The elution is enhanced with increasing concentration of the competing ions in the desorption liquid. In solutions of strong acids, H_3O^+ ions participate in these competitive interactions. When a dilute acid is used to elute solutes sorbed on a weak cation exchanger, the desorption effect is further enhanced by suppression of the dissociation of weak cation exchange (carboxylic)

TABLE 1

Characteristics of the Sorption and Desorption of n-Butylamine on Cartridges Packed with Various Sorbents

Separon SGX C18 (1), Separon SGX C18 conditioned with OPA (2), Separon HEMA-BIO SB (3), Separon HEMA-BIO CM (4), Spheron SB 1000 (5), Spheron C 1000 (6)
Desorption liquid: methanol acidified to pH = 3 (1), ethanol (2), 0.1 M perchloric acid (3-6)

V_D in ml, volume of the desorption liquid necessary for quantitative elution
V_R in ml, retention volume evaluated from the inflexion point on the breakthrough curve
V_B in ml, breakthrough volume
n_s in mmole, adsorbed amount of the amine, calculated from V_R and concentration of the amine in the sample (1 mmole.l^{-1})
n_e in mmole, recovered amount of the amine in the desorption step
R in %, recovery
f_e enrichment factor $f_e = V_R/V_D$

Cartridge	1	2	3	4	5	6
V_D [ml]	6.0	11.0	24.0	6.0	25.0	14.0
V_R [ml]	130	55	440	155	705	770
V_B [ml]	25	44	275	140	640	727
n_s [mmole]	0.130	0.055	0.440	0.155	0.705	0.77
n_e [mmole]	0.127	0.038	0.468	0.123	0.586	0.708
R [%]	97.7	69.1	106.4	79.4	83.10	91.9
f_e	4.2	4.0	11.5	23.3	24.9	52

groups of the exchanger at low pH, which decreases its ion-exchange capacity.

As 14 ml of 0.1 M $HClO_4$ necessary to achieve quantitative recovery of n-butylamine sorbed on the Spheron C 1000 cartridge is too large a volume for practical enrichment of the samples of amines, we investigated possibilities of decreasing this volume by increasing the concentration of perchloric acid and by adding methanol to the desorption liquid. When 0.33 M $HClO_4$ in 50% methanol was used, the volume necessary for quantitative desorption was decreased to 6 ml (Table 2). Further increasing the concentration of perchloric acid

TABLE 2

The Effect of the Composition of the Desorption Liquid on the Recovery and Enrichment Factor of n-Butylamine for the Spheron C 1000 Cartridge

1: 0.1 M $HClO_4$ in water; 2: 0.33 M $HClO_4$ in 50% methanol; 3: 1.0 M $HClO_4$ in 50% methanol.
Symbols as in TABLE 1.
f_e calculated using V_B = 730 ± 50 ml

desorption liquid	1	2	3
V_D [ml]	14.0	6.0	3.0
n_s [mmole]	0.72	0.82	0.77
n_e [mmole]	0.66	0.83	0.74
R [%]	91.7	101.2	96.1
f_e	52.1 ± 3.6	121 ± 8.3	243 ± 16.7

to 1.0 M reduced of the necessary volume of the desorption liquid to 3 ml, with resulting enrichment factor of 240 (Table 2), which we found satisfactory for practical enrichment purposes. Fig.5 compares the volumes of various desorption liquids necessary to accomplish quantitative recovery of n-butylamine from the Spheron C 1000 cartridge when the amine is sorbed to the full saturation capacity of the exchanger.

HPLC Determination of Amines after Sample Enrichment by SPE

The breakthrough volume of 10^{-3} M n-butylamine on the Spheron C 1000 cartridge was 730 ml and significantly higher breakthrough volumes can be expected for lower concentrations of alkylamines. Therefore we used "safe" volume of 500 ml of aqueous samples of amines and 3 ml of 1 M perchloric acid in 50% methanol as the desorption liquid in the enrichment step before the HPLC analysis in all the experiments. The enrichment factor related to 10 ml of the sample after the derivatization procedure was 50.

To verify the HPLC method, 20 µl samples of n-butylamine in the concentration range of 10^{-8} to 9.10^{-7} M were analyzed by HPLC with fluorimetric detection at the highest settings of the detector sensitivity (PMT GAIN = 15 and 16). The minimum detectable concentration corresponding to the peak height equal three times the baseline noise was 7.10^{-9} M, which is comparable with the data published by Mellbin and Smith (11). The calibration curve was linear in the concentration range tested, with correlation coefficient of 0.9997.

This calibration curve was used to test the HPLC method of determination of n-butylamine after the

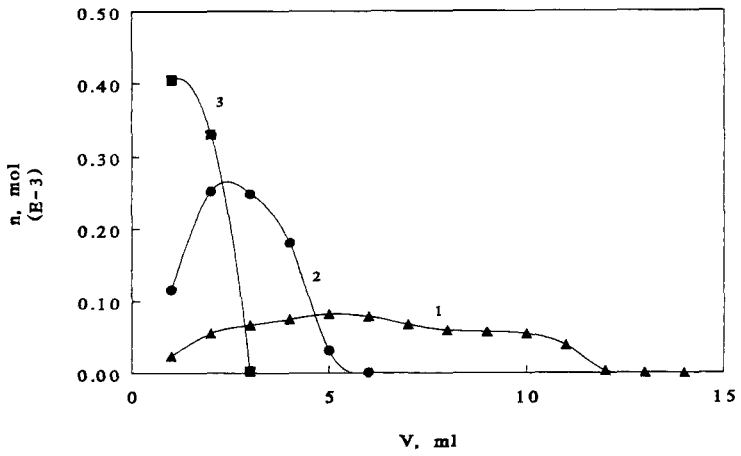

FIGURE 5

Desorption curves of n-butylamine from a cartridge packed with Spheron C 1000 using 0.1 M $HClO_4$ in water (1), 0.33 M $HClO_4$ in 50% methanol (2) and 1.0 M $HClO_4$ in 50% methanol (3) as the desorption liquid.
V - volume desorbate; n - mass of the amine in fractions.

enrichment step. The recovery of the method for n-butylamine in the concentration range from 1.10^{-9} to 1.10^{-8} M was 98-102% (Table 3). The limit of detection calculated from the baseline noise corresponds to $1.4.10^{-10}$ M. Because of the small peak of the impurity found in the experiments with blank sample, probably originating from the chemicals, practical limits of determination correspond to approximately 1.10^{-9} M, i.e., 75 ppt.

The method was tested on an artificial mixture of six alkylamines. 20 µl of a sample containing ethyl-, n-propyl-, n-butyl-, n-pentyl-, n-hexyl and n-heptylamine derivatives with OPA in concentrations $2.6.10^{-7}$ - $3.8.10^{-7}$ M was injected directly into the 1090M HP liquid chromatograph with fluorimetric detector. The

TABLE 3

Enrichment of Aqueous Solution of n-Butylamine on a Spheron C 1000 Cartridge

Sample volume 500 ml; desorption with 3 ml 1.0 M $HClO_4$ in 50% methanol (enrichment factor f_e=50 after the derivatization step, final volume of derivatized sample = 10 ml)

c_0 in nmole.l^{-1}, concentration of the amine in the original sample
c_1 in nmole.l^{-1}, expected concentration of the amine in the sample after enrichment and derivatization
c_2 in nmole.l^{-1} - experimentally found concentration of the amine in the enriched sample by HPLC
S sensitivity settings of the fluorimetric detector (PMT GAIN)
R in %, recovery of the method

c_0	c_0, ppb	c_1	c_2	S	R
11.0	0.80	550	564	15	102.5
4.6	0.34	230	231	15	100.5
1.2	0.09	60	58.9	16	98.2
0.0	0.03	-	18.6	16	-

resulting chromatogram (Fig.6) was compared with the separation of an aqueous sample containing the amines in concentrations $5.2.10^{-9}$ - $7.6.10^{-9}$ M, after enrichment from 500 ml to the final volume of 10 ml after derivatization with OPA, as described above (Fig.7).

The amounts of the amines in 20 µl samples should be theoretically equal in the two experiments (5.2-7.8 pmoles of the individual amines). Theoretical (c_1) and

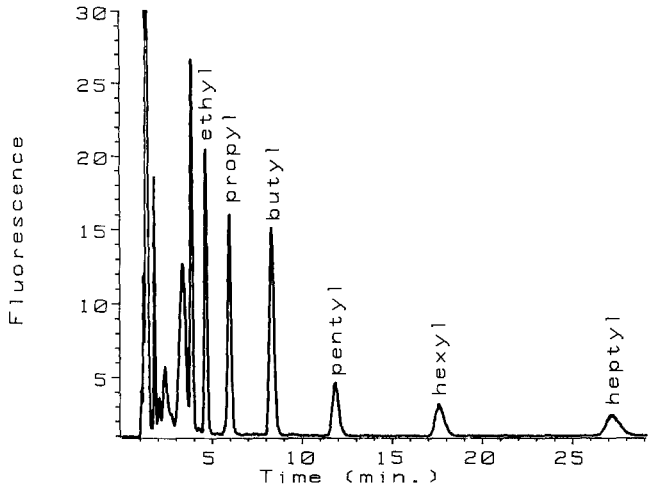

FIGURE 6

Chromatographic separation of 20 µl of a mixture of n-alkylamines ($2.6.10^{-7}$–$3.8.10^{-7}$ M each) after derivatization with OPA and fluorimetric detection. Column: Separon SGX C18, 7 µm (150x3 mm); mobile phase: methanol in water, 70:30, 0.5 ml.min^{-1}. λ_{ex}=223 nm; λ_{em}=435 nm.

experimentally found (c_i) concentrations of the individual amines, recoveries and minimum detectable concentrations equivalent to the signal threefold the baseline noise are given in Table 4.

The recoveries were between 90 and 114%, which is sufficient for practical applications. The detection limits for n-butylamine are approximately three times lower than those found for the instrumental setup with the CI 100 integrator, which can be attributed to better performance of the data station (and possibly, of the pump) in the 1090M liquid chromatograph in comparison with the modular instrument built from the individual parts. The practical limits of determination, however,

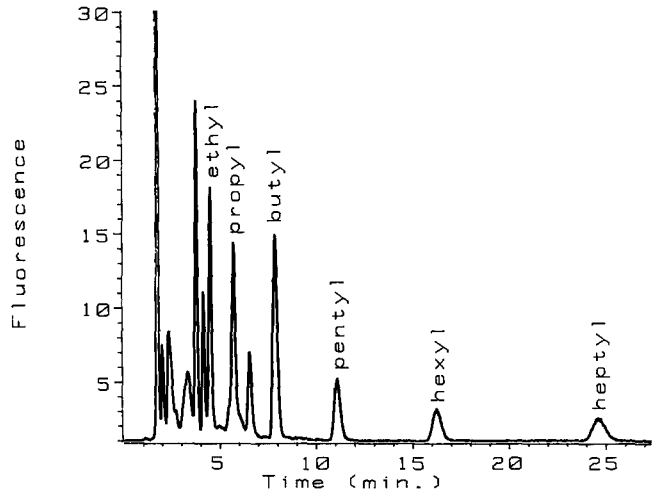

FIGURE 7

Chromatographic separation of a mixture of n-alkylamines ($5.2.10^{-9}$–$7.6.10^{-9}$ M each) after enrichment of 500 ml of the sample on a Spheron C 1000 cartridge and derivatization with OPA (volume of the sample after derivatization = 10 ml; 20 µl injected). Other conditions in Fig.6

are likely to be limited by impurities present in the sample, desorption liquid, mobile phase, or derivatizing reagent rather than by the baseline noise. The detection limits increased with increasing molecular weight of the amine because of decreasing peak height of more retained solutes. The detection limits of more strongly retained compounds could probably be slightly improved by using gradient elution.

CONCLUSIONS

Octadecyl silica Separon SGX C18 is not suitable sorbent for enrichment of aqueous samples of alkylamines

TABLE 4

Enrichment of Aqueous Solution of a Mixture of n-Alkylamines on a Spheron C 1000 Cartridge

MDC in nmole.l^{-1}, minimum detectable concentration other symbols as in TABLE 3.

AMINE	c_0	c_0,ppb	c_1	c_2	R	MDC
ETHYL	7.50	0.38	375	337	89.8	2.4
PROPYL	7.20	0.42	360	372	103.4	3.0
BUTYL	6.0	0.43	300	292	97.3	2.7
PENTYL	5.24	0.46	262	297	113.4	8.7
HEXYL	5.44	0.55	272	266	97.9	15
HEPTYL	5.66	0.65	283	310	109.6	24

by solid-phase extraction, as the breakthrough volumes are too low for a satisfactory enrichment factor. The attempts to use this material as the support for in-situ derivatization during the enrichment step were also unsuccessful. The breakthrough volumes were significantly increased when polymeric cation exchangers with hydrophilic matrix in the H^+ form were used as the sorbents for solid-phase extraction. Untreated cation exchangers show larger breakthrough volumes than the exchangers with the matrix subject to additional hydrophilization procedure .

Weak cation exchangers show steeper desorption curves and lower desorption volumes than the strong

cation exchangers tested. Based on the results of the sorption-desorption tests, weak cation exchanger Spheron C 1000 with carboxylic acid ion-exchange groups was selected as the best sorbent for enrichment of aqueous samples of alkylamines by solid-phase extraction. With aqueous-methanolic perchloric acid as the desorption liquid, the enrichment factor of 50 can readily be achieved in the sample pre-treatment step, including adsorption, desorption and derivatization of amines with o-phthaldialdehyde reagent.

The solid-phase extraction enrichment procedure was combined with reversed-phase HPLC and fluorimetric detection for determination of aliphatic amines in aqueous samples. The method was linear over the concentration range of at least two orders of magnitude and limits of determination below 100 ppt of the amine in the original sample could be achieved, with recoveries from 90 to 115%.

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DETERMINATION OF NEUTRAL SUGARS IN MYCOBACTERIAL CELL WALLS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A high-performance liquid chromatography method for the determination of neutral sugars in mycobacterial cell walls was developed. Cell wall samples were hydrolysed in either 1 N H_2SO_4 for 5 h at 100±2°C or 4 N TFA for 4 h at 100±2°C. The hydrolysed samples were applied to an ion moderated partition column using water as the eluent, and the column effluent was monitored by refractive index detection. Using acid treated neutral sugar standards the minimum calibration curve r^2 value was 0.996, the coefficient of variation of multiple determinations averaged 5.2% and the detection limit for each analyte was 0.2 μg.

INTRODUCTION

Mycobacterial cell walls have been shown to exhibit unique immunomodulating and anti-tumor properties, which are being exploited in the development of human pharmaceuticals [1]. The cell walls of mycobacteria

are composed of three types of polymers: peptidoglycan, cell wall associated proteins, and arabinogalactan (or arabinomannan) substituted with mycolic acids. The measurement of cell wall neutral sugars is necessary in structure/activity studies and bulk drug quantification during pharmaceutical development.

The neutral sugars in mycobacterial cell wall preparations have been measured by gas chromatography (GC), after pre-column derivatization of the sugars to their corresponding alditol acetates [2,3].

An comprehensive literature review revealed no high-performance liquid chromatography (HPLC) protocols for the analysis of mycobacterial cell wall neutral sugars. Consequently, a relatively simple HPLC technique utilizing refractive index (RI) detection was developed, which did not require pre-column derivatization of the reducing sugars.

MATERIALS AND METHODS

All of the chemicals used were of the highest available commercial purity. Arabinose (ARA), galactose (GAL), glucose (GLC) and trifluoroacetic acid (TFA) were from Sigma (St.Louis, MO, USA). *Mycobacterium phlei* and *M. fortuitum* cell wall skeleton (CWS) preparations were from Ribi ImmunoChem Research, Inc. (Hamilton, MT, USA). HPLC grade water was supplied by a Modulab type I water system from Continental (San Antonio, TX, USA).

Samples were hydrolyzed with 4 N TFA or 1 N H_2SO_4 (4.00 mg CWS/ml acid) in vacuum sealed glass tubes at 100±2°C. Working standards consisting of ARA, GAL and GLC (each at 0.500 mg/ml) in 4 N TFA or 1 N H_2SO_4 were similarly prepared. After TFA hydrolysis, standard and sample aliquots were desiccated, then re-suspended in equal volumes of HPLC water. After H_2SO_4 hydrolysis, standard and sample aliquots were neutralized with 0.5 N NaOH.

The HPLC (except for the column) was from Waters (Milford, MA, USA), and consisted of: a 700 autoinjector (200 μl sample loop); a 510 pump; a temperature control module (operated at 85°C); a 410 RI detector (40°C internal); computer control (via a system interface module) by Maxima (ver. 3.3). The column (Aminex HPX-87P, with a matching guard column) was from Bio-Rad (Richmond, CA, USA). For the analysis of H_2SO_4 treated standards and samples, a Bio-Rad deashing column was also used. The eluent for all of the analyses was degassed HPLC water, delivered isocratically at 0.5 ml/min.

RESULTS AND DISCUSSION

Based on previous analyses of bacterial polysaccharides, TFA and sulfuric acids were chosen for sample hydrolysis [2-4]. Experiments were conducted to determine the optimal hydrolysis conditions for neutral sugar release from the CWS preparations. It was found that hydrolysis in 1 N

H_2SO_4 for 5 h at 100±2°C, or in 4 N TFA for 4 h at 100±2°C resulted in optimal sugar release (data not shown). A typical chromatogram of TFA hydrolysed *M. phlei* CWS (50 μl injection) is shown in Figure 1.

The calibration curves for GLC, GAL and ARA in the working standards, whether H_2SO_4 or TFA treated, were consistently linear (minimum r^2 of 0.996) over the range of 1 to 20 μg per injection for each analyte (Table 1). In an additional test, linearity was demonstrated to at least 100 μg per

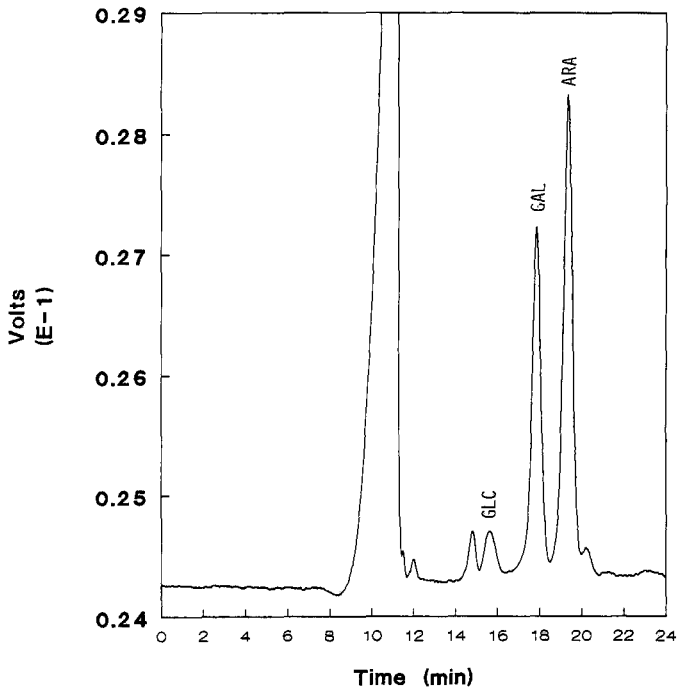

FIGURE 1. A typical chromatogram of TFA hydrolysed *M. phlei* CWS (50 μl injection).

TABLE 1

The standard curve linear regression data for the 1 N H_2SO_4 (5 h at 100±2°C) and 4 N TFA (4 h at 100±2°C) treated working standards is shown below. Standard curves were generated by making multiple analyses of each working standard, which represented 1, 2.5, 5, 10, 15 and 20 μg injections of each neutral sugar.

Analyte	Acid Trt	Slope	y-intercept	r^2
GLC	H_2SO_4	6232	-1264	0.998
GLC	TFA	5761	-1248	0.998
GAL	H_2SO_4	6079	-862	0.998
GAL	TFA	5325	-1167	0.996
ARA	H_2SO_4	5885	-620	0.999
ARA	TFA	5223	-1663	0.997

injection for each neutral sugar. The detection limit for GLC, GAL and ARA was determined to be 0.2 μg, corresponding to a signal-to-noise ratio of 2.

Precision, as measured by the coefficient of variation, averaged 5.2% and ranged from 0.6 to 14.3% (Table 2). The accuracy of the method was assessed through several standard addition experiments, by adding GLC, GAL and ARA to CWS preparations prior to acid hydrolysis. Recovery of standards after H_2SO_4 hydrolysis averaged 91%, while recovery of GLC, GAL and ARA after hydrolysis in TFA averaged 103% (Table 3).

Hydrolysis with H_2SO_4 resulted in lower neutral sugar values, especially for ARA, in comparison to TFA treatment (Table 2). Thus, the ARA/GAL ratios were also lower for H_2SO_4 treated samples (Table 2). The

TABLE 2

The neutral sugars in *M. fortuitum* and *M phlei* CWS preparations were determined after hydrolysis in 1 N H_2SO_4 for 5 h at 100±2°C, or hydrolysis in 4 N TFA for 4 h at 100±2°C. The average μg of each neutral sugar (± one standard deviation, four determinations) per miligram of CWS are shown.

CWS Prep	Acid Trt	GLC	GAL	ARA	ARA/GAL
M fort.	H_2SO_4	4 ± 0.2	137 ± 4	126 ± 5	0.91
M fort.	H_2SO_4	9 ± 1	96 ± 3	118 ± 5	1.23
M phlei	TFA	7 ± 1	148 ± 6	158 ± 15	1.06
M phlei	TFA	18 ± 0.1	101 ± 2	144 ± 2	1.42

TABLE 3

The percent recoveries of standards added to *M. fortuitum* and *M. phlei* CWS preparations were determined for both the 1 N H_2SO_4 (100±2°C for 5 h) and 4 N TFA (100±2°C for 4 h) hydrolysis treatments. The average percent recoveries (± one standard deviation, four determinations) for each neutral sugar are shown.

CWS Prep	Acid Trt	GLC	GAL	ARA
M phlei	H_2SO_4	95 ± 4	90 ± 7	87 ± 8
M phlei	TFA	98 ± 2	109 ± 8	109 ± 8
M fort.	TFA	98 ± 3	100 ± 4	102 ± 6

lower sugar values found after H_2SO_4 hydrolysis correlated with the lower overall recovery of standards added to CWS before H_2SO_4 hydrolysis (Table 3).

Previous GC determinations of the ARA/GAL ratio of several mycobacterial species, after 1 N H_2SO_4 hydrolysis for 5 h at 100°C, ranged from 2.33 to 2.76 [2]. In a more recent paper, the ratio for *M. tuberculosis*, after hydrolysis in 2 M TFA for 1 h at 121°C, was found to be 1.38 by GC analysis [3]. This latter value compares more favorably to the ratios reported in this paper (Table 2).

The low levels of GLC measured by the HPLC method (Table 2) were probably derived from glycogen which may have been trapped in the cell wall during CWS preparation. Additionally, the CWS preparations were checked for mannose (MAN) content with this method, by adding MAN (Sigma) to the working standards. Based on previous data [2,3] no MAN was expected to be present in CWS from *M. fortuitum* or *M. phlei*, and none was detected.

For the analysis of mycobacterial CWS preparations with the HPLC method, hydrolysis in 4 N TFA for 4 h at 100±2°C is recommended for two reasons. First, standard recoveries were higher in comparison to 1 N H_2SO_4 treatment (Table 3). Second, and ARA/GAL ratios in TFA treated samples (Table 2) more closely matched recent published data [3].

The HPLC method described herein should be widely applicable to the analysis of bacterial cell wall neutral sugars, and it is simpler to perform than the GC technique which has been described elsewhere [2-3].

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REVERSED-PHASE RETENTION BEHAVIOR OF FLUORESCENCE LABELED PHOSPHOLIPIDS IN AMMONIUM ACETATE BUFFERS

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ABSTRACT

Subcomponents of fluorescent derivatives of phosphatidylethanolamine (PE) and phosphatidylserine (PS) were resolved by reversed-phase high-performance liquid chromatography (HPLC) with mobile phases containing acetonitrile, methanol, water, and ammonium acetate. The fluorescence labeled phospholipids (PL) include N-(rhodamine B sulfonyl)-PE, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-PE, N-(5-fluoresceinthiocarbamoyl) (FL)-PE, N-(1-pyrenesulfonyl)-PE, and N-(5-dimethylaminonaphthalene-1-sulfonyl)-PE. Among the compounds studied, FL-PE exhibited the highest degree of selectivity for component resolution. The HPLC behavior of the five PE derivatives was examined under variable concentrations of ammonium acetate. Capacity factors of the PL subcomponents increased with increasing concentrations of the acetate buffers. Incorporation of triethylamine into the mobile phase alleviated peak

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broadening and improved detection sensitivity of polar PL (FL-PE and PS). Fatty acid structures of molecular species in FL-PE were studied by particle beam (PB)-LC-mass spectrometry (MS). Compatibility of the HPLC method with PB-LC-MS is demonstrated.

INTRODUCTION

In general, phospholipids (PL) can be enzymatically or chemically transphosphatidylated into phosphatidylethanolamine (PE). The presence of an amino group in a PE molecule facilitates conversion of the parent compound to various derivatives. Incorporation of a fluorigenic reagent into PE yields a fluorescent derivative which can be sensitively measured with a fluorescence detector.

Molecular species or subcomponents of PL have been separated by reversed-phase high-performance liquid chromatography (HPLC) (1-9). Structurally intact PL subclasses have been analyzed by reversed-phase ion-pair HPLC-UV detection (10-13). HPLC analyses of fluorescent derivatives of diradylglycerols obtained by enzymatic hydrolysis of PL have been documented (14-19). A few reports on HPLC assays of fluorescent derivatives of PL are available in the literature (20-22). In a recent study (23), a new reversed-phase HPLC-fluorescence detection technique has been developed for the direct quantitative determination of compositions of PL molecular species without isolation of components for phosphorus analysis.

Conventionally, molecular species of PL are characterized by fatty acid analyses of isolated individual PL subcomponents. Simultaneous separation and characterization of the lipid subcomponents requires hyphenated techniques such as LC-mass spectrometry (MS) or

LC-nuclear magnetic resonance spectrometry. In LC-MS methodology, mobile phases containing tetraalkyl ammonium phosphates (23) suffer from severe limitations because of their incompatibility with a LC-MS interface. Fortunately, mobile phases with ammonium acetate buffers are vaporizable and therefore amenable to LC-MS analyses. An HPLC study on the retention behavior of various fluorescent derivatives of PE in acetate buffers should pave the way to subsequent structural studies of PL molecular species by on-line LC-MS.

A number of publications have appeared in the literature dealing with off-line MS analyses of PL using various soft ionization techniques (24-30). Although there are two published reports concerning on-line LC-MS studies with moving belt- and thermospray interfaces (31,32), particle beam (PB)-LC-MS analyses of PL molecular species have not been investigated. In this paper, the results of structural verification of subcomponents of a sample of FL-PE by PB-LC-MS are presented.

Our continuous studies of the deterioration of soybean PL during storage and the composition of the polar lipids in modified oil required the development of analytical methods for the separation of PL subcomponents. Analysis of PL in soybean oil provides useful information on the oil stability and the impact of genetic modification on the distribution of plant constituents. In view of the analytical capability of the LC-MS technique for the characterization of individual PL molecular species, the HPLC behavior of several derivatives of PE was studied under mobile phase conditions simulating the LC-MS interface systems. The HPLC results along with the optimization data are reported in this paper.

EXPERIMENTAL

Materials:

Fluorescent derivatives of phosphatidylethanolamine (PE) [N-(rhodamine B sulfonyl) (NRD)-PE, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) (NBD)-PE, N-(5-fluoresceinthiocarbamoyl) (FL)-PE, N-(1-pyrenesulfonyl) (PY)-PE, and N-(5-dimethylaminonaphthalene-1-sulfonyl) (DAN)-PE] were obtained from Avanti Polar Lipids, INC. (Pelham, AL). All these polar lipids were derived from egg phosphatidylcholine. Egg DAN-PE was obtained from Molecular Probes, Inc. (Eugene, OR). Plant DAN-PE was prepared from plant PE, dansyl chloride and triethylamine in chloroform solution as described previously (23). Brain dansylated phosphatidylserine (DAN-PS) was the product of Avanti. Commercial phospholipids (PL) were used as received without further purification. PL samples were stored in a freezer at -30°C whenever not in use. Dodecyltriethyl ammonium phosphate and triethylamine were obtained from Regis Chemicals (Morton Grove, IL). HPLC grade ammonium acetate and HPLC solvents acetonitrile and methanol were obtained from J. T. Baker, Inc. (Phillipsburg, NJ). Ultrapure HPLC grade water was obtained by filtering distilled water through a Milli Q water purifier system (Millipore Inc., Bedford, MA).

Methods:

High-performance liquid chromatography: A Model SP8700 liquid chromatograph (Spectra Physics, San Jose, CA) interfaced with a Model 980 Programmable Fluorescence detector (Applied Biosystems Inc., Foster City, CA) was

used for most of the HPLC separations unless otherwise specified. Wavelength parameters for various fluorophores were: DAN-PE (and DAN-PS), excitation 338 nm, emission 470 nm; FL-PE, excitation 489 nm, emission 550 nm; PY-PE, excitation 342 nm, emission 398; NRD-PE, excitation 563 nm, emission 585 nm; NBD-PE, excitation 460 nm. emission 534 nm. Mobile phases were acetonitrile-methanol-water containing 5-50 mM ammonium acetate and were prepared immediately before use. Aliquots of freshly prepared analytical samples (10-20 ul of 2 mg/ml solutions) were injected via a Model 7125 injector (Rheodyne Co., Cotati, CA) (25-ul loop) onto a reverse-phase HPLC column. The column packings were of NovaPak C18 prepacked in a stainless steel column (300 mm x 3.9 mm I.D., 4 um) as supplied by Waters Associates (Milford, MA). In all HPLC analyses, mobile phases were filtered, degassed, and pumped through the column at a flow rate of 1 ml/min. Capacity factors (k') were determined from the equation $k' = t/t_o - 1$, where t and t_o are the retention times of an analyte and an unretained solute, respectively.

Capillary gas chromatography: Molecular species of fluorescent PL were isolated by collecting individual HPLC peak components which were then treated with methanol and hydrochloric acid to yield fatty acid methyl esters using a published procedure (33). The fatty acid methyl esters were analyzed with a Varian (Palo Atto, CA) Model 3400 gas chromatograph equipped with a flame ionization detector. A fused silica capillary column (0.25 mm x 30 m) coated with 0.2 um SP 2330 (Supelco Inc., Bellefonte, PA) was used throughout the analyses. In a typical GC analysis, the column temperature was initially held at 200°C for 15 min and then increased from 200 to 220°C at a rate of 10°C/min.

Particle beam liquid chromatography-mass spectrometry: PB-LC-MS analyses were performed in the EI mode on a Vestec Model 201 LC-MS (Vestec Corp., Houston, TX), equipped with a Universal Interface. The chromatographic system used was a Kratos Spectroflow 400 Ternary Pumping System (Kratos Analytical, Ramsey, NJ) equipped with a 400 nm UV detector. A sample (100 ug) of FL-PE was injected onto an unused NovaPak C18 column (different from the one used in HPLC analyses) and eluted with a mobile phase of acetonitrile-methanol-water (70:20:10) containing 35 mM ammonium acetate at a flow rate of 1.2 ml/min. The Teknivent Vector/One Mass Spectrometry Data System (Teknivent Corp., St. Louis, MO) was used for acquiring and processing data.

RESULTS AND DISCUSSION

Fig. 1 shows structures of the five fluorescence labeled PE investigated. Of these derivatives, FL-PE (Fig. 1B) and NRD-PE (Fig. 1A) are of particular interest for comparative study of their HPLC behavior because of the similarity in ring structures. In the absence of mobile phase electrolyte, the polar lipids each having a negative charge at the phosphoryl moiety exhibited little retention on a reversed-phase column. However, HPLC with mobile phases containing ammonium acetate buffers led to variable degrees of analyte retention as well as component separations, depending on the nature of fluorophores attached to the amino group of PE. The observed retention of PL components on the hydrocarbonaceous stationary phase as a result of adding ammonium acetate to mobile phases is parallel to that found in HPLC with and without tetraalkyl ammonium phosphates in mobile phases (23).

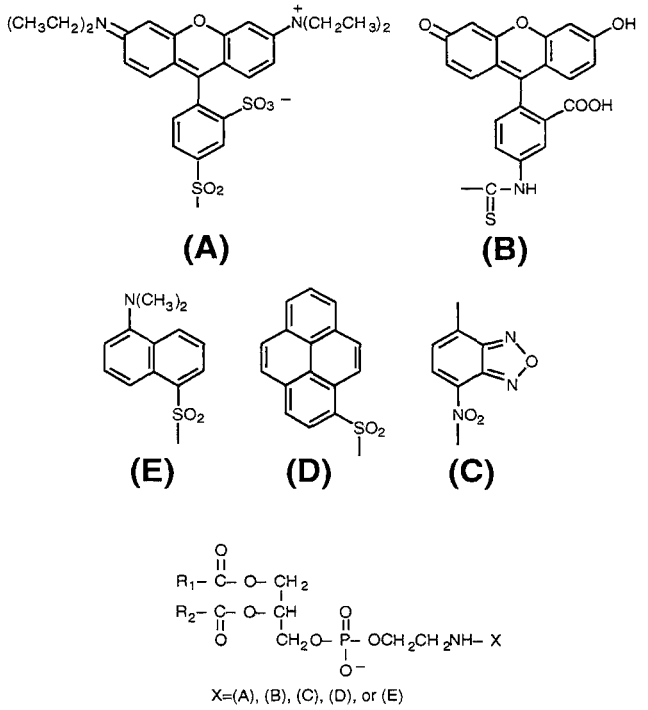

Fig. 1. Structures of fluorescent derivatives of phosphatidylethanolamine (PE) investigated: (A) NRD-PE, (B) FL-PE, (C) NBD-PE, (D) PY-PE, and (E) DAN-PE.

Fig. 2 shows separations of molecular species of different fluorescent derivatives of PE derived from egg PC. The three major components 1-3 in Fig. 2A-2E correspond to those listed in Tables I-III. It is noteworthy that each of the three major components in FL-PE were further split into a-b doublets (Fig. 2B and 2B'). Although the rhodamine fluorophore in NRD-PE possesses the same aryl tricyclic ring structure as the fluorescein counterpart in FL-PE (Fig. 1A vs 1B), only three major

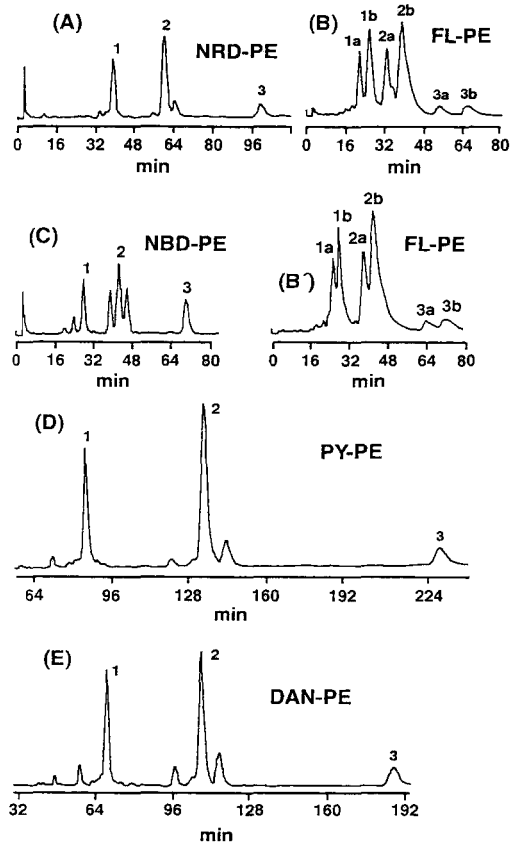

Fig. 2. HPLC separations of subcomponents of fluorescent derivatives of phosphatidylethanolamine derived from egg phosphatidylcholine. Mobile phase: acetonitrile-methanol-water (70:25:5) containing 50 mM ammonium acetate. Triethylamine (50 mM) was added to the mobile phase in (B'). Column: used NovaPak C18. Flow rate: 1 ml/min.

peaks are visible in the chromatogram of the former derivative (Fig. 2A vs 2B). The differences in the types of substituents on the ring system evidently brought about the differences in the chromatographic outcome. Nonetheless, the exact nature of the structural effect of the fluorescein moiety on component resolution is unclear. Incorporation of triethylamine into the mobile phase used in Fig. 2B led to a nearly four-fold increase in detection sensitivity at the slight expense of selectivity for component resolution as shown in Fig. 2B. This may be explained in terms of reduced adsorption of FL-PE analytes on the stationary phase used. The acidic carboxyl- and hydroxy groups in fluorescein appeared to cause adsorption of analyte solutes on the octadecylsilica (ODS) phase.

Retention of all resolved components of PE derivatives on ODS was quite sensitive to the variation in water content of mobile phases employed. As demonstrated in Table I, a small increase in the percentage of water in mobile phases resulted in a drastic increase in capacity factors (k') values. The retention data in Table I provide optimal ranges of mobile phase solvent compositions so that k' values of individual analyte components can be measured within reasonable limits of retention times during routine PL assays. The observed short retention times of all the compounds studied in HPLC without ammonium acetate (at zero concentration) suggested that the PL solutes were in the form of ionized species in the separation processes. Addition of variable amounts of ammonium acetate to mobile phases resulted in retention of the polar lipid on ODS, which is indicative of diminished ionic characteristics (or increased hydrophobicity) of the analyte species. Table II shows the general trend of concentration effects of

Table I. Effect of mobile phase solvent composition on k' values of various fluorescence labeled PE derived from egg PC

Subcomponent	k'*				
Mobile phase NH_4OCOCH_3 (25 mM)	PL compound				
	FL-PE	DAN-PE	PY-PE	NBD-PE	NRD-PE
Acetonitrile-methanol-water (70:25:5)					
1	8.0 (9.2)	23.0	26.6	12.2	19.4
2	12.2 (14.4)	36.0	42.3	18.5	30.2
3	20.8 (25.4)	58.4	70.0	31.0	50.4
Acetonitrile-methanol-water (70:28:2)					
1	4.6 (5.1)	11.0	13.3	6.3	8.1
2	7.0 (7.8)	17.4	20.4	9.0	12.0
3	10.8 (12.2)	27.4	32.6	13.2	19.8

* Values in parentheses are for the additional FL-PE peaks further resolved.

ammonium acetate on k' values of the major components of various PE derivatives. Higher k' values (longer retention times) were obtained with mobile phases containing higher concentrations of ammonium acetate. The mobile phase variables (solvent compositions and acetate concentrations) discussed above can be used for optimization of retention parameters to meet separation requirements for specific HPLC experiments.

Table III compares the HPLC results obtained with different mobile phase electrolytes, ammonium acetate and

Table II. Effect of ammonium acetate concentration on k' values of various fluorescence labeled PE derived from egg PE

Subcomponent	k'**				
Mobile phase* NH_4OCOCH_3 concentration	PL compound				
	FL-PE	DAN-PE	PY-PE	NBD-PE	NRD-PE
50.0 mM					
1	6.0 (7.25)	13.6	16.4	7.2	9.7
2	9.1 (10.6)	22.0	25.2	10.6	14.4
3	14.2 (17.2)	34.6	40.2	16.5	23.4
12.5 mM					
1	3.4	7.2	9.0	5.1	6.5
2	5.0	11.2	13.8	7.7	10.0
3	7.4	17.4	21.2	11.8	16.2

* Mobile phase:acetonitrile-methanol-water (70:28:2) containing variable concentrations of ammonium acetate.
**Values in parentheses are for the additional FL-PE peaks further resolved.

dodecyltriethyl ammonium phosphate (DTAP). The presence of a hydrocarbonaceous electrolyte (DTAP) at a relatively low concentration (5 mM) in the mobile phase considerably enhanced hydrophobic interactions between PY-PE (and DAN-PE) solutes and the octadecylsilica phase. For three other derivatives (FL-PE, NBD-PE, and NRD-PE), much higher concentrations of electrolytes were required to show significant differences between the k' values obtained with

Table III. Comparison of retention data for fluorescence labeled PE studied in two different mobile phase electrolyte systems

Subcomponent	k'**				
Mobile phase* electrolyte (5 mM)	PL compound FL-PE	DAN-PE	PY-PE	NBD-PE	NRD-PE
Ammonium acetate					
1	3.6 (4.0)	10.4	12.6	6.4	10.2
2	5.6 (6.2)	17.0	20.5	10.4	16.2
3	9.4 (10.6)	28.2	35.4	16.8	27.8
Dodecyltriethyl ammonium phosphate					
1	3.2	27.4	39.8	9.4	13.0
2	5.4	45.0	64.2	14.8	19.8
3	8.6	78.1	111	25.6	33.3

* Mobile phase:acetonitrile-methanol-water (70:25:5) containing an electrolyte (indicated above) at pH 6.5.
**Values in parentheses are for the additional FL-PE peaks further resolved.

the two electrolyte systems. The ammonium acetate mobile phase appeared to have a greater tendency for resolving the a-b subcomponents than the tetraalkyl ammonium phosphate mobile phase. Examination of HPLC data compiled in Tables I-III revealed that variations in the magnitude of k' values among the PL derivatives reflect disparity in polarity of different PL structures. Accordingly, the retention order (k'-PY-PE > k'-DAN-PE > k'-NRD-PE >

k'-NBD-PE > k'-FL-PE) for the PE compounds in the series follows an increasing order of polarity of the polar lipids (PY-PE < DAN-PE < NRD-PE <NBD-PE < FL-PE).

Examples for analyses of dansylated PL derived from other natural sources are presented in Fig. 3. For separations of dansylated egg PE (Fig. 3A) and dansylated soybean PE (Fig. 3B), there was a striking similarity between the separation profiles obtained with mobile phases containing ammonium acetate and those with mobile phases containing tetraalkyl ammonium phosphate reported previously (23). On the other hand, HPLC of dansylated brain PS with ammonium acetate mobile phase produced peak broadening and tailing (Fig. 3C). The carboxyl group in PS seemed to cause severe adsorption of the PS components on the reversed-phase column. However, separations of components were substantially improved by the addition of triethylamine to the ammonium acetate mobile phase (Fig. 3C).

Capillary GC fatty acid analyses of hydrolysates of individual fractions 1a, 1b, 2a, 2b, 3a, and 3b isolated from HPLC of FL-PE (Fig. 2B) showed that these components were attributed to molecular species containing fatty acids 16:0-18:2 (1a-b), 16:0-18:1 (2a-b), and 18:0-18:1 (3a-b). When the same sample of FL-PE was analyzed under different HPLC conditions such as those used in the later LC-MS experiment with a new NovaPak column, different mobile phase conditions (see Experimental on LC-MS procedure), two additional minor components 2c and 2d were resolved (Fig. 4).

Initial attempts at using a thermospay-LC-MS technique for the analysis of FL-PE were unsuccessful. Subsequent

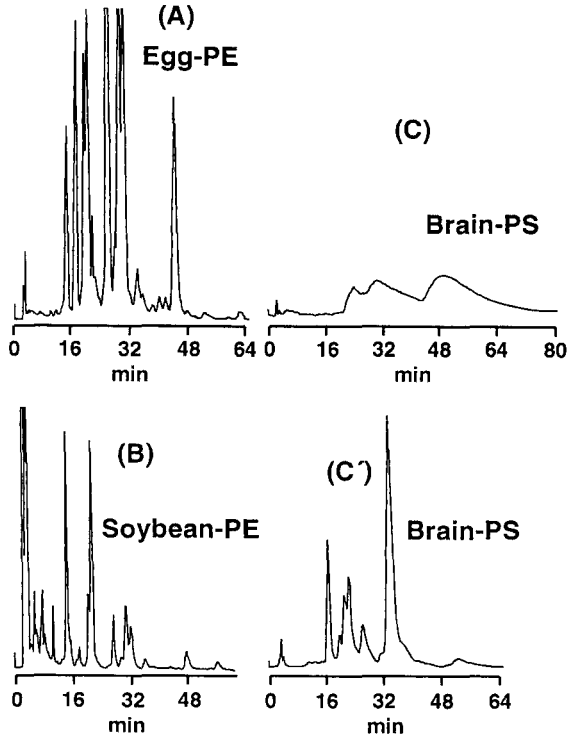

Fig. 3. HPLC separations of subcomponents of dansylated phospholipids derived from animal and plant sources. Mobile phases: acetonitrile-methanol-water (70:28:2) containing 25 mM (A, B) or 5 mM (C, C') ammonium acetate. Triethylamine (15 mM) was added to the mobile phase in (C'). Column: used NovaPak C18. Flow rate: 1 ml/min.

elaboration of a particle beam (PB)-LC-MS instrument system on the FL-PE sample met with some success. With the exception of components 3a and 3b whose intensities were too weak to be measurable, the total ion chromatogram showing the separation of components 1a, 1b, 2a, 2b, 2c,

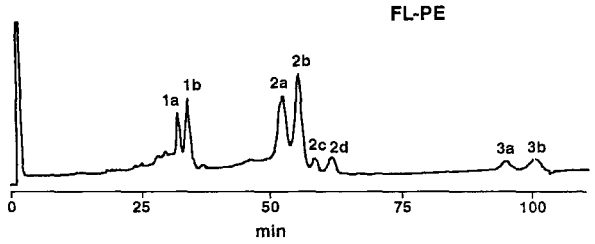

Fig. 4. HPLC separation of molecular species of fluorescein (FL) labeled phosphatidylethanolamine (PE) derived from egg phosphatidylcholine. Column: unused NovaPak C18. Mobile phase: acetonitrile-methanol- water (70:20:10) containing 35 mM ammonium acetate at a flow rate of 1.2 ml/min.

and 2d was very similar to the HPLC profile in Fig. 4. Since the PB-LC-MS system was operated in the EI mode, molecular ions of the polar lipid components were not detected. Each of the EI mass spectra of ion peaks exhibited major mass fragments of fatty acid chain moieties and those of the fluorescein fluorophore. The fragmentation patterns for the fatty acid region were particularly informative for the characterization of PL molecular species. The individual EI mass spectra of molecular species were closely superimposable with composite spectra of two standard fatty acids. The MS data (not shown here) indicated that the EI fragmentation patterns between a- and b-components (or between c- and d-component) were indistinguishable.

In summary, molecular species of fluorescence labeled PL can be analyzed by reversed-phase HPLC with ammonium acetate mobile phases. For enhancing detection of polar PL (FL-PE and DAN-PS), adsorption of analytes on a column can

be eliminated by adding triethylamine to the ammonium acetate mobile phase. The LC-MS technique used for the characterization of fatty acid structures has proven useful and may be applied to the analysis of other PL compounds.

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A NON-ION-PAIRING HPLC METHOD FOR MEASURING NEW FORMS OF ASCORBATE AND ASCORBIC ACID

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ABSTRACT

A new high-performance liquid-chromatography (HPLC) method separates vitamins L-ascorbic acid (C_1) and L-ascorbyl-2-sulfate (C_2). Previous HPLC reverse phase methods utilized ion-pairing reagents and resulted in co-elution with other substances. Vitamin C_1 preceded C_2 in retention time.

In this new method, which deletes the ion-pairing reagent, vitamin C_2 precedes the elution of vitamin C_1. This new direct method allows for a better separation from other conflicting metabolites and constituents of extraction mixtures. In addition, this method provides a way of identifying ascorbyl-2-monophosphate (C_3), a form of vitamin C that may be found in animal diets.

Examples will be shown of HPLC profiles of extractives from tissue and diets known to contain these new forms of vitamin C.

INTRODUCTION

There have been recent advances in the development of more stable forms of L-ascorbic acid, bringing increased demands for more precise and direct methods of analysis for these esters. A number of methods, which are available in the literature, offer reliable assays for vitamins C_1 and C_2 if the mixtures are not too complex (1,2,3,4,5). A recent comparison found positive and negative aspects in three different methods for the measurement of C_1 and C_2 in shrimp tissue (6).

There are a number of vitamin C_1 methods that employ ion-pairing reagents (IPR) to obtain delayed retention times. Schüep et al. (4) demonstrated very effectively the influence that IPR (1,5-dimethylhexylamine) exerted upon retention times of vitamin C_2 on an ODS Hypersil column. Using a concentration range from 0.2 to 1.0 ml/L of IPR in the mobile phase, the retention times ranged from 5.62 min. to 22.58 min.

Between 1985 and1989, this laboratory used two 10-micron C_{18} μBondapack columns(250mm x 4.6mm) in tandem. The solvent system used was a 0.1 M sodium acetate at pH 5.0 with an n-octylamine concentration of 0.17 ml/L plus 200 mg/L disodium ethylene-diamine tetra-acetic acid (EDTA). A flow rate of 1.25 ml/min was maintained. Utilizing this protocol, it was possible to maintain consistent retention times for vitamins C_1 and C_2. In a 1987 publication (3), a method was reported using the above protocol for the separation of C_1 and C_2. The retention times for that method were 6.0 min. for C_1 and 11.0 min. for C_2. In contrast, two years later, retention times were 4.8 min. for C_1 and 8.0 min. for C_2, as reported at a meeting in New Orleans (7). These retention times remained consistent for a period of about two years.

However, in 1990, when this system was put into use again for C_1 and C_2 analyses, the μBondapack column no longer produced the same results. Two additional C_{18} type columns were tried, also with varying results. Since 1990, when using n-octylamine as an IPR and C_{18} reverse-phase types of columns, this laboratory has experienced variability of retention times for C_1, and particularly C_2. Using a concentration of 0.17 ml/L and a static equilibration period of one night, as well as a mobile equilibration of 2.5 hours, the retention times varied from 5.75 min. to 6.75 min. for C_1 and from 7.5 min. to 14.5 min. for C_2. The result was an overlap of peaks, making it more difficult to quantify. The purpose of this paper is to present a way to circumvent some of the difficulties encountered when using this type of technology.

A new development in column technology eliminates the need for ion-pairing reagents, offering the possibility of an HPLC method to separate smaller, less complex molecules. In addition, this column retains many of the characteristics of existing C_{18} reverse-phase columns.

METHOD AND MATERIALS

The analytical column used is an Alltima (Alltech Associates) 5 μm C_{18} reverse-phase column, 250 mm x 4.6 mm. A hand-packed Alltima guard column is used to protect the analytical column.

The HPLC equipment used is a Perkin-Elmer Corporation(P & E) model 250 isocratic pump with an in line P&E solvent filter, a P&E model 290 UV/Vis detector, set at 254 nm, and a Bioanalytical Systems Inc. (BAS) electro-chemical detector, set at +0.72 volts. The data system is linked to a PE Nelson model 950 interface and an Epson III+ computer. The software used was the PE Nelson 2100.

Special chemicals used were n-octylamine and sweet potato acid phosphatase enzyme (Sigma Chemical Co.).

Tissue was homogenized with a Brinkmann polytron homogenizer and centrifuged with an Eppendorf minifuge. The extracts were denatured by microwave (MW) with a Panasonic commercial 600-watt oven and with trichloroacetic acid (TCA).

Two solvent systems were utilized: (1) 0.10 M sodium acetate pH 5.0 with 200 mg of sodium EDTA per liter, (2) 0.10 M ammonium acetate pH 5.0. For comparative purposes, n-octylamine at 0.17 ml/L was added to solvent (1). The first solvent was utilized whenever the need was only for quantification, whereas the second solvent was used when further purification of the peaks was desired in addition to quantification. The flow rate was 0.75 ml per minute at a pressure of 2200 psi. Flow rate should not exceed 1.0 ml/min.

Tissue extracts were made as follows: (1) The MW extract was made by homogenizing 1 volume of tissue to 2 volumes of glass distilled (GD) water for 30 sec. The homogenate was placed in a sealed Teflon tube and microwaved for 1 min. at a power setting of med-low, and then at medium setting for another 30 sec. The denatured extract was diluted to a total volume of 10 ml, re-homogenized for 1 min., and centrifuged for 4 min. at 15,000 RPM. Supernatant solution was decanted and filtered through a 0.45 µm syringe filter. The filtered solution was used for the HPLC determination. (2) The TCA extract was made by homogenizing 1 vol. of tissue to 4 vols. of GD water for 30 sec., and while homogenizing, 5 vols. of 10% TCA was added and homogenizing was continued for another 60 sec. This extract was centrifuged and filtered as above and then injected into HPLC.

RESULTS AND DISCUSSION

Three very significant results were obtained with this new method:

(1) Very consistent retention times, as well as a rapid equilibration time, were found for vitamins C_1 and C_2. Table 1 compares the retention and equilibration times between the Alltima and conventional C_{18} reverse-phase columns. The day-to-day retention times varied

TABLE 1

Equilibration time comparisons of Vitamins C_1 and C_2

STANDARD C_{18} COLUMN

HPLC Run	#1	#2	#3	#4	#5	#6	#7	#12
VIT C_1	5.6	5.7	5.75	5.78	5.8	5.83	5.92	5.95
VIT C_2	6.0	6.2	6.5	6.62	6.73	6.9	7.06	7.40

ALLTIMA COLUMN

HPLC Run	#1	#2	#3	#4	#5	#6	#7	#8
Vit C_2	4.71	4.72	4.72	4.72	4.71	Equil.	Equil	Equil.
Vit C_1	6.2	6.23	6.22	6.20	6.21	Equil.	Equil.	Equil.

Columns were eluted with 0.1 M sodium acetate pH 5.0 with 200 mg/L EDTA. The standard C_{18} column required an added 0.085 ml/L of n-octylamine as an IPR. The Alltima column did not require the IPR addition. Note in the standard C_{18} column an additional 7 runs were required before equilibrium was achieved (#6 to #12). Time in minutes.

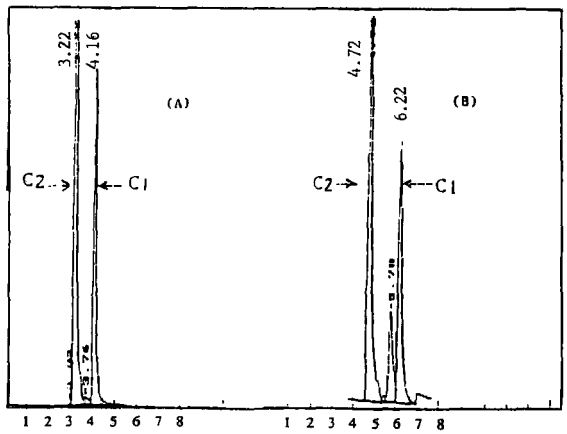

Figure 1. A. L-ascorbyl-2-sulfate and L-ascorbic acid eluting with ammonium acetate solvent. Time scale in minutes. B. Same standards eluting with sodium acetate solvent.

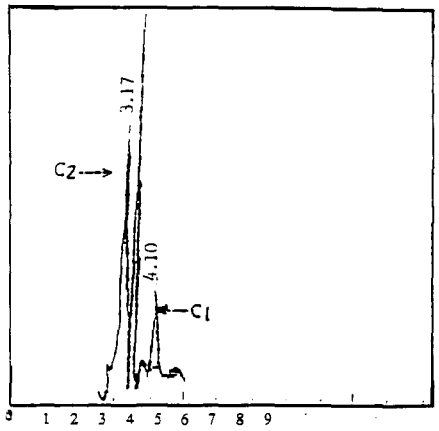

Figure 2. Detection limits for L-ascorbyl-2-sulfate and L-ascorbic acid eluted with ammonium acetate solvent (2 ng and 1 ng, respectively). Time scale in minutes.

less than 0.02 min. for both vitamins on the Alltima column. Figures 1A and 1B illustrate the retention times for the two vitamin standards in two different solvent systems on the Alltima column. Figure 2 represents the detection limits of vitamins C_1 and C_2 eluted from the Alltima column with ammonium acetate solvent (C_1 = 1 ng; C_2 = 2 ng).

(2) Using the Alltima column, vitamin C_2 elutes almost immediately after the injection volume has eluted, as compared with the longer retention time when using the IPR, n-octylamine, and the standard C_{18} column. This shorter retention time for C_2 allows for a reduced chance of co-elution with other substances. Vitamin C_1 unlike C_2 can be easily verified by electro-chemical detection as well as UV. Therefore C_1's later elution presents a lesser problem. Figure 3 illustrates the early elution of vitamin C_2 in a typical microwave extract of rainbow trout muscle.

(3) Even though their retention times are only 0.2 min. apart, vitamins C_2 and C_3 may be distinguished from one another in this system with the use of sweet potato acid phosphatase. The subsequent measurement of C_3 is indicated by an increase of C_1 concentration. The increase occurs when C_3 is dephosphorylated. This method is useful when analyzing diets containing both vitamins or when determining the stability of vitamins C_2 and C_3. Figure 4A is the HPLC profile of standards C_1, C_2 and C_3 eluted with ammonium acetate as the mobile phase. Figure 4B is the profile of the same standards pre-incubated at

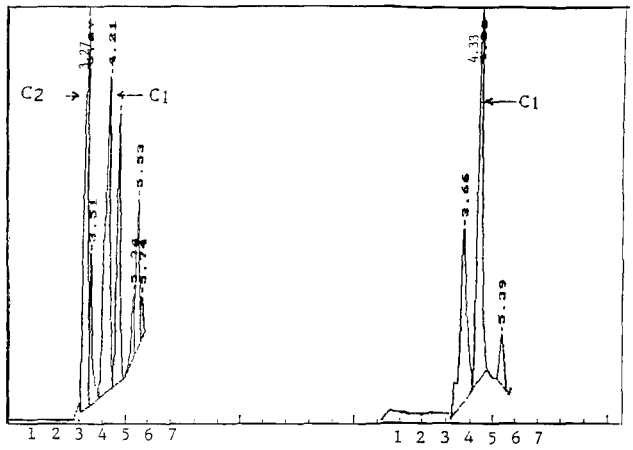

Figure 3. A typical microwave(MW) denatured extract profile(UV detector) from rainbow trout muscle which had been fed a normal vitamin C concentration of 120 mg/Kg; on the right an EC detector profile. Both were eluted with ammonium acetate. Time scale in minures.

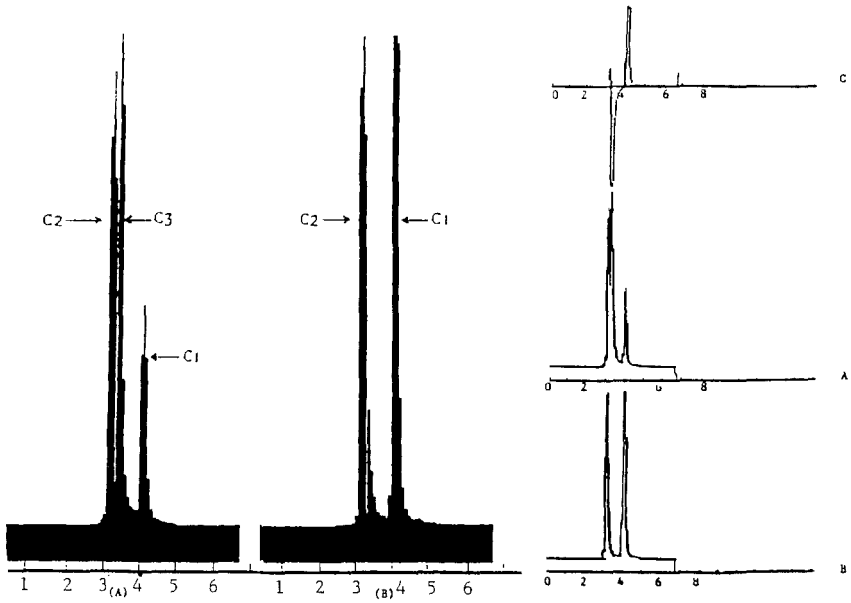

Figure 4. A. The solid profiles of standards L-ascorbyl-2-sulfate, L-ascorbyl-2-monophosphate and L-ascorbic acid eluted with ammonium acetate solvent. B. The same standards after exposure to sweet potato acid phosphatase enzyme (exposure for 30 min. at room temperature).
(Compilation) The same data shown in figure 4 A and 4 B. In the top profile (C) the difference is shown after the enzyme treatment. Note the negative profile which graphically depicts the loss of L-ascrobyl-2-monophosphate. Time scale in minutes.

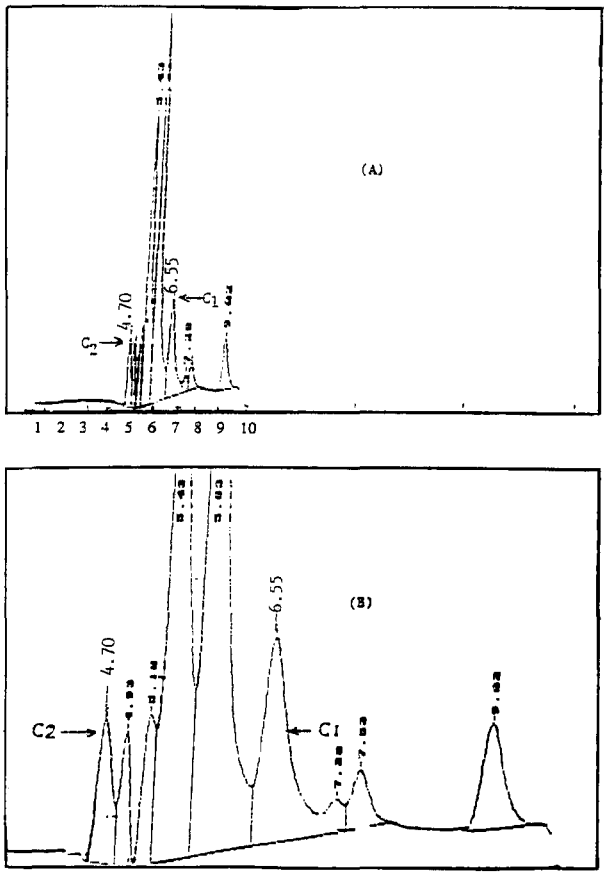

Figure 5. A. A profile of a 5% trichloroacetic acid(TCA) extract from the carcass of a coho salmon smolt fed a diet containing 120 mg/Kg L-ascorbic acid and eluted with ammonium acetate. B. An enpanded portion of the profile showing the vitamin C_2 and C_1 peaks. Time scale in minutes.

room temperature with sweet potato acid phosphatase enzyme. Note that the C_1 component of the profile has increased, whereas the C_2/C_3 double peak has disappeared. Figure 4 (compilation) is a computer-generated profile of the standards shown in the previous Figures 4A and 4B. A ratio differential after the enzyme treatment is shown in the top profile.

Figures 5A and 5B show additional uses of the Alltima column for determining C_1 and C_2 concentrations in fish tissue. Figure 5A represents a typical 5% TCA extract of freeze-dried powder from whole-body tissue of a coho salmon reared on a diet containing 120 mg/kg of C_1. Solvent system was sodium acetate. Figure 5B is a computer expansion profile of the retention time periods of C_1 and C_2. Note that the two peaks of interest are well differentiated.

CONCLUSIONS

(1) Retention times for L-ascorbyl-2-sulfate and L-ascorbate are more consistent when using the Alltima column (without an ion-pairing reagent) than when using a standard C_{18} column (with the reagent).

(2) Equilibrium is more quickly achieved using the Alltima column than when using the standard C_{18} which uses the ion-pairing reagent.

(3) L-ascorbyl-2-sulfate elutes almost immediately after the injection volume has eluted from the Alltima column (as compared with the longer time required when using the ion-pairing reagent of the standard C_{18} column). This C_2 shortened retention time reduces the chance of confusion with other eluted peaks. L-ascorbate unlike L-ascorbate-2-sulfate can be differentiated by dual detection of UV and EC. Therefore, L-ascorbate's longer elution time presents less of a problem.

(4) Salt-free eluates of desired peaks may be obtained with the use of ammonium acetate mobile phase rather than sodium acetate and the ion-pairing reagent. These eluates may then be further purified for more rigorous identification.

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THE ANALYSIS OF RO 24-4736 IN HUMAN PLASMA BY MULTIDIMENSIONAL REVERSED PHASE MICROBORE HPLC/UV

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ABSTRACT

A highly sensitive HPLC method for Ro 24-4736, 5-[3-[4-(2-chlorophenyl)-9-methyl-6H-thieno[3,2-f]=[1,2,4]triazolo[4,3-a][1,4]diazepin-2-y-1]-2-propynyl]=phenanthrydin-6(5H)-one, in human plasma was developed employing multidimensional reversed phase microbore HPLC. First dimension chromatography with a (3 cm x 2 mm i.d.) base deactivated C8 stationary phase column followed by second dimension chromatography on a diisopropyl octyl silane bonded phase HPLC column (15 cm x 1.0 mm i.d.) was used. Detection was by UV (239 nm, $\epsilon \approx 61{,}000$). Differences in the stationary bonding chemistry and pore size provide remarkable selectivity, yielding an assay method limited mainly by the inherent detector sensitivity. Differences in analyte retention caused by differences in the stationary phases allowed use of a weak mobile phase for the first dimension, focusing the analyte on the head of the second column. Use of smaller i.d. second dimension columns (2.0 mm and 1.0 mm) further concentrated the analyte and decreased the limit of quantitation to 0.05 ng/ml. Plasma concentration versus time profiles of Ro 24-4736 following oral doses in man were obtained using this method. The use of

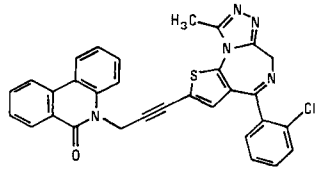
multidimensional chromatography with two reversed phase columns of different selectivity may provide a general technique for exploiting the advantages of microbore chromatography.

INTRODUCTION

Ro 24-4736 (I) (Figure 1) is a highly potent platelet aggregating factor receptor antagonist. A multidimensional reversed phase HPLC assay procedure with UV detection for the quantitation of I in human plasma using Ro 24-3729 (II) (Figure 1) as the internal standard has been developed.

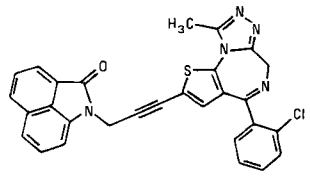
A very sensitive HPLC method for I in human plasma was needed to support drug development studies. As is so often the case, single column HPLC detection was limited not by inherent detector sensitivity, but rather by co-elution of endogenous components. A multidimensional HPLC method was developed (1,2), employing sample cleanup by liquid-liquid extraction (3-9) into hexane/methylene chloride. First dimension chromatography included a short base deactivated C8 HPLC column (3 cm x 2 mm i.d.), then second dimension chromatography on an RX-C8 microbore HPLC column (15 cm x 1 mm i.d.). Direct transfer, using a switching valve, was used as a means of transferring I and II from the first dimension column to the second dimension column. The effect of stationary phase chain length in the first dimension, mobile phase composition (1,2), column temperature, and the use of

I



Ro 24-4736

II



Ro 24-3729

Figure 1. Molecular Structures for Ro 24-4736 (I) and Ro 24-3729 (II).

conventional bore, narrowbore, and microbore columns in the second dimension were investigated. With UV detection at 239 nm, the limit of detection was 1 ng/ml using a 4.6 mm i.d. column, 0.1 ng/ml using a 2.0 mm i.d. column, and 0.05 ng/ml using a 1.0 mm column.

EXPERIMENTAL

Chemicals and Materials

Ro 24-4736 (I) was obtained from the Quality Control Department and Ro 24-3729 (II) was obtained from the Medicinal Chemistry Department, Hoffmann-La Roche, Inc., Nutley, NJ 07110 (U.S.A.). All solvents were HPLC grade

and obtained from Fisher Scientific, Fairlawn, NJ 07410. Ammonium acetate, ACS grade, was obtained from J.T. Baker Inc., Phillipsburg, NJ 08865. Distilled water was purified with a Milli-Q UF Plus water purification unit, Millipore Corp., Bedford, MA 01730. Human plasma was obtained from Rockland Inc., Gilbertsville, PA 19525.

Instrumentation

A multidimensional liquid chromatography system is used for this assay (Figure 2). The HPLC instrument consisted of a WISP 712 autosampler, Waters Div., Millipore Corp., Milford, MA 01757; two Beckman model 126 gradient HPLC pumps, Beckman Instruments, San Ramon, CA 94583; two SpectraFocus UV detectors, Spectra-Physics, San Jose, CA 95134; a Chrompak HPLC column oven, Chrompak Inc., Raritan, NJ 08869; and a Rheodyne model 7163-031 (120V AC) solenoid valve kit, Rheodyne Inc., Cotati, CA 94928. Data collection was carried out by a P.E. Nelson 3000 series chromatography software with a model 960 Intelligent Interface, Perkin-Elmer Systems Inc., Cupertino, CA 95014. A relay module consisting of a 5 volt power supply and two single pole double throw relays allowed contact closures on the A/D module to control the solenoid valves, and thereby change the position of the switching valve during the chromatographic run.

It is essential that the first dimension column be thermostated in a column oven to allow accurate

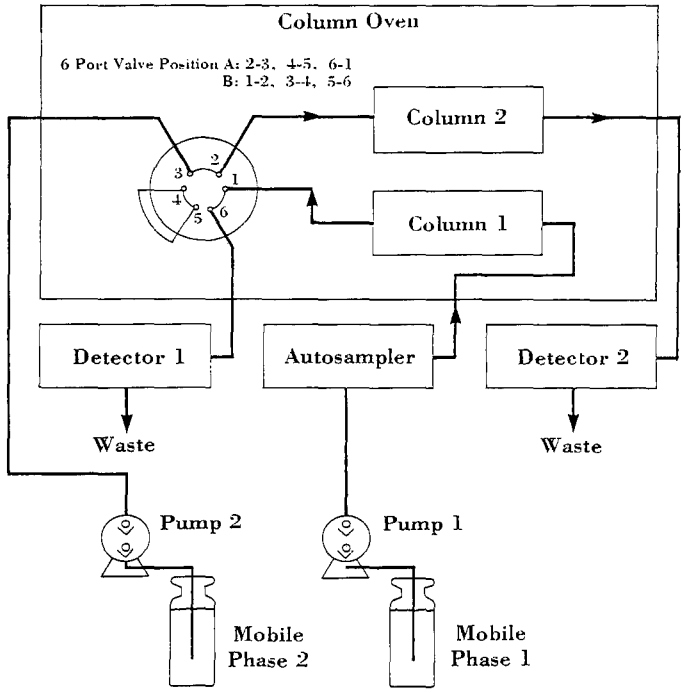


Figure 2. Schematic Representation of the Multidimensional HPLC System.

collection of the peaks of interest within the time window set by the analyst. The column switching valve and the second dimension column are also contained in the column oven.

The switching valve is activated using compressed air controlled through dual Rheodyne 120V AC air solenoids switched on and off by contact closures on the Nelson Analytical chromatography data system. Timed event commands in the chromatography data system method

control the contact closures. A switching valve can often be ordered as a built in feature on integrated instruments and controlled through the instrument data system.

Chromatographic Procedure and Conditions

The microbore chromatographic system employed a narrowbore reverse phase column (column 1), C8-BD, 3 cm x 2 mm i.d., ES Industries, Berlin, NJ 08009, and a microbore column (column 2), Zorbax RX-C8, packing from MacMod Inc., Chadds Ford, PA 19317, 15 cm x 1 mm, column packed by ES Industries.

The conventional bore assay used a 4 mm x 5 cm C8-BD column in the first dimension and a 4.6 mm x 15 cm RX-C8 column in the second dimension. The narrowbore assay utilized a 3 mm x 3 cm C8-BD column in the first dimension and a 2.1 mm x 15 cm RX-C8 column in the second dimension.

The mobile phase for the first dimension column of the microbore system was acetonitrile/ammonium acetate buffer (pH 4.8; 0.1 M) (40:60 v/v) (mobile phase 1) with a flow rate of 0.2 ml/min.; for the second dimension column, the mobile phase was acetonitrile/ammonium acetate buffer (pH 4.8; 0.1 M) (60:40 v/v) (mobile phase 2) with a flow rate of 0.05 ml/min. Flow rates for the narrow bore and conventional bore systems were scaled up based on the ratio of the square of radii for the

respective columns of the respective systems, relative to the column radii and flow rates of the microbore system. The HPLC columns were thermostated at 42 °C. Both detectors were set for UV detection at 239 nm.

With the six port valve in position A (Figure 2), the sample was injected onto the C8-BD column (column 1). Just before the analytes started to elute from column 1, the valve was timed to switch to position B. This allowed the direct transfer of the eluent from column 1 to column 2, the microbore reverse phase column, where the analytes were further separated from endogenous components. As soon as the analytes were completely transferred to column 2, the valve was switched back to position A. A synthetic mixture containing 20 ng of **I** and **II** in reconstitution solvent was injected before each set of samples to determine correct valve timing, and the data system timed events file controlling the relays was modified accordingly. Valve timing was set so as to minimize collection of the first dimension eluent before and after the elution of the peaks of interest.

Sample Preparation

Calibration standards were prepared in duplicate by adding 1.0 ml aliquots of heparinized human plasma, 50 µl aliquots of **I** spiking solutions and 50 µl of **II** (internal standard) working solution (in acetonitrile) to 16 x 100 mm disposable culture tubes. The tubes were then vortex

mixed. Standards contained 0.10, 0.20, 0.50, 1.0, 2.0, 5.0, 10.0, and 20.0 ng/ml of **I** and 20 ng/ml of **II**. For experimental samples, 50 μl of **II** (internal standard) working solution and 50 μl of acetonitrile were added.

Sample Extraction

To each sample, 5.0 ml of hexane/methylene chloride (9:1 v/v) were added. The samples were vortex mixed vigorously for 15 minutes. They were then centrifuged for 5 minutes @ 1500 RPM @ 10 °C. The organic layer from each sample was transferred to a 16 x 100 mm disposable culture tube. The samples were then evaporated to dryness under a stream of nitrogen in a 40 °C water bath. Each sample was reconstituted with 50 μl of acetonitrile/ammonium acetate buffer (pH 4.8; 0.1 M) (25:75 v/v). These reconstituted samples were vortex mixed for 2 minutes on a multitube vortex mixer and transferred to autosampler vials with limited volume inserts, ready for injection.

Sample Analysis and Calculation

One each of the duplicate set of calibration standards was injected at the beginning and the end of each sample analysis run, bracketing the quality assurance standards, control blanks and experimental samples. From the peak height ratios of **I** to **II** (internal standard), a linear calibration curve was constructed. The slope and the intercept of the

calibration curve were calculated by using weighted ($1/y^2$) linear regression. Concentrations of **I** were calculated from the peak height ratio of samples with the use of the parameters obtained above.

Sample Stability

The stability of **I** in human plasma was evaluated at room temperature over four time periods. (**I**) was spiked into drug free human plasma, which had been collected in the presence of sodium heparin anticoagulant. Replicates of six samples were left on the benchtop for periods of 24, 6, 3, and 0 hours. They were then all analyzed simultaneously.

The stability of **I** in human plasma through three cycles of daily freezing and thawing was investigated. (**I**) was spiked into drug free human plasma which had been collected in the presence of sodium heparin anticoagulant. Each day, triplicate samples stored at -20 °C were removed and allowed to thaw and equilibrate at room temperature. Aliquots were then analyzed and the samples refrozen. The procedure was repeated on the second day. The control samples were only thawed once for analysis.

Recovery

Absolute recovery of **I** from human plasma was determined by comparing the mean of the peak heights from a set of six replicate extracted samples to a set of six

replicate unextracted samples. The extracted samples were prepared by spiking **I** into human plasma at a level of 4 ng/ml and extracting as described above. The unextracted samples were made by spiking **I** into extracted control blanks prior to reconstitution.

RESULTS AND DISCUSSION

Method Development

The initial, single dimensional chromatographic system failed to separate **I** from endogenous components. Co-elution of endogenous components with the analyte limited sensitivity to 25 ng/ml. With multidimensional chromatography, differences in stationary phase bonding chemistry and pore size provide remarkable selectivity, yielding an assay method limited mainly by the inherent detector sensitivity.

Variations of sample preparation and HPLC conditions were examined. Two sample preparations were investigated. An acetonitrile protein precipitation used in initial method development, which failed to provide adequate sample cleanup and resulted in practically no first dimension column life, was changed to the presently accepted liquid-liquid extraction. A multidimensional HPLC system was required to separate **I** from co-eluting endogenous peaks. The original multidimensional assay had a lower limit of quantitation of 1.0 ng/ml using

conventional bore columns in the first and second dimension. A more sensitive method was needed to measure plasma levels of **I** following low doses to support drug development studies. Multidimensional narrowbore and microbore HPLC/UV assays were developed with a lower limit of quantitation of 0.1 ng/ml and 0.05 ng/ml, respectively.

In each case, the analyte was preconcentrated on the head of the second column, since the first dimension mobile phase had a much lower organic content than the second dimension mobile phase. This resulted in peak compression, and therefore, increased sensitivity. Since the compressed peak gave very good resolution, it was possible to further enhance sensitivity through improved signal to noise by using a relatively long flow cell (6 mm, 9 μl) with a wide bore (1.4 mm).

Figure 3 shows first dimension chromtograms, matrix free synthetic mixture of **I** and **II**, and a sample human plasma injection. Figure 4 shows chromatograms of both spiked and experimental samples. A typical run time for plasma samples was 20 minutes. Typical retention times for **I** and **II** on column 2 were 12.9 and 11.5 minutes, respectively. Control blanks showed no interfering peaks from endogenous components of the plasma matrix, and the analyte peak at the low concentration limit was easily detectable. Baseline resolution of **I** and **II** at the high

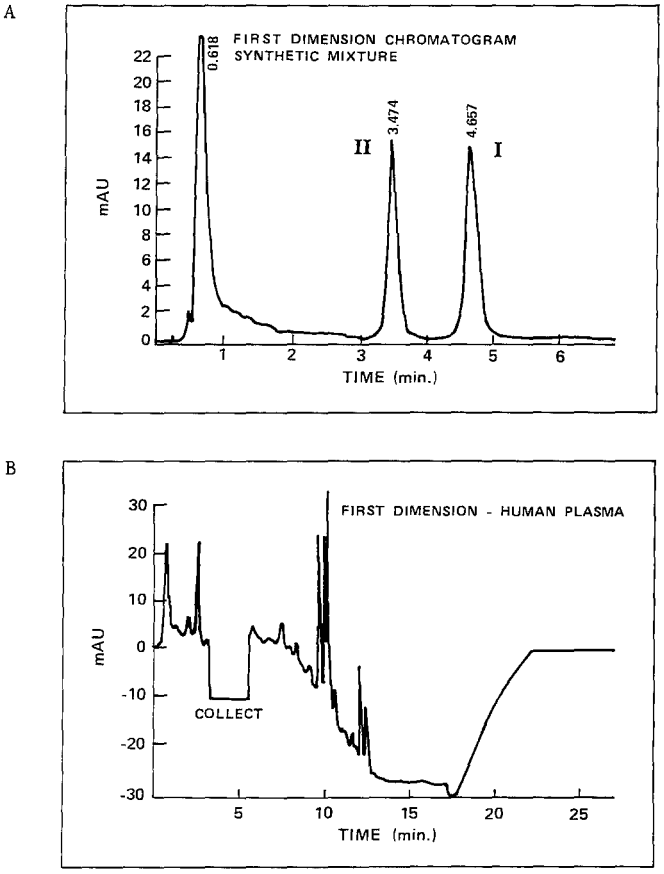


Figure 3. First Dimension Chromatograms: A) Synthetic Mixture of **I** and **II**; B) Human Plasma Injection Showing Collection of Analyte and Internal Standard into Sample Loop.

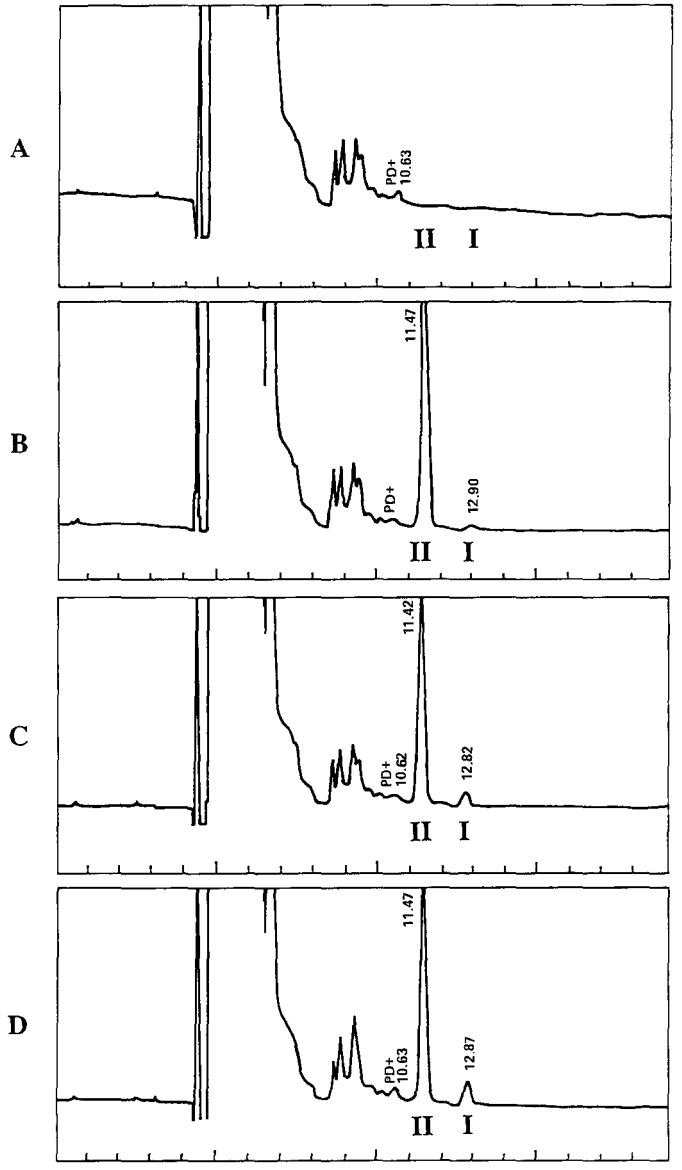


Figure 4. Typical Second Dimension Chromatograms of both Spiked and Experimental Samples: A) Human Plasma Blank; B) 0.1 ng/ml of **I** and 20 ng/ml of **II** in Human Plasma; C) 3.0 ng/ml of **I** and 20 ng/ml of **II**; D) Low Dose Experimental Sample.

concentration limit was still obtained. Operation of the column oven at 42 °C provided a stable temperature environment and high column efficiency through improved mass transfer.

Linearity

The response factor (analyte peak height ÷ [internal standard peak height x analyte concentration]) differed by less than 5% from the mean response factor for all calibration levels, indicating good linearity for the method.

Stability

The objective was to determine if **I** was stable under conditions in which plasma samples may be subject to during normal sample preparation. Analysis of plasma which had been allowed to stand for 0, 3, 6, and 24 hours at room temperature yielded mean ± S.D. values of 7.87 ng/ml ± 0.39 (n=6), 7.78 ng/ml ± 0.04 (n=6), 7.87 ng/ml ± 0.07 (n=6), and 7.72 ng/ml ± 0.07 (n=6), respectively. The mean concentration of **I** did not appear to change for as long as 24 hours under benchtop conditions. The percent difference from time zero to 24 hours is only 1.8%.

The stability of **I** to three cycles of daily freezing and thawing was intended to reproduce the conditions which plasma samples may be subject to if reanalysis was required. The difference between the first and third

cycle was -4.3%. (I) is stable in human plasma after three freeze/thaw cycles.

Recovery

Recovery of I was found to be acceptable at 79.3%. The recovery of II was not determined.

Precision

The inter-assay precision was estimated by determining the mean and the percent relative standard deviation for the values obtained for the high and low quality assurance samples on three separate days. The %R.S.D. for the low (0.35 ng/ml) quality assurance sample was 4.60%, while the %R.S.D. for the high (3.0 ng/ml) quality assurance sample was 8.15%. The overall %R.S.D. (average of %R.S.D.'s for the high and low standards) was 6.38%, indicating excellent day to day reproducibility.

Reliability

No problems in reliability were observed related to the increased complexity of the multidimensional system. Changing the precolumn filter element after every third tray was required to keep back pressure low on the first dimension system. No problems were encountered with the column switching valve. The first dimension column must be replaced when resolution between I and II is being reduced due to severe peak tailing. The first dimension column life is estimated to be approximately 250 injections. First dimension column life can be further

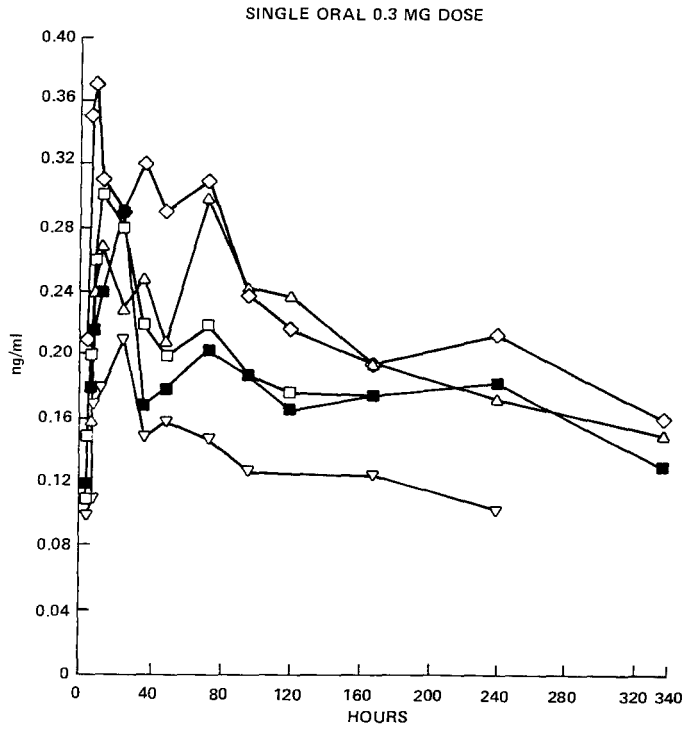


Figure 5. Plasma (I) Concentration vs. Time Profile: 0.3 mg Dose Given to Five Subjects.

extended by employing gradient elution after **I** and **II** have eluted from the column. This also prevents late eluting endogenous components from co-chromatographing with the analyte or internal standard in subsequent chromatographic runs.

Application to Experimental Samples

The plasma concentration versus time curves for 5 subjects given a 0.3 mg oral dose of compound **I** are shown in Figure 5.

CONCLUSIONS

A sensitive and reliable multidimensional HPLC/UV analytical method has been developed for the determination of **I** in human plasma. Liquid-liquid extraction of **I** and an internal standard, **II**, into hexane/methylene chloride (9:1 v/v) serves to separate the analytes from the bulk of the endogenous plasma components. The sample is injected onto the first dimension column containing a C8-BD packing. The portion of the eluent from this column containing **I** and **II** are transferred by switching a Rheodyne 7010P pneumatically actuated six port valve onto the second dimension reverse phase column, which separates **I** and **II** from endogenous components co-eluting with them on the first column. UV detection, using a wavelength of 239 nm, provides excellent sensitivity and selectivity. The high sensitivity of the method allows quantitation of **I** to 0.05 ng/ml using a 1.0 mm i.d. column; 0.1 ng/ml using a 2.0 mm i.d. column; and 1.0 ng/ml using a 4.6 mm column.

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DETERMINATION OF CARBAMATE INSECTICIDES IN WATER BY C-18 SOLID PHASE EXTRACTION AND QUANTITATIVE HPTLC

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ABSTRACT

N-Methylcarbamate insecticides were extracted from water using solid phase extraction with a C-18 column. The column eluate was chromatographed on a high performance preadsorbent silica gel plate, the pesticides were detected with p-nitrobenzenediazonium fluoborate reagent, and quantification was performed by densitometric scanning. Recoveries of carbaryl, carbofuran, methiocarb, and propoxur at 0.5 to 5 ppm fortification concentrations averaged 96.8% with a range of 82.5 to 112% and an average standard deviation of 7.5% for triplicate determinations.

INTRODUCTION

In previous papers, methods based on solid phase extraction (SPE) and quantitative silica gel TLC were reported for the determination of chlorophenoxy acid and triazine herbicides (1), organochlorine insecticides (2), and organophosphorus insecticides (3). Earlier TLC analyses of water for N-methylcarbamate insecticides used conventional sequential solvent extraction methods (4) and were often based on visual estimation of spot area (5). This paper extends the SPE/quantitative TLC methodology to

four N-methylcarbamate insecticides. C-18 SPE columns were used for extraction, preadsorbent high performance silica gel layers for separation, and p-nitrobenzenediazonium fluoborate reagent for detection prior to densitometric scanning.

EXPERIMENTAL

Standards

Standards of carbaryl (Sevin), carbofuran (Furadan), methiocarb (Mesurol), and propoxur (Baygon) were obtained from the EPA Pesticide and Industrial Chemicals Repository (Las Vegas, NV). Individual stock standard solutions were prepared with concentrations of 5.00 mg/ml in ethyl acetate. These solutions were diluted 1:50 with ethyl acetate to prepare TLC standards with concentrations of 100 ng/ul. Stock solutions were stored in a freezer and TLC standards in a refrigerator when not in use.

Thin Layer Chromatography

TLC was carried out on 10 x 10 cm Whatman LHPKDF laned, high performance preadsorbent silica gel plates. Standards and samples from the C-18 SPE column were applied to the preadsorbent using a Drummond (Broomall, PA) digital microdispenser, and plates were developed for a distance of 7 cm beyond the preadsorbent in a paper-lined, vapor saturated Camag (Wilmington, NC) HPTLC twin-trough chamber with toluene-acetone (4:1) for carbaryl, carbofuran, and methiocarb or hexane-acetone-chloroform (75:15:10) for propoxur. After drying with warm air from a hair drier for ca. 5 min., pesticide zones were detected by dipping the plate into 1.0 M KOH in methanol held in a Desaga (Whatman, Clifton, NJ) dipping chamber, drying again with the hair drier, and dipping into freshly prepared chromogenic reagent, prepared by dissolving 25 mg of p-

nitrobenzenediazonium fluoborate in 90 ml of acetone plus 10 ml of diethylene glycol (4). Zone areas were measured by scanning with a Shimadzu (Columbia, MD) CS-930 densitometer in the single beam, reflectance mode at the wavelength of maximum absorption for each compound, as determined from the in situ spectra recorded between 400 and 700 nm with the spectral mode of the densitometer. Percent recovery was determined by comparing the areas of samples with standards representing 100% recovery.

Analysis of Samples

Samples were analyzed by use of J.T. Baker (Phillipsburg, NJ) 6 ml Bakerbond light loaded octadecyl SPE columns (no. 7189-07) and a J.T. Baker glass manifold no. 7018-00 operated with a vacuum of 15-20 mm of mercury to produce a flow rate of ca. 8 ml/min.. Each column was prewashed with two column volumes of ethyl acetate, one of methanol, and one of deionized water. The manifold stopcock below the column was adjusted to prevent the column from becoming dry during or after the conditioning stages. Sample was added through a 75 ml plastic reservoir attached to the column, followed by 1 column volume of deionized water. The column was dried by drawing vacuum for 15 minutes, and removed from the manifold. Pesticides were eluted into a 4 or 5 ml graduated vial with a tapered bottom by forcing 2 ml of ethyl acetate through the column with gentle pressure from a rubber bulb. The vial was placed in a 30-50°C water bath, and the ethyl acetate was evaporated within 10 min. by a flow of nitrogen gas. The residue was reconstituted in 2.00 ml of ethyl acetate, and three 2.00 ul aliquots of sample (representing 500 ng if recovery is 100%) and three 5.00 ul aliquots of standard (containing 500 ug of pesticide) were spotted

for TLC. The averages of the two closest standard and sample areas were compared to calculate percent recovery.

Initial recovery studies were carried out by analyzing deionized water fortified with the pesticides, and the applicability of the method to a real sample was tested by spiking a pond water sample that was preanalyzed and found not to contain any of the carbamate pesticides. Water was fortified at concentration levels of 5.00, 2.00, 1.00, and 0.50 ug/ml (ppm) by adding 100 ul of the stock pesticide solution (500 ug) from a 100 ul Drummond digital microdispenser to 100, 250, 500, and 1000 ml, respectively.

RESULTS AND DISCUSSION

Carbaryl, carbofuran and methiocarb appeared as flat, compact bands with respective R_f values of 0.55, 0.49, and 0.52 in toluene-acetone (4:1), while the propoxur band had a value of 0.30 in hexane-acetone-chloroform (75:15:10). Visible zone colors and wavelengths of maximum absorption from in situ spectra were as follow: carbaryl-blue, 610 nm; carbofuran and propoxur-purple, 550 nm; and methiocarb-pink, 510 nm. The plate background ranged from white to pale yellow. The variation in R_f values and zone colors aid in identifying unknown carbamates.

Aliquots of 3.00, 5.00, and 7.00 ul of carbaryl standard solution (containing 300, 500, and 700 ng) were spotted and developed, and the calibration equation of scan area versus weight, calculated using a linear regression program on a personal computer, had a linear regression coefficient of 0.999. This relationship permitted the use of a quantification method for determination of recovery based on area comparison between samples

and standards within this linear range on each plate. Areas of duplicate aliquots typically agreed within 3-5%. Plates were normally scanned immediately after zone detection. They could be stored if wrapped in aluminum foil, but colors faded within a few hours if exposed to light. To quantify unknown samples, apply extract samples and standards in the range of 1.00-10.0 ul on each plate, and compare the areas of the sample and standard zones that compare most closely, preferably within +/-25%.

Ethyl acetate was found to be a superior eluting solvent for a range of pesticides by Junk and Richard (6). The ethyl acetate column eluate contained 0.1 to 0.3 ml of water as a second phase. This water remained in the vial after nitrogen blowdown and reconstitution but was of no concern. Water could not be completely eliminated by prolonged vacuum drying nor by passing hexane through the column prior to the ethyl acetate eluent.

Recovery tests for the four carbamates in deionized water were carried out in triplicate. Respective average percent recovery values for carbaryl, carbofuran, methiocarb, and propoxur were 99.2, 97.1, 82.5, and 100.6 at 5 ppm and 96.5, 90.3, 94.6, and 112 at 2 ppm. Recovery of carbaryl at 1 ppm averaged 99.4%, and at 0.5 ppm 95.5%. Standard deviations ranged from 2.0 to 12%, with a mean of 7.5%. These recovery and precision values are acceptable for trace residue analysis at the low ppm level (7).

Separate 250 ml samples of pond water from a local farm, which was shown to contain no carbamate pesticides by prior analysis, were spiked with 1 ppm of carbaryl and carbofuran and analyzed in duplicate by the SPE/HPTLC method. Recoveries averaged 95.0 and 91.5 %, respectively. There were no interfering zones detected in the chromatograms of this sample. The selectivity of the detection

reagent was further demonstrated by chromatographing the related pesticides BPMC (fenocarb), aminocarb, aldicarb, and methomyl. None of these compounds was detected at the at the 1 ug level.

The purpose of this research was to demonstrate with a limited number of pesticides and samples that the SPE/HPTLC approach can be utilized successfully for determination of carbamate insecticides in water samples. Extraction efficiency, accuracy, and precision are adequate for routine use in water analysis. The method combines the convenience and low solvent consumption of SPE with the simplicity and high sample throughput of preadsorbent quantitative HPTLC. It will be applicable to the analysis of any water sample not containing co-extractable impurities that interfere with the chromatography, detection, or scanning of the analyte. It can be used to analyze lower concentrations of pesticides by passing greater volumes of water through the SPE column.

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HPLC SEPARATION AND DETERMINATION OF PHENOLPHTHALEIN AND ITS GLUCURONID AS MARKERS OF ENTEROHEPATIC CIRCULATION USING ACETONITRILE-METHANOL AS ORGANIC MODIFIER AND APPLICATION TO RAT PLASMA

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ABSTRACT

An isocratic-HPLC method for the separation and determination of phenolphthalein and its metabolite phenolphthalein-glucuronide, using bromocresol purple as an internal standard, a mobile phase methanol-cetonitrile-50 mM phosphate buffer (pH 7.6) (35:10:55, v/v/v) and a 3 μm reversed-phase C_{18} column (50 x 4.6 mm i.d.), is described. The flow-rate was 1 ml/min and the UV detector wavelength 230 nm. This method extends a prior study of the most important variables that can affect the retention of these compounds (i.e., concentration of acetonitrile as organic modifier, buffer concentration and pH). The proposed method has been applied to the determination of phenolphthalein and phenolphthalein-glucuronide in rat plasma using solid phase extraction.

INTRODUCTION

Phenolphthalein (P), its metabolite phenolphthalein-glucuronide (PG), and bromocresol purple (BCP) are compounds derived from triphenylmethane skeleton. The presence of polar subtituent groups enhance their water solubility and their existence as neutral, anionic or cationic species depending on the pH of the medium[1].

In a previous paper[2], a HPLC separation of P and PG as markers of enterohepatic circulation, using BCP as an internal standard (IS), was reported. This method uses a mobile phase MeOH-50 mM phosphate buffer (pH 7.7) (47.5:52.5, v/v) and a reversed-phase C_{18} column, with UV detection. Moreover, an inversion in the elution order of BCP and P was observed depending on the pH buffer and phosphate buffer concentration used or when other additives such as triethylamine or acetic acid were added to the mobile phase. In this paper a HPLC method with isocratic elution for the separation and determination of P and PG using a C_{18} column, is described. This method involves a previous study of the most important variables that can affect separation of this compounds, using acetonitrile (AcCN) as organic modifier. After optimizing these variables and taking into account the behavior of these compounds in MeOH[2], a mobile phase MeOH-AcCN-phosphate buffer (pH 7.6) was finally used. This method

has some advantage with regard to the method developed for methanol[2] as organic modifier (the elution order of these compounds is independent of pH buffer and buffer concentration, the peak resolution is better, the number of possibilities to be applied to biological samples increases with regard to using MeOH or AcCN alone, the expent of solvent are lower and the analysis times are very similar). The proposed method has been applied to the determination of P and PG in rat plasma using solid phase extraction (SFE).

MATERIALS AND METHODS

Apparatus

The chromatographic system consisted of the following components: a Rabbit-HP solvent delivery system equipped with two pumps and a pressure module (Rainin Instrument Co Inc., Woburn, MA, USA), a Rheodyne 20-μl loop injector (Rheodyne, Berkeley, CA, USA); a reversed-phase guard column (Rainin Microsorb C_{18}; 15 x 4.6 mm i.d.; 3μm) and a reversed-phase Rainin Microsorb C_{18} column (50 x 4.6 mm i.d., 3μm); a Knauer UV-VIS variable-wavelength monitor operating between 190 and 400 nm (Knauer, Hambourg, Germany).

An Apple Macintosh SE 30 Computer interfaced to the HPLC equipment using the Dynamax HPLC Method manager, Version 2.1 (Rainin Instrument Co. Inc. Woburn, MA, USA), was also used.

A Jouan centrifugal concentrator vacuum system (Jouan, Inc. Winchester, VA, U.S.A.) was used to evaporate samples under reduced pressure in centrifuge tubes.

For pH measurements, an Orion Digital pH-meter equipped with a Fisher 13/620/91 combined glass calomel electrode was used.

Reagents

P, PG and BCP were provided by Sigma Chemical Co (St. Louis, MO, U.S.A.). β-Glucuronidase was provided from Boehringer Mannheim Biochemicals (IN, U.S.A.). HPLC-grade Potassium Phosphate Monobasic, Sodium Acetate, AcCN and MeOH were purchased from Fisher Scientific (Fair Lawn, NJ, USA). The water used to prepare the mobile phase and all aqueous solutions was purified using a Milli-Q water purification system from Millipore (Bedford, MA, USA).

Mobile Phase

The mobile phase was prepared daily by mixing the acetate or phosphate buffer solution with AcCN (or the

mixture of MeOH and AcCN) at the required volume ratio. The pH of buffer solutions was adjusted by the addition of 1 M sodium hydroxide or 1 M hydrochloric acid.

After mixing of buffer solution and AcCN (or the mixture of AcCN and MeOH), the mobile phase was filtered and degassed using vacuum and ultrasonication, and the corresponding pH was also monitored.

In all experiments % AcCN (or % AcCN and % MeOH) and % Buffer equal 100 (v/v) (or v/v/v).

Chromatographic Analysis

After conditioning the column with mobile phase, HPLC chromatograms were obtained at room temperature with a injection of a solution of 20 µl containing 10 µg/ml of an aqueous solution of PG, and P and 20 µg/ml BCP(IS). The flow-rate was 1.0 ml/min; UV detection: 230 nm.

The composition of mobile phase, control of pumps and data acquisition for each experiment was coordinated using a computer program (see apparatus). [Two different reservoirs containing AcCN - phosphate buffer (pH fixed) (10:90, v/v), and AcCN - phosphate buffer (pH fixed) (60:40, v/v), were used. In the case of employing mixtures of AcCN and MeOH as organic modifier the reservoirs contain 10% AcCN and 40% AcCN respectively and a fixed concentration of MeOH in each one, were used].

All chromatographic measurements were performed in triplicate.

RESULTS AND DISCUSSION

Effect of AcCN Concentration

The effect of organic modifier, AcCN on the retention of the three compounds under study PG, P and BCP has been carried out using phosphate buffer (pH 6.7). Phosphate buffer concentration was varied from 10 to 70 mM. Fig. 1 shows a plot of capacity factors [$k' = (t_R - t_o)/t_o$, where t_R is the retention time of the compound and t_o the retention time of an unretained compound] for the compounds versus AcCN concentration at 50 mM phosphate buffer concentration. Other plots k' versus AcCN concentration at different phosphate buffer concentration were very similar behavior and depends slightly on the AcCN concentration.

In Fig.1 can be observed that the elution order was P/BCP/PG, with PG and BCP overlapping at higher concentrations of AcCN. A gentle increase of k' values for P and BCP, and a exponential increase for P when the AcCN concentration is decreased, were also observed.

Different separations can be carried out specially at lower AcCN concentrations, though the k' values for P are very large.

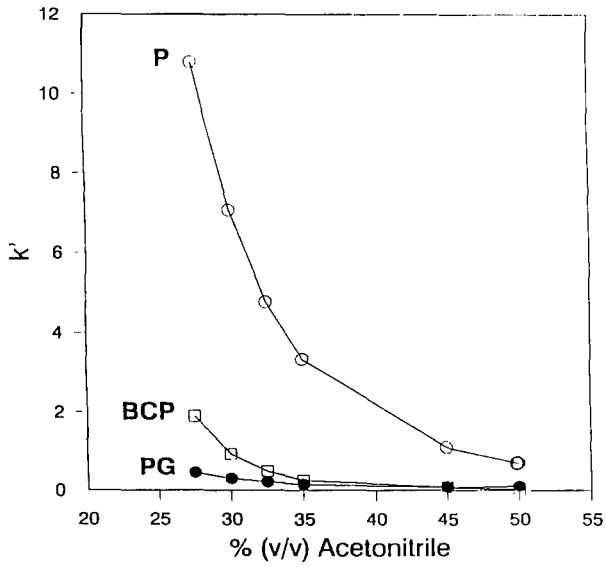

FIGURE 1. Effect of AcCN concentration on the retention of PG, P and BCP at 10 mM phosphate buffer (pH 6.7). P and PG 10 μg/ml, and BCP 20 μg/ml. Conditions: Column (see Experimental); Flow-rate, 1.0 mil/min; UV detection at 230 nm; Sample size 20 μl.

Effect of Buffer Concentration

The effect of phosphate buffer concentration on the retention of PG, P (pK_a 9.3)[3] and BCP (pK_a 6.1)[3]has been studied at pH 6.7. The phosphate concentration was varied in the range 10-70 mM at pH. 6.7. At a given buffer concentration in the range studied the elution order was always PG/BCP/P, and the retention can be explained by the rule of hydrophobic interactions when the concentration of AcCN is varied. With these experimental

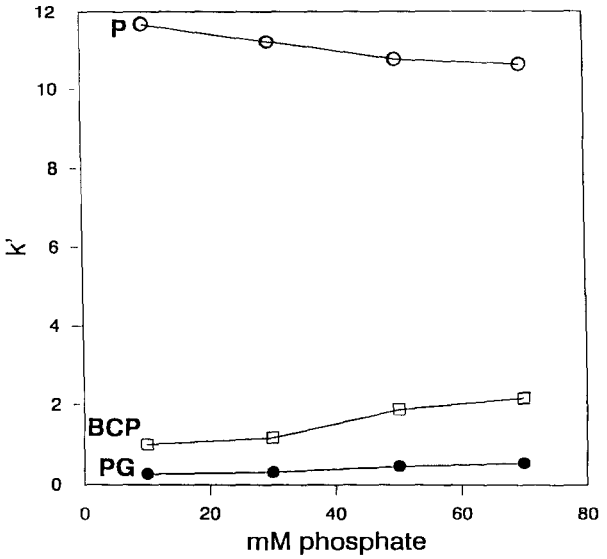

FIGURE 2. Effect of phosphate buffer concentration on the retention of PG, P and BCP. Mobile phase: AcCN-phosphate buffer (pH 6.7) (27.5:72.5, v/v). The remainder conditions as in Figure 1.

conditions different separations can be carried out. As an example of this effect, in Fig. 2 it can be observed that for 27.5% AcCN the k' values of PG and BCP increased slightly when the phosphate concentration is increased (probably due to the low buffering capacity of buffer used). However, the k' values to P decreased (i.e., k' values in the range 10-70 mM phosphate varied between 7.45 and 6.33 for 30% AcCN, and between 11.70 and 10.66 for 27.5% AcCN respectively). This indicates that the retention of P is due to a slight increase in the

dissociation process of P (salt effect) which is more important than the buffering capacity effect.

For the remainder of experiments, a 50 mM phosphate buffer concentration was selected due to the acceptable buffering capacity.

Effect of pH

The pH effect on the retention of P, PG, and BCP was studied at different pH values: pH 4.5 (50 mM acetate buffer), and pH 6.7 and 7.6 (50 mM phosphate buffer). The AcCN concentration was varied for each pH. At any constant pH, the retention decreased when AcCN concentration increased according to hydrophobic interactions.

In Fig. 3 is shown the influence of pH on the retention and separation of the compounds using 30% AcCN as a representative example. It can be observed that the elution order is PG/BCP/P. When the pH value increased a slight decrease of the k' values for PG is observed, however this decrease is more noticeable for P and specially for BCP which is practically linear, as a consequence of its dissociation process. In the case of P, this decrease can be explained by taking into account two simultaneous effects: the proximity of the pK_a value of P and the salt effect mentioned above (i.e., at pH 7.6

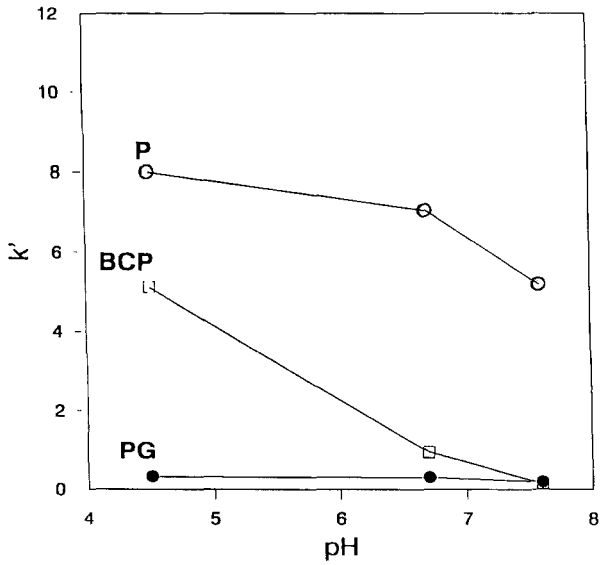

FIGURE 3. Effect of pH on the retention of PG, P and BCP. Mobile phase: AcCN-50 mM phosphate buffer (30:70, v/v). The remainder conditions as in Figure 1.

in comparison to pH 6.7 the concentration of the basic species of phosphate increases and consequently the concentration of its counter-ion).

Effect of Mixtures of AcCN/MeOH and Other Modifiers

From the results obtained above using AcCN as organic modifier it is possible to establish different analytical procedures for the separation and determination of these type of compounds. As it can be observed throughout this study, the k' values of PG are very short from the point

of view of analyzing this compound in biological samples. For increasing k' values of PG it was necessary to decrease pH or % AcCN (v/v). However, the retention of P increased greatly. The use of AcCN and MeOH mixtures as organic modifier was studied because AcCN as mobile phase elutes PG and BCP at pH 7.6 (see pH effect) at the same k' values while MeOH does not[2].

This study was performed using 50 mM phosphate buffer (pH 7.6), by fixing a given % MeOH in the range 20-35%, and varying the %AcCN in the range 10-22%. By combining simultaneously MeOH and AcCN effects on the separation of these compounds at pH 7.6, the elution order did not change in any case.

In this way, a large number of complete separations were obtained, which were always better than using MeOH or AcCN alone. In Table I is shown a summary of the k' values for PG, BCP and P at pH 7.6, at several concentrations of mixtures MeOH and AcCN. With the purpose of analyzing biological samples a mobile phase consisting of MeOH-AcCN-50 mM phosphate buffer (pH 7.6) (35:10:55, v/v/v) was finally chosen. In Fig.4, a typical chromatogram for the separation of PG, BCP and P is shown.

Appropriate mobile-phase modifiers, such as triethylamine (TEA) or acetic acid (AcOH) were added to the buffer solution above chosen to eliminate problems

TABLE 1

Capacity Factors, k', of PG, BCP and P in MeOH:AcCN: [50 mM Phosphate buffer(pH 7.6) and 50 mM Phosphate Buffer Containing AcOH* or TEA(pH 7.6)**] Mobile Phase.

Compounds	%(v/v)MeOH	%(v/v)AcCN							
		10	11.5	13	14.5	16	17.5	19	20.5
PG BCP P	20						0.90 2.31 10.0	0.70 1.41 7.21	0.41 0.92 5.61
PG BCP P	25				0.77 2.22 7.41	0.63 1.51 5.80	0.36 0.82 3.21		
PG BCP P	30			0.68 2.20 6.06	0.55 1.74 5.06	0.35 1.04 3.80			
PG BCP P	35	0.81 3.93 7.10	0.72 3.11 6.10	0.50 2.16 4.62	0.43 1.41 3.62				
PG BCP P	35*	0.82 4.12 6.52	0.65 2.92 5.52	0.53 2.21 3.61	0.41 1.60 3.01				
PG BCP P	35**	0.90 5.30 6.93	0.83 4.32 5.94	0.63 3.40 4.80	0.52 2.43 4.01				

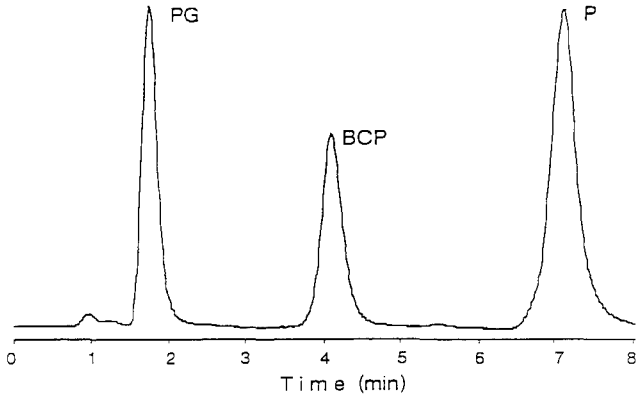

FIGURE 4. HPLC-UV chromatogram of PG, P and BCP standards. P and PG 10μg/ml, and BCP 20 μg/ml. Mobile phase MeOH-AcCN-50 mM phosphate buffer (pH 7.6) (35:10:55, v/v/v). The remainder conditions as in Figure.1.

due to secondary interactions that cause broad and tailing peaks[4]. After adding TEA or AcOH to buffer, the pH was adjusted again to pH 7.6. In this situation the effects about broad and tailing peaks are similar to those observed when only MeOH was used as organic modifier[2]. However using mixtures of AcCN and MeOH, there was no inversion in the retention order of P and BCP (see also data of k' in Table 1).

Calibration Graphs

Calibration samples were prepared in the mobile phase. Standards containing mixtures of P, PG and BCP

(IS) were made in triplicate at four different concentrations in the ranges P (0.2-20 μg/ml) using 20 μg/ml BCP. These solutions were analyzed using a mobile phase composed of MeOH - AcCN - 50 mM phosphate buffer (pH 7.6)(35:10: 55, v/v/v), a flow-rate of 1 ml/min, and UV detection at 230 nm.

The results were analyzed by linear regression. Plotting the peak area ratio (PAR) of PG or P to BCP (IS) versus the concentration (c) of PG or P, the calibration equations can be expressed in the following way: PG, PAR = 0.0142 + 0.129 c [regression coefficient (r^2 = 1.000)] and P, PAR = 0.0899 + 0.221 c (r^2 = 0.996). In both cases intercepts were not significantly different from zero.

Precision and Accuracy

The precision was examined by analysis of ten samples (n=10) of four concentrations of P and PG in the range (2-20 μg/ml), which were calculated by means of the calibration graphs. The standard deviation (SD) were in the range 0.05-0.17 μg/ml (mean 0.10 μg/ml) for PG and 0.08-0.15 μg/ml (mean 0.13 μg/ml) for P. The relative standard deviation (RSD) were in the range 0.9-2.8% (mean 1.8%) for PG and 1.1-4% (mean 2.2%) for P.

The accuracy[5-6] was assessed in the range 2-20 μg/ml for PG and P. The Barlett and Harley test[7] was applied to

the results to corroborate the randomness of the variances. A linear regression analysis was carried out on the values obtained on known concentrations and the corresponding calculated values obtained. A t-test was applied to the results and the value of the intercept was obtained. This study confirmed that the present method does not present a systematic error (i.e. it has slope value equal to unity) and does not require a blank correction (i.e. it has an intercept equal to zero).

Plasma Analysis

Samples containing P, PG and BCP (IS) were evaporated to dryness. 1 ml blank rat plama was added to the resulting residue. After vortexing for 1 min, to the sample containing plasma and compounds, 3 ml of acetone acidified with acetic acid solution was added to precipitate proteins. After vortexing for 1 min, the plasma proteins were pelleted by centrifugation for 15 minutes. An aliquot of supernatant (3 ml) was transferred to a clean tube and evaporated to dryness. The residue was resuspended in 1 ml of 0.1 M phosphate buffer (pH 7.4) and sonicated for 10 min. The resulting solution was further processed by SFE using a C_{18} (1 ml) column that was previously conditioned with two aliquots of MeOH (1 ml) followed by two aliquots of water (1 ml). The buffer

solution containing the analytes was aspirated through the conditioned column and washed with two aliquots of water (1 ml). Elution of the analytes into a clean test tube was accomplished using 1 ml of MeOH. After evaporating the methanolic eluate to dryness, the final residue was resuspended in 1 ml of HPLC mobile phase, centrifuged to remove particulates, and 20 μl injected onto HPLC.

Using this procedure, four rat plasma samples were analyzed and recoveries were in the range (81.6-88.6%) [mean 83.6% ± 2.4% (RSD)] for BCP, and 91.4-101.2% [mean 93.6% ± 5.4 (RSD) for P (based on 10 μg/ml for P and 20 μg/ml for BCP mobile phase standards and corrected for volume changes). PG was not evaluated by this extraction procedure because the blank of plasma contains a serious interference (there is a endogenous compound in plasma blank chromatograms which coelutes with the peak of PG). In order to obtain separate P and PG levels, the analysis of rat plasma samples was performed for P before and after treatment with β-glucuronidase[8]. The PG levels can can be obtained by sustraction. The recovery for PG was 87% ± 3.2 (RSD).

The method previously developed[2] and the proposed method may be applied to the study of enterohepatic recirculation in other biological fluids such as rat urine or bile, and for developing other chromatographic methods especially for determining PG an P in rat plasma

within a single run (i.e. gradient elution of PG, and isocratic one for P and BCP).

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SUCCESSFUL AND RAPID VERIFICATION OF THE PRESENCE OF A PHOSPHATE GROUP IN SYNTHETIC PHOSPHOPEPTIDES USING THE CONDITIONS OF STANDARD DABS-CL AMINO ACID ANALYSIS

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ABSTRACT

The increased importance of phosphopeptides, and the currently used post-assembly phosphorylation protocol in synthetic peptide laboratories, requires a rapid and sensitive method to verify the presence of the phosphate group in synthetic phosphopeptides. A reversed-phase high-performance liquid chromatography protocol has been developed to verify the success of the phosphorylation reaction in synthetic phosphopeptides after hydrolysis and derivatization with 4-dimethylamino-azobenzene-4'-sulphonyl chloride. Phosphoamino acid standards and model phosphopeptides were used to study the optimal elution and hydrolysis conditions of phosphoamino acids. A 1.5-hour, gas-phase acidic hydrolysis condition liberated the phosphoamino acids from the phosphopeptides, and still did not destroy them. After hydrolysis, the dabsylated free phosphoamino acids were baseline separated from the other acidic amino acids and were eluted from the reversed-phase column in the following order: phosphoserine, phosphothreonine, and phosphotyrosine. The utility of the approach was demonstrated by the phosphoamino acid analysis of several synthetic phosphopeptides, in which the amino acid environment of the phosphorylated serine or tyrosine was different. This method may not only be applicable for phosphopeptides, but also for verifying the presence of phosphate groups in phosphoproteins.

INTRODUCTION

In the last decade, understanding the biological functions of post-translationally modified proteins has become the focus of interest, and it has elevated the importance of synthetic phospho- and glycopeptides. The negatively charged phosphoryl groups are on the surface of proteins, and are known to play a crucial role in the recognition processes at both the macromolecular and cellular levels (1-5). Our laboratory is particularly interested in the physical, chemical, and biological properties of phosphorylated peptides. One series of peptides we have studied corresponds to various regions of the low molecular-weight, microtubule-associated protein τ. The neurofibrillary tangles (one of the hallmark lesions of Alzheimer's disease) are made of hyperphosphorylated forms of protein τ (6). Using synthetic phosphopeptides, we have demonstrated that conformational and immunological alterations in the protein are due to the incorporation of phosphate groups into amino acids that lack the phosphate in normal τ (7-10).

Recognition of the increasing importance of phosphopeptides demands the development of appropriate analytical methods. Analysis of unmodified peptides is most often accomplished by amino acid analysis, fast atom bombardment mass spectroscopy (FAB-MS), nuclear magnetic resonance (NMR) spectroscopy, and sequencing. All these methods are equally appropriate to verify the presence of phosphate groups on synthetic peptides, but all have drawbacks. FAB-MS and NMR are not common in the peptide synthesis laboratories. Although sequencing is more often available, all of the above listed methods tend to be expensive for the analysis of a series of phosphopeptides. Moreover, phosphopeptides tend to remain in the matrix during FAB-MS spectroscopy, especially when other charged groups are present (11). Loss of the phosphate group is also reported during NMR spectroscopy in an acidic aqueous solution (12). The conventional organic phosphate analysis (13) is a good alternative, but it requires samples in the several hundred μg range. Alternatively, the hydrophilic character of the phosphate group presents a chromatography-based analysis, since the phosphopeptides exhibit a decreased retention time compared to their non-phosphorylated analogs on reversed-phase high-performance liquid chromatography (RP-HPLC) (11,14). This may apply to the phosphorylated amino acids as well.

The standard procedure for amino acid analysis of synthetic peptides follows hydrolysis, post- or precolumn derivatization and HPLC analysis. Verification of the presence of the phosphate group on the phosphopeptides requires the detection of phosphorylated amino acids in the hydrolyzate utilizing the different conditions of amino acid analysis. The stability of phosphoamino acids under acidic conditions

is well characterized (15-16), and various derivatization methods are used for identification of phosphoamino acids, such as O-phthalaldehyde (17-21), 9-fluorenylmethyl chloroformate (FMOC) (22), phenyl isothiocyanate (PITC) (23) and 4-dimethyl-aminoazobenzene-4'-sulphonyl chloride (DABS-Cl) (24-25).

Most of the currently used phosphopeptide synthetic protocols utilize a post-assembly phosphorylation procedure (26). In an ideal case, only the phospho-peptides are present after the phosphorylation reaction, but in our experience the reaction is never complete and two peaks, corresponding to the phosphorylated and non-phosphorylated peptides, are detected after cleavage from the resin. The growing number of synthetic phosphopeptides requires a rapid, unambiguous and sensitive method to verify the success of this phosphorylation reaction. Since it was previously reported that partial acidic hydrolysis of phosphopeptides can liberate phosphoserine from the peptides (15), our aim was to work out a method to analyze phosphopeptides using standard amino acid analysis conditions. Precolumn derivatization offers a sensitivity range at a low-picomole level (27), and, in addition to high sensitivity, the advantages of the DABS-Cl method include the use of visible wavelength detection and the stability of the derivatized amino acids (28-29). Our efforts were boosted by our most recent successful development of DABS-Cl amino acid analysis of the similar acid-sensitive glycopeptides (30).

We report here the advantageous application of DABS-Cl amino acid analysis for the compositional study of synthetic phosphopeptides. Most of the studied peptides correspond to phosphorylated fragments of protein aggregates of Alzheimer's disease.

MATERIALS AND METHODS

Chemicals

Unmodified and phosphorylated peptides were synthesized and purified as previously described (26). The following peptides were investigated (* marked amino acids were phosphorylated):

GS: Z-Gly-Ser[OP(OBzl)$_2$]-OMe;

PKSPV: H-Pro-Lys-Ser*-Pro-Val-NH$_2$;

GDSKG: H-Gly-Asp-Ser*-Lys-Gly-NH$_2$;

GDRSG: H-Gly-Asp-Arg-Ser*-Gly-NH$_2$;

GDSRG: H-Gly-Asp-Ser*-Arg-Gly-NH_2;

T3: H-Gly-Ala-Glu-Ile-Val-Tyr-Lys-Ser*-Pro-Val-Val-Ser-Gly-Asp-NH_2;

T3Ala: H-Gly-Ala-Glu-Ile-Val-Tyr-Lys-Ser*-Pro-Val-Val-Ala-Gly-Asp-NH_2;

Ac-TGV11: Ac-Gly-Asp-Thr-Ser*-Pro-Arg-His-Leu-Ser-Asn-Val-NH_2;

TDG16: H-Asp-Ala-Gly-Leu-Lys-Glu-Ser*-Pro-Leu-Gln-Thr-Pro-Thr-Glu-Asp-Gly-NH_2;

HNFM 1-17: H-Glu-Glu-Lys-Gly-Lys-Ser*-Pro-Val-Pro-Lys-Ser*-Pro-Val-Glu-Glu-Lys-Gly-NH_2;

T1NM-GSR: H-Gly-Asp-Arg-Ser*-Gly-Tyr-Ser*-Ser*-Pro-Gly-Ser*-Pro-Gly-Thr-Pro-Gly-Ser*-Arg-NH_2;

TR2: H-Val-Lys-Ser-Lys-Ile-Gly-Ser*-Thr-Glu-Asn-Leu-Lys-His-Gln-Pro-Gly-Gly-Gly-NH_2;

T3+9: H-Gly-Ala-Glu-Ile-Val-Tyr-Lys-Ser-Pro-Val-Val-Ser-Gly-Asp-Thr-Ser*-Pro-Arg-His-Leu-Ser-Asn-Val-NH_2;

AT8C: H-Tyr-Ser-Ser-Pro-Gly-Ser*-Pro-Gly-Thr-Pro-Gly-Ser-Arg-Ser-Arg-Thr-NH_2;

T3TYR: H-Gly-Ala-Glu-Ile-Val-Tyr*-Lys-Ser-Pro-Val-Val-Ser-Gly-Asp-NH_2;

TGNL: H-Asn-Gln-Leu-Tyr*-Asn-Glu-Leu-NH_2;

TGRL: H-Arg-Glu-Glu-Tyr*-Asp-Val-Leu-NH_2;

Ac-PRH-Camk: Ac-Pro-Arg-His-Leu-Ser*-Asn-Val-Ser-Ser-Thr-Gly-Ser-Ile-Asp-Met-Val-Asp-NH_2, and

APP645-661: H-Leu-Val-Met-Leu-Lys-Lys-Lys-Gln-Tyr-Thr-Ser*-Ile-His-His-Gly-Val-Val-NH_2.

All peptides were characterized by conventional amino acid analysis and organic phosphate analysis (13). Phosphoamino acid standards, O-Phospho-L-Serine (L-2-Amino-3-hydroxypropanoic acid 3-phosphate), O-Phospho-L-Threonine (L-2-Amino-3-hydroxybutanoic acid 3-phosphate), and O-Phospho-L-Tyrosine (L-3-[4-Hydroxyphenyl]alanine 4'-phosphate), were purchased from Sigma Chemical Company (St. Louis, MO, USA). Hydrolysis and dabsylating reagents were purchased from Beckman (San Ramon, CA, USA), HPLC solvents and all the rest of the chemicals were from Aldrich (Milwaukee, WI, USA).

Gas-Phase Hydrolysis

Lyophilized samples (12 in 600 µl vials) and 700 µl of 6 M HCl were placed in a hydrolysis vessel (provided by Beckman; volume 113 cm^3), and then flushed with argon and evacuated at 0.1 millibar (0.00145 p.s.i.; 1 p.s.i. = 6894.76 Pa) for 1 to 2 min. The vessels were placed in a drying oven at 110 °C for 1.5 hours.

Dabsylation

The amino acid mixture (the result of hydrolysis of 2 to 5 nmol peptide or phosphoamino acid standards) was dissolved in 20 µl $NaHCO_3$-NaOH buffer (pH = 8.3), and 40 µl DABS-Cl solution (40 µg in 40 µl acetonitrile) was added (27-29). The vials were closed and placed in a drying oven at 70 °C for 12 to 14 min. After derivatization, samples were diluted with 440 µl of ethanol:water mixture (1:1), and 8% of the diluted sample was injected for HPLC analysis.

High-Performance Liquid Chromatography

The Beckman System Gold HPLC apparatus consisted of a 126 programmable solvent delivery module, a 167 scanning UV-visible detector module operating at 436 nm, an Altex 210A injector and a C_{18} Ultrasphere-DABS column (4.6 x 250 mm). The system was controlled by an IBM system 2 model 55SX personal computer with Beckman System Gold Personal Chromatography software version 6.0. The chromatographic conditions were as follows: Solvent A (final pH 6.50 ± 0.05) contained 100 ml of 0.11 M sodium citrate (pH 6.51), 860 ml HPLC water, and 40 ml N,N-dimethyl formamide (DMF). Solvent B contained 300 ml solvent A, 672 ml acetonitrile, and 28 ml DMF. The modified solvent A contained 115 ml of 0.11 M sodium citrate buffer, 845 ml HPLC water, and 40 ml DMF. The flow rate was 1.4 ml/min. The gradient conditions were as listed in Table 1. The solvent vessels were continuously flushed with argon. All runs were done at room temperature.

RESULTS AND DISCUSSION

Phosphoamino Acid Standards and Hydrolysis Conditions

We studied the elution profile of the dabsylated phosphoamino acids in our system, utilizing the regular amino acid analysis conditions. The unhydrolized and

TABLE 1
Solvent Composition During Reversed-Phase HPLC

	Time (min)	Solvent A %	Solvent B %	Duration (min)
Gradient	Start	71	29	
	0	49	51	24
	24	14	86	10
	40	0	100	1
	47	71	29	0.25
	55			End of run

derivatized phosphoamino acids and amino acid peaks were eluted in the following order: phosphoserine, aspartic, phosphothreonine, glutamic, and phosphotyrosine, as has been reported before (24). When the amino acids and phophoserine standards were co-injected, the peaks were baseline separated. In order to fully separate the dabsylated phosphothreonine and phosphotyrosine from dabsylated aspartic (Asx) and glutamic (Glx), respectively, a modification of the content of solution A was necessary. When the concentration of citric acid in the eluents was increased from 11 mM to 12.6 mM, the dabsylated phosphothreonine and phosphotyrosine peaks were baseline separated. In addition, when different amounts of unhydrolized phosphoamino acid standards were derivatized, the peak areas were directly proportional to the measured amounts.

In order to find an optimal hydrolysis time for studying the phosphopeptides, we examined the effect of hydrolysis on the recovery of the phosphoserine standard. We found that 1.5 hours was optimal, a value slightly less than that previously reported (18) 2- to 4-hour period. It needs to be mentioned that the longer hydrolysis time was proposed based on experiments with 6 N HCl in solution, in contrast to our gas-phase conditions. In our hands, hydrolyzing the phosphoserine standard as much as 3 hours caused a 25 to 50% decrease in the final quantity of phosphoserine.

The chromatogram of the hydrolized phosphoserine standard (Figure 1A) contained the dabsylated serine peak, indicating that the decrease of phosphoserine began after 1.5 hours of hydrolysis. Co-injecting the hydrolyzed amino acid and phosphoserine standard produced peaks that were baseline separated (Figure 1B), and no extra peak was found compared to the blank (Figure 1C), except the peak of phosphoserine at 8.09 min. The hydrolized and dabsylated aspartic was eluted at 8.59 min, and the glutamic was eluted at 9.44 min (Figure 1B).

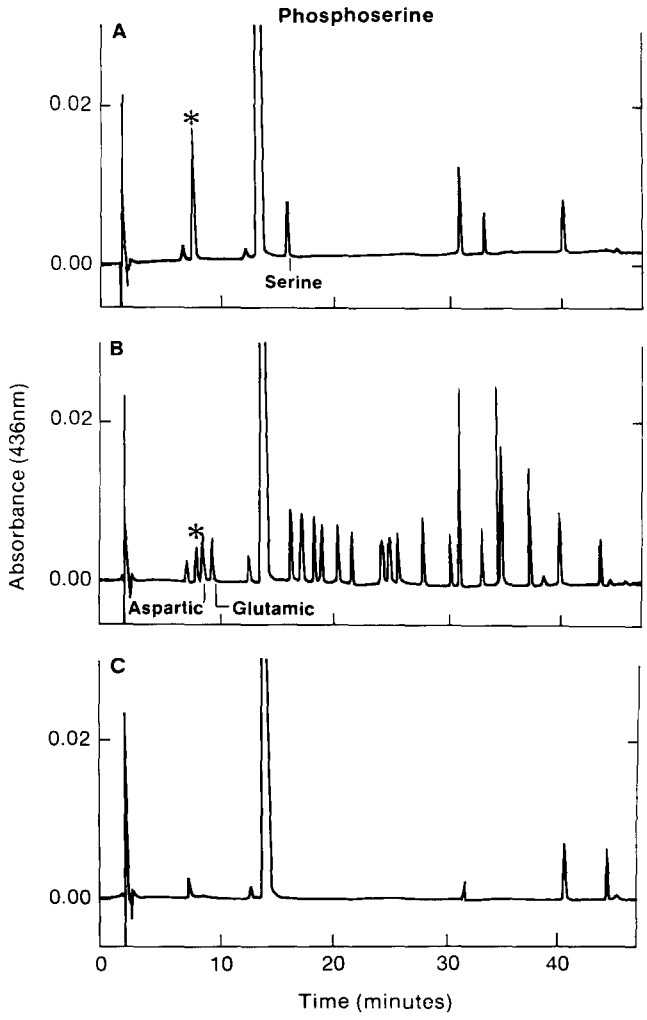

Figure 1. Reversed-phase chromatography of hydrolyzed and dabsylated phosphoserine standard (A), hydrolyzed and dabsylated amino acid standard coinjected with phosphoserine standard (B), and blank (C). The peaks of the phosphoserine were marked with an asterisk. On chromatogram A, 8% of 5 nmol phosphoserine standard was injected; on chromatogram B, 1 nmol of each amino acid and 0.8 nmol of phosphoserine were mixed for coinjection, and 8% of the mixture was injected. On chromatogram B the dabsylated phosphoserine peak was eluted at 8.09 min, the Dabs-aspartic (Asx) was eluted at 8.59 min and the Dabs-glutamic (Glx) peak was observable at 9.44 min.

Model Peptides

Five well-characterized synthetic model phosphopeptides (Table 2) and their non-phosphorylated analogs were examined in preliminary studies. All of these peptides were synthesized in our laboratory. We selected short, phosphoserine-containing peptides because (i) they can be reliably characterized by FAB-MS; (ii) they can be hydrolyzed without major difficulties; (iii) serine-phosphorylation is much more frequent in cytoskeletal proteins than the other two most commonly phosphorylated amino acids; and (iv) phosphoserine is often adjacent to glycine or positively charged amino acids (31).

Using 1.5 hours for hydrolysis of each of the 10 peptides, we found an extra peak only on the chromatograms of phosphopeptides eluting before the Dabs-aspartic. The chromatograms of non-phosphorylated analogs lacked this extra peak. Figure 2 demonstrates this result. The extra peak was eluted at the same position as the dabsylated and hydrolyzed phosphoserine standard. Co-injecting the dabsylated hydrolyzate of phosphopeptides with Dabs-phosphoserine standard, the extra peak overlapped with the peak of the standard. However, because of the partial hydrolysis of peptide bonds, 1.5 hours of hydrolysis time was not enough for quantitative analysis. Nevertheless, it was still good enough to verify the presence of the phosphate group in hydrolyzates of phosphopeptides.

Phosphopeptides

Based on these results, we extended the analysis for immunologically active, medium-sized phosphorylated fragments of protein aggregates of Alzheimer's disease. As Table 3 shows, these peptides contained either phosphoserine or phosphotyrosine. We hydrolyzed and dabsylated several different phosphopeptides. The results were the same as with the model peptides. The extra peak was observed in the dabsylated hydrolyzates of the phosphopeptides (indicating the presence of a phosphate group in the peptides), but was absent from the chromatograms of non-phosphorylated analogues. Figure 3 illustrates this result. The Dabs-phosphoserine peak was eluted before the Dabs-aspartic, while the chromatogram of the non-phosphorylated peptide lacked the Dabs-phosphoserine peak. The Dabs-phosphotyrosine eluted after the Dabs-glutamic in an almost baseline separated manner (Figure 4). The retention times were in agreement with the amino acid and phosphoamino acid standards. The dabsylated aspartic was eluted at 8.55 min, the glutamic was eluted at 9.41 min and the extra peak was found at 9.77 min (Figure 4), while the peak of the dabsylated phosphotyrosine standard was observable at 9.78 min (see insert on Figure 4).

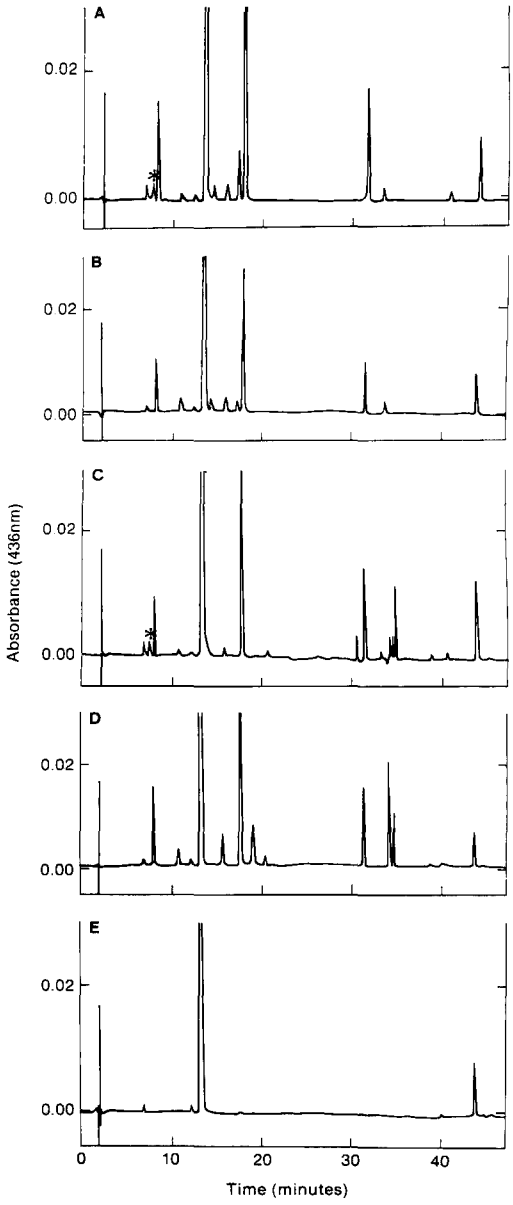

Figure 2. Reversed-phase chromatograms of hydrolyzed and dabsylated model peptides. Panels A and B show the chromatograms of phosphorylated and nonphosphorylated analogs of peptide GDSRG, respectively, panels C and D show the chromatograms of phosphorylated and non-phosphorylated GDSKG peptide, respectively. The phosphoserine peaks are marked with an asterisk. The peak that elutes after the labelled peak corresponds to the dabsylated aspartic. The peak of Dabs-aspartic is observed also on the chromatograms of non-phosphorylated peptides (B, D). Chromatogram E is the blank.

TABLE 2

Detection of Phosphoserine in Synthetic Model Phosphopeptides

Sequences	Peak of phosphoserine	FAB-MS ([M+H$^+$],m/z)
GS(P)*	+	571
PKS(P)PV	+	605, 627[M+Na$^+$]
GDS(P)KG	+	542
GDRS(P)G	+	570
GDS(P)RG	+	570

* This peptide contained three protecting groups (see Materials and Methods).

TABLE 3

Detection of the Presence of a Phosphate Group in Synthetic Phosphopeptides

Sequence	Peak of phosphoserine	Peak of phosphotyrosine
GAEIVYKS(P)PVVSGD	+	
GAEIVYKS(P)PVVAGD	+	
GDTS(P)PRHLSNV	+	
DAGLKES(P)PLQTPTEDG	+	
EEKGKS(P)PVPKS(P)PVEEKG	+	
GDRS(P)GYS(P)S(P)PGS(P)PGTPGS(P)R	+	
VKSKIGS(P)TENLKHQPGGG	+	
GAEIVYKSPVVSGDTS(P)PRHLSNV	+	
YSSPGSPGTPGSRSRT	+	
GAEIVY(P)KSPVVSGD		+
NQLY(P)NEL		+
REEY(P)DVL		+

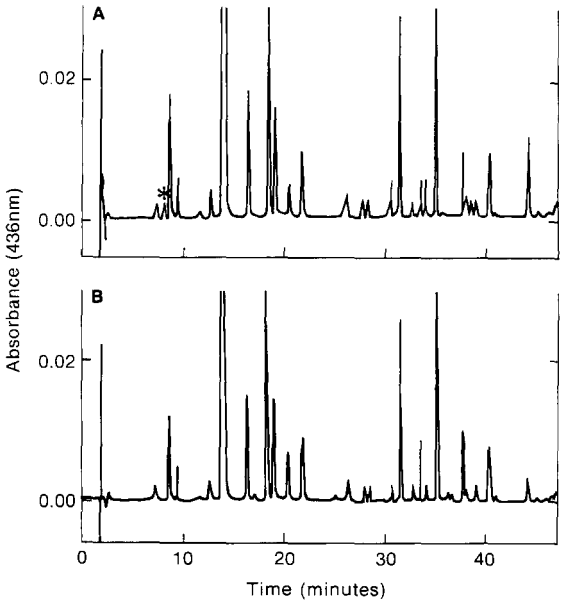

Figure 3. Chromatograms of hydrolyzed and dabsylated peptide T3. Panel A shows the chromatogram of the phosphorylated peptide, panel B its nonphosphorylated analog. The asterisk marks the phosphoserine peak. The peaks, eluted after the marked peak, are the dabsylated aspartic and glutamic, respectively. These two peaks are observed also on the chromatogram of the nonphosphorylated peptide (B). In both cases, the same amounts of peptides (3 nmol) were hydrolyzed and dabsylated, and 8% of them were injected.

The period of hydrolysis is important because short hydrolysis does not liberate the phosphoserine, while longer hydrolysis destroys it very quickly. We found that less than 1 hour of hydrolysis did not liberate the free phosphoamino acids. After 3 hours of hydrolysis of the T3 peptide (see Materials and Methods), we still could determine the presence of phosphoserine, although the amount of it (calculated by peak areas) was two thirds of that obtained by 1.5 hours of hydrolysis. Studying APP and Ac-PRH-Camk phosphopeptides (see Materials and Methods) the presence of phosphoserine could not be verified after 1.5 hour hydrolysis. In these peptides one of the neighboring amino acids of phosphoserine was leucine or isoleucine. The Ile-Ser and the Leu-Ser peptide bonds are likely to be resistant to acidic hydrolysis similar to the earlier reported resistance of Leu-Thr (15), and the bonds probably could not be destroyed during the 1.5 hour hydrolysis. By increasing

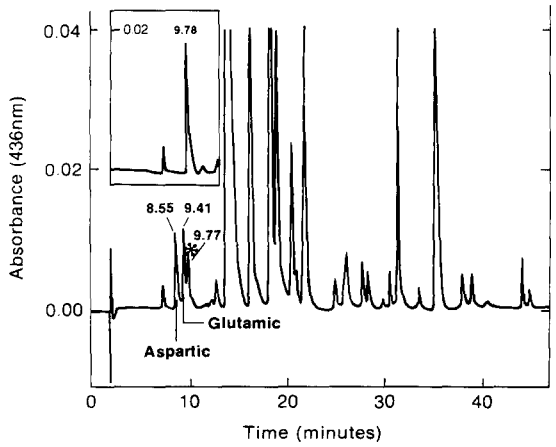

Figure 4. Chromatogram of hydrolyzed and dabsylated T3TYR phosphopeptide. This peptide was phosphorylated on the tyrosine residue. The dabsylated aspartic was eluted at 8.55 min, the dabsylated glutamic was eluted at 9.41 min, and the extra peak (marked with an asterisk) was found at 9.77 min. The dabsylated phosphotyrosine standard (see insert) was eluted at 9.78 min using the modified solvents (see Materials and Methods). In both cases, 5 nmol of standard or peptide were hydrolyzed and dabsylated, and 8% each was injected.

the hydrolysis time to 3 hours, a low intensity phosphoserine peak was observed, but this could not be considered as a reliable detection. Three hours of hydrolysis, which partially destroyed the Ile-Ser and Leu-Ser bonds, has already destroyed the liberated phosphoserine. These results seem to support the idea that each individual peptide sequence has a specific influence on the detectability of phosphoserine. Different sequences may require different hydrolysis times to demonstrate the presence of phosphoserine.

The partial hydrolysis of peptide bonds does not offer optimal circumstances for quantitative analysis, because short hydrolysis does not destroy the peptide bond entirely, and the sensitivity of phosphoserine similarly increases the analytical difficulties. Quantitative analysis of phosphoamino acid standards and phosphopeptides after hydrolysis and derivatization showed a high variability. As Table 4 illustrates, the peak area of the phosphoserine standard was less variable than that of phosphoserine from phosphopeptides. However, upon hydrolyzing numerous standards, we observed more than 40% variability in the standard phosphoserines. As far as phosphoserine from phosphopeptides is concerned, the

TABLE 4

Recovery of Phosphoserine in Different Phosphopeptides and in Phosphoamino Acid Standard

Name	Peak area of 80 pmole phosphoserine
Phosphoserine standard	0.32
DAGLKES(P)PLQTPTEDG	0.18
GDRS(P)G	0.07
GDS(P)RG	0.12
Phosphoserine standard	0.33
VKSKIGS(P)TENLKHQPGGG	0.03
GDTS(P)PRHLSNV	0.05

area of phosphoserine peaks showed very different results. Since the detected amount of phosphoserine in phosphopeptides seems to be sequence-dependent, we did not use the results for quantitative analysis. Correction based on the amount of phosphoamino acid standard is also useless because a longer time is required for the cleavage of peptide bonds, while the phosphoamino acid is free during the short hydrolysis. As Table 4 shows, after hydrolyzing and dabsylating the same amounts of phosphoserine and phosphopeptides, the observed peak area of phosphoserine originating from the phosphopeptide is far less than the peak area of the phosphoserine standard. Nevertheless, the quantitative analysis is appropriate when the peptide sequences are the same, but the number of incorporated phosphate groups differs. Quantitative analysis may be used to verify mono- or diphosphorylated synthetic peptides.

In conclusion, we have demonstrated our ability to verify the presence of phosphoserine and phosphotyrosine in synthetic phosphopeptides and, consequently, to verify the success of a phosphorylation reaction on synthetic phosphopeptides using DABS-Cl amino acid analysis. This method may not only be applicable for phosphopeptides, but also for analysis of phosphoproteins.

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PROCEDURES FOR THE ANALYSES OF DOLASTATINS 10 AND 15 BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY[1]

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ABSTRACT

A series of HPLC procedures were evaluated for assessing the purity of dolastatin 10 (1) and dolastatin 15 (2) samples. Interestingly two readily interconvertible (ambient temperature) dolastatin 10 (1) conformers were detected using a potassium dihydrogen phosphate buffered solvent (methanol-water) with a C8 reversed-phase column. A solvent system composed of acetonitrile-2-propanol-water containing sodium 1-hexanesulfonate was found especially useful for evaluating the purity of dolastatin 10 and 15 specimens. Useful HPLC procedures were also found for detecting diastereomeric isomers in the key dolastatin 10 synthetic intermediate Boc-(S,R,R)-Dap-(S)-Doe using β-cyclodextrin in 3:2 methanol-water.

In 1972 we found extracts of the Western Indian Ocean sea hare *Dolabella auricularia* to produce over 100% life extension in the U.

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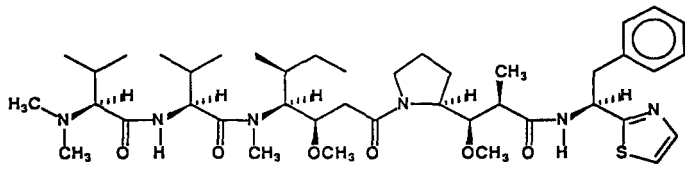
S. National Cancer Institute murine P388 lymphocytic leukemia. Because of this very promising antineoplastic activity we proceeded to isolate and determine structures for the various cytostatic and antineoplastic components.[2-6] The more potent of these components proved to be the two linear depsipeptides dolastatin 10 (1) and dolastatin 15 (2). The isolation,[2,5,6] structure determination[2,7] and initial syntheses[7-10] of these two remarkable peptides have been reported. Because both have been selected for clinical trials, gram-scale quantities were required. Since the marine animal produces only trace ($\sim 10^{-6}$% yields) quantities of these substances, synthetic routes were devised and completed for producing both dolastatins 10[7] and 15[8]. Development of suitable HPLC techniques for ascertaining purity became a very necessary part of these investigations. A variety of HPLC conditions were investigated and resulted in suitable methods for analyses of dolastatins 10 and 15 as well as for several key synthetic intermediates.

EXPERIMENTAL

The β-Cyclodextrin (2.7 g) used in the mobile phase was obtained from Sigma-Aldrich Co. and dissolved in water (120 ml) at 40-50°C. Upon cooling to room temperature, methanol (180 ml) was added. The solution became warm and was allowed to cool (overnight) to room temperature. The precipitated β-cyclodextrin (1.04 g, dry) was removed. The filtrate was a saturated solution of β-cyclodextrin (0.57%) in CH_3OH-H_2O and was used as a mobile phase. The HI-Chrom (reversible) HPLC column (250 x 4.6 mm) containing Pirkle covalent phenylglycine packing (modified Spherisorb S5NH 5 μm particle) was supplied by REGIS Chemical Co.



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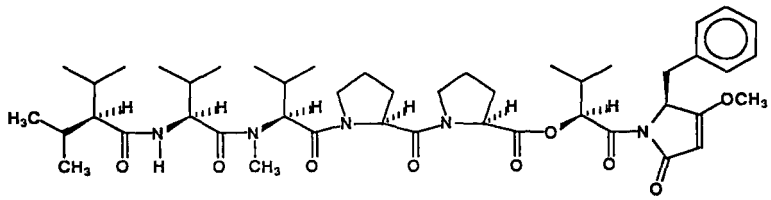

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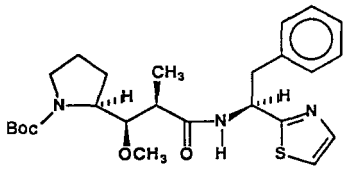

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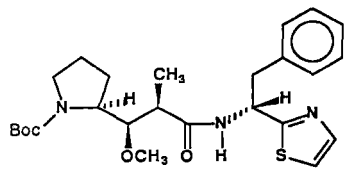

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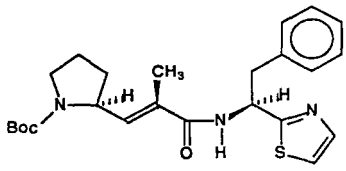

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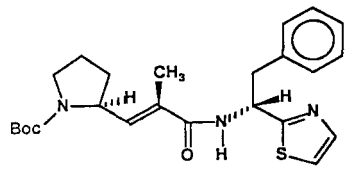

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The HPLC analyses were performed (unless noted otherwise) using a reversed phase Phenomenex Ultremex 3 C8 column (100 x 4.6 mm) with an analytical Gilson HPLC (802B, 811, 2 x 302) instrument equipped with a Rheodyne injection valve (7125 with a 20 μl loop working, pressure 94-101 bar). Control of the HPLC unit was performed with an Apple IIe gradient manager (V 1.2 Gilson). Detection was accomplished with the Hewlett-Packard 1040A, and 9000-300 UV diode array detection system set at 230 nm. Chromatographic displays and data analyses were plotted with a Hewlett-Packard ColorPro plotter.

METHODS AND DISCUSSION

In the stereoselective synthesis of dolastatin 10 (1),[7] synthesis of the (S)-dolaphenine unit proved to be suprisingly challenging. In addition, some reaction conditions led to racemization. Poor yields were usually obtained in a penultimate dehydrogenation step. Protection of (S)-dolaphenine as the BOC derivative improved stability somewhat, but this derivative was still sensitive to oxidation and polymerization upon exposure to air. Due to this instability, HPLC analyses were complicated. However, when (S)-dolaphenine was immediately coupled with BOC-(S)-dolaproine, the resulting dipeptide, BOC-(S,R,R)-dolaproyl-(S)-dolaphenine [Boc-(S,R,R)-Dap-(S)-Doe], (3), was considerably more stable and allowed HPLC analysis of this dolastatin 10 intermediate. Thus, our efforts were initially directed not only to the HPLC analyses of dolastatin 10 and dolastatin 15 alone, but also to the resolution and analysis of dipeptide 3 and its diastereomeric

isomer, Boc-(S,R,R)-Dap-(R)-Doe (4). In addition, two minor side products formed in the dolastatin 10 synthesis, the dehydration products Boc-(S)-dehydro-Dap-(S)-Doe (5) and Boc-(S)-dehydro-Dap-(R)-Doe (6), were also examined. Finally, we explored various HPLC conditions for the resolution of dolastatin 10 and its diastereoisomer, (R)-Doe-*iso*-dolastatin 10.

Initial HPLC analysis of synthetic dolastatin 10 (1) was conducted with a reverse-phase C8 column using a solvent system of 3:1 acetonitrile-H_2O containing 1% acetic acid. Although this eluting system often seemed to provide good resolution between peptide **1** and impurities, reproducible results proved difficult to obtain. The lack of consistent results was attributed to precipitation of the compound(s) during elution. As a consequence, buffered mobile phases containing phosphate salts were investigated. Such mobile phases have been used in chromatographic separations of various synthetic and natural peptides.[11] Use of KH_2PO_4 as a buffer on the C8 column (mobile phase 3:1 CH_3OH-H_2O containing 50mM KH_2PO_4) for chromatography of dolastatin 10 yielded a well defined peak with a longer retention time (3.8 min) than in the previous solvent system (Rt 1.6 min). But this solvent system was found to have limitations. A major problem was precipitation of the phosphate in the pumps, tubing and column during a slight change in the ratio between methanol and buffer, causing severe blockage of the system with concomitant high working pressure (>160 bar). An even more severe drawback was the complete failure of the buffer system to resolve a mixture of authentic dolastatin 10 and the important

diastereoisomer, (R)-Doe-*iso*-dolastatin 10. Since this chiral isomer might be a possible contaminant in synthetic dolastatin 10, the phosphate buffer system was discontinued for evaluating dolastatin 10 purity. However, it was of use in recognizing two room temperature conformers of dolastatin 10, as described below.

During HPLC analysis of dolastatin 10 (1) using the phosphate buffer system, two peaks with Rt of 3.8 min and 4.6 min respectively, would often be observed. The relative ratios of these two peaks would change as shown in Fig. 1, depending upon the samples history. HPLC analysis of several synthetic dolastatin 10 specimens in the CH_3OH phosphate buffer system (reversed phase column) all revealed a single major peak, Rt of ~3.8 min, with a purity of >98%. These fractions were subsequently combined and dissolved in methanol. The solution was passed through a column of Sephadex LH-20 for the purpose of obtaining a pure homogeneous sample for anticancer evaluation. The resulting homogeneous dolastatin 10 was reexamined by HPLC using the phosphate buffer mobile phase. As shown in Fig. 1b, two distinct peaks were initially exhibited, the longer (Rt 4.6 min) peak predominating over the first in the approximate ratio of 2:3. After allowing the HPLC sample solution to stand over various time intervals followed by reinjection, a change in the ratios of the two peaks was noted. Eventually, over time, the shorter (Rt 3.8 min) peak became the dominant peak in a ratio of 95:5. The 3.8 min peak was identical both in Rt and UV spectrum to that of the original dolastatin 10 samples prior to the steric exclusion chromatography on Sephadex LH-

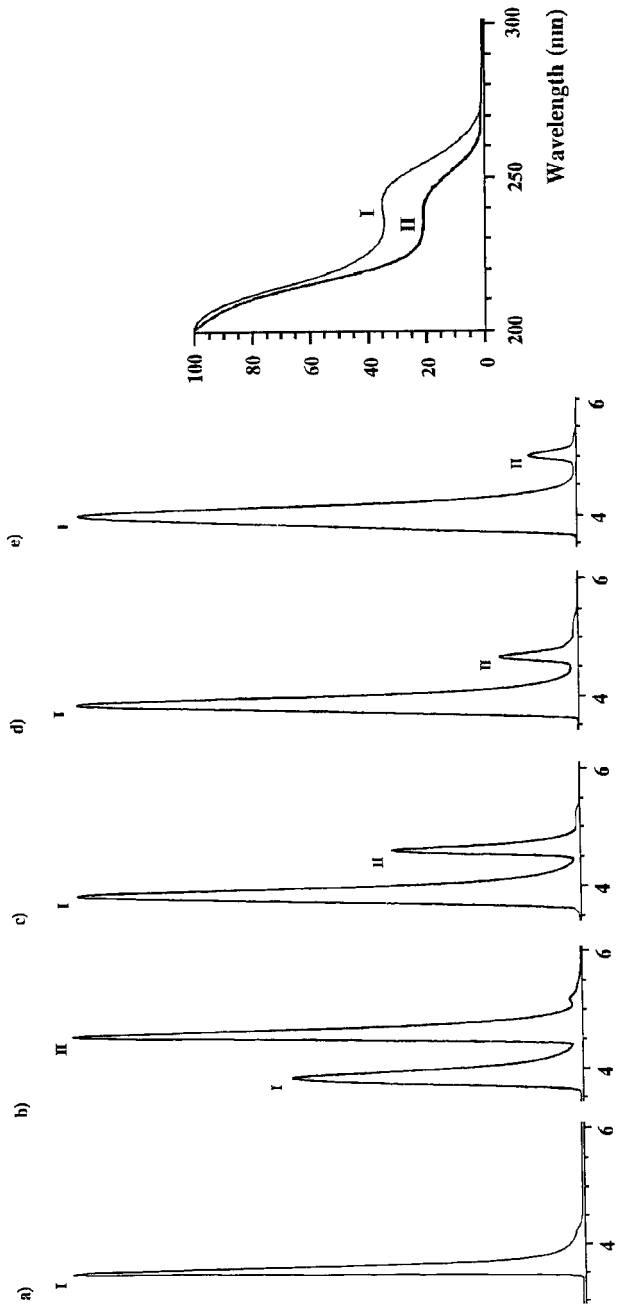

Figure 1. The HPLC (phosphate buffer) of dolastatin 10 (1) showing conformational changes with time in the 3.8 to 4.6 min. retention time range.

a) Pure dolastatin 10 prior to LH-20 chromatography.
b) Same sample immediatly after LH-20 chromatography and solution in eluting solvent (MeOH).
c) Same sample as in b) but after standing for 30 minutes.
d) Same sample as in b) but after standing for 90 minutes.
e) Same sample as in b) but after standing for 14 hours.

20. Such phenomena is typical of the conversion of one conformational isomer to another.

Different obstacles were encountered during the HPLC analysis of synthetic dolastatin 15 (2). Elution of peptide 2 with the 3:1 CH_3OH-H_2O buffer (50mM KH_2PO_4) system on the C8 reversed phase column gave a Rt of 5.5 min, as compared to dolastatin 10 (3.8 min). Because of the problems noted above, the phosphate buffer was abandoned and other systems were investigated. With dolastatin 15, a mobile phase consisting of 1:1 CH_3CN-H_2O on the reversed phase column seemed to provide excellent resolution of dolastatin 15 (2) and its impurities. Final purification of synthetic peptide 2 produced a single major peak with a Rt of 4.3 min and >97% purity.

To find improved analytical HPLC conditions for ascertaining purity of both dolastatin 10 and 15, as well as their synthetic dipeptide intermediates, attention was focused on other mobile phase additives used in the HPLC analyses of peptides.[11] The possibility of substituting 0.1% H_3PO_4 or CF_3COOH for the phosphate buffer system was abandoned when it was found that dolastatin 10 could not be eluted from the column. Sodium dodecyl sulfate (SDS) and β-cyclodextrin[12] (β-CD) were next examined as mobile phase additives. Again dolastatin 10 was not eluted when 1mM SDS in CH_3OH was used as the mobile phase on the reverse phase C8 column. A mobile phase containing β-cyclodextrin (0.57%) produced very broad peaks and this inclusion-complex modifier was generally unsatifactory.

The use of sodium 1-hexanesulfonate ($HexSO_3Na$) as a mobile phase additive gave the most promising results with both dolastatins

10 and 15. A solvent mixture composed of 3:1 CH_3OH-5mM $HexSO_3Na/H_2O$ gave broad but well defined peaks. By substituting acetonitrile for methanol, the peak shape was improved along with the retention time. Further experimentation using the simplex optimization procedure[13] resulted in a much improved solvent system consisting of CH_3CN-2-propanol-5mM $HexSO_3Na/H_2O$ 65:15:20. Because it can be mixed with various solvents in a broad range of concentrations without precipitation, use of $HexSO_3Na$ has distinct advantages over KH_2PO_4.

Optimal concentrations of $HexSO_3Na$ in the CH_3CN- 2-propanol-H_2O (65:15:20) solvent system were determined (Table 1). The optimal concentration of $HexSO_3Na/H_2O$ was about 10 mM or less for separation of dolastatin 10 from dolastatin 15. But this solvent system did not resolve a mixture of dolastatin 10 and (R)-Doe-*iso*-dolastatin 10. When analyzed separately, a ΔRf of 0.19 min was noted for these two compounds. However, when an equimolar mixture of the two was chromatographed, even at low sample concentrations, only peak broadening was observed. A concentration of 20 mM $HexSO_3Na$ led to the best resolution of synthetic dolastatin 10 from its impurities.

A prime objective in the synthesis of dolastatin 10 was optical purity and it became necessary to determine the extent of any racemization of Boc-(S)-dolaphenine. HPLC of a Boc-(R,S)-dolaphenine racemic mixture using CH_3OH-H_2O (3:2) containing 0.57% β-CD[12] (0.8 ml/min) gave no resolution of the R and S isomers. On the other hand, when racemic dolaphenine was converted to Boc-(S,R,R)-Dap-(R,S)-Doe (derivatives 3 and 4), excellent resolution of the two diastereoisomers was achieved, providing a useful method for measuring optical purity of the starting Boc-dolaphenine (Table II).

TABLE I

Retention times for dolastatin 10 and 15 *vs* the concentration of sodium 1-hexanesulfonate in 65:15:20 acetonitrile-2-propanol-water-$HexSO_3Na$

	Rt(min)	
mM $HexSO_3Na$	D-10	D-15
5	10.23	9.40
10	6.15	5.78
15	4.27	4.57
20	3.91	3.75

TABLE II

Retention times for dipeptides 3-6 in CH_3OH-H_2O containing 0.57% β-CD (0.8 ml/min).

Compound	R_t(min)
3	11.11
6	11.84
4	12.23
5	12.75

Here it should be noted that resolution of Boc-(R,S)-dolaphenine was also not realized using a Pirkle chiral column with either *n*-hexane- 2-propanol (9:1, Rt 10.89 min, 0.6 ml/min, 37 bar) or with a 95:5 ratio (Rt 15.23 min, 0.60 ml/min, 47 bar). In addition, dolastatins 10 and 15 were not eluted at all from this column using *n*-hexane 2-propanol (4:1) as mobile phase.

In addition to HPLC analyses, other methods must be employed to accurately evaluate purity of the synthetic dolastatins, *i.e.*, optical rotation and careful high field nmr analyses. The new procedure described for the analysis of dolastatin 15 using $HexSO_3Na$ as an additive in the CH_3CN- 2-propanol (65:15:20) solvent system should assist significantly in detection of possible diastereomeric contaminants. In addition, the HPLC conditions described using β-cyclodextrin and use of the Pirkle type chiral column for resolution of closely related dipeptides should prove to be generally useful.

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DETERMINATION OF GLYOXAL, METHYLGLYOXAL, DIACETHYL, AND 2,3-PENTANEDIONE IN FERMENTED FOODS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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ABSTRACT

A highly sensitive and rapid high-performance liquid chromatographic method for the determination of glyoxal, methylglyoxal, diacethyl, and 2,3-pentanedione in fermented foods is described. After extraction of the compounds with methanol, the compounds in the extract are converted into the corresponding fluorescent derivatives by reaction with 1,2-diamino-4,5-methylenedioxybenzene, a fluorogenic reagent for α-dicarbonyl compounds. The derivatives are separated on a reversed-phase column (L-column ODS) with isocratic elution using acetonitrile - 0.5M ammonium acetate, and are detected fluorimetrically. The detection limits are 11.6 - 13.8 fmol per 10-μl injection for all the compounds at a signal to noise ratio of 3.

INTRODUCTION

Diacethyl and 2,3-pentanedione are well known to be one of the important fragrant components in fermented foods such as alcoholic drinks and dairy products, and to be produced by microorganisms during fermented processes. Thus, it is very important to quantify the compounds in the final products for reasons of quality control. On the other hand, it is stipulated that glyoxal and methylglyoxal also are present in the fermented foods and are important for quality check of the foods. However, the compounds have never been successfully determined. This may be partially due to the lack of a sensitive and selective method for the simultaneous determination of the four α-dicarbonyl compounds described above.

Some methods including gas chromatography (1-4), spectrophotometry (5-7), and high-performance liquid chromatography (HPLC) (8,9) have been developed for the determination of diacethyl and/or 2,3-pentanedione in fermented foods. However, the methods have limited sensitivity and do not allow the simultaneous determination of the four α-dicarbonyl compounds described above.

We previously developed 1,2-diamino-4,5-methylenedioxybenzene (DMB) as a highly sensitive and selective fluorogenic reagent for α-dicarbonyl compounds in HPLC (10). The reagent reacts with the α-dicarbonyl compounds in the presence of β-mercaptoethanol and sodium dithionite to produce the corresponding fluorescent quinoxalines (Fig. 1). In this paper, we applied the reaction to the simultaneous determination of glyoxal, methylglyoxal, diacethyl, and 2,3-pentanedione which are very important for quality check of fermented foods.

EXPERIMENTAL

Reagents and solutions

All chemicals and solvents were of analytical-reagent grade, unless stated otherwise. Distilled water, purified with a Milli Q II system (Japan Millipore, Tokyo, Japan) was used for all aqueous solutions. Glyoxal, methylglyoxal, diacethyl, and 2,3-pentanedione were purchased from Wako Pure Chemicals

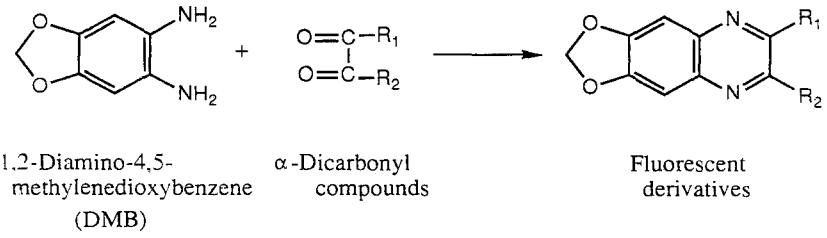

FIGURE 1. Derivatization of α-dicarbonyl compounds with 1,2-diamino-4,5-methylenedioxybenzene (DMB).

(Tokyo, Japan). DMB was prepared as described previously (11); it is now commercially available from Dojindo Labs. (Kumamoto, Japan). DMB solution (7.0 mM) was prepared in water containing 0.2 M β-mercaptoethanol and 0.25 M sodium dithionite. The DMB solution could be used for more than 1 week when stored in a refrigerator at 4 °C.

Instrumentation

A Hitachi (Tokyo, Japan) 655-A11 high-performance liquid chromatograph equipped with a sample injector (10-μl loop) was used. A Shimadzu (Kyoto, Japan) RF-535 fluorescence spectromonitor fitted with a 12-μl flow-cell operating at an excitation wavelength of 350 nm and emission wavelength of 390 nm. The column was a L-column ODS (100x4 mm i.d.; particle size, 5 μm)(Chemical Inspection and Testing Institute, Tokyo, Japan). The mobile phase was acetonitrile - 0.5 M ammonium acetate (35:65, v/v). The flow-rate was 1.0 ml/min. The column temperature was ambient (ca. 25 °C). Uncorrected fluorescence excitation and emission spectra of the eluates were measured with a Hitachi 650-60 fluorescence spectrophotometer fitted with a 20-μl flow-cell; the spectral bandwidths were 5 nm in both the excitation and emission monochromators.

Food samples

Portions (ca. 0.1 g or 0.1 ml) of yoghurt, beer, and wine were diluted with 5.0 ml of methanol, and the mixtures were centrifuged at 1000 g for 5 min. The supernatants were used as food sample solutions.

Derivatization procedure

A portion (100 μl) of a sample solution in a screw-capped 1-ml vial was diluted with 10-μl of water and 100 μl of the DMB solution. The vial was tightly closed and warmed at 60 °C for 40 min in the dark. After cooling, 10 μl of the resulting mixture were injected into the chromatograph.

The amounts of α-dicarbonyl compounds were calibrated by means of the standard addition method: water (10 μl) added to the sample solution was replaced by the standard solution (10 μl) containing 1.0 - 150 pmol each of the α-dicarbonyl compounds. The net peak heights in the chromatogram were plotted against the concentrations of the individual α-dicarbonyl compounds spiked.

RESULTS AND DISCUSSION

The derivatization conditions were the same as described previously (10).

HPLC conditions

The separation of the DMB derivatives of glyoxal, methylglyoxal, diacethyl, and 2,3-pentanedione was studied on a reversed-phase column (L-column ODS) using methanol, acetonitrile, water, 0.5 M ammonium acetate and their mixtures as mobile phase. The best separation was achieved using acetonitrile - 0.5 M ammonium acetate (35:65, v/v). The individual α-dicarbonyl compounds tested gave single peaks. Figure 2 shows a typical chromatogram obtained with a standard mixture of the four α-dicarbonyl compounds [retention time (min): glyoxal, 6.1; methylglyoxal, 7.7; diacethyl, 9.2; 2,3-pentanedione, 17.6].

Determination of glyoxal, methylglyoxal, diacethyl, and 2,3-pentanedione in food samples

Representative chromatograms obtained with yoghurt and beer are shown in Figs. 3(A) and (B), respectively. The components of peaks 1, 2, 3, and 4 (Figs. 3) were identified as the DMB derivatives of glyoxal, methylglyoxal, diacethyl, and 2,3-pentanedione, respectively, on the basis of their retention times and fluorescence excitation and emission spectra. This was achieved by comparison

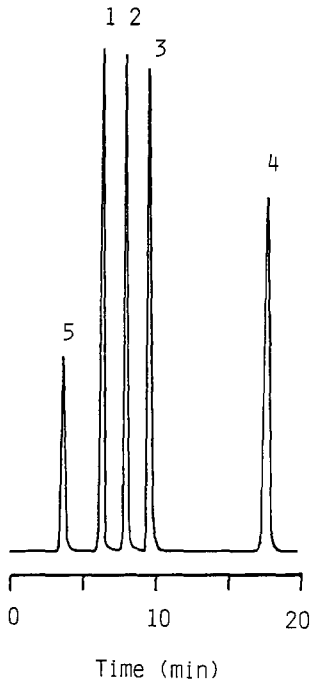

FIGURE 2. Chromatogram obtained with a standard mixture of α-dicarbonyl compounds. A portion (100 μl) of a standard mixture of glyoxal, methylglyoxal, diacethyl, and 2,3-pentanedione ($1.0x10^{-5}$ M each) was treated as in the procedure. Peaks: 1=glyoxal, 2=methylglyoxal, 3=diacethyl, 4=2,3-pentanedione, 5=DMB.

of the spectra with the standards, and also by co-chromatography of the standards and the foodstuff samples with aqueous 10 - 80 % acetonitrile or methanol as the mobile phase. Peak 6 may be due to the endogenous unknown α-dicarbonyl compounds in foodstuffs. This was suggested by the following results. Peaks 6 increased in height in proportion to the sample size of the foods. No peaks were detected in the chromatograms when the foodstuff samples were treated without DMB. Moreover, all the eluate from peaks 6 exhibited fluorescence excitation and emission maxima around 350 and 390 nm, respectively, almost identical with those of the DMB derivatives of the four α-dicarbonyl compounds tested.

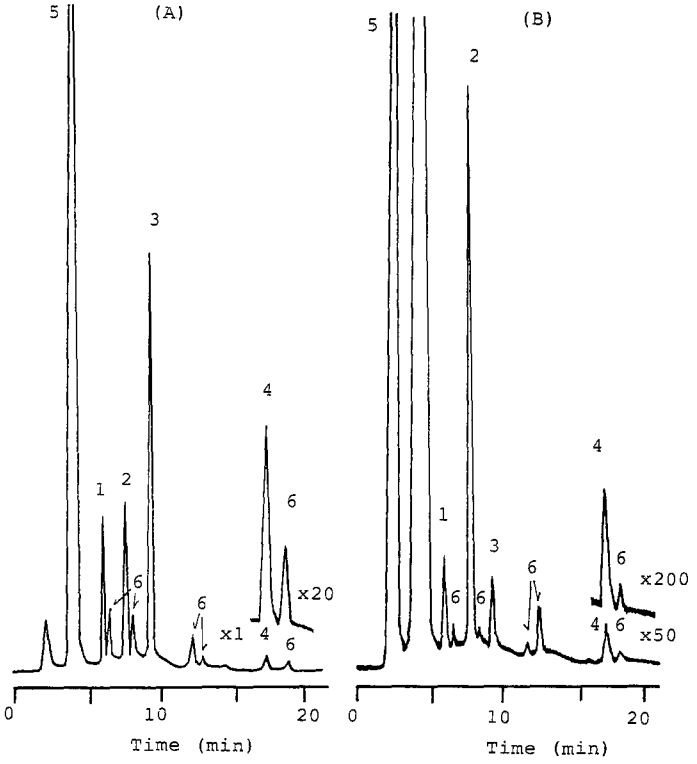

FIGURE 3. Chromatograms obtained with foodstuffs. Portions (100 µl) of (A) yoghurt and (B) beer sample solutions were treated as in the procedure. Peaks: 1 - 5 = see in Fig. 2, 6=unknown.

Many substances such as carboxylic acids, alcohols, sugars, aldehydes, ketones, phenols, amines, and amino acids gave no fluorescent derivatives under the described conditions. On the other hand, DMB reacts with α-keto acids (α-ketoglutaric, pyruvic, α-ketoisovaleric, α-ketoisocaproic, and α-keto-β-methylvaleric acids) to produce fluorescent derivatives. However, the DMB derivatives of α-keto acids have fluorescence excitation (maximum, 367 nm) and emission (maximum, 446 nm) spectra different from those of the α-dicarbonyl compounds and were eluted at retention times of 3 - 5 min. Thus,

α-keto acids did not interfere with the sensitive determination of the four α-dicarbonyl compounds tested.

Linear relationships were observed between the net peak heights and the amounts of the individual α-dicarbonyl compounds spiked to foodstuff samples, up to at least 90 nmol/g (or ml) of the foods. The correlation coefficients of the calibration curves were higher than 0.998 for all the compounds. The detection limits for glyoxal, methylglyoxal, diacethyl, and 2,3-pentanedione were 260, 270, 400, and 330 pmol/g (or ml) of foods (12.0, 12.5, 13.8, and 11.6 fmol/10 μl injection volume), respectively, at a signal to noise ratio of 3. The within-day precision of the methods were established by repeated determination (n=10) using the individual food samples. The relative standard deviations did not exceed 4.0 % for all the compounds in all food samples. The recoveries of the α-dicarbonyl compounds added to 100 μl of the individual food samples in the amounts of 5.0 nmol were 94.2 - 99.6 %.

The amounts of the four α-dicarbonyl compounds in foodstuffs were determined by this method (Table 1). The mean values of diacethyl and 2,3-pentanedione in beer, wine, and yoghurt were not very different from those listed by other workers (4,7,8,12-14). The levels of glyoxal and methylglyoxal in foodstuffs are first determined by the present method.

TABLE 1

Concentration (nmol/ml or g) of α-Dicarbonyl Compounds in Foodstuffs

Foodstuffs		Glyoxal	Methylglyoxal	Diacethyl	2,3-Pentanedione
Yoghurt	A	15.8	17.7	10.5	5.4
	B	12.3	14.4	22.5	6.2
	C	10.9	8.4	25.9	4.8
Beer	D	0.6	3.3	0.5	0.4
	E	0.4	1.3	0.6	0.4
	F	0.7	1.2	0.6	0.4
Wine	(red)	12.8	12.6	32.0	1.1
	(white)	8.7	40.2	11.2	1.4

This work provides the first fluorimetric HPLC method for the simultaneous quantification of glyoxal, methylglyoxal, diacethyl, and 2,3-pentanedione in fermented foods. This method has a satisfactory sensitivity in the determination of the compounds in a small amount of the foods. This is of advantage in the measurement of the α-dicarbonyl compounds for the quality control of fermented foods such as alcoholic drinks and dairy products. This method is rapid and simple to perform and can, therefore, be applied to routine analyses in the investigations of fermentation technology and food chemistry.

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LIQUID CHROMATOGRAPHIC DETERMINATION OF OXYTETRACYCLINE AND CHLORTETRACYCLINE RESIDUES IN ANIMAL TISSUES

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ABSTRACT

A simple and sensitive method has been developed for the simultaneous determination of oxytetracycline and chlortetracycline in animal tissues by liquid chromatography(LC). Tissue samples were extracted with EDTA-McIlvaine buffer(ph4.0)/methanol(3/7). The extracts were purified with Sep-pak C18 and Bond Elut SCX cartridges. Two tetracyclines were separated with a synthetic polymer based-ODS column under basic condition. They were determined by LC with programmable fluorometric detector.

INTRODUCTION

Oxytetracycline(OTC) and chlortetracycline(CTC) belong to groups of tetracycline antibiotics(TCs) that are used most frequently with respect to veterinary medicine, animal nutrition and feed additives. Therefore, monitoring of residual TCs is important from the viewpoint of veterinary food hygiene. Many methods have been developed for the determination of simultaneous or individual OTC and CTC in biological samples (1-8). These meth-

ods have been based mainly on liquid chromatography(LC) with UV(1-6) or fluorometric(7,8) detection. But they lacked sensitivity required to detect TCs residues and involved tedious manipulations. This paper describes a rapid and sensitive method for the determination of OTC and CTC in tissues by LC with programmable fluorescence detection and ODS based poly(styrene divinylbenzen) copolymer as the stationary phase. This method is suitable for routine analysis of residual OTC and CTC in animal tissues.

EXPERIMENTAL

Chemicals

OTC hydrochloride and CTC hydrochloride were kindly donated by Pfizer Pharmaceuticals Inc.(Tokyo,Japan) and Takeda Chemical Industry Ltd.(Osaka,Japan), respectively. Acetonitrile was of LC grade and all other chemicals were of analytical grade from Cica-Merck(Tokyo,Japan). Sep-pak C18 and Bond Elut SCX cartridges were purchased from Waters Association(Milford,MA,U.S.A.) and Analytichem International(Harbor City,CA,U.S.A.),respectively. LC grade water was obtained by purifying reversed osmosis water in a Milli-Q II system(Millipore,Bedford,MA,U.S.A.).

Apparatus and chromatographic conditions

An LC system consisted of two 6AD pumps, a SIL-6B auto injector, a SCL-6B system controller, a CTO-6A column oven, a RF-550A spectrofluorometer, a C-R4AX integrator(Shimadzu,Kyoto,Japan) and a KT-35 degasser(Shodex,Tokyo,Japan). All analyses were carried out using a ODP-50 5-um 250mm x 4.6mm(I.D.)column, which was purchased from Asahi chemical ind.(Tokyo,Japan). The mobile

phase was prepared by mixing 900 ml of Sorensen buffer(pH12.0) and 100 ml of acetonitrile. The flow rate was 1ml/min and the column temperature was maintained at 40°C. The wave length of spectrofluorometer was changed to ex. 350nm, em. 420nm from ex.374nm,em.508nm 7 min later, by using time programming technique.

Sample preparation procedure

Minced 10g tissue was homogenized with 30ml of 10mM Na_2EDTA-McIlvaine buffer(pH 4.0)-methanol(3:7) mixture and centrifuged twice. The supernate was combined and concentrated to ca.5 ml under vacuum. The concentrated solution was offered onto a Sep-pak C18 cartridge column. The cartridge was rinsed with 5 ml of water and eluted with 10 ml of methanol. The eluate was offered onto the next Bond Elut SCX cartridge. The cartridge was rinsed with 10 ml of water and eluted with 10 ml of 1N HCl-methanol(2:8). The eluate was adjusted to pH 12 with 10N sodium hydroxide and injected to the LC after an hour.

RESULTS AND DISCUSSION

Variation of the emission spectra of OTC(I) and CTC(II) for different pH(9-12) solution are shown in Fig.1. The maximum fluorescence wave length for OTC is at 390nm on excitation, and 495-500nm on emission, and that for CTC is 350nm on excitation, and 405-410nm on emission,respectively. As CTC decomposes in alkaline medium to form isochlortetracycline(ISOCTC)(7), the fluorescence intensities of TCs are strongly dependent on the pH of the solution. Perhaps, the same phenomenon may happen on OTC to that on as CTC. The reduced fluorescence intensities in alkaline medium are due to the decomposition of these two TCs.

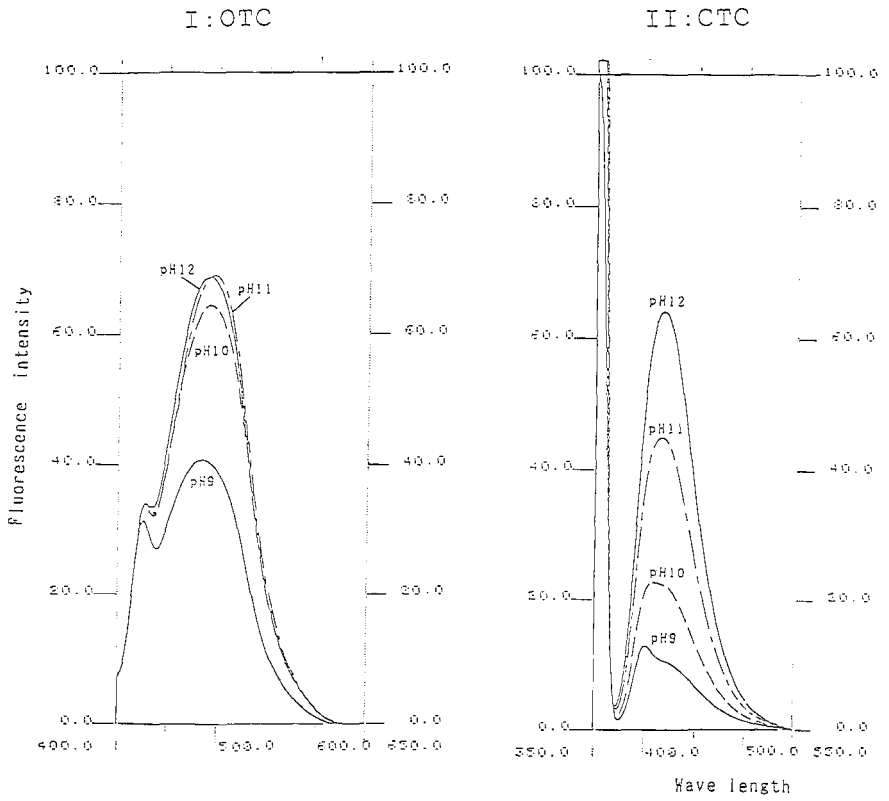

Fig. 1. Emission spectra of TCs (I:OTC, II:CTC) for different PH(9-12) solutions.

The time-dependent changes of decomposed TCs are shown in Fig.2,3. The fluorescence intensity of OTC reached the maximum value immediately and was held constant for long time. That of CTC increased slowly and reached plateau after an hour, to maintain its intensity for moreover 24 hrs. The reacted time was of one hour was necessary for the simultaneous determination of these two TCs.

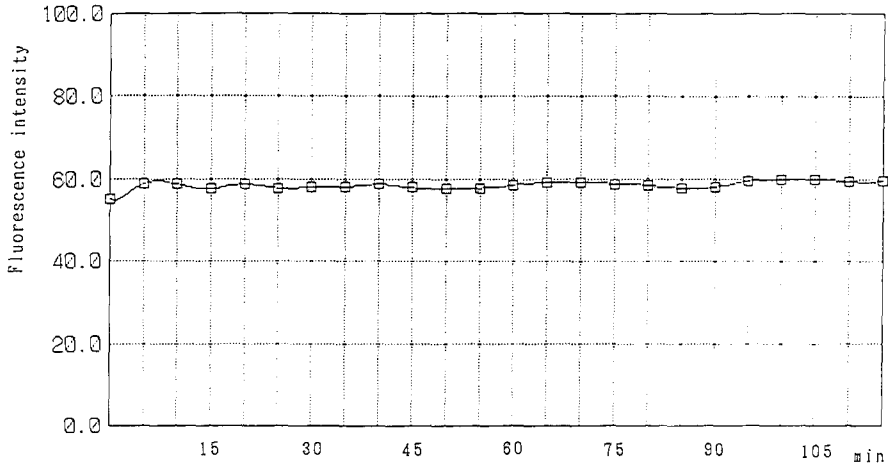

Fig.2. Fluorescence intensity versus time profile of OTC in basic condition(mobile phase).

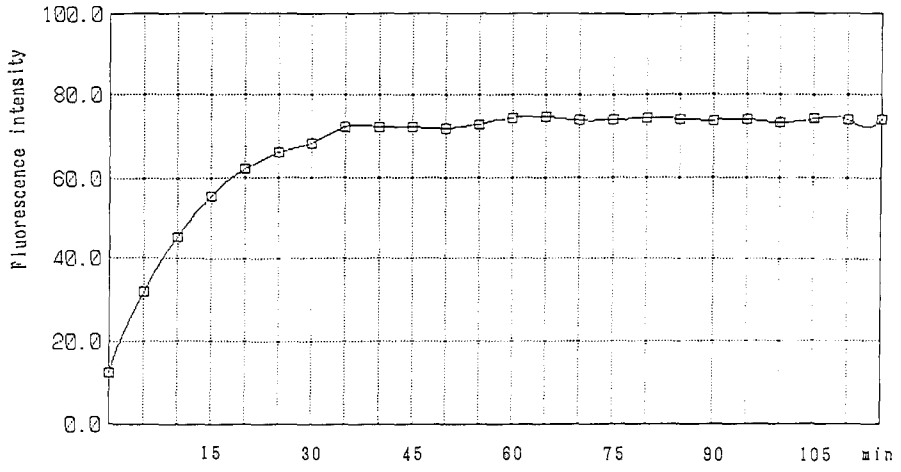

Fig.3. Fluorescence intensity versus time profile of CTC in basic condition(mobile phase).

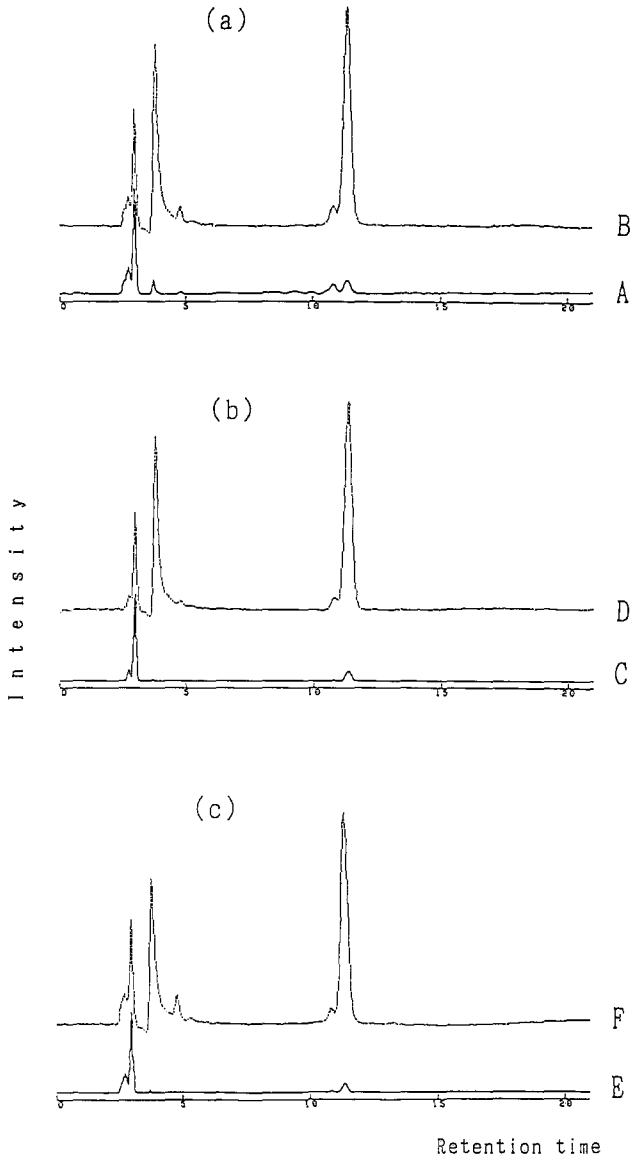

Fig. 4. Liquid chromatograms of drug free & fortified samples; A, C, E:drug free samples; B, D, F:fortified samples; (a):bovine; (b):swine;(c):chicken; LC conditions are described in literature.

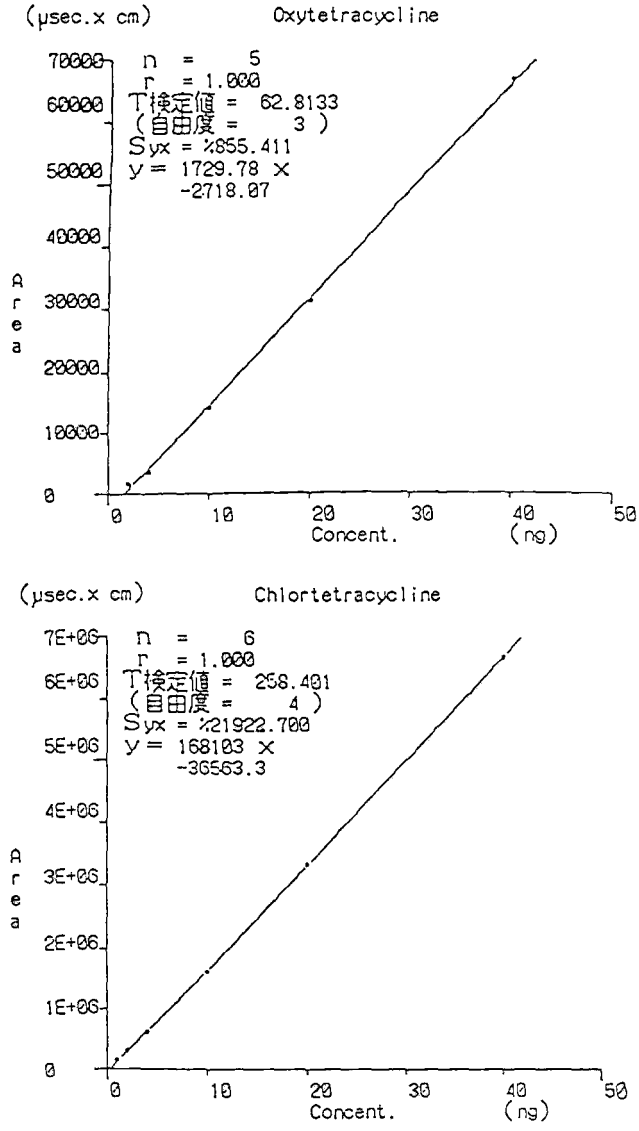

Fig. 5. Calibration curves for OTC and CTC.

Table 1 Recoveries of OTC and CTC from Bovine, Swine and Chicken.

	Added(μg/g)	Recovery±S.D., C.V.(%)		
		Bovine	Swine	Chicken
OTC	0.2	79.5±5.4, 6.8	74.4±4.3, 5.8	63.4±1.6, 2.5
	1.0	81.3±7.4, 9.1	85.6±3.9, 4.6	74.8±4.6, 6.1
CTC	0.2	78.5±6.1, 7.8	72.3±6.3, 8.7	61.8±3.6, 5.8
	1.0	86.5±9.7, 11.2	73.1±6.0, 8.2	70.5±4.5, 6.4

n=5

Some large interfering peaks derived from the matrix appeared before OTC and CTC peaks in the case of using an only ODS cartridge. The second cartridge of SCX was therefore connected with the ODS cartridge to remove the interfering peaks. Although they still appeared after the use of double cartridges, this method was fairly effective in minimizing the interference with co-extracted components of the meat. Both peak height of 5ng OTC 1ng CTC were more than 4 times that of the interfering peaks. Consequently, the matrix peaks were estimated to be negligible. These results are shown in Fig.4.

Results of calibration runs for the validation range of 2-40ng (OTC) and 1-40ng(CTC), respectively seen in Fig.5, showed excellent linearity (r=0.999).

Two amounts(0.2&1ug) of TCs were added to the drug free minced meat(10g), and their recoveries were measured by the procedure described above. The results are summarized in Table 1, which shows recovery ranges from 61.8 to 86.5% and coefficients of variation (C.V.) from 2.5 to 11.2%.

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SEPARATORY DETERMINATION OF BILIARY METABOLITES OF EQUILIN IN RAT BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A method for the separation and determination of biliary metabolites of equilin in rat by high-performance liquid chromatography with electrochemical detection has been developed. After extraction of equine estrogens in bile with a C_{18} bonded silica cartridge, conjugated metabolites were hydrolyzed with sulfatase containing β-glucuronidase and free estrogens liberated were then separated on a reversed phase C_{18} column, the limit of detection being in the range of 5-20 pg. The transformation of equilin administered orally into 2-methoxy derivatives of equilin, equilenin and their 17β-reduced metabolites is also discussed.

INTRODUCTION

Equine estrogens are ring B unsaturated steroids formed through a squalene-cholesterol independent pathway and are

excreted exclusively into urine of pregnant mares as their sulfates (1). These estrogens have widely been used for treatment of postmenopausal and estrogen deficient women (2,3). Recently, a strong association between equine estrogen replacement therapy and endometrial cancer has been demonstrated (4-7). To assess the biological and endocrine potencies, clarifying the biotransformation of these estrogens is needed. However, metabolism of these equine estrogens still remains unclear, though that of classical estrogens such as estrone and estradiol in man and rodent has been well established (8). Bhavnani et. al have shown that after administration of [^{3}H] equilin and its sulfate into postmenopausal women and men, polar metabolites were excreted in urine (9,10). But the precise knowledges on the transformation of equine estrogens during circulation in the body has been spaced. It is generally accepted that both endogenous and exogenous estrogens are excreted to a considerable extent into bile and are partly reabsorbed from the intestine. Accordingly, a reliable method for the qualitative and quantitative analysis of metabolites in bile is needed for clarifying the metabolic fate of equine estrogens.

In recent years, various chromatographic methods, gas-liquid chromatography (GC), GC-mass spectrometry and high-performance liquid chromatography (HPLC), have been used for the simultaneous determination of steroid hormones in biological fluids. Among these methods, HPLC with electro-

chemical detection (ECD) is believed to be a powerful tool for the separation and determination of phenolic steroids with reliable sensitivity and specificity. The present paper deals with the high-performance liquid chromatographic determination of biliary metabolites of equilin in rat.

EXPERIMENTAL

Instruments

The apparatus used for this work was a Waters Model 510 solvent delivery system (Millipore-Waters, Milford, MA, U.S.A.) equipped with a Coulochem Model 5100A electrochemical detector (Environmental Sciences Assoc. Inc., Bradford, MA, U.S.A.). The potential voltage of the detector for 1st electrode for screening was set at 0.1 V *vs.* Ag/AgCl electrode. A Cosmosil 5C18-AR (5μm) column (15 cm x 4.6 mm i.d.) (Nacarai Tesque Inc., Kyoto, Japan) was used at ambient temperature.

Materials

Equilin (Eq) and equilenin (Equ) were kindly donated by Ayerst Laboratories (New York, NY, U.S.A.). Estrone (E_1) and estradiol (E_2) were purchased from Teikoku Hormone Mfg. Co. (Tokyo, Japan). 17β-Dihydroequilin (17β-DHEq) and 17β-dihydroequilenin (17β-DHEqu) were obtained by sodium borohydride reduction of Eq and Equ, respectively. 1-Methylestrone

was prepared in these laboratories. 2-Methoxyestrone (2-$MeOE_1$), 2-methoxyestradiol (2-$MeOE_2$), 2-methoxyequilin (2-MeOEq), 2-methoxyequilenin (2-MeOEqu) and their 17β-hydroxyl derivatives (2-MeODHEq and 2-MeODHEqu) were synthesized as reported previously (11). The reagents used were of analytical reagent grade. Solvents were purified by distillation and degassed by vacuum evacuation prior to use. The sulfatase (EC 3.1.6.1) preparation derived from Helix pomatia (Type H-5) was supplied by Sigma (St. Louis, MO, U.S.A). Piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20, 0.6 meq./g) was prepared as reported (12). A Sep-Pak C_{18} cartridge (Millipore-Waters) was washed successively with methylene chloride (5 ml), ethanol (10 ml), H_2O (15 ml), 5% bovine serum albumin (3 ml), H_2O (15 ml), ethanol (10 ml) and then H_2O (15 ml) prior to use. All glasswares used were silanized with trimethylchlorosilane.

Collection of rat bile

Male Wistar rats weighing ca. 250 g were anesthetized with diethyl ether, cannulated to the bile duct with polyethylene tube by surgical operation and housed in Bollman cages for collection of bile. All animals were starved overnight prior to administration of equilin. A suspension of equilin (10 mg) per head in dimethylsulfoxide (50 μl)-saline (350 μl)-Tween 80 (100 μl) was given orally to rat, and bile was collected in a test

tube containing 500 μl of 30 mM ascorbic acid in 0.15 M acetate buffer (pH 5.0) over a period of 24 h following administration of equilin.

Determination of metabolites in bile

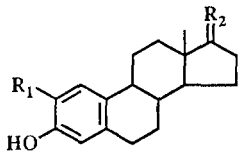
To a 10 μl aliquot of rat bile was added 1-methylestrone (100 ng) and following dilution with 0.15 M acetate buffer (pH 3.0, 2 ml) containing 2% ascorbic acid, the mixture was passed through a Sep-Pak C_{18} cartridge. After washing with distilled water (10 ml), estrogens were eluted with methanol (5 ml) and the eluate was evaporated *in vacuo*. The residue was dissolved in 0.15 M acetate buffer containing 2% ascorbic acid (pH 5.0, 2 ml) and incubated with sulfatase (500 Fishman units) at 37 ^{o}C for 15 h. The whole was applied to a Sep-Pak C_{18} cartridge in the manner described above, for extraction of liberated steroids. The hydrolyzate in 90% ethanol (1 ml) was applied to a column (18 mm x 6 mm i.d.) of PHP-LH-20 (100 mg). Estrogens were eluted with 90 % ethanol (5 ml) and the eluate was evaporated *in vacuo*. The residue was redissolved in acetonitrile (1 ml) and then subjected to the HPLC analysis.

Recovery test

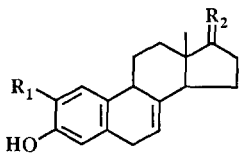
A synthetic mixture of each estrogen (100 ng) dissolved in 10 μl of bile and the whole was then subjected to the proposed method. Recoveries were calculated against a standard mixture of estrogens carried through the procedure.

RESULTS AND DISCUSSION

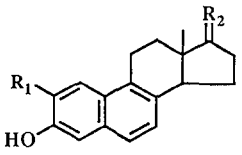
Our initial effort was directed toward the development of efficient chromatographic conditions for the determination of E_1, Eq, Equ, related 2-methoxy derivatives and their 17β-hydroxylated compounds listed in Fig. 1 with a reversed phase column. The separation of estrogens was influenced by species of salts in a mobile phase. The addition of disodium hydrogen or ammonium dihydrogen phosphate in a mobile phase resulted in the insufficient resolution of estrone, equilin and/or equilenin. On the other hand, these estrogens were satisfactorily resolved with a mobile phase including sodium acetate. Therefore, the effect of pH of a mobile phase on k' values of estrogens were examined on a Cosmosil 5C18-AR column with 0.5% sodium acetate-acetonitrile as a mobile phase. The k' values obtained were plotted against pH of the buffer in a mobile phase. As shown in Fig. 2, increasing k' values with increasing pH in the range of 3.0-6.0 were observed for all the steroid examined. The almost identical elution order was found for guaiacol estrogens (2-MeOEqu>2-MeOEq>2-MeOE_1 and 2-MeODHEqu>2-MeODHEq>2-MeOE_2) and related phenolic compounds (Equ>Eq>E_1 and DHEqu>DHEq>E_1) according to the structure of ring B and the substituent at C-17. This may be due to hydrophobicity of a double bond in the B ring for Eq and a naphthalene ring for Equ. Although almost all compounds were



E_1 : R_1=H, R_2=O
E_2 : R_1=H, R_2=β-OH
2-MeOE_1 : R_1=OCH_3, R_2=O
2-MeOE_2 : R_1=OCH_3, R_2=β-OH



Eq : R_1=H, R_2=O
17β-DHEq : R_1=H, R_2=β-OH
2-MeOEq : R_1=OCH_3, R_2=O
2-MeODHEq : R_1=OCH_3, R_2=β-OH



Equ : R_1=H, R_2=O
17β-DHEqu : R_1=H, R_2=β-OH
2-MeOEqu : R_1=OCH_3, R_2=O
2-MeODHEqu : R_1=OCH_3, R_2=β-OH

Fig. 1. Structures of equine estrogens and related compounds.

well resolved by the use of a binary solvent system with 0.5% sodium acetate-acetonitrile or -methanol, insufficient resolution of DHEq and 2-MeODHEqu was attained. Accordingly, a ternary solvent system consisting of 0.5% sodium acetate, acetonitrile and tetrahydrofuran (THF) or methanol were investigated. The effect of the content of THF or methanol in acetonitrile on a k' value relative to that of 1-methylestrone

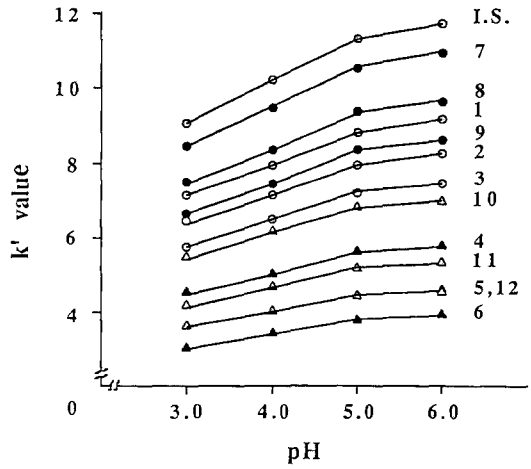


Fig. 2. Effect of pH of mobile phase on k' values of estrogens. Conditions: column, Cosmosil 5C18-AR; mobile phase, 0.5% sodium acetate-acetonitrile (60:40, v/v), flow rate, 1.0 ml/min. 1=E_1, 2=Eq, 3=Equ, 4=E_2, 5= 17β-DHEq, 6= 17β-DHEqu, 7=2-MeOE_1, 8=2-MeOEq, 9=2-MeOEqu, 10=2-MeOE_2, 11=2-MeODHEqu, 12= 2-MeODHEqu, IS=1-methylestrone.

was examined with the constant ratio of an organic solvent and a buffer solution (40:60, v/v). As depicted in Fig. 3, the remarkable variance was found for THF with respect to the feeble effect for methanol. The relative k' values of E_2, DHEq, DHEqu and corresponding 17-oxo compounds increased remarkably with a increasing the ratio of THF to acetonitrile. On the contrary, the decreasing values for 2-methoxy derivatives having a 17-oxo group were found, while those with a 17β-hydroxyl group showed almost constant k' values.

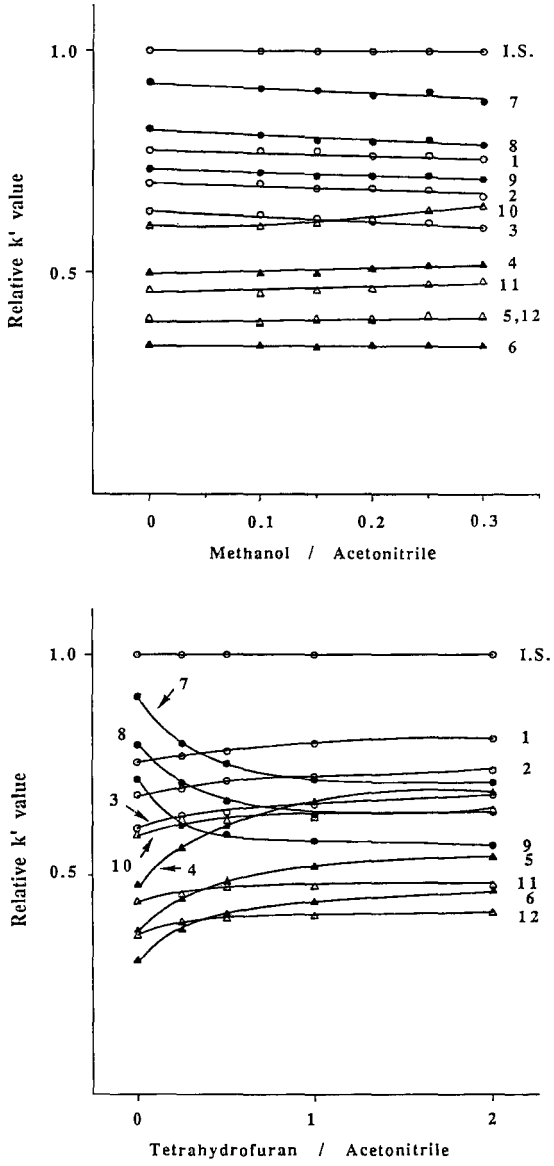


Fig. 3 Effect of content of methanol (A) or tetrahydrofuran (B) in CH_3CN on relative k' values of estrogens. Abbreviations and conditions the same as in Fig 2.

These chromatographic behaviors would be helpful for structural elucidation of equine estrogens in biological fluids. The suitable ratio of THF to acetonitrile in a mobile phase for the separation of DHEq and 2-MeODHEq was found to be in the range of 0.25-1.0. The combined use of binary and ternary solvent systems may serve for the qualitative and quantitative analysis of these estrogens with high reliability. A typical chromatogram obtained with authentic specimens is shown in Fig. 4.

The next effort was directed to investigate the electrochemical properties of equine estrogens. The effect of the applied potential on the sensitivity was tested in the range of 0.3 - 0.8 V *vs.* the silver-silver chloride electrode. The hydrodynamic voltammograms obtained with 1 ng each of these compounds per injection are shown in Fig. 5. It was found that responses of guaiacol estrogens increased linearly up to 0.5 V and then reached a plateau, and, on the other hand, phenolic compounds showed the increasing responses up to 0.8 V(13). From these results, the applied potential was set at 0.7 V. A calibration graph was constructed by plotting the peak area of each compound to that of the internal standard, a linear response to each compound being observed up to 1 ng. The detection limits were estimated to be 5 pg for Equ, DHEq and 2-MeOEqu, and to be lower than 20 pg for other steroids (signal to noise ratio = 5 at 10 nA full-scale).

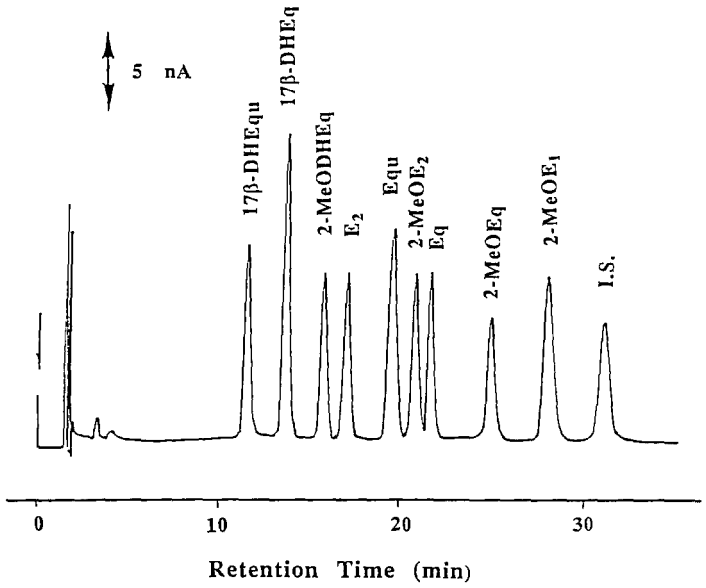


Fig. 4 Typical high-performance liquid chromatogram of the mixture of standard estrogens.
Conditions: column, Cosmosil 5C18-AR; mobile phase, 0.5% acetate buffer (pH 5.0)-methanol-acetonitrile (60:9:30, v/v/v); flow rate, 1.0 ml/min.

The present method was then applied to the characterization and determination of biliary metabolites in rat following oral administration of Eq. The separation and determination of trace compounds in biological fluids is markedly influenced by the clean-up procedure employed. For this purpose, an octadesylsilyl bonded silica cartridge was used for extraction and concentration of estrogens in bile prior to the HPLC analysis. A hundredth milliliter of a bile specimen was subjected to the

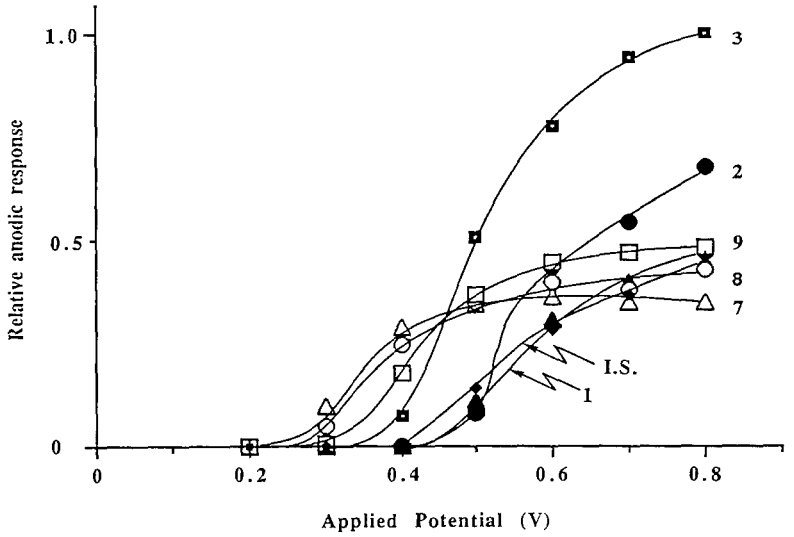


Fig. 5. Hydrodynamic voltamograms of typical estrogens. The maximum response of each steroid is arbitrarily taken as 1.0.
Abbreviations and conditions the same as in Fig. 2 and 4, respectively.

clean-up procedure with a Sep-Pak C_{18} cartridge and the enzymatic hydrolysis with sulfatase containing β-glucuronidase. The liberated steroids were further purified by ion exchange chromatography on a lipophylic gel, PHP-LH-20 to remove co-existing substances and then subjected to the HPLC separation with ECD. The recovery rates carried through the procedure described above were estimated by determining the amount of representative compounds added to a bile specimen. As listed in Table 1, each compound spiked in bile was recovered at a rate of more than 89%.

TABLE 1 Recovery of Equine Estrogens Added to Rat Bile

Compound	Equine estrogen ($ng \cdot ml^{-1}$)		Recovery ± S.D.[a]
	Added	Found	(%)
Eq	50.0	44.6	89.2 ± 4.6
17β-DHEq	50.0	44.5	88.9 ± 3.9
2-MeOEq	50.0	44.3	88.5 ± 5.1
2-MeODHEq	50.0	46.5	93.0 ± 5.7
Equ	50.0	49.7	99.4 ± 3.5
17β-DHEqu	50.0	49.0	98.0 ± 5.6
2-MeOEqu	50.0	49.7	99.4 ± 1.8
2-MeODHEqu	50.0	47.1	94.2 ± 2.6

[a] n=6

A chromatogram of equine estrogens in rat bile is illustrated in Fig. 6. Equ, as a main component, 2-MeOEqu and their 17β-reduced compounds are observed with Eq. The result with the observation of ring B dehydrogenated metabolites is the same with previous findings demonstrated with human endometrium (14) and rat liver (15), and strongly indicates the presence of enzyme, 6,8(9) steroid dehydrogenase. In accordance with the previous study with the pregnant mare (16), formations of B ring saturated estrogens, namely E_1 and E_2, were not found. A conspicuous feature is the new finding that equilin can be metabolized to 2-MeOEq and 2-MeOEqu, which are further

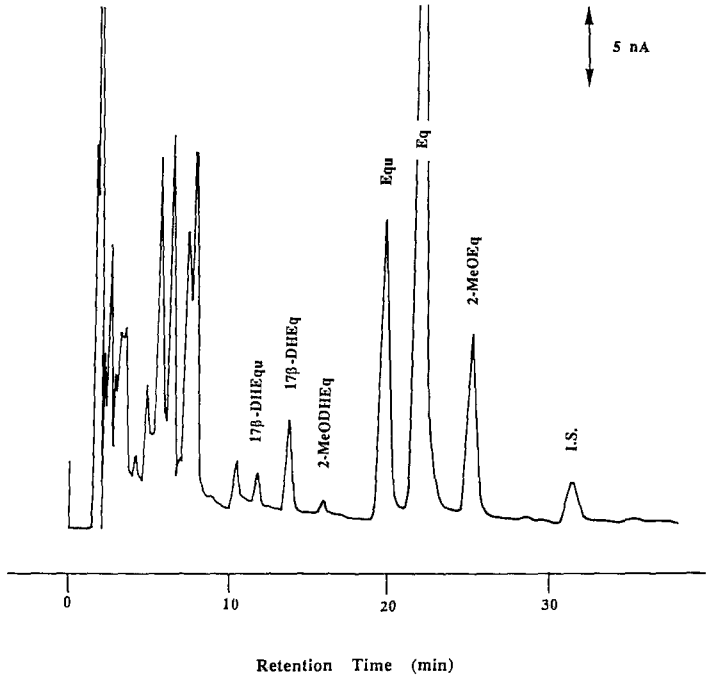


Fig. 6. High-performance liquid chromatogram of estrogens in rat bile after administration of equilin. Conditions the same as in Fig. 4.

metabolized to 17β–reduced compounds in a certain degree. This is the first demonstration of *in vivo* formation of 2-methoxylated metabolites from equilin administered, though the *in vitro* 2- and 4-hydroxylation of equine estrogens have been previously indicated in baboon liver (17). At present, it remains unknown whether 2-MeOEqu is formed from 2-MeOEq or Equ. It is well documented that conversion of phenolic

steroids to catecholic estrogens is major metabolic pathway in man and rodents (8). From these points of view, the present results strongly suggest that the catecholic Eq and/or Equ are formed as intermediary products during circulation in the body. The metabolism of catechol equine estrogens involving O-methylation appears to be an attractive to be resolved.

Recently, it has been shown that administration of [^{3}H]Eq to postmenopausal women and men resulted in the formation of DHEq, DHEqu, and Equ, which were isolated from plasma and urine (10,18). The present investigation indicates that all three metabolites can be formed in rat. It is still unknown that the enzyme involved in interconversion of equilin and equilenin into their 17β-reduced estrogens is identical with 17β-hydroxysteroid dehydrogenase.

Further studies are being conducted to clarify metabolic disposition of equine estrogens.

ACKNOWLEDGEMENT

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COMPUTATIONAL CHEMICAL ANALYSIS OF THE RETENTION OF SACCHARIDES ON AMINO PHASE

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SUMMARY

A model adsorbent of primary amino bonded phase was constructed by molecular design and the structure was optimized by molecular mechanics (MM2) of the CAChe™ program. The properties of standard molecules were calculated by MOPAC-BlogP of the CAChe™. Furthermore, the capacity ratios of saccharides measured on a primary amino bonded phase in aqueous acetonitrile was analyzed by the molecular interaction energies calculated by MM2. The capacity ratios were basically related to summation of calculated van der Waals and hydrogen bonding energies.

INTRODUCTION

Primary amino bonded phases and quaternary amine ion-exchange resins have been used for the separation of saccharides in liquid chromatography. The retention mechanism however has not been well discussed, and it was suggested that the number and the steric position of hydroxy groups are important [1]. The recovery from amino bonded phases was however poor for some saccharides such as mannose, ribose, arabinose and galactose. The reason for the poor recovery may be due to the in-column glycation of saccharides with the amino phase. The reaction mechanism of glycation was analyzed by comparison of the reactivity of saccharides with p-toluidine and computational chemical analysis. The reactivity could be related to the hydrogen bonding energy of the amino group of aromatic amines and hydroxy groups of saccharides as calculated by MM2 of the CAChe™ program [2]. On the other hand, the calculation of molecular interaction made it possible to explain the alkyl chain length effect on hydrogen bonding of alkanols and the chiral recognition mechanism [3]. The retention mechanism of saccharides on the amino phase was further analyzed by computational chemical analysis of molecular interaction between a saccharide and a model amino phase constructed by a computational chemical method

EXPERIMENTAL

The molecular design and computational chemical calculation were performed by the CAChe™ program from Sony-Techtronix (Tokyo-Beaverton(OR)). The computer used was a Macintosh IIfx.

RESULTS AND DISCUSSION

A honeycomb type layer was first constructed, and the two layers were bonded together in parallel to diminish the flexibility

of the hydrocarbon phase. The selectivity of the hydrocarbon phase was examined by the energy value of molecular interaction calculated by molecular mechanics (MM2) of the CAChe™ program, then one surface of the hydrophobic phase was hydroxylated. The hydroxylated phase demonstrated properties similar to those of a vinylalcohol copolymer gel in liquid chromatography and an ethylene glycol phase in gas chromatography where polyaromatic hydrocarbons were retained more than expected from their Van der Waals volumes calculated by MOPAC-Blog P of the CAChe™ program. This may be due to weak hydrogen bonding [4]. The basic hydrocarbon phase was modified by bonding amino groups on one surface as shown in Fig. 1. The dark of circles decreases in order of nitrogen > carbon > hydrogen atoms.

The molecular weight of the amino phase was 5,154 which was constructed of 368 carbons, 30 nitrogens and 318 hydrogens. The basic selectivity of the modified bonded phase having amino

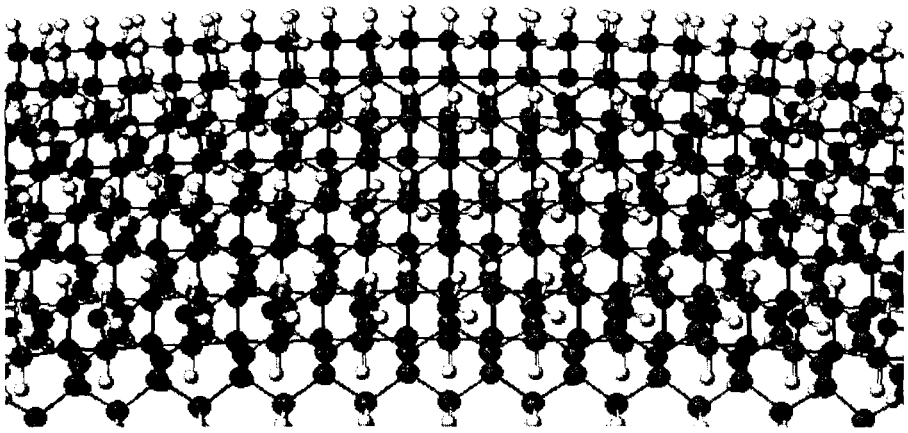

Fig. 1 Computer designed amino phase

Table 1 Molecular interaction energies of standard compounds with amino phase

chemicals	VWV Å^3/mole	TE Kcal/mole	HB Kcal/mole	ES Kcal/mole	VW Kcal/mole
amino phase		1507.85	-40.12	-1.56	376.78
benzene	83.79	1489.94	-47.81	-1.36	372.27
naphthalene	127.60	1473.60	-52.94	-1.32	370.63
anthracene	171.49	1457.24	-58.32	-1.20	369.46
hexane	112.79	1498.46	-40.20	-1.56	366.76
decane	180.01	1496.51	-40.55	0.62	361.64
tetradecane	247.26	1491.06	-40.50	0.11	356.01
pentanol	104.06	1495.49	-45.53	-1.49	368.59
nonanol	171.44	1490.51	-45.43	-1.43	363.10
tridecanol	238.62	1485.40	-45.26	-1.50	357.12
cyclohexane	101.40	1504.50	-40.18	-1.51	370.72
perhydronaphthalene	157.27	1503.55	-40.13	-1.52	367.21
perhydroanthracene	212.88	1516.67	-40.25	-1.51	367.49

VWV: van der Waals volume, TE: total energy, HB: hydrogen bonding energy, ES: electro static energy, VW: van der Waals energy

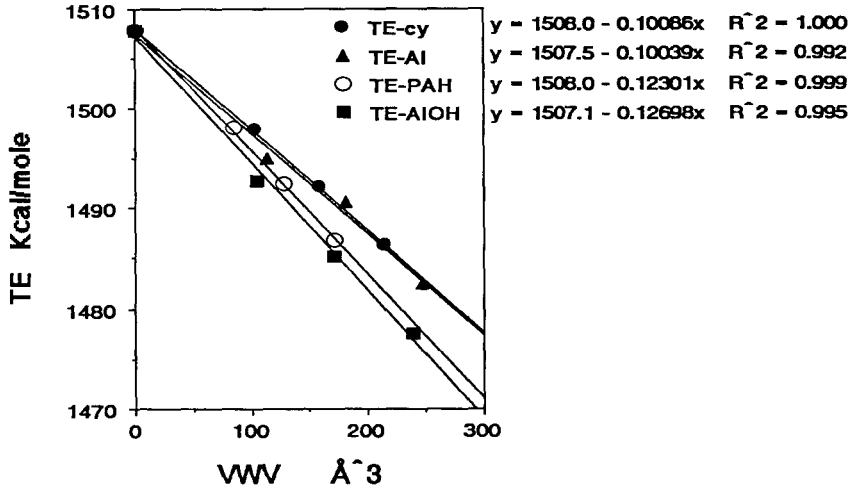

Fig. 2 Selectivity of amino phase based on total energy and van der Waals volume

groups was first examined from the molecular interaction between this phase and standard compounds used for the evaluation of the model hydrophobic adsorbent. The chemicals are given in Table 1 with their van der Waals volumes calculated by MOPAC-BlogP of the CAChe™ program and their molecular interaction energies calculated by MM2 of the CAChe™ program.

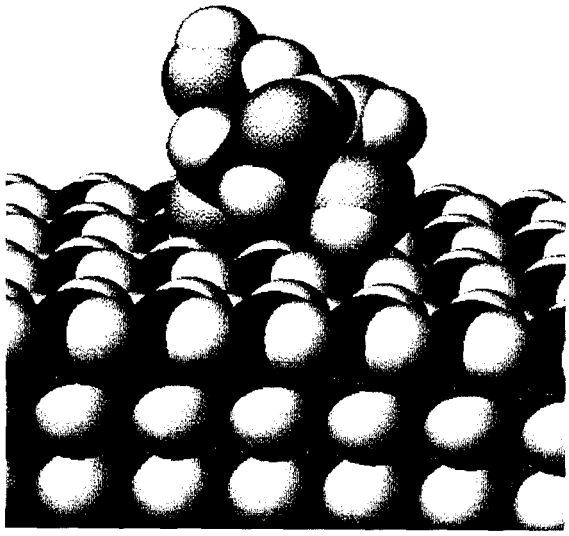

Fig. 3 Adsorption of glucose on the amino phase

The selectivity of this amino phase was examined by comparison of slopes between their van der Waals volumes and calculated energy values as shown in Fig. 2.

The selectivity was enhanced in the order: alkanes ≈ alkanols > cyclic hydrocarbons > aromatic compounds from van der Waals energy calculated by MM2, and alkanols ≈ aromatic compounds > cyclic hydrocarbons ≈ alkanes from total energy calculated by MM2. The selectivity was similar to those for the hydroxylated phase as compared to the result obtained on two hydrophobic phases. The order of selectivity was: alkanes ≈ alkanols > aromatic compounds ≈ cyclic hydrocarbons by van der Waals energy and : aromatic compounds > alkanols > alkanes ≈ cyclic hydrocarbons by total energy.The selectivity was different from that of hydrophobic phases on which aromatic compounds demonstrated the most selectivity by van der Waals energy and the least selectivity by total energy [4].

Amino groups are usually bonded on silica gels, vinylalcohol gels and methyl silicone phase coated on silica gels. The molecular interaction between this amino phase and saccharides was therefore directly examined by MM2 calculation of the CAChe™ program.

The optimized form, a saccharide adsorbed on the amino phase, indicated that the number of hydrogen bonds between a saccharide and the amino phase can be related to their capacity ratios measured by liquid chromatography on a propylamine bonded vinylalcohol copolymer gel in 70% aqueous acetonitrile.

The adsorption form of glucose on the amino phase is shown in Fig. 3. The dark of circles decreases in order of nitrogen > carbon > oxygen > hydrogen atoms. The hydroxy groups of carbon 1 and 2 of glucose made hydrogen bonding with the amino groups of surface, and that of carbon 6 of glucose protruded toward space as seen in Fig. 3. The hydroxy groups of carbon 2, 3 and 4 of mannose made hydrogen bonding with the amino groups of surface, and that of carbon 1 of manose protruded toward space. The capacity ratio was further related to total, hydrogen bonding, electrostatic and van der Waals energies as calculated by MM2.

After subtracting the individual energy of saccharides from the molecular interaction energy listed in Table 2, the capacity ratio demonstrated a good relation with the summation of van der Waals energy and hydrogen bonding energies as given in Fig. 4. Hence, a smaller van der Waals energy means stronger steric forces, and a larger hydrogen bonding energy means strongerhydrogen bonding. An exception was ribose. These saccharides may be adsorbed by their reversed form, therefore, the molecular interaction energy was calculated. The molecular interaction energy of the reversed-form was generally less than that of normal form that was first calculated and listed in Table 2. The smaller value of summation of van der Waals energy and hydrogen bonding energy calculated first and that done later was also related to the capacity ratio. The

Table 2 Molecular interaction energy of saccharides with amino phase

saccharide	k'	TE*	HB*	ES*	VW*	NH2/TE#	NH2/HB#	NH2/ES#	NH2/VW#
Arabinose	0.93	16.66	-3.04	5.83	2.32	1507.00	-51.35	3.90	370.75
Fructose	1.03	19.81	-3.30	10.60	2.24	1509.65	-51.17	8.46	369.18
Galactose	1.28	18.99	-2.28	9.03	5.11	1503.99	-58.63	8.75	372.20
Glucose	1.38	15.36	-3.94	8.24	4.62	1501.84	-64.90	9.07	374.12
Mannose	1.25	31.93	-3.18	9.11	8.27	1512.58	-53.36	7.37	373.14
Ribose	0.83	16.12	-2.65	5.94	1.89	1505.64	-60.29	3.64	373.36
Xylose	1.01	15.99	-3.36	5.07	2.30	1508.91	-51.23	4.26	370.27

unit: Kcal/mole, * energies of saccharides , # energies of molecular interaction

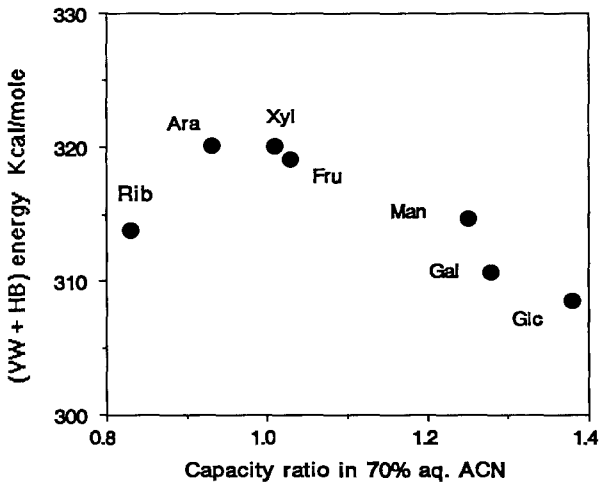

Fig. 4 Capacity ratio related to calculated energy

smaller energy showed a good relation with the capacity ratio, however glucose was an exception in this case.

If the retention form in chromatography and the surface structure of adsorbent are known, the difference of retentiontime, hence that of molecular interaction can be related to energy values calculated by computational chemistry.

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MEETING ANNOUNCEMENT

1994 PREP SYMPOSIUM & EXHIBIT

sponsored by the
Washington Chromatography Discussion Group

June 12 - 15, 1994

at the
Georgetown University Conference Center by Marriott
Washington, DC

Prof. Georges Guiochon, Chairman

This PREP symposium series has become an important meeting for presenting application and new advances in the area of preparative chromatography and related techniques. As a result of strong interest in this series of symposia, the PREP Symposium will be held every year in the United States and will include a major exposition.

Information about the Prep Symposium Series may be obtained from Ms. Janet E. Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(1), 251-257 (1994)

LIQUID CHROMATOGRAPHY CALENDAR

1994

JANUARY 10 - 14: Supercritical Fluid Chromatography and Extraction, Hyatt Regency Hotel on the Inner Harbor, Baltimore, Maryland. Contact: Larry T. Taylor, Dept of Chemistry, Virginia Polytechnic Institute & State University, Blacksburg, VA 24061, USA.

JANUARY 16 - 20: 19th IUPAC Symposium on the Chemistry of Natural Products, Karachi, Pakistan. Contact: Prof. Atta-Ur-Rahman, Director H. E. J. Research Institute of Chemistry, University of Karachi, Karachi-75270, Pakistan.

FEBRUARY 2 - 3: AOAC Southeast Section Meeting, Ramada Hotel & Convention Center, Atlanta, GA. Contact: Doug Hite, Technical Services, P. O. Box 40627, Melrose Station, Nashville, TN 37204, USA.

FEBRUARY 28 - MARCH 4: PittCon'94: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, Chicago, Illinois. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd, Pittsburgh, PA 15235-9962, USA

MARCH 22 - 24: PrepTech '94, A New Conference on Industrial Bioseparations, Meadowlands Hilton Hotel, S3ecaucus, New Jersey. Contact: Symposium Manager, PrepTech '94, ISC, Inc., 30 Controls Drive, Shelton, CT 06484, USA.

APRIL 10 - 15: 207th ACS National Meeting, San Diego, Calif. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036, USA.

APRIL 19 - 22: Rubber Division ACS, 145th Spring Technical Meeting, Palmer House Hotel, Chicago, Illinois. Contact: C. Morrison, Rubber Division, P.O. Box 499, Akron, OH 44309-0499, USA.

MAY 8 - 13: HPLC '94: Eighteenth International Symposium on High Performance Liquid Chromatography, Minneapolis Convention Center, Minneapolis, Minnesota. Contact: Mrs. Janet Cunningham, Barr Enterprises, P.O. Box 279, Walkersville, MD 21793, USA.

JUNE 1 - 3: Joint 26th Central Regional/27th Great Lakes Regional Meeting, ACS, Kalamazoo and Huron Valley Sections. Contact: H. Griffin, Univ of Michigan, Ann Arbor, MI 48109, USA.

JUNE 1 - 3: International Symposium on Hormone & Vetrinary Drug Residue Analysis, Congress Centre Oud Sint-Jan, Brugge, Belgium. Contact: Prof. C. Van Peteghem, University of Ghent, L:aboratory of Food Analysis, Harelbekestraat 72, B-9000 Ghent, Belgium.

JUNE 5 - 7: VIth International Symposium on Luminescence Spectrometry in Biomedical Analysis - Detection Techniques & Applications in Chromatography and Capillary Electrophoresis, Congress Centre Oud Sint-Jan, Brugge, Belgium. Contact: Prof. Dr. Willy R. G. Baeyens, University of Ghent, Pharmaceutical Institute, Dept. of Pharmaceutical Analysis, Harelbekestraat 72, B-9000 Ghent, Belgium.

JUNE 12 - 15: 1994 Prep Symposium & Exhibit, sponsored by the Washington Chromatography Discussion Group, at the Georgetown University Conference Center by Marriott, Washington, DC. Contact: Janet E. Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

JUNE 13 - 15: Fourth International Symposium on Field-Flow Fractionation (FFF'94), Lund, Sweden. Contact: Dr. Agneta Sjogren, The Swedish Chemical Society, Wallingatan 24, 2 tr, S-111 24 Stockholm, Sweden.

JUNE 19 - 23: 24th National Medicinal Chem. Symposium, Little America Hotel & Towers, Salt Lake City, Utah. Contact: M. A. Jensen & A. D. Broom, Dept. of Medicinal Chem, 308 Skaggs Hall, Univ of Utah, Salt Lake City, UT 84112, USA.

JUNE 19 - 24: 20th International Symposium on Chromatography, Bournemouth International Centre, Bournemouth, UK. Contact: Mrs. Jennifer A. Challis, The Chromatography Society, Suite 4, Clarendon Chambers, 32 Clarendon Street, Nottingham, NG1 5JD, UK.

JUNE 24 - 27: BOC Priestly Conference, Bucknell University, Lewisburg, PA, co-sponsored with the Royal Soc of Chem. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

JULY 31 - AUGUST 5: American Society of Pharmacognosy Annual Meeting, International Research Congress on Natural Products, joint meeting with the Assoc. Français pour l'Enseignement et la Recherche en Pharmacognosie, and the Gesellschaft fur Arzneipflanzenforschung, and the Phytochemical Society of Europe, Halifax, Nova Scotia, Canada. Contact: D. J. Slatkin, P. O. Box 13145, Pittsburgh, PA 15243, USA.

AUGUST 21 - 26: 208th ACS National Meeting, Washington, DC. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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.

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

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 13 - 16: 50th Southwest Regional Meeting, ACS, Fort Worth, Texas. Contact: H. C. Kelly, Texas Christian Univ, Chem Dept, Ft. Worth, TX 76129, 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.

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 15 - 17: 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.

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 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 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.

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.

The Journal of Liquid Chromatography will publish, **at no charge**, announcements of interest to liquid chromatographers in every issue of the Journal. To be listed in the LC Calendar, we will need to know: Name of the meeting or symposium, sponsoring organization, when and where it will be held, and whom to contact for additional details. Incomplete information will not be published. You are invited to send announcements to **Dr. Jack Cazes, Editor, Journal of Liquid Chromatography, P.O. Box 2180, Cherry Hill, NJ 08034-0162, USA.**

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

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

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

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MATERIALS
METHODS
RESULTS
DISCUSSION
ACKNOWLEDGEMENTS
REFERENCES

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

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

5. The first word of each **paragraph** within the body of the text should be indented five spaces.

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

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

Following are acceptable reference formats:

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

Book:

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

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.

9. Only standard symbols and nomenclature, approved by the International Union of Pure and Applied Chemistry (IUPAC) should be used.

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

Additional Typing Instructions

1. The manuscript must be prepared on good quality **white bond paper**, measuring approximately 8½ x 11 inches (21.6 cm x 27.9 cm). The typing area of the first page, including the title and authors, should be 5½ inches wide by 7 inches high (14 cm x 18 cm). The typing area of all other pages should be no more than 5½ inches wide by 8½ inches high (14 cm x 21.6 cm).

2. The **title, abstract, tables and references** are typed single-spaced. All other text should be typed 1½-line spaced or double line spaced.

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

4. **Tables** should be typed on separate pages, one table to a page. A table may not be longer than one page. If a table is larger than one page, it should be divided into more than one table. The word **TABLE** (capitalized and followed by an Arabic number) should precede the table and should be centered above the table. The title of the table should have the first letters of all main words in capitals. Table titles should be typed single line spaced, across the full width of the table.

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

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