JOURNAL OF LIQUID CHROMATOGRAPHY

VOLUME 6 NUMBER 9 1983

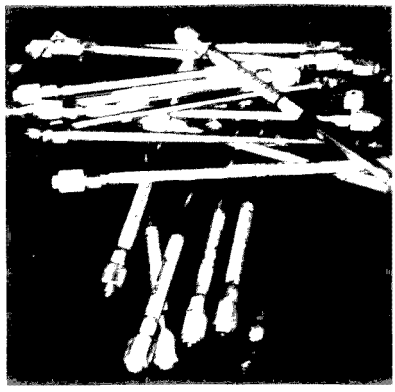
CODEN: JLCHD8 6(9) i-viii, 1557-1726 (1983)
ISSN: 0148-3919

GPC

COLUMN REGENERATION SERVICE

μSTYRAGEL™ TOYO SODA ULTRA STYRAGEL™

SHODEX μBONDAGEL™ μSPHEROGEL™



All of these GPC columns give excellent performance. However, when they fail for various reasons (many of which are beyond your control) you must replace them at great expense. The GPC gels themselves NEVER WEAR OUT and may be used over and over as often as necessary!

ASI can clean and repack any 10 micron GPC packing to ***exceed*** new column specifications. All columns are unconditionally guaranteed for ***in use*** performance. Regardless of responsibility, if an ASI repacked column fails ***for any reason*** within the warranty period, it will be repaired at no additional cost.

Compare the performance, warranty and price and you will see that ASI offers a truly remarkable value.

REPACKED BY ASI			REPLACEMENT COST			
PERFORMANCE	WARRANTY	PRICE	COLUMN TYPE	PERFORMANCE	WARRANTY	PRICE
>20,000 p m	120 days	$250	WATERS ASSOC μSTYRAGEL™	9000-12000 p m	?	$595
>30,000	90	$350	WATERS ASSOC ULTRASTYRAGEL™	>46,000	?	$675
>20,000	120	$250-500	TOYO SODA (TSK)	20,000	?	$630-1050
>20,000	120	$250-500	SHODEX	20,000	30 DAYS	$750-1080
>20,000	120	$250	BECKMAN μSPHEROGEL™	20,000	30 ?	$620
>15,000	90	$175	WATERS ASSOC μBONDAGEL™	?	?	$405-495

asi ANALYTICAL SCIENCES INCORPORATED

SUITE B-24 AIRPORT PARK • 1400 COLEMAN AVENUE • SANTA CLARA CALIF 95050 • (408) 779-0131

. . . . THE WORLD'S FINEST LIQUID CHROMATOGRAPHY COLUMNS

Circle Reader Service Card No. 101

JOURNAL OF LIQUID CHROMATOGRAPHY

Editor: DR. JACK CAZES Editorial Secretary: ELEANOR CAZES
P. O. Box 1440-SMS
Fairfield, Connecticut 06430

JOURNAL OF LIQUID CHROMATOGRAPHY

JOURNAL OF LIQUID CHROMATOGRAPHY

July 1983

Aims and Scope. The journal publishes papers involving the application of liquid chromatography to the solution of problems in all areas of science and technology, both analytical and preparative, as well as papers that deal specifically with liquid chromatography as a science within itself. Included will be thin-layer chromatography and all modes of liquid chromatography.

Indexing and Abstracting Services. Articles published in *Journal of Liquid Chromatography* are selectively indexed or abstracted in:

• Analytical Abstracts • ASCA • BioSciences Information Service of Biological Abstracts (BIOSIS) • Chemical Abstracts • Current Contents/Life Sciences • Current Contents/Physical and Chemical Sciences • Engineering Index • Excerpta Medica • Journal of Abstracts of the All-Union Institute of Scientific and Technical Information of the USSR • Physikalische Berichte • Science Citation Index

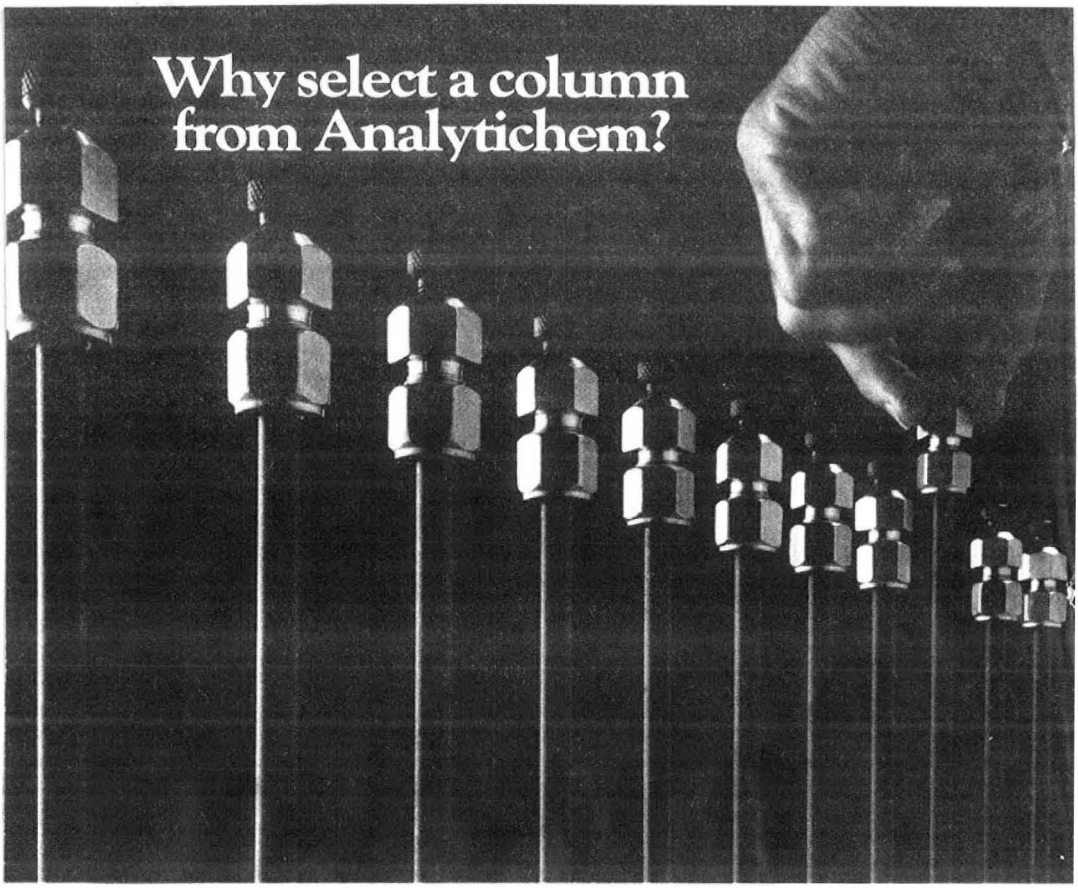
Manuscript Preparation and Submission. See the last page of this issue.

Subscription Information. *Journal of Liquid Chromatography* is published in fourteen numbers and two supplements in January, February, March (3 numbers), April, May, June (2 numbers), July, August, September (2 numbers), October, November, and December by Marcel Dekker, Inc., 270 Madison Avenue, New York, New York 10016. The subscription rate for Volume 6 (1983), containing fourteen numbers and two supplements, is $298.00 per volume (prepaid). The special discounted rate for individual professionals and students is $149.00* per volume. To secure this special rate, your order must be prepaid by personal check or may be charged to MasterCard or VISA. Add $36.80 for surface postage outside the United States. For airmail to Europe, add $72.32; to Asia, add $91.52.

Mailing Address. *Please mail payment with order to:* Marcel Dekker Journals, P. O. Box 11305, Church Street Station, New York, New York 10249.

Contributions to this journal are published free of charge. Second-class mailing permit pending at New York, New York and at additional mailing offices.

*THIS REFLECTS A 50% DISCOUNT GIVEN TO INDIVIDUAL SUBSCRIBERS.



Because the greater the selection the greater the selectivity.

The new line of HPLC columns from Analytichem provides selectivity unequalled by anyone. The reason is simple. Analytichem offers a wider selection of phases than anyone.* Now you can choose the phase that is precisely suited to your particular application. These new columns, packed with our unique Sepralyte™ 5μm spherical media, set an unprecedented standard of chromatographic efficiency... regardless of the phase you select.

The performance of each new Analytichem column is fully guaranteed and backed by the industry's strongest customer service and technical support teams. Our technical advisors have the training and hands-on experience to assist you in solving virtually any separation problem. Next time you're considering HPLC columns, be selective. Call Analytichem. You'll find the columns you need and the service you deserve.

Analytichem International
24201 Frampton Ave., Harbor City,
CA 90710, USA. (800) 421-2825.
In California (213) 539-6490
TELEX 664832 ANACHEM HRBO

*C18, C8, C2, Phenyl, Cyano, Amino, Diol, Silica, Quaternary Amine, Carboxylic Acid, Sulfonic Acid

JOURNAL OF LIQUID CHROMATOGRAPHY

Volume 6, Number 9, 1983

CONTENTS

Catecholamines & Metabolites

or other neurochemically significant compounds

WITHIN

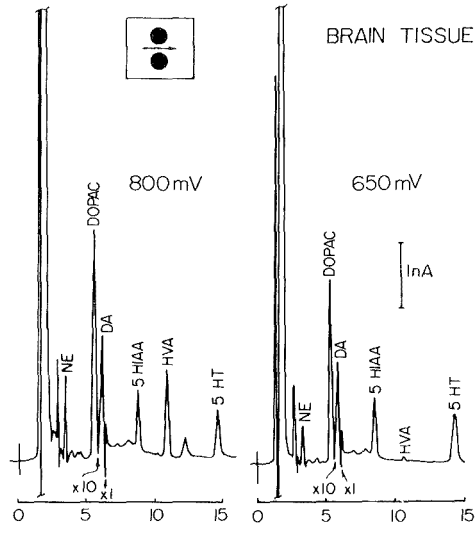
Brain Tissue
Adrenal Tissue
Body Fluids
Dosage Forms
Plant Matter
Insect Tissue

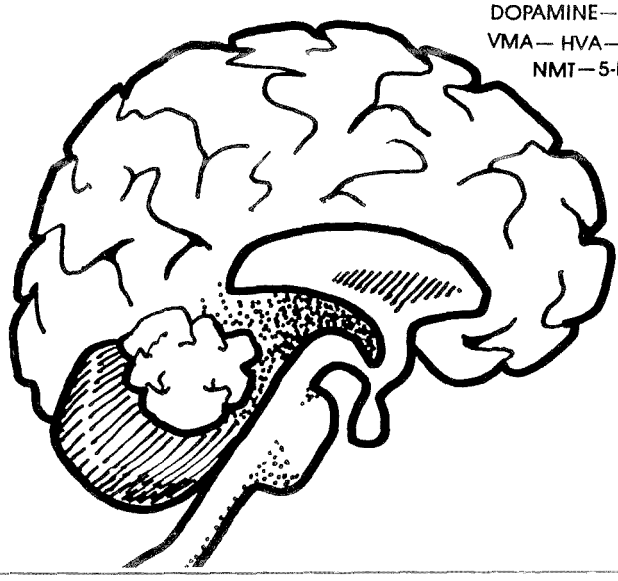
WITHOUT

Derivatives
Radioisotopes
Fluorescence
Gas Chromatography
Mass Spectrometry
A Rich Uncle

BAS Analyzers based on liquid chromatography/electrochemistry (LCEC) have replaced radiochemical and fluorescence procedures in all of the above areas. Detailed procedures, sample preparation materials, and support from our own analytical laboratory compliment each complete instrument package. These systems are capable of monitoring many phenolic natural products and drugs. All of the catecholamine metabolites and many tryptophan metabolites can be determined. In addition, the activity of many neurologically important enzymes can be measured. Let us know your specific requirements.

DON'T CIRCLE THE NUMBER! Call or write today for a detailed reply!





DOPAMINE—NOREPINEPHRINE—EPINEPHRINE
VMA— HVA—DOPAC—MHPG—M—NM 3-MT
NMT—5-HT—5-HIAA — TH—DBH— COMT
PHENOTHIAZINE —MORPHINE
TRICYCLIC ANTIDEPRESSANTS
GLUTATHIONE—GABA

BAS

1205 KENT AVENUE
WEST LAFAYETTE, IN
47906
317 463 2505

Circle Reader Service Card No. 105

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(9), 1557-1558 (1983)

LEST WE EXAGGERATE.....

How often do we run across phrases in technical publications that refer to quantity or degree of ..... which are obviously not quantitative at all, but are merely used to impress someone or emphasize an experimental conclusion? Non-specific terms are used to represent real quantities. One wonders, for instance, how many experts were consulted when an author concluded that, "most authorities agree that ....." I recently came across a list known as Ried's Table that attempts to assign absolute values to non-specific descriptions.

Here's an adaptation of Ried's Table.

Description	Value
One	1
Only one	1
A couple	2 - 4
A few	3 - 5
Quite a few	3 - 6
Several	3 - 9
Many	3 - 8
Most ("most authorities")	4 - 6
Half a dozen	5 - 7
About half a dozen	4 - 8
A lot	6 - 10
Quite a lot	7 - 11
A whole lot	8 - 17
Ten	9 - 11
Around ten	7 - 13
A dozen	11 - 13
About a dozen	9 - 15
A bunch	8 - 15
A whole bunch	9 - 19
Two dozen	22 - 26
About two dozen	21 - 27
A few hundred	101 - 201
A couple of hundred	101 - 202
Two or three hundred	140 - 275
Most	10 - 20%
A majority	50% + 1

0148-3919/83/0609-1557$3.50/0

A clear majority	51%
A vast majority	52 - 60%
An overwhelming majority	61 - 70%
Almost all	71 - 75%
Practically all	76 - 80%
All	81 - 85%
Absolutely all	86 - 90%
100% of those tested	91 - 95%

Jack Cazes,
Editor

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(9), 1559-1576 (1983)

SEDIMENTATION-FLOTATION FOCUSING FIELD-FLOW FRACTIONATION IN CHANNELS WITH MODULATED CROSS-SECTIONAL PERMEABILITY. I. THEORETICAL ANALYSIS

Josef Janča and Věra Jahnová
Institute of Analytical Chemistry
Czechoslovak Academy of Sciences
611 42 Brno, Czechoslovakia

ABSTRACT

A new-shaped cross-section of a channel with modulated permeability used in Sedimentation-Flotation Focusing Field-Flow Fractionation is proposed. The shape of the resulting velocity profile inside the channel is described as a function of the geometric channel characteristics. Basic separation parameters such as retention, efficiency and the related resolution are discussed. The principle of a channel with modulated permeability can also be applied to other subtechniques of Field-Flow Fractionation.

INTRODUCTION

Field-Flow Fractionation (FFF) is a separation method conceptually proposed by Giddings (1) who, together with his coworkers, elaborated the theory, the experimental techniques and a number of applications for the purpose of separating macromolecules and particles.

The principle of the method bases upon the simultaneous influence of concentration and flow inhomogeneities. The separation is due to differences

 0148-3919/83/0609-1559$3.50/0

in the migration velocity of the solute species separated in a narrow channel of rectangular cross-section through which a fluid is flowing. The physical field applied perpendicularly to the channel longitudinal axis, for instance, a thermal, electrical or magnetic field or gravitational forces, etc., produces a concentration gradient across the channel, commonly along the direction of the forces of the field. The streamline velocity of the fluid passing through the channel also changes across the channel. This velocity gradient is caused by viscosity effects associated with flow processes.

In our preliminary communication (2) we described the principle of a new separation method called Sedimentation-Flotation Focusing Field-Flow Fractionation (SFFFFF). This new method is based on the well-known physical phenomenon owing to which suspended particles or dissolved macromolecules sediment or float in the density gradient of the liquid phase. The sedimentation-flotation processes cause the solute of a given density to concentrate to a layer wherein the density of the environment is the same as that of the solute. Diffusion processes or the Brownian migration act in the opposite direction tending to disperse the concentrated solute zone that was formed. After a while dynamic equilibrium is reached, the solute flux due to sedimentation-flotation forces being just equilibrated by the flux resulting from dispersive processes and, as a result, the solute is focused to a narrow zone whose concentration distribution corresponds to the local density gradient. Upon attaining this dynamic equilibrium or even in the course of equilibration, the liquid in the narrow fractionation channel moves in a direction perpendicular to the density gradient

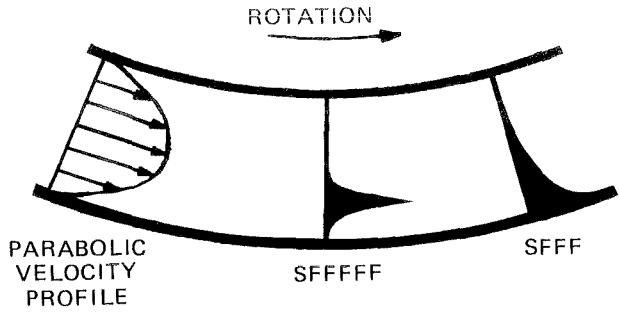


FIGURE 1. Section of the rotating channel with parabolic velocity profile. The concentration distribution across the channel is Gaussian in case of sedimentation-flotation focusing field-flow fractionation (SFFFFF) and exponential in case of sedimentation field-flow fractionation (SFFF).

formed. Under laminar flow conditions a steady velocity profile is produced and the solute zone moves along the channel with a linear velocity corresponding to the streamline velocity in the relevant coordinate. In our initial proposal (2) we supposed that the channel across which a density gradient and, consequently, a concentration distribution of the solute is formed, was narrow enough and that the velocity profile generated was parabolic as shown in Figure 1. This figure compares, at the same time, the Gaussian concentration distribution of the solute for SFFFFF and the exponential distribution for the classical Sedimentation Field-Flow Fractionation method (SFFF) (3).

This paper examines the theoretical aspects of SFFFFF for the case of a separating channel with modulated cross-sectional permeability that leads to a more complex distribution of the streamline velocities inside the channel. When chosing the appropriate shape of the cross-section, improved separation characteristics can be achieved by means of the SFFFFF method.

ANALYSIS

The distribution of the streamline velocities of a unidirectional steady flow in a channel of given cross-section can be expressed by solving the general equation

$$\frac{\delta^2 u}{\delta y^2} + \frac{\delta^2 u}{\delta z^2} = - \frac{G}{\mu} \qquad (1)$$

where u is the linear streamline velocity along the x-axis, y and z are coordinates in the plane of the channel cross-section, $G = \Delta P/L$ is the pressure gradient throughout the channel length L, and μ is the viscosity of the fluid. The analytical solution of Equation (1) for a given shape of the channel cross-section is known for certain simple cases only (4). One of the latter is a channel of rectangular cross-section. The positions of the walls of a rectangular channel are determined by the coordinates $y = \pm b$ and $z = \pm c$, $c > b$, where $a = c/b$ is called aspect ratio. Since the expression

$$u = \frac{1}{2} G \, (b^2 - y^2) \, / \mu \qquad (2)$$

is an even function of the both variables y and z that satisfies the Laplace equation and is zero for $y = \pm b$, it can be written as the Fourier series for y of the form

$$\sum_{n \text{ odd}} A_n \cosh \frac{n \pi z}{2b} \cos \frac{n \pi y}{2b} \qquad (3)$$

while the coefficients A_n can be found from the

boundary condition for $z = \pm c$. This results in a relatively complex function describing the shape of the velocity profile in the coordinates y and z of a rectangular channel (5). An approximate simplified solution (5) suited to describe the shape of the velocity profile, if $c \gg b$ and $a \gg 1$, respectively, is given by

$$U = (1 - Y^2)\left[1 - \frac{\cosh\,(\sqrt{3}\,a\,Z)}{\cosh\,(\sqrt{3}\,a)}\right] \qquad (4)$$

where $U = u/u_{max}$ (u_{max} is the maximum streamline velocity in the channel centre), $Y = y/b$ and $Z = z/c$.

Figure 2 demonstrates the course of the dependence of U on Z for $Y = 0$ and for two different values of $a = 20$ and 100. It is obvious from the figure that the velocity profile formed in the plane of the z and x axes is flat in the central part while it is steeply bending near the side walls of the channel only. The higher the value of a, the steeper the curvature that begins relatively nearer to the wall. On the other hand, the velocity profile formed in the plane of the x and y axes or in parallel planes is parabolic as shown in Figure 3 for illustration.

Let us examine now the changes of the velocity profile in the plane of the x and z axes or in parallel planes in the case of the channel being of other than rectangular cross-section so that its permeability may change continuously along the z axis. We shall consider two cases that may be of practical importance: a channel whose two longer walls consist of planes containing the angle α (see Figure 4a), and a channel whose two longer walls consist of parabolic surfaces (see Figure 4b).

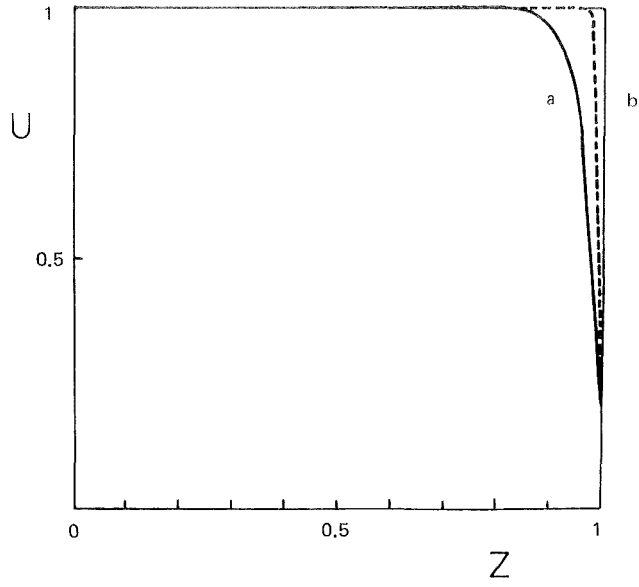


FIGURE 2. Velocity distribution in a rectangular channel. Aspect ratio a: a = 20 b: a = 100.

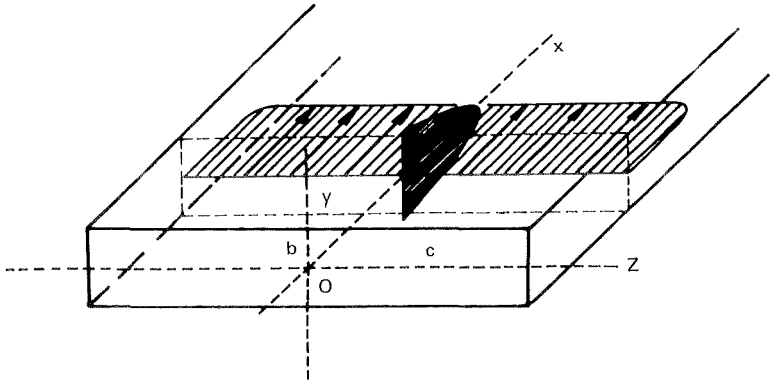


FIGURE 3. Schematic illustration of velocity distributions in a rectangular channel.

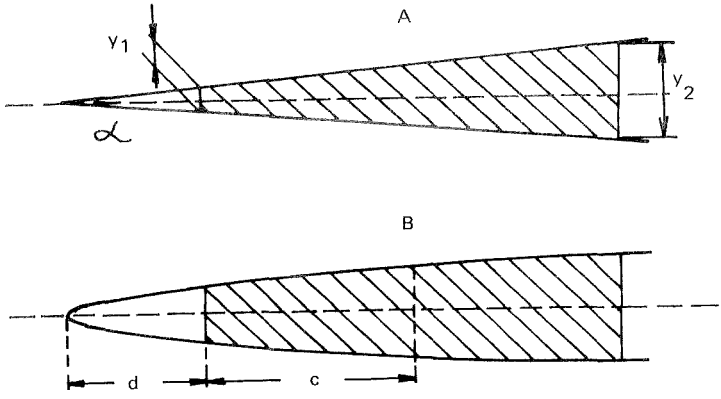


FIGURE 4. Cross-sections of two different model channels.

In case that channel permeability changes along the z axis, Equation (4) can be re-written as follows

$$U = \varphi(Z)\ (1 - Y^2)\left[1 - \frac{\cosh\ (\sqrt{3}\ a\ Z)}{\cosh\ (\sqrt{3}\ a)}\right] \quad (5)$$

where $\varphi(Z)$ is the permeability in the given point Z. In order to calculate and quantitatively express permeability in point Z, we can suppose with fair approximation that, in the close vicinity of point Z, relations describing the flow of a fluid between two parallel planes are valid and that, as a consequence, velocity U_Z in point Z, normalized relative to Z=0 is

$$U_Z = \frac{b^2(Z)}{b^2(0)}\ (1 - Y^2) \quad (6)$$

Therefore, linear velocity U is a function of the square of the distance of the channel walls in point Z. For instance, it holds for the velocity ratio in points A and B,

$$\frac{U_A}{U_B} = \left[\frac{b_A}{b_B}\right]^2 \qquad (7)$$

In view of the above facts, the following relation holds for a channel formed by two planes containing a given angle α i.e. for a channel geometrically determined by the length ratio of the two shorter walls y_1 and y_2 (see Figure 4a) and by the aspect ratio,

$$U^* = \left[1 + Z \left(\frac{y_2 - y_1}{y_2 + y_1}\right)\right]^2 (1 - Y^2) \left[1 - \frac{\cosh(\sqrt{3}\, aZ)}{\cosh(\sqrt{3}\, a)}\right] \qquad (8)$$

where U^* is the normalized velocity $U^* = U/U_{Z=0}$ ($U_{Z=0}$ being the velocity for Z=0).

The aspect ratio in Equation (8) is also a function of the coordinate Z. Since variability a(Z) affects the resulting form of the function $U^* = U(Z)$ only in the marginal parts when Z is approaching ± 1, constant values of $\underline{a}$ for calculating U^* in both marginal parts can be applied in Equation (8) without substantially affecting the course of the function $U^* = U(Z)$ in the important central part. Figure 5 shows the course of the functions $U^* = U(Z)$ for two different channels differing in the y_2/y_1 ratio, that means in the modulated permeability along the z-axis. The calculations were made for two different values of a = 20 and 100 in order to evaluate the influence of variability a(Z) on the results of the calculations. In this case, if the longer channel walls consist of two planes containing a given angle, the central parts of the velocity profiles are shaped like parabolic sections with the maximum velocity in the vicinity of one of the shorter side walls of the channel.

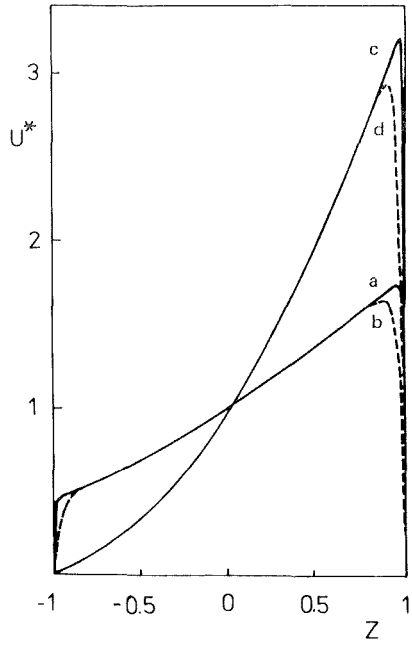


FIGURE 5. Normalized velocity distributions in channels with plane walls. a: $y_2/y_1 = 2$, $a = 100$, b: $y_2/y_1 = 2$, $a = 20$, c: $y_2/y_1 = 10$, $a = 100$, d: $y_2/y_1 = 10$, $a = 20$.

In case the channel consists of two surfaces parabolic in shape as shown in Figure 4b, the following relation holds instead of Equation (7)

$$\frac{U_A}{U_B} = \frac{c + d + c\,Z_A}{c + d + c\,Z_B} \tag{9}$$

and it holds for U^*

$$U^* = \left(\frac{cZ}{c+d} + 1\right)(1-Y^2)\left[1 - \frac{\cosh(\sqrt{3}\,a\,Z)}{\cosh(\sqrt{3}\,a)}\right] \tag{10}$$

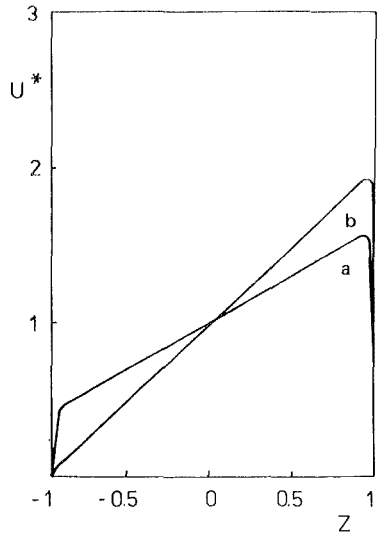


FIGURE 6. Normalized velocity distributions in channels with parabolic walls. a: c = 1.5, d = 1, a = 100, b: c = 49.5, d = 1, a = 100.

where d is the distance from the apex of the parabola to the shorter of the side walls (see Figure 4b).

The central part of the velocity profiles in the planes parallel to the x and z axes is linear in this case (as schematically depicted in Figure 6). The velocity profiles in the planes parallel to the plane where the x and y axes are situated are parabolic again.

RETENTION

If centrifugal forces are applied across the channel along the z axis, a steady-state or quasi-stationary density gradient can be formed in the two

or multicomponent system of the liquid phase under appropriate conditions. For the thermodynamic equilibrium, this system should satisfy the condition

$$M_i \left(1 - \bar{v}_i \rho(Z)\right) \omega^2 r \, dr - \sum_k \left(\delta\mu_i/\delta c_k\right) dc_k = 0 \quad (11)$$

The quantities M_i, $\bar{v}_i$, μ_i, and c of Equation (11) are molecular weight, partial specific volume, the chemical potential and the concentration of the i-th component, respectively, while r is the distance from the axis of rotation, $\rho(Z)$ is the density of the system at coordinate Z and ω is the rotational velocity of the centrifuge rotor. If the substance to be separated - the solute - is injected into the channel as a short puls, solute migration along the z axis takes place due to the density gradient. The concentration distribution of the solute at equilibrium or at steady-state condition along the z axis in the channel can be described with the aid of a Gaussian function of the form, for instance,

$$c(Z) = c_{max} \exp\left[- \frac{V \omega^2 Z_{max}}{2 RT} \frac{d\rho}{dZ} \left(Z_{max} - Z\right)^2 \right] \quad (12)$$

where c(Z) is the concentration in the coordinate Z, V is the molar volume of solute, R is the gas constant, and T is the absolute temperature. The coordinate Z_{max} corresponds to the maximum concentration of the solute, that means to the position where the solute density equals the density of the environment in the liquid phase. Equations (11) and (12) and the way of deriving them were discussed in detail in a previous paper by the author (2).

If two or more different solutes differing in density are simultaneously present in the channel,

their concentrating is effected due to sedimentation-flotation forces in various coordinates Z_{max}, in accordance with the course of the dependence of the liquid-phase density on coordinate Z. As soon as the liquid phase begins to move along the channel in the direction of the x axis, a velocity profile is formed along the z axis, that means along the density gradient or in the direction of the separation of the individual solutes. As a consequence, various solutes will be entrained at different linear velocities along the x axis, and this leads to their effective separation as can be seen from the schematic illustration in Figure 7. The individual separated solute species are detected at the channel outlet by means of a suitable detector, in a similar way as in chromatography.

EFFICIENCY

In chromatography, separation quality is generally characterized by the height equivalent to the theoretical plate H. If supposing, again, that, in the channel coordinate Z, H can be described with the aid of relations valid for two parallel infinite planes and that the starting solute concentration along the y axis is homogeneous, it holds (6)

$$H = 2\,D/\bar{u}(Z) + w^2(Z)\,\bar{u}(Z)/105\,D \qquad (13)$$

where D is the diffusion coefficient of the solute, $w(Z) = 2b(Z)$ is the distance of the channel walls along the y axis in the coordinate Z, and $\bar{u}(Z)$ is the average velocity of the liquid flowing in the channel at the coordinate Z. The highest efficiency, that means

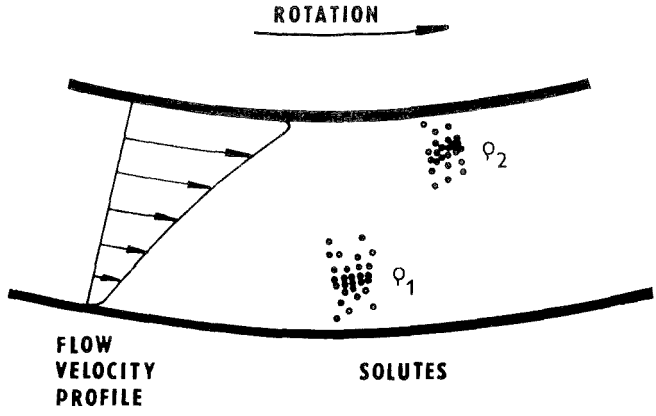


FIGURE 7. Separation by SFFFFF of two solute species in a rotating channel with modulated cross-sectional permeability.

minimum values of H, can be attained in the minimum of the function described by Equation (13). This minimum can be found in putting the first derivative of $dH/d\bar{u}(Z)$ equal to zero

$$\frac{dH}{d\bar{u}(Z)} = - \frac{2D}{\bar{u}^2(Z)} + \frac{w^2(Z)}{105\ D} = 0 \qquad (14)$$

The optimum velocity $\bar{u}_{opt}$

$$\bar{u}_{opt} = \frac{D}{w(Z)} \sqrt{210} \qquad (15)$$

corresponds to the minimum of this function.
The minimum value of H_{min} is given by the relation

$$H_{min} = \frac{2\ w(Z)\ \sqrt{210}}{105} = 0.276\ w(Z) \doteq \frac{w(Z)}{4} \qquad (16)$$

RESOLUTION

The chromatographic resolution R_s of two solutes 1 and 2 has been defined by the known equation

$$R_s = \frac{2(t_{R1} - t_{R2})}{t_{W1} + t_{W2}} \qquad (17)$$

where t_{Ri} are the retention times of solutes 1 and 2, and t_{Wi} are the widths of the elution curves of solutes 1 and 2 expressed in time units. Alternatively, an expression in elution volume units instead of time t, can be used in Equation (17). The height equivalent to the theoretical plate can be written as

$$H = \frac{\sigma^2}{L} \qquad (18)$$

where L is the length of the channel and σ is the standard deviation of the elution curve given in units of length. For a Gaussian elution curve it holds that $4\sigma = W$, where W is the width of the elution curve expressed in units of length. In view of Equations (16) and (18) it holds

$$W_{min} = 2\sqrt{w(Z)L} \qquad (19)$$

The trivial relation $u(Z) = L/t(Z)$ is valid for the linear velocity of the fluid phase. By substituting from Equations (7) and (19) into Equation (17) and by rearrangement we arrive at

$$R_s = \left[\frac{1 - \left(\frac{b_1}{b_2}\right)^2}{1 + \left(\frac{b_1}{b_2}\right)^{1.5}}\right] \left(\frac{L}{2b_1}\right)^{0.5} \qquad (20)$$

Equation (20) demonstrates which parameters describing the geometric and dimensional characteristics of the channel and to which quantitative degree decide on the resolution attainable when separating two solutes. Thus, resolution increases with the root of the channel lenght-to-thickness ratio in a given coordinate Z where the centre of the focused concentrated zone of the solute is situated. Or, a higher resolution can be attained if elongating the channel and decreasing its dimension along the y axis. The term in square brackets at the right-hand side of Equation (20) demonstrates to which degree the difference in channel widths in the positions of the centres of the zones of the separated solutes 1 and 2, affects the resolution. If denoting the term in square brackets in the right-hand side of Equation (20) as ψ , we can rewrite Equation (20) as follows

$$R_s = \psi \left(\frac{L}{2b_1}\right)^{0.5} \qquad (21)$$

Figure 8 shows the plot of the dependence ψ on the b_1/b_2 ratio. As is evident from the figure, the value of ψ increases with the decreasing b_1/b_2 ratio, asymptotically approaching unity. This means, in practice, that increasing the b_1/b_2 ratio is meaningful only up to a certain value; above this value, no substantial improvement in resolution can be attained.

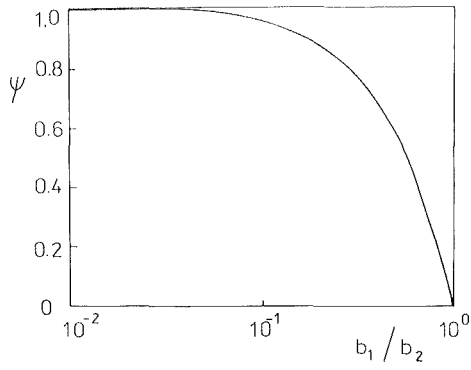


FIGURE 8. Dependence of resolution parameter ψ on ratio b_2/b_1.

EXPERIMENTAL

A very simple apparatus was constructed to demonstrate the formation of the flow velocity profile in a channel with modulated cross-sectional permeability. Two strips of PVC foil of different thickness (0.15 and 0.45 mm) were placed longitudinally between two glass plates of 2.5 x 1 x 30 cm so as to form a channel of trapezoidal cross-section. The dimensions of the channel were 1.5 x 30 cm, the thickness increasing from 0.15 to 0.45 mm. One of the ends of the channel was tightened and provided with stainless steel capillary tube for the inlet of solvent (ethanol). The whole system was clamped between two perspex plates and tigthened with several screws. The experiment proper was made by injecting a short pulse of ethanol-soluble dye into a steady stream of solvent flowing down the channel. The zone produced of the dye was recorded photographically at various phases of its development. These phases are shown in Figure 9. At the wall on the broader side of the channel the zone moves faster.

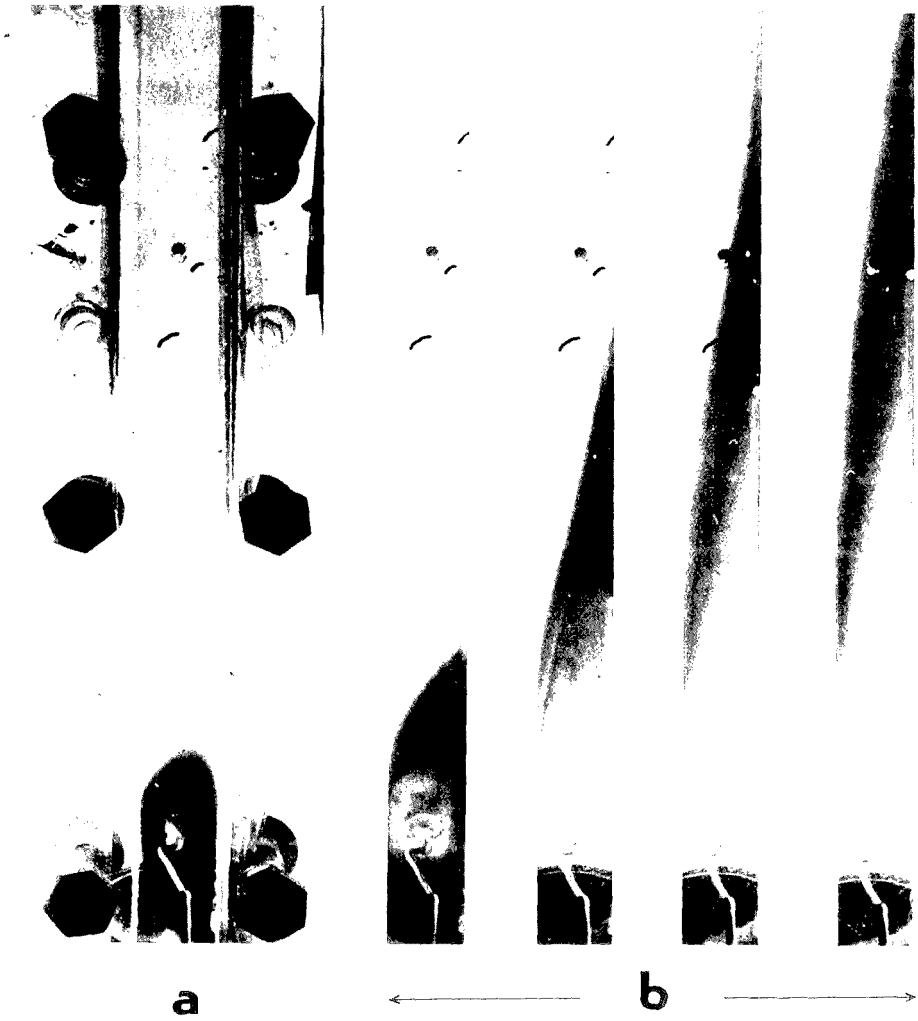


FIGURE 9. Development of the zone shape in the channel of trapezoidal cross-section.
a - top view on the channel at the moment of injection of dye
b - section of the channel with various phases of the zone movement.

The shapes of the front and the rear of the zone both correspond to a parabolic field of velocities in the core of the channel, which agrees with the theoretical analysis.

CONCLUSION

In this first part of a series of contributions dealing with SFFFFF we have demonstrated in theory the basic potentials that can be accomplished when using channels with modulated cross-sectional permeability. Even though we have restricted ourselves to applying this channel type solely to SFFFFF, it is obvious that the properties of this channel - as far as the potential formation of the shape of the velocity profile is concerned - can also find use in other subtechniques of FFF. We shall further examine this potential use. Investigations are continuing in order to establish the practical utilization of the described principle related to a channel with modulated permeability. Other aspects of this principle and of the SFFFFF method will be the subjects of later papers.

REFERENCES

1. Giddings, J. C., Separ. Sci., 1, 123, 1966.
2. Janča, J., Makromol. Chem., Rapid Commun., 3, 887, 1982.
3. Giddings, J. C., Yang, F. J. F. and Myers, M. N., Anal. Chem., 46, 1917, 1974.
4. Batchelor, G. K., An Introduction to Fluid Dynamics, Cambridge University Press, Cambridge, 1967.
5. Takahashi, T. and Gill, W. N., Chem. Eng. Commun., 5, 367, 1980.
6. Giddings, J. C., J. Chromatogr., 5, 46, 1961.

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(9), 1577-1590 (1983)

DETERMINATION OF CARBOHYDRATES BY ANION EXCHANGE CHROMATOGRAPHY WITH PULSED AMPEROMETRIC DETECTION

Roy D. Rocklin* and Christopher A. Pohl

Dionex Corp., 1228 Titan Way,
Sunnyvale, CA 94086 USA

ABSTRACT

Carbohydrates such as sugar alcohols, monosaccharides, disaccharides, and other oligosaccharides are separated as anions by ion exchange chromatography with a sodium hydroxide eluent. Retention time and selectivity are controlled by varying eluent strength and column temperature. The carbohydrates are detected by oxidation at a gold electrode. A repeating sequence of three potentials electrochemically cleans the electrode surface of oxidation products and other interfering species. Detection limits are as low as 30 ppb for sugar alcohols and monosaccharides, and about 100 ppb for oligosaccharides. Other species containing CHOH groups can also be detected, such as alcohols and glycols.

INTRODUCTION

The determination of carbohydrates by chromatography has been hampered by two factors: the lack of a suitable high performance separation method and the inability to detect the carbohydrates at low levels. A number of separation methods have been used. Carbohydrates can be determined by gas chromatography, however, their non-volatile nature requires a time consuming derivitization procedure (1). The most common method is the use of high capacity strongly acidic cation exchange columns in the metal form using water as the eluent (2). Calcium is the most common metal, although silver and lead have also been used.

 0148-3919/83/0609-1577$3.50/0

These columns suffer from several drawbacks. First, the organic acids present in many samples elute metals from the cation exchange resin. This results in resin bed shrinkage and requires frequent regeneration of the column back to the original metal form. Second, these columns exhibit poor selectivity for higher oligosaccharides. Low crosslink resin is required for resolution of even the lower oligosaccharides such as DP2 through DP4. The resin exhibits high compressibility, making it unsuitable for high flow rates and rapid analysis. Third, high column temperatures (85°C) are needed for optimum column performance. These high temperatures present a severe test of the performance of the detector, particularly for a refractive index detector. Finally, since only water or water organic solvent mixtures can be used as an eluent, the methods of retention and selectivity control left open to the chromatographer are severely limited.

A second major method of carbohydrate analysis involves the use of micro-particulate silica with an amino bonded phase (3). While this system successfully separates mono and disaccharides, the higher oligosaccharides (DP3-DP10) are eluted as broad peaks and require extended analysis times. Furthermore, amino stationary phases are subject to reaction with aldehydes or ketones in samples (frequently present in foods and beverages) to form a Schiff's base. Because of this, amino bonded phases tend to exhibit reduced lifetimes with certain samples.

A third method is the separation of sugars via their borate complexes (4). While the selectivity of these systems are superior to the above two methods, the kinetics of the complex formation process is slow, thus requiring low flow rates and long analysis times.

We report here an alternative to the above method; the direct separation of carbohydrates by anion exchange. Because carbohydrates have pK values ranging from 12 to 14 (5), retention is possible on a strongly basic hydroxide form anion exchange column with highly alkaline eluents. Such a system is previously

unreported, although retention of methylglycosides on a strongly basic hydroxide form anion exchange column has been reported (6).

Due to the absence of a strongly absorbing UV chromophore, carbohydrates are usually detected by a refractive index detector. R.I. detectors have low sensitivity and are difficult to use. Low UV detection (< 200 nm) has been employed, but the need for ultra clean eluents and interference problems have limited its utility.

The detection method reported here uses an electrochemical detector which supplies a triple potential sequence to a gold working electrode. Both potentiometric and single potential (D.C.) amperometric detectors have been used to detect carbohydrates (7). Potentiometric detectors suffer from slow response times. Single potential oxidation of sugars causes the electrode surface to become contaminated by the products of the reaction, thus rendering the detector unusable. Triple pulse amperometry of sugars was first reported by Hughes and Johnson using a platinum electrode (8). Although the detection method is successful, the potential at which the oxidation current is measured (E1,-0.40V) is sufficiently negative to cause the reduction of oxygen. Gold was investigated as an alternative working electrode material.

EXPERIMENTAL

All chromatography was performed on a Dionex System 2011 (P/N 35295) Ion Chromatograph. The sample loop size was 50 μL and the eluent flow rate 1.0 mL/min. The detector was a breadboard version of a Dionex IonChrom™/Pulsed Amperometric Detector (P/N 35227) consisting of an amperometric flow-through cell and a potentiostat. The cell is a thin layer design with a gold working electrode, a silver/silver chloride reference electrode, and a glassy carbon counterelectrode. The potentiostat applies a series of up to three potentials (E1, E2, E3) in a repeating waveform. The pulse durations (t1, t2, t3) are selectable by the user.

The cell current is measured only during E1 for 16.67 ms (to cancel 60 hz line noise). A 40 ms delay following the potential step allows charging current to decay.

The anion exchange column used was a prototype HPIC-AS6 column (P/N 35391). The ion exchange resin used in the column consisted of 10 micron substrate coated with a monolayer of anion exchange latex similar to that used in other HPIC anion Ion Chromatography columns.

The eluent consisted of NaOH in deionized water (0.15 N). 0.2 M CH_3CO_2Na was added as a pusher to elute oligosaccharides. Eluents were prepared from carbonate free 50% NaOH solutions and protected from carbon dioxide contamination with Ascarite traps. Sugar samples were purchased from Pfanstiehl Laboratories (Waukegan, IL) and Sigma Chemicals Co., St. Louis, MO. The maltose oligomers were a generous gift of the A.E. Staley Company.

RESULTS & DISCUSSION

Cyclic Voltammetry of Glucose

The choice of potentials and pulse durations for chromatographic detection is most easily determined from voltammetric information. Accordingly, the cyclic voltammetry of sugars on gold was studied and is illustrated in Figure 1 by glucose. The dashed line is a background scan of the 0.1 N NaOH supporting electrolyte. It shows irreversible oxidation of gold beginning at approximately 0.25 V. The reduction of the surface oxide back to gold is shown as a cathodic peak at approximately 0.1 V.

With glucose added to the solution, oxidation begins at -0.5 V on the positive going scan. The current rises slightly and remains unchanged until it rises at -0.15 V towards a peak at 0.26 V. On the reverse scan, the current actually reverses from cathodic to anodic at the onset of the gold oxide reduction. This implies that glucose oxidation which occurs at the bare gold surface is inhibited upon the formation of gold oxide. As soon as the reduction of gold oxide back to gold begins,

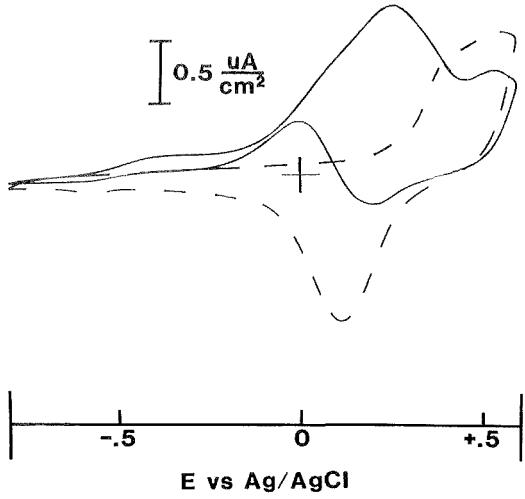


Figure 1. Cyclic voltammetry of 0.92 mM (166 ppm) glucose on a gold working electrode. Dashed line is 0.10 M NaOH supporting electrolyte. 0.20 V/sec sweep rate.

oxidation of glucose that had diffused toward the electrode while the surface was covered with oxide takes place.

Cyclic voltammetry of the sugar alcohols xylitol and sorbitol and the disaccharide sucrose were also studied. They were similar to glucose, except the height of the anodic peak at 0 V on the negative going scan was larger with the sugar alcohols and smaller with sucrose.

Choice of Applied Potentials

Single potential (D.C.) amperometry with E_{app} set to 0.1 V was attempted. 0.1 V was chosen because at this potential, there should be a large fraction of unoxidized gold on the electrode surface. Although detection was possible, the response decreased rapidly as products from the oxidation reaction deactivated the surface. A large improvement in reproducibility was obtained by pulsing the applied potential from -0.8 V to 0.2 V and measuring the oxidation current at 0.2 V. The negative potential apparently serves as a

cleaning step, ensuring the reduction of gold oxide back to gold. Also, the magnitude of the sugar oxidation current from a voltage pulse is greater than that caused by the hydrodynamics of flow when only a single potential is used. The use of both negative and positive cleaning potentials was found to provide the most reproducible chromatograms, as well as minimum interference from other species in the samples. The following potentials and times (shown in Figure 2) have been chosen as optimum for carbohydrate determination: E1: 0.20 V, 60 ms; E2: 0.60 V, 60 ms; E3: -0.80 V, 240 ms.

The signal-to-noise ratio is affected the most by the choice of the detection potential, E1. The peak heights for three sugars and the background current are shown in Figure 3. The large increase in background current from 0.2 V to 0.4 V is accompanied by a 4X increase in noise. The optimum value for E1 is 0.2 V. Due to the similarity of the electrochemistry of the different sugars, this value may be optimum for most, if not all, sugars.

Small (0.1 V) changes in E2 and E3, as well as their durations, have only a minor effect on response.

Chromatography

One of the main advantages of the anion exchange chromatography of carbohydrates as compared to metal form cation exchange is the ability to influence retention time and retention order. The primary operating variables are eluent strength and column temperature. The separation of a mixture of sugar alcohols and saccharides is shown in Figure 4. The addition of acetate ion results in reduction in the K' of all solutes. This enables the elution of the oligosaccharides DP2 through 10, shown in Figure 5.

In general, the observed anion exchange affinity follows the trend: sugar alcohols < monosaccharides < disaccharides < oligosaccharides, although in some cases overlap is observed in the elution order of mixtures of disaccharides and oligosaccharides. For a homologous series of carbohydrates, retention

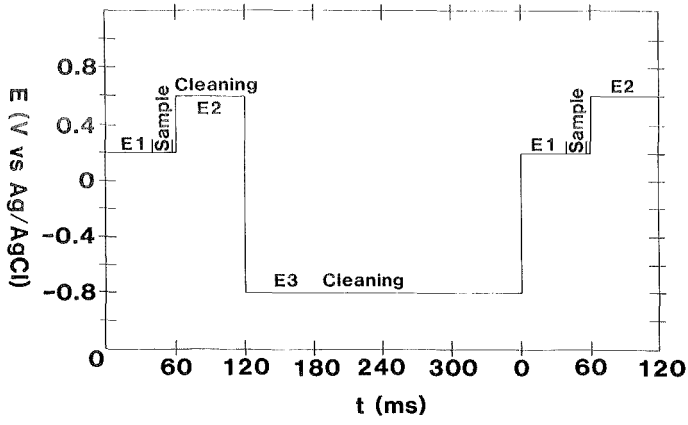


Figure 2. Triple potential program applied to the gold working electrode for the detection of carbohydrates. Oxidation current is sampled from 40 to 56.7 ms after the beginning of E1.

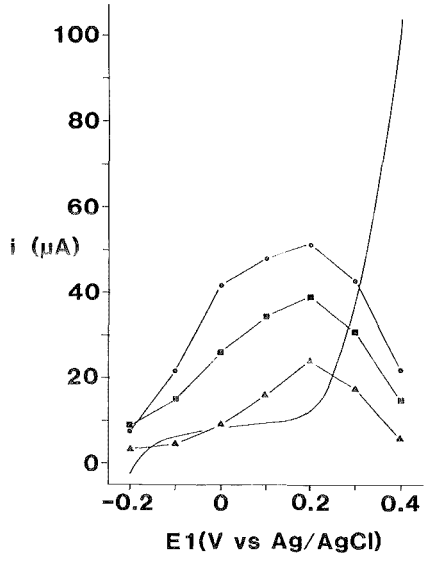


Figure 3. i_p as a function of E1 for 100 ppm xylitol (dots), 100 ppm glucose (squares), and 316 ppm sucrose (triangles). Background current as a function of E1 is also shown.

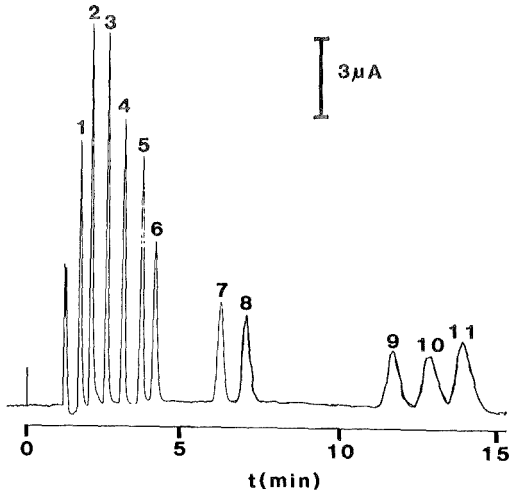


Figure 4. Separation of sugar alcohols and saccharides. Listed in order, they are: 25 ppm xylitol (1); 50 ppm sorbitol (2), rhamnose (3), arabinose (4), glucose (5), fructose (6), lactose (7); 100 ppm sucrose (8), raffinose (9), stachyose (10); 150 ppm maltose (11). 0.15 M NaOH eluent at 36°C.

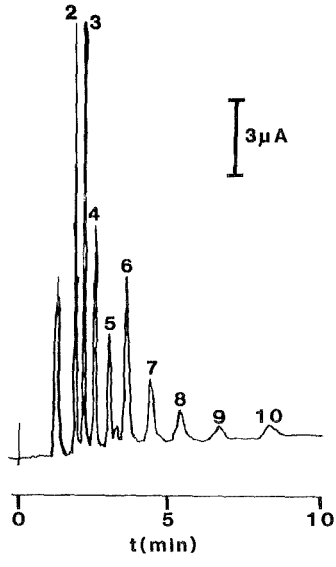


Figure 5. Separation of oligomers of maltose, DP 2 (maltose) through DP 10 (maltodecose). 0.2 M NaOH, 0.2 M CH_3CO_2Na eluent at 34°C.

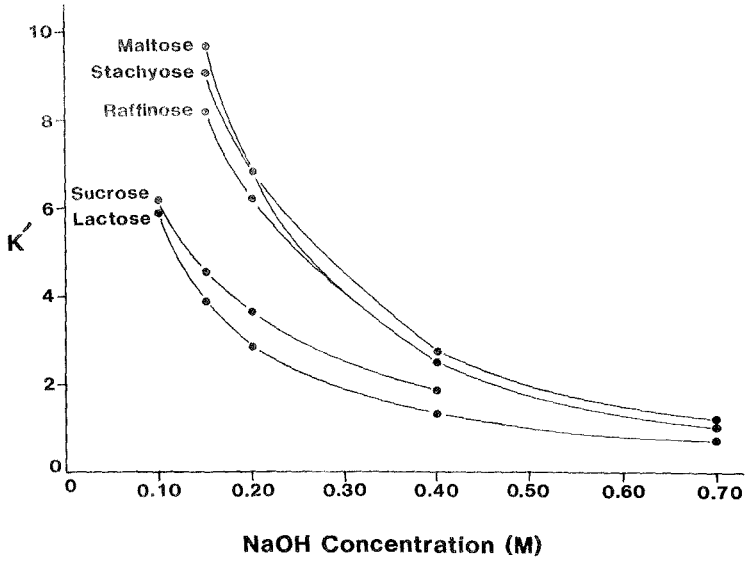


Figure 6. Capacity factor versus NaOH concentration. Column temperature: 34°C; sample concentrations: 100 ppm each.

increases as the degree of polymerization (DP) increases. Selectivity changes are often observed as the hydroxide ion concentration is varied. Typical selectivity changes observed for oligosaccharides are illustrated in Figure 6. For the oligosaccharides shown, optimum selectivity is observed at 0.150 M NaOH. Similar chromatographic behavior is observed for monosaccharides and sugar alcohols. While in some cases these selectivity changes may be used to advantage, in general hydroxide ion concentration is not a useful method for controlling selectivity. Often, the desired selectivity for a particular mixture of carbohydrates is only observed at K' values which are unacceptably large or small.

The hydroxide ion concentration has a significant effect on the observed efficiency for a particular solute. It is generally observed that column efficiencies increase as the

hydroxide ion concentration is increased. For glucose, efficiency nearly doubles when hydroxide ion concentration is increased from 0.10 M NaOH to 1.0 M NaOH.

A second variable useful for control of selectivity and retention is column temperature. Retention decreases for all solutes as the temperature increases. The degree to which temperature alters retention is loosely related to the carbohydrate size; the observed magnitude of the temperature effect being: oligosaccharides > disaccharides > sugar alcohols > monosaccharides. This can be used to advantage for adjusting the elution order for a given mixture of carbohydrates. The effect of temperature on the selectivity observed for some common carbohydrates is shown in Figure 7. Note that an elution order reversal is observed for maltose (a disaccharide) and stachyose (a tetrasaccharide) as the temperature is increased.

Three factors serve to limit the effective upper range of temperature in the retention of sugars on hydroxide form anion exchange columns:

(1) Tailing and secondary peaks are observed for some carbohydrates (particularly glucose and mannose) when the temperature of the column exceeds 45°C. This is probably caused by rearrangement about the anomeric carbon atom, the Lobry de Bruyn-Van Ekenstein transformation.
(2) Efficiencies tend to decrease when temperatures exceed 45°C.
(3) Anion exchange materials in the hydroxide form are subject to Hoffman degradation at elevated temperatures.

Because of the above, columns are normally operated at temperatures between 20°C and 45°C.

A third variable for control of selectivity and retention is the addition of anions other than hydroxide to the eluent. The addition of other anions is sometimes desirable due to the fact that high levels of NaOH (>0.2 M) can result in increased detector noise as well as peak assymetry for the sugar

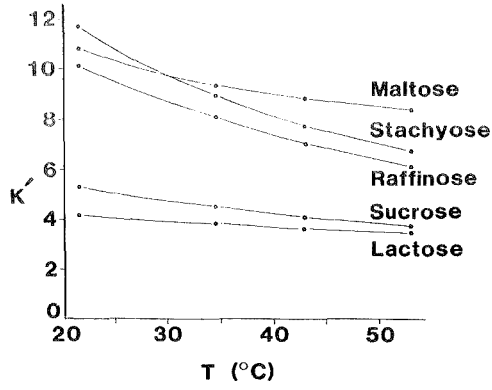


Figure 7. Capacity factor versus temperature. Eluent: 0.15 M NaOH; sample concentrations: 100 ppm each.

alcohols. Acetate, carbonate, nitrate, and sulfate have been evaluated as eluent additives. In our work, acetate was found to be the preferred eluent additive due to the fact that its affinity for the anion exchange resin is similar to that of hydroxide. The other anions were found to be compatible with the detector but their high affinity for the anion exchange resin resulted in a substantial reduction of the column loading capacity.

Because of the elution power of carbonate, it is important that carbonate be excluded from the eluent system. This is particularly true for eluent systems used for the analysis of sugar alcohols and monosaccharides. Proper precautionary measures include the use of an Ascarite trap on the eluent bottle and ASMT Class I deionized water when making up eluents.

Sensitivity, Linearity and Reproducibility

The detection limits for the carbohydrates shown in Figure 4 range from 30 ppb for the sugar alcohols and monosaccharides to 100 ppb for the oligosaccharides. Plots of the log of peak height vs the log of concentration are linear up to several hundred to 1,000 ppm, where overloading of the capacity of the

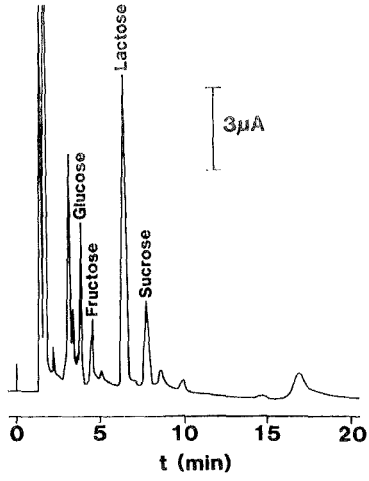


Figure 8. Extract from flavored potato chips. Lactose concentration in the extract is 70 ppm.

column occurs. Although the log/log plots are linear, the slopes for several sugars are: xylitol, 1.07; glucose, 1.03; sucrose, 1.16. The near linearity of i_p *vs* C plots, rather than $1/i_p$ *vs* 1/C as on platinum (8) implies that adsorption of carbohydrate molecules during t3 (E3 = -0.80 V) is not part of the reaction mechanism.

Reproducibility of peak heights for multiple injections of a single sample is generally better than 1%. Peak heights have a tendency to increase with time, beginning with the time at which the working electrode was cleaned. This increase has been observed to be approximately 5 to 15% after 8 hours of operation. Errors in concentration measurements can be minimized by the frequent use of standards.

CONCLUSION

The anion exchange separation of carbohydrates with alkaline eluent systems provides a powerful new tool in the analysis of carbohydrates. The combination of this chromatographic system

with triple potential detection provides a highly selective and sensitive method particularly well suited to complex samples. For example, the determination of lactose in flavored potato chips, shown in Figure 8, was easily accomplished even though the solution to be analyzed contained a high concentration of potentially interfering salts.

The triple potential detection method can also be used to determine other species containing the alcohol functional group. Using different chromatographic conditions, simple alcohols and glycols have been detected. NOTE: The analytical methods described in this paper are the subject of pending patents.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Dennis C. Johnson of Iowa State University and Dr. William E. Rich for their help and advice, and Karen Pohl for performing the initial experimentation.

REFERENCES

1. Knapp, D.R. Handbook of Analytical Derivitization Reactions, Wiley, N.Y. 1979, p. 539

2. Scobell, H.D.; Brobst, K.M.; Steele, E.M. Cereal Chem., 1977, 54, 905
   Fitt, L.E.; Hassler, W.; Just, D.E. A Rapid and High Resolution Method to Determine the Composition of Corn Syrup by Liquid Chromatography. J. Chromatogr. 1980, 187, 381
   Ladish, M.R.; Heubner, A.L.; Tsao, G.T. High Speed Liquid Chromatography of Cellodextrins and other Saccharide Mixtures using Water as Eluent, J. Chromatogr., 1978, 147, 185

3. Yang, R.T.; Milligan, L.P.; Mathison, G.W., Improved Sugar Separation by High Performance Liquid Chromatography Using Porous Microparticle Carbohydrate Columns. J. Chromatogr., 1981, 209, 316

Hendrix, D.L.; Lee, R.E.; Baust, J.G.; James, H., Separation of Carbohydrates and Polyols by a Radially Compressed High Performance Liquid Chromatographic Silica Column Modified with Tetraethylenepentamine, J. Chromatogr., 1981, 210, 45

4. Sinner, J.; Puls, J., J. Chromatogr., 1978, 156, 197, Non-Corrosive Dye Reagent for Detection of Reducing Sugars in Borate Complex Ion-Exchange Chromatography
Verhaar, L.A.; Dirkx, J.M.H., The Analysis of Mixtures Containing Glucose, Fructose, and Mannose, Carbohyd. Res., 1977, 53, 247

5. Rendleman, J.A., Ionization of Carbohydrates in the Presence of Metal Hydroxides and Oxides. Carbohydrates in Solution, Advances in Chemistry, Series #117, American Chemical Society, 1973, p. 51

6. Neuberger, A.; Wilson, B.M., The Separation of Glycosides on a Strongly Basic Ion-Exchange Resin: An Interpretation in Terms of Acidity, Carbohyd. Res., 1971, 17, 89

7. Brunt, K., Comparison Between the Performances of an Electrochemical Detector Flow Cell in a Potentiometric and an Amperometric Measuring System Using Glucose as a Test Sample, Analyst, 1982, 107, 1261

8. Hughes, S.; Johnson, D.C., Amperometric Detection of Simple Carbohydrates at Platinum Electrodes in Alkaline Solutions by Application of a Triple-Pulse Potential Waveform, Anal. Chim. Acta, 1981, 132, 11
Hughes, S.; Johnson, D.C., High Performance Liquid Chromatographic Separation with Triple Pulse Amperometric Detection of Carbohydrates in Beverages., J. Agric. Food Chem., 1982, 30,, 712

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(9), 1591-1601 (1983)

HPLC SEPARATION AND CHARACTERIZATION OF SYNTHETIC AND NATIVE CYTOCHROME C DERIVED PEPTIDES

N.E. Rankin, R.A. Earl, R.S. Young, L.E. Barstow,
M.A. Cusanovich, and G.S. Wilson+
Department of Chemistry and Department of Biochemistry
University of Arizona
Tucson, Arizona 85720

ABSTRACT

The use of HPLC for the characterization of peptides used in semisynthesis of cytochrome c is discussed. HPLC provided a convenient method for assessing the purity and structure of the peptides used. HPLC was also useful in monitoring the progress of solid phase peptide synthesis and in purifying the final product.

INTRODUCTION

The preparation, purification, and characterization of relatively large peptides (30-50 residues), in particular those produced by solid phase synthesis techniques, present a considerable analytical challenge. Conventional techniques such as amino acid analysis (AAA) and peptide mapping are frequently insufficient indicators of purity and can consume excessive amounts of time and material without providing definitive answers. High performance liquid chromatography (HPLC) has proven very valuable in providing such information (1, 2), and we report on its application to the preparation of semisynthetic cytochrome c.

Harbury and co-workers (3, 4) demonstrated that horse heart cytochrome c, a 104 residue protein, could be cleaved at met-65 to produce two essential pep-

+Author to whom reprint requests should be sent at the Dept. of Chemistry

 0148-3919/83/0609-1591$3.50/0

tides, a 65 residue peptide to which a heme group is attached (HP1-65) and a non-heme peptide containing the remaining residues (NHP 66-104). As a result of the cleavage reaction with cyanogen bromide, the met-65 of the heme peptide is converted into homoserine lactone. This latter moiety will react with NHP 66-104 under appropriate conditions to reestablish a covalent linkage between residues 65 and 66. A fully biologically active protein results which differs only from the native at residue 65, now homoserine. The effect of individual residues on structure-function relationships in the intact protein may be established by making individual substitutions in the NHP 66-104 sequence followed by fragment recombination. The success of such a venture clearly depends upon the efficacy of the purification and characterization methods employed particularly in distinguishing changes in individual residues.

The HPLC has proven useful in the purification and characterization of peptides and proteins (1, 2). This is in spite of the still not completely understood mechanism for retention. Several groups (6-10) have established empirical relationships between amino acid composition and retention. Others (11-13), including this paper, have found it convenient and successful to use the values calculated by employing Rekker's hydrophobic values (5) or by Meek (6, 7) to predict elution order.

EXPERIMENTAL PROCEDURES

Materials. All reagents and buffers used were reagent grade unless otherwise specified. Reagents were obtained from the following sources: Horse heart cytochrome c (type III), cyanogen bromide from Sigma Chemical Company; HPLC grade acetonitrile, dipotassium hydrogen phosphate from Matheson, Coleman and Bell; ammonium acetate, formic acid, and 1-butanol from Fisher Scientific; t-butoxycarbonyl and g-Fluoromethyloxycarbonyl protected amino acids from Vega Biochemicals.

Methods. Heme peptides 1-80 and 1-65 were prepared by limited cyanogen bromide cleavage and were purified by chromatography on Sephadex G-75 with acetic acid:formic acid:H_2O, 40:10:50 v/v as the mobile phase (14). Non-heme peptides

66-104, 66-80 and 81-104 were prepared as described by Corradin and Harbury (3, 4), except that purfication was performed on the Sephadex G-75 column described above. The appropriate fractions were pooled, lyophilized, and then purified in a final step by gel chromatography on Sephadex G-50-F with 7% formic acid as solvent.

A Model 332 Altex gradient liquid chromatograph was used for all HPLC applications. An Altex Ultrasphere-ODS column (5um, 4.6 mm i.d. x 25 cm, octadecylsilane bonded silica) or an Altex Ultrasil-ODS (10um, 4.6mm i.d. x 25 cm, octadecylsilane bonded silica) was used. Solvents were first filtered through 0.5 Millipore filters (type fH for organic solvents, type HA for aqueous solvents). Acetonitrile and ammonium acetate were used in all separations. 0.1 M ammonium acetate at pH 4.5 was used for most peptides while a 0.2 M solution at pH 5.0 was used for some, indicated on the individual figures. Flow rates and gradient conditions used are shown on the individual chromatograms. All peptide samples were filtered through 0.45um type HA Millipore filters prior to injection or centrifuged for 2 minutes in a Fisher micro-centrifuge model 235A.

RESULTS AND DISCUSSION

Separation of Heme Peptides. It has been our experience that the successful synthesis and recombination of peptide fragments is greatly facilitated by careful monitoring of purity at designated stages of the synthesis. Figure 1 shows the two important heme peptides which are obtained from the cyanogen bromide cleavage of cytochrome c. These correspond to attack at met-65 and met-80 to produce HP 1-65 and HP 1-80 which elute in both the open (homoserine carboxylate) (Peaks A and C) or in the synthethetically active lactone (Peaks B and D) forms. It has been previously pointed out (4) that the lactone form will open in the presence of base. Under such conditions Peaks A and C can be shown to grow at the expense of B and D thus confirming the peak assignments. This treatment does not result in modification of the heme moiety.

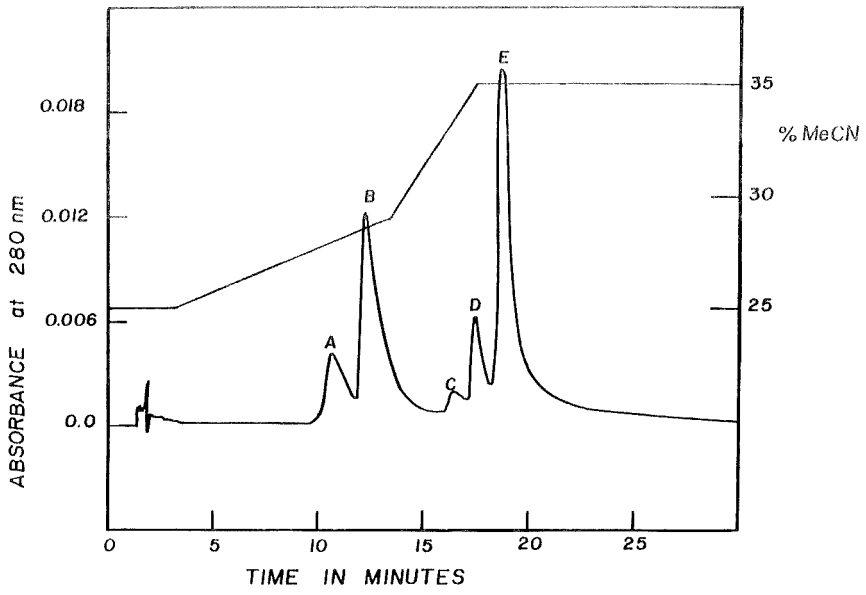


Figure 1 - HPLC of Heme Peptides from CNBr Cleavage.
A-HP1-65 homoserine; B-HP1-65 homoserine lactone; C-HP1-80 homoserine; D-HP1-80 homoserine lactone; E-native cytochrome c. Conditions: Ultrasil Column, Flow rate 2 ml/min. acetonitrile/ammonium acetate buffer gradient as shown.

The ability to predict retention times based on peptide size or amino acid composition is quite important because peak assignment is a tedious process and consumes considerable time and material. It has been previously suggested (10, 16) that hydrophobic interactions with the stationary phase determine retention on ODS columns. In this case the larger peptides have longer retention times even though the overall positive charge is also higher. The striking influence of C-terminal residue modification is clearly seen in the comparison of Peaks A and B or C and D. The opening of the homoserine lactone creates a localized negative charge and reduction in the overall positive charge on the peptide. The resulting lowered retention time with otherwise constant amino acid composition does not agree with the predictions of the models (6, 7) based solely on hydrophobic interactions but suggests instead the strong individual

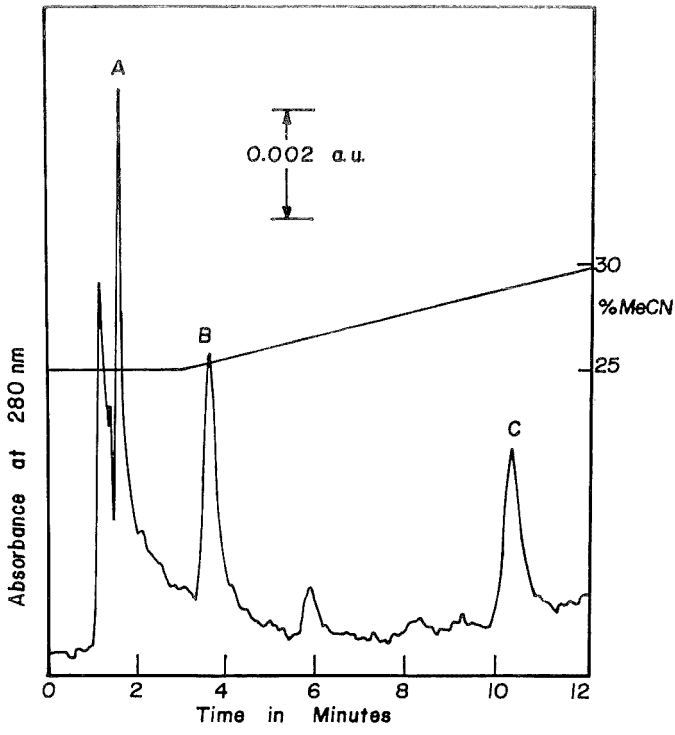


Figure 2 - HPLC of Non-Heme Peptides from CNBr Cleavage.
A-NHP66-80 homoserine lactone and NHP66-80 homoserine; B-NHP81-104;
C-NHP66-104 Conditions: Ultrasphere-ODS column, flow rate 2 ml/min, Same as acetonitrile/ammonium acetate gradient as shown.

influence on retention of the charged carboxyl group In any case, the ability to quickly establish the presence of the lactone at position 65 is significant because the lactone is essential for the formation of a covalent bond between residues 65 and 66 in the recombination studies.

Separation of Non-Heme Peptides. Figure 2 shows a chromatogram of the three non-heme peptides obtained from the digestion of horse heart cytochrome c. The identity of the various peaks was established by separate injections of the various components as well as amino acid analysis of the eluted peaks. Again as in the case of the heme peptides, the elution order is determined primarily by

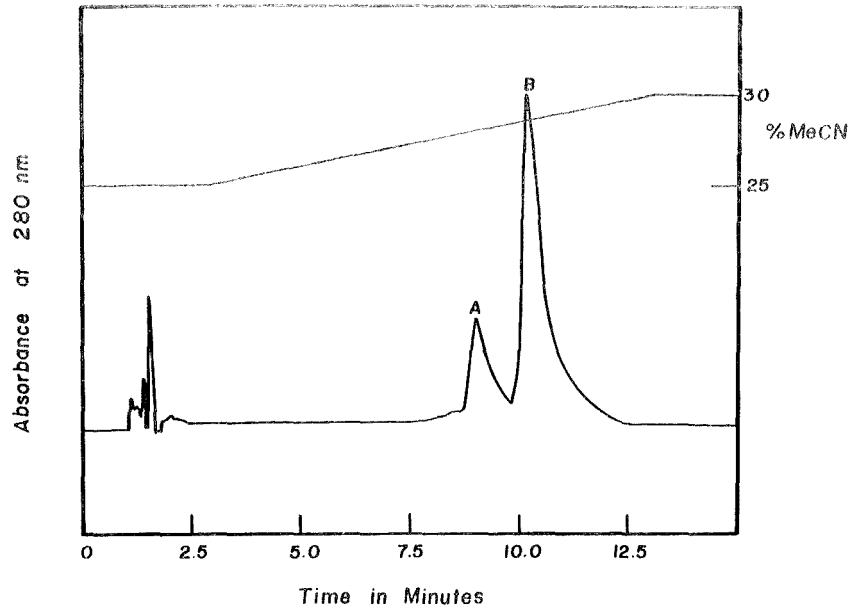


Figure 3 - HPLC of Non-Terminal Homoserine Non-Heme Peptide A-NHP66-104 (homoserine-80); B-NHP66-104(methionine-80) Conditons: Same as Figure 2, gradient as shown.

peptide length rather then by charge. If the NHP 66-80 peak is rechromatographed at a lower acetonitrile concentration (13%) the two peptides containing the C-terminal homoserine lactone and carboxylate with net charges of +2.52 and +1.52 respectively can be resolved. The elution order is analogous to that of the heme peptides. If NHP 66-104 is prepared with a 50-fold excess of CNBr instead of the normal 3-fold excess, part of the NHP 66-104 formed will possess met-80 which has been converted into homoserine without amide bond cleavage (3, 4). This is shown in Figure 3 where the distinction between these two closely related peptides is essential to the subsequent peptide reconstruction. The substitution of homoserine for methionine may render the peptide more polar thus resulting in more rapid elution.

Separation of Synthetic Cytochrome c-Derived Peptides. We have previously demonstrated the preparation of semi-synthetic cytochrome c from the native

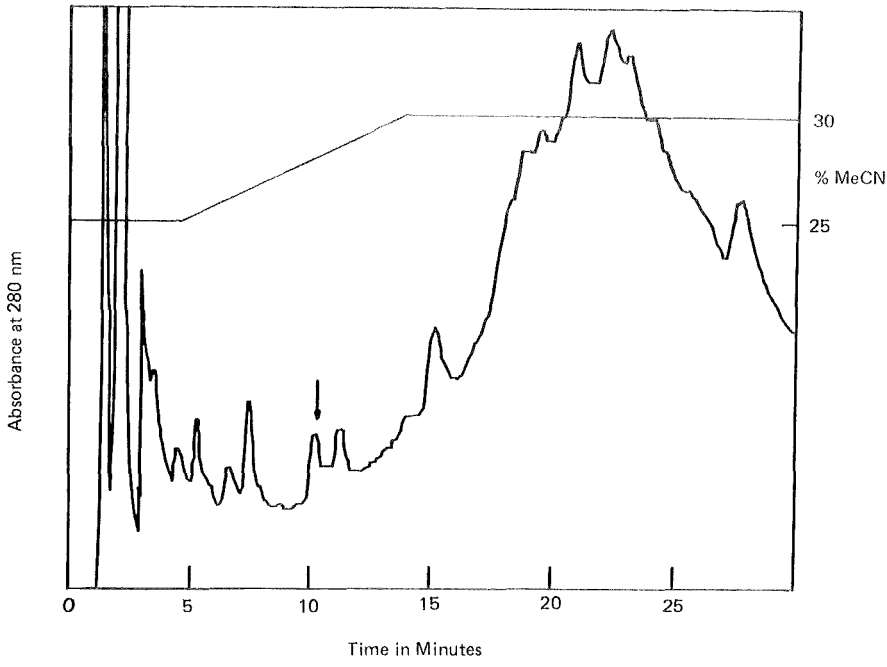


Figure 4 - HPLC of t-Boc Synthesized NHP66-104
Arrow indicates retention time of native peptide. Conditions: Same as Figure 2, gradient as shown.

HP1-65 and NHP 66-104 prepared by solid phase peptide synthetic techniques described elsewhere (15). Conventional t-butoxycarbonyl (t-Boc) derivatives were used for α-amino group protection (17, 18). Standard amino acid analysis of synthetic NHP 66-104 samples give the expected results within experimental error. Yet, as shown in Figure 4, HPLC analysis of the same peptide shows a high degree of heterogeneity. Indeed very little synthetic peptide is eluted at the retention time of the corresponding native peptide. Enzymatic hydrolysis of the synthetic material followed by amino acid analysis showed that the large very broad chromatographic peak (Figure 4) was due primarily to incomplete removal of blocking groups at the conclusion of the synthesis. A variety of conditions were examined for the resin-peptide cleavage step following synthesis

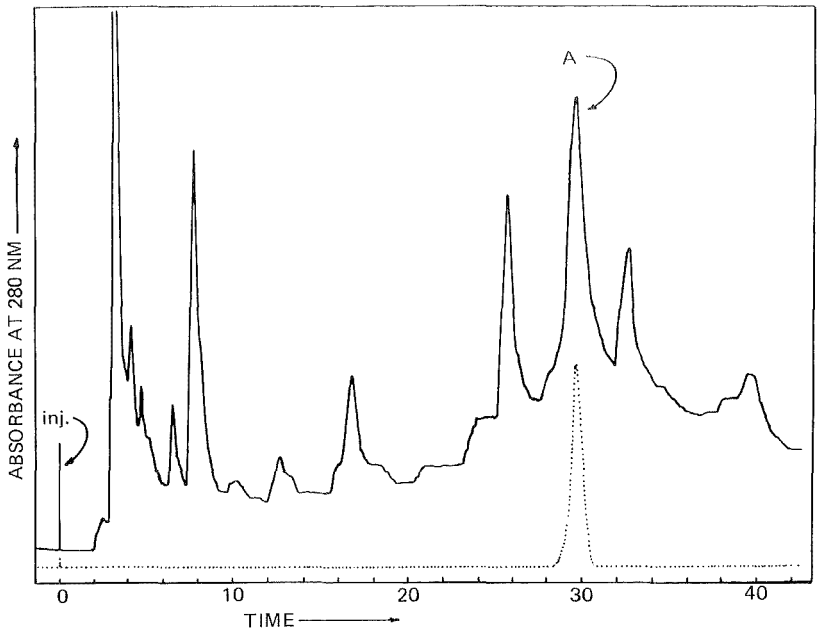


Figure 5 - HPLC of F-moc Synthesized NHP66-104
Arrow indicated retention time of native peptide. Conditions: Ultrasphere ODS column, flow rate 1ml/min, acetonitrile/ammonium acetate gradient as shown. Curve A - HPLC after HF cleavage, Curve B - HPLC after preliminary HPLC purfication.

that also removes side-chain blocking groups. It was not possible to achieve complete deblocking in anhydrous HF without destruction of the peptide. Further, the constant washing of the growing peptide with mild acids in the α-amino deprotection step can also have detrimental effects. This has encouraged the use of the base labile α-amino protecting group 9 fluoromethyloxycarbonyl (F-moc) (19). Figure 5 Curve A shows the chromatogram of F-moc synthesized NHP 66-104 before HPLC purification. The presence of a small number of well-defined peaks supports a "cleaner" higher yield synthesis. The fraction eluting at ca. 29 minutes was rechromatographed, giving Curve B. The characteristics of this latter fraction were identical to those of the native peptide.

HPLC has also been used to monitor the course of the solid phase reactions. This is quite important for the synthesis of relatively large peptides because

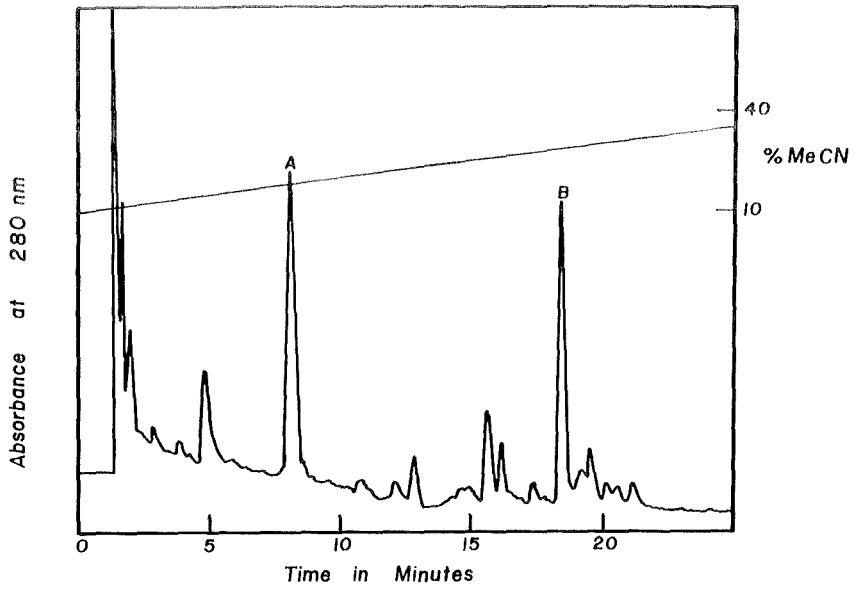


Figure 6 - HPLC of F-moc Synthesized NHP81-104
A-Truncated Peptide; B - NHP81-104 Conditions: Same as Figure 2, gradient as shown.

errors occurring early in the sequence will result in low yields and complicated mixtures which are time consuming and expensive to evaluate and purify. Figure 6 shows the chromatogram of synthetic NHP 81-104. Peak B is identical to native NHP 81-104. leaving Peak A as a major contaminant. It can be presumed to be a shorter or truncated peptide resulting from incomplete reaction. By comparing the peptide product at various stages of synthesis with native peptides derived from cytochrome c by limited enzymatic hydrolysis, it is possible to establish the step at which extraneous peaks appear. In this case, it was established that truncation occurs in the Arg-91 region. It has been suggested that resin support collapse is the cause of the problem (21). Correction of the factors causing this problem now makes possible synthesis of the NHP 66-104 peptide in reasonable yields.

Retention times are reproducible on a single column (within 4%), however, columns of the same type from the same manufacturer do not give identical reten-

tion behavior. This makes the availability of suitable standards essential. The retention of cytochrome c derived peptides is very sensitive to the acetonitrile concentration of the mobile phase. Changes as small as 5% can result in the extremes of complete retention or non-retention on the column.

It is of interest in the present study to be able to qualitatively predict retention times, or at least retention order for eluting peptides. Such predictions do not eliminate the need for peptide isolation and identification, but they can help considerably in the optimization of elution conditions. The peptides in question are relatively large (15-104 residues) and the separations are carried out at around pH 4.5. This means that the peptides are not in an open chain configuration and consequently not all residues are freely accessible for interaction with the stationary phase. The simplest approach is to consider the retention order to be determined by peptide size (number of residues). We find that for our peptides this latter method works as well as those of Rekker (5), Meek (6, 7) and Sasagawa (10). These methods all produce about 10% uncertainty in the prediction retention time which becomes quite important when peptides of comparable size but different sequence must be separated. In some cases the effect of individual amino acid substitutions can be correctly predicted (66-104 vs 66-104 homoserine) but this approach cannot be described as totally reliable.

The ability of HPLC for the separation, purification and identification of cytochrome c-derived peptides has been demonstrated. Separation of relatively large peptides with single residue modification has been achieved. The monitoring of the growing peptide product during solid phase synthesis has enabled the detection of synthesis error thus saving considerable time and expense.

ACKNOWLEDGEMENTS

This work was supported in part by National Science Foundation Grants PCM 80-22064 and PCM-80-11555.

REFERENCES

1. K. A. Walsh, L. H. Ericsson, D. C. Parmlee and K. Titani, Annual Rev. Biochem., 50, 261, 1981.
2. F. E. Regnier and K. M. Gooding, Anal. Biochem., 103, 1, 1980.
3. G. Corradin and H. A. Harbury, Biochem. Biophys. Acta, 221, 489, 1970.
4. G. Corradin and H. A. Harbury, Proc. Natl. Acad. Sci. USA, 68, 3036, 1971.
5. R. F. Rekker, The Hydrophobic Fragmental Constant, Elsevier, Amsterdam, 1977, 301.
6. J. L. Meek, Proc. Natl. Acad. Sci. USA, 77, 1632, 1980.
7. J. L. Meek and Z. L. Rossetti, J. Chromatogr., 211, 15, 1981.
8. S. J. Su, B. Grego, B. Niven and M. T. W. Hearn, J. Liq. Chromatogr., 4, 1381, 1981.
9. K. J. Wilson, A. Honeggar, R. P. Stotzel, G. J. Hughes, Biochem. J., 199, 31, 1981.
10. T. Sasagawa, T. Okuyama and D. C. Teller, J. Chromatogr., 240, 329, 1982.
11. J. J. L'Italien and R. A. Laursen, J. Biol. Chem., 256, 8092, 1982.
12. P. McPhie and F. T. Chiu, Arch. Biochem. Biophys., 211, 471, 1981.
13. M. T. W. Hearn and B. Grego, J. Chromatogr., 203, 349, 1981.
14. R. A. Earl, Ph.D. Dissertation, University of Arizona, 1982.
15. L. E. Barstow, R. S. Young, E. Yakali, J. J. Sharp, J. C. O'Brien, P. W. Berman and H. A. Harbury, Proc. Natl. Acad. Sci. USA., 74, 4248, 1977.
16. I. Molinar and C. S. Horvath, J. Chromatogr., 142, 623, 1977.
17. R. B. Merrifield, Fed. Proc. Fed. Amer. Soc. Exp. Biol., 21, 412, 1962.
18. R. B. Merrifield, J. Am. Chem. Soc., 85, 2149, 1963.
19. C. D. Chang and J. Meienhofer, Int. J. Peptide Protein Res., 11, 246, 1978.
20. J. Meienhofer, M. Waki, E. P. Heimer, T. J. Lambros, R. C. Makofske, and C. D. Chang, Int. J. Peptide SProtein Res., 13, 35, 1979.
21. E. Atherton, V. Wooley, and R. C. Sheppard, J. Chem. Soc. Chem. Commun., 970, 1980.

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(9), 1603-1616 (1983)

A CHEMILUMINISCENCE DETECTOR FOR TRACE DETERMINATION OF FLUORESCENT COMPOUNDS

G. Mellbin
Technical Analytical Chemistry, Chemical Center
P.O.B. 740, S-220 07 Lund 7, Sweden

ABSTRACT

A detector making full use of the advantages offered by chemiluminiscence has been constructed. Light measurement was performed with modern electronic equipment using photon counting and the system was tested using dansyl derivatives of adrenaline, noradrenaline and of some amino acids. The detection limits for the two catecholamines were 6 and 16 fmol, respectively, and less than 0.5 fmol for the amino acids. To reach these low levels all reagents were purified or selected for ultra-high purity. The effects of reagent contaminants and of various optical filters have been studied using adrenaline as the test substance.

INTRODUCTION

Introduction of chemiluminiscence in liquid chromatography can be interpreted as an attempt to increase the sensitivity and selectivity of fluorescence methods, and thus extend their applicability to the assay of chemical compounds of various kinds. The effective detection limit in fluorescence analysis using a high-energy light source, e.g. a xenon lamp, and a photomultiplier tube is restricted to about 100 pmol for dansyl derivatives, due to problems with stray light and intensity variations of the light source. Application of UV- and tunable dye lasers have improved the situation. However, UV-lasers are limited to a few

 0148-3919/83/0609-1603$3.50/0

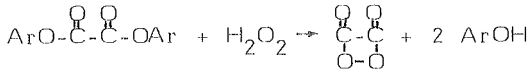
wavelengths and tunable lasers are rather expensive, making them available to a small number of laboratories.

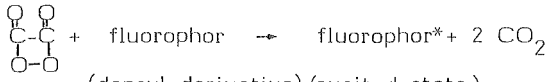
Another problem when trying to decrease the detection limit arises from the light measurement. In conventional fluorescence systems, light is detected with a photomultiplier tube connected to an electric circuitry measuring the photon induced current in a continous manner. Even when no photons strikes the light sensitive surface of the photomultiplier, a small current arises from thermally excited electrons in the dynode chain. This dark current restricts the low limit of detection, unless precautions are taken to reduce it.

Due to the fact that photons propagate as distinct quanta, every photon striking the first dynode gives rise to a very short pulse of electrons, which is amplified in the dynode chain. Pulses generated by thermally excited electrons are amplified to a smaller extent, making them distinguishable from true light pulses. By electronic discrimination and counting, one can register only those pulses having an energy above a preset value. As the background can be kept as low as a few photons per sec., it is possible to count the number of photons interacting with the first dynode. This is the principle of photon counting.

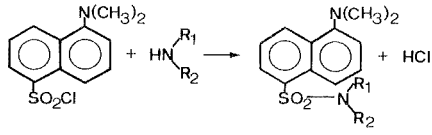
Usually the number of pulses is counted during a preset period of time, e.g. 1 sec., then the result is displayed and a new counting cycle is started. By using this method the instrument background can virtually be kept at zero, making it possible to measure very low sample concentrations. In previous applications of chemiluminiscence to thin-layer chromatography (1) and high performance liquid chromatography (HPLC) (2,3) use has not been made of photon counting for light measurement, whereby the sensitivity has been restricted by dark current fluctuations in the photomultiplier.

In clinical work, bio- and chemiluminiscence have been used for numerous analytical purposes (4) utilizing different methods for chemical excitation. The method used in this work was of the peroxylate type with the proposed mechanism:





The structure of the oxalate substituents (Ar) has been shown to influence both the reagent stability and the fluorescence intensity (5). The use of bis(2,4,6-trichlorophenyl)oxalate (TCPO) as the oxalic ester gives good reagent stability together with high quantum yields. The reaction of dansyl chloride with amines and phenols occurs according to the general equation:



reagent (dansyl chloride) reactant (amine) derivative

EXPERIMENTAL

Apparatus

HPLC was performed with a 5.0 mm I.D. x 200 mm column packed with 5 µm Nucleosil C_{18} particles (Macherey-Nagel & Co., Düren, G.F.R.). The pump used for chromatography was a LDC Constametric III double piston HPLC pump (Laboratory Data Control, Riviera Beach, Fl., U.S.A.) and the injector was a Rheodyne 7120 (Rheodyne, Berkeley, CA., U.S.A.) equipped with a 20 µl sample loop. The photon counter was developed and constructed by Auratronic Electronic Consultant, Stockholm, Sweden. It utilizes a Hamamatsu type R268UH-HA-P photomultiplier tube (Hamamatsu TV. Co. Ltd., Hamamatsu-City, Japan). Filters were manufactured by Carl Zeiss, Oberkochen/Würft, G.F.R. Catecholamine samples were prepared using a Bond Elut C_{18} column (Analytichem International, Harbour City, CA., U.S.A.).

The photomultiplier tube was mounted as shown in Fig. 1. Immediately in front of the tube is a filter holder with a filter followed by a spiral-type flow cell. To avoid signal deterioration the pre-amplifier is placed as close to the photomultiplier tube as possible, inside the light-proof box. A μ-metal magnetic shield prevents disturbance from laboratory equipment. On top of the box is a carefully sealed cover, giving a background count of only 50-150 photons per sec. The design of the flow cell is shown in Fig. 2. It was constructed from borosilicate glass and on the revers side it was covered with aluminium foil to direct all light towards the photon counter.

Chemicals

l-Adrenaline, l-noradrenaline, dansyl chloride and cyklohexylamine salts of dansyl-l-glutamic acid, dansyl-l-methionine, dansyl-d,l--norleucin and dansyl-l-α-alanine were obtained from Sigma Chemical Company, St. Louis, MO., U.S.A. Ethyl acetate, acetone, hydrogen peroxide, tris(hydroxymethyl)aminomethane (TRIS) and hydrochloric acid, all of analytical grade, and ethyl acetate, acetone and acetonitrile of spectroscopic grade were obtained from E. Merck, Darmstadt, G.F.R. Toluene of HPLC-grade was supplied by the same source. Acetonitrile for HPLC was from Rathburn Chemicals Ltd., Walkerburn, Gt. Britain and from BDH Chemicals Ltd., Parkstone, Gt. Britain. Hydrogen peroxide "Aristar" was also supplied by BDH. TCPO was prepared by the method of Mohan and Turro (6). All water was doubly distilled.

Purification of chemicals

Analytical grade ethyl acetate and acetone were distilled using a 1-m filled column. The TCPO was recrystallized from HPLC-grade toluene, the solution being treated with charcoal, and TRIS was recrystallized from aqueous ethanol (7).

Analytical conditions

In the determination of catecholamines the mobile phase consisted of 85% (v/v) acetonitrile and 15% (v/v) 0.05 mol l^{-1} TRIS-HCl--buffer, pH 7.7. The flow-rate was 1 ml min^{-1} . TCPO was dissolved

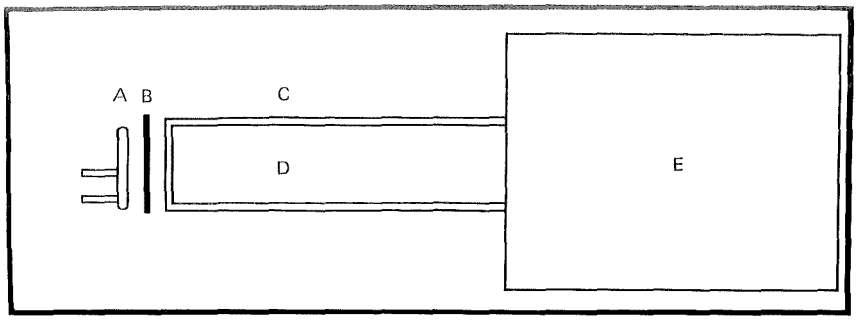


FIGURE 1

Light measurement compartment with A) Flow cell, B) Filter, C) μ-metal shield, D) Photomultiplier and E) Pre-amplifier.

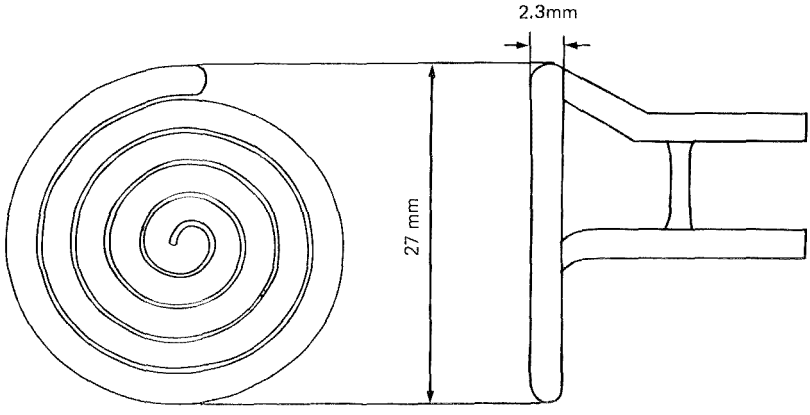


FIGURE 2

Design of flow cell.

in ethyl acetate at a concentration of 5 mmol l^{-1} and 30% (v/v) H_2O_2 was diluted with acetone to a final concentration of 0.5 mol l^{-1}. The flows were 0.3 ml min^{-1} for the TCPO solution and 0.5 ml min^{-1} for the H_2O_2 reagent. In order to analyse the amino acids the buffer content of the mobile phase was increased to 65% (v/v) and acetonitrile made up the final 35% of the mobile phase. The flow-rate was maintained at 1 ml min^{-1}. To avoid phase separation due to the low solubility of TCPO in aqueous systems, the TCPO reagent was pumped at 0.5 ml min^{-1} and the H_2O_2 reagent at 1.3 ml min^{-1}.

Sample preparation

Dansyl amino acid samples were freshly prepared by dissolving the derivatives in doubly distilled water. Dansyl derivatives of catecholamines were prepared as follows: Adrenaline and noradrenaline were dissolved in 0.1 mol l^{-1} HCl. A 100-µl volume of this solution was mixed with 330 µl of $NaHCO_3$ buffer (pH 8.5) and 2 ml of 0.1% (w/v) dansyl chloride in acetone. After vigorous mixing the samples were allowed to react at 55°C for 10 min. To remove the excess reagent, 2 ml of 0.5% (w/v) l-α-alanine was added and the sample mixture was once again placed at 55°C for 10 min. After application on a Bond Elut C_{18} column and rinsing with 5 ml of 50% ethanol, the sample was eluted with 500 µl of acetone and diluted to 10 ml with mobile phase. Care was taken to prevent UV-irradiation during sample preparation.

Procedure

The entire system outline is shown in Fig. 3. The eluent was kept in a glass container and was pumped through the injector and the column at a pressure of 1500 psi. The TCPO and H_2O_2 reagents, dissolved in ethyl acetate and acetone, were placed in two 500-ml stainless steel containers pressurized with helium and forced through a 1-m x 0.15 mm I.D. stainless steel or teflon capillary tubing giving a flow of 2 ml min^{-1} or less. The exact flow rates were adjusted by carefully regulating the helium pressure. This method gives a constant and absolutely pulse free delivery, which is important to achieve a low background noise.

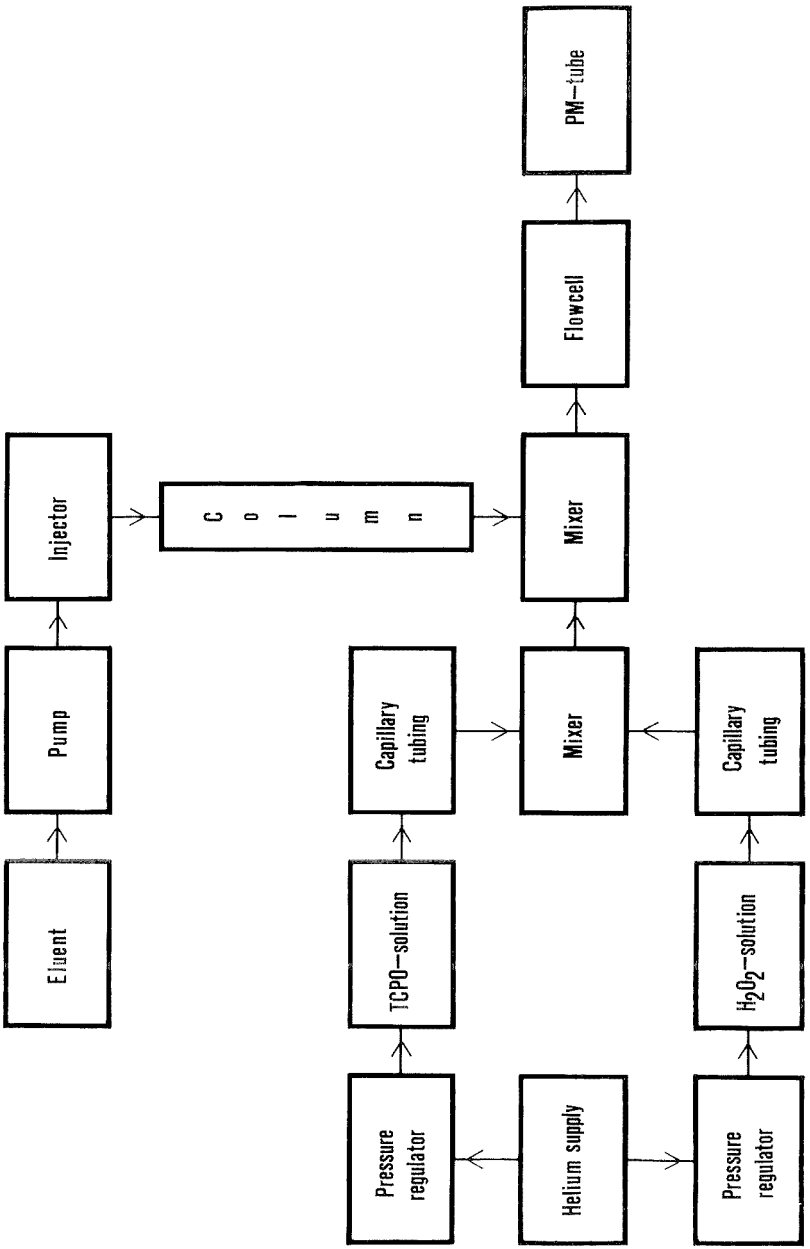


FIGURE 3

Block diagram of the complete analytical system.

The TCPO and H_2O_2 solutions were mixed in a static mixer according to Kobayashi and Imai (3), and then immediately brought in contact with the column effluent using an identical mixer. Upon mixing, 1,2-dioxetanedione is formed which excites fluorescent molecules when encountered in the column effluent. Since the chemiluminiscent radiation declines very rapidly, the flow was conducted directly to the flow cell where the light intensity was measured by photon counting. The number of photons striking the photomultiplier tube during 1 sec. was counted, the digital reading was converted automatically to an analog result and the chromatogram registered on a recorder in conventional manner. When starting up the system, it was allowed to operate for 10 to 15 min. so that the base line became stable. If the system has been turned off less than a couple of hours, it can be used within a few min.

RESULTS AND DISCUSSION

Effect of reagent purity

Table 1 lists the effect of mobile phase purity on signal and background readings. It can be seen that acetonitrile may contain relatively large amounts of fluorescent contaminants and still fulfil the UV-absorbance criterion of a HPLC-grade solvent. Therefore a careful choice of acetonitrile supplier is essential. The purification of TRIS is of smaller importance but still affects the results. During the remainder of this work BDH HPLC acetonitrile has been used in the mobile phase together with recrystallized TRIS.

In Table 2 the effects of purity of TCPO and H_2O_2 reagents are summarized. It is evident that the make of H_2O_2 has little influence on the peak height to background ratio, while distillation of the reagent solvents is essential.

Test of optical filters

In an attempt to increase the sensitivity of the detector system, 25 different filters were tested using the dansyl derivative of adrenaline as the test substance. A filter may be used to reduce the background level since the sample and the contaminants emit light at different

TABLE I

Effect of Mobile Phase Purity on the Peak Height to Background Ratio*

TRIS	Aceto-nitrile	HCl	Background (cps)	Peak height/ background x1000
P.A. recryst.	BDH HPLC	P.A.	15600	1.67
P.A.	BDH HPLC	P.A.	18000	1.50
P.A. recryst.	Rathburn HPLC	P.A.	36700	0.75
P.A. recryst.	Merck Uvasol	P.A.	15200	1.71

*Constant amounts of approximately 0.9 pmol of tridansyl-adrenaline were injected.

TABLE II

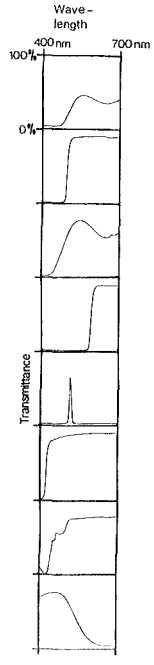
Effect of Purity of TCPO and H_2O_2 Reagents on the Peak Height to Background Ratio*

Acetone	Ethyl acetate	H_2O_2	Background (cps)	Peak height/background x1000
P.A. dest.	P.A. dest.	Merck P.A.	84100	1.080
P.A. dest.	P.A.	Merck P.A.	190000	0.500
P.A.	P.A. dest.	Merck P.A.	235000	0.404
P.A. dest.	P.A. dest.	BDH Aristar	95000	0.989

* Constant amounts of approximately 0.9 pmol of tridansyl-adrenaline were injected.

TABLE III

Effect of Filters in the Optical Path on the Peak Height to Background Ratio

Filter	Background (cps)	Peak height/background x1000	Characteristics
None	142900	0.308	
GG16	13600	0.750	
GG14	33900	0.697	
GG8	43000	0.588	
OG3	810	0.543	
Inter-ference	1905	0.522	
GG15	94700	0.394	
GG17	108200	0.333	
BG23	77000	0.252	

wavelengths. The filters were placed next to the photomultiplier tube immediately in front of the flow cell. As can be seen from Table 3, the best choice (GG16) increases the signal to background ratio more than twice compared to measurements without a filter. As the chemiluminiscence from the dansyl derivative of adrenaline gives a wide wavelength distribution with a maximum at 512 nm, a 90 nm bandwith interference filter with its peak transmittance at 519.5 nm was expected to increase the signal to background ratio significantly. The

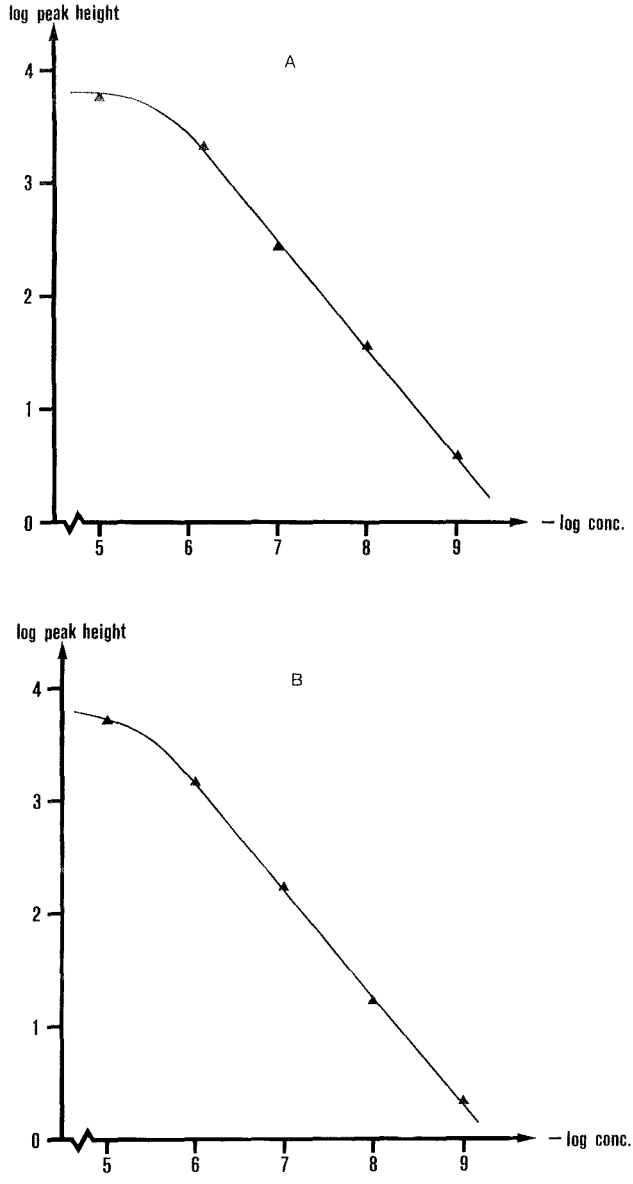


FIGURE 4

Correlation between sample concentration (mol l^{-1}) and peak height for A) adrenaline and B) noradrenaline.

gain was, however, not as large as accomplished with some of the other filters.

Linear detection range and detection limit for catecholamines

A plot of peak height versus concentration for adrenaline and noradrenaline displays a linear correlation over more than three orders of magnitude (Figs. 4A and B). At the high concentration end the peaks were flattened at the top probably due to reagent shortage. The peak intensities were in the order of 3 million cps. Extrapolating to the noise level gives a detection limit of 6 fmol for adrenaline and 16 fmol for noradrenaline. This is to be compared with the value of approximately 12 pmol obtained for the same derivatives by Frei, Thomas and Frei using a conventional fluorescence detector (8).

Assay of amino acids

In order to make a further comparison between this detector and a conventional fluorescence detector (2), some amino acids were analysed under almost identical conditions to those in ref. (2). Dansyl derivatives of glutamic acid, alanine, methionine and norleucine were dissolved in water and analysed using the same system as for the catecholamines. A VG10 filter turned out to be the best choice in order to supress the effect of background radiation. The sensitivity varied somewhat for different derivatives but the methionine derivative, being the least sensitive, had a detection limit of less than 0.5 fmol (Fig. 5). Using a fluorescence detector with the lamp turned off, Kobayashi and Imai (2) reached a detection limit of 10 fmol for the same dansyl amino acids, that is, the present method is more than 20 times as sensitive. This brings chemiluminiscence combined with photon counting up to and possibly beyond the limits of laser induced fluorescence, making it the most sensitive optical method of detection available at present.

CONCLUSIONS AND FURTHER WORK

Chemiluminiscence in combination with photon counting affords a high-sensitive analytical method for the assay of many substances, especially in the biochemical field. The considerable decrease in de-

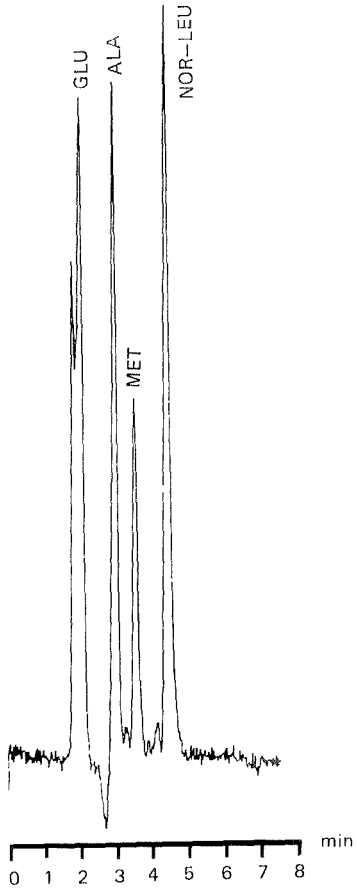


FIGURE 5

Chromatogram of amino acid dansyl derivatives. Injected volume: 20 µl. Sample concentrations: dansyl-l-methionine (MET) $2.07 \cdot 10^{-10}$ mol l^{-1}, dansyl-l-glutamic acid (GLU) $1.73 \cdot 10^{-10}$ mol l^{-1}, dansyl-l-α-alanine (ALA) $2.37 \cdot 10^{-10}$ mol l^{-1} and dansyl-d,l-norleucin (NOR-LEU) $2.15 \cdot 10^{-10}$ mol l^{-1}. The chromatographic conditions are given in the text.

tection limits for dansyl derivatives of catecholamines and amino acids in comparison with those attained with conventional fluorescence methods, should be possible to realize for many other compound classes. On that account it is our intention to apply the present method to other fields of interest e.g. to the assay of amines as o-phtaldialdehyde derivatives.

ACKNOWLEDGEMENTS

The author is indebted to Professor Bengt Smith, Head of the Department of Technical Analytical Chemistry, for valuable discussions during the course of this work.

REFERENCES

1. Curtis, T.G. and Seitz, W.R., J. Chromatogr., 134, 343 (1977).

2. Kobayashi, S. and Imai, K., Anal. Chem., 52, 424 (1980).

3. Kobayashi, S., Sekino, J., Honda, K. and Imai, K.,Anal. Biochem., 112, 99 (1981).

4. Gorus, F. and Schram, E., Clin. Chem., 25, 512 (1979).

5. Rauhut, M.M., Bollyky, L.J., Roberts, B.G., Loy, M., Whitman, R.H., Ianotta, A.V., Semsel, A.M. and Clarke, R.A., J. Amer. Chem. Soc., 89, 6515 (1978).

6. Mohan, A.G., and Turro, N.J., J. Chem. Educ., 51, 528 (1974).

7. Perrin, D.D., Armarego, W.L.F. and Perrin, D.R., "Purification of Laboratory Chemicals", Pergamon Press, Oxford, 1966.

8. Frei, R.W. Thomas, M. and Frei, I., J. Liq. Chromatogr., 1, 443 (1978).

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(9), 1617-1630 (1983)

COMPARISON OF VARIOUS COLUMNS FOR THE HIGH-SPEED HPLC ANALYSIS OF DRUGS OF FORENSIC INTEREST

Ira S. Lurie and Susan M. Carr
Special Testing and Research Laboratory
Drug Enforcement Administration
McLean, Virginia 22102-3494

ABSTRACT

A comparison of the use of various commercially available columns for the high-speed reverse-phase ion-pair high performance liquid chromatographic separation of drugs of forensic interest is discussed. The columns include a Partisil 5 ODS-3 RAC, a Partisil 5 C8 RAC, a Radial Pak microBondapak C18 cartridge, a Perkin-Elmer HS/5 C18 and a Perkin-Elmer HS/3 C18. The mobile phases employed contain water, acetonitrile, phosphoric acid, and sodium hydroxide, with or without hexylamine. When a mobile phase without an amine modifier is employed, retention times were at least halved, except with a HS/3 C18 column, over those obtained with conventional columns. Basic drugs did not elute when the above mobile phase is used with a HS/3 C18 column. In addition, the selectivities of the other high speed columns were similar. Further reductions in retention times and different selectivities were obtained when an amine modifier is utilized. Column performance parameters such as n, V and v are presented for the columns examined. A new column performance parameter S which is $(n/V)^{\frac{1}{2}}$ is introduced and discussed.

INTRODUCTION

Reverse-phase ion-pair HPLC has been employed for the analysis of drugs of forensic interest (1, 2). The microBondapak C18 column (30cmx3.9mm) and Partisil 10-ODS-3 (25cmx4.6mm) column have been shown to exhibit similar selectivities (2). The goal of the present study was to investigate ways of obtaining faster separations using systems designed for high-speed analysis. There are three ways of obtaining faster separations with a given mobile phase assuming the column packing material to be employed is of a similar nature to the previously utilized column.

 0148-3919/83/0609-1617$3.50/0

1) An increased number of plates per second could be obtained by using 7-15cm length and 4-5mm id columns with 3 or 5 micron particle size packing material. The use of injectors, detectors and connecting tubing which minimize band spreading would be required. The Perkin-Elmer HS/5 C18 and Perkin-Elmer H8/3 C18 are examples of such columns.

2) An increased number of plates per second could be obtained by using 10cm length and 9.5mm id columns with 5micron particle size packing material. Unlike the columns in Category 1) conventional equipment would be employed. The Partisil 5 ODS-3 RAC and Partisil 5 C8 RAC are examples of such columns.

3) An increased number of plates per second could be obtained by using 10cm length and 5-8mm id cartridges with 5 or 10 micron particle size packings. A compression module is required for these cartridges of which the Radial Pak microBondapak C18 is an example.

Another way of obtaining faster separations is to modify the mobile phase. For the reverse-phase ion-pair chromatography of basic drugs, the addition of a competing amine to the mobile phase will reduce k' values (3, 4).

In this paper various ways of performing reverse-phase ion-pair high-speed HPLC analysis for drugs of forensic interest are compared. Columns were employed from each of the above categories utilizing mobile phases with or without the presence of a competing amine. Only one column from each commercial type was employed and therefore this study did not take into account variations between columns from the same manufacturer.

EXPERIMENTAL

Equipment

Two liquid chromatographs were employed in this study. One chromatograph consisted of the following components: Model 6000A pump (Waters Associates); Model U6K injector (Waters) or Model 7125 injector (Rheodyne); Model 440 fixed wavelength uv detector at 254 nm with a 12.5 µl flow cell (Waters); Systems 1VB data system (Spectra Physics) or Sigma 15 data system interfaced with a Model 3600 data station (Perkin-Elmer). Columns employed on the first chromatographic system consisted of two prepacked 8mmx10cm stainless steel columns each with 5 micron C18 and 5 micron C8 packing material (Partisil 5 ODS-3 RAC and Partisil 5 C8

RAC, Whatman); a prepacked 4.6mmx25cm stainless steel column with 10 micron C18 packing material (Partisil 10-ODS-3, Whatman); and a prepacked 8mmx10cm polyethylene cartridge with 10 micron C18 packing material (Radial Pak microBondapak C18 cartridge, Waters) mounted inside a radial compression chamber (Z-Module, Waters).

The other liquid chromatograph consisted of the following components: Model 8800 4-solvent gradient system (DuPont); Model LC85 variable UV detector, containing a 2.5 microliter flow cell or 1.5 microliter flow cell at 214 or 254nm (Perkin Elmer); Sigma 15 Data System interfaced with a Model 3600 Data Station (Perkin-Elmer). A prepacked 4.6mmx12.5cm stainless steel column with 5 micron C18 packing material (Perkin Elmer HS/5 C18) and a prepacked 4.6mmx10cm stainless steel column with 3 micron C18 packing material (Perkin-Elmer HS/3 C18) were used with this system.

Materials

The following chemicals were used: hexylamine (Eastman Chemicals, Rochester, N.Y.); methenamine (Merck, Rahway); acetonitrile (Burdick and Jackson). Other chemicals were reagent grade. Authentic drug standards of USP/NF quality were employed.

One mobile phase consisted of 20% acetonitrile, 79% water, and 1% phosphoric acid adjusted to pH 2.2 with 2N sodium hydroxide. This was prepared by filtering and degassing through a 0.45 micron filter a solution consisting of 1580ml water, 400ml acetonitrile, 20ml phosphoric acid and 60ml 2N sodium hydroxide.

The second mobile phase consisted of 12% acetonitrile, 87% water, and 1% phosphoric acid adjusted to pH 2.2 with 2N sodium hydroxide. This mobile phase was prepared as previously reported (2).

Hexylamine was added to mobile phases after they were filtered and degassed resulting in a final molarity for amine of 0.072M with the pH raised to approximately 3.

RESULTS AND DISCUSSION

Table 1 presents a comparison of retention times (RT's) and relative retention times (RRT's) for 4 of the high speed columns and a conventional column, the Partisil 10-ODS-3 using a mobile phase without an amine modifier. The high speed columns gave RT's at least one-half those obtained with the conventional column in addition to similar selec-

TABLE 1

Retention Data for Drugs of Forensic Interest Using Various Alkylsilica Stationary Phases With Mobile Phase Without Hexylamine

Column	HS/5 C18 flow 2.5 ml/min.		5 ODS-3 RAC flow 8.0 ml/min.		5 C8 RAC flow 8.0 ml/min.		RADIAL PAK flow 8.0 ml/min.		10 ODS-3 flow 2.0 ml/min.	
Mobile phase: see Figure 1.										
Drug	RT	RRT	RT	RRT	RT	RRT	RT	RRT	RT	RRT
Heroin	3.0	1.0	3.1	1.0	2.8	1.0	2.5	1.0	7.8	1.0
Caffeine	1.2	.42	1.2	.38	1.2	.44	.88	.35	3.2	.41
Phenobarbital	6.0	2.0	6.4	2.1	6.1	2.1	4.1	1.6	12.1	1.6
Methamphetamine	1.3	.42	1.2	.40	1.2	.43	1.1	.42	3.1	.40
Acetyl-codeine	2.9	.97	3.0	.95	2.8	.98	2.4	.94	7.4	.96
Noscapine	6.0	2.0	6.3	2.0	5.8	2.1	4.9	2.0	15.0	1.9
Papaverine	5.7	1.9	5.9	1.9	5.6	2.0	4.6	1.8	16.0	2.1
Cocaine	3.8	1.3	3.9	1.2	3.8	1.4	3.2	1.3	10.4	1.3
PCP	7.3	2.4	7.7	2.5	7.3	2.6	6.1	2.4	18.3	2.4
LSD	5.2	1.8	5.5	1.8	5.4	1.9	4.4	1.8	14.0	1.8

tivities. Therefore these high speed columns should be applicable to the analysis of drugs of forensic interest using mobile phases which had been previously utilized with the microBondapak C18 and Partisil ODS-3 columns.

Results from the HS/3 C18 column were omitted from Table 1 because basic drugs did not elute from the column under these conditions. The Perkin-Elmer HS/3 C18 column required a much more liphophilic mobile phase to elute heroin and the resulting peak is very assymetrical* with only 75 theoretical plates.

Although the addition of methenamine to the mobile phase resulted in reduced tailing and shortened retention time for heroin, the peak shape was still unsatisfactory and the plate count was only 300. There appears to be unbonded silanol sites on the HS/3 C18 column. It has been reported that using a similar mobile phase, with alkyl-bonded columns containing residual silanol groups, the addition of a competing amine to the eluent reduced peak tailing and decreased RT's for basic drugs (3, 4). The nature of the added amine affects both peak shape

* Satisfactory peak shape is taken to be an Assymetry Factor (A) of 1.8 or less, measured at 10% of peak maximum. (A) is computed by dividing the distance from peak center to trailing edge by the distance from peak center to leading edge.

and retention time for basic drugs. Since the addition of methenamine to the mobile phases did not result in a satisfactory peak shape for heroin, the effect of hexylamine was investigated since it has been shown to be one of the best blocking agents for silanol groups (4). Satisfactory peak shape was obtained with over 1000 theoretical plates for the heroin peak.

Table 2 shows results from measurements made under the same conditions as Table 1, except for the addition of hexylamine to the mobile phase. The addition of hexylamine further decreased RT's for the HS/5 column by a factor of 1.5-2. In addition, the amine reduced RT's with the HS/3 column to values less than those obtained with the HS/5 column.

Hexylamine in the mobile phase also affects column selectivity, as illustrated by the changes in RRT's for the HS/5 column for caffeine, phenobarbital, PCP, and LSD. RRT's for other substances changed little. Although satisfactory peak shape was obtained for heroin on the 3 micron column, some bases exhibited excessive tailing.

The parameter, v, described by Desty, et al. (5), was evaluated for the columns studied and is defined by

$$v = n/RT \qquad (1)$$

where n = theoretical plates/column

RT = retention time (min)

The number of theoretical plates (n) was calculated as follows:

$$n = 25(RT/W_{4.4})^2 \qquad (2)$$

where $W_{4.4}$ is the width of the peak at 4.4% of peak height.

The performance parameter, S, which is introduced in this paper is defined by

$$S = (n/V)^{1/2}$$

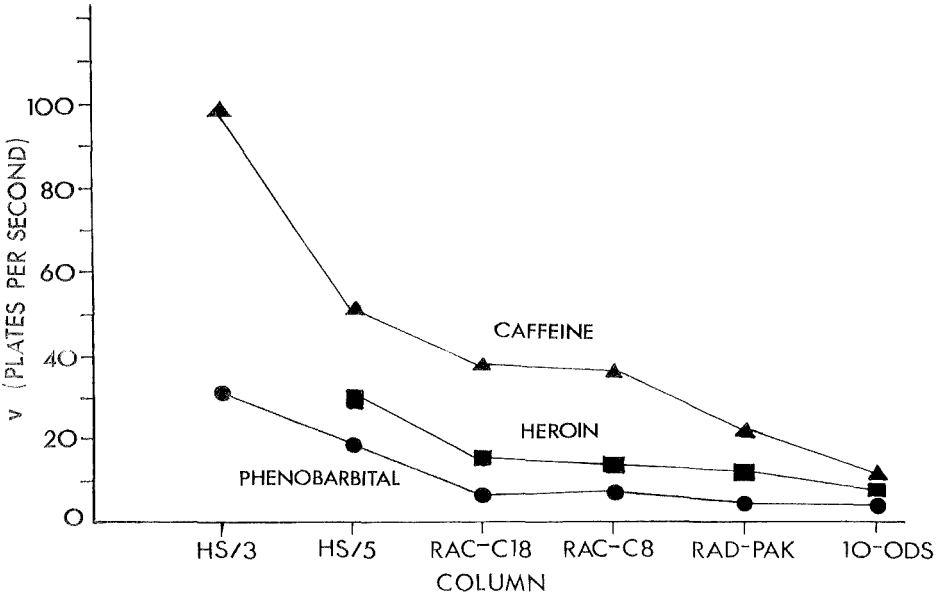
where V = retention volume (ml)

The parameter S is useful for the evaluation of column performance because it is a function of both the separating power of the column and of solvent consumption. The rate of production of theoretical plates, v, is of use where separating power is of primary interest. Graphical presentations of the v factor (Figure 1) and S factor (Figure 2) were obtained from experimental results for three compounds of forensic interest (caffeine, phenobarbital and heroin), using the five high speed columns and a conventional column. The column performance parameters

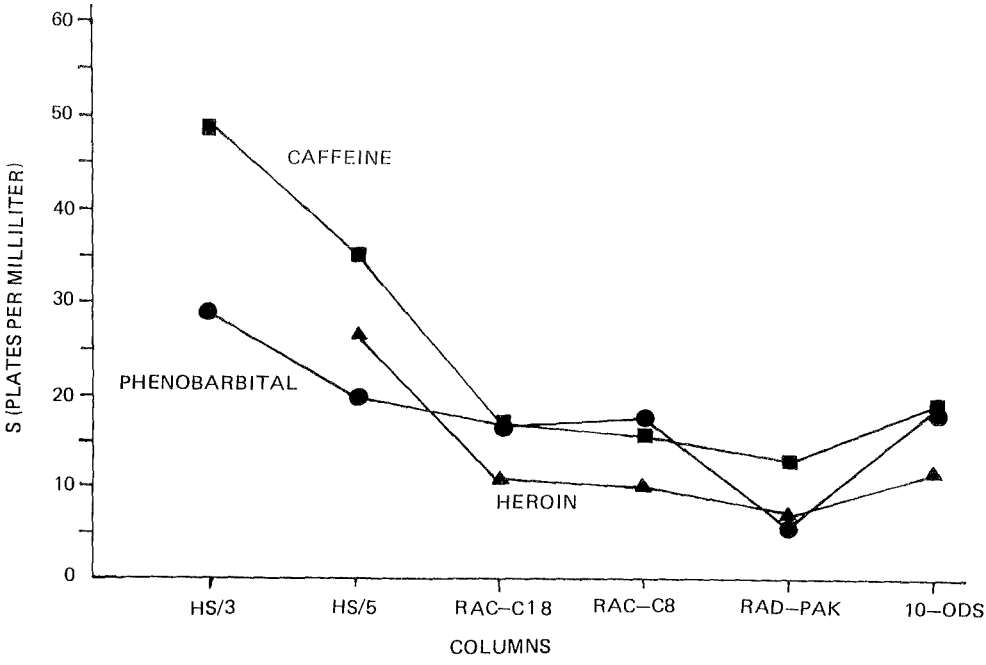
TABLE 2

Retention Data for Drugs of Forensic Interest Using the HS/5 C18 and HS/3 C18 Columns With Mobile Phase Containing Hexylamine

Column	HS/5 C18 flow 2.5 ml/min.		HS/3 C18 flow 2.5 ml/min.	
Mobile phase: same as Figure 1 except .072M hexylamine				
Drug	RT	RRT	RT	RRT
Heroin	1.6	1.0	1.1	1.0
Caffeine	1.2	.75	.70	.61
Phenobarbital	4.8	3.0	3.1	2.7
Methamphetamine	.83	.52	.62	.54
Acetyl-codeine	1.6	1.0	1.1	1.0
Noscapine	3.4	2.1	2.2	1.9
Papaverine	2.8	1.8	1.9	1.6
Cocaine	1.7	1.0	1.6	1.36
PCP	2.8	1.8	4.6	4.0
LSD	2.3	1.4	1.8	1.5



1. Plot of plates per second (v) versus column type for various solutes in mobile phase consisting of 20% acetonitrile, 79% water, 1% phosphoric acid adjusted to pH 2.2 with 2N sodium hydroxide.

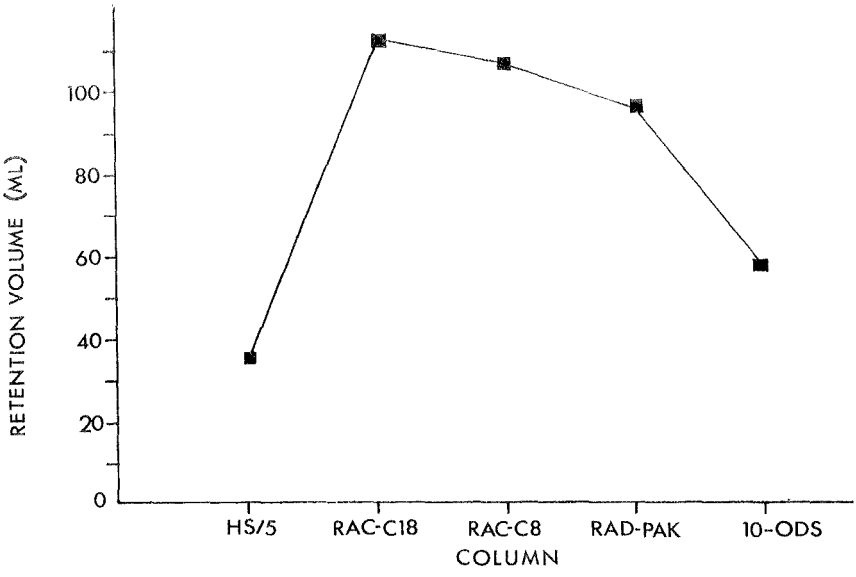


2. Plot of square root of plates per volume (S) versus column type for various solutes. See Figure 1 for mobile phase.

TABLE 3

Performance Parameters for Caffeine, Phenobarbital and Heroin On Various Alkyl-Silica Stationary Phases With Mobile Phase Without Hexylamine

Drug	Caffeine				Phenobarbital				Heroin			
Mobile phase and flow rates: see Tables 1 and 2												
Column	V	n	v	S	V	n	v	S	V	n	v	S
See text for description of performance parameter symbols												
HS/3 C18	2.1	4900	98	48	10.8	8425	32	28				
HS/5 C18	3.1	3802	51	35	14.9	6289	18	20	7.5	4960	28	26
5 ODS-3 RAC	9.6	2644	37	17	51.5	2633	7	17	24.9	2844	15	11
5 C8 RAC	9.9	2640	36	16	48.6	2900	8	18	22.7	2203	13	10
Radial Pak	7.0	1150	22	13	33.0	1080	4	6	20.0	1010	7	7
10 ODS-3	6.4	2258	12	19	24.3	2860	4	19	15.5	2243	5	12



3. Plot of retention volume (V) versus column type for heroin. See Figure 1 for mobile phase.

n, v, V and S for these compounds are presented in Table 3. A mobile phase without hexylamine was used at the same linear velocity. For caffeine and phenobarbital the highest values for v and S were obtained with the Perkin-Elmer HS/3 C18 column. The high value for v using the HS/3 column is consistent with the 3 micron particle size. This is a result of the lower value of the A and the C terms in the Van Deempter equation which are proportional to the particle diameter (6). Similar results have been reported by other authors using columns with 3 micron packing (7-9). The appreciably higher values for S obtained with the Perkin-Elmer HS/3 and HS/5 C18 columns as compared to the other high speed columns tested apparently resulted from their greater efficiencies and lower solvent consumption. The lower solvent consumption of the Perkin-Elmer high speed columns, illustrated in Figure 3, is due of course to their smaller internal diameters. As indicated in Figure 2, the S value of the conventional 10-ODS-3 column is higher than some of the high speed columns as a result of reduced solvent consumption. When

TABLE 4

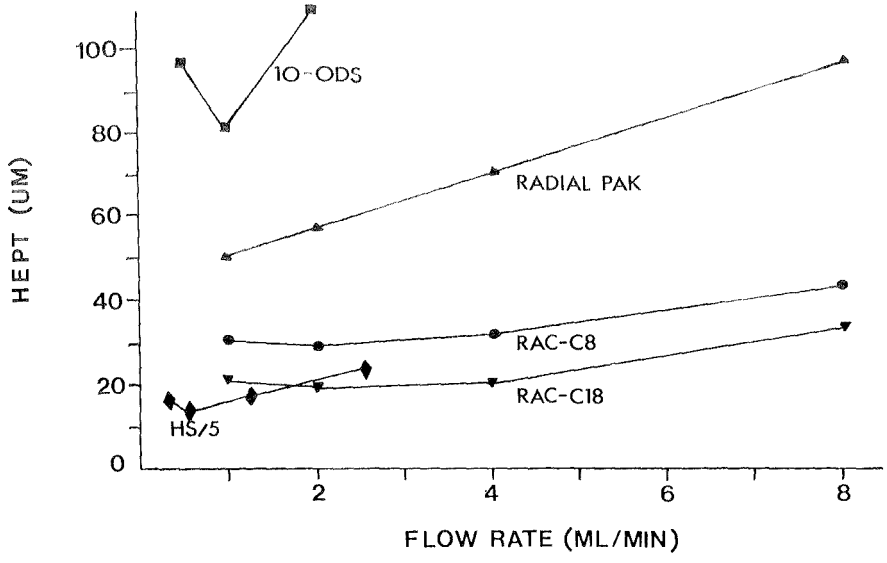
Performance Parameters for Caffeine, Phenobarbital and Heroin On the HS/5 C18 and HS/3 C18 Columns With Mobile Phase Containing Hexylamine

Drug	Caffeine				Phenobarbital				Heroin			
Mobile phase and flow rates: see Table 2												
Column	V	n	v	S	V	n	v	S	V	n	v	S
See text for description of performance parameter symbols												
HS/3 C18	1.7	2889	67	41	7.8	8742	47	34	2.8	1427	21	22
HS/5 C18	3.0	3540	49	34	12.0	6356	22	23	4.0	3053	32	28

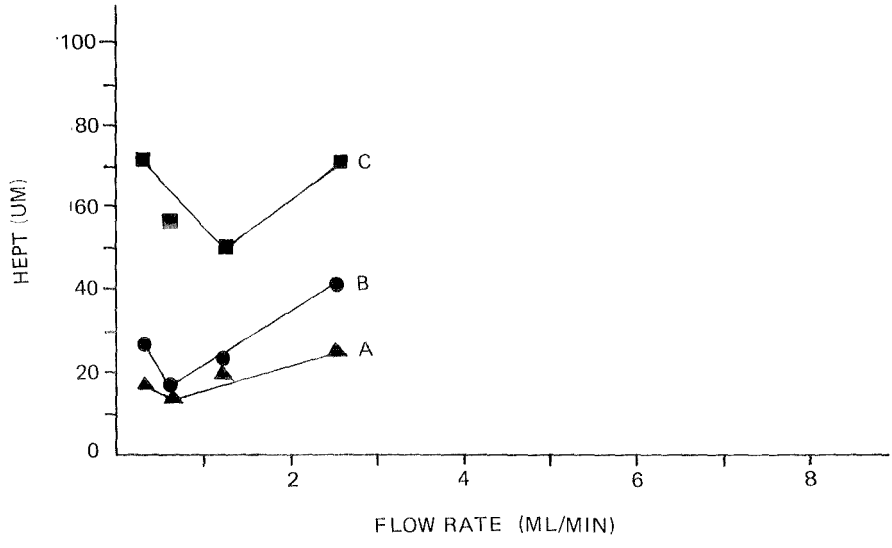
employing a mobile phase without hexylamine, the highest values for v and S are obtained for heroin with the Perkin-Elmer HS/5 C18 column.

A comparison of Table 3 and 4 indicates that the addition of hexylamine to the mobile phase has little effect on v or S for the compounds studied with the HS/5 column. The number of theoretical plates of the heroin peak is reduced on the HS/5 column when hexylamine is added to the mobile phase. The number of theoretical plates of heroin and caffeine on the HS/3 column is significantly lower than that of the HS/5 column using the latter mobile phase. For the HS/5 column using a mobile phase containing hexylamine, there is a slight increase in both v and S for heroin due to the column's significantly reduced retention volume.

As Figure 1 and Table 3 indicate, the v and n values are considerably higher for the HS/5 C18 column than the RAC-C18 and RAC-C8 columns. Neglecting extra-column effects (which is reasonable for phenobarbital due to its large k' value) one would expect as a result of the infinite diameter phenomenon that the larger diameter RAC columns would be closer in efficiency to the narrower HS/5 column. Indeed, it has been reported that for 10cm length columns packed with 5 micron packing material the 9.5 mm id column showed marked increases in efficiencies over a 4.5mm id column (10). Therefore it would appear that the RAC columns are not as well packed as the HS/5 column which results in a higher A term in the van Deemter equation.



4. Plot of HETP versus flow rate for heroin on various columns. See Figure 1 for mobile phase.



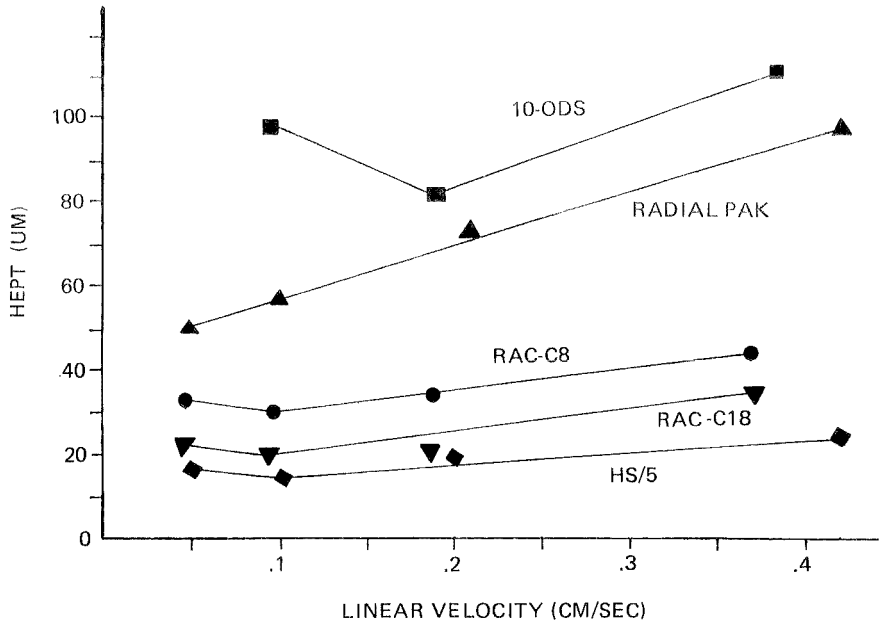
5. Plot of HETP versus flow rate for heroin; on a HS/5 C18 column with mobile phase without hexylamine (A) (see Figure 1); same column as (A) except mobile phase contains .072M hexylamine (B); on a HS/3 C18 column with mobile phase containing .072M hexylamine (C).

Figures 4 and 5 are plots for heroin of HETP versus flow rate for the various chromatographic systems. In order to obtain lower analysis times, higher than optimum flow rates were used and found to result in a small sacrifice in the number of theoretical plates.

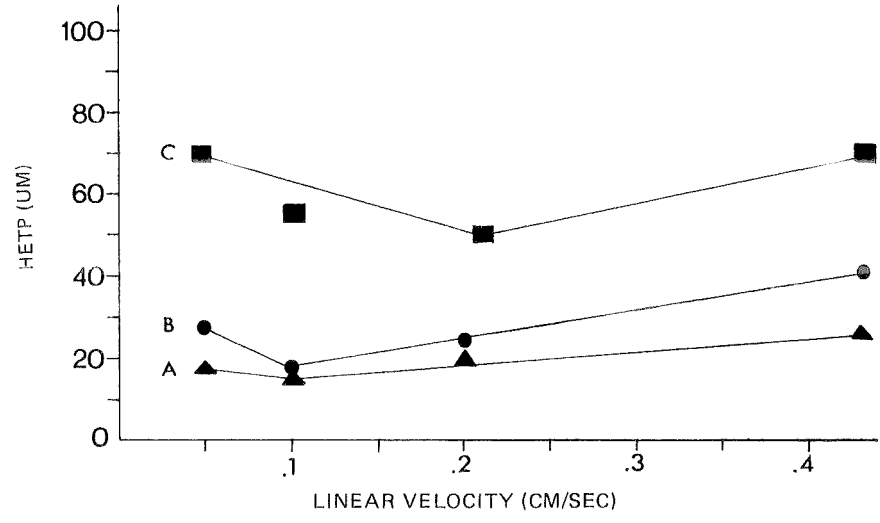
Figure 6 gives van Deemter plots for heroin using various columns without hexylamine. Van Deemter plots for the HS/5 C18 and ODS-3 RAC with other solutes have been reported and the curve shape is very similar to those illustrated in Figure 6 (7, 9). The smaller HETP values and smaller increase in HETP with linear velocity for the columns con-containing 5 micron particle sizesis a result of the effect of the smaller particle's size on the A and C term of the van Deemter equation. The shape of the curve for the Radial Pak microBondapak cartridge is very similar to that obtained for the microBondapak C18 column (11). The lower values for HETP on the Radial Pak microBondapak C18 column than on the 10-ODS-3 column are due to a smaller A term in the van Deemter equation as a result of the elimination of wall effects.

Figure 7 illustrates the effect of the addition of hexylamine to the mobile phase on the van Deemter plot for heroin on the HS/3 and HS/5 C18 columns. The higher HETP values and greater increase in HETP with linear velocity when an amine is added may be a result of the contribution of the D term in the van Deemter equation. This term relates to mass transfer effects in the stationary phase and could increase because of the presence of two differing absorption sites which result when a competing amine is added to the mobile phase (12). The two kinds of sites consist of bonded C18 groups and unbonded silanol groups (11). The higher HETP values for the HS/3 C18 column are consistent with a larger number of unbonded silanol sites and therefore a greater inhomogeneity of absorption sites. The HS/3 C18 column because it contains the smaller 3 micron particle size packing material would be expected to contain lower HETP values than the HS/5 C18 column (7).

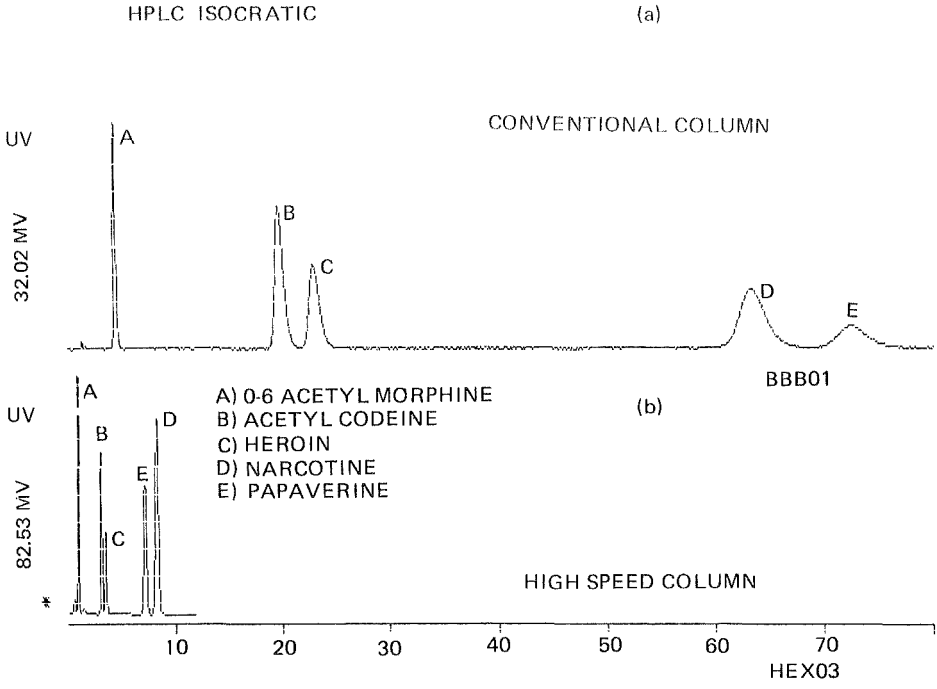
The HS/5 C18 column with a mobile phase containing hexylamine gave the best values of v and S for heroin. The use of this column with a mobile phase like one that has been previously reported for heroin analysis (2), except that methanesulfonic acid is replaced by hexylamine, gave a greatly improved separation for heroin and its major by-products. It was found that O^6-acetylmorphine, acetylcodeine, heroin,



6. Plot of HETP versus linear velocity for heroin on various columns. See Figure 1 for mobile phase.



7. Plot of HETP versus linear velocity for heroin. For description of (A), (B), and (C) see Figure 5.



8. Comparison of the separation of O^6-acetylmorphine (A), acetylcodeine (B), heroin (C), noscapine (D) and papaverine (E) on two chromatographic systems. System a consists of a Partisil 10-ODS-3 column with mobile phase which doesn't contain hexylamine. System b consists of a HS/5 C18 column with the same mobile phase as system a except that hexylamine is present. Flow rate on both systems is 3.0ml/min. The mobile phases are described in text.

noscapine and papaverine were resolved in almost one-tenth the previously reported (2) time, as illustrated in Figure 8. The addition of methanesulfonic acid or hexylamine to the mobile phase was necessary to produce a linear isotherm for heroin below a concentration of 2 mg/ml. The use of the above chromatographic system for the analysis of both uncut and adulterated samples is being studied.

ACKNOWLEDGEMENTS

The authors would like to thank Waters Associates for providing the Z-Module with a Radial Pak microBondapak C 18 cartridge.

REFERENCES

1. Lurie, I. S., Improved Isocratic Mobile Phases for the Reverse-Phase Ion-Pair Chromatographic Analysis of Drugs of Forensic Interest. J. Liq. Chromatogr., 4, 399 (1981).

2. Lurie, I. S., Sottolano, S. M. and Blasof, S., High Performance Liquid Chromatographic Analysis of Heroin by Reverse-Phase Ion-Pair Chromatography. J. Forensic Sci., 27, 519 (1982).

3. Sokolowski, A. and Wahlund, K. G., Peak Tailing and Retention Behaviour of Tricyclic Antidepressant Amines and Related Hydrophobic Ammonium Compounds in Reverse-Phase Ion-Pair Liquid Chromatography on Alkyl-Bonded Phases. J. Chromatogr., 189, 299 (1980).

4. Gill, R., Alexander, S. P. and Moffat, A. C., Comparison of Amine Modifiers Used to Reduce Peak Tailing of 2-Phenethylamine Drugs in Reversed-Phase High Performance Liquid Chromatography. J. Chromatogr., 247, 29 (1982).

5. Desty, D. H., Goldup, A. and Swanton, W. T., Performance of Coated Capillary Columns, In Brenner, N., Callen, J. E. and Weiss, M. D., eds., Gas Chromatography (1961 Lansing Symposium). Academic Press, New York, 105 (1962).

6. Snyder, L. R. and Kirkland, J. J., Introduction to Modern Liquid Chromatography, 2nd Edition, Wiley Interscience, New York, 168 (1979).

7. DiCesare, J. L., Dong, M. W. and Ettre, L. S., Introduction to High Speed Liquid Chromatography. Perkin-Elmer, Norwalk, CT, 24 (1981).

8. Cooke, H. C. N., Archer, B. G., Olsen K. and Berick, A., Comparison of Three- and Five-Micrometer Column Packings for Reversed-Phase Liquid Chromatography. Anal. Chem., 54, 2277 (1982).

9. McCoy, R. W. and Pauls, R. E., Comparison of Commercial Types in Liquid Chromatography. J. Liq. Chromatogr., 5, 1869 (1982).

10. Webber, T. J. N. and McKerrel, E. H., Optimization of Liquid Chromatographic Performance on Columns Packed With Microparticulate Silica. J. Chromatogr., 122, 243 (1976).

11. Fallick, G. J. and Rausch, C. W., Radial Compression Separation System. American Lab, November 1979.

12. Rudzinski, W. E., Bennett, D. and Garcia, V., Retention of Ionized Solutes in Reversed-Phase High Performance Liquid Chromatography. J. Liq. Chromatogr., 5, 1295 (1982).

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(9), 1631-1643 (1983)

EVALUATION OF MICRO-HPLC AND VARIOUS STATIONARY PHASES IN REVERSED-PHASE MODE FOR THE SEPARATION OF STYRENE OLIGOMERS

K.Jinno and T.Sato
School of Materials Science
Toyohashi University of Technology
Toyohashi 440
Japan

ABSTRACT

The potential of the micro-HPLC technique and various reversed-phase packing materials for the separation of styrene oligomers is evaluated. The results indicate that longer carbon chains and/or copolymer materials with small-diameter particles are superior to other type packings for the separation of oligomers with methanol as mobile phase. Micro-HPLC promises the possibility of separating conformational isomers of styrene oligomers.

INTRODUCTION

Over the past few years, much effort has been directed toward increasing both selectivity and efficiency in liquid chromatography (LC). The introduction of various types of micro-HPLC columns (1-5) and smaller size packing materials represents a revolution in column technology (6-8).

In light of recent interest in micro-HPLC and small-dimension packing materials, it seems appropriate at this

 0148-3919/83/0609-1631$3.50/0

time to assess the utility of the new column technology for separation problems in polymer chemistry.

The purpose of this communication is to evaluate their ability for the separation of styrene oligomers, which is one of the typical chromatographic problems in polymer chemistry. The discussion, here, is restricted to an isocratic mode with methanol as mobile phase, using teflon micro columns.

EXPERIMENTAL

All measurements of micro-HPLC separation were made with the MF-2 microfeeder (Azuma Electric, Tokyo, Japan) to control the mobile phase flow. A Jasco (Tokyo, Japan) Uvidec-100 II ultraviolet spectrophotometer was used. Conventional HPLC measurements were made with a system consisting of a Jasco Trirotar III and Uvidec-100 III ultraviolet spectrophotometer.

The column packing materials evaluated here are listed in Table 1, in which carbon chain-length, particle size, column length and commercial name are shown for each. All of the micro columns used were constructed with teflon tubing, 0.5 mm inner diameter, packed with materials listed in Table 1 by a slurry technique. The conventional HPLC column was a 4.6 mm i.d. x 25 cm stainless steel tube packed with FineSIL C18 (Jasco, 10 µm).

The mobile phase was HPLC-grade methanol purchased from Kanto Chemicals (Tokyo, Japan). Flow rates were 8 µl/min for micro-HPLC and 0.5 ml/min for conventional HPLC, respectively.

Table 1

Packing Materials Used in This Work

Column No.	Carbon Chain Length	Particle Diameter	Column Length	Commercial Name
1	C 1	10 μm	162 mm	FineSIL C1
2	C 2	10	220	FineSIL C2
3	C 8	10	193	FineSIL C8
4	C 8	5	175	Develosil C8-5
5	C18	5	185	FineSIL C18-5
6	C18	10	211	FineSIL C18-10
7	C18	10	210	FineSIL C18 T*
8	C18	7	160	Chemcosorb ODS/H-7
9	C18	3	169	Develosil C18-3
10	C18	5	196	Develosil C18-5
11	C18	10	179	Develosil C18-10
12	C18	5	232	SC-01
13	copolymer, styrene-divinylbenzene	10	44	HP-01

* trimethylsililated after chemical bonding.

Test solute for column evaluation was styrene oligomers A-500 (purchased from Toyo Soda, Tokyo, Japan) which has an average molecular weight of 500 with a butyl side chain.

RESULTS AND DISCUSSION

The chromatograms obtained under identical chromatographic conditions, with micro-HPLC, are shown in Figures 1-A, 1-B and 1-C. The number of each chromatogram corresponds to that shown in Table 1. The chromatogram recorded with conventional HPLC is shown in Figure 2.

Effect of chain length: Obviously, materials bonded with shorter carbon chains will have smaller capacity factors

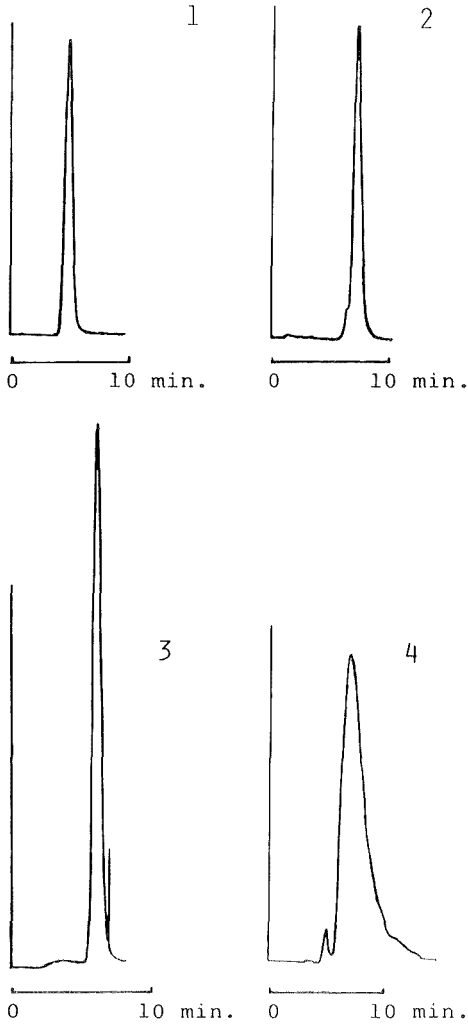


Figure 1 Separation of styrene oligomers with micro-HPLC
A: column No. 1-7 in Table 1.
B: column No. 8-11
C: column No. 12, 13
Chromatographic conditions:
Mobile phase; methanol 100 %
Flow rate; 8 µl/min
Sample injection; 0.3 µl
Column temperature; 25 °C
Detection; UV 207 nm
(n=2 means dimer).

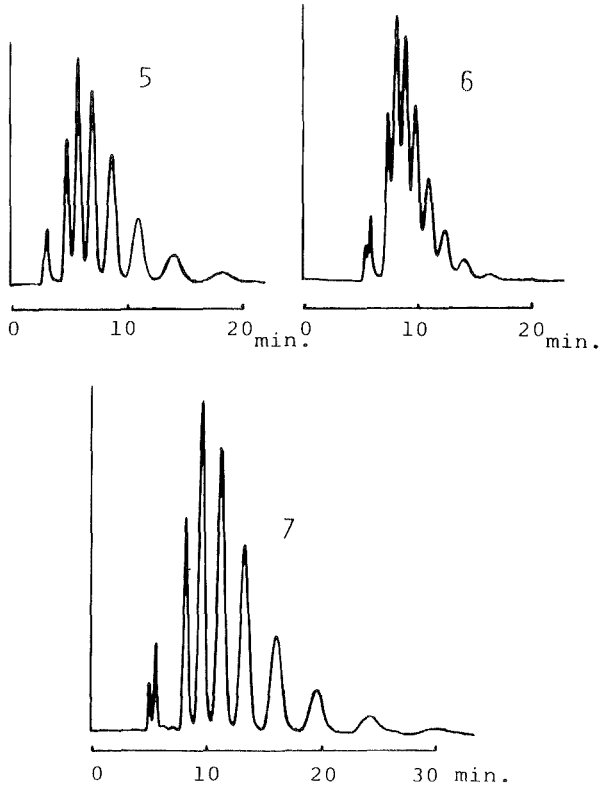


Figure 1 - continued

than those with longer carbon chains when used with the same mobile phase. However, in this instance, the retention times of oligomers with C2, C8 and C18 columns (No. 2, 3, 6 and 7 in Figure 1, all materials have the same particle diameter) are almost the same; this means that longer carbon-chain columns are more likely to resolve styrene oligomers. From the chromatograms shown in Figure 1, it is apparent that the separations with C1, C2 and C8 packings (group-1) are poor, whereas those with C18 and copolymer (group-2) are good.

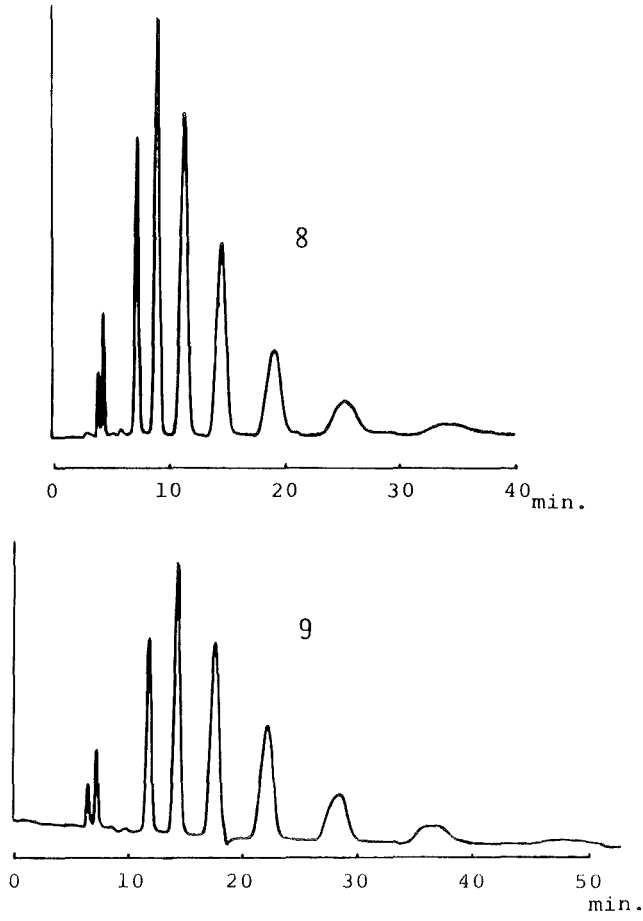
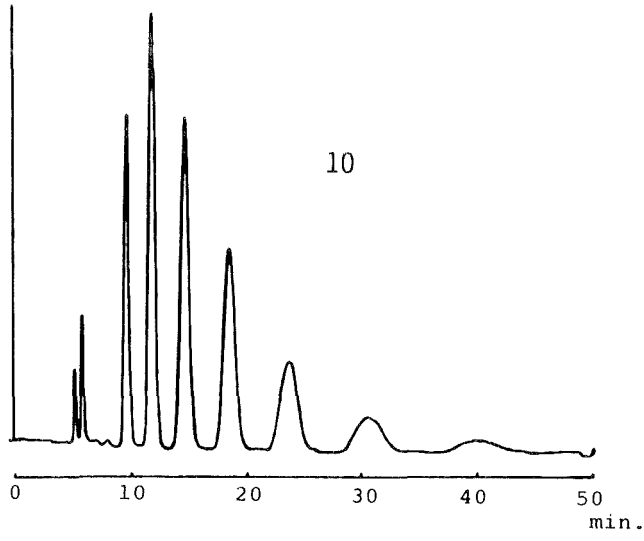


Figure 1 - continued

There have been a number of discussions concerning the retention mechanism in reversed-phase separation (9-24). Despite the popularity and wide applicability of reversed-phase separations, a complete description of the retention mechanism for bonded phases has not yet been formulated. However, it is well known that a major factor in determining



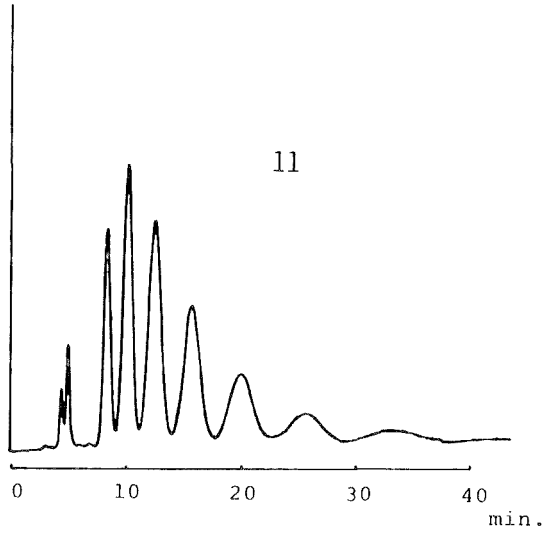


Figure 1 - continued

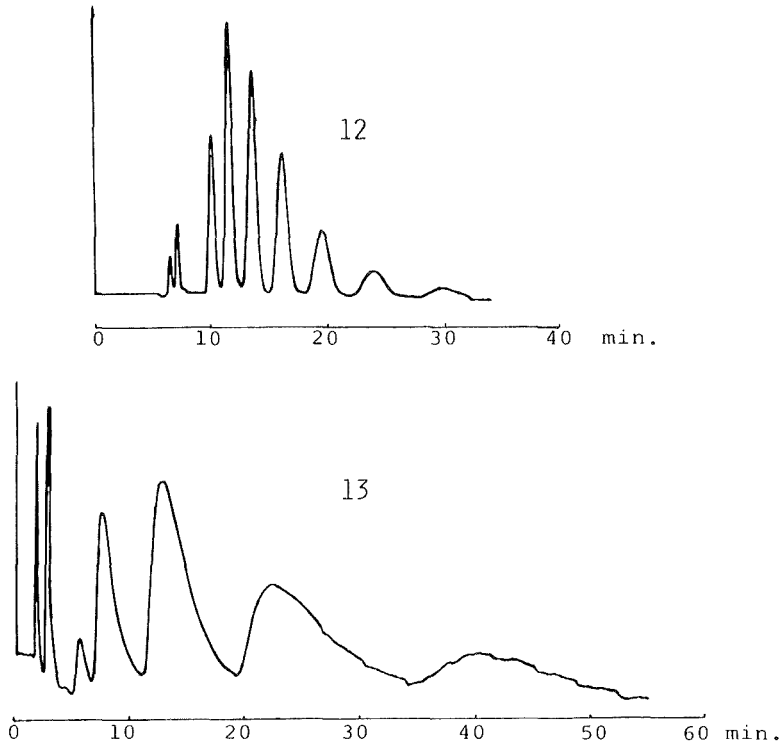


Figure 1 - continued

the selectivity of reversed-phase LC is the degree of aqueous character of the mobile phase; solute distribution has been modeled by invoking a solvophobic interaction (24-26).

In our previous paper (27), it was reported that the interactions between solute and stationary phases such as C2, C8 and C18 are similar to each other with mixtures of acetonitrile and water as mobile phases. However, it seems, in this instance, that some differences are present in retention mechanism between the stationary phases of group-1 and -2.

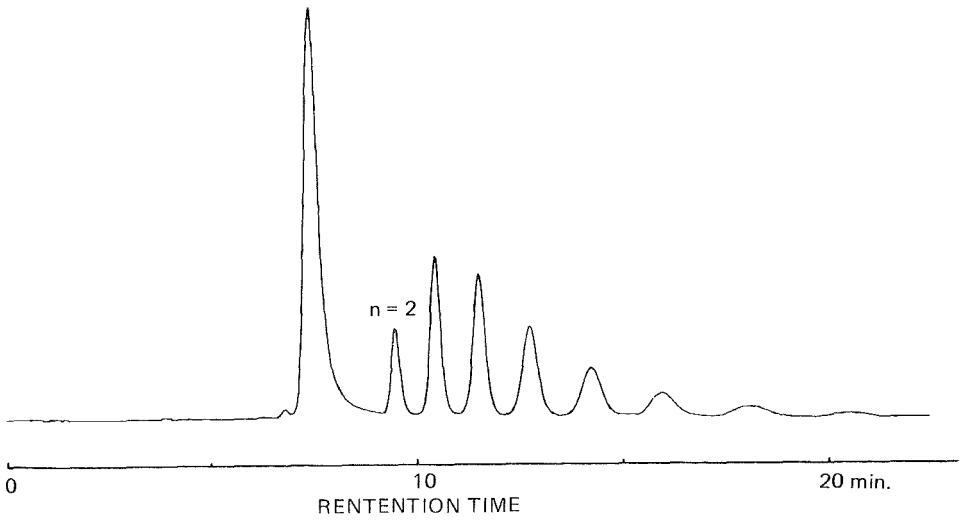


Figure 2 Separation of styrene oligomers with conventional HPLC
Chromatographic conditions:
Column; 4.6 mm i.d. x 25 cm, FineSIL C18
Mobile phase; methanol 100 %
Flow rate; 0.5 ml/min
Sample injection; 1 µl
Column temperature; 30 °C
Detection; UV 207 nm

Tanaka et.al. (28) have investigated the effect of the structure of the stationary phase on retention and selectivity in reversed-phase LC using chemically bonded stationary phases on silica gels. Their conclusions are as follows; in addition to the solvophobic interaction and the solvation of the solutes in the mobile phase, effects such as steric recognition and $\pi - \pi$ interaction between solute and the stationary phase were found to be important in determining retention in a reversed-phase system. Extended, longer carbon groups and large aromatic rings in the stationary phase contribute to the preferential retention of more planar solutes.

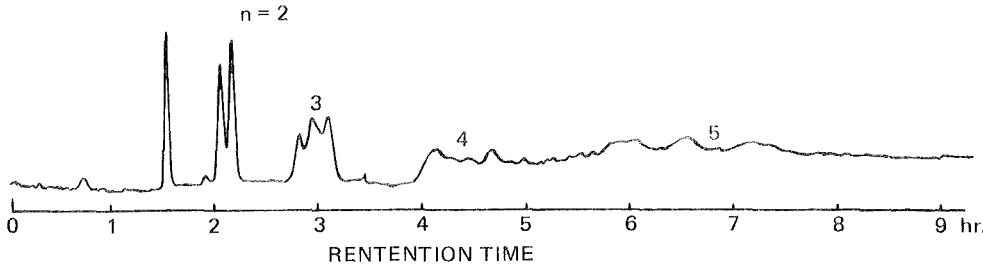


Figure 3 Separation of styrene oligomers with best optimized micro-HPLC
Chromatographic conditions:
Column; connected two columns of No. 8 and 10.
Mobile phase; acetonitrile 100 %
Flow rate; 2 ul/min
Sample injection; 0.1 µl
Column temperature; 25°C
Detection; UV 207 nm

The results we report exhibited a similar mechanism to that described above. The stationary phases with the longer carbon-chain (C18) and the aromatic rings (copolymer) can retain styrene oligomers more than those with shorter carbon chains. This means that there exists an effect of molecular shape and size to their retention.

The differences of separation ability between the stationary phases with the same carbon chain length might be caused from their other characteristics, e.g., internal porosity, specific surface area, treatments for chemical bonding, etc., which have not been determined for the materials investigated here.

Effect of particle size: The effect of particle size is seen in the results. Calculated k' values (capacity factor) for each oligomer are tabulated in Table 2 for Column No. 9, 10 and 11. The k' values are almost consistent each

Table 2

Logarithm of Capacity Factor (k') for Styrene Oligomers.

Column No.	Particle Diameter	log k' n=2	n=3	n=4	n=5	n=6
9	3 um	-0.198	-0.012	0.158	0.328	0.468
10	5	-0.215	-0.015	0.156	0.314	0.464
11	10	-0.194	-0.004	0.164	0.318	0.467

other for these columns. It is apparent that the decrease of particle diameter improves the resolution but does not change the retention. Smaller particles need more analysis time in some instances, although it is realized that the analysis time generally decreases in proportion to the square of particle diameter (29).

Performance of micro-HPLC: Micro-HPLC promises great improvements in selecting chromatographic conditions due to its advantageous features: smaller flow rates (in the order of microliters per minute), smaller required sample quantity (less than one microliter), and easier procedures for preparation of columns.

In order to evaluate the potential of micro-HPLC, a comparison of the chromatograms obtained with a conventional HPLC column and the micro-HPLC columns was examined.

It is clear, from No. 7 in Figure 1-A and Figure 2, that the resolution of the conventional column is not higher than those of the micro columns, and that there are no disadvantages in performance caused by column miniaturization.

It is concluded that micro-HPLC offers high potential for separations in polymer chemistry because of the above

features. Also, this technique drastically reduces system and running costs.

Figure 3 shows an optimized chromatogram for styrene oligomers by micro-HPLC in which conformational isomers of each oligomer can be resolved. Recent developments in micro-column technology in capillary LC (30-32) will bring higher resolution separations of isomers with shorter analysis times.

The authors would like to show their sincere thanks to Dr. Y.Hirata and C.Fujimoto for their encouragement.

REFERENCES

1) D.Ishii, K.Asai, K.Hibi, T.Jonokuchi and M.Nagaya; J.Chromatogr., 144, 157 (1977).

2) R.P.W.Scott and P.Kucera; J.Chromatogr., 125, 251 (1976).

3) T.Tsuda and M.Novotny; Anal.Chem., 50, 271 (1978).

4) M.Novotny; Anal.Chem., 53, 1294A (1981).

5) K.Jinno and Y.Hirata; J.High Resolu.Chromatogr.Chromatogr. Commun., 5, 85 (1982).

6) J.L.DiCesare, N.W.Dong and L.S.Ettre; Chromatographia, 14, 257 (1981).

7) J.L.DiCesare, N.W.Dong and J.G.Atwood; J.Chromatogr., 217, 369 (1981).

8) M.W.Dong and J.L.DeCesare; J.Chromatogr.Sci., 20, 49 (1982).

9) H.Hemetsberger, W.Maasfeld and H.Riken; Chromatographia, 9, 303 (1976).

10) J.H.Knox and A.Pryde; J.Chromatogr., 112, 171 (1975).

11) C.H.Lochmüller and D.W.Wilder; J.Chromatogr.Sci., 17, 574 (1979).

12) R.E.Majors and M.J.Hopper; J.Chromatogr.Sci., 12, 767 (1974).

13) R.K.Gilpin, J.A.Korpi and G.A.Janicki; Anal.Chem., 47, 1498 (1975).

14) K.Karch, I.Sebastian and I.Halasz; J.Chromatogr., 122, 3 (1976).

15) K.K.Unger, N.Becker and P.Roumeliotis; J.Chromatogr., 125, 115 (1976).

16) E.J.Kikta, Jr. and E.Grushka; Anal.Chem., 48, 1098 (1976).

17) H.Hemetsberger, M.Kellerman and H.Riken; Chromatographia, 10, 726 (1977).

18) P.Roumeliotis and K.K.Unger; J.Chromatogr., 149, 211 (1981).

19) G.E.Berendsen and L.de Galan; J.Liq.Chromatogr., 1, 561 (1978).

20) M.C.Hennion, C.Picard and M.Cande; J.Chromatogr., 166, 21 (1978).

21) N.Tanaka, K.Sakaguchi and M.Araki; J.Chromatogr., 199, 327 (1980).

22) C.H.Lochmüller, H.H.Hangac and D.W.Wilder; J.Chromatogr. Sci., 19, 130 (1981).

23) K.Jinno, T.Ohshima and Y.Hirata; J.High Resolu.Chromatogr. Chromatogr.Commun., 5, 621 (1982).

24) B.Karger, J.R.Grant, A.Hartkopf and P.Weiner; J.Chromatogr., 128, 65 (1976).

25) C.Horvath and W.Melander; J.Chromatogr.Sci., 15, 393 (1977).

26) C.Horvath, W.Melander and I.Molnar; J.Chromatogr., 125, 129 (1976).

27) K.Jinno; Chromatographia, 15, 667 (1982).

28) N.Tanaka, Y.Tokuda, K.Iwakuchi and M.Araki; J.Chromatogr., 239, 761 (1982).

29) G.Guiochon; "High Performance Liquid Chromatography." C.Horvath, Ed., Academic Press, New York, Vol.2, 1(1980).

30) Y.Hirata and K.Jinno; J.High Resolu.Chromatogr.Chromatogr.Commun., in press.

31) F.J.Yang; J.Chromatogr.Sci., 20, 241 (1982).

32) D.Ishii and T.Takeuchi; J.Chromatogr.Sci., 18, 462 (1980).

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(9), 1645-1671 (1983)

THE INFLUENCE OF PEPTIDE STRUCTURE ON THE RETENTION OF SMALL CHAIN PEPTIDES ON REVERSE STATIONARY PHASES

Donald J. Pietrzyk, Ronald L. Smith,
and William R. Cahill, Jr.
Department of Chemistry
The University of Iowa
Iowa City, Iowa 52242

ABSTRACT

Several characteristic structural features of peptides were considered in a study on the retention of small chain peptides on C_8 and polystyrene-divinylbenzene reverse stationary phases. These include the effects of subunit nonpolar, polar, acidic, and basic side chains, the influence of the location (terminal or interior) of a nonpolar subunit in the peptide chain, effects of two or more nonpolar subunits and their location (terminal or interior) in the peptide chain, and the effect of two chiral centers and their location (terminal and/or interior) in the peptide chain. LC data, which were collected in an acidic, zwitterion pH, and basic mobile phase where possible, indicated that location of the side chains in the peptide relative to the terminal charge sites is also an important factor in determining peptide retention. Peptides with side chains containing acidic or basic groups were studied as a function of mobile phase pH. Whether these groups are ionized or not and their location relative to the terminal charged sites strongly influences peptide retention.

INTRODUCTION

Amino acids (AA) and peptides in the past have often been separated by ion exchanger procedures. Recently, modern reversed

 0148-3919/83/0609-1645$3.50/0

phase liquid chromatography (RPLC), which offers improved versatility, efficiency, and shorter analysis times has been widely used for these separations. Although most investigations have utilized alkyl-modified silica as the stationary phase (1-4), separations by RPLC are also possible on polystyrene-divinylbenzene (PSDV) copolymers which act as nonpolar adsorbents (5,6). With both stationary phases key mobile phase variables are pH, ionic strength, water-organic solvent ratio, and the addition of counterions. Since the PSDV stationary phases are stable throughout the entire pH range they permit a basic mobile phase condition to be used where the AA and peptides can be separated in their anionic form. In contrast alkyl-modified silicas are stable only up to pH 8.

Similarities exist between the two types of stationary phases when applied to AA and peptide separations. For example, manipulation of the mobile phase variables to improve resolution or to alter selectivity is similar; that is, retention decreases as organic modifier concentration increases, eluting strength is in the order CH_3CN > EtOH > MeOH, and the analyte retention is lowest when the mobile phase is at the zwitterion pH. For both stationary phases retention from a buffered mobile phase free of hydrophobic counterions is dominated by interactions between hydrophobic centers within the AA and peptides and the hydrophobic surface of the stationary phase (1-6). If hydrophobic counterions are present, then a double layer type interaction, which is strongly influenced by an ion exchange selectivity prevails under defined experimental conditions (7).

Control of the elutropic properties of the mobile phase and the hydrophobic nature of the stationary phase focuses attention on how intrinsic hydrophobicities exhibited by AA and peptides influences their retention. Success in this goal has already been achieved. For example, AA retention on alkyl-modified silica (2) and PSDV (5,6) can be correlated to carbon number in the AA side chain or to hydrophobicity (8) for the side chain. Retention also correlates to AA partition coefficients in octanol-water distribution (2). For dipeptides, where one AA subunit varies, retention can be correlated to the hydrophobicity of the side chain for the variable AA unit (2,3,5,6,9) while for peptides with repeating AA units, log retention increases linearly as the repeating AA units increase (2,5,6). The effects of mobile phase pH on dipeptide retention appears to be systematic (5,6,10) while di-and tripeptide diastereomer separations follow a structure related elution order (4).

Retention coefficients for individual AA subunits have been estimated via numerical analysis procedures from retention data obtained for a series of long chain peptides of known structure using a known, fixed mobile phase gradient (11-15). These constants are then used to predict the retention time for other peptides providing the same gradient is used and the AA composition of the peptide is known. In general, the correlation between actual and predicted retention times have been favorable (11-15), although there is some disagreement about the coefficient values for certain subunits (15).

The goal of this investigation is to evaluate how individual AA subunits within the peptide chain influence retention of the peptide. Since the AA side chain can be polar, nonpolar, acidic, or basic its influence on retention can be large. The approach taken was to study the retention of a series of peptides whose structures were systematically modified with respect to chirality, type, and location of individual AA subunits (terminal or peptide interior) in the peptide chain. Both alkyl-modified silica and PSDV reverse stationary phases were used with acidic, zwitterion pH, and basic (only with the PSDV stationary phase) mobile phases where the solvent is a water-organic modifier mixture. Since the terminal and side chain acidic or basic groups can be ionized, depending on mobile phase pH, the combined effect of these charge sites and the peptide structure can be evaluated.

EXPERIMENTAL

Chemicals and Instrumentation

Amino acids and many peptides were obtained from Sigma Chemical Co., Chemalog, Vega Biochemicals, and Research Plus. Several peptides were synthesized by reaction of a specific AA or peptide of known structure with (tert-butoxycarbonyl)-L-amino acid N-hydroxysuccinimide ester (BOC-AA). This reaction adds the AA from the BOC-AA to the N-terminus of the starting AA or peptide through the formation of a peptide bond (16,17). Organic solvents and water were LC quality while all inorganic salts were analytical reagent grade.

The PRP-1 (4.1 mm x 150 mm) column, which is a spherical, 10 μm, PSDV copolymer of large surface area and porosity, was obtained from Hamilton Co. The alkyl-modified silica columns were 3.2 and 4.6 mm x 250 mm, 10 μm, Lichrosorb C_8 (Altex and E/M) and 4.6 mm x 250 mm, 10 μm μ-Bondapak C_{18} (Waters). Both Altex Model 332 and Waters Model 202 LC instruments equipped with fixed wavelength detectors or with a Tracor Model 970 or Spectra Physics 770 variable wavelength detector were used.

Procedures

Sample solutions (about 1 mg/ml) were prepared by dissolving mg quantities of the AA or peptide in H_2O, EtOH, or their combination in 6 ml Hypovials fitted with Hycar Septa (Pierce Chemical). Samples were refrigerated when not in use. Operating conditions generally involved sample aliquots of 10 μl, 1 ml/min flow rate, inlet pressures of about 500 to 1400 psi depending on column and eluting condition, detection at either 254 or 208 nm, and controlled temperature at 25°C. Mixed solvents are expressed as per cent by volume. The mobile phase pH was maintained with HCl, NaOH, and phosphate buffer while ionic strength was controlled with added NaCl. Details of these basic LC procedures and calculation of column void volume and capacity factors are provided elsewhere (4-6,7).

RESULTS AND DISCUSSION

Amino acids and peptides change from cations (pH < 2), to zwitterions, and finally to anions (pH > 10) in solution as the pH is increased from an acidic to a basic condition. The charge

form of the AA or peptide strongly influences their retention on reverse stationary phases; in general, a well-defined retention minimum is found when the mobile phase is at the zwitterion pH (5,6,10). If the side chains contain acidic or basic groups, ionization at these sites will contribute to the overall charge on the AA or peptide depending on the pH and ionization constants of the sites. When the mobile phase pH is adjusted so that these groups are also ionized retention is greatly reduced. If all contributing ionization sites are considered the influence of pH on retention can be quantitatively described and predicted (5,10).

Initial LC experiments focused on the retention of AA and a series of dipeptides that differed in only one of the AA subunits. These data were consistent with previous results (5,6) in that retention was found to increase as the hydrophobic property of the side chain increased. The effect was the largest from an acidic or basic mobile phase where the charge resides at either the AA or dipeptide terminal $-NH_2$ or $-CO_2H$ group, respectively, and the smallest from a mobile phase at the zwitterion pH where both groups are charged. These trends were observed for both the PSDV and alkyl-modified silica stationary phases. It should be noted that in these and subsequent studies, LC experiments on the latter stationary phase where restricted to a mobile phase pH of 2 to 8. Thus, the full effect of the presence of anionic sites within the AA and peptides on their retention is only realized with the PSDV stationary phase.

If the side chains also contain acidic or basic sites retention is significantly reduced at mobile phase pH conditions

TABLE I

Retention of Acidic and Basic Dipeptides on PRP-1 as a Function of Mobile Phase pH

	Capacity Factor, k'			
	pH			
Dipeptide	2.0	5.8	8.6	11.6
L-Tyr-L-Tyr	29.3	6.35	35	0.04
L-Tyr-L-Glu	3.38	0.20	0.48	0
L-Glu-L-Tyr	7.88	0.31	0.24	0
L-Tyr-L-Val	15.4	2.03	19.3	0.48
L-Val-L-Tyr	15.4	1.65	16.5	0.43
L-Tyr-L-Arg	1.67	0.91	6.18	0.31
L-Arg-L-Tyr	4.31	0.82	2.54	0.19

A 10 μm, 150 x 4.1 mm, PRP-1 column using a 1:99 CH_3CN:H_2O, phosphate buffer, μ=0.10M mobile phase at 1.0 ml/min.

where these sites are also ionized. Data illustrating this are shown in Table I (only results for a PSDV stationary phase are shown) where retention of dipeptides with two acidic side chains (Tyr-Glu and Tyr-Tyr), one acidic and one hydrophobic (Tyr-Val), and one acidic and one basic (Tyr-Arg) can be compared.

The first case provides the greatest change in retention since the dipeptides in basic solution (pH = 11.6) are multivalent anions; that is, both side chain acidic sites as well as the terminal carboxyl groups are ionized extensively. In acidic

solution the hydrophobic effect of the side chain, where the order is Tyr > Glu, increases retention since only the terminal amine group is ionized. Furthermore, the hydrophobic effect of Tyr is greater when it is in position 2 in the dipeptide or when it is furthest from the terminal charged site. Introducing Val into a Tyr dipeptide eliminates one potential ionization site and thus, the retention increases in a basic mobile phase due to the hydrophobic influence of the Val side chain. In an acidic mobile phase the hydrophobicity of the Val side chain and its effect on retention are intermediate relative to the Tyr and Glu side chains.

For the dipeptides in Table I that contain both an acidic and a basic side chain a suppressed retention due to additional ionization at these sites is seen in both an acidic and basic (pH = 11.6) mobile phase. The higher retention of L-Arg-L-Tyr over L-Tyr-L-Arg from an acidic mobile phase and the opposite for a basic mobile phase is consistent with the fact that the hydrophobic side chain is the furthest from the charged site in this order.

The Glu acidic side chain (pK_a = 4.36) ionizes at a lower pH than the Tyr side chain (pK_a = 10.47). This difference has a large effect on retention at mobile phase pHs between the zwitterion pH and a very basic pH. In this region (see data at pH = 8.6 in Table I) the Glu side chain is readily ionized and retention is suppressed. In contrast a higher mobile phase pH is required to bring about appreciable ionization of the Tyr side chain. Thus, retention of the Tyr containing dipeptides increases as the mobile phase pH is increased from the zwitterion pH to a pH where ionization is

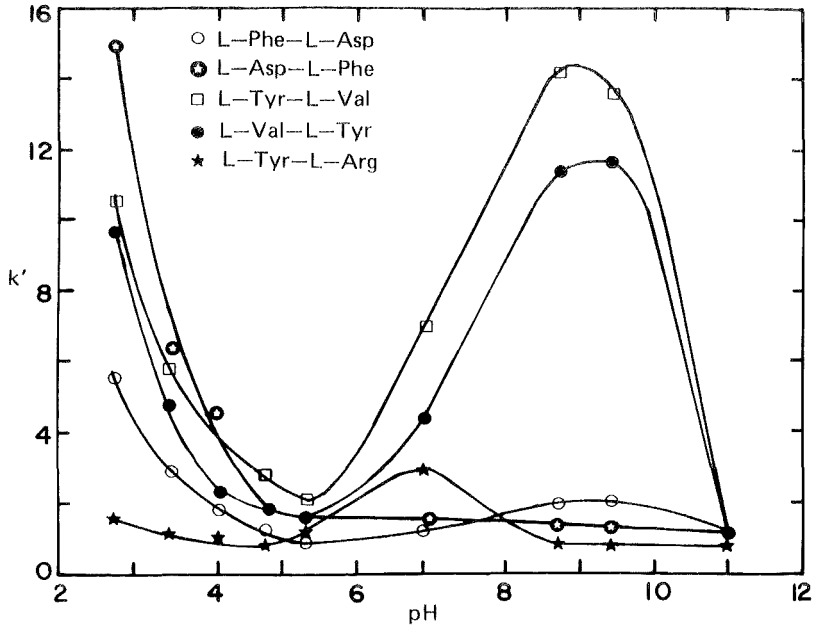


FIGURE 1

Retention of Acidic and Basic Dipeptides on PRP-1 as a Function of pH

A 4.1 x 150 mm, 10 μm, PRP-1 column and a 2:98 $CH_3CN:H_2O$, 1.0 x 10^{-2}M buffer (phosphate), μ = 0.10M (NaCl) mobile phase at a flow rate of 1.10 ml/min.

appreciable. At this point retention decreases sharply. Several examples illustrating this sharp change in retention with mobile phase pH are shown in Figure 1. These data clearly point out the need to carefully control mobile phase pH in order to maintain the expected selectivity when separating peptides with acidic and basic side chains. They also indicate that pH is a powerful tool for changing selectivity when separating peptides of this type from each other or from those without acidic or basic side chains.

No attempt was made to investigate retention of long chain peptides containing several acidic or basic side chains under pH conditions where these groups are and are not ionized. However, the data in Table I (see also Table VI and VII), as well as other data not reported here, indicate that retention would sharply decrease as the number of ionized sites increase, while under mobile phase pH conditions where the sites are not ionized, retention would increase according to the number and hydrophobicity of the side chains present in the peptide.

In order to focus on the effect of individual side chains in longer chain peptides, peptides were obtained or synthesized that have structures which systematically change with respect to side chain hydrophobicity, charge site, and/or chirality. Subsequently, retention data for these peptides were determined on the PSDV and alkyl-modified silica stationary phases as a function of mobile phase pH. Since the former stationary phase permits a wider mobile phase pH only data for this stationary phase are reported here (18).

The effect of a repeating hydrophobic side chain on retention from an acidic, zwitterion pH, and basic mobile phase is shown in Figure 2 where retention of a series of $(L\text{-}Ala)_x$ peptides is correlated to the peptides hydrophobicity. This was determined by a summation of the assigned hydrophobicity (0.53) for each Ala subunit according to Rekker (8). Similar results were obtained on the C_8 column at pH conditions where column stability is maintained. As hydrophobicity increases, retention increases

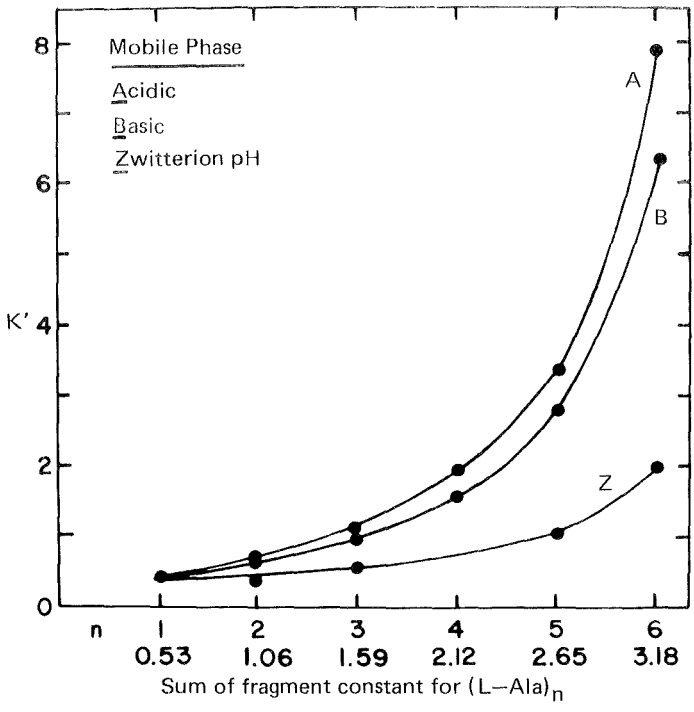


FIGURE 2

Retention of a Series of $(L\text{-}Ala)_x$ Peptides as a Function of Hydrophobicity

Conditions are the same as Figure 1 except the solvent is 100% H_2O and pH = 1.75, 5.20, and 11.00.

systematically whether the analyte is an anion, a cation, or a zwitterion. However, the rate of change is the lowest for the latter condition or where both the terminal $-NH_2$ and $-CO_2H$ groups are ionized.

Table II lists retention data as a function of mobile phase pH for a series of $L\text{-}Phe\text{-}(Gly)_x$ peptides where the Phe unit is systematically shifted through the peptide. Since the charge on

TABLE II

Retention of L-Phe-$(Gly)_x$ Peptides on PRP-1 as a Function of Mobile Phase pH

	Capacity Factor, k'		
	pH		
Peptide	2.1	5.9	10.0
L-Phe	2.32	0.98	1.58
Gly-L-Phe	6.44	1.05	2.34
L-Phe-Gly	3.00	1.40	3.29
L-Phe-Gly-Gly	2.59	1.33	4.13
Gly-Gly-L-Phe	8.07	1.10	1.78
L-Phe-Gly-Gly-Gly-Gly	2.40	1.63	5.27
Gly-L-Phe-Gly-Gly-Gly	5.03	2.81	5.95
Gly-Gly-L-Phe-Gly-Gly	6.50	3.13	4.18
Gly-Gly-Gly-L-Phe-Gly	6.75	2.60	3.60
Gly-Gly-Gly-Gly-L-Phe	9.27	1.42	1.87

Column-mobile phase conditions are the same as Table I except the mobile phase solvent is 5:95 $CH_3CN:H_2O$.

the peptide can be shifted from one end (terminal $-NH_2$ group) in the peptide to the other (terminal $-CO_2H$ group) or at both ends (at the zwitterion pH), the effect of the position of the hydrophobic side chain relative to the charged site can be ascertained. As the chain length of the L-Phe-$(Gly)_x$ peptides in Table II increases, the trend becomes more obvious and the effect is clearly

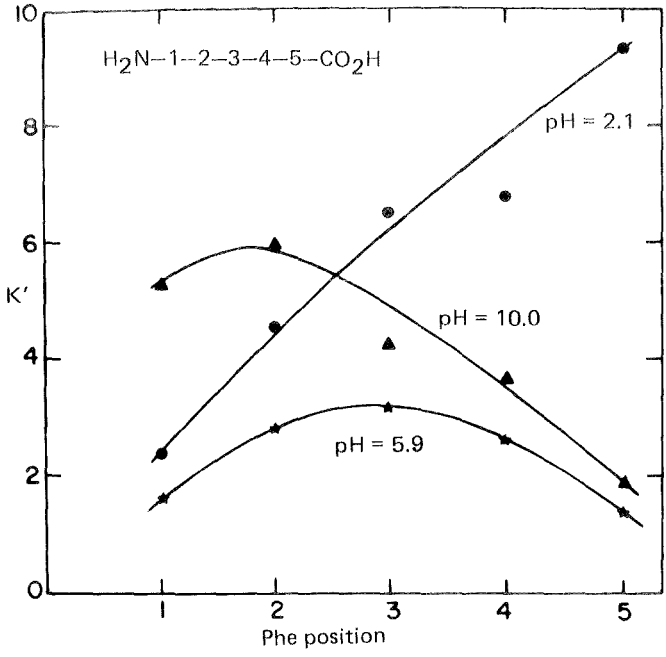


FIGURE 3

Effect of the Location of the Phe Subunit on the Retention of a Series of L-Phe-$(Gly)_4$ Peptides on PRP-1 as a Function of pH

Column conditions are given in Table II.

illustrated in Figure 3 where retention of the 5-unit peptides are plotted for the three mobile phase pH conditions as a function of the position of the L-Phe in the peptide. Whether the mobile phase is basic (terminal $-CO_2^-$ group) or acidic (terminal $-NH_3^+$ group), retention increases as the hydrophobic side chain is moved away from the charge site. In the former case this is when the side chain is at subunit 1 while in the latter this is at subunit 5. For a zwitterion pH, where both terminal groups are charged, the

highest retention is favored when the side chain is at subunit 3 or midway to the two charge centers.

The effects of two and three hydrophobic centers are shown in Table III and Table IV, respectively, where retention data for a series of $(L\text{-Phe})_2\text{-}(Gly)_x$ and $(L\text{-Phe})_3\text{-}(Gly)_x$ peptides are shown. Additional hydrophobic centers increase retention and consequently the mobile phase eluting power was increased (% CH_3CN increased) in order to reduce retention times. Although not all peptide combinations were available, the general trends in retention, even though more complex, are consistent with those observed for peptides with only one hydrophobic center (Table II). For example, the highest retention is favored by PPG and PPGG in basic solution where both hydrophobic centers are as far as possible from the $-CO_2^-$ terminal charged site and lowest in acidic solution where they are as close as possible to the $-NH_3^+$ terminal charged site. Similarly,at the zwitterion pH the highest retention is favored by those peptides where the hydrophobic centers are in the interior positions (GPPG, GPPGG) and lowest retention when these centers are at the terminal ends (for example, compare PGP to PGGP).

The correlation between peptide retention and a systematic peptide structure change is also illustrated in Table V. Whether Gly (set 2 peptides),Phe (set 3 peptides),or Phe-Phe (set 4 peptides) is added to the N-terminus of set 1 peptides, the retention order is essentially the same as found in their absence (compare to set 1 peptides). Other similar trends are apparent when comparing the peptides in Table II to IV.

TABLE III

Retention of $(L\text{-Phe})_2(Gly)_x$ Peptides on PRP-1 as a Function of Mobile Phase pH

	Capacity Factor, k'		
	pH		
Peptide	2.2	6.4	10.05
L-Phe-L-Phe	3.39	1.46	3.95
L-Phe-L-Phe-Gly	1.80	1.56	3.99
Gly-L-Phe-L-Phe	5.49	1.68	2.02
L-Phe-Gly-L-Phe	4.86	1.32	2.11
L-Phe-Gly-L-Phe-Gly	3.09	1.69	2.77
L-Phe-Gly-Gly-L-Phe	3.24	1.08	2.79
Gly-L-Phe-Gly-D,L-Phe[a]	5.02 5.53	1.64	1.90
Gly-L-Phe-L-Phe-Gly	3.69	2.84	2.52
L-Phe-L-Phe-Gly-Gly	1.45	1.57	3.94
Gly-L-Phe-Gly-L-Phe-Gly	3.64	1.87	2.31
Gly-L-Phe-L-Phe-Gly-Gly	3.20	2.34	2.40
Gly-L-Phe-Gly-Gly-L-Phe	4.36	1.31	1.39

Column-mobile phase conditions are the same as Table I except that the mobile phase solvent is 1:4 $CH_3CN:H_2O$.

a. Data are for LL and LD, respectively, where resolved.

TABLE IV

Retention of $(L\text{-Phe})_3(Gly)_x$ Peptides on PRP-1 as a Function of Mobile Phase pH

	Capacity Factor, k'		
	pH		
	2.4	6.4	10.2
L-Phe-L-Phe	0.765	0.333	0.602
L-Phe-L-Phe-L-Phe	2.96	1.63	2.82
L-Phe-L-Phe-L-Phe-Gly	2.91	1.50	2.72
L-Phe-L-Phe-Gly-D,L-Phe	2.24 2.69	1.31 1.82	2.14
L-Phe-L-Phe-L-Phe-Gly-Gly	1.24	1.28	2.43
L-Phe-L-Phe-Gly-Gly-L-Phe	1.59	1.00	1.61
L-Phe-L-Phe-Gly-L-Phe-Gly	2.02	1.76	2.37

Column-mobile phase conditions are the same as Table I except the mobile phase solvent is 3:7 $CH_3CN:H_2O$. a. Data are for LLL and LLD, respectively, where resolved.

Several $(L\text{-Leu})_y\text{-}(Gly)_x$ peptides were also studied (17). Although this series did not cover as great a range as with the $(L\text{-Phe})_y\text{-}(Gly)_x$ peptides, the available data indicated similar retention trends that correlated to the number and location of the Leu hydrophobic side chains within the peptide chain.

Table VI lists retention data for several $(L\text{-Tyr})\text{-}(Gly)_x$ peptides as a function of pH. In this series the single Tyr subunit introduces an acidic side chain which provides a charged site in addition to the terminal $-CO_2^-$ group providing the mobile phase

TABLE V

Correlation of Peptide Structure and Retention on PRP-1 for $(L\text{-Phe})_y(Gly)_x$ Peptides

Set	Acidic Mobile Phase
1	PGG < GGP
2	PPGG < PGGP ≃ PGPG
3	GPPGG < GPGPG < GPGGP
4	PPPGG < PPGGP < PPGPG
	Zwitterion pH Mobile Phase
1	GGP < PGG
2	PGGP < PGPG ≃ PPGG
3	GPGGP < GPGPG < GPPGG
4	PPGGP < PPPGG < PPGPG
	Basic Mobile Phase
1	GGP < PGG
2	PGGP ≃ PGPG < PPGG
3	GPGGP < GPGPG ≃ GPPGG
4	PPGGP < PPGPG < PPPGG

See Tables I to IV; P=L-Phe, G=Gly.

TABLE VI

Retention of $(L\text{-Tyr})(Gly)_x$ Peptides on PRP-1 as a Function of Mobile Phase pH

	Capacity Factor, k'		
	pH		
Peptide	2.0	5.9	11.1
L-Tyr	4.72	1.14	0
L-Tyr-Gly	5.60	1.94	0.10
Gly-L-Tyr	11.0	1.10	0
Gly-Gly-L-Tyr	15.6	1.46	0
Gly-L-Tyr-Gly	10.5	2.68	0.18
L-Tyr-Gly-Gly	5.34	2.28	0.16
Gly-Gly-Gly-L-Tyr	18.8	1.99	0.11
Gly-Gly-L-Tyr-Gly	12.8	3.83	0.13
Gly-L-Tyr-Gly-Gly	10.9	4.74	0.31
L-Tyr-Gly-Gly-Gly	5.96	2.97	0.24

Column-mobile phase conditions are the same as Table I except that the mobile phase solvent is 100% H_2O.

pH is high enough. Thus, in a strongly basic mobile phase retention is sharply reduced due to the additional charge. At the zwitterion pH and in an acidic mobile phase the Tyr subunit imparts its own hydrophobic contribution to the retention; as expected retention is low from the former and high from the latter mobile phase. Several other retention trends are apparent and are

consistent with those already indicated in the previous discussion when the influence of the additional charged sites are accounted for. For example: 1) In acid solution retention increases as the Tyr subunit is shifted further away from the charged N-terminus: 2) At the zwitterion pH the highest retention is favored when the Tyr subunit is moved to the chain interior. Also, higher retention occurs when the Tyr subunit is at the N-terminus rather than at the terminal $-CO_2H$ group. 3) In basic solution, even though retention is barely detectable, the data indicate that higher retention is favored when the Tyr subunit is located further away from the charged terminal $-CO_2H$ group. 4) Although not shown in Table VI,retention passes through a maximum between the zwitterion and strongly basic pH which correlates to the stepwise ionization of the terminal $-CO_2H$ group and side chain acidic site (see Figure 2) (5,10).

Table VII lists retention data for several $Trp(Gly)_xTyr$ peptides as a function of pH. The low retention at a basic pH and the high retention at a pH intermediate to the zwitterion and very basic pH is consistent with the ionization of the acidic side chain of the Tyr subunit. In basic solution retention increases as the number of Gly units increases but at the intermediate pH retention decreases. Retention is low at the zwitterion pH and high in acidic solution due to the hydrophobic contribution of Trp and Tyr. Unlike the $(Ala)_x$ series in Figure 2, retention passes through a maximum as the number of Gly units increases. The reason for this is not readily apparent since retention (see Table VI) increases in the order G-G-G-Tyr > G-G-Tyr > G-Tyr and the addition of the Trp subunit to the N-terminus of these chains

TABLE VII

Retention of L-Trp$(Gly)_x$ L-Tyr Peptides on PRP-1 as a Function of Mobile Phase pH

	Capacity Factor, k'			
	pH			
Peptide	2.0	5.9	9.5	11.1
L-Tyr	0.6	0.2	0.2	0
L-Trp	5.30	2.12	1.85	1.61
L-Trp-L-Tyr	28.1	7.43	15.9	1.31
L-Trp-Gly-L-Tyr	42.8	9.48	13.0	1.38
L-Trp-$(Gly)_2$-L-Tyr	34.8	9.92	11.6	1.41
L-Trp-$(Gly)_3$-L-Tyr	29.6	7.29	11.0	1.56
L-Trp-$(Gly)_4$-L-Tyr	27.1	7.10	10.8	1.77
L-Tyr-$(Gly)_2$-L-Trp	4.62	4.43	4.27	

Column-mobile phase conditions are the same as Table I except the mobile phase solvent is 1:9 CH_3CN:H_2O.

results in the retention maximum at Trp-G-G-Tyr. The effect of reversing Trp and Tyr is seen by comparing data for Tyr-G-G-Trp to Trp-G-G-Tyr in Table VII. Since the charged sites in the former are at both ends in a basic mobile phase, retention is much lower. In an acidic and zwitterion pH mobile phase switching the Tyr and Trp to N-terminus and carboxyl-terminus, respectively, reduces retention significantly.

Separation of dipeptide diastereomers on a C_8 (4) and PSDV (6) stationary phase has been reported previously. For dipeptides

with hydrophobic and hydrophilic side chains the data indicate that the L-L and D-D enantiomers always coelute first followed by the coelution of the D-L and L-D enantiomers regardless of mobile phase pH. Table VIII lists retention data for a series of $(Leu)_2(Gly)_x$ peptides where the two chiral centers provided by the two Leu subunits occupy different positions in the peptide chain. The effect of the hydrophobic side chain provided by the Leu subunits is consistent with the trends already described. However, the nature and location of the chiral centers also strongly influence peptide retention. When the Leu chiral centers are adjacent the L-L and D-D enantiomers coelute first as in the case of dipeptide diastereomers (4,6). When the chiral centers are not adjacent a significant change in selectivity is observed. That is, the L-L and D-D enantiomers coelute either second or very close to or with the L-D and D-L enantiomers. This trend is independent of mobile phase pH. However, the selectivity for the separation of the diastereomers, in general, favors the order acidic > basic > zwitterion mobile phase pH (see Table VIII). The trends shown in Table VIII were also observed on the C_8 stationary phase (18) at mobile phase pH conditions where column stability is maintained.

The observed elution order for the diastereomeric dipeptides can be correlated to their preferred molecular conformation (4). Since the two hydrophobic side chain groups and the two terminal groups, which are charged depending on the mobile phase pH, are on the same side in the D-L and L-D dipeptide enantiomers and on opposite sides for the L-L and D-D enantiomers, the retention of

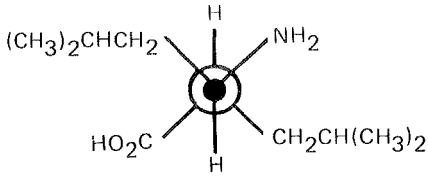
TABLE VIII

Retention of L-Leu-D,L-Leu(Gly)$_x$ Peptides on PRP-1 as a Function of Mobile Phase pH

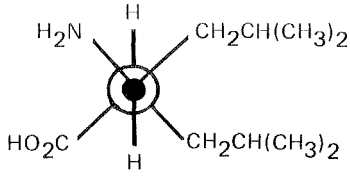
	Capacity Factor, k'					
	pH					
	2.20		6.25		10.1	
Peptide[a]	LL	LD	LL	LD	LL	LD
L-Leu-D,L-Leu	1.87	6.02	0.46	1.52	1.67	2.08
L-Leu-D,L-Leu-Gly	0.93	3.23	0.51	1.83	2.02	2.90
L-Leu-Gly-D,L-Leu	2.89	2.14	0.61	0.48	1.15	1.05
Gly-L-Leu-D,L-Leu	3.23	7.90	0.86	1.67	0.89	1.83
L-Leu-Gly-Gly-L,D-Leu	2.10	1.84	0.56	0.42	1.01	0.94
Gly-Gly-L-Leu-D,L-Leu	3.21	7.32	0.80	1.44	0.67	1.25
L-Leu-D,L-Leu-Gly-Gly	0.85	2.89	0.65	2.08	2.07	2.92
Gly-L-Leu-Gly-D,L-Leu	3.63	3.51	0.82	1.00	0.93	1.04
Gly-L-Leu-Gly-Gly-D,L-Leu	3.19	2.92	0.83	0.67	0.90	0.80
L-Leu-Gly-Gly-Gly-D,L-Leu	1.63	1.69	0.39	0.44	0.71	0.71
L-Leu-D,l-Asp	3.93	9.41	0	0	0	0.73
L-Leu-D,L-Arg	4.29	8.31	1.95	8.27	30	34

Column-mobile pahse conditions are the same as Table I except the mobile phase solvent is 15:85% CH_3CN:H_2O; for Leu-Asp and Leu-Arg it was 100% H_2O and the pH was 2.1, 5.9, and 9.5. a. Peak assignments were made from chromatographic data obtained for each enantiomer.

the former is greater due to the higher overall hydrophobic concentration of the side chains. This is illustrated below



L–L



L–D

where the conformation for the L-L and L-D dipeptide diastereomers for Leu-Leu are shown looking down the peptide chain. (The D-D and D-L forms would be the mirror images of the conformations shown.) This preferred conformation prevails when the two chiral centers are adjacent to each other in longer chain peptides. If the chiral centers are separated by a nonchiral subunit, such as Gly, the concentration of hydrophobic side chains in the preferred conformation changes. Construction of models using the criteria discussed elsewhere (4) indicates a slightly greater concentration of side chain hydrophobicity for separated chiral centers rather than for adjacent ones. For example, models of Leu-Gly-Leu indicate side chains on adjacent sides for L-G-L and D-G-D enantiomers and opposite sides for L-G-D and D-G-L enantiomers. However, the change is not large enough to produce a retention reversal but rather a similarity in retention (see Table VIII). If additional Gly subunits are inserted between the chiral centers the retention difference is reduced further. This is due to the increased separation of the

chiral centers and to the change in the preferred molecular conformation.

Several workers (19,20) have suggested that elution orders are reversed (D-L and L-D elute first followed by the coelution of the D-D and L-L enantiomers) for the separation of diastereomeric dipeptides containing basic side chains which are in the ionized form. The data in Table VIII do not agree with this and indicate that the elution order for diastereomers containing either ionized acidic or basic side chains is consistent with the retention order observed for other diastereomeric dipeptides (4,6).

The data reported here clearly indicate that modest structural differences in small chain peptides often lead to major changes in peptide selectivity, particularly when coupled with control of mobile phase pH. Often elution orders can be reversed by a suitable change in mobile phase pH. Consider the L-Phe-$(Gly)_4$ pentapeptide series in Figure 3 (see also Table II). In an acidic mobile phase retention with favorable resolution follows the order GGGGP > GGGPG > GGPGG > GPGGG > PGGGG while in a basic one the elution order is essentially reversed. If a zwitterion mobile phase pH is used GGPGG is retained the most, however, resolution of the mixture is poor compared to either an acidic or basic mobile phase.

Prediction of peptide elution time (11-15) is possible by the establishment of a coefficient characteristic of each AA as being either a terminal or an interior subunit. A mixed solvent

mobile phase gradient and a constant pH was usually used in establishing the AA coefficient (11-15). Since the alkyl-modified silica was the stationary phase, the constants were established only at an acidic pH (pH < 3) and/or near zwitterion pH conditions (pH 5 to 7). The data shown here clearly indicate, at least for small chain peptides, that the location of the subunit within the peptide interior significantly influences peptide retention. For example, GPGG, GGPGG, and GGGPG would be predicted to have identical elution times when using the AA coefficients. Figure 3 clearly indicates that this is not the case and the location of the Phe within the peptide significantly influences retention. Other examples demonstrating this are apparent from Tables II to VIII.

Several other factors, in addition to those indicated by Sasagawa et al. (15), may account for the variance in the reported AA coefficients. 1) The mixed solvent gradient usually covers a wide range of added organic modifier (often from 0 to 60%). This can influence the ionization of the weak acids and their salts and subsequently change the apparent pH of the mobile phase; the effect should be more noticeable in the acidic mobile phase. 2) The type of co-anion used for the buffer components can have a strong influence on the retention of the peptides, particularly if the peptides are retained in a cation (or anion) form via a double layer interaction (7,21-22). 3) The data reported here indicate that the retention is not independent of the location of a given AA subunit within the

peptide. However, it is important to note that for short chain peptides like those used in this study, peptide shape is probably not yet a significant factor. For example, approximately 4.3 AA subunits are required for one helix turn. In previous studies (11-15) long chain peptides were used and it is reasonable to suggest that peptide shape factors will tend to reduce and perhaps normalize individual contributions of AA side chains within the peptide.

ACKNOWLEDGEMENTS

This investigation was supported by Grant CHE 79-13203 awarded by The National Science Foundation. Initial results were reported at the Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Atlantic City, March, 1981, as paper number 366.

REFERENCES

1. Majors, R.E., Barth, H.G. and Lochmüller, C.H., Anal. Chem., 54, 323R (1982).
2. Molnar, J. and Horvath, C., J. Chromatogr., 142, 623 (1977).
3. Lundanes, E. and Greibrokk, T., J. Chromatogr., 149, 241 (1978).
4. Kroeff, E.P. and Pietrzyk, D.J., Anal. Chem., 50, 1375 (1978).
5. Kroeff, E.P. and Pietrzyk, Anal. Chem., 50, 502 (1978).
6. Iskandarani, Z. and Pietrzyk, D.J., Anal. Chem., 53, 489 (1981).
7. Iskandarani, Z. and Pietrzyk, D.J., Anal. Chem., 54, 1065 (1982).
8. Rekker, R.F., The Hydrophobic Fragmental Constant, pg. 301, Elsevier, Amsterdam, 1977.

9. Terabe, S., Konaka, R., and Inouye, K., J. Chromatogr., 172, 163 (1979).

10. Pietrzyk, D.J., Kroeff, E.P. and Rotsch, T.D., Anal. Chem., 50, 497 (1978).

11. O'Hare, M.J. and Nice, E.C., J. Chromatogr., 171, 209 (1979).

12. Meek, J.L., Proc. Natl. Acad. Sci., USA, 77, 1632 (1980).

13. Su, S.J., Grego, B., Niven, B., Hearn, M.T.W., J. Liq. Chromatogr., 4, 1745 (1981).

14. Wilson, K.J., Honegger, A., Stotzel, R.P., and Hughes, G.J., Biochem. J., 199, 31 (1981).

15. Sasagawa, T., Okuyama, T., and Teller, D.C., J. Chromatogr., 240, 329 (1982).

16. Bodanszki, M., Klausner, Y.S., and Ondetti, M.A., Peptide Synthesis, 2nd Ed., J. Wiley, New York, 1976.

17. Cahill, Jr., W.R., Kroeff, E.P., and Pietrzyk, D.J., J. Liq. Chromatogr., 3, 1319 (1980).

18. Cahill, Jr., W.R., Ph.D. Thesis, University of Iowa, Iowa City, Iowa, December, 1981.

19. Rivier, J. and Burgus, R., Biological/Biomedical Applications of Liquid Chromatography, Vol. I, G.L. Hawk, Ed., M. Dekker, New York, 1979, pg. 1.

20. Hearn, M.T.W., Advances in Chromatography, Vol. 20, J.C. Giddings, E. Grushka, J. Cazes, P.R. Brown, Eds., M. Dekker, New York, 1982, pg. 1.

21. Cantwell, F.F. and Puon, S., Anal. Chem., 51, 623 (1979).

22. Smith, R.L. and Pietrzyk, D.J., Unpublished results, see Abstract ANYL 29, 184th American Chemical Society Meeting, Sept. 1982, Kansas City, Missouri.

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(9), 1673-1691 (1983)

REVERSED-PHASE SEPARATION OF THE MAJOR DEOXYRIBONUCLEOSIDES AND THEIR MONONUCLEOTIDES USING TETRABUTYLAMMONIUM HETAERONS

John P. Caronia, Jonathan B. Crowther

and Richard A. Hartwick

Department of Chemistry

Rutgers University

New Brunswick, N.J. 08854

ABSTRACT

Simultaneous separation of the four major deoxyribonucleosides and their monophosphate nucleotides was achieved using tetrabutyl ammonium phosphate hetaerons with a reversed-phase (C8) packing material. Baseline resolution for all eight solutes was achieved within 48 minutes, using a 7.5% methanol mobile phase, 2.0 mM in TBA, buffered with 50 mM phosphate at pH 4.8. The effect of methanol and TBA concentrations upon the retention of neutral and anionic solutes was studied in detail. It was determined that changes in solute k' with increasing methanol could be explained by essentially independent phenomena. These are: 1) a decrease in the partition coefficient of the TBA cation with increasing organic concentration, resulting in lower surface charge densities, and 2) a decrease in the hydrophobic interactions of the solutes with the reversed-phase HPLC. The overall effect was a log-linear decrease in k' with increasing methanol concentration. An empirical equation was derived for the above model which was found to be helpful in determining the optimal separation conditions for the nucleosides and nucleotides.

 0148-3919/83/0609-1673$3.50/0

INTRODUCTION

The simultaneous separation of the nucleosides and their mononucleotides is of significance in many areas of nucleic acid research. Reversed-phase HPLC has become the method of choice for the separation of the nucleosides and their bases (1-2), while ion-exchange is most effective for nucleotide analyses (3). Some success has been achieved in the simultaneous separation of nucleosides and nucleotides using polystyrene based exchangers (4). Such resins however exhibit inherently poor mass transfer properties, resulting in slow analyses and poor efficiencies.

Ion-pairing, or hetaeric HPLC as coined by Horvath (5-7), is uniquely suited for the simultaneous separation of both neutral and ionic solutes (8-11). The apparent separation mechanism accounting for this unique selectivity is the adsorption of hetaerons to the stationary phase (12-15), resulting in a mixed-mode phase with significant ionic and hydrophobic properties. Such dynamic ion-exchange resins produce unique selectivities for separations such as the catecholamines and their metabolites (16,17) and would seem to be equally suited for the simultaneous analysis of the nucleosides and their mononucleotides.

Theory

Relatively few systematic studies into the effect of organic modifier concentration in ion-pair separations have been presented. Therefore, a systematic investigation into

the changes in k' occuring with changes in methanol and tetrabutylammonium phosphate concentrations on a reversed-phase (C8) system for the the deoxyribonucleosides and their nucleotide monophosphates was undertaken. It has been found that the surface concentration of the hetaeron decreases with increasing methanol (18-20). This results in a decreasing retention for ionic solutes (14). At the same time, a neutral molecule normally separated by hydrophobic mechanisms will also decrease retention, usually in a log-linear form (18-20). The net result is a decrease in retention of both neutral and ionic solutes with increasing methanol but for different mechanistic reasons.

If the k' contributions from ionic and hydrophobic forces are essentially independent, then the total k' can arise from two contributions;

$$k'_{OBS} = k'_{HYD} + k'_{IONIC} \qquad \text{Eq. 1}$$

It is well documented that the first part of equation 1 can be expressed as;

$$k'_{HYD} = k'_{o} e^{A \cdot C} \qquad \text{Eq. 2}$$

where A is the slope of the decrease in k' with increasing organic, and k' is the intercept.

The latter half of equation 1 represents the ionic contribution of a solute/hetaeron pair. This can be described in terms of hetaeron surface concentration, or

$$k'_{IONIC} = \Theta(C_s) \qquad \text{Eq. 3}$$

where Θ is some interaction index, and Cs is the stationary phase concentration of adsorbed hetaeron. This adsorbed surface concentration of hetaeron (or TBA in this experiment) can be accurately predicted by the Freundlich isotherm;

$$C_s = \alpha \cdot C_m^{\beta} \qquad \text{Eq. 4}$$

The basic Freundlich equation concerns the increase in Cs with Cm, the mobile phase concentration of TBA, at constant methanol. It was found experimentally in this study that the intercept of a ln-ln plot of the Freundlich isotherm decreased exponentially with methanol, but that the slope was constant within experimental error. Thus;

$$\alpha = \alpha_o \cdot e^{a \cdot C} \qquad \text{Eq. 5}$$

where a and α_o are the slope and intercept of a plot of the ln α versus the percent methanol.

The above equations are straightforward and are based upon normal adsorption behavior for small molecules on a solid surface. In sum they should describe the decrease in surface TBA concentration as a function of methanol concentration. A further empirical refinement was necessary. The slope of the k' versus Cs plot for each solute was not constant with increasing methanol, but rather decreased exponentially. Thus,

$$\Theta = \Theta_o e^{\nu \cdot C} \qquad \text{Eq. 6}$$

where Θ_o is the intercept of $\ln \Theta$ versus methanol percent, and ν is the slope. With this final refinement, the overall equation for k' as a function of methanol and TBA mobile phase concentration becomes;

$$k'_{OBS} = k'_o e^{A \cdot C} + \Theta_o e^{\nu \cdot C} (\alpha_o \cdot e^{a \cdot C} \cdot C_m^{\beta}) \qquad \text{Eq. 7}$$

Equation 7 describes the retention of a solute in terms of seven system constants. Three of these, α, β and a, are basic thermodynamic constants for a given pairing ion/stationary/mobile phase combination and need be determined only once. Of the remaining four, k_b' and A are constants for a given solute on a simple reversed-phase system and have been discussed in the literature previously (18-20). The remaining two, Θ_o and ν, are solute specific for a given pairing ion system.

Equation 7 was found to fit quite accurately the retention of the deoxyribonucleosides and their monophosphate nucleotides from 0 to 15% methanol concentrations. While equation 7 is empirical, it is nevertheless useful in optimizing and predicting the qualitative and quantitative effects of the two primary variables in the ion-pair system.

EXPERIMENTAL

Instrumentation

Chromatographic instrumentation consisted of a Waters M 6000 pump, (Waters Associates, Milford, MA) and either a

Waters U6K injector or a Rheodyne 7125 injection valve (Rheodyne Inc., Cotati, CA). The detectors used were either a Waters M 440 detector at 254 nm or a Kratos FS 770 variable wavelength detector (Kratos Analytical Instruments, Ramsey, NJ). Detection of TBA breakthrough curves was accomplished using a detection wavelength of 222 nm., while 254 nm was used for detection of the nucleosides and nucleotides. Data were recorded using either strip chart recorders, or a Hewlitt-Packard 3390A electronic integrator (Hewlitt-Packard, Avondale, PA). A Haskel DSTV 300 pneumatic intensifier pump was used for the packing of all columns.

Columns

Whatman Partisil 5-C8 packing material (Whatman Inc., Clifton, N.J.) was used throughout the ion-pair studies. Particle size for all experiments was 5 micron. The columns were packed in the laboratory using the slurry method. For the 10 cm columns, 1.2g of material was slurried in 10 ml of isopropyl alcohol (IPA), and was packed upwards at 5000 psi pressure for 10 minutes using acetone as the packing solvent. Without releasing the pressure, the columns were inverted, and packed downwards for another 5 minutes. The pressure was shut off with a high-pressure valve, and the apparatus allowed to equilibrate for 5-10 minutes before removal of the column. After assembly, columns were washed and equilibrated using 60% methanol/water mixtures and were tested for reproducibility of k' values and for efficiencies

using test solutes of interest. Commercially prepacked columns were also used, in which cases the accuracy of k' values were again confirmed.

Preparation of Reagents and Chemical Standards

The eluent used consisted of HPLC grade methanol (Burdick & Jackson,Muskegon MI), double distilled water, with a 0.05M buffer prepared from ACS grade KH_2PO_4 (Fisher Scientific Co., Fairlawn NJ) and NaOH (Fisher) at a pH of 4.80. The concentration of buffer remained constant for all the eluents. The pairing ion used was tetrabutyl ammonium phosphate (TBAP) (Eastman Kodak Co, Rochester NY). All eluents were filtered through 0.45 micron membrane filters, then degassed with helium before use. Small changes in total ionic strength occurred as the concentration of TBA was varied. Such changes were minimized through the use of relatively high ionic strengths. Working standards of the solutes were prepared at 0.025mg/ml.

Determination of Adsorption Isotherms

Frontal elution breakthrough curves were used in order to determine directly the adsorption of the pairing ion TBA on the stationary phase surface. Absorbance measurements at 222nm proved to be sufficiently sensitive to allow for the monitoring of eluting TBA concentration profiles. The identity of the breakthrough fronts were confirmed by using a simple water-methylene chloride extraction system. An anionic dye (in this case a water soluble blue ink)

partitioned into the organic phase in the presence of TBA. When determining the breakthrough volumes, a solvent system was first run with a given percent methanol and buffer but no TBA ion. The ion-pair containing eluent made from the same batch of methanol-water buffer was then flushed through the system up to the column union. Initial time was taken as the start-up of the carefully calibrated pump.

RESULTS

Effect of Pairing Ion Concentration on Retention

The k' values of the nucleosides and their monophosphate nucleotides were found to be a linear function when plotted against the surface concentration of adsorbed TBA, as shown in Figure 1 for the nucleoside dGuo, and the nucleotide dAMP. This direct relationship between adsorbed hetaeron and k' has been reported previously (14). A maximum in k' is observed for the charged solutes, followed by a decrease in retention. It is likely that this maximum results from mobile phase pairing, fitting the general model presented by Horvath et al (5-7). However, in the region prior to this maximum, i.e., up to about 0.18 umole m for dAMP, or 4-5 mM TBA in the mobile phase, it seems likely that the dynamic ion-exchange model most accurately describes the data (12-15). The slope of this curve is labelled as $_{\theta}$. It is interesting to note that the k' values of the nucleosides decrease linearly, while the k' of the charged nucleotides increase linearly with surface TBA concentration.

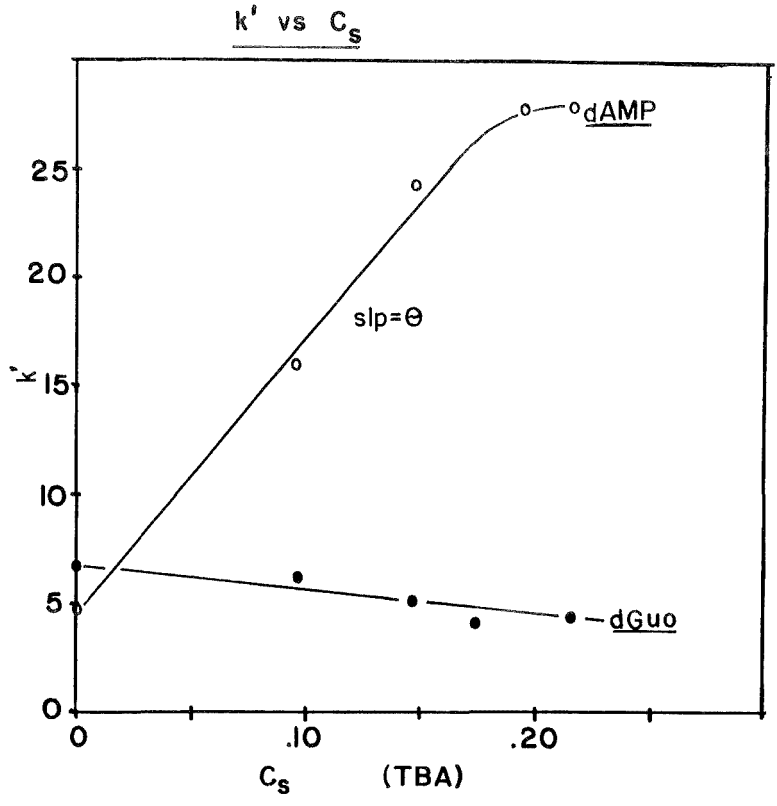


Figure 1

Plot of k' vs. concentration of pairing ion Cs, on the stationary phase, for the negatively charged nucleotide dAMP, and the nucleoside dGuo (for conditions see experimental).

Effect of Methanol Concentration

The k' values for all eight of the nucleosides and nucleotides were found to decrease logarithmically with increasing volume percent of methanol in the mobile phase. Figure 2 shows this log-linear relationship for the dAMP solute over the range of 0-10% (v/v) methanol/water, using a

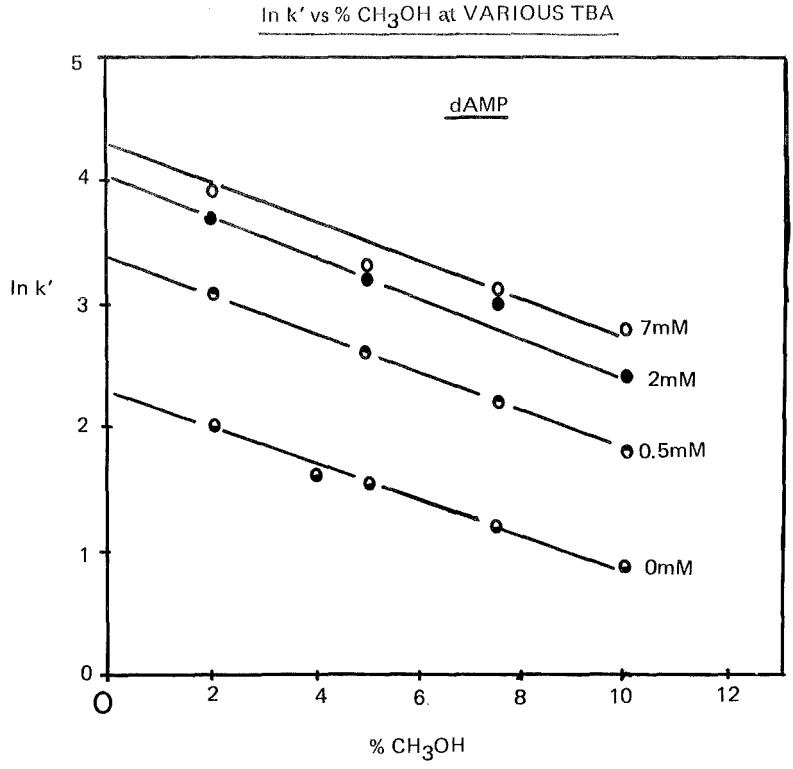


Figure 2

Plot of ln k' vs. % methanol for a negatively charged nucleotide, dAMP, at several TBA concentrations.

50 mM phosphate buffer eluent. The lower curve at 0 mM TBA represents a simple reversed-phase (C8) system. The linear correlation coefficients at 0 mM TBA was found to average 0.9293 for all solutes, with no coefficient being less than 0.9000.

When TBA is present in the mobile phase, this same log-linear decrease in retention is observed, but at higher or lower k' values. Thus, the ln k' values for dAMP

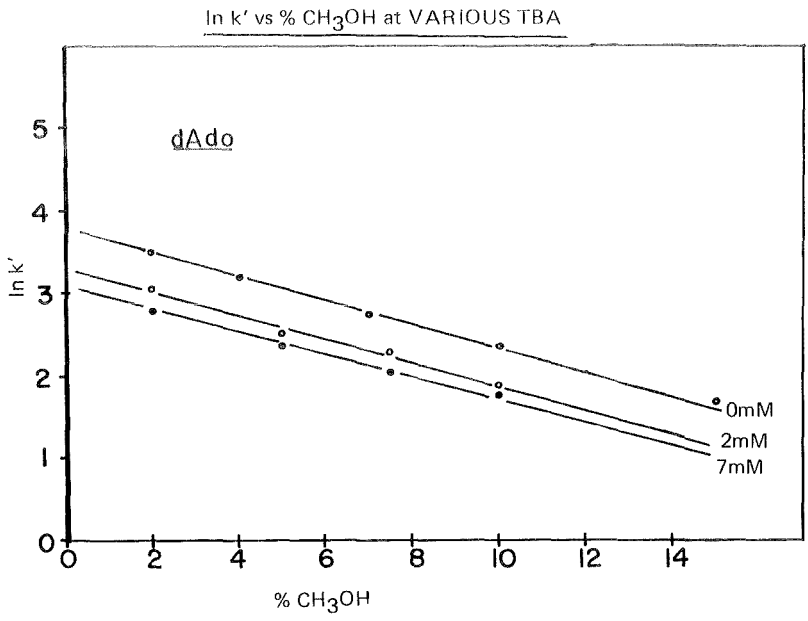


Figure 3

Plot of lnk' vs. % methanol for a nucleoside, deoxyadenosine, at various TBA concentrations

increase steadily with increasing TBA, with equal slopes but greater intercepts.

For the nucleoside dAdo, as shown in Figure 3, the same parallel ln k' lines are observed. The intercepts however decrease with increasing TBA. This is presumably due to repulsion of the solutes from the increasingly cationic stationary phase.

The TBA adsorption to the stationary phase was found to fit a Freundlich isotherm, Eq. 4 . Stationary phase concentrations were determined using frontal elution techniques. Within experimental error, the Freundlich

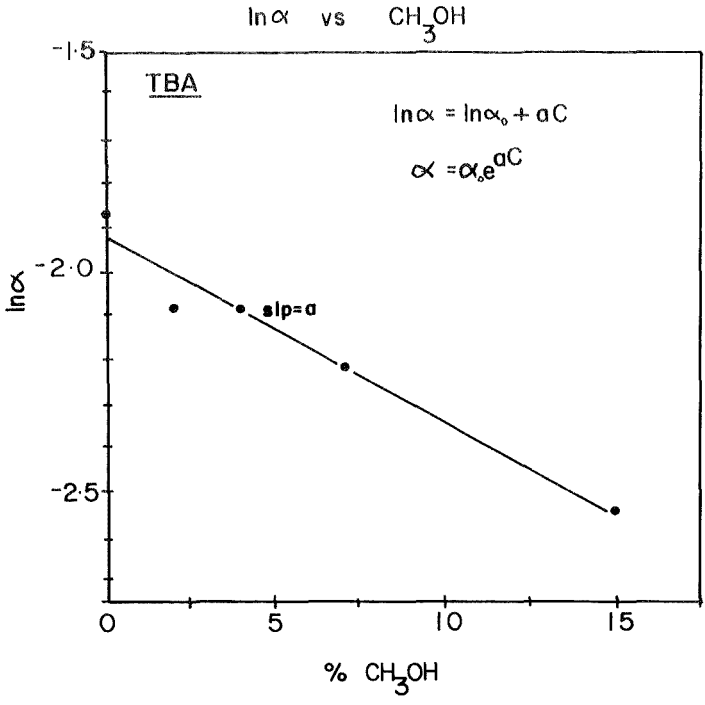


Figure 4

Plot of ln α vs. % methanol in the mobile phase. Alpha is the intercept of the Freundlich isotherm for the adsorption of TBA onto the stationary phase. The slope of the plot is a, and the intercept, α_o. All other conditions the same as figure 1.

slopes for TBA on the C8 stationary phase were found to be constant with increasing methanol, while the intercepts decreased exponentially, as shown in Figure 4.

The slopes of the k' versus Cs curves or θ, decreased exponentially with increasing methanol, as shown in Figure 5 for the deoxynucleotides. The same log-linear relationship was also observed for the deoxynucleosides.

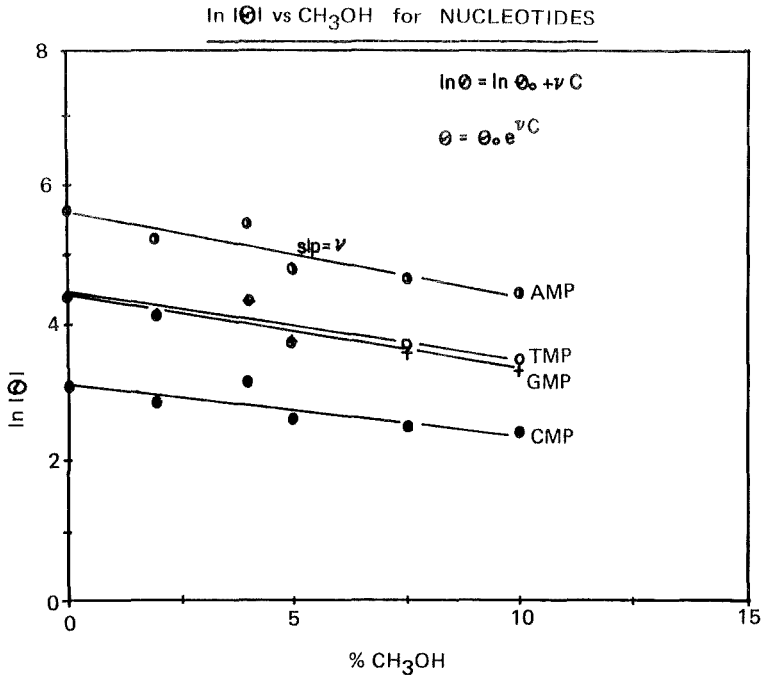


Figure 5

Plot of k' of dNMP, vs. Cs at various methanol concentrations. The slope $_{\Theta}$ varies systematically with increasing organic modifier concentrations.

Net Effect of Methanol and TBA Concentration

The overall effect of TBA and methanol upon the k' of the deoxynucleosides and their mononucleotides can be described by equation 7 for mobile phase concentrations of up to 15% methanol and at TBA concentrations of up to 7 mM TBA. The net effect of increasing methanol at constant TBA is to decrease retention for all solutes. At constant methanol, the deoxynucleosides will decrease in retention, while the deoxymononucleotides will increase. The rate of increase or decrease can be predicted with reasonable

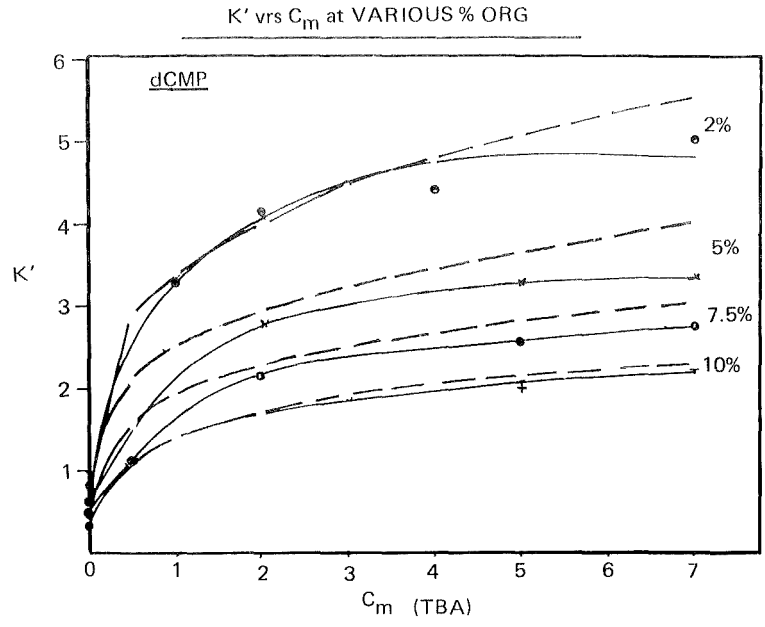


Figure 6

Comparisons of the calculated (broken lines) and observed (solid lines) k' values for the negatively charged mononucleotide dCMP. Experimental conditions the same as in Fig. 1.

Table 1

Summary of retention constants for equation 7 for the eight deoxynucleosides and their mononucleotides. Stationary phase: Whatman C8. Mobile phase: methanol/water, 50 mM in phosphate. Temperature:ambient. Valid range: 0-15% (v/v) methanol, 0-7 mM TBA.

	dCyd	dGuo	dThd	dAdo	dCMP	dGMP	dTMP	dAMP
k'_o	3.42	13.74	15.33	48.42	0.88	3.42	3.74	9.97
A	-.122	-.145	-.137	-.150	-.072	-.188	-.125	-.130
Θ_o	-6.75	-26.58	-40.44	-121.51	22.64	88.23	84.77	267.70
ν	-.145	-.197	-.220	-.221	-.074	-.116	-.101	-.121

for TBA: α_o =0.146 a= -0.0418 β = 0.308

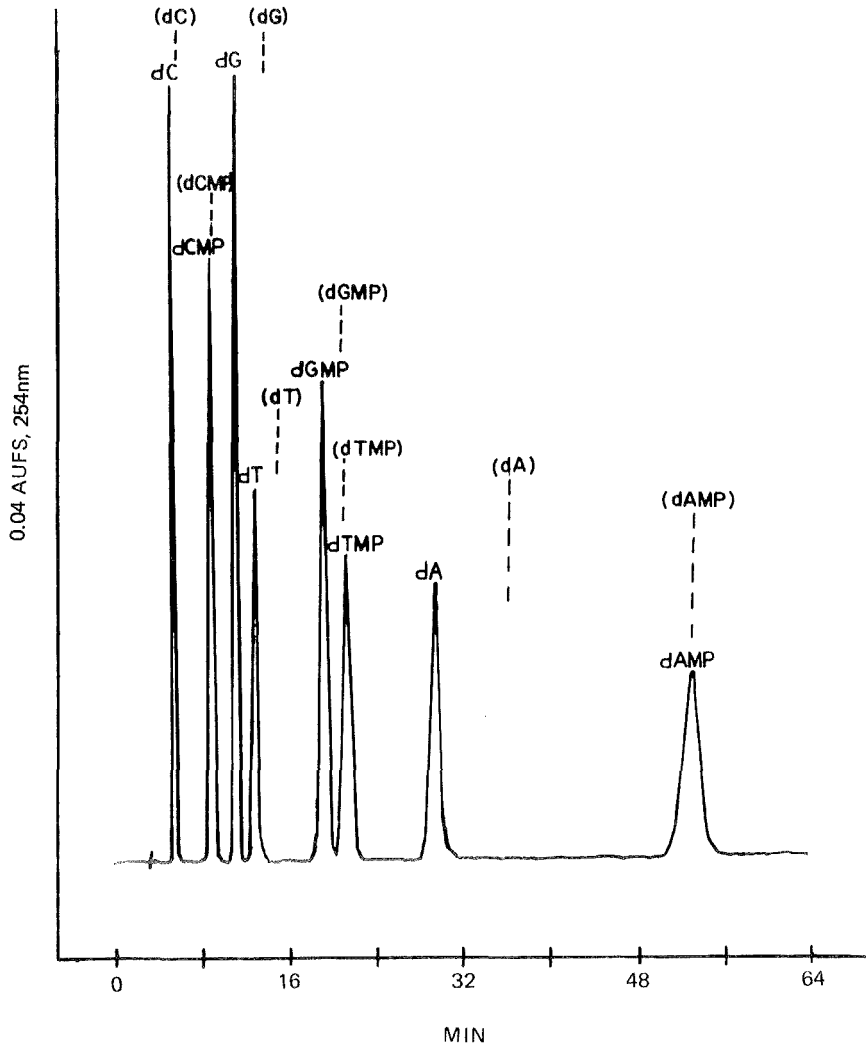
accuracy according to equation 7. Figure 6 shows the predicted and observed values for dCMP, using the system constants presented in Table 1.
Excellent qualitative accuracy, and reasonable quantitative fits were observed for all eight solutes using equation 7. Over the range of conditions studied, equation 7 proved to be accurate to within 5-10% for all eight of the deoxynucleosides and their nucleotide monophosphates.

Final Separation Conditions

Figure 7 presents the simultaneous separation of the eight deoxyribonucleosides and their monophosphate nucleotides. Baseline separation was achieved for all solutes, with a total analysis time of 50 minutes. The retention times as predicted by equation 7 are presented as broken lines. This separation offers considerable flexibility in conditions. Changing the methanol or TBA concentration will drastically alter the elution pattern, according to EQ. 7. Thus, if only several of the solutes studied need be separated, new conditions can be readily calculated and form the basis for at least the initial starting conditions. These conditions can then be fined tuned empirically at the instrument.

CONCLUSIONS

An ion-pair separation of the major deoxyribonucleosides and their monophosphate nucleotides has been presented. Complete separation of all solutes was achieved in under 1 hour, with excellent efficiencies. An



Figures 7

Chromatogram of the eight test solutes comparing the observed and predicted retention times according to Eq. 9. Mobile phase: 0.050 M phosphate buffer, 2.0 mM TBA, 7% (v/v) methanol in water. Temperature, ambient. Column: Whatman Partisil 5 C8 (4.6 mm id X 250 mm).

empirical function was derived to aid in the optimization of separation conditions. It appears that ion-pair HPLC is uniquely suited for the separation of the nucleic acid constituents, since in many cases both charged and non-charged solutes are involved. Ion-pairing offers the flexibility of changing the retention of the charged solutes at a much greater rate than the neutral solutes, resulting in excellent control over the selectivity of the system.

The mechanism of separation appears to fit a dynamic ion-exchange model most accurately. If this is indeed the case, then it ought to be feasible to synthesize stationary phases with properties similar to those observed in ion-pairing systems, but without the problems inherent in ion-pairing. These shortcomings include the addition of foreign ions to the mobile phase, long equilibration times and diminished column lifetimes. The synthesis of such phases are being studied in our laboratory and will be reported in the literature.

Acknowledgements

The authors wish to acknowledge the many helpful discussions with Professor Phyllis R. Brown, at the University of Rhode Island and with Dr. Fred Rabel, of Whatman Inc. The technical and scientific assistance of Joseph Sweeney, of Waters Associates, Paul B. Champlin, of Kratos Instruments, and Klaus Lohse of Farrand Optical are gratefully acknowledged.

This research is supported by the Rutgers Research Council, a Biomedical Research Support Grant,the Petroleum Research Fund and by the National Science Foundation, grant number CHE-8100224.

REFERENCES

1. C.W. Gehrke, K.C. Kuo, G.E. Davis, R.D. Suits, T.P. Waalkes and E. Borek, J. Chromatogr. 150, 455 (1978).

2. R.A. Hartwick and P.R. Brown, Crit. Rev. Anal. Chem., 10(3), 279 (1981).

3. R.A. Hartwick and P.R. Brown, J. Chromatogr., 112, 650 (1975).

4. N.E. Hoffman and J.C. Liao, Anal. Chem., 49, 2231 (1977).

5. C.S. Horvath, W. Melander, I. Molnar, and P. Molnar, Anal. Chem., 49 (1977) 2295

6. W.R. Melander, K. Kalghatgi and Cs. Horvath, J. Chromatogr., 201, 201 (1980).

7. W.R. Melander and Cs. Horvath, J. Chromatogr., 201 211 (1980).

8. C.P. Terweij-Groen, S. Heemstra and J.C. Kraak, J. Chromatogr., 161, 69 (1978).

9. A.T. Melin, M. Ljungcrantz and G. Schill, J. Chromatogr., 185, 225 (1979).

10 A.O. Konijnendijk and J.L.M. van de Venne, J. Chromatogr., 186, 373 (1979).

11. C.M. Riley, E. Tomlinson and J.M. Jefferies, J. Chromatogr., 185, 197 (1979).

12. P.T. Kissinger, Anal. Chem., 49, 883 (1977).

13. R.A. Bidlingmeyer, S.N. Deming, W.P. Price, Jr., B. Sachok and M. Petrusek, J. Chromatogr., 186, 419 (1979).

14. J.H. Knox and R.A. Hartwick, J. Chromatogr., 204 3 (1981).

15. R.P.W. Scott and P. Kucera, J. Chromtogr., 175, 51 (1979).

16. A.M. Krstulovic, S.W. Dziedzic, L. Bertani-Dziedzic and D.E. DiRico, J. Chromatogr., 217, 523 (1981).

17. A.M. Krstulovic, Adv. in Chromatogr., (J. Calvin Giddings, ed.), Vol. 17, p. 279; Marcel Dekker, New York, 1979.
Chromatogr., 161, 69 (1978).

18. P. Jandera and J. Churacek, J. Chromatogr., 91 207 (1974)

19. R.A. Hartwick, C.M. Grill and P.R. Brown, Anal. Chem., 51, 34 (1979).

20. P. Jandera, H. Colin and G. Guiochon, Anal. Chem., 54, 435 (1982).

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(9), 1693-1704 (1983)

HPLC SEPARATION OF DIASTEREOMERIC ADDUCTS OF GLUTATHIONE WITH SOME K-REGION ARENE OXIDES.

Oscar Hernandez, Ashok B. Bhatia and Michael P. Walker

Laboratory of Environmental Chemistry

National Institute of Environmental Health Sciences

P.O. Box 12233

Research Triangle Park, N.C. 27709

ABSTRACT

The diastereomeric glutathione (GSH) adducts of the K-region arene oxides phenanthrene 9,10-oxide, pyrene 4,5-oxide, (±)-benz[a]anthracene 5,6-oxide, and (±)-benzo[a]pyrene 4,5-oxide were separated by reversed phase liquid chromatography. Chromatographic conditions involved an organic base, Tris-base or diethylenetriamine (DETA), neutralized to pH 3 with phosphoric acid, and an alcohol, methanol or 1-propanol, as modifier. For (±)-benzo[a]pyrene 4,5-oxide, the use of DETA and 1-propanol provided a complete stereochemical profile of the thioether conjugates derived from this arene oxide. For the GSH adducts of (±)-benz[a]anthracene 5,6-oxide complete separation was achieved under two sets of chromatographic conditions; methanol and 1-propanol enhanced the selectivity of the system for different sets of diastereomers. For both arene oxides, the GSH adducts with S-configuration eluted earlier than the R-diastereomers.

INTRODUCTION

A growing body of evidence indicates that a close relationship exists between the stereochemistry of oxides derived from polynuclear aromatic hydrocarbons and the expression of mutagenic and carcinogenic acitivity (1). This stereo-dependence is also manifested in the detoxication processes involving these metabolites. Stereopreference in the hydrolysis of

0148-3919/83/0609-1693$3.50/0

arene oxides to trans-dihydrodiols by epoxide hydrolase has been reported (7). Equally important, the glutathione transferase enzymes have also been shown to be highly stereospecific in catalyzing the reaction of epoxides with glutathione (3). The ability to analyze for the different stereoisomers formed in the reaction of arene oxides with glutathione becomes a limiting factor in the interpretation of experimental data.

Analytical conditions for the glutathione (GSH) adducts of (±)-benzo[a]-pyrene 4,5-oxide (BPO) have been reported (3). This HPLC analysis has been successfully employed in the stereochemical analysis of the enzymatic reaction of GSH with this arene oxide. More recently these analytical conditions were applied in the determination of the stereochemistry of oxidation of benzo[a]pyrene by a purified cytochrome P-450 preparation (4). In the present report we describe conditions for the analysis of diastereometric GSH adducts of phenanthrene 9,10-oxide, pyrene 4,5-oxide, (±)-benz-[a]anthracene 5,6-oxide (BAO) and improved conditions for the separation of thioethers derived from (±)-BPO.

MATERIALS AND METHODS

Phenanthrene 9,10-oxide and pyrene 4,5-oxide were prepared by literature procedures (5). Racemic BPO and BAO were obtained from the Midwest Research Institute, Kansas City, MO. Samples of optically pure BPO were prepared by a published procedure (1). The GSH adducts were prepared by reaction of GSH with the corresponding epoxide in methanol solution (6). The structures were verified by ^{13}C-nmr and mass spectral analysis with the exception of the benz[a]anthracene products. Supporting evidence for the latter comes from mechanistic considerations and similarities with (±)-BPO. A sample of purified GSH adducts of (+)- and (-)-BAO were generously provided by Dr. Richard N. Armstrong, Department of Chemistry, University of Maryland, College Park, MD 20742. Assignment of absolute stereochemistry to the GSH adducts of pyrene 4,5-oxide and phenanthrene 9,10-oxide was not complete at this time.

The chromatographic conditions employed involved two systems. System 1, Waters Associates M6000A pump, model U6K injector, model 440 UV absorbance detector (254 nm), RCM-100 radial compression system equipped with a 5 micron C_{18} Radial-PAK (8 mm ID x 10 cm) and a Brownlee 5 micron RP-18 pre-column. The eluent was 75 mM phosphoric acid adjusted to pH 3 with either Tris-base or diethylenetriamine (DETA). Methanol was used as modifier in the concentration indicated in the figure legends. System 2, DuPont Gradient LC system 8822 with absorbance detector (254 nm), model 7125 Rheodyne injector, 5 micron Zorbax C_8 column (4.6 mm ID x 25 cm) equipped with a Brownlee RP-8 5 micron pre-column. The eluent consisted of 5 mM DETA neutralized to pH 3 with concentrated phosphoric acid. 1-Propanol was used as modifier in the concentrations indicated in the figure legends.

RESULTS AND DISCUSSION

The structures of the compounds of interest are shown in Fig. 1. The relative stereochemistry of the hydroxyl and thioether groups is depicted as trans in accordance with the known mechanism of nucleophilic ring opening of epoxides (7). The symmetric nature of phenanthrene 9,10-oxide and pyrene 5,6-oxide excludes the possibility of positional isomers while for the unsymmetrical epoxides (±)-BPO and (±)-BAO two positional isomers are possible. The nomenclature used in Fig. 1 is as follows: The numbers identify regioisomers, e.g., for (±)-BPO 3 refers to a 5-glutathionyl-adduct and 4 to a 4-glutathionyl isomer; for (±)-BAO 5 refers to a 5-glutathionyl and 6 to a 6-glutathionyl regioisomer. Because of the chiral nature of GSH each regioisomer will consist of a pair of diastereomers, identified by the letters a and b following the compound number.

For the symmetrical K-region arene oxides of phenanthrene and pyrene, the diastereomeric adducts formed on reaction with GSH are 1a,b and 2a,b (Fig. 1). In both cases, separation of the two possible diastereomers was readily achieved as shown in Fig. 2. Assignment of relative stereochemistry to the individual diastereomers was not available.

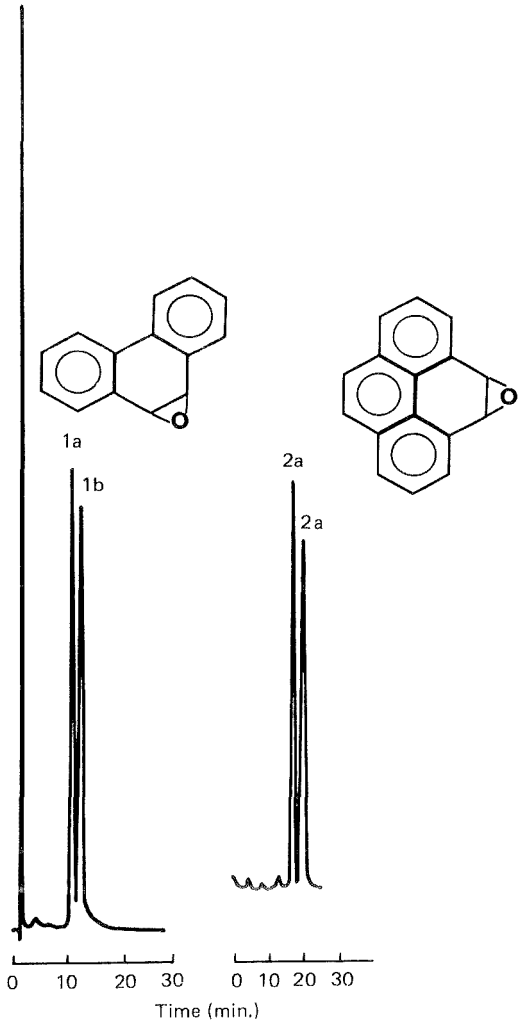
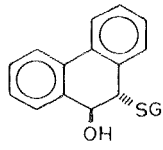
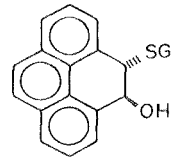


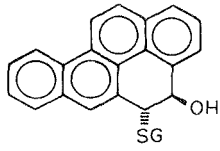
Figure 1. Structures of the GSH adducts of phenanthrene 9,10-oxide (1), pyrene 4,5-oxide (2), (±)-benzo[a]pyrene 4,5-oxide (3 and 4), and (±)-benzo[a]anthracene 5,6-oxide (5 and 6). Absolute stereochemistry is not implied. The letters designated diastereomers for each regioisomer.



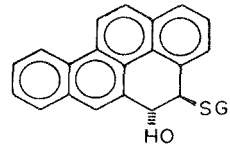
1a,b



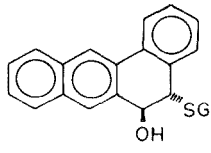
2 a,b



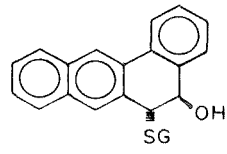
3 a,b



4 a,b



5 a,b



6 a,b

Figure 2. HPLC profile for the diastereomeric GSH adducts of phenanthrene 9,10-oxide (1a and 1b) and pyrene 4,5-oxide (2a and 2b). System 1, Tris-phosphate pH 3/30% MeOH, flow rate 3 ml/min.

For (±)-BPO there are two possible positional isomers (e.g. 3 and 4, Fig. 1). Because this arene oxide is capable of existing in enantiomeric forms, a total of four diastereomers are formed on reaction of racemic BPO with GSH. The separation originally developed for the GSH adducts of (±)-BPO provided baseline separation for two of the four diastereomers (Fig. 3a). These analytical conditions found immediate application in establishing the stereochemical course of the enzymatic reaction of GSH with this arene oxide (3). The first two eluting peaks, 3a and 4a in Fig. 3a, represent a single diastereomer each originating from a different enantiomer of (±)-BPO (8). Thus, compound 3a, a 5-glutathionyl regioisomer,

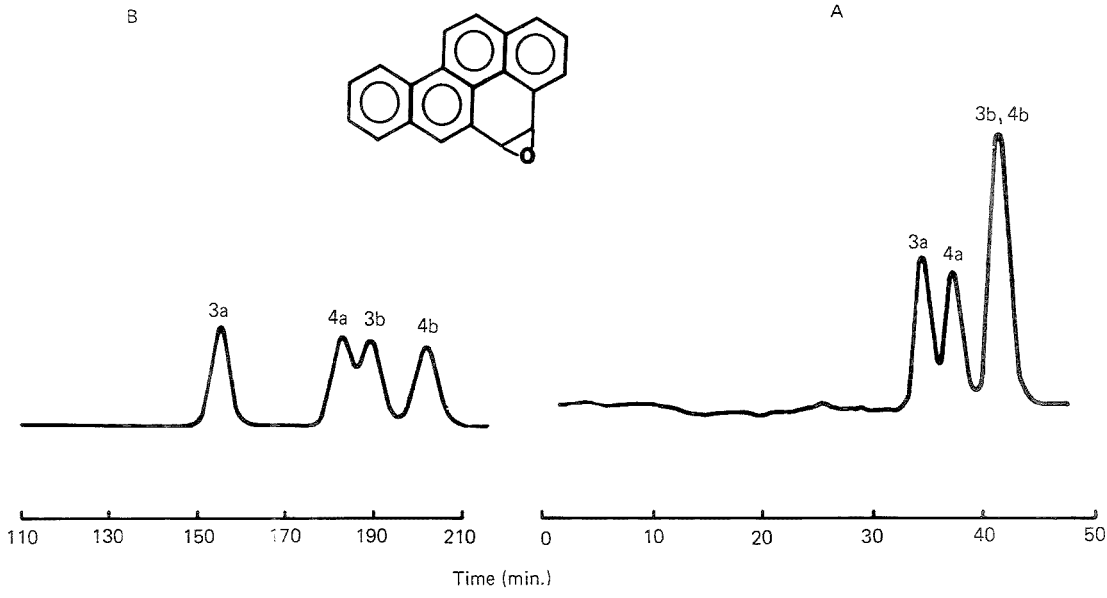


Figure 3. HPLC profile for the diastereomeric GSH adducts of (±)-benzo[a]-pyrene 4,5-oxide. Trace A, Tris-phosphate pH 3/40% MeOH, flow rate 3 ml/min. Trace B, system 2, DETA-phosphate pH 3/9% 1-propanol, flow rate 2 ml/min. Peak identification: 3a, (4S,4S)-5-SG; 4a, (4S,5S)-4-SG; 3b, (4R,5R)-5-SG; 4b, (4R,SR)-4-SG.

originates from (4S,5R)-BPO and it follows that the absolute configuration of 3a is (4S,5S); compound 4a, a 4-glutathionyl regioisomer, originates from (4R,5S)-BPO and it has absolute configuration (4S,5S). The third eluting peak in Fig. 3a contains the remaining two diastereomers, a 4-glutathionyl isomer (from (4S,5R)oxide) and a 5-glutathionyl isomer (from (4R,5S)oxide), both with absolute configuration (4R,5R). Separation of the latter set of diastereomers was required for further studies on the stereochemical aspects of the enzyme catalyzed reaction. The approach followed was to affect the selectivity of the system by changes in the eluent. Two modifications were introduced: first, the use of diethylenetriamine (DETA) in the preparation of the buffer was found beneficial (9); second, the use of 1-propanol as organic modifier had a profound effect on the selectivity of the column. As shown on Fig. 3b separation of all the diastereomeric GSH adducts of (±)-BPO was achieved with the modifications described above. The diastereomers shown coeluting in Fig. 3a are now identified as 3b, a 5-glutathionyl isomer with absolute configuration (4R,5R), and 4b, a 4-glutathionyl isomer with absolute configuration (4R,5R). The HPLC on Fig. 3b represents a complete stereochemical profile of the GSH adducts of (±)-BPO.

The situation with (±)-BAO was as complicated as with BPO with two positional isomers and a total of four diastereomers. As shown in Fig. 4, conditions were developed which allow the analysis of all four diastereomers. Trace 4a shows separation of three peaks in the ratio of 2:1:1, the modifier used in this case was methanol. Trace 4b shows a different profile, three peaks in the relative ratio 1:1:2, when 1-propanol was used as modifier. Structural and stereochemical assignments for the GSH adducts of BAO, based on HPLC analysis of authentic GSH conjugates of optically pure BAO (see Materials and Methods), were as follows: (5R,6S)-BAO on reaction with GSH gave rise to a 5-glutathionyl isomer, absolute configuration (5S,6S), identified as 5a, and a 6-glutathionyl isomer, absolute configuration (5R,6R), identified as 6b; (5S,6R)-BAO

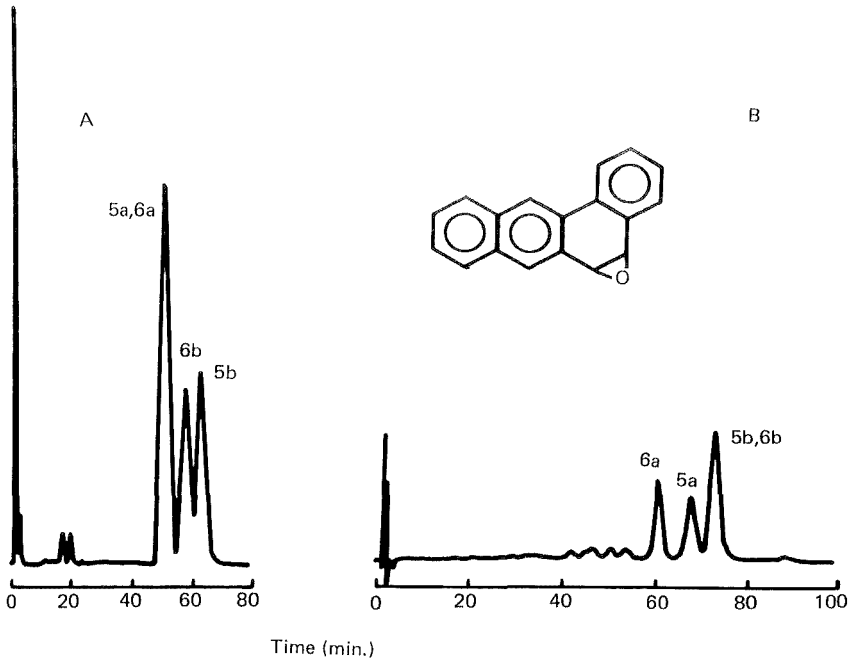


Figure 4. HPLC profile for the diastereomeric GSH adducts of (±)-benz[a]-anthracene 5,6-oxide. Trace A, system 1, Tris-phosphate pH 3/30% MeOH, flow rate 3 ml/min. Trace B, system 2, DETA-phosphate pH 3/9% 1-propanol, flow rate 2 ml/min. Peak identification: 6a, (5S,6S)-6-SG; 5a, (5S,6S)-5-SG; 6b, (5R,6R)-6-SG; 5b, (5R,6R)-5-SG.

formed a 5-glutathionyl adduct, absolute configuration (5R,6R), identified as 5b, and a 6-glutathionyl adduct, absolute configuration (5S,6S), identified as 6a. Elution profiles for all these diastereomers is illustrated in Fig. 4. A recent report in the literature described an HPLC separation of the GSH conjugates of BAO (10). The profile reported in that paper resembled that obtained in the present work with methanol as modifier. A direct extrapolation to the present work is not possible since we have shown that the separation is quite sensitive to solvent effects. The conditions used in that report (10) (3 micron ODS-2 column, 8% CH_3CN/20%

MeOH/72% 100 mM Tris-phosphate pH 2.5) are substantially different from the ones used in the present study. The conditions described here allow separation of all diastereomers by choosing the appropriate solvent conditions.

The two bonded phases used is this study, C-8 Zorbax and Waters C-18, were comparably effective in resolving these compounds. The differences observed in selectivity are more directly correlated to the effect of the organic modifiers. The use of an organic base remains a prime requirement (9), and DETA would seem to be the base of choice for these compounds.

The observed change in column selectivity as a function of organic modifier is the combined result of two effects: 1) the nature of the interaction between the solvent and the bonded phase; 2) solute-solvent interactions. The first effect involves a comparison between methanol and 1-propanol. Higher molecular weight alcohols are reported to change the selectivity of reversed-phase bonded phases by a loading effect, the hydrophobic alcohols effectively saturate the HPLC column (11). How this effect may help discriminate among stereoisomers is not predictable. The second effect is perhaps more easily correlated with the problem at hand. Diastereomers differ in the spatial arrangement of their substituents and consequently are expected to have different stable conformations. This stable conformer population may be significantly affected by solvent interactions, and it has been reported (7) that for dihydro derivatives of polynuclear aromatic hydrocarbons a shift from diequatorial to diaxial conformers occurs in high polarity solvents. The use of 1-propanol, a stronger eluent than methanol, decreases the amount of organic modifier (9% vs 30% methanol) required for elution, with the resulting mobile phase being more aqueous and hence more polar. This change in polarity could have an effect on the relative conformer populations of the GSH adducts of (±)-BPO and (±)-BAO (12).

Although it is not possible at this time to clarify the observed selectivity effects, two important observations emerge from these studies.

First, the order of elution of sets of regioisomers, 3 and 4 for BPO and 5 and 6 for BAO is consistent in that diastereomers with S-configuration emerge earlier than those with R-configuration; 3a and 4a for BPO (Fig. 3b), and 5a and 6a for BAO (Fig. 4b). Second, the order of elution of diastereomers with the same absolute configuration at the carbon-bearing sulfur, i.e. 3a and 4a for BPO, 5a and 6a for BAO, is determined by the proximity of the peptide residue to the most hydrophobic portion of the molecule. Thus, on 3a the peptide residue is adjacent, and presumably interacts by inhibiting binding of the "naphthalene residue" to the bonded phase (13), while in 4a this group is more available for binding; similarly, 3b elutes earlier than 4b. A similar analysis in BAO shows that 5a elutes earlier than 6a, and 5b is less retained than 6b. These observations on the influence of stereochemistry in the separation of thioether derivatives of K-region arene oxides are in accordance with those reported for the GSH adducts of styrene oxide (14). In the latter, thioethers with S-configuration eluted earlier than the R-diastereomers. It is possible that the order of elution under the conditions described here, represent a general rule for this type of compounds. If such were the case, it should be possible to assign relative configuration, based on the HPLC elution profile of GSH adducts of epoxides of unknown stereochemistry. A prerequisite would be the ability to separate the anticipated number of diastereomers. For phenanthrene 9,10-oxide and pyrene 4,5-oxide this conditions is met and it is tempting to assign the S-configuration to the first eluting diastereomer in each case (Fig. ?).

The conditions described here provide a tool for the stereochemical analysis of the glutathione transferase catalyzed reactions with arene oxides and, in the case of metabolically formed epoxides, for the stereochemistry of oxidation of polynuclear aromatic hydrocarbons by the cytochrome P-450 dependent monoxygenase system (4).

REFERENCES

1. Chang, R.L., Wood, A.W., Levin, W., Mah, H.D., Thakker, D.R., Jerina, D.M., and Conney, A.H. Proc. Natl. Acad. Sci. USA, 76, 4280 (1979).

2. Armstrong, R.N., Levin, W., and Jerina, D.M. J. Biol. Chem. 255, 4698 (1980).

3. Hernandez, O., Foureman, G.L., Cox, R.H., Walwer, M., Smith, B., and Bend, J.R. Fifth International Symposium on Polynuclear Aromatic Hydrocarbons, Cooke, M., and Dennis, A.J., eds. Battelle Press, Columbus, Ohio, 1981, p. 667.

4. Armstrong, R.N., Levin, W., Ryan, D.E., Thomas, P.E., Mah, H.D., and Jerina, D.M. Biochem. Biophys. Res. Commun., 100, 1077 (1981).

5. Krisham, S., Kuhn, D.G., and Hamilton, G.A. J. Amer. Chem. Soc., 99, 8121 (1977).

6. Hernandez, O., Bhatia, A., Foureman, G., Walker, M.P., and Bend, J.R. Synthesis and stereochemical analysis of glutathione adducts of arene oxides, 184th ACS National Meeting, Kansas City, MO, September, 1982.

7. Beland, F.A. and Harvey, R.G. J. Amer. Chem. Soc., 98, 4963 (1976).

8. Hernandez, O., Walker, M., Cox, R.H., Foureman, G.L., Smith, B.R., and Bend, J.R. Biochem. Biophys. Res. Commun., 96, 1494 (1980).

9. Melander, W.R., Stoveken, J., and Horvath, C. J. Chromatogr. 185, 111 (1979).

10. van Bladeren, P.J., Armstrong, R.N., Cobb, D., Thakker, D.R., Ryan, D.E., Thomas, P.E., Sharma, N.D., Boyd, D.R., Levin, W., and Jerina, D.M. Biochem. Biophys. Res. Commun., 106, 602 (1982).

11. Scott, R.P.W. and Kucera, P.J. Chromatogr., 142, 213 (1977).

12. The development of more efficient procedures for the synthesis of these compounds will eventually allow verification of these observations on the conformation of thioether metabolites of epoxides by the appropriate nuclear magnetic resonance experiments.

13. Hernandez, O., Yagen, B., Cox, R.H., Bend, J.R., and McKinney, J.D. J. Liquid Chromatogr. 5, 345 (1982).

14. Hernandez, O., Bhatia, A.V., and Walker, M. J. Liquid Chromatogr. (in press).

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(9), 1705-1716 (1983)

SEPARATION OF ORGANIC ACIDS IN PLANT TISSUE BY HPLC WITH A TWIN PHASE, ION EXCHANGE AND REVERSE PHASE, COLUMN

A. CLEMENT* and B. LOUBINOUX**

*Station de Recherches sur les Sols Forestiers
et la Fertilisation
Centre National de Recherches Forestières
Champenoux 54280 Seichamps - France

**Laboratoire de Chimie Organique III ERA-CNRS n° 558
Université de Nancy I - B.P. 239
54506 Vandoeuvre-les-Nancy Cédex - France

ABSTRACT

No single isocratic chromatographic technique allows the complete separation of common organic aliphatic and alicyclic acids of plants. In order to obtain a better isocratic separation with a single HPLC method we combined in one chromatography assay the Ion Exchange and Reverse Phase technics in building a twin phase column. The first attempts are promising. This double chromatography based on the polarity of molecule (Reverse Phase) and on its acidic characteristics (Ion Exchange) has the advantages of both methods and allows good separations of the acids.

INTRODUCTION

The number of chromatographic techniques used reflects the difficulty of natural organic acid separation. The most common methods are Gas Liquid (1,2,3,4,5) and Liquid-Liquid Chromatogra-

 0148-3919/83/0609-1705$3.50/0

phy. High performance Liquid Chromatography (HPLC) has recently been uses in three forms : Reverse Phase (6,7,8,9,10,11), Ion Exchange (12,13,14,15,16,17,18,19,20) and Counter Ion Chromatography (21). No single method allows the complete separation of common organic aliphatic and alicyclic acids present in plants and consequently each problem needs a specific solution. Chromatographic columns with specific characteristics (12,13) are available commercially but they often involve expensive and delicate procedures, particularly due to the high cost of columns and the need of a gradient apparatus.

In order to obtain a good isocratic separation of organic acids with a single HPLC method, we combined in one chromatography assay, the Ion Exchange and Reverse Phase techniques in building a twin phase column. For this purpose we took advantage of two main properties of the organic acid molecules :

- their polarity (brought out by the Reverse Phase)
- their acid dissociation constant (brought out by the Ion Exchange).

MATERIALS

Choice of supports

The reverse phase support, Lichrosorb RP 18 (Merck) particle size 10 µ, was chosen because of the silica-bonded octadecyl groups (C_{18}) which are the best support for the retention of polar organic acids. The ion exchange support, Lichrosorb AN (Merck) particle size 10 µ, was chosen because of the silica-bonded ammonium quaternary groups which are strongly anionic.

Column characteristics

Supports	RP 18	Ion Exchange
Length	320 mm	80 mm
Diameter	4 mm	4 mm
Particle size	10 µ	10 µ

Choice of eluents

We used as mobile phases aqueous phosphate buffers at different pH and concentrations.

Chromatographic equipment

The chromatographic system included the following equipment :

. a rotary solvent distribution valve (Reodyne 50-03)

. an injector valve (Teflon rotary valve Reodyne 50)

. a high pressure pump (ORLITA D MP-AE 10-4)

. an UV detector (PHILIPS Pye Unicam LC), 210 nm

. a pulse damper (TOUZART et MATIGNON)

. a flow rate and pressure control unit (TOUZART et MATIGNON)

. a recorder (KIPP and ZONEN BD 41)

. recorder-computer (SHIMADZU)

. column packing equipment (TOUZART et MATIGNON)

. a temperature regulator (JULABO).

METHODS

The column was first partially filled with the Reverse Phase support and then totally filled with the Ion Exchange support. The following solvents were used :

- for Reverse Phase : a mixture of ethanol (8 ml) and bromoform (15 ml), with similar densities of the support and solvent (d = 2.1)
- for Ion Exchange : the phosphate buffer later used as the chromatographic mobile phase.

The suspension of each support was homogenized and degassed by ultrasonics during several minutes and then poured into the filling apparatus precolumn TOUZART et MATIGNON (23 ml capacity). The suspension was pushed from the precolumn to the chromatographic column using a 450 bars filling pressure, with ethanol for the Reverse Phase, and phosphate buffer for the Ion Exchange part. The required time to built a twin phase column was about 3 hours.

The functional direction was the same as the filling direction; thus the column was used first in the Ion Exchange part and second in the Reverse Phase part.

After preparation the column was stabilized with the chromatographic mobile phase and tested for its efficiency before final attatchment to the chromatograph.

RESULTS AND DISCUSSION

Our chromatographic studies were directed toward the separation of organic acids (quinic, shikimic, tartaric, malic, fumaric, succinic, citric and oxalic) which contribute to the leaf tissue acidity of trees. HPLC chromatography performed either by Ion Exchange or with Reverse Phase did not give good results. The table gives representative results. Using the twin phase column, we

TABLE : Average values of the capacity coefficients of acids in increasing order for a 30 cm length Lichrosorb AN 10 µ diameter column (k'_{AN}) and a 15 cm length Lichrosorb RP_{18} 5 µ diameter column ($k'_{RP_{18}}$).

These coefficients were obtained with a potassium orthophosphate buffer as eluent (concentration 0.1 M, pH = 2.5)

ACIDS	SHIKIMIC	QUINIC	TARTARIC	MALIC	SUCCINIC	CITRIC	OXALIC	FUMARIC
k'_{AN}	0.50 ⟷	0.54	1.50 ⟷	1.62	2.08	5.5	11.7 ⟷	27

ACIDS	OXALIC	TARTARIC	QUINIC	MALIC	SHIKIMIC	CITRIC	FUMARIC	SUCCINIC
$k'_{RP_{18}}$	0.10 ⟷	0.28 ⟷	0.33	0.60 ⟷	0.75	2.20 ⟷	2.26 ⟶	2.33

⟷ Non separated acids : common part > 1/2 the height of the smaller peak

⟷ Partially separated acids : common part < 1/2 the height of the smaller peak

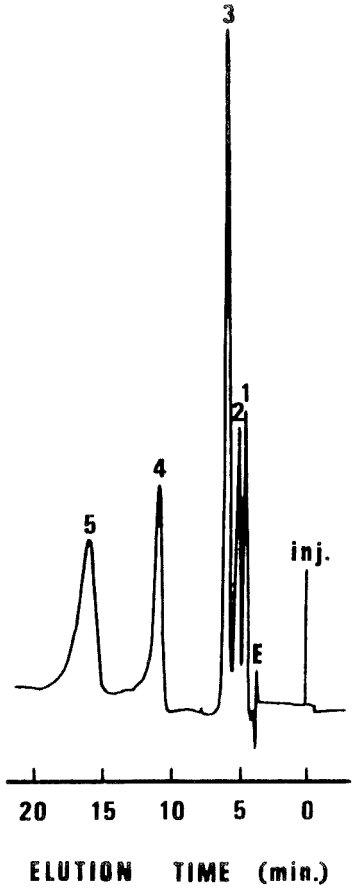


FIG. 1

Acids	Concentration µg/ml	k'
1. Quinic	225	0.26
2. Tartaric	200	0.39
3. Shikimic + Malic	11 + 700	0.98
4. Succinic + Citric	200 + 200	1.98
5. Fumaric	10.5	3.36

Sensitivity : S = 0.08 A.U.F.S.
Temperature : 20°C
Flow rate : 1.45 ml/minute
Pressure : 81 bars
Buffer : (KH_2PO_4-H_3PO_4) c = 0.33 M ; pH = 2.5
PEAKS : E = Eluent

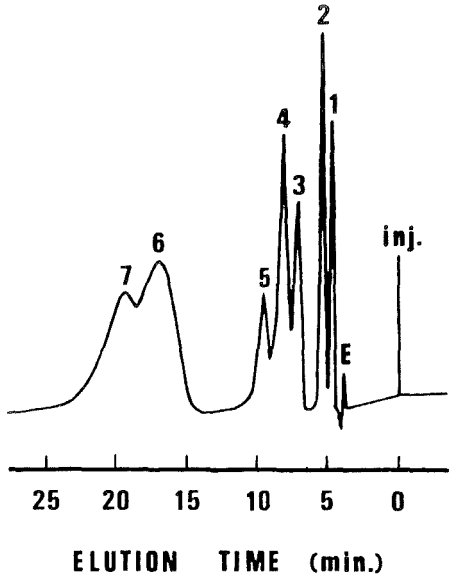


FIG. 2

Acids	Concentration µg/ml	k'
1. Quinic	300	0.25
2. Shikimic	11	0.43
3. Malic	300	0.86
4. Tartaric	300	1.14
5. Succinic	300	1.54
6. Oxalic + Fumaric	300	≈ 3.5
7. Citric	300	≈ 4.5

Sensitivity : S = 0.08 A.U.F.S.
Temperature : 20°C
Flow rate : d = 1.29 ml/minute
Pressure : 71 bars
Buffer : (KH_2PO_4) c = 0.2 M ; pH = 4.5
PEAKS : E = Eluent

expected to obtain both the advantages of the two kinds of chromatography :

- a good separation of quinic and shikimic acids brought by the Reverse Phase
- an increase by the Anion Exchange support of the capacity coefficients (k') of oxalic, and perhaps tartaric, acids observed with Reverse Phase, in order to displace their chromatographic peaks, respectively, away from those of quinic and shikimic acids.

At first, we studied the effects of eluent pH, and ionic strength on the retention of each acid by a 40 cm length twin phase column (RP 18 and A.N. in the proportion 1/4). It appeared that the acids fell into two groups :

- those whose capacity coefficient k' were little influenced by pH and ionic strength ; they are quinic, shikimic, malic and succinic acids ;
- those whose capacity coefficient k' were more dependant on pH and ionic strength ; they are citric, fumaric, oxalic and tartaric acids.

Generally, k' decreases when ionic strength increases at constant pH, and k' increases with increasing pH at constant ionic strength. The pH effect is more noticeable for tartaric and citric acids, especially with low eluent concentrations (0.1 M). At pH = 2.5 and above oxalic acid is completely dissociated and consequently strongly retained by the Anion Exchange support. We obtained a very high k' coefficient for low eluent concentrations.

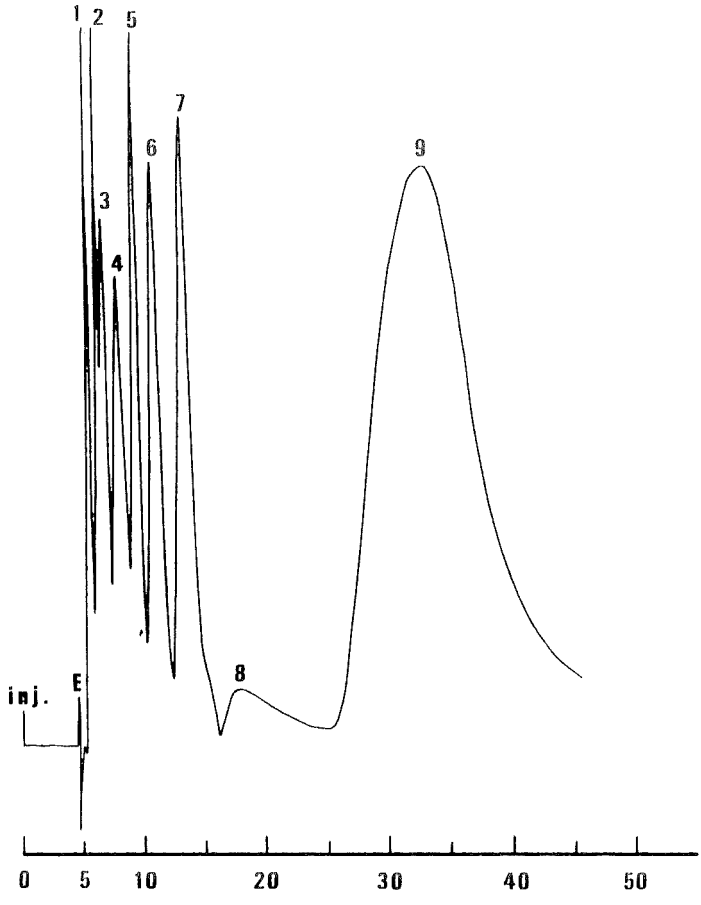


FIG. 3

Acids	Concentration µg/ml	k'
Quinic	250	0.21
Shikimic	7.5	0.38
Malic	250	0.50
Tartaric	250	0.75
Succinic	250	1.05
Malonic	250	1.37
Fumaric	5.0	1.91
Citric	250	3.01
Oxalic	250	6.22

Sensitivity : S = 0.04 A.U.F.S.
Temperature : 40°C
Flow rate : d = 0.99 ml/minute
Pressure : 41 bars
Buffer : (KH_2PO_4-H_3PO_4)
c = 0.2M, pH = 3.8

With this data in hand, we used the same column to analyse mixtures of acids using different experimental conditions. Our results show that flow rate, temperature, and sample mixture composition play a prominent part in the acids separations. The figures give representative examples of the separation obtained. Figure 3, for instance, shows the separation of 9 organic acids at a temperature of 40°C, which is a very promising result. It must be noted that although, an increase of temperature improves acid separation, good results were obtained at 25°C for more less complex cases (Fig. 1,2).

The choice of the proportion of the support in the column (Reverse Phase 3/4 - Anion Exchange 1/4) was not well suited to oxalic acid identification. Increasing the ratio of the Reverse Phase support would give better results. Another way improvement would be obtained with the use of very low pH ; such a pH suppresses an important dissociation of this acid, and consequently it would strongly lower its retention time. Unfortunately these low pH's are uncompatible with silicon supports. An Anion Exchange Chromatography at very low pH (pH < 2.5) has been used by others to identify oxalic acid (13,14). An ammonium styrene support type, which does not allow the use of high solvent pressure, was used.

CONCLUSION

The first attempts to separate aliphatic and alicyclic acids of tree foliar tissue on a twin phase column, using Reverse Phase and Ion Exchange supports, are promising. This double chromatogra-

phy, based on the polarity of the molecule (Reverse Phase) and on its acid characteristics (Ion Exchange), uses the advantages of both methods. The possibility of varying the proportions of the two supports in a single column allows the researcher a personalized column whose properties can be modulated complementarly by the eluent ionic strength, the eluent pH, and the temperature conditions.

ACKNOWLEDGEMENTS

The authors would like to thank the technical assistance of Mr's BITSCH, MARSEU, MINEL, and MM. BRECHET and HENRION.

REFERENCES

1. A. Boudet, G. Alibert, J.L. Puech, Bull. Soc. Biol., 52, 119 (1970)

2. P. Morard, E. Bourrier, Chimie Analytique, 53, 315 (1971)

3. R.D. Phillips, D.H. Jennings, New Phytol., 77, 333 (1976)

4. J.C. Rutter, W.R. Johnston and C.M. Willmer, J. Exp. Bot., 28, 1019 (1977)

5. S.K. Sarkar and S.S. Malhotra, J. Chromatogr., 171, 227 (1978)

6. E.D. Coppola, E.C. Conrad and R. Cotter, J. Assoc. Off. Anal. Chem., 61, 1490 (1978)

7. R. Farinotti, M. Caude, G. Mahuzier and R. Rosset, Analusis, 7, 449 (1979)

8. C. Gonnet, M. Marichy, W. Philippe, Analusis, 7, 370 (1979)

9. M. Nelson, Altex Scientific Brief, Organic Acid Separation (Limited Distribution)

10. B.Y. Ong, C.W. Nagel, J. Chromatogr., 157, 345 (1978)

11. P. Symonds, Ann. Nutr. Alim., 32, 957 (1978)

12. Bio-Rad Laboratories, The Liquid Chromatographer, n° 2 EG, August (1979)

13. Bio-Rad Laboratories, The Liquid Chromatographer, n° 4 EG, Spring (1980)

14. U.J. Kaiser, Chromatographia, 6, 387 (1973)

15. K.J. Palmer, M.D. List, J. Agr. Food Chem., 21, 903 (1973)

16. C. Israelian, Waters Associates S.A. Paris, Personal Communication

17. A. Rapp, A. Ziegler, Chromatographia, 9, 148 (1976)

18. V.T. Turkelson, M. Richards, Analytical Chem., 50, 1420 (1978)

19. C.T. Wehr, Liquid Chromatography at work, 83, Varian Instrument Division, Palo-Alto (California)

20. C.T. Wehr, Liquid Chromatography at work, 85, Varian Instrument Division, Palo-Alto (California)

21. L.E. Vera-Avila, Thèse de Doctorat de 3e cycle, Paris (1980)

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(9), 1717-1719 (1983)

LC NEWS

PROGRAMMABLE UV-VIS DETECTOR FOR HPLC may be optionally equipped with fast scanning and wavelength capabilities. It is capable of wavelength speeds up to 100 nm per second. The system is microprocessor-based and accessed via an HP-41 programmable calculator. Automatic baseline zero is featured for offsetting wavelength-induced baseline shift. Kratos Analytical Instruments, Inc., JLC/83/9, 170 Williams Drive, Ramsey, NJ, 07446, USA.

PNEUMATIC CLAMPING SYSTEM is for use with HPLC columns and cartridges. No-fault connections ensure consistent results. Columns are added or removed easily by relatively untrained operators without altering analytical integrity. They are useful to 5000 psi. EM Science, Inc., 480 Democrat Rd., Gibbstown, NJ, 08027, USA.

AUTOMATIC LC SAMPLE HANDLER is designed for use with almost any type of commercial LC unit; it functions as an independent module with a wide choice of user-programmable parameters and instructions. Segmented trays hold 80 vials in any combination. They revolve in both directions providing random access to all vials, and an automatic calibration cycle returns the standard vial to the injection position when required, regardless of its location. IBM Instruments, Inc., JLC/83/9, Orchard Park, P. O. Box 332, Danbury, CT, 06810, USA.

PUMP PRIMING VALVE is designed to simplify purging of HPLC pumps when change of solvent is made. When fitted between pump and injector, it permits new solvent to be purged through the pump to waste via a drain tube. Only half a turn is required to open the valve fully. Negretti & Zambra (Aviation), Ltd., JLC/83/9, The Airport, Southampton, SO9 3FR, Hampshire, England.

ION CHROMATOGRAPHY determines mono-, di-, and oligosaccharides up to DP 12 in beer and wort. Separations of both fermentable sugars and higher saccharides can be obtained with greater selectivity than conventional techniques. Parts-per-million levels are determined with an amperometric detector. Dionex, Inc., JLC/83/9, 1228 Titan Way, Sunnyvale, CA, 94086, USA.

UNIVERSAL HPLC SOLVENT CLARIFICATION MEMBRANE accomplishes filtration of all commonly used HPLC solvents. The membranes have an extensive range of chemical compatibility, are hydrophilic, and do not require a pre-wetting step. They also exhibit an extremely low extractables content, thereby eliminating concern of contamination from the membrane. Millipore Corp., JLC/83/9, 80 Ashby Road, Bedford, MA, 01730, USA.

HPLC FILTER is made of a new fluoropolymer membrane and is housed in solvent resistant polypropylene. It is pressure rated at 75 psi and is available in a 0.45 micron pore size. Gelman Sciences, Inc., JLC/83/9, 600 S. Wagner Rd., Ann Arbor, MI, 48106, USA.

FLAME IONIZATION DETECTOR FOR LC is said to overcome general limitations of prior "universal" LC detectors. A rotating disc, "ringed" by a fibrous quartz belt, conducts samples to a dual-flame ionization detector for analysis. Volatile solvent is vaporized and removed by vacuum. Solutes are carried into the FID, and residual sample is then removed by hotter hydrogen/oxygen cleaning flames. Tracor Instruments, JLC/83/9, 6500 Tracor Lane, Austin, TX, 78721, USA.

HPLC REAGENTS & COLUMNS are described in a new brochure. Included are buffers, ion pair reagents, derivatizing reagents, hardware, and solvents. Fisher Scientific Co., JLC/83/9, 711 Forbes Ave., Pittsburgh, PA, 15219, USA.

LAB AUTOMATION SYSTEM FOR SAMPLE PREPARATION combines robotics and lab stations to automate procedures. The controller interfaces the operator with the robot. Software is menu-based and uses familiar laboratory terms. Zymark Corp., JLC/83/9, Zymark Center, Hopkinton, MA, 01748, USA.

APPLICATIONS GUIDE deals with sample preparation, highlighting background, principles, and techniques of solid phase extraction. The guide contains over 40 detailed procedures for preparing environmental, pharmaceutical, biological, food, and cosmetic samples, such as priority pollutants, crude oil, trace metals, aflatoxins, steroids, etc. J. T. Baker Chem. Co., JLC/83/9, 222 Red School Lane, Phillipsburg, NJ, 08865, USA.

HPTLC/TLC BIBLIOGRAPHY SERVICE is available free of charge. Publications may be included by mailing to the publisher. Applied Analytical Industries, Inc., JLC/83/9, Route 6, Box 55, Wilmington, NC, 28405, USA.

DETERGENT REMOVING GEL is an affinity chromatographic support that selectively removes detergents from protein solutions, with proteins being recovered in virtually 100% yields. The support can be regenerated for repeated use. Pierce Chemical Co., JLC/83/9 P. O. Box 117, Rockford, IL, 61105, USA.

AUTOMATED LC SAMPLE PREP/INJECTION SYSTEM combines solid-phase sample preparation and syringeless injection into a single operation. Samples are extracted by sorbent cassettes that selectively isolate and concentrate compounds of interest. Analytichem, International, Inc., JLC/83/9 24201 Frampton Avenue, Harbor City, CA, 90710, USA.

ANALYSIS OF FATTY ACIDS in cooking oils, lipid extracts, etc. necessitate an efficient separation approach prior to their quantitation in the laboratory. Previously accomplished by gas chromatography with derivatization, high resolution results are obtained with HPLC, particularly in analyses of rape seed oil and tall oils. Free acids are determined at the microgam level, and derivatized acids are determined at the nanogram level. Pye Unicam, Ltd., JLC/83/9 York Street, Cambridge CB1 2PX, England.

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(9), 1721-1724 (1983)

LC CALENDAR

1983

AUGUST 10-12: 22nd Canadian High Polymer Forum, Univ. of Waterloo, Canada. Contact: A. Garton, NRC of Canada, Div. of Chem., Ottawa, Ont., Canada, K1A OR6.

AUGUST 14-19: 25th Rocky Mountain Conference, Denver Convention Complex, Denver, Colorado. Contact: E. A. Brovsky, Rockwell International, P. O. Box 464, Golden, CO, 80401, USA.

AUGUST 15-19: Coal Science: 1983 Int'l Conference, Pittsburgh, PA. Contact: N. Maceil, JWK Int'l Corp., 275 Curry Hollow Road, Pittsburgh, PA, 15236, USA.

AUGUST 22-26: 7th Australian Symposium on Analytical Chemistry, Adelaide, Australia. Contact: D. Patterson, AMDEL, P.O.Box 114, Eastwood S.A. 5063, Australia.

AUGUST 26 - SEPTEMBER 2: Int'l. Symp. on Solvent Extraction, Denver, CO. Contact: D. Nowak, AIChE, 345 E. 47th St., New York NY, 10017, USA.

AUGUST 28 - SEPTEMBER 2: 11th World Petroleum Congress, London. Contact: Amer. Petrol. Inst., 2101 L St., N.W., Washington, DC, 20037, USA.

AUGUST 28 - SEPTEMBER 2: ACS 186th Nat'l Meeting, Washington, DC. Contact: A. T. Winstead, ACS, 1155 16th St., NW, Washington, DC, 20036, USA.

AUGUST 29 - SEPTEMBER 2: 4th Danube Symposium on Chromatography & 7th I'nt'l. Sympos. on Advances & Applications of Chromatography in Indudtry, Bratislava, Czech. Contact: Dr. J. Remen, Anal. Sect., Czech. Scientific & Technical Soc., Slovnaft, 823 00 Bratislava, Czechoslovakia.

SEPTEMBER 22-23: Symposium: "Columns in High Performance Liquid Chromatography," Lady Mitchell Hall, University of Cambridge. Contact: A. G. W. Mulders, Hewlett-Packard, GmbH, Postfach 1280, D-7517 Waldbronn 2, West Germany.

SEPTEMBER 25-30: Federation of Anal. Chem. & Spectroscopy Societies (FACSS) Conf., Philadelphia. Contact: M. O'Brien, Merck, Sharp & Dohme Res. Labs., West Point, PA, 19486, USA.

OCTOBER 2-6: 97th Annual AOAC Meeting, Shoreham Hotel, Washington, DC. Contact: K. Fominaya, AOAC, 1111 N. 19th St., Suite 210, Arlington, VA, 22209, USA.

OCTOBER 3-5: Chemexpo '83, Harbor Castle Hilton Hotel, Toronto, Ont., Canada. Contact: ITS Canada, 20 Butterick Rd., Toronto, Ont., Canada, M8W 3Z8.

OCTOBER 3 - 6: Advances in Chromatography: 20th Int'l Symposium, Amsterdam, The Netherlands. Contact: A. Zlatkis, Chem. Dept., University of Houston, Houston, TX, 77004, USA.

OCTOBER 12-13: 8th Annual Baton Rouge Anal. Instrum. Disc. Grp. Sympos., Baton Rouge, LA. Contact: G. Lash, P. O. Box 14233, Baton Rouge, LA, 70898, USA.

OCTOBER 12-14: Analyticon'83 - Conference for Analytical Science, sponsored by the Royal Society of Chemistry and the Scientific Instrument Manufacturers' Ass'n of Great Britain, Barbican Centre, London. Contact: G. C. Young, SIMA, Leicester House, 8 Leicester Street, London WC2H 7BN, England.

NOVEMBER 3-4 ACS 18th Midwest Regional Meeting, Lawrence, Kansas. Contact: W. Grindstaff, SW Missouri State Univ., Springfield, MO, 65802, USA.

NOVEMBER 9-11: ACS 34th SE Regional Meeting, Charlotte, NC. Contact: J. M. Fredericksen, Chem. Dept., Davidson College, Davidson, NC, 28036, USA.

NOVEMBER 14-16: 3rd Int'l. Sympos. on HPLC of Proteins, Peptides and Polynucleotides, Monte Carlo, Monaco. Contact: S. E. Schlessinger, 400 East Randolph, Chicago, IL, 60601, USA.

NOVEMBER 16-18: Eastern Analytical Symposium, New York Statler Hotel, New York City. Contact: S. David Klein, Merck & Co., P. O. Box 2000, Rahway, NJ, 07065, USA.

NOVEMBER 22-23: Short Course: "Sample Handling in Liquid Chromatography," sponsored by the Int'l. Assoc. of Environmental and Biological Samples in Chromatography, Palais de Beaulieu, Lausanne, Switzerland. Contact: Dr. A. Donzel, Workshop Office, Case Postale 130, CH-1000 Lausanne 20, Switzerland.

NOVEMBER 24-25: Workshop: "Handling of Environmental and Biological Samples in Chromatography," sponsored by the Int'l. Assoc. of Environmental Anal. Chem., Palais de Beaulieu, Lausanne, Switzerland. Contact: Dr. A. Donzel, Workshop Office, Case Postale 130, CH-1000 Lausanne 20, Switzerland.

1984

FEBRUARY 12-16: 14th Australian Polymer Symposium, Old Ballarat Travel Inn, Ballarat, Australia, sponsored by the Polymer Div., Royal Australian Chemical Inst. Contact: Dr. G. B. Guise, RACI Polymer Div., P. O. Box 224, Belmont, Victoria 3216, Australia.

APRIL 8-13: National ACS Meeting, St. Louis, MO. Contact: Meetings, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

MAY 20 - 26: 8th Intl. Symposium on Column Liquid Chromatography, New York Statler Hotel, New York City. Contact: Prof. Cs. Horvath, Yale University, Dept. of Chem. Eng., P. O. Box 2159, Yale Stn., New Haven, CT, 06520, USA.

AUGUST 26-31: National ACS Meeting, Philadelphia, PA. Contact: Meetings, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

OCTOBER 1-5: 15th Int'l. Sympos. on Chromatography, Nurenberg, West Germany. Contact: K. Begitt, Ges. Deutscher Chemiker, Postfach 90 04 40, D-6000 Frankfurt Main, West Germany.

1985

FEBRUARY 11-14: Polymer 85, Int'l Symposium on Characterization and Analysis of Polymers, Monash University, Melbourne, Australia, sponsored by the Polymer Div., Royal Australian Chemical Inst. Contact: Polymer 85, RACI, 191 Royal Parade, Parkville Victoria 3052, Australia.

APRIL 28 - MAY 3: 189th National ACS Meeting, Miami Beach. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

SEPTEMBER 8-13: 190th National ACS Meeting, Chicago. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA

1986

APRIL 6-11: 191st National Am. Chem. Soc. Mtng., Atlantic City, NJ. Contact: A. T. Winstead, ACS, 1155 16th Streeet, NW, Washington, DC, 20036, USA.

SEPTEMBER 7-12: 192nd National Am. Chem. Soc. Mtng., Anaheim, Calif. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA

1987

APRIL 5-10: 193rd National Am. Chem. Soc. Mtng., Denver, Colo. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

AUGUST 30 - SEPTEMBER 4: 194th National Am. Chem. Soc. Mtng., New Orleans, LA. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

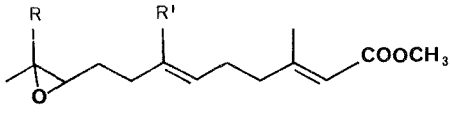
The Journal of Liquid Chromatography will publish announcements of interest to liquid chromatographers in every issue of the Journal. To be listed in the LC Calendar, we will need to know: Name of the meeting or symposium, sponsoring organization, when and where it will be held, and whom to contact for additional details. You are invited to send announcements to Dr. Jack Cazes, Editor, Journal of Liquid Chromatography, P. O. Box 1440-SMS, Fairfield, CT, 06430, USA.

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(9), 1725 (1983)

ERRATUM

E. S. Chang, J. Liq. Chrom., 6(2), 291-299 (1983)
"Analysis of Insect Hormones by Means of a Radial Compression Separation System."

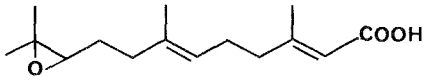
Figure 1 in this paper was incorrect. The correct structures for Figure 1 are:



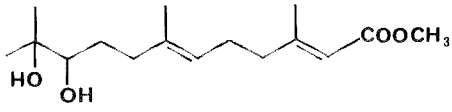
JH I: R: Et, R': Et

JH II: R: Et, R': Me

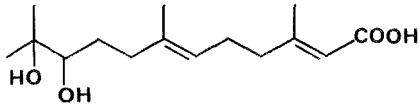
JH III: R: Me, R': Me



JH III acid



JH III diol



JH III acid–diol

Corrected A.P. 29.10.

INSTRUCTIONS FOR PREPARATION OF MANUSCRIPTS FOR DIRECT REPRODUCTION

Journal of Liquid Chromatography is a bimonthly publication in the English language for the rapid communication of liquid chromatographic research.

Directions for Submission

One typewritten manuscript suitable for direct reproduction, carefully inserted in a folder, and two (2) copies of the manuscript must be submitted. Since all contributions are reproduced by direct photography of the manuscripts, the typing and format instructions must be strictly adhered to. Noncompliance will result in return of the manuscript to the authors and delay its publication. To avoid creasing, manuscripts should be placed between heavy cardboards and securely bound before mailing.

Manuscripts should be mailed to the Editor:

Dr. Jack Cazes
Journal of Liquid Chromatography
P. O. Box 1440-SMS
Fairfield, Connecticut 06430

Reprints

Owing to the short production time for articles in this journal, it is essential to indicate the number of reprints required upon notification of acceptance of the manuscript. Reprints are available in quantities of 100 and multiples thereof. For orders of 100 or more reprints, twenty (20) free copies are provided. A reprint order form and price list will be sent to the author with the notification of acceptance of the manuscript.

Format of Manuscript

1. The general format of the manuscript should be as follows: title of article; names and addresses of authors; abstract; and text discussion.

2. Title and Authors: The entire title should be in capital letters and centered on the width of the typing area at least 2 inches (5.1 cm) from the top of the page. This should be followed by three lines of space and then by the names and addresses of the authors in the following way (also centered):

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

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

3. Abstract: Three lines below the addresses, the title ABSTRACT should be typed (capitalized and centered on the page). This should be followed by a single-spaced, concise, abstract comprising less than 10% of the length of the text of the article. Allow three lines of space below the abstract before beginning the article itself.

4. Text Discussion: Whenever possible, the text discussion should be divided into such major sections as INTRODUCTION, MATERIALS, METHODS, RESULTS, DISCUSSION, ACKNOWLEDGMENTS, and REFERENCES. These major headings should be separated from the text by two lines of space above and one line of space below. Each heading should be in capital letters, centered, and underlined. Secondary headings, if any, should be flush with the left margin, underscored, and have the first letter of all main words capitalized. Leave two lines of space above and one line of space below secondary headings.

5. Paragraphs should be indented five (5) typewriter spaces.

6. Acknowledgment of collaboration, sources of research funds, and address changes for an author should be listed in a separate section at the end of the paper.

7. References (including footnotes) in the text will be numbered consecutively by numbers in parentheses. All references (and footnotes) should then be aggregated in sequence at the end of the communication. No footnotes should be shown at the bottom of pages. The reference list follows immediately after the text. The word REFERENCES should be capitalized and centered above the reference list. It should be noted that all reference lists should contain initials and names of all authors; *et al.* will not be used in reference lists. Abbreviations of journal titles and styles of reference lists will follow the American Chemical Society's Chemical Abstracts List of Periodicals. References should be typed single-spaced with one line space between each reference.

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

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

10. Any material that cannot be typed, such as Greek letters, script letters, and structural formulae, should be drawn carefully in black India ink (do not use blue ink).

Typing Instructions

1. The manuscript must be typewritten on good quality white bond paper measuring approximately 8½ x 11 inches (21.6 cm x 27.9 cm). Do not use Corrasible bond or its equivalent. The typing area of the article opening page, including the title, should be 5½ inches wide by 7 inches deep (14 cm x 18 cm). The typing area of all other pages should be no more than 5½ inches wide by 8½ inches deep (14 cm x 21.6 cm).

2. In general, the chapter title and the abstract, as well as the tables and references, are typed single-spaced. All other text discussion should be typed 1½-line spaced, if available, or double-spaced. Prestige elite characters (12 per inch) are recommended, if available.

3. It is essential to use black typewriter ribbon (carbon film is preferred) in good condition so that a clean, clear impression of the letters is obtained. Erasure marks, smudges, creases, etc., may result in return of the manuscript to the authors for retyping.

4. Tables should be typed as part of the text but in such a way as to separate them from the text by a three-line space at both top and bottom of each table. Tables should be inserted in the text as close to the point of reference as possible, but authors must make sure that one table does not run over to the next page, that is, no table may exceed one page. The word TABLE (capitalized and followed by an Arabic number) should precede the table and be centered on the page. The table title should have the first letters of all main words in capitals. Titles should be typed single-spaced. Use the full width of the type page for the table title.

5. Drawings, graphs, and other numbered figures should be professionally drawn in black India ink (do not use blue ink) on separate sheets of white paper and placed at the end of text. Figures should not be placed within the body of the text. They should be sized to fit within the width and/or height of the type page, including any legend, label, or number associated with them. Photographs should be glossy prints. A typewriter or lettering set should be used for all labels on the figures or photographs; they may not be hand drawn. Captions for the pictures should be typed single-spaced on a separate sheet, along the full width of the type page, and preceded by the word FIGURE and a number in arabic numerals. All figures and lettering must be of a size to remain legible after a 20% reduction from original size. Figure numbers, name of senior author, and arrow indicating "top" should be written in light blue pencil on the back or typed on a gummed label, which should be attached to the back of the illustration. Indicate approximate placement of the illustrations in the text by a marginal note in light blue pencil.

6. The reference list should be typed single-spaced although separated from one another by an extra line of space. Use Chemical Abstract abbreviations for journal titles. References to journal articles should include (1) the last name of all author(s) to any one paper, followed by their initials, (2) article title, (3) journal, (4) volume number (underlined), (5) first page, and (6) year, in that order. Books should be cited similarly and include (1) author, surname, first and middle initials, (2) title of book, (3) editor of book (if applicable), (4) edition of book (if any), (5) publisher, (6) city of publication, (7) year of publication, and (8) page reference (if applicable). E.g., Journals: Craig, L. C. and Konigsber, W., Use of Catechol Oxygenase and Determination of Catechol, Chromatogr., 10, 421, 1963. Books: Albertsson, P. A., Partition of Cell Particles and Macromolecules, Wiley, New York, 1960. Article in a Book: Walter, H., Proceedings of the Protides of Biological Fluids, XVth Colloquim, Pteeters., H., eds., Elsevier, Amsterdam, 1968, p. 367.

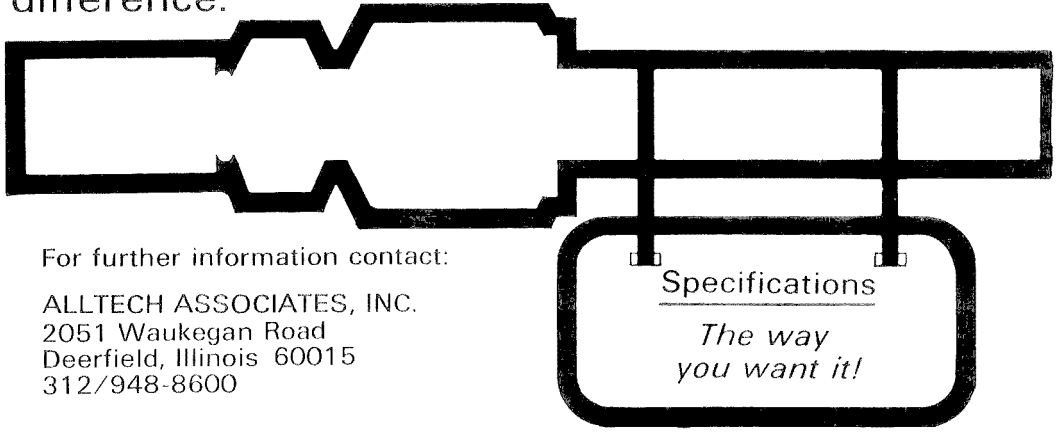
Custom packing HPLC columns has become our specialty. Any length, several ID's (including 3.2mm) and almost any commercially available packing material may be specified. We'll supply the column others won't.

With each column, you will receive the original test chromatogram plus a vial of the test mixture. Our advanced technology and computer testing is your assurance of a quality product.

When custom packing and testing is your special concern, we make the difference.

Each one is our special concern

CUSTOM PACKED HPLC COLUMNS



For further information contact:

ALLTECH ASSOCIATES, INC.
2051 Waukegan Road
Deerfield, Illinois 60015
312/948-8600

ALLTECH ASSOC

Circle Reader Service Card N

Fluorescence & HPLC— GILSON is the new choice.

When your applications require the increased sensitivity and detection capabilities of fluorometry—add the affordable Model 121 Fluorometer to your system.

Key features of the Model 121 include:

- Innovative design to greatly increase light collection efficiency and reduce scatter
- Dynamic range through five orders of magnitude
- Modular assemblies for easy change of filters
- Analytical and microbore flow cells
- Auto-zero function for one-step baseline zeroing
- Switch-selectable time constants
- Standard and auxiliary outputs for connection to rec... or data systems

Whether you nee... ...nd-alone fluorometer o... ...con... ...with ... ...S detectors, Model 121 ...

...form... contact us or ...

...ese...