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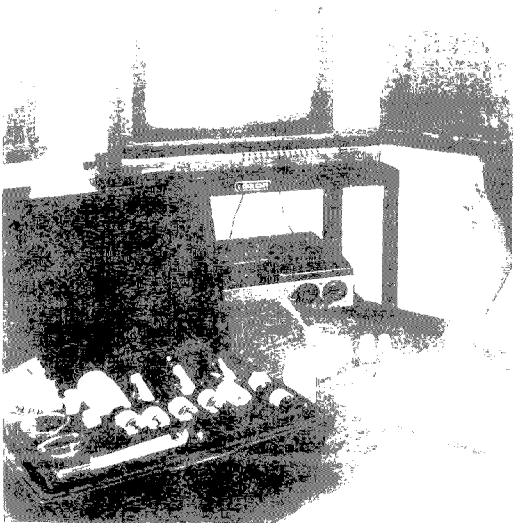

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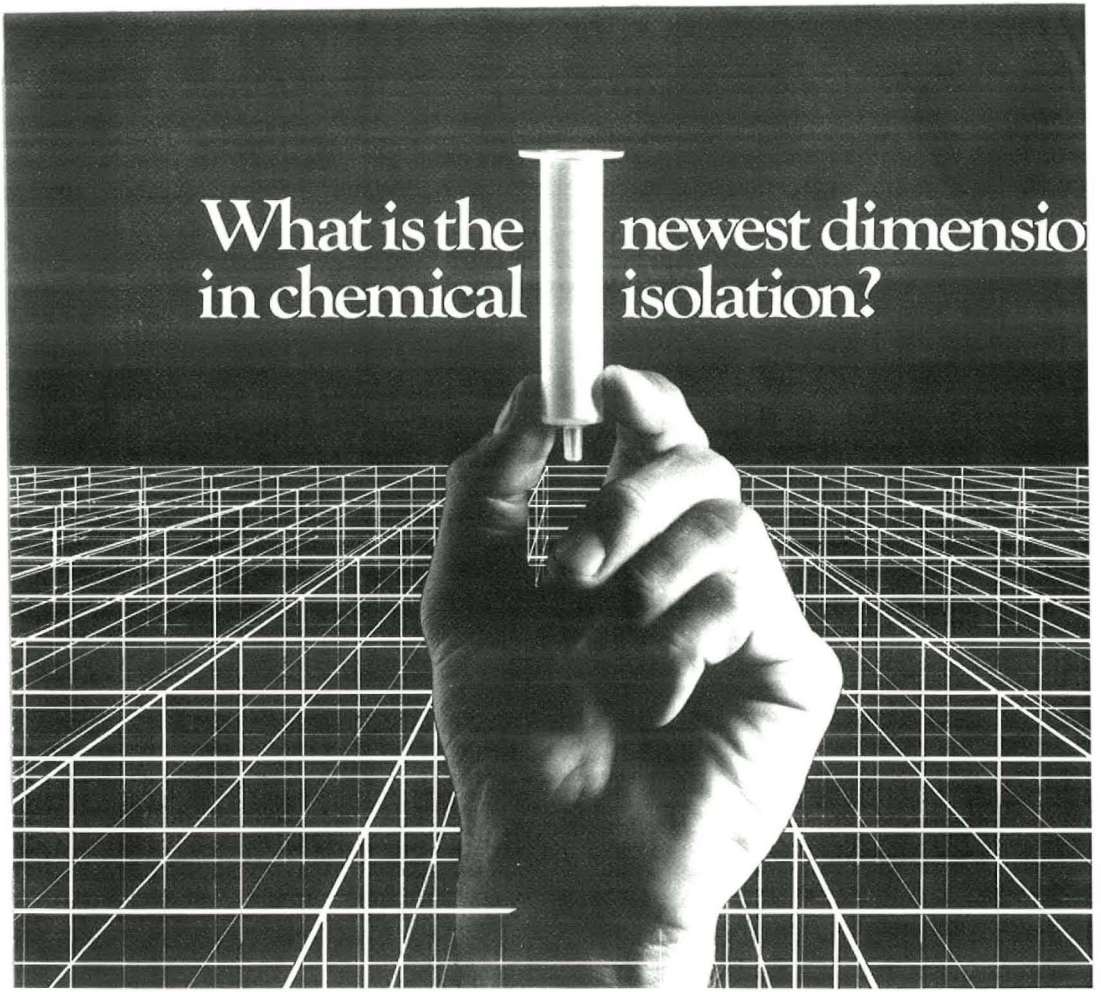

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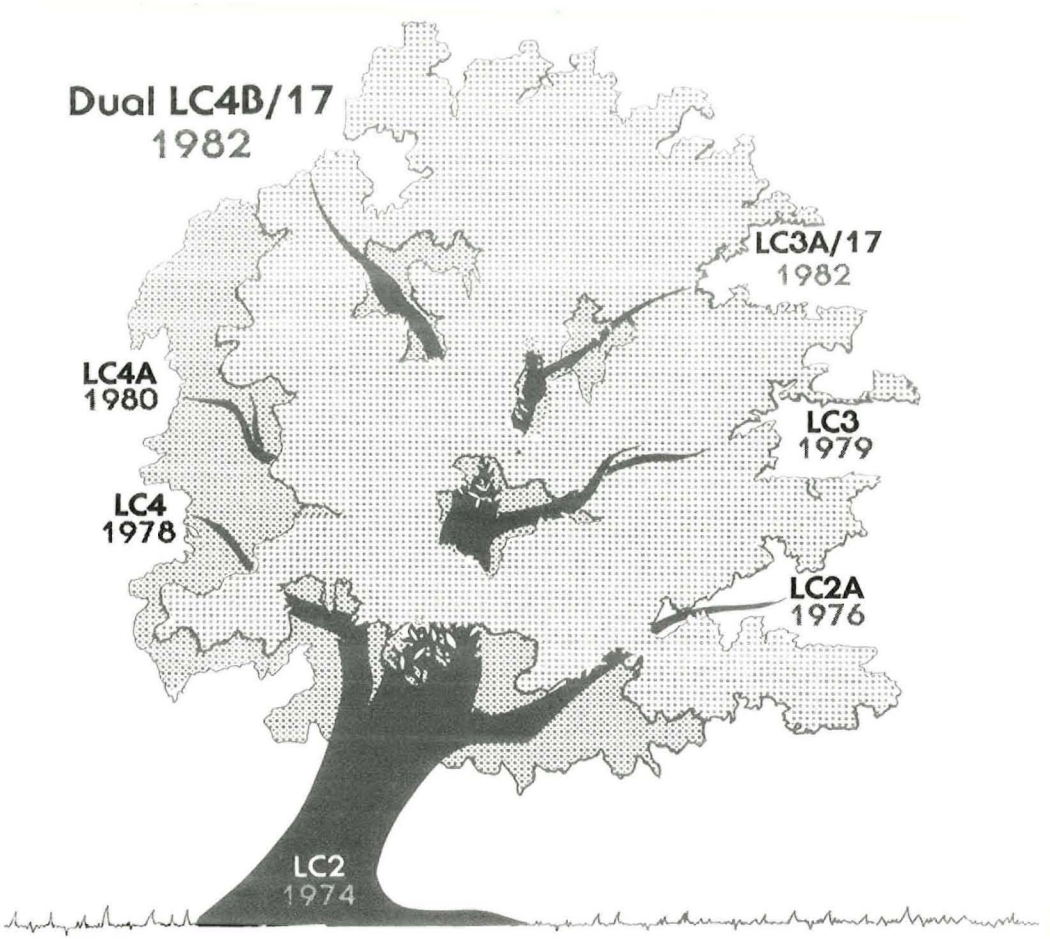

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JOURNAL OF LIQUID CHROMATOGRAPHY, 5(12), 2227-2239 (1982)

MEASUREMENT OF THE DEAD VOLUME BETWEEN CONCURRENT DETECTORS IN GEL PERMEATION CHROMATOGRAPHY

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ABSTRACT

Multiple detection of gel permeation chromatography effluent is going to become a routine way of polymer characterization in most laboratories. It is then necessary to give attention to the dead volume connecting the cells of concurrent detectors. If neglected, considerable errors may occur in data handling.

We report, in this paper, a method of dead volume measurement. It firstly concerns the coupling of a high pressure gel permeation chromatograph and the "Ouano"-type continuous viscometer, but its general utility is demonstrated. The main conclusion is that the geometric estimate of the dead volume is not suitable, even when an accurate calculation is possible. The method we describe here leads to an experimental dead volume. It is greater than the geometric one (as theoretically proved recently) but it gives the best results.

INTRODUCTION

With the increasing use of Gel Permeation Chromatography (GPC) as a characterization tool for complex polymeric samples, a fast development in dual detection techniques for the effluent is observed. Indeed, it has been known for a long time, except for linear homopolymers, that the size exclusion mechanism does not lead to the separation of macromolecules according to their

molecular weights. As a result, the response of a single concentration detector is often inadequate to give reliable average molecular weights. For the analysis of copolymers with variable composition, the differential refractometer (DRI)-UV detector coupling is particularly used (see the review of Janca (1)). On another hand, the Benoit's universal parameter $[\eta].M$ as a calibration concept (2) requires the fitting of GPC with a molecular size detection, i.e. viscometry (3-4) or light scattering (5-6). But till now, the two detections have never been performed in a single cell. Consequently, accurate data treatment requires the knowledge of the connecting dead volume, ΔV, between detectors so that any pair of experimental points really corresponds to the same species.

From the study of Bruessau (7), the geometric determination of the ΔV value leads to unsatisfactory results, because changes occur in the peak shape during transfer form one cell to the other. That is why this author recommends a method which is suitable to the particular case of two concentration detectors (DRI and UV for instance). According to Cantow (8), the analysis of homopolymers under such conditions gives two similar chromatograms, one of them has to be shifted till their ratio is a straight line parallel to the abscissa. The resulting ΔV value is valid for further calculations. Nevertheless, this method is presented as invalid in a concentration detector-molecular size detector coupling since there is no specific case where the two responses are proportional.

In the laboratory, we have been dealing for a few years (9) with the coupling of a high pressure GPC and an "Ouano"-type continuous viscometer (10). Such a system enables one to rapidly characterize various macromolecular samples (11), even at high temperatures, as recently proved (12). But, the accuracy of the results (especially the viscosity laws) is dependent on the ΔV value. For this reason, we have improved the above described method of ΔV measurement for the concentration detector-molecular size detector coupling.

EXPERIMENTAL

The continuous viscometric detector is an application of the Poiseuille's law. Its principle has been previously described (10-11). Assuming laminar flow through a capillary tube, we can employ the following relationship :

$$P = \frac{8}{\pi} \cdot \frac{l}{r^4} \cdot Q \cdot \eta$$

with - P : pressure drop of the fluid
- Q : flow rate
- l,r : length and radius of the capillary tube
- η : absolute viscosity.

Accordingly, the continuous pressure drop measurement (besides the concentration, C, detection) enables the determination of the intrinsic viscosity $[\eta]$ along with the elution volume (for very low C values) :

$$[\eta] = \frac{1}{C} \ln \frac{P}{P_o}$$

where subscrip, o, refers to pure solvent.

A scheme of the apparatus is given in Figure 1. A stainless steel capillary tube is inserted between the column set and the refractometer (Waters R 401). Two pressure transducers CMAC 5 (Sedeme, 11, rue Simonet - F-75013 PARIS), performing the differential measurement of the pressure drop, are connected by "T" fittings into the system. If it is necessary to select the viscometer length over a wide range, it is important to recognize that the inner diameter of the commercially available 9/1,000-inch capillary fluctuates greatly. By measuring the pressure drops through several capillaries with equal lengths, we observed

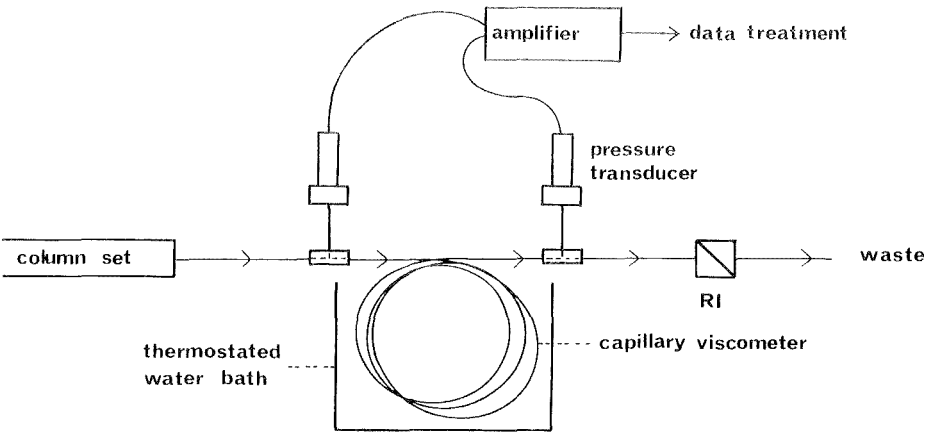

Figure 1 : Scheme of the apparatus.

large discrepancies, the ratio between the extremes being about 3. Poiseuille's law leads to the following range :

$$0.10 < r < 0.14 \text{ mm}$$

Thus, the continuous viscosmeter is required for a precise measurement of the capillary radius. If not, errors in the geometric determination of dead volumes may be as large as 100%.

For our purpose, we have chosen a capillary with r = 0.135 mm and l = 3m. The difference between the coupling of two detectors with cells of negligible volume (I) and the particular case of DRI-continuous viscometer (II) is shown in Figure 2. For the latter, the ΔV value is the sum of two terms : the dead volume of connecting capillaries and half the volume of the viscometer itself. From the Poiseuille's law, we deduced the geometric estimate :

$$\Delta V_G \simeq 0.15 \text{ ml}$$

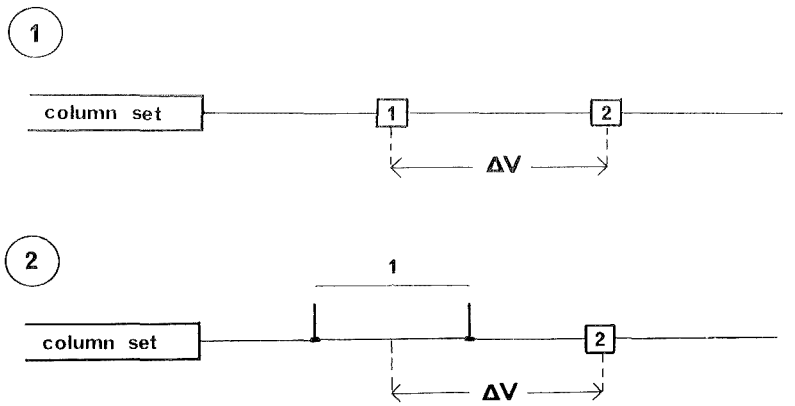

Figure 2 : Difference between the coupling of two detectors with cells of negligible volume (1) and the DRI-continuous viscometer coupling (2).

Routinely, experiments were run under the following conditions :

Solvent : THF ; Flow rate : 1ml/mn ; Temperature : 30°C.
Column set : 4 μ styragel 10^3, 10^4, 10^5 and 10^6 Å

The apparatus was equipped with a system of automatic recording, data treatment and graphic output (12) whose performances are demonstrated in Figure 3, 4 and 5 with a polyurethane sample. After smoothing of the chromatograms (Figure 3), the viscosity law was deduced from universal calibration (Figure 4) as well as the molecular weight distribution shown in Figure 5. In this case, we obtained :

$\bar{M}n$ = 37,000
$\bar{M}w$ = 58,000
$[\eta]$ = 109 ml/g
α = 0.68 (Mark-Houwink exponent)

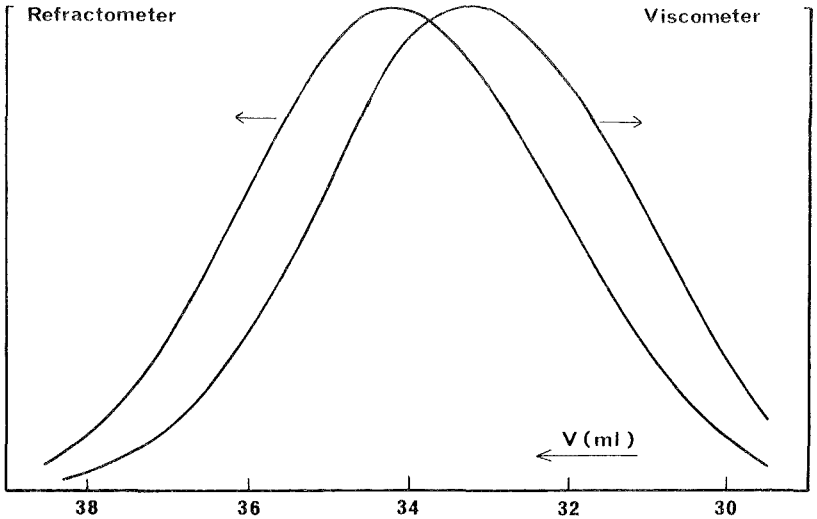

Figure 3 : Polyurethane sample : refractometric and viscometric responses.

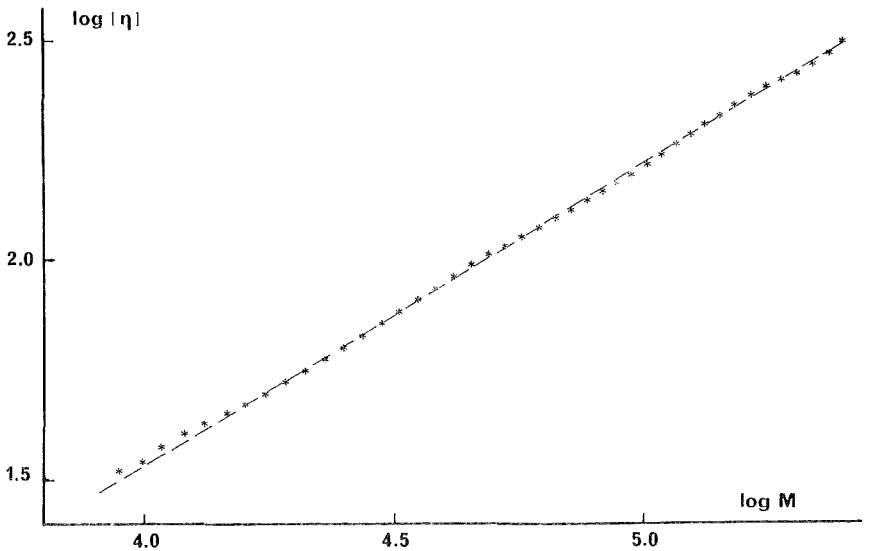

Figure 4 : The experimental Mark-Houwink relationship.

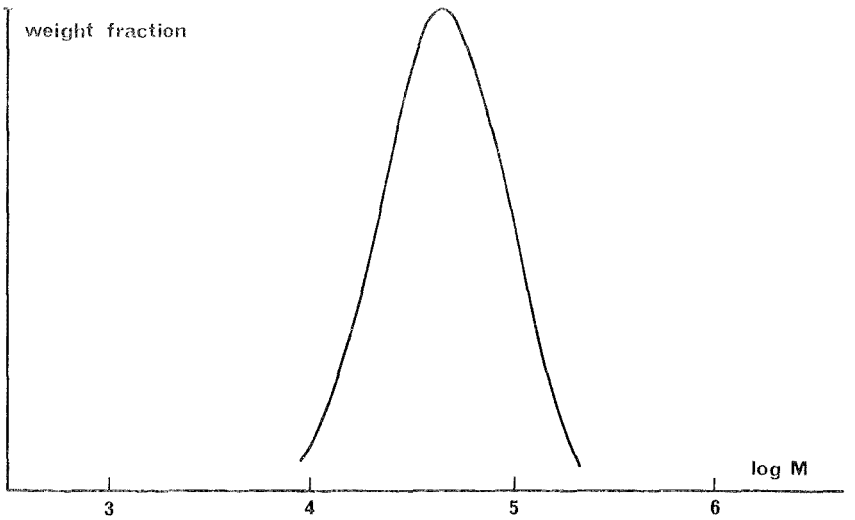

Figure 5 : The molecular weight distribution.

The above mentioned values are linked to a certain ΔV. But before describing the method which provides this ΔV value, we will demonstrate, using a simple model, that a good accuracy is required.

DISCUSSION

From a theoretical viewpoint, we will assume, a column set with a linear calibration curve of molecular weight, M, versus the elution volume, V :

$$\ln M = a - bV$$

Let us consider a macromolecular sample with a molecular weight distribution of the Wesslau type :

$$w\ (\ln M) = \frac{1}{\beta\sqrt{\pi}} \exp\ (-\frac{1}{\beta^2} \ln^2 \frac{M}{M_p})$$

M_p is the peak molecular weight with peak elution volume V_p and β is a function of polydispersity I :

$$\beta^2 = 2 \ln I$$

The viscosity law for this sample is : $[\eta] = KM^{\alpha}$

Using the following notations :

C(V) concentration chromatogram
S(V) pressure drop chromatogram ($S = P - P_o$)

we obtain, after a few steps :

$$C(V) = Cm \cdot \exp\left(-\frac{b^2}{\beta^2}(V - V_p)^2\right)$$

$$S(V) = Sm \cdot \exp\left(-\frac{b^2}{\beta^2}(V - (V_p - \frac{\alpha \ln I}{b}))^2\right)$$

where Cm and Sm are the respective apexes of the two chromatograms. It appears, when the concentration chromatogram has a gaussian shape, that the pressure chromatogram has also a gaussian equation. The standard deviation is the same but the peak apex of the pressure drop curve is shifted towards lower elution volumes :

$$V'_p = V_p - \frac{\alpha \ln I}{b}$$

(even if the two detections are performed in a single cell). Under routine conditions ($\alpha = 0.7$ and $b \simeq 0.4$), we obtain :

$$V_p - V'_p \simeq 1.75 \ln I$$

and, for instance $V_p - V'_p \simeq 1.2$ ml for $I = 2$.

The above mentioned example for polyurethane is not very far from the Wesslau distribution and we can observe in Figure 3 that

the experimental shift is in satisfactory agreement with the theoretical one.

It is, therefore, easy to predict the consequences of a bad estimate of ΔV : it leads to a wrong value of the expression $\frac{\alpha \ln I}{b}$. If ΔV is underestimated (as when neglected), α will be too high, and vice versa. In addition, the smaller the polydispersity index, the more important the errors become.

Such results can be experimentally confirmed with the assistance of a computer. By varying the ΔV value in the calculations, we obtained, for the polyurethane sample :

- $\Delta V = 0$: $\alpha \simeq 0.8$
- ΔV correct : $\alpha \simeq 0.7$
- $\Delta V \times 2$: $\alpha \simeq 0.6$

with the subsequent errors in the average molecular weights. Consequently, it is very important to use a reliable method of ΔV measurement.

DESCRIPTION OF THE METHOD

The method we describe is based on Cantow's method (7), but modified because of the nature of specific responses of molecular size detectors. Their responses, indeed, are not proportional to the concentration but are proportional either to C.[η] (viscometer) or C.M. (LALLS). The only way to consider them as concentration detectors is to avoid the size exclusion mechanism so as to obtain two similar signals. In our particular case (DRI-viscometer coupling), the first trials were performed by injecting a polymeric sample after removing the columns. But this method is not suitable, since polymer elution is too fast and the chromatograms are very distorted. It is therefore necessary to keep a column set in the system, but one has to select low porosity

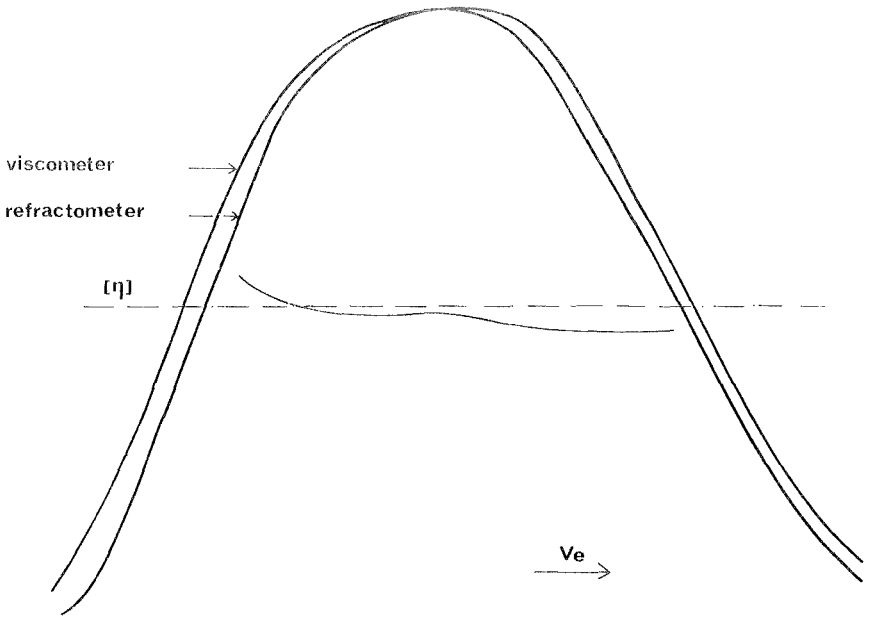

Figure 6 : Underestimated shift volume. After linear regression, [η](V) exhibits a negative slope.

packings so that a high molecular weight polymer is totally excluded and axial dispersion is the only factor responsible for spreading area. Measurements were performed under the following experimental conditions :

- Column set : 2 μ styragel (100 Å and 500 Å)
- Solvent : THF ; Flow rate : 1ml/mn ; Temperature : 30°C
- Sample : polystyrene standard $\bar{M}w \simeq 10^6$ (I < 1.1)
- Injected volume : 100μl ; Concentration 0.5%

In order to precisely measure the dead volume in our system, about twenty injections were made. All parameters were kept constant, except the shift volume (converted in terms of delay

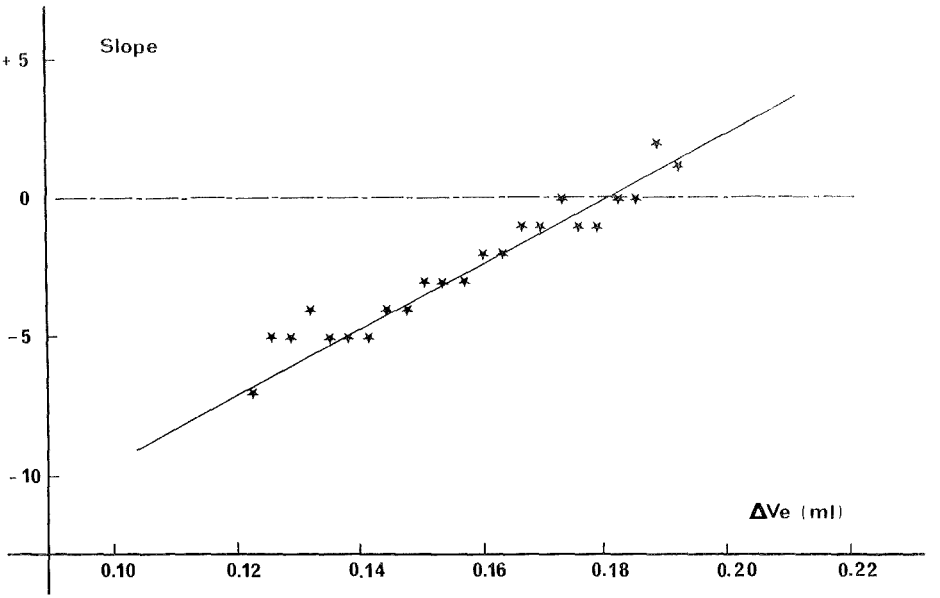

Figure 7 : The experimental shift volume is the abscissa of the intercept.

time in the automatic dual data acquisition system) which was gradually increased in the range of the geometric estimate. In each case, we plotted the apparent intrinsic viscosity $[\eta](V) = \frac{1}{C} \ln \frac{P}{P_o}$ (with the already mentioned notations) versus the elution volume (see Figure 6 as an example). The slope is calculated in arbitrary units using a linear regression method. The experimental ΔV value (ΔV_E) is defined by the zero slope : $[\eta](V)$ is constant and equal to the true intrinsic viscosity $[\eta]$. The slope measurement is relatively imprecise but a linear regression on slopes versus shift volume (shown in Figure 7) enabled us to reduce the uncertainty to a few percent. It leads to :

$$\Delta V_E = 0.18 \text{ ml}$$

This value, greater than the geometric one (ΔV_G = 0.15 ml), confirms the recent result of Bruessau (7). In addition, we compared these two values as delay time for the dual data acquisition in the case of routine experiments. The use of ΔV_E leads to correct viscometric results, when ΔV_G gives Mark-Houwink exponents α systematically overestimated. Both these reasons bring us to recommend the use of this method in preference to the geometric estimate.

CONCLUSION

The results presented in this paper clearly indicate that the shift volume between the detectors in DRI-viscometer coupling has to be carefully taken into account in the treatment of GPC data. Model simulation as well as experimental evidence confirm the need for good accuracy. Therefore, we developed an easy method of dead volume measurement, which can be used for any kind of GPC dual detection, but is especially adapted to a molecular size detector (viscometer, LALLS) coupling. The resulting value appears to lead to satisfactory results in our system. Moreover, it is in good agreement with a recent theoretical model which shows that the geometrical estimate is systematically smaller than the value to be used in the data acquisition, probably due to change in the peak shape during transfer between the two detectors.

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MOLECULAR WEIGHT DISTRIBUTIONS AND MONODISPERSE INTRINSIC VISCOSITY-MOLECULAR WEIGHT EQUATION OF SILICONE ELASTOMER*

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ABSTRACT

The experimental curves of Gel Permeation Chromatography of methyl vinylsilicone elastomer polymerized by a basic catalyst and containing a small amount vinyl groups are given.

They are in consistent with the theoretical curves calculted from logarithmic-normal distribution function. Thereby, the molecular weight distribution of the samples determined belong to the logarithmic-normal distribution. With GPC, $[\eta]$ and $\langle M \rangle_n$ of the polydispersed sample the momodispersed $[\eta]$ - M relationship of the methyl vinyl silicone elastomer was established.

This relation agrees with the momodispersed $[\eta]$ - M relation established by Qian Ren-yuan, et al.[1]

*Presented at the GPC Symposium, Chinese Chemical Soc., Guilin, People's Republic of China, June 2-6,1981.

INTRODUCTION

The Mark-Houwink equation of the intrinsic viscosity-molecular weight relationship of high polymer solution shows,

$$[\eta] = K M^{\alpha} \qquad (1)$$

The molecular weight obtained only from the parameter K and α in the monodispersed equation for the $[\eta]$ value of a polydispersed sample is in accordance with the viscosity average molecular weight of which the definition is well-known.

$$\langle M \rangle_{\eta} = \left(\sum_{i=1}^{n} W_i M_i^{\alpha} \right)^{1/\alpha} \qquad (2)$$

In determining the parameter in the equation, monodispersed samples are necessary. But they are difficult to obtain, even with careful fractionation of a polydispersed sample. Therefore, a correction of dispersed effect must be made on the equation obtained experimentally.[2,3,4]

The relation between intrinsic viscosity $[\eta]$ and average molecular weight, $\langle M \rangle_w$ or $\langle M \rangle_n$, for a polydispersed sample is:

$$\langle [\eta] \rangle = K_w \langle M \rangle_w^{\alpha} = q_w K \langle M \rangle_w^{\alpha} \qquad (3)$$

or

$$\langle [\eta] \rangle = K_n \langle M \rangle_n^{\alpha} = q_n K \langle M \rangle_n^{\alpha} \qquad (4)$$

Where q_n and q_w are the correction factors polydispersed. They are related with types of distribution of the sample and the distribution breadth.[2,3,4] Therefore, to obtain monodispersed Mark-Houwink equation, a definite knowledge of the molecular weight distribution of the sample must be known.

Qian Ren-yuan et al.[1] established monodispersed $[\eta]$ - M relationship of polydimethylsiloxane after determining the distribution of sedimentation coefficients, $\langle M \rangle_w$, $[\eta]_{\theta}$ and $[\eta]_{toluene}$.

However, the molecules of the commercial silicone elastomer contain a small amount of vinyl groups. In order to study their influence on the $[\eta]$ - M relationship, we determined the molecular weight

distribution of some polydispersed samples of polymethyl vinyl siloxane by Gel Permeation Chromatograph, as well as the number-average molecular weights and intrinsic viscosities in toluene solution for each sample. The monodispersed equation of polymethyl vinyl siloxane is so obtained. It agrees with the monodispersed equation of polydimethylsiloxane proposed by Qian Ren-yuen et al. Thus, the existence of a small amount of vinyl groups has no obvious influence on the solution properties of polysiloxane.

EXPERIMENTAL

SAMPLES

The silicone elastomer samples used are non-fractionated polydispersed samples from our laboratory. They were obtained by polymerizing the dimethylcyclosiloxane containing a small amount of cyclic vinyl siloxane at the presence basic catalyst.

VISCOMETRY

The intrinsic viscosity of the polydimethyl vinyl siloxane was determined in toluene at 25 °C by ubbelonde dilution viscometer.

OSMOTIC PRESSURE

The number-average molecular weight of the polymethyl vinyl siloxane was determined in toluene at 27 °C with ANAUER membrane osmometer.

GEL PERMEATION CHROMATOGRAPH

The determination of Gel Permeation Chromatograph was accomplished in an equipment with the column length 2.5m, interal diameter 9 mm, syphon volume 3.0 ml and porous silica beads of 120 - 140 mesh as the packing (treated with hexamethyldilazane). Whole elution volume of the column was 130 ml. Eluted solvent was toluene. Operated at room temperature with a flow rate of 0.7 ml / min. The concentration of the injected samples of polystyrene and polymethyl vinyl siloxane were 1 mg / ml and 4 mg / ml respectively. Injected volume was 1 ml. The concentra-

tions of the eluted solution were determined by the turbidity mothed.

The column was caliorated with a series of narrowly distributed polystyrene samples. The viscosity equation of the polystyrene is

$$[\eta] = 1.1 \times 10^{-4} M^{0.73} \qquad (5)$$

The universal calibration relation of the column is obtained:

$$\log J = \log[\eta] M$$

$$\log J = 13.08 - 0.079 V \qquad (6)$$

The universal calibration curves is shown in Figure 1.

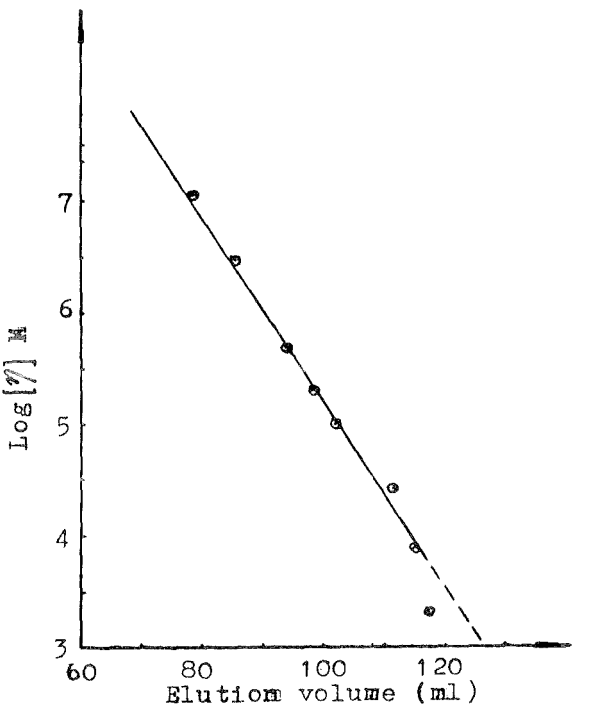

Figure 1. Universal calibration curve

RESULTS AND DISCUSSION

The GPC curves of all the samples are shown in Figure 2. According to the following equation,

$$\bar{V} = \sum_{i=1}^{n} W_i \, V_i$$

$$\sigma^2 = \sum_{i=1}^{n} W_i \, (V - \bar{V})^2$$

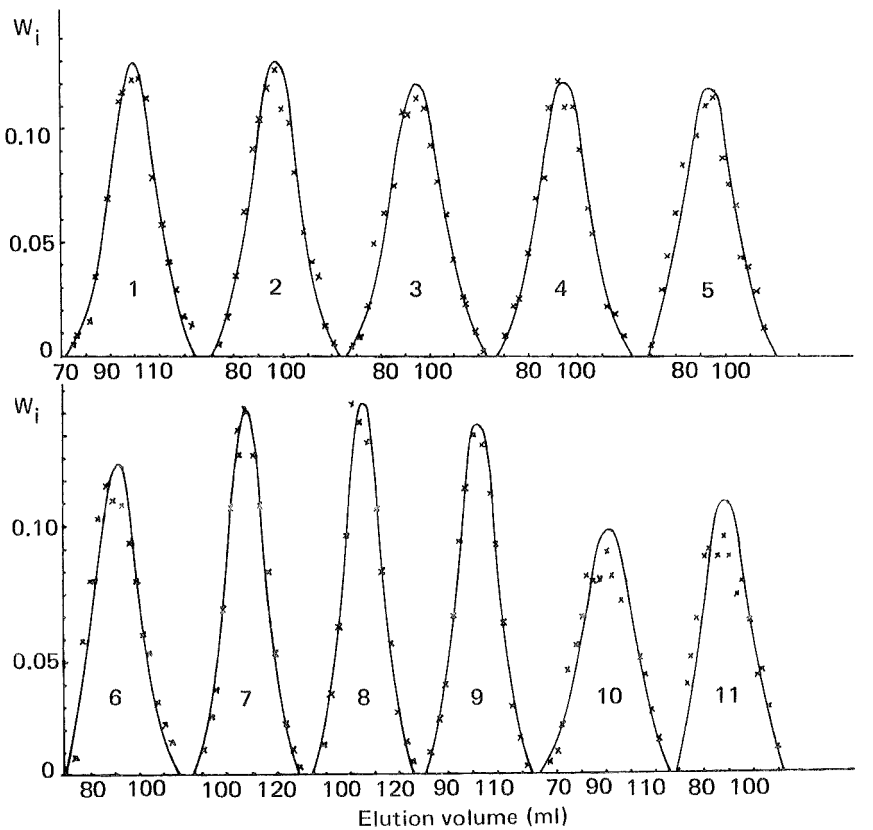

Figure 2.

Table 1.

$\bar{V}$, σ^2 , $[\eta]$, $\langle M \rangle_n$, $\langle M \rangle_w$ / $\langle M \rangle_n$ of the samples

sample	$\bar{V}$ (ml)	σ^2	$[\eta]$ (dl/g)	$\langle M \rangle_n \times 10^{-4}$ (calc.)	$\langle M \rangle_w / \langle M \rangle_n$
1	99.51	91.53	0.84	14.7	2.80
2	97.17	90.83	1.03	20.0	2.72
3	94.85	112.26	1.17	22.5	3.43
4	94.95	102.65	1.24	25.4	3.10
5	93.60	107.32	1.39	30.0	3.18
6	91.83	82.70	1.60	39.2	2.48
				average	2.95
7	107.86	56.74	0.42	8.7	1.89
8	105.77	54.13	0.45	9.2	1.84
9	102.60	59.56	0.58	13.2	1.95
10**	90.93	99.43	1.11	34.8	1.81
11**	89.52	82.04	1.24	42.2	1.63
				average	1.82

: The data for 10 and 11** were determined by another column with the universal calibration equation as

$$\log J = 11.06 - 0.058\ \bar{V}$$

and from our experimental curves, calculted the mean elution volume $\bar{V}$ and standard deviation σ^2 as shown in Table 1. In the equation, W_i and V_i are the weight fraction and elution volume of each individual fraction. $[\eta]$ and $\langle M \rangle_n$ of each sample obtained from the viscosity and osmotic pressure measurements are also listed in Table 1.

Log $[\eta]$ values were plotted against log $\langle M \rangle_n$ for each sample in Figure 3.

Line a in Figure 3 is for the samples 1 to 6, and line b for the samples 7 to 11. $\alpha = 0.70$ and $K_m = 2.0 \times 10^{-4}$ are taken from the slope and the intercept of the line a, $\alpha = 0.70$ and $K_m = 1.51 \times 10^{-4}$ from the slope and intercept of the line b.

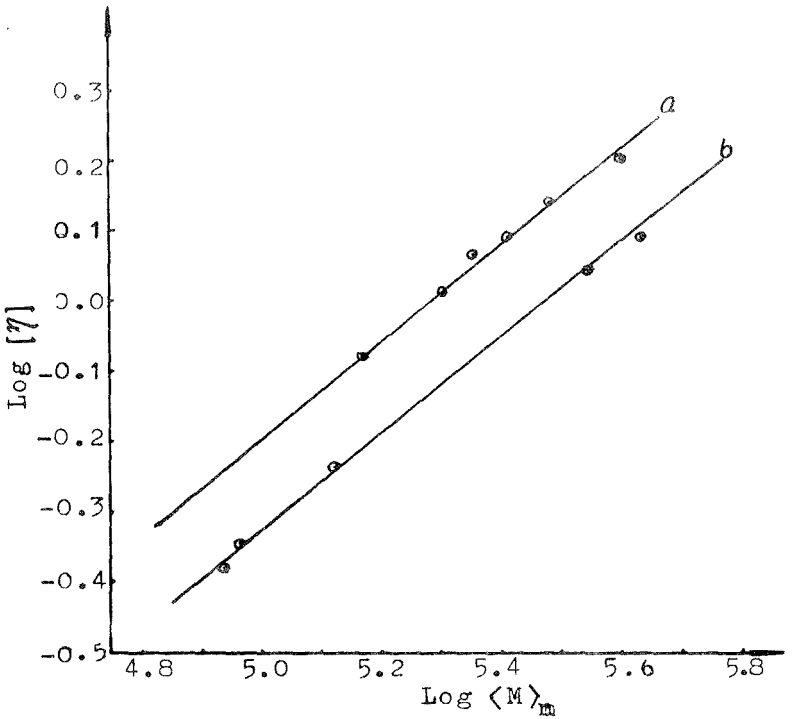

Figure 3. Log $[\eta]$ vs. log $\langle M \rangle_m$

In determining the average molecular weight of polymers by GPC, the principle of the universal calibration may be used, i.e.

$$\langle M \rangle_w = K^{-\frac{1}{1+\alpha}} \cdot \sum_{i=1}^{n} W_i J_i^{\frac{1}{1+\alpha}} \qquad (7)$$

$$\langle M \rangle_m = K^{\frac{1}{1+\alpha}} \Big/ \sum_{i=1}^{n} W_i J_i^{-\frac{1}{1+\alpha}} \qquad (8)$$

$$\frac{\langle M \rangle_w}{\langle M \rangle_n} = \sum_{i=1}^{n} W_i J_i^{\frac{1}{1+\alpha}} \cdot \sum_{i=1}^{n} W_i J_i^{-\frac{1}{1+\alpha}} \qquad (9)$$

Therefore, distribution breadth index, $\langle M \rangle_w / \langle M \rangle_n$, obtained are independent of the parameter K. From the experimental curve of each sample take $\alpha = 0.70$ (the slope of a, b lines in Figure 3). The distribution breadth index $\langle M \rangle_w / \langle M \rangle_n$ of the eleven samples are obtained by the equation (9). See the results in Table 1. Take the samples 1 to 6 as one group and the average value of the distribution breadth index is 2.95, while another group, the samples 7 to 11, gives the average value of the distribution breadth index as 1.82 also shown in Table 1.

The experimental curve of each sample shows good symmetry, so Gauss normal distribution function

$$F(V) = \frac{1}{\sqrt{2\pi}\,\sigma} \exp\left[-\frac{1}{2\sigma^2}(V - \bar{V})^2 \right] \qquad (10)$$

can be used.

The normalized distribution curves calculated from the average value and the standard deviation of each sample (in Table 1) and with equation (10) agrees well with the experimental results, as shown in Figure 2. As the calibration curve of our GPC column is linear, the molecular weight distribution of the samples studied may be described by log-normal distribution function. For the polydispersed sample with log normal distribution,[2,3,4]

$$q_w = \left(\frac{\langle M \rangle_w}{\langle M \rangle_n} \right)^{\frac{1}{2}\alpha(\alpha - 1)} \qquad (11)$$

$$q_m = \left(\frac{\langle M \rangle_w}{\langle M \rangle_n} \right)^{\frac{1}{2}\alpha(\alpha + 1)} \qquad (12)$$

Substitute into the equation (12) the average values of the distribution index of two groups and α values. The q_n values of two groups so obtained are 1.903 and 1.428 respectively. From the K_n and q_n of two groups, we obtain:

$K = 1.05 \times 10^{-4}$ and $K = 1.06 \times 10^{-4}$

Therefore, $[\eta]$ - M relation in the monodispered solution may be expressed as

$$[\eta] = 1.06 \times 10^{-4} \; M^{0.70} \tag{13}$$

Since

$$[\eta] = K \langle M \rangle_\eta^\alpha = K \left(\frac{\langle M \rangle_w}{\langle M \rangle_n} \right)^{1/2} \alpha(\alpha + 1) \cdot \langle M \rangle_n^\alpha$$

$$= K \left[\left(\frac{\langle M \rangle_w}{\langle M \rangle_n} \right)^{1/2} (\alpha + 1) \cdot \langle M \rangle_n \right]^\alpha$$

then

$$\langle M \rangle_\eta = \left(\frac{\langle M \rangle_w}{\langle M \rangle_n} \right)^{1/2} (\alpha + 1) \cdot \langle M \rangle_m \tag{14}$$

The viscosity average molecular weight $\langle M \rangle_\eta$ of each sample may be obtained by substituting $\langle M \rangle_n$, α and $\frac{\langle M \rangle_w}{\langle M \rangle_n}$ of eleven samples into the equation (14)

Plot $\log[\eta]$ against $\log \langle M \rangle_\eta$ as shown in Figure 4. It can be seen that the experimental points of two sample groups fall on the line for the monodispersed $[\eta]$ - M relation (13). It is independent of the size of the distribution breadth.

Qian Ren-yuan, Ying Qi-zong et al, obtained the monodispersed $[\eta]$ - M relationship for polydimetnyl-siloxane as

$$[\eta] = 9.53 \times 10^{-5} \; M^{0.71} \tag{15}$$

shown by dotted line in Figure 4. The solid line and dotted line overlap each other.

It can be seen that the presence of a small amount of the vinyl groups in the molecular chain of poly-dimethylsiloxane has shown no evident change in the property of solution and the flexibility of the molecular chain.

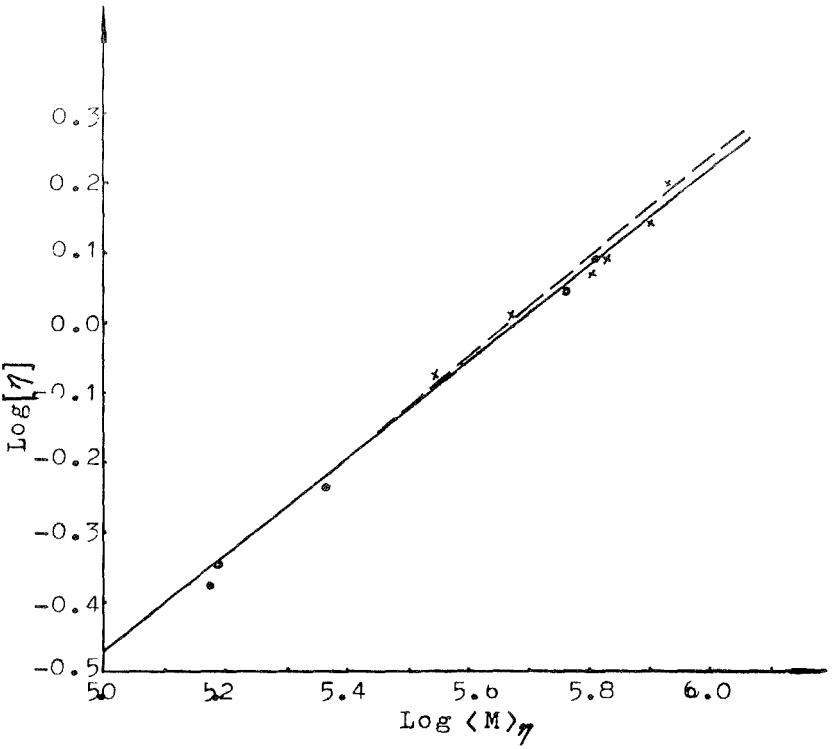

Figure 4. $\log[\eta]$ vs. $\log\langle M\rangle_\eta$

o and x : equation (14)
solid line : equation (13)
dotted line : equation (15)

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Characterization of Copolymers and Polymer Mixtures by Gel Permeation Chromatography

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ABSTRACT

Estimation of molecular weights from GPC data is complicated when the polymer sample consists of a mixture of homopolymers or of statistical copolymers with nonuniform compositions. This is because sizes of solvated polymer coils depend on solvent interaction with both the homo- and hetero-units of the copolymers and because the extent of solvation of different homopolymers can differ. The overall degree of solvation may change effectively with composition and use of a single "average" set of Mark-Houwink constants in calibration procedures will then produce false molecular weight data from the GPC data. A new molecular weight average, $\bar{M}_x$, is defined to overcome this problem. This average can be determined from the GPC chromatogram and intrinsic viscosity of the sample in the GPC solvent. Mark-Houwink coefficients are not needed. $\bar{M}_x$ lies between $\bar{M}_w$ and $\bar{M}_z$.

Hydrodynamic volumes of solvated polymers are fundamental in determining the gel permeation chromatographic (GPC) elution volumes of the species in a mixture (1-3). All species with the same hydrodynamic volume

appear with the same elution volume. For homopolymers in a given GPC solvent, hydrodynamic volumes can be related directly to molecular weights, with allowance where necessary for concentration effects (4-6). When the sample consists of a mixture of homopolymers or statistical copolymers with non-uniform compositions the estimation of molecular weight information from GPC data is much more complicated.

The major difficulty in analyzing the GPC chromatogram of a compositionally heterogeneous polymer sample involves conversion of hydrodynamic volume (Vh_i) of species i to molecular weight (M_i). This is because the size of the solvated polymer coil (and hence the Mark-Houwink constants (7)) depends on the solvent interaction with both the homo- and hetero-units in the case of copolymers (8-10). In some cases, as when the copolymer structure tends toward alternation, the influence of hetero segments may predominate. As a consequence, the values of the Mark-Houwink constants would be relatively insensitive to composition within certain limits. However, in instances where the copolymer composition tends to be more random, or when the sample is a blend of copolymers or homopolymers, the overall degree of solvation will change effectively with blend composition at a given molecular weight, and the use of a single "average" set of Mark-Houwink constants will produce false molecular weight parameters from GPC data. The alternative is to assign a set of effective Mark-Houwink constants at each elution volume, but this is an intractable experimental problem.

We, therefore, propose the use of a new molecular weight average, $\bar{M}_x$, to characterize polymer samples of heterogeneous composition by GPC in cases where a single set of Mark-Houwink constants is unsatisfactory. $\bar{M}_x$, the hydrodynamic volume average, is defined according to:

$$\bar{M}_x = \frac{\Sigma w_i [\eta]_i M_i}{\Sigma w_i [\eta]_i} = \frac{\Sigma w_i [\eta]_i M_i}{[\eta]} \qquad (1)$$

where w_i and $[\eta]_i$ are the weight fraction and intrinsic viscosity, respectively, of all species which exit the GPC columns with elution volume Ve_i. The denominator in equation (1) is equal to the intrinsic viscosity, $[\eta]$, of the whole sample in the GPC solvent. This parameter is measured separately. The values in the numerator are available from the GPC chromatogram. At infinite dilution of the species in the sample (5,11), the product $[\eta]_i M_i$ can be read directly from the universal calibration curve and w_i is equated to the ratio of the area of the GPC detector response at elution volume Ve_i to the total area under the chromatogram. This molecular weight average is particularly useful for mixtures of homopolymers or for copolymers in which composition may vary with molecular weight. The latter materials are produced in free radical batch copolymerizations that exhibit a significant composition drift with conversion and an autoacceleration-induced increase in molecular weights at higher conversions.

The value of $\bar{M}_x$ will fall between $\bar{M}_w$ and $\bar{M}_z$ of the sample. The Mark-Houwink relation for a monodisperse species is:

$$[\eta] = KM^a \tag{2}$$

where K and a are the M-H constants. Substitution of equation (2) into equation (1) gives:

$$\bar{M}_x = \frac{\Sigma w_i K M_i^{a+1}}{\Sigma w_i K M_i^{a}} = \frac{\Sigma w_i M_i^{a+1}}{\Sigma w_i M_i^{a}} \tag{3}$$

When a = 1

$$\bar{M}_x = \frac{\Sigma w_i M_i^{2}}{\Sigma w_i M_i} = \bar{M}_z \qquad (a = 1) \tag{4}$$

and when a = 0

$$\bar{M}_x = \frac{\Sigma w_i M_i}{\Sigma w_i} = \bar{M}_w \qquad (a = 0) \qquad (5)$$

For most random coil polymers in the relatively nonpolar solvents which are commonly used $0.5 \leq a \leq 0.8$ (7) and $\bar{M}_w < \bar{M}_x < \bar{M}_z$. Because of its dependence on the Mark-Houwink exponent a, $\bar{M}_x$ of a homopolymer will be somewhat solvent dependent. The more familiar $\bar{M}_v$ also exhibits some solvent dependence, for similar reasons.

When the composition of a polymer sample varies along with the molecular weights of the component species characterization of the molecular weight distribution is as formidable experimental problem. Some of the difficulties can be circumvented by use of the $\bar{M}_x$ average which can be estimated from the GPC chromatogram and the intrinsic viscosity of the polymer without a calibration for the components of the sample. Although a single average must convey less information than knowledge of the whole distribution, $\bar{M}_x$ may be expected to correlate with some mechanical and rheological properties of mixtures since its magnitude lies between $\bar{M}_w$ and $\bar{M}_z$. It is recommended that when $\bar{M}_x$ is used to characterize a copolymer or polymer blend, both the parameters $\bar{J}_w$, which is proportional to the weight average hydrodynamic volume of the sample, and the standard deviation σ_J should also be reported:

$$\bar{J}_w = \Sigma w_i [\eta]_i M_i \qquad (6)$$

$$\sigma_J = (\Sigma w_i (J_i - \bar{J}_w)^2)^{1/2} \qquad (7)$$

where $J_i = [\eta]_i M_i$. This procedure provides a molecular weight average to characterize the sample and a measure of the mean and breadth of the distribution of hydrodynamic volumes in the sample.

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HPSEC OF CATIONIC POLYMERS ON A POLYAMINE SUPPORT

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ABSTRACT

High performance steric exclusion chromatography of polyvinylpyridines (PVP) on silica particles coated with polymerized amine (SynChropak CATSEC) is described. PVP standards were analyzed first on a conventional neutral support (SynChropak GPC), and the results were compared with the amine coated silica. Conditions for analysis were then examined for optimization. Linear recovery was observed for a PVP standard of M.W. 3,000.

INTRODUCTION

Over the past few years, high performance steric exclusion chromatography (HPSEC) on silica based supports has become an accepted technique for polymer characterization. Methodologies for analyzing neutral and anionic polymers have been established by numerous research groups; however, the analysis of cationic polymers has been a problem. Positively charged molecules ionically bind to negative silanols on silica surfaces. When silica has a chemically-bound neutral layer covering the surface, the negative character of the silica is reduced but not eliminated (1-4). When Pfannkoch et al. examined this phenomena for a glycerylpropylsilyl support (SynChropak GPC), they found that

adjusting the pH or the ionic strength of the mobile phase would allow certain cationic proteins such as lysozyme and cytochrome c to elute by size (1). Alternatively, organic solvents have been used by Rand et al. to aid elution (2). Barth found that he could analyze cationic polymers on SynChropak GPC columns by preconditioning them with a cationic homopolymer (3). Highly charged polymers such as polyvinylpyridines (PVP) exhibited adsorption or, at best, tailing, under most conditions on neutrally bonded columns (4).

One way to eliminate the negative silanol groups and the adsorptive effects of cationic polymers is to bond an amine to the silica surface to neutralize the silanols. The resulting support would have a net positive charge and would exhibit anion exchange characteristics for appropriate molecules. Talley and Bowman used such an approach when they reacted controlled porosity glass with 3-aminopropyltriethoxysilane which was subsequently quaternized (5). PVP standards eluted from this support without tailing when an acid eluent was used.

This paper describes the use of SynChropak CATSEC columns which are composed of high performance silica coated with polymerized amine. PVP standards elute from these columns according to size, and they exhibit Gaussian peak shapes when appropriate operating conditions are used.

EXPERIMENTAL

Apparatus: SynChropak CATSEC 100 and CATSEC 1000 (250 x 4.6mm ID) and SynChropak GPC 100 (250 x 10mm ID) were obtained from SynChrom,

Inc. (Linden, IN). A ConstaMetric IIG liquid chromatograph (Laboratory Data Control, Riviera Beach, FL) with a Model 7125 injection valve (Rheodyne, Berkeley, CA) and a Chem Research Model 2020 multiple wavelength detector (Instrumentation Specialties Company, Lincoln, NE) were used for the analyses.

Reagents: Trifluoroacetic acid (TFA) was purchased from Pierce Chemical Company (Rockford, IL). Sodium chloride and sodium phosphate monobasic were from Mallinkrodt (Paris, KY). The glycyl-l-tyrosine and cytidine were from Sigma Chemical Company (St. Louis, MO). The poly (2-vinylpyridine) (PVP) standards were obtained from Larry Rosen at Pressure Chemical Company (Pittsburgh, PA).

Procedure: The 0.1% trifluoroacetic acid/0.2M sodium chloride solution, the 1.0% trifluoroacetic acid/0.2M sodium chloride solution, and 0.1% trifluoroacetic acid solution used for buffers were prepared in water. The 0.02M sodium phosphate/0.2M sodium chloride solution was adjusted to pH 2.0 with hydrochloric acid. The glycyltyrosine and cytidine standards and poly (2-vinyl-pyridine) standards were prepared in buffer and sonicated where necessary to effect solution. Duplicate samples of 2.5-10µl were injected into the liquid chromatograph with the isocratic buffer at a flow rate of 0.5ml/min. Full scale absorbance of the detector was set at 0.16 and at a wavelength of 254 nm.

RESULTS AND DISCUSSION

Analysis of PVP standards on a neutral support: In order to evaluate the chromatography of the cationic polymers on the

polyamine support, it was first necessary to run them on silica with a neutral bonded layer. A SynChropak GPC 100 column which has a glycerylpropylsilyl bonded layer was chosen for the study. A mobile phase of 0.1% trifluoroacetic acid was used to suppress ionic effects. The PVP standards did elute under these conditions; however, some adsorption was occurring. Initial injections of the samples seemed to coat the reactive sites but these adsorbed polymers appeared to leach off over time. Subsequent samples

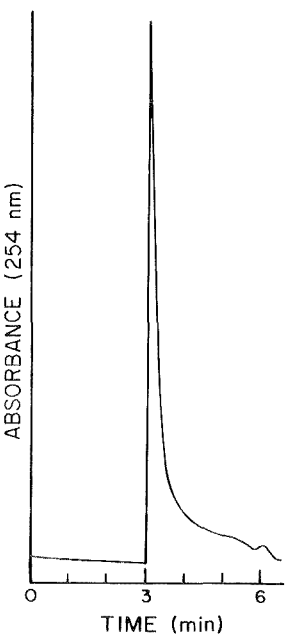

FIGURE 1

Analysis of a PVP standard (MW 20,000) on a SynChropak GPC 100 column (25 cm x 1 cm I.D.). Mobile phase: 0.1% trifluoroacetic acid; flowrate: 3.0 ml/min.

did elute according to size; however, some tailing of the peaks was present as seen in Fig. 1. Although these neutral supports could be used for the analysis of cationic polymers, the adsorption, sample leaching, and tailing made them less than ideal for this application.

Analysis on a polyamine support: A new column packing material, SynChropak CATSEC, was developed for the SEC of cationic polymers. This is a porous silica which has a thin layer of polyamine polymerized with a neutral hydrophilic crosslinker to increase stability. When PVP standards were run on these columns, linear recovery of the PVP was observed as seen in Fig. 2. With lower

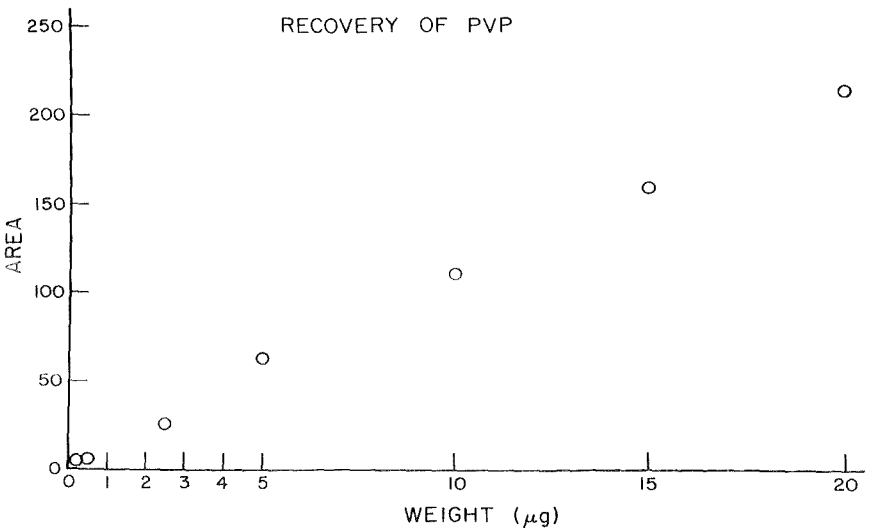

FIGURE 2

Recovery of a PVP standard (MW 3,000) from a SynChropak CATSEC 1000 column (250 mm x 4.6 mm I.D.). Mobile phase: 0.1% trifluoroacetic acid, 0.2M sodium chloride; flowrate: 0.5 ml/min.

concentrations, slight adsorption was observed. Once the feasibility of using these columns was determined, conditions for analysis were examined for optimization.

Mobile phase selection: When a mobile phase of 0.1% trifluoroacetic acid was used for analysis, the standards appeared to have

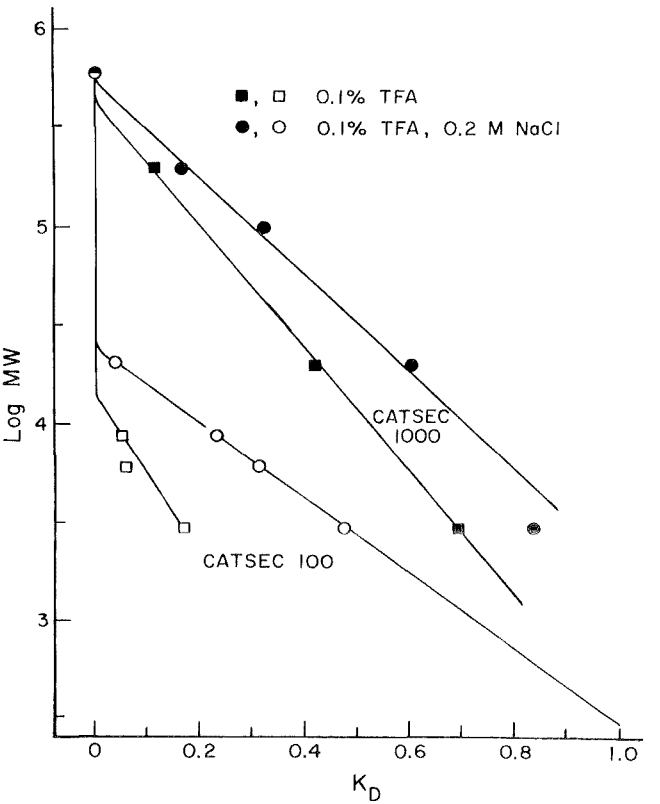

FIGURE 3

Ion-exclusion effect shown by the calibration curves for PVP standards on SynChropak CATSEC 100 and 1000 columns.

lower retention times than would be expected on columns of these pore diameters (100 Å and 1000Å, respectively). This phenomenon would be symptomatic of ion-exclusion, which would be expected with pores containing a charged surface and an eluent with inadequate ionic strength. The density of positive charges within the pore repel the positively charged polymers and prevent them from penetrating properly. The effect was more severe for the 100 Å than the 1000 Å column due to the much higher surface area of the 100 Å support. Figure 3 illustrates the ion-exclusion effect on the calibration curves of each support. A mobile phase of 0.1% trifluoroacetic acid containing 0.2M sodium chloride was used for comparison.

Eluents containing 0.2M salt appeared to obviate ion exclusion on columns of all pore diameters. Mobile phases of several different pH values were used and all gave similar results when 0.2M sodium chloride was added: 1) 1.0% trifluoroacetic acid, 0.2M sodium chloride (pH 1.4), 2) 0.1% trifluoroacetic acid, 0.2M sodium chloride (pH 1.6), and 3) 0.02M sodium phosphate, 0.2M sodium chloride (pH 2.0). Figure 4 shows the analysis of three PVP standards on a 100Å SynChropak CATSEC 100 column using 0.1% trifluoroacetic acid and 0.2M sodium chloride. Figure 5 exhibits the analysis of four PVP standards using the 0.02M sodium phosphate (pH 2.0) buffer with 0.2M sodium chloride. No differences are seen between the eluents as regards peak shape. When 0.5M sodium chloride was used, some added retention was seen. This was probably due to a hydrophobic interaction or a salting-out

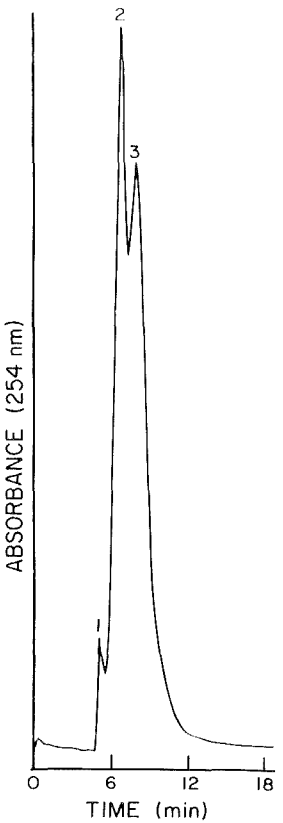

FIGURE 4

Analysis of PVP standards on a SynChropak CATSEC 100 column (250 mm x 4.6 mm I.D.). Mobile phase: 0.02M sodium phosphate, 0.2M sodium chloride, pH 2.0; flowrate: 0.3 ml/min; PVP standards: 1. MW 600,000; 2. MW 11,000; 3. MW 3,000.

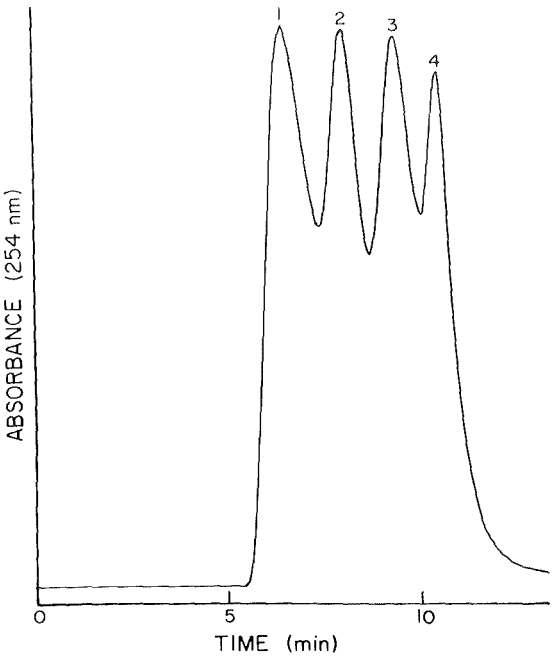

FIGURE 5

Analysis of PVP standards on a SynChropak CATSEC 1000 column (250 mm x 4.6 mm I.D.). Mobile phase: 0.1% trifluoroacetic acid, 0.2M sodium chloride; flowrate: 0.3 ml/min; PVP standards: 1. MW 600,000; 2. MW 100,000; 3. MW 20,000; 4. MW 3,000.

phenomenon and has been observed elsewhere (3). Some folding and extending of charged polymers occurs with changes in salt concentrations so it is important to maintain the same operating conditions for all analyses in order to get valid molecular weight comparisons and calibration curves.

Choice of totally included solute: One problem which occurred during these studies concerned the choice of a probe used to deter-

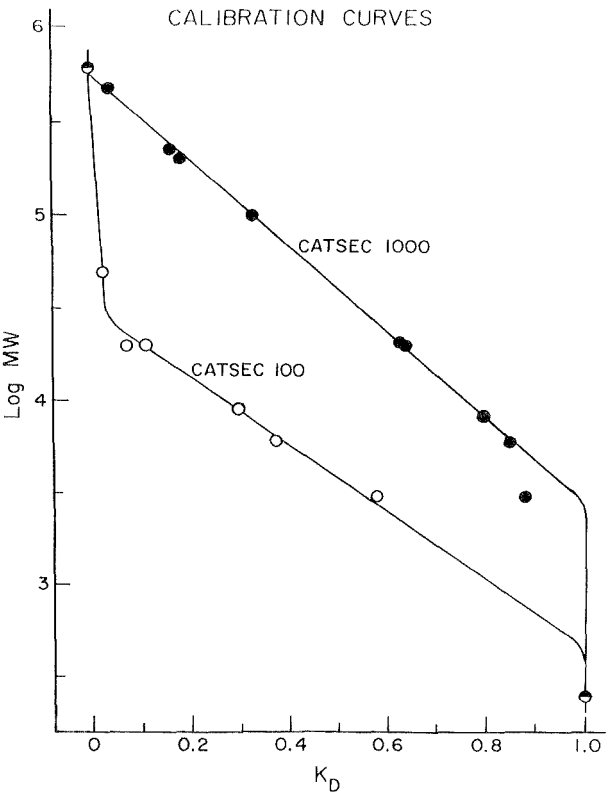

FIGURE 6

Calibration curves for SynChropak CATSEC 100 and 1000 columns using cytidine as the total volume indicator. Mobile phase: 0.1% trifluoroacetic acid, 0.2M sodium chloride; flowrate: 0.5 ml/min.

mine the total column volume. Pfannkoch et al. stressed the importance of finding a molecule which exhibits no interaction whatsoever with the column (1). Glycyltyrosine, which had been successfully used with glycerylpropyl columns, was initially used for these columns. The amphoteric character of this dipeptide caused it to be retained slightly on this polyamine column in all cases except when the mobile phase with the lowest pH (pH 1.4) was used. This phenomenon was not immediately obvious since the calibration curves were linear. Changes in the structure of the PVP polymers or the polyamine coating were blamed for the observed changes in K_D or internal volume of the support. When a more suitable small molecule, cytidine, was chosen, parameters such as internal volume and K_D were reproducible for mobile phases with different pH. Figure 6 shows the calibration curve for the PVP standards using cytidine instead of glycyltyrosine, which was used in Fig. 1.

CONCLUSIONS

Cationic polymers such as polyvinylpyridines can be analyzed by steric exclusion chromatography on SynChropak CATSEC columns. The polyamine coating on these columns neutralizes negative silanol groups and prevents adsorption of the polymers. Acidic eluents containing 0.2M salt eliminate ion-exclusion and interaction between the polymers and the supports. Cytidine was shown to be a good indicator of the total volume of the column.

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UTILIZATION OF WEAK ACID-BASE INTERACTIONS TO IMPROVE SEPARATIONS IN NORMAL PHASE LIQUID PREPARATIVE CHROMATOGRAPHY

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ABSTRACT

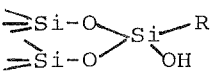
Three 40 μm derivatized silicas, two aminoalkyl and one carboxyalkyl, have been compared to 40 μm silica gel for performance in normal phase preparative liquid chromatography. The three derivatives showed higher selectivity for compounds capable of hydrogen-bond formation or acid-base interactions. Retention times were related to the basicity of the amine bonded derivatives or the acidity of the carboxylic acid derivative and the pK values of the solutes. The advantages for each of the four sorbents for neutral, acidic and basic compounds have been described.

INTRODUCTION

In aqueous systems hydrogen bonding capabilities and acid-base interactions between organic moieties are minimized because of the polarity of the aqueous medium and the strong hydrogen bonding properties of the water molecules themselves. In organic solvents or organic solvent-water mixtures, however, these properties become important and can be used to chromatographically resolve compounds with interacting functional groups.

We have explored the capabilities of three siloxane bonded polar phases in normal phase chromatography, wishing to exploit the enhancement of weak acid-base interactions in solvents most often utilized by the preparative chemist. We have limited ourselves to isocratic elution, relying for resolution enhancement upon solvent composition and the nature of the bonded phase.

The three bonded phases were siloxanes in which R



was 3-aminopropyl,3-(2-aminoethylamino)propyl or 2-carboxyethyl. All derivatives were bonded to silica of 40 μm average particle size. These bonded phases have recently become available commercially as bulk packing and seemed to offer good possibilities for selective retention and purification of compounds possessing weakly basic or weakly acidic sites for hydrogen bonding.

The hydrogen bonding capabilities of the aminopropyl bonded phase have been exploited extensively in analytical HPLC (in the so-called "carbohydrate column") to separate mono, di and trisaccharides (1). The resulting complexes formed between the amine groups of the bonded phase and the very weakly acidic hydroxyl groups of the carbohydrates result in different reten-

tion times in an acetonitrile-water mobile phase and, therefore, resolve the carbohydrates.

The other end of the scale of acid-base interactions in organic media with this bonded phase is the use by Pirkle of the stable ionic bond between R-N-3,5-dinitrobenzoylphenylglycine and the amine group to create an immobilized chiral phase to chromatographically separate optical isomers (2).

We have limited our exploration of the preparative capabilities of the amine bonded phases primarily to the intermediate range of interactions between these two extremes, though a brief examination of carbohydrate separation is also included.

EXPERIMENTAL

MATERIALS

Ethyl acetate, hexane, acetonitrile, and methylene chloride were 'Baker Analyzed'® solvents. The solutes were Baker organic reagents.

The silica and the three bonded phase packings were obtained from Baker as "flash chromatography" grade silica and Baker amino (NH_2), Baker 1°,2°-amino (NH_2-NH) and Baker carboxyl (COOH) bonded phases for preparative liquid chromatography. The 3-aminopropyl bonded phase contained 2.45% N; 1.75 meq N/g; loss on drying at 110°C was 6.3%; loss on ignition at 600°C

after drying at 110°C was 10.72%. The diamine bonded phase contained 2.86% N; 2.04 meq N/g; loss on ignition at 600°C after drying at 110°C was 16.06%. The carboxyl bonded phase contained 4.6% C; 1.0 meq acid/g groups by titration.

The weight of the support loading was determined by heating 1 g of the siloxane derivative in a crucible at 600°C in an oven for 3 hours.

$$\% \text{ loading} = \frac{\text{wt of loss at } 600° \times 100}{\text{wt of phase after heating at } 110°C}$$

In order to show that the weight loss above 110°C is due primarily to the support loading and not to the elimination of water from vicinal hydroxyl groups, silica itself was first ignited at 600°C. From 100-600°C a loss of 3.3% took place. We can conclude, therefore, that the weight loss due to water formation is below 3.3% in the amine bonded derivatives because of the reduced content of unreacted hydroxyls.

Support loadings of 10.72% and 16.06% for the monoamine and diamine derivatives suggest a small amount of polymer formation for both derivatives (1).

APPARATUS

An Altex glass chromatography column, 9 mm x 250 mm, equipped with a stainless steel plunger fitted with Teflon® tubing was used in all experiments. An Altex Tefzel slider injection valve with a 0.5 mL loop was

connected directly to the plunger. A laboratory pump (1/4" piston, flow rate 0-19 mL/min, Fluid Metering Company, Oyster Bay, NY), was the pressure source. A pulse dampener (Fluid Metering Company) was inserted between the pump and the injection valve. The eluate from the column was monitored by a Waters 403 refractive index detector which, in turn, was connected to a Hewlett-Packard strip chart recorder.

PROCEDURE

Column packing: The irregular-shaped particles had an average diameter of 40 μm with a narrow distribution of 30-65 μm. Columns were dry packed by first pouring enough adsorbent into the column to fill a 30 mm height. The column was then carefully "bounced" on the table top to settle the adsorbent. Repetitive additions of similar small aliquots were added and the columns were "bounced" or tapped until a 200 mm height of settled adsorbent was added. The plunger was then inserted and adjusted carefully to contact the surface of the adsorbent.

Chromatographic Evaluation of Phases

Capacity factors (k') were determined by injecting 100 μL samples of solutions containing 1 g of solute per 100 mL of mobile phase. The flow rate of mobile phase was 7.5 mL/min.

DISCUSSION

Since no analytical TLC plates of the three bonded phases exist, chromatography on a small column is the best method available for optimizing mobile phase composition for later scale-up separations of a variety of solutes. When larger preparative columns are packed with the 40 μm adsorbents used in this study, similar capacity factors are routinely obtained (results reported elsewhere).

Monoamine Bonded Phase

The chromatographic medium most commonly utilized for normal phase chromatographic purification of organic compounds is silica. Therefore, for purposes of providing a baseline for better understanding the chromatographic properties of the relatively new bonded phase packings, it seemed useful to do comparative studies under identical conditions. Table I illustrates that with a mobile phase of low solvent strength (3) silica exhibits relatively non-specific interactions with acidic materials having a wide range of pK values. Specific interactions occasionally credited to silica can probably be ascribed to trace impurities such as sodium, aluminum or sulfate; these impurities impart to silica acidic or basic properties as evidenced by the 2-9 pH range of various silicas in water

(4). The silica used in these experiments gave a neutral pH when slurried in water.

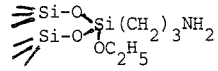
With the monoamine phase the range of retention times with the same mobile phase used for silica increases considerably for the series of alcohols, with retention times of 4-5 minutes for very weak aliphatic alcohols having pKa's of 19, increasing to retention times of greater than 20 minutes for the substituted phenols having pKa's of 7 or less. It is reasonable to assume that for this series of alcohols the acid-base interaction between the hydroxyl hydrogen of the solute and the amine nitrogen of the solid phase determines retention time.

Even with alcohols such as chloroethanol and phenethyl alcohol showing high pKa values of 18 or greater, the results are consistent with those expected from electronic effects; for example, the increased retention of chloroethanol over ethanol or benzyl alcohol over phenethyl alcohol can be predicted on relative acidities, even through actual pKa values do not appear in the literature.

It is interesting to note, however, that retention times for both mixtures are reversed on silica. With these alcohols (pKa about 18) the major interactions with silica occur between the silanol hydrogen and the hydroxyl oxygen (5). In the case of benzyl and phen-

TABLE 1

Separation of Alcohols and Amines on Silica and 3-Aminopropyl-Derivatized Silica



Adsorbent		Silica*							
wt g:		5.6				6.6			
Mobile Phase		Hexane/Ethyl Acetate 3/1		Hexane / Ethyl Acetate (3/1)		Hexane / Ethyl Acetate (3/7)		Acetonitrile / Water (7/3)		Acetonitrile / 0.05M Aqueous Sodium Bicarb. (7/3)
Flow Rate 7/5 mL/min										
E^{O}***		0.23		0.23		0.34		>0.75		>0.75
Solute	pKa	Retention Time (min)	k'	Retention Time (min)	k'	Retention Time (min)	k'	Retention Time (min)	k'	Retention Time (min)
benzene	37	1.5	--	1.4	--	1.3	--			
benzyl alcohol	~18	5.0	2.3	10.4	6.3	3.4	1.6	1.3	--	
1-decanol	19	3.6	1.4	4.4	2.1	2.4	0.7			
hexanol	19	4.2	1.8	4.8	2.4	2.5	0.7			
ethanol	18	8.8	4.9			3.0	1.3			
chloroethanol	<18	7.5	4.0			4.2	2.2			

phenethyl alcohol	>18	5.8	3.1	9.0	5.4	2.8	1.2			
eugenol	10	2.4	0.6	13.0	8.3	4.5	2.5			
phenol	9.9	2.2	0.5			7.5	4.8	1.3	--	
p-nitrophenol	7.2	4.8	2.2	>20		>20		11.0	7.4	2.2
2,4-dinitro-phenol	4.0	>20		>20		>20		>20		1.6
aniline****	4.6	4.8	2.2	3.5	1.5	2.0	0.5			
o-nitroaniline	0.5	3.5	1.3	6.5	3.6	2.5	0.8			
m-nitroaniline	2.5	6.8	3.5	9.8	6.0	2.7	1.1			
p-nitroaniline	1.0	10.8	9.8	6.2		4.7	2.6			
N-methylaniline	4.84					1.7	0.3			
N-dimethylaniline	5.07					1.5	0.15			

*J. T. Baker Catalog No. 7030-0. **J. T. Baker Catalog No. 7034-0. ***Solvent strength determined on silica; see ref. 3.
****For aniline and its derivatives the pKa represents the dissociation of the conjugate acid BH+ of the listed base B.
Experimental Conditions: Altex glass column (9 mm x 25 cm) containing an adjustable plunger; FMI laboratory pump; FMI pulse dampener; Altex loop injector; Waters refractive index detector R403; Hewlett-Packard strip chart recorder.

ethyl alcohols the greater basicity of the oxygen in phenethyl alcohol is responsible for a stronger hydrogen bond with the silica silanol group. The concomitant decreased acidity of the phenethyl alcohol hydroxyl hydrogen affords a weaker hydrogen bond with the nitrogen of the amino bonded phase.

Even though the relative retention times of both aromatic and aliphatic alcohols on the amine phase are a function of the acidity of the hydroxyl hydrogen, the absolute retention times are not a function of aqueous pKa alone. The electronic effects of the aromatic ring in the organic mobile phase are far stronger than in the primarily aqueous solutions used for pKa determinations. Hence the aromatic alcohols as a group bind more tightly to the amine bonded phase than the aliphatic alcohols.

As the acidity of the solute increases, solvents of ever-increasing solvent strength are required to achieve reasonable elution times from the amine phase. The strongest acid eluted with an aqueous organic mix was 2,4-dinitrophenol with a pKa of 4; in this case elution was achieved only by including sodium bicarbonate in the aqueous component of the mobile phase to form the dinitrophenolate ion. The inclusion of 0.05M sodium bicarbonate (pH 8) has a greater effect on the elution time of 2,4-dinitrophenol than on the less

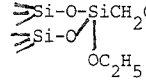
acidic p-nitrophenol because the former ionizes more readily. Such control of elution times by mobile phase composition is highly desirable in preparative chromatography, since purification in preparative liquid chromatography is best achieved when the desired compound is eluted after the removal of impurities. The amine bonded phase can be used to advantage for early separation of materials incapable of hydrogen bonding and subsequent controlled elution of acidic materials.

Diamine Bonded Phase

The dominant interacting amine group of the diamine bonded phase is the terminal primary amine which is less basic than the comparable amine group in the monoamine derivative; a pKb of 7.0 for the diamine phase was determined by acid titration. The lower basicity explains the lower retention times of the alcohols in Table II; for example, retention times of 7.5 and 4.5 minutes for phenol and eugenol on the monoamine phase are reduced to 4.1 and 2.6 minutes, respectively, on the diamine phase. The differences in retention time increase as the pKa of the interacting aromatic or aliphatic alcohol decreases. Eugenol is a somewhat special case since its methoxy group can intramolecularly hydrogen bond with the adjacent hydroxyl group, thereby competing with the bonded

TABLE II

Separation of Alcohols and Amines on 3-(2-Aminoethylamine)propyl-Derivatized Silica



Bonded Phase+

Adsorbent wt g: 6.5 g

Mobile Phase:	Hexane / Ethyl Acetate (3/1)		Hexane / Ethyl Acetate (3/7)		Methylene Chloride / Acetonitrile (2/8)	
E^o	0.23		0.34		0.5	
Solute	Retention Time (min)	k^1	Retention Time (min)	k^1	Retention Time (min)	k^1
benzene	1.4	--	1.3	--	1.2	--
benzyl alcohol	9.6	5.9	3.0	1.3	1.8	0.5
1-decanol			1.9	0.5		
hexanol	4.0	1.9	2.1	0.6		
ethanol			2.6	1.0		

chloroethanol			3.4	1.6		
phenethyl alcohol	7.8	4.6	2.6	1.0		
eugenol	7.6	4.4	2.6	1.0		
phenol			4.1	2.2	2.6	1.2
p-nitrophenol	>20		>20		>20	
2,4-dinitrophenol	>20		>20		>20	
aniline	4.8	2.4	2.0	0.5		
o-nitroaniline	8.3	4.9	2.6	1.0		
m-nitroaniline	14.5	9.4	3.2	1.5		
p-nitroaniline	>28.0	>19.0	5.7	3.4	2.1	0.8
N-methylaniline			1.6	0.2		
N-dimethylaniline			1.4	0.1		

*J. T. Baker Catalog No. 7042-o.
Experimental Conditions: See Table I.

phases for hydrogen-bond interactions. Thus, the retention times for eugenol on both phases are significantly less than expected by considerations of pKa alone.

Recently Becker and Unger (6) related the capacity factors of various phenols to their pKa values on a similar diamine bonded silica. Their results clearly showed that an increased binding of the phenols with the polar support paralleled increased acidity of the solute. Our data are in agreement with this conclusion, but the binding capacity of the two diamine derivatives are strikingly different in a manner that correlates directly with the nitrogen contents of the bonded phases of 2.32 and 2.86%, respectively. Our bonded phase gave higher k' values with the same solvent system (dichlormethane/acetonitrile 20/80; E = 0.50); for example, phenol showed a k^1 value of 1.2 rather than 0.2. More importantly, 4-nitrophenol and 2,4-dinitrophenol could only be eluted from our column with a much more polar mobile phase. The higher nitrogen content and increased capacity of our derivative reflects a more highly polymerized coating on silica.

Comparison of the Amine Bonded Phases

It is clear that retention on both derivatives increases as pKa decreases. Hydrogen bonding falls off

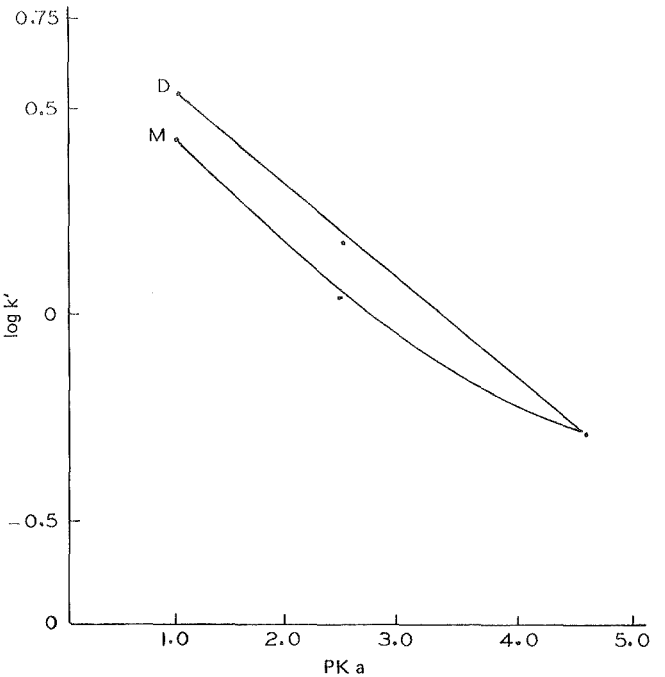


Figure 1. Plot of Log k^1 Versus pKa for Weak Metallic Bases

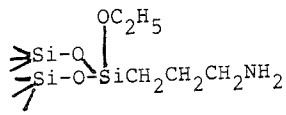
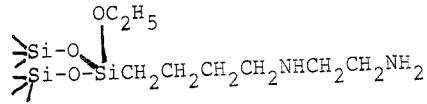
Chromatographic Conditions: Column, M = Monoamine Bonded Phase; D = Diamine Bonded Phase; Mobile Phase, Ethyl Acetate-Hexane (7/2); Flow Rate, 7.5 mL/min

so sharply at pKa 18 that no difference between hexanol and decanol can be detected. Although the monoamine is more retentive for alcohols, the diamine binds more tightly to weak aromatic bases (Figure 1) that have two hydrogen atoms available for interaction with the amine nitrogens of the adsorbent. No difference is seen with bases having pKb's less than 2 pK units different from the adsorbents; aniline and methylated anilines behave

similarly with both adsorbents, but differences become apparent with the more acidic m- and p-nitroanilines. The intramolecular hydrogen bonding in o-nitroaniline precludes consideration in this plot (Figure 1).

Since 5 and 10 μm aminopropyl silica are extensively used analytically for HPLC analysis of carbohydrates, it seemed useful to test the two 40 μm bonded phases for preparative separation capabilities of these compounds. A closely related series of carbohydrates was resolved on both amine bonded phases (Table III). As the relative basicity of the two bonded phases predicts, the aminopropyl silica binds more strongly to carbohydrates than the diamino derivative. Arabinose, for example, elutes in 1.8 minutes from the diamino column with an acetonitrile-water (4/1) mobile phase. Under the same conditions arabinose was still retained on the aminopropyl column after 18 minutes. When the mobile phase was changed to acetonitrile-water (3/1), however, arabinose eluted in 4.5 minutes; the k^1 values for all the solutes then approximate the data obtained on the diamine column with the solvent mixture of lower solvent strength. The incorporation of water in the mobile phase substantially increased the pressure in the analytical columns (65-80 psi), but analysis of carbohydrate systems with both 40 μm bonded derivatives continued to

TABLE III
Separation of Carbohydrates

Adsorbent		
A. Analytical Column 9 mm x 20 cm		
Adsorbent wt g:	6.6	6.5
Mobile Phase	Acetonitrile/Water 3/1	Acetonitrile/Water 4/1
Flow Rate: mL/min	7.2	7.6
Pressure: psi	65	80
Solute	Retention Time (min) / k^1	Retention Time (min) / k^1
Solvent front	1.3 / --	1.2 / --
thymidine	2.6 / 1.0	1.8 / 0.5
arabinose	4.5 / 2.4	3.8 / 2.2
fructose	5.0 / 2.8	4.4 / 2.6
glucose	6.2 / 3.7	5.2 / 3.3
sucrose	9.0 / 5.9	8.0 / 5.7
maltose	11.6 / 7.9	9.6 / 7.0
lactose	12.2 / 8.3	11.0 / 8.2

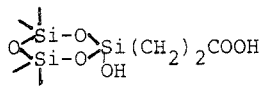
be an unusually simple procedure. Acetonitrile-water mobile phases with carbohydrate solutes must be categorized as normal phase chromatography, since in this case water is used to expedite elution.

The acid-base properties of amino bonded derivatives can exhibit wide variations, possibly dependent on the nitrogen content and the amount of cross-linked aminosilane coating on the silica. Whereas our monoamine bonded silica is clearly more basic than the diamine derivative, Jesorek et al (7) described products with quite different behavior. For example, the pH of an aqueous slurry of their mono and diamino derivatives is 8-9 and 9-10, respectively; our comparable pH values are 8.2 and 6.9. Further, they reported the apparent (pKb) of the mono and diamino bonded phases to be 7.0 and 6.4; we found 6.2 and 7.0 after a very slow attainment of equilibrium in a titration with 0.1N HCl. The relative retentivities of our two amine phases correlated with our pK measurements.

2-Carboxyethyl Bonded Phase

The pH of a suspension of the bonded phase in water was 4.1. Table IV shows the behavior of this polar bonded phase under the conditions reported with the amine-bonded derivatives. Again the dominant mechanism appears to be an acid-base interaction, in this case between the carboxylic acid of the solid

TABLE IV
Separation of Amines on Silica and
2-Carboxyethyl-Derivatized Silica

Adsorbent		Silica (5.6 g)		 (6.8 g)			
Mobile Phase		A		A		B	
Flow Rate		7.5 mL/min		7.5 mL/min		7.5 mL/min	
	pKb	Retention Time (min)	k^1	Retention Time (min)	k^1	Retention Time (min)	k^1
Solute							
benzene		1.4	--	1.4	--	1.3	--
aniline	9.42	2.4	0.71	1.5	0.07		
N-N-dimethyl-aniline	8.94	1.6	0.14	1.4	--		
pyridine	8.77	6.1	3.35	2.4	0.71		
n-butylamine	3.39	>20	--	>20	--	2.5	0.9
diethylamine	2.9	>20	--	>20	--	2.8	1.15
piperidine	2.8	>20	--	>20	--	3.6	1.77

Mobile Phase

A = Ethyl Acetate/Hexane (7/3)
B = Acetonitrile/Methanol/Acetic Acid 9/1/0.12

phase and the basic group of the solute. As would be predicted, retention times increase with a decrease in the pKb of the solute, but the interaction becomes important only with moderately strong bases. A weak base such as aniline is practically unretained; pyridine (pKb = 8.77) binds more tightly and is easily separated from aniline (pKb = 9.42). With the increased basicity of aliphatic amines, however, strong interaction with the carboxyl groups of the sorbent occurs; retention times beyond 20 minutes are observed.

A mobile phase of substantially stronger elution strength (acetonitrile/methanol/acetic acid 9/1/0.12) removes n-butylamine, diethylamine and piperidine at 2.5, 2.8 and 3.6 minutes, respectively. These amines elute in order of their basicities; the α value of 1.97 for piperidine and n-butylamine represents a fairly easy separation.

As Table IV shows, this carboxylic acid bonded phase is less retentive than free silica for weakly basic compounds. For example, under comparable conditions, aniline and pyridine are eluted from silica at 2.4 and 6.1 minutes, but in 1.5 and 2.4 minutes from the acid derivative. Since a suspension of silica in water gave a neutral pH, its bonding properties can be attributed to the polar properties of the free silanol groups rather than the acidic nature of silica.

As one inspects all the retention data for the three bonded phases plus silica, it becomes clear that collectively they offer the preparative chromatographer a new dimension in optimizing practical separations on a preparative scale. By selecting the appropriate solid phase for his compound, he can maintain the convenience of isocratic elution with readily volatile solvents. For example, ethyl acetate/hexane mobile phases resolve moderately acidic compounds on the diamine phase, weakly acidic on the monoamine phase and neutral and weakly basic compounds on silica. Moderately to strongly basic compounds can be resolved on the carboxylic acid bonded phase with a volatile mobile phase of strong solvent strength. Ultimately, the limit to this approach is solubility. Strongly acidic and basic materials usually require aqueous systems and the advantage of a volatile solvent is lost.

ACKNOWLEDGEMENT

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THE EFFECT OF SILANOL MASKING ON THE RECOVERY OF PICLORAM AND OTHER SOLUTES FROM A HYDROCARBONACEOUS PRE-ANALYSIS EXTRACTION COLUMN

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ABSTRACT

The recoveries of picloram, picloram-methylester, hexazinone, benzene, and acetophenone from aqueous samples were studied using a commercially available hydrocarbonaceous pre-analysis extraction cartridge, both with and without tetrabutylammonium hydrogen sulfate (TBAHS) in the eluent. Extraction efficiency was found to be dependent on sample loading volume. The results suggest a mixed mechanism of retention involving both "silanophilic" and "hydrophobic" interactions in the absence of tetrabutylammonium ion. The ability of TBAHS to mask surface silanol groups and/or ion-pair with counterionic solutes is invoked to explain the observations. Chromatograms of the solutes obtained on a C_{18} bonded analytical column in both the presence and absence of TBAHS are also presented.

INTRODUCTION

Some organic compounds are efficiently removed from solution by contact with an adsorbent via adsorption trapping (1). Silica gel bonded with hydrocarbonaceous ligands, i.e. reversed-phase material, is a potentially useful adsorbent for extraction and concentration of organic compounds from aqueous samples. The sample adsorption/desorption process from hydrocarbonaceous stationary phase material consists of three steps: (a) an organic solvent followed by aqueous prewash, (b) charging the sample to

the column from an aqueous matrix, and (c) sample desorption involving the elimination of low or high polarity contaminants which may or may not precede removal of the compound of interest. Retention for this process is reflected by the percent recovery or efficiency.

However, chromatographic effects attributed to unmasked silanol groups in reversed-phase analytical columns (2-6) may also affect the recovery of adsorbate from reversed-phase pre-analysis extraction columns. Several manufacturers now offer C_{18} bonded phases in convenient-to-use disposable cartridges, but these products vary as to the extent of free silanol groups remaining in the bonded phase material.

The presence of solvent-accessible silanol groups in chemically bonded stationary phases for reversed-phase liquid chromatography (RPLC) can lead to a dual retention mechanism (2-4). Horvath et al. (5,6) postulate that both "hydrophobic" and "silanophilic" interactions occur. Depending on the manner in which a hydrocarbonaceous bonded phase is produced (7-9), some silanol groups will remain unreacted due to steric inhibition; furthermore, SiOH groups may be reformed in the work-up procedure if bifunctional or trifunctional modifers are used (10,11). These silanol groups can produce undesirable effects on column stability, retention, and peak symmetry due to the reduced hydrophobic character of the surface (2,3).

The technique of "capping" accessible silanol groups with a small silane such as trimethylchlorosilane has been used to improve retention characteristics (3,4). Noudek and Vyskocil (12) demonstrated that the concentration of hydroxyl groups could be greatly reduced by this procedure. Protonated alkylamines (6,13) and lipophilic quaternary ammonium salts (6) have been added to the mobile phase in RPLC to reduce the silanophilic influence upon retention by preferential adsorption of these charged species on residual silanol sites.

The previously reported work has dealt with the problem of silanol groups remaining on reversed-phase material used in ana-

lytical columns. The purpose of this paper is to report results observed when TBAHS was used to minimize silanophilic interactions in solute adsorption/desorption from a commercially available C_{18}-bonded pre-analysis extraction column.

MATERIALS AND METHODS

Apparatus

The liquid chromatograph consisted of a Waters (Milford, MA) Model 6000A solvent pump, Model 710B intelligent sample processor, Model 440 UV absorbance detector, and a Houston Instrument (Austin, TX) recorder. Hypodermic syringes with Luer Lok tips were purchased from Becton-Dickson (Rutherford, NJ) and a Filtrator from Fisher Scientific (Pittsburgh, PA).

Reagents and Chemicals

Spectrophotometric grade acetonitrile (ACN) and glacial acetic acid (HOAc) were obtained from Burdick and Jackson Laboratories, Inc. (Muskegon, MI) and J.T. Baker Chemical Co. (Phillipsburg, NJ), respectively. Water was processed with a Bion Exchange system purchased from Pierce Chemical Company (Rockford, IL). Tetrabutylammonium hydrogen sulfate (TBAHS) and acetophenone were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI), and benzene from Fisher Scientific. Picloram (99.0%) and picloram-methyl ester (99.7%) were obtained from the Dow Chemical Company (Midland, MI) and hexazinone (analytical standard, 99+%) from E.I. DuPont de Nemours & Co. (Wilmington, DE).

Chromatographic Procedures

SEP-PAK C_{18} cartridges were purchased from Waters Associates, Inc. House vacuum (530 mm Hg) was used to pull the samples through the reversed-phase cartridges. Preparation of the cartridges and charging of the sample was accomplished by using a side arm filtering flask. For component elution, a Fisher Filtrator was used to collect the sample directly into a 10.0 mL volumetric flask. Individual sample solutions of acetophenone, benzene, picloram, picloram-methyl ester and hexazinone were prepared by adding 1.0 uL, 2.5 uL, 4.3 ug, 57.5 ug and 13.0 ug

respectively to the appropriate volume of 4% acetic acid with or without 10 mM TBAHS. The following procedures were used for sample concentration of C_{18} SEP-PAK cartridges:

1) Prewash of cartridges with 5 mL ACN followed by 10 mL of 4% HOAc or 4% HOAc/10 mM TBAHS.
2) Loading of sample in 1.0 mL of 4% HOAc or 4% HOAc/10 mM TBAHS.
3) Desorption by 9.0 mL of 25% HOAc or 25% HOAc/10 mM TBAHS.
4) Samples were then diluted to 10.0 mL and analyzed by RPLC.

Two Ultrasphere ODS (5 μm, spherical porous particle) columns (1UE732N and 1UE747N) were obtained from Altex Scientific, Inc. (Berkeley, CA) and used in this study. The analytical column was preceded by a guard column, 7 cm by 2.1 mm i.d. dry packed with Whatman CO:PELL ODS (30-38 μm). After equilibration, mobile phase solvent mixtures were degassed in an ultrasonic bath. The mobile phase consisted of mixtures of water, acetic acid, and acetonitrile with or without the presence of 10 mM TBAHS, at a flow rate of 1.5 mL/min. The ultraviolet detector was operated at 254 nm.

Calculations

Determinations of retention times and integration of peak areas were performed by a Perkin-Elmer Model Sigma 10B data handling system (Norwalk, CT). Direct comparison of peak areas with external standards was used to determine sample concentration.

RESULTS AND DISCUSSION

The recovery of the herbicide picloram from aqueous environmental samples following concentration by hydrocarbonaceous extraction cartridges is of current interest in this laboratory. Bushway (14) demonstrated quantitative recoveries of carbaryl and 1-naphthol following trace enrichment of one liter water samples by passage through C_{18} SEP-PAK cartridges. Beier and Greenblatt (15) recovered up to 80% of some lacinilines and cadalenes by extraction and concentration of one liter aqueous samples with SEP-PAK C_{18} cartridges. However when attempts were made to effect the trace enrichment of large volume water samples contain-

ing picloram using SEP-PAK C_{18} cartridges, recoveries were found to be very dependent upon sample loading volume. A profile of the dependence of percent recovery upon sample loading volume for picloram (Fig. 1) shows that less than 50% recovery was obtained for a sample throughput of 25 mL. A similar non-linear dependence of extraction efficiency upon sample loading volume was demonstrated by Saner et al. (16) for some simple aromatic solutes and Nyagah (17) for the recovery of the herbicide pyrazon from SEP-PAK C_{18} cartridges.

Since silanophilic interactions have been implicated in chromatography on analytical reversed-phase columns, the participation of silanol groups in the recovery of adsorbate from hydrocarbonaceous cartridges is also possible. To establish the nature of this contribution, TBAHS was chosen as a silanol masking agent

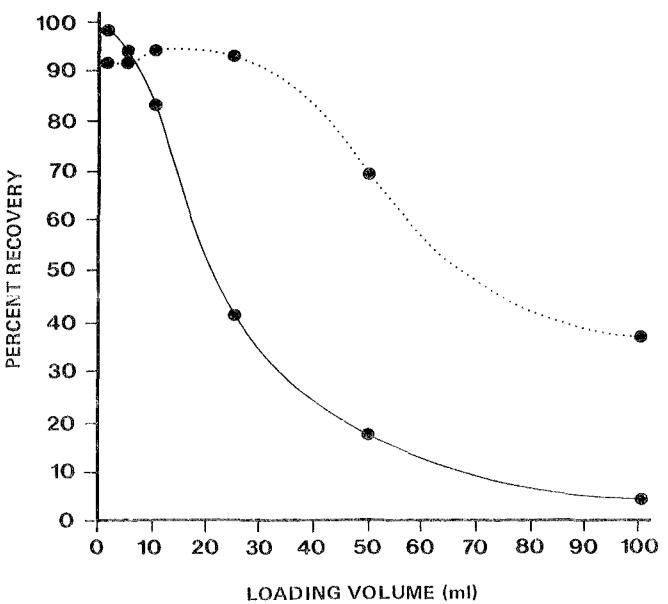


FIGURE 1. Percent recovery of picloram as a function of sample loading volume either with (•••) or without (——) TBAHS.

(6). The recovery profile for picloram as a function of sample volume was obtained with TBAHS (10 mM) added to each of the three steps of the adsorption/desorption process and is shown in Fig. 1. In the presence of TBAHS, the recovery of picloram was still curvilinear and dependent upon sample loading volume, but the efficiency was much improved for sample volumes greater than 10 mL.

To elucidate the results observed during the determination of picloram, the percentage recovery of other solutes (Fig. 2) representing diversity in polarity was studied as a function of the loading volume of the sample. All experiments were conducted under optimal conditions for picloram recovery for purposes of comparison and may not produce the best recovery for the other solutes. Acetic acid (4%) was used for pH control to enhance the retention of picloram (pKa=1.97) (18) by ion suppression (19). Acetic acid (25%) was optimal for the desorption of picloram. Experiments were performed both with and without 10 mM TBAHS in the prewash, sample loading, and desorption eluents. When samples containing TBAHS in the desorption solvent were analyzed on a C_{18} analytical column, the HPLC eluent was also modified with 10 mM TBAHS. The presence of TBAHS produced a slower drip rate from the cartridges, as well as a slightly higher back pressure in the chromatograph, presumably due to an increase in viscosity.

For the non-polar solute benzene, the alteration of the recovery plot upon addition of a silanol masking agent should be minimal. Indeed, the offset of the plots in Fig. 3B is likely due to the slight increase in the carbon coverage of the surface from adsorbed TBAHS resulting in a small increase in extraction efficiency. The maxima observed toward low loading volumes in Fig. 3 are curious, and may be dependent upon the manner in which the data were collected. The aqueous wash of the cartridge prior to charging of the sample may have been insufficient to remove all of the adsorbed acetonitrile.

Acetophenone represents a moderately polar, non-ionizable solute. The recovery of acetophenone versus loading volume is

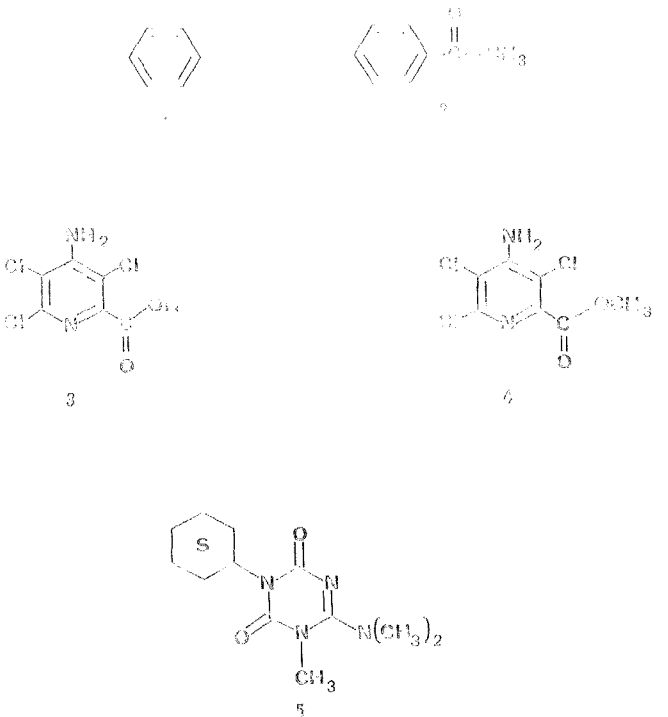


FIGURE 2. Compounds included in this study: (1) benzene, (2) acetophenone, (3) picloram (4-amino-3,5,6-trichloropicolinic acid), (4) picloram-methyl ester, and (5) hexazinone (3-cyclohexyl-6-(dimethylamino)-1-methyl-1,3,5-triazine-2,4-(1H, 3H)-dione)

illustrated in Fig. 3A. At sample loading volumes greater than 22 mL, the recovery of acetophenone was greater without the silanol masking agent. In the absence of TBAHS, hydrogen bonding between surface silanol groups and the polar carbonyl function in acetophenone enhances the overall adsorption to the stationary phase and thereby increases the resultant recovery. Therefore, a dual retention mechanism, i.e. silanophilic as well as hydrophobic, is not always detrimental to an analysis.

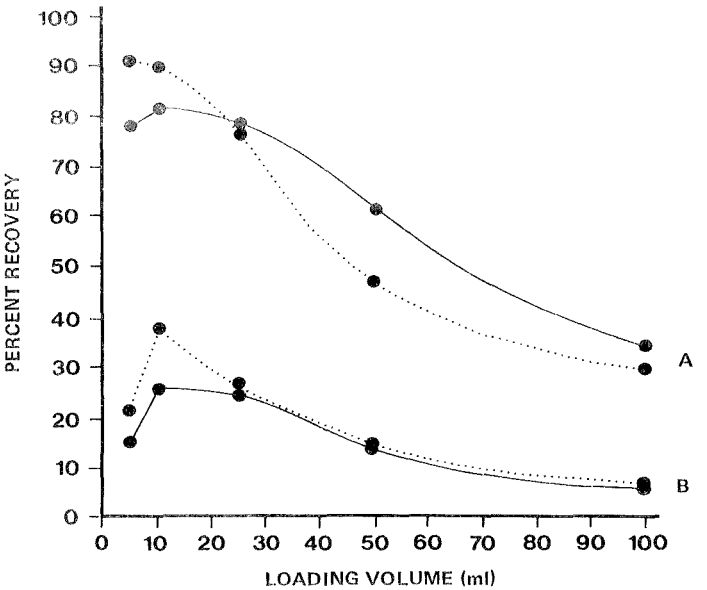


FIGURE 3. Percent recovery as a function of sample loading volume for (A) acetophenone and (B) benzene, either with (•••) or without (——) TBAHS.

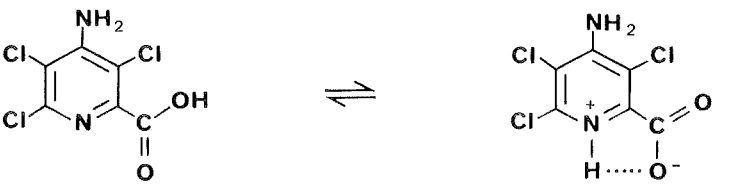
The chromatographic consequences of masking silanol groups with TBAHS are two-fold. The number of electrical charges on the bonded phase surface (20) is reduced by the adsorption of the tetrabutylammonium moiety on unreacted silanol groups. The interaction also slightly increases the total hydrocarbonaceous content of the stationary phase.

Tanaka et al. (2) have shown on C_{18} bonded analytical columns that relative to benzene, retention of toluene decreased and retention of acetophenone increased with increased exposure to silanol groups. Cooke and Olsen (3) illustrated the capacity factor changes (d=decreased, i=increased) for acetophenone (d), nitrobenzene (d) benzene (i), and toluene (i) after the end-capping of a C_{18} analytical column. These changes in capacity factor are

analogous to the changes in efficiency observed for benzene and acetophenone in these recovery experiments.

Results obtained on analytical columns (2) suggest that silanols interact most with functional groups made up of electron rich atoms, such as hydroxyl, carbonyl, nitrile, and nitro moieties, and therefore are expected to exert the greatest effect upon compounds containing such groups. Even the most commonly used organic modifiers for the mobile phase, methanol and acetonitrile, are also competing for adsorption on silanol sites (2) with methanol apparently being bound in preference to acetonitrile (6).

For ionizable compounds even greater effects would be expected upon masking the electrically charged surface with TBAHS. Picloram, 3A, can exist as: (a) the neutral molecule, (b) the conjugate acid (formed by protonation of the base functionality), (c) the conjugate base (due to ionization of the carbonyl group), and (d) the zwitterion, 3B, (18,21). Zwitterion formation is also



3A 3B

observed in other pyridine carboxylic acids (22). Intramolecular hydrogen bonding helps to reinforce structure 3B. This complicates measurement of the acid dissociation constant for picloram. Reported pKa values range from 2.8 to 4.1. The most reliable value seems to be that of Osteryoung and Whittaker (18) who report a pKa' value of 1.97 for picloram, representing a combined equilibrium constant for the dissociation of both the neutral and zwitterionic forms. The pKa for the amine group of picloram is estimated at -2.0 to -1.3 (18), so the concentration of the conjugate acid is negligible under the experimental conditions used here. In 4% acetic acid (pH=2.5), some equilibrium exists between

the neutral molecule, the zwitterion, and the conjugate base. The presence of negative sites on the bonded phase surface greatly reduces retention (and therefore recovery) by inhibiting interaction with the bonded phase ligand (C_{18}).

The possible contribution of ion-pair formation to the improvement of the recovery observed for picloram cannot be ignored. Strong acids can be retained throughout the usable pH range for bonded phase materials (pH2-8) by ion-pairing with lipophilic quaternary ammonium salts. Complexation with the tetrabutylammonium counterion (in the mobile phase or at the bonded phase surface) would increase the hydrophobicity of the picloram anion and increase retention on a reversed-phase adsorbent.

In order to pursue this problem, the dependence of recovery upon loading volume was plotted for the methyl ester of picloram, 4, in Fig. 4. Zwitterion formation is blocked in the ester and ion-pairs with TBAHS would not occur. Fig. 4 demonstrates the improvement in recovery of picloram-methyl ester due to silanol masking. The comparison of Figs. 1 and 4 cannot be expected to be additive, because the methyl group not only blocks ionization, but also contributes its own hydrophobicity. The conclusion then, is that the improvement in the recovery of picloram (Fig. 1) is probably due to a combination of silanol masking and ion-pairing, while the improvement for the methyl ester (Fig. 4) is attributed to silanol masking alone.

The recent results of Nyagah (17) in developing a recovery analysis for the herbicide pyrazon (5-amino-4-chloro-2-phyenyl-3-pyridizone) lend further support to these observations. The recovery of pyrazon from C_{18} cartridges was shown to be dependent upon the concentration of NaCl in the sample. Nyagah found that NaCl added to water samples containing pyrazon greatly improved the recovery of this solute from SEP-PAK C_{18} cartridges, and proposed that the added salt decreased the water solubility of pyrazon thereby increasing adsorption onto the C_{18} bonded ligand. Palusova et al. (23) have shown that the addition of sodium chloride to water containing pyrazon increased the yield of this

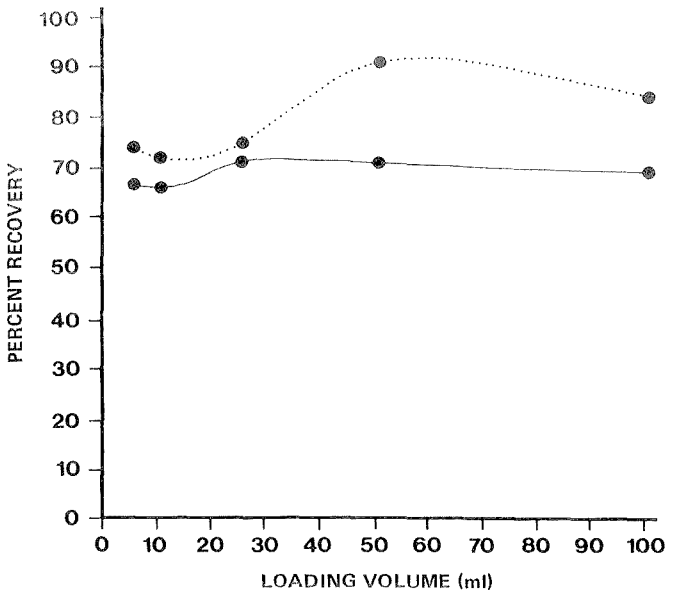


FIGURE 4. Percent recovery of picloram-methyl ester as a function of sample loading volume either with (•••) or without (—) TBAHS.

herbicide in the organic solvent because this decreased the solubility of the extracting solvent in water. The quantitative recovery of pyrazon from 500 mL water samples containing 25% NaCl demonstrated by Nyagah (17) may actually be another example of silanol masking. Saturation of the silanol groups on ODS (Octyldecasilyl) silica columns by inorganic salts added to the eluent was first established by Knox and Jurand (24). The ability of a potassium phosphate buffer to attenuate the untoward effects of the Donnan equilibrium and ion exclusion due to electrical charges on the bonded phase surface of analytical columns has been shown (25). In this lab, the mean values for the extraction of 800 mL water samples containing picloram demonstrated no difference by Student's t ($P>0.20$) whether the sample was acidified

to pH2 with H_2SO_4 or acidified with H_3PO_4 and NaCl (4%) added prior to extraction with diethyl ether. Andrews and Good (26) tested the recovery of pesticides from various types of water samples with C_{18} Bond Elut columns and found that recovery improved for 6 of the 7 compounds examined when distilled water samples (100 mL) were compared to water samples containing salt or up to 10% methanol. These results are compatible with the argument presented here as both methanol (6) and inorganic salts (24) adsorb on silanol sites.

The ionic species of picloram is negatively charged in 4% acetic acid, while the dimethylamino group of hexazinone, 5, is protonated resulting in a positive charge. This positive charge should effect a reduction in hexazinone recovery. However, the increase in recovery found for hexazinone upon addition to TBAHS (Fig. 5) suggests that the effects of silanol masking outweigh the expected reduction in recovery due to like charge competition.

A chromatogram of the five solutes further emphasizes the positive effects of silanol masking in this instance. The analytical column used in this experiment was end-capped by the manufacturer, therefore solvent-accessible silanol groups are minimal. Alteration in the chromatogram of the solutes compared in this study upon the addition of TBAHS is shown in Fig. 6. Table 1 lists the solute capacity factors for the chromatograms in Fig. 6. Each k' value represents the average of four injections. The chromatograms were recorded using the same solvent batch, half of which had been made 10 mM in tetrabutylammonium hydrogen sulfate. The capacity factors for benzene, acetophenone, and picloram-methyl ester were each decreased approximately 3% by the addition of TBAHS. The retention of acetophenone and picloram-methyl ester relative to benzene, as given by the α_x value, did not change however. Since these three diverse solutes behaved similarly, there appear to be no observable SiOH interactions in this analytical column.

The capacity factor of picloram nearly doubled when TBAHS was added to the eluent while k' for hexazinone decreased by about

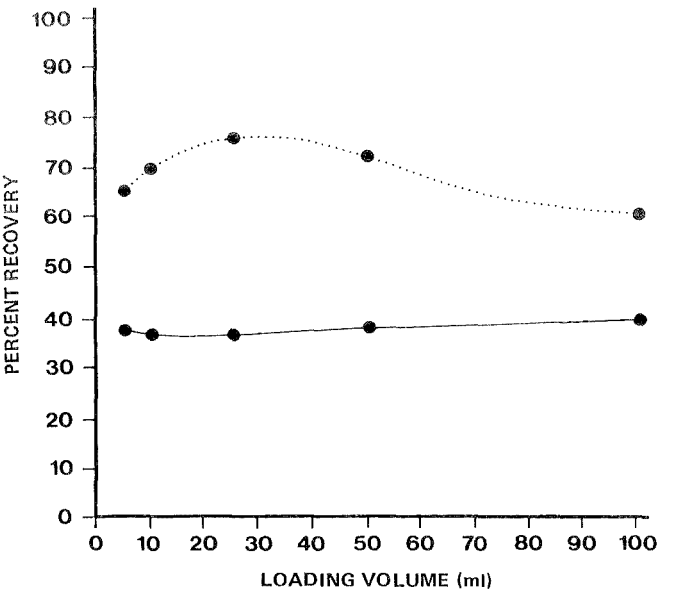


FIGURE 5. Percent recovery of hexazinone as a function of sample loading volume either with (···) or without (—) TBAHS.

TABLE 1

Experimentally Determined Capacity Factors, k', on Ultrasphere ODS (1UE747N)

$\alpha_x = k'_x/k'_{benzene}$

	4% Acetic Acid/Acetonitrile (70:30)			
	No Added TBAHS		TBAHS (10 mM)	
Solute (x)	k'	α_x	k'	α_x
picloram	1.13	0.10	2.03	0.19
hexazinone	3.51	0.32	2.77	0.26
acetophenone	4.42	0.40	4.25	0.40
picloram-methyl ester	6.88	0.63	6.67	0.63
benzene	10.93	1.00	10.60	1.00

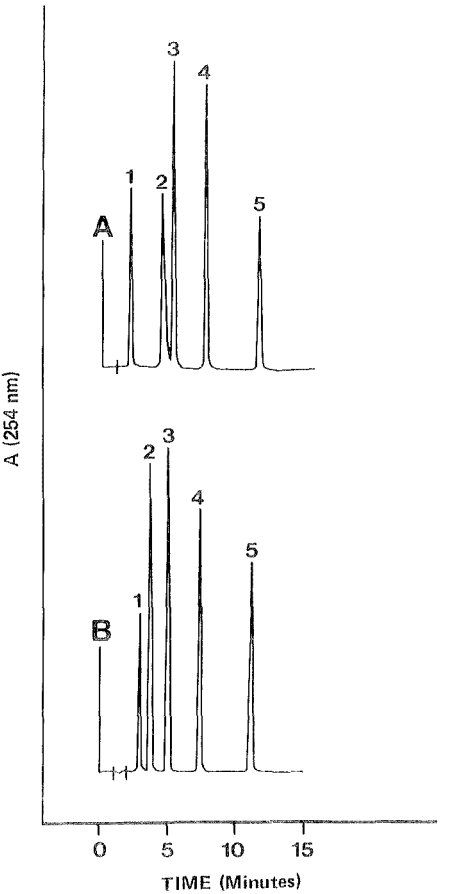


FIGURE 6. RPLC elution on Ultrasphere ODS (1UE747N) in 4% acetic acid/acetonitrile (70:30): (A) without TBA, (B) containing 10 mM TBA. Peaks: (1) picloram, (2) hexazinone, (3) acetophenone, (4) picloram-methyl ester, and (5) benzene.

TABLE 2

Summary of Chromatographic Results Produced by Addition of TBAHS (10 mM).

Solute	Sep-Pak Recovery	Analytical Column Retention
benzene	increased	slight decrease
acetophenone	decreased	slight decrease
picloram	increased	increased
picloram-methyl ester	increased	slight decrease
hexazinone	increased	decreased

20%. Since there seems to be little or no interaction attributable to silanol groups in this column, the observed increase for picloram must be the result of ion-pair formation between picloram and the TBAHS added to the mobile phase. Based on this result and those for picloram-methyl ester, it appears that the improvement in recovery for picloram observed in Fig. 1 is due to both silanol masking and ion-pair formation. The reduction in k' for hexazinone is produced by like-charge competition between the hexazinone and TBAHS moieties. Table 2 summarizes the results of both the extraction column and analytical column studies.

CONCLUSIONS

The heterogeniety of covalently bonded hydrocarbonaceous stationary phases can lead to a mixed mechanism of retention. The potential problem of residual silanol groups is common to all hydrocarbonaceous bonded materials whether used as analytical columns or preconcentration cartridges. End-capping appears to successfully reduce the number of solvent-accessible silanol groups in reversed-phase sorbents, but may not be economically feasible for pre-analysis extraction columns. Silanol masking with lipophilic quaternary ammonium ions is a viable alternative.

For complicated molecules having a diversity of functional groups, the interactions become more complex and masking SiOH groups may or may not improve recovery. Ion-pairing techniques, already well developed for use in reversed-phase analytical columns, can be used to improve extraction efficiency from hydrocarbonaceous recovery columns.

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PREPARATION AND PURIFICATION OF TRITIATED DEXTROMETHORPHAN

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ABSTRACT

Tritiated dextromethorphan (^{3}H-DM) was prepared from dextrorphan and purified by reverse-phase high performance liquid chromatography (HPLC). The tritiated drug was found to be homogeneous and identical to authentic unlabeled DM by both HPLC and thin layer chromatography. The specific radioactivity of ^{3}H-DM was 26 Ci/mmole.

INTRODUCTION

Dextromethorphan (DM) is a non-narcotic antitussive which suppresses cough by an action on the central nervous system. Although little is known concerning the mechanism of action of this drug, the structural similarity of DM to codeine has suggested that DM acts at the same central sites as codeine to suppress cough. However, Cavanagh et al. (1) have recently demonstrated that naloxone antagonizes the antitussive effects of codeine and other opiate analgesics but does not antagonize the antitussive activity of DM. These findings indicate different modes of action for the cough suppressant effects of DM and codeine.

Since DM does not elicit its antitussive effects by an opiate receptor interaction, as evidenced by the lack of effect of naloxone, the possibility exists that this drug acts at other specific central nervous system sites which can be detected by an appropriate binding assay procedure. To study such a possibility, we have prepared tritium labeled DM (^{3}H-DM) of high specific activity, and, as initially reported (2-4), we have found specific high affinity binding sites for DM in guinea-pig brain which may be related to the antitussive effects of the drug.

The present report describes the preparation of ^{3}H-DM and its purification by reverse-phase high performance liquid chromatography (HPLC). Since it is necessary that the radioligands used in binding studies be at the minimum 95 percent pure (5), it was essential for us to develop simple chromatographic systems in which we could obtain pure ^{3}H-DM in good yield. Dixon et al. (6) have described a method to purify ^{3}H-DM using column chromatography and thin layer chromatography (TLC) but the purity and recovery of ^{3}H-DM achieved by their procedures were not stated.

MATERIALS AND METHODS

Dextrorphan tartrate and dextromethorphan hydrobromide were generously supplied by Hoffman-La Roche (Nutley, NJ). N-methyl-N'-nitro-N-nitroso guanidine and the diazomethane-generating apparatus were obtained from Aldrich Chemical Co. (Milwaukee, WI). n-Nonylamine was purchased from Sigma (St. Louis, MO). Acetonitrile and n-propanol were HPLC grade (Burdick and Jackson) and all other reagents were Fisher ACS reagent grade. Acetic acid, acetonitrile, triethylamine and n-propanol were distilled over ninhydrin prior to use.

Deionized water (Hydro Systems, Durham, SC) was used in the preparation of all aqueous buffers.

Chromatography

The HPLC equipment consisted of a Milton-Roy Simplex minipump, model 396-31 (Riviera Beach, FL) and a Rheodyne #70-10 sample injection valve with a 1 ml loop (Rainin Instrument Co., Inc., Ridgefield, NJ); gradients were generated by an LKB Ultragrad 11300 gradient maker (Rockville, MD). Separations were performed on an Altex Lichrosorb RP-18 column (10 µm particle size, 4.6 x 250 mm) or an Altex Ultrasphere-cyano column (5 µm particle size, 4.6 x 250 mm). UV determinations of the column effluents were made with a Beckman System 25 spectrophotometer and radioactivity was determined in a Beckman LS-230 liquid scintillation counter using a phase-combining scintillant (Liquiscint) from National Diagnostics (Somerville, NJ). The presence of n-nonylamine in the column effluents was monitored by an automated fluorescamine-based detection system (7) using a Spectra/Glo Model FL-1B Fluorometer (Gilson Medical Electronics, Inc., Middleton, WI) coupled to a Linear dual channel recorder (Irvine, CA). During the preparation of mobile phase solvents, aqueous buffers were filtered through Millipore HA 0.45 µm filters and organic solvents through millipore FH 0.5 µm filters. Prior to use all solvents were thoroughly degassed.

TLC was carried out on Whatman LK5D silica gel G plates and DM standards were visualized with iodoplatinate.

Fluorescence Spectroscopy

Fluorescence spectroscopy of labeled and unlabeled DM was performed with a Perkin-Elmer Model 650-10S spectrofluorometer coupled to a Perkin-Elmer recorder (Norwalk, CT).

Preparation of Diazomethane

Diazomethane was generated by the action of alkali on N-methyl-N'-nitro-N-nitroso guanidine using the procedure and apparatus described by Fales et al. (8).

Preparation of ^{3}H-DM from dextrorphan

Iodination of dextrorphan [(+)-3-hydroxy-17-methylmorphinan] was carried out by the method of Grewe et al. (9). Briefly, 255 mg of dextrorphan tartrate were dissolved in water and the free base (0.6 mmole) obtained by the addition of 1 N sodium hydroxide. A solution of iodine (0.6 mmole) in potassium iodide (0.9 mmole) was added dropwise and with constant stirring to the dextrorphan solution. After 10 minutes the solution was bubbled with 100% CO_2 and filtered. The precipitate was washed twice with water, dried under vacuum and taken up in chloroform. The chloroform was then evaporated at 50°C under a stream of N_2 and iodinated dextrorphan obtained by crystallization from methanol. 60 mg of the iodinated compound were sent to New England Nuclear Corp. (Boston, MA) and tritiated by catalytic reduction with 25 Ci of tritium gas to a specific activity of 60.4 mCi/mg. Methylation of ^{3}H-dextrorphan to form tritiated (+)-3-methoxy-17-methylmorphinan (^{3}H-DM) was carried out by reacting 2-8 mCi of ^{3}H-dextrorphan with diazomethane in ether for 30 minutes at 0°C. At the end of the reaction, ether and residual diazomethane were removed by a stream of N_2 and the components of the reaction mixture subjected to HPLC as described in the next section. The yield of ^{3}H-DM varied between 20 and 50 percent.

RESULTS AND DISCUSSION

HPLC systems were initially designed to achieve a good separation of DM from dextrorphan. Because DM is more lipophilic than dextrorphan, such a separation on an

octadecylsilane column (RP-18) could be readily obtained. However, DM exhibited long retention times with asymmetric peaks and severe tailing and this behavior was observed even when ion-pairing reagents were present in the mobile phase. Several investigators who observed similar phenomena during ion-pair HPLC of hydrophobic cationic drugs found that retention time and peak symmetry could be dramatically improved when long-chain alkyl ammonium compounds were included in the mobile phase (10,11). This approach was investigated and the nine-carbon ammonium compound, n-nonylamine, was incorporated into the mobile phase. The results were quite satisfactory in that retention time of DM was significantly reduced and peak shape greatly improved.

As Fig. 1 illustrates, an excellent separation of DM from dextrorphan was obtained using a 2-30% acetonitrile gradient in 0.1 M acetic acid, 0.01 M n-nonylamine. In addition, this HPLC system also resolved ^{3}H-DM from several tritiated side products of the methylating reaction (Fig. 1). The radioactive profile of an aliquot from a typical diazomethane reaction mixture shows not only a distinct peak of radioactivity eluting in the same position as unlabeled DM but also two other radioactive peaks with significantly greater retention times. Recovery of radioactivity in this system was greater than 90 percent.

When an entire reaction mixture was subjected to HPLC using the system just described, the elution volume corresponding to the DM peak was collected as four individual fractions (similar to #20,21,22 and 23 in Fig. 1) which were not pooled. Aliquots of each of these fractions were analyzed for radiochemical purity by rechromatographing on the same HPLC system and by TLC in two solvent systems: (chloroform:methanol:ammonium hydroxide, 75:25:0.3; ethanol:acetic acid:water, 60:30:10). The results from these chromatographic analyses indicated that the earliest

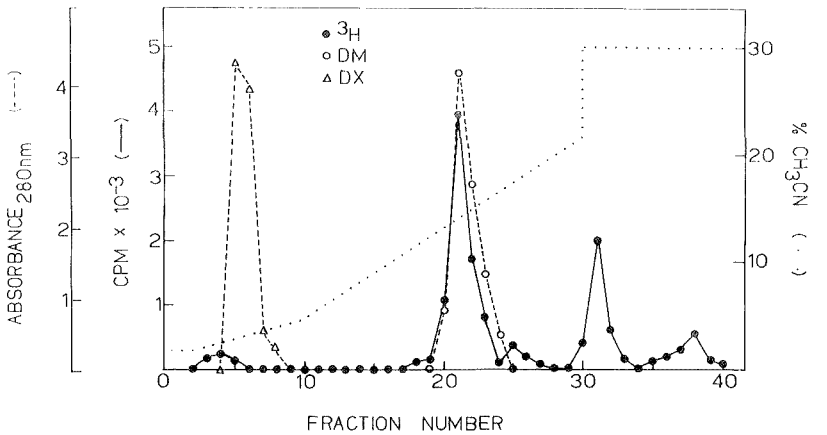

FIGURE 1. Reverse-phase HPLC of ^{3}H-DM prepared by methylation of ^{3}H-dextrorphan. An aliquot from a typical diazomethane reaction mixture was applied to an RP-18 column and the radioactivity eluted with a 2-30% acetonitrile gradient in 0.1 M acetic acid, 0.01 M n-nonylamine. Two ml fractions were collected at a flow rate of 0.67 ml/min. Recovery of radioactivity was 91 percent. Also shown is the elution profile for 0.7 µmoles of unlabeled dextrorphan (DX) and 0.7 µmoles of unlabeled DM.

eluting fraction (#20) was impure. During the HPLC analysis only 60 percent of the radioactivity of fraction #20 co-eluted with the peak of unlabeled DM. TLC analysis yielded a similar estimation of purity since it was observed that less than 50 percent of the radioactivity of fraction #20 migrated with unlabeled DM. In contrast to the heterogeneous nature of the radioactivity in the early DM peak fraction (#20), the radioactivity in the next three fractions (#21-23) appeared homogeneous and behaved identical to that of authentic unlabeled DM during HPLC and TLC analysis.

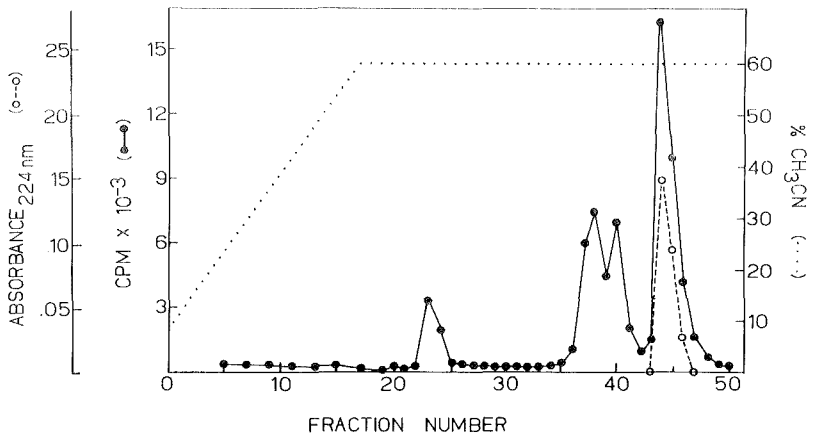

FIGURE 2. Reverse-phase HPLC of the radioactivity of fraction #20. An aliquot of fraction #20, mixed with 50 nmoles of unlabeled DM, was applied to an Ultrasphere-cyano column and eluted with a 10-60% acetonitrile gradient in 7.5 mM H_3PO_4, 10 mM triethylamine, pH 4.0. One ml fractions were collected at a flow rate of 0.6 ml/min. Recovery of radioactivity from the column was 99%.

The radioactive homogeneity of fractions #21-23 was further established by reverse-phase HPLC on an Ultrasphere-cyano column using a 10-60% acetonitrile gradient in 7.5 mM H_3PO_4, 10 mM triethylamine. The resolving power of this system is demonstrated in Fig. 2 where it can be seen that three tritiated species in addition to ^{3}H-DM are present in fraction #20. In agreement with the results obtained by TLC analysis, ^{3}H-DM accounts for less than 50 percent of the radioactivity of this fraction. When fractions #21-23 of the ^{3}H-DM peak were analyzed by this same HPLC system, one homogeneous peak of radioactivity co-eluting with authentic unlabeled DM was found for all three fractions. Based on these results as well as the

previous analyses by HPLC and TLC, ^{3}H-DM in the three later peak fractions was considered radiochemically pure and used for subsequent binding studies without further purification.

Having achieved the purification of tritium labeled DM, it was necessary to remove n-nonylamine from the ^{3}H-DM preparations since it interfered with the binding assay. Although n-nonylamine could be removed by evaporation under reduced pressure, this procedure resulted in significant losses of the tritiated drug. Therefore, n-nonylamine was separated from ^{3}H-DM by reverse-phase HPLC on an octadecylsilane column using a 10-40% n-propanol gradient in 10 mM KH_2PO_4, pH 4.0. Recovery of ^{3}H-DM during this procedure was greater than 95 percent.

Tritiated DM was stored at 4°C in the propanol-phosphate buffer with no chemical deterioration in one year. The concentration of labeled drug was determined by fluorescence spectroscopy using excitation and emission wavelengths of 230 nm and 305 nm respectively. A specific activity of 26 Ci/mmole was calculated for the final ^{3}H-DM product. During the fluorescence determinations it was observed that the excitation and emission spectra of radioactive DM were identical to that of authentic unlabeled DM.

In conclusion, we have described the preparation of tritium labeled DM and the chromatographic procedures used to obtain ^{3}H-DM in radiochemically pure form. Our results have clearly demonstrated that reverse-phase HPLC is an excellent method to purify ^{3}H-DM. By incorporating n-nonylamine into the mobile phase buffer, a significant portion of the ^{3}H-DM peak eluted from the RP-18 column during the first HPLC step was pure. Even though n-nonylamine was an interfering substance in our binding assay and had to be removed from the ^{3}H-DM preparation by an additional HPLC step, the overall methods we have reported are fast, simple and yield excellent recoveries.

Since TLC is a standard method for purifying radioligands used in binding studies, it is perhaps relevant to mention that this chromatographic procedure was our preliminary method for isolating ^{3}H-DM. However, we found that as a routine preparative procedure, TLC provided neither the recovery nor the purity of ^{3}H-DM that was subsequently achieved by HPLC. Nevertheless, TLC served as a useful analytical procedure for assessing the purity of ^{3}H-DM isolated by HPLC.

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EVALUATION OF THE USE OF LITHIUM NITRATE AS A TEST SUBSTANCE FOR THE DETERMINATION OF THE HOLD-UP TIME ON A REVERSED-PHASE PACKING

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ABSTRACT

The use of lithium nitrate as a test substance for the determination of the hold-up time on μBondapak C18 columns has been evaluated by comparison with the hold-up time calculated with the homologous series of n-alcohols. The influence of charge exclusion effects on the retention time of lithium nitrate is investigated. The addition of phosphoric acid to the eluent appears to be an effective method to reduce charge exclusion effects. The existence of deviations from linearity are demonstrated for the homologous series of n-alcohols in eluents with a low methanol content.

INTRODUCTION

In the investigations of the relation between structure and retention or between eluent composition and retention, retention is usually expressed as the capacity factor or the logarithm of the capacity factor.

For the calculation of the capacity factor the hold-up time is used. Large errors in the value of the capacity factor can result from imprecise and/or inaccurate measurements of the hold-up time, particularly in the case of compounds which show little retention.

Lithium nitrate has been used in the laboratory of the authors for the measurement of the hold-up time in reversed-phase high performance liquid chromatography (HPLC) for a number of

years. It had been chosen because of its good solubility in water-methanol mixtures and its detectability with UV detectors. However, some doubts arose about its use due to the fact that differences in retention times were observed between some very polar compounds, the constituents of the eluent and lithium nitrate. It was then decided to compare the retention times of those substances with the theoretical hold-up time calculated from the homologous series of n-alcohols, a method which has been in use for a long time in gas chromatography (1-7), and which has more recently been described for HPLC (8-10).

In a recent publication Berendsen et al. (8) objected to using salt solutions for the determination of the hold-up time. They describe a strong dependence of the retention time of bromide on the quantity injected. They explain this phenomenon by the charge exclusion effects from the pores of the column packing and the suppression of this effect at higher salt concentrations.

For this reason the dependence of the retention time of lithium nitrate on the quantity injected was also investigated. As charge exclusion effects will depend on the charge of the packing, i.e. the dissociation of the free silanol groups, the effect of the injection of acidic and basic samples (i.e. solutions of phosphoric acid and ammonium carbonate in the eluent) as well as the addition of phosphoric acid or lithium chloride to the eluent on the retention time of lithium nitrate were included in this study. The results of these investigations are given together with the previous ones.

In this study some attention is also paid to the occurrence of deviations from the well-known linear relationship between the logarithm of the capacity factor and the number of carbon atoms for the series of n-alcohols in eluents with low methanol content.

EQUIPMENT AND CHEMICALS

Chromatographic experiments were performed using a 6000A solvent delivery system, a U6K injection system and an R401 differential refractometer (all from Waters Assoc., Milford, MA, U.S.A.).

The 2 ml sample loop of the U6K injector was replaced by a loop of approximately 100 µl in order to reduce errors in the retention time measurements caused by decompression and subsequent compression of the contents of the sample loop upon injection of the sample. A µBondapak C18 column, 30 cm x 3.9 mm I.D., particle size 10 µm, (Waters Assoc.) was used. The column was thermostatted by means of a metal block which was shielded from the surroundings by isolating material and which was connected in series with the housing of the refractive index detector to a circulating waterbath. Due to the limited column life time not all experiments were carried out using the same column. When data were obtained on different columns this is indicated in the tables. The temperature of the column and the refractive index detector was kept at 25° C.

The retention times were measured and recorded by the combination of an SP 4000 central processor, an SP 4020 data interface and an SP 4050 printer plotter (all from Spectra Physics, Santa Clara, CA, U.S.A.).

The pH shifts in the eluent were studied with a Radiometer (Copenhagen, Denmark) G 299 A capillary glass pH electrode which was connected to the outlet of the refractive index detector. The outlet of the capillary glass electrode was connected to a small glass vessel (provided with an outlet for the excess of eluted solvent) in which a Type 373-90 reference electrode (Ingold, Zürich, Switzerland) was placed. The capillary glass electrode and the reference electrode were connected to a Radiometer PHM 64 pH meter. The jacket around the capillary glass electrode was filled with potassium nitrate solution which was electrically connected to the reference electrode to avoid instability of the signal of the pH electrode (11, 12).

Water was purified by deionization and subsequent double destillation from glass. Methanol (pro analysis) was obtained from Merck (Darmstadt, G.F.R.). The other chemicals were purchased from various suppliers.

INSTRUMENTAL AND ANALYTICAL PROCEDURES

Retention times were corrected for the extra column volume by replacing the column by a low dead volume union and measuring the retention time of an equal volume of a test substance. All retention times were corrected by subtraction of the "extra column hold-up time". Retention times were measured using the SP 4000 integrator. The peak width parameter and the slope sensitivity for the integration process were adapted in such a way that precision in the retention time measurements of 0.1 to 0.2% could be obtained.

To avoid cavitation in the pump heads methanol and water were filtered directly before the preparation of the eluents by vacuum filtration through filters with a pore size of 0.2 μm. After mixing the eluents were degassed further ultrasonically. In order to avoid long term flow fluctuations the pump was continuously operated at the same flow (1.0 ml/min).

The hold-up time was studied with the eluents methanol, methanol-water (8:2, w/w), methanol-water (6:4, w/w), methanol-water (4:6, w/w), methanol-water (2:8, w/w) and water. A limited number of experiments has also been done with eluents containing 50% w/w methanol.

The theoretical value of the hold-up time was calculated from the retention times of the alcohols methanol, ethanol, 1-propanol, 1-butanol, 1-pentanol, 1-hexanol, 1-heptanol, 1-octanol and 1-decanol.

The following test substances for the determination of the hold-up time were compared: water, methanol, formamide, glycerol, 1,3,5-trihydroxybenzene (THB) and lithium nitrate.

In the eluents containing water-methanol (5:5, w/w), water-methanol (8:2, w/w), and water, lithium nitrate, urea and thiourea were compared. All compounds were dissolved in or diluted with the eluent. Solutions of lithium nitrate were prepared in a concentration of 10^{-1} gmol·kg^{-1}.

Sufficiently symmetrical peaks and constant retention times were obtained by injection of approximately 20 μl. The other compounds were injected in such quantities that they could be well detected

at 8 to 16x attenuation on the refractive index detector. The amounts injected were generally 100 to 200 µg.

MATHEMATICAL PROCEDURES

The calculation of the hold-up time is based upon the assumption that in reversed-phase liquid chromatography the relationship between the retention of the members of the homologous series of n-alcohols and their number of carbon atoms is given by the following well-known equation

$$t_R - t_d = t_d \times 10^{(A+B\times N)} \qquad (I)$$

(in which t_d, t_R, N, A and B are the hold-up time, the retention time of the alcohol, its number of carbon atoms and two constants respectively).

After measurement of the retention times of the n-alcohols the values of the hold-up time and both constants that fit best in the equation are computed. For the calculation of the hold-up time a calculation method based upon minimizing of the function

$$\sum_m \left(\frac{t_{R_m} - t_d - t_d \times 10^{A+B\times N_m}}{t_{R_m}} \right)^2 \qquad (II)$$

according to Levenberg and Marquard (13,14) was used. The calculation was done by means of a program based upon the FORTRAN subroutine ZXSSQ (15). This method should be preferred to other possible techniques for in this calculation method the separate retention times contribute to the calculated hold-up time according to the reproducibility in their measurements.

The accuracy in the hold-up time calculated from the retention time of a number of members of a homologous series depends on the following factors:

1. The number of homologues involved in the calculation
2. The number of carbon atoms of the homologue involved in the calculation

3. The precision in the retention times measured (which is a function of the number of measurements)
4. The values of the constants A and B in Equation I
5. Deviations from the assumed valid relationship of Equation I.

The computer program used for the calculation of the hold-up time also calculates the standard deviation in the hold-up time. This standard deviation results from the observed differences between the measured retention times and the calculated relationship of Equation I.

It therefore results both from the reproducibility of the retention times measured and possible systematic deviations from Equation I. When the assumption of the validity of Equation I is correct, the following results should be obtained on the calculation of the hold-up time:

1. The measured retention times should exhibit a random distribution round the values obtained with the calculated relationship of Equation I
2. This distribution should have a standard deviation which corresponds with the precision of the measurement of the retention times of the homologues
3. No significant differences should occur between the hold-up times calculated from different combinations of the members of the homologous series.

When one or more of these results are not obtained, deviations of Equation I are likely to occur.

RESULTS AND DISCUSSION

Calculation of the hold-up time from the homologous series of n-alcohols

In the eluents with lower methanol contents only the lower homologues of the series of alcohols have been chromatographed, because the higher members of the series gave too long retention times and were only detectable if rather large quantities were injected.

TABLE 1

Retention Times of Alcohols

	Eluent Composition % w/w Methanol					
	100%	80%	60%	40%	20%	0%
Methanol		158.1	154.9	156.4	163.1	200.9
Ethanol	166.8	168.6	171.9	183.7	205.6	301.2
Propanol	167.0	174.3	188.5	225.9	305.4	667
Butanol	169.6	181.2	214.9	320.9	603	2042
Pentanol	172.1	192.8	262.5	538	1525	
Hexanol	175.1	208.8	336.3	1038		
Heptanol	178.9	231.3	462.6			
Octanol	182.4	261.1				
Decanol	193.0	358.8				

The reproducibility in the measurements of the retention times was found to be dependent on the solvent composition. The following relative standard deviations were found for the eluents investigated: For the eluents containing 100% and 80% methanol 0.2%, for 60% methanol 0.4%, for 40% methanol 0.5-0.7%, for 20% methanol 1-1.5% and for 0% methanol 1-2%. These higher relative standard deviations with the eluents with a lower methanol content are mainly caused by long term fluctuations in the retention times. Short term reproducibility was found to be considerably better. The long term fluctuations may possibly be caused by the increased solubility of air in the eluents at lower methanol contents, resulting in an increased tendency to cavitation in the pump head. The retention times found for the n-alcohols are given in Table 1.

In the literature deviations from Equation I for the lower members of a homologous series have been described (16). Although it is possible that such observations result from the use of a wrong value for the hold-up time, this is not necessarily the cause of this deviation. It is therefore necessary to evaluate the validity of Equation I.

TABLE 2

Hold-up Times Calculated from the Retention Times of Alcohols

Eluent Composition % w/w Methanol	I	II
100%	———	155.2 (1.5)
80%	148.8 (2.2)	152.8 (0.9)
60%	138.9 (3.7)	146.8 (1.2)
40%*	142.6 (3.6)	151.0 (0.3)
20%*	147.2 (4.6)	157.9 (1.3)
0%*	164.8 (1.5)	169

I Hold-up time calculated from all alcohols
II Hold-up time calculated from all alcohols except methanol
* These values have been obtained on a second column

The effect of the inclusion of the retention time of methanol in the calculation is shown in Table 2.
The standard deviations in Table 2 are obtained with the computer program. The strong decrease in the standard deviation when the retention time of methanol is excluded from the calculation reflects the contribution of methanol to the deviation from Equation I.
It was therefore concluded that the retention time of methanol should not be included in the calculation of the hold-up time.
This phenomenon of systematic deviations due to methanol is not unexpected as methanol is in these experiments both solute and modifier.
The methanol in the eluent is known to be incorporated in the stationary phase according to a non-linear distribution isotherm (17,18). The retention time of the concentration pulse caused by the injected methanol will be influenced by the curvature of this distribution isotherm.
This will result in a chromatographic behaviour which deviates from the relationship of Equation I. When methanol was excluded from the calculation no indication for deviations from Equation I were

TABLE 3

Average Hold-up Times Calculated from 12 Series of Retention Times of n-Alcohols with the Eluent containing 40% MeOH

Hold-up Time calculated from	Averaged Hold-up Time	Standard Deviation in the Averaged Hold-up Time
Methanol-Hexanol	142.6	0.3
Ethanol-Hexanol	151.0	0.3
Propanol-Hexanol	152.0	0.3
Butanol-Hexanol	153.7	0.7
Ethanol-Pentanol	150.5	0.2
Propanol-Pentanol	150.7	0.3
Ethanol-Butanol	150.3	0.2

obtained with eluents with a methanol content higher than 40%. With eluents containing 40% methanol, however, indications for a slight non-linearity were obtained. For further examination of this phenomenon 12 series of retention times of n-alcohols from methanol to hexanol were determined (the injected quantity was 100-200 µg). For each of the series the hold-up time was calculated from different combinations of alcohols. The corresponding hold-up times were averaged and the standard deviations in the averaged hold-up time were calculated. The results obtained are given in Table 3. The data clearly indicate the existence of a deviation from Equation I, even when methanol is excluded from the calculations. The data also suggest that the non-linearity is more likely to be caused by deviation of hexanol than deviations of ethanol.
(The standard deviations in Table 3, in contrast to those in Table 2, result only from the reproducibility of the retention times measured and not from systematic deviations from Equation I).
Analogous results were obtained with 14 series of retention times of series of alcohols from ethanol to pentanol with an eluent containing 20% methanol (Results not shown). With eluents containing

0% methanol no such investigations could be done because only the retention times of ethanol, propanol and butanol were available for calculation.

Examination of the peak shape of the n-alcohols revealed that the peaks showed an increased tendency to tail when the methanol content of the eluents was lowered from 40% to 20% and 0%. This tendency to tail was stronger with the alcohols with longer chains.

The change in peak shape on changing the quantities injected for the eluents containing 20% and 0% methanol is illustrated by Fig. 1. The chromatograms were recorded in such a way that the changes in the quantities injected were compensated by a change in the

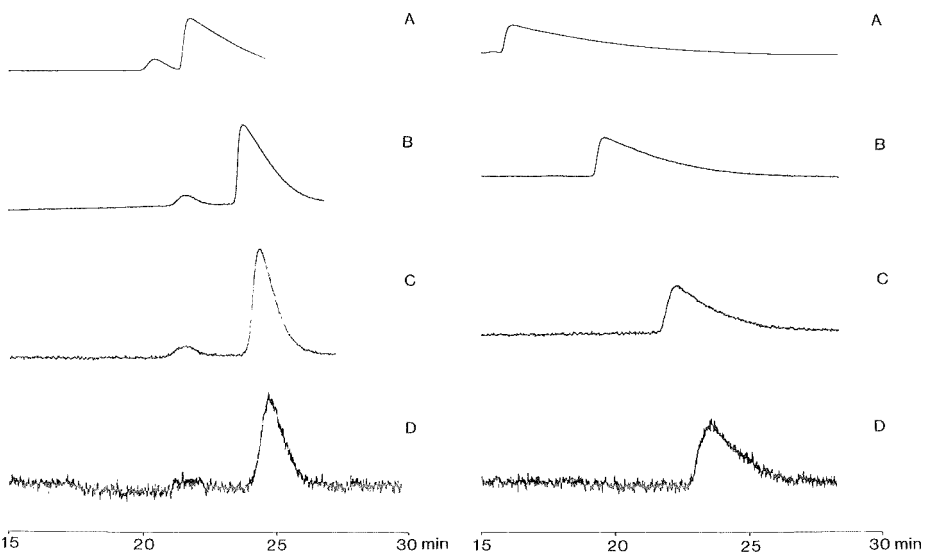

FIGURE 1

Influence of amounts injected on the peak shape and the retention time of pentanol (left, eluent 20% methanol w/w) and butanol (right, eluent water). The amounts injected and the detector attenuation are a: 1280 µg, 64; b: 320 µg, 16; c: 80 µg, 4 and d: 20 µg, 1.

attenuation of the refractive index detector. In this way a better comparison of the peak shape is possible.

The changes in the retention times and the peak shapes on varying the quantities injected clearly indicate that some overload ing occurs. (For the peak chape improves on decreasing the quantity injected and the retention times measured seem to approach a constant value).

Although reduction of the quantities improves the linearity, no sufficient linearity (i.e. no constant retention times) could be obtained with the eluents containing 0% and 20% methanol, due to the limited sensitivity of the refractive index detector, which made it impossible to reduce the quantities injected beyond the range studied. In the eluents containing 40% methanol the linearity may be regarded as acceptable when quantities injected are kept sufficiently low and when the retention of hexanol is excluded from the calculation.

The possibility that the phenomena observed arose from imperfections in an individual column or from instrumental imperfections could be excluded by demonstrating that peaks with identical shape were obtained on other commercial μBondapak C18 columns, user packed μBondapak C18 columns and user packed (10 μm) Lichrosorb RP 18 columns and that no imperfections in peak shape were observed when the column was replaced by a low dead volume union. The suggestion of Berendsen et al. (8) that n-alcohols can be used for the calculation of the hold-up time even with eluents containign 0% methanol seems therefore not to be justified at least not for μBondapak C18 columns. A careful check whether deviations from linearity of Equation I occur is therefore necessary whenever the hold-up time is calculated from a series of n-alcohols. This makes this method for the determination of the hold-up time a very time-consuming and tedious one. In our opininion it should therefore only be regarded as an independent method to evaluate whether a test substance has a retention time which corresponds to the actual hold-up time.

Determination of the hold-up time with test substances

The use of test substances is particularly important when frequent determinations of the hold-up time are necessary to eliminate the effect of long term fluctuations in the flow, when very precise measurements of capacity factors are needed.
To determine the hold-up time by means of a test substance this test substance should not be retained by, nor be excluded from the column packing.

Very polar uncharged test substances can be expected to interact with residual silanol groups. Such interactions should be expected to be more important when the eluent is less polar i.e. contains more organic modifier. When an uncharged test substance is less polar, hydrophobic retention should be expected to occur (at least with eluents containing little or no modifier). It is debatable, whether an uncharged test substance for the hold-up time can be at the same time both sufficiently polar (not to be retained by hydrophobic effects) and at the same time be sufficiently apolar (not to be retained by adsorption at residual silanol groups). It seems to be even more debatable that such an uncharged test substance can fulfil these requirements at all possible concentrations of the modifier. With charged test substances no retention should be expected to occur unless the ions involved have a sufficiently hydrophobic character to be adsorbed with the formation of an electrical double layer or as ion pairs.

However, when the packing itself is charged, charge exclusion effects for ions of the same charge, and ion exchange effects for ions of opposite charge should be expected to occur. Reversed-phase packings have been demonstrated to possess considerable ion exchange properties (due to the dissociation of residual silanol groups) over a large range of pH values (12). This dissociation results in a negative charge at those pH values.

Charged test substances can be used only for the determination of the hold-up time when the charge of the dissociated silanol groups can be masked by large concentrations of electrolyte.

The following factors should therefore be regarded as important for the behaviour of test substances:

1. The amount of residual silanol groups
2. The degree of dissociation of the residual silanol groups
3. The affinity of the test substance for the residual silanol groups
4. The affinity of the components of the eluent for the residual silanol groups and their concentration in the eluent
5. The hydrophobicity of the test substance
6. The nature and the concentration of the modifier
7. The presence of components in the eluent which modify the character of the hydrophobic layer.

From these factors it appears that test substances have to be evaluated (with an independent method) for each particular set of experimental conditions. Comparison with the hold-up time calculated from a homologous series should be regarded as a standard method of evaluation (provided that no deviations from Equation I are observed).

The retention times of some test substances are given in Table 4 together with the calculated hold-up times. From this

TABLE 4

Retention Times of Test Substances and the Values Calculated for the Hold-up Time from the Series of n-Alcohols

	Eluent Composition % w/w Methanol					
	100%	80%	60%	40%*	20%*	0%*
Water	166.8	160.3	157.5	157.5	162.6	-
Methanol	-	158.1	154.9	156.4	163.1	200.9
Formamide	162.5	160.7	160.8	156.8	159.9	178.4
Glycerol	158.9	158.7	155.5	153.6	156.8	175.1
THB	153.6	155.8	152.8	168.8	222.9	779.5
Lithium Nitrate	153.4	152.7	144.0	149.8	150.9	155.4
Calculated	155.1	152.8	146.8	150.5	157.9**	169**

*These values have been obtained on a different column
**These values have been calculated from tailing peaks

table it appears that the retention time of lithium nitrace corresponds well with the calculated hold-up times at methanol concentrations above 20%.
According to the concentration pulse concept no differences in the retention times of methanol and water should occur.
The differences found might possibly be explained by the dependence of the retention time of the vacancy peak on the concentration of the modifier in the injected sample as described by McCormack and Karger (17).

The values of the retention times of the concentration pulses of the components of the eluents, water and methanol, are found to be significantly higher than the calculated hold-up time, which is in accordance with the well-established fact that the modifier is incorporated in the stationary phase (17-21) and which corresponds with the results obtained by Berendsen et al. (8).
The differences between the calculated hold-up time and the retention times of formamide and glycerol could possibly also be explained by hydrophobic effects or by adsorption at residual silanol groups.
Water was found to be retained considerably with the eluent containing 100% methanol. Although this effect may be of less importance with eluents with lower methanol contents deuterium oxide was therefore not considered to be a safe marker for the hold-up time. It was therefore not included in this study.

The possibility of incorporation of components of the eluent into the stationary phase makes total porosity measurements (based upon the differences in weight after filling the column with solvents of different densities) useless as a method for the determination of the hold-up time. No such measurements are therefore presented in this study.
Differences in the incorporation of the components of the eluents at different eluent compositions may explain the variation of the calculated hold-up time on the variation of the eluent composition. The fact that the retention time of lithium nitrate follows the same pattern of variation as the calculated hold-up time strongly sug-

gests that its retention time corresponds indeed with the actual hold-up time.

At high methanol contents the retention time of THB corresponds with the retention time of lithium nitrate and the calculated hold-up time. At low methanol contents THB is obviously retained by a hydrophobic retention mechanism.

The influence of charge exclusion effects on the retention time of lithium nitrate

As charge exclusion effects have extensively been described by Berendsen et al. (8), it was investigated whether such effects occurred with the quantities injected in this study and whether the retention times measured were influenced by them.
As charge exclusion effects will depend on the charge of the column packing (i.e. the degree of dissociation of the redisual silanol groups) such effects should be expected to be dependent on the pH of the eluent when buffered eluents are used. When non-buffered eluents are used the charge of the column packing will be influenced by previously used eluents and/or the nature of previously injected samples.

When charge exclusion effects are studied the condition of the column packing should therefore be well defined in this respect. In this study this has been done by injection of measured quantities of solutions of phosphoric acid or ammonium carbonate in the eluent onto the column before the series of injections of lithium nitrate. The retention times measured are given in Table 5. The data in this table indicate that only with the injection of small amounts of lithium nitrate charge exclusion effects are observed. These effects are more pronounced when basic samples have been injected previously.
On the injection of 140 µg lithium nitrate (∿ 20 µl 0.1 M) no significant charge exclusion effects are observed.
Upon addition of a small amount of phosphoric acid to the eluent no charge exclusion effects could be observed over the entire range of quantities studied.

TABLE 5

Retention Time of Lithium Nitrate

µl injected (10^{-1} M. $LiNO_3$ in the Eluent)	Eluent Composition I a)	I b)	II a)	II b)	III
100	154.9	153.2	153.7	153.1	153.9
50	154.1	150.7	153.7	151.1	153.7
20	152.4	143.7	151.4	144.4	152.1
10	151.8	135.4	149.8	139.2	153.0
5	141.1	120.5	146.0	131.2	153.3
2	125	111.6	139.3	125.5	152.9
1	117	105.2	138.3	121.7	153.3

I 50% methanol w/w
II 50% methanol w/w containing 10^{-3} mol·kg^{-1} lithium chloride
III 50% methanol w/w containing 10^{-3} mol·kg^{-1} phosphoric acid
a) series measured after two injections of 100 µl 0.1 M. phosphoric acid in the eluent
b) series measured after two injections of 100 µl 0.1 M. ammonium carbonate in the eluent

These effects are more pronounced when basic samples have been injected previously.

On the injection of 150 µg lithium nitrate no significant charge exclusion effects are observed.

Upon addition of a small amount of phosphoric acid to the eluent no charge exclusion effects could be observed in the range of quantities studied.

When the elution of injected lithium nitrate was monitored with a flow-through pH electrode a decrease of the pH was observed during the elution of the peak of lithium nitrate. Obviously some lithium ions are exchanged against protons. The liberated protons will influence the charge of more distant parts of the column thereby reducing charge exclusion effects. This mechanism will be one of the causes for the variation of the retention times of salts with the injected quantity. Whether this mechanism is more important than the suppression of the charge exclusion effect by

ionic strength of the injected sample cannot be concluded from the data in this study. However, the data in Table 5 indicate that addition of phosphoric acid to the eluent is much more effective than the addition of an equivalent amount of lithium chloride.
It is therefore probable that the effect of buffers to suppress charge exclusion effects will in some cases be largely due to the reduction of dissociated silanol groups instead of the masking of their charge as has been suggested by Parker et al. (23) and by Wells and Clark (24).
Buffers of low pH should therefore be expected to be more effective than those of high pH. This was confirmed by an experiment in which the retention time of lithium nitrate was measured in eluents composed of equal weights of methanol and 0.01 M. aqueous phosphate buffers of different pH.
When buffers of pH 4.5 , 5.0 and 5.5 were used the retention times of lithium nitrate were found to be constant. With buffers of a higher pH lower retention times were found and the precipitation of lithium phosphate occurred.
The non-linearity of the chromatographic distribution process in eluents containing 20% and 0% methanol makes it impossible to use the calculation of the hold-up time as an independent method to determine whether the retention time of lithium nitrate does correspond with the actual hold-up time or not. The fact that no increase in the retention time with increasing amounts of injected lithium nitrate could be found for these eluents suggests, however, that these retention times indeed correspond with the actual hold-up times.

Comparison of lithium nitrate with urea and thiourea as test substances for the hold-up time

In a recent publication the use of thiourea has been proposed as a test substance for the determination of the hold-up time (10). We have therefore made a comparison between the retention times of thiourea, urea, lithium nitrate and the hold-up time calculated from a series of n-alcohols in eluents containing 50%, 20% and 0% methanol. The values obtained are given in Table 6.

TABLE 6

Comparison of the Retention Times of Lithium Nitrate, Urea and Thio Urea and the Hold-up Time Calculated from a Series of n-Alcohols

Eluent Composition % w/w Methanol	Hold-up Time Calculated	Lithium Nitrate	Urea	Thiourea
50%	158.3	160.7	164.7	168.1
20%	-	167.7	168.6	177.0
0%	-	166.8	179.2	200.0

From the values in Table 6 it appears that the retention time of thiourea is significantly higher than those of urea with all three eluents. It is therefore highly improbable that the retention time of thiourea corresponds with the hold-up time. The fact that the difference between the retention times of thiourea and urea increases with decreasing methanol content strongly suggests that the retention of thiourea is hydrophobic in character.
The fact that urea gives significantly higher retention times than lithium nitrate in eluents containing 0% and 50% methanol makes its use as a test substance rather dubious. For the latter eluent this is confirmed by comparison with the hold-up time calculated from the n-alcohols.

CONCLUSION

Lithium nitrate was found to be the only test substance for the hold-up time that gave correct values over the whole range of eluents investigated, provided a sufficient quantity was injected to overcome charge exclusion effects. The occurrence of charge exclusion effects was found to be dependent on the history of the column, i.e. pretreatment with acidic or basic samples. The occurrence of charge exclusion effects could be reduced considerably by the addition of small amounts of phosphoric acid in the eluent.

The retention time of lithium nitrate being the correct value for the hold-up time could be confirmed by comparison with the

hold-up time calculated from the homologous series of n-alcohols for eluents with a methanol content of 40% or more.
In eluents with a low methanol content of 20% and 0% significant deviations from Equation 1 were found, probably due to a non-linear chromatographic distribution process.

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HIGH RESOLUTION SEPARATION OF URINARY ORGANIC ACIDS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Reverse phase, anion exchange, and two-dimensional HPLC techniques were studied in order to increase resolution of organic urinary acids for eventual quantitative measurements. Reverse phase HPLC with a phosphate buffer/acetonitrile gradient yielded a separation of over 85 components in forty minutes and a peak area reproducibility of better than 5%. Connecting two reverse phase columns together resulted in the separation of 110 components. Anion exchange chromatography was determined to be of little use in resolving urinary acids in a resonable time except as the first stage in two-dimensional chromatography where fractions from the anion exchange column were injected into a reverse phase column. Over 139 components were separated by this two-dimensional method.

INTRODUCTION

Separation of the components present in complex mixtures, such as urine and other biological fluids, presents unique chromatographic problems because of the large variety and number of compounds in these samples. One such group of components, the low molecular weight organic acids found in human urine, is of particular interest since it includes intermediates and end products of various metabolic pathways which, in turn, can be affected by diseases (3,4). In most cases, correlations between the presence of a specific disease and urinary acids are related to concentration variations rather than to the presence or

absence of a specific component (5,6). For diseases which have not been thoroughly investigated, it is much easier to first separate all of the components in the sample and then to identify and quantify those which show variations compared to healthy individuals than it is to isolate and identify all of the components. This former technique has been termed "quantitative metabolic profiling (6)". To be successful this technique requires highly efficient chromatographic methods capable of completely resolving all of the components in the sample in order to facilitate peak integration.

Anion exchange chromatography was one of the first methods to be used in separating urinary components; however, in order to separate 100 of more constituents, long columns and separation times in excess of 20 hours were necessary (7,8,9). More recently, over 130 urinary acids were separated by gas chromatography (GC) (10) and over 230 acids have been separated by capillary GC (11). Although they are very efficient and allow coupling to a mass spectrometer for identification, GC methods are limited to components of high volatility; otherwise, derivatization steps are required which may decrease recovery of the components. High performance liquid chromatography (HPLC) is not limited by component volatility nor by the presence of various salts which often accompany the extraction procedure. Molnár and Horváth recently reported the separation of over 100 urinary organic acids in 30 minutes by reverse phase HPLC (12). However, many of the peaks were not sufficiently resolved to be useful for quantitative metabolic profiling.

Two-dimensional chromatography is a technique in which fractions from one chromatographic mode are transferred to another chromatographic mode to increase resolution (13). Some applications of this technique have been reported for separating urinary acids (14,15), most notably by Spiteller and Spiteller who separated about 500 acid components using a two-dimensional system employing thin layer chromatography and then capillary GC-mass spectrometry (16).

This paper describes some refinements on the reverse phase HPLC method and also presents the results of a two-dimensional study employing anion exchange and then reverse phase HPLC for the separation of urinary organic acids.

MATERIALS

Apparatus

An Altex 322 MP microprocessor controlled gradient HPLC (Beckman Instrument Co., Berkeley, CA) equipped with two model 100A pumps, a model 400-12 mixer, a model 210 injection valve with a 20 µL sample loop, a model 100-10 Hitachi variable VIS-UV wavelength detector set at 280 nm, and a Linear model 385 chart recorder were used throughout this study. Peak areas were determined by a Hewlett Packard model 3380-S electronic integrator.

Columns

Analytical columns were packed by the manufacturer; they included: RSiL-AN 5 µm anion exchange, 25 cm x 4.6 mm I.D. (Alltech Associates, Deerfield, IL), SynChropak AX300, 10 µm anion exchange, 25 cm x 4.1 mm I.D. (SynChrom, Inc., Linden, IN), and Beckman Ultrasphere ODS, 5 µm reverse phase, 25 cm x 4.6 mm I.D. Guard columns consisting of either the RSiL-An anion exchange material (5.0 cm x 4.6 mm I.D.) or Lichrosorb RP-18, 10 µm (E. M. Merck) (7.0 cm x 2.2 mm I.D.) were packed in this laboratory with a model 704 Micromeritics slurry packer (Micromeritics, Norcross, GA). No guard column was used with the SynChropak column. The guard and analytical columns were kept at the appropriate temperature controlled by a Thermomix Model 1460 constant temperature water bath.

METHODS

Sample Preparations

Urine samples were collected from an apparently healthy adult male, combined, then frozen in 30 mL vials at -80°C until ready for use. The acids were extracted from urine either by concentra-

tion on a DEAE Sephadex A-125-120 (Sigma Chemical Co., St. Louis, MO) anion exchange column followed by elution with pyridinium acetate and then lyophilization to dryness (for anion exchange and reverse phase chromatography studies) or by extraction into an ether-ethyl acetate mixture followed by evaporation of the organic layer (for two-dimensional chromatography studies). Details of these procedures are given by Rehman et al. (17).

HPLC

Aqueous buffers were prepared either from reagent grade potassium dihydrogen phosphate (Mallincrodt, St. Louis, MO) with the pH adjusted using 85% orthophosphoric acid (Fisher Scientific Co., Fair Lawn, NJ) or from glacial acetic acid with the pH adjusted using sodium hydroxide. Water was purified on a Milli-Q system (Millipore Corp.). Buffers and HPLC grade solvents (acetonitrile and methanol, Burdick and Jackson Laboratories, Muskegon, MI) were filtered through a 1.2 μm (aqueous solvents) or 0.5 μm (organic solvents) filter and then deaerated before using.

For ion exchange chromatography a concave gradient was used starting with either 0.02 M pH 3.0 phosphate buffer or 0.10 M pH 7.0 acetate buffer and increasing to 1.13 M phosphate or 4.0 M acetate, respectively, followed by a reverse gradient to the initial conditions. The column was equilibrated at the initial conditions for fifteen minutes before injecting another sample. The flow rate was 1.0 mL/min.

For reverse phase chromatography the initial mobile phase was the appropriate aqueous buffer. The acetonitrile or methanol concentration was increased to 39% in 36 minutes in a concave fashion, then linearly to 70% in 15 minutes (to insure complete elution of all components), followed by a reverse gradient and equilibration under initial conditions for 15 minutes (Figure 1). The flow rate was maintained at 2.0 mL/min.

In the studies involving two-dimensional chromatography, fractions were taken from the RSiL-AN anion exchange column, con-

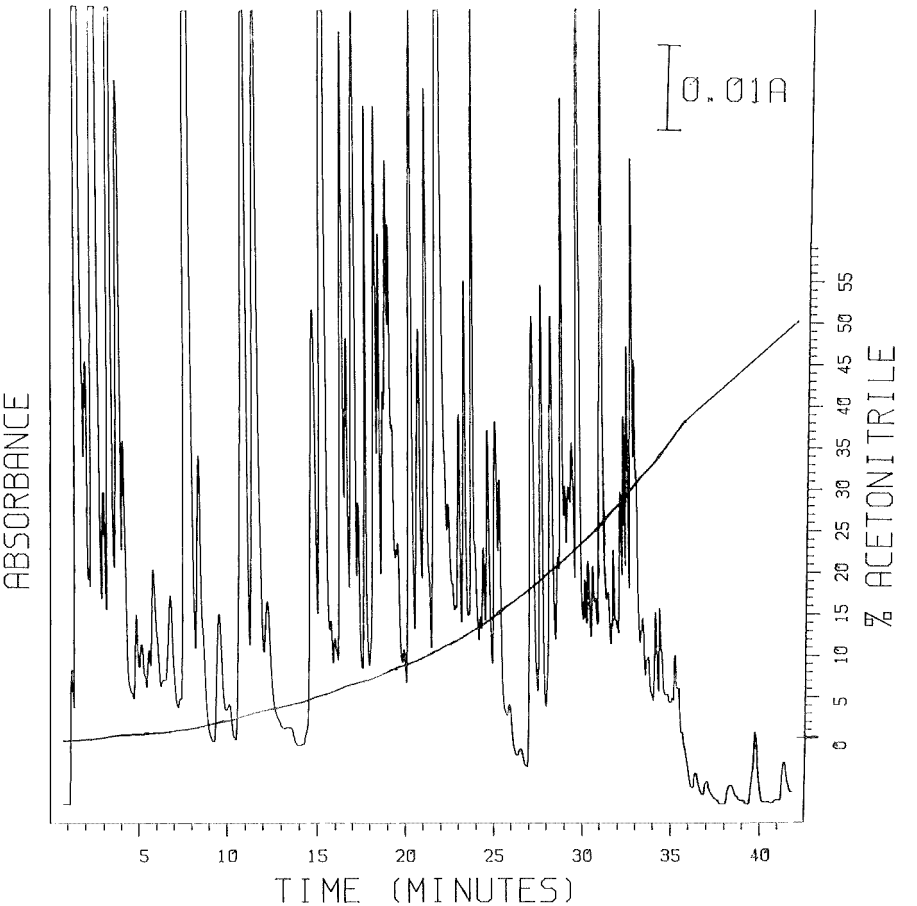


FIGURE 1. Separation of urinary acids on a reverse phase column using a concave pH 2.1 0.2M phosphate buffer/ acetonitrile gradient.

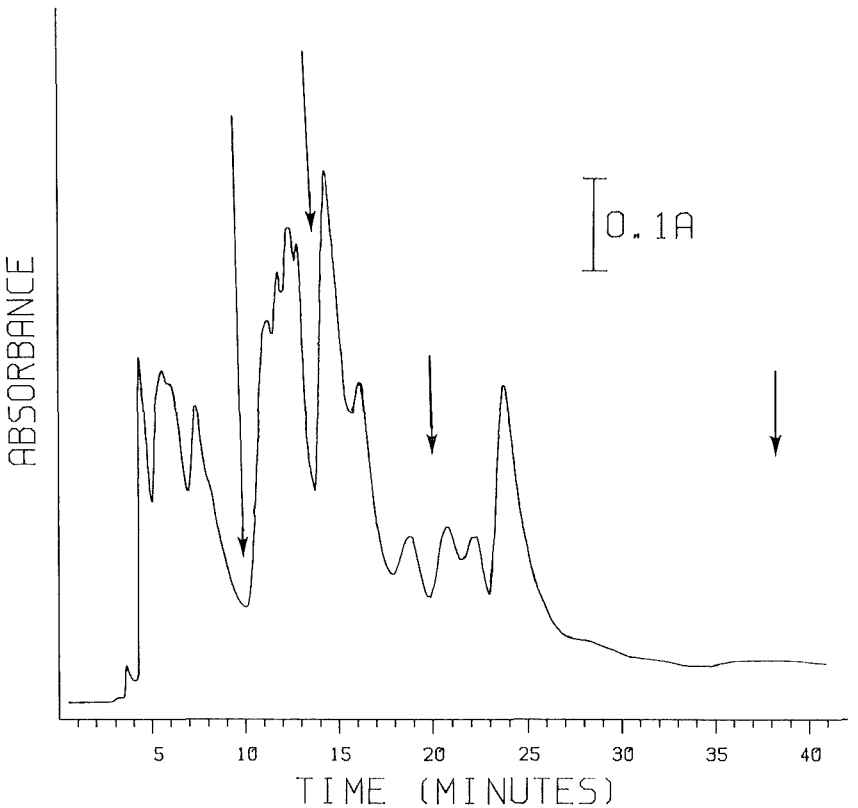


FIGURE 2. Separation of urinary acids on an anion exchange column using a linear gradient from 0.05 M phosphate buffer at pH 3.0 to 0.5 M phosphate in 50 minutes. Fractions were taken at the times indicated by arrows.

centrated, then injected into the reverse phase column, as follows. For the anion exchange separation, 100 µL of sample was injected, and a linear gradient from 0.05 M phosphate buffer (pH 3.0) to 0.5 M phosphate in 50 minutes was employed. Four fractions were collected without stopping the flow (Figure 2). The fractions

were extracted into ether-ethyl acetate, the organic layer evaporated, and the residue dissolved in 50 µL of 0.20 M (pH 2.1) phosphate buffer. A twenty µL aliquot of each fraction was then injected into the reverse phase column using the same conditions given above.

RESULTS AND DISCUSSION

Ion Exchange Chromatography

Two types of anion exchange column packings were investigated: RSiL-AN using an aqueous pH 3.0 phosphate buffer gradient with increasing phosphate concentration at 60°C and SynChropak AX300 using an aqueous pH 7.0 acetate buffer gradient with increasing acetate concentration at 30°C. Since most of the urinary acids in the extract have pK's between 4 and 5 (18), the predominate mode of separation at pH 3.0 is probably sorption rather than ion-exchange (19); ion exchange would predominate at pH 7.0 or above. Neither column gave a good separation of urinary acids; only approximately 25 resolved or partially resolved peaks were observed for both columns. Although the peaks from the SynChropak column were sharper, the RSiL-AN column and phosphate mobile phase were chosen for the studies involving two-dimensional chromatography because the pH was more compatible with that of the aqueous phase for the reverse phase column (see below).

Reverse Phase Chromatography

Preliminary studies with standard acid solutions and urinary acid extracts indicated that the sharpest, most symmetrical peaks and the best separation of urinary acids on an Ultrasphere column are obtained with a pH 2.1 phosphate buffer (0.2 M) at 50°C using a concave gradient from 0 to 70% acetonitrile. Under these conditions, approximately 85 resolved and partially resolved peaks were obtained (Figure 1). These results are comparable to those of Molnár and Horváth (12), who reported 104 peaks under similar conditions using a 5 µm Lichrosorb RP-18 column.

The peak area reproducibility of the method was determined by three injections of the same urine extract. Twenty-eight of

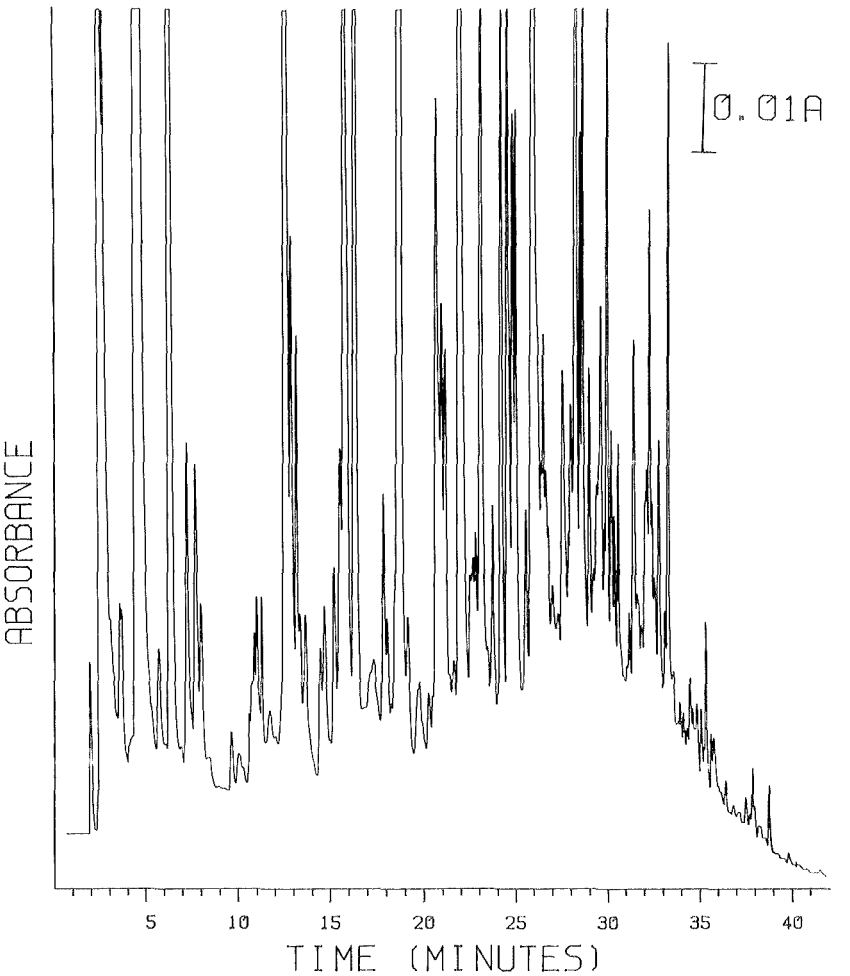


FIGURE 3. Separation of urinary acids on two reverse phase columns connected together. The gradient is shown on Figure 1.

the larger and better resolved peaks were chosen for comparison. The mean and median percent standard deviations of each peak for the raw areas were 5.8 and 4.4, respectively and 5.0 and 3.5, respectively for normalized areas.

The peak retention times generally had standard deviations from 0.07 to 0.18 minutes, although somewhat larger deviations were sometimes observed for peaks having retention times between 5 and 12 minutes.

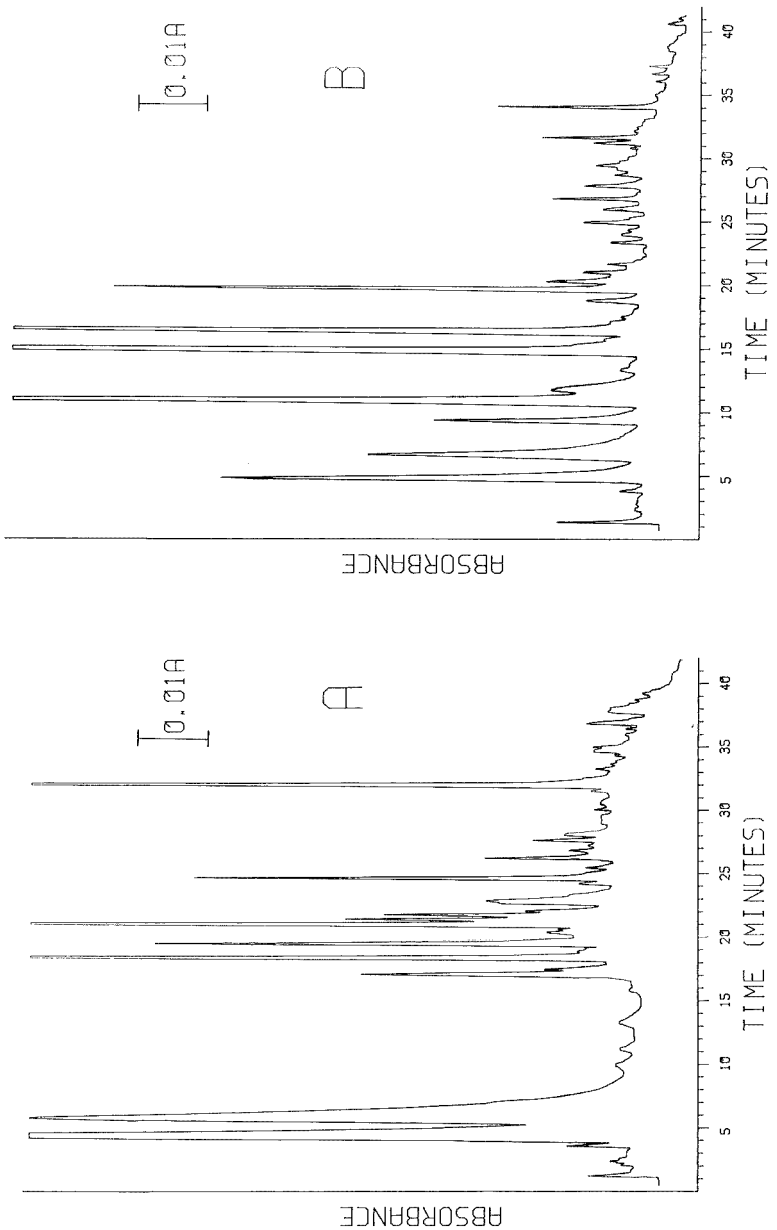
A similar separation and number of peaks (although a different peak pattern) were observed when methanol was substituted for acetonitrile in the gradient; however, the pressure reached 5000 psi compared to 3500 psi with acetonitrile. Likewise, a similar chromatogram was obtained when 0.1 M acetic acid (pH 2.9) was substituted for phosphate. Although not a buffer, acetic acid may be appropriate for use when isolation of separate peaks or fractions from the reverse phase column is required, since acetic acid can be removed by lyophilization.

As stated above, in order for quantitative metabolic profiling to be useful, it is necessary for all of the components in the sample to be as completely resolved as possible. While the optimum conditions for the Ultrasphere column probably have been attained, it is apparent from Figure 1 that many of the peaks remain unresolved.

One means of increasing resolution is to increase column length by connecting two or more columns together. Under identical conditions as with one column, connecting two and three columns together produced nearly 110 (Figure 3) and 90 peaks respectively. The apparent decrease in resolution with the three column system is probably due to the increase in interconnective tubing. In addition, using three columns resulted in an extremely high pressure (8000 psi).

Two-Dimensional Chromatography

A urine extract was separated first on the RSiL-AN anion exchange column at pH 3.0. Four fractions were taken during the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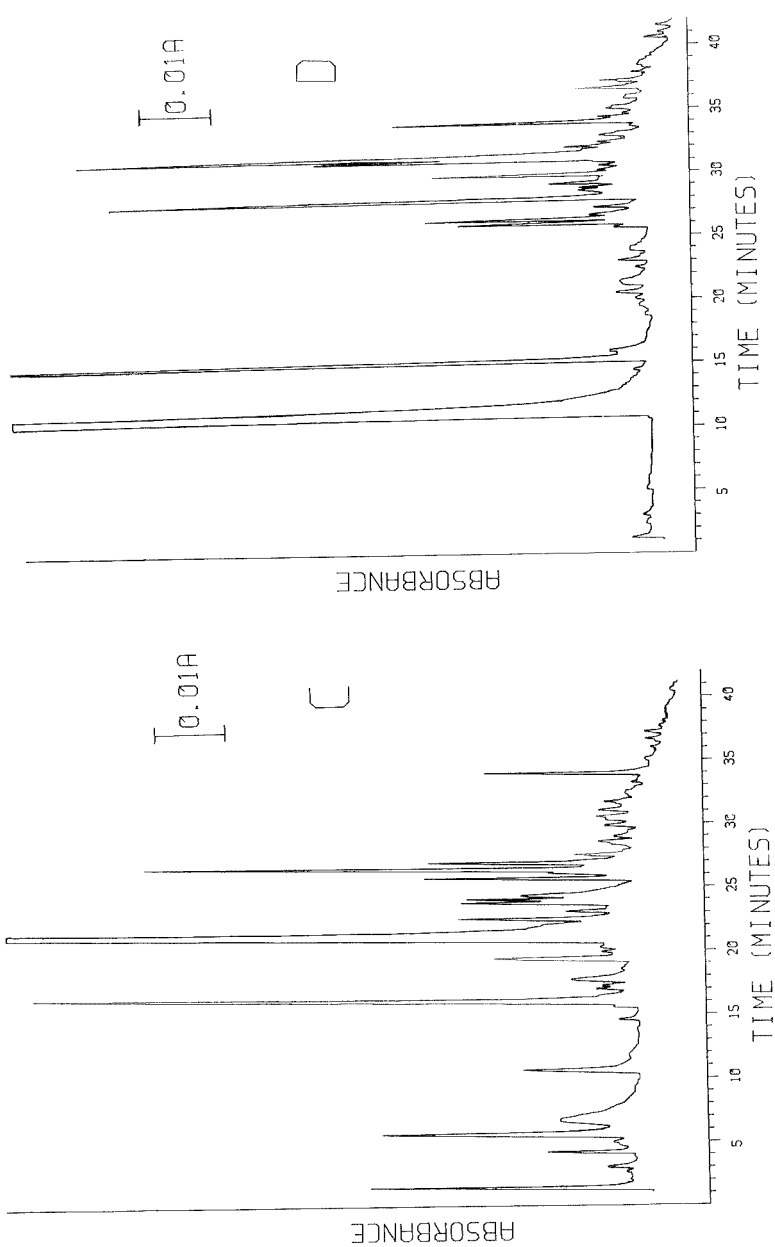

FIGURE 4. Separation of fractions collected from the anion exchange column (as indicated in Figure 2) on a reverse phase column. The gradient is shown in Figure 1. A. Fraction 1. B. Fraction 2. C. Fraction 3. D. Fraction 4.

separation (indicated by the arrows in Figure 2). The acids were extracted, concentrated, and then injected into the Ultrasphere reverse phase column. Fractions one to four yielded 55, 46, 48, and 56 peaks, respectively (Figure 4A-D). Considering overlap from adjacent fractions, of the 205 total peaks, 139 appear to be due to different components.

Identification of Components

When the quantitative metabolic profiling approach is used to investigate diseases, it is not necessary to identify every

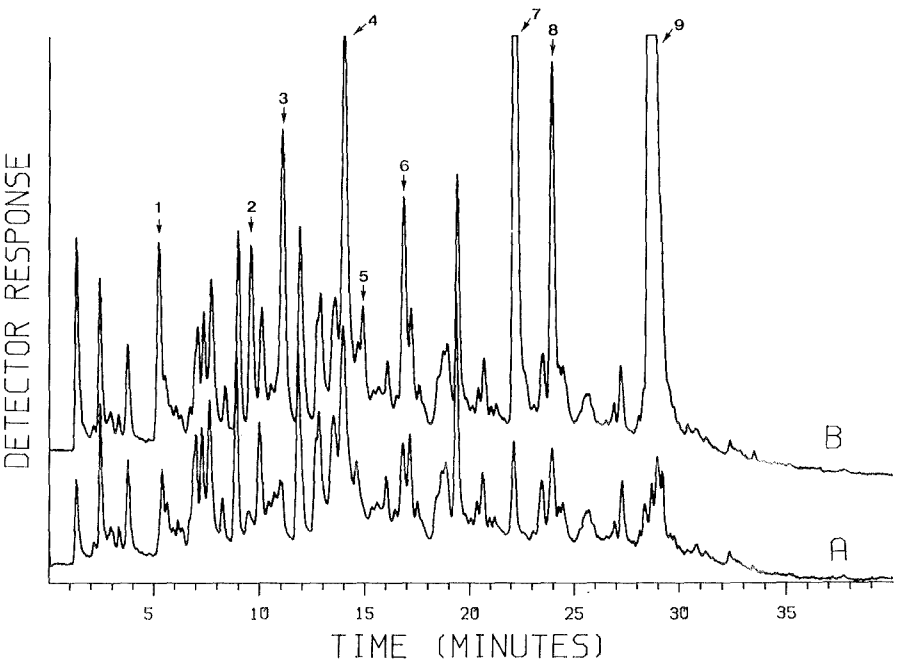

FIGURE 5. A. Chromatogram of a urinary acid extract diluted 1/1 with pH 2.1 phosphate buffer.
B. Chromatogram of the extract from Figure 5A but diluted 1/1 with a solution containing nine organic acids at 100ppm each. The identity of each numbered peak is given in Table 1. Both chromatograms were obtained using the conditions given in Figure 1 and in the text.

TABLE 1

Identification of Some Organic Acids Present in Urine

Peak Number*	Compound
1	4-hydroxy-3-methoxymandelic
2	3,4-dihydroxyphenylacetic acid
3	p-hydroxybenzoic acid
4	m-hydroxybenzoic acid
5	m-hydroxyphenylacetic acid
6	4-hydroxy-3-methylphenylacetic acid
7	4-hydroxy-3-methoxycinnamic acid
8	indole-3-acetic acid
9	transcinnamic acid

*Refers to those peaks which are numbered in Figure 5

component in the chromatogram. Only those components need be identified which show statistically significant differences between samples taken from diseased *versus* normal subjects while positive identification of these components can be made by retention time comparison and/or by spiking the sample with known compounds. This is shown in Figure 5 which is a comparison of chromatograms of a urinary organic acid extract *vs* the same extract spiked with nine organic acids which are commonly found in urine (10). Table 1 lists each peak number and its identity. Clearly, this tentative peak identification is facilitated by a highly efficient and reproducible HPLC separation.

CONCLUSIONS

Reverse phase HPLC yields a rapid and reproducible separation of urinary acids; however, in order to increase resolution for quantitative measurements, more efficient columns are required. Efficiency may be increased by connecting columns together; however, it is somewhat limited by interconnective tubing and increase operating pressure. As more efficient ODS

columns are developed, especially microbore columns, a greater resolution may eventually be realized.

Although tedious and time consuming, two-dimensional chromatography results in a separation of the largest number of urinary acids yet reported by HPLC. In addition, a greater degree of baseline separation is achieved over one dimensional HPLC since fewer components are present in each fraction than are present in the entire sample. Its use is suggested if components of a specific fraction are to be identified and/or quantified. With the development of on-line techniques; i.e. automatic column switching, this method may eventually become more practical.

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SEPARATION OF BACTERIAL MENAQUINONES BY HPLC USING REVERSE PHASE (RP18) AND A SILVER LOADED ION EXCHANGER AS STATIONARY PHASES

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ABSTRACT

Bacterial menaquinones were separated isocratically on a reverse phase Li Chrosorb RP18 5 µm and a silver loaded ion exchanger.
On octyldecylsilica support the separation of the menaquinones depends on their lipophilic character, on the silver column mainly on the number of double bonds in the isoprenyl chain. Comparing the runs of both columns the menaquinones were easily differentiated.

INTRODUCTION

Menaquinones (2 methyl 3 polyisoprenyl 1,4 naphthoquinones) play an important role in the bacterial electron transport, oxidative phosphorylation (10) and formation of endospores (5). The menaquinones are not evenly distributed among bacteria. They may vary in the length of their isoprenyl side chain and the degree of saturation. This variation is taxa specific. The value of menaquinones in bacterial taxonomy was clearly shown by Yamada (11), Minnikin (9), and Collins (3).

For the separation and identification of the different menaquinone species, various methods were employed. Thin layer chromatography on different layers (4), mass spectrometry (9), and gas chromatography after hydrogenation (2). Recent reports point up the value of high performance liquid chromatography for the separation of menaquinones. In this regard, reverse phase RP8 as stationary phase and methanol as mobile phase proved very effective (Collins, personal communication).
Also, RP18 and dichloromethane/acetonitrile (6) and RP18 and methanol/chloroform with silver ions as modifier (8) have been used sucessfully. Similar substances have been separated on silver loaded silicagel supports (1).

For routine analyses these methods have some disadvantages. The mobile phases containing silver nitrate are quite corrosive and have a high absorbance, and the silver of the silver impregnated silicagel bleeds from the support after some time, leading to a reduction of the retention time of the menaquinones. The separation of the ordinary menaquinones (menaquinones with isoprenyl side chains lacking saturated isoprenyl units) is sufficient with reverse phases (RP8 and RP18). However, these reverse phases cause problems in the separation of natural mixtures of menaquinones with isoprenyl side chains differing in degree of saturation and chain length. I now report the separation and identification of diverse menaquinone mixtures using stationary phases of different separation behaviours.

MATERIAL AND METHODS

The method of isolation of menaquinones has been described previously (7). Menaquinone mixtures of fully unsaturated menaquinones were obtained from Dr. D. Collins (Reading G.B.). Partly saturated menaquinones were iso-

lated from bacterial strains in which the type of menaquinone was known from the literature. Their identities were varified by mass spectrometry. Cis-menaquinones which arise by photoisomerisation were identified and isolated from the naturally occuring trans-menaquinones by silicagel thin layer chromatography using hexane/dibutylether 85:15 v/v as mobile phase (12). The chromatogramms shown in fig. 1 were obtained by injecting 1-5 µl of the menaquinone mixture in 2-chlorobutane. The following conditions were employed: Apparatus Desapro Milton Roy mininpump simplex with pulse dampening device and a Rheodyne 7125 injection valve. The stainless steel column 250x4 mm (Merck 50333) was filled with Li Chrosorb RP 18 5 µm. It was protected by a precolumn (Brownlee Labs. MPLC RP18) and kept at 30° C with a column oven (Jones Chromatography). The flow rate was 1 ml/min acetonitrile/tetrahydrofurane 70:30 v/v (Promochem Chrom AR), at a pressure of 80 atm. A Uvicord S detector (LKB) was used at 254 nm with a sensivity set at 0,005 abs. range. The chart speed was 1 cm/min using a Kontron W+W 600S recorder. The separations presented in fig. 2 were obtained by injecting 1 to 20 µl of the menaquinone mixture in methanol. The following conditions were employed: Apparatus Perkin Elmer LC-pump (series 1) with Rheodyne 7105 injection valve and stopflow fittings. The stainless steel silver column (250x4,6 mm i.d.) was custom-packed with silver loaded Nucleosil^{R} 10SA (Macherey, Nagel & Co, Düren F.R.G.) by Chrompack Nederland, Middleburg, The Netherlands. It was kept at 65° C by use of a precision thermostat (Lauda) and protected by a column inlet filter (Rheodyne). Methanol (Merck 6007) was used as eluat at a flow rate of 1,5 ml/min and 40 atm pressure. A Perkin Elmer LC-75 UV-detector with autocontrol was used at 269 nm, with sensivity set at 0,02 abs. range. The chart speed was 0,5 cm/min with a Varian A-25 recorder.

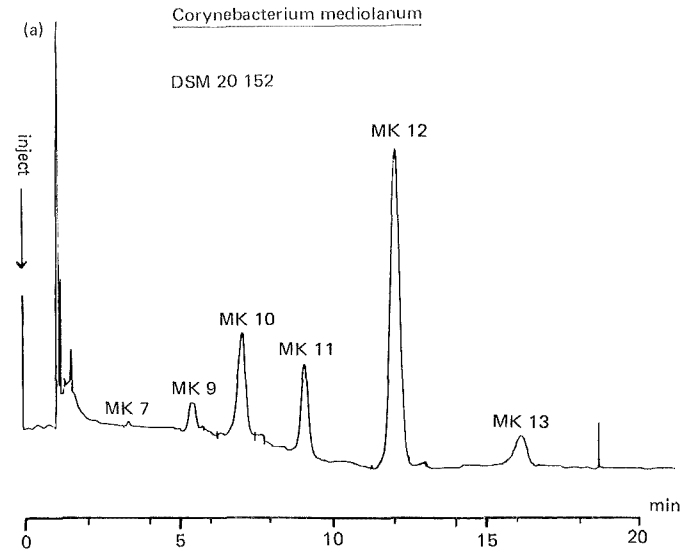


FIGURE 1A

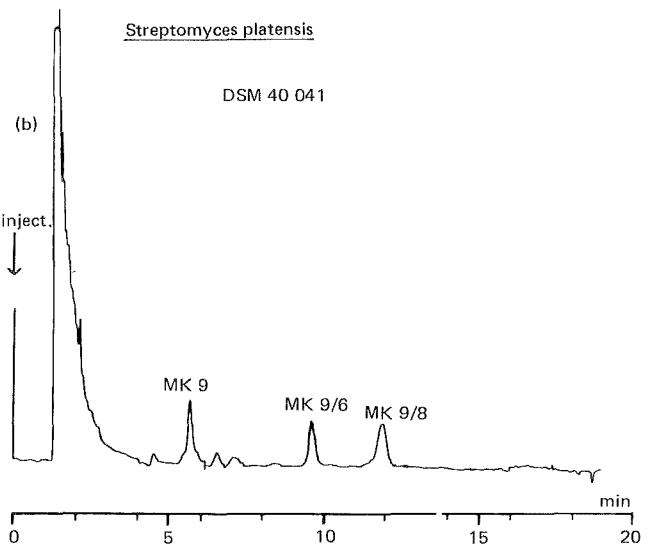


FIGURE 1B

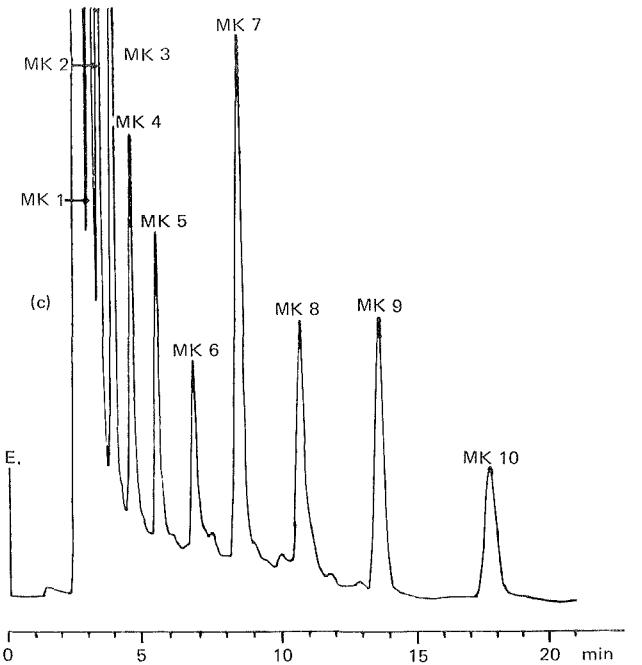


FIGURE 1

Separation of menaquinones (MK) on 5 µm Li Chrosorb^{R} RP18, column 250x4 mm, mobile phase ACN/THF, 70:30 v/v, flow rate 1 ml/min, temperature 35° C, sample size 5 µl.
(a) menaquinones with an ordinary isoprenyl side chain (7-13 isoprenyl-units)
(b) menaquinones with nine isoprenyl units, three (MK9/6) or four (MK9/8) of them beeing saturated; MK9 internal standard
(c) menaquinone standard mixture MK1-MK10, conditions as before except the column, Beckman Ultrasphere ODS 250x4,6 mm

$^{+}$DSM Deutsche Sammlung von Mikroorganismen (German Collection of Microorganisms)

RESULTS AND DISCUSSION

The best resolution of menaquinone homologues were obtained by separation on RP18 phase with acetonitrile/tetrahydrofurane 70:30 v/v at a flow rate of 1 ml/min. Homologues differing in number of isoprenyl units (MK1 to MK13, fig. 1a and 1c) as well as those with the same chain length but different degree of saturation (MK9(H6), MK9(H8), fig 1b) could be well separated. The theoretical plate heigth for MK9(H2) was 27 µm (9300 plated per 25 cm column). Under these conditions the loss of one double bond in the isoprenyl side chain resulted in an increase in retention time equivalent to 0,7 of an additional isoprenyl unit. This separation behaviour caused problems in differentiating menaquinones with a partly unsaturated isoprenyl side chain from those with a fully unsaturated one e.g. MK9(H6) and MK11 (3x0,7=2; 9+2=11, fig. 1a and 1b).

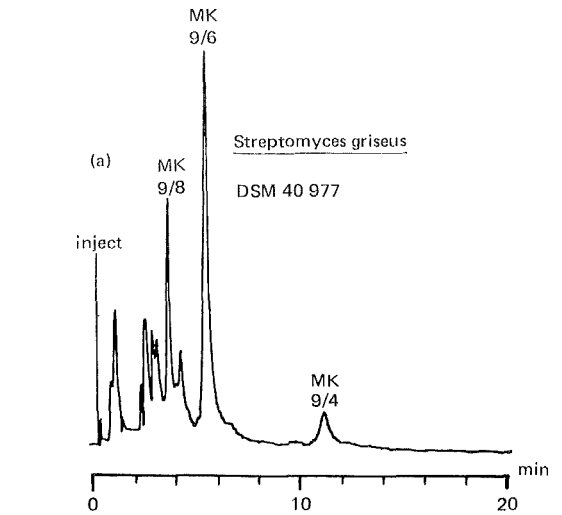
Therefore, it became necessary to separate the menaquinones on an additional stationary phase which had a different separation behaviour. The separation of menaquinones on octyldecylsilica (RP18) depends mainly on their lipophilic character. An increase in the retention time occurs by adding isoprenyl units or pairs of hydrogen atoms (eliminating double bonds).

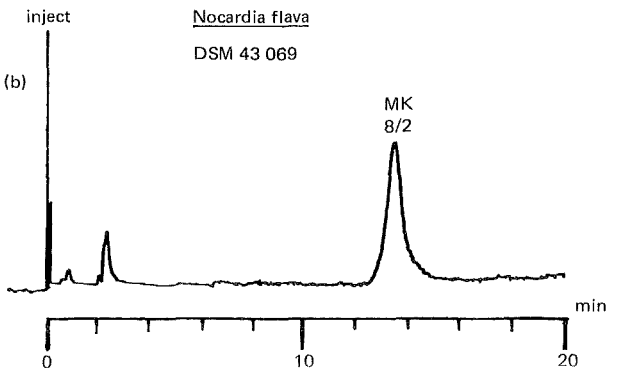
FIGURE 2

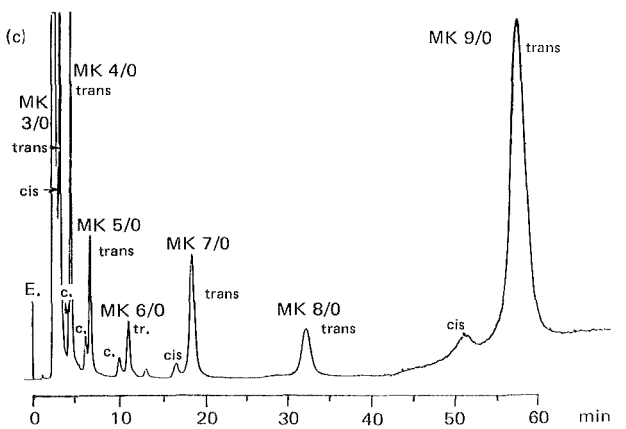
Separation of menaquinones on a silver loaded ion exchanger Nucleosil^{R} 10SA(Ag^{+}) column 250x4,6 mm, mobile phase methanol 100%, flow rate 1,5 ml/min, temperature 65^{o} C, sample size 10 µl.

(a) + (b) bacterial menaquinones with partly saturated isoprenyl side chains, MK9/4 (a) shows a shorter retention time than MK8/2 (b), both have the same number of double bonds

(c) menaquinone standard mixture MK1 - MK9


MK
9/6
Streptomyces griseus
(a)
MK
9/8
DSM 40 977
inject
MK
9/4
min
0
10
20


inject
Nocardia flava
DSM 43 069
(b)
MK
8/2
min
0
10
20


(c)
MK 4/0
trans
MK
3/0
trans
cis
MK 5/0
trans
MK 7/0
trans
E.
c.
c.
MK 6/0
tr.
c.
cis
MK 8/0
trans
cis
MK 9/0
trans
0
10
20
30
40
50
60
min

A totally different separation behaviour is obtained by using a silver loaded ion exchanger. In this case the bonds of double bond silver complexes compete with the solubility of the organic mobile phase. These bonds are so strong that only menquinones with 9 or less double bonds are eluted in reasonable retention times (MK9, 57 min at 65° C). Increasing the temperature resulted in boiling of the methanol use of a more lipophilic mobile phase had no effect on the retention time. The theoretical plate height for MK9(H2) was 60 µm (4100 plates per 25 cm column). As expected (Ag-TLC data), menaquinones with the same number of double bonds but longer isoprenoid chain length were eluted earlier thus, MK9(H4) eluted before MK8(H2) even though both contained seven double bonds (fig. 2a and 2b). Cis-menaquinones had shorter retention times than trans-menaquinones (fig. 2c). Menaquinones with the same number of isoprenyl units and double bonds but different points of saturation (isomers) were very well separated on the silver column; e.g. MK8(H4) of Micropolyspora brevicatena DSM 43024 and MK8(H4) of Nocardia autotrophica DSM 40011. The mass of both was varified by mass-spectrometry.

The results clearly show the value of HPLC for the separation and identification of menaquinones. By comparing the different elution profiles of the menaquinones isolated from bacteria and separated on both columns, the single peaks can easily be identified.

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QUANTITATIVE HPLC ANALYSIS OF PLASMA AMINO ACIDS AS ORTHOPHTHALDIALDEHYDE/ETHANETHIOL DERIVATIVES

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ABSTRACT

A reverse phase high performance liquid chromatographic method for the analysis of plasma amino acids is described. The method employs pre-column derivatization with o-phthaldialdehyde using ethanethiol as the reducing agent. The analysis shows good linearity and reproducibility. An average overall difference of 12% was seen for results obtained by the HPLC method versus those obtained with an amino acid analyzer. The chromatographic parameters of buffer concentration and column temperature were also examined.

INTRODUCTION

More than 70 diseases associated with defects in amino acid metabolism have been reported (1). Since most of these conditions result in abnormal plasma concentrations of one or several amino acids, plasma amino acid concentration profiles have been useful in the diagnosis of these disorders.

Amino acids were first determined in physiological fluids by the classic method of Stein and Moore (2). This method utilized ion-exchange chromatography and the reaction with ninhydrin for the separation and detection of amino acids. Several modifications of this method have been developed (3,4); however, separation of amino acids in physiological fluids requir at least 4 1/2 hours.

Amino acids in hydrolysates of proteins have recently been analyzed by HPLC as phenylthiohydantoin (5,6), dansyl (7) and o-phthaldialdehyde/thiol derivatives (8,9). Hill et al.(10) and Lindroth et al.(8) have shown that amino acids in plasma can be determined in less than an hour by HPLC as o-phthaldialdehyde/ethanethiol (OPA/EtSH) or o-phthaldialdehyde/2-mercaptoethanol derivatives. The purpose of the present study was to characterize the optimal parameters necessary for the quantitation of plasma amino acids by HPLC as OPA/EtSH derivatives.

MATERIALS AND METHODS

Chromatographic System

Waters Associates Liquid Chromatographic Systems with two Model 6000A pumps were used for all experiments. One system utilized a Model 660 solvent programmer, a Model U6K injector and a Hewlett Packard HP3352D data system, whereas the other system utilized a Model 720 microprocessor solvent programmer, a WISP 710B automatic injector system and a Model

730 data module. Both systems were equipped with Schoffel FS-970 fluorometers adjusted to the following parameters: excitation wavelength - 229 nm; emission cut-off filter - 470 nm; time constant - 0.5 s. The sensitivity setting was adjusted so that tne photomultiplier tube had an absolute sensitivity of approximately 40 A/lm.

A μBondapack C_{18}, 30 cm x 3.9 mm i.d., reverse phase column along with a C_{18}/Corasil, 2 cm x 3.9 mm i.d. guard column was used for all analyses. New columns were conditioned before analysis of OPA/EtSH amino acids by equilibrating the column with 70% CH_3OH/H_2O and then programming to 100% CH_3OH in 15 min and allowing approximately 100 mL of CH_3OH to flow over the column. The solvent was then changed to 100% water over a 15 min period. The water was allowed to flow over the column for approximately 5 min. The solvent system was then changed to 0.1% (v/v) H_3PO_4 and approximately 200 mL of the acid was allowed to flow through the column. The column was flushed with water to remove the phosphoric acid prior to equilibrating with the solvent system described below. This procedure was found to be necessary to remove from the column an acid soluble component that reacted with the OPA/EtSH reagent and caused rapid degradation of the column. The acid wash of the columns was required only once.

The temperature of the column was maintained at 28° by circulating water through an aluminum water jacket that en-

circled the column. A Haake Model FJ heater/circulator and a Haake K11 cooling system was used to produce the required water temperature and pump the water through the column jacket.

A stock buffer of 300 mM phosphate/518 mM sodium was prepared by dissolving 11.59 g $NaH_2PO_4 \cdot H_2O$ and 30.66 g Na_2HPO_4 in reagent grade water and diluting to 1 L with reagent grade water. The buffer was filtered through a 0.45μ filter prior to preparing the HPLC solvent systems. Solvent system A (15.0 mM PO_4/25.9 mM Na) was prepared by diluting 50.0 mL of the 300 mM PO_4/518 mM Na stock buffer to 1 L with reagent grade water. Solvent system B_1 (15.0 mM PO_4, 25.9 mM Na, 45% H_2O/CH_3CN) was prepared by diluting 50.0 mL of 300 mM PO_4/518 mM Na stock buffer and 400 mL of reagent grade water to 1 L with CH_3CN. Solvent System B_2 (15.0 mM PO_4, 25.9 mM Na, 45% H_2O/tetrahydrofuran) was prepared by the same procedure described for solvent system B_1 except the solution was diluted to volume with tetrahydrofuran instead of acetonitrile.

Analysis of the OPA/EtSH amino acid derivatives on a new column resulted in a gradual, parallel decrease in the retention time of all the amino acid derivatives except arginine. This resulted in a loss of resolution between arginine and tyrosine. To regain this resolution, the concentration of the buffer in solvent A and B was increased by using an addi-

tional 10.0 mL of stock buffer. Additionally, 10.0 mL less water was added to solvent systems B so that the concentration of water remained 45%.

For separation of the amino acid derivatives (except Gly and Thr) a solvent program consisting of a linear gradient from 15% (or 10%) solvent B_1/solvent A to 75% solvent B_1/solvent A over a period of 60 min was used (System I). For the separation of OPA/EtSH Gly and Thr derivatives an isocratic system of 30% B_2/A was used. After 12 min the solvent was changed to 75% B_2/A to remove the other amino acid derivatives from the column (System II). A flow rate of 2.0 mL/min was used for both solvent programmed systems. The columns were reequilibrated to initial solvent conditions by a 10 min reverse linear gradient.

Preparation of Standards

Individual crystalline samples of L-amino acids were obtained from Pierce Chemical Co. (AMAC Standard Kit, No. 20065), Aldrich Chemical Co. (DL citrulline, No. 85,572-3; DL-p-fluorophenylalanine, No. F1-380-D; Taurine, No. 15,224-2) and Sigma Chemical Co. (β-alanine, No. A-7752; DL-α-aminoadipic acid, No. A-0637; L-α-amino-n-butyric acid, No. A-1879: DL-β-aminoisobutyric acid, No. A-8504; L-1-methylhistidine, No. M-9005; L-3-methylhistidine, No. M-3879; L-ornithine, No. O-2375). A mixture of 0.4μmol/mL of each of the following amino acids was

prepared in 0.1 N HCl: Ala, Arg, Asn, Glu, Gln, Gly, His, Ile, Leu, Lys, Met, Orn, Phe, Ser, Tau, Thr, Trp, Tyr and Val. Five mL (2.00 μmol each amino acid) of this solution was placed in several 150 mm x 14 mm test tubes, lyophylized to dryness and glass sealed under the original vacuum. Fresh standards were prepared daily by dissolving the residue from one of the lyophylized standards with 4.0 mL of 0.1 N HCl to produce a 500 nmol/mL stock standard. Other amino acids standards were prepared by diluting aliquots of this solution with 0.1 N HCl.

A stock solution of the internal standard (2.00 mol/mL fluorophenylalanine) was prepared by dissolving 37.7 mg of 97% DL-fluorophenylalanine in 0.1 N HCl and diluting to 100 mL with 0.1 N HCl. The working internal standard solution (20.0 nmol/mL fluorophenylalanine) was prepared by diluting 1.0 mL of the 2.00 μmol/mL fluorophenylalanine solution to 100 mL with methanol.

Preparation of OPA/EtSH Amino Acid Derivatives

Two methods, a macro and a micro method were used to prepare OPA/EtSH derivatives of amino acids in plasma and standards. In the macro preparation, 2.00 mL of methanol or internal standard solution were placed into a 15 mL glass-stoppered centrifuge tube. Three-tenths mL of standard amino acid solution or plasma (or serum) was slowly added to the methanol

with intermittent mixing. The solution was mixed on a vortex-type mixer for 15 s and then centrifuged at 1200 x g for 5 min. One mL of the supernatant phase was transferred to a 5.00 mL volumetric flask. The reaction was initiated by adding, in order, 0.5 mL of saturated borate buffer (pH 9.5), 1.0 mL of 20 µL/mL ethanethiol (in methanol) and 1.0 mL 20 mg/mL o-phthaldialdehyde (in methanol). The total was diluted to 5.00 mL with methanol and allowed to remain at room temperature for at least 2 min for completion of reaction before analysis of 5 to 10 µL on the HPLC system.

In the micro method, 30 µL of amino acid standard or plasma (or serum) sample were added to 200 µL of internal standard solution in a 5 mL glass-stoppered centrifuge tube. The solution was mixed on a vortex-type mixer for 15 s and then centrifuged at 1200 x g for 5 min. One hundred µL of the supernatant phase was transferred to a 15 mL centrifuge tube. The reaction was initiated by adding, in order, 20 µL saturated borate buffer (pH 9.5) and 10 µL/mL ethanethiol/20 mg/mL o-phthaldialdehyde in methanol. The solution was allowed to stand at room temperature for at least 2 min before analysis of 5 to 10 µL on the HPLC system.

Quantitative Analysis

Mixtures of three or four of the standard amino acids were analyzed on the HPLC system at concentrations ranging

from 500 nmol/mL to 10 nmol/mL. The peak area (A_s) of each amino acid derivative was divided by the concentration (c_s) to produce the response factor (k). The response factor for each concentration of a given amino acid was averaged to determine the k that was used to calculate the concentration of the amino acid in the sample (C_u) as follows:

$$C_u = \frac{A_u}{k}$$

A_u= Area of amino acid derivative in sample

Optimization of OPA/EtSH Reagent Concentration

A plasma sample was prepared for amino acid analysis by mixing 1.5 mL of plasma with 10.0 mL of methanol in a culture tube. Following centrifugation at 1200 x g for 5 min, 1.0 mL of the supernatant phase, 1.0 mL of 300 nmol/mL amino acid standard and 0.5 mL saturated borate buffer (pH 9.5) were added to six 5.00 mL volumetric flasks. One of the following volumes of a solution containing 100 mg OPA/100 µL EtSH per mL methanol was added to one of the reaction flasks: 0.005, 0.025, 0.050, 0.100 or 1.00 mL. The solutions were diluted to 5.00 mL with methanol. Five µL of each sample were analyzed by the described HPLC system 2 min after initiating the reaction. To determine the optimal amount of OPA/EtSH necessary for complete reactivity with the amino acids in plasma, the

sum of the area of the peaks for all amino acids in a profile was plotted against the concentration of OPA/EtSH.

Effect of Buffer Concentration on Retention of OPA/EtSH Amino Acids

Six sets of A and B_1 solvent systems were prepared containing different concentrations (3.00/5.16, 6.00/10.3, 9.00/15.5, 12.0/20.6, 15.0/25.8 and 18.0/31.0 mM PO_4/mM Na) of sodium phosphate buffer, while maintaining a 45% concentration of water in solvent B_1 and the same pH in all systems. A sample of OPA/EtSH derivatized amino acids (19.6 pmoles each) was analyzed by the described HPLC system using each set of A and B_1 solvent systems in turn. The effect of the change in buffer concentration was visualized by plotting the retention time for each amino acid derivative against the buffer concentration.

Effect of Column Temperature on the Retention of OPA/EtSH Amino Acids

Using the described HPLC system, 19.6 pmol of OPA/EtSH amino acid standard was analyzed using column temperatures of 15°, 20°, 25°, 28°, 30° and 40°. The effect of column temperature on the retention of each amino acid derivative was observed by plotting the retention time of each amino acid against the column temperature.

Comparison of Plasma Amino Acid Concentrations Determined by an Amino Acid Analyzer and by the Proposed Method

Samples of plasma were collected from six healthy rats and were analyzed for Thr, Ser, Pro, Glu, Gly, Ala, Val, Met, Ile, Leu, Tyr, Phe, Lys, His and Arg on a Beckman 119C Amino Acid Analyzer. The samples were also analyzed by the procedure described in the present study. The data from both methods were compared by taking the absolute difference in the values obtained by the two methods and dividing by the value obtained by the amino acid analyzer. This number was multiplied by 100 to obtain the percent difference.

RESULTS AND DISCUSSION

Figures 1 and 2 show chromatograms of OPA/EtSH derivatives of standard amino acids, using system I and system II respectively. Attempts were made to resolve the Gly and Thr derivatives in one system along with the other amino acid derivatives shown separated in Figure 1. As indicated by Larsen et al. (11), Jones et al. (9) and Figure 2, the use of tetrahydrofuran in the solvent system is effective in resolving Gly and Thr, however, small amounts of tetrahydrofuran added to either solvent A or solvent B_1 in system I resulted in either the loss of resolution between Ser and Gln or Ile and Trp or the loss of resolution betw en both sets of these amino acids. Since system II gives baseline resolution between Gly and Thr, it was decided to sacrifice the

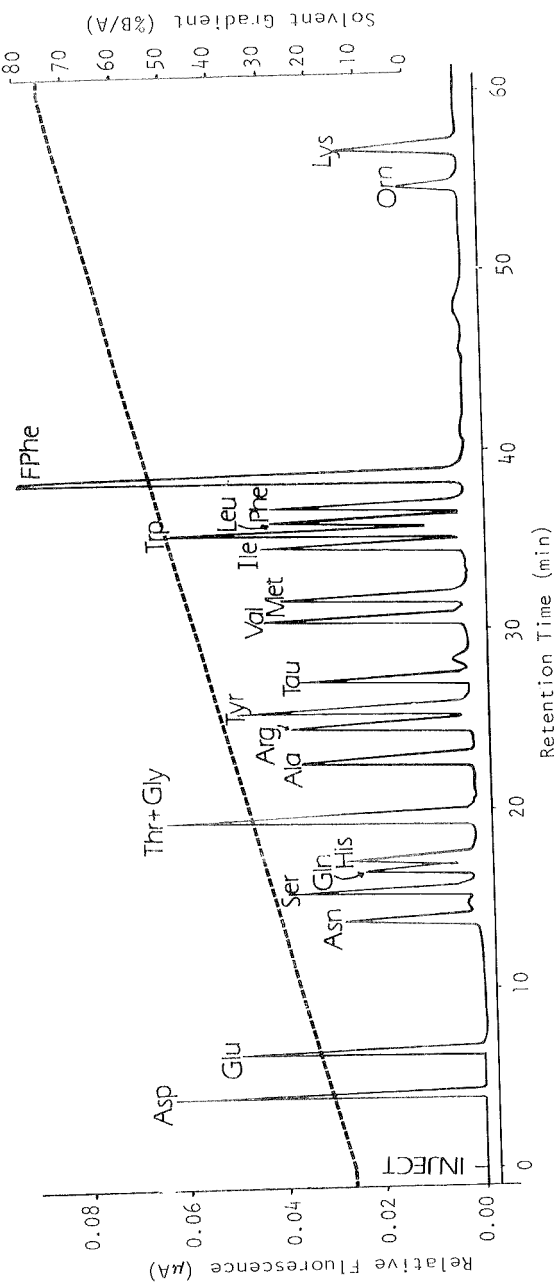

Figure 1. Chromatogram of OPA/EtSH Amino Acid Standards. Chromatographic System I. (FPhe = Fluorophenylalanine, Internal Standard)

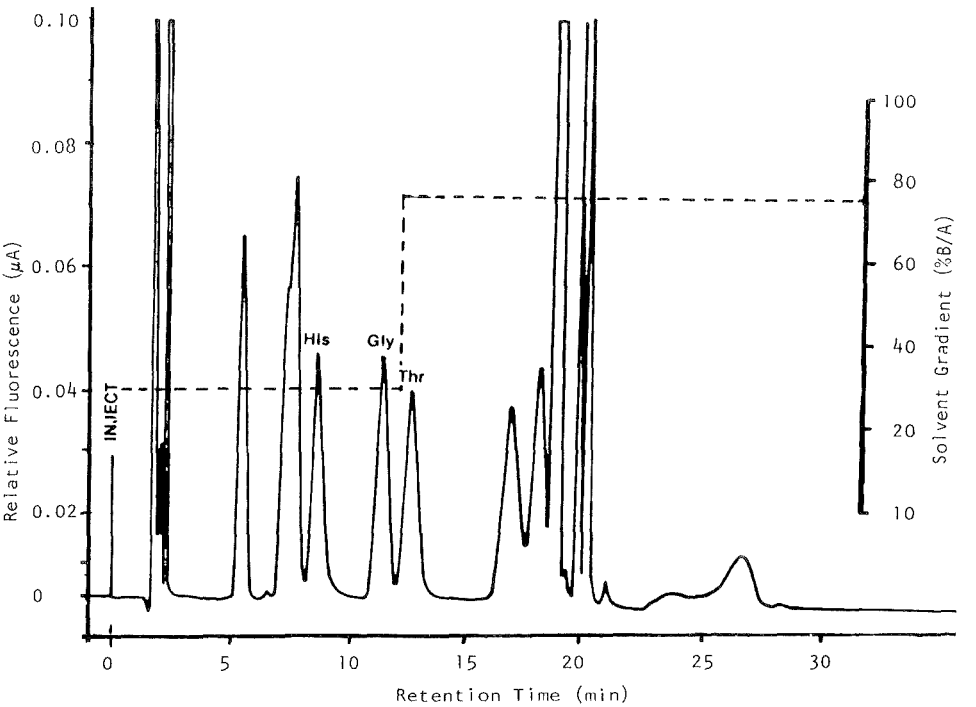

Figure 2. Chromatogram of OPA/EtSH Amino Acid Standards. Chromatographic System II.

separation of this pair of amino acids in system I, in order to maintain good baseline resolution of all other amino acids and to use system II to analyze Gly and Thr separately.

In addition to those amino acids shown in the chromatogram in Figure 1, the elution order of several other amino acids in system I is listed in Table 1. A comparison of retention times of amino acids relative to fluorophenylalanine is listed. These sets of data were obtained six months

TABLE 1

Elution Order of OPA/EtSH Amino Acids on Bondapak C_{18} in System I

Amino Acid	RR_t [1,2]	RR_t [1,3]
Aspartic Acid	0.101	0.106
Glutamic Acid	0.166	0.168
αAminoadipic Acid	0.226	---
Asparagine	0.355	0.357
Serine	0.398	0.399
Glutamine	0.426	0.430
Histidine	0.448	0.445
Citrulline	0.490	---
Threonine	0.505	0.506
Glycine	0.505	0.506
1-Methylhistidine	0.541	---
Alanine	0.588	0.590
Arginine	0.610	0.639
β-Alanine	0.646	---
Tyrosine	0.664	0.663
Taurine	0.702	0.706
β-Aminoisobutyric Acid	0.702	---
α-Amino-n-butyric Acid	0.702	---
Valine	0.789	0.795
Methionine	0.817	0.826
Isoleucine	0.891	0.902
Tryptophan	0.914	0.920
Leucine	0.926	0.939
Phenylalanine	0.947	0.957
p-Fluorophenylalanine	1.000	1.000
Ornithine	1.385	1.427
Lysine	1.437	1.483

[1] RR_t = Retention time of OPA/EtSH amino acid relative to the retention time of OPA/EtSH p-fluorophenylalanine.

[2] RR_t of OPA/EtSH amino acids determined on column number 113134 on 11/80.

[3] RR_t of OPA/EtSH amino acids determined on column number 139094 on 5/81.

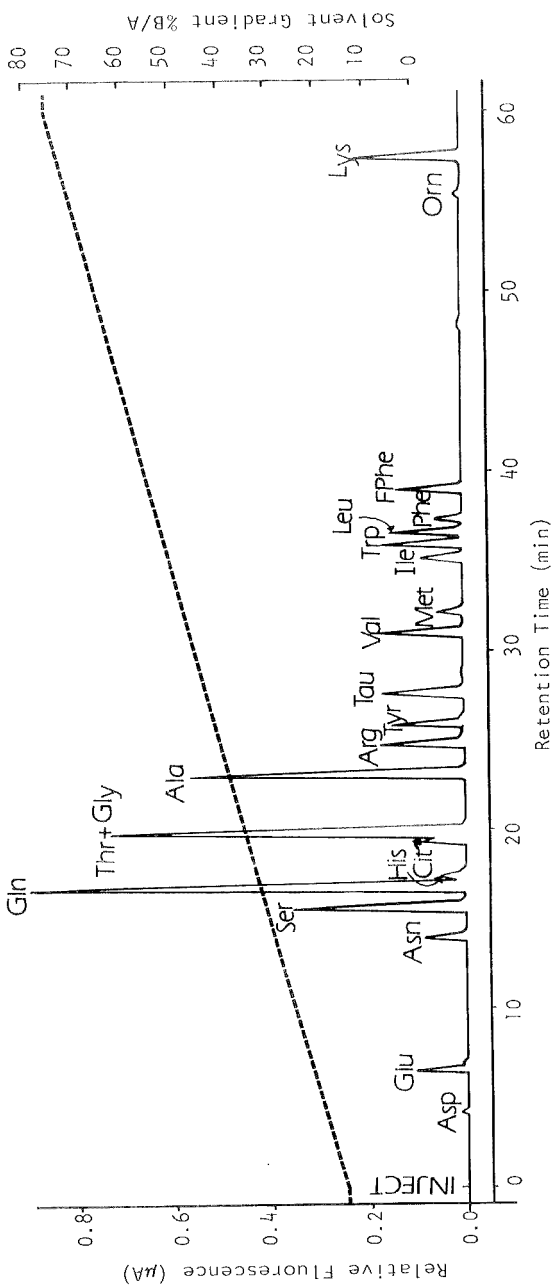

Figure 3. Chromatogram of Rat Plasma Amino Acids as OPA/EtSH Derivatives. Chromatographic System I. (FPhe = Fluorophenylalanine, Internal Standard)

apart on two columns containing different batches of μBondapak C_{18} column material. Relative retention times between the two runs are proximal. Note that Gly and Thr co-elute. α-Amino-n-butyric acid and β-aminoisobutyric acid co-elute with Tau.

A chromatogram of OPA/EtSH derivatives of amino acids extracted from a normal rat plasma is shown in Figure 3. The amino acid derivatives were identified by comparing the relative retention times of the peaks in the sample profiles to the relative retention times of the amino acid standards.

The average variation in the retention time of the 20 amino acids in the standard solution when analyzed six times was 1.9% CV (range 1.6% to 2.4%). For the same set of samples the average relative retention time varied by 0.3% CV (range 0.1% to 0.6%). The retention data suggest that the identification of amino acids should be evaluated on the basis of the relative retention time.

Linear regression analysis of the peak area of each amino acid versus the amino acid concentration over a range of 500 to 5 nmol/mL gave a correlation coefficient of 0.999 or better for all the amino acids in the standard solution. Figure 4 shows a standard curve for 3 representative amino acids. The curve illustrated for Ala is representative of the slope that was obtained for most of the amino acids. The curves obtained for Orn and Lys had a lower slope value than the other amino

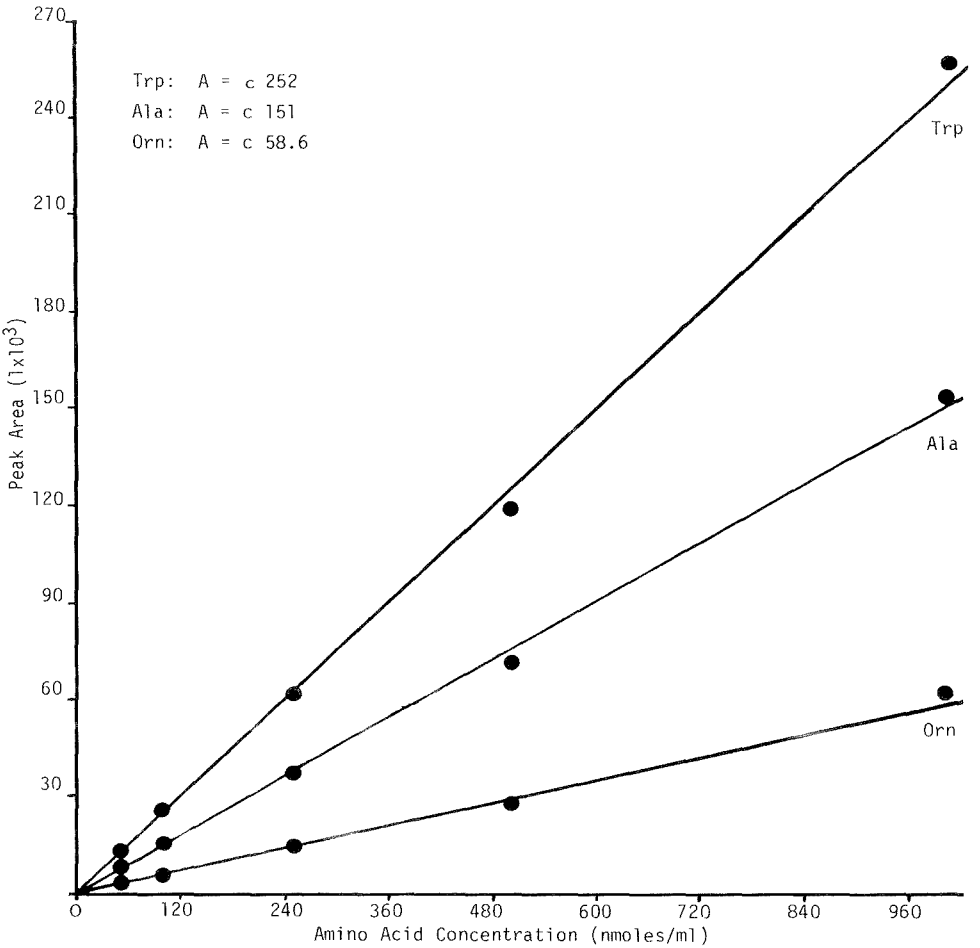

Figure 4. Representative Standard Curves for OPA/EtSH Amino Acid Response versus Concentration.

acid derivatives. This has been observed by other investigators in both post-column and pre-column derivatization with OPA/mercaptoethanol (8, 9, 12, 13). The cause may be due to the presence of two fluorescent isoindole structures, as occurs for both Orn and Lys, causing an internal quenching of the fluroescence of each structure. The fact that the slope for Lys was greater than the slope for Orn suggests that a greater distance between the two isoindole structures results in less internal quenching effects. The slope for Trp has a larger value than that of the other amino acid derivatives. This was probably due to the natural fluorescence of Trp enhancing the fluorescence of the adduct.

The relationship between the peak area response and the amount of derivatizing reagent used in the reaction of a plasma extract containing 2.3 μmol/mL additional concentration of each amino acid is shown in Figure 5. For the proposed derivatization procedure (utilizing 0.3 mL of plasma), the data indicated that 1 mL of a solution containing 5 mg OPA/5 μL EtSH was sufficient for complete reactivity of all amino acids in plasma even when present at a concentration several times normal. In order to be sufficiently upon the plateau of the reactivity curve a concentration of 20 mg OPA/20 μL EtSH per mL was chosen for routine applications. The ratio of OPA/EtSH was maintained at approximately 1:2 to insure

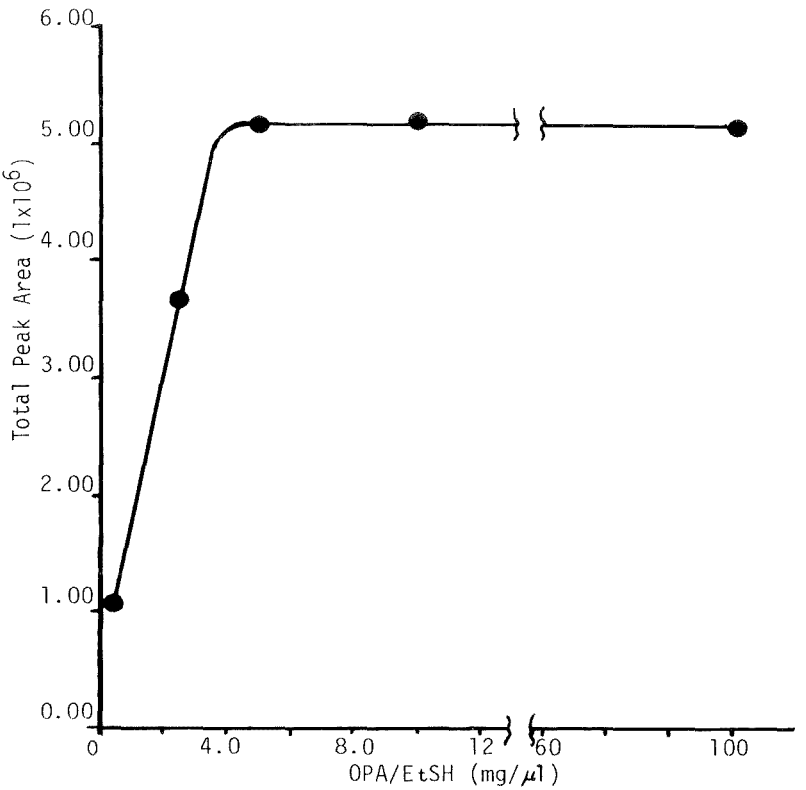

Figure 5. Optimum Concentration of o-Phthaldialdehyde and Ethanethiol for Complete Reaction of Amino Acids in Plasma.

that ethanethiol was the only thiol present in sufficient quantity to form the fluorescent derivative.

Hill, et al. (10) demonstrated that a change in the buffer concentration in the HPLC system affected the retention of the Tau and Tyr OPA/EtSH derivatives but not the retention of Arg. It was suggested that the sodium ion was re-

sponsible for the effect. Lindroth et al. (8) demonstrated that changing the buffer concentration in the solvent system affected the retention of all the OPA/mercaptoethanol amino acid derivatives except Arg or ammonia. Data were presented that suggested that the phosphate ion was responsible for the effect and not the sodium ion. Figure 6 shows the effect of changing the buffer concentration on the retention time of several of the OPA/EtSH amino acid derivatives. As observed for the OPA/mercaptoethanol amino acid derivatives an increase in buffer concentration results in an increase in the retention time of all OPA/EtSH amino acids except the Arg derivative. Additionally, Larsen et al. (11) have shown this same effect to occur by increasing the concentration of the counter ion triethylamine in the solvent system.

It was observed that as samples or standards were analyzed, the retention time of each amino acid, except Arg, decreased. The resolution between all amino acid derivatives remained constant except between Arg and Tyr which deteriorated. As suggested by the data in Figure 6, increasing the concentration of the Na/PO_4 buffer in the solvent system resulted in regaining the original retention time of all the amino acids as well as the resolution between Arg and Tyr. Changing the buffer concentration to maintain Arg-Tyr resolution is described in the Materials and Methods section. The similarity in the effects of column aging and decreasing

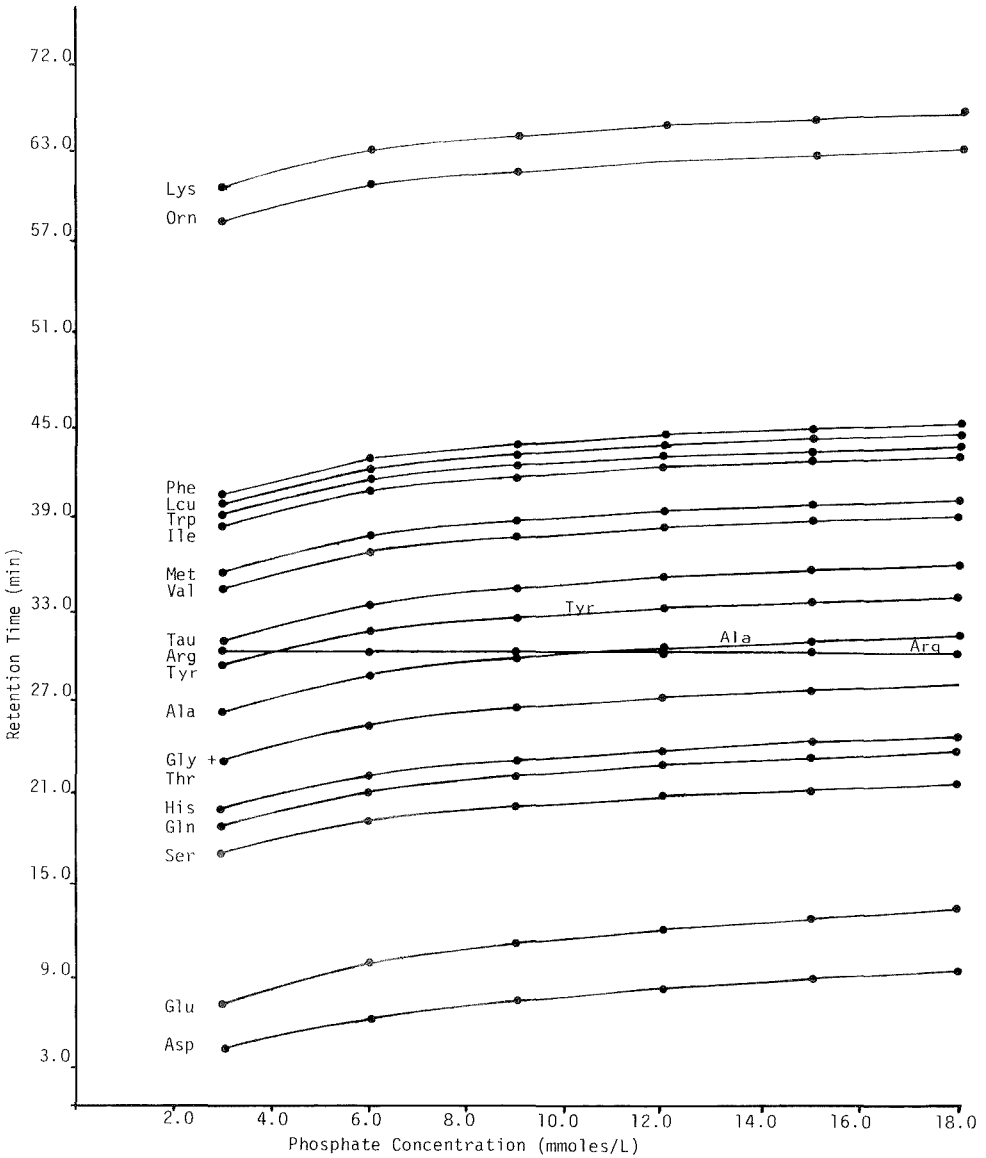

Figure 6. Effect of Buffer Concentration on the Retention of OPA/EtSH Amino Acids on a μC_{18} Column.

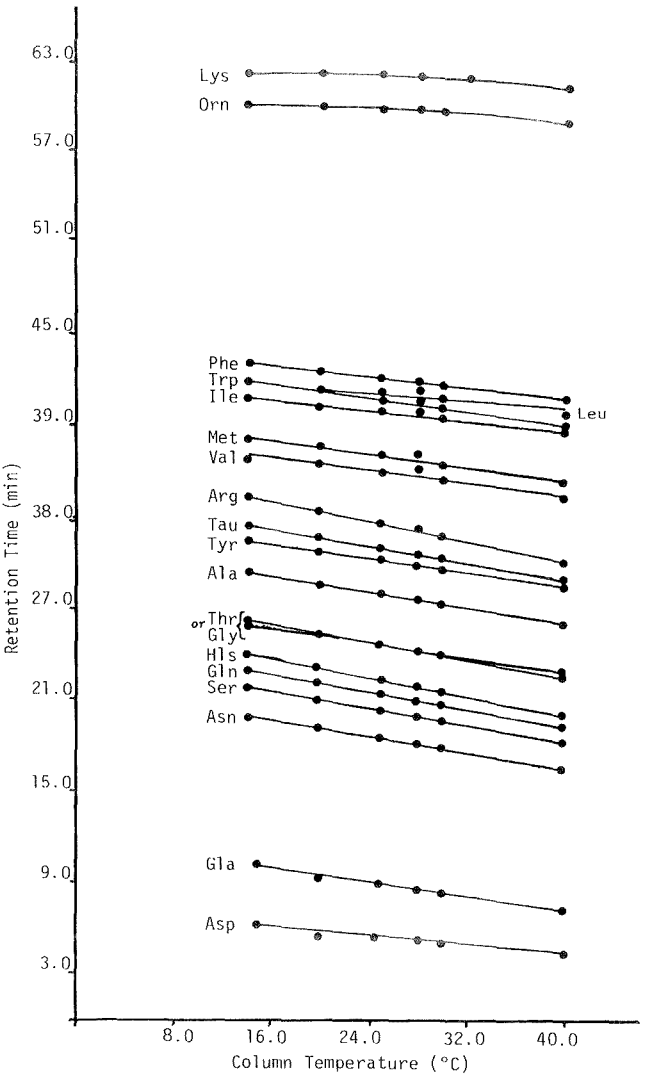

Figure 7. Effect of Column Temperature on the Retention of OPA/EtSH Amino Acids on a μC_{18} Column.

buffer concentration on the retention of the amino acid derivatives suggested that the two phenomena were related.

As expected, increasing the temperature of the column (Figure 7) caused a decrease in the retention of all the amino acids; however, there was little change in the resolution between most peaks. Exceptions to this were in the resolutions of Trp and Leu which tend to co-elute at low temperatures; Phe and Leu, and Trp and Ile which tend to co-elute at high temperatures; and Thr and Gly which begin to separate at either high or low temperatures. The Thr-Gly resolution was slight and losses in resolution of the other amino acid pairs prevented high column temperature from being used to resolve Thr-Gly in a total system. Temperatures low enough to resolve Thr and Gly resulted in peak broadening and loss of resolution between the Trp-Leu amino acid pair. A temperature of 28° appeared to be optimal for resolving the greatest number of amino acid pairs.

Table 2 lists a comparison between rat plasma amino acid concentrations obtained by the amino acid analyzer and the HPLC method presented in this study. Of the amino acids studied, the values differed by as much as 31% and as little as 0%. The average difference for all the amino acids was 12.3%.

Recently, Fernstrom and Fernstrom (14) reported reasonable correlation between amino acid profiles obtained by the

TABLE 2

Comparison of Plasma Amino Acid Concentrations Determined by Amino Acid Analyzer and HPLC/OPA Methods

Amino Acid	Plasma 1		Plasma 2		Plasma 3		Plasma 4		Plasma 5		Plasma 6		Average
	OPA[a]	IE[b]	OPA	IE	OPA	IE	OPA	IE	OPA	IE	OPA	IE	%DIF[c]
Ala	465	538	551	609	548	552	651	651	528	564	525	571	6.4
Arg	177	229	221	256	178	192	205	231	161	207	159	200	16.3
Gly + Thr	802	963	990	1061	990	1056	993	1061	747	902	748	869	11.6
His	74	80	87	89	93	90	94	80	67	72	85	95	7.9
Ile	71	82	83	91	112	117	92	86	76	89	87	93	9.1
Leu	123	148	147	166	162	168	156	157	134	148	144	160	8.7
Lys	352	511	346	466	454	535	352	535	282	363	417	503	24.3
Met	54	64	55	59	78	78	62	69	54	65	51	57	10.2
Phe	57	62	60	64	71	67	61	56	58	63	59	62	6.9
Ser	296	374	328	396	390	407	410	430	283	330	330	397	13.0
Tyr	129	119	95	99	152	123	167	132	125	114	101	90	14.1
Val	152	196	183	213	225	254	181	233	167	224	185	233	19.4

a. Plasma concentrations in nmoles/mL obtained by the HPLC/OPA method described in the present study

b. Plasma concentrations in nmoles/mL obtained by amino acid analyzer

c. $\%\ DIF = \frac{\Sigma\left|100(OPA-IE)/IE\right|}{N}$; where N = number of samples analyzed

HPLC method presented here with those obtained by an amino acid analyzer. Additionally, they have demonstrated that amino acid concentration profiles obtained by the HPLC method on plasma from rats with experimental diabetes was diagnostic for the disease.

The amino acid analysis presented in this study offers a rapid and sensitive alternative to the time-consuming ion-exchange methods currently in use for determining plasma amino acid concentrations. Further studies are in progress to determine the accuracy of the HPLC method.

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A COMPARISON OF RADIALLY-COMPRESSED AND STAINLESS STEEL COLUMNS FOR THE REVERSED-PHASE ION-PAIR SEPARATION OF PHENCYCLIDINE SYNTHETIC MIXTURES

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ABSTRACT

The reversed-phase ion-pair HPLC separation of phencyclidine synthetic mixtures was optimized utilizing Radial-Pak radially compressed columns. Variables examined in the optimization included column type (C-18, C-8, or CN), pairing ion (methane-, pentane-, hexane-, or octane sulfonates) and mobile phase composition (varying concentrations of methanol or acetonitrile in water). The chromatographic behavior of the phencyclidine mixtures in the various systems utilizing radially compressed columns is compared and contrasted to a similar previous study which examined similar variables on stainless steel columns. The optimum system for radially compressed columns was found to consist of a Radial-Pak C-18 column and a mobile phase of 85:15 MeOH:H_2O, 2.5% acetic acid, 1% triethylamine and 5mM sodium hexane sulfonate.

INTRODUCTION

In a previous study (1), an optimized HPLC separation for crude synthetic mixtures of phencyclidine (1-(1-phenylcyclohexyl) piperidine, PCP, 1) was reported. In that study, the separation of 1 and cosynthetics 1-[1-(1,1'-biphenyl-4-yl)cyclohexyl]piperidine (2), 1-[1-phenylethyl)cyclohexyl]piperidine (3), and 1,1'-(1,4-phenylenedicyclohexylidine)bis[piperidine] (4) (see Figure 1

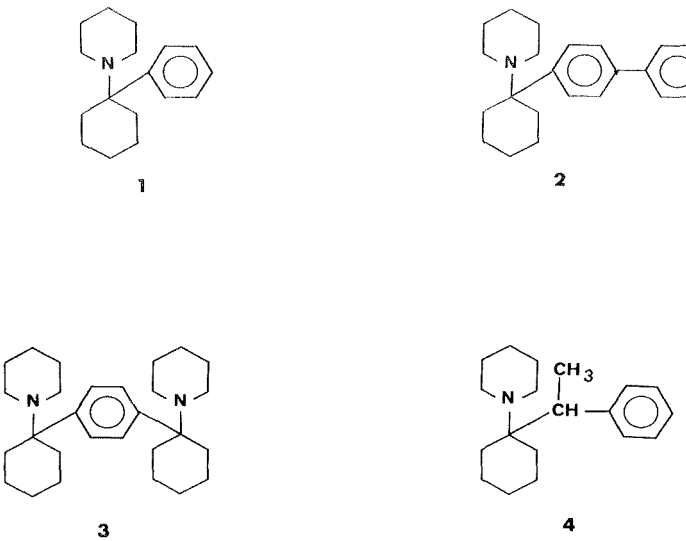


FIGURE 1

Structures of PCP (1) and some identified co-synthetics.

for structures) was optimized utilizing stainless steel analytical (SSA) columns and a reversed-phase paired ion method. The success of this study prompted us to investigate the efficacy of radially-compressed columns for a similar analysis, and we wish to now report the results of this study and compare them with the previously reported results for SSA columns (1).

In the original study (1), compounds 1 and 2 were used as the probe compounds for the determination of the selectivity factor, α, since they constituted the major components of the mixture (2). A total of 11 peaks were observed using the optimized conditions (C-18 column, 70:30 MeOH:H_2O, 5m$\underline{M}$ $C_6H_{13}SO_3Na$, pH 3.5 with CH_3COOH and a flow rate of 2.5 ml/min); the later

eluting peaks (larger k's) being small and not well-resolved. In the studies reported herein, it was noted that these strongly retained compounds were not observed except in organic-rich mobile phase mixtures, suggesting a high degree of hydrophobic character for these later eluting compounds. Since these compounds were of minor consequence, our study was limited to the compounds eluting before that peak attributed to compound 2.

During the determination of the k'- and α-values of 1 and 2 as a function of solvent strength, it was observed that as the concentration of the organic component of the mobile phase decreased, the peak previously attributed to 2 began to separate into two peaks, the smaller being the more retained (*vide infra*). Some preliminary evidence that this peak did indeed consist of two compounds was previously obtained by collecting the peak semi-preparatively from a 5/8" C-18 column and, following workup, comparing the mass and NMR spectra. Slight differences in these spectra were observed compared to the synthetic 2, and it is suspected that the second peak represents an isomer of 2. Important, however, is the demonstrated increased resolution of the Radial-Pak columns over that of the SSA columns.

EXPERIMENTAL SECTION

Apparatus

A Waters Associates (Milford, MA) ALC 244 Liquid Chromatograph was used for all analyses. Mobile phase delivery was accomplished by twin M6000A pumps interfaced through the Waters model 660 solvent programmer. Detection was by UV at 254 nm at a sensitivity of 0.02 AUFS. Chromatograms were obtained using a Houston Instruments Omniscribe strip chart recorder (Houston, TX). Columns were radially compressed in the Waters Radial Compression Module. Columns examined included the 10cm x 8mm id Radial-Pak C-18, C-8, and CN (all Waters).

Reagents and Samples

All solvents were prepared exactly as described previously (1) except that 1% triethylamine (TEA) was added. Crude PCP was synthesized according to the method of Kalir (3).

Analysis and Calculations

The standard solution used for the determination of k' and the selectivity ratio, α, was 0.30 µg/µL of crude PCP in MeOH. Each injection was 10.0 µL at a mobile phase flow rate of 2.5 mL/min. Column void times (to) were obtained by injection of pure MeOH or H_2O. The k' values for PCP and $\underset{\sim}{2}$, and $\alpha_{\underset{\sim}{2}/\underset{\sim}{1}}$ (the selectivity between $\underset{\sim}{1}$ and $\underset{\sim}{2}$), were calculated in the usual manner (4).

RESULTS AND DISCUSSION

Table 1 contains the values of least-square slopes and intercepts for log $k'_{\underset{\sim}{1}}$ vs. % organic in the mobile phase for each of the pairing agents examined on the Radial-Pak C-18, C-8 and CN columns. With MeOH as the organic modifier, both the absolute value of the slope (|slope|) and the y-intercept decrease in the order C-18 > C-8 > CN column for a given pairing agent. The value of |slope| is an indication of the sensitivity of log k' to changes in %MeOH, i.e. the steeper the slope (larger |slope|) the more effect a small change in %MeOH will have on the retention of the solute (1). The y-intercept, corresponding to log k' at 0% MeOH, is an indicator of the ability of a particular column (or pairing agent) to retain the analyte (i.e. large values for y-intercept mean greater retention). Since both y-intercept and |slope| decrease as column polarity is increased, it is obvious that the more non-polar column (i.e., C-18) permits more control of analyte retention for a given pairing agent with MeOH containing mobile phases.

TABLE 1

Least Squares Equations[a] for log k'_1 vs. % MeOH and vs. % MeCN in Mobile Phase.[b]

Pairing Ion	MeOH/H_2O			MeCN/H_2O		
	C-18 Slope/Intercept	C-8 Slope/Intercept	CN Slope/Intercept	C-18 Slope/Intercept	C-8 Slope/Intercept	CN Slope/Intercept
C_1SO_3Na	-0.0293/2.59	-0.0277/2.17	-0.0210/1.34	-0.0119/1.10	-0.0148/1.06	-0.0132/0.769
C_5SO_3Na	-0.0300/2.66	-0.0290/2.28	-0.0193/1.19	-0.0144/1.21	-0.0146/1.02	-0.00910/0.499
C_6SO_3Na	-0.0299/2.68	-0.0315/2.53	-0.0186/1.13	-0.0148/1.28	-0.0147/1.05	-0.0133/0.767
C_8SO_3Na	-0.0317/2.85	-0.0310/2.49	-0.0202/1.28	-0.0132/1.19	-0.0157/1.11	- .0208/1.33

[a] Correlation coefficients in all cases were >0.96. The equation is of the form log k'_1 = m (% organic) + b where m = slope and b = y-intercept.

[b] These equations were linear over the range of 50-90% MeOH.

TABLE 2

Least Squares Equations[a] for[b] log k'_2 vs. % MeOH and vs. % MeCN in Mobile Phase as a function of Pairing Ion and Column Type.

Pairing Ion	$MeOH/H_2O$			$MeCN/H_2O$		
	C-18 Slope/Intercept	C-8 Slope/Intercept	CN Slope/Intercept	C-18 Slope/Intercept	C-8 Slope/Intercept	CN Slope/Intercept
C_1SO_3Na	-0.0445/4.30	-0.0565/5.01	-0.0394/3.27	-0.0235/2.31	-0.0233/2.00	-0.0280/2.04
C_5SO_3Na	-0.0445/4.32	-0.0427/3.84	-0.0303/2.36	-0.0241/2.27	-0.0237/1.96	-0.0180/1.26
C_6SO_3Na	-0.0463/4.50	-0.0484/4.37	-0.0302/2.35	-0.0265/2.50	-0.0252/2.10	-0.0226/1.58
C_8SO_3Na	-0.0470/4.56	-0.0430/3.85	-0.0313/2.45	-0.0246/2.34	-0.0247/2.05	-0.0270/1.88

[a] Correlation coefficients in all cases were >0.94. The equation is of the form log k'_1 = m (% Organic) + b where m = slope and b = y-intercept.

[b] These equations were linear over the range of 50-90% MeOH in all cases.

As Table 1 shows, log k'_I varies less predictably when MeCN-containing mobile phases are used. For the C_1, C_5, and C_6SO_3Na pairing agents, the y-intercept decreases as column polarity is increased, but a sharp increase in intercept is noted for C_8SO_3Na on the CN column. It is to be noted that the y-intercepts are different on the same column for MeOH and MeCN mobile phases. Since the y-intercept represents k' at 0% organic, this difference would appear to be a contradiction to the concept that k' should be the same in 100% aqueous mobile phase. However, in mobile phase compositions of less than 30% organic, log k' vs. % organic begin to deviate from linearity. The extrapolation to give the log k' values shown in Tables 1 and 2 utilized the linear range of values. The change in |slope| is even less predictable in MeCN mobile phases with no systematic trend being noted. At this time, no explanation presents itself to account for the apparent randomness of analyte retention in MeCN mobile phases. However, similar differences in the behavior of log k' in MeOH- and MeCN- containing mobile phases were observed in the previous study using SSA columns (1).

The least-square slopes and intercepts were determined for compound 2 on each column and with each pairing agent (Table 2), and for any given column type and pairing agent combination, both |slope| and y-intercept were larger for 2 than for 1 under the same conditions. This is not unexpected, since 2 contains an additional (relatively non-polar) phenyl group.

The experimental and predicted values for k'_1 on both the SSA C-18 column (from the previous study (1)) and on the Radial-Pak C-18 column are compared in Table 3. It is interesting to note that for each pairing agent/% MeOH combination, k'_1 is smaller for the SSA column than for the Radial-Pak column. A possible explanation for the greater retentivity of the Radial-Pak column relative to the SSA column lies in the fact that the

TABLE 3

Experimental and Predicted[a] k'_1-Values as a Function of % MeOH and Pairing Ion Using SSA[b] and Radial-Pak C-18 Columns.

	SSA C-18		Radial-Pak C-18	
% MeOH	Exptl. k'_1	Pred. k'_1	Exptl. k'_1	Pred. k'_1
		C_5SO_3Na		
45	4.35	3.80	- -	- -
50	2.68	2.55	15.24	- -
60	1.07	1.14	7.18	7.31
65	0.72	0.77	- -	- -
70	- -	- -	3.48	3.69
80	0.17	0.23	1.84	1.86
90	- -	- -	0.95	1.06
		C_6SO_3Na		
45	7.19	6.81	- -	- -
50	- -	- -	- -	- -
55	2.60	2.75	- -	- -
60	1.60	1.75	7.80	7.82
65	1.10	1.11	- -	- -
70	0.71	0.71	3.92	3.89
80	- -	- -	1.95	1.94
85	- -	- -	1.41	1.37
90	- -	- -	0.98	1.04
		C_8SO_3Na		
55	8.45	7.12	- -	- -
60	3.49	4.09	9.13	8.93
70	- -	- -	4.11	4.33
75	0.75	0.77	- -	- -
80	0.41	0.44	2.23	2.09
85	- -	- -	1.39	1.46
90	0.16	0.15	1.01	1.01

[a] Predicted values are as predicted using equation (1).

[b] Data taken from Ref. 1

Radial-Pak packing material is not end-capped after the reaction which bonds the octadecysilane moiety to the silica-gel (the C-18 SSA column utilized in (1) was end-capped). The lack of end-capping exposes the solute to free silanol groups present on the silica base. Interaction of the positive, protonated nitrogen in the piperidyl ring of 1 with these silanol sites could result in increased retention. As noted in the Experimental Section, triethylamine (TEA) was added to the mobile phases used on Radial-Pak columns in an attempt to minimize these secondary "polar" interactions. Mobile phases which did not contain TEA were found to be unacceptable due to severe peak tailing. The fact that addition of TEA significantly improved peak shapes is indicative of these proposed "polar" interactions. It should be noted that the non-end-capped silica and the addition of TEA to the mobile phases results in a fundamentally different system for the Radial-Pak columns as opposed to the SSA columns. The comparisons between Radial-Pak and SSA columns made herein reflect not only the differences due to the compression of the Radial-Pak columns, but primarily the differences due to mobile phase and packing material chemistry.

The log k' values for 1 and 2 were fitted to an equation of the form

$$\log k' = a\ (\%\ \mathrm{MeOH}) + b\ (\mathrm{nC}) + c\ (\%\mathrm{MeOH})\ (\mathrm{nC}) + d \qquad (1)$$

using the GLM procedure of SAS (5), where %MeOH is the concentration of MeOH in the mobile phase and nC is the chain-length of the sulfonate pairing-ion (i.e. for hexane sulfonate nC=6). The derived a, b, c, and d parameters are tabulated in Table 4 for each of the columns examined.

In order to determine the optimum system for the separation of PCP and its cosynthetics, $\alpha_{2/1}$ (the selectivity ratio between 2 and 1) was maximized since our previous study (1) had shown that as the separation of 1 and 2 increased, all components were

TABLE 4

Coefficients for Equation 1 for PCP (1) and 2 on Three Different Radially Compressed Columns.[a]

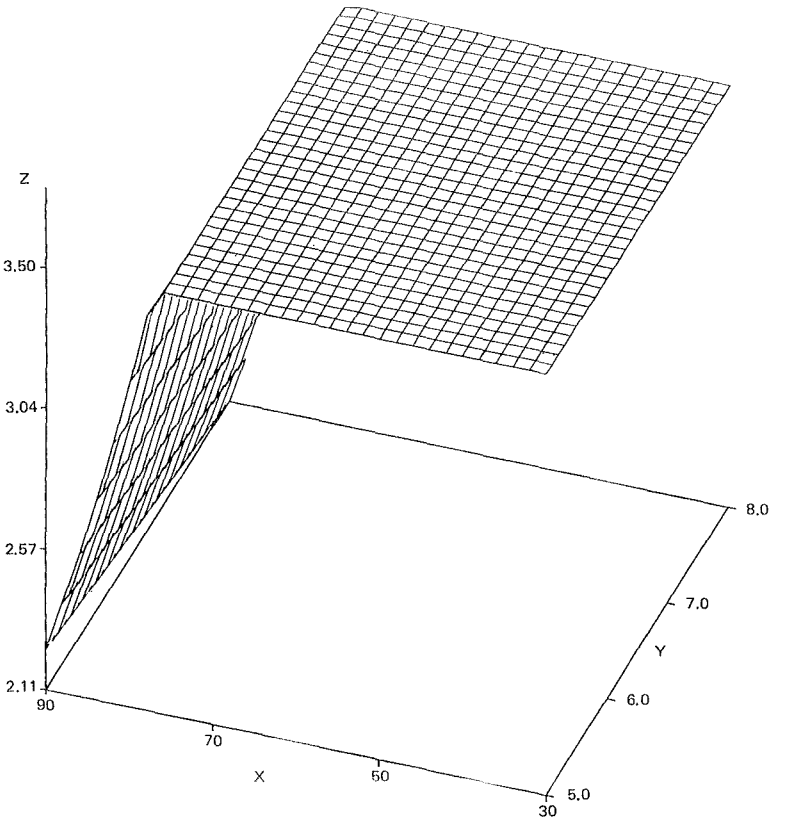
Column	a	b	c	d
For PCP(1) –				
C-18	-0.0267	0.0646	-0.000598	2.32
C-8	-0.0259	0.0722	-0.000718	1.97
CN	-0.0172	0.0359	-0.000354	0.980
For 2 –				
C-18	-0.0413	0.0678	-0.000733	4.03
C-8	-0.0410	0.00764	-0.000224	3.77
CN	-0.0298	0.0223	-0.000159	2.26

[a] Correlation coefficients for the equations on each of the columns were >0.995 for 1 and >0.993 for 2.

increasingly well-resolved. Figures 2, 3 and 4 are three-dimensional plots of $\alpha_{2/1}$ as a function of both % MeOH and nC. The plots were generated using the SAS/graph procedure (6) and solving

$$\alpha_{2/1} = \frac{10\uparrow \text{ eq 1 for compound 2}}{10\uparrow \text{ eq 1 for compound 1}}$$

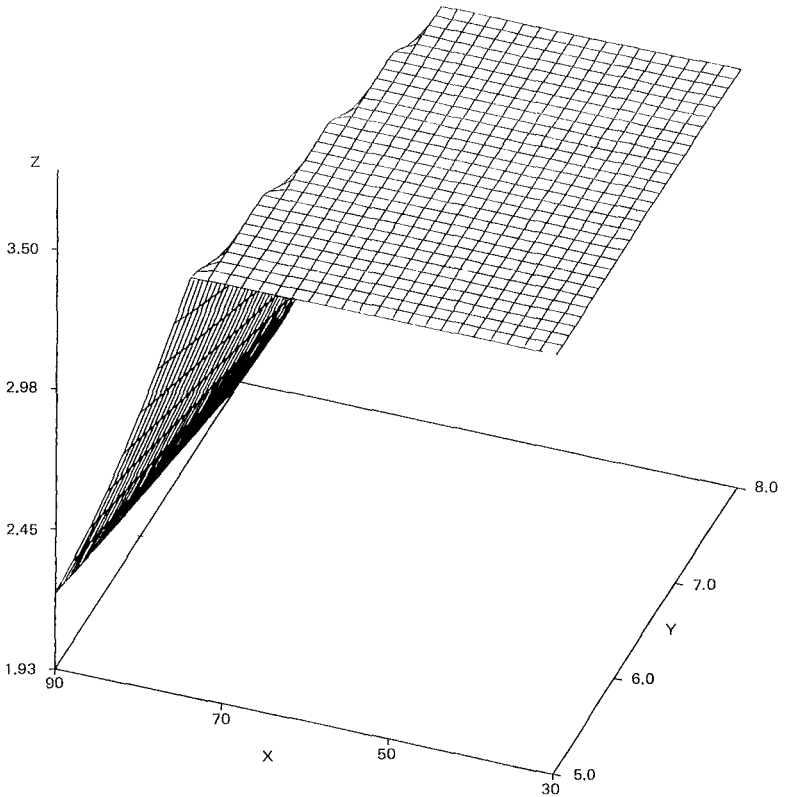
for MeOH percentages of 30-90% at 2% intervals and pairing ion chain lengths of 5 to 8 at 0.1 intervals. The $\alpha_{2/1}$ (z) axis in each plot was arbitrarily clipped at $\alpha = 3.5$ since qualitative examination of the chromatograms showed that an α value of 3.5 was more than sufficient to result in resolution of all peaks of interest while keeping analysis times relatively short.



X IS %MEOH, Y IS PAIRING-ION CHAIN LENGTH, & Z IS ALPHA
ALPHA VALUES GREATER THAN 3.5 *SET EQUAL TO* 3.5

FIGURE 2

Plot of $\alpha_{2/1}$ (z axis) as a function of % MeOH (x axis) and nC (y axis) on the radially compressed C-18 column.

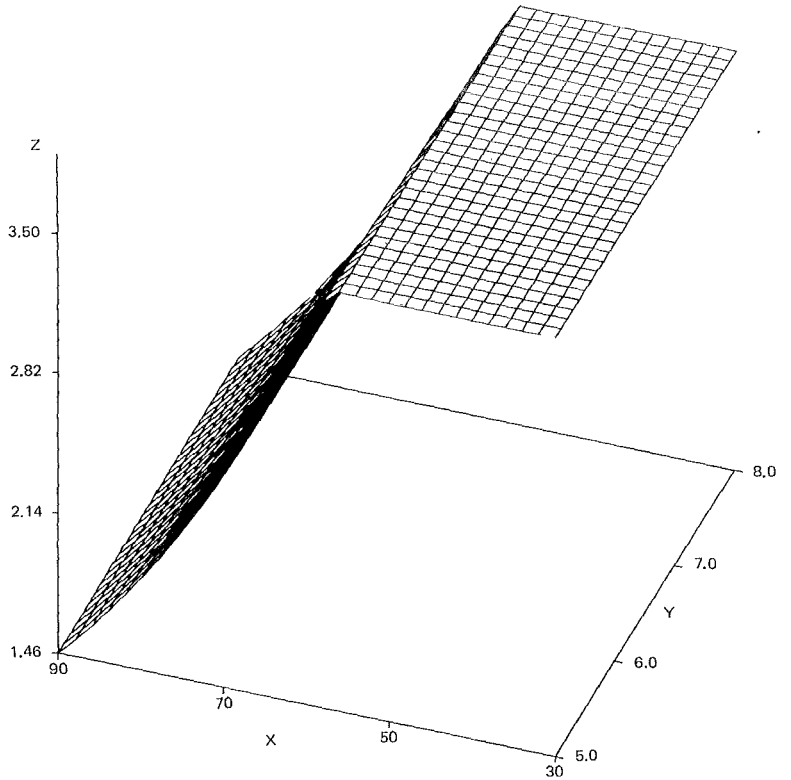


X IS %MEOH, Y IS PAIRING-ION CHAIN LENGTH, & Z IS ALPHA ALPHA VALUES GREATER THAN 3.5 SET EQUAL TO 3.5

FIGURE 3

Plot of $\alpha_{\tilde{2}/\tilde{1}}$ as a function of % MeOH and nC on the radially compressed C-8 column.

Examination of Figs. 2-4 shows that all three columns are capable of achieving α values of 3.5 within the range of conditions examined. However, the "steepness" of the plots increases in the order C-18 (Fig 2) > C-8 (Fig 3) > CN (Fig 4). The fact that the C-18 column exhibits the steepest slope indicates that,



X IS %MEOH, Y IS PAIRING-ION CHAIN LENGTH, & Z IS ALPHA ALPHA VALUES GREATER THAN 3.5 SET EQUAL TO 3.5

FIGURE 4

Plot of $\alpha_{2/1}$ as a function of % MeOH and nC on the radially compressed CN column.

for a given % MeOH, the C-18 column would yield the largest α value of the three columns examined. It is interesting to note that this steepness occurs only in the % MeOH (x) direction. The plots are all relatively flat in the nC (y) direction, indicating that pairing-ion chain length exerts little effect on the separation of 1 and 2.

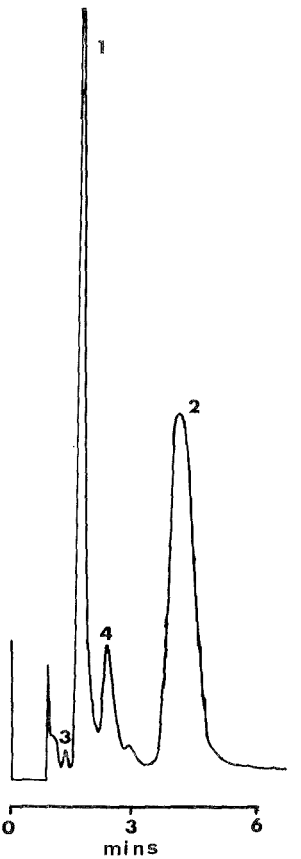


FIGURE 5

Chromatogram of crude synthetic PCP mixture. Numbers correspond to structures in Figure 1. Conditions: 85:15 MeOH:H_2O, 5m$\underline{M}$ C_6SO_3Na + 1% TEA and 2.5% HOAc; Flow rate 3.5 mL/min; UV @ 254 nm, 0.1 AUFS; Radially compressed C-18 column.

Figure 5 illustrates the separation of a crude PCP mixture obtained on a Radial-Pak C-18 column. The chromatogram shown in Fig. 5 was obtained using conditions which were predicted from Fig. 4 to yield approximately $\alpha_{\underset{\sim}{2}/\underset{\sim}{1}} = 3.5$ (i.e. 85:15 MeOH:H_2O with C_6SO_3Na as pairing agent). The actual value of $\alpha_{\underset{\sim}{2}/\underset{\sim}{1}}$ as measured from the chromatogram is approximately 3.6. As demonstrated in Figures 3 and 4, an equivalent $\alpha_{\underset{\sim}{2}/\underset{\sim}{1}}$ can also be obtained on the C-8 or CN columns by decreasing the amount of MeOH in the mobile phase. However, the more MeOH-rich mobile phase is preferred since MeOH-rich mobile phases are less viscous, allowing higher flow rates at lower pressures, thereby reducing analysis time.

Close examination of the peak attributed to compound $\underset{\sim}{2}$ in Fig. 5 shows a slight deformation on the trailing edge of the peak. This shoulder is attributed to the possible presence of an isomer of $\underset{\sim}{2}$ (*vide supra*). Further separation of this shoulder can be achieved by further decreasing the % MeOH in the mobile phase at the cost of substantially increased analysis time. Figure 6 illustrates a chromatogram in which the shoulder is more completely resolved utilizing %MeOH = 75. However, the increase in resolution required an approximate two-fold increase in analysis time, substantially increased tailing for the compound $\underset{\sim}{1}$ peak, and resulted in a greatly decreased detection efficiency for $\underset{\sim}{3}$, $\underset{\sim}{4}$, and other (unidentified) peaks in the mixture.

In summary, the liquid chromatographic behavior of $\underset{\sim}{1}$ has been determined under reversed-phase paired ion conditions on radially compressed columns. An optimized system (consisting of a C-18 Radial-Pak column and a mobile phase of 85:15 MeOH:H_2O, 5 m$\underline{M}$ hexane sulfonate, 2.5% acetic acid and 1% TEA) is presented for the separation of $\underset{\sim}{1}$ and cosynthetics $\underset{\sim}{2}$, $\underset{\sim}{3}$, and $\underset{\sim}{4}$ on radially compressed columns and the method demonstrated on a crude synthetic PCP mixture. It was shown that the Radial-Pak C-18 column could provide superior resolution (relative to the SSA

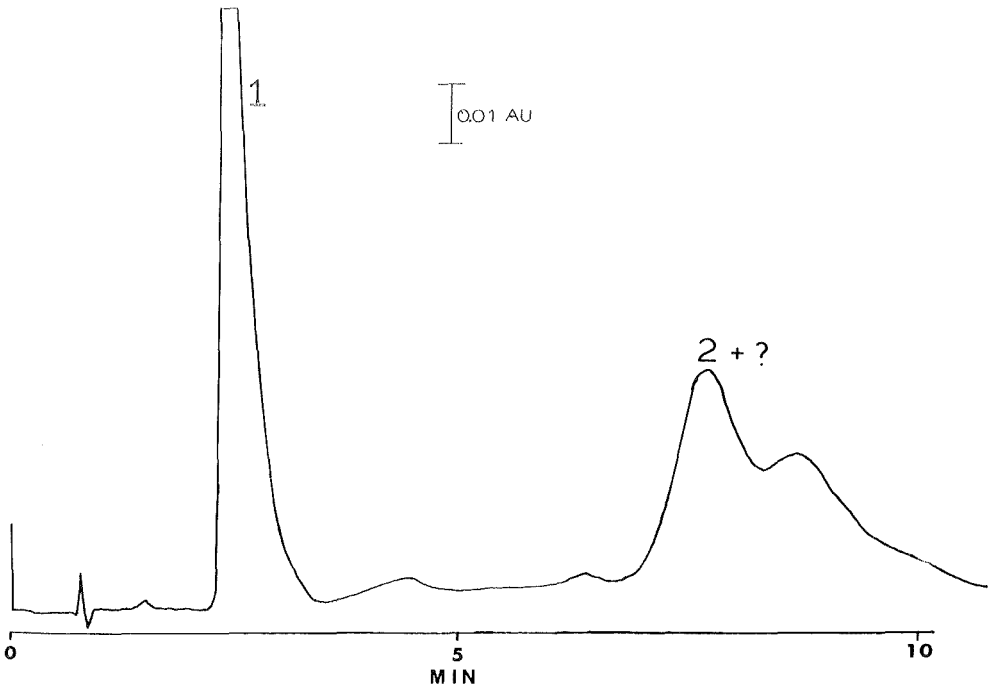


FIGURE 6

Chromatogram of crude synthetic PCP mixture illustrating increased resolution of shoulder from peak attributed to 2. Conditions: same as Fig. 1 except 75:25 MeOH:H_2O.

C-18 column) for a suspected isomer of 2. Comparisons of log k' vs. % MeOH plots for the Radial-Pak C-18 column and the SSA C-18 column showed that PCP has a much higher affinity for the Radial-Pak C-18 column. Whether this increased affinity is due to hydrophobic or polar interactions is not clear at the present time. However, we suspect that the differences in affinity are due to polar interactions since it was necessary to dope the

mobile phases used on Radial-Pak columns with TEA to obtain acceptable peak symmetries.

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ANALYSIS OF CIMETIDINE IN BIOLOGICAL FLUIDS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The clean-up and analysis of cimetidine in human urine and blood is described. Samples were prepared by adsorption on Waters Sep-pak C-18 disposable pre-columns in basic solution followed by elution in 1 ml methanol. For blood samples, the eluate was concentrated under a stream of nitrogen; urine samples required no further concentration. The separation was performed on a reversed phase column using a mixture of methanol-1 mM sodium dodecyl sulphate in a 10 mM phosphate buffer of pH 3.0 (35:65) as mobile phase. Procaine was used as internal standard. Detection was by UV at 228 nm yielding a minimum detectable quantity of 20 ng with linearity over three decades of concentration.

INTRODUCTION

Cimetidine (Tagamet^{R}, SKF) (N"-cyano-N-methyl-N'-[2(5-methyl-1H--imidazol-4-yl)methyl-thioethyl]guanidine), a histamine H_2 receptor antagonist, inhibits the secretion of gastric acids and has been used very effectively in the treatment and maintenance of patients with duodenal and peptic ulcers and in the treatment of Zollinger-Ellison syndrome (1-3).

Although adverse side effects are generally minor and gastrointestinal in nature (4,5), there are reports of more serious reactions including CNS adverse effects (6,7). Furthermore, the action of cimetidine has been shown to be dose dependent (8). For these reasons, the analysis of cimetidine in biological fluids, particularly blood and plasma is of clinical interest.

Several high performance liquid chromatography (HPLC) analyses of cimetidine have appeared in the literature (9-15) utilizing both normal phase and reversed phase separations. Reversed phase separations have been performed with a variety of mobile phases, including highly basic solutions with high percentages of organic modifier (9) and acidic solutions with low modifier concentrations (14). While the use of mobile phase of pH 8 or above easily leads to column degradation via silica dissolution, the acidic mobile phases used offer limited range of capacity factors due to the low retention of cimetidine under acidic conditions. Sample preparation procedures reported have required double or triple extractions (9,10) or at least single extraction steps followed by concentration steps (13-15).

This paper describes a reversed phase separation using a mobile phase containing ion pair reagent offering improved control over capacity factors. The clean-up procedure utilizes Waters Sep-pak C-18 cartridges for a rapid clean-up and concentration.

EXPERIMENTAL

Reagents and Samples

Analytical grade methanol was obtained from E. Merck (Darmstadt, GFR). HPLC-grade water was prepared using a Milli Q water system (Waters Millipore, Millford, MA, USA). Potassium phosphate and sodium dodecyl sulphate (SDS) were obtained from Baker (Deventer, The Netherlands). Cimetidine (CMT) was obtained from Smith, Kline and French Labs (Philadelphia, PA, USA) and procaine hydrochloride from Sigma (St. Louis, MO, USA).

Blood and urine samples were taken from a duodenal ulcer patient receiving cimetidine in 400 mg doses at 12 hour intervals. Blood samples were prevented from coagulating by the addition of NaEDTA. All samples were stored in a refrigerator at $5^{o}C$.

HPLC Apparatus

The HPLC system consisted of a Gilson (Villers le Bel, France) Model 302 single piston pump with a Kontron (Zürich, Switzerland) Model 812 pulse dampener. A Valco Instruments (Houston, TX, USA) high pressure six port model CV UH Pa valve with a 20 µl loop was used for sample introduction. A Pye Unicam (Cambridge, UK) Model LC 3 variable wavelength UV detector was used as a detector at 228 nm. A Kipp and Zonen (Delft, The Netherlands) BD-8 multi range recorder was used to record detector out-put. The HPLC column consisted of a 10 cm x 4.6 cartridge column system packed with 10 µm C-18 material from Brownlee Labs (Clara, CA, USA) Sep-pak^{R} C-18 cartridges were obtained from Waters.

METHODS

The HPLC mobile phases consisted of mixtures of methanol and 1 mM sodium dodecyl sulphate in a 10 mM phosphate buffer of pH 3.0. Routine analyses were performed using 35% methanol. The use of mixtures of methanol and 0.1% solution of ammonium hydroxide was also investigated. The mobile phases were filtered using a 0.5 µm Waters Millipore solvent filtration system and degassed under vacuum in an ultrasonic bath.

Samples were prepared as follows. To 1-5 ml of blood or urine was added one drop of ammonium hydroxide giving the sample a pH of approximately 10. An appropriate amount of the internal standard procaine (1 mg/ml for urine and 20 µg/ml for blood) was also added. The sample was passed through a Sep-pak C-18 cartridge which had previously been washed with a dilute ammonium hydroxide solution. The cartridge was then washed with 10 ml of a dilute solution of ammonium hydrox-

ide (pH 10) and 10 ml of the 1 mM sodium dodecyl sulphate in 10 mM phosphate buffer at pH 3.0. Following the washes, the cimetidine was eluted from the cartridges with 1 ml methanol. For the analysis of blood, this eluent was concentrated to 50 µl under a stream of nitrogen. In the case of urine samples this concentration step was not required.

RESULTS AND DISCUSSION

Initial tests were run using a mobile phase consisting of mixtures of methanol and 0.1% aqueous solution of ammonium hydroxide. Figure 1 shows a plot of log k' vs % methanol. This system provided adequate retention, but rapid degradation of the analytical column resulted due to silica dissolution. Although this effect could be circumvented via the use of silica presaturation of mobile phases, we preferred to develop a simpler system using ion pair chromatography. Using sodium dodecyl sulphate to form ion pairs with the protonated amino groups in cimetidine, retention behaviour similar

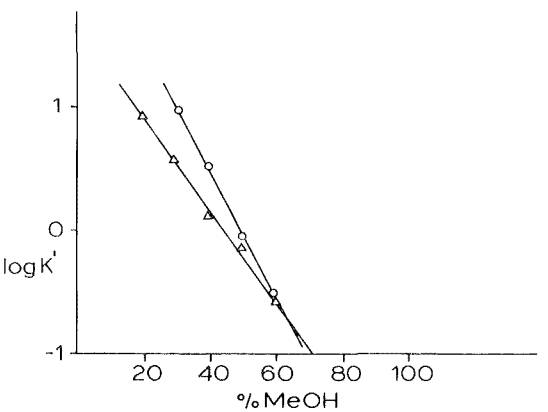

Figure 1. Retention behaviour of Cimetidine on reversed phase (C-18) material. Δ; CH_3OH: 1% NH_3 in H_2O, pH 10, O; CH_3OH: 20 mM KH_2PO_4, 1 mM SDS in H_2O, pH 3.0.

to the ammonium hydroxide mobile phase was obtained. This is illustrated also in Figure 1. The amount of methanol added to the sodium dodecyl sulphate solution was dictated in both blood and urine samples by the resolution of cimetidine from the internal standard, procaine. At 35% methanol, the resolution of these two compounds was found to be 1.3 with cimetidine eluting in 9 ml at a flow-rate of 2 ml/min.

The clean-up procedure was found to be adequate for both blood and urine samples. Figure 2 shows a plot of the concentration of ci-

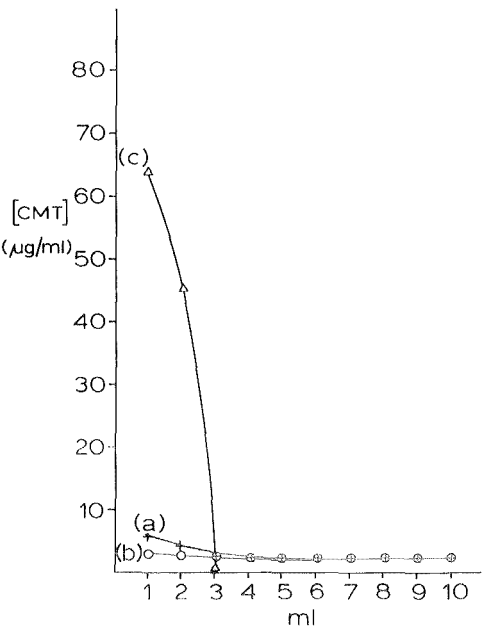

Figure 2. Concentration and elution of cimetidine on Sep-pak C-18 cartridges. (a) loading sample with equilibration if Sep--pak with 0.1% NH_3 in water, (b) loading sample without equilibration with 0.1% NH_3 in water, (c) elution of sample (10 ml loaded) by methanol. Original sample concentration: 20 µg/ml.

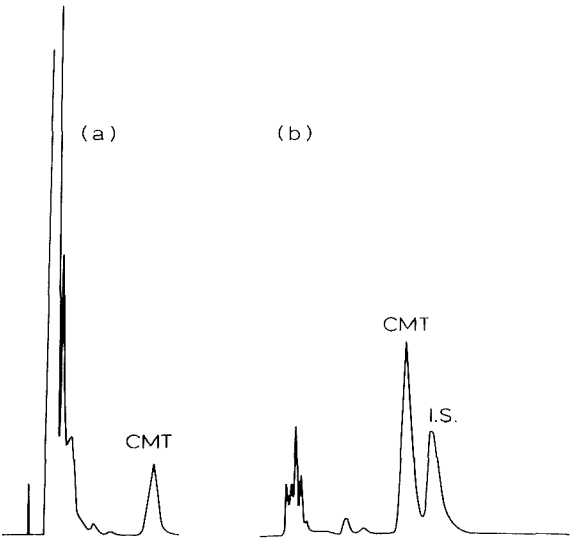

Figure 3. Analysis of cimetidine (CMT) in urine: Column: 10 cm x 4.6 mm C-18. Mobile phase: 35:65 CH_3OH: 20 mM KH_2PO_4 1 mM SDS in H_2O, pH 3.0; Flow-rate 2 ml/min; Detection UV at 228 nm; 20 µl loop injection. a) Raw urine, without Sep-pak clean--up: No internal standard added b) Urine with Sep-pak clean--up. Original sample volume: 50 ml with 1 mg/ml procaine internal standard (1.5.) added.

metidine eluting from the Sep-pak as a function of the volume of sample (curve A) or eluent (curve C) passed through the Sep-pak. Curve A represents the cimetidine eluting from the Sep-pak during sample loading. A relatively constant low level of cimetidine elutes throughout the loading step. The mechanism of this phenomenon is not known; however, the use of the internal standard which behaves similarly negates any adverse effect on the quantitation. Curve B represents the elution of cimetidine during sample loading when the Sep-pak is not initially washed with a basic solution. A larger concentration of cimetidine elutes in the first 2 ml of sample due to a lack of equilibration. Curve C shows the elution of the analyte after loading 10 ml of sample from the Sep-pak with methanol. As can be seen all the cimetidine is eluted in 3 ml.

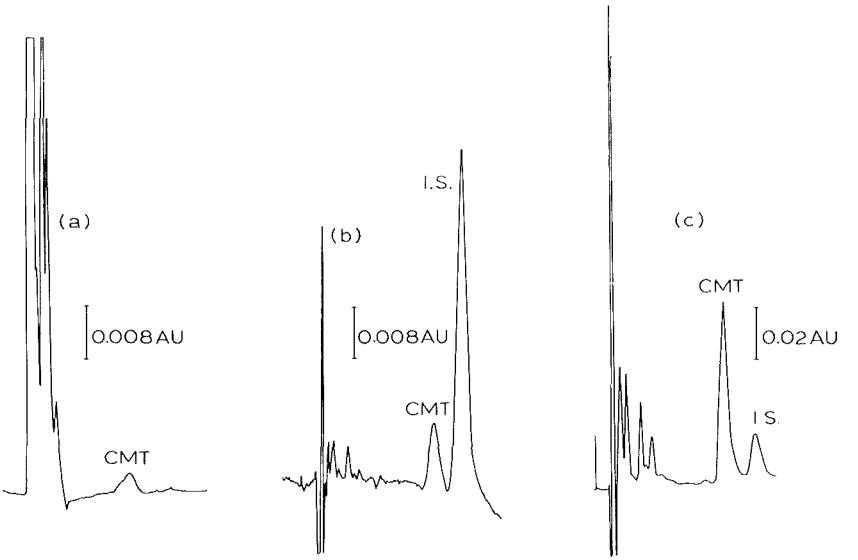

Figure 4. Analysis of cimetidine in blood. Conditions as in figure 3. a) Untreated whole blood. No internal standard added. b) whole blood after Sep-pak clean-up. Original sample: 1 ml blood + 20 µg/ml procaine internal standard. c) Whole blood after Sep-pak clean-up and concentration of eluent to 50 µl. Original sample as in (b).

Figure 3 shows the analysis of cimetidine in urine with and without the clean-up procedure. No procaine is present in the untreated samples. As can be seen, in the case of urine, the clean-up is not absolutely necessary since the analyte and internal standard are well resolved from the rest of the matrix. Figures 4 a, b and c shows the analysis of blood; without clean-up, with clean-up and with clean-up and concentration, respectively. The amount of internal standard added was less in the concentrated sample to keep the procaine peak on scale. Again it can be seen that, in general, the major components in the sample are well resolved from the analyte, but the concentration in the blood is approximately 1000 times less than in urine. Even with the clean-up on a Sep-pak cartridge which involves a concentration of approximately five fold, the cimetidine is

present at a low level (Fig. 4b). In the routine method further concentration by evaporation of the solvent was used and the cimetidine was easily detected at therapeutic levels (Fig. 4c). Lower detection limits could also be achieved by using larger injection volumes with little loss in resolution.

Using ultraviolet absorbance detection at 228 nm, a minimum detectable quantity of 20 ng was obtained. Data from a calibration plot yielded a correlation coefficient of 0.9988 over three decades of concentration.

The concentration of cimetidine in blood following administration of the drug was examined as a function of time. This is shown graphically in Figure 5. The samples were taken at 2 hour intervals for 10 hours following a 400 mg dose of cimetidine. Although the blood concentration reaches a maximum of approximately 3.3 µg/ml 6 hours after dosage, there appears to be a basal concentration of at least 1.5 µg /ml maintained by the dosage schedule. These analyses were preformed in duplicate and the resulting values were within 2.1% of each other.

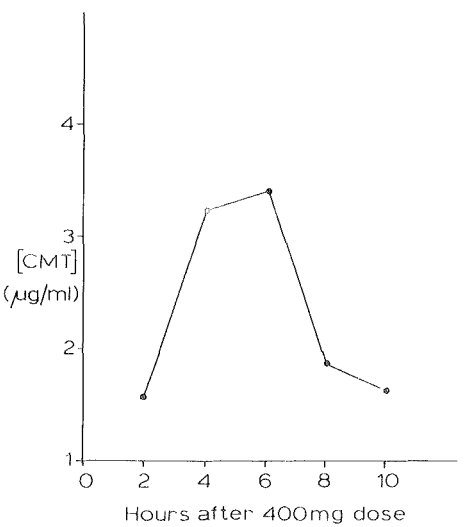

Figure 5. Blood concentration of cimetidine following 400 mg dose. 5 ml blood samples taken at 2 hour intervals.

CONCLUSION

The method described offers a rapid, easy and sensitive technique for the analysis of cimetidine in biological fluids. The use of ion pair chromatography has significant advantages over previously reported approaches to this analysis, in that it avoids the use of highly basic mobile phases which lead to short column lifetimes and offers greater control over the retention than the use of acidic buffered mobile phase. The clean-up technique described has the advantages of speed and simplicity. It has been shown to be useful for both blood and urine samples. According to Larsen et al. (9) cimetidine undergoes metabolic conversion in blood (but not in plasma) to the sulphoxide, and it may be advantageous to assay cimetidine in plasma rather than whole blood. The technique described here should be applicable for such samples as well.

Subsequent work is under way to place the analysis on-line in an automated column switching system which should supply rapid automated clean-up and analysis of cimetidine in plasma and urine.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 5(12), 2423-2434 (1982)

LIQUID CHROMATOGRAPHIC ANALYSIS OF TRYPTOPHAN AND SEROTONIN METABOLITES. COMPARISON OF UV, ELECTROCHEMICAL AND SPECTROFLUORIMETRIC DETECTION.

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ABSTRACT

Tryptophan and five of its indolic metabolites have been separated by reversed-phase high performance liquid chromatography. The isocratic chromatographic system consisted of a column (20 x 0.46 cm i.d.) packed with RSil C18 HL5 and a methanol/$NaClO_4$ 0.2 M -$HClO_4$ (pH 1.4) mobile phase.

Three detection methods were tested : UV detection, fluorometric detection and electrochemical detection.

The limits of detection were found to be 4 picomoles (serotonin, 5 HTrp) and 20 pmoles (Trp, 5 HIAA, IAA , N-acetyl Trp) in the case of UV detection ; 1 pmole (serotonin), 4 pmoles (5H-Trp) and 10 pmoles (5 HIAA, Trp, IAA, N-acetyl Trp) for fluorometric detection ; and 1 pmole for electrochemical detection.

Analysis of human plasma were carried out using the above three detection methods to compare their relative specificity.

INTRODUCTION

Tryptophan is metabolized via two major pathways, the kynurenine pathway and the indolic pathway. The second one, quantitatively

less important, presents however a great physiological importance, mainly because it is the precursor of serotonin (5-hydroxy tryptamin), fundamental brain neurotransmitter.

Serotonin is predominantly metabolized in 5-hydroxy indolacetic acid, an urinary metabolite, variations of which are dependent on those of serotonin.

Abnormal metabolism of tryptophan has been associated with a number of disease states in man, including Hartnup disease, Down's syndrome, schizophrenia, depression (1-6)... Aberrations of tryptophan metabolism are also associated with some cancerous tumors.

These metabolic disorders result in modification of normal concentrations of tryptophan metabolites in an organism ; therefore it seemed of interest to possess a sensitive, reliable, specific and rapid technique for measuring tryptophan (Trp), serotonin, 5-hydroxy indolacetic acid (5 HIAA), and other indol derivatives such as 5-hydroxy tryptophan (5-HTrp), indolacetic acid (IAA) and N-acetyl tryptophan (N-acetyl Trp).

The chromatographic technique generally used for aminoacid analysis is ion exchange (7-9), a time-consuming method requiring relatively large sample volumes.

HORVATH (10-12) has demonstrated the interest of aqueous eluents used simultaneously with nonpolar stationary phases, for the separation of small polar molecules. This method, previously used by different authors (14-17), was adapted and optimized for the separtion of Trp and its indolic metabolites.

Reported in this paper are the separation of these compounds using reversed-phase HPLC, and the comparative results of three detection methods : UV, fluorometric and electrochemical detection.

EXPERIMENTAL

. Apparatus

The chromatographic equipment consisted of a Chromatem 380 pump (Touzart & Matignon, France), a Rheodyne Model 71-20 sample injection valve, a Pye Unicam variable wavelength UV monitor (Philips, France), a JY 3 fluorimeter (Jobin Yvon, France), a DELC "Thin layer" electrochemical detector (Tacussel, Lyon France) and a PRG 5 polarograph (Tacussel) as potentiostat. The cell of the electrochemical detector, adjusted to a volume of 8 µl, was equiped with a glassy carbon working electrode, the reference potential being supplied by a silver-silver chloride electrode. The cell was used in the d.c. mode throughout.

Peak surfaces were integrated with an Autolab System I integrator (Spectra Physics, USA).

The signal output of the detectors was displayed on a 0470 L Linseis recorder and an EPL Tacussel recorder (in the case of electrochemical detection).

. Chromatographic system

A 20 x 0.46 cm i.d. column was packed (13) with RSIL C18 HL5 (Interchim, Montluçon, France), particle size 5 µm).

The solvents used for the slurry and the packing procedure were respectively 1-butanol and methanol. The composition of the mo-

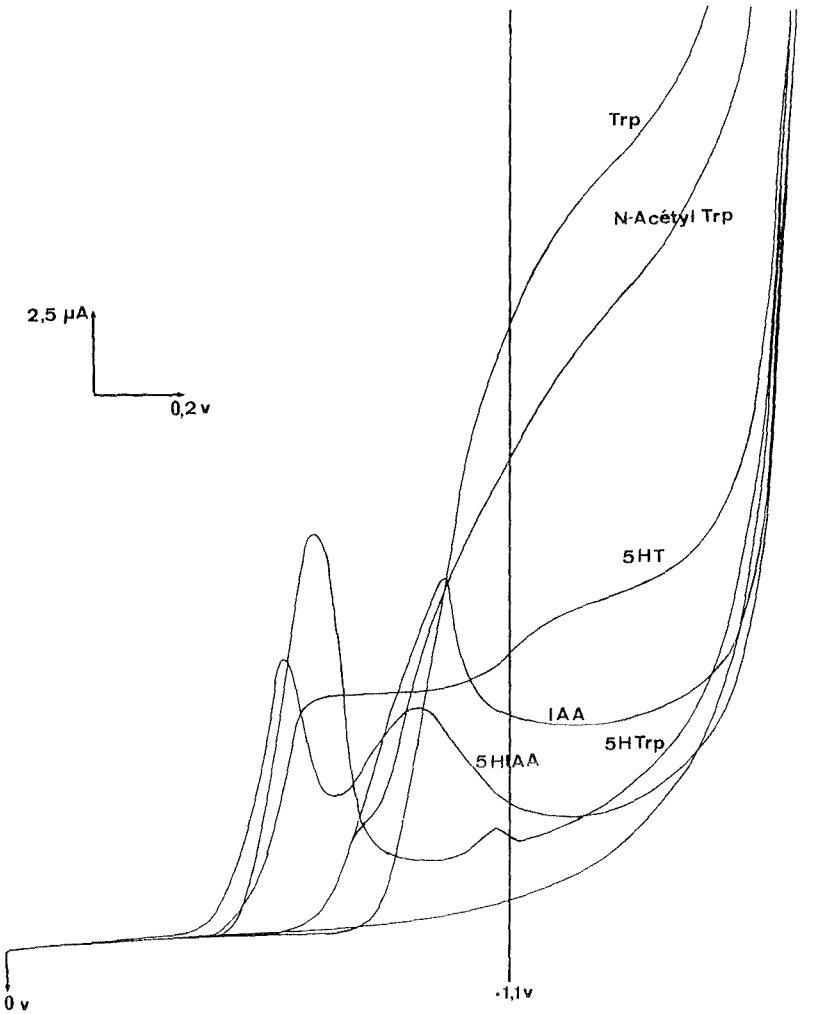

Figure 1 - Intensity-potential curves of tryptophan and indolic metabolites (concentrations 2.10^{-4}M). Glassy carbon rotating disc electrode, 3 mm diameter 3000 r.p.m.

bile phase consisted of methanol and $NaClO_4$ 0.2 M - $HClO_4$, pH 1.4 (30:70). The mixture was degassed for 5 minutes in an ultrasonic bath before use.

. Reagents

Standard solutions were prepared by dissolving tryptophan and metabolites (SERVA, Analytical grade) in the mobile phase.

Methanol was purchased from MERCK (Chromatographic grade), 1-butanol, $NaClO_4$ and $HClO_4$ were purchased from PROLABO (NORMAPUR guaranteed reagent).

Healthy human blood was collected in heparinized tubes and centrifugated. The plasma was defecated with a 100 g/l sulfosalycilic acid solution (4 vol. plasma/1 vol. acid), then stored at -25°C prior to analysis.

. Procedure

The compounds under study were detected at 280 nm, which corresponds to the maximum absorbance of tryptophan.

The excitation and fluorescence wavelength used in fluorometric detection were respectively 300 nm and 335 nm.

The oxidation potential used in electrochemical detection was +1.1 V. It was determined by plotting the current vs potential curves for each solute (fig.1).

The detection limits ($\frac{\text{signal}}{\text{background noise}} = 5$) were determined using standard solutions of compounds.

. Results and discussion

The reversed-phase HPLC separation of a synthetic mixture of tryptophan and its metabolites is shown in figure 2.

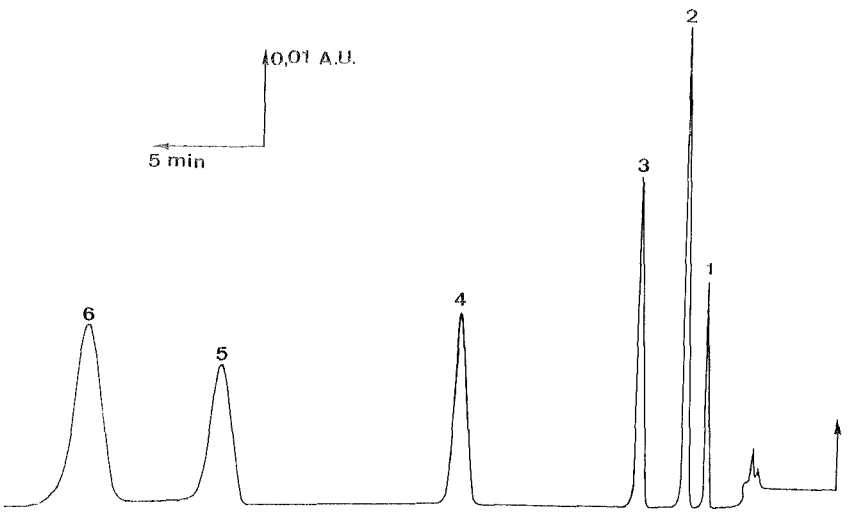

Figure 2 - Chromatographic separation of tryptophan and its indolic metabolites. Column : RSil C18 HL5 (20 x 0.46 cm i.d.). Mobile phase : methanol/$NaClO_4$ 0.2 M-$HClO_4$ pH 1.4 (30:70). UV detection 280 nm. Flow rate 0.6 ml/min. Peaks : (1) serotonin, (2) 5 HTrp ; (3) 5 HIAA , (4) Trp ; (5) N-acetyl Trp ; (6) IAA.

The phenolic compounds (serotonin, 5-HTrp, 5 HIAA) are eluted more rapidly than tryptophan and other metabolites, a fact which shows an important decrease in the stationary phase affinity due to phenolic ring.

The linearity of detectors was demonstrated in the 5-100 µM concentration range, by measurement of the peak surface.

Figures 3, 4 and 5 show the injection of standard solutions of 4 and 10 pmoles in the three detection modes, making it possi-

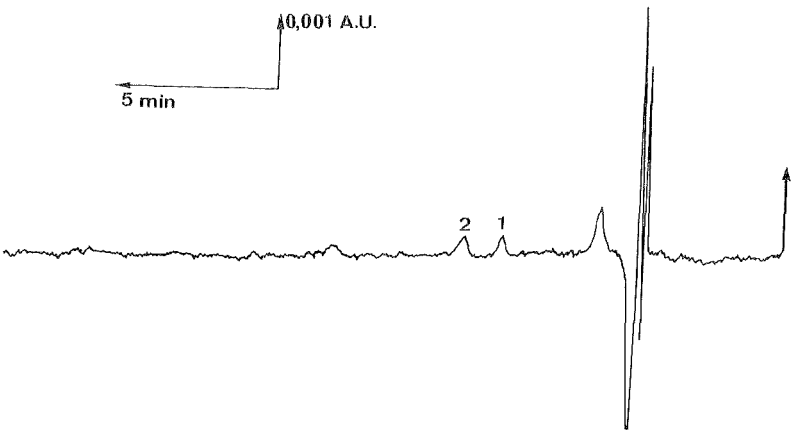

Figure 3 - UV detection wavelength 280 nm. Injection of 4 pmoles. column : Rsil C18 HL 5 (20 x 0.46 cm i.d.) , mobile phase : methanol/$NaClO_4$ 0.2 M - $HClO_4$ pH 1.4 (20:80). Flow rate : 0.6 ml/mn. Peaks : (1) serotonin ; (2) 5-HTrp.

ble to demonstrate their relative sensitivity. Due to a non reproducibility in the different batches of RSil C 18, the mobile phase composition had to be modified from 20 to 30 % methanol.

Figure 5 shows the improvement in electrochemical detection sensitivity which became 10 times greater due to a decrease in the cell joint thickness from 500 µm to 200 µm.

200 µm thickness (8 µl) is, in this case, the best compromise between sensitivity and baseline stability.

Detection limits in UV detection are 4 pmoles for serotonin and 5-HTrp ; and 20 pmoles for Trp, 5HIAA, IAA and N-acteyl Trp.

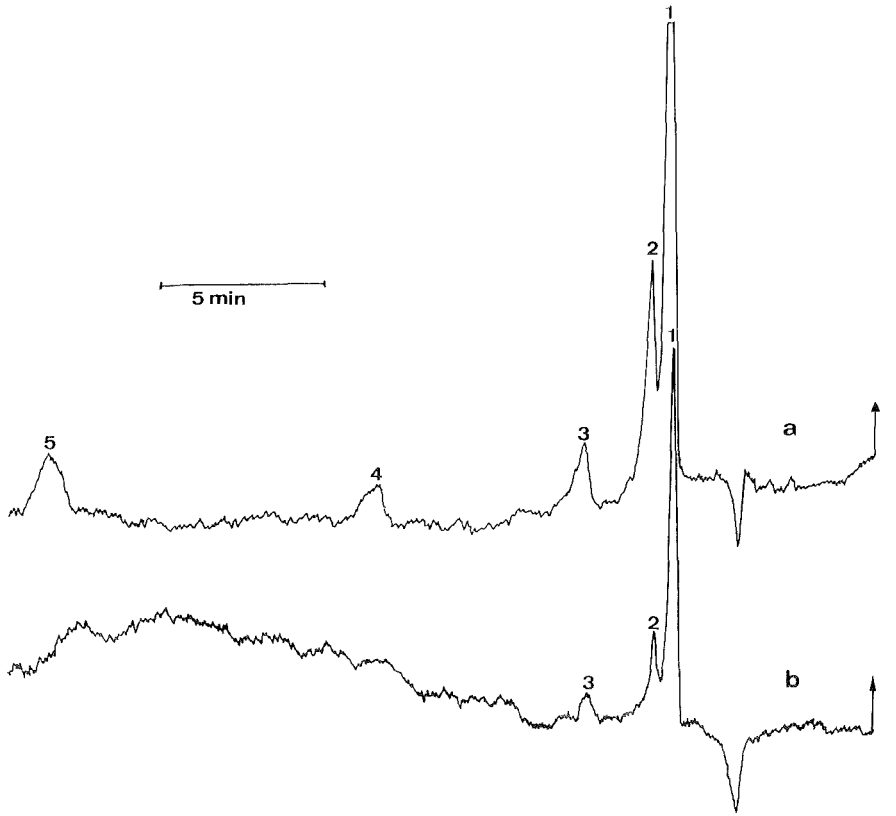

Figure 4 - Fluorometric detection, λ_{exc} = 300 nm ; λ_{fluo} = 335 nm (gain x 1000). Injection of (a) 10 pmoles , (b) 4 pmoles. Column : RSil C18 HL5 (20 x 0.46 cm i.d.) ; mobile phase : méthanol/$NaClO_4$ 0.2 M - $HClO_4$ pH 1.4 (30 : 70). Flow rate : 0.6 ml/min. Peaks : (1) serotonin ; (2) 5 HTrp ; (3) 5 HIAA ; (4) Trp ; (5) N-acetylTrp.

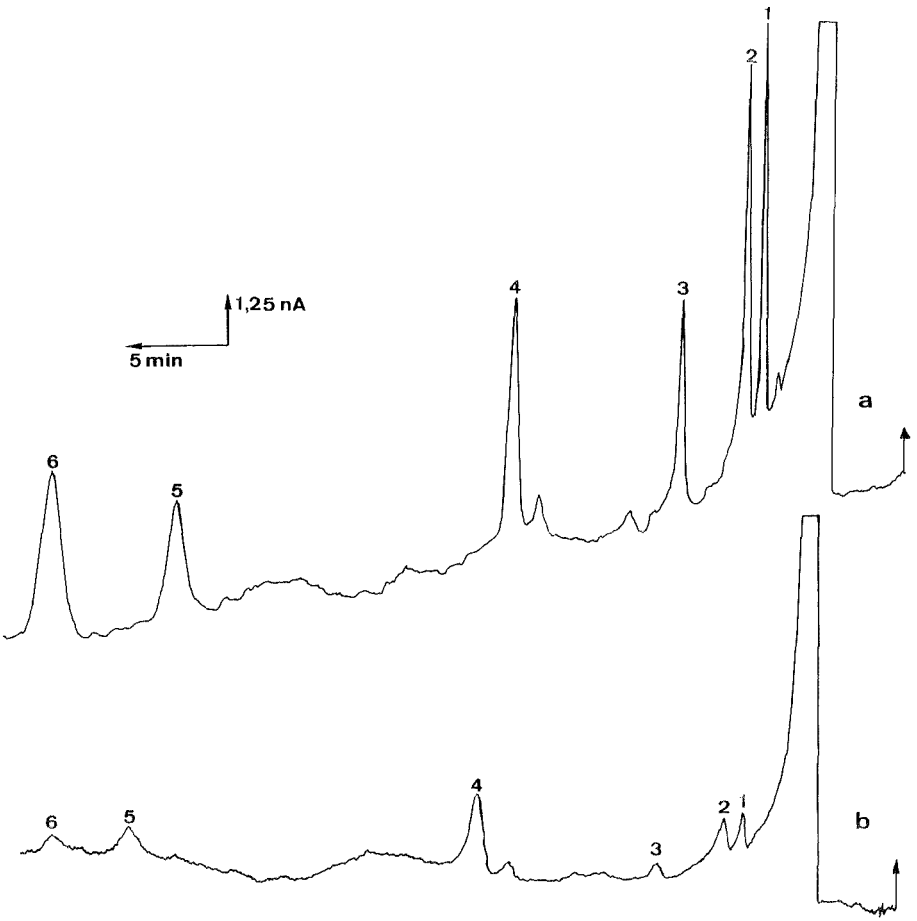

Figure 5 - Electrochemical detection, detector operating potential + 1,1 V.Electrochemical cell thickness : (a) 200 µm ; (b) 500 µm. Injection of 4 pmoles. Column : RSil C 18 HL 5 (20 x 0.46 cm i.d.); mobile phase : methanol/ $NaClO_4$ 0.2 M - $HClO_4$ pH 1.4 (25:75). Flow rate : 0.6 ml/min. Peaks : (1) serotonin ; (2) 5 HTrp ; (3) 5 HIAA ; (4) Trp ; (5) N-acetylTrp ; (6) IAA.

In fluorometric measurements the detection limits have been determined to be 1 pmole for serotonin ; 4 pmoles for 5-HTrp ; and 10 pmoles for 5 HIAA, Trp, IAA and N-acetyl Trp.

Electrochemical detection appears to be the most sensitive detection mode, the detection limits being 1 pmole for all the six compounds studied.

Fluorometric and electrochemical detection modes are particularly specific in the cases mentioned above, because most biological compounds, like other aminoacids, are not naturally fluorescent nor electrochemically active, and therefore will not interfere with either of these detection modes.

It has been possible to compare the relative importance of interferences in each mode of detection when analyzing tryptophan and its metabolites in biological fluids.

Figure 6 shows the chromatogram obtained after injection of 4 µl of blood plasma. The three detectors were monitored at their maximum sensitivity.

The UV and fluorometric detection sensitivity are comparable for the determination of tryptophan (concentration 40 µM). Interferences are less important in fluorometric detection. Nevertheless the peaks corresponding to hydroxylated indolic metabolites are not separated from the interference peak.

The major advantage of UV detection is its relatively easy use. Its use is particularly indicated in determining tryptophan and compounds in the case of higher concentrated samples.

Figure 6 c confirms that electrochemical detection provides the best sensitivity and specificity among the three methods studied.

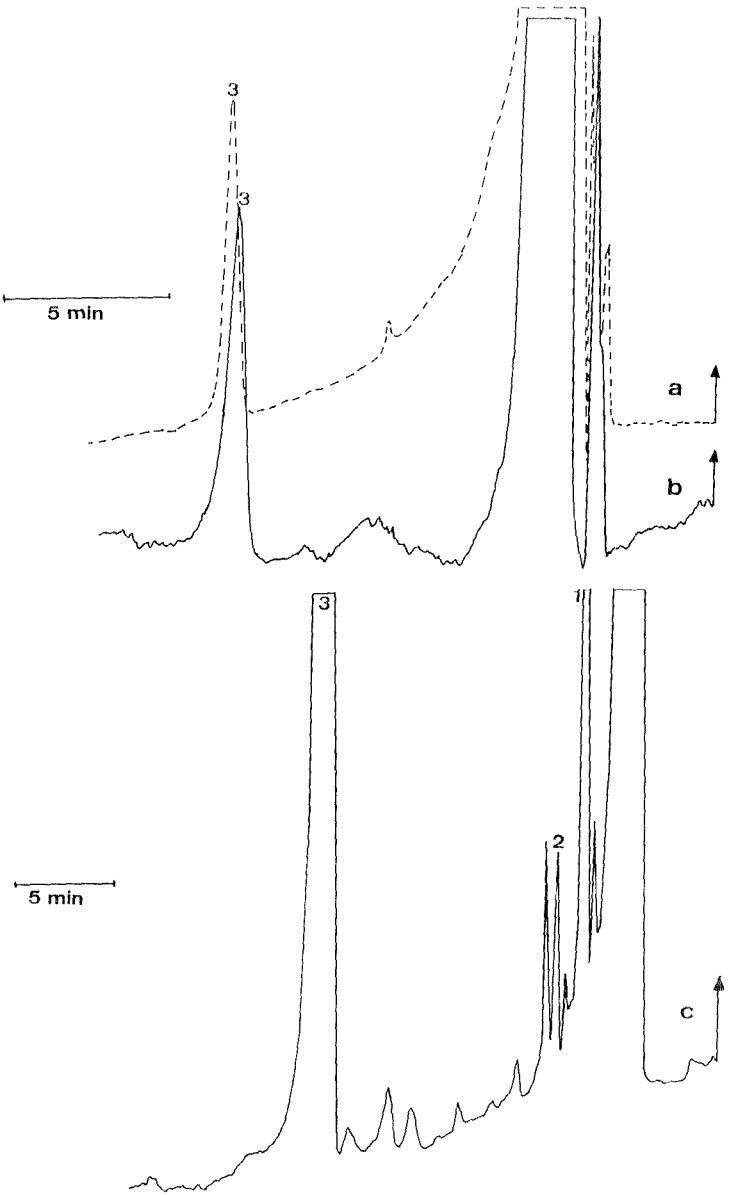

Figure 6 - Chromatogram of a 4 µl serum using (a) UV detection , (b) fluorometric detection , (c) electrochemical detection. Peaks : (1) serotonin ; (2) 5-HTrp ; (3) Trp.

With this technique the peak of interferences is comparatively the lest important. Electrochemical detection is, among the three methods tested ,the only one allowing the detection of the peaks of serotonin and 5-HTrp , which are not overlapped by the interference peak.

Therefore, electrochemical detection is,in the present case, the most interesting detection method, mainly because of its detection limit (1 pmole) and its specificity.

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SIMULTANEOUS DETERMINATION OF DOPAMINE, NOREPINEPHRINE, TYRAMINE AND OCTOPAMINE BY REVERSE-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

A method is described for the simultaneous separation and estimation of the catecholamines, norepinephrine and dopamine and their monohydroxy-equivalents, octopamine and tyramine. The method employs high-performance liquid chromatographic separation of the compounds on a C18 reverse-phase column with a mobile phase containing methanol as the organic modifier, octane sulphonate as an ion-pair reagent and acetic acid/ammonium hydroxide buffer. The influences of electrode potential and solvent pH on detector response were studied, and the optimal conditions identified as detector potential of 0.95 volts and pH 6.0. The technique of post-column mixing was introduced to provide optimal pH conditions for detector response without the constraint of on-column oxidation of catecholamines. The effects of buffer ionic strength on retention factors and detector response were also investigated and, on the basis of the results obtained, the optimal buffer strength was identified as 0.08-0.09 molar. The described procedure can be used for simultaneous estimation of catecholamines and monohydroxyphenolamines at concentrations between 200-5000 pg.

INTRODUCTION

The monohydroxyphenolamines, tyramine and octopamine have been implicated in a number of nervous disorders including migraine, epilepsy, Parkinsonism, schizophrenia and hepatic encephalopathy (1), and octopamine has been identified also as a putative neurotransmitter in invertebrates (2). The obvious biomedical importance of monohydroxyphenolamines has resulted in the development of several analytical procedures to determine concentrations in nervous tissue. These diverse procedures include the use of radioenzymatic methods (3), gas chromatography mass spectrometry (4), fluorometric techniques (5), and reverse-phase liquid chromatography with detection of derivatised compounds by fluorescence (6) or using amperometric detection of natural compounds (7,8). The relative merits and constraints of the various analytical procedures have been discussed (4,9) and it is apparent that the utility of any technique depends upon the analytical requirements of the particular experimental procedure. One shortcoming in the existing arsenal of analytical techniques for monohydroxyphenolamines is the lack of a procedure that permits rapid, estimation of monohydroxyphenolamines and the equivalent dihydroxy compounds in a single sample.

The present study examines high-performance liquid chromatographic separation of the catecholamines dopamine and norepinephrine and the monohydroxy-equivalents tyramine and

octopamine using an isocratic aqueous mobile phase flowing over a bonded octadecylsilane solid phase with electrochemical detection of the eluate. The report describes the effects of electrode potential, solvent pH and buffer ionic strength on the separation and detection of the standard compounds and defines optimal chromatographic conditions for estimation of these amines in a single sample.

MATERIALS AND METHODS

Chemicals

Norepinephrine bitartrate, Dopamine HCl and Tyramine HCl were purchased from Calbiochem (La Jolla, Ca., U.S.A.). Octopamine HCl was obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) and other chemicals, except when otherwise indicated, were also obtained from this source and were of reagent grade. Stock solutions of standards were prepared in 0.1 M $HClO_4$, 5.0 mM sodium bisulfite at a concentration of 40 µg/ml and stored at 4°C. Working standard solutions were prepared freshly each week by diluting stock solutions in methanol to give a final concentration of 200 ng/ml for each compound.

Equipment

Chromatographic separations were performed on a 15 cm x 3 mm I.D. stainless steel column packed with 5 µm Ultrasphere RP-18

ion pair material (Beckman). The mobile phase comprised acetic acid/ammonium hydroxide buffer containing 0.1 mM sodium EDTA, 2.5 mM sodium octane sulphonic acid (Aldrich Chem., Milwaukee, Wisc., U.S.A.; Bioanalytical Systems, Lafayette, Ind., U.S.A.; or Helix Associates, Newark, Del., U.S.A.) and 20% methanol (HPLC grade, Caledon Laboratories, Ont.). Prior to the addition of methanol, the buffer was filtered through a 0.22 µm millipore filter. The mobile phase was helium degassed and pumped at 2.0 ml/min by a Spectra Physics Model 740B pump through a Waters WISP 710A autosampler onto the analytical column. A Brownlee MPLC RP-18 SPHERI-5 guard column was installed between the injector and analytical column. Chromatographic separations were performed at ambient temperatures.

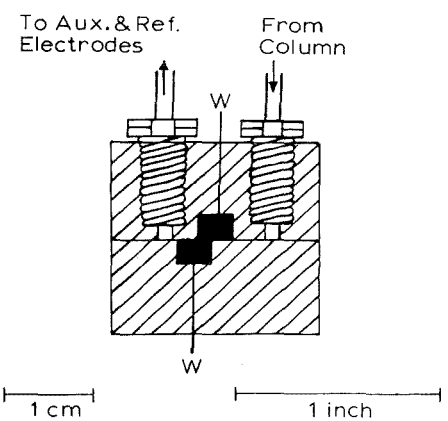

Figure 1. Modified thin-layer amperometric detector showing position of working electrodes.

Detection was accomplished electrochemically by means of a modified Bioanalytical Systems (Layfayette, Ind., U.S.A.) Model TL-5 glassy carbon detector cube and a Model LC-3 potentiostat. The electrochemical potential was adjusted to desired potentials against a silver-silver chloride reference electrode. The detector cube was modified to include a second glassy carbon working electrode surface as illustrated in Figure 1. This modification greatly increased the signal response without any appreciable increase in background current.

Peak height and area calculations of individual peaks were accomplished with the aid of a Spectra Physics Model 4100 computing integrator.

Experimental

Determination of optimal detector potential was achieved by making repeated injections of standard solutions at increasingly positive electrode potentials. This was continued until a maximum peak height for the component of interest was obtained. The current ratio at any potential was calculated by dividing the current (peak height) by that obtained at the most positive potential. The electrochemical response for monophenolamines at different pH values was determined by adjusting the buffer components, acetic acid and ammonium hydroxide, to a specific pH while maintaining the total ionic strength at approximately 0.06 Molar.

Changes in capacity factor (k') and detector response were analysed by altering the ionic strength of the buffer while maintaining a constant pH at 6.0.

Following the determination of the buffer conditions required to provide optimal response and capacity factors for all sample components, the linearity of the detector response was measured and approximate limits of detection for the sample components calculated.

RESULTS AND DISCUSSION

The estimation of catecholamines by HPLC/EC procedures is widely accepted (10), however the concomitant measurement of monohydroxyphenolamines has not yet been developed and, indeed, the application of amperometric detection to monohydroxyphenolamines separated by HPLC is limited to a few reports (7,8). An essential condition for detection of a compound electrochemically is that the electrode potential should be sufficiently high to effect electro-oxidation of the compound. The effect of electrode potential on the detector response to tyramine and octopamine is illustrated in Fig. 2. It is apparent that octopamine is not detected when the applied potential of the electrochemical cell is less than 0.7 volts whereas a slight response for tyramine is evident at this potential. The observed response for tyramine at detector potential of 0.7 volts appears to be at variance with a previous report which indicates no

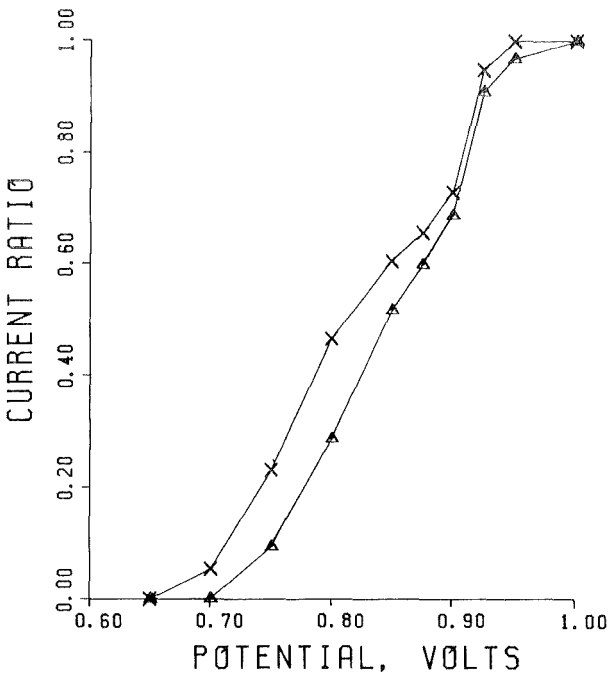

Figure 2. Hydrodynamic voltammograms of tyramine (x) and octopamine (Δ) standards.

detector response for this compound at 0.7 volts (11), although the overall pattern of the hydrodynamic voltammograms in the two studies are very similar. The slight difference in the minimum electrode potential that is required for detector response may be explained by the different pH employed in the two investigations. The pH (6.0) used in the present study facilitates oxidation at the electrode surface and enables octopamine and tyramine to be detected at a lower detector potential than under more acidic conditions. The results presented in Fig. 2 indicate also that

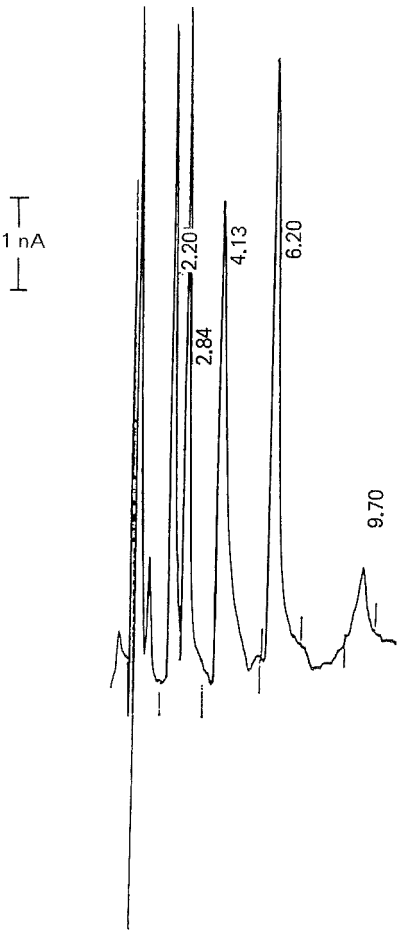

Figure 3. High-performance liquid chromatogram of a standard solution containing 5 ng norepinephrine (RT:2.20), octopamine (RT:2.84), dopamine (RT:4.13), tyramine (RT:6.20). Chromatographic conditions are described in the text.

the maximal detector response for octopamine and tyramine is obtained with an applied potential of 1.0 volts. However, for practical purposes it is convenient to operate the detector potential at 0.95 volts. Reduction of the potential from 1.0 volt to 0.95 volts results in an appreciable decrease in background current and noise, and also eliminates the necessity for frequent cleaning of the detector surface that is apparent at 1.0 volts.

A typical chromatogram for separation of 5 ng samples of dopamine, norepinephrine, tyramine and octopamine with detector potential set at 0.95 volts is shown in Fig. 3. The buffer used to obtain this chromatograph contained 0.04 M acetic acid and 0.04 M ammonium hydroxide and was adjusted to give a final pH of 6.0. The peak appearing at 9.70 minutes is a contaminant associated with sodium octane sulphonic acid and was present in material obtained from several manufacturers.

Monohydroxyphenolamines have a higher oxidation potential than their dihydroxy-equivalents, and this difference is reflected in the effect of pH on detector response to the compounds under investigation. Fig. 4 demonstrates the effect of pH on detector response, as measured by peak height, and indicates increased oxidation of octopamine and tyramine at the electrode surface with increasing pH. The reduced signal for norepinephrine and dopamine at a pH above 5.75 is also apparent and confirms an earlier report by Moyer and Jiang (12). The

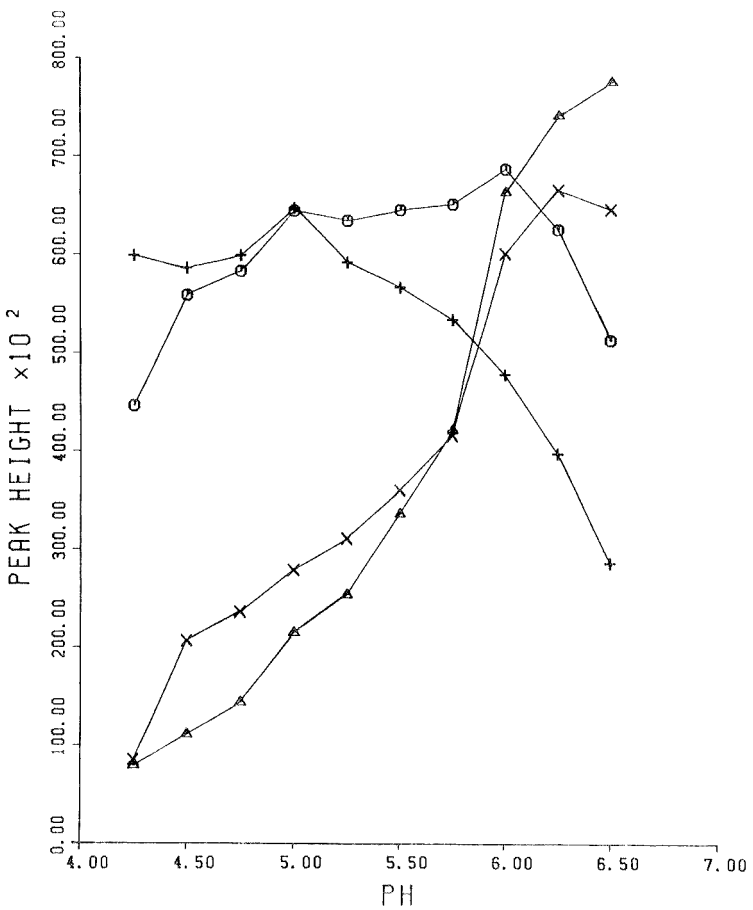

Figure 4. Effect of solvent pH on detector response to 5 ng samples of norepinephrine (O), octopamine (Δ), dopamine (+) and tyramine (x) following separation by HPLC. Coefficients of variation were calculated for the 7-10 determinations that comprise each point and range from 0.445-2.898 (norepinephrine), 1.044-7.329 (octopamine), 0.528-2.371 (dopamine) and 1.082-5.791 (tyramine).

reduction in detector response for catecholamines at higher pHs may be due to auto-oxidation of the compounds prior to their arrival at the detector cell. The data presented in Fig. 3 indicate that the optimal solvent pH at which to obtain an acceptable level of detector response for all compounds included in this study is pH 6.0; however, if only monohydroxyphenolamines are being estimated the solvent pH may be increased above 6.0.

In addition to promoting on-column oxidation of catecholamines the use of more alkaline solvents also increases the amount of detector background current. These constraints can be overcome by the use of post-column mixing, a technique that has previously been described by Kissinger et al. (13). This requires assembly of a three-way T-union between the analytical column and the detector cell. In the present system one inlet port admits a solvent with a higher pH than that of the mobile phase buffer, which enters through the second inlet port following passage through the column. The two solvents mix in a short teflon mixing coil prior to their entry into the detector cell. A pulse-free pumping source for the post-column solvent is provided by an adapted reservoir with flow restrictor attached to a pressurised nitrogen cylinder. The use of post-column mixing permits the pH for chromatography and electrochemical detection to be optimised separately.

The influence of ionic strength on the retention of catechol compounds has been demonstrated (12,14), and the theoretical

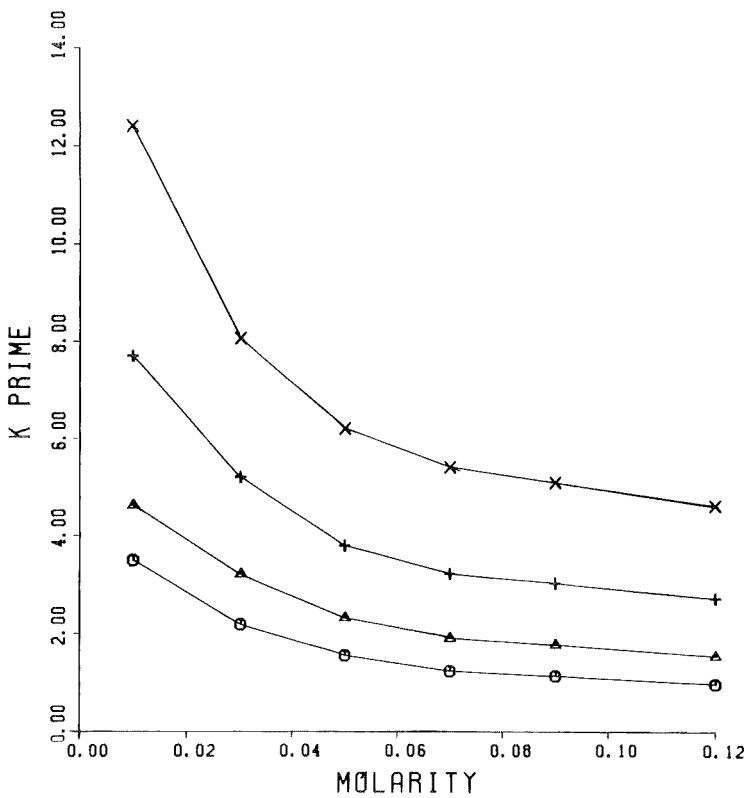

Figure 5. Effect of molarity of solvent buffer on retention of norepinephrine (O), octopamine (Δ), dopamine (+) and tyramine (x) during HPLC separation on RP-C18 column. Coefficients of variation were calculated for the 6-10 determinations that comprise each point and range from 0.441-1.390 (norepinephrine), 1.096-3.986 (octopamine), 0.681-2.840 (dopamine) and 1.004-2.975 (tyramine).

implications of solvophobic interactions have been discussed (15). In the present study, the effect of changes in molarity of acetic acid and ammonium hydroxide on the capacity factor (k') was investigated and the results are illustrated in Fig. 5. It is evident that k' tends to decrease as the molarity of the buffer increases, and this effect may be explained in terms of alterations in hydrophobicity of solute molecules due to changing amounts of acetic acid in solution. Thus, the results presented in Fig. 5 should not be equated with earlier studies on absolute ionic strength (13,15).

Fig. 6 illustrates the effect of molarity on detector response as indicated by measurement of peak height. The data presented in this figure must be considered in association with that of Fig. 5, which demonstrates the greater retention time of the standard compounds at low molarities. The increased retention time results in broadening of peaks and reduced peak height, and also increases the possibility of on-column auto-oxidation at pH 6.0; thus, the observed reduction in detector response at low molarities may be due in part to the change in k'.

On the basis of the data contained in Figs. 5 and 6 the buffer strength selected to achieve optimal chromatographic conditions was between 0.08 and 0.09 molar. At this molarity the capacity factor for each amine is within the optimal limits of between 1 and 6 (Fig. 5) and a high detector response is also

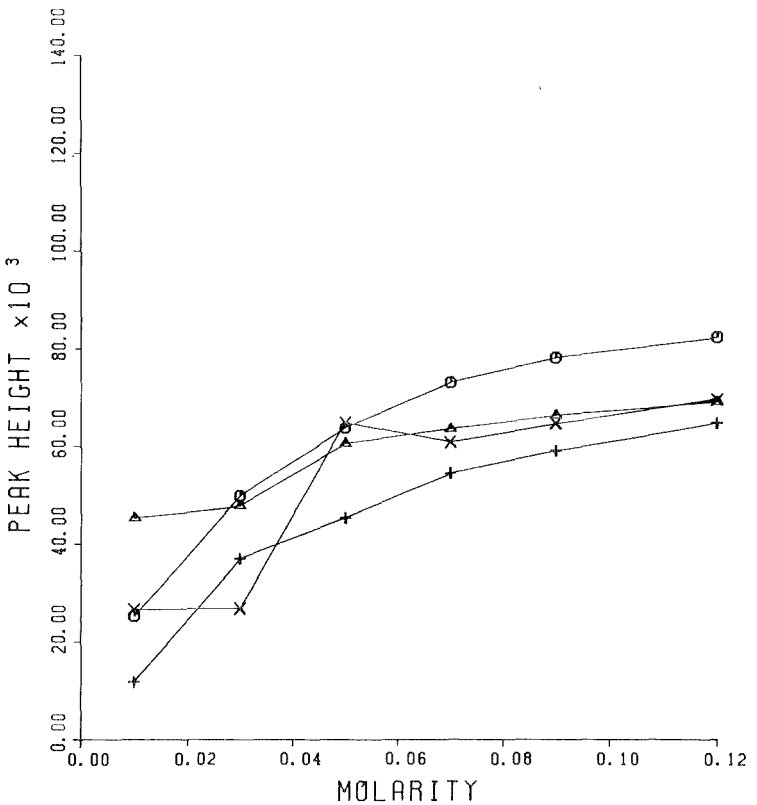

Figure 6. Effect of molarity of solvent buffer on detector response to norepinephrine (O), octopamine (Δ), dopamine (+) and tyramine (x) as measured by peak area following separation by HPLC. Coefficients of variation were calculated for the 7-10 determinations that comprise each point and range from 1.073-1.884 (norepinephrine), 2.149-4.615 (octopamine), 2.479-5.334 (dopamine) and 1.246-6.295 (tyramine).

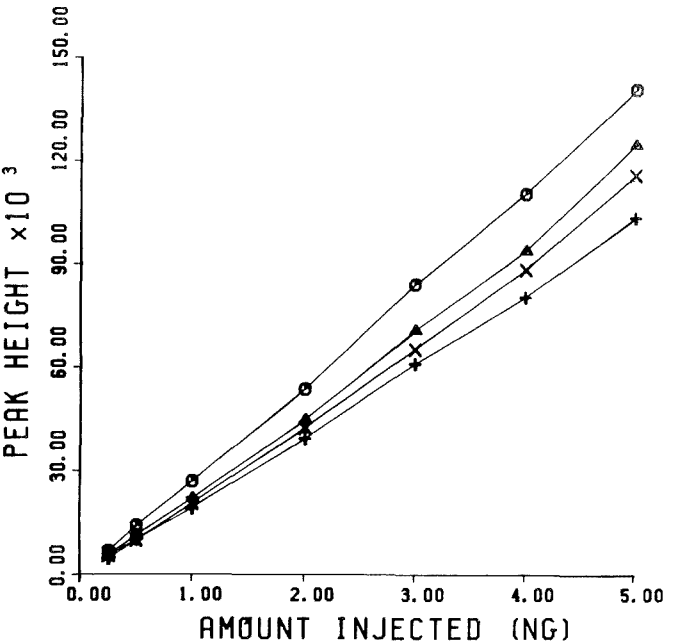

Figure 7. Linearity of estimation of norepinephrine (O), octopamine (Δ), dopamine (+) and tyramine (x) by HPLC with electrochemical detection. Linear regression analyses are presented in Table 1.

obtained (Figs. 6). The detector response continues to increase at molarities above 0.1 molar, but associated with the increased response is an excessive increase in background current.

Fig. 7 demonstrates the linearity of detector response to the four test amines using concentrations ranging from 250 to 5000 picograms. Linear regression analyses of these data are given in Table 1 and confirm that the chromatographic procedure described in this report permits accurate and simultaneous

TABLE 1

Linear regression analysis of effect of amine concentration on electrochemical detection following separation by high-performance liquid chromatography.

COMPOUND	SLOPE ± S.E.	Y-INTERCEPT ± S.E.	CORRELATION COEFFICIENT (R SQUARE)	APPROXIMATE DETECTION LIMIT (PG)*
Norepinephrine	2.826 ± 0.025	-0.069 ± 0.065	.999	131
Octopamine	2.469 ± 0.052	-0.172 ± 0.136	.997	191
Dopamine	2.074 ± 0.023	-0.086 ± 0.059	.999	186
Tyramine	2.306 ± 0.043	-0.155 ± 0.112	.998	197

*limit established with signal:noise ratio of 4:1.

determination of catecholamines and monohydroxyphenolamines at concentrations below 200 picograms. Indeed, as the present experiments were conducted with detector sensitivity at only 5 nA full scale, the potential for even greater sensitivity is considerable. The application of this procedure to extracted biological samples will be reported in a subsequent publication.

ACKNOWLEDGEMENTS

Supported by grants from the Natural Sciences and Engineering Research Council of Canada to RGHD.

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LC NEWS

LIQUID CHROMATOGRAPH/MASS SPECTROMETER INTERFACE continuously concentrates the effluent from a conventional liquid chromatograph and delivers the concentrated solution into the mass spectrometer. Combination with a mass spectrometer/data system provides capability for analysis of complex and intractable biological, environmental, and petrochemical samples. Extranuclear Laboratories, Inc., JLC/82/12, P. O. Box 11512, Pittsburgh, PA, 15238, USA.

PROTEIN SEQUENCING SOLVENTS eliminate solvent impurities that contribute to losses in PTH amino acid derivatives during protein sequencing even at the picomole level. Each solvent undergoes recovery testing using a representative mixture of 16 different PTH amino acid derivatives. This assures higher yields for Edman Degradation procedures. Burdick & Jackson Laboratories, JLC/82/12, 1953 S. Harvey St., Muskegon, MI, 49442, USA.

PROGRAMMABLE WAVELENGTH DETECTOR is microprocessor controlled. It permits selection of any number of wavelengths in one-nanometer increments from 190 to 370 nm for optimal detection of all components. Utilizes keyboard entry that may be changed at any point and as often as desired. Varian Instrument Group, JLC/82/12, 2700 Mitchell Drive, Walnut Creek, CA, 94598, USA.

HIGH PRESSURE FLUID CELLS for UV monitors are rated at 1000 psi back pressure and incorporate

enhanced chromatographic flow characteristics to optimize the plate count in microparticulate columns without sacrificing detector sensitivity. The cells also improve bubble clearing and allow additional detectors to be added downstream without excessive band spreading. LDC/Milton Roy Co., JLC/82/12, P. O. Box 10235, Riviera Beach, CA, 33404, USA.

APPLICATIONS DEVELOPMENT KIT permits application of solid phase technology in the development of new sample preparation methods. One can experiment with new methods or solve existing problems with the phases included in the kit. Included are octadecyl, phenyl, cyanopropyl, aminopropyl, benzenesulfonic acid, quaternary amine, diol, and unbonded silica. Analytichem Internat'l, Inc., JLC/82/12, 24201 Frampton Avenue, Harbor City, CA, 90710, USA.

HPLC GRADIENT PROGRAMMER/SYSTEM CONTROLLER uses a microcomputer based on the Zenith/Heath Z-89 and controls up to 3 pumps to produce virtually any type of gradient or flow profile. Control functions include sample injection, solvent selection, integration, fraction collection, and recorder speed. The Anspec Co., JLC/82/12, P. O. Box 7044, Ann Arbor, MI, 48107, USA.

POST COLUMN REACTOR can be used to determine metals. It features a pneumatic pump, mixing tee, and a packed bed reactor in a self-contained unit. Dionex, JLC/82/12, 1228 Titan Way, Sunnyvale, CA, 94086, USA.

HPLC GRADE ETHANOL is now available. This solvent has been previously difficult to obtain. A new flow check has been developed for use with capillaries, as well as a digital flow meter for GC and HPLC. Alltech Associates, JLC/82/12, 2051 Waukegan Rd., Deerfield, IL, 60015, USA.

DUAL ELECTRODES FOR LC/EC capable of handling applications in single, dual-series, and dual-parallel modes. The dual parallel mode permits ratioing for identification of

chromatographic peaks and also enhances selectivity and saves time. Dual-series assays are possible for reversible redox couples and, in many cases, can enhance both selextivity and detection limits. Bioanalytical Systems, Inc., JLC/82/12, 111 Lorene Place, West Lafayette, IN, 47906, USA.

DIGITAL DISPLAY PRESSURE MONITOR is ideal for modular HPLC systems and is universally adaptable. Available for two ranges: 0-1000 and 0-10,000 pounds with accuracy within +/- 1% of actual pressure. High and low pressure limits are infinitely adjustable with audable warning when preset limits have been reached. Low 9-volt operation with remote transducer location reduce hazards associated with flammable solvents. Microbore Technology, Inc., JLC/82/12, P. O. Box 10875, Reno, NV, 89510, USA.

HIGH SPEED ION CHROMATOGRAPHY COLUMNS separate 8 ions in 5 minutes. Based on Single column ion chromatography (SCIC) technology, they can be adapted to virtually any existing HPLC system. They cvan analyze chloride, nitrate, bicarbonate, and sulfate in acid rain within 3 minutes; phosphate, chloride, nitrite, bromide, nitrate, bicarbonate, sulfate, and iodide in food samples within 5 minutes. Wescan Instruments, Inc., JLC/82/12, 3018 Scott Blvd, Santa Clara, CA, 95050, USA.

GEL FILTRATION COLUMNS are in widespread use for the separation of enzymes, proteins, polysaccharides, nucleic acids, water-soluble polymers and oligomers. A wide range of pore sizes accomodates a broad range of molecular weights. Kratos Analytical Instruments, JLC/82/12, 170 Williams Drive, Ramsey, NJ, 07446, USA.

HPLC OF CNBr CLEAVAGE FRAGMENTS of a bacterial toxin "parent" protein have been successfully separated with a Wide-Pore Octadecyl C-18 column. The 5 major fragments and several intermediates resulting from cyanogen bromide treatment were

well resolved in less than 20 minutes. J. T. Baker Research Products, JLC/82/12, 222 Red School Lane, Phillipsburg, NJ, 08865, USA.

CHROMATOGRAPHY DATA SYSTEM FOR APPLE II fits into an empty slot of the Apple and receives analog signals from the chromatograph's recorder output and converts it to digital with 12-bit precision up to 20 times/sec. Signals are smoothed, then peaks identified and integrated. Chromatogram is displayed on the CRT in real time using the high resolution graphics mode. Analytical Computers, JLC/82/12, P. O. Box 285, Elmhurst, IL, 60126, USA.

TANDEM ENRICHMENT INJECTOR VALVES perform sample enrichment and injection, sample clean-up, and other column switching tasks at pressures up to 7000 psi, and have narrow flow passage to minimize band spreading. Rheodyne, JLC/82/12, P. O. Box 996, Cotati, CA, 94928, USA.

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CRT-INTERACTIVE GRADIENT PUMP CONTROLLER simplifies setup and control of up to 3 solvent delivery systems for applications from microbore to analytical to preparative LC. Conditions may be set up or change conditions while an analysis is running. Waters Associates, Inc., JLC/82/12, P. O. Box 795, Avon, CT, 06001, USA.

LABORATORY AUTOMATION is the subject of a newsletter that provides information of new products and techniques for preparing and handling laboratory samples. Included is a calendar of scientific meetings and technical presentations on automated sample preparation using laboratory

robotics. Zymark Corp., JLC/82/12, 102 South Street, Hopkinton, MA, 01748, USA.

LC TEACHING SOFTWARE presents a clear, succinct text supported by graphical presentations designed to instruct the student in a particular area of LC. Perkin-Elmer Corp., JLC/82/12, Main Avenue, Norwalk, CT, 06856, USA.

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LC CALENDAR

1982

DECEMBER 6 - 8: 2nd International Symposium on HPLC of Proteins, Peptides, and Polynucleotides, Hyatt Regency Hotel, Baltimore, MD. Contact: S. E. Schlessinger, Mgr., 2nd Intl. Symposium on HPLC, 400 E. Randolph, Chicago, IL, 60601, USA.

DECEMBER 6 - 8: 3rd Biennial TLC Symposium-Advances in TLC, Hilton Hotel, Parsippany, NJ. Contact: J.C. Touchstone, Hospital of the University of Pennsylvania, Philadelphia, PA, 19104, USA.

1983

MARCH 7 - 12: Pittsburgh Conference on Anal. Chem. & Applied Spectroscopy, Convention Hall, Atlantic City, NJ, USA. Contact: Mrs. Linda Briggs, Program Secretary, 437 Donald Rd., Pittsburgh, PA, 15235, USA.

MARCH 20 - 25: National Amer. Chem. Soc. Meeting, Seattle, WA, USA. Contact: A. T. Winstead, Amer. Chem. Soc., 1155 Sixteenth St., NW, Washington, DC, 20036, USA.

MAY 2 - 6: VIIth International Symposium On Column Liquid Chromatography, Baden-Baden, West Germany. Contact: K. Begitt, Ges. Deutscher Chemiker, Postfach 90 04 40, Varrentrappstrasse 40-42, D-6000 Frankfurt (Main), West Germany.

MAY 30 - JUNE 3: International Conference on Chromatographic Detectors, Melbourne University. Contact: The Secretary, International Conference on Chromatographic Detectors, University of Melbourne, Parkville, Victoria, Australia 3052.

JUNE 1 - 3: The Budapest Chromatography Conference, Budapest, Hungary. Contact: Dr. T. Devenyi, Institute of Enzymology, Hungarian Academy of Sciences, Budapest, Hungary or Dr. H. Issaq, Frederick Cancer Research Facility, P.O.Box B, Frederick, MD, 21701, USA.

JULY: 3rd Int'l. Flavor Conf., Amer. Chem. Soc., The Corfu Hilton, Corfu, Greece. Contact: Dr. S. S. Kazeniac, Campbell Inst. for Food Research, Campbell Place, Camden, NJ, 08101, USA.

JULY 17 - 23: SAC 1983 International Conference and Exhibition on Analytical Chemistry, The University of Edinburgh, United Kingdom. Contact: The Secretary, Analytical Division, Royal Society of Chemistry, Burlington House, London W1V 0BV, United Kingdon.

1984

OCTOBER 1 - 5: 15th International Symposium on Chromatography, Nurenberg, West Germany. Contact: K. Begitt, Ges. Deutscher Chemiker, Postfach 90 04 40, Varrentrappstrasse 40-42, D-6000 Frankfurt (Main), West Germany.

The Journal of Liquid Chromatography will publish announcements of LC meetings and symposia in each issue of The Journal. To be listed in the LC Calendar, we will need to know: Name of 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 for inclusion in the LC Calendar to Dr. Jack Cazes, Editor, Journal of Liquid Chromatography, P. O. Box 1440-SMS, Fairfield, CT, 06430, USA.

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