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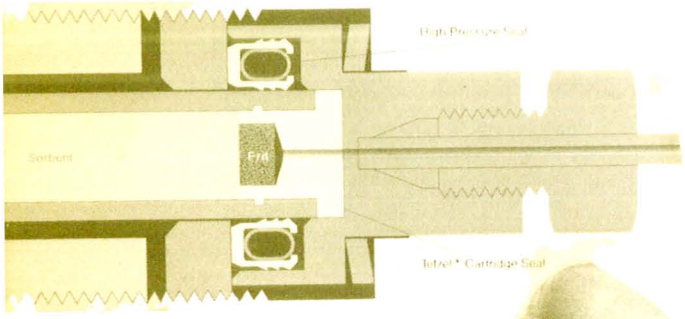

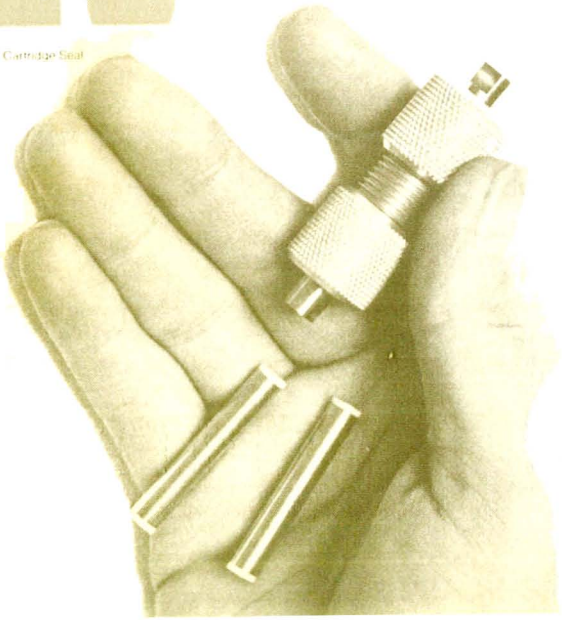

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Volume 4, Number 2, 1981

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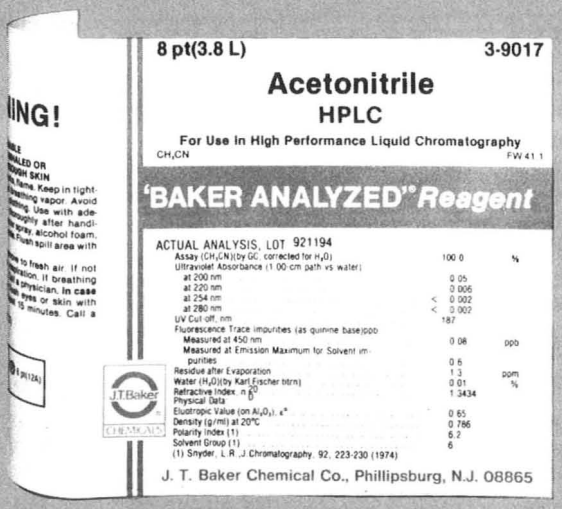

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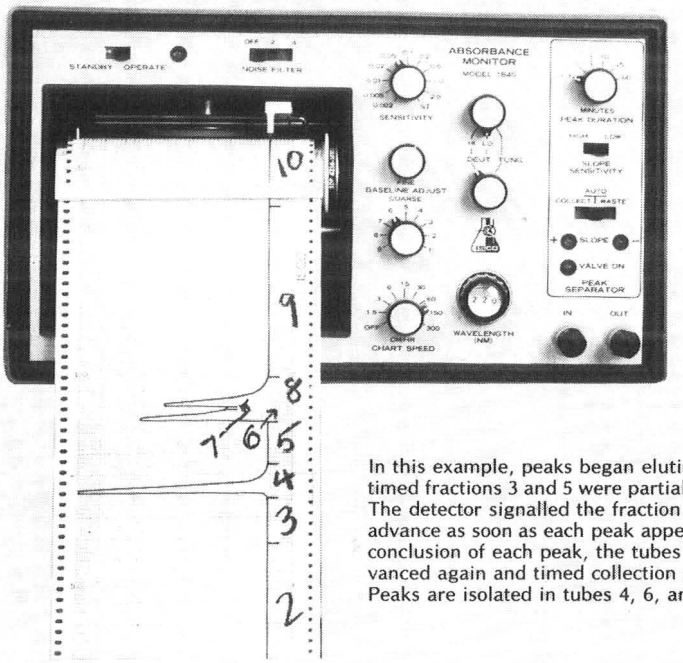

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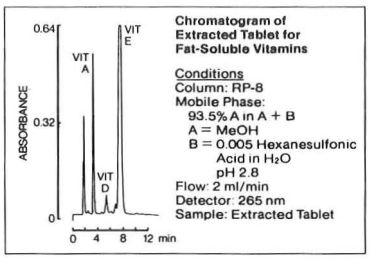

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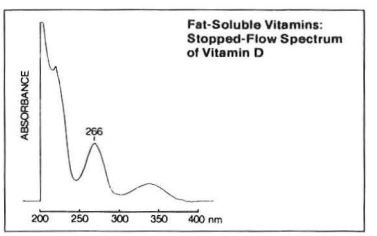

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THE EFFECT OF THE CONCENTRATION AND INJECTED VOLUME OF POLYMER SOLUTION IN SIZE EXCLUSION CHROMATOGRAPHY

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ABSTRACT

The concentration and total injected volume of polymer solution affect elution volumes in size exclusion chromatography. When selecting optimal experimental conditions for both analytical and preparative separation, it must be considered wheter it is more advantageous to inject a smaller volume of solution of higher concentration, or vice versa. Theoretical analysis by means of derived equations and experimental results showed that if the injection conditions are chosen so that the contribution to the total width of the elution curve due to injection width is negligible within the limits of experimental error, and the total amount of the injected polymer is constant, the ratio of concentration to the injected solution volume may be chosen within broad limits without affecting the results of separation to any considerable extent.

INTRODUCTION

Important experimental factors which should be considered in choosing optimal experimental conditions in size exclusion chromatography (SEC), particularly in the separation of polymers, are the concentration

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and total injected volume of polymer solution. In analytical separations it is important to reach a high-quality signal of the detector used, i.e. a sufficient difference between the detector response to the eluating polymer solution, on the one hand, and the noise of the base-line on the other, to make results of the calculation of molecular weight distribution sufficiently reproducible. In preparative separation it is desirable to have the highest possible amount of the fractionated polymer sample in one injection, so that it would not be necessary to inject a limited amount of the polymer repeatedly, which makes the fractionation considerably more labour- and time-consuming. It is generally known that the injection of the sample must be as short as possible, or in other words, the injected volume of polymer solution must be as small as possible to prevent an additional broadening of the elution curve. In such a case the concentration must be sufficiently high to satisfy the requirement on the detector response. It is also well known that with increasing concentration of the injected polymer solution the elution volume also increases. This phenomenon is usually called the concentration effect and has been investigated by many authors (cf.ref.1). A complete theoretical model quantitatively describing concentration effects in the SEC of polymers has been reported in an

earlier paper (1). Hence, for experimental purposes one must choose an optimal compromise between the contradictory requirements just outlined.

Let us start with the maximal acceptable injected volume of polymer solution. For the overall width of the elution curve, w_T, we have, according to the rule of additivity of second central moments or variations

$$w_T^2 = w_I^2 + w_P^2 + w_S^2 \qquad (1)$$

where w_I is a contribution due to injection, proportional to the injected volume, w_P is a contribution due to the polydispersity of the sample, and w_S is a contribution due to spreading in separation columns, connecting capillaries and in the detector.

Instead of w, the width of the chromatogram may alternatively be described by using the standard deviation, σ. If the elution curve and injection function may be approximated by the Gaussian curve, the width of each contribution, w, is equal to fourfold standard deviation σ. If we consider a certain per cent experimental error in SEC, we may calculate the highest w_I so as to put its contribution to w_T within the limits of this experimental error. First, we determine the upper limit of w_I, and thus the maximal injected volume, which is still accep-

table with respect to experimental error. Further, we find out whether it is better to inject such a maximal solution volume at a minimal concentration at which the ratio of detector response to the noise of the base-line is sufficient, or a smaller solution volume at a higher concentration, and examine the role of concentration effects in this case.

Thus, for analytical SEC we have to determine if in the case of injection of a certain amount of polymer in solution acceptable with respect to the sensitivity of detection one has to prefer injection of a larger solution volume at a lower concentration, or vice versa. For preparative fractionation by means of SEC in those cases where the maximal capacity of the separation should be employed, one has to determine the upper limits of concentration and injected volume at which the influence of spreading caused by injection and of concentration effect on the results of fractionation is still acceptable. Similarly to the analytical SEC, in those cases where it is not necessary to employ the maximal capacity of the separation system, one has to determine optimal conditions with respect to the concentration and total volume of the injected polymer solution.

In order to express quantitatively the dependence of the elution volume V_e on concentration g_I and on the

total amount of the injected polymer solution (which may be characterized by the standard deviation of the injection function σ_I), we employ theoretical relations derived in our earlier papers (1-3). At infinite dilution, the linear part of the dependence of the elution volume V_{eo}, on the hydrodynamic volume, $v \cdot \varepsilon_o$, of separated macromolecules may be described by an empirical equation

$$V_{eo} = P + Q \ln (v \cdot \varepsilon_o) \qquad (2)$$

where P,Q are constants, v is the volume of an unswollen coil and ε_o is a dimensionless swelling factor at infinite dilution (4). The elution volume V_e depends on concentration according to a relation

$$V_e = P+Q\Big[\ln v + \frac{B^2 g_I^2 \sigma_I^2}{(\sigma_T^2-\sigma_I^2)A^2} \cdot \ln \Big(\frac{\sigma_T A + Bg_I\sigma_I}{\sigma_I A + Bg_I\sigma_I}\Big) - \frac{Bg_I\sigma_I}{(\sigma_T+\sigma_I)A} + \frac{\sigma_I^2}{\sigma_T^2-\sigma_I^2} \cdot \ln (A + Bg_I) - \frac{\sigma_T^2}{\sigma_T^2-\sigma_I^2} \cdot \ln\Big(\frac{\sigma_T A + Bg_I\sigma_I}{\sigma_T}\Big)\Big] + k'\Big(\frac{2[\eta]g_I\sigma_I}{\sigma_T+\sigma_I} + \frac{2\,k_H[\eta]^2 g_I^2\sigma_I^2}{\sigma_T^2 - \sigma_I^2} \cdot \ln \frac{\sigma_T}{\sigma_I}\Big) \qquad (3)$$

derived in our earlier papers (2,3) for a change in V_e with concentration due to a change in the effective dimensions of separated macromolecules and dynamic vis-

cosity phenomena. In Eq.(3), A and B are constants defined by

$$A = \frac{1}{\varepsilon_o} , \qquad B = \frac{1}{g_x}\left(\frac{\varepsilon_o - 1}{\varepsilon_o}\right) \tag{4}$$

where g_x is a critical concentration (4) at which dimensions of the macromolecules in solution are the same as under the theta thermodynamic conditions; $[\eta]$ is intrinsic viscosity, k_H is the Huggins constant and k' is a constant characterizing the given separation system with respect to the contribution of the dynamic viscosity phenomenon (1-3) and still having an empirical character. In addition to the mentioned contributions, i.e. to the change in the dimensions of separated macromolecules and viscosity phenomena, some complementary contributions may also be taken into account (1). Since the physical meaning and the absolute value of these contributions have not yet been explained unambiguously (5), they are not considered here. The above relations may be employed for a theoretical estimate of the effect of concentration, g_I, or of the injected volume of polymer solution characterized by σ_I on the elution volume V_e while maintaining the total constant amount of the injected sample characterized by the product $g_I \times \sigma_I$.

EXPERIMENTAL

All SEC measurements were performed using an apparatus built at this Institute. A differential refractometer R-403 (Waters Associates, Inc., Milford,Mass. USA) was used as the concentration detector. The separation columns were packed with silicagel Porasil (Waters), types B, D, E, and connected in a series consisting of two columns of each type in the order 2xE, 2xD, and 2xB. The whole separation column system was thermostated at 25± 0.5°C. Tetrahydrofuran distilled from cuprous chloride and potassium hydroxide was used as solvent, with the flow-rate 0.375 ml/min. The elution volumes were measured by using a siphon, 1.704 ml in volume. The separation system was calibrated by means of polystyrene (PS) standards (Waters). The calibration plot is shown in Fig. 1. Solutions of PS standards at concentrations of 0.05 % (w/v) were injected using a six-way injection valve (Waters) provided with a loop, 1.636 ml in volume. The effect of the ratio of concentration to the injected volume at a total constant injected amount of the polymer was determined with a PS standard, molecular weight M_w= 694 000 (Knauer, Oberursel, FRG). The injected concentrations in this case were 0.2, 2, and 4% (w/v), the corresponding injected volumes were 1.25, 0.125, and 0.0625

ml,respectively. These volumes of solutions of the PS standard were injected with the loop switched into a hydraulic circuit for 200, 20, and 10 sec.Hence, the total injected amount of the PS standard was 2.5 mg in all cases.

RESULTS AND DISCUSSION

The attainable reproducibility of elution volumes in liquid chromatography, and hence in SEC, lies approximately in the limits ± 0.1%. This means that if the width of the injection w_I is to affect the resulting w_T only within the limits comparable with experimental errors of the elution volumes, w_T/w_{To} must be equal to the maximal value 1.001. w_{To} is a hypothetical width of the elution curve for a case where $w_I = 0$. With respect to Eq.(1) it holds that

$$\frac{w_I^2}{w_{To}^2} + 1 = (1.001)^2 \qquad (5)$$

Since it holds approximately that $w_T \approx w_{To}$, we obtain after the rearrangement of Eq.(5)

$$\frac{w_I}{w_T} = \sqrt{(1.001)^2 - 1} = 0.0447 \qquad (6)$$

Hence, under the given conditions the maximal injection

width characterized by w_I is approximately 4.5% of w_T.

In a theoretical investigation of the effect of the ratio of concentration g_I to the injected solution volume characterized by the standard deviation of the injection function σ_I, Eq.(3) was employed. The total weight amount of the injected polymer was constant. The model calculations were performed with the experimental parameters of Eq.(3) from our earlier paper (3). These values are summarized in Table 1. The separation system used in this paper was designed in order to cover a wide range of molecular weights of the polymers (cf. calibration plot in Fig.1), and has already been employed because of its high efficiency in the determination of molecular weight distribution of many polymers. At the same time, such a system is most frequently used in many laboratories. In our case we had no suitable polymer standard with narrow molecular weight distribution and with a sufficiently high molecular weight which would allow us to determine reliably the constant k´necessary for the calculation of V_e using Eq.(3). This empirical constant is determined by measuring the concentration dependence of V_e for a totally excluded polymer sample (2). The results of model calculations are summarized in Table 2, which also gives changes (in per cent) of the elution volumes ΔV_e due to the particular contributing processes, as well as the total change (again in

TABLE 1

Values of the parameters used in the calculation of the elution volumes from Eq.(3) of the PS standard with M_w=867 000

P	Q	vx10^{17}	A	B	σ_T	k´	[η](ml/g)	k_H
-85.89	-3.01	8.855	0.357	75.48	1.75	7.957	211.71	0.362

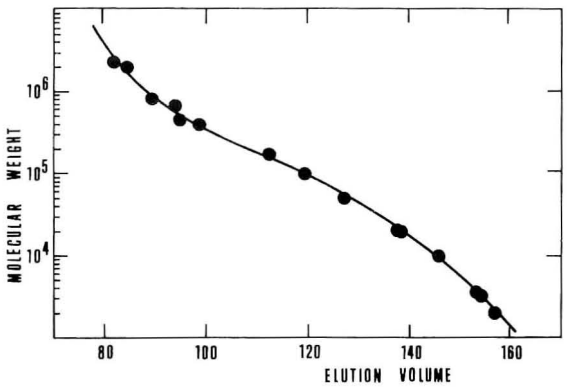

FIGURE 1. Calibration plot of the column separation system

per cent) of the elution volume ΔV_e caused by a change in g_I/σ_I at a constant $g_I x \sigma_I$. In the first set of ΔV_e values in Table 2, the value of $g_I x\ \sigma_I = 0.05$; up to $\sigma_I = 0.05$ the requirement that σ_I should be approximately 4.5% of $\sigma_T = 1.75$ is fulfilled. It can be seen that within the whole range $0.001 \leqq \sigma_I \leqq 0.1$ or in the respective concentration range covering two orders of magnitude, $0.5\% \leqq g_I \leqq 50\%$ (w/v), the total change ΔV_e and the particular changes corresponding to the contributing processes are almost constant; ΔV_e slightly decreases from approximately 6.5%

TABLE 2

Change in the elution volume caused by the injection of a solution of the PS standard of the given concentration and volume with respect to zero concentration or volume expressed in per cent

g_I(%)	σ_I	ΔV_e (%)		
		Change in effective dimensions	Viscosity phenomena	Total
50	0.001	1.470	5.025	6.495
10	0.005	1.470	4.863	6.333
5	0.01	1.467	4.787	6.254
2	0.025	1.461	4.666	6.127
1	0.05	1.452	4.540	5.992
0.5	0.1	1.427	4.361	5.788
0.1	0.5	1.210	3.489	4.699
10	0.025	5.839	31.367	37.206
5	0.05	5.818	29.437	35.255
2	0.125	5.733	26.451	32.184
1	0.25	5.539	23.617	29.156
0.5	0.5	5.127	20.038	25.165

to approximately 5.8% with concentrations ranging between 50% and 0.5% (w/v). Only if σ_I distincly exceeds the limit of 4.5% from σ_T, i.e. for σ_I = 0.1 and 0.5 at σ_T = 1.75, and if the concentrations are lower than 0.5% (w/v) the decrease in ΔV_e with concentration is distincly steeper. In the second set of ΔV_e values in Table 2, $g_I x \sigma_I$ = 0.25, which is five times higher than in the preceding case. The ΔV_e values expressed in per cent are therefore also correspondingly higher. In this case the dependence of ΔV_e on concentration is evidently steeper than in the preceding case, even though, e.g., an increase in concentration from g_I = 5% to 10% (w/v) causes a change in ΔV_e from c. 35% to c. 37%, i.e. a relatively small change. Similarly to the preceding case, a steeper decrease in this dependence occurs in the range of low concentrations or of high σ_I. Of course, the σ_I values in the second set are higher in most cases than 4.5% from σ_T. It may be inferred from the results of the theoretical calculation that a rise in concentration compensated by a simultaneous decrease in the injected volume of polymer solution (with the same total injected wight amount of the polymer) leads to an increase in the elution volume. However this change in most of the practical applications is almost comparable with experimental errors,and

hence almost negligible, providing that σ_I compared with σ_T lies within the limits determined in advance with respect to the required precision of fractionation.

The results of an experimental study of the effect of the ratio of concentration to the injected volume of the PS standard at a constant total weight amount of the injected polymer are summarized in Table 3. The experimental procedure has been desribed in the Experimental. The concentration range exceed one order of magnitude from 0.2% (w/v) to 4% (w/v), with the respective injected volumes of the solution of the PS standard 1.25 to 0.0625 ml. As documented by the results in Table 3, the elution volume V_e remained the same within the limits of experimental error in all these cases. A similar experimental finding has been described by Moore (6). Also, the polydispersity M_w/M_n of this standard calculated from direct experimental data without corrections is virtually unaffected by variation of the experimental conditions of injection. For the sake of comparison, we also injected 1.25 ml of a solution of the PS standard at the concentration 2% (w/v), i.e. a tenfold weight amount of the polymer (25 mg). The resulting elution volume is higher by more than 9% compared with the preceding measurements. The experimental results confirmed

TABLE 3

Effect of the injected volume and concentration on the elution volume of the PS standard with M_w=694 000

Concentration of injected solution (% w/v)	Injected volume (ml)	V_e	M_w/M_n
0.2	1.25	94.5	1.10
2.0	0.125	94.6	1.12
4.0	0.0625	94.3	1.13
2.0	1.25	103.5	1.19

excellent agreement with the preceding theoretical calculations. They also demonstrated that in the range where the width of injection within the limits of experimental error is negligible, (compared with the width of the elution curve), the injection conditions, i.e. concentration and the total volume of injected solution, may be broadly varied (if the total weight amount of the polymer remains constant), without any pronounced effect on the results of fractionation by the SEC method.

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UNIVERSAL CALIBRATION AND MOLECULAR WEIGHT AVERAGES IN GEL PERMEATION CHROMATOGRAPHY ILLUSTRATED BY CELLULOSE NITRATE AND POLY(OXYPROPYLENE)

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ABSTRACT

The nature of the averaging process in the analysis of gel permeation chromatograms was examined for cases where the molecules in the detector cell of the apparatus were of different molecular weight and of the same molecular weight. When the molecules have the same molecular weight, the hydrodynamic volume (1), $[\eta]M$, averaged across a chromatogram was found to become KM^{a+1} for any molecular weight average at the elution volume corresponding to that average. $[\eta]$ is intrinsic viscosity, M is molecular weight, and K and a are the appropriate Mark-Houwink constants. Thus when size separation is by molecular weight, the universal GPC calibration functions include KM_n^{a+1} where M_n is the number average molecular weight.

Cellulose nitrate and poly(oxypropylene) were analyzed using three sets of columns and two GPC instruments. KM_n^{a+1}, KM_w^{a+1}, and $[\eta]M_w$ were found to represent the hydrodynamic volume since these functions fell on the universal calibration plot for nearly nonodisperse polystyrene standards. The function $[\eta]M_n$ was displaced from the polystyrene universal calibration plot by factor which equaled M_w/M_n. The slopes and intercepts of the universal calibration plots were found to be completely consistent with the slopes and intercepts of the molecular weight calibration plots showing that the Mark-Houwink constants were correct. Intrinsic viscosity - molecular weight relations were presented for 12.0-12.6%N cellulose nitrate and for low molecular weight poly(oxypropylene), the latter relation being a correction of that of Sholtan and Lie (18).

INTRODUCTION

Since the introduction of the concept of universal gel permeation chromatography (GPC) calibration by Grubisic, Rempp, and Benoit (1) the nature of the hydrodynamic volume used in this calibration has been the subject of a number of papers (2-8). The expression for the hydrodynamic volume of the polymer molecules in the detector cell of the GPC apparatus at any one time was shown by A.E. Hamielec and A.C. Ouano (2) and Hamielec, Ouano, and Nebenzahl (3) to be $[\eta]M_n$ where $[\eta]$ is intrinsic viscosity and M_n is number average molecular weight. These workers employed what in effect were mixtures of polymers which had widely different shapes at the same molecular weight. Their results showed that the separation factor for this polymer mixture was also $[\eta]M_n$ across the whole chromatogram. On the other hand as shown by much experience there is no doubt that for a chromatogram the use of $[\eta]M_w$, where M_w is the weight average molecular weight, for hydrodynamic volume is ordinarily correct. This is because in most cases only one type of polymer of one size is present. However, on thinking about the subject it became apparent that our understanding of the averaging process in GPC statistics was incomplete. On looking at the matter in detail it was found, as will be shown below, that for a single kind of polymer the GPC separation factor averaged for a chromatogram should be KM^{a+1} at the elution volume corresponding to M, where M is molecular weight of any type average and K and a are the appropriate Mark-Houwink constants, rather than only $[\eta]M_w$ or KM_w^{a+1} at the peak elution

volume. This conclusion was tested using GPC results on Cellulose Nitrate and Poly(oxypropylene) samples.

ARGUMENT

Several papers (2-8) have appeared concerning the nature of "the" hydrodynamic volume to use in the so-called universal calibration procedure for Gel Permeation Chromatography (GPC) devised by Grubisic, Rempp, and Benoit (1). Recent work by Hamielec and Ouano (2) has shown without any doubt that the calibration constant should be $[\eta]M_n$ where $[\eta]$ is the intrinsic viscosity and M_n is the number average molecular weight at any elution volume.

The universal calibration for GPC was based on Einstein's relation for the viscosity of a suspension of spheres (9):

$$[\eta] = \frac{kNV_e}{M} \qquad \text{- - - - - - - - - - - - -} \quad 1$$

where $[\eta]$ = intrinsic viscosity

k = a constant

N = Avagadro's number

V_e = volume of a sphere equivalent to that of one solute molecule

M = molecular weight of solute

See Flory (10), p. 606. From Equation 1 the "hydrodynamic volume", NV_e is proportional to $[\eta]M$. $\text{Log}[\eta]M$ was found to be a linear function of elution volume for a number of polymers (1). $\frac{[\eta]M}{k}$ is an average of the size of all the solute molecules. Each molecule will have a molecular weight, M_i, and a characteristic intrinsic viscosity, $[\eta]_i$, so that:

$$[\eta]M = \frac{[\eta]_i M_i}{m} \qquad \text{- - - - - - - - - - -} \quad 2$$

where m is the number of molecules.

Following Hamielec and Ouano (2), if we define $[\eta]M$ as J, at any point on the elution volume curve J will be constant although M_W/M_η may vary. For that point let $[\eta] = \Sigma w_i[\eta]_i$ where w_i is the weight fraction of the ith species. That is, the intrinsic viscosity of the polymer is the weight, or better - viscosity, average of that of the fractions. But $J_i = [\eta]_i M_i$ and $[\eta]_i = J_i/M_i$. Therefore $[\eta] = \Sigma w_i J_i/M_i$. Hence $M = \frac{J}{[\eta]} = \frac{J}{\Sigma w_i J_i/M_i}$. But J is constant. Therefore $M = \frac{J}{J\Sigma w_i/M_i} = \frac{1}{\Sigma w_i M_i}$ which is the number average molecular weight. Hence M is M_η and J is $[\eta]M_\eta$.

In the general case as shown by Hamielec and Ouano (2) where the Mark-Houwink relation is not the same for all molecules, hydrodynamic volume varies with chromatographic elution volume in accordance with:

$$\text{Log } [\eta]M_\eta = \text{Log } A - bC \qquad \text{- - - - - - - - - -} \quad 3$$

where A and b are constants and C is the elution volume, a relation which is linear or nearly so. If one is satisfied with molecular size, the average hydrodynamic volumes may be calculated statistically from h_i, the height of the elution curve at any point, and $J_i = [\eta]_i M_i$ read from the universal calibration curve determined for standard monodisperse samples.

$$J_n = \frac{\Sigma h_i}{\Sigma h_i/J_i} \quad \text{and} \quad J_W = \frac{\Sigma h_i J_i}{\Sigma h_i} \quad \text{in volume per mol.}$$

However, Hamielec, Ouano, and Nebenzahl (3) have devised an approximation method to obtain M_η and M_W from h_i and J_i.

The statistical treatment of GPC results has always assumed that molecules of the same size in the detector cell have the same molecular weight. For most linear polymers the assumption

is justified. If all the molecules are of the same size and obey the same Mark-Houwink relation, their molecular weights are the same.

$$[\eta]_1 M_1 = [\eta]_2 M_2$$
$$K M_1^a M_1 = K M_2^a M_2$$
$$M_1 = M_2$$

In such a case in the detector cell of the chromatograph and at any point on the elution volume axis, $M_n = M_w = M_v$. If for the whole sample the Mark-Houwink relation is the same for all the molecules, then size separation by GPC is proportional to molecular weight. Then if the same Mark-Houwink relation holds between different samples, comparison can be made between samples of various average molecular weights on the basis of elution volumes at these averages.

When a high molecular weight solute is polydisperse, we write the Mark-Houwink relation:

$$[\eta] = K M_v^a$$

where K and a = constants

M_v = viscosity average molecular weight

At a single elution volume if several species are present each obeying its own intrinsic viscosity - molecular weight relation, there will be a composite relation of the form $K M_v^a$. Thus $[\eta] M_n$ becomes $K M_v^a M_n$ which can be placed in terms of one molecular weight average only through the introduction of a dispersity term. If, however, at a single point on the elution

volume curve all the molecules obey the same Mark-Houwink equation then they are all of the same molecular weight and the expresions M_v, M_w, and M_n have no meaning at that point and $[n]M_n$ becomes KM^{a+1}. If the same Mark-Houwink equation holds for all the polymer then KM^{a+1} becomes the size separation expression all along the elution volume curve. Separation is point by point along the chromatographic curve. If each point is associated with one molecular weight the type of average involved in separation does not enter the picture. When we then average a single curve, since various amounts of polymer are associated with different points, we will in general have various sizes and molecular weights each of which must at least approximately fit

$$\text{Log } KM^{a+1} = \text{Log } A - bC \quad \text{- - - - 4}$$

$$\text{and} \quad \text{Log } M = \text{Log } \left(\frac{A}{K}\right)^{\frac{1}{a+1}} - \frac{b}{a+1} C \quad \text{- - - - 5}$$

$$\text{Let} \quad \left(\frac{A}{K}\right)^{\frac{1}{a+1}} \equiv d \quad \text{- - - - 6}$$

$$\text{and} \quad \frac{b}{a+1} \equiv e \quad \text{- - - - 7}$$

$$\text{Then} \quad \text{Log } M = \text{Log } d - eC \quad \text{- - - - 8}$$

If the molecular weight of the fractions is known one may then calculate M_w, M_v, M_n statistically for the whole polymer in the usual manner from the observed heights of the chromatogram and read off corresponding values of C_w, C_v, and C_n from a plot of Equation 8 so that one has:

$$\text{Log } M_{w,v,n} = \text{Log } d - eC_{w,v,n} \quad \text{- - - - 9}$$

With a single polymer the three Equations 9 each connect two variables only and have the same intercept and slope and therefore form one curve for various samples of one kind of

polymer. They must, since as the area of a chromatogram approaches zero, the three molecular weight averages approach each other.

If we substitute Equations 6 and 7 in Equation 9 and rearrange we obtain:

$$\text{Log } KM_n^{a+1} = \text{Log } A - bC_n \quad \text{- - - - - - - - -} \quad 10$$

$$\text{Log } KM_w^{a+1} = \text{Log } A - bC_w \quad \text{- - - - - - - - -} \quad 11$$

$$\simeq \text{Log } [\eta]M_w \quad \text{- - - - - - - - -} \quad 12$$

$$\text{Log } KM_v^{a+1} = \text{Log } A - bC_v \quad \text{- - - - - - - - -} \quad 13$$

$$= \text{Log } [\eta]M_v$$

If Equation 10 is substracted from Equation 11:

$$\text{Log } M_w/M_n = \frac{b(C_n-C_w)}{a+1} = e(C_n-C_w) \quad \text{- - -} \quad 14$$

Here M_w/M_n is the dispersity of the whole polymer.

EXPERIMENTAL

The ideas expressed above were tested using results obtained from eleven cellulose nitrate samples with nitrogen content varying from 12.0% to 13.1% and twelve poly(oxypropylene) mixtures of known dispersity varying from 1.03 to 1.53 (11). Two different instruments and three sets of columns were used.

a. Materials

Cellulose Nitrate was ideally suited for our purpose since it has a broad molecular weight distribution which varies greatly between samples. Eleven cellulose nitrate (N/C) samples were selected for characterization. The samples described in Table 1 were obtained from Hercules, Inc., Parlin, New Jersey and

TABLE 1

Cellulose Nitrate Samples

Hercules Lot	Type	Nitrogen Content, % (a)	Mol Wt. M_n Osm. (b)
3569	RS-0.5"	11.95	
4106	RS-5.4"	12.03	26133
1696	RS-15"	11.86	39985
4874	RS-33"	11.97	33515
4569	RS-60"	11.97	55908
4419	RS-125"	12.00	52428
7715	6"	12.60	39489
2234	10-15"	12.64	72200
7719	20"	12.61	52428
2238	36"	12.56	57700
9038	12"	13.13	57695

(a) Nitrogen was determined by the nitrometer method according to Mil. Std. 286B

(b) Osm - Membrane Osmometer

contained 30% ethanol. Viscosity grades of the samples varied from 0.5 to 125 seconds. Molecular weights were determined by the ArRo Laboratories, Joliet, Illinois in acetone solution using a Hewlett-Packard membrane osmometer. The membrane osmometer method employed

would best be described as dynamic rather than static and low molecular weight impurities in the samples may not have diffused through the membrane. The cellulose nitrate solutions were allowed to stand two to three days to effect complete solution.

Polystyrene standards were obtaned from Waters Associates (12)and the Pressure Chemical Company (17).

Poly(oxypropylene) samples of nominal molecular weight 400, 1000, and 2000 were obtained from BASF-Wyandotte while 4000 molecular weight material was obtained from the Dow Chemical Co. Formulation and treatment of these materials are described in Reference 11.

The solvent for all measurements was tetrahydrofuran (THF).

b. Equipment

Two chromatographic instruments were used, a Waters Associates Model 200 and a modified Waters Associates 202/401 with a high pressure pump capable of reaching 2000 psi, equivalent to a Model 244. Cellulose nitrate was analyzed using both instruments. On the Waters 200 the columns employed were 2.5×10^4 AU (Cat. No. 39715), 1×10^5 AU (Cat. No. 39716), 3×10^5 AU (Cat. No. 39717), 1×10^6 AU (Cat. No. 39718), and 1×10^7 AU (Cat. No. 39719) designated column set B, while with the 202/401 the columns were 10^2, 10^3, 10^4, 10^5 and 10^6 AU pore size called column set C. Poly(oxypropylene) was run with the Waters 200 instrument using columns of 2.25×10^2, 1.4×10^3, 1.4×10^3 and 3.5×10^3 AU called column set II.

Column sets B and II were packed with Styragel while column set C was packed with Micro-Styragel of smaller particle size. In all cases a flow rate of 1.0 ml/minute was used at 25°C. The Waters 200 instrument was used at a sensitivity of four for the NC and a sensitivity of one for the Poly(oxypropylene).

c. Calibration with Polystyrene Standards

The GPC curves were analyzed by the successive approximation method described by Adams et al (13). The results for the column sets are shown in Tables 2, 3 and 4 and Figures 3, 4, and 5. The equations describing the polystyrene curves were:

Column set B, Waters 200: $\text{Log } M_n = 11.79 - 0.0317C$ - - 16

Column set C, Waters 202/401:
$\text{Log } M_n = 10.27 - 0.156C$ - - - - 17

Column set II, Waters 200:
$\text{Log } M_n = 7.91 - 0.0336C$ - - - - 18

where C is elution volume in ml at the number average molecular weight(M_n).

Polystyrene intrinsic viscosities were calculated from the equation of Spatorico and Coulter (8). These authors reviewed previous work and concluded that the intrinsic viscosity of polystyrene in THF at 25°C was best represented by

$$[\eta] = 1.11 \times 10^{-4} M_w^{0.725}, \text{ dl/g} \quad - - - - - - \quad 19.$$

The product of the weight average molecular weight of the standards and their calculated intrinsic viscosity at that

TABLE 2

Analysis of GPC Curves, Cellulose Nitrate and Standard Polystyrene
Waters200 Instrument and Column Set B

	Lot No.	M_n (GPC)	M_w (GPC)	M_w/M_n	C_n 5 ml	C_w 5 ml	C_n-C_w 5 ml	$[\eta]$ dl/g	$[\eta]M_w$ dl/mol	KM_n^{a+1} dl/mol	$[\eta]M_n$ dl/mol
Cellulose Nitrate	3569	22133	57900	2.62	45.0	42.4	2.6	0.851	49273	8610	18835
	4106	34163	177800	5.20	43.8	39.4	4.4	1.61	286258	18808	55000
	1696	44293	180904	4.08	43.1	39.3	3.8	1.87	338290	30016	82828
	4874	45707	234769	5.14	43.0	38.6	4.4	2.82	662048	31762	128893
	4569	54007	303073	5.61	42.5	32.9	4.6	3.12	945588	42890	168502
	4419	54000	298890	5.53	42.5	38.0	4.5	3.20	956448	42890	172822
	7715	72557	330200	4.55	41.8	37.7	4.1	3.51	1.16×10^6	72974	254675
	2234	66800	302700	4.53	42.0	37.9	4.1	4.40	1.33×10^6	62884	293920
	7719	76419	421200	5.51	41.6	37.0	4.6	4.10	1.73×10^6	80265	313646
	2238	57039	478900	8.40	42.4	36.7	5.7	4.81	2.3×10^6	47321	274358
	9038	59896	265300	4.43	42.3	38.3	4.1	3.84	1.02×10^6	51762	230000
Poly-styrene	4190038	773000	865760	1.12	37.1	37.0		2.24	1939302	1731520	
	4190037	392000	411600	1.05	39.2	39.1		1.31	539196	513520	
	41984	164000	173020	1.055	41.8	41.8		0.70	121114	114800	
	41995	96200	100048	1.04	43.0	42.9		0.47	47023	45214	
	21570	49000	50960	1.04	44.8	44.6		0.29	14778	14210	
	4190039	19086	19849	1.04	46.8	46.4		0.145	2878	2767	

* Measured Intrinsic Viscosity for Cellulose Nitrate, Calculated for Polystyrene
Cellulose Nitrate, 12.0-12.6%N: $K = 0.00013$, $a = 0.80$
Polystyrene: $K = 0.00011$, $a = 0.725$

TABLE 3

Analysis of GPC Curves, Cellulose Nitrate and Standard Polystyrene
Waters 202/401 Instrument and Column Set C

	Lot No.	M_n (GPC)	M_w (GPC)	M_w/M_n	C_n 1 ml	C_w 1 ml	C_n-C_w 1 ml	[η]* dl/g	[η]*M_w dl/mol	KM_n^{a+1} dl/mol
Cellulose Nitrate	3569	20686	43000	2.08	37.1	34.8	2.3	0.851	36593	7624
	4106	28877	86742	3.00	36.1	32.9	3.2	1.61	100637	13898
	1696	30803	98414	3.19	35.9	32.5	3.4	1.87	126312	15610
	4569	49320	133536	2.71	34.5	31.7	2.8	3.12	218786	36424
	9038	57962	178838	3.09	34.1	30.9	3.2	3.84	370144	48708
Poly-styrene	4190038	773000	842570	1.09	29.05	28.70	0.35	2.24	1.89×10^6	1.73×10^6
	41984	164010	177120	1.08	32.79	32.56	0.23	0.70	123984	114800
	41995	96200	101010	1.05	34.11	33.91	0.20	0.47	47475	45214
	4190039	19750	21330	1.08	38.48	38.40	0.08	0.149	3178	2943

* Measured Intrinsic Viscosity for Cellulose Nitrate, Calculated for Polystyrene
Cellulose Nitrate, 12.0-12.6%N: K = 0.00013, a = 0.80
Polystyrene: K = 0.00011, a = 0.725

TABLE 4

Analysis of GPC Curves, Poly(oxypropylene) and Standard Polystyrene
Waters 200 Instrument and Column Set II

	Lot No.	M_n (GPC)	M_w (GPC)	M_w/M_n	C_n 5 ml	C_w 5 ml	C_n-C_w 5 ml	$[\eta]*M_w$ dl/mol	KM_n^{a+1} dl/mol	$[\eta]M_n^*$ dl/mol
Polypropylene		500	527	1.05	29.80	29.65	0.15	15.0	13.7	14.2
Oxide	31	1030	1082	1.05	27.90	23.75	0.15	50.0	46.1	47.7
	21	1935	2012	1.04	26.05	25.95	0.10	142	133	137
		4000	4120	1.03	24.10	24.00	0.10	473	450	459
	22	1969	2185	1.11	26.02	25.70	0.32	163	137	147
	23	1902	2350	1.24	26.07	25.50	0.57	184	129	149
	24	2024	2598	1.28	25.95	25.30	0.65	218	143	170
	25	1991	2816	1.41	26.00	25.05	0.95	250	140	177
	26	1913	2922	1.53	26.10	24.95	1.15	266	130	174
	32	1099	1259	1.15	27.70	27.30	0.40	64.6	51.4	56.4
	33	1096	1350	1.23	27.70	27.10	0.60	72.6	51.2	59.0
	34	1122	1476	1.32	27.60	26.85	0.75	84.4	53.2	64.1
	35	1164	1621	1.39	27.50	26.55	0.95	98.7	56.6	70.9
	36	1198	1756	1.47	27.40	26.35	1.05	113	59.4	77.1
Polystyrene	12a	2027	2513	1.24	27.4	27.0	0.4	80.7	55.7	65.1
	11a	3659	4244	1.16	26.0	25.4	0.6	199	154	172
	8b	9966	11162	1.12	23.3	23.0	0.3	1056	868	943
	2a	18000	19800	1.10	21.8	21.6	0.2	2839	2408	2581

* Calculated Intrinsic Viscosity
Poly(oxypropylene): K = 0.00040, a = 0.68
Polystyrene: K = 0.00011, a = 0.725

TABLE 5

Analysis of GPC Calibration Curves in Terms of Linear Equations

Polymer	Waters GPC	Column* Set	Mark-Houwink Constants k $dl/g \times 10^4$	a	Intercept Log d Fig. 3,4,5	Slope e ml^{-1} Fig. 3,4,5	Intercept Log Kd^{a+1}	Slope e (a+1) ml^{-1}	Intercept Log A Fig. 6,7,8	Slope b_{-1} ml Fig. 6,7,8	Slope e_{-1} ml^{-1} Fig. 9	Slope e (a+1) ml^{-1}
Cellulose Nitrate	200	B	1.3	0.80	11.83	0.0333	17.40	0.0599	17.20	0.0588	0.0325	0.0586
Cellulose Nitrate	202/401	C	1.3	0.80	9.99	0.154	14.10	0.277	14.05	0.275	0.154	0.277
Poly(pxypropylene)	200	II	4.0	0.68	7.42	0.0317	9.07	0.0533	9.49	0.0565	0.0332	0.0558
Polystyrene	200	B	1.1	0.725	11.79	0.0317	16.37	0.0548	17.20	0.0588		
Polystyrene	202/401	C	1.1	0.725	10.27	0.156	13.76	0.269	14.05	0.275		
Polystyrene	200	II	1.1	0.725	7.91	0.0336	9.69	0.0580	9.49	0.0565		

* Column Set B 2.5×10^4, 10^5, 3×10^5, 10^6, 10^7 A.U., Styragel packing

C 10^2, 10^3, 10^4, 10^5, 10^6 A.U., Micro-Styragel packing

II 2.25×10^2, 1.4×10^3, 1.4×10^3, 3.5×10^3, A.U., Styragel packing

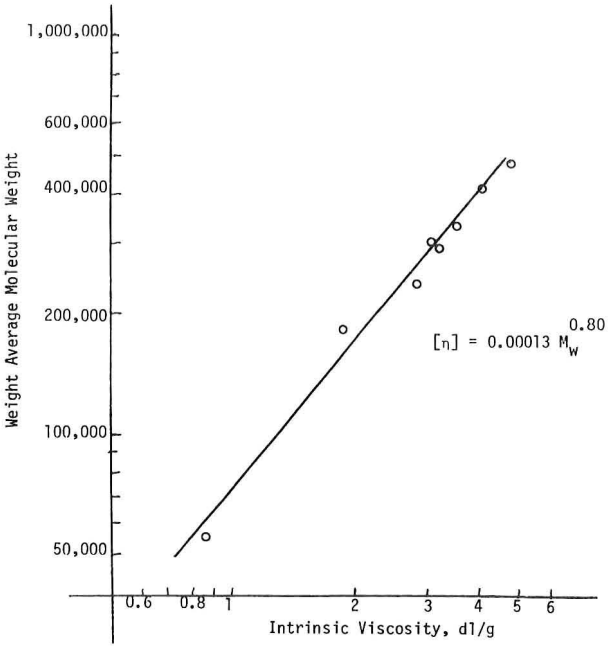

Figure 1: Mark-Houwink Relation for 12.0-12.6% N Cellulose Nitrate in THF at 25°C

molecular weight were plotted as the hydrodynamic volume against elution volume at peak height. The results are shown in Tables 2 , 3 , and 4 and as lines without points in Figures 6, 7, and 8.

d. Cellulose Nitrate and Poly(oxypropylene) Analysis

The GPC curves for cellulose nitrate and poly(oxypropylene) were analyzed using the method described above. In the case of cellulose nitrate the elution volumes at the peak heights were plotted against the number average molecular weight (Mn) that had

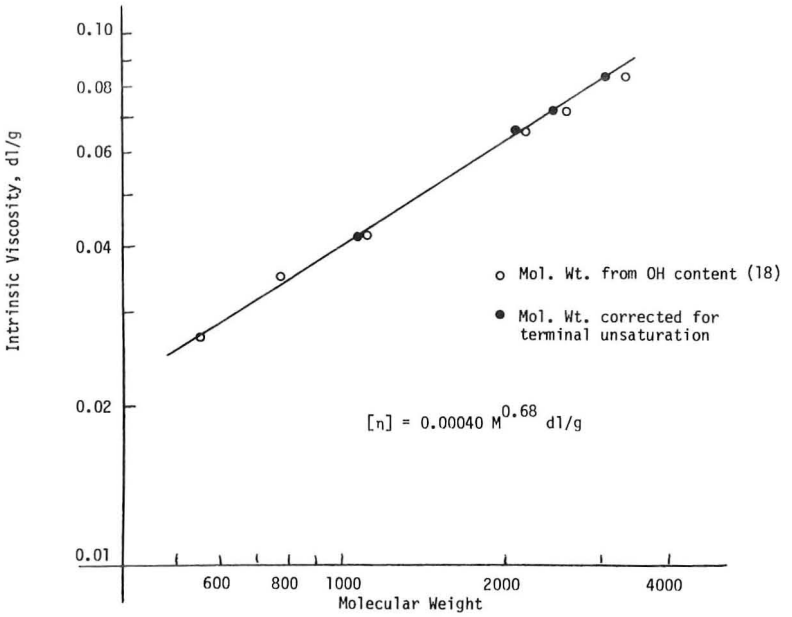

Figure 2: Intrinsic Viscosity - Molecular Weight Relation for Polypropylene Oxide of Narrow Molecular Weight Distribution in THF at 20°C

been determined independently by membrane osmometry. From the first approximate curve, the elution volumes at Mn were calculated. These elution volumes were in turn plotted against Mn (osmometer) forming a new calibration curve. The process was repeated and the final calibration curve approached by successive approximations. Elution volumes and molecular weights from fractionated N/C samples were also used to calculate calibration curves using data of Carignan and Turngren (14).

Results on cellulose nitrate using the Waters 200 and column set B are given in Table 2 and Figure 3 while those using the Waters 202/401 and column set C are shown in Table 3 and Figure 4. The scatter in the points is attributed mainly to the molecular weight procedure.

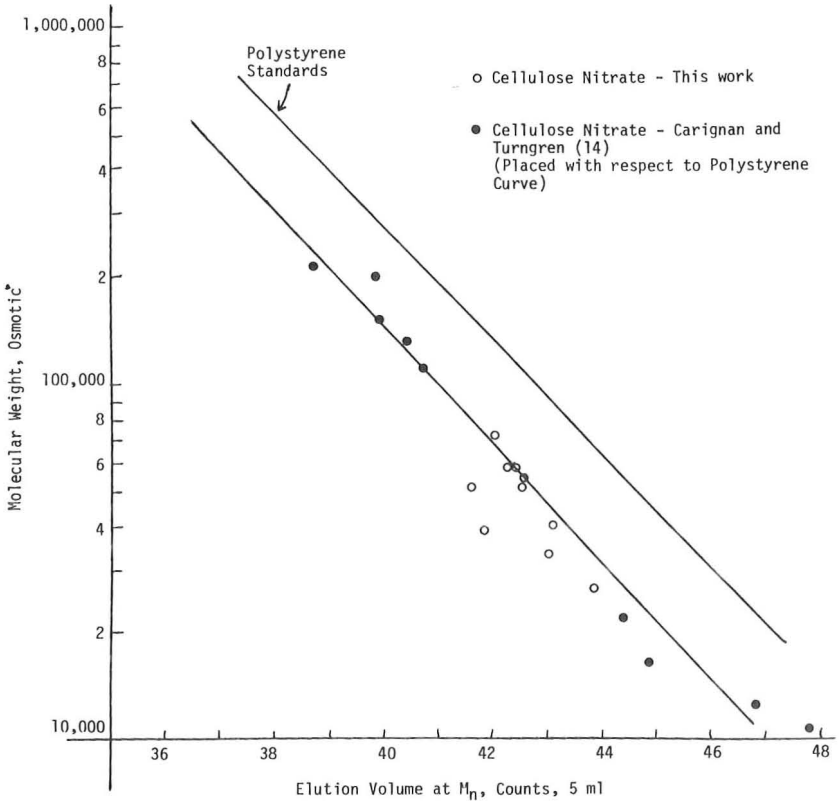

Figure 3: GPC Calibration Curve for Cellulose Nitrate Using Waters 200 Instrument and Column Set B

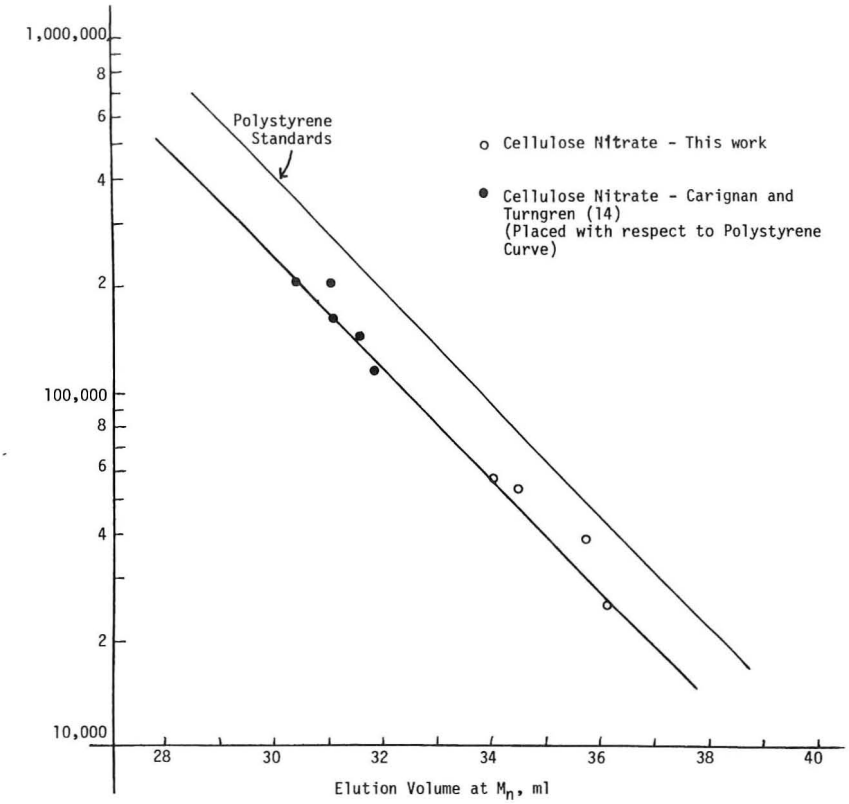

Figure 4: GPC Calibration Curve for Cellulose Nitrate with Waters 202/401 High Pressure Instrument and Column Set C

Results of Carignan and Turngren (14) were used to give a sufficient molecular weight range. Their data were placed with respect to the two polystyrene curves from their paper and this work. The cellulose nitrate calibration using column set B and

the Waters 200 instrument was:

$$\text{Log } M_n = 11.83 - 0.0333C \quad - - - - - - - - - - \quad 20.$$

With the high pressure Waters 202/401 and column set C the calibration was:

$$\text{Log } M_n = 9.99 - 0.154C \quad - - - - - - - - - - \quad 21$$

where C is in milliliters.

Molecular weights calculated from this calibration curve (Eq. 21) for five cellulose nitrate lots are given in Table 3 . As expected, the number average molecular weights are comparable with those obtained using the Waters 200 and column set B which was packed with Styragel as opposed to Micro-Styragel in column set C. However, in all cases the dispersity values, M_w/M_n, found with the Micro-Styragel in the high pressure instrument were lower than found with Styragel in the Waters 200. Since standard number average molecular weight values were used to calibrate each instrument, the two instruments must in principle yield the same number average molecular weights. The weight averages and dispersity may be different since different column sets were used. Columns of 10^7 AU pore size can be used with the low pressure Waters 200 instrument but can not be made for the 202/401. Hence exclusion results at high molecular weights with the latter instrument.

Results on Poly(oxypropylene) using the Waters 200 GPC and column set II are given in Table 4 and Figure 5. The line in Figure 5 obeyed the relation:

$$\text{Log } M_n = 7.42 - 0.031C \text{ - - - - - - - - - - -} \quad 22$$

where C is in milliliters.

It must be noted that this relation is given incorrectly in the previous paper (11) which, however, does not invalidate the other data and conclusions reached therein.

Intrinsic viscosities [η] of cellulose nitrate were determined on the samples shown in Table 1 in tetrahydrofuran (THF) at

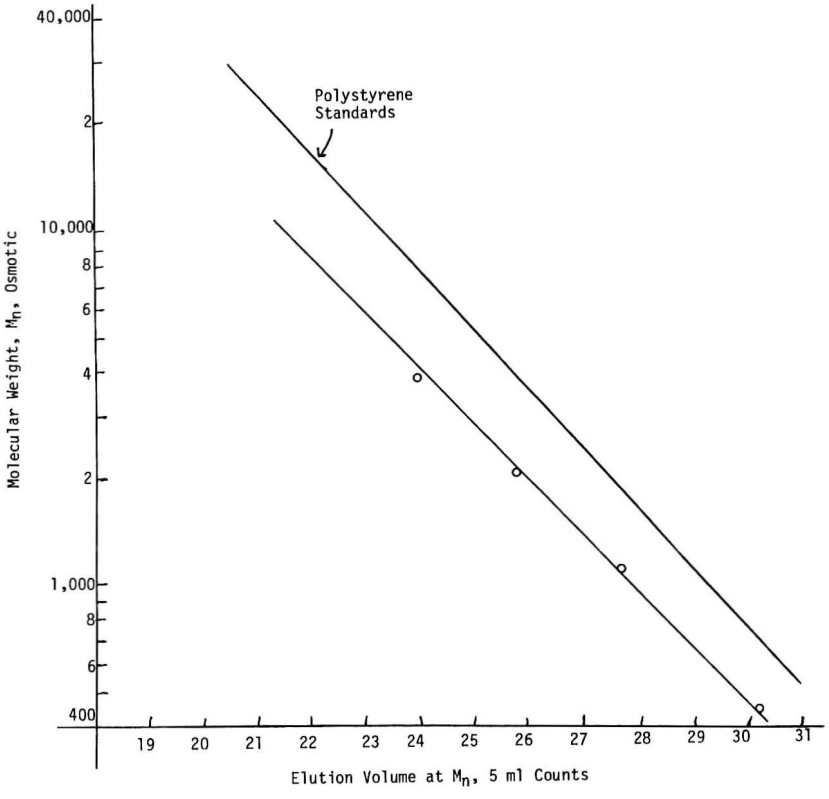

Figure 5: GPC Calibration Curve for Polypropylene Oxide Using Waters 200 Instrument and Column Set II

25°C with results given in Tables 2 and 3 . A plot of the data in Figure 1 gave the relation:

$$[\eta] = 0.00013\ M_w^{0.80} \quad - - - - - - - - - - - - \quad 23.$$

This result compares with $0.00023\ M_w^{0.84}$ found by Timpa and Segal (15) for cellulose trinitrate in THF at 25°C. Concentration in both cases was in g/dl. If intrinsic viscosity in Figure 1 is corrected to give intrinsic viscosity of cellulose trinitrate using the method of Lindsley and Frank (16) one obtains

$$[\eta] = 0.00018\ M_w^{0.84} \quad - - - - - - - - - - - - \quad 24.$$

Intrinsic viscosities of the poly(oxypropylene) mixtures in THF were calculated from the equation

$$[\eta] = 0.00040\ M_w^{0.68}, \text{ dl/g at } 20°C \quad - - - - - \quad 25.$$

This relation is a modification of that of W. Scholtan and W.Y. Lie (18). These workers determined intrinsic viscosity as a function of molecular weight calculated from hydroxyl number. They did not correct for the presence of terminal unsaturation which becomes of some importance for poly(oxypropylene) above molecular weight 2000. Using results found previously (11) terminal unsaturation can be estimated as a function of molecular weight and used to correct equivalent weight from hydroxyl analyses. At molecular weight 1000 terminal unsaturation is 0.022 meq/g; at molecular weight 2000 it is 0.033 meq/g; and at molecular weight 4000 it is 0.074 meq/g. Figure 2 shows the results of Scholtan and Lie (18) after such a correction.

DISCUSSION

To summarize what was done as a result of work outlined in the previous section:

1. From known values of M_n and from elution volumes were determined calibration relations between Log M_n and elution volumes at M_n in the form of Equations 15, 17, 18, 20, 21, and 22 as shown in Figures 3, 4, and 5.

2. These calibration curves were used to calculate GPC values of M_n, M_w, M_w/M_n, C_n, C_w, and (C_n-C_w) which are given in Tables 2 , 3 , and 4.

3. Using experimental values of intrinsic viscosity or known Mark-Houwink constants given in Equations 19 and 25 the functions $[\eta]M_w$, KM_n^{a+1}, and $[\eta]M_n$ were calculated and are shown in Tables 2, 3, and 4 .

4. The functions Log $[\eta]M_w$, Log KM_n^{a+1}, and Log $[\eta]M_n$ were plotted against elution volume as shown in Figures 6, 7, and 8 and the Logarithm of the dispersity, M_w/M_n, was plotted against (C_n-C_w) as shown in Figure 9.

5. The slopes and intercepts of the linear curves in Figures 6, 7, 8 and 9 were calculated and tabulated in Table 5 together with those from Equations 15, 17, 18, 20, 21 and 22 taken from Figures 3, 4, and 5.

Since straight line calibration curves were used to relate Log M with elution volume, if Equations 10 and 11 hold, then plots of Log KM_n^{a+1} and Log KM_w^{a+1} versus elution volume must form straight

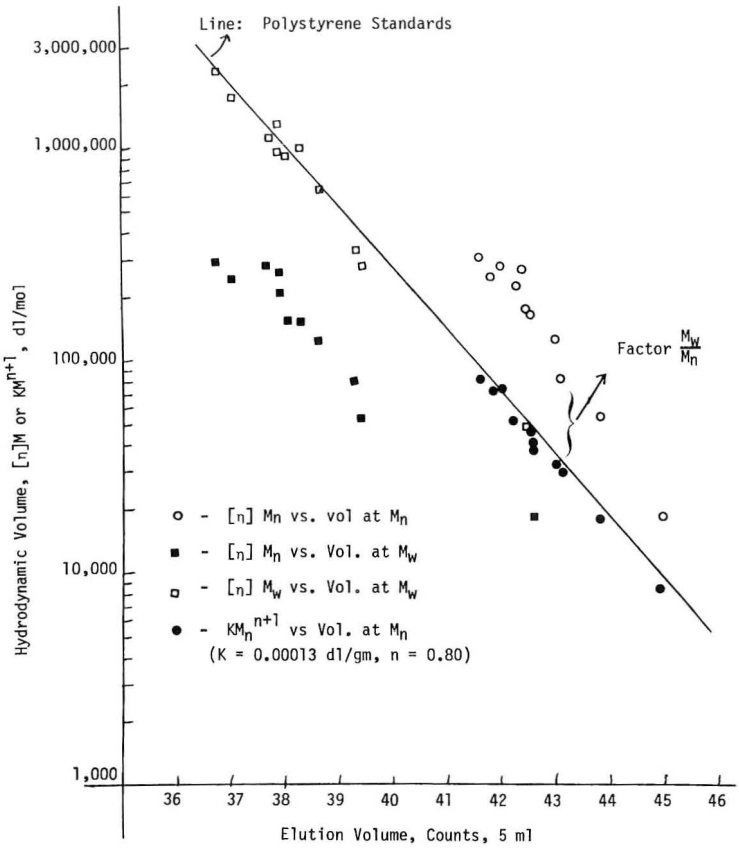

Figure 6: Comparison of Number and Weight Average Molecular Weights for Universal GPC Calibration using Nitrocellulose Samples and Measured Intrinsic Viscosities.

lines. The line formed, however, will lie on that of the polystyrene standards only if the functions KM_n^{a+1} and KM_w^{a+1} represent true hydrodynamic volumes.

From the results we wish to see several things:

a. First, does Log $[\eta]M_\eta$ vary directly with elution volume for different samples and does the curve formed lie on the Log $[\eta]M$ versus

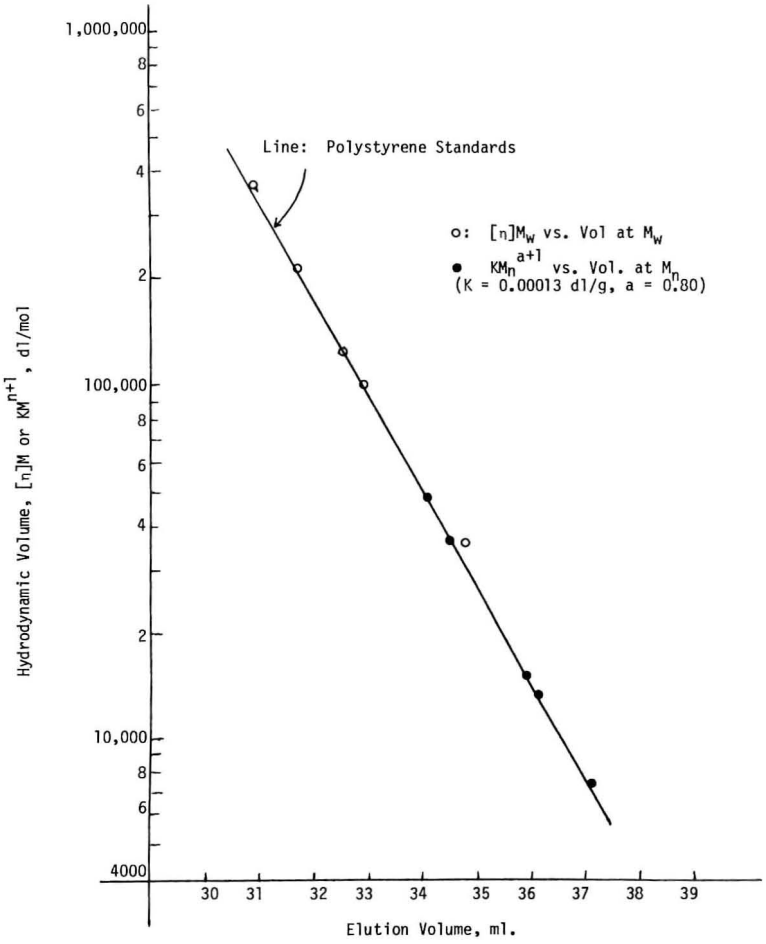

Figure 7: Low Resolution Universal GPC Plot for Cellulose Nitrate with the Waters 202/401 Instrument Using Measured Intrinsic Viscosities

elution volume curve for nearly monodisperse standard polystyrene samples? If it does not lie on the polystyrene curve, is the factor separating the two curves the dispersity M_w/M_n, in accordance with the factor between Equation 3 and Equations 10 and 11?

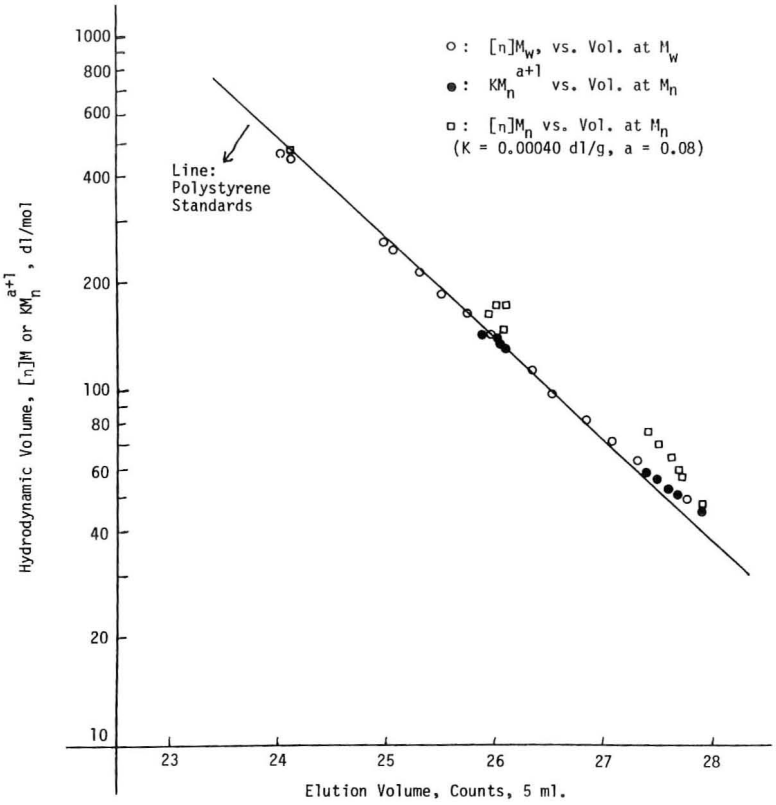

Figure 8: Comparison of Different Average Molecular Weights in Universal GPC Calibration Using Polypropylene Oxide Samples and Calculated Intrinsic Viscosities

An examination of Figures 6 and 8 shows that Log [η]M_n plotted against either volume at M_n or M_w does not lie on the polystyrene universal plot. The factor between [η]M_n for cellulose nitrate and [η]M for polystyrene at the same elution volume is in fact equal to M_w/M_n. In Figure 8 a clear progression is shown between [η]M_n for poly(oxypropylene) and the polystyrene curve as the dispersity increases from 1.03 to 1.53.

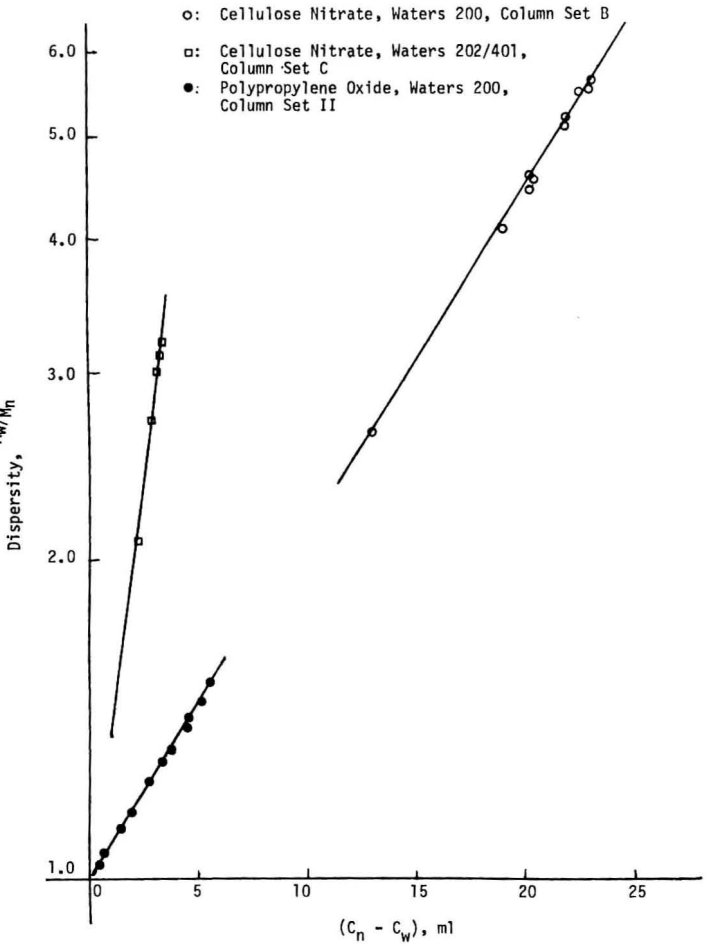

Figure 9: Relation Between GPC Dispersity and Elution Volume Separation, C_n-C_w.

b. Secondly, do Log KM_n^{a+1} and Log $[\eta]M_w$ (or Log KM_w^{a+1}) versus M_n and M_w respectively fall on the polystyrene curve in accordance with Equations 10, 11, and 12? Figures 6, 7, and 8

show that these equations are certainly obeyed within the limits of error.

c. Thirdly, is the dispersity related to the difference between elution volume at M_n and M_w in accordance with Equation 14 and does the constant in Equation 14 have the same value as e in Equation 9? The logarithm of the dispersity is plotted against (C_n-C_w) in ml in Figure 9. Linear relations result whose slope is given in the next to the last column of Table 5. In the three cases the slopes are essentially the same as found from Equation 9 in Figures 3, 4, and 5. As above, the excellence of the fit of the points to the lines is because linear calibration curves were employed and not because of the quality of the experimental techniques.

d. Fourth, do the relations between the constant in Equation 9 and Equations 10 and 11 satisfy Equations 6 and 7? The intercept, Log d, and slope, e, from Equation 9 taken from Figures 3, 4, and 5 are given in Table 5 for the various systems. In accordance with Equations 6 and 7 these were converted to Log Kd^{a+1} and e(a+1) to give values of Log A and b. These values are compared in Table 5 with Log a and b from Equations 10, 11, and 12 determined from Figures 6, 7, and 8. Close agreement is found.

The numbers in Table 5 may be used to compare the column sets used with cellulose nitrate in the Water's 200 and 202/401 instruments. The resolution of the systems is $-\frac{dc}{dJ} = \frac{1}{2.3026bJ}$ where J is the hydrodynamic volume. That is, the higher the molecular

weight the poorer the resolution. For the 200 instrument $-\frac{dc}{dJ} = \frac{7.39}{J}$ and for the 202/401 $-\frac{dc}{dJ} = \frac{1.58}{J}$. These values for the particular column sets employed would be higher for the 202/401 if a higher porosity column could have been used.

CONCLUSION

When the molecules in the detector all obey the same Mark-Houwink relation, Equations 10, 11, and 13 constitute a "Universal GPC Calibration" in term of KM^{a+1} of any molecular weight average, of $[\eta]M_v$, or approximately of $[\eta]M_w$. None of these expressions, however, is $[\eta]M_n$ which indeed has no meaning other then $[\eta]M$ when only one species is present. When the dispersity of the whole sample is large, use of KM_n^{a+1} doubles the number of points available for determination of the calibration curve because the points placed by KM_n^{a+1} will be at the low molecular weight end of the universal curve and those from KM_w^{a+1} will be at the upper. In theory one such sample might suffice for a determination of the curve. The factor between Equations 10, 11, and 13 and Equation 3 is the dispersity of the sample. As the dispersity approaches 1.0 a point placed by Equation 3 will approach the curve formed by Equations 10, 11 or 13.

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DEPENDENCE OF THE CAPACITY RATIO UPON TYPE OF THE EXCESS ADSORPTION ISOTHERM

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ABSTRACT

Model calculations of the capacity ratio for binary mobile phase "1+2" and the excess adsorption isotherm of 1-st solvent have been made by assuming nonideality of mobile and surface phases and energetic heterogeneity of the adsorbent surface. These calculations make posible the study of correlation between shapes of the folloving functions: the capacity ratio and the excess adsorption isotherm as functions of the mobile phase composition.

INTRODUCTION AND THEORETICAL

According to the theoretical results of Jaroniec and al. (1-2) the capacity ratio of the r-th substance in binary mobile phase "1+2" can be evaluated as follows:

$$k_r = k_{1r}\, y_1^{1/m} + k_{2r}(1-y_1)^{1/m} + A_{12}(y_1 - x_1) \qquad (1)$$

where k_{1r} and k_{2r} are the capacity ratios of the r-th substance in 1-st and 2-nd solvent, respectively;

y_1 and x_1 are the mole fractions of 1-st solvent in the surface and mobile phases; m is the constant characterizing energetic heterogeneity of the adsorbent surface; A_{12} is the parameter describing the solute-solvent interactions. The mole fraction of 1-st solvent in the surface phase y_1 may be calculated according to the following equation(3):

$$y_1 = \left\{ 1 + \left[K_{12} x_1 / (1 - x_1) \right]^{-m} \exp \left[m q^l (M-1)(1 - 2x_1) + m L q^s (1 - 2y_1) \right] \right\}^{-1} \qquad (2)$$

where the parameters L, M, q^l and q^s have their usual meaning, i.e., q^l and q^s are the parameters characterizing the interactions in the bulk and surface phases, respectively; L and M are the parameters describing the lattice model of solution; K_{12} is the equilibrium constant for exchange reaction between molecules of 1-st and 2-nd solvents in both phases.

However, the excess adsorption isotherm of 1-st solvent $n^e_{1,t}$ is equal to:

$$n^e_{1,t} = n^s (y_1 - x_1) \qquad (3)$$

where n^s is the total number of moles in the surface phase.

Basing on equations 1,2 and 3, the following factors determining the liquid adsorption chromatography, can be studied:

(a) energetic heterogeneity of solid surface of random distribution of adsorption sites - the parameter m ,
(b) nonideality of the surface phase - the parameter q^s,
(c) nonideality of the bulk phase - the parameter q^l
(d) lattice model of the solution - the parameters L and M ,
(e) solute - solvent interactions - the parameter A_{12}.

Parameters q^s and q^l characterize the surface and bulk regular solution, respectively.

Now, we shall discuss the correlation between the excess adsorption isotherm of 1-st solvent equations 3 and 2 and the capacity ratio of the r-th solute in the binary mobile phase "1+2" equations 1 and 2 . This discussion gives answer on the following question: in such degree type of the excess adsorption isotherm determines shape of the curve k_r vs.x_1.

RESULTS AND DISCUSSION

Studies of Dąbrowski and al.(3) and Roe(4) showed that influence of lattice model of solution on the shape of the theoretical adsorption isotherms is small. Thus, a change of the parameters L and M i.e., change of the lattice model of solution, changes slightly the values of k_r. In Figures 1 - 3 the comparative model calculations of the excess adsorption isotherms $n^e_{1,t}(x_1)$ (equations 2 and 3) and capacity ratios $k_r(x_1)$ (equations 1 and 2) with $A_{12} = 0$ are presented. All model calculations have been made for the close-packed hexagonal

lattice, i.e., L = 0.5 and M = 0.25. For k_{1r} and k_{2r} the values 1.0 and 3.0 were assumed, respectively. The other parameters, characterizing the curves k_r vs.x_1 presented in Figures 1-3 have been summarized in Table 1. The excess adsorption isotherms $n^e_{1,t}(x_1)$ have been evaluated according to equations 3 and 2, whereas, the capacity ratios $k_r(x_1)$ were calculated according to equations 1 and 2 . For n^s the value 1.0 was assumed.

Table 1

PARAMETERS m, q^1 AND q^s USED IN CALCULATIONS OF THE FUNCTIONS $n^e_{1,t}(x_1)$ AND $k_r(x_1)$ PRESENTED IN FIGURES 1-3

The other parameters are equal to: K_{12}= 2.718 , k_{1r} =1.0 , k_{2r}=3.0 , L= 0.5, M=0.25, A_{12}=0, n^s=1.0

Figure	Code of the curve	m	q^1	q^s
1A, 1B	a	0.3	0	0
	b	0.6	0	0
	c	0.9	0	0
2A, 2B	a	0.9	-1	0
	b	0.9	0	0
	c	0.9	1	0
2C, 2D	a	0.9	-1	0
	b	0.9	-1	-1
3C, 3D	a	0.9	1	0
	b	0.9	1	1
3A, 3B	a	0.6	-1	-1
	b	0.6	0	0
	c	0.6	1	1

Now, we will describe succesively Figures 1-3. In Figure 1 the dependence of k_r and $n^e_{1,t}$ upon the heterogeneity parameter m is showed. It follows from this figure that for homogeneous surfaces, i.e., m = 1, the excess adsorption isotherm $n^e_{1,t}(x_1)$ is the U-shape isotherm according to Schay and Nagy clasification (5). However, for heterogeneous surfaces. i.e., for m belonging to the interval $(0,1)$, the S-shape excess adsorption isotherm may be obtained. The relationship k_r vs.x_1, corresponding to the U-shape excess adsorption isotherms are a decreasing functions. In the case of the S-shape adsorption isotherms, they show a minimum (see Figure 1).

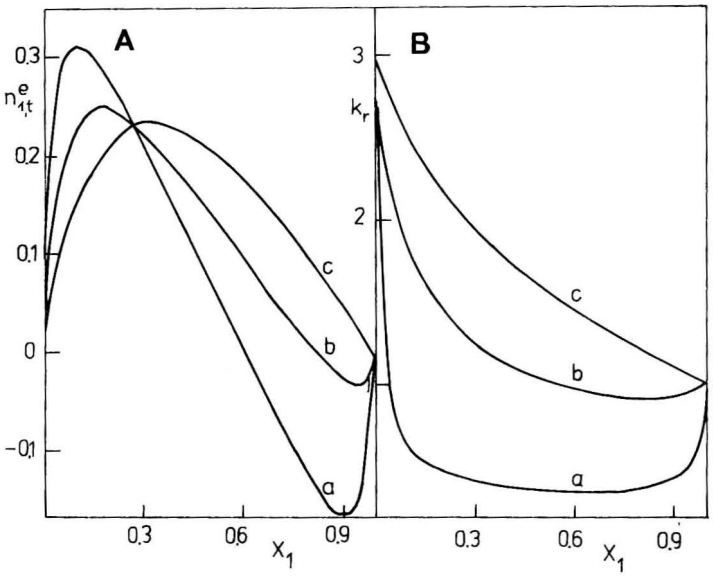

Figure 1
The functions $n^e_{1,t}(x_1)$ (part A) and $k_r(x_1)$ (part B) for different values of m . Parameters used in calculations are summarized in Table 1.

Thus, energetic heterogeneity of the adsorbent surface may be one of the reasons of minimum on the curves k_r vs.x_1.

Figures 2A and 2B shows the influence of the parameter q^1, characterizing the regular mobile phase, on the excess adsorption isotherm and the relationship k_r vs.x_1. It follows from these Figures that for different values of q^1 the curves $n^e_{1,t}(x_1)$ and $k_r(x_1)$ intersect at the same value of x_1. For negative values of q^1 the U-shape excess adsorption isotherms are observed, wereas, the positive values of q^1 can give the S-shape isotherms. For values of q^1 belonging to the interval $(-1,1)$ the curves $k_r(x_1)$ are a decreasing functions. For low concentrations of x_1 $k_r\left(q^1<0\right) > k_r\left(q^1 = 0\right)$, whereas, after the inflection point high values of x_1 this inequality is reversed. In the case of $q^1 > 0$, $k_r < k_r\left(q^1 = 0\right)$ at low concentrations of x_1 and $k_r > k_r\left(q^1 = 0\right)$ after the inflection point.

Figures 2C, 2D, 3C, 3D show the influence of the parameter q^s on the curves $k_r(x_1)$. The calculations were made for two cases: (a) the parameters q^1 and q^s are identical, (b) the parameter $|q^1|$ is grater than $|q^s|$. Such model is usually used in adsorption from solutions. The curves $k_r(x_1)$, presented in Figures 2C, 2D, 3C, 3D have similar shape as those showed in Figures 2A and 2B.

Figures 3A and 3B show the model calculations for heterogeneous solid surface m=0.6 and regular both phase $q^1 = q^s = (-1,0.1)$. It follows from these figures that effects of energetic heterogeneity of adsorbent surface are more significant that those connected with regularity of both phases (6,7).

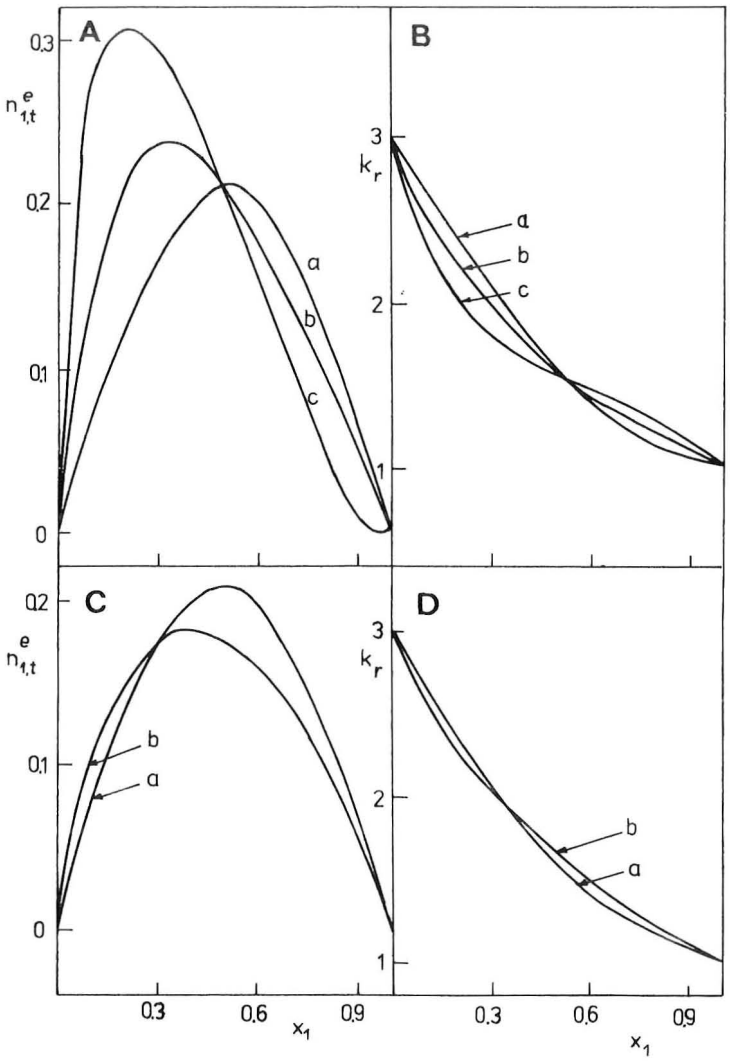

Figure 2
The functions $n^e_{1,t}(x_1)$ (parts A, C) and $k_r(x_1)$ (parts B, D). The parameters are summarized in Table 1.

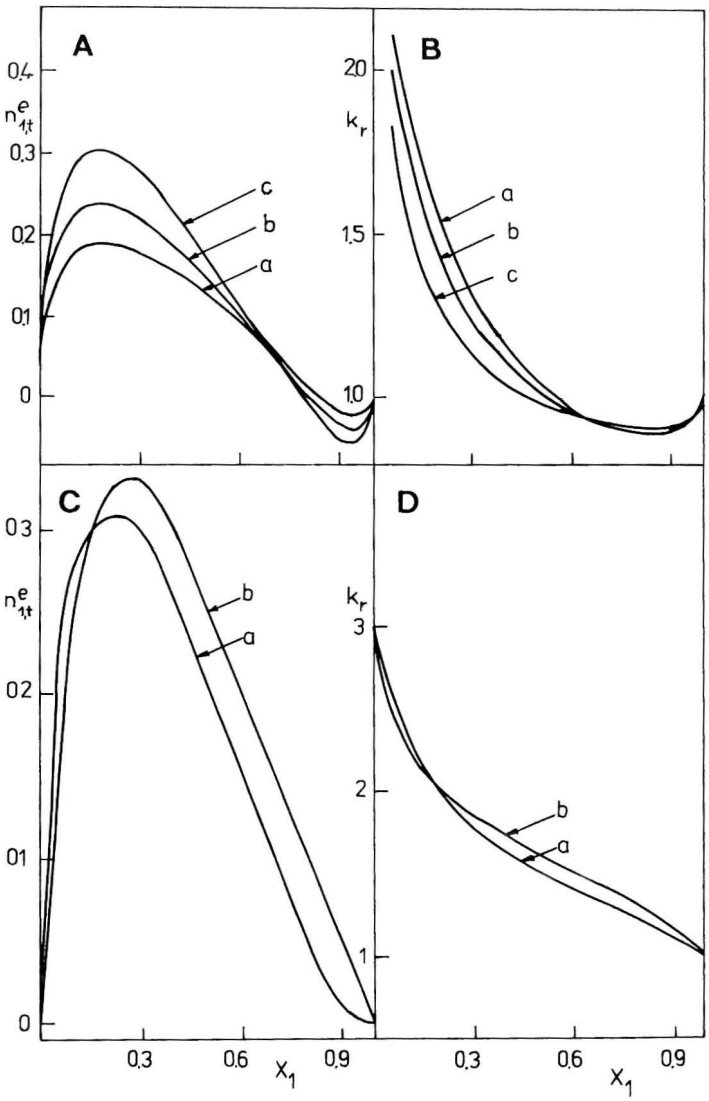

Figure 3
The function $n^e_{1,t}(x_1)$ (parts A, C) and $k_r(x_1)$ (parts B, D). The parameters are summarized in Table 1.

The conclusions resulting from presented model calculations are following:

(a) a correlation exists between the excess adsorption isotherm of 1-st solvent and the function $k_r(x_1)$: U-shape isotherms correspond to a decreasing k_r - functions without inflection point, whereas, S-shape isotherms relate to k_r- functions with inflectionpoint.

(b) k_r- curves with inflection point are most frequently obtained for strongly heterogeneous surfaces and regular solutions of positive deviations from Raoult's law.

(c) k_r- curves without inflection point are obtained for homogeneous adsorbent and regular solutions of negative deviations from Raoult's law.

The knowledge of excess adsorption isotherm, characterizing the system: mobile phase-adsorbent, is very important in liquid adsorption chromatography. The experimental values of $n^e_{1,t}$ may be directly used to determine the parameters m and A_{12}, according to the following equation :

$$k_r = k_{1r}\left(n^e_{1,t}/n^s + x_1\right)^{1/m} + k_{2r}\left(1 - n^e_{1,t}/n^s - x_1\right)^{1/m} + A_{12}/n^s \; n^e_{1,t} \qquad (4)$$

which is obtained from equations 1 and 3 . Application of equation 4 creates a new possibilities for characterization of chromatographic systems. Such investigations have been intiated in the paper (8) .

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RECYCLING TECHNIQUE IN PREPARATIVE LIQUID CHROMATOGRAPHY

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ABSTRACT

A theoretical and practical study of recycling technique is developped for preparative liquid chromatography. The optimal cycle number, n_{opt}, is determined for the separation of two solutes in such a way that the resolution between the second peak of cycle (n-1) and the first one of cycle n is equal to the resolution between the peaks of cycle n. A simple relationship is propounded to determine n_{opt} which only depends on retention volumes and external volume, but it does not depend on band spreading. With this recycling technique it is shown that the maximum injection volume under optimal recycling conditions is greater than n_{opt} times the maximum injection volume for one cycle. So the use of recycling can be opportune and beneficial to increase the throughput in preparative liquid chromatography.

INTRODUCTION

Preparative liquid chromatography is probably one of the separation methods that requires much effort in order to optimize its

peculiar parameters such as recovery ratio, throughput, costs .... For the separation and collection of a component by liquid chromatography, the method to be used must fit the purpose of the work, the available apparatus and staff. For routine work, it is necessary to optimize the method. On the other hand, for an occasional preparative work, the chosen method will be the faster one and will use an available device. For the recycling the column eluent (mobile phase and sample) is flowed through the column head until the resolution between the components is sufficient. From an industrial point of view, such a separation has the advantage to be more economical, with respect to the consumption of solvent and stationary phase, than an identical one obtained with a single elution. For an occasional operation, recycling may allow an immediate work with the available column even if its dimensions seem not to be appropriate for the preparative problem without recycling. However recycling is chiefly limited by two factors : firstly, the sample is not always a pure binary mixture but it often is a much more complex solution for which interferences may occur between the various compounds. In this case a pretreatment of sample makes it possible to isolate the two compounds of interest and to eliminate the other solutes. Secondly, the increase of the difference between the retention volumes resulting from the n passages of the compounds through the column is counterbalanced by the bandspreading originating in the external volume. So the recycling technique is not always possible and, in this work, we try to emphasize the conditions under which it can be used for a preparative work and its advantages in comparison with successive injections on the same column.

Up to now, many investigations dealt with the advantages of this technique in analytical chromatography and in preparative chromatography as well.

Martin (1) made an exhaustive study of the parameters that affect the quality of recycling and particularly the bandspreading

in connecting tubes and pump. This latter phenomenon had not been taken into account by Lesec (2,3) and Kalash (4) because it was probably negligible in their cases. This interesting simplification is quite justified by using columns packed with large particles,but it is no longer valid in modern preparative liquid chromatography, with the use of microparticles for which chromatographic dispersion decreases.

THEORY

If the column is volume overloaded and the phase system operates in the linear part of the solute partition isotherm, the maximal sample load, $Q_{i,max}$, depends on two terms : the maximal injection volume, $V_{i,max}$, and the maximal injection concentration, $C_{i,max}$. The maximal injection volume, $V_{i,max}$, is given by the next relationship (5) :

$$V_{i,max} = V_o \left[k'_1 (\alpha - 1)/R_{s,mini} - 1.25 (2 + k'_1 + \alpha k'_1)/\sqrt{N} \right] \quad (1)$$

where V_o is the column dead volume, N is the theoretical plate number of the column, k'_1 is the capacity factor of the first eluted solute, α is the selectivity factor between the two solutes, and $R_{s,mini}$ is the minimal resolution at preparative scale to obtain the recovery ratio and the purity desired. To a first approximation $V_{i,max}$ can be estimated with $R_{s,mini} = 1$.

The purpose of recycling is, on the one hand, the increase of the apparent dead volume V_o, on the other hand, the increase of the apparent plate number, N.

Equation 1 shows that the maximal injection volume, $V_{i,max}$, as well increases under these conditions. In order to examine how such aims can be reached, it is necessary to describe the recycling pro-

blem in terms of chromatographic parameters and apparatus. For this study the sample is assumed to contain two solutes and the flow rate is kept constant. The resolution between peaks 1 and 2 after a single passage, R_1, can be written :

$$R_1 = (V_{R2} - V_{R1})/2\ (\sigma_{c,1} + \sigma_{c,2}) \qquad (2)$$

where V_{R1} and V_{R2} are the retention volumes of peaks 1 and 2 after a single passage i.e. the volumes of mobile phase flowing through the detector between sample injection and passage of the maxima of the peaks 1 and 2 in the detector respectively. $\sigma^2_{c,1}$ and $\sigma^2_{c,2}$ are the volume variances of these peaks after a single passage. Let V_A be the "recycling volume" i.e. the volume of the liquid phase in the apparatus between the outlet of the detector cell and the inlet of the injection device, and σ^2_A the volume variance of the peak broadening originating in this volume V_A. The "pseudo retention volumes" after n cycles for peaks 1 and 2 are respectively :

$$V_{1,n} = n\ V_{R1} + (n - 1)\ V_A \qquad (3)$$

$$V_{2,n} = n\ V_{R2} + (n - 1)\ V_A \qquad (4)$$

and the volume variances are :

$$\sigma^2_{1,n} = n\ \sigma^2_{c,1} + (n - 1)\ \sigma^2_A \qquad (5)$$

$$\sigma^2_{2,n} = n\ \sigma^2_{c,2} + (n - 1)\ \sigma^2_A \qquad (6)$$

These preceeding parameters are summarized in Figure 1 (for n = 2).

If we assume that, for two adjacent peaks, the contribution of the column to the volume variance does not depend on the retention :

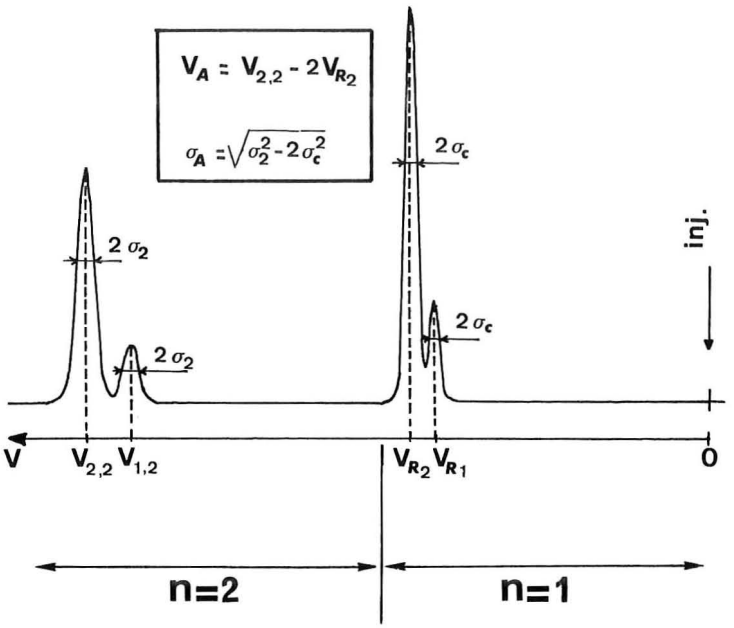

Figure 1 : Principal parameters of interest in recycling technique.

$$\sigma_{c,1} = \sigma_{c,2} = \sigma_c \tag{7}$$

and therefore :

$$\sigma_{1,n} = \sigma_{2,n} = \sigma_n \tag{8}$$

with $\lambda = \sigma_A^2/\sigma_c^2$, Martin (1) showed that the resolution after n cycles is given by :

$$R_n = R_1 \sqrt{n} \,/\, \sqrt{1 + \frac{n-1}{n}\,\lambda} \tag{9}$$

R_n is larger than R_1 if n is larger than λ; under such conditions the recycling technique becomes profitable.

The resolution between the peak 2 of cycle (n-1) and the peak 1 of cycle n, R_m, decreases while the cycle number n increases because the total volume of the system is limited and the second peak of cycle (n - 1) tends to overtake the first peak of cycle n. In the same way as Equation 1, R_m can be written (for n > 1) :

$$R_m = (V_{1,n} - V_{2,n-1})/2\ (2\sigma_n + \sigma_{n-1}) \qquad (10)$$

or :

$$R_m = \frac{V_{R2} + V_A - n\ (V_{R2}-V_{R1})}{2\sigma_c \left(\sqrt{n}\ \sqrt{1 + \frac{n-1}{n}\lambda} + \sqrt{n-1}\ \sqrt{1 + \frac{n-2}{n-1}\lambda}\right)} \qquad (11)$$

For a value of n greater than 5, Equation 11 can be simplified by using the next approximation :

$$\sqrt{\frac{n-2}{n-1}} \simeq \sqrt{\frac{n-1}{n}} \simeq 1 - \frac{1}{2n} \qquad (12)$$

and Equation 11 becomes :

$$R_m = \frac{V_{R2} + V_A - n\ (V_{R2} - V_{R1})}{2\sigma_c\ \sqrt{n}\ \sqrt{1 + \frac{n-1}{n}\lambda}\ \left(2 - \frac{1}{2n}\right)} \qquad (13)$$

The optimal cycle number, n_{opt}, can be defined from a given value of R_m (1) or by the cycle number for which R_m is equal to R_n (4). For a preparative purpose, the latter criterion is better to define n_{opt}, and Equations 2, 11 and 13 give :

$$n_{opt} = (5\ V_{R2} - V_{R1} + 4\ V_A)\ /\ 8\ (V_{R2} - V_{R1}) \qquad (14)$$

The value of n_{opt} differs from the one proposed by Kalash (4). This difference originates in the fact that we take into account

the volume and the band broadening in external region out of the column. n_{opt} can be expressed in terms of the chromatographic parameters (k'_1 and α) and $\mu = V_A/V_o$:

$$n_{opt} = \frac{4\ (1 + \mu) + (5\alpha - 1)\ k'_1}{8\ k'_1\ (\alpha - 1)} \qquad (15)$$

When the capacity factor, k'_1, increases the optimal cycle number, n_{opt}, decreases down to a minimal value, $(5\alpha - 1)/8(\alpha - 1)$, which only depends on the selectivity factor, α.n_{opt} increases while α decreases. Futhermore n_{opt} increases with μ i.e., for a given column, the external volume, V_A, acts as a reservoir for the peaks. But, from Equation 9, it is difficult to know the variation of the final resolution, $R_{n_{opt}}$, with V_A because V_A and σ_A^2 (i.e.λ and μ) are each other dependent ; the relationship between V_A and σ_A^2 depends on the geometrical characteristics of the external hydraulic circuit. Recycling is interesting only if :

$$n_{opt} > \lambda \qquad (16)$$

For the calculation of the maximum injection volume in recycling technique, $(n_{opt}\ V_o)$ and N_{opt} are substituted for V_o and N respectively in Equation 1 :

$$(V_{i,max})_{n_{opt}} = n_{opt}\ V_o \left[k'_1(\alpha-1)/R_{S,mini} - 1.25\ (2+k'_1 + \alpha k'_1)/\sqrt{N_{opt}}\right] \qquad (17)$$

with :

$$N_{opt} = N \frac{n_{opt}}{1 + \frac{n_{opt} - 1}{n_{opt}}\ \lambda} \qquad (18)$$

$(n_{opt}\ V_o)$ and N_{opt} are the apparent dead volume of the column and the apparent plate number respectively after the optimal cycle number, n_{opt}. If Inequality 16 is verified, the combination of Equa-

tions 1, 17 and 18 enables to deduce :

$$(V_{i,max})_{n_{opt}} > n_{opt} V_{i,max} \quad (19)$$

The result is very interesting : the maximal injection volume with the recycling technique under optimal conditions is larger than n_{opt} times the maximal injection volume with a single passage ; moreover, the operation times for n_{opt} cycles and for n_{opt} successive injections are roughly equivalent, so the recycling technique allows the increase of throughput in comparison with successive injections.

EXPERIMENTAL

The liquid chromatograph consists of the following components :

- a Waters M 6000 A reciprocating pump (Waters, Paris, France) as elution pump which allows recycling operation by means of a special manifold.
- An Orlita AE 10 - 4 diaphragm pump (Touzart et Matignon, Vitry, France) to supply the sample solution.
- A Rheodyne 7010 valve (Touzart et Matignon) as commutation valve between the two pumps.
- A stainless steel column, 100 cm x 0.47 i.d., slurry packed with silica gel Partisil 20 (Whatmann, Ferrières, France), 19 µm mean particle size.
- A Pye Unicam LC 55 spectrophotometer (Pye Unicam, Paris, France) or an Altex model 153 analytical UV photometer (Touzart et Matignon) operating at 254 nm.

The scheme of chromatograph is given in Figure 2. The solvents and chemicals are of high purity and were purchased from various suppliers.

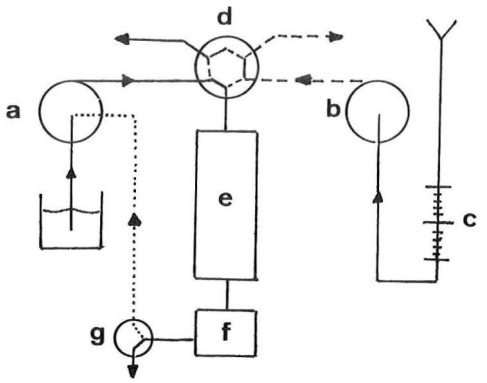

Figure 2 : Scheme of the chromatograph.
a : elution pump ; b : injection pump ; c : precision burette ; d : 6 way valve ; e : column ; f : detector ; g : recycling manifold.

RESULTS AND DISCUSSION

The validity of Equation 15 is examined for different values of the parameters k'_1, α and μ. For this study the samples are equimolar mixtures of butyl phtalate and isobutyl phtalate at about 10^{-4} mg/ml dissolved in the mobile phase. The mobile phases are binary mixtures of isooctane and ethyl acetate (from 98/2 v/v up to 90/10 v/v) in order to vary the capacity ratio values of solutes. The flow rate was 2.5 ml/mn. Experimental determinations give μ values of 0.40 and 0.23 when the experiments are performed with the Altex photometer and the Pye Unicam spectrophotometer respectively. The n_{opt} values are experimentally determined by checking the variation of R_n and R_m versus n. Figure 3 examplifies such a determination. We can notice the rapid decrease of R_m during the first cycles. On the other

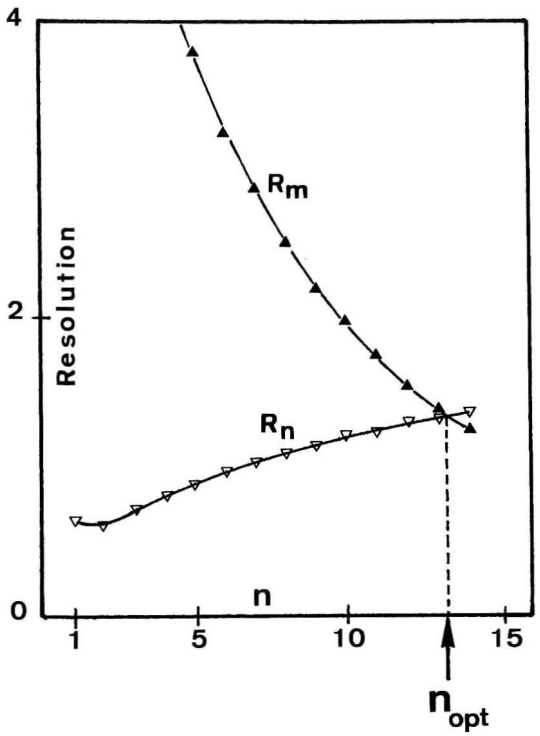

Figure 3 : Resolutions R_n and R_m versus the cycle number n.
λ = 2.09
μ = 0.23
k_1' = 1.23
α = 1.08

hand, R_n slowly increases during the same time, and even keeps constant until the third passage ; it is quite logical because λ is slightly larger than 2 and so R_n must become larger than R_1 only after the second passage. The optimal cycle number determined experimentally in this way is 13.6 ; it is in good agreement with the one calculated from Equation 15 i.e. 13.9.

Experimental and theoretical values of n_{opt} and $R_{n_{opt}}$ for different values of α , k_1', μ and λ are summarized in Table 1.

TABLE 1

Optimal cycle number under different experimental conditions.

	Experiment N°	1	2	3	4	5	6
	α	1.08	1.10	1.07	1.11	1.08	1.47
	k'_1	4.39	2.88	1.22	5.05	1.25	0.88
	μ	0.40	0.23	0.23	0.40	0.23	0.23
	λ	0.41	0.36	0.40	0.21	2.09	2.06
	R_1	1.28	0.95	0.60	1.21	0.65	1.78
n_{opt}	Experimental Value	8.6	7.6	15	5.9	13.6	4.4
	Theoretical Value	8.8	7.8	16	6.4	13.9	3.2
$R_{n_{opt}}$	Experimental Value	2.9	2.2		2.5	1.4	2.2
	Theoretical Value	2.7	2.0		2.6	1.4	2.3

Experimental measurements generally agree very well with theoretical values, the latter are always larger than the former except for the experiment N°6 in which the optimal cycle number is smaller than 5 ; in this case the approximations of Equation 12 is no longer valid.

So Equation 15 allows the determination of the optimal cycle number, n_{opt}, and consequently the calculation of resolution, $R_{n_{opt}}$, and standard deviation $\sigma_{n_{opt}}$, for this cycle number.

In the second part of this work, the validity of Equation 17 which gives the maximal injection volume after n_{opt} cycles, is illustrated with an example of separation : the two solutes are nitrated isomers of o-terphenyl : x-nitro o-terphenyl and 4-nitro

o-terphenyl (ponderal ratio : 1/4), eluted with a binary mixture of isooctane and dichloromethane (80/20 v/v) at a flow rate of 2.1 ml/mn. The concentration of the injected samples dissolved in the mobile phase is 1.1 mg/ml. The operating conditions determined after two passages are : x-nitro o-terphenyl retention volume V_{R1}= 30.5 ml, 4-nitro o-terphenyl retention volume V_{R2}= 32.9 ml, column standard deviation σ_c= 0.57 ml, external volume V_A= 3.48 ml, external standard deviation σ_A= 0.47 ml, resolution after a single passage R_1= 1.05. Under these conditions the calculated optimal cycle number is equal to 7.7 ; practically we chose the nearest smaller integer. After 7 cycles and for a small injected volume, the resolution, R_7, and the standard deviation, σ_7, are respectively equal to 2.2 and 1.83 ml. The maximum injection volume calculated from Equation 17 with $R_{s,mini} = 1$, is $(V_{i,max})_{n_{opt}}$ = 12.3 ml i.e. 6.7 σ_7. This sample volume is injected on the column and the solutes are collected : the end of the collection of the first peak and the beginning of the collection of the second one is performed at the valley between the two peaks. Analysis of the second collected fraction shows a 98 % purity for the 4-nitro o-terphenyl and demonstrates the validity of the maximum feed volume in recycling calculated according to Equation 17.

For the comparison between recycling technique and successive injection technique, sample volumes varying from V_i = 0.5 σ_c up to V_i = 3.08 σ_c are injected on the column and solute collection at the valley is performed after a single passage. The 98 % purity for the 4-nitro o-terphenyl is obtained for V_i = 1.2 σ_c which corresponds to V_i = 0.68 ml. 7 successive injections of the latter volume give an overall injected volume of 4.75 ml i.e. 2.6 times smaller than the maximum injected volume with the recycling operation. The recycling technique (7 cycles) and the successive technique (7 injections) require 266 ml and 240 ml respectively for the complete elution of the two solutes. So, in this case and under identical flow rate conditions, the throughput reached in recycling is 2.3 times larger than in usual elution.

CONCLUSIONS

For an occasional preparative work, the recycling technique can be used under volume overload conditions. There is an optimal cycle number, n_{opt}, for which the injection volume is maximum. This optimal cycle number only depends on the retention volumes of the two solutes and on the external volume out of the column. Furthermore the recycling technique under optimal conditions (after n_{opt} cycles) allows the increase of the throughput in comparison with n_{opt} successive injections.

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DETERMINATION OF MICROGRAM AMOUNTS OF COPPER BY PEAK PAPER CHROMATOGRAPHY

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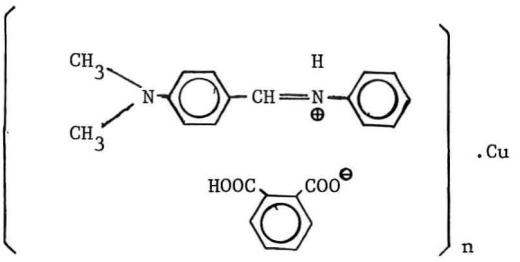
ABSTRACT

A chromatographic method has been developed for determining 1.00 to 6.00 μg l^{-1} of copper. The test solution (1 μl) was applied to paper impregnated with 0.1% phthalate salt of aniline and p-dimethylaminobenzaldehyde (p-DAB) based schiff's base solution and the chromatogram was developed with methanol, then immersed in aqueous 5% solution of potassium ferrocyanide for 35. The heights of the reddish brown peak shaped zones produced were related to concentration of copper. Equal amounts of silver, nickel and cobalt did not interfere. The error was $\leq \pm 0.20$ with standard deviation 0.105.

INTRODUCTION

Peak paper chromatography is a useful tool for the quantitative determination of microgram amounts of ions especially when one has to carry out a large number of estimations on small volume of samples (1-4). Ions are determined by peak chromatography on paper which has been impregnated with a sparingly soluble compound containing a precipitant ion for the test ion, the compound formed between the two being even less soluble than the impregnant itself. Microgram amounts of sulfate (5) and chloride (6) has been determined using barium chloride and mercury nitrate as precipitant respectively. Bismuthol-2 impregnated papers have been used for the determination of tellurium in presence of cadmium (7). Copper forms with phthalate salt of aniline and p-dimethylaminobenzaldehyde based schiff's base a bright green complex which is sparingly soluble in methanol.



In the present report we used the formation of this complex as the basis of a method for determining microgram amounts of copper in presence of silver, nickel and cobalt by peak paper chromatography.

EXPERIMENTAL

Reagents and Apparatus

Standard Stock solutions: The standard solution of copper was prepared by dissolving 1.0 g of copper metal of grade BDH (AR) in conc. HNO_3 on heating. Nitric acid was removed by evaporation until the moist-salt was reached, finally the residue was dissolved in water and transferred to a 100ml volumetric flask. The concentration of this solution was 10 mg cu/ml. Weaker solutions were prepared by diluting this stock solution.

Aqueous silver, nickel and cobalt salt solutions were prepared from BDH (AR) silver nitrate, nickel sulphate and cobalt sulfate respectively. The stock solution contained 10 mg of metal/ml. Weaker solutions were prepared by diluting the stock solution. Synthetic mixture of Cu-Ag, Cu-Ni and Cu-Co were prepared from solution of appropriate concentrations.

Schiff's base solution: The schiff's base was obtained by mixing equimolar solution of aniline phtalate and p-DAB (1:1) and finally recrystallized from methanol. 0.1% solution of this schiff's base was prepared in methanol and used for impregnation.

Preparation of papers: Chromatography was performed on Whatman No.1 paper strips of size 20x3 cm using 25x5 cm glass jars. Paper strips were impregnated in 0.1% solution of schiff's base for 3 to 5 seconds and then dried at room temperature.

Detection reagent: A 5% solution of potassium ferrocyanide was used for locating the peak of copper on paper.

Solvent used for development: Double distilled methanol was used as developing solvent.

Procedure

Determination of Copper: A sample of test solution (1 μl) was applied by means of a microsyringe to a impregnated paper strip. The chromatograpms were developed by ascending technique as usual. The ascent of solvent in each case was 15 cms., then the strips were dried at room temperature for 15 minutes and finally impregnated with potassium ferrocyanide to locate the peaks. The height of the peaks was measured in millimeters.

Determination of copper in presence of silver nickel and cobalt: Synthetic mixtures containing equal amounts (1 to 5 μg) of Cu-Ag, Cu-Ni and Cu-Co were applied by means of microsyringe to a impregnated paper strip. Rest of the procedure was same as in case of copper.

RESULTS AND DISCUSSION

The method is based on the fact that a circular spot of the precipitate is formed when test solution is applied to the paper strip. On ascending via capillaries in the paper, methanol elutes from the spot amount of test ion in excess of the stoichiometric amount of the precipitant on the area occupied by the spot. The excess of test ion reacts with fresh portions of impregnant along the methanol ascent line leaving behind on the paper a trace in the form of a precipitate peak with a reasonably correct shape. The curves relating copper concentration to the height of the peak formed, calculated by the least square method, are linear for the copper concentraion. Table-1 gives the experimental and calculated data. The error and standard deviation was formed to be $\pm$0.20 μg and 0.105 respectively.

Silver, nickel and cobalt also forms complexes with schiff's base whose solubility products are less than that of the copper complex with the same reagent. Experimental data showed that silver, nickel and cobalt taken in 1:1 ratio and in small excess with respect to copper (up to 5 μg) does not interfere with copper determination.

TABLE I. Experimental And Calculated Data For The Determination Of Copper In Chromatograms (n = 10).

Concentration of precipitant (%)	Mean Cu peak height (mm), $\bar{y}i$	Cu concentration in the drop applied (ug)	Amount of Cu determination (ug)	Error (ug)	Standard deviation (ug)
0.1	16.2	1.00	0.88	-0.12	
0.1	22.5	1.50	1.61	+0.11	
0.1	29.1	2.00	2.13	+0.13	
0.1	42.6	3.00	2.94	-0.06	0.105
0.1	51.6	4.00	3.88	-0.12	
0.1	57.2	5.00	5.00	0.00	
0.1	62.8	6.00	6.06	+0.06	

We analyzed mixtures (1:1) of Cu-Ag, Cu-Ni and Cu-Co at levels 1.0 to 5.0 μg in a drop of solution (1 μl). With this concentration range there is a linear relationship between copper concentration and the height of the peaks formed. The respective results are given in Table II. The error in determining copper in presence of silver, nickel and cobalt lies within the same limits as where these are absent.

To assess the accuracy of determination of paper impregnated with 0.1% solution of schiff's base we performed seven replicate determinations of copper solution of known concentration (Table III). After the chroma-

TABLE II. Seven Replicate Determinations of Copper (4.0 μg Cu Taken)

	Cu peak height (mm), $\bar{y}i$		Cu detected (μg) Xi calc.	Error (μg)	Standard deviation (μg)
	40.00		3.84	-0.16	
	40.0		3.84	-0.16	
	43.0		4.12	+0.12	
	41.0		3.93	-0.07	0.245
	44.5		4.27	+0.27	
	45.5		4.36	+0.36	
	44.0		4.22	+0.22	
Mean	41.14	Mean	4.14		

togram had been developed the copper concentration was found from a calibration curve plotted within the coordinates:
Copper content (μg) verses peak height (mm).

The calibration curve calculated by the least square method is of the form

$$X = \frac{yi + 564.55}{767.13}$$

Thus this method which is simple and rapid to carry out, can be used to analyze small amounts of material (a few μg). to work with small volumes of solution (a few ml) and to carry out a large number of determinations on such amounts.

TABLE III Experimental And Calculated Data For The Determination Of Copper In The Presence Of Equal Amounts Of Silver, Nickel And Cobalt.

Impurity added, μg	Amount of Cu applied μg, xi	Mean peak height, mm, yi	Yi	Amount of Cu determined μg	Error μg
1.0 Ag(I)	1.0	14.5	14.5	1.02	0.02
2.0 Ag(I)	2.0	29.7	31.1	1.91	0.09
3.0 Ag(I)	3.0	42.5	41.7	3.06	0.06
4.0 Ag(I)	4.0	49.0	50.0	3.92	0.08
5.0 Ag(I)	5.0	56.6	57.2	4.95	0.05
1.0 Ni(II)	1.0	16.4	14.2	1.15	0.15
2.0 Ni(II)	2.0	28.3	31.1	1.82	0.18
3.0 Ni(II)	3.0	42.3	41.7	3.04	0.04
4.0 Ni(II)	4.0	51.1	50.0	4.09	0.09
5.0 Ni(II)	5.0	57.1	57.2	4.99	0.01
1.0 Co(II)	1.0	17.2	14.2	1.21	0.21
2.0 Co(II)	2.0	29.5	31.1	1.90	0.10
3.0 Co(II)	3.0	42.2	41.7	3.04	0.04
4.0 Co(II)	4.0	52.1	50.0	4.17	0.17
5.0 Co(II)	5.0	57.1	57.2	4.99	0.01

Standard deviations for the determination of copper in presence of Ag(I), Ni(II) and Co(II) are 0.07, 0.13 and 0.145 respectively.

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GEL CHROMATOGRAPHIC BEHAVIOR OF LABILE METAL COMPLEXES

TRIMETA- AND TETRAMETAPHOSPHATE COMPLEXES WITH BIVALENT METAL CATIONS

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ABSTRACT

The gel chromatographic behavior of metal ions in a labile complex formation system was expressed as a function of the ligand concentration in an eluent and the stability constants of the complexes. Trimeta- and tetrametaphosphate complexes with bivalent metal ions were used as examples. The retention volumes of the metal complexes were found to be always greater than those of the corresponding free ligands.

INTRODUCTION

Much attention has been drawn to the gel chromatographic behavior of metal complexes. In some cases inert complexes have been used as samples(1-3). Labile systems have also been investigated, and it was recognized that the complex equilibria among solutes play an important role(4-8). The distribution coefficients of EDTA complexes and monomeric oxoanions of phosphorus have been found to be dependent on pH of an eluent(4,7). The mutual separation of these oxoanions were performed by adjusting eluent pH at a desired value(8).

In a previous paper(5) the authors reported a theoretical consideration on the gel chromatographic behavior of labile metal complexes. It was shown that the retention volume of magnesium in gel chromatography can be expressed as a function of tetrametaphosphate concentration in an eluent and the stability constant of the complex. To confirm the versatility of this theoretical approach the present paper describes the gel chromatographic behavior of magnesium, calcium, strontium, nickel and zinc in the presence of trimetaphosphate (P_{3m}) and tetrametaphosphate(P_{4m}). It can be expected from the previous work(9,10) that only one-to-one complexes are predominantly formed under the present experimental conditions. A solution containing a ligand was used as an eluent in order to prevent dissociation of a complex during elution. The stability constants of metal complexes were estimated and compared with those in the literature. The retention volumes of the metal complexes were found to be greater than those of the corresponding ligands.

MATERIALS AND METHODS

Sodium trimetaphosphate trihydrate and sodium tetrametaphosphate tetrahydrate were prepared according to the literature(11), and the purity was checked by paper chromatography.

A liquid chromatograph with an atomic absorption detector (Perkin-Elmer 403) previously described in detail (12-14) was used. A Sephadex G-15 column (8x650 mm) was equipped with a constant temperature jacket controlled at 25°C. A flow rate was maintained at 1.7 ml/min.

An eluent contained sodium salt of P_{3m} or P_{4m} of a desired concentration, ca. 10^{-3} M hydrochloric acid and 0.1 M sodium chloride as background electrolytes(pH 3.4). Hydrochloric acid was added to the eluent in order to prevent the adsorption of some kinds of bivalent metal ions to a Sephadex gel(15). In this eluent P_{3m} and P_{4m} acids are almost completely dissociated. Sample solutions which contain sodium salt of P_{3m} or P_{4m}, sodium chloride and hydrochloric acid of the same composition as that of the eluent were

prepared so as to contain bivalent metal ion of specific concnetrations, i.e., $2x10^{-5}$ M for magnesium and zinc and $1x10^{-4}$ M for calcium, strontium and nickel. After the column was sufficiently equilibrated with the eluent, one ml of sample solution was applied to the column.

RESULTS AND DISCUSSION

Representative elution patterns are shown in Fig. 1. It is evident that the retention volume of a metal is greatly dependent on the ligand concentration of an eluent. In order to describe the change in the apparent retention volume of a metal, $\bar{V}_M$, with ligand concentration in an eluent, a one-to-one complex formation reaction is considered

$$M + L \rightleftarrows ML \qquad (1)$$

where M, L and ML represent metal ion, ligand ion and complex, respectively. The stability constant of ML is expressed as follows

$$K = \frac{[ML]_m}{[M]_m[L]_m} \qquad (2)$$

where subscript m represents a mobile phase.

The apparent distibution coefficient of M, $\bar{K}_d$, can be expressed by eq.(3)

$$\bar{K}_d = \frac{[M]_s + [ML]_s}{[M]_m + [ML]_m} \qquad (3)$$

where subscript s represents a stationary phase. $\bar{V}_M$ can be expressed using the volume of the mobile phase, V_m, and that of the stationary phase in a given column, V_s, and $\bar{K}_d$.

$$\bar{V}_M = V_m + \bar{K}_d V_s \qquad (4)$$

Equation (4) can be rearranged to eq.(5) using eq.(2) and eq.(3), and V_M and V_{ML},

$$\bar{V}_M = \frac{V_M + K[L]_m V_{ML}}{1 + K[L]_m} \qquad (5)$$

where V_M is the retention volume of the free metal, and V_{ML} is that

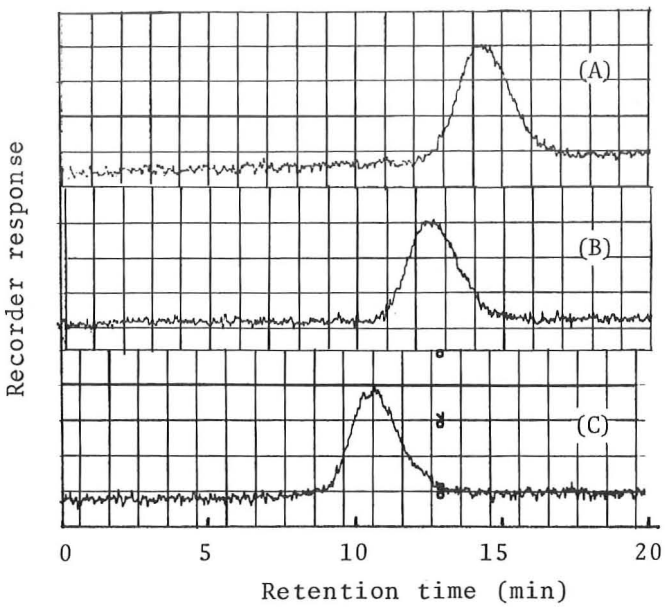

FIGURE 1.

Representative elution patterns for the Ca-P_{4m} system. The compositions of the eluents:

(A) 0.1 M NaCl + ca.10^{-3} M HCl

(B) 0.1 M NaCl + ca.10^{-3} M HCl + 4x10^{-4} M $Na_4P_4O_{12}$

(C) 0.1 M NaCl + ca.10^{-3} M HCl + 7x10^{-3} M $Na_4P_4O_{12}$

of the metal complex for the hypothetical system, in which the complex is present and the free metal is absent. It can be concluded that the apparent retention volume of the metal is expressed in terms of the free ligand concentration in the mobile phase and the stability constant of the complex in the mobile phase. This enables us to treat the gel chromatographic behavior of the labile complex by the theory similar to one applied in electrophoresis in which solutes migrate in a single phase(16,17).

V_M was determined by the elution of metal ion with 0.1 M sodium chloride and ca.10^{-3} M hydrochloric acid solution. Equation

(5) is valid only when V_M and V_{ML} are constant under these experimental conditions. In order to check the constancy of V_M, sample concentration dependence of V_M was examined (Fig. 2). It is clear that V_M values of magnesium, calcium, strontium, zinc and nickel

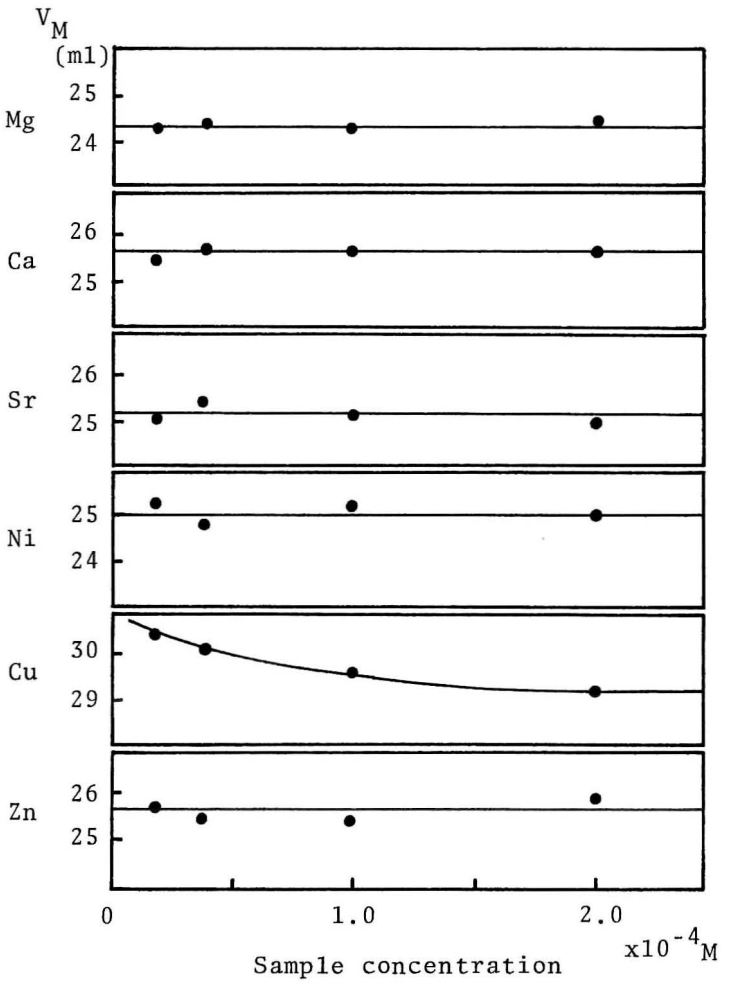

FIGURE 2.
Sample concentration dependence of V_M.

do not vary with sample concentrations. A V_M value of copper decreases with the increase in sample concentrations, which may be attributed to the adsorption of copper ions to the gel matrix.

Equation (5) can be rearranged to eq.(6).

$$\bar{V}_M = V_{ML} + \frac{1}{K} \cdot \frac{V_M - \bar{V}_M}{[L]_m} \qquad (6)$$

When the metal concentration in a sample is much smaller than the total ligand concentration, $[L]_m$ can be replaced by the total ligand concentration.

The plots of V_M vs. $(V_M - \bar{V}_M)/[L]_m$ for the calcium-trimetaphosphate and calcium-tetrametaphosphate systems are shown in Fig. 3.

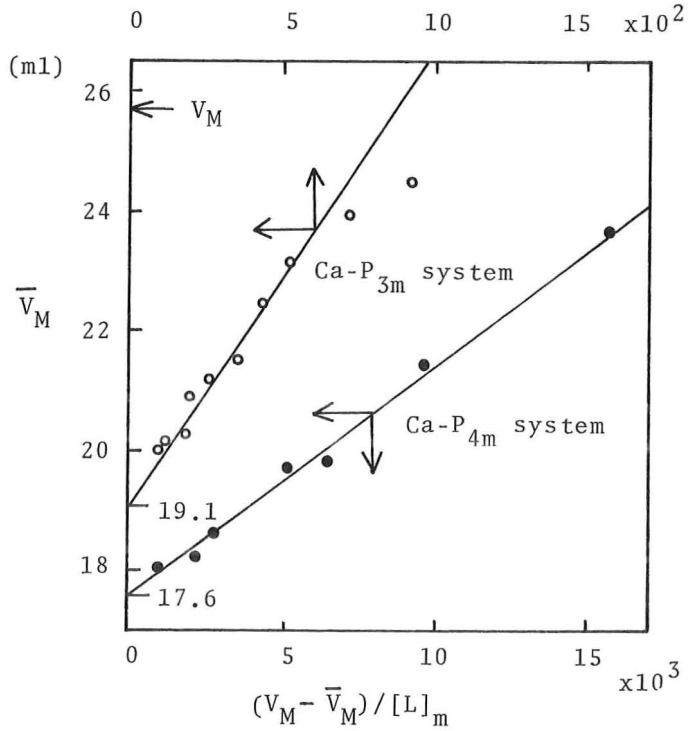

FIGURE 3.
Plots of $\bar{V}_M$ vs. $(V_M - \bar{V}_M)/[L]_m$ for the $Ca\text{-}P_{3m}$ and $Ca\text{-}P_{4m}$ systems.

The linear relationships thus obtained indicate the validity of the theory mentioned above including the constancy of V_M and V_{ML}. The y-intercepts gave the retention volumes of the complexes(V_{ML}), and from their slopes, the stability constants(K) were obtained. The experimental uncertainty greatly influences the value of $(V_M - \bar{V}_M)/[L]_m$, when the difference between V_M and $\bar{V}_M$ is small.

Equation (5) can also be altered to eq.(7), in which $\bar{V}_M$ can be expressed as a function of $\log[L]_m$. A similar equation has been employed in the analysis of electrophoretic mobility data of ineteracting systems(16,17).

$$\bar{V}_M = \frac{V_M + V_{ML}}{2} + \frac{V_M - V_{ML}}{2} \tanh\frac{2.303}{2}(-\log K - \log[L]_m) \quad (7)$$

K and V_{ML} were obtained by the least squares method. In Fig.4,

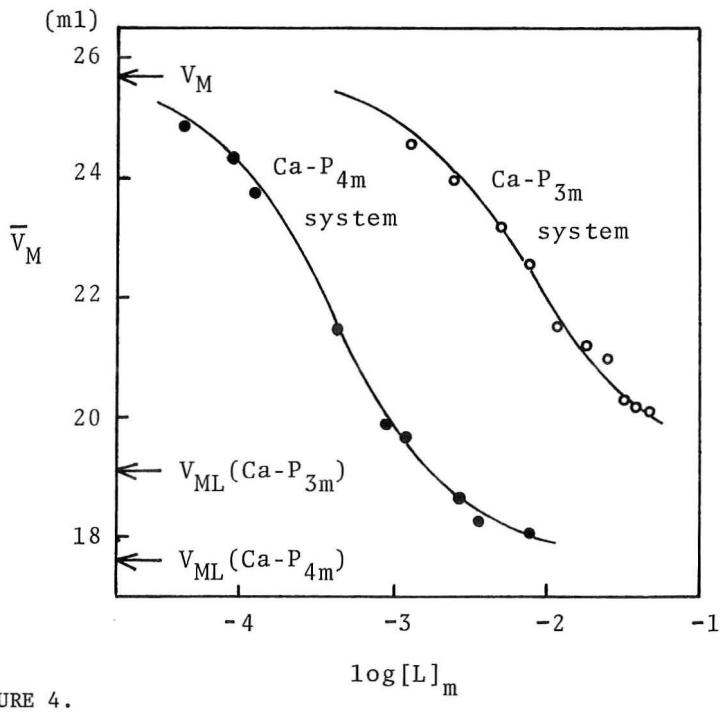

FIGURE 4.

Plots of $\bar{V}_M$ vs. $\log[L]_m$ for the Ca-P_{3m} and Ca-P_{4m} systems. The solid line refer to the calculated curves based on equation(7).

the plots of $\bar{V}_M$ vs. $\log[L]_m$ for the calcium-trimetaphosphate and calcium-tetrametaphosphate systems are shown. In Table 1 the stability constants thus obtained are listed together with the reported values obtained by other methods. Even though the methods are based on quite different principles the values are well consistent with each other, which supports the theory mentioned above.

In Table 2, K_{av} values of the metal complexes and the ligands are shown. It is noteworthy that the K_{av} values of the complexes are always greater than those of the corresponding ligands. A similar phenomenon was observed for EDTA complexes(20). The conformational change in these ligands by chelate formation and/or the dehydration of the ligands accompanying with the complexation may be the main reasons.

It has been pointed out that ion-pair formation should be taken into account when elution behavior of ionic species is examined in gel chromatography(3). The K_{av} values of trimetaphosphate

TABLE 2

K_{av} Values of Trimeta- and Tetrametaphosphate and Their Complexes

	free ligand	complexes				
		Mg	Ca	Sr	Ni	Zn
P_{3m}	0.14	0.27	0.28	0.30	0.28	0.31
P_{4m}	0.11	0.19	0.21	0.18	0.22	0.20

$K_{av}=(V_r - V_0)/(V_t - V_0)$; V_r is the retention volume, V_t is the total bed volume and V_0 is the void volume determined with Blue Dextran 2000.

and tetrametaphosphate obtained in this work with 0.1 M sodium chloride solution may be affected by the ion-pairing with sodium ions. In order to correlate the gel chromatographic behaviors of phosphorus oxoanions with their complexation reactions, more precise study is now in progress in our laboratory.

TABLE 1-1

Stability Constants of Trimetaphosphate Complexes

	This work	Other works					
	logK [a]	logK	Method [b]	Medium	Ionic Strength	Temp. (°C)	Ref.
Mg	2.51	1.80	A	NMe_4Cl	0.1	25	(18)
Ca	2.11	1.64	A	NMe_4NO_3	1.0	25	(18)
		2.50	B	NaCl	0.15	37	(19)
		2.06	B	NaCl	0.1	20	(9)
Sr	2.23	1.91	A	NMe_4NCl	0.1	25	(18)
		1.95	B	NaCl	0.15	20	(19)
		2.03	B	NaCl	0.23	20	(9)
Ni	2.29	1.82	A	NMe_4NO_3	0.1	25	(18)
Zn	2.29	2.00	A	NMe_4NO_3	0.1	25	(18)
		1.94	B	$NaClO_4$	0.23	20	(9)

TABLE 1-2

Stability Constants of Tetrametaphosphate Complexes

	This work	Other works					
	logK [c]	logK	Method [b]	Medium	Ionic Strength	Temp. (°C)	Ref.
Mg	3.39	3.47	A	NMe_4NCl	0.1	25	(18)
Ca	3.39	3.04	A	NMe_4NO_3	1.0	25	(18)
		3.36	B	NaCl	0.15	37	(19)
		3.28	B	NaCl	0.1	20	(9)
Sr	3.18	2.80	B	NaCl	0.15	20	(19)
		2.70	B	NaCl	0.23	20	(9)
Ni	3.15	3.38	A	NMe_4NO_3	0.1	25	(18)
Zn	3.37	3.63	A	NMe_4NO_3	0.1	25	(18)
		2.86	B	$NaClO_4$	0.23	20	(9)

a) I = 0.10 ∿ 0.38 , b) A:potentiometry(ion-selective electrode) B:ion-exchange method, c) I = 0.10 ∿ 0.18

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ESTIMATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC RETENTION INDICES OF GLUCURONIDE METABOLITES

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ABSTRACT

The retention indices of several glucuronide metabolites and their parent compounds were measured using a reversed-phase HPLC system. It was found that the typical glucuronide metabolite had a retention index 244 ± 31 units lower than the parent compound.

INTRODUCTION

Though glucuronide formation is the most common pathway to the production of mammalian urinary metabolites of drugs and organic compounds, surprisingly little is known about the chromatographic properties of this important group of compounds. The most common approach to the identification and quantitation of these metabolites is to assay for the free aglycon following enzymatic or chemical hydrolysis of the sample and then subtract the quantity of free aglycon that was originally in the sample. Because of the high polarity of these metabolites, it is extremely difficult to extract these compounds from urine samples. Despite these difficulties, the intact glucuronides have been analyzed by

gas chromatography following derivatization of the carboxylic acid group with diazomethane and derivatization of the hydroxyl groups with N,O-bis(tri-methylsilyl) acetamide (1).

Direct analysis of the intact glucuronide without derivatization using high-performance liquid chromatography is a much more promising approach to the problem. The major objective of this study was to determine if the glucuronides could be chromatographed satisfactorily using reversed-phase columns and if the retention index of the metabolite can be predicted.

EXPERIMENTAL

Materials

The 2-keto alkane standards were obtained from Analabs (North Haven, Connecticut). Morphine and codeine were obtained from the Theta Corporation (Media, Pennsylvania). Morphine-3-glucuronide and codeine glucuronide were obtained from Applied Science Inc. (State College, Pennsylvania). Testosterone, testosterone glucuronide, 6-Bromonaphthol, 6-Bromonaphthol glucuronide, phenolphthalein, phenolphthalein glucuronide, 8-hydroxyquinoline, 8-hydroxyquinoline glucuronide, p-nitrophenol, and p-nitrophenol glucuronide were obtained from Sigma Chemical Company (Saint Louis, Missouri). All other chemicals and solvents were of reagent grade and were used without further purification.

Chromatography

A 3.9 mm i.d. x 30 cm C_{18} reversed-phase column (μ-Bondapak

C_{18}, Waters Associates, Inc., Milford) with a 10μm particle size was used for the study. The mobile phase flow rate was 2.0 ml/min and was comprised of 6.6 g K_2HPO_4, 8.4 g KH_2PO_4, 1.6 l CH_3OH, and 2.4 l H_2O. The pH of the mobile phase was 7.0 before the addition of the methanol.

A Waters Associates, Inc. M-6000 pump, U6K injector, and Model 440 dual wavelength ultraviolet detector were used. The first detector was operated at 254 nm, while the second detector (in series) was operated at 280 nm.

Retention Index Measurements

The basic construction and properties of the retention index scale have been previously reported (2). The capacity factor (k'_x) of the test compounds and standards were determined from the observed retention time (t_x) and the retention time of the solvent front (t_o). The index (I) of a given 2-keto alkane standard was, by definition, equal to 100 times the number of carbons in the standard. Thus, acetone was assigned a value of 300, and 2-butanone, 400. The index of a drug was calculated from the capacity factor observed for the drug (k'_x), the capacity factor for a 2-keto alkane standard eluting just before the test compound (k'_N), and the capacity factor of the next higher homologue (k'_{N+1}) using Equation 2.

$$k'_x = \frac{t_x - t_o}{t_o} \qquad \text{Eq. 1}$$

$$I = 100 \frac{\log k'_x - \log k'_N}{\log k'_{N+1} - \log k'_N} + 100N \qquad \text{Eq. 2}$$

RESULTS AND DISCUSSION

The retention times of the compounds and their glucuronide metabolite varied from about one minute for p-nitrophenol glucuronide to over one hour for testosterone. The difference in the retention time of the glucuronide metabolite and the aglycon varied considerably from compound to compound and this parameter would not be useful for the prediction of the retention time of the glucuronide derivative of some new test compound. However, when the HPLC retention index of the glucuronide and its aglycon were examined, a very clear pattern was observed (Table I). The retention index of the glucuronide was found to have a much lower retention index as one might expect for the highly polar glucuronide group and it was observed that the shift in retention index was very nearly the same for all of the glucuronides. The average value for the shift in retention index was found to be 244 ± 31 units. If this trend were to be extended to other drugs and organic compounds, one would predict that retention index of the glucuronide metabolite would be 244 units lower than that experimentally observed for the parent compound. The ability to predict the retention properties of the metabolite would be extremely useful because reference standards for the metabolites are difficult to obtain and they are rarely available in the early stages of the metabolism studies of a new compound.

TABLE I

Effect of Glucuronide Formation on the HPLC Retention Index of Drugs

Drug	Index	Shift
morphine	625	
morphine-3-glucuronide	361	-264
codeine	712	
codeine glucuronide	489	-223
testosterone	920	
testosterone glucuronide	710	-210
6-bromonaphtol	871	
6-bromonaphtol glucuronide	617	-254
phenolphthalein	740	
phenolphthalein glucuronide	508	-232
8-hydroxyquinoline	612	
8-hydroxyquinoline glucuronide	311	-301
p-nitrophenol	493	
p-nitrophenol glucuronide	270	-223
	Average =	-244 ± 31

In previous studies, it was found that the retention index of a test compound (I_x) could be predicted from the retention index experimentally observed for a related reference compound (I_{ref}) and the sum of the Hansch substituent constants (π_x) than are readily obtained from reference tables. The estimation of the retention index of the new compound was made using Equation 3 which was previously developed (3,4).

$$I_x = 200\ \pi_x + I_{ref} \qquad \text{Eq. 3}$$

Though this equation was not developed specifically for metabolism studies, the retention index of a metabolite could be estimated if the retention index of the parent compound were known and if the Hansch π values for all of the substituent changes were

available. Unfortunately, the π value for the glucuronide group was not available. However, if Equation 3 is used in the reverse manner, a 244 unit retention index shift would indicate that the glucuronide group would have a π value of - 1.22 ± 0.16 units. If more than one structural change occurred during the metabolic transformation of the drug, one would use the sum of the π values for each of the changes to estimate the retention of the final metabolite. All of the aglycons in Table I contain a hydroxyl group which was than conjugated to form the glucuride. For simple aromatic compounds that do not contain a hydroxyl group, the π value would be equal to the sum of the glucuronide group and the aromatic hydroxyl group (-0.67(5)) which would be -1.89. Thus, the estimated retention index of the glucuronide metabolites of non-hydroxylated aromatic compounds would be 378 units lower than the parent compound rather than the 244 units shown in Table I.

One of the major difficulties in the detection of drug metabolities in urine samples is that there are a large number of natural components in the urine that have strong UV chromophores. Unless one uses extremely selective extraction procedures, one is almost assured of finding a natural component with nearly the same retention time as the drug metabolite. If dual UV detectors are used in series, the absorbance ratio at the two wavelengths for the compound can be obtained with good precision and this measurement has been shown to be an extremely useful aid in the identification of the compound (6, 7).

TABLE II

The Relative Response of 254 nm and 280 nm Ultraviolet Detectors to Glucuronide Metabolites

Compound	A_{254}/A_{280}
morphine	0.77
morphine-3-glucuronide	0.50
codeine	1.27
codeine glucuronide	1.17
testosterone	ca.35
testosterone glucuronide	33.8
6-bromonaphtol	0.96
6-bromonaphtol glucuronide	1.08
phenolphthalein	1.23
phenolphthalein glucuronide	1.26
8-hydroxyquinoline	1.49
8-hydroxyquinoline glucuronide	0.56
p-nitrophenol	1.11
p-nitrophenol glucuronide	0.38

The absorbance ratio of the compounds and their glucuronide metabolites (Table II) was also found to be very useful in the characterization of the compounds in the present study. For those aglycons that have the hydroxyl group removed from the UV chromophore (codeine and testosterone) one would expect the glucuronide metabolite to have the same absorbance ratio which was observed. Most of the phenolic metabolite either showed a reduction in the absorbance ratio (morphine, 8-hydroxyquinoline, and p-nitrophenol) or remained unchanged (6-bromonaphthol and phenolphthalein) when conjugated with glucuronic acid.

Preliminary studies with codeine-6-glucuronide using the 40% methanol mobile phase given in the experimental have indicated that the glucuronide is poorly resolved from the natural constituents in

human urine. If the methanol content of the mobile phase was reduced to 20%, the glucuronide could be detected at 30 μg/ml in directly injected urine samples. If lower levels of the glucuronide are to be measured it will be necessary to remove interfering peaks that elute before and after the glucuronide.

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HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF N-(2-PROPYL)-N'-PHENYL-p-PHENYLENEDIAMINE (IPPD) AND N-(1,3-DIMETHYLBUTYL)-N'-PHENYL-p-PHENYLENEDIAMINE (DBPD) AND ITS APPLICATION TO THE BIOMONITORING OF EXPOSED INDIVIDUALS.

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ABSTRACT

A rapid method based on reverse-phase high-pressure liquid chromatography (HPLC) is described for the separation and quantitation of N-(2-propyl)-N'-phenyl-p-phenylenediamine (IPPD) and N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine (DBPD) in urine.Sample preparation is based on a simple extraction procedure and analysis is carried out on a chromatographic system using a LiChrosorb RP 18 column and buffered aqueous methanol as the mobile phase.The relationship between peak heights and amount injected was linear over a range of 0.05-5 µg for both compounds.Retention times and peak heights were highly reproducible.Detection was very sensitive, allowing quantitation of 5 ng of either compound.The application of the techniques for biomonitoring body fluids as an indicator of exposure to aromatic amines is discussed.

INTRODUCTION

The detection and determination of trace concentration of aromatic amines is of extreme importance,as many of these compounds have been demonstrated during the past few decades to be carcinogenic (1).

The development of a specific and sensitive method for their determination in the environment and in body fluids is therefore important and necessary.

Several procedures for quali-quantitative determination of aromatic amines, which provide a varying degree of sensitivity (TLC,GLC,HPLC) have been published (2-7).

IPPD and DBPD are two of the aromatic amines most widely-used as antioxidant agents in rubber industries.

The approach taken in this study was to develop a system which exploits the HPLC method to detect,in the nanomole range,aromatic amines in the urine of the exposed individuals.

MATERIALS AND METHODS

Standard IPPD and DBPD (Bayer) of known purity (greater than 98%) were used without further purification.The stock solution of 5 µg/ml was prepared by dissolving 5 mg of each standard,exactly weighed on a Cahn electrobalance mod. G2,in water purified by a MILLY-Q-System (Millipore Corp.,Bedford,Mass., U.S.A.) and diluting to volume into a 1000 ml volumetric flask.

Standard solutions were analyzed immediatly after preparation and kept in refrigerator (4 °C) to retard amines oxidation.

An appropriate quantity of aromatic amines standards were added to urine samples to yield urine standards containing 0.005,0.01,0.1,1,2 µg/µl.

These urine samples (100 ml) were extracted in order to isolate aromatic amines by shaking with analytical grade diethyl ether (50 ml) into a separatory funnel.The extraction was repeated twice,then the combined extracts were,if necessary,centrifuged (5 minutes at 2000 r.p.m.).The diethyl ether extracts were dried over anhydrous sodium sulfate,then the anhydrous extracts were eva-

porated to dryness under reduced pressure in a rotary evaporator.The residues were redissolved in methanol (LiChrosolv,Merck),transferred to 10 ml volumetric flasks and brought to volume with methanol.The volume of solution injected ranged from 1 to 10 µl.

HPLC analyses were run on a Perkin-Elmer Series 3B liquid chromatograph. Component elution was monitored with a LC-75 variable wavelength detector (160-600 nm) equipped with a LC-75 Autocontrol.The column used was a Hibar--LiChrosorb RP 18 10 µm (Merck,25 x 0.26 cm I.D.).The separations reported were achieved under the following conditions : mobile phase,methanol-water (78:12), adjusted to pH 6.7 with ammonium hydroxide,0.02 M in ammonium acetate;flow rate,2 ml/min;temperature,28 °C;wavelength,290 nm;chart speed,0.5 cm/min. Graphs were generally obtained with an attenuation setting corresponding to 0.04 AUFS on a 10 mV recorder and peak areas were determined by a Perkin-Elmer Sigma 10 integrator.

RESULTS AND DISCUSSION

Figure 1 shows a chromatogram obtained on a synthetic mixture of IPPD and DBPD.IPPD and DBPD peaks are symmetrical with baseline resolution.Under the employed conditions the retention times were higly reproducible;20 injections of each compound over a period of two weeks gave mean retention times of 156 and 324 sec,with coefficients of variation of 0.72 and 1.19% for IPPD and DBPD respectively.Calibration curves of peaks area (or height) against the amounts of IPPD and DBPD injected were constructed from triplicate injections of six standard solutions of IPPD and DBPD,they were found to be linear over a 0.05--5.0 µg range for both compounds.The calibration curves obtained with IPPD and DBPD in water and urine showed no significant differences and standard solutions

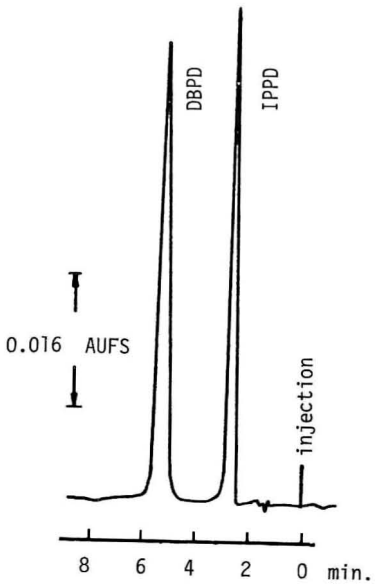

FIGURE 1

HPLC chromatogram of an aqueous solution (3µl) of IPPD and DBPD standard Conditions : column,LiChrosorb RP 18 10 µm (Merck,25 x 0.26 cm I.D.);mobile phase,methanol-water (78:12),adjusted to pH 6.7 with ammonium hydroxide,0.02 M in ammonium acetate;flow rate,2 ml/min;temperature,28 C;wavelength,290 nm;chart speed,0.5 cm/min.

were thus made up in water for convenience.Figure 2 illustrates the HPLC graph of urine containing IPPD,in which it can be seen that the matrix gives no interference at the retention time of the peak of IPPD.

The precision of the outlined method was studied by injecting fifteen 10 µl aliquots of the IPPD and DBPD synthetic mixture containing 0.25 and 0.35 µg respectively.Reproducibility measured as peak height was good,with coefficients of variation of 1.025-1.210% due to the combined errors of HPLC resolution,injection and detection.

With instrument sensitivity of 0.02 AUFS,the minimum detectability was 5--10 ng;below this level baseline detector noise exceeded peak height.

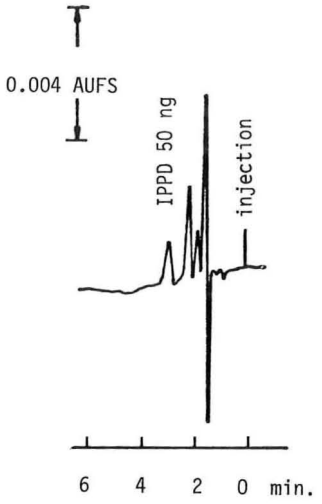

FIGURE 2

HPLC chromatogram of human urine containing IPPD standard (10µl).Conditions same as in FIGURE 1.

Human urine containing IPPD and DBPD showed recovery for IPPD in the range of 93-98.5% with an average recovery of 95.5% and for DBPD in the range of 89--98% with an average recovery of 93.5%.Recoveries were calculated by comparing peak heights from volumetric dilutions of equivalent amounts of IPPD and DBPD.

CONCLUSION

The method described is suitably accurate,rapid,selective and sensitive for determining IPPD and DBPD in water and urine samples.Many other separations of closely related compounds can be performed in a similar way and may be used in routine analysis.The choice of the mobile phase composition (percentage of water in methanol,ammonium acetate concentration and pH) permits the simple regulation

of solute capacity factors,thus providing a rapid and effective solution for specific problems.A one-step extraction procedure was chosen and developed to reduce the amount of sample handling;consequently the potential for loss of material and possible contamination is minimized.No elevated or irregular baselines nor pressure build-ups have been encountered while working with these partially purified samples.

Furtheremore the case under examination,illustrated above (human urines containing exogenous aromatic amines) demonstrates that the method employed is a useful approach to the biomonitoring of IPPD and DBPD in the urines of exposed individuals.

Investigations into the presence of IPPD and DBPD in body fluids of rubber workers are currently in progress in our laboratories.

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DETECTION OF FURFURAL AND 2-FUROIC ACID IN BACTERIAL CULTURES BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

The Furfural and 2-furoic acid present in bacterial cultures were extracted and detected by High pressure Liquid Chromatogrphy (HPLC). Using the methanol/water solvent (70/30), the column, μ Bondapak C18 (Waters Associates) seperated these compounds well. The detection was performed at 254 nm where binary mixtures were absorbed. This method provided a rapid and simultaneous detection for the conversion of furfural into 2-furoic acid followed by the utilization of 2-furoic acid during bacterial growth.

INTRODUCTION

A number of methods are available for determining furan derivatives. Furfural was quantitatively determined by titration with bromine monochloride (1). Paolo(2) described that the mixtures of furfural and 5-hydroxymethyl furfural (HMF) were seperated by a thin layer chromatography (TLC). The spots were cut and developed with Dische reagent and than estimated by spectrophotometer. Furfuryl alcohol and furoic acid were analysed by Morimoto et al (3), using gas chromatography. Kostenco(4) described a method for determination of furfural and methylfurfural on silica

gel modified by fluorohydrocarbon radicals by High Performance Liquid Chromatography (HPLC).

However, the methods described above were not suitable for the determination of furfural and 2-furoic acid for the following reasons: no selectivity, time-consuming and complicated sample preparation in individual determination of biological mixtures.

This paper describes a new method, using HPLC for the determination of furfural and 2-furoic acid present in biological mixtures.

MATERIALS AND METHODS

Bacterial Culture Medium

One litre of culture medium contained: KH_2PO_4, 1.0g; K_2HPO_4, 2.0g; KNO_3, 1.0g; $MgSO_4 7H_2O$, 0.2g; NaCl, 0.2g; $CaCl_2$, 0.01g; furfural, 0.5g; yeast extract, 0.3g. Fifty ml of culture medium was poured into 250 ml Erlenmeyer flasks. After the medium was autoclaved at 121 °C for 15 min and cooled down to room temperature, the test microorganism was inoculated.

Sample Preparation

Pseudomonas FS1 which was isolated from soil of University Campus was cultivated at 37 °C for three days in G24 Environmental Incubator Shaker (New Brunswick Scientific Co., Inc., Edison N.J. USA).

Five ml samples were withdrawn at appropriate time interval during the culture development and centrifuged at 5,000 rpm for 15 min at 4 °C (Rotor Model RPR20-2, Hitachi Automatic Refrigerated Centrifuge, Hitachi Koki Co., Ltd) to obtain cell-free sample.

This sample was filtered through 0.45 μm porosity milipore filter, 47 mm diameter (Millipore Corp.). The pH of sample was adjusted to 3 with 2N HCl. Two ml of

acidified sample was mixed with 1 ml of diethyl ether in a capped tube and left at cold room (4 °C) until two phases were clearly seperated.

Nonagueous phase was decanted into another capped tube and evaporated at 35 °C. An appropriate amount of this sample was redissolved in the corresponding methanol solvent for HPLC analysis and the other in distilled water for scanning of UV spectrophotometer.

Analysis by UV Spectrophotometer and HPLC

Prepared samples were scanned within the range of 210 nm to 330 nm in wavelength by Perkin-Elmer Model 139 UV-VIS spectrophotometer (Hitachi, Ltd., Tokyo, Japan).

Operational conditions of HPLC (Waters Associates Inc., Milford, Mass. 01757, USA) were as follow: column, μ-Bondapak C18; solvens, methanol/water (100/0, 70/30, 50/50, 10/90); flow rate, 1.0 ml/min; detector, UV Model 440, 254 nm; injection, 10 μl; temperature, room temperature. Methanol solvents (for chromatographic grade, Merck) were degassed and filtered through Millipore filter prior to usage.

RESULTS AND DISCUSSION

Growth of Pseudomonas FS1 was observed in the culture medium containing yeast extract and furfural as a carbon source. A simple method to determine the quantity of furfural and possible amount of its metabolite(s) in this bacterial culture was needed. Furfural is an aromatic compound and has UV absorbance at 277 nm of wavelength(5). The metabolite(s) would be either furan derivatives such as 2-furoic acid and 2-furfuryl alcohol(3) or glutamate which does not have UV absorbance(6).

If metabolites are furan derivatives, it is certain

that it has UV absorbance. In order to resolve above presumptions UV scanning was carried out and the results are shown in Figure 1.

The peak at 277 nm was the furfural (A,B) and showed the gradual decrease (A,B,C,D). In addition a new peak at 245 nm appeared after 12 hr of growth and thereafter disappeared. A substance which peaks at 245 nm (E_{245}) seems to be a metabolite formed extracellularly. The purified E_{245} substance was identified as 2-furoic acid when compared with a standard 2-furoic acid by IR spectrophotometer. 2-Furoic acid was produced due to the oxidation of furfural by an exoenzyme(7).

For simultaneous determination of furfural and

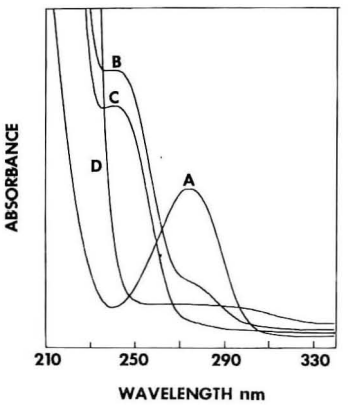

FIGURE 1

UV scanning spectra of culture filtrate during the growth of Pseudomonas FS1. (A) a peak of furfural in culture medium, (B) (C) peaks after 18 hr and 30 hr of growth, respectively, (D) disappearance of two peaks after 55 hr of growth.

2- furoic acid in bacterial cultures, culture filtrate was prepared to be analyzed by HPLC with different mobile phases of methanol as shown in Figure 2.

Furfural and 2-furoic acid could be separated well when solvents other than absolute methanol were used. The optimum condition for retention time of each mobile phase occurred only when 70% methanol was used.

Thus, transformation of furfural into 2-furoic acid and the utilization of 2-furoic acid during the culture development of Pseudomonas FS1 could be detected by HPLC, using 70% methanol solvent.

Pairs of peaks in chromatograms in Figure 3 demonstrated the gradual increase and decrease of 2-furoic

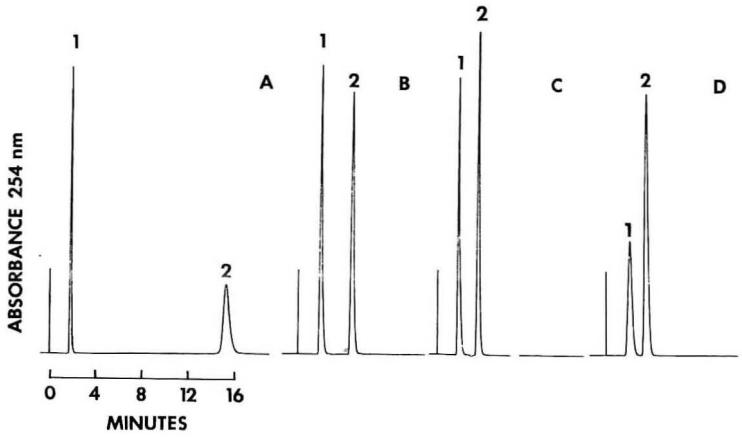

FIGURE 2

Chromatograms of 2-furoic acid(1) and furfural(2) at various concentrations of methanol solvent.
(A) 10% methanol, (B) 50% methanol, (C) 70% methanol, (D) 100% methanol.

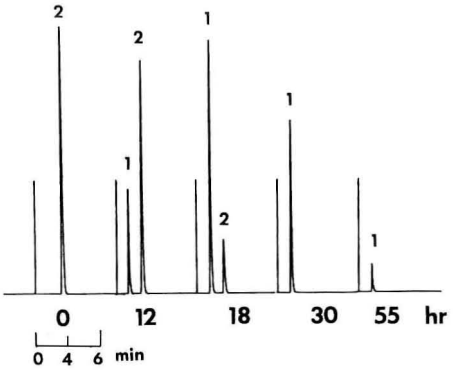

FIGURE 3

HPLC chromatograms of 2-furoic acid(1) and furfural(2) in culture filtrate during the culture development of Pseudomonas FS1. Solvent: 70% methanol. Other conditions are listed in text.

acid and furfural. The peaks that disappeared were completely utilized at the end of growth. As shown in Figure 1, spectrophotometric analysis was not suitable for quantitative determination of each compound in the binary mixtures with high concentration, since absorbances of the two compounds overlapped each other which resulted in error. However, HPLC analysis allowed binary mixtures of furfural and 2-furoic acid to be fractionated and detected rapidly and simultaneously.

Using HPLC method it is posible to estimate the amounts of furfural and 2-furoic acid by measuring peak heights or peak areas with reference to a calibration curve of standard compounds.

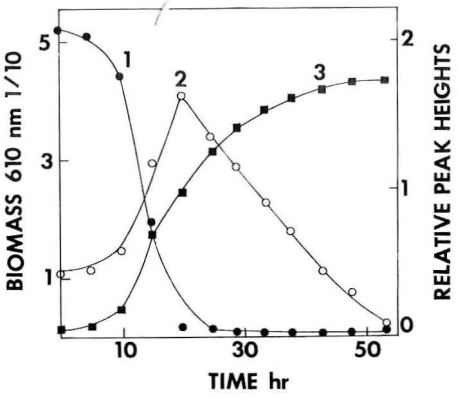

FIGURE 4

Quantitative changes of furfural (1) and 2-furoic acid (2) depending on the increase of cell mass(3).

The quantitative changes of furfural and 2-furoic acid, depending on cell mass expressed as turbidity at the optical density of 610 nm were shown in Figure 4.

Using the HPLC method, we have found unique physiological properties of Pseudomonas FS1 which is of interest in view of elucidating the metabolism of furfural as a toxicant.

2-Furoic acid was not utilized until furfural was completely converted into 2-furoic acid. This indicated that furfural was subjected to oxidation into 2-furoic acid followed by the reutilization of 2-furoic acid as a carbon source. Therefore Pseudomonas FS1 could decompose furan rings by converting furfural into 2-furoic acid.

In summary, the HPLC method described above can be useful for the detection as well as quantitative deter-

mination of furfural and 2-furoic acid present in bacterial cultures.

ACKNOWLEDGEMENTS

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ANALYSIS OF DEXAMETHASONE ACETATE IN PHARMACEUTICAL FORMULATIONS BY HPLC

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ABSTRACT

A rapid and effective high-pressure liquid chromatographic method has been developed for the quantitative determination of dexamethasone 21 acetate in pharmaceutical formulations.Sample preparation employs a simple extraction procedure and analysis is carried out on a reverse-phase chromatographic system using a LiChrosorb RP 18 column and a water-acetonitrile as mobile phase.The extraction procedure gives quantitative recovery and chromatographic results show that drug levels of as 0.1 ppm can be conveniently analyzed without significant background interferences.

INTRODUCTION

The action,uses and side effect of dexamethasone 21 acetate are those of dexamethasone,a syntetic adrenal steroid derived from prednisolone.Dexamethasone acetate is particularly employed as a repository form of dexamethasone,for systemic or intralesional use.

Current analytical techniques for quantitative dexamethasone

determination in various biological and non biological media are based on colorimetric methods with bleu tetrazolium or with a modified Oorter-Silber reagent (1).Problems associated with the analyses are usually complex;colorimetric methods do not seem sufficiently quantitative when dexamethasone incorporated into a complicated matrix is being analyzed, furthermore they are time consuming for routine work and rather tedious to perform.

High-pressure liquid chromatography (HPLC) has been recently proposed as an alternative procedure for the determination of dexamethasone phosphate sodium salt (2,3).

The present paper is an application of reverse-phase HPLC to routine analyses of dexamethasone acetate in pharmaceutical formulations, in the range of 10 ng level.

MATERIALS AND METHODS

Pharmaceutical formulations (dermal ointments and suppositories) containing dexamethasone acetate,were prepared for chromatographic analyses as follows:

ointments: ointment sample (5 g) exactly weighed were suspended in 100 ml of twice-distilled warm water (40 °C),quantitatively poured into a 250 ml separatory funnel and then extracted with 50 ml of chloroform.The extraction was repeated three times.The lower (chloroform) layers were transferred into a 500 ml separatory funnel and washed twice with 100 ml of redistilled water.The washed extract was filtered through 25 g of anhydrous sodium sulfate (held in 60 ml coarse fritted-glass funnel).The anhydrous

extract was evaporated to dryness under reduced pressure in a rotary evaporator.The residue was redissolved in acetonitrile (LiChrosolv,Merck) filtered through a Gelman Acrodisc-CR 0.45 μm filter and then diluting to volume into a 25 ml volumetric flask;
suppositories: four suppositories (8 g) were dissolved in 100 ml of warm ethanol (40 °C).The clear ethanolic solution was stored overnight in a refrigerator (4 °C).This treatment removes a substantial proportion of inactive lipid suppository base which was subsequently separated by filtration through a Whatman GF/C filter.The filtered solution was evaporated to dryness,redissolved in 100 ml of diethyl ether,transferred into a 500 ml separatory funnel and then extracted twice with 100 ml of redistilled water.This treatment is useful to eliminate the water solubile ingredients.The diethyl ether layer was dried and evaporated to dryness.The residue was redissolved in acetonitrile,filtered and diluting to volume into a 25 ml volumetric flask.

Dexamethasone acetate standard was obtained from commercial suppliers.The stock solution of 0.5 mg/ml was made by dissolving in acetonitrile 5 mg of dexamethasone acetate,exactly weighed on a Cahn electrobalance mod. G 2 and diluting to volume into a 50 ml volumetric flask. An aliquot (5 ml) of the stock solution was accurately measured and transferred into a 50 ml volumetric flask and diluted to volume with acetonitrile.Injections of 10 μl of dexamethasone acetate standard solution (0.11 μg/μl) were made for quantitation.

The chromatographic separations were performed with a Perkin-Elmer Series 3B liquid chromatograph.Component elution was monitored with a LC-75 variable wavelength detector (190-600 nm) equipped with a LC-75

Autocontrol.The column was a Hibar-LiChrosorb RP 18 10 µm (Merck, 25 x 0.26 cm I.D.).The separations reported were achieved under the following conditions: mobile phase,50% acetonitrile (LiChrosolv,Merck) in deionized redistilled water;flow rate,1.5 ml/min;temperature,25 °C; wavelength,238 nm;chart speed,0.5 cm/min.Graphs were generally obtained with an attenuation setting corresponding to 0.16 AUFS on a 10 mV recorder and peak areas were determined by a Perkin-Elmer Sigma 10 integrator.

RESULTS AND DISCUSSION

A chromatogram of ointment extract containing dexamethasone acetate is shown in Figure 1.The dexamethasone acetate peak is completely resolved and is symmetrical.Detector response, as measured from standard peak heights,was linear up to at least 0.1 µg.The minimum detection limit was 10 ng;belowe this level baseline detector noise exceeded peak height.The accuracy of the proposed method was further checked by means of recovery experiments carried out on one rappresentative pharmaceutical preparation (ointment and suppository) in which a measured quantity of dexamethasone acetate was added to the sample.The mean recovery of dexamethasone acetate in dermal ointment was 98% with a relative deviation of ±1.5%, the mean recovery in suppositories was 96% with a relative deviation of ±2%.With the proposed method there was no interference from propyl gallate,butyl hydroxyanisole,tween 80 and other active or inactive ingredients present in ointment and suppositories.

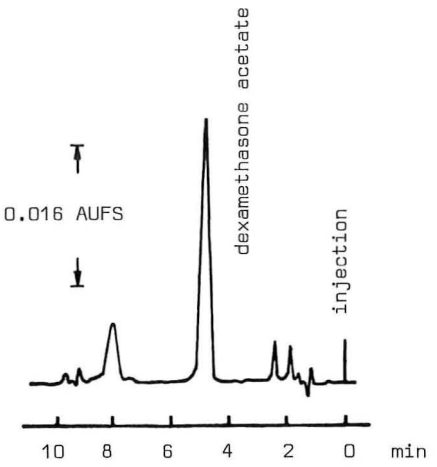

Figure 1

HPLC chromatogram of an acetonitrile extract (10 µl) of a dermal ointment sample containing dexamethasone acetate.Conditions:column,LiChrosorb RP 18 10 µm (Merck,25 x 0.26 cm I.D.);mobile phase,water-acetonitrile (1:1);flow rate,1.5 ml/min;temperature,25 OC;chart speed,0.5 cm/min;wavelength,238 nm.

CONCLUSION

The proposed method for the assay of dexamethasone acetate in pharmaceutical formulations confirms that the HPLC technique has several distinct advantages over the conventional technique currently in use.It is simple,fast and has a precision and accurancy greater than those of the more tedious colorimetric methods.We believe that the technique described here can be used,with a slight modification of the extraction procedure,for measured dexamethasone acetate,in other pharmaceutical formulations,in biological materials (plasma) and for analysing samples where interference is too great for the use of other techniques.

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LIQUID CHROMATOGRAPHIC ANALYSIS OF 4-NITROQUINOLINE 1-OXIDE AND METABOLITES

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ABSTRACT

High pressure liquid chromatographic (HPLC) methods for separation of the carcinogen 4-nitroquinoline 1-oxide (NQO) from its reduced metabolites, 4-hydroxyaminoquinoline 1-oxide (HAQO) and 4-aminoquinoline 1-oxide (AQO), are described. Simultaneous fluorescence and U.V. absorbance analysis, using a gradient system with reversed phase HPLC, gave good resolution of the metabolites.

INTRODUCTION

4-nitroquinoline 1-oxide (NQO) is a carcinogen that has been widely used in both *in vivo* and *in vitro* systems (4). 4-hydroxyaminoquinoline 1-oxide (HAQO), which is produced by reduction of NQO in mammalian cells (14) appears to be a more potent carcinogen than NQO on an equimolar basis (3,6,11). Reduction of HAQO yields 4-aminoquinoline 1-oxide (AQO), which has not demonstrated substantial carcinogenic activity (4).

Both NQO and HAQO have been reported to act directly on DNA *in vitro* (7,9). However, recent data indicate that the carcinogenic effects of NQO may be due, to a large extent, to HAQO

formed by the enzymatic reduction of NQO (13,14). In our studies, which use NQO or HAQO to inactivate the infectivity of cytomegalovirus (CMV) (1,2) or herpes simplex virus (HSV) (12), we have followed the reduction of NQO to HAQO and AQO in lysates of CMV- or HSV- infected cells. HPLC analysis of NQO-treated cell lysates has been an effective method for following the metabolism of NQO to HAQO and AQO.

MATERIALS AND METHODS

Chemicals

NQO and HAQO were supplied by the NCI Carcinogenesis Research Program through IIT Research Institute, Chicago, Illinois. AQO was the generous gift of Dr. Yutaka Kawazoe of the Faculty of Pharmaceutical Sciences, Nagoya City University, Nagoya, Japan. Solvents were from Burdick and Jackson (Muskegon, Michigan).

Chromatography

For TLC, Brinkmann Instruments (Westbury, New York) 20 cm x 20 cm, 0.25 mm thick Silica G plates without fluorescence indicator were used. Whatman (Clifton, New Jersey) 7.6 cm x 7.6 cm, 0.20 mm thick KC_{18} plates were used for reverse phase TLC. HPLC was performed on a Waters Associates, Inc. (Milford, Mass.) model GPC/LC-204 instrument equipped with a model 660 Solvent Programmer, two model M6000A pumps and a model U6K injector system. HPLC separation of the ethanol extracts of NQO-treated cell lysates was accomplished on a 3.9 mm x 30 cm μBondapak C_{18} reverse phase column (Waters Assoc.). A model SF-770 variable wave-length spectrophotometric detector and a model FS-970 fluorescence

detector (Schoeffel Inst. Corp., Westwood, New Jersey) were connected in series to the outlet of the column. The signals from the detectors were recorded on a Houston Instruments (Austin, Texas) model A5211-1 two pen recorder. U.V. absorbance was monitored at 360 nm. The excitation wavelength of the fluorescence detector was set at 360 nm and a KV-470 emission filter was used. In the HPLC system, a 15 min linear gradient at 1.5 ml per minute was produced from 10 or 20% methanol in water to 90 or 100% methanol.

Fractions of the HPLC eluant were collected at 1 min intervals and analyzed on an Aminco-Bowman spectrophotofluorometer equipped with a 416-992 xenon lamp, a R136 photomultiplier tube and a 1620-809 X-Y recorder. The fractions were scanned in 1 x 4 cm fused quartz cells at sensitivity 39, slit arrangement 3 and meter multiplier readings of 0.03 to 0.001.

Sample Preparation

Details of cell culture, virus propagation, carcinogen treatment and virus inactivation have been previously published (1). NQO, when used to treat virus stocks, was dissolved immediately prior to use in dimethylsulfoxide and diluted in maintenance medium (1). HAQO was dissolved in 0.50N HCl. After dilution of the HAQO solution in maintenance medium (1:20), the pH was adjusted to 7.2 by the addition of 7.5% sodium bicarbonate ($NaHCO_3$). When used as standards for TLC or HPLC, the carcinogens were dissolved in the indicated organic solvent immediately prior to use.

For TLC, NQO-treated virus stocks were lightly sonicated, added to an equal volume of the first TLC solvent, and 5 µl volumes were repeatedly spotted on the TLC plates. Fluorescence of the carcinogens was observed with a model B-100A longwave U.V. lamp (Ultra-Violet Products, Inc., San Gabriel, Calif.).

Samples from NQO-treated, virus-infected cell lysates were prepared for HPLC by heating in boiling water for 1 min and sedimenting the precipitated protein at 240 x G for 10 min. Ethanol (95%) was added to the supernatant to reach a 65% ethanol concentration. The specimen was then maintained on ice for 15 min and clarified by centrifugation as before.

RESULTS

In our previous data with virus inactivation by NQO, no convincing photodynamic component was evident in virus survival curves (1,12). NQO, however, has been shown to be photodynamically activated and such a component would be anticipated based on the structure of NQO (7,8). Reduction of NQO to HAQO and AQO had been observed in rat cells (13,14) and, therefore, might be expected in human cells. HAQO, in contrast to NQO, is not photodynamically activated (4) and has been shown to inactivate both transforming DNA (10,15) and bacteriophage T_4 (5). Thus, it appeared that HAQO, rather than NQO, might be responsible for the observed inactivation of virus infectivity. NQO-treated lysates of virus-infected cells were, therefore, examined for the presence of HAQO.

TLC separation of NQO, HAQO and AQO was first performed by the method of Sugimura *et al.* (13) using silica gel plates and a solvent

system of sec-butanol:ethyl acetate:water (1:1:1). Comigration of samples with known standards was observed, suggesting that detectable amounts of HAQO and AQO were formed in the lysates of virus-infected cells. However, variability of the Rf values of both standards and samples, as observed by Sugimura et al. (13), was noted. Silica gel plates developed first with methanol and then with ethyl acetate gave somewhat more consistent results. Reverse phase TLC with acetonitrile:water (75:25) as the solvent was used to confirm results with the above systems. Unfortunately, during TLC procedures, degradation of HAQO was noted (13) and recovery of standards and samples was poor.

HPLC separation of NQO, HAQO and AQO standards (Fig. 1) was developed to permit reproducible separation and recovery of these chemicals from cell lysates. HPLC separation of extracts of NQO-treated virus-infected cells demonstrated significant amounts of HAQO and AQO (Fig. 2). The fluorescent and U.V. peaks observed during the chromatography of the NQO-treated lysates of virus-infected cells corresponded to the retention times of the NQO, HAQO and AQO standards. Addition of standards to NQO treated lysates of virus-infected cells resulted in the expected increase in the size of the corresponding peak on the chromatogram.

The use of HPLC separation allowed for ready spectrofluorometric examination of the eluate. The maximum excitation of the HAQO containing fractions was at 360 nm and maximum emission was at 478 nm (Fig. 3). These values and the

shape of the emission and excitation scanning curves are in good agreement with the published values for HAQO (4,13,14) and data obtained using a HAQO standard on the same equipment.

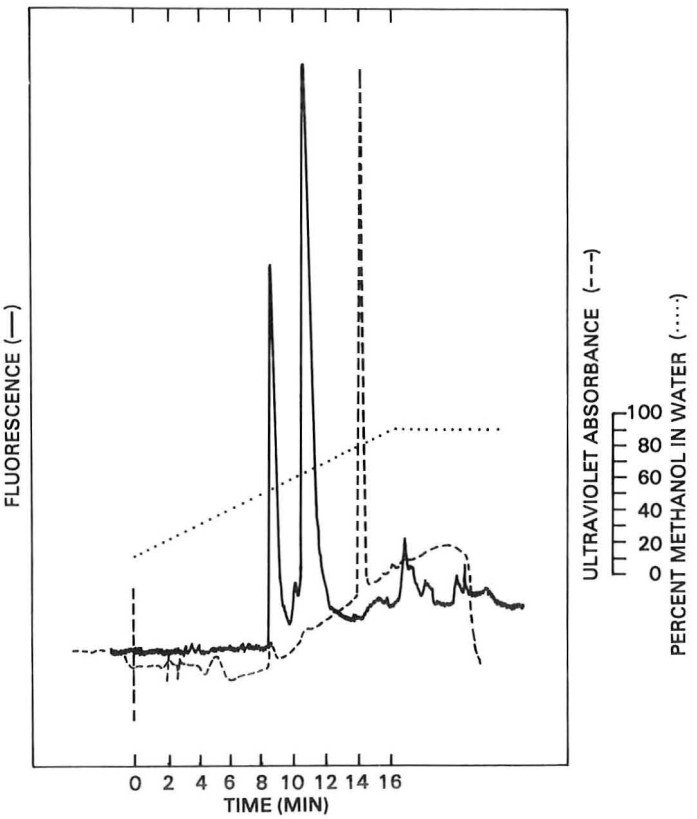

Figure 1. HPLC analysis of NQO, HAQO and AQO standards. A reverse phase column was developed with a 10 to 90% methanol gradient (····) in water. Fluorescence (——) was measured with excitation at 360 nm and emission at 470 nm. U.V. absorbance (----) was measured at 360 nm.

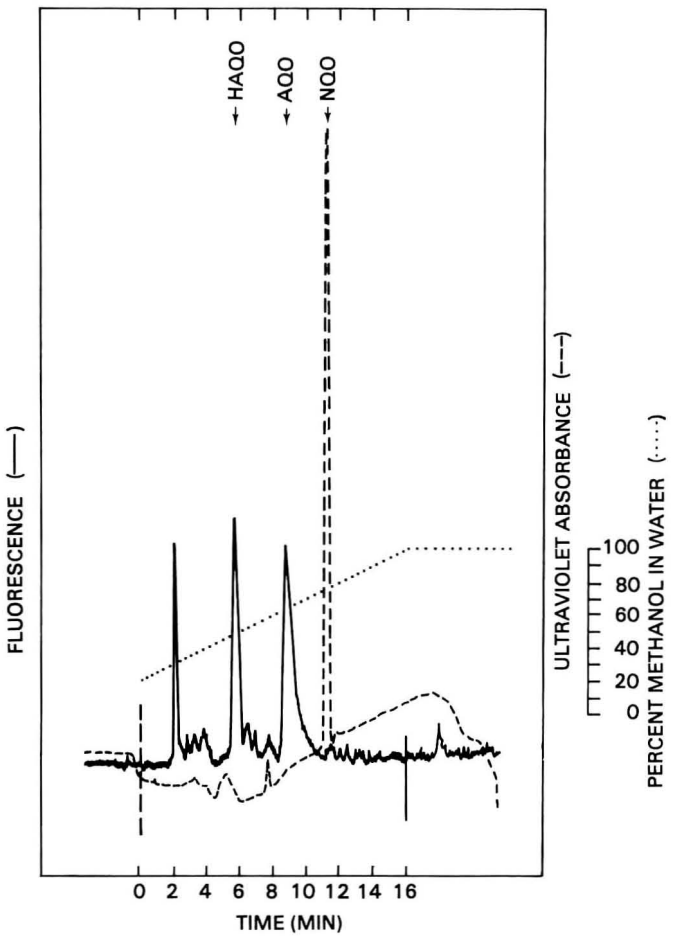

Figure 2. HPLC analysis of an ethanol extract of a NQO-treated (50 μg/ml) cytomegalovirus (strain AD-169) stock. A reverse phase column was developed with a 20 to 100% methanol gradient (····) in water. Fluorescence (——) was measured with excitation at 360 nm and emission at 470 nm. U.V. absorbance (----) was measured at 360 nm. The retention times of NQO, HAQO, and AQO standards are indicated ( ).

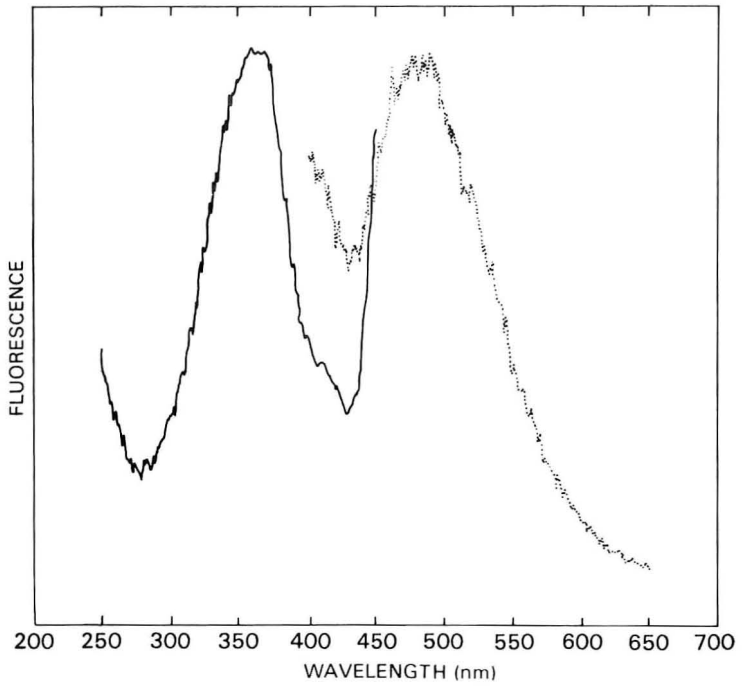

Figure 3. The fluorescence excitation (——) and emission spectra (....) of the fraction from Fig. 2 with the retention time of HAQO. For the excitation spectra, the emission was measured at 478 nm. For the emission spectra, the excitation was at 360 nm.

DISCUSSION

HPLC provided several advantages for examination of the metabolism of NQO in lysates of virus-infected cells. The separation of NQO, HAQO and AQO was readily achieved. The fluorometric assay of the HPLC eluate was highly sensitive. The retention times, peak areas and elution profiles of these compounds were reproducible. Preparation for HPLC did not appear to cause degradation of the standards as was noted with TLC.

HPLC also allowed for the simple recovery of the separated compounds for further analysis by spectrofluorometry, and thus obviated the problems encountered in the recovery of compounds from TLC plates.

The HPLC separation of these compounds for analysis and quantification should facilitate the study of these carcinogenic compounds in established models of carcinogenesis and cocarcinogenesis.

ACKNOWLEDGEMENTS

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TRIBUTYL PHOSPHATE AS STATIONARY PHASE IN REVERSED PHASE LIQUID CHROMATOGRAPHIC SEPARATIONS OF HYDROPHILIC CARBOXYLIC ACIDS, AMINO ACIDS AND DIPEPTIDES

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ABSTRACT

Tributyl phosphate (TBP) has been used as stationary liquid phase in reversed phase liquid column chromatography with aqueous buffers or aqueous buffers + methanol as the mobile phase. Hydrophobic microparticulate (5 μm) alkyl-derivatized silica was used as support. The stability of the columns is very high as is their separating efficiency.

The strong hydrogen accepting properties of TBP makes retention possible of highly polar solutes containing hydrogen donating groups. Retention of carboxylic acids is obtained by the distribution of the uncharged form into TBP and it is regulated by the pH of the mobile phase. No influence from the support on the retention of carboxylic acids has been seen. Benzoic, phenylacetic, mandelic, indole-3-acetic and glucuronic acid derivatives, amino acids and dipeptides have been chromatographed and the influence of structural effects on the separation factors is discussed.

INTRODUCTION

In reversed phase partition chromatography, where an organic liquid is used as the stationary phase in combination with an aqueous mobile phase, the retention of highly polar organic molecules may require a stationary phase that can form strong hydrogen

bonds to these molecules. A few liquids have been studied for use as stationary phases in modern liquid chromatography, namely 1-pentanol [1, 2] and butyronitrile [3]. They have been used in systems for separations of nonionic compounds as well as ion-pairs of ionic compounds. Many compounds, e.g. carboxylic acids of biological origin, were very slightly retained in these systems. We therefore investigated the properties of tributyl phosphate (TBP) as a stationary liquid phase. Being a strong hydrogen acceptor it should bind hydrogen donating samples, like carboxylic acids, strongly.

TBP is a well-known and powerful extractant for metal ions and inorganic acids but the extraction of organic acids has also been noticed [4]. TBP has been extensively used as stationary phase in reversed phase liquid chromatographic separations of metal ions [5], but its use for the separation of organic compounds in similar systems seems not to have been studied. Some work has been done using TBP as additive in a mobile organic phase in paper chromatography [6] and column chromatography [7].

This paper introduces TBP as a stationary phase on modern microparticulate alkyl-modified silica as support. The method of coating is discussed and the use of the system is illustrated by separations of very polar carboxylic acids of biological importance.

EXPERIMENTAL

Materials

The chromatographic system consisted of an LDC Solvent Delivery System 711-26, a sample valve injector, either a Valco CV-6UHPa or a Rheodyne 7120, with 10 and 20 μL loops respectively, a Cecil 212 variable wavelength UV-detector with 10 μL cell and set at 254 nm. A Heto waterbath 02 PT 923 (HETO, Birkerød,

Denmark) with external circulation was used to thermostate the chromatograph.

Stainless steel columns of 100 mm length and 4.5 mm inner diameter were used with a 2 μm Altex filter in each end fitting. The columns were slurry packed with 5 μm particles of either LiChrosorb RP-8 or LiChrosorb RP-18 (Merck).

Tributyl phosphate (TBP) and methanol were analytical grades from Merck. Sodium dihydrogenphosphate, disodium hydrogenphosphate and phosphoric acid were used to prepare the buffers and were analytical grades from Merck. Water was purified in a Milli-Q apparatus (Millipore). All chemicals were used without purification.

Batch Distribution Experiments

Batch distribution studies were performed in centrifuge tubes with equal volumes of TBP and aqueous phase. Equilibrium was attained by mechanical shaking for 20 min at 25.0 °C. The TBP phase was pre-saturated twice with an equal volume of water + methanol (9:1). The distribution ratio of carboxylic acids was determined with buffer pH 6.98 + methanol (9:1) as aqueous phase. The initial and the equilibrium concentration in the aqueous phase was determined by UV photometry.

The distribution ratio of methanol was measured in the pre-saturation step by a gas chromatographic determination of methanol in the aqueous phase. Compensation was made for a change in the phase volume ratio.

Chromatographic Technique

The eluent reservoir, immersed in a water-bath, and the column, which was jacketed, were thermostated at 25.0 °C by circu-

lating water.

The eluent contained aqueous phosphate buffers of ionic strength 0.1. In most cases methanol was added to the buffer in the ratio 1:9.

To ensure column stability the eluent was partly saturated with TBP. This was achieved by gently mixing a large volume of pre-thermostated solvent (usually 3 L) with 10 mL of TBP. After phase separation, which usually required 48 hours, partially saturated eluents were prepared by mixing the TBP-saturated solvent with an appropriate amount of unsaturated solvent of the same composition.

Coating of the columns with the stationary liquid phase (TBP) was achieved by two methods: 1. The equilibration method. The eluent was pumped through the column until constant retention volumes were obtained for sodium nitrate (unretained compound) and a retained compound. At completed coating the column porosity, $\varepsilon_m = V_m/V_o$, is ≤ 0.44. V_m is the hold-up volume and V_o is the volume of the empty column calculated from its length and inner diameter. 2. With the injection method TBP is injected directly into the column by the injection valve. Increments of 10 - 50 µL of TBP were injected at a frequency of ca. 10 µL/min. The total volume of stationary phase, V_s, that should be injected is calculated by

$$V_s = V_{m,1} - V_{m,2} = V_{m,1} - \varepsilon_{m,2} \times V_o \qquad (1)$$

$V_{m,1}$ is the hold-up volume before injections have started while $V_{m,2}$ and $\varepsilon_{m,2}$ are the hold-up volume and the desired porosity after injections of stationary phase. After the loading, the columns were conditioned over-night with a recirculating eluent.

Columns once loaded can be stored if filled with an eluent free from salts.

No precolumn needs to be used.

Capacity ratios, k', were calculated from the retention

volume, V_R, and the hold-up volume by $k' = (V_R - V_m)/V_m$.

RESULTS AND DISCUSSION

Solvent Properties

Tributyl phosphate is slightly soluble in water. The solubility at 25.0 °C in phosphate buffer pH 6.05 was determined, by gas chromatography, to be 0.034 % w/v. In pure water the solubility is 0.039 % w/v [8]. It is important to note that the solubility decreases with increasing temperature [8] since this can influence the stability of the chromatographic columns.

The solubility of water in the TBP phase is 4.67 % w/v [8], which corresponds to 3.58 M [9].

TBP, equilibrated with aqueous buffers containing 10 % methanol, contains 2 % v/v methanol since the distribution ratio of methanol was found to be 0.2.

Coating with TBP

Alkyl-modified silica supports can be coated with organic liquids as stationary phases by the equilibration method [1]. In this method the stationary liquid is adsorbed from an eluent which is saturated or partly saturated with the liquid. Fig. 1 gives results for the coating of LiChrosorb RP-18 with TBP. As TBP occupies the pores of the support the porosity decreases while the retention increases. The results indicate that TBP has filled the pores completely since the porosity reaches 0.39 which equals the normal value for the interparticle porosity of 0.4 $\pm$ 0.03. Due to the low solubility of TBP the coating procedure becomes very time-consuming.

Much faster coating was obtained by the injection method. The

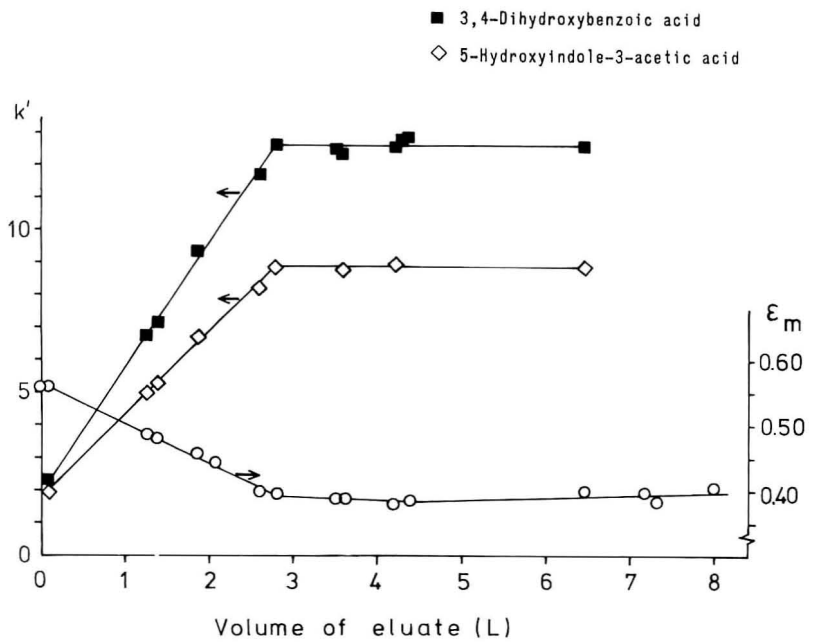

FIGURE 1. Change of retention (k') and porosity (ε_m) during coating by the equilibration method. At the start of this experiment the column was already partly coated with TBP. Column: LiChrosorb RP-8, 5 µm, 100x4.5 mm. Eluent: phosphate buffer pH 6.05, 95 % relative saturation of TBP, 0.5 ml/min.

columns could be coated within one hour but conditioning overnight was necessary in order to get good efficiency. The actual volume of injected TBP was not more than 20 % higher than that calculated from eq. (1), which shows that the uptake is rather efficient. No droplets were observed in the eluate during the coating. However, care should be taken not to over-load the column since an excess is very time-consuming to get rid of.

Variation of the amount of stationary phase can be obtained by adjusting the saturation degree of the eluent [1]. In the present work, however, only maximally loaded columns were used.

The phase-ratio, V_s/V_m, in the columns can be calculated from measured porosity values [1] and was found to be 0.6 based on an ε_m-value of 0.43 and a total porosity of 0.7 for LiChrosorb RP-18 [1].

Stability and Column Efficiency

The TBP-coated columns have a high long-term stability. One column has been in continuous use for 9 months with several changes of the pH of the buffer. The retention volumes changed less than 5 %.

It is suitable to use eluents of 90 % relative saturation with TBP since they gave stable columns with a porosity of 0.42 - 0.44 at equilibrium.

The use of completely saturated eluents always caused detection disturbances in the UV detector due to the presence of micro-drops of TBP in the eluate. It seems that the solubility of TBP was lower in the column than in the eluent. Similar effects have been seen in other cases [1].

The presence of methanol in the eluent was necessary in order to avoid a drastic increase in the flow resistance which otherwise occurred, probably caused by particles produced in the sample injection valve and a break-down of the column.

The column efficiency is illustrated in Fig. 2. The plate height tends to decrease with increasing capacity ratios.

Retention Model

Samples which are retained by distribution to the TBP-layer on the columns will get capacity ratios according to

$$k' = (V_s/V_m) \times D \qquad (2)$$

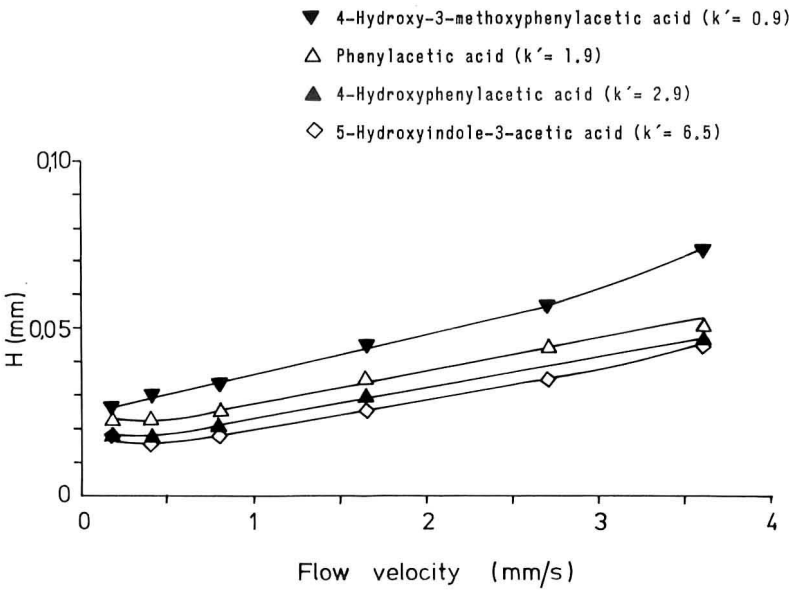

FIGURE 2. Dependence of column efficiency on flow velocity. Column: LiChrosorb RP-18, 5 µm, coated with TBP by the equilibration method, ε_m = 0.42 - 0.43. Eluent: phosphate buffer pH 5.72 + + methanol (9:1), 90 % relative saturation of TBP.

where D is the distribution ratio of the sample. For an acid, HA, its distribution ratio will be given by

$$D = \frac{C_{org}}{C_{aq}} = \frac{[HA]_{org}}{[HA]_{aq} + [A^-]_{aq}} = \frac{K_D}{1 + \frac{K'_a}{a_{H^+,m}}} \qquad (3)$$

which takes into account the distribution of the acid into TBP and its protolysis in the mobile phase. C_{org} and C_{aq} are the total concentrations of the acid in the stationary and mobile phases respectively, and K_D is the distribution constant of the acid. K'_a is the apparent acid dissociation constant and $a_{H^+,m}$ is the hydronium ion activity in the mobile phase. Accordingly, the capacity ratio can be regulated by the pH of the mobile phase. As

the pH was measured in the buffer before the addition of methanol, the relationship between the logarithm of the capacity ratio and the pH in the buffer of the eluent can be expressed by the following function after combination of equations (2) and (3)

$$\log k' = \log (K_D \times V_s/V_m) - \log (1 + K_a^*/10^{-pH}) \qquad (4)$$

K_a^* is an apparent acid dissociation constant which includes the medium factor for the hydronium ion (cf. [11]). The function has two asymptotes, one with a slope of 0 and the other with a slope of -1, which intersect at a pH which equals pK_a^* for the acid. Some examples of these relations are given in Fig. 3 where

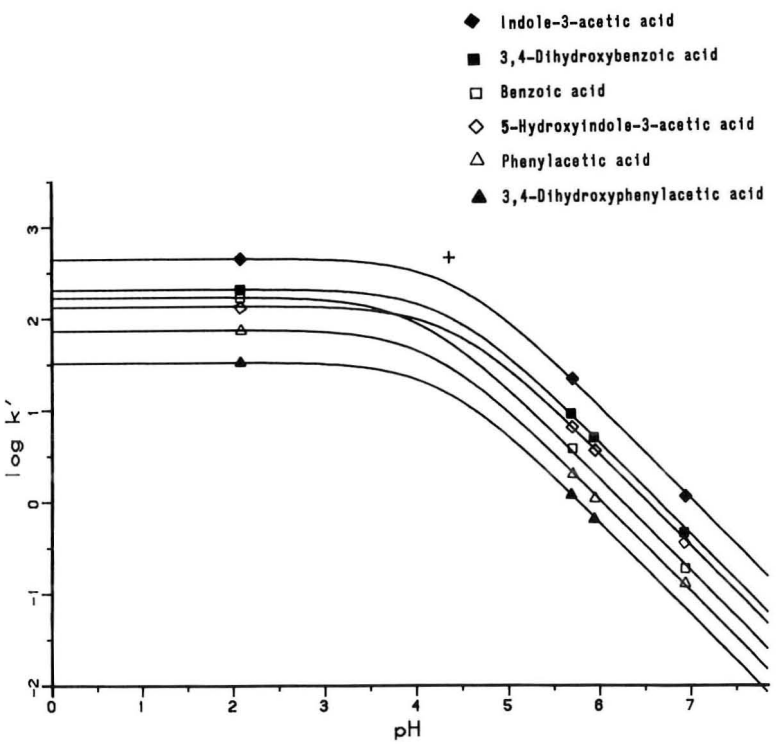

FIGURE 3. Dependence of the retention of acids on the pH of the buffer. Conditions as in Fig. 2. + = intersection point for the asymptotes of indole-3-acetic acid.

the curves have been obtained by non-linear regression according to eq. (4). The experimental points fit well to the curves.

Plots of this kind would be obtained also when the acids interact with the support. A comparison of the found k'-values with those calculated from known distribution ratios between the free liquid phases shows, however, that the influence of the support on the retention is almost negligible. Results are shown in Table 1. The found k'-values were smaller than the calculated by a factor of 0.8. The deviation from unity may be due to errors in the phase-ratio and to a different composition between the chromatographic stationary phase and the TBP-phase used in the batch experiments. Furthermore, the separation factors, α, obtained in chromatography and in distribution between free phases, agree rather closely.

TABLE 1

Comparison of Chromatographic Retention with Batch Distribution
α, the separation factor = quotient between the D-value or k'_{found}-value of an acid and the corresponding data for benzoic acid. Chromatographic conditions; Eluent: phosphate buffer pH 6.94 + + methanol (9:1), 90 % relative saturation with TBP. Porosity (ε_m): 0.42-0.44. Stationary phase: TBP. Support: LiChrosorb RP-18.

Acid	Batch		Chromatogr.		Comparison chrom.-batch
	D	α	k'_{found}	α	k'_{found}/k'_{calc} *)
	(pH 6.98)		(pH 6.94)		(pH 6.94)
5-Hydroxyindole-3--acetic acid	0.78	1.77	0.35	1.94	0.83
Indole-3-acetic acid	2.45	5.58	1.12	6.22	0.85
Benzoic acid	0.44	1.00	0.18	1.00	0.76
3-Hydroxybenzoic acid	0.98	2.24	0.42	2.33	0.79
4-Hydroxybenzoic acid	2.60	5.93	1.09	6.06	0.78

*) $k'_{calc} = (V_s/V_m) \times D \times 0.9$ is the capacity ratio calculated from the batch extraction experiments. The phase-ratio is taken to be 0.6. The factor 0.9 compensates for the pH difference between batch and chromatographic experiments.

Retention and Separation of Various Compounds

The strong electron donating ability of the phosphate oxygen in TBP enables the formation of strong hydrogen bonds to acidic solutes and a liquid chromatographic system with TBP as the stationary phase can then give considerable retention of hydrophilic acids and a separation selectivity which is different from that obtained in the conventional reversed phase systems with alkyl--bonded phases.

A survey of the retention obtained for acidic compounds is given in Table 2. It includes derivatives of benzoic, phenylacetic, mandelic and indoleacetic acids. Also included are some glycine and glucuronic acid conjugates, one amino acid and some dipeptides. All peaks had good symmetry except for compounds that contain amino groups i.e. the amino acids and the dipeptides.

TABLE 2

Retention of Acids

Chromatographic conditions; Eluent: phosphate buffer + methanol (9:1), other conditions as in Table 1.
pK_a^o = thermodynamic acid dissociation constant from ref [13].

Sample	pK_a^o	k'		
		pH of the buffer		
		2.08	5.70	6.94
Benzoic acid derivatives				
Benzoic acid	4.20	164	3.72	0.18
Salicylic acid	3.03	700-800	1.36	
3-Hydroxybenzoic acid	4.16	415	8.35	0.42
4-Hydroxybenzoic acid	4.58	420-430	22.1	1.09
3,4-Dihydroxybenzoic acid		203	9.06	0.44
3,5-Dihydroxybenzoic acid		680	11.6	0.57
2,5-Dihydroxybenzoic acid		900-950		
3,4,5-Trihydroxybenzoic acid	4.33	86	3.42	0.14
4-Aminobenzoic acid	2.38, 4.89	21.6	5.30	0.29
4-Hydroxy-3-methoxy-benzoic acid			5.12	0.30
3-Hydroxy-4-methoxy-benzoic acid		107	4.07	0.21

TABLE 2 (continued)

Sample	pK_a^o	k'		
		pH of the buffer		
		2.08	5.70	6.94
Phenylacetic acid derivatives				
Phenylacetic acid	4.32	73.7	1.98	0.13
2-Hydroxyphenylacetic acid		79.4	2.84	0.20
3-Hydroxyphenylacetic acid		93.6	2.73	0.17
4-Hydroxyphenylacetic acid		80.4	2.88	0.22
3,4-Dihydroxyphenylacetic acid		32.5	1.16	0.03
4-Hydroxy-3-methoxy-phenylacetic acid		26.8	0.92	
Mandelic acid derivatives				
Mandelic acid	3.41	14.9	0.05	
3-Hydroxymandelic acid		18.7	0.07	
4-Hydroxymandelic acid		14.7	0.07	
3,4-Dihydroxymandelic acid		5.76	0.03	
4-Hydroxy-3-methoxy-mandelic acid		5.31		
3-Hydroxy-4-methoxy-mandelic acid		4.87		
Indoleacetic acid derivatives				
Indole-3-acetic acid		439	21.5	1.12
5-Hydroxyindole-3-acetic acid		133	6.56	0.35
Miscellaneous carboxylic acids				
Phtalic acid	2.95, 5.41	29.6	0	
Hippuric acid		9.77	3.73	
Salicyluric acid		156		
Fumaric acid	3.02, 4.38	23.1		0
Maleic acid	1.94, 6.23	0.63		
Glucuronic acid derivatives				
4-Nitrophenyl-β-D-glucuronic acid		9.77		
2-Naphtyl-β-D-glucuronic acid		40.6		
Amino acids and dipeptides				
Phenylalanine	2.16, 9.12	0.87		
Phenylalanyl-Alanine		1.57		
Phenylalanyl-Leucine		23.5		
Leucyl-Phenylalanine		21.9		
Phenylalanyl-Serine		0.55		
Phenylalanyl-Phenylalanine		44.7		
Valyl-Phenylalanine		5.99		
Lysyl-Phenylalanine		0.09		

Their peaks showed pronounced tailing. The influence of pH on retention is in accordance with eq. (4). A typical chromatogram is shown in Fig. 4.

Retention data obtained at acidic pH have been used to calculate the separation factors for some simple structural changes. Increase in retention was obtained for hydroxylation (log α = 0.12, phenol/benzene) and carboxylation (log α = 0.14, benzoic acid/benzene) in benzene while hydroxylations in the benzoic, phenylacetic, mandelic and indoleacetic acids gave either an increase or a decrease depending on the position for hydroxylation.

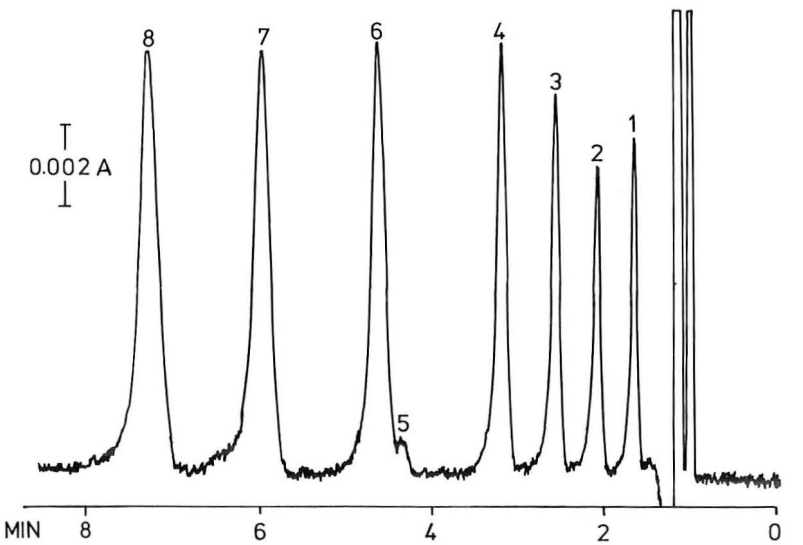

FIGURE 4. Separation of a mixture of carboxylic acids. Conditions as in Fig. 2 but pH = 6.02.
Samples: 1. 3,4-Dihydroxyphenylacetic acid
2. Phenylacetic acid
3. 2-Hydroxyphenylacetic acid
4. 3-Hydroxy-4-methoxybenzoic acid
5. Impurity
6. 5-Hydroxyindole-3-acetic acid
7. 3,4-Dihydroxybenzoic acid
8. 3,5-Dihydroxybenzoic acid

Increase of the carbon chain length increased the retention (log α = 0.47, methylpropylketone/methylethylketone).

Even very hydrophilic acids like fumaric acid and maleic acid are retained. A very high separation factor, 37, is obtained between these cis-trans isomers.

Phenylalanine and the dipeptides were only studied with pH 2 buffer in the eluent. In such a medium they will have a net positive charge and they might be retained as the phosphate ion-pairs.

The phase system can also be used for separations of ion--pairs of hydrophilic amines, like the catecholamines, which will be discussed in forthcoming publications [13].

ACKNOWLEDGEMENTS

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SEPARATION OF BIOLOGICAL PYRIDINES BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

The separation of the six pyridine compounds which comprise the pyridine nucleotide cycle, nicotinamide adenine dinucleotide phosphate and para-aminobenzoic acid, a compound biologically related to these pyridines, can be achieved rapidly utilizing high pressure liquid chromatography. Optimum separation is accomplished using ion-ion pairing in reverse phase chromatography with a C_{18} stationary phase and an aqueous mobile phase of 5 mM pentanesulfonic acid and 25 mM KH_2PO_4. The effect of temperature on the separation is minimal. As little as 10 ng of these compounds is detected via absorption of ultraviolet light at a wavelength of 254 nm.

INTRODUCTION

The pyridine nucleotide cycle has been found in some form, in every living organism in which it has been searched for (1). This cycle allows the conservation and recycling of the pyridine ring in cellular metabolism. The cycle itself is central to all of cellular metabolism since the intermediates of the cycle are involved in over 300 oxidation-reduction reactions (2), repair of UV light-induced damage to DNA (3), synthesis of vitamin B_{12} (4) and control of cellular differentiation via the synthesis of poly adenosine diphosphate ribose (5). Many physiological studies require the quantitation of one or more components of this cycle. In order to study the cycle

quantitatively, a rapid procedure for the isolation of the various components of the cycle is necessary. Earlier work on the separation on these intermediates relied on the use of multiple paper chromatographic separations. Thus, Withold (6) had to run multiple solvents to separate the various intermediates. Averett and Tritz (7) published a two-dimensional paper chromatographic method which uniquely separated the various pyridines but the process required a total of 24 h for complete development. In each of these separation procedures quantitation was difficult owing to the necessity of eluting the compound of interest from the paper prior to quantitation.

This report describes a high pressure liquid chromatography procedure which separates the six components of the pyridine nucleotide cycle and allows their quantitation, all in a matter of minutes. In addition, the system separates out p-aminobenzoic acid, a biologically related and sometimes contaminating substance and nicotinamide adenine dinucleotide phosphate, a metabolic product of one of the components of the pyridine nucleotide cycle.

EXPERIMENTAL

Instrumentation

A Waters Associates (Milford, MA) ALC/GPC Model 244 high pressure liquid chromatograph with a M6000A solvent delivery system, U6K injector and Model 440 dual wavelength UV absorbance detector was used in the separation system. The columns evaluated were commercial stainless steel columns pre-packed with C_{18}-μ-Bondapak (30 cm x 4.6 mm, 10 μ) for reverse phase chromatography or with ZIPAX SCX (30 cm x 4.6 mm pellicular) for ion exchange chromatography (both purchased

from Alltech Associates, Arlington Heights, IL) or with spherosorb (25 cm x 4.0 mm, 5 μ) for ion exchange chromatography utilizing functional nitrile groups (Chromanetics Corp., Baltimore, MD).

Retention times and concentrations of eluants were electronically calculated using a CSI Supergrator-3A (Columbia Scientific Industries, Austin, TX). The program used to determine these values is shown in Figure 1. The signal from the UV detector was fed through the integrator to a Fisher Recordall dual pen recorder (Fisher Scientific Co., Pittsburgh, PA).

PROGRAM

RUN PARAMETERS

initial peak width	0.1
minimum peak size	0.
sensitivity	3.

TIMED EVENTS

type	time	value
1	15.00	—

CALCULATION PARAMETERS

type calculation	3.
type results	2.
system factor	1.
sample amount	2.
minimum report	0.

IDENTITY PARAMETERS

Calibration Table Number	1.
Time Reference peaks	
minimum value	1.
percent window	1.
minimum window	0.01
Unknown factor	1.
Calibration Table Percent Update	
normal run	0.
calibration run	50.

Figure 1. Program used to determine retention times and concentration of eluants.

Chemicals and Reagents

The mobile phase for reverse phase chromatography consisted of reagent grade potassium dihydrogen phosphate, and distilled water to which was added either 1-pentanesulfonic acid sodium salt or 1-heptanesulfonic acid sodium salt (Eastman Organic Chemicals, Rochester, NY) to a concentration of 5 mM. The pH of these solutions was adjusted with phosphoric acid to either 2.5 or 3.5. The mobile phase used in ion exchange chromatography with both ion exchange columns consisted of a 2.5 mM phosphate buffer containing the proper ratio of KH_2PO_4 and K_2HPO_4 to give a pH of 2.5, 3.5 or 7.5. At the lower pH ranges it was necessary to adjust the pH with phosphoric acid. All solvents were filtered through a 0.45 μm pore size nitrocellulose filter (Millipore Corp., Bedford, MA).

Stock solutions of the pyridine nucleotide cycle intermediates, obtained from Sigma Chemical Co., St. Louis, MO,were made up in distilled water at a concentration of 1 μg/μl and were stored at -20^{o} C until used. At the time of use, each solution was diluted 1:50 with distilled water and 0.2 μg samples (10 μl) were chromatographed to obtain retention times. Finally, a mixture of the compounds was chromatographed and the retention times compared.

Preparation of C_{18} Column

Prior to use, the reverse phase column was prepared by washing with 50 ml of 0.1 M oxalate followed by 150 ml distilled water and then 50 ml of 0.1% triethanolamine. Finally, the column was equilibrated with the solvent system to be used in the separation for 30 minutes. Equilibration was established by obtaining similar results

in duplicate runs at a 15 minute interval.

RESULTS AND DISCUSSION

The pyridine nucleotide cycle consists of the intermediates nicotinic acid, nicotinic acid mononucleotide, nicotinic acid adenine dinucleotide, nicotinamide adenine dinucleotide, nicotinamide mononucleotide, and nicotinamide. Nicotinamide adenine dinucleotide phosphate, although not in the pyridine nucleotide cycle, is a metabolic product of nicotinamide adenine dinucleotide; p-aminobenzoic acid, although not a pyridine, has biologic effects which influence the concentration of the pyridine nucleotide cycle components.

Initial attempts to separate the above mentioned eight compounds were carried out using ion exchange high pressure liquid chromatography. Neither cation exchange chromatography using a nitrile column and mobile phases of phosphate buffer at pH values of 2.5, 3.5 or 7.5 nor cation exchange chromatography with a ZIPAX SCX column and mobile phases of phosphate buffer at pH values of 2.5, 3.5 or 7.5 successfully separated the compounds of interest. It became evident that ion exchange chromatography would result in a separation of these compounds only with gradient elution techniques or with elution by multiple solvent systems. In order to attempt to develop a more simple system, the separation of these compounds via ion-ion pairing in reverse phase high pressure liquid chromatography with isocratic elution was investigated.

Preliminary attempts at reverse phase chromatography of these compounds met with difficulty due to the inability to separate nicotinic acid adenine dinucleotide, nicotinamide adenine dinucleotide

and nicotinamide; nicotinamide adenine dinucleotide phosphate, nicotinamide mononucleotide and nicotinic acid mononucleotide were also not resolved. This initial attempt at separation utilized pentanesulfonic acid at a pH of 3.5. Addition of 5 mM KH_2PO_4 to the pentanesulfonic acid or use of heptanesulfonic acid, pH 3.5, with 5 mM KH_2PO_4 did not enhance the separation (Figure 2, panels (a) and (b) respectively). These data were interpreted to mean that the pH of 3.5 was not low enough to suppress a majority of the ionization of these compounds and, therefore, we were not dealing with a complete ion pair system. This problem was overcome by lowering the pH to 2.5. Figure 3, panels (a) and (b), illustrates the result of lowering the pH of the mobile phase.

Evaluation of these results led to the postulation that the optimum separation would be achieved with a mobile phase of pentanesulfonic acid at a pH of 2.5 with added KH_2PO_4. The function of the phosphate ions was to increase the polarity of the mobile phase with the concomitant increase in retention time of the pyridine compounds. In order to determine the most efficient concentration of phosphate, the separation of nicotinamide mononucleotide from nicotinamide adenine dinucleotide phosphate was investigated; these two compounds had been the most difficult to separate. Table I tabulates the results of the effect of phosphate ions on this separation. As is evident, a solvent system of 5 mM pentanesulfonic acid, pH 2.5, containing 25 mM KH_2PO_4, separated nicotinamide mononucleotide from nico-

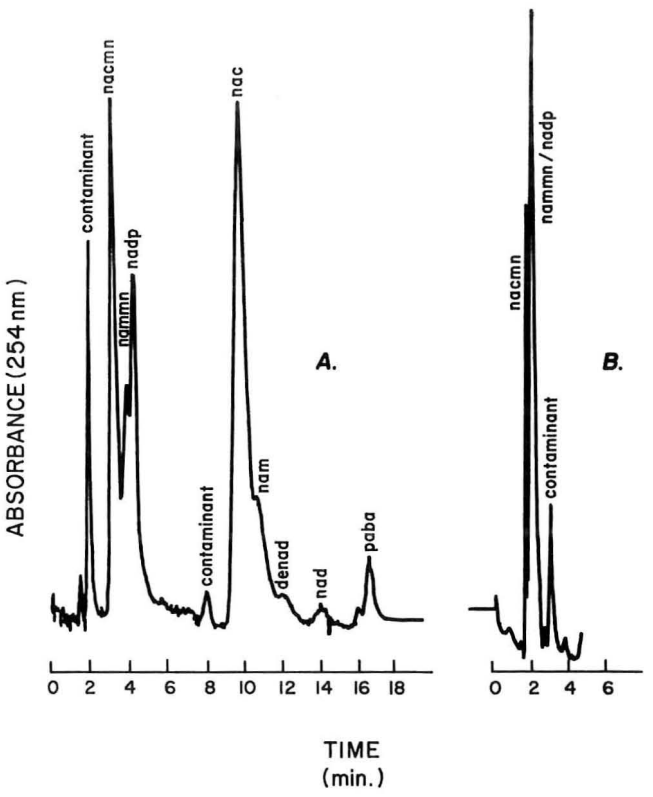

Figure 2. Separation of biological pyridines in reverse phase high pressure liquid chromatography. Conditions: column, C_{18}-μ-Bondapak (30 cm x 4.6 mm, 10 μ); temperature, ambient; mobile phase, 5 mM pentane sulfonic acid containing 5 mM KH_2PO_4, pH 3.5 (panel A) or 5 mM heptane sulfonic acid containing 5 mM KH_2PO_4, pH 2.5 (panel B).

Abbreviations: nacmn, nicotinic acid mononucleotide; nammn, nicotinamide mononucleotide; nadp, nicotinamide adenine dinucleotide phosphate; nac, nicotinic acid; nam, nicotinamide; denad, nicotinic acid adenine dinucleotide; nad, nicotinamide adenine dinucleotide; paba, para-aminobenzoic acid.

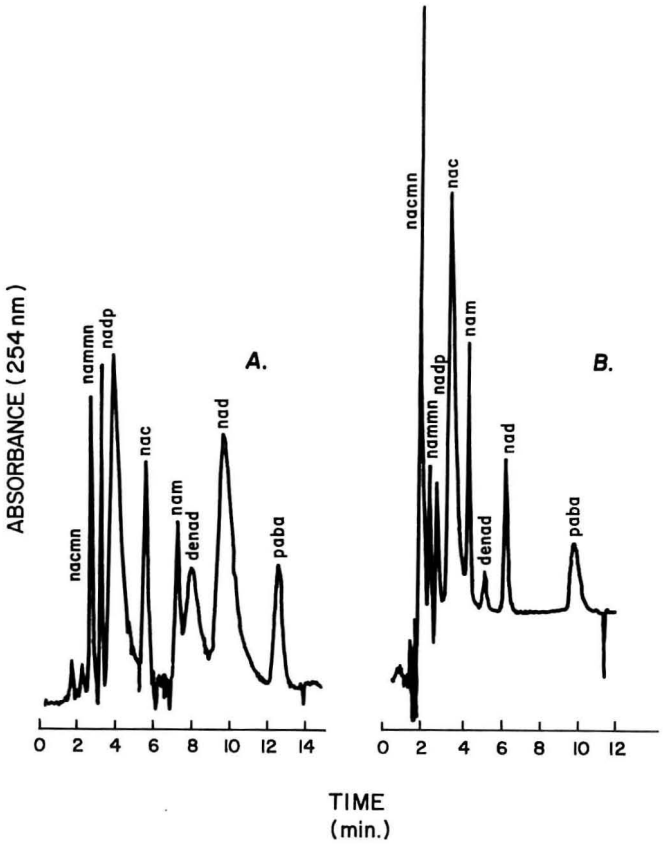

Figure 3. Effect of pH 2.5 on the separation of biological pyridines in reverse phase high pressure liquid chromatography. Conditions: same as described in Fig. 2 except that the pH was lowered to 2.5; abbreviations, same as listed in Fig. 2.

TABLE I

Variation in Minutes of Retention Time of NAMMN[1] and NADP[2] with Changing Phosphate Concentrations.

Intermediate	Phosphate Concentration 0 mM	5 mM	10 mM	25 mM	50 mM
NAMMN	2.93	2.79	2.75	2.73	2.73
NADP	3.23	3.07	3.16	3.52	3.51

Conditions were identical to those listed in Figure 1 except only the aqueus mobile phase of 0.005 M pentanesulfonic acid at pH 2.5 was used.

[1]NAMMN - Nicotinamide mononucleotide
[2]NADP - Nicotinamide adenine dinucleotide phosphate

tinamide adenine dinucleotide. Using this mobile phase, all of the biological pyridines of interest plus p-aminobenzoic acid were chromatographed. The resulting separation is shown in Figure 4.

The effects of temperature upon the separation shown in Figure 4 was investigated. The original separation was run at ambient temperature. At no temperature tested (0°, 10°, 15°, 20° C) was there sufficient enhancement of the separation to warrant the use of closely controlled temperatures.

The final parameter investigated was the limits of detectability via absorption of UV light. By calibrating the integrator with known concentrations of the various compounds and obtaining peak areas, it was possible to estimate the ultimate limits of detection. For all of the compounds except nicotinic acid, the level of detection was approximately 10 ng. For nicotinic acid this value was 1 ng.

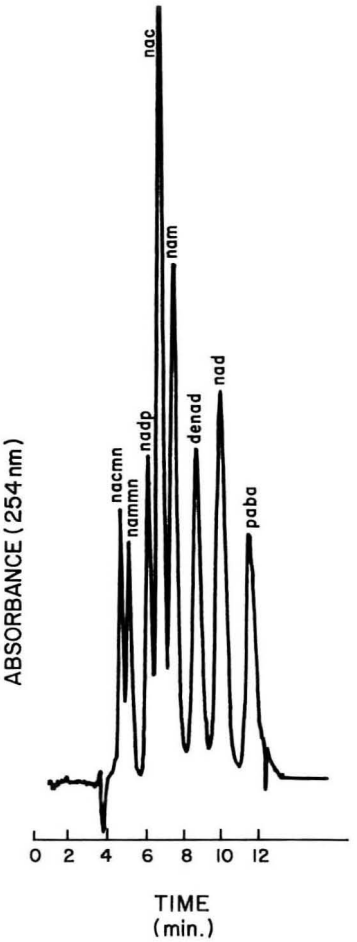

Figure 4. Effect of 25 mM KH_2PO_4 on the separation of biological pyridines in reverse phase high pressure liquid chromatography. Conditions: same as described in Fig. 2 except that the solvent system contained 5 mM pentane sulfonic acid and 25 mM KH_2PO_4, pH 2.5; abbreviations, same as listed in Fig. 2.

ACKNOWLEDGMENTS

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OPTIMIZATION OF A REVERSE PHASE ION-PAIR CHROMATOGRAPHIC SEPARATION FOR DRUGS OF FORENSIC INTEREST PART I - VARIABLES EFFECTING CAPACITY FACTORS

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ABSTRACT

A study is presented employing approximately 50 drugs of forensic interest to determine the effect of stationary phase, water-methanol ratio, alkyl length and concentration of counter-ion and basicity of the compound chromatographed on capacity factors utilizing a reverse phase ion-pair separation. Microbondapak-C18, Microbondapak-Alkyl Phenyl and Microbondapak-CN are the columns examined. The mobile phases used contain water, methanol, acetic acid and an alkylsulfonate salt. Horvath's solvophobic theory is a useful model for explaining many of the chromatographic trends.

INTRODUCTION

Recently it was demonstrated that reverse phase ion-pair chromatography is a most versatile technique when applied to drugs of

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forensic interest[1]. This mode utilized a buffered aqueous-organic mobile phase containing a counter-ion which is available to form a lipophilic complex with the salt of a drug. This technique allows the simultaneous analysis of basic, acidic and neutral compounds. The methodology depicted by Lurie used a single isocratic system utilizing a Microbondapak-C18 column and a mobile phase consisting of 40% methanol, 59% water, 1% acetic acid and 0.005M heptanesulfonic acid at a pH of approximately 3.5. In general, by using this system, basic drugs are analyzed via ion pairing and acidic drugs by ion suppression.

This isocratic system is applicable to ergot alkaloids, phenethylamines, opium alkaloids, local anesthetics, barbiturates as well as other drugs of forensic interest. Although this technique approached the ideal situation of using a single HPLC system for a wide range of drugs of forensic interest, certain drawbacks existed. First of all the phenethylamines, amphetamine and methamphetamine were poorly resolved. In the case of opium alkaloids, heroin and acetylcodeine co-eluted. Although compounds related to cocaine were well resolved, their retention times were longer than optimum. LSD and Iso-LSD (a common component in LSD exhibits) had retention times that were fifteen and eighteen minutes respectively. PCP had a retention time of approximately seventeen minutes. Therefore, it was desired to optimize these and other various separations for resolution and speed. When dealing with semi-preparative reverse phase ion-pair chromatography, conditions must be optimized in order to obtain satisfactory resolution of the analytical separation[2].

With this goal in mind, a study was undertaken to determine the effect of column type, water-methanol ratio, counter-ion size, counter-ion concentration and basicity of drugs chromatographed on a reverse phase ion-pair chromatographic separation for drugs of forensic interest. This paper will discuss the effect of the above variables on the capacity factor for the various compounds chromatographed. The drugs include barbiturates, local anesthetics, phenethylamines, opium alkaloids, ergot alkaloids and other drugs of forensic interest. A subsequent paper will discuss the effect of the above parameters on selectivity factors[3].

EXPERIMENTAL

The liquid chromatograph consisted of the following components: Model 6000A pump (Waters Associates, Milford, MA); Model U6K injector (Waters); prepacked 3.9 mm x 30 cm stainless steel columns: Microbondapak-C18, Microbondapak-Alkyl Phenyl and Microbondapak-Cyanide (Waters); Model 770 variable UV detector at 254 nm (Schoeffel Instruments, Westwood, NJ) or Model 440 fixed UV detector at 254 nm (Waters); Systems IVB integrator (Spectra Physics, Santa Clara, CA).

Materials

The following chemicals were used: methanesulfonic acid, butanesulfonic acid sodium salt, heptanesulfonic acid sodium salt (Eastman Chemicals, Rochester, NY); methanol, distilled in glass (Burdick and Jackson, Muskegon, MI); distilled water and other chemicals were reagent grade. Authentic drug standards of USP/NF quality were employed.

Procedures

The capacity factor k' was calculated according to the formula $k' = \frac{(tRi - tRo)}{tRo}$ where tRi is the retention time of component i and tRo is the retention time of a non-retarded component which in this instance was approximated to be methanol. The selectivity factor (α) is obtained from the formula $\alpha = k'j/k'i$, where k'j and k'i are the capacity factors of the jth and ith sample component.

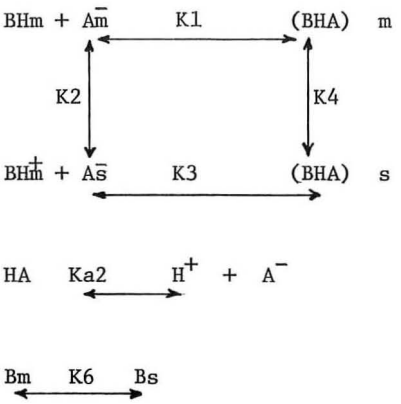
All mobile phases were prepared by dissolving an alkylsulfonic acid or alkylsulfonic acid salt in a solution consisting of glacial acetic acid, methanol and distilled water. After filtering and degassing the solution through a Millipore 0.50 micron filter (Millipore Corporation, Bedford, MA), the pH was adjusted to 3.5 with 2N NaOH.

All standards were dissolved in methanol. For the determination of capacity factors, co-injections consisting of 5 ml of drugs of interest and of methamphetamine (meth) were used. The k' values were then determined based on the above mentioned selectivity factor and the k' of methamphetamine in a given mobile phase based on the relationship $k'j = \alpha k'meth$ or $k'j = k'meth/\alpha$. The concentrations of these drugs were 0.5 mg/ml except for LSD, LAMPA and Iso-LSD.

THEORETICAL

The separations that take place can be described by the following equations:

$$BH^+ \quad \underset{\longleftarrow}{\overset{Kal}{\longrightarrow}} \quad B + H^+$$



B and HA represent basic and acidic drugs respectively while A^- refers to a negative counter ion. The subscript m depicts mobile phase while s refers to the stationary phase. The mechanism of ion pairing is very much in dispute. The ion pair mechanism has been shown by Horvath et. al.[4] to proceed by equation Kal, K1 and K4 which represent ion pair formation in the mobile phase followed by adsorption of the ion pair on the stationary phase. Kissinger[5] and Scott and Kucera[6] believe the mechanism proceeds via equation Kal, K2 and K3 which depicts the counter ion being adsorbed onto the stationary phase and ion pairing occuring by an ion exchange mechanism.

At a low pH equation Ka2 is shifted to the left which would favor formation of the free acid. This situation represents ion suppression. Free base, if present in the mobile phase, can be adsorbed onto the stationary phase as represented by equation K6.

RESULTS AND DISCUSSION

Approximately 50 drugs of forensic interest were chromatographed using three different stationary phases; namely a Microbondapak-C18,

a Microbondapak-Alkyl Phenyl and a Microbondapak-Cyanide. For each column, mobile phases consisting of water, methanol, 1% acetic acid and a 0.005M alkylsulfonate counter ion at pH 3.5 were employed. For methanol concentrations of 40% and 30%, the counter ion was varied from heptanesulfonate to methanesulfonate in increments of three carbons. For 20% methanol only methanesulfonate was used because of excessive retention of many bases with butanesulfonate or heptanesulfonate counter ion. Retention data for the various drugs is presented elsewhere[3].

Effect of Counter Ion Size on k'

The effect of varying the counter ion size from heptanesulfonate to methanesulfonate on the various classes of drugs is described below. For any given column and water-methanol ratio, the k' of barbiturates was independent of counter ion. This was expected since barbiturates are weak acids with pka values greater than 7[7]. This means these compounds would exist as the free acid at pH 3.5 and wouldn't be expected to ion pair.

For most bases studied at a constant water-methanol ratio, the k' increased with increasing size of counter ion with both the C18 and alkylphenyl columns. This effect has been well documented[4, 8]. Also, for most of the bases studied, the ratio of k's for any given set of counter ions is fairly constant. This relationship is demonstrated in Figure 1 & 2 by the constant slopes of the curves between any two data points. Since most of these compounds have the same charge, their ratios are independent of elute surface area. Quinine and quinidine in the majority of cases have ratios of k' values that

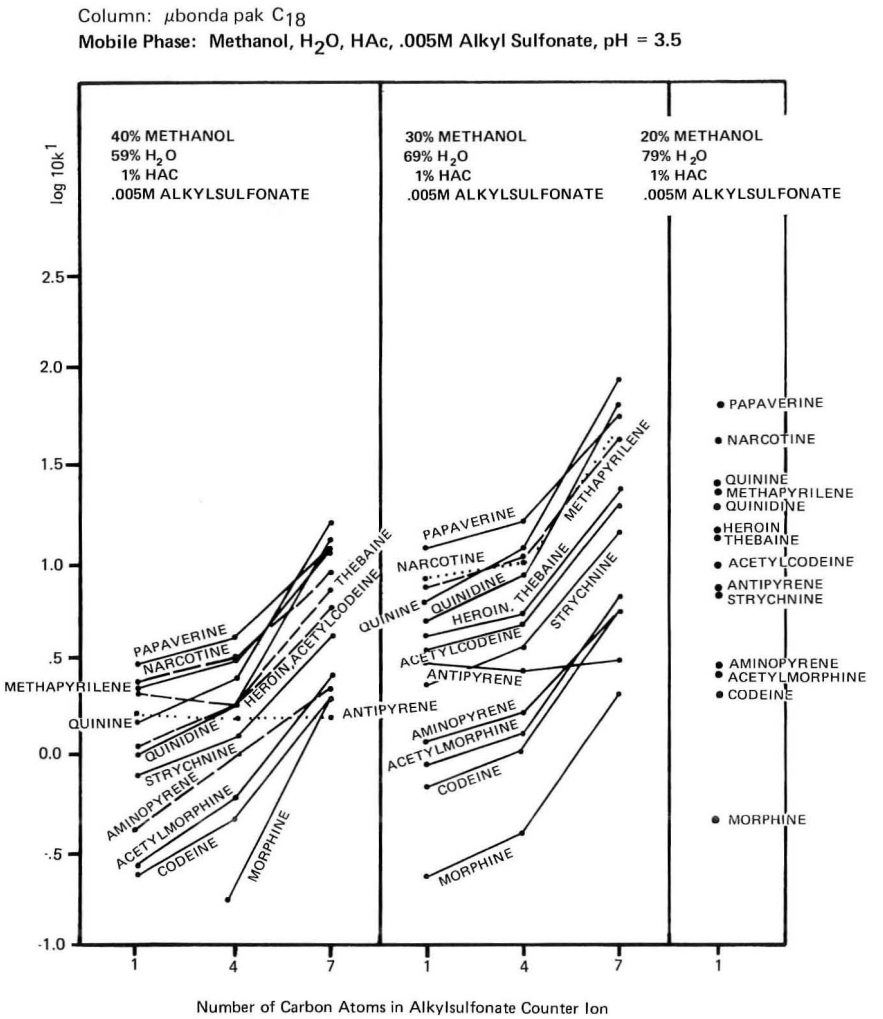


Figure 1 - Plot of log k' of opium alkaloids and related compounds versus number of carbon atoms in alkylsulfonate counter ion utilizing various methanol, water, 1% acetic acid, 0.005M alkylsulfonate mobile phases at pH 3.5; Column Microbondapak C-18.

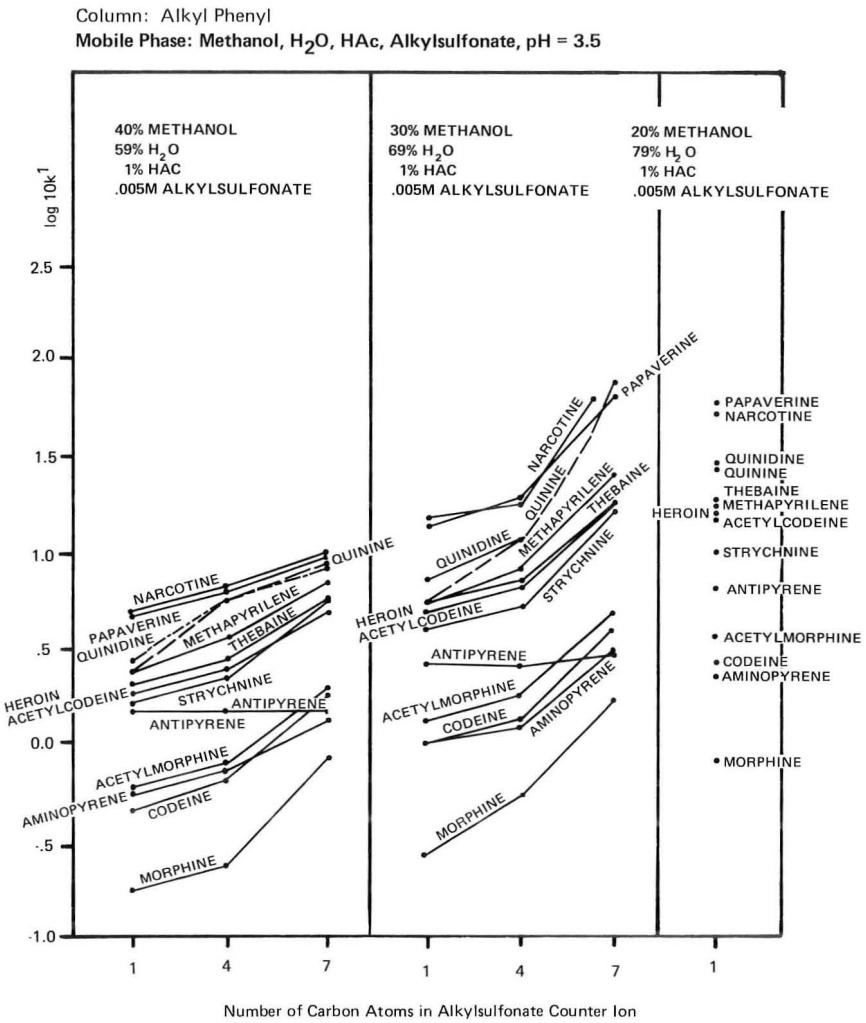


Figure 2 - Same as Figure 1 except column is a Microbondapak Alkyl Phenyl.

are approximately double that of the other bases. This is to be expected since both compounds have two basic pka values[7] representing two ionizable sites. We cannot explain why morphine and aminopyrene in certain instances exhibit anomalous behavior in relationship to their variation of k's with counter ion.

Tomlinson et. al.[9], who studied ion pairing between anionic solutes and alkylbenzyldimethylammonium chlorides, observed that a divalent solute exhibits twice the change in retention with increased size of counter ion than a monovalent solute. Tomlinson further points out that a 2:1 stoichiometric interaction between the counter ion and anionic solute would be sterochemically unfavorable if ion pairing occured by an ion exchange mechanism. Horvath et. al.[4] showed for cationic solutes with anionic counterions such as alkylsulfates and alkylsulfonates that the increase in retention with size of counterion is independent of the size of the solute but depends on its charge. Horvath's conclusions were based on working at counter ion concentrations where the solute's retention would be at a maximum. In our study, 0.005M does not represent for methanesulfonate, butanesulfonate and heptanesulfonate in most cases a counter ion concentration for which solute retention is at a maximum.

In general, higher variations of k' with counter ion were observed on the C18 column than the alkylphenyl column as is shown in Figure 1 & 2. A possible explanation for this effect can be derived from Horvath's et. al. solvophobic theory for retention in reverse phase ion pair chromatography[4]. According to this theory, if we assume ion pairing occurs in the mobile phase followed by adsorption

of the ion pair onto the stationary phase, the equilibrium constant for the binding of the neutral ion pair is expressed as $\ln K = a - b + c \text{ delta } A$ where a, b and c are constants depending on solvent and column properties and delta A is the contact area which is the difference between the molecular surface area of the ion pair stationary phase complex and the surface areas of the stationary phase ligand and the ion pair. This contact area is proportional to the molecular surface area of the complex formed between the ion pair and hydrocarbonous ligand. Similarly, the C18 column which consists of 18 carbon bonded groups has more hydrocarbon character than the Alkyl Phenyl column which consists of ethylbenzene groups and thus a greater contact area with the ion pair. Thus the greater increase in retention with size of counter ion on the C18 column would be expected.

In addition, the variation of k' with counter ion on the C18 and alkyl phenyl columns appear to be fairly independent of water-methanol ratio (Figures 1-2). On both columns, k' increases exponentially with the carbon number of the counter ion. Although antipyrene, benzocaine, caffeine, diazapam, mecloqualone, methaqualone and theophylline all have basic functional groups, they exhibit no significant variation of k' with counter ion size as is illustrated in Figure 1 for antipyrene. For antipyrene, benzocaine, caffeine and methaqualone who have basic pka values of 1.4, 2.8, 0.6 and 2.5 respectively[7], no appreciable protonation of these bases would be expected at the mobile phase pH of 3.5. Equation Kal shows that this protonation would be required for ion pair formation Diazapam has a basic pka value of 3.4 while theophylline has a basic pka of

3.5 and an acid pka of 8.6[7]. At a pH of 3.5, some protonation of the basic functional group would be expected. The unionized compound, of which a significant amount would exist at pH 3.5 could be adsorbed by the stationary phase. This chromatographic process could be represented by the equation K5. The non-variation of k' with counter ion for diazapam and theophylline could be explained by domination of process K5 over K1 and K4 which represent ion pairing. Although we could not ascertain for sure, it is probable that these pKa values were determined in water. Pka values for bases tend to be lower when alcohol greater than 20% is present in the mobile phases[9]. A lower pKa value would mean that less ionized base would be present and could account for the above behavior of diazapam and theophylline. No pKa data was available for mecloqualone. Glutethimide, which is a weak acid with a pka of 4.52[7], as expected, exhibits no appreciable variation of k' with counter ion size.

No significant variation of k' with counter ion size was observed for any drugs on the cyanide column as is shown in Figure 3. This is probably related to the small aliphatic character of this column. As stated earlier, retention is proportional to the molecular surface area of the complex formed between the ion pair and the hydrocarbonous stationary phase.

Effect of Stationary Phase on k'

For any mobile phase the retention order of barbiturates on a given stationary phase was C18 ≈ alkylphenyl > cyanide (Figure 4). This is consistent with the work of Scott and Kucera[6] and Hennion et. al.[10] who show that a constant surface coverage of the parent

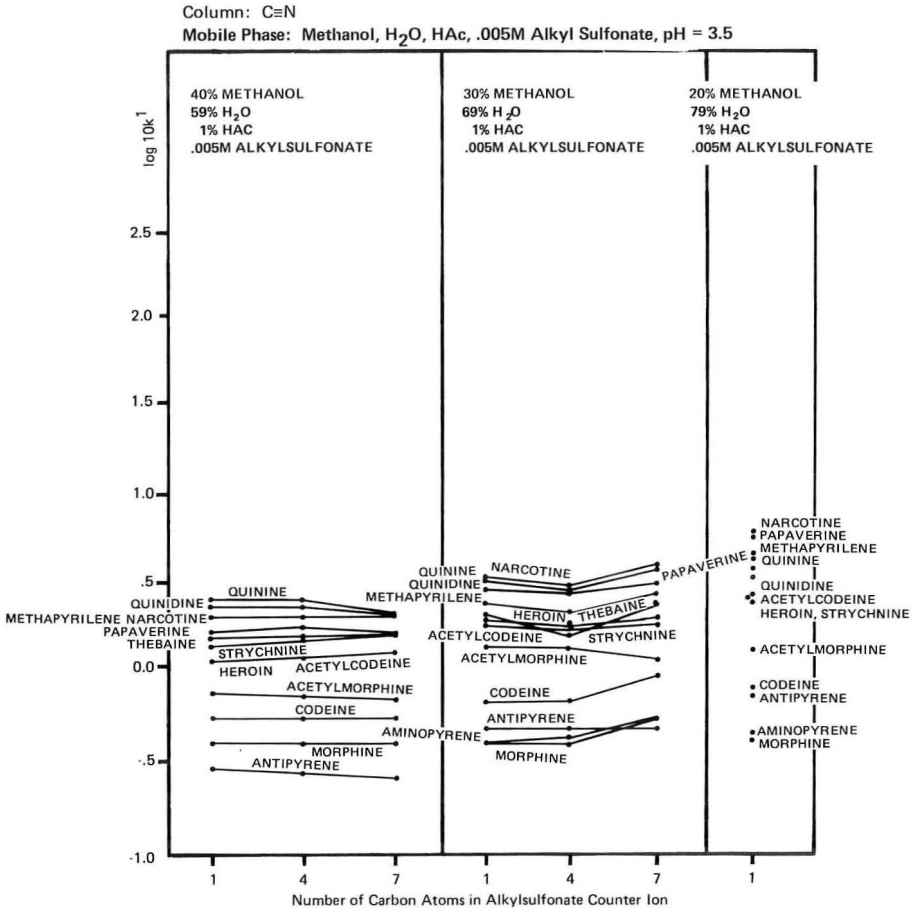


Figure 3 - Same as Figure 1 except column is Microbondapak CN.

silica increases retention with the carbon chain length of the bonded group. In general, the Microbondapak-C18, Microbondapak-Alkyl Phenyl and Microbondapak-Cyanide have constant surface coverage[11]. The carbon chain lengths of the C-18, alkylphenyl and cyanide columns are

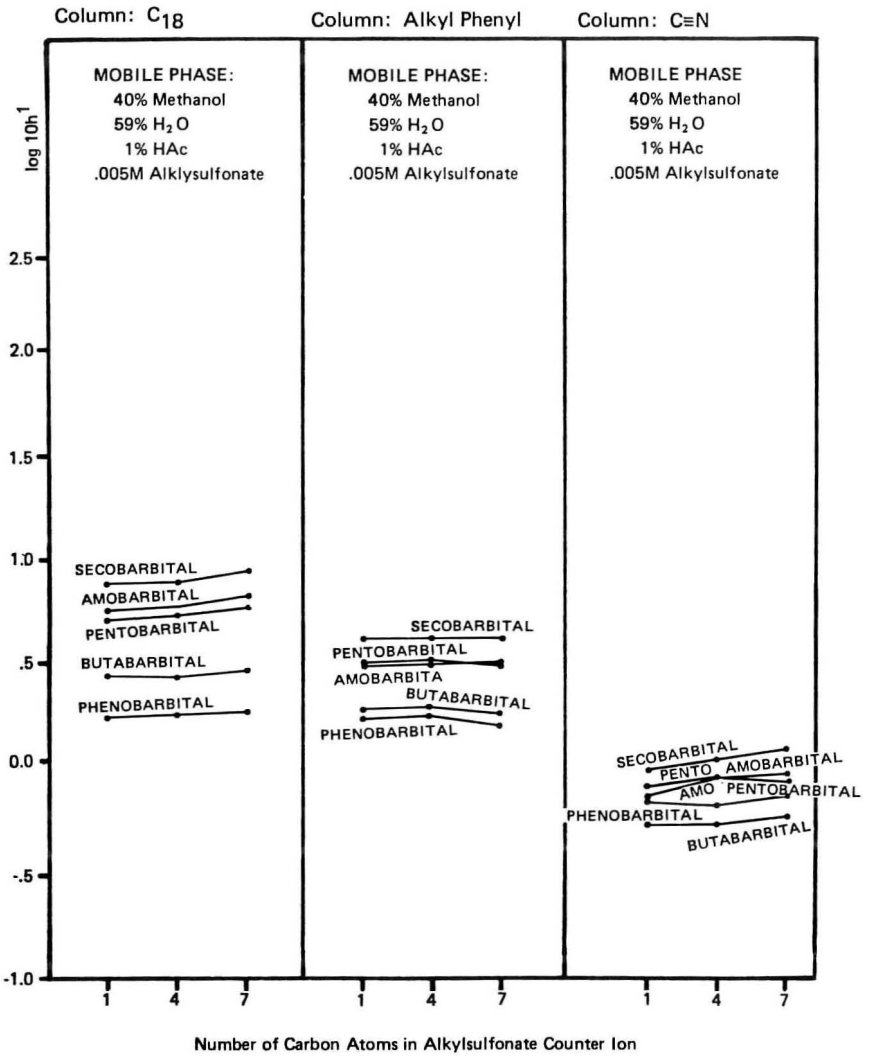


Figure 4 - Plot of log k' of barbiturates versus number of carbon atoms in alkylsulfonate counter ion utilizing mobile phase of 40% methanol, 59% water, 1% acetic acid, 0.005M alkylsulfonate at pH 3.5.

18, 8 and 4 respectively. The other workers were refering to linear chains. The alkylphenyl column contains a benzene ring with an attached ethyl group bonded to the silica while the cyanide column contains a cyanide group attached to propyl group. Phenobarbital, which is the only barbiturate studied to contain a benzene ring, has the greatest retention on the alkylphenyl column relative to the C-18 column. Both dipole[12] and pi orbital interactions[13] have been hypothesized to occur between the alkylphenyl column and solutes. This could explain the behavior of phenobarbital. Retention for phenobarbital on the cyanide column is greatest relative to its retention on the C-18 and alkylphenyl columns. This effect could also be attributed to dipole and pi orbital interactions.

In comparing retention of basic drugs, whose k' increases with counter ion size, some interesting trends were observed. When heptanesulfonate was used as a counter ion, an appreciable variation of k' with stationary phase was observed. The order of retention was C-18 > alkyl phenyl > cyanide. Archari and Jacob[14] obtained a similar result with a mobile phase consisting of 49% methanol, 50% water, 1% acetic acid and 0.005M heptanesulfonic acid at a pH of 4.0 in a study involving seventeen bases, seven of which are studied in this report. However, when butanesulfonate or methanesulfonate were used as counter ions a much smaller variation of k' with stationary phase was observed. In many instances retention was actually greater on the cyanide or alkylphenyl column than on the C-18 column. Horvath et. al.[4] has shown that when assuming ion pairing in the mobile phase mechanism, retention is proportional to the difference in the molecular

surface area of a complex formed between the non-polar hydrocarbonous ligand of the stationary phase and the non-polar moiety of the ion pair. This interaction is hydrophobic in nature, meaning it is based on repulsion between the ion pair and ligand with water causing the ion pair and ligand to associate. Thus a smaller hydrocarbonous ligand on a stationary phase would retain an ion pair less. Based on the size of their hydrocarbonous ligands, retention of the ion pairs on the three columns studied would be expected to be C-18 > alkylphenyl > cyanide with an appreciable difference of magnitude. It appears that when using a larger counter ion the above relationship was observed for the bases studied. The lesser variation of k' with stationary phase that was observed for bases when smaller counter ions were used could be explained by the decreased hydrophobic interactions between the ion pair and the ligand. These smaller interactions could favor the dipole and pi orbital interactions that occur on the cyanide and alkylphenyl columns. All of the bases whose retention times vary with counter ion have one or more benzene rings or multiple sites of unsaturation.

Effect of Water-Methanol Ratio on Retention

Increasing the ratio of water to methanol increases retention on all three columns for all the barbiturates studied. This is typical of retention in reversed phase systems where chromatography is based on hydrophobic interactions[15], [16]. For all three columns studied, the increase in retention with the water-methanol ratio was linear. A similar result was reported by Tjaden et. al.[17] for a methyl silica column. This increase is independent of barbiturate and column

type for the C-18 and Alkyl Phenyl column. This result is consistent with the findings of Karch et. al[18] using alcohols and phenols on C-18 and C-4 straight chain bonded columns. The increase in retention with water was independent of barbiturate on the CN column.

On the C-18 and alkylphenyl column all bases exhibited an increase in k' with an increase in water-methanol ratio as is illustrated in Figures 1 & 2 for opium alkaloids. Archari and Jacob[14] reported similar results using a Microbondapak C-18 column. According to Horvath et. al.[4], adsorption of the ion pair onto the stationary phase increases with increased surface tension of the mobile phase. The surface tension of a mobile phase increases by adding water.

The increase in retention with increased water concentration for all compounds studied was considerably less on the cyanide column. In certain instances there was no increase in retention. This result is consistent with Horvath et. al.'s[15] theory of hydrophobic interactions which depends on a complex being formed between a hydrocarbonous ligand and the non-polar substituents on an eluite. This interaction is proportional to the contact area between the ligand of the stationary phase and the solute. Since the cyanide column has a small hydrocarbonous ligand, the hydrophobic effect would be expected to be smaller than on the C-18 and alkylphenyl columns.

Effect of Counter Ion Concentration on Retention

Using eleven drugs of the original fifty, a limited study of the effect of varying counter ion concentration on retention was conducted. On both the C-18 and Alkyl Phenyl columns, the counter ion concentrations of 0.005M and 0.02M methanesulfonate, butanesulfo-

nate and heptanesulfonate were employed. The drugs included butabarbital, methamphetamine, procaine, lidocaine, cocaine, ephedrine, codeine, heroin, quinidine, LSD, and PCP. On the C-18 column the k' for the various solutes did not vary with the change in methanesulfonate concentration. The k's of methamphetamine, lidocaine, and ephedrine increased by approximately 10% with a four fold increase of butanesulfonate concentration while the retention of the other solutes did not change. All basic solutes, except quinidine, increased by a factor of approximately 1.5 with an increase in heptanesulfonate concentration from 0.005M to 0.02M. The k' of quinidine increased by a factor of approximately 2.2. Butabarbital, since it does not ion pair, exhibited no change of k' with counter ion concentration.

On the Alkyl Phenyl column, the k' of the various solutes (except for butabarbital) increased approximately 12% with increased concentration of methanesulfonate. The k' of most solutes increased by approximately 1.45 with a four fold increase of butanesulfonate or heptanesulfonate concentration. For quinidine and PCP k' increased by a factor of approximately 1.15 with an increase in butanesulfonate concentration. When quadroupling the heptanesulfonate concentration, the k' of quinidine increased by a factor of 1.7. Butabarbital did not vary with butanesulfonate or heptanesulfonate concentration. Both parabolic and hyperbolic relationships have been observed for the increase of k' with counter ion concentration for bases[6]. According to the solutes studied by Horvath et. al.[6] on the Microbondapak-C-18 column, 0.02M butanesulfonate and heptanesulfonate is near the concentration that k' does not vary with counter ion concentration. The

greater increase in k' for quinidine with heptanesulfonate concentration is consistent with having two ionizable sites available for ion pairing. Why this effect for quinidine was not observed for butanesulfonate is not apparent at this time.

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OPTIMIZATION OF A REVERSE PHASE ION-PAIR CHROMATOGRAPHIC SEPARATION FOR DRUGS OF FORENSIC INTEREST PART II - FACTORS EFFECTING SELECTIVITY

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ABSTRACT

Variables effecting selectivity for a reverse phase ion-pair chromatographic separation are examined for various drugs of forensic interest. Factors studied include type of stationary phase, ratio of water to methanol, size and concentration of counter-ion anc basicity of drug chromatographed. Most of the selectivity effects can be explained by Horvath's solvophobic theory.

INTRODUCTION

In a recent paper on reverse phase ion-pair chromatography for drugs of forensic interest, the resolution and time for analysis obtained were less than optimum[1]. Thus, it was desired to optimize

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the various separations for resolution and speed. For compounds with similar k' values, resolution can be expressed by the following relationship: $R = \frac{1}{4} (\alpha-1\alpha) (k'/k'+1) N^{\frac{1}{2}}$. This equation shows that small increases in alpha, the selectivity factor, affords an appreciable increase in resolution. Thus selectivity is an important parameter in optimizing a separation. This manuscript discusses the effect of column type, water-methanol ratio, counter ion size, counter ion concentration and basicity of a drug chromatographed on the selectivity factor for a reverse phase ion-pair chromatographic separation.

EXPERIMENTAL

The experimental conditions and procedures used are identical to those reported previously[2].

RESULTS AND DISCUSSION

Approximately 50 drugs of forensic interest including barbiturates, local anesthetics, phenethylamines, opium alkaloids, ergot alkaloids and other drugs of forensic interest were chromatographed using various stationary and mobile phases as previously indicated[2]. Tables 1-4 represent retention data for these drugs using the various mobile phases studied.

Effect of Stationary Phase on Selectivity

The role of the stationary phase on selectivity for three different columns was examined. They are Microbondapak C-18, Microbondapak Alkyl Phenyl and Microbondapak Cyanide. Wikby et. al.[3] has shown that for closely related solutes having different aliphatic character, selectivity would decrease in the order C18 > alkylphenyl > cyanide. This observa-

tion was based on the Horvath et. al.[4] model for retention in a reverse phase system from which can be derived that in a given mobile phase selectivity increases with the contact area between the non-polar portion of the solute and the stationary phase. Butabarbital and pentobarbital differ in their aliphatic character where an isobutyl group is replaced by an isopentyl group (Figure 1). In agreement with Wikby et. al.[3], the selectivity factor of butabarbital and pentobarbital decrease in the order C-18 < alkylphenyl < cyanide (Figure 2).

Phenobarbital and butabarbital do not follow this relationship (Figure 2), probably due to the presence of the phenyl ring in phenobarbital (Figure 1). Dipole and pi orbital interactions with the alkylphenyl and isopropyl cyano columns could enter into selectivity effects as well. On all three columns amobarbital and pentobarbital have a selectivity of approximately one which indicates equal retention as depicted in Figure 2. This is to be expected since both compounds have identical aliphatic character. Amobarbital and pentobarbital differ only in a pento group versus an isopento group (Figure 1). Pentobarbital and secobarbital have nearly identical selectivity on both the C-18 and alkylphenyl column (Figure 2). The selectivity of these barbiturates is slightly less on the cyano column which is illustrated in Figure 2. These compounds differ in that an ethyl group in pentobarbital is replaced by a propylene group (Figure 1). In this instance, interactions between the pi orbital in secobarbital and those in alkylphenyl and the cyano column could lead to higher selectivities than would be expected based on the Horvath et. al.[4]

TABLE 1

Capacity factors k'i and selectivity factors αji for the column Microbondapak-C18 and mobile phase methanol, water, 1% acetic acid and .005M alkylsulfonate.

Drug	40% Methanol MSA[1]		40% Methanol BSA[2]		40% Methanol HSA[3]	
	k'i	αji		αji		αji
Phenobarbital	1.85		1.87		1.97	
Butabarbital	2.83	1.53	2.81	1.50	2.98	1.51
Pentobarbital	5.76	2.03	5.61	2.00	6.11	2.05
Amobarbital	5.80	1.01	5.71	1.02	6.24	1.02
Secobarbital	8.02	1.38	7.99	1.40	8.56	1.37
Benzocaine	2.85		2.74		3.18	
Procaine	.237		.431		2.03	
Lidocaine	.819	3.46	1.27	2.95	5.68	2.80
Cocaine	1.77	2.17	2.66	2.09	9.29	1.64
Tetracaine	5.04	2.85	7.49	2.80	28.8	3.10
Acetominophen	.262		.260		.236	
Theophylline	.440	1.68	.441	1.70	.417	1.77
Caffeine	.744	1.69	.754	1.71	.726	1.74
Phenylpropanolamine	.263		.492		2.59	
Ephedrine	.328	1.25	.577	1.17	2.92	1.13
Amphetamine	.581	1.77	.935	1.62	4.13	1.41
Methamphetamine	.581	1.00	.935	1.00	4.30	1.04
Phentermine	.808	1.39	1.26	1.35	5.85	1.36
Methylphenidate	1.34	1.66	2.05	1.63	8.47	1.45
Antipyrene	1.42		1.41		1.52	
Morphine	0		.163		1.09	
Codeine	.225		.419	2.57	1.99	1.83
Acetylmorphine	.269	1.20	.500	1.19	2.31	1.16
Aminopyrene	.387	1.44	.834	1.67	2.04	
Strychine	.732	1.89	1.19	1.43	4.73	2.32
Acetylcodeine	1.05	1.43	1.58	1.33	6.19	1.31
Heroin	1.08	1.03	1.56	1.01+	6.11	1.01+
Thebaine	1.07	1.01+	1.61	1.03	6.24	1.02
Quinidine	1.02	1.05+	1.75	1.09	15.1	2.42
Quinine	1.38	1.35	2.30	1.31	18.6	1.23
Methapyrilene	2.12	1.54	3.05	1.33	14.0	1.33+
Narcotine	2.16	1.02	3.03	1.01+	11.3	1.24+
Papaverine	2.71	1.25	3.80	1.25	13.8	1.22
Mescaline	.307		.557		.270	
DMT	.501	1.63	.799	1.43	4.30	1.59
LSD	2.53		3.67		15.3	
Lampa	2.83	1.12	4.10	1.12	16.6	1.08
Iso-LSD	4.17	1.47	5.88	1.43	20.7	1.25
TCP	4.08	1.21	5.81	1.18	22.2	1.19
PCP	3.36		4.93		18.7	
Glutethimide	5.21		5.13		5.46	
Methaqualone	9.82		9.82		10.8	
Mecloqualone	12.1	1.23	12.3	1.25	13.0	1.20
Diazapam	21.6		20.9		23.0	
Phenmetrazine	.581		.820		3.64	
Phendimetrazine	.581	1.00	.874	1.07	3.87	1.06
MDA	.581		.935		4.13	
Diethylpropion	.819		1.24		4.73	

TABLE 1 (CONTINUED)

Capacity factors k'i and selectivity factors αji for the column Microbondapak-C18 and mobile phase methanol, water, 1% acetic acid and .005M alkylsulfonate.

Drug	30% Methanol MSA[1]		30% Methanol BSA[2]		30% Methanol HSA[3]	
	k'i	αji	k'i	αji	k'i	αji
Phenobarbital	3.43		3.39	1.47	3.93	
Butabarbital	5.10	1.49	4.99	2.19	5.90	
Pentobarbital	12.2	2.39	10.9	1.05	13.4	1.50
Amobarbital	12.3	1.01	11.5	1.40	13.8	2.27
Secobarbital	17.9	1.45	16.1		19.9	1.03
Benzocaine	5.73		5.76		6.84	1.44
Procaine	.845		1.21		4.89	
Lidocaine	1.90	2.25	2.56	2.11	13.5	2.76
Cocaine	6.09	3.20	6.91	2.70	31.0	2.30
Tetracaine	17.0	2.79	20.3	2.94	*	3.44
Acetominophen	.443		.424		.492	
Theophylline	.833	1.88	.860	2.03	.858	1.74
Caffeine	1.52	1.82	1.48	1.72	1.58	1.84
Phenylpropanolamine	.492		.796		4.50	
Ephedrine	.679	1.38	1.09	1.37	5.52	1.23
Amphetamine	1.01	1.49	1.47	1.35	8.28	1.50
Methamphetamine	1.25	1.24	1.72	1.17	9.44	1.14
Phentermine	1.65	1.32	2.29	1.33	13.3	1.41
Methylphenidate	3.65	2.21	4.68	2.04	26.1	1.96
Antipyrene	3.05		2.79		3.07	
Morphine	.225		.384		2.14	
Codeine	.753	3.35	1.04	2.71	4.87	2.28
Acetylmorphine	.906	1.20	1.25	1.20	6.17	1.26
Aminopyrene	1.25	1.38	1.64	1.31	4.97	1.24
Strychine	2.48	1.98	3.32	2.02	15.1	3.04
Acetylcodeine	3.40	1.37	4.45	1.34	19.9	1.32
Heroin	3.66	1.08	4.73	1.06	20.9	1.05
Thebaine	3.59	1.02+	4.70	1.01+	21.0	1.00
Quinidine	4.56	1.27	8.34	1.77	62.0	2.95
Quinine	6.41	1.41	11.5	1.38	85.1	1.38
Methapyrilene	7.38	1.15	10.6	1.08	44.9	1.90+
Narcotine	8.62	1.17	11.0	1.04	46.5	1.04
Papaverine	12.3	1.43	15.4	1.40	59.5	1.28
Mescaline	.850		1.19		6.34	
DMT	1.25	1.47	1.72	1.44	8.82	1.39
LSD	9.69		12.5		62.1	
Lampa	10.4	1.07	13.7	1.10	65.5	1.05
Iso-LSD	18.8	1.81	20.8	1.37	89.3	1.37
TCP	15.8	1.32	19.3	1.28	*	
PCP	12.0		15.1		65.6	
Glutethimide	11.3		10.8		13.4	
Methaqualone	28.1		24.8		30.2	
Mecloqualone	35.9	1.28	30.1	1.21	38.7	1.28
Diazapam	*		*		*	
Phenmetrazine	1.25		1.46		8.43	
Phendimetrazine	1.25	1.00	1.72	1.18	8.50	1.01
MDA	1.25		1.72		9.25	
Diethylpropion	2.32		2.92		12.8	

*retention greater than two hours
+successive capacity ratios are reversed

1-msa - methanesulfonate
2-bsa - butanesulfonate
3-hsa - heptanesulfonate

TABLE 2

Capacity factors k'i and selectivity factors αji, for the column Microbondapak Alkyl Phenyl and mobile phase methanol, water 1% acetic acid and .005M alkylsulfonate.

Drug	40% Methanol MSA[1]		40% Methanol BSA[2]		40% Methanol HSA[3]	
	k'i	αji	k'i	αji	k'i	αji
Phenobarbital	1.51		1.61		1.59	
Butabarbital	1.73	1.15	1.80	1.12	1.80	1.13
Pentobarbital	3.01	1.73	3.16	1.75	3.18	1.77
Amobarbital	2.96	1.02+	3.15	1.00	3.13	1.02+
Secobarbital	3.93	1.33	4.21	1.33	4.22	1.35
Benzocaine	2.16		2.37		2.27	
Procaine	.525		.628		1.63	
Lidocaine	.781	1.49	1.02	1.62	2.72	1.67
Cocaine	2.04	2.61	2.72	2.67	5.97	2.19
Tetracaine	3.68	1.80	5.09	1.87	11.4	1.91
Acetominophen	.294		.252		.282	
Theophylline	.620	2.11	.589	2.34	.575	2.04
Caffeine	1.14	1.69	1.13	1.71	1.02	1.74
Phenylpropanolamine	.310		.419		1.35	
Ephedrine	.395	1.27	.544	1.30	1.59	1.18
Amphetamine	.574	1.46	.737	1.35	2.03	1.27
Methamphetamine	.620	1.08	.848	1.15	2.27	1.12
Phentermine	.787	1.27	1.04	1.23	2.77	1.22
Methylphenidate	1.72	2.19	2.28	2.19	5.52	1.99
Antipyrene	1.46		1.51		1.46	
Morphine	.163		.242		.857	
Codeine	.456	2.80	.628	2.60	1.67	1.95
Aminopyrene	.500	1.10	.737	1.17	1.45	1.15+
Acetylmorphine	.554	1.11	.750	1.02	1.92	1.32
Strychine	1.67	3.01	2.28	3.04	5.31	2.77
Heroin	1.76	1.05	2.33	1.02	5.31	1.00
Acetylcodeine	1.80	1.02	2.38	1.02	5.45	1.02
Thebaine	1.85	1.03	2.52	1.06	5.61	1.03
Methapyrilene	2.43	1.31	3.71	1.47	7.49	1.34
Quinine	2.66	1.09	5.32	1.43	10.4	1.39
Quinidine	2.80	1.05	5.32	1.00	10.1	1.03+
Narcotine	4.08	1.46	5.42	1.02	11.4	1.13
Papaverine	4.25	1.04	5.55	1.02	10.6	1.08+
Mescaline	.388		.562		1.54	
DMT	.620	1.60	.848	1.51	2.27	1.47
LSD	3.52		4.80		10.5	
Lampa	3.93	1.12	5.26	1.12	11.5	1.10
Iso-LSD	4.72	1.20	6.15	1.16	12.1	1.05
TCP	4.09		5.32		11.8	
PCP	4.69	1.15	6.08	1.14	13.5	1.14
Glutethimide	4.25		4.45		4.34	
Methaqualone	9.67		9.92		9.22	
Mecloqualone	11.3	1.17	11.2	1.13	10.5	1.14
Diazapam	17.2		19.3		17.4	
Phenmetrazine	.620		.848		2.14	
Phendimetrazine	.620	1.00	.848	1.00	2.14	1.06
MDA	.688		.950		2.38	
Diethylpropion	.856		1.12		2.68	

TABLE 2 (CONTINUED)

Capacity factors k'i and selectivity factors αji, for the column Microbondapak Alkyl Phenyl and mobile phase methanol, water 1% acetic acid and .005M alkylsulfonate.

Drug	30% Methanol MSA[1]		30% Methanol BSA[2]		30% Methanol HSA[3]	
	k'i	αji	k'i	αji	k'i	αji
Phenobarbital	2.75		2.87		3.23	
Butabarbital	3.02	1.10	3.16	1.10	3.53	1.09
Pentobarbital	5.80	1.92	6.10	1.93	6.99	1.98
Amobarbital	5.80	1.00	6.04	1.01+	5.80	1.20+
Secobarbital	7.64	1.32	8.11	1.35	9.76	1.40
Benzocaine	4.28		4.52		5.02	
Procaine	1.00		1.38		4.19	
Lidocaine	1.26	1.26	1.72	1.25	6.00	1.43
Cocaine	4.82	5.02	6.32	3.66	19.2	3.2
Tetracaine	9.62	2.03	12.8	2.03	43.2	2.25
Acetominophen	.392		.468		.478	
Theophylline	1.00	2.55	1.07	2.32	1.11	2.32
Caffeine	1.95	1.82	2.10	1.72	2.21	1.84
Phenylpropanolamine	.398		.603		2.25	
Ephedrine	.599	1.50	.841	1.39	2.91	1.32
Amphetamine	.794	1.33	1.10	1.31	3.81	1.31
Methamphetamine	1.00	1.26	1.38	1.25	4.69	1.23
Phentermine	1.22	1.22	1.68	1.22	5.67	1.21
Methylphenidate	3.51	2.88	4.65	2.77	15.6	2.75
Antipyrene	2.65		2.69		3.09	
Morphine	.305		.498		1.79	
Codeine	1.00	3.28	1.38	2.77	4.26	2.38
Aminopyrene	1.00	1.00	1.23	1.12+	3.26	1.31+
Acetylmorphine	1.23	1.23	1.63	1.32	5.21	1.60
Strychine	3.92	3.19	5.19	3.18	17.6	3.38
Heroin	4.58	1.17	5.96	1.15	18.8	1.07
Acetylcodeine	4.65	1.02	5.78	1.03+	19.0	1.01
Thebaine	4.96	1.07	6.43	1.11	19.8	1.04
Methapyrilene	5.31	1.07	7.89	1.23	27.1	1.37
Quinine	7.00	1.32	12.1	1.53	56.3	2.08
Quinidine	6.71	1.04+	11.9	1.02+	55.3	1.02+
Narcotine	13.1	1.95	16.7	1.40	53.5	1.03+
Papaverine	14.6	1.11	18.2	1.09	51.6	1.04+
Mescaline	.714		1.02		3.37	
DMT	1.19	1.67	1.66	1.63	5.25	1.56
LSD	10.2		15.5		45.8	
Lampa	11.2	1.10	17.2	1.11	49.7	1.09
Iso-LSD	15.5	1.38	21.4	1.24	54.9	1.10
TCP	9.91		14.6		47.8	
PCP	11.2	1.13	17.0	1.16	51.6	1.08
Glutethimide	8.92		9.02		10.0	
Methaqualone	23.8		25.2		26.2	
Mecloqualone	27.4	1.15	30.4	1.21	32.8	1.17
Diazapam	50.6		64.2		60.0	
Phenmetrazine	.962		1.38		4.47	
Phendimetrazine	1.00	1.04	1.38	1.00	4.69	1.05
MDA	1.14		1.56		5.25	
Diethylpropion	1.58		2.06		6.38	

*retention greater than two hours
+successive capacity ratios are reversed

1-msa - methanesulfonate
2-bsa - butanesulfonate
3-hsa - heptanesulfonate

TABLE 3

Capacity factors k'i and selectivity factors αji, for the column Microbondapak-CN and mobile phase methanol, water, 1% acetic acid and .005M alkylsulfonate.

Drug	40% Methanol MSA[1]		40% Methanol BSA[2]		40% Methanol HSA[3]	
	k'i	αji	k'i	αji	k'i	αji
Butabarbital	.513		.495		.618	
Phenobarbital	.704	1.37	.614	1.24	.804	1.30
Amobarbital	.744	1.10	.817	1.33	.981	1.22
Pentobarbital	.774	1.04	.817	1.00	.933	1.05+
Secobarbital	.944	1.22	.997	1.22	1.19	1.27
Benzocaine	.929		.915		1.03	
Procaine	.774		.817		.804	
Lidocaine	.913	1.18	.956	1.17	.957	1.19
Cocaine	1.64	1.80	1.76	1.84	1.54	1.61
Tetracaine	2.80	1.71	2.93	1.67	3.06	1.98
Theophylline	.134		.131		.122	
Caffeine	.177	1.32	.183	1.40	.179	1.47
Acetominophen	.199	1.12	.188	1.03	.203	1.13
Phenylpropanolamine	.493		.514		.522	
Ephedrine	.561	1.14	.584	1.14	.587	1.12
Amphetamine	.774	1.38	.817	1.39	.804	1.38
Methamphetamine	.774	1.00	.817	1.00	.804	1.00
Phentermine	.774	1.00	.817	1.00	.804	1.00
Methylphenidate	1.07	1.38	1.14	1.39	1.10	1.37
Antipyrene	.288		.280		.260	
Morphine	.410		.419		.412	
Aminopyrene	.416	1.01	.430	1.03	.412	1.00
Codeine	.561	1.35	.584	1.36	.562	1.36
Acetylmorphine	.774	1.38	.756	1.29	.758	1.35
Heroin	1.08	1.39	1.15	1.52	1.09	1.44
Acetylcodeine	1.11	1.03	1.17	1.02	1.13	1.03
Strychnine	1.29	1.16	1.32	1.13	1.33	1.18
Thebaine	1.44	1.12	1.42	1.08	1.36	1.02
Papaverine	1.50	1.04	1.58	1.11	1.41	1.04
Methapyrilene	1.88	1.25	1.85	1.17	1.87	1.33
Narcotine	1.89	1.00	1.98	1.07	1.95	1.04
Quinidine	1.95	1.03	2.13	1.08	1.83	1.07+
Quinine	2.07	1.06	2.24	1.05	1.91	1.04
Mescaline	.506		.560		.540	
DMT	1.22	2.41	1.23	2.20	1.20	2.22
LSD	2.26		2.46		2.29	
Lampa	2.49	1.10	2.70	1.10	2.48	1.08
Iso-LSD	2.69	1.08	2.92	1.08	2.55	1.03
TCP	1.52		1.63		1.53	
PCP	1.73	1.14	1.83	1.11	1.70	1.11
Glutethimide	1.01		.980		1.14	
Methaqualone	1.39		1.36		1.41	
Mecloqualone	1.76	1.27	1.74	1.28	1.86	1.32
Diazapam	2.35		2.34		2.56	
Phenmetrazine	.774		.817		.804	
Phendimetrazine	.774	1.00	.817	1.00	.804	1.00
Diethylpropion	.774		.817		.804	
MDA	.851		.907		.949	

TABLE 3 (CONTINUED)

Capacity factors k'i and selectivity factors αji, for the column Microbondapak-CN and mobile phase methanol, water, 1% acetic acid and .005M alkylsulfonate.

Drug	30% Methanol MSA[1]		30% Methanol BSA[2]		30% Methanol HSA[3]	
	k'i	αji	k'i	αji	k'i	αji
Butabarbital	.859		.870		.833	
Phenobarbital	1.18	1.37	1.08	1.24	1.10	1.32
Amobarbital	1.45	1.23	1.33	1.23	1.36	1.24
Pentobarbital	1.43	1.01+	1.31	1.01+	1.31	1.04+
Secobarbital	1.82	1.27	1.65	1.26	1.69	1.29
Benzocaine	1.86		1.63		2.56	
Procaine	.859		.870		1.10	
Lidocaine	1.06	1.23	1.03	1.18	1.34	1.22
Cocaine	2.45	2.32	2.35	2.28	2.78	2.07
Tetracaine	5.36	2.19	4.91	2.09	6.34	2.28
Theophylline	.198		.174		.187	
Caffeine	.312	1.58	.283	1.58	.285	1.52
Acetominophen	.318	1.02	.286	1.01	.290	1.02
Phenylpropanolamine	.445		.487		.614	
Ephedrine	.537	1.21	.572	1.19	.738	1.20
Amphetamine	.810	1.50	.791	1.38	1.01	1.37
Methamphetamine	.859	1.06	.870	1.10	1.10	1.09
Phentermine	.859	1.00	.870	1.00	1.10	1.00
Methylphenidate	1.42	1.65	1.42	1.63	1.75	1.59
Antipyrene	.480		.451		.466	
Morphine	.407		.414		.534	
Aminopyrene	.407	1.00	.431	1.04	.542	1.01
Codeine	.636	1.56	.649	1.51	.809	1.49
Acetylmorphine	.859	1.35	.870	1.34	1.10	1.36
Heroin	1.64	1.91	1.54	1.77	1.87	1.70
Acetylcodeine	1.68	1.02	1.60	1.04	1.94	1.04
Strychnine	1.85	1.10	1.52	1.05+	2.11	1.09
Thebaine	2.28	1.23	2.15	1.41	2.58	1.22
Papaverine	2.84	1.25	2.65	1.23	3.15	1.22
Methapyrilene	2.77	1.02+	2.67	1.01	2.82	1.12+
Narcotine	3.35	1.21	3.12	1.17	3.70	1.31
Quinidine	2.85	1.18+	2.72	1.15+	3.08	1.20+
Quinine	3.18	1.12	3.04	1.12	3.44	1.12
Mescaline	.558		.565		.719	
DMT	1.50	2.69	1.50	2.65	1.82	2.53
LSD	4.27		3.93		4.69	
Lampa	4.80	1.12	4.38	1.11	5.15	1.10
Iso-LSD	5.41	1.13	4.90	1.13	5.62	1.09
TCP	2.25		2.18		2.74	
PCP	2.55	1.13	2.48	1.14	3.12	1.14
Glutethimide	2.07		1.85		1.86	
Methaqualone	3.21		2.80		2.86	
Mecloqualone	4.45	1.39	3.98	1.42	3.92	1.37
Diazapam	5.69		4.92		5.05	
Phenmetrazine	.859		.870		1.10	
Phendimetrazine	.859	1.00	.870	1.00	1.10	1.00
Diethylpropion	.859		.870		1.10	
MDA	1.06		1.04		1.29	

*retention greater than two hours
+successive capacity factors are reversed

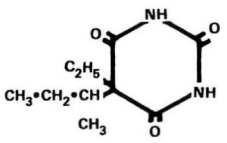
1-msa - methanesulfonate
2-bsa - butanesulfonate
3-hsa - heptanesulfonate

TABLE 4

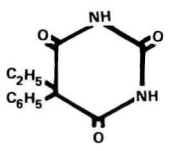
Capacity factors k'i and selectivity factors αji for mobile phase 20% methanol, 79% water, 1% acetic acid and 0.005M methanesulfonate acid using various columns.

Drug	Microbondapak C-18		Microbondapak Alkyl Phenyl		Microbondapak CN	
	k'i	αji	k'i	αji	k'i	αji
Phenobarbital	7.21	1.51	6.60		1.61	
Butabarbital	10.9	2.47	7.01	1.06	1.20	1.34+
Pentobarbital	26.9	1.05	15.2	2.17	2.04	1.70
Amobarbital	28.2	1.49	14.8	1.03+	2.11	1.03
Secobarbital	42.0		22.8	1.54	2.66	1.26
Benzocaine	12.7		10.1		2.56	
Procaine	2.17		2.65		1.09	
Lidocaine	4.10	1.90	2.73	1.03	1.29	1.18
Cocaine	17.1	4.17	14.7	5.39	3.43	2.67
Tetracaine	52.1	3.05	32.5	2.22	8.17	2.38
Acetominophen	.829		.863		.395	
Theophylline	1.76	2.12	2.02	2.34	.256	1.54+
Caffeine	3.58	2.03	4.34	2.03	.386	1.51
Phenylpropanolamine	.840		.743		.471	
Ephedrine	1.23	1.46	1.10	1.48	.587	1.25
Amphetamine	1.87	1.53	1.50	1.36	.860	1.46
Methamphetamine	2.47	1.32	2.02	1.36	.980	1.14
Phentermine	3.26	1.32	2.46	1.22	.980	1.00
Methylphenidate	9.36	2.92	8.95	3.64	1.82	1.86
Antipyrene	7.73		6.52		.726	
Morphine	.536		.849		.464	
Codeine	2.04	3.81	2.79	3.29	.778	1.68
Acetylmorphine	2.59	1.27	3.58	1.28	1.14	1.46
Aminopyrene	2.94	1.14	2.38	1.50+	.469	2.43+
Strychine	7.43	2.53	12.2	5.13	2.40	5.12
Acetylcodeine	12.3	1.66	16.4	1.34	2.40	1.00+
Heroin	13.9	1.13+	16.7	1.02	2.31	1.04+
Thebaine	13.0	1.07+	17.2	1.03	3.45	1.49
Quinidine	18.0	1.38	30.9	1.80	3.20	1.08+
Quinine	24.6	1.37	29.9	1.03+	3.64	1.14
Methapyrilene	21.7	1.13+	17.6	1.70+	3.57	1.02+
Narcotine	41.5	1.91	52.7	2.99	5.17	1.45
Papaverine	64.0	1.54	65.6	1.24	5.03	1.03+
Mescaline	2.47		1.77		.681	
DMT	2.77	1.12	2.67	1.51	1.85	2.72
LSD	37.1		37.2		7.10	
Lampa	40.0	1.08	42.6	1.15	8.01	1.13
Iso-LSD	74.1	1.85	58.0	1.36	9.33	1.13
TCP	41.5		31.1		3.27	
PCP	44.5	1.07	38.8	1.25	3.81	1.16
Glutethimide	29.6		24.2		3.15	
Methaqualone	*		*		5.51	
Mecloqualone	*		*	*	8.50	1.54
Diazapam	*		*		9.56	
Phenmetrazine	2.02		1.92		.980	
Phendimetrazine	2.47	1.22	2.26	1.18	.980	1.00
MDA	2.38		2.30		1.18	
Diethylpropion	5.80		3.68		1.04	

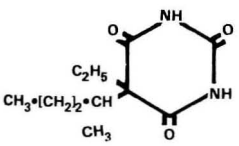
BUTABARBITAL



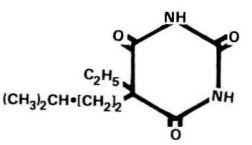
PHENOBARBITAL



PENTOBARBITAL



AMOBARBITAL



SECOBARBITAL

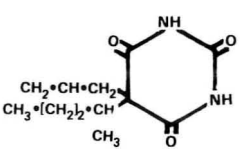


Figure 1 - Structures of barbiturates.

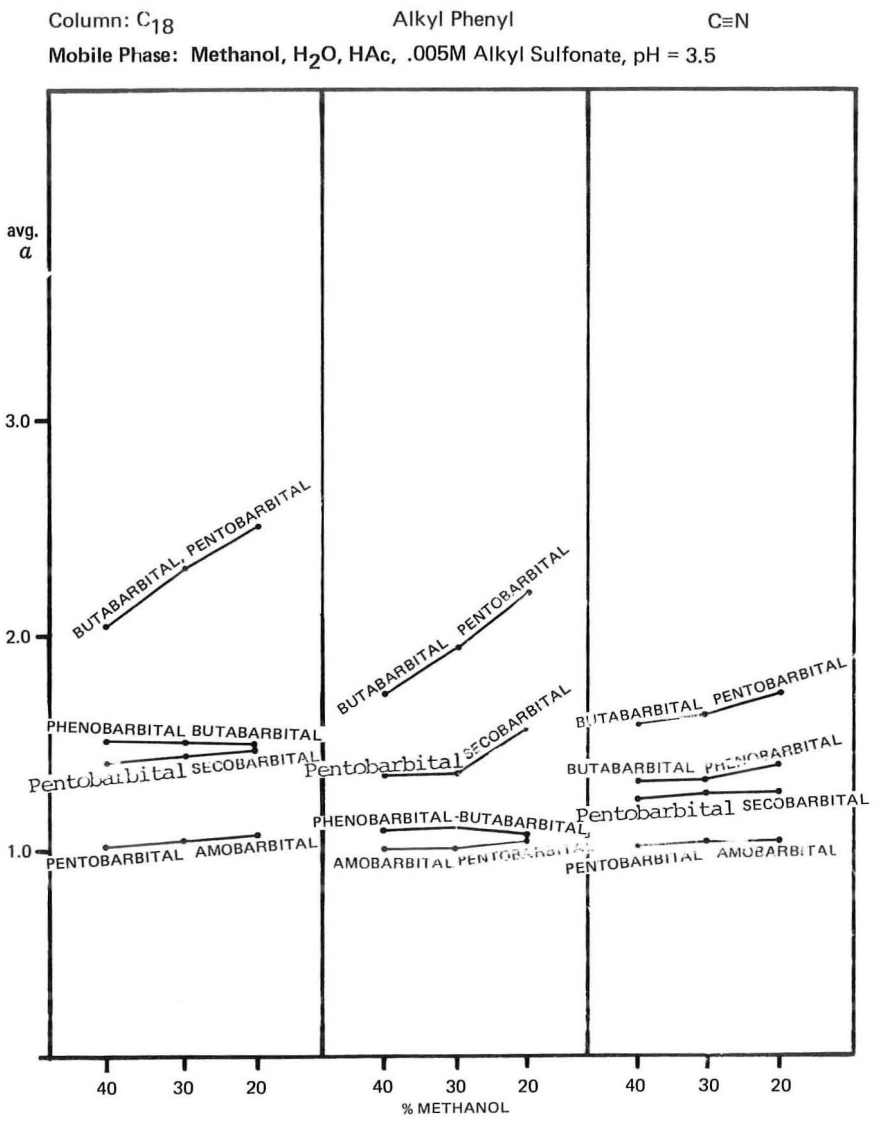


Figure 2 - Plot of average selectivity factors of barbiturates versus percent methanol for the Microbondapak C-18, Microbondapak Alkyl Phenyl and Microbondapak CN columns.

The decrease in selectivity on the cyanide column versus the alkylphenyl column could be explained by the small pi bonding character of the isopropyl cyano column.

As was shown earlier, based on the Horvath et. al.[4] model for reverse phase chromatography, for a given mobile phase selectivity increases with the contact area between non-polar substituents on the solute and the hydrocarbon ligands on stationary phase[3]. Horvath's theory for retention in reverse phase ion pair chromatography via an ion pair in mobile phase mechanism is also based on this model. Thus for solutes having different aliphatic character in a given mobile phase, selectivity would be expected to decrease in the order of C-18 < alkylphenyl < isopropyl cyano. For most bases studied the above relationship was not observed. No explanation could be offered for the above effect at this time.

Effect of Water-Methanol Ratio on Selectivity

It can be deduced from the Horvath et. al.[4] theory of retention in reverse phase chromatography that the selectivity of two solutes differing in aliphatic character on a given stationary phase will increase primarily with the surface tension of the eluent[3]. Therefore, increasing the ratio of water to methanol in the mobile phase should effect an increase in selectivity between two solutes differing in non-polar character. A similar effect is predicted based on the Karger et. al.[5] hydrophobic theory. Butabarbital and pentobarbital which differ in aliphatic character show an increase in selectivity with increased water in the mobile phase for all three

columns (Figure 2). The selectivity factor for amobarbital and pentobarbital which have identical aliphatic character is invariant to the water-methanol ratio (Figure 2). The phenobarbital:butabarbital:secobarbital pairs have selectivities that are also independent of the water-methanol ratio. The above effects are shown in Figure 2. No explanation is available for these effects. Tjaden et. al.[6] demonstrated that when using a water-methanol system on a silanized silica column, selectivity increases with the water-methanol ratio for barbiturates. The structures of these compounds differed only by non-polar substituents.

Horvath et. al.'s[5] theory for reverse phase ion pair chromatography would predict that the selectivity of two closely related solutes differing in aliphatic character would increase with the surface tension of the eluent. Earlier it was shown that the equilibrium constant for the binding of the neutral ion pair is proportional to the contact area between the ion pair and the stationary phase. This equilibrium constant would be proportional to the capacity factor. Wikby et. al.[3] showed that based on Horvath's theory of retention for reversed phase chromatography the following relationship: $d\log k'/d\,\Delta A = \alpha/940.7$ where gama is the surface tension and ΔA is the contact area of solute with the stationary phase. A similar relationship could be derived from the equilibrium constant for the adsorption of the ion pair on to the stationary phase: $\ln K = a - b + c\,\Delta A$, where the magnitude of this term depends primarily on the surface tension of the mobile phase and the cntact area. For a given counter ion, an increase in selectivity

with increased water concentration was observed on all three columns for phenylpropanolamine and ephedrine, amphetamine and methamphetamine, (Figure 3) morphine and codeine, morphine and acetylmorphine, and codeine and acetylcodeine. The small increase in selectivity with the water-methanol ratio for heroin and acetylcodeine only occured on the C-18 column (Figure 3). In this instance the increased hydrocarbon character in heroin which has one more carbon atom than acetylcodeine is offset by the affinity of the polar carbonyl function in heroin for the polar mobile phase, see Figure 4. Positional isomers, methamphetamine and phentermine, LSD and LAMPA, and the stereoisomers quinine and quinidine, having identical aliphatic character exhibit no changes in selectivity with water-methanol ratio (Figure 3). LSD and Iso-LSD, diastereoisomers with identical aliphatic character, have selectivity values which increase with greater water-methanol ratios (Figure 3). The structures of these compounds are given in Figure 4.

Effect of Counter Ion Size on Selectivity

The retention of strong basic drugs on the C-18 and alkylphenyl column significantly increases with the size of the alkylsulfonate counter ion. The counter ion size has little effect on the k' values of weakly basic and acidic drugs. Therefore, for drug combinations consisting of strong and weak bases and acids, large changes in selectivity could result by changing the size of the counter ion. For certain base pairs on C-18 and alkylphenyl columns at a constant water-methanol ratio, the selectivity increases with decreasing size of the counter ion. Examples are codeine and acetylcodeine, phenyl-

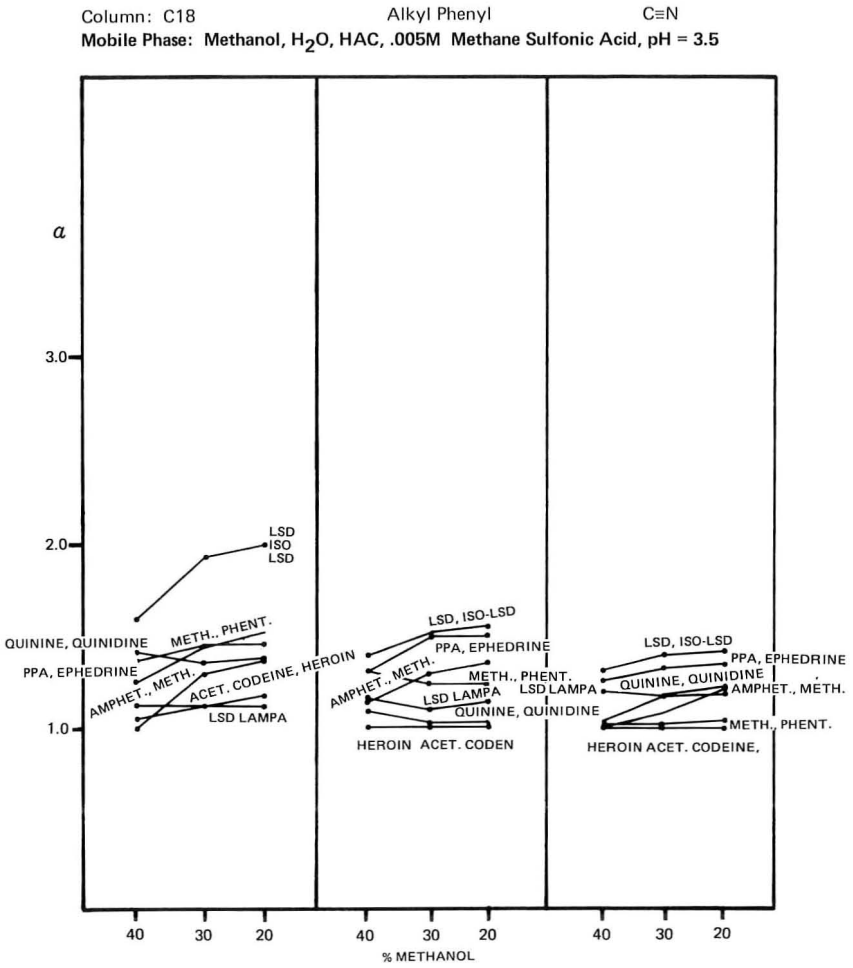
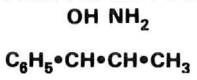
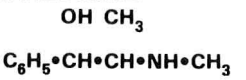


Figure 3 - Plot of selectivity factors with methanesulfonate counter ion versus percent methanol for the Microbondapak C-18, Microbondapak Alkyl Phenyl and Microbondapak CN columns.

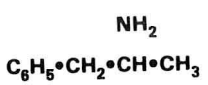
PHENYLPROPANOLAMINE



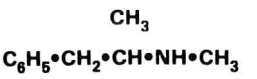
EPHEDRINE



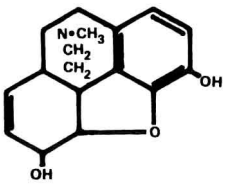
AMPHETAMINE



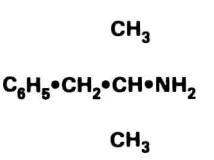
METHAMPHETAMINE



MORPHINE



PHENTERMINE



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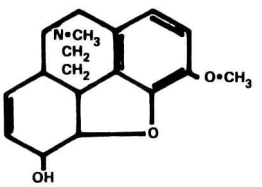

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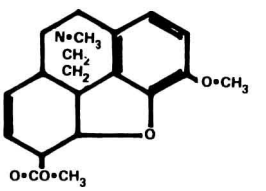

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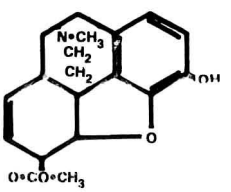
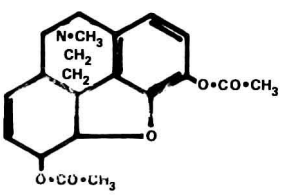
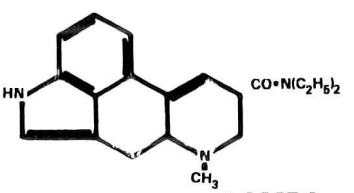


HEROIN



LSD, ISO-LSD



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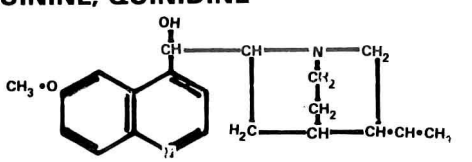

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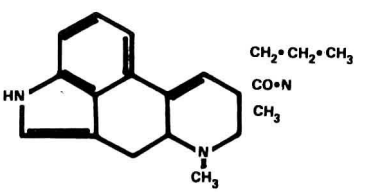

Figure 4 - Structures of basic drugs.

propanolamine and ephedrine, and lidocaine and cocaine. At the present time this effect can not be explained.

Effect of Counter Ion Concentration on Selectivity

For the ten basic solutes studied[2] only the selectivity of quinidine with other bases varied with counter ion concentration on both the C-18 and Alkyl Phenyl columns. The behavior of quinidine could be explained, as stated earlier[2], based on the two ionizable sites this compound has to interact with counter ion.

ACKNOWLEDGEMENTS

The authors are grateful to Mr. Jeffrey Weber for his review of the manuscript and to Dr. Mary Chaklos for her helpful discussions.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 4(2), 375-376 (1981)

LC NEWS

PROTEIN SEPARATION SYSTEM that accomplishes high-resolution in minutes. System efficiently separates acidic, neutral, and mildly basic proteins with molecular weights from 2000 to 80,000 with high recovery, speed, and easy operation. Waters Associates, Inc., JLC/81/2, 34 Maple Street, Milford, MA, 01757, USA.

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LC CALENDAR

1981

February 2-9	"First Chem'l. Eng. Exhibition - KORCHEM", KOEX Exhibition Center, Seoul, Korea. Contact: Int'l. Chem. Eng. Exhibition, KOEX Exhibition Center, Seoul, Korea.
March 9-13	"Pittsburgh Conference on Anal. Chem. & Appl. Spectroscopy", Convention Hall, Atlantic City, NJ USA. Contact: The Pittsburgh Conference, Mrs. Linda Briggs, 437 Donald Rd., Pittsburgh, PA 15235, USA.
March 29-April 3	"National Am. Chem. Soc. Meeting", Atlanta, GA, USA. Contact: Am. Chem. Soc., 1155 Sixteenth Streeet, NW, Washington, DC 20036, USA.
March 29-April 3	"Advances in Separation Technology", Nat'l ACS Meeting, Atlanta, GA, USA. Contact: N. Li, Exxon Res. & Eng. Co., P. O. Box 8, Linden, N. 07036, USA.
March 29-April 3	"Chromatographic Separations of Coal-Derived Materials", "Nat'l ACS Meeting, Atlanta, GA USA. Contact: L. Taylor, Chem. Dept., Virginia Polytechnic Inst. & State Univ., Blacksburg, VA 24601, USA
March 29-April 3	"Standardized Materials for Chromatography", Nat'l ACS Meeting, Atlanta, GA USA. Contact: L. S. Ettre, Perkin-Elmer Corp., Main Avenue, Norwalk, CT 06856, USA.
April 28	"Detectors in Chromatography", Chrom. & Electrophoresis Grp. The Royal Society of Chemistry, College of Technology, Southend, U.K. Contact: Dr. D. Simpson, Anal. for Industry, Bosworth House, High Street, Thorpe-le-Soken, Essex CO 16 OEA, U.K.
May 11-15	"5th International Symposium on Column Liquid Chromatography", Avignon, France. Contact: G. Guiochon, Lab de Chim. Anal. Phys., Ecole Polytechnique, Rte. de Saclay, 91128 Palaiseau, France.
May 17-19	"Symposium on Environmental and Industrial Applications of LCEC and Voltammetry", Indianapolis, Indiana, USA. Contact: LCEC Symposium, 1205 Kent Avenue, West Lafayette, IN 47906, USA.
May 20-22	"Symposium on the Anal. of Steroids", sponsored by The Hungarian Chemical Society, Eger, Hungary. Contract: Prof. S. Gorog, Hungarian Chem. Soc., 1061 Budapest VI, Anker koz 1, Hungary.
June 4-5	"4th World Chromatography Conference", Aerogolf Sheraton Hotel, Luxembourg. Contact: V.M. Bhatnagar, Alena Enterprises of Canada, P.O. Box 1779, Cornwall, Ontario, K6H 5V7, Canada.
June 22-26	"4th Int'l Symposium on Affinity Chromatography and Related Techniques", Katholieke Universiteit, Nijmegen, The Netherlands. Contact: Dr. T.C.J. Gribnau, Organon Scientific Development Group, P.O. Box 20, 5340 BH OSS, The Netherlands.
July 20-24	"Second International Flavor Conference", National Hellenic Research Foundation, Athens, Greece. Contact: Dr. S.J. Kazeniac, Campbell Institute for Food Research, Campbell Place Camden, N.J. 08101, USA.

August 23-28 "National Am. Chem. Soc. Meeting", New York, NY, USA. Contact: Am. Chem. Soc., 1155 Sixteenth Street, NW, Washington, DC 20036, USA.

August 30-September 5 "XI Int'l Congress, IV European Congress of Clinical Chemistry", Vienna, Austria. Contact: 11th Int'l Congress of Clinical Chem., P. O. Box 105, A-1014 Wien, Austria.

September 20-25 "8th Annual FACSS Meeting", Philadelphia, PA USA. Contact: R. A. Barford, USDA, 600 E. Mermaid Lane, Philadelphia, PA 19118, USA.

October 1-2 "Japan Conference on Chromatography", Palace Hotel, Tokyo, Japan. Contact: V.M. Bhatnagar, Alena Enterprises of Canada, P.O. Box 1779, Cornwall, Ontario, K6H 5V7, Canada.

October 4-9 "Symposium on Novel Separation Processes", at the 2nd World Congress of Chemical Engineering, Montreal, Canada. Contact: Congress Secretariat, 151 Slater Street, Suite 906, Ottawa, Ont., Canada, K1P 5H3.

October 12-15 "EXPOCHEM '81", Houston, TX. Contact: Dr. A. Zlatkis, Chem. Dept., University Houston, Houston, TX 77004, USA.

November 19-20 "1981 International Chromatography Conference", Carillon Hotel, Miami Beach, Florida, USA. Contact: V.M. Bhatnagar, Alena Enterprises of Canada, P.O. Box 1779, Cornwall, Ontario, K6H 5V7.

1982

March 28-April 2 "National American Chem. Soc. Meeting", Las Vegas, NV USA. Contact: A. T. Winstead, Am. Chem. Sco., 1155 Sixteenth St., NW, Washington, DC 20036, USA.

April 14-16 "12th Annual Symposium on the Anal. Chem. of Pollutants", Amsterdam, The Netherlands. Contact: Prof. R. W. Frei, Congress Office, Vrije Universiteit, P. O. Box 7161, 1007-MC Amsterdam, The Netherlands.

June 28-30 "Analytical Summer Symposium", Michigan State Univ., East Lansing, MI USA. Contact: A. I. Popov, Chem. Dept., Michigan State Univ., East Lansing, MI 48824, USA.

July 12-16 "2nd Int'l Symposium on Macromolecules", - IUPAC, University of Massachusetts, Amherst, Massachusetts, USA.

August 15-21 "12th Int'l Congress of Biochemistry", Perth, Western Australia. Contact: Brian Thorpe, Dept. of Biochemistry, Faculty of Science Australian National University, Canberra A.C.T. 2600, Australia.

September 12-17 "National American Chem. Soc. Meeting", Kansas City, MO USA. Contact: A. T. Winstead, American Chem. Soc., 1155 Sixteenth St., NW, Washington, DC 20036, USA.

1983

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

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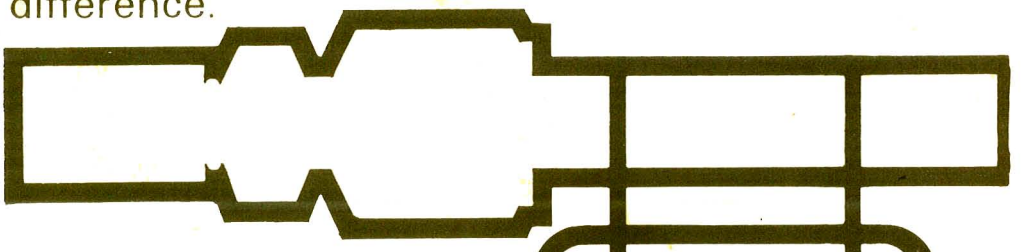

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