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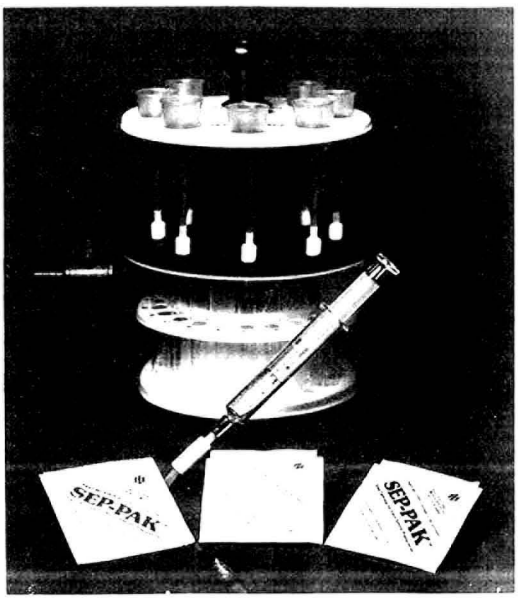

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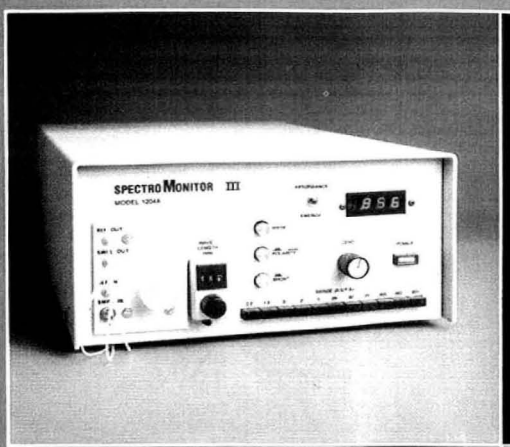

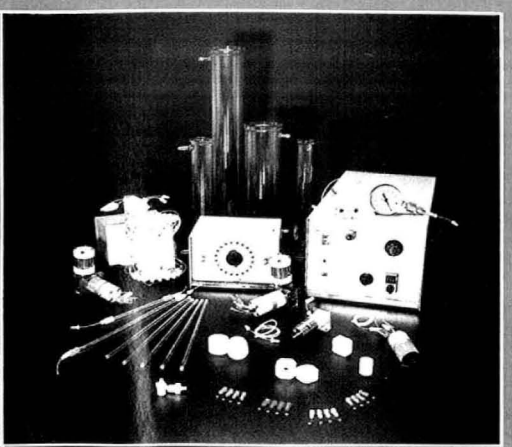

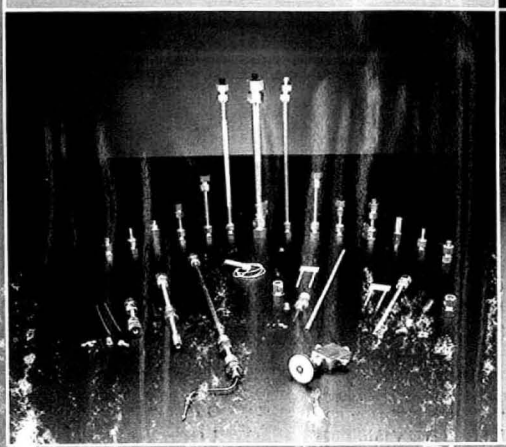

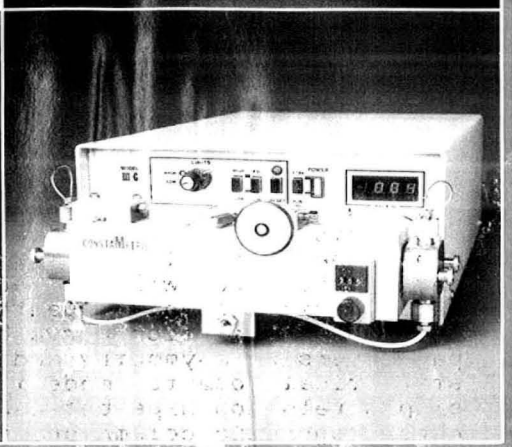

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RELATION BETWEEN THE ASYMMETRY OF THE ELUTION CURVE AND THE EFFICIENCY OF THE SEPARATION SYSTEM IN SIZE EXCLUSION CHROMATOGRAPHY

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ABSTRACT

The shape of the elution curve provides information on different processes contributing to separation and dispersion. This information also includes the relation towards the concentration profile in the chromatographic column. If, e.g., the concentration profile is symmetrical, the resulting tailing of the elution curve is caused by the passage of the chromatographic profile through the column end with continuing zone broadening. The tailing will be the more marked the lower will be the efficiency of the chromatographic column. A quantitative description of the shape of the elution curve by means of the defined parameters of asymmetry and efficiency, based on statistical moments, made it possible to obtain very simple relationships between the efficiency of the chromatographic column and the asymmetry of the elution curve. In addition, the asymmetry of the elution curve was evaluated by a slope analysis, which can be less complicated for practical application than the evaluation with the aid of statistical moments and which also permits a graphical evaluation of an experimental chromatogram. It was found that a bijective and regular relationship exists between the asymmetry parameters defined on the basis of statistical moments and those defined on the basis

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of the tangent slopes at the point of inflexion of the elution curve. The defined parameters are recommended for an objective evaluation of the shapes of elution curves instead of diverse, entirely empirical parameters, described in literature without physical basis. The results obtained are relevant particularly in those instances when the efficiency of the separation system for a given solute is relatively low, such as, e.g., in size exclusion chromatography of macromolecules.

INTRODUCTION

Various theoretical models describing chromatographic processes predict almost Gaussian elution curve in the limit of infinite elution time (1). A record of the response of a differential detector at the end of chromatographic columns (i.e., in a fixed length coordinate of the longitudinal axis of of chromatographic column or of their system) to an eluting solute as a function of the time (or of the volume of the mobile phase passed through) is called the elution curve. The limiting condition of the infinite elution time is a simplifying assumption which makes it possible to approximate the resulting elution curve by the Gaussian function. Taylor (2) derived mathematically and proved experimentally that if a soluble low-molecular-weight substance is introduced into a solvent flowing slowly in a tube of a small diameter (in a capillary), almost a Gaussian concentration profile originates in the limit of infinite time in the direction of the longitudinal axis. This is a consequence of combined influences of molecular diffusion and variation in the velocity across the capillary cross-section.

The concentration distribution along the longitudinal axis of the capillary or the chromatographic column at a given fixed time or towards a given fixed elution volume is called the concentration profile. If the concentration profile of a simple monodisperse solute is symmetrical, the resulting tailing of the elution curve originates due to the passage of this symmetrical concentration profile through the column end (detector) while zone broadening continues. Whereas a given point of the zone has already been detected, the part of the zone following this point in time development still moves in the column and is subjected to additional broadening. In other words, broadening of zone front is less than that of zone back, owing to a shorter time for which the front is present in the column. The greater will be the spreading of the concentration profile on its movement along the unit length of the column, the more marked will be the tailing of the elution curve. Hence it is obvious that the concentration profile and the elution curve are in mutual relationship (with respect to symmetry or asymmetry) associated with the efficiency.

The shape of the elution curve provides information on different processes contributing to separation and dispersion. In a number of instances, information of this kind is of high relevance. The information on molecular-weight distribution of the polymer sample under study is encoded in the shape of the elution curve in size-exclusion chromatography (SEC) of polymers. In the case of the flow in capillary systems (2), it is the information on the character of the concentration profile on the basis of which it is possible to estimate the shape

of the velocity profile in the capillary or the value of the diffusion coefficient. A number of other possible informative data provided by the elution curve were assumed by Grushka and coworkers (3), e.g., the information on overlap and contamination of the elution curve, on conditions in the column etc. In principle it is possible to obtain some of the information mentioned above from the shape of the concentration profile, e.g., by column scanning in a given relevant instant. This way of obtaining information is, however, fairly complicated as far as the experimental equipment is concerned and cannot be realized under any arbitrary conditions.

The evaluation from the elution curves is easier. It is, however, necessary that internal relationship between the shape of the concentration profile and the shape of the elution curve should be known. The present work continues the previous study on the relationship between the efficiency of the chromatographic column system and the asymmetry of the resulting elution curves (4), generalizes the preceding conclusions and, besides the analysis by means of statistical moments, utilizes also the slope analysis, which simplifies the evaluation of experimental elution curves asymmetry by a graphical method.

Though the derived relationships are valid for elution chromatography in general, they are of practical importance only when mean or small number of theoretical plates is generated on the passage of a monodisperse solute through a separation system; in other words, when the efficiency characterized by the number of theoretical plates is relatively low for a given solute as in SEC of polymers.

THEORETICAL CONSIDERATIONS

Solution for a Gaussian Model of the Concentration Profile

In order to make mathematical operations easier, it is convenient to define the following dimensionless quantities

$$L = x/l, \quad T = t/t_R = V/V_R \qquad (1)$$

where x is a longitudinal coordinate in the direction of the chromatographic column, l is the total column length or the coordinate of the detector position, t and V are the time and the elution volume coordinates, t_R and V_R are the corresponding retention time and volume, resp. Assuming a Gaussian injection function for which it holds that $\xi_I \rightarrow 0$, the concentration profile or the elution curve can be described by the function (4)

$$F(L,T) = (1/\sqrt{\xi_T^2\, 2\pi T})\exp\left[-(L - T)^2/2T\xi_T^2\right] \qquad (2)$$

where ξ_T is the total standard deviation of the concentration profile provided that the maximum lies in the coordinate L = 1 (x = l). The concentration profile and the elution curve were described analogically in our earlier work (4). Function F(1,T) is the elution curve whereas function F(L,t) is the concentration profile at a given time, t = const.

Statistical moments (5) can conveniently be used to describe shapes of elution curves quantitatively. Let the n-th statistical moment

with respect to zero $/u_n'$ of the function F(1,T) in Eqn. 2, be defined by the following equation

$$/u_n' = \int_0^\infty T^n \, F(1,T) \, dT \qquad (3)$$

then the n-th central moment, $/u_n$ is defined by the relationship

$$/u_n = \int_0^\infty (T - /u_1')^n \, F(1,T) \, dT \qquad (4)$$

The zeroth statistical moment with respect to zero is equal to one by definition

$$/u_0' = \int_0^\infty F(1,T) \, dT = 1 \qquad (5)$$

The first statistical moment with respect to zero $/u_1'$ of the function F(1,T), defined by Eqn. 2, has, as the only one, a direct and simple analytical solution (4)

$$/u_1' = 1 + \xi_T^2 \qquad (6)$$

Of interest are also the second and the third central moments, $/u_2$ and $/u_3$, respectively, which can be described by

$$/u_2 = \int_0^\infty T^2 \, F(1,T) \, dT - (/u_1')^2 \qquad (7)$$

and

$$/u_3 = \int_0^\infty T^3 \, F(1,T) \, dT - 3/u_1'/u_2 - (/u_1')^3 \qquad (8)$$

Physical meaning of the above moments is as follows: the first statistical moment with respect to zero, $/u_1'$, means the average elution time or volume, the second central moment, $/u_2$, is equal to the variation

of the elution curve (i.e., to the second power of the standard deviation) and the third central moment, $/u_3$, is a measure of the elution curve asymmetry. For a symmetrical elution curve is $/u_3 = 0$, for a tailed elution curve the value of $/u_3$ is positive and for an elution curve with a fronting it is negative.

In addition, normalized parameters were defined, making it possible to describe quantitatively the shapes of the elution curves and their mutual correlations even in such cases when they were obtained under different conditions. Parameter designated (4) asymmetry A is defined by the relationship

$$A = /u_3/(/u_1')^3 \qquad (9)$$

Asymmetry A is in formal analogy with the reciprocal value of the theoretical plate number, N, which can be expressed in a similar manner

$$N = (/u_1')^2/u_2 \qquad (10)$$

Skew parameter, γ, can be defined by analogy (6)

$$\gamma = /u_3/u_2^{1.5} \qquad (11)$$

A series of the values of asymmetry A of elution curves was calculated (4) numerically for given efficiencies of the chromatographic system, characterized by the number of theoretical plates, N. The resulting values of asymmetry A were correlated with the efficiencies, N, and the following relationship (4) was obtained by using the linear regression method.

$$A = c_1 N^{-c_2} \qquad (12)$$

The values obtained for constants c_1 and c_2 were 2.86816... and 1.99497..., respectively. The achieved regression coefficient, expressing the reliability of the calculated correlation, was high; r = 0.999997.

For practical applications the use of the approximative relationship will be feasible

$$A = 3\ N^{-2} \tag{13}$$

Correlation between the values of γ and N led to the relationship (4)

$$\gamma = c_1\ N^{-c_3} \tag{14}$$

where c_1 = 2.86816..., c_3 = 0.49497... with regression coefficient r = 0.99994. The quantitative description of the shapes of elution curves with the aid of the above parameters, based on the statistical moments, can be rather difficult in some cases of practical applications. In order to obtain a sufficient precision in the determination of the values of A, N or γ , it is necessary that experimental chromatograms should be read fairly precisely particularly in both of their extreme parts. The use of elution curve in digital form, obtained with the aid of a converter of analogue output signal of the differential detector is advantageous. Sometimes the use of slope analysis may be more beneficial for both evaluation of the efficiency of the chromatographic system, characterized by parameter N, and the evaluation of the asymmetry of the elution curve obtained. Graphical evaluation of the chromatogram is also used for this purpose. In order to calculate the asymmetry, the slope of the tangent values at the points of inflexion of the frontal and back parts of

the elution curve are utilized. This quantity will be designated as slope asymmetry SA

$$SA = (1 + \frac{S_B}{S_F}) = (1 - \frac{|S_B|}{S_F}) = (1 - \frac{a}{b}) \qquad (15)$$

where S_F is the slope of the tangent at the point of inflexion of the elution curve front and S_B is the slope of the tangent at the point of inflexion of the elution curve back. This evaluation may be carried out graphically in the manner demonstrated in Fig. 1.

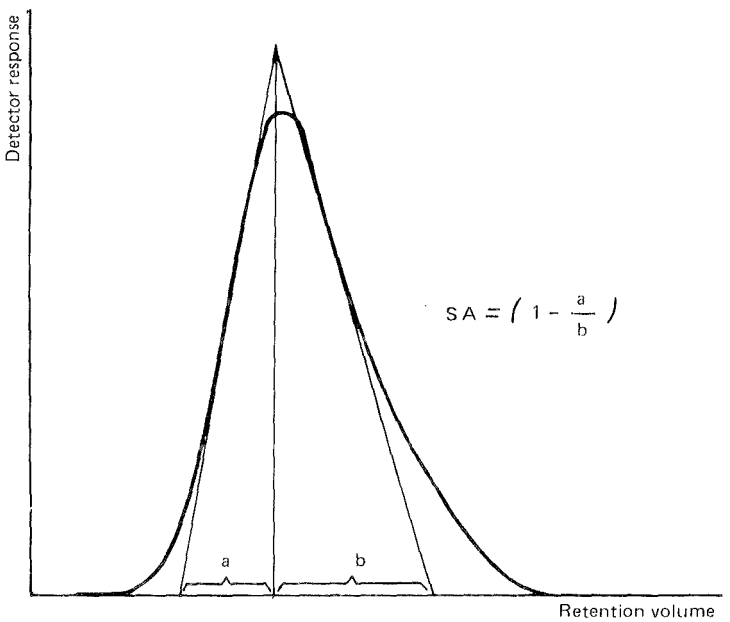

FIGURE 1.
Graphical evaluation of the slope asymmetry of the elution curve

For slope analysis one needs to know the slopes of tangents at the points of inflexion of the elution curve. First the positions of these points of inflexion in T coordinate must be found. This can be effectuated by making the second derivative of function F(1,T) equal to zero, hence

$$d^2 F(1,T)/(dT)^2 = 0 \qquad (16)$$

By differentiating it is obtained

$$F(1,T)\left(\left(\frac{1}{2\xi_T^2 T^2} - \frac{1}{2T} - \frac{1}{2\xi_T^2}\right)^2 + \frac{1}{2T^2} - \frac{1}{\xi_T^2 T^3}\right) = 0 \qquad (17)$$

It holds at the points of inflexion that $F(1,T) \neq 0$ and thus after the rearrangement it is obtained that

$$T^4 + 2\xi_T^2 T^3 + (3\xi_T^4 - 2)T^2 - 6\xi_T^2 T + 1 = 0 \qquad (18)$$

For coordinates T of the points of inflexion, calculated by using Eqn. 18, the slopes of the elution curve are found from the first derivative of function F(1,T)

$$dF(1,T)/dT = F(1,T)(1/2\xi_T^2 T^2)(1-T^2-\xi_T^2 T) \qquad (19)$$

By correlation analysis of the set of A and SA values, obtained by calculation for different values of the efficiencies of chromatographic systems it was found that

$$SA = c_4 A^{c_5} \qquad (20)$$

Numerical values of constants are $c_4 = 1.140018$ and $c_5 = 0.234163...$, with the regression coefficient being $r = 0.9991$. Eqn. 20 can again be replaced for practical application with the approximative relationship

$$SA = 1.1 \sqrt[4]{A} \qquad (21)$$

Although constants c_n are not integers and the procedure for their determination was based on numerical methods, the equations obtained have the meaning of a fundamental relationship between the efficiency of the chromatographic system and the contribution to the shape or asymmetry of the elution curve. The reliability of the constants obtained, expressed in terms of regression coefficient r, approaching unity, indicates justification of this statement.

The above calculations were based on the initial assumption that the injection function has a zero width and that the system of separation columns acts as a Gaussian operator. This approximation is accepted in the case of SEC when, e.g., correction of molecular-weight distribution for a longitudinal spreading is performed by solving a convolution integral the root of which is a Gaussian distribution function.

Solution for a General Dispersion Model of the Concentration Profile

Starting from a general mass balance of the infinitesimal segment of the separation column which can be considered homogeneous from the viewpoint of dispersion characteristics we can write

$$\frac{\delta C}{\delta T} = \frac{1}{Pe} \frac{\delta^2 C}{\delta L^2} - \frac{\delta C}{\delta L} \qquad (22)$$

where C is a dimensionless quantity, $C = c/c_o$, where c is the actual concentration, c_o is the concentration at the column inlet at the moment of injection and $Pe = ul/D_m$ is Peclet's number, u is the linear velocity of the mobile phase and D_m is the diffusion coefficient of the solute in the mobile phase. Eqn. 22 can be solved with the boundary conditions

$$C(L,0) = 0 \qquad L \geq 0 \qquad (23)$$

and for the injection δ-function

$$C(T,0) = \delta(T) \qquad T \geq 0 \qquad (24)$$

For a bounded solution of function C(L,T) it must further hold

$$C(L,T) \varepsilon < 0, +\infty > \qquad L \geq 0 \qquad (25)$$

The known solution is obtained by employing the Laplace transformation

$$C(L,T) = \frac{L\sqrt{Pe}}{2T\sqrt{\pi T}} \exp\left(-\frac{Pe}{4T}(L - T)^2\right) \qquad (26)$$

The n-th derivative of the Laplace transform is equal to the n-th statistical moment of the function with the variables of the transformation approaching zero. This fact can formally be expressed by the relationship

$$/u_n' = \lim_{p \to 0} \left((-1)^n \left[d^n \bar{C}(p)/dp^n \right] \bar{C}(p)^{-1} \right) \qquad (27)$$

where the Laplace transformation $\bar{C}(p)$ of the function $C(1,T)$ is defined by the relationship

$$\bar{C}(p) = \int_0^\infty \exp(-pT) F(1,T) dT \qquad (28)$$

By solving Eqn. 27 for function $C(1,T)$, the moments are obtained

$$/u_1' = \int_0^\infty C(1,T) T \, dT / \int_0^\infty C(1,T) dT = 1 \qquad (29)$$

where

$$\int_0^\infty C(1,T) dT = 1 \qquad (30)$$

Hence for the second and the third central moments it is obtained

$$/u_2 = \int_0^\infty C(1,T)(T-1)^2 dT = \int_0^\infty C(1,T) T^2 dT - 1 = 2/Pe \qquad (31)$$

$$/u_3 = \int_0^\infty C(1,T)(T-1)^3 dT = \int_0^\infty C(1,T) T^3 dT - 6/Pe - 1 = 16/Pe^2 \qquad (32)$$

By substituting from Eqns. 29-32 to definition relationships 9-11, the precise relations are obtained

$$A = 4N^{-2} \qquad (33)$$

and

$$\gamma = 4N^{-0.5} \qquad (34)$$

For slope analysis the second derivative of the function $C(1,T)$

$$\frac{d^2C(1,T)}{dT^2} = C(1,T)\left(\frac{Pe^2}{16T^4} - \frac{5Pe}{4T^3} + \left(\frac{15}{4} - \frac{Pe^2}{8}\right)\frac{1}{T^2} + \frac{3Pe}{4T} + \frac{Pe^2}{16}\right) = 0 \qquad (35)$$

must again be known.

As at the points of inflexion it holds that $C(1,T) \neq 0$, after the rearrangement it is obtained that

$$T^4 + \frac{12T^3}{Pe} + \left(\frac{60}{Pe^2} - 2\right)T^2 - \frac{20T}{Pe} + 1 = 0 \qquad (36)$$

Coordinates of the points of inflexion are obtained by solving Eqn. 36. The slopes of tangents at these points of inflexion are obtained from the first derivative of the function C(1,T)

$$\frac{dC(1,T)}{dT} = C(1,T)\left(\frac{Pe}{4T^2} - \frac{3}{2T} - \frac{Pe}{4}\right) \qquad (37)$$

Hence for the relationship between SA and A it is finally valid

$$SA = 1 + \frac{2-3T_B\sqrt{A}-2T_B^2}{2-3T_F\sqrt{A}-2T_F^2}\sqrt{\left(\frac{T_F}{T_B}\right)^7}\exp\left(\frac{-1}{\sqrt{A}}\left(\frac{1+T_B^2}{T_B} - \frac{1+T_F^2}{T_F}\right)\right) \qquad (38)$$

CONCLUSIONS

It follows from the above results that the relation between the shapes of the elution curve and of the concentration profile is given by a simple dependence of the contribution to the elution curve asymmetry on the efficiency of the chromatographic system. Addi-

tionally it can be stated that slope asymmetry parameter, SA, as defined, shows an obvious and precise functional dependence on the objective asymmetry parameter, A, defined on the basis of the statistical moments. This important finding permits a simple evaluation of an experimental chromatogram (also by graphical method) in an objective manner, i.e., by means of the parameter which has not (in contrast to different graphical methods used till now) only an empirical definition character. The use of SA for the evaluation of the elution curve asymmetry will be necessary particularly in those cases when the integration of the chromatogram in both marginal regions, required for the calculation of the statistical moments, would, with respect to a given chromatogram, bring about substantial inaccuracies into the calculation of the third central moment, and would thus make the accuracy of the resulting value of A doubtful.

REFERENCES

1 Giddings, J. C., Dynamics of Chromatography, Marcel Dekker, New York, 1965.

2 Taylor, G., Proc. R. Soc., A 219, 186, 1953.

3 Grushka, E., Myers, M. N., Schettler, P. D. and Giddings, J. C., Anal. Chem., 41, 889, 1969.

4 Janča, J., J. Liq. Chromatogr., 1, 731, 1978.

5 Bennett, C. A. and Franklin, N. L., Statistical Analysis in Chemistry and Chemical Industry, Wiley, New York, 1954.

6 Grushka, E., Anal. Chem., 44, 1733, 1972.

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CONCENTRATION DEPENDENCE OF DISPERSION OF POLYMER SOLUTIONS FLOWING IN CAPILLARIES

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ABSTRACT

Dispersion of polymer solutions flowing in capillaries is an important phenomenon the knowledge of which is necessary for quantitative evaluation of polymer separations by chromatographic methods. The papers published in this field present a number of contradictory results. At various concentrations and various molecular weights also some other phenomena occur in the case of polymers, such as, e.g., non-Newtonian behaviour, which can affect dispersion.

In the present work influences of polymer molecular weight, specific viscosity of the polymer solution injected as a pulse or as a step into a capillary and influence of the flow-rate were studied. Experimental conditions were selected, so as to avoid possible interfering effects. The results confirmed our theoretical considerations and numerical calculations of other authors.

INTRODUCTION

The flow of polymer solutions in narrow capillaries also occurs in a number of natural and technological processes. Among them, chromatographic separation methods, such as, e.g., Steric-Exclusion

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Chromatography (SEC), occupy an important place. The movement of the chromatographic zone through a random spatial porous structure of the column packing is a complicated process. In microscopic view, elementary transport phenomena occur in short pore segments between packing particles and are controlled by the same laws as the flow in the capillary. This is why the knowledge of elementary phenomena appearing during the flow of polymer solutions through narrow capillaries makes it possible to undersand also more complicated phenomena that occur in porous materials.

A number of authors have already studied dispersion of polymer solutions flowing in capillaries and its consequences for the separation by SEC method. Billmeyer and Kelley (1) observed considerable tailing in the chromatogram of a polystyrene (PS) solution. They did not notice this phenomenon with low-molecular weight solutes. They explained it by a worse mixing of PS solution which is more viscous. Besides this, they found some other anomalies, such as occurrence of negative peaks. These anomalous effects were suppressed by the use of a refractometric detector with a smaller volume of the measuring cell.

Biesenberger and Ouano (2) and Ouano and Biesenberger (3,4) carried out in detail both theoretical and experimental study on the dispersion of polymer solution in capillaries. The differential mass balance describing the dispersion of the solute was the starting point for their theoretical study

$$\frac{\delta c}{\delta t} = - v \frac{\delta c}{\delta z} + D_R \frac{1}{r} \frac{\delta}{\delta r} \left(r \frac{\delta c}{\delta r} \right) + D_L \frac{\delta^2 c}{\delta z^2} \qquad (1)$$

c is the concentration of the solute, z is the longitudinal coordinate, t is the time, v is the velocity

of the mobile phase, D_R and D_L are the coefficients of the radial and longitudinal diffusion, respectively. Solving this equation under the given boundary conditions, they obtained the functions describing the shape of the chromatographic zone under various flow conditions. Namely for the case of segregated flow regime, when the influences of both axial and radial diffusion are very weak with respect to low diffusion coefficients (as with macromolecules) or with respect to the characteristics of the capillary (short length of the capillary, L, or large r for the given mean linear velocity, $\bar{v}$) and for the case of Taylor's regime, when $r \ll L$ and D is great and hence only the influence of axial diffusion is negligible. However, the experimental results were not in accord with theoretical considerations. The chromatograms showed anomalous shape, they were bimodal. These anomalies were the more marked the longer was the capillary length and the higher was the molecular weight of the used model PS standard.

Hess and Tierney (5) published a very important paper in which they treated solution of eqn. (1) numerically in connection with the study of the dispersion during the flow of a solution of macromolecules in a capillary. One of the important findings of their study is that the flattening of the velocity profile occurs which is the more marked the higher is the molecular weight of the polymer or the higher is the viscosity of its solution. Another important factor, discussed in their paper, is the influence of possible non-Newtonian behaviour of the polymer solution in consequence of which the velocity profile will also be flattened. Hess and Tierney stated that their numerical results indicate lower decrease in the dispersion than the one that was observed experimentally by other authors mentioned above.

In their subsequent work, Ouano and Biesenberger (6) substantially confirmed their earlier experimental findings by repeated experiments of their preceding works, however with the use of dual detection (a UV detector and a differential refractometer in series), i.e., under the conditions where it was possible to exclude assumed artefacts.

Mayock and coworkers (7), as well as Golay and Atwood (8), dealt with the problems of the dispersion in the capillary in the initial stages of the flow. They found out by numerical methods applied to the solution of eqn. (1) that in these initial stages, i.e., in the transient range between the segregated flow regime and Taylor´s flow regime, bimodal concentration profiles or bimodal elution curves may occur.

On the basis of the published experimental data and in the absence of physically clear theory on the molecular level, which would describe quantitatively various phenomena occurring in the discussed transport processes, neither the possibility can be excluded that, in the case of soluble polymers, the same phenomena occur as those observed by Daily and Bugliarello (9) for the flow of particles. These authors observed the tendency of dilute fibre suspensions to concentrate during the flow in the centre of the capillary and to establish conditions for non-Newtonian behaviour by their orientation in the flow. Similar observations were made by Goldsmith and Mason (10, 11).

In the present paper experimental study of the dispersion of polymer solutions during the flow in the capillary was extended with the aim of approaching a capillary model to real flow conditions in the chromatographic column so that these results might be used in future works to explain dispersion phenomena in SEC of polymers. Concentration dependence of the

dispersion of polymer solutions flowing in the capillary is studied for the reason that considerable concentration gradients exist in the chromatographic column which result in viscosity gradients making appreciable contributions to the total zone broadening. Furthermore, the viscosity phenomena show considerable influence on the polymer separation by SEC, and from this point of view have already been evaluated in a number of papers (for review see ref. 12). Explanation of their nature will require additional studies.

EXPERIMENTAL

A very simple equipment was used for the measurements. A constant flow-rate of the solvent was effectuated by means of a home-made syringe pump. Solutions were injected with a six-port valve (Waters Assoc., Milford, Mass., U.S.A.). Stainless-steel capillaries (Knauer, Oberursel, FGR), of various lengths, 1 mm in the inside diameter, were used. The eluate was detected with a differential refractometer, model 2025/50 (Knauer, Oberursel, FGR) with the cell volume of 8 µl in the case of the pulse injection experiment and with an UV detector at the wavelength of 254 nm (Development Works, Czech. Acad. of Sci., Prague, Czech.) in the case of step injection experiment. The UV detector was modified so that the measuring cell was composed of a quartz capillary with the inside diameter of 1 mm, to which the stainless-steel capillary was directly connected. UV radiation passed perpendicularly to this cell, the effective volume of which was 8 µl. By this manner the flow disturbance on the passage from the stainless-steel capillary to the measuring cell of the detector was excluded. For registration a Linear

recorder, model 355 (Linear Instruments Corp., Irvine, Ca., U.S.A.), was applied. The measurement of the elution volume was derived from the movement of mechanical parts of the pump and thereby it was fairly precise. Elution volumes were recorded in various volumetric intervals - counts - the volume of which was 2.5×10^{-2} ml in the case of the step injection and 2.0×10^{-1} ml in the case of the pulse injection. The injected volume was 30 ul in the case of pulse injection. In the step injection experiment, the injection loop was replaced with a capillary, 1 mm in the inside diameter and 5 m in the total length, being equal in the length to the capillary in which dispersion measurement was performed. With the pulse injection, the length of the used stainless-steel capillary was 248 cm. Toluene and tetrahydrofuran (THF) were used as solvents for the pulse injection and the step injection experiments, respectively. Toluene of analytical grade (Lachema, Brno, Czechoslovakia) was used directly, THF was distilled from cuprous chloride and potassium hydroxide in nitrogen atmosphere.

Solutions of naphthalene in toluene and polystyrene (PS) standards in toluene were injected in the pulse injection experiment and solutions of benzene in THF and PS standard in THF in the step injection experiment. Molecular weights of the used PS standards (Waters Assoc., Milford, Mass., U.S.A.) are listed in Table 1.

The stainless-steel capillaries in which the experiments were performed were straight, without any coiling. They were inserted into a rubber tubing 1 cm in the inside diameter, in such a way that these capillaries passed through the perforated wall of the rubber tubing closely behind the injection valve and

TABLE 1

Molecular Weights of PS Standards Used for the Study (Manufacturer's Data)

Standard	Molecular Weight
PS 1	2 700 000
PS 2	2 610 000
PS 3	867 000
PS 4	670 000
PS 5	470 000

just before the detector. Water, thermostated to the temperature of 25 $\pm$ 0.05 ^{o}C, circulated in the tubing. Thereby it was secured that the conditions of the experiment were precisely defined, constant and without any influences disturbing the hydrodynamic flow.

RESULTS AND DISCUSSION

The authors of the referred works (2-4, 6) performed their measurements in capillaries, 1 mm in the inside diameter, under the flow-rates used in practical SEC separations of polymers, i.e., 1 ml/min. This flow-rate corresponds to the linear velocity of the displacement of approximately 2 cm/sec. Columns for SEC are usually 4-8 mm in diameters and have the interstitial porosity $\emptyset$ = 0.35 - 0.40. It follows from these data that linear velocities of the displacement in the interstitial volume are 5 to 25 times less under common chromatographic conditions than in the referred papers. In order to approach the conditions of linear

velocities of the displacement in the column in this respect, when using the capillary with the inside diameter of 1 mm, we must operate within approximate range of the flow-rates of 0.04 - 0.20 ml/min. This is why we carried out our measurements at the two different flow-rates, 0.038 ml/min and 0.334 ml/min.

For the evaluation of the dispersion regime under the present experimental conditions, Peclet's number, Pe,

$$Pe = L \bar{v} / D \qquad (2)$$

and Taylor's number (2), Ta,

$$Ta = R^2 \bar{v} / LD \qquad (3)$$

were used, where R is the radius of the capillary. For the given experimental conditions (particularly L and $\bar{v}$) Pe is always $Pe \gg 1$, which means that the contribution of the longitudinal diffusion is small. At the higher of the both studied flow-rates $Ta \gg 1$ for polymer solutions which means that segregated flow regime (2) is concerned, in which the radial diffusion is small. At the lower flow-rate $Ta > 1$ (approximately $Ta \doteq 8$), which means that rather a transient range between the segregated flow and Taylor regimes (2) is concerned. For low-molecular weight solutes (naphthalene, benzene) $Ta \ll 1$, with respect to a higher diffusion coefficient, i.e., at both of the flow-rates the condition of Taylor flow regime holds true.

In order to evaluate experimental data quantitatively from the viewpoint of the agreement or discrepancies between them and the theoretically assumed model of the flow regime, statistical moments of elution curves, u_1' (the first statistical moment with respect

to zero) and $/u_n$ (the n-th central moment), defined by the equations

$$/u_1' = \Sigma V_i \; h_i \; / \; \Sigma h_i \qquad (4)$$

$$/u_n = \Sigma (V_i - /u_1')^n \cdot h_i \; / \; \Sigma h_i \qquad n=2,3,\ldots \qquad (5)$$

were used, where V_i is the elution volume, h_i is the height of the chromatogram from the base-line. With the aid of Eqns. (4) and (5) it was possible to calculate the corresponding statistical moments of the elution chromatograms resulting from the pulse injection experiment. The statistical moments of the frontal chromatograms obtained by the step injection were calculated with the use of Eqns. (6) and (7)

$$/u_1' = \Sigma V_i \cdot (h_i - h_{i-1}) \; / \; H \qquad (6)$$

$$/u_n = \Sigma (V_i - /u_1')^n \cdot (h_i - h_{i-1}) \; / \; H \qquad (7)$$

where H is the total height of the frontal chromatogram from the base-line up to the plateau for which it holds true that

$$H = \Sigma (h_i - h_{i-1}) \qquad (8)$$

Based on these statistical moments, the criterion of elution curve asymmetry, A, was defined in the preceding work (13)

$$A = /u_3 \; / \; (/u_1')^3 \qquad (9)$$

Additionally, for the case of the dispersion which can be described by Eqn. (1), the relationship (14)

between the number of theoretical plates, N, of the given system

$$N = (/u_1')^2 / /u_2 \qquad (10)$$

and the asymmetry, A,

$$A = 4\ N^{-2} \qquad (11)$$

was derived.

The agreement between the values of A_{exp}, calculated from the experimental chromatograms with the use of Eqn. (9), and theoretical ones, A_{theor}, calculated with the aid of Eqn. (11) for the values of N found experimentally (Eqn. (10)), can then serve as a suitable criterion for the evaluation of the accord between the theoretical assumption of the dispersion and the real experimental observations. In other words, the differences between A_{exp} and A_{theor} indicate that the real character of the dispersion differs from the one that is assumed by the used theoretical model. The above interpretation was used to evaluate both the pulse and the step injection experiments.

For the height equivalent to a theoretical plate, HETP, the known relationship can be written

$$HETP = L / N \qquad (12)$$

For a circular capillary it then holds (15)

$$HETP = \bar{v}\ R^2 / 24\ D \qquad (13)$$

Eqn. (13) results from the solution of Eqn. (1) under the pertaining boundary conditions with axial diffusion being neglected and with the assumption of a parabolic velocity profile (16).

Diffusion coefficient is a function of dimensions or molecular weight of the solute molecules and the viscosity of the medium according to Stokes-Einstein equation

$$D = R^{\bullet} T / 6 \pi r_1 \eta N_A \qquad (14)$$

where $R^{\bullet}$ is the gas constant, T absolute temperature, r_1 the radius of the solute molecule, η viscosity of the medium and N_A Avogadro number. If a spherical solute macromolecule and a direct proportion between the volume of the macromolecule and its molecular weight can be assumed, Stokes-Einstein equation can be written in a more general form

$$D = \text{const} \cdot 1 / (\eta M^{1/3}) \qquad (15)$$

Some empirical expressions describe the relationship between D and M increased to a higher power (up to $M^{2/3}$), such as, e.g., Wilke/Chang equation (17). The above relationships and equations became the basis for the quantitative evaluation of the experimental results.

Results of both pulse and step injection experiments, obtained at the two different flow-rates for a series of samples of PS standards having the given initial value of the specific viscosity, η_{spec} are listed in Tables 2-5. These specific viscosities of the injected solutions of PS standards were calculated from the known concentration of the solutions of PS standards of the given molecular weights with the use of the Mark-Houwink equations

$$[\eta] = 8.81 \cdot 10^{-3} M^{0.75} \qquad (16)$$

valid for PS in toluene at 25 ^{o}C (18) and

TABLE 2

Results of the Pulse Injection Experiment Measured at a Flow-Rate of 0.038 ml/min

Sample	η_{spec}	$/u'_1$	$/u_2$	$/u_3$	N	A_{exp}	A_{theor}
Naphthalene 0.0625-4%	—	10.84	0.267	0.02	440	0.000016	0.000021
PS 1	9.458	15.94	20.82	67.95	12.2	0.0168	0.0269
	3.366	14.11	17.80	30.50	11.2	0.0109	0.0320
	1.342	12.38	11.45	21.69	13.4	0.0114	0.0223
PS 3	3.366	13.46	10.20	27.29	17.8	0.0112	0.0127
	1.342	11.24	8.85	21.11	14.3	0.0149	0.0196
	0.586	12.56	9.57	25.04	16.5	0.0126	0.0147
PS 5	3.366	12.11	5.13	9.92	28.6	0.0056	0.0049
	1.342	12.09	6.27	15.09	23.3	0.0085	0.0074
	0.586	11.92	6.37	15.26	22.3	0.0090	0.0080
	0.272	11.48	5.63	7.22	23.4	0.0048	0.0073

$$[\eta] = 1.17 \cdot 10^{-2} M^{0.717} \qquad (17)$$

valid for PS in THF at 25 °C (19) and with the use of the Huggins equation

$$\eta_{spec} = [\eta] c + k_H [\eta]^2 c^2 \qquad (18)$$

c in the above equations is concentration, $[\eta]$ is the intrinsic viscosity, M is the molecular weight and

TABLE 3

Results of the Pulse Injection Experiment Measured at a Flow-Rate of 0.334 ml/min

Sample	η_{spec}	$/u'_1$	$/u_2$	$/u_3$	N	A_{exp}	A_{theor}
Naphthalene 0.0625-4%	—	10.92	1.49	0.38	80.0	0.00029	0.00062
PS 2	9.458	25.71	107.5	882.6	6.1	0.0519	0.1058
	3.366	17.95	57.11	288.5	5.6	0.0499	0.1257
	1.342	14.50	23.60	25.5	10.6	0.0177	0.0358
PS 3	3.366	19.00	48.94	271.0	7.4	0.0395	0.0735
	1.342	14.90	25.36	100.8	8.8	0.0305	0.0522
	0.586	13.76	24.97	97.9	7.6	0.0376	0.0696
PS 5	3.366	16.67	25.19	71.2	11.0	0.0154	0.0329
	1.342	14.08	18.74	64.4	10.6	0.0231	0.0357
	0.586	11.40	8.12	17.8	16.0	0.0120	0.0156

the Huggins constant, $k_H = 0.362$, was measured in our previous work (20) for THF; for toluene it is $k_H = 0.34$ (18).

Let us notice now in detail the results summarized in Tables 2 and 3, i.e., the dispersion in the elution mode in the pulse injection. At the both, higher and lower flow-rates a marked dependence of $/u'_1$, i.e., of the average elution volume, on η_{spec} of the injected polymer solution was found. For better illustration it is shown in Fig. 1. This dependence

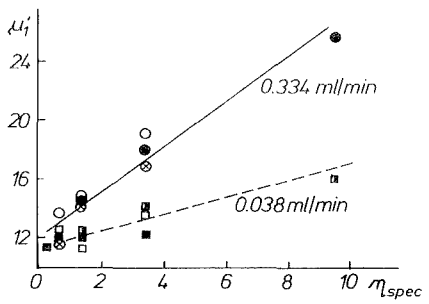

FIGURE 1
Dependence of the average elution volume on the specific viscosity of solutions of various PS standards injected as pulses.
Flow-rate: 0.334 ml/min: ● PS 2, ○ PS 3, ⊗ PS 5
Flow-rate: 0.038 ml/min: ◧ PS 1, □ PS 3, ⊠ PS 5

is only slightly influenced by the molecular weight of the solute. So far similar observation is concerned as with packed columns (21). In contradiction to the experiments with packed columns the dependence of u_1' on η_{spec} is markedly less steep at the lower flow-rate. In order to demonstrate illustratively circumstances of the dependence of u_1' on η_{spec}, Fig. 2 shows the chromatograms obtained by the injection of solutions of PS standards at different concentrations and thus at different values of η_{spec} at the higher flow-rate. The identical feature of a similar figure in our earlier work (21) is the fact that the frontal part of the chromatogram has almost identical course and origin for all of the three injections, whereas the back part is retarded more significantly as η_{spec} increases. Thereby the width of the chromatographic zone extends and tailing apparently increases. The values of A in

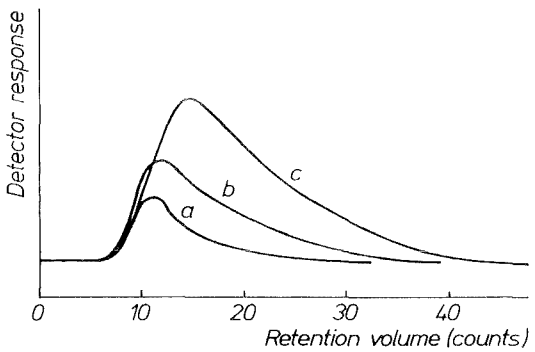

FIGURE 2
Chromatograms of PS 3 standard injected as a pulse at various concentrations at a flow-rate of 0.334 ml/min. a... η_{spec}= 0.586, b... η_{spec}= 1.342, c... η_{spec}= 3.366

Tables 2 and 3, however, show (again with a certain experimental variance) practical independence on η_{spec}. Thus an enlarged tailing declared by the authors of (2) is only apparent as it is a simple consequence of the increase in the zone width, characterized by $/u_2$ with an increasing η_{spec}. This dependence of $/u_2$ on η_{spec} is also entirely obvious in Tables 2 and 3 in spite of the variance of the experimental results being greater in consequence of experimental inaccuracies increased to the second power (see definition Eqn. (5)).

At the higher of the both investigated flow-rates the ratio A_{theor}/A_{exp} is approximately 2.0 for all of the injected PS standards and concentrations, with non-systematic variations in the range from 1.3 to 2.5. Even for naphthalene injected in a wide range of concentrations the ratio A_{theor}/A_{exp} = 2. The explanation obviously follows from the fact that although the injected volume is about 1.5 % of the total volume of

the capillary, it is not infinitely small as it would be necessary for the ideal case, which causes the observed differences between A_{theor} and A_{exp}.

At the lower flow-rate the observed ratios A_{theor}/A_{exp} are lower; on average $A_{theor}/A_{exp} = 1.4$. The contribution to the broadening caused by the injection is doubtlessly less significant, regardless the fact that, with the exception of PS 1 standard, the other differences are assentially within the framework of experimental errors. It is obvious from Eqn. (11) that the error of 5 % in the reading of N will result in the error of about 10 % in the value of A.

With regard to Eqns. (10), (12), (13) and (15) it must hold true for the given constant viscosity of various injected PS standards

$$\frac{(/u_2)_{M_1}}{(/u_2)_{M_2}} = \left(\frac{M_1}{M_2}\right)^{1/3} \qquad (19)$$

and for the injection of PS standards of the given molecular weights and viscosities it then holds

$$\frac{(/u_2)_{\eta_1}}{(/u_2)_{\eta_2}} = \frac{\eta_1}{\eta_2} = \eta_{rel} = \eta_{spec} - 1 \qquad (20)$$

In consideration of the dilution of the polymer solution due to the zone broadening in the course of its movement along the column, all of the viscosities in Eqn. (20) have the meaning of average values which, however, are proportional to the values of viscosities of the injected solutions and therefore the latter ones can be used for the correlation.

The results presented in Table 2 and 3 show (with the variance given by experimental errors) basic agreement of experimental data with Eqns. (19) and (20). It means that in the case when diffusion takes place in a high degree (in the first place radial diffusion) the dependence of $/u_2$ on η_{spec} is primary. The dependence of $/u_1'$ on η_{spec} is, in this case, only a consequence of the preceding dependence. The correlation of $/u_2$ values measured at various flow-rates with Eqn. (13) shows a good agreement between the theoretical assumption and the experiment for the low-molecular weight solute - naphthalene. An increase in HETP values of polymer solutions at the higher flow-rate is, however, substantially less than it corresponds to Eqn. (13). These facts, in the same way as the character of the chromatographic curves in Fig. 2, confirm that, at least at the higher of the both studied flow-rates, segregated flow regime takes place in the first place in consequence of which convective character of diffusion predominates. However, it is obvious that other phenomena of hydrodynamic character, such as establishment of other than parabolic velocity profile during the flow of polymer in the capillary, can take place.

The results of the step injection experiment are presented in Tables 4 and 5, again for the two above mentioned flow-rates. Sign "plus" in column "Specific Viscosity Step" indicates that the solution of PS 4 standard was injected into THF, the change in η_{spec} was thus positive and, reversely sign "minus", means that THF was injected into the solution of PS standard.

At the lower flow-rate, an inexpressive tendency to an average higher elution volume $/u_1'$ can be observed if the solution of PS standard is injected into THF when

TABLE 4

Results of the Step Injection Experiment Measured at a Flow-Rate of 0.038 ml/min

Specific Viscosity Step	$/u_1'$	$/u_2$	$/u_3$	N	A_{exp} $.10^{-3}$	A_{theor} $.10^{-3}$
Benzene into THF	151.5	12.59	4.402	1823	0.0013	0.0012
+0.392	152.0	290.1	4847	79.6	1.38	0.63
+0.872	154.1	285.0	4227	83.3	1.16	0.58
+2.133	150.4	289.6	5266	78.1	1.55	0.66
-0.239	146.0	315.7	3226	67.5	1.04	0.88
-0.514	144.5	309.4	4336	67.5	1.44	0.88
-1.170	150.5	357.0	7029	63.4	2.06	0.99

compared with $/u_1'$ of benzene injected into THF and the tendency to an average lower $/u_1'$ with the injection of THF into the solution of the PS standard. This may be an indication of the establishment of the plug velocity profile with the injection of PS solution into THF on the zone boundary and "break-through" of the zone boundary by the less viscous THF on its injection into the PS solution, leading to the velocity profile elongated more than the parabolic one. Flattening of the velocity profile may be caused on the one hand by non-Newtonian behaviour of the polymer solution, which, however, appears more marked only at higher molecular weights and at higher polymer concentrations, and, on

TABLE 5

Results of the Step Injection Experiment Measured at a Flow-Rate of 0.334 ml/min

Specific Viscosity Step	$/u_1'$	$/u_2$	$/u_3$	N	A_{exp} $.10^{-3}$	A_{theor} $.10^{-3}$
Benzene into THF	152.2	134.6	360	172	0.102	0.135
+0.404	140.7	468.6	8001	42.2	2.87	2.24
+1.319	146.6	525.6	14567	40.9	4.62	2.39
+2.224	147.4	340.3	5487	63.8	1.71	0.98
-0.239	133.0	459.4	8077	38.5	3.43	2.70
-0.514	130.1	409.7	6442	41.3	2.92	2.35
-1.170	134.2	857.2	42575	21.0	17.6	9.07

the other, by the tendency of macromolecules to concentrate in the centre of the capillary. Thereby the viscosity in the centre of the capillary increases in consequence of which the velocity in the capillary centre decreases. This phenomenon can cause "by-pass" of the cloud of the polymer solution at higher concentration by that having a lower concentration in the proximity of the wall. In accord with this is also a steeper slope of the dependence of $/u_1'$ on η_{spec} at the higher flow-rate for the pulse injection experiment the explanation of which was presented above on the basis of Eqns. (19) and (20).

The tendency of macromolecules or particles to concentrate in the centre of the capillary can be

explained by the lift forces (22) which were observed experimentally by several authors (9-11, 23), as mentioned already above.

The theoretical value of asymmetry is in a very good agreement with the experimental value for the elution of benzene. The ratio of other values A_{theor}/A_{exp} is on average ca. 0.5, i.e., tailing is greater than assumed by the theoretical model.

Much more marked are differences in $/u_1'$ at the higher flow-rate when the influence of the radial diffusion of polymer solute is almost neglectable, under the given conditions of the segregated flow. On the injection of the solution of PS standard into THF $/u_1'$ is by 5 % lower in comparison with $/u_1'$ on the injection of benzene into THF. On the injection of THF into the solution of PS standard the values of $/u_1'$ are by 13 % lower than the values of $/u_1'$ for benzene. These facts confirm that the influence of the radial diffusion is really slight and therefore the zone front moves, owing to a higher linear velocity in the capillary centre, at a much higher speed. Further it is obvious that in the case of the injection of THF into the solution of PS standard, occurs "break-through" of the zone of the PS solution by much less viscous and thus more mobile THF. The ratio A_{theor} / A_{exp} lies in the range 0.52 - 0.80 and is on average by 15 % higher than at the lower flow-rate. Examples of the elution curves from the step injection experiment are shown in Figs. 3 and 4.

Slight and non-systematic influence of η_{spec} on $/u_1'$ in the both instances of the low and the high flow-rates indicates that the shape of the velocity profile is a dominant factor and hence it is convection that governs transport processes at the both boundaries

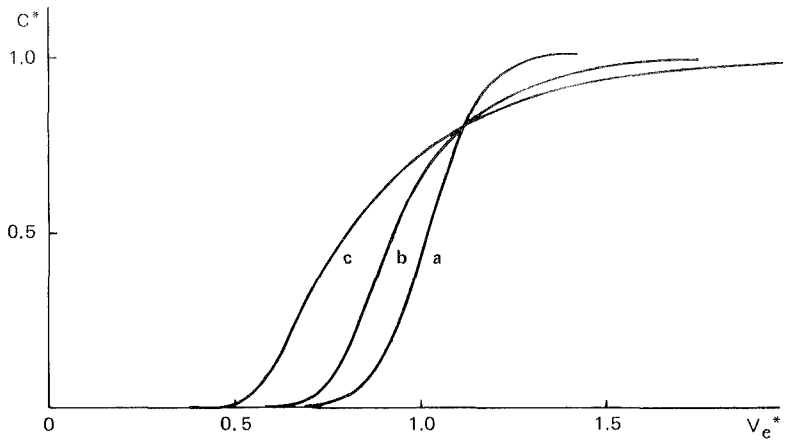

FIGURE 3
Normalized chromatograms of benzene and PS 4 standard injected as steps at various concentrations at a flow-rate of 0.334 ml/min.
a...benzene, b... η_{spec} = +2.224, c... η_{spec} = -1.170
C^*, Ve^* normalized concentration and elution volume

and in the polymer zone under the given conditions of the flow.

The above results are in a good agreement with the calculations by Hess and Tierney (5) who found out that a decrease in the maximal velocity in the capillary centre, caused by the flattening of the originally parabolic velocity profile during the flow of polymer solutions, is of the order of per cents (depending on the molecular weight and the concentration).

Further experimental and theoretical studies of the shape of the velocity profile on the solvent-

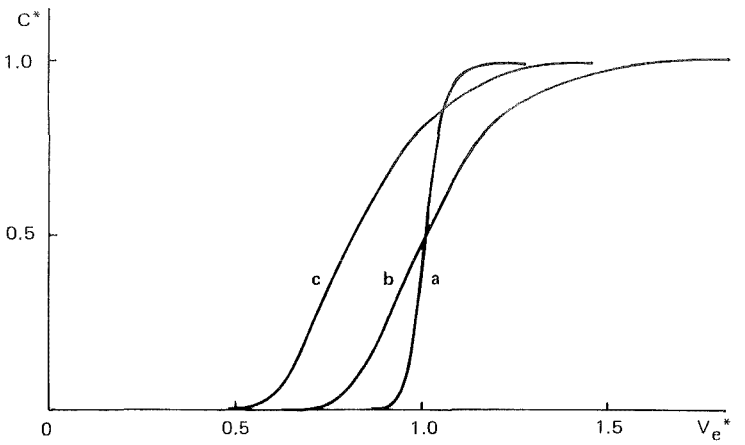

FIGURE 4
Normalized chromatograms of benzene and PS 4 standard injected as steps at various concentrations at a flow-rate of 0.038 ml/min.
a...benzene, b... η_{spec}= +0.872, c... η_{spec}= -0.514.
C^*, Ve^* normalized concentration and elution volume

-polymer solution or polymer solution-solvent boundary are in progress.

ACKNOWLEDGEMENT

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REFERENCES

1. Billmeyer, F. W. and Kelley, R. N., J. Chromatogr., 34, 322, 1968.

2. Biesenberger, J. A. and Ouano, A. C., J. Appl. Polym. Sci., 14, 471, 1970.

3. Ouano, A. C. and Biesenberger, J. A., J. Appl. Polym. Sci., 14, 483, 1970.

4. Ouano, A. C. and Biesenberger, J. A., J. Chromatogr., 55, 145, 1971.

5. Hess, M. and Tierney, J. W., J. Chromatogr., 55, 165, 1971.

6. Ouano, A. C. and Biesenberger, J. A., Proceedings, 9th International Seminar on Gel Permeation Chromatography, Miami Beach, 1970, p. 465.

7. Mayock, K. P., Tarbell, J. M. and Duda, J. L., Separ. Sci. Technol., 15, 1285, 1980.

8. Golay, M. J. E. and Atwood, J. G., J. Chromatogr., 186, 353, 1979.

9. Daily, J. W. and Bugliarello, G., Ind. Eng. Chem., 51, 887, 1959.

10. Goldsmith, H. L. and Mason, S. G., Nature, 190, 1095, 1961.

11. Goldsmith, H. L. and Mason, S. G., J. Colloid. Sci., 17, 448, 1962.

12. Janča, J., Pokorný, S., Vilenchik, L. Z. and Belenkii, B. G., J. Chromatogr., 211, 39, 1981.

13. Janča, J., J. Liq. Chromatogr., 1, 731, 1978.

14. Janča, J., J. Liq. Chromatogr., in press.

15. Scott, R. P. W., Contemporary Liquid Chromatography, Wiley, New York, 1976.

16. Crank, J., The Mathematics of Diffusion, Clarendon Press, Oxford, 1956.

17. Giddings, J. C., Dynamics of Chromatography, M. Dekker, New York, 1965.

18. Brandrup, J. and Immergut, E. H., Polymer Handbook, 2nd ed., Wiley, New York, 1975.

19. Kolínský, M. and Janča, J., J. Polym. Sci., Polym. Chem. Ed., 12, 1181, 1974.

20. Janča, J., J. Chromatogr., 134, 263, 1977.

21. Janča, J. and Pokorný, S., J. Chromatogr., 148, 31, 1978.

22. Saffman, P. G., J. Fluid. Mech., 22, 385, 1965.

23. Caldwell, K. D., Nguyen, T. T., Myers, M. N. and Giddings, J. C., Separ. Sci. Technol., 14, 935, 1979.

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CRUDE OIL HYDROCARBON GROUP SEPARATION QUANTITATION

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Introduction

The petroleum industry like most other industrial segments are continuously developing new and sophisticated techniques of analysis and at the same time are working to automated routine techniques. In the petroleum industry, the source of supply has generally become less stable and economics has dictated new thinking, both in processing and in the analysis of composition to support these new trends. In addition, this industry is faced with having a new raw material, shale oil, and coal liquids, in the near future.

The traditional techniques for hydrocarbon group composition were developed when different crudes were in common supply. Today, not only do the sources of crude tend to change more often, but these crudes are different than those used in the past. High Performance Liquid Chromatography (HPLC) is making major intrusion into the separation needs of the petroleum chemist. A major hurdle that this technique faces is that of quantitation. The traditional techniques have generally been solvent-nonsolvent or open column step gradient procedures using gravimetric quantitation procedures. The detectors in HPLC equipment provide an analog signal from specific (ultraviolet) or nonspecific (differential refractometer) detectors. Such is the case in the hydrocarbon group separation of crude oils. An early use of HPLC for the separation of crude oil into hydrocarbon groups was published by Sautoni.[1] In this technique, the asphaltenes are removed and the sample is separated into saturated, aromatics, and polar aromatics using n-hexane as the solvent and an alkyl amine modified silica gel column packing. (Figure 1).

The n-hexane mobile phases across this amine modified packing provides unique selectivity for the paraffinic and aromatic hydrocarbons in crude oils (Table 1). All of the normal, iso, and cyclo paraffins elute with k' = 0.1. This includes such paraffinic structures as cholstane. Yet the k' of a long alkyl branched mono aromatic is at a k' = 0.16. If alkenes or cycloalkenes are present, as in shale oil, they will elute with the saturates at k' of 0.1. This resolution is in part obtained by using relatively small sample loads (sample loads of 0.2 micrograms is typical) on the column. These small sample loads necessitate the use of an ultraviolet photometer to monitor the aromatics and polar aromatics where k' are between one and five.

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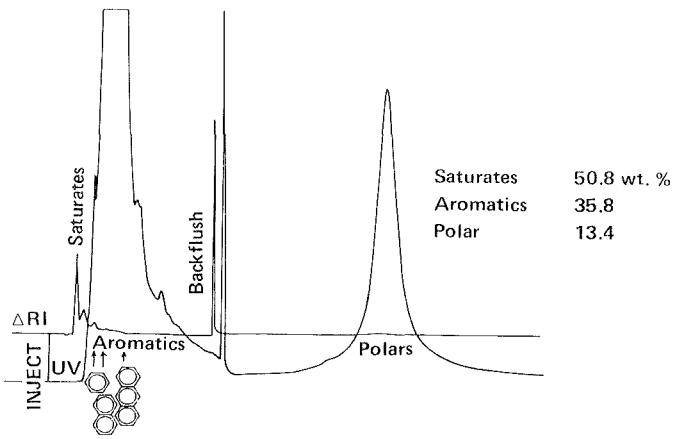

Figure 1 Hydrocarbon Group separation of Alaskan Crude Oil. n-Hexane at 2.0 ml/min. across a 3.9mm x 30cm Energy Analysis Column.

Instrumentation

All separations were performed on a Hydrocarbon Group Analyzer System. The column, 3.9mm x 30cm long, is an Energy Analysis (NH_2) column. The mobile phase is n-hexane at 2.0 ml/min. The crude oil samples were prepared by dissolving 0.5 gms/20 ml of n-hexane. The sample is deasphalted by filtration across a 0.5 micron Millipore Millex-SR® fluorocarbon membrane filter assembly. A 10 microliter injection is used.

The crude oil samples were also characterized by ASTM D-2007.[2] In addition, some crude oils were preparatively separated into hydrocarbon groups for gravimetric quantitation using a 57mm x 30cm NH_2 preparative cartridge in the Waters LC/500A preparative system.

Experimental

The separation scheme for the hydrocarbon group separation of crudes, resids, and syncrudes was modified to ensure baseline separation of the three major groups, saturates, aromatics, and polar aromatics (Table 1). The modifications made to the published separation technique include:

- Optimization of mobile phase linear velocity for column diameter.
- Evolving packing chemistry to ensure complete elution of the polar aromatics.
- Optional time for backflush routine.
- Automated data handling and baseline routines.

The areas of the aromatic envelope and the polar aromatic envelope and the polar aromatic envelope were obtained using a UV monitor at 254nm at 0.5 absorbance

Table I

Retention (k') of a variety of hydrocarbons using n-hexane as the mobile phase at 2.0 ml/min. or, with n-hexane at a linearity velocity of 0.010 to 0.012 cm/sec.

	(k')		
	Alumina	NH2	Silica
hexadecane		0.10	
dodecane		0.10	
heptadecane		0.10	
1-heptadecene		0.12	
1-octene		0.13	
1,5 cyclooctadecene		0.22	
1-octadecene		0.12	
cycloheptane		0.10	
pristane	0.04	0.10	0.06
cholestane	0.04	0.10	0.06
benzene	0.07	0.16	0.24
n-butylbenzene	0.07	0.13	0.34
toluene	0.08	0.15	0.48
n-decylbenzene	0.07	0.13	0.38
mestylene	0.08	0.13	--
phenylundecane	0.07	0.13	0.37
biphenyl	0.28	0.41	1.27
naphthalene	0.24	0.37	0.54
2 methylnapthalene	0.25	0.35	0.71
2,3 dimethylnaphthalene	0.99	0.36	0.55
2,3,5 trimethylnaphthalene	0.57	0.36	1.34
acenaphthylene	0.48	0.60	1.00
acenaphthlene	0.38	0.40	0.94
triphenylene	8.30	1.85	2.58
anthracene	1.22	0.83	1.45
phenanthrene	1.02	0.84	1.46
1 methylphenanthrene	1.53	0.81	1.74
fluoranthene	2.46	1.15	1.28
chrysene		2.88	
benzo(a)pyrene		3.78	
ocresol		11.16	
phenol		11.00	
2,2'bipyridine		11.23	
pyridine		11.00	
thiophene		13.00	

The Energy Analysis (NH2) column is backflushed at k' = 5.0 and the run is complete at k' = 14.50.

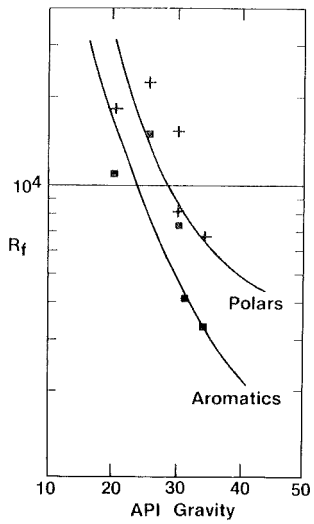

Figure 2 Whole Crude.
Relationship between Response Factor (Rf) and API Gravity.

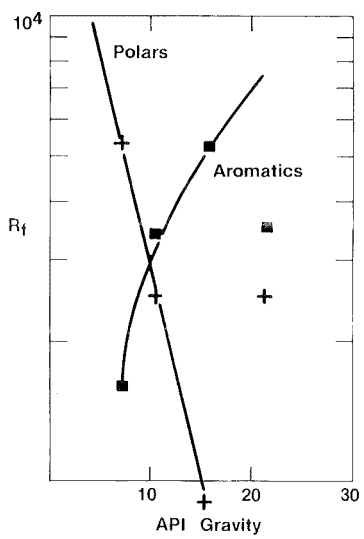

Figure 3 Topped Crude.
Relationship between Response Factor (Rf) and API Gravity.

Table II

API GRAVITY AND Rf VALUES OF AROMATICS AND POLAR AROMATICS

API GRAVITY	AROMATICS Rf	POLAR AROMATICS Rf
Whole Crude		
30.3	0.7342×10^{-4}	0.8143×10^{-4}
26.0	1.496×10^{-4}	2.219×10^{-4}
20.4	1.098×10^{-4}	1.813×10^{-4}
34.5	0.3322×10^{-4}	0.6723×10^{-4}
31.4	0.4171×10^{-4}	1.534×10^{-4}
Topped Crude		
10.4	0.3405×10^{-4}	0.2507×10^{-4}
15.8	0.5271×10^{-4}	0.0642×10^{-4}
21.4	0.3521×10^{-4}	0.2514×10^{-4}
7.3	0.1599×10^{-4}	0.5277×10^{-4}
Asphalt		
---	0.6534×10^{-4}	0.7475×10^{-4}

units full scale. Peak grouping techniques were used so that a single area was obtained for each hydrocarbon group. The response of the saturate peak was obtained from a differential refractometer using peak height techniques, or areas as determined electronically.

Several crude oils were characterized by the traditional ASTM D-2007 to obtain hydrocarbon group composition by an accepted technique.

Having determined the hydrocarbon group composition by accepted techniques and obtained areas of these hydrocarbon groups by LC techniques, response factors (Rf) were obtained. These factors will convert the areas obtained from HPLC to wt%. A relationship was found between Rf values and API gravity[3], (Table 2, Figures 2 and 3). The mathematical expression for these relationships is:

Whole Crudes

- Aromatics

$$Rf = -6.0568 \times 10^{-3} + 6.851 \times 10^{-4} (API) - 2.4587 \times 10^{-5} (API)^2 + 2.8535 \times 10^{-7} (API)^3$$

- Polars
  $Rf = -5.0235 \times 10^{-3} + 5.7482 \times 10^{-4}(API) - 2.0426 \times 10^{-5}(API)^2 + 2.3322 \times 10^{-7}(API)^3$

Topped Crudes

- Aromatics
  $Rf = 7.353 \times 10^{-5} + 1.5731 \times 10^{-5}(API) - 4.9658 \times 10^{-7}(API)^2$

- Polars
  $Rf = 1.6477 \times 10^{-4} - 1.9930 \times 10^{-5}(API) + 6.2648 \times 10^{-7}(API)^2$

  Where API = API Gravity at $60^{\circ}F$.

Three samples of deasphalted bright stock were analyzed using this technique for quantitation. The API gravity was determined on the whole sample, from this data the Rf values for the aromatics and polar aromatics was calculated. The three bright stock samples were then preparatively separated on the 57mm x 30cm NH_2 cartridge. The saturates, aromatics, and polar aromatics of a 5-gram injection were collected and quantitated gravimetrically. (Table 3).

The API gravity on a second series of crude oils and resids was determined and characterized by this technique. In addition these crudes were ana-

Table III

COMPARISON OF ANALYTICAL QUANTITATION AND GRAVIMETRIC QUANTITATION FROM PREP

Analytical Data

API GRAVITY	22.6	30.2	31.7
Saturates	88.00±0.2 wt.%	65.32±0.2 wt.%	99.36±0.10wt.%
Aromatics	6.39±0.16	33.97±0.15	8.41±0.07
Polar Aromatics	5.61±0.33	0.71±0.01	1.23±0.05
Asphaltenes	nil	nil	nil

5-gram Preparative Separation

Saturates	74.25wt.%	58.85wt.%	76.07wt.%
Aromatics	5.36	27.61	6.48
Polar Aromatics	3.27	0.8	1.79
Asphaltenes	nil	nil	nil
Recovery	82.87wt.%	87.26wt.%	84.34wt.%

Table IV

COMPARISON OF QUANTITATION FROM ASTM 2007 AND HPLC

ASTM 2007

	Saturates	Aromatics	Polar Aromatics
Uvalde Crude	24.5wt%	71.9wt%	3.6wt%
Bunker C	21.1	34.2	30.3
South Louisiana	74.9	18.6	8.4
Luwait	54.3	25.7	17.9
Agentina Heavy	54.0	19.1	11.0

HPLC

	Saturates	Aromatics	Polar Aromatics
Uvalde	22.19±0.9wt%	74.15±1.63wt%	3.73±0.08wt%
Bunker C	20.98±0.9wt%	33.83±0.38wt%	30.79±1.43wt%
South Louisiana	74.59±0.75wt%	16.21±0.16wt%	9.00±0.1wt%
Kuwait	54.58±0.8wt%	30.06±0.25wt%	11.57±0.35wt%
Argentina Heavy	48.49±1.0wt%	24.99±0.39wt%	10.62±0.29wt%

lyzed by ASTM D-2007 (Table 4). Each of the crudes was analyzed 10 times, each a separate sample preparation, to obtain data on repeatability of this HPLC technique of quantitation (Table 4).

The best repeatability and correspondence of the saturates was obtained when the saturates were obtained by difference. The refractive index differential between the mobile phase, n-hexane, and the saturate peak is so small the major measurement errors are obtained.

Conclusions

An LC technique is shown that will provide the separation and quantitation of hydrocarbon groups, from whole crudes and resids from a wide range of sources. The relationship between API gravity and Rf (response factor) appears valid even when compared against gravimetric techniques.

References

1. J.C. Sautoni, R.E. Swab, "Rapid Hydrocarbon Group Type Analysis by High Performance Liquid Chromatography" J. Chrom. Sci. 13, 361 (1975).

2. American Society of Testing and Material
1916 Race Street
Philadelphia, PA 19103

3. ASTM D-287-67
American Society of Testing and Material
1916 Race Street
Philadelphia, PA 19103

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ANALYSIS OF A MIXTURE OF POLYCHLORINATED BIPHENYLS, DDT AND ITS ANALOGUES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A simple and rapid method for separation and quantitative analysis of polychlorinated biphenyls (PCBs) and chlorinated pesticides (DDT and its analogues DDE and DDD in their o,p'- and p,p'-isomers) is described. The procedure consists of two steps:a)transformation of DDT and its analogues in o,p'- and p,p'-dichlorobenzophenone (DCBP); b)determination of the amount of PCBs and ΣDDT as DCBP by HPLC. Results obtained confirm that HPLC can be considered as an alternative or a supplementary methodology to conventional methods such as gas chromatography. The method is applied to marine organisms.

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INTRODUCTION

PCBs are widely used in modern technology and are very persistent and cumulative environmental pollutants. They, along with DDE*, are reported to be the most abundant of the chlorinated aromatic pollutants in the ecosystem (1). It has been reported (2) that they are more stable than DDT and its metabolites, probably because the PCBs lack the C_2 residue linking the aromatic rings, which is the action site of most DDT biodegradation. The most relevant toxicological aspects resulting from exposure to the PCBs are chloroacne (3), liver injury (4), hydropericardial edema (5) and teratogenic effects (6). Therefore, the rapid analysis of PCBs is an important problem which has stimulated the development of many procedures employing TLC and GC. However, HPLC has only rarely been considered as a useful methodology to detect PCBs and ΣDDT (7-9). On the other hand, it is known that PCBs yield many individual peaks in GC analysis with widely different retention times. For this reason,

*DDE = 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene;DDT = 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane;DDD = 1,1-dichloro-2,2-bis(p-chlorophenyl)ethane;DDMU = 1-chloro-2,2-bis(p-chlorophenyl)ethylene and isomers o,p'- respectively;DBU = 1,5-diazabicyclo-[5.4.0]undec-5-ene.

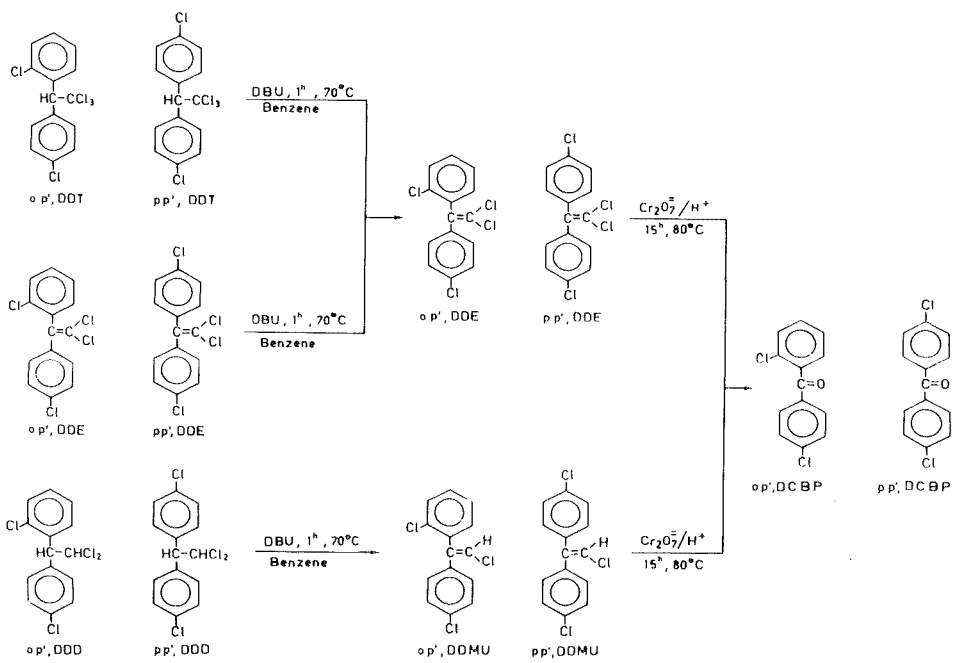
DDT and PCBs peaks overlap; this makes it necessary to remove PCBs PCBs from the samples prior to analysis. This problem is generally overcome by chromatography of cleaned-up extracts from chromatography on Florisil (10), silicic acid-Celite (11), activated coal (12), or silica gel (13) before analysis by GC. To simplify the analysis of a heterogeneous mixture of PCBs, it has been suggested that these should be converted to a single derivative. Usually, perchlorination is the preferred route (14).

The aim of this work was to investigate the use of HPLC to determine the contamination of environment by PCBs in the presence of DDT and its metabolites.

MATERIALS AND METHODS

A Perkin-Elmer Series 3B liquid chromatograph, equipped with an LC-75 Spectrophotometric Detector at 254 nm and an LC Autocontrol, was used. The stainless-steel column (Perkin-Elmer Silica-A) was 25 cm long and had an i.d. of 0.26 cm. Chromatograms were recorded and integrated with a Perkin-Elmer Sigma 10B Chromatography Data Station. The mobile phase consisted of n-hexane/$CHCl_3$ (95:5, 12:2 v/v) and n-hexane/Et_2O (9:1, 95:5 v/v). As a standard, PCBs sample Aroclor 1242,

purchased from Monsanto (St.Louis, Mo.,USA), was used. The chlorinated pesticides were all from Aldrich-Europe (Beerse,Belgium). The dehydrohalogenation and oxidation reactions were carried out according to Miles (15) but using different reaction times (Scheme 1). Standard curves were obtained by plotting peaks areas against the amount of compound injected. All separations were carried out at ambient temperature.



Scheme 1.

RESULTS AND DISCUSSION

A procedure to avoid the preliminary removal of PCBs from DDT and its analogues was set up to speed up the analysis. The chemical transformation of DDT and its metabolites in DCBP is performed by dehydrohalogenation, followed by oxidation. Analysis of the reaction products by HPLC leads to elimination of a chromatographic step; PCBs, recovered unaltered from the reactions, are in fact eluted as a single peak. Moreover, under the experimental conditions used, PCBs are completely separated from DCBP (Fig. 1).

The most important parameters characterizing the method have been calculated.

The minimum detectable amounts (Fig. 2) of PCBs, p,p'-DCBP and o,p'-DCBP were 2.57 ng, 0.62 ng and 1.30 ng respectively. The standard deviation, evaluated from eight repeated injections with four different volumes of standard mixture, was in the range of $\pm$ 2%.

PCBs and DCBP could be determined in a concentration range of 10-100 ng with no loss in linearity (Fig. 3).

In four different experiments the average recovery was

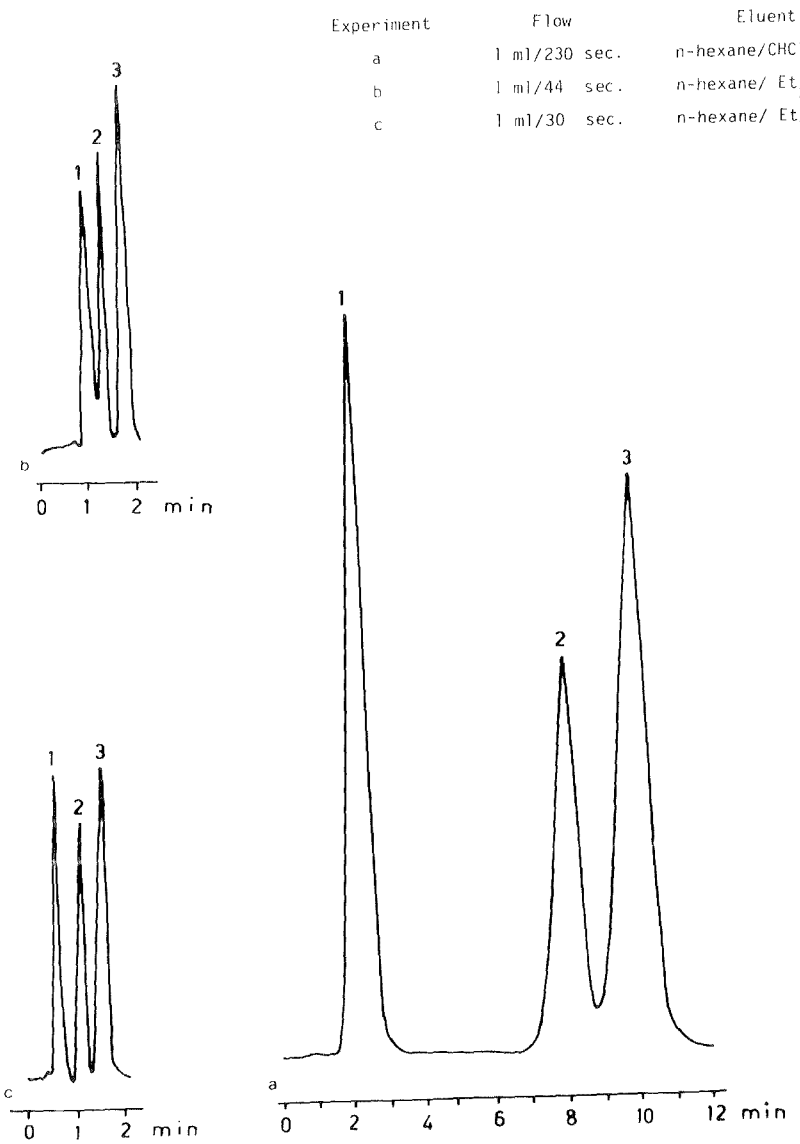


Figure 1. Chromatograms of standard mixtures of PCBs (1), p,p'-DCBP (2) and o,p'-DCBP (3).

Figure 2. Minimum detectable sample.

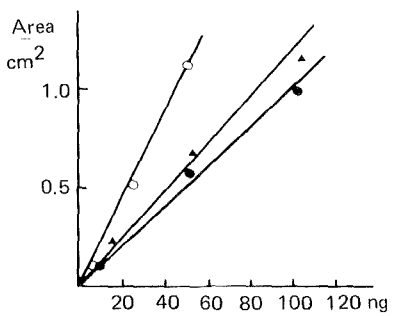


Figure 3. Calibration graphs for: ○ p,p'-DCBP, ● o,p'-DCBP, ▲ PCBs.

82.3 ± 1.2% for PCBs, 87.5 ± 1.8% for p,p'-DCBP and 88.2 ± 2.2% for o,p'-DCBP.

After standardization with pure samples, the method was applied to marine organisms, which are excellent indicators of the contamination level of the environment. Two species

of organisms were used: tuna (Thunnus thynnus thynnus from Catania bay) and snail (Murex brandaris from Castellabate bay); the specimens were immediately frozen and kept at -20°C until use.

The tuna samples were purified as already described (16), including the chromatographic step and then subjected to the dehydrohalogenation and oxidation reactions; snail extracts, obtained as already reported (17), were freed from fats by repartition with acetonitrile, and directly submitted to the dehydrohalogenation and oxidation reactions.

The results (Fig. 4) show that the chromatographic step can, indeed, be by-passed.

The nature of compounds responsible for the individual peaks was confirmed by co-injection of the above extracts with reference samples.

The concentrations of PCBs and ΣDDT are reported in Table 1. Reported values, calculated from standard curves obtained with external standard, are consistent with those determined by other procedures (16), thus demonstrating the excellent sensitivity and specificity of the method.

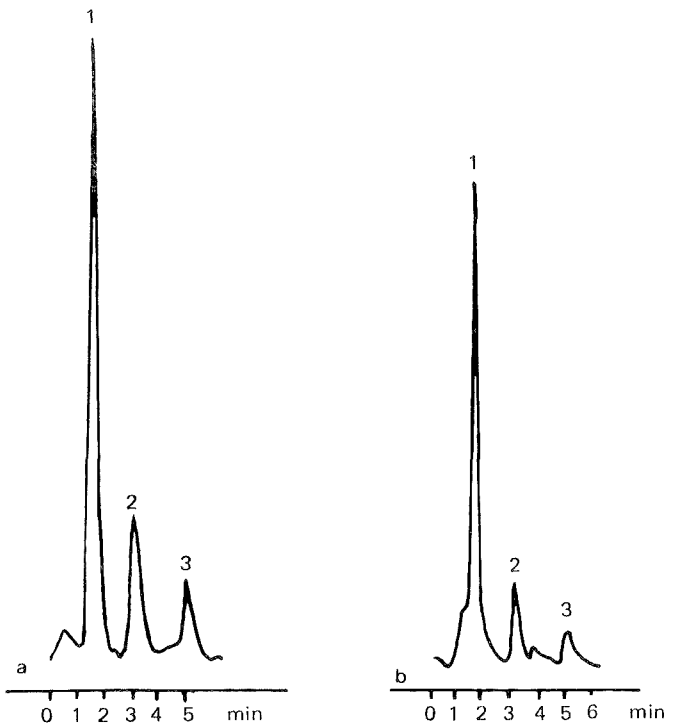


Figure 4. Separation of PCBs (1) from p,p'-DCBP (2) and o,p'-DCBP (3) in snail (a) and tuna (b).

TABLE 1

Concentrations of PCBs and ΣDDT in marine organisms expressed as ng g^{-1} of dry tissue.

Species	PCBs	ΣDDT
tuna	42.1	4.6
snail	63.6	5.2

The results show that HPLC can be an excellent procedure for the analysis of a mixture of PCBs and chlorinated pesticides. By this method, the need to separate PCBs from DDE is eliminated; this is the most significant interfering substance in the analysis. Moreover, PCBs eluted as a single peak can be easily determined. Furthermore, preparation of the sample is more rapid than in the case of GC, while the most important factors that influence the validity of the method are comparable with those of GC.

ACKNOWLEDGEMENTS

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REFERENCES

1) R.W.Risebrough,P.Reiche,D.B.Peakall,S.G.Herman and M.N.Kirven, Nature, 220, 1098(1968).

2) D.B.Peakall and J.L.Lincer, BioScience, 20, 958(1970).

3) L.Schawartz, J.Amer.Med.Ass., 122, 158(1943).

4) J.W.Miller, US Pub.Healt.Rep., 59, 1085(1944).

5) D.F.Flick,C.D.Douglass and L.Gallo, Poultry Sci., 42, 855(1963).

6) J.McLaughlin,Jr.,J.P.Morliac,M.J.Verrett,M.K.Mutchler and O.G.Fitzhugh, Toxical.Appl.Pharmacol., 5, 760(1963).

7) F.Eisenbeiss and H.Sieper, J.Chromatogr., 83, 439(1973).

8) U.A.TH.Brinkman,J.W.F.L.Seetz and H.G.M.Reymer, J.Chromatogr., 116, 353(1976).

9) S.H.Byrne,J.A.Schmit and P.E.Johnson, J.Chromatogr. Sci., 9, 592(1971).

10) L.M.Reynolds, Bull.Environ.Contam.Toxicol., 4, 128(1969).

11) J.A.Armour and J.A.Burke, J.Ass.Offic.Anal.Chem., 53, 761(1970).

12) O.W.Berg,P.L.Diosady and G.A.V.Ress, Bull.Environ. Contam.Toxicol., 7, 338(1972).

13) A.V.Holder and K.Mordesen, J.Chromatogr., 44, 481(1969).

14) J.A.Armour and J.A.Burke, J.A.O.A.C., 56, 987(1973).

15) J.R.W.Miles, J.A.O.A.C., 55, 1039(1972).

16) V.Amico,G.Oriente,M.Piattelli and C.Tringoli, Mar. Pollut.Bull., 10, 177(1979).

17) Pesticide Analytical Manual, Food and Drug Administration, vol. 1, section 211(1970).

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DETERMINATION OF THE MOLECULAR WEIGHT DISTRIBUTION OF POLYETHYLENE TEREPHTHALATE BY GEL PERMEATION CHROMATOGRAPHY *

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ABSTRACT

The determination of the molecular weight distribution of polyethylene terephthalate by GPC at room temperature is described. The solvent used is o-chlorophenol-chloroform (1:9) and pure chloroform is used as eluent. K and alpha of the Mark-Houwink equation of polyethylene terephthalate and of polystyrene in this solvent system were obtained. It was shown that the universal calibration can be applied.

INTRODUCTION

The determination of the molecular weight distribution of polyethylene terephthalate (PET) is a very important problem. m-Cresol was formerly used as the

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GPC solvent. Since m-cresol has a very high viscosity, the GPC experiment must be performed at a rather high temperature. This is not only inconvenient but it is also very difficult to avoid partial degradation of the sample during the experiment (1). Recently, Paschke (2) developed a solvent system for GPC determination at room temperature, in which degradation of PET was eliminated. The procedure was troublesome, since high temperature filtration had to be used for sample preparation. Jiang (3) reported the use of o-chlorophenol - chloroform as a solvent for GPC.

This paper describes the use of o-chlorophenol - chloroform as a solvent and chloroform as eluent at room temperature for the determination of molecular weight distribution of PET. It was shown that, in this system, the universal calibration method can be applied. The intrinsic viscosity and molecular weight relationship for PET and for PS were obtained, providing the values of K and alpha that must be used in the universal calibration.

MATERIALS AND METHODS

A standard sample of PET was prepared in our laboratory. Several PET samples were dissolved in phe-

nol - tetrachloroethane (1:1) and were fractionated by adding petroleum ether (90-120 deg. C.). Intrinsic viscosities of fractions thus obtained were determined in phenol - tetrachloroethane (1:1). The molecular weights were calculated according to the following equation (4):

$$[\eta] = 2.1 \times 10^{-2} M^{0.82}$$

The range of molecular weights of fractions was from 4000 to 30,000.

Standard samples of polystyrene were supplied mostly by Knauer, Inc., West Germany and by Waters Associates, Milford, Mass., USA.

Chloroform, A.R., was dried over anhydrous sodium sulfate or by passage through a silica gel column.

Two gel permeation chromatographic systems were used for GPC measurements. The first system was a Knauer device. Experimental parameters were as follows: Two columns in series (0.75 X 60 cm P-100) packed with Spheron gel of 20-40 microns; detector, UV, at 254 nm; flow rate 0.6 ml/min; temperature approx. 25 deg. C.; injection volume, 20 microliters (0.25%).

The second system was a Waters Model 244 GPC. Three columns of Microstyragel of 10,000, 10,000, and

1,000 Angstroms were selected. The total number of theoretical plates was 17,000. Detector, UV, at 254 nm; flow rate 1.0 ml/min; temperature approx. 25 deg. C.; injection volume, 20-40 microliters (0.1-0.2%).

The sample solutions were prepared as follows: PET samples were weighed into 10 ml volumetric flasks and 1 ml o-chlorophenol was added to each. The flask was warmed to 80 deg. C. until they were dissolved. They were then diluted with chloroform to 10 ml.

RESULTS AND DISCUSSION

Selection of Eluent and Solvent

From the thermodynamic point of view, a polymer will dissolve in a solvent only if the free energy of mixing is negative. Since

$$\Delta G_M = \Delta H_M - T\Delta S_M, \text{ and } \Delta H_M$$

usually adopts a positive value, a dissolution process will be favored when ΔH_M is very small or approaches zero. The value of ΔH_M can be related to the difference between the solubility parameters of the polymer and solvent. When the solubility parameter of a solvent is similar to that of the polymer, dissolution

will be favored. The solubility parameter of PET was reported in the literature as approximately 9.7 to 10.7. We expect that chloroform, with a solubility parameter of 9.3, should be a potential solvent. Due to the high crystallinity of PET, it was found to be insoluble in pure chloroform. The sample was, therefore, first dissolved in a small amount of o-chlorophenol. Following complete solution, chloroform was added and a rather stable clear solution was obtained which would remain clear long enough for GPC measurements.

To select a suitable proportion for the mixed solvent, PET and PS sample solutions in a variety of different proportions of mixed solvent were prepared and their retention volumes were measured under identical conditions. The data obtained are given in Table 1 and

TABLE 1

The Influence of Different Proportions of Solvent for PET and PS on Their Retention Volumes

PET ($\bar{M}_\eta = 2.99\times10^4$)			PS ($\bar{M}_n = 3.5\times10^5$)		
o-Chlorophenol:Chloroform		V_e,ml	o-Chlorophenol:Chloroform		V_e,ml
1	4	18.2	1	4	15.2
1	9	18.2	1	9	15.2
1	19	18.3	1	13	15.1
			0	100	15.2

TABLE 2

The Retention Volumes of PS of Different Molecular Weight in Mixed Solvent and Chloroform

$\bar{M}_n$	o-Chlorophenol:Chloroform(1:9) V_e,ml	Chloroform V_e,ml
3.5×10^5	15.2	15.1
2.18×10^5	16.0	16.0
1.11×10^5	17.7	17.7
3.6×10^4	19.8	19.8
9.05×10^3	23.8	23.9
3.57×10^3	25.1	25.0
1.79×10^3	25.9	25.9
8.11×10^2	27.6	27.4
6.0×10^2	28.1	28.2

Table 2, which show that a proportion of one volume of o-chlorophenol to nine volumes of chloroform is good, since around this proportion, any variation of the ratio of the components changes the retention volumes of PET and PS within 1%. This will ensure the reliability of the experimental result. The presence of the o-chlorophenol in the mixed solvent does not interfere with the GPC chromatogram of the PET sample, because

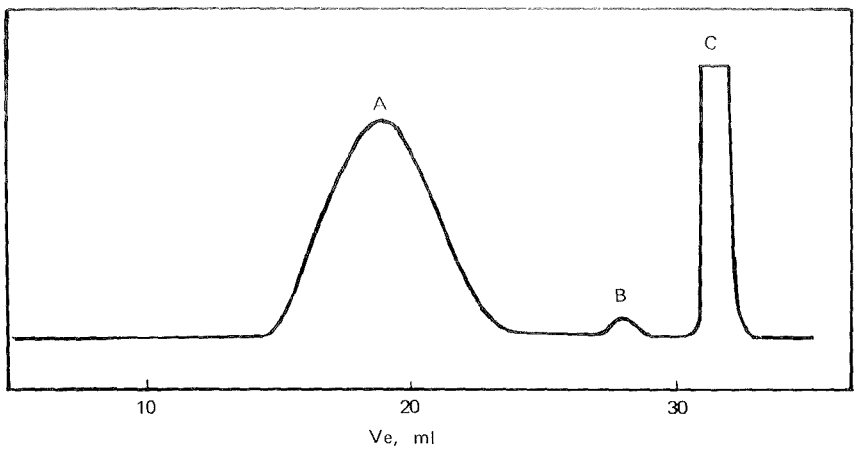

Figure 1. A typical chromatogram of PET in mixed solvent. (A - PET Polymer: B - PET oligomer; C - o-Chlorophenol).

the peak for o-chlorophenol is far behind that of the PET sample. A typical chromatogram is shown in Figure 1.

GPC Calibration Curves

The elution volumes of two sets of PET standards and PS standards, measured in two GPC systems, under conditions described above, are listed in Table 3 and Table 4.

The corresponding calibration curves for the two column systems are illustrated in Figure 2 and Figure 3.

TABLE 3

Data for Calibration Curve of Spheron Column

Standard Sample of PS	$\bar{M}_w$	V_e, ml	Standard Sample of PET	$\bar{M}_\eta$	V_e, ml
1	2.0×10^6	16.0	1	3.43×10^4	26.0
2	4.98×10^5	16.0	2	2.78×10^4	28.0
3	1.10×10^5	23.0	3	2.46×10^4	29.0
4	5.0×10^4	28.0	4	2.14×10^4	30.0
5	3.7×10^4	30.0	5	1.61×10^4	32.5
6	1.75×10^4	34.5	6	1.13×10^4	35.0
7	4.0×10^3	44.0	7	8.85×10^3	37.0
8	2.2×10^3	45.0	8	6.70×10^3	38.5
			9	5.93×10^3	40.5

Universal Calibration

Standard samples of PET are not available commercially. The preparation of PET standard samples by fractionation is troublesome and time-consuming. If the universal calibration proposed by Benoit is applicable in this new solvent system, we can then use PS samples, which are commercially available, to calibrate our column. In the universal calibration treatment, Mark-Houwink equations for both polymers in the same

TABLE 4

Data for Calibration Curve of Styragel Column

Standard Sample of PS	$\bar{M}_n$*	V_e,ml	Standard Sample of PET	$\bar{M}_\eta$	V_e,ml
1	1.50×10^6	13.3	1	2.99×10^4	18.2
2	3.50×10^5	15.2	2	2.11×10^4	19.6
3	2.18×10^5	16.0	3	1.75×10^4	20.0
4	1.11×10^5	17.7	4	7.67×10^3	21.5
5	3.60×10^4	19.8	5	4.57×10^3	23.6
6	9.05×10^3	23.8	6	4.22×10^3	23.8
7	3.57×10^3	25.1			
8	1.79×10^3	25.9			
9	8.11×10^2	27.6			
10	6.00×10^2	28.1			

* Peak Molecular Weight by GPC.

solvent are required. Therefore, intrinsic viscosities of some standard samples of PET and of PS were measured in the mixed solvent, o-chlorophenol - chloroform (1:9) at 25 deg. C. The log[η] vs log M plots are shown in Figure 4. The corresponding Mark-Houwink equations were calculated by a least square method:

$$[\eta]_{PET} = 5.84 \times 10^{-5} M_{PET}^{0.91}$$

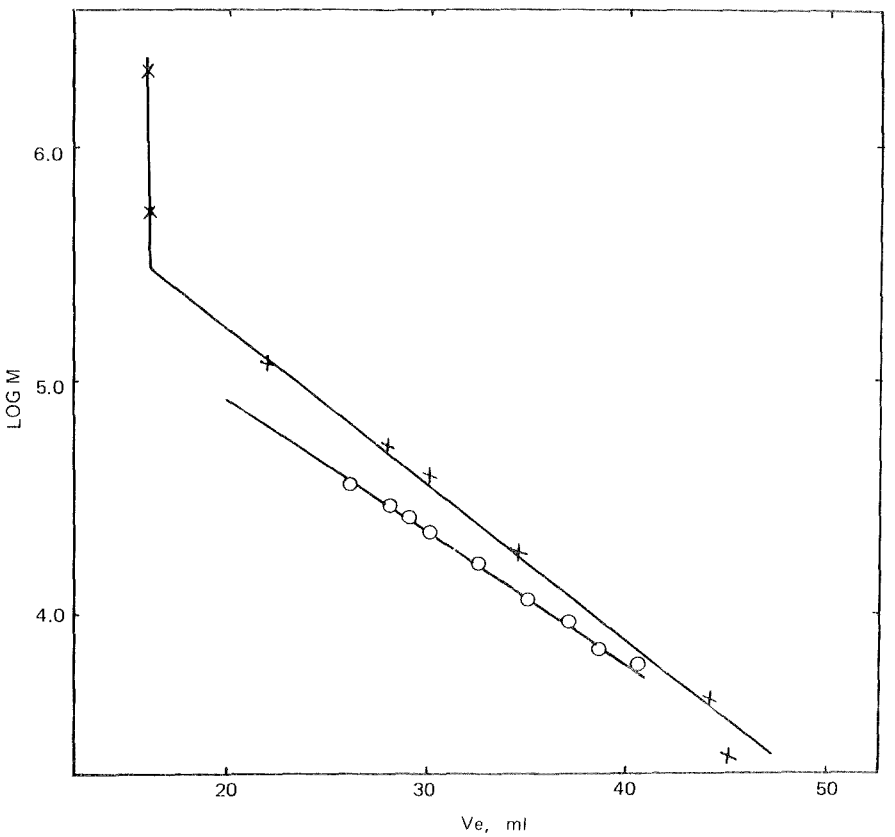

Figure 2. GPC calibration curves for Spheron column. (o PET x PS)

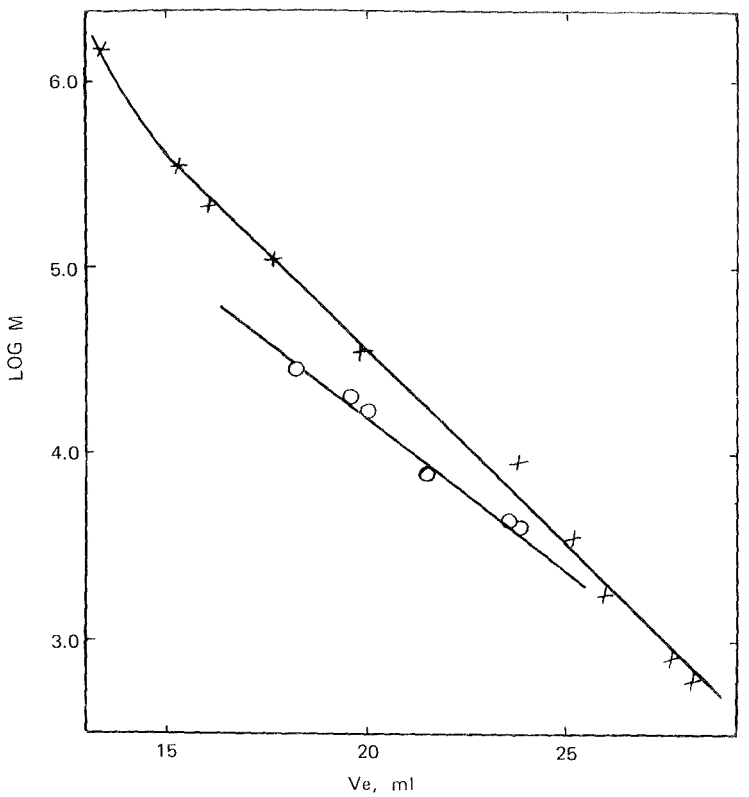

Figure 3. GPC curves for Styragel column. (o PET x PS)

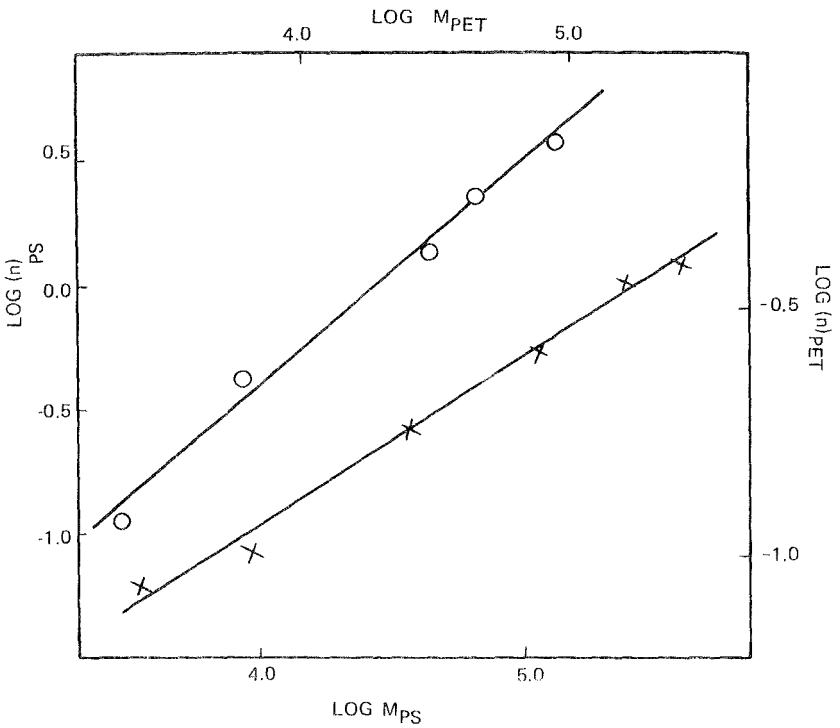

Figure 4. The log [η] vs log M plots for PET and PS in a mixed solvent of o-chlorophenol and chloroform (1:9).
(o PET x PS)

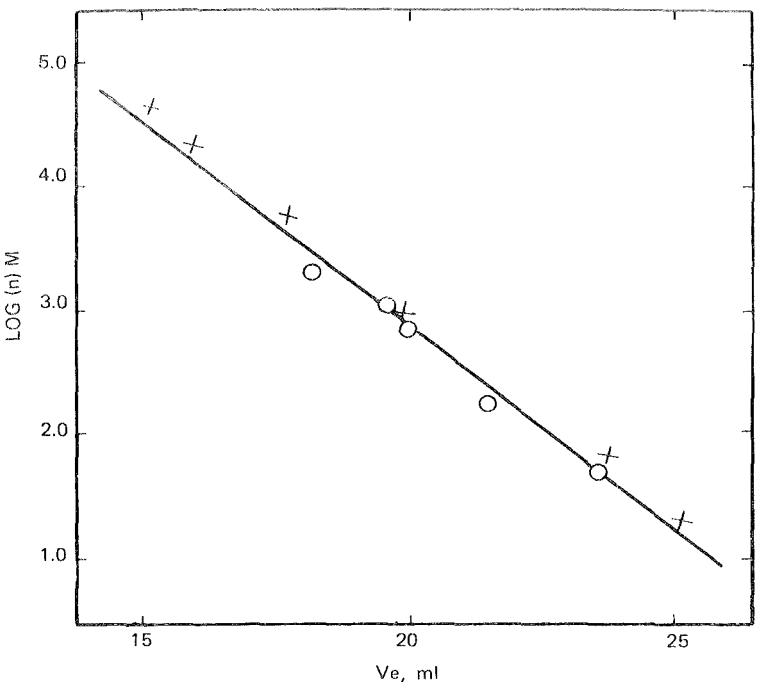

Figure 5. Log [η] vs Ve plot for PET and PS in a mixed solvent of o-chlorophenol and chloroform (1:9). (o PET x PS).

$$[\eta]_{PS} = 2.0 \times 10^{-4} M_{PS}^{0.68}$$

The log [η] vs Ve plots of PET and PS were obtained from the data given above. All the points fall on the same straight line as shown in Figure 5. This shows that universal calibration is applicable in this system.

REFERENCES

1. Shaw, G., Gel Permeation Chromatography Seminar, 1969, pp. 12-15.

2. Paschke, E. E., Bidlingmeyer, B. A. and Bergman, J. G., A New Solvent System For GPC of Poly-(Ethylene Terephthalate), Polymer Preprints, ACS, 17 (No. 2), 440 (1976).

3. Jiang Er-Fang, Determination of the Molecular Weight Distribution of Poly(Ethylene Terephthalate) by GPC, Gaofenzi Tongxun, 51, (1980).

4. Conix, A., On the Molecular Weight Determination of Poly(Ethylene Terephthalate), Makromol. Chem., 26, 226 (1958).

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ENZYMATIC DETERMINATION OF TRIGLYCERIDES IN CONJUNCTION WITH HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The glycerol kinase (GK) catalyzed reaction involving the conversion of glycerol and adenosine triphosphate (ATP) to glycerol-3-phosphate and adenosine diphosphate (ADP) has been used in conjunction with HPLC for the determination of triglycerides. After alkaline hydrolysis of the triglycerides to glycerol, the enzyme reaction was carried out. The ADP formed and the remaining ATP were then separated by HPLC and the ADP peak area correlated to the concentration of triglycerides originally present in the sample. Linearity of the method was established from 28-180 mg/dl with a reproducibility of 6.5% RSD. A comparison between the HPLC method and the standard coupled enzyme system for triglycerides in real serum indicated a correlation coefficient of 0.977.

INTRODUCTION

Triglycerides (esters of glycerol and fatty acids) make up a general type of lipid found in plasma. They combine with proteins in the form of water soluble complexes termed lipoproteins. Abnormalities in lipid metabolism are characterized by an excess of one or more of the lipoproteins. These can be detected by plasma cholesterol and triglyceride levels. Elevated triglyceride levels are likely to be found in diabetes mellitus, liver disease and atherosclerosis (1).

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Several methods for the determination of glycerol or glycerol derived from serum triglycerides have been reported. A two enzyme system involving glycerokinase (GK) and glycerophosphate dehydrogenase (GPD) has been employed for the determination of glycerol, however the equilibrium constant for the GPD-catalyzed reaction was unfavorable requiring the use of the trapping agent hydrazine (2). Another coupled enzyme system for glycerol, involving GPD and diaphorase, indicated good selectivity but the procedure was somewhat lengthy (3). Hercules and Sheehan (4) coupled the chemiluminescent luciferase reaction to the GK enzyme reaction and related the production of light due to the residual ATP to the initial glycerol present in the sample.

Probably the most common method for the assay of serum triglycerides is that basically reported by Bucolo and David (5). It is based on four coupled enzyme reactions.

$$\text{Triglycerides} \xrightarrow{\text{lipase}} \text{Glycerol} + \text{Fatty Acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol-P} + \text{ADP}$$

$$\text{ADP} + \text{PEP} \xrightarrow{\text{PK}} \text{Pyruvate} + \text{ATP}$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^{+} \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}.$$

where ATP is adenosine triphosphate, ADP is adenosine diphosphate, PK is pyruvate kinase, PEP is phosphoenolpyruvate, LDH is lactate dehydrogenase, and NADH and NAD^{+} are the reduced and oxidized forms, respectively, of nicotinamide adenine dinucleotide. The amount of NADH oxidized is represented by a decrease in absorbance at 340 nm.

Previously, we have used high performance liquid chromatography (HPLC) to facilitate the assay of kinase enzymes such as creatine kinase (6). To avoid the necessity of a coupled enzyme system for the assay of glycerol, we have used HPLC to separate and quantitate the ADP produced in the GK reaction. The amount of ADP can be related to the glycerol formed after alkaline hydrolysis of the triglycerides present in the sample. Besides avoiding the instability problems of NADH, the expense of enzymes and substrates required in the coupled systems is eliminated.

MATERIALS

Apparatus

The liquid chromatograph consisted of an Altex Model 110-A high pressure pump (Altex, Berkely, CA), a Rheodyne Model 7125 injector (Rheodyne, Berkely, CA) and an Altex Model 153 UV detector (254 nm). A 5 cm x 4.1 mm I.D. precolumn packed with octadecyl (C-18) derivatized pellicular silica and a 25 cm x 4.1 mm I.D. R-Sil C-18 working column (Alltech Associates, Deerfield, Illinois) were used for the separations. Peaks were recorded by an Omniscribe Model B-5000 recorder (Houston Instruments, Austin, TX.) Peak areas were calculated using a HP 3000 minicomputer equipped with a HP-7221A plotter (Hewlett-Packard, Palo Alto, CA).

Chemicals

All water used was distilled and deionized. Tetrabutylammonium hydrogen sulfate was supplied by either Aldrich (Milwaukee, WI) or Sigma (St. Louis, MO). Trizma buffer [tris(hydroxymethyl)aminomethane], ATP (sodium salt), GK (lyophilized from E. coli), control serum (type 1A normal), mercapto-ethanol, and triolein were all obtained from Sigma. All substrates and enyzmes were stored refrigerated at 0-5^{o}C or frozen in a desiccator as recommended. Real serum samples were obtained from Mercy Hospital, Hamilton, OH.

METHODS

The triolein standard solutions, ranging from about 25-280 mg/dl, were prepared by dissolving the appropriate amount in isopropanol and then diluting to 25 ml with absolute ethanol. The glycerokinase was reconstituted to a concentration of 8 units/ml with 0.02 M, pH 7.0, sodium phosphate buffer containing 0.001 M 2-mercaptoethanol. Stock ATP and magnesium acetate solutions of 0.033 M and 2.5 mM, respectively, were prepared using 0.1M Tris buffer, pH = 7.0 . An aliquot no greater than 3 ml of the triglyceride

standard was added to 0.5 ml of ethanolic KOH and diluted to 3.5 ml with ethanol. Saponification of this solution was allowed to occur for 30 minutes at 70°C. The sample tubes were covered with Parafilm to decrease evaporation. After cooling, 1 ml of 0.15 M $MgSO_4$ was added to precipitate the free fatty acids and the sample tubes were then centrifuged. Volumes of 0.5 ml were taken of either the control serum or the real serum samples for saponification and treated as described previously for the triolein standards.

The assay mixture consisted of 3.0 ml buffer/Mg^{+2} solution, 0.1 ml ATP solution, and 0.5 ml hydrolyzate supernatant. For the serum samples the hydrolyzate volume was either 0.5 ml or 1.0 ml. To initiate the reaction, 0.3 ml of the GK solution was pipetted into the test tube and the mixture was swirled. The test tubes were immediately placed in a 25°C bath for 10 minutes.

The ADP formed in the assay was separated from the excess ATP by the use of HPLC. The mobile phase consisted of 88% 0.1 M KH_2PO_4, 0.025 M tetrabutylammonium hydrogen sulfate and 12% methanol, adjusted to a pH 7.1 with NaOH and was pumped at a flow rate of 1.5 ml/min. The sample injection volume was 20 µl. The detector was generally set at 0.04 absorbance units full scale (a.u.f.s.), however occasionally 0.08 a.u.f.s. was required. The temperature was ambient and the chart speed was 0.25 cm/min.

The Sigma Triglyceride Diagnostic Kit (No.335-UV) based on the 4 enzyme system was employed as a reference triglyceride technique and was used as written. The Sigma assays were carried out using a Hewlett-Packard 8450A UV-VIS spectrophotometer.

RESULTS

The glycerokinase used for the assay contained several minor enzyme impurities, two of which were hexokinase and myokinase. The enzyme hexokinase can catalyze the reaction shown below.

$$\text{Glucose} + \text{ATP} \longrightarrow \text{Glucose-6-P} + \text{ADP}$$

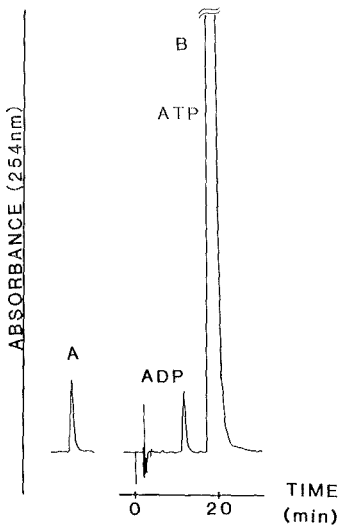

FIGURE 1. HPLC check for possible interferences by hexokinase. (A)-Blank assay containing buffer/Mg^{+2} solution, ATP and GK as described in METHODS section. (B)-Blank assay as in A with glucose (200 mg/dl). Both enzyme reactions were incubated for 10 minutes and then quenched by immersing the test tube in boiling water for 2 minutes. This quenching procedure was determined not to be necessary and in fact increased the blank ADP peak due to ATP decomposition.

In this case the amount of ADP formed would be larger than the true value. Figure 1 shows the comparison of a blank sample with and without the presence of 200 mg/dl of glucose. This level of glucose is higher than that typically formed in serum. It can be concluded that the presence of hexokinase would not be expected to interfere with the glycerokinase catalyzed reaction. Myokinase can catalyze the following reaction.

$$2\ ADP \longrightarrow AMP + ATP$$

Since ADP was the product monitored in the assay, the presence of myokinase could contribute a negative error. Since it has been shown that the presence of AMP will inhibit myokinase (7), assays were performed comparing the amount of ADP formed with and without the presence of AMP in the assay mixture. As

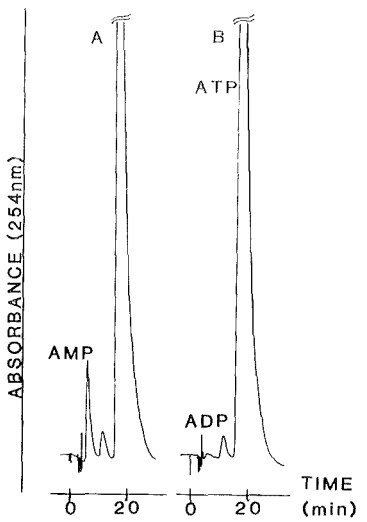

FIGURE 2. HPLC check for possible interference by myokinase. (A)-Blank assay as in Figure 1A. (B)-Blank assay as in A with AMP (2 mM). Both enzyme reactions were incubated 30 minutes and no quenching was carried out.

shown in Figure 2, any possible myokinase impurity did not appear to interfere with the assay of glycerol.

A typical separation of ADP and ATP obtained for a 28 mg/dl range of triolein is given in Figure 3. Other constituents present either in the standards or the serum samples were either nonabsorbing or retained on the precolumn and did not interfere with the separation. The time for complete separation of ADP and ATP was about 20 minutes. Assuming the detection limit is twice the response of the blank ADP peak, a value of about 10 mg/dl can be calculated. A plot of triolein concentration versus the ADP peak area given in Figure 4 demonstrates the linearity of the assay. A slope of 0.27 cm^2/10 mg/dl with an intercept of -0.64 and a correlation coefficient of 0.9944 were calculated. The relative average standard deviation for 3 determinations was found to be 6.5%.

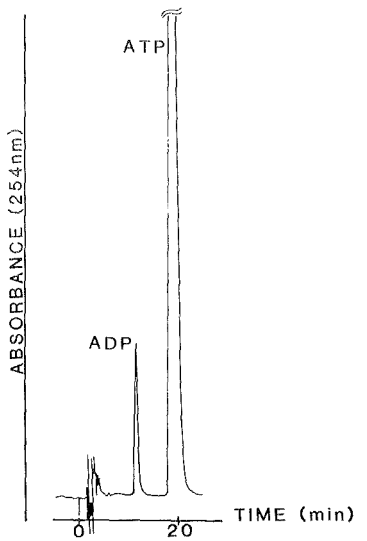

FIGURE 3. Chromatogram obtained for a sample originally 28 mg/dl in triolein.

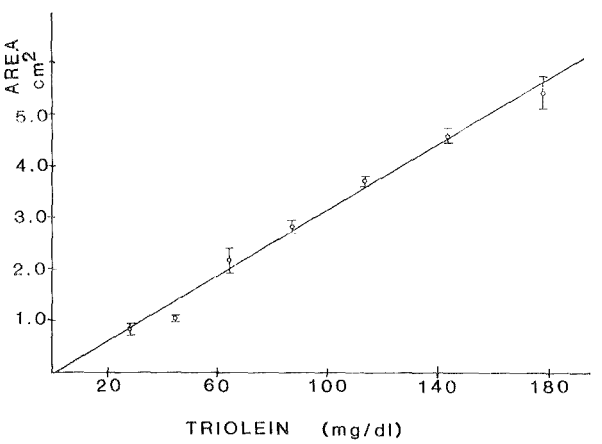

FIGURE 4. ADP peak area as a function of mg/dl of triolein. Error bars represent the standard deviation of 3 determinations.

TABLE 1. RECOVERY OF TRIGLYCERIDES ADDED TO SERUM

Triglycerides Added mg/dl	Total Triglycerides Found	Recovery %
0	78 ± 10	---
94	153 ± 2.0	88
136	230 ± 9.8	107
176	256 ± 0.1	100
214	308 ± 0.5	105
250	376 ± 7.5	114
	Avg.	103%

TABLE 2. COMPARISON OF THE HPLC METHOD WITH THE SIGMA ENZYMATIC METHOD

Sigma*	Range*	n	HPLC*	Range*	n
71.6	2.6	2	79.7	10.8	2
121.0	2.6	2	136.6	20.7	3
213.9	4.3	2	249.5	1.2	2
85.9	---	1	111.7	0.4	2
151.9	19.1	2	158.6	25.7	3
69.0	13.0	2	78.9	0.7	2
79.9	---	1	73.9	16.8	3
60.8	---	1	99.0	4.4	2
151.0	---	1	164.7	1.1	2

*All values expressed in mg/dl of triglycerides.

A study of the percent recovery of triglycerides added to control serum is summarized in Table 1. The % recovery ranged from 88 to 114% with an average value of 103%. The average standard deviation in the total triglycerides found was about $\pm$ 5 mg/dl.

Results of the comparison of the HPLC method with the Sigma Reference assay for triglycerides is given in Table 2. The average range for both methods was about 8 mg/dl. A plot of the HPLC values versus the Sigma results gave a correlation coefficient of 0.9770.

DISCUSSION

The linearity of the system essentially covered the normal range for triglycerides in serum. This range is 40-160 mg/dl for males and 35-135 mg/dl for females (8). However, samples up to 380 mg/dl could be determined with good accuracy (Table 1) if diluted first or by reducing the sensitivity of the HPLC detector.

The triglyceride determinations using the HPLC method tended to be slightly higher than those found by the reference method. This may be due to a volume displacement error that occurs when proteins are precipitated because the serum volume containing the triglycerides decreases by the volume of proteins precipitated (8). Since larger sample aliquots were used in the HPLC method than the reference method, a consistent discrepency could result. Smaller sample aliquots could probably be used particularily if the injection volume and/or the detector sensitivity were increased. Total analysis time for the HPLC method was about 25 minutes discounting the time required for saponification. The sample throughput could probably be increased substantially by using a computer-controlled HPLC that could switch a valve between the injector and column to divert the ATP band to waste instead of the column before subsequent automatic injection of the next sample.

Application of the assay for other samples such as antifungal creams containing the triglyceride triacetin should be straightforward. It is also

expected this general method would be applicable to the determination of other important clinical substrates that are involved in kinase enyzme systems such as the glucose-hexokinase catalyzed reaction.

ACKNOWLEDGMENT

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REFERENCES

1. White, A., Handler, P., Smith, E. L., Hill, R. L. and Lehman, I. R., "Principles of Biochemistry", 6th ed., McGraw Hill, New York,NY, 1978, p. 568.
2. Spinella, C. J. and Mager, M., Modified Enzymatic Procedure for the Routine Determination of Glycerol and Triglycerides in Plasma, J. Lipid Res., 7, 167, 1966.
3. Frings, C. S. and Pardue, H. L., An Automatic Spectrophotometric Method for the Enzymatic Determination of Glycerol, Anal. Chim. Acta, 34, 225, 1966.
4. Hercules, D. M. and Sheehan, T. L., Chemiluminescent Determination of Serum Glycerol and Triglycerides, Anal. Chem., 50, 22, 1978.
5. Bucolo, G. and David, H., Quantitative Determination of Serum Triglycerides by the Use of Enzymes, Clin Chem., 19, 476, 1973.
6. Danielson, N. D. and Huth, J. A., Direct Assay for Creatine Kinase by Reversed-Phase High Performance Liquid Chromatography, J. Chrom, 221, 39, 1980.

7. Walter, B., AMP Increases the Lag Period for the Oliver-Rosalki Assay for Creatine Phosphokinase, Clin. Chem., 24, 1034, 1978.

8. Tietz, N. W., "Fundamentals of Clinical Chemistry", W. B. Saunders Co., Philadelphia, PA, 1976, p. 500.

9. Ko, H. and Royer, M. E., Automated Determination of Glycerol in Plasma, Anal. Biochem., 26, 18, 1968.

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RAPID HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PHYSOSTIGMINE IN PLASMA

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ABSTRACT

A new method is described for the quantitative determination of physostigmine in human plasma. The drug is isolated from human plasma utilizing a C_{18} SEP PAK Cartridge, and quantified by liquid chromatography with ultraviolet detection. The average recovery is 54.3 $\pm$ 4.3% (S.D.) with a day to day coefficient of variation of 4%.

INTRODUCTION

Physostigmine is a potent acetylcholinesterase inhibitor (1) that has been used clinically for the treatment of glaucoma (2), myasthenia gravis (3), and as an antidote against certain hallucogenic agents (4). Recent work has suggested a role for the drug in senile dementia of the Alzheime's type (5). Existing methods including colorimetry (6), fluorometry (7), gas-liquid chromatography (8), high-performance liquid chromatography (HPLC, 9) and thin-layer chromatography (10) can only determine μg quantities of physostigmine, and are not applicable to plasma samples. Although an enzymatic method (11) has the adequate sensitivity for the determination of physostigmine in human whole blood, it requires a tedious sample preparation. Recently an HPLC method (12) with uv detection has been described for the assay of physostigmine in brain tissue.

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A new method for the determination of physostigmine in human plasma using C_{18} SEP PAK clean-up procedure and HPLC is described here. The method is simple, rapid, sensitive, and suitable for routine analyses.

MATERIALS AND METHOD

Chemicals

Physostigmine standard and sodium phosphate (monobasic) were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium dodecyl sulfate (SDS) was purchased from Regis Chemical Co. (Morton Grove, IL). C_{18} SEP PAK cartridges were obtained from Waters Assoc. Inc. (Milford, MA). Methanol was purchased from Burdick and Jackson Labs. (Muskegon, MI). Water was deionized and then double-distilled in glass.

Apparatus

The liquid chromatograph was constructed from four components = M 45 solvent delivery system (Wates Assoc., Inc.); LC-6 UV detector (Bioanalytical Systems, West Lafayette, IN); Model 7125 injection Valve (Rheodyne Inc., Cotati, CA); and Model LS - 44 recorder (Linseis Inc., Princeton Jct. N.J.) A μ-Bondapak C_{18} reverse-phase column (Waters Assoc., Inc.) was used with an in-line guard column of 5 μm RP-18 (Brownlee Labs., Santa Clara, CA). The mobile phase was prepared by mixing 500 ml of 0.005 M NaH_2PO_4 (pH = 5.7) and 500 ml of MeOH. The flow-rate was fixed at 1.5 ml/min at ambient temperature and the wave length set at 254 nm. At the end of each day, the system was flushed with 200 ml of MeOH - H_2O (3:1 ; V/V).

C_{18} SEP PAK procedure

The cartridge was activated by passing 5 ml of H_2O and then 5 ml of MeOH by pressurizing through a glass syringe followed by 10 ml of H_2O.

Sodium dodecylsulfate (SDS, 0.6% W/V) 1 ml in an aqueous solution was added to the aqueous solution or 1 ml of human plasma

spiked with known amounts of physostigmine prepared in the mobile phase. The mixture was thoroughly mixed on a vortex and passed through the cartridge via a syringe at a flow-rate not greater than 2 ml/min. The cartridge was consecutively washed with 15 ml of H_2O, 4 ml of the mobile phase, and 0.5 ml of MeOH. An additional 0.5 ml of MeOH was passed through the cartridge, and the elute collected in a 10 ml glass disposable tube. The methanol elute was evaporated to dryness under a stream of dry N_2 at 30°C. The dried residue was dissolved in 100 µl of the mobile phase. The cartridge can be regenerated by flushing with 10 ml of MeOH and 15 ml of H_2O.

RESULTS AND DISCUSSION

Figure 1 shows representative chromatograms of human plasma spiked with O ng (1A) and 200 ng (1B) of physostigmine.

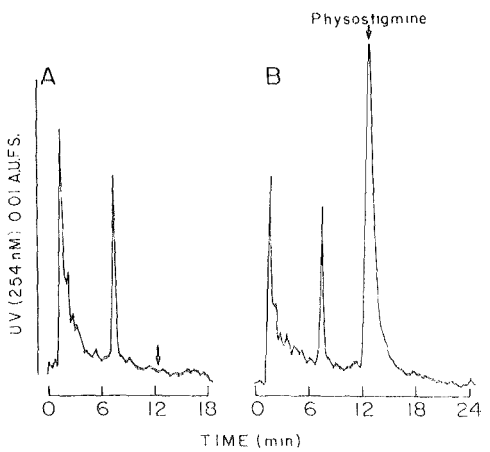

FIGURE 1

Chromatograms of human plasma samples: (A) Physostigmine -free (blank) plasma; (B) plasma containing 200 ng of physostigmine. Conditions were as given in the Materials and Method section.

Identification of the physostigmine peaks observed in the plasma samples was based on its retention time and cochromatography with the authentic compound. The effects of column temperature and pH on the chromatographic properties of the physostigmine peak were investigated. Higher temperatures (>40°C) or lower pH values (< 4.0) significantly reduced the retention time and improved the shape of the physostigmine peak. However, we elected to use ambient temperature and pH 5.7 to maximize stability (7) and minimize interferences from plasma constituents.

The clean-up procedure using the C_{18} SEP PAK cartridge was found to yield more reproducible results and cleaner chromatograms than the conventional extraction procedure using chloroform, ether, ethyl acetate, and a mixture of cyclohexane - pentanol (4 : 1; V/V) (4). SDS must be added to aqueous solutions or plasma samples spiked with physostigmine otherwise low recoveries (3 ~ 5%) will be obtained.

Calibration curves for physostigmine in human plasma were linear over the concentration range 100 to 10,000 ng/ml. The average recovery of physostigmine added to plasma when concentrations between 100 ng and 400 ng/ml were studies was 54.3 $\pm$ 4.3% (mean $\pm$ S.D., n = 20). The average recovery could be increased to 75% when the first (0.5 ml) and additional (1 ml) methanol fractions collected and combined. However, under these circumstances more interfering peaks were observed. The within-run and day-to-day precision data (CV) were 2.4% (n = 5) and 4.0% (n = 5), respectively for plasma samples spiked with 200 ng of physostigmine. The detection limit of physostigmine was 50 ng/ml of plasma.

Plasma samples from patients, with either on an unrestricted diet or L-Dopa apo-Morphine, Haloperidol and Probenecid were examined. All of the chromatograms were free from overlapping peaks.

It is also worth noting that the C_{18}SEP PAK clean-up procedure has been successfully utilized in our laboratory for the sample preparation of acetylcholine in rat brain tissue, polypeptides and tetrahydroaminoacridine in human plasma.

In conclusion, this newly developed HPLC method is sensitive, simple and can be adapted directly for the determination of physostigmine levels in CSF, serum and urine.

ACKNOWLEDGEMENT

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REFERENCES

1. Coxworth, E., The Alkaloids, VIII, Manske, R.H.F., ed., Academic Press, New York, 1965, p. 43.
2. Rodin, F.H., Am. J. Opthalmol., 30, 19, 1947.
3. Walker, M.D., Lancet, 1, 1200, 1934.
4. Christensen, I., Acta. Pharm. Suec., 6, 287, 1969.
5. Davis, K.L., Mohs, R.C., Tinklenberg, J., NEJM., Enhancement of Memory Physostigmine, 301, 346, 1979.
6. Khalil, S.K.W. and Patterson, B.D., J. Pharm. Sci., 60, 1387, 1971.
7. Taylor, K.M., J. Pharm. Pharmac., 19, 770, 1967.
8. Teare, F.W. and Borst, S.I., J. Pharm. Pharmacol., 21, 277, 1969.
9. Kneczke, M., J. Chromatogr., 198, 529, 1980.
10. Rogers, A.R. and Smith, G., J. Chromatogr., 87, 125, 1973.
11. Groff, W.A., Ellin, R.I., and Skalsky, R.L., J. Pharm. Sci. 66, 389, 1977.
12. DeWildt, D.J., Porsius, A.J., and Van Roog, H.H., J. Chromatogr., Biomed. Appl., 225, 381, 1981.

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LIQUID CHROMATOGRAPHIC DETERMINATION OF VALPROIC ACID IN HUMAN SERUM

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ABSTRACT

The concentration of the antiepilepsy drug valproic acid (2-propylpentanoic acid) was determined in both a processed freeze dried human serum material and patient serum samples obtained from a clinical laboratory. The freeze dried material is being issued by the National Bureau of Standards as Standard Reference Material 1599. The analytical procedure developed involves organic extraction of valproic acid and an internal standard (cyclohexane-carboxylic acid) from the serum matrix; derivatization of the carboxylic acids to phenacyl esters; measurement of the analyte and internal standard species by reversed-phase high performance liquid chromatography. The results obtained on both types of samples compare favorably with results obtained using more conventional gas chromatographic approaches.

INTRODUCTION

In the late 1970's valproic acid (2-propylpentanoic acid) became an important drug in the treatment of epilepsy (1,2), and consequently, the need arose for analytical procedures capable of providing accurate and precise measurements of the drug in a serum matrix. The work of Durst and Grushka (3,4) in 1975 showed that phenacyl esters of carboxylic acids can be easily prepared through the use of a crown ether catalyst. The phenacyl ester

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derivative can then be chromatographed by reversed-phase high performance liquid chromatography (HPLC).

Schmidt and Slavin (5) first applied the techniques of phenacylation and HPLC to the analysis of valproic acid in 1978. They only worked with organic solutions of pure compounds, however, and did not address the problems associated with the analysis of the drug in a biological matrix. The potential of applying the method of phenacylation to the analysis of valproic acid in serum has, for the most part, been over-looked. Although many procedures for the analysis of valproic acid have been published since 1975, the predominant scheme involves a liquid-liquid extraction of the free acid and an internal standard, and analysis by gas chromatography (GC) (6-11).

One advantage of GC procedures is that they permit the direct analysis of valproic acid whereas HPLC procedures require a derivatization step such as phenacylation to enhance the UV absorbance. A disadvantage of working with the free acid is that extreme care must be taken to avoid losses due to volatilization when concentrating the sample extract (6,7). Several investigators have avoided the need to concentrate the extract by utilizing a back extraction technique to remove interferences (8,9), while others have used micro scale extraction followed by the direct injection of an aliquot of the extract (10). Fellenberg and Polland (11) addressed this problem by extracting with a nonpolar solvent (n-heptane) which yielded a cleaner extract. The detection limit for each of these procedures is thus a function of its ability to produce a clean sample extract. An additional problem associated with GC procedures is that the free acid does not chromatograph well on most conventional GC packing materials (7). Severe peak tailing is often observed unless special packings capable of handling polar compounds are used. Finally, the heavily loaded packings commonly used for polar compounds often produce excessive column bleed and deteriorate quickly (12).

Our interest in the HPLC determination of valproic acid arose from the need for an accurate and precise method for the certification of three concentration levels of this drug in a freeze-dried human serum matrix that will be issued by the National Bureau of Standards (NBS) as Standard Reference Material (SRM) 1599.

The human serum matrix is a processed pooled serum lot specially prepared for NBS. Although not discussed in this paper, SRM 1599 also contains three concentration levels of carbamazepine, another antiepilepsy drug. This drug was determined by separate analytical procedures and its presence in the serum matrix did not interfere with the analysis of valproic acid.

The certification of an SRM requires the use of a "definitive" method, where the analyst has complete knowledge of all sources of error, or at least two independent analytical procedures. In this paper we will describe the modifications to the procedure of Schmidt and Slavin that we found necessary to provide us with one of the two procedures required for certification. We found this to be an accurate and precise method for the analysis of valproic acid in both a freeze-dried serum matrix and in serum samples obtained from a clinical laboratory.

MATERIALS AND METHODS

Reagents

HPLC grade methanol, acetonitrile, water, and methylene chloride were used. The derivatizing reagent was Phenacyl-8 obtained from the Pierce Chemical Co.*, Rockford, Il., and

*Certain commercial equipment, instruments, or materials are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

contained 0.1 mmol/mL phenacylbromide and 0.005 mmol/mL crown ether in acetonitrile. The stock Phenacyl-8 was diluted 1-5 with acetonitrile prior to use. The phosphoric acid used in this work was reagent grade. Valproic acid was purchased from Saber Laboratories, Morton Grove, Il., and cyclohexane carboxylic acid was obtained from Eastman Kodak Co., Rochester, N.Y. The reaction vessels and sealing tool used in this work can be obtained from most chromatographic supply houses.

Processing of Human Serum for Use as SRM 1599

The serum base was procured from Seraplex, Inc. (Arcadia, CA.). Following procurement of this serum, the material was further processed at a local biological production plant where it was pooled into a single lot (approximately 24 L) and stirred to achieve homogeneity. The serum was then passed through depth and membrane filters, having a final pore size of 0.22 μm to achieve sterility, into four sterile containers, each marked for a volume of 4 L. Samples of the filtrate were tested by HPLC for suitability and freedom from interfering peaks prior to spiking with valproic acid and carbamazepine.

One of the four containers was designated as Blank Serum. The three remaining containers were spiked with valproic acid and carbamazepine to produce three concentration levels roughly corresponding to human sub-therapeutic, therapeutic, and toxic serum levels of the drugs. The three spiked containers were labeled low, medium, and high, respectively.

The contents of each container were dispensed in 5.00 mL aliquots into vials that were sequentially numbered and appropriately labeled. The contents of each vial was then freeze-dried and the vial stoppered under nitrogen.

Instrumentation

The HPLC system used in this work consisted of a variable volume injector, auto-sampling accessory, two reciprocating

diaphragm pumps, variable wavelength UV detector, and an integrator-printer/plotter. The column used in this work was monomeric octadecylsilane (C-18).

SAMPLE PREPARATION

SRM 1599

Samples of freeze-dried human serum (SRM 1599) were reconstituted with 5.00 mL of water. The samples were allowed to remain at room temperature for 1 h with occasional rotation (by hand) to ensure complete dissolution.

A 1.00 mL aliquot of each sample was placed in a 15 mL screw-top vial. Known amounts of cyclohexane carboxylic acid were added to each vial to serve as an internal standard. The serum aliquot was acidified by adding approximately 100 µL of concentrated phosphoric acid, then extracted with 2.0 mL of methylene chloride. The extraction step was accomplished by placing the capped vials on a wrist-action automatic shaker for 30 min. The vials were than centrifuged for 10 min at 2500 rpm to separate the aqueous and organic layers.

Approximately 0.5 mL of the sample extract was placed in a 1 mL reaction vessel with 0.5 mL of the derivatizing reagent solution and approximately 0.5 mg of sodium bicarbonate. The vessels were sealed and heated at 75 °C for 2 h. Upon completion of the reaction period, each vessel was opened and its contents concentrated with nitrogen purge to approximately 0.5 mL. The vessels were then filled to ~1 mL with acetonitrile before aliquots were taken for analyses by HPLC.

Figure 1 shows the reaction scheme which was used to produce the phenacyl ester derivatives of valproic acid and cyclohexane carboxylic acid. A 30 min. gradient from 40 to 80 percent acetonitrile in water was used for analyses.

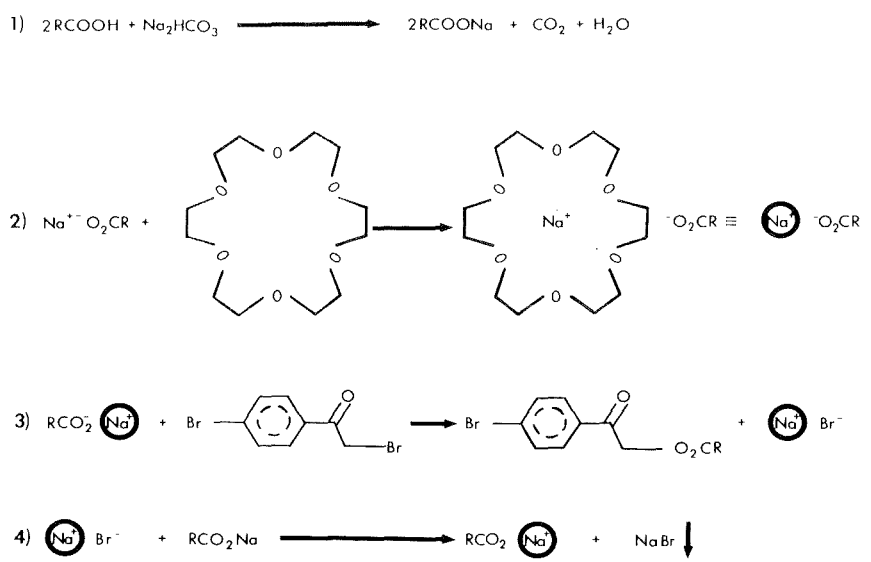

FIGURE 1. Phenacylation of valproic acid. R = C_7H_{15}.

Patient Serum Samples

Essentially the same sample preparation procedure was used for both the processed freeze dried SRM sample and the samples of human sera obtained from the University of North Carolina. The modifications that were made were as follows: 0.1 mL instead of 0.5 mL of derivatizing reagent solution was used; the incubation time was reduced from 2 h to 0.5 h.

The patient serum samples were analyzed under isocratic conditions, using a 65 percent acetonitrile in water mobile phase. The HPLC analysis time was 10 min.

Preparation of Standards

Stock solutions of valproic acid and cyclohexane carboxylic acid (1 mg/mL level) were prepared by accurately weighing appro-

priate amounts of the crystalline material into 100 mL volumetric flasks and diluting to the mark with 10 percent methanol in water.

Working standards, appropriate to each sample concentration level, were prepared by adding aliquots of the stock solutions to blank serum. The working standards were then processed as described in the sample preparation section above.

Six working standards were prepared and processed with the samples from each concentration level. The standards ranged in concentration from 9-20, 50-80, and 120-185 μg/mL of valproic acid for the low, medium, and high concentration levels respectively. The internal standard, cyclohexane carboxylic acid, was added to samples and working standards to produce concentrations of 50 μg/mL for the low and medium concentration levels and 100 μg/mL for the high level.

RESULTS

Fifteen samples from each concentration level of SRM 1599 were analyzed in duplicate by reversed-phase HPLC. A typical chromatogram of a medium level sample is shown in figure 2. Figure 3 is a standard curve containing area response ratios (valproic acid to internal standard) versus the concentration of valproic acid in working standards ranging from 5-200 μg/mL. Although this curve appears linear over the entire concentration range investigated, we preferred to quantitate using calibration curves that covered the smaller ranges mentioned earlier.

The mean value obtained from the HPLC analysis of each concentration level of SRM 1599 is shown in table 1 along with results obtained using two additional methods of analysis. The NBS-GC values were obtained during the course of certifying this processed serum as an SRM. The NC-GC values were provided by Dr. Ken Dudley, University of North Carolina School of Medicine.

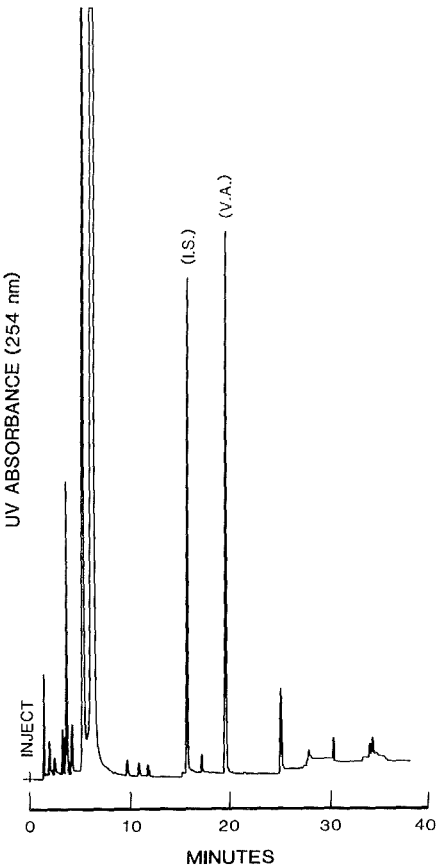

FIGURE 2. HPLC chromatogram of SRM 1599, medium level sample. I.S. = cyclohexane carboxylic acid, V.A. = valproic acid.

TABLE 1

Determination of Valproic Acid in SRM 1599 by Three Methods of Analysis

	Concentration (µg/mL)		
	Low Level	Medium Level	High Level
NBS-HPLC	14.6 ± 0.3[a]	67.1 ± 0.9	145 ± 1
NBS-GC	14.3 ± 0.1	71.2 ± 0.4	141 ± 1
NC-GC	14.7 ± 0.4	67.5 ± 1.5	141 ± 1

[a]Uncertainties reported represent the standard deviation of the mean.

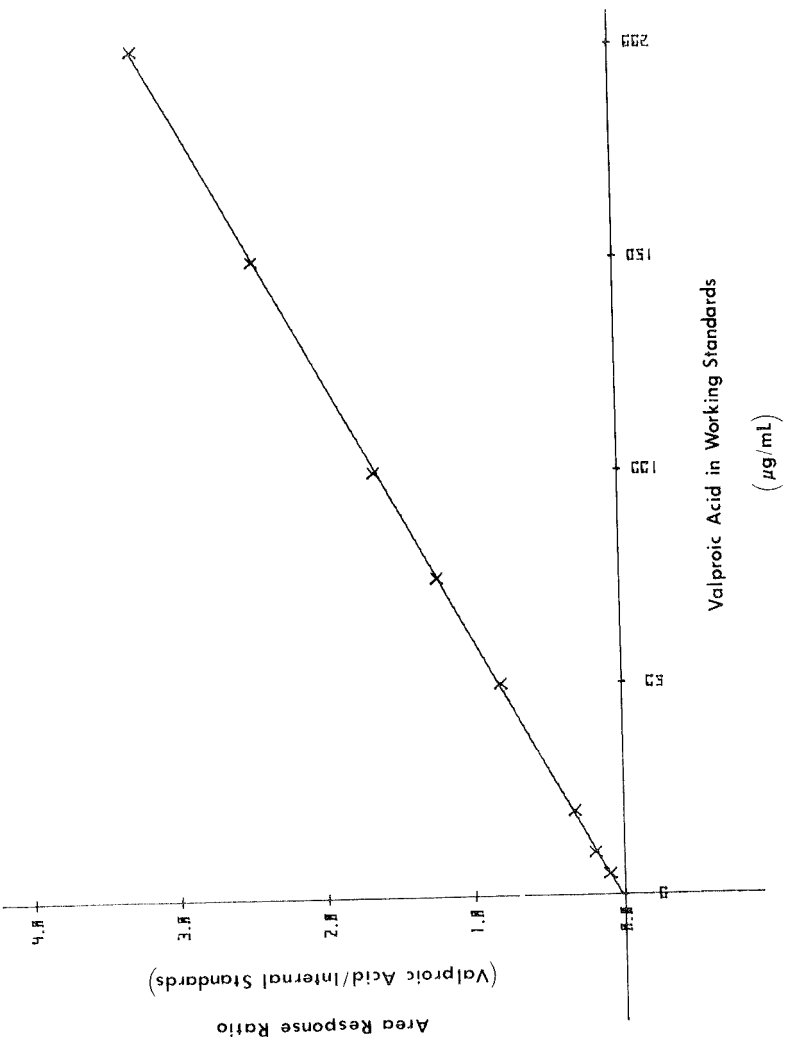

FIGURE 3. Valproic acid calibration curve. Response ratio is the detector response for valproic to that of cyclohexane carboxylic acid added at the 50 (µg/g) level as an internal standard.

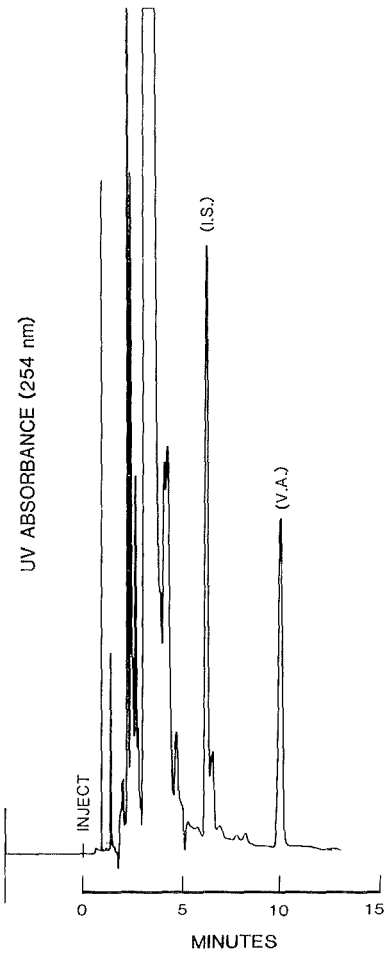

FIGURE 4. HPLC chromatogram of a patient serum sample. I.S. = cyclohexane carboxylic acid, V.A. = valproic acid.

The results obtained from the HPLC analysis of several patient serum samples are compared to the original clinical analysis supplied by Dudley in table 2. Figure 4 is a chromatogram of one of the patient serum samples.

TABLE 2

HPLC Analysis of Patient Serum Samples

	Valproic Acid Concentration (μg/mL)	
Sample No.	NBS-HPLC	NC-GC
1	85 ± 2[a]	79
2	40 ± 2	37
3	56 ± 2	67
4	67 ± 2	69
5	54 ± 3	58

[a]Uncertainties reported represent one standard deviation (n = 6).

SUMMARY

The procedure that we have described was developed for the analysis of valproic acid in a processed freeze dried human serum sample that is being issued as NBS SRM 1599. We feel, however, that this method has the simplicity, rapidity, accuracy, and precision necessary to be used routinely in the clinical laboratory.

A clinical laboratory faced with the analysis of large numbers of samples would view the procedure used for analysis of SRM 1599 as too time-consuming. We found three key areas where we could substantially reduce the analysis time, thereby making the method sufficiently rapid for a clinical laboratory. First, we found that the reaction incubation time could be reduced from 2 h to as short as 30 min. Secondly we found that, we could reduce the amount of derivatizing reagent from 0.5 to 0.1 mL, thereby eliminating a number of large non-analyte peaks from the chromatogram. This allowed us to use isocratic conditions and thereby reduce the HPLC run time from 40 min to 10 min (see figures 2 vs. 4.).

The data given in tables 1 and 2 show this method to yield results comparable to those of the GC methods commonly used for this assay. Therefore we feel that this method is a valuable additional tool that can be confidently used by laboratories faced with the problem of analyzing valproic acid in biological samples. In addition, this method is also applicable to the analyses of other carboxylic acids. For example, we have recently applied this procedure to the analysis of butyric, pentanoic and hexanoic acid in wastewater samples.

ACKNOWLEDGEMENTS

The authors acknowledge partial financial support from the National Institute for Neurological, Communicative Diseases and Stroke. The authors wish to thank Dr. Ken Dudley, University of North Carolina, Department of Pharmacology, School of Medicine, for performing analyses on samples of SRM 1599 and for providing several patient serum samples. We also thank Dr. Robert Paule, NBS, for the statistical analysis of data given in table 1.

REFERENCES

(1) Pinder, R. M., Brogden, R. N., Speight, T. M., and Avery, G. S., Drugs, 13, 81, 1977.

(2) Simon, D. and Penry, J. K., Epilepsia, 16, 549, 1975.

(3) Durst, H. D., Milano, M., Kikta, Jr., E. J., Connelly, S. A., and Grushka, Eli, Anal. Chem., 47, 1797, 1975.

(4) Grushka, Eli, Durst, H. D., and Kikta, Jr., E. J., J. Chromatogr. 112, 673, 1975.

(5) Schmidt, G. J. and Slavin, W., Chromatogr. Newslett. 6, 2, 1978.

(6) Godolphin, W., Thoma, J., Clin. Chem., 24/3, 483, 1978.

(7) Bulletin, 778, Supelco, Inc., "Analysis of Valproic Acid", 1978.

(8) Dusci, L. J. and Hackett, L. P., J. Chromatogr. 132, 145, 1977.

(9) Jensen, C. J. and Gugler, R., Chromatogr., 137, 188, 1977.

(10) Berry, D. J. and Clarke, L. A., J. Chromatogr., 156, 301, 1978.

(11) Fellenberg, A. J. and Pollard, A. C., Clin. Chim. Acta, 81, 203, 1977.

(12) Chesler, S. N., personal communication.

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THE RAPID DETERMINATION OF NEUTRAL SUGARS IN BIOLOGICAL SAMPLES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A procedure for the analysis of neutral sugars in biological specimens is described. The method entails acid hydrolysis of the sample to liberate monosaccharides, which are subsequently derivatized with dansyl hydrazine. The sugar-dansyl hydrazones are separated and quantitated by hplc on a 5μ C18 RadialPak column with a gradient of acetonitrile in 10mM ammonium sulfate at pH 7. Fluorescent detection of the derivatized sugars permits 100-fold increased sensitivity compared to previously published glc methods.

This procedure was applied to the neutral sugar analysis of a glycoprotein of known composition (thyroglobulin) and to hard keratin fibers. The latter substance served as a model to critically evaluate the method on a highly resistant biological matrix containing low concentrations of neutral sugars.

INTRODUCTION

Analysis of carbohydrates in biological materials is a demanding and time-consuming task. Classically, gas-liquid chromatography (glc) has been used for carbohydrate analysis of glycoproteins. Since sugars are not volatile, they must first be converted to suitable volatile derivatives. These techniques uti-

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lize alditol acetates (1), aldonitrile acetates (2), or trimethylsilyl ethers of methyl glycosides (3), and are lengthy procedures to complete. Liquid chromatography on ion-exchange columns with post-column derivation (4-6) has also been used. While these procedures provide adequate sensitivity for the detection of 0.1 μg of carbohydrate, they are complicated by the necessity for large quantities of starting material, cumbersome volumes of hydrolytic and pretreatment solutions and, in many cases,multiple peaks from discrete sugar residues.

A recent technique (7) employs 5-dimethylamino-naphthalene sulphonyl (dansyl) hydrazine to selectively label reducing sugars and the resulting derivatives are separated by high-performance liquid chromatography (hplc). This procedure is demonstrably more rapid and sensitive than earlier glc or hplc methods. In the present communication, we have applied this method to the analysis of neutral sugars of a characterized glycoprotein (thyroglobulin), and to human hard keratin fibers. We show that this is the method of choice for neutral sugar analyses of biological materials.

MATERIALS AND METHODS

Solvents, standards, and hplc equipment have been described previously (7). Acetonitrile was purchased fresh and stored at 4°C. Bovine thyroglobulin (Type I) was purchased from Sigma Chemical Co. (St. Louis, MO). Human hard keratin fibers were from a single donor. The anion-exchange resin was AG1x8 (acetate

form) 200/400 mesh from Bio-Rad Laboratories (Richmond, CA). Before use, the resin was converted to the carbonate form by washing first with 2N sodium carbonate (200 ml), then with water (200 ml). For cation-exchange chromatography, AG50Wx8 (hydrogen form) 200/400 mesh resin (Bio-Rad) was used after purification. The resin was purified by converting it first to the sodium form and then back to the hydrogen form (8). This treatment removed an unidentified yellow substance (probably a resin degradation product), which interfered with the hplc separation. The final product was suspended in two volumes of water and 5ml was pipetted into a 1 x 30 cm chromatographic tube.

Glycoproteins and keratin fibers were hydrolyzed with 1N or 2N HCl at 100°C for various times (see Figures 2 and 4). After cooling, maltose was added as an internal standard. The sample was neutralized by adding an excess of AG1 resin (carbonate form, 0.5g per equivalent H+) and then transferred to a 1 x 30 cm chromatographic tube, whose outlet fed into the cation-exchange column. After rinsing the columns with 10ml water, the effluent was dried on a rotary evaporator, then redissolved in a small volume of water.

The previously described procedure (7) for the hplc analysis of reducing sugars was modified as follows. The sugar-dansyl hydrazone derivation product (40 μl) was diluted only 7.5-fold and used without Sep-Pak treatment. For increased resolution, separations were performed on a RadialPak 5μ C18 cartridge (0.8 x 10 cm) with a 2 ml/min flow rate and a concentration

gradient of acetonitrile (solvent B) in 10mM ammonium sulfate at pH 7. (The gradient was isocratic at 22%B for 8 min, increase linearly in 9 min to 28% B, step to 50% B for 5 min.) These modifications optimized the separation of the neutral sugars commonly found in mammalian glycoproteins.

RESULTS

A typical separation of standard sugar-dansyl hydrazones is shown in Figure 1. The procedure provided baseline resolution of nearly all derivatives over a twenty-fold range of sugar concentrations. Dansoic acid, a by-product of the derivation reaction, appears at the front of the chromatogram. An unidentified contaminant from the derivation reagent elutes after xylose. It is sufficiently resolved so as not to hamper the quantitation of xylose. Unreacted dansyl hydrazine is removed from the column with 50% acetonitrile, and elutes after fucose (not shown).

Separation of the sugar derivatives from a hydrolysate of thyroglobulin is shown in Figure 2. With our sample pretreatment scheme, the hexoses elute with no interference. If preparative ion-exchange chromatography is omitted, additional unidentified peaks appear which elute near glucose and complicate the results (data not shown).

Recovery of neutral sugars from thyroglobulin hydrolysates is shown in Figure 3. Four hours hydrolysis produced linear recoveries of each hexose independent of the glycoprotein concentration. Fucose, however, required a shorter hydrolysis

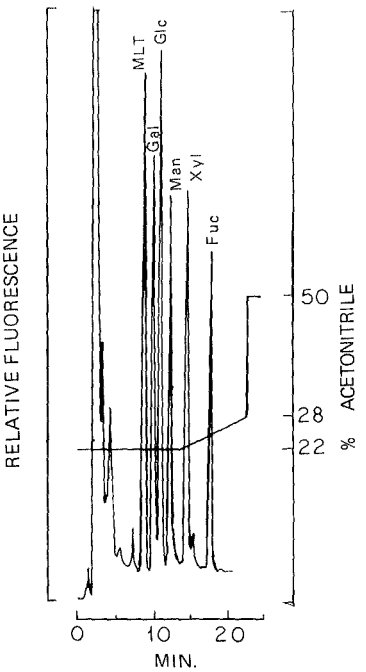

FIGURE 1. Chromatographic separation of standard sugar-dansyl hydrazones. Conditions for separation as described in text. Detector was Schoeffel FS970 Spectrofluoro Monitor (excitation wavelength, 240nm; emission longpass filter, 550nm; range, 0.2 μAFS; sensitivity, 42%; time constant, 2.5 sec). Sample was 1 nmole each standard: mlt, maltose; gal, galactose; glc, glucose; man, mannose; xyl, xylose; fuc, fucose.

time (1 hour) to obtain a linear recovery. The concentration of each sugar in this thyroglobulin sample is given by determining the slope of the appropriate curve (Table 1).

To demonstrate the sensitivity and versatility of our method, it was applied to the analysis of neutral sugars in

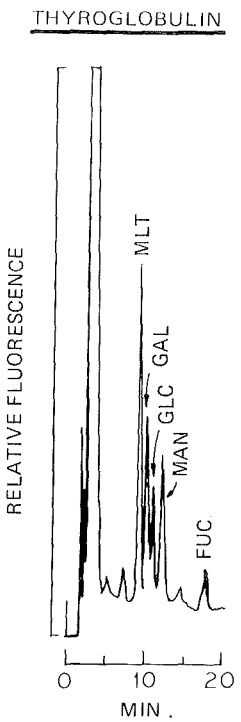

FIGURE 2. Sugar dansyl hydrazones from a thyroglobulin hydrolysate (4 hr at 100°C in 2 ml 1N HCl). Conditions as described in Figure 1.

hard keratin fibers. Human hair provided an ideal model for the analysis of small amounts of sugar in a resistant biological matrix. Figure 4 shows a chromatogram of the sugar-dansyl hydrazones produced from a keratin hydrolysate. Even though the sugars were present in very low concentrations in keratin fibers, there was still no observable interference from peptide or melanin by-products formed during hydrolysis.

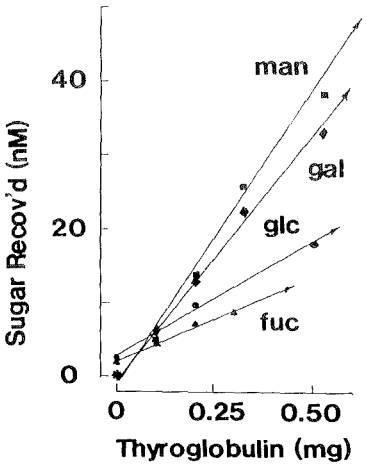

FIGURE 3. Proportional recovery of neutral sugars from various amounts of thyroglobulin hydrolyzed in 1 ml 1N HCl at 100°C (fucose for 1 hr., remainder for 4 hr.) Each point is the mean of 3 determinations.

TABLE 1.

Concentration of Neutral Sugars in Bovine Thyroglobulin

Saccharide	Concentration	
	Found *	Reported **
	(μmoles per g)	
Galactose	66.4	72
Mannose	79.9	128
Fucose	23.1	24
Glucose	31.4	--

* - from the slopes of the curves in Figure 3 by linear regression analysis

** - Reference (9)

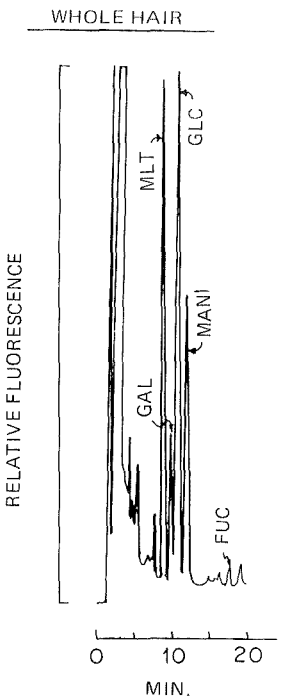

FIGURE 4. Separation of the sugar-dansyl hydrazones from a hard keratin fiber hydrolysate (2 hr at 100°C in 2 ml 1N HCl). Chromatographic conditions as described in Figure 1.

The kinetics of sugar release from keratin fibers is shown in Figure 5. Maximal release was obtained at 2 hours. Hydrolysis in 2N HCl was more effective in releasing saccharide from keratin than lower concentrations of HCl (data not shown). As with thyroglobulin, recovery of sugars from the hydrolysates was linear and independent of keratin concentration (Figure 6). The sugar content of hard keratin fibers was readily determined from the slopes of these curves (Table 2).

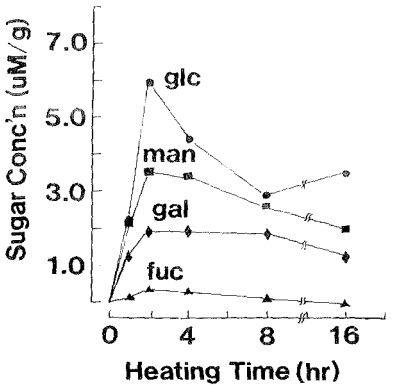

FIGURE 5. Rate of hydrolysis of neutral sugars from hard keratin fibers in 2 ml 2N HCl at 100°C. Analyzed as their dansyl hydrazones in triplicate; chromatographic conditions as in Figure 1.

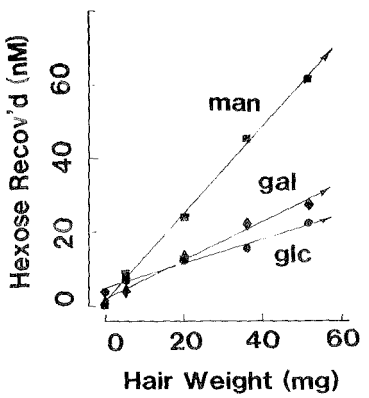

FIGURE 6. Proportional recovery of neutral sugars from human hard keratin fiber hydrolysates (2.5 h at 100°C in 2 ml 2N HCl). Each point is the mean of 3 determinations.

TABLE 2.

Concentration of Neutral Sugars in Hard Keratin Fibers

Saccharide	Concentration *
	(μmoles per g)
Galactose	0.53
Mannose	1.18
Glucose	0.34

* - from the slopes of the curves in Figure 6 by linear regression analysis

DISCUSSION

In a previous manuscript, we demonstrated that neutral sugars commonly found in mammalian tissues can be separated and quantitated by hplc of their dansyl hydrazone derivatives (7). This method offers greatly increased speed, versatility, and sensitivity of detection over previously published methods (1-6). Glc methods for sugar analysis currently in use (1-3) are some 100-fold less sensitive than our present method and require many more hours to complete (7). The versatility of our method is demonstrated by noting that previous methods of analysis require mg amounts of glycoprotein starting material (1-4), whereas we characteristically use 10μg of glycoprotein for hydrolysis and derivation. Trace determinations (e.g. in keratin sugar analysis) can accomodate several mg of protein without interference.

In the present communication, we have improved the resolution of galactose, glucose and mannose dansyl hydrazones by using a 5μ C18 column. In conjunction with a specific gradient of acetonitrile, the 5μ C18 column also gave improved efficiency for xylose and fucose. These late eluting sugars can now be quantitated with even greater sensitivity and accuracy than was previously reported (7).

It is worthwhile noting that the integrity of the acetonitrile is crucial to the success of our method. By storing the solvent at 4°C, we have insured its integrity for periods in excess of 6 months. When stored at room temperature for periods more than a few weeks, deleterious effects on our chromatographic separations were repeatedly observed. While we have not investigated the source of this effect, it seems likely that a decomposition product from the acetonitrile alters the retention of a derivation by-product and thereby disrupts the chromatogram. We have observed that the effect is usually reversible, but on one occasion has irreversibly altered the stationary phase.

Thyroglobulin served in the critical evaluation of this procedure for glycoprotein analysis. The linear correlations obtained in Figure 3 demonstrate that this method provides quantitative results. Galactose and fucose were found (Table 1) in concentrations similar to reported values for calf thyroglobulin, while mannose gave two-thirds of the reported levels (9). If hydrolysis conditions were optimized, the mannose content may reach reported values. However, after one hour hydrolysis, we

have recovered 2/3 of the mannose liberated in four hours of hydrolysis. This suggests that we are approaching the optimal hydrolysis time with one hour heating. More likely, this preparation of thyroglobulin probably contains a lower concentration of mannose. Since thyroglobulin does not normally contain glucose (9), the presence of a significant amount of glucose indicates the presence of contaminants in the commercially available thyroglobulin sample.

In the quantitative carbohydrate analysis of any sample, conditions must be optimized for the hydrolytic release of each component sugar. The different hydrolysis times required for the hexoses and fucose is probably related to the lability of fucose to acid hydrolysis. In fact, the bimolecular nature of likely fucose degradation pathways supports the concentration-dependency observed for its recovery after four hours of hydrolysis (10).

Hard keratin fibers present a challenging subject for carbohydrate analysis. The tenacity of the keratin protein structure, its low carbohydrate content, and the presence of various pigments introduce special complications. However, we have shown that the application of this procedure overcomes these obstacles without difficulty. Indeed, the results demonstrate that ours is the method of choice for the analysis of neutral sugars in biological materials.

ACKNOWLEDGEMENT

The authors wish to sincerely thank John and Paula Meehan for their continued support of research on keratinized tissues.

REFERENCES

1. Niedermeier, W., Anal. Biochem., 40, 465 (1971)

2. Mawhinney, T.P., Feather, M.S., Barbero, G.J., and Martinez, J.R., Anal. Biochem., 101, 112 (1980)

3. Bhatti, T., Chambers, R.E., and Clamp, J.R., Biochim. Biophys. Acta, 222, 339 (1970)

4. Barr, J., and Norton, P., Anal. Biochem., 108, 313 (1980)

5. Mopper, K., Dawson, R., Liebezeit, G., and Hansen, H.P., Anal. Chem., 52, 2018 (1980)

6. Honda, S., Takahashi, M., Kakehi, K., and Ganno, S., Anal. Biochem., 113, 130 (1981)

7. Alpenfels, W.F., Anal. Biochem., 114, 153 (1981)

8. Boas, N.F., J. Biol. Chem., 204, 533 (1953)

9. McQuillan, M.T., and Trikojos, V.M., Glycoproteins (Gottschalk, A. ed.), Elsevier, Amsterdam, 1966, p. 516

10. Marshall, R.D., and Neuberger, A., Glycoproteins (Gottschalk, A. ed.), Elsevier, Amsterdam, 1972, p. 246

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(9), 1725-1748 (1982)

RAPID HPLC METHODS FOR THE SEPARATION AND QUANTITATION OF A MONO-, DI-, AND TRI-SACCHARIDES MIXTURE AND APPLICATIONS.

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ABSTRACT

A simple, rapid method has been developed for the separation and quantitation of mono-,di-, and tri-saccharides. The method utilizes a 30cmx 3.9mm i.d. Microbondapak NH_2 column, refractive index detection and water-acetonitrile elution. Two chromatographic systems are described. The isocratic mode was necessary to develop a procedure.20 Carbohydrate's retention times were evaluated. To optimize the separation of nine water-soluble sugars , a gradient mode flow-programming was used. Separation was achieved within 28 minutes. The low detection limit (4 micrograms) of the above chromatographic procedure and its different possibilities could be of great interest to the analyst. The method has been successfully applied to quantify the major carbohydrates found in two types of commercial honey.

INTRODUCTION

There are mainly two techniques for the separation and quantitation of carbohydrates in HPLC.

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Derivatized sugars, soluble in the non-polar solvents and detected by UV absorption at an appropriate wavelength; water-soluble sugars which can be directly eluted, without pretreatment, with colorimetric, refractive index and/or UV absorption detections.

As pointed out so often, the gain in sensitivity and the resolving power are advantages of the first method. Isomerization can even be avoided by working under extremely rigorous conditions (1). However, the derivatization is cumbersome; derivative preparation is time-consuming, and derivative recovery is seldom quantitative. Moreover, other physico-chemical methods of analysis are needed to identify the by-products which very often result from derivatization.

For the second method, numerous modes of HPLC have been investigated and reported in the literature. Ion-exchange columns for HPLC have been evaluated (2); they require long chromatographic runs. The trend to fast separations in carbohydrate analysis can be seen from the increasing use of siloxane-bonded phases as stationary phase.

The most widely used stationary phase is an amino-propyl group bonded to silicagel (3-8). Besides this amino phase, cyano and combinations of amino and cyano have been used successfully (9). With the moderately polar stationary phases, the most suitable polar mobile phase remains

water-acetonitrile in different ratios. In these chromatographic systems, the carbohydrates are analyzed without prior chemical transformation . The means of detections usually employed are : refractive index, and UV detection at 188 nm (10) or at 192 nm (11).

The aim of this work was to study the chromatographic behaviour of a range of mono-, di-, and tri-saccharides on a microbondapak NH_2 column with refractive index detection. The separation and quantitation were also evaluated to determine the major sugars contained in two types of Algerian honeys.

MATERIALS

Reagents :

The acetonitrile, spectrophotometric grade, was obtained from E. Merck, Darmstadt (GFR), and Eastman (USA);acetic acid from E. Merck; sugars and sugars alcohols are reference substances for chromatography, purchased from E. Merck.

The studied honeys were available from Sogedia(ALGERIA). The standards were prepared by dissolving amounts of carbohydrates in distilled water in the range 20-100 mg/ml (2-10%w/v). The weighed honey samples were diluted with water-acetonitrile mixture (50/46 v/v), filtered and injected directly into the chromatograph.

Apparatus :

Chromatograms were run on :

a Waters Associates liquid chromatograph model ALC/GPC 244 equipped with two M 6000 solvent delivery systems, a M 660 solvent programmer, a U-6K universal injector and a MR 401 refractive index detector.

Two recorders were employed :

Houston Instruments Omniscribe (Austin, Texas)operated at 10 mV sensitivity (Attenuation : 8x) and a chart speed of 0.1 in/min for separations;

Sefram Servotrace (type PE Paris) used at variable voltage range (Attenuation : 256-16 x) and a chart speed of 0.1 in/min for quantitations (e.g., calibrations curves). The separations were conducted with a 30cm x 3.9mm i.d. Microbondapak NH_2 stainless steel column (Waters Associates). The samples were injected with a 10 microliter syringe (Scientific glass Engineering P.T.Y./Ltd, AUSTRALIA) for separations and 1, 5, 10 and 20 microliter syringes (Hamilton Co., Reno,Nevada) for quantitations. Filtration of carbohydrate standards and honey solutions was carried out using Waters Associates sample clarification kit (0.45 microns pore size,aqueous solvent filtration disk). The solvents were filtered using a Pyrex filter holder; 0.45 microns pore size,type HA of the filter membrane (Millipore Corp., Bedford, Mass.).

METHODS

The chromatographic system was employed in the single-pump isocratic mode for development of a procedure and in the single-pump gradient flow rate mode to establish the best chromatographic conditions for the routine analysis. All chromatographic analyses were carried out at room temperature (ca. 20-22°c). The solvent mixtures were degassed before each assay. After gradient flow-rates, the gradients were reversed for 10 min to reestablish initial conditions. Flow-rates were maintained between 0.8 and 2.5 ml/min. Water-acetonitrile mixtures were used as elution solvent and all quantitations in this report were made on the basis of peak height measurements.

RESULTS AND DISCUSSION

The porous support of the used column is made by chemically bonding an amino group to silicagel microparticles (microporasil) at 9% by weight (12). With this stationary phase, the first approach was to study the polarity effect of the water-acetonitrile mobile phase (by increasing the percentage of water)with a 5-monosaccharide separation. The best elution-with, however, some broad peaks - had been obtained by using the above mobile phase in the ratio 10/90 (Figure 1a). To improve

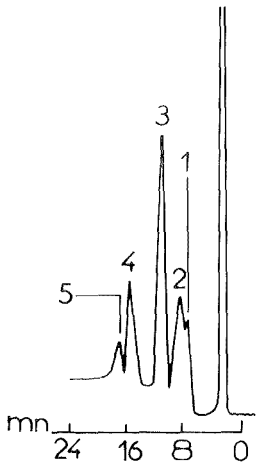

FIGURE Ia : Isocratic mode sepation of 5 monosaccharides: I.D (+)-xylose; 2.L(+)-arabinose; 3.D(-)-fructose; 4.D(+)-glucose; 5.D(+)-galactose. Solvent : Water/Acetonitrile 10/90. Chromatographic conditions were the same as in Table I.

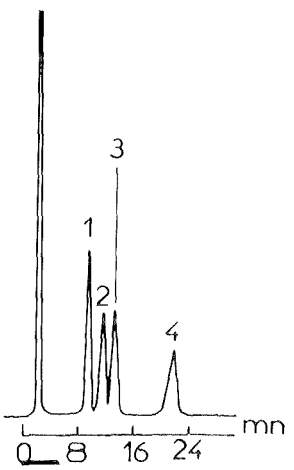

FIGURE 1b : Isocratic mode elution of di-, and tri- saccharides with the same conditions as in Table 2. The peaks 1,2,3 and 4 correspond to : sucrose, maltose, lactose and raffinose respectively.

the peaks symmetry, we refered to TLC.Bolliger and co-workers (13) advise modification, in this case,of the eluting solvent pH by small amounts of buffers such as Na_3PO_4, KH_2PO_4, CH_3COOH, etc.

In the present study, a variable amount of CH_3COOH was added to modify the mobile phase pH. Thereby,the best efficiency and resolution were achieved by acetonitrile-water-acetic acid (90/09/01) elution. With this mixture, 17 carbohydrates were eluted sequentially. Their retention times are indicated in Table 1. We can conclude that the di-,and tri-saccharides are strongly retained by the stationary phase. Indeed, the elution times of these sugars exceed one hour. This same eluting solvent allowed us to separate 6 monosaccharides (Figure 2a) and a fructose-glucose-mannose mixture whose difficulty of separation is often mentioned in the literature (14).The elution of this mixture was carried out by recycling the partially fractionated components (Figure 2b). With regard to di-,and tri-saccharides, they cannot be eluted with reasonable retention times using acetonitrile-water-acetic acid in the ratio 90/9/1. We attribute this fact to the poor solubility of di-and tri-saccharides in the above solvent. This can be improved by increasing the polarity of the mobile phase(i.e. by increasing the water content). After many assays, a satisfactory separation of four di-and tri-saccharides was

TABLE 1

Isocratic Mode Elution of 17 Carbohydrates

a°)Operating Conditions

Column	:	microbondapak NH_2
Attenuation	:	8x
Pressure	:	400 psi.
Solvent	:	$CH_3CN/H_2O/CH_3COOH$ (90/09/01)
Flow-rate	:	1.5 ml / min.
Chart speed	:	0.1 in/mn.
Injections	:	10 microliters of each of samples at 10%(w/v).

b°)Retention Times :

Compound	Retention Times (min.)
2-Desoxy-D-ribose	4.4
L(+)-Rhamnose	6.2
D(-)-Ribose	6.2
Meso-Erythritol	7.2
D(+)-Xylose	8.2
L(+)-Arabinose	9.2
L(-)-Sorbose	11.8
D(-)-Fructose	11.8
D(+)-Mannose	14.6
D(+)-Glucose	16.5
D(-)-Sorbitol	22.5
D(+)-Galactose	17.8
D(-)-Mannitol	23.4
Meso-Inositol	61.3 (peak tailing)
Sucrose	59.3 (peak tailing)
Cellobiose	
Maltose	83.5 (peak tailing)
Trehalose	
Lactose	90.8 (peak tailing)
Raffinose	

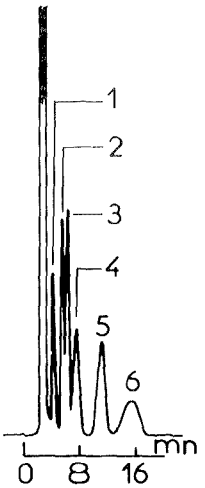

FIGURE 2a : Separation of monosaccharides under isocratic conditions of Table 1; 1.2-deoxy-D-ribose; 2.D(-)-ribose; 3.meso-erythritol; 4.D(+)-xylose; 5.D(-)-fructose; 6.D(+)-glucose.

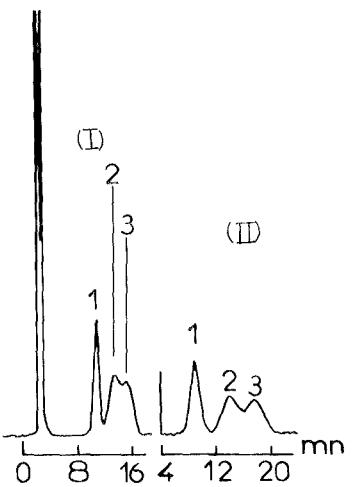

FIGURE 2b : Elution of a fructose (1)-mannose (2)-glucose (3) mixture (I). Elution profile of the same mixture by recycling the mobile phase (first passage), 4 mn after injection of the sample (II). Other conditions were as in Table I.

TABLE 2

Isocratic Mode Elution of Di-,and Tri-saccharides

a°)Operating Conditions :

Column : Microbondapak NH_2
Attenuation: 8x
Pressure : 420 psi.
Flow-rate : 1.5 ml/min.
Chart Speed: 0.1 in/min.
Solvent : water-acetonitrile (20/80).
Injections : 5 microliters of each of samples at 10%(w/v).

b°)Retention Times :

Compound	Retention Times (min.)
Sucrose	9.6
Cellobiose	11.8
Maltose	11.7
Trehalose	12.8
Lactose	13.6
Raffinose	22.0

obtained with water-acetonitrile (20/80) as eluent (Figure 1b). The operating conditions and retention times of these sugars are shown in Table 2.

From these results, it appears that an effective separation of a mono-, di-, and tri-saccharide mixture is carried out in two stages :

1) Elution of monosaccharides with $CH_3CN/H_2O/CH_3COOH$ at 90/09/01; the retention times of di-, and tri-saccharides are long and their peaks too broad to be measured accurately.

2) Elution of di-, and tri-saccharides with CH_3CN/H_2O at 80/20 as mobile phase. The retention times of monosaccharides are very short; the resolution between their different peaks is poor.

In order to elute, over the same chromatogram, a mono-, di-, and tri-saccharides complex mixture, we considered using a flow-rate gradient.

The flow-rate gradient allows us to change the mobile phase flow-rate with time. Such possibilities are made possible by manipulating the ratio of water to acetonitrile in the eluent and programming the flow-rate. The elution of nine carbohydrates was programmed over a 20 min. period by using linear and concave gradient curves.

1) According to a linear flow profile; CH_3CN/H_2O are in the ratio 85/15. When the flow-rate is increased from 1 to 2 ml/min., we notice a poor separation of the first 3 monosaccharides (i.e., ribose, meso-erythritol, xylose) and good resolution for the di-, and tri-saccharides. The complete elution did not exceeded 32 minutes (Figure 3a).

2) With a concave flow profile; in this case, we modified only the flow-rate (from 1 to 2.5 ml/min.) and

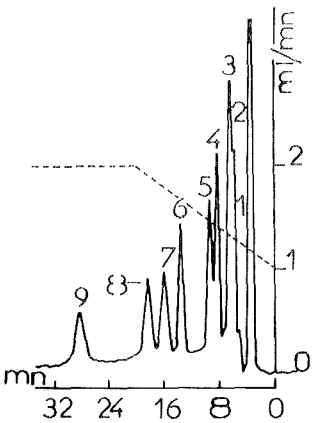

FIGURE 3a : Linear flow-gradient H_2O/CH_3CN (15/85) elution of 9 carbohydrates. 1.D(-)-ribose; 2. meso-erythritol; 3.D(+)-xylose; 4.D(-)-fructose; 5.D(+)-glucose; 6. sucrose; 7. maltose; 8.lactose; 9. raffinose.Conditions: flow-rate; from I to 2 ml/mn. over a 20 mn period. Injection amount : 0.2 mg of each of 9 sugars. Attenuation : 32mV.

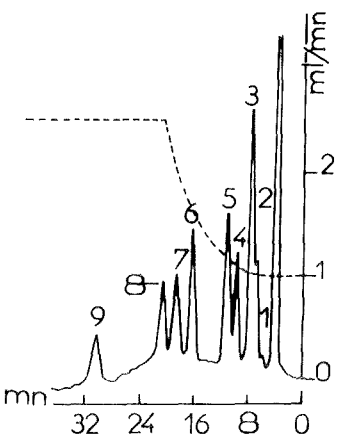

FIGURE 3b : Concave gradient flow-rate mode separation of 9 mono-, di-, and tri-saccharides. 1.D(-)-ribose (0.1 mg); 2. meso-erythritol (0.1 mg); 3.D(+)-xylose (0.2 mg); 4.D(-)-fructose (0.1 mg); 5.D(+)-glucose (0.2 mg); 6. sucrose (0.2 mg); 7. maltose (0.2 mg); 8. lactose (0.2 mg); 9. raffinose (0.2 mg). Conditions; solvent : H_2O/CH_3CN(15/85), flow-rate : from 1 to 2.5 ml/mn over a 20mn period, Attenuation : 32 mV.

the flow gradient profile. No detectable influence has been noticed with this approach (Figure 3b).

3) In a third stage, the polarity of the mobile phase was decreased (H_2O/CH_3CN, 10/90). The gradient was programmed with a linear scan and the period of flow-rate maintained unchanged. Under these conditions a good separation of 8 mono-, and di-saccharides was obtained. However, Raffinose, after one hour running, was still not eluted (Figure 4b).

4) With a linear flow profile, the composition of the eluent (H_2O/CH_3CN, 12.5/87.5) and the variation of flow-rate (from 0.8 to 2.5 ml/min.) were modified. No improvement was noted in relation to the above case Figure 4a).

5) To elute over a same chromatogram, the mine carbohydrates, several systems were investigated. The best separation was achieved under the following conditions : the gradient flow-rate system consisted of 13.5% water and 86.5% acetonitrile,programmed with concave scan from 1 to 2.5 ml/min. (Figure 5).

In conclusion, if a good separation of mono- and di-saccharides was desired, alternatives 3) or 4)might be the answer. For satisfactory resolution of di-, and tri-saccharides, the conditions given in 1) or 2) will be more appropriate. For an intermediate solution,case 5) will be the method of choice. However, the resolution

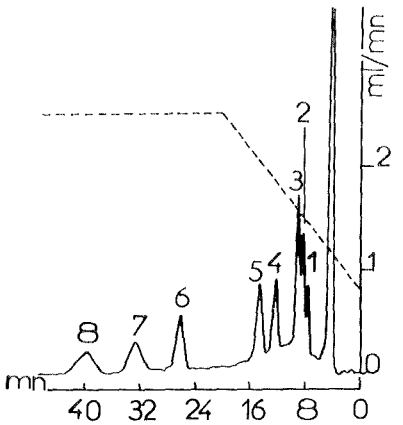

FIGURE 4a : Linear flow-gradient H_2O/CH_3CN(12.5/87.5) elution of 8 mono-, and di-saccharides. 1.D(-)-ribose (0.1 mg); 2. meso-erythritol (0.1 mg); 3.D(+)-xylose (0.2 mg);4.D(-)-fructose (0.1 mg); 5.D(+)-glucose(0.2 mg); 6. sucrose (0.2 mg); 7. maltose (0.2 mg); 8. lactose (0.2 mg). Conditions: 0.8 to 2.5 ml/mn over a 20 mn period. Attenuation: 32 mV.

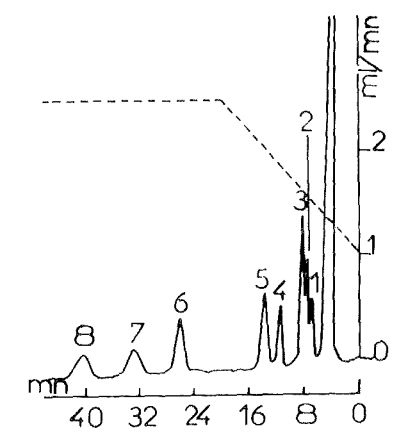

FIGURE 4b : Separation of 8 carbohydrates. Solvent : H_2O/CH_3CN(10/90), flow-rate : from 1 to 2.5 ml/mn over a 20mn period. Other conditions were as in Figure 4a.

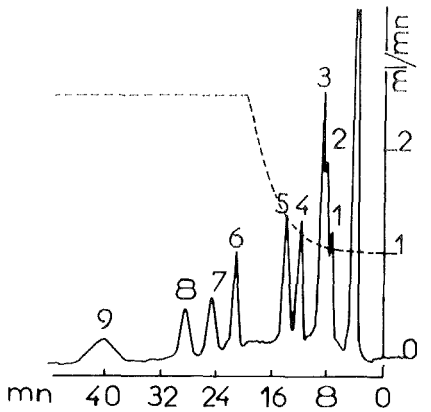

FIGURE 5 : Flow-gradient mode separation of 9 mono-, di-, and tri-saccharides with H_2O/CH_3CN (13.5/86.5). Other conditions were as in Figure 3b.

between the first three monosaccharides remains weak and the analysis time longer (45 min.).

To improve these two important parameters, and taking into account the retention times of different monosaccharides, we replaced in the studied carbohydrates mixture, ribose and meso-erythritol with two monosugars most commonly encountered in food-chemistry: rhamnose and arabinose. In a second stage, several operating conditions were considered to optimize the separation. With a linear gradient flow profile, the flow-rate was increased from 1 to 2 ml/min. over a 5 minute period. The eluting solvent being H_2O/CH_3CN in the ratio 15/85, a satisfactory separation of a mono-, di-, and tri-saccharides

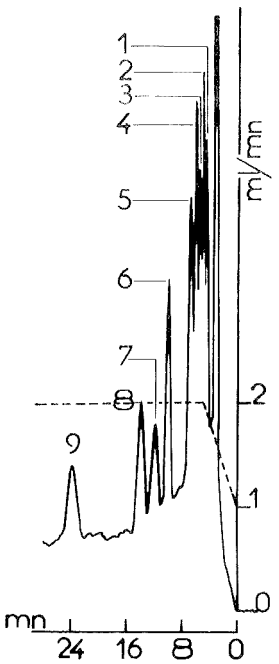

FIGURE 6 : Liear gradient flow elution profile of a synthetic carbohydrates mixture.1.L(+)-rhamnose; 2.D(+)-xylose; 3.L(+)-arabinose;4.D(-)-fructose; 5.D(+)-glucose;6.sucrose; 7.maltose; 8.lactose; 9.raffinose. Conditions;solvent : H_2O/CH_3CN(15/85),flow-rate: from 1 to 2ml/mn over a 5mn period, attenuation: 16mV,injection: 3 microliters of a mixture at 2%(w/v).

mixture was carried out within 28 min. Figure 6 shows that the 9 carbohydrates are eluted from the column in order of their molecular weights (i.e., mono-, di-, and tri-saccharides with the following sequence :

1) Pentoses (rhamnose, xylose, arabinose)

2) Hexoses (fructose, glucose)

TABLE 3

A - Resolution and HETP Calculated from Figure 5.

Carbohydrates	Resolution	Efficiency (plates/m)
Fructose Glucose	1.1	-
Sucrose	-	11000

B - Resolution and HETP Calculated from Figure 6.

Carbohydrates	Resolution	Efficiency (plates/m)
Fructose Glucose	0.6	-
Sucrose	-	5300

3) Disaccharides (sucrose, maltose, lactose)

4) Trisaccharides (raffinose).

To evaluate the degree of separation and efficiency of the column used, we compared the resolutions and HETP's obtained from chromatograms of figures 5 and 6. For the-

se calculations, three carbohydrates were considered : D(-)-fructose, D(+)-glucose, whose separation is often difficult, and sucrose, which gives a sharp, symmetrical peak. The results obtained are shown in Table 3.
As these tables indicate, if we want to obtain a satisfactory separation within 28 min. of a mono-, di-, and tri-saccharides mixture with an uniform chromatogram, the operating conditions of Figure 6 will be suitable . If, on the other hand, enhanced resolution and efficiency required for the hexoses and disaccharides, we would choose the chromatographic conditions of Figure 5.

QUANTITATIVE EVALUATION AND DETECTION LIMIT

Often, only very dilute solutions of sugars are available, so the detection limit for this system is therefore of interest. Figure 7 shows the calibration plots for five mono-, and four di-, and tri-saccharides which were established with reference solutions of different concentrations, using the RI detector. The operating conditions were those of Figure 6, with different attenuations. Standard solutions were prepared as follows : 100 mg of each of 9 sugars were mixed together and dissolved in 5 ml of distilled water.
0.2; 0.3; 0.5; 1; 3; 5 and 10 microliters of that solution were injected directly. The averages of peak heights (cm) from triplicate sample injections were plotted

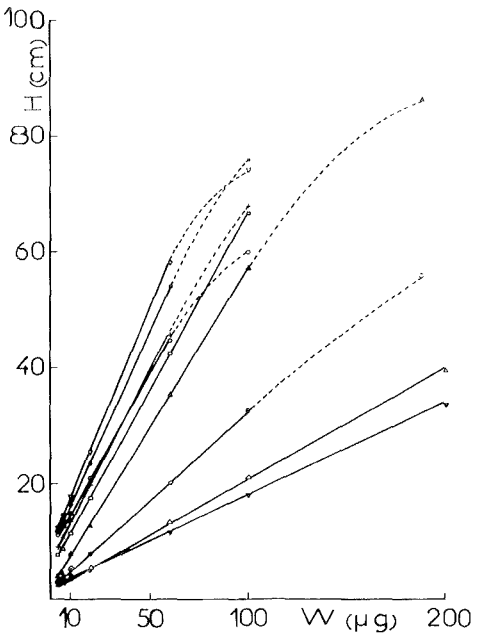

FIGURE 7 : Calibration curves. Peak height, H(cm) versus weight, W(µg) of injected carbohydrate. (▽) xylose; (●). fructose; (✚). rhamnose; (○). arabinose; (□). glucose; (▲). sucrose; (⊙). lactose; (△). maltose; (▼). raffinose. The chromatographic conditions were those of Figure 6.

against weight (microgram) of carbohydrates to obtain the standard curves. It can be seen that there are linear relationships between peak height and component weight in the investigated region from about :

- 4 to 60 micrograms for xylose, fructose, arabinose and rhamnose;
- 4 to 100 micrograms for glucose, sucrose and lactose;

- 4 to 200 micrograms for maltose;
- 6 to 200 micrograms for raffinose

for the injected samples.

The low detection limit for monosaccharides (4 micrograms), carried out with the Microbondapak NH_2 column and using a RI detector, is comparable to those obtained (2 to 12 micrograms) for the same underivatized monosaccharides on a chemical bonded amino stationary phase by Binder with UV detection at 188 nm (10).

APPLICATION

In order to further illustrate our above study, we determined the main carbohydrates found in commercial food products in Algeria. We chose two types of honey : one, which is widely distributed, the other was locally marketed variety. The honey samples were prepared according to the method of Thean and co-workers (15).The resultant solutions were analyzed with a Microbondapak NH_2 column under the experimental conditions outlined in Figure 6. The results showed that the aliquot from the first type of honey contained, primarily, four carbohydrates : fructose (35.6%), glucose (38.6%), sucrose(4%) and maltose (2.4%) -Figure 8a - ; while in the second aliquot , the major components were : fructose, glucose and sucrose, respectively, in the percentages : 21.9% ; 31.7% and 35.3%.

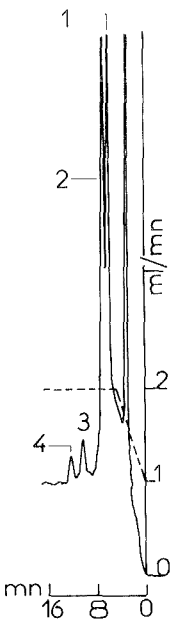

FIGURE 8a: Linear gradient flow elution profile of major components of commercial honey. 1.fructose; 2.glucose; 3.sucrose; 4.maltose. The analysis conditions were as in Figure 6.

It appears that the second type of honey is adulterated with a high sucrose content which reduces the levels of other sugars (Figure 8b). With regard to the first type of honey, the results found are comparable to those obtained by other authors for Orange Blossom Brand honey which was analyzed by HPLC (15) , and by the official methods of the AOAC (16).

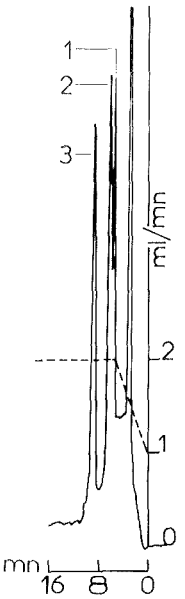

FIGURE 8b: Adulterated honey with a high sucrose content. 1. fructose; 2. glucose; 3. sucrose. The elution conditions were the same as in Figure 8a.

CONCLUSION

A high performance liquid chromatographic (HPLC) procedure based on a Microbondapak NH_2 column, an aqueous acetonitrile solvent and a gradient flow-rate mode has been developed. The method is rapid and no further treatment of the sample is necessary, except for the separation of undissolved solids contained in the solution. Depending upon the sugars' variety in the sample, many chromatographic conditions have been suggested

to obtain sufficient resolution and/or a satisfactory analysis time. If, for instance, raffinose is not present in the sample, only 16 minutes are necessary to elute an eight mono-, and di-saccharide mixture.Compared with the normally used detection by refractive index measurement, this system has the advantages of a lower detection limit and the possibility of shortening the analysis time for a complex mixture containing nine mono-, di-, and tri-saccharides. On the other hand, this procedure shows better performance than those employing UV detection for aqueous sugar mixture analysis without derivatization or pretreatment.

AKNOWLEDGEMENTS

This work was supported by project n° 76/AA/03/01 of O.N.R.S.

REFERENCES

1. THOMSON, R.M., J. Chromatog., 166, 201 (1978).
2. THOMAS, J., and LOBEL, L.H., Anal. Biochem., 73, 222 (1976).
3. CHROMPACK, Chromatography, NR. 9,173 (1978).
4. JONES, A.D., BURNS, I.W., SELLINGS, S.G., and COX, J.A., J. Chromatog., 144,169 (1977).
5. CONRAD, E.C.,FALLICK, G.J., Brewers Digest, 49, 72 (1974).

6. PALMER, J.K., Anal. Lett., 8,215 (1975).

7. HUNT, D.C., JACKSON, P.A., MORTLOCK, R.E., and KIRK, R.S., The Analyst, 102, 917 (1977).

8. SCHWARZENBACH, R., J.Chromatog.,117, 206 (1976).

9. RABEL, F.M., CAPUTO, A.G., and BUTTS, E.T., J. Chromatog., 126, 731 (1976).

10. HANNO, B., J. Chromatog., 189, 414 (1980).

11. HETTINGER, J., and MAJORS, R.E., Varian Instruments Applications, 10,6 (1976).

12. Waters Associates S.A., Inc., D.13, 16 (1977).

13. BOLLIGER, H.R., and KONIG, A., T.L.C., 2nd Ed.,Egon Stahl, New-York, 807 (1978).

14. VAN OLST, H., and JOOSTEN, G.E.H., J. Liquid Chromatog., 2, 111 (1979).

15. THEAN, J.E., FUNDERBURK, W.C. Jr., J.A.O.A.C., 60, 838 (1977).

16. Official Methods of Analysis, 12th Ed., AOAC, Waschington D.C., secs. 31.108, 31.011, 31.025(a), 31.026(c) (1975).

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ON LINE DETECTION OF PLASMA BORNE VASOCONSTRICTOR BY THE USE OF HIGH PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

An on line detection system for a plasma borne vasoconstrictor was developed using a rat heart bioassay and a reversed-phase high pressure liquid chromatograph (HPLC). Partially purified plasma borne vasoconstrictor, which is yet to be characterized, was fractionated by HPLC, and the output from the unit was introduced into the rat heart bioassay system directly. The on line HPLC-rat heart bioassay system detected the active fraction from approximately 20 substances in the partially purified plasma. This system enabled rapid and reproducible identification of the active vasoconstrictor in plasma.

INTRODUCTION

The existence of a vasoconstrictor component in blood plasma has been suggested but never proven (1, 2,3). This component apparently is not a commonly known vasoconstrictor like norepinephrine or angiotensin (5,6). One reason for the difficulty in

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isolating and characterizing this plasma component may be due to the fact that the design of a separation process which is coupled to a meaningful biological assay is quite difficult for this type of material. Such a separation coupled to a biological assay is a requisite for successful purification of components of complex biological mixtures.

For the isolation of this vasoconstrictor component from human plasma, Bohr employed Sephadex gel G-15, and collected the elution fractions, and tested their biological activities using arterial strips from rats and rabbits (7,8). Moretti and others recently reported a similar substance and partially purified it using chemical procedures and thin layer chromatography (9-11). However, the purification methods utilized by the above-mentioned researchers are not effective and often require tedious preparation of the sample for the bioassay (1,7).

We have developed a system for the above purpose in which the powerful separation capability of reversed-phase HPLC is directly incorporated into a rat heart bioassay. In this system the biological activity of the components eluted from the chromatography column can be directly determined by a rat heart perfusion bioassay.

One of the properties of the plasma borne vasoconstrictor is the production of vasoconstrictions in the coronary, skeletal muscle, and perhaps renal circulations (12). Because many known vasoconstrictors do not produce vasoconstriction in the coronary circulation, even though they do produce constrictions in other organs, and because the preparation of the rat heart bioassay is relatively easy, we used the rat heart as the detector organ (13-15).

MATERIALS

As for the mobile phase, one liter of glass distilled water was filtered with millipore filter (Millipore Corp.) under faucet vacuum and then degassed for approximately 20 minutes by faucet vacuum. A similar treatment was done for the methanol used as the elution solution. HPLC grade methanol (J.T. Baker) was used for chromatography and all other chemicals were ACS reagent grade. The Tyrode's solution contains 151 mM NaCl, 4.4 mm KCl, 2.0 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.39 mM Na_2HPO_4, 13.2 mM $NaHCO_3$, 6.2 mM glucose, pH 7.4, 37^{o}C, saturated with 95% O_2, 5% CO_2 in distilled water. In order to establish iso-osmolarity at the perfusion

inlet to the coronary circulation, the osmolarity of Tyrode's solution is increased to approximately 10% above that of normal Tyrode's solution.

A Cole Parmer peristaltic pump (Master Flex - 7562) provided constant perfusion of Tyrode's solution to the coronary circulation. The pressure changes were detected by a Statham pressure transducer (PB-23) and recorded on a dual channel recorder (Versagraph 885).

The HPLC used in the experiment was a Perkin Elmer dual pump (Series 2/2) HPLC with a 4.6 mm x 25 cm reversed-phase column (Perkin Elmer C-18) and a syringe loaded injection valve (Rheodyne Model 7125 with a 20 µl loop). A variable wave length UV-visible detector (Perkin Elmer LC-75) was set at 254 nm for optimum peak detection. Chromatograms were recorded on the dual channel strip chart recorder.

METHODS

Extraction The original chemical extraction procedure for the vasoconstrictor substance was established by Moretti et al. (2) and modified for use in this study. One thousand ml of human platelets were obtained from a local blood bank. After the adjust-

ment of the pH to 3.5 by acetic acid they were washed with 2000 ml of ethyl ether in order to remove neutral lipids. The ether layer was discarded. After repeating the ether wash, the pH was adjusted to 2.5 with hydrochloric acid and the solution saturated with NaCl. An equal volume of chloroform: methanol (2:1, volume) was added to the aqueous phase to precipitate proteins. The solution was centrifuged and the resulting chloroform: methanol layer was removed by a 100 ml syringe. The chloroform: methanol extraction was repeated three times, and the fractions were combined and dried in vacuo. To the residue was added ether: methanol: water (1:1:1, pH = 3.0, adjusted by HCl). The aqueous phase was collected, dried, dissolved in methanol and stored in a cold room. Since the quantity of the vasoactive substance extracted is unknown, we designated this extract as 1000 ml equivalent of plasma (ml eq.).

Chromatography and Bioassay One thousand ml eq. of crude plasma extract was condensed to 1 ml volume in methanol. In order to evaluate the quality of vasoconstrictor extraction 10 µl of the extract was injected onto the reversed-phase column and eluted with a 10% methanol-water mobile phase at a rate of

1 - 2 ml/min. These prebioassay chromatograms were obtained without using the rat heart.

The coronary circulation of excised rat heart was connected to the perfusion system shown in figure 1. The heart was perfused with oxygenated Tyrode's solution at constant flow (9 ml/min). Before starting any measurements, the heart was perfused for 20 minutes to remove residual plasma.

One thousand ml eq. of crude extract was completely dried, freed from methanol, and dissolved in 1 ml of distilled water. Ten µl of this methanol-free extract solution was injected onto the reversed

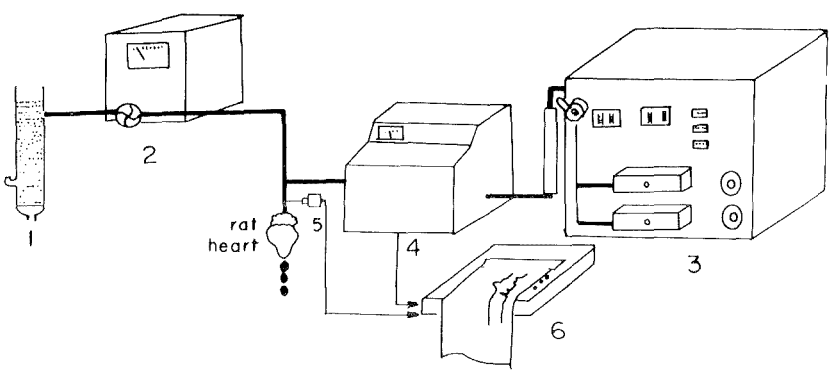

Figure 1. A schematic presentation of a rat heart bioassay system utilizing HPLC. Bubbled Tyrode's solution 38°C (1) was perfused into a rat heart by a peristaltic pump (2). The effluent from the HPLC (3) was also infused into the heart. The perfusion pressure measured by a pressure transducer (5) and UV absorption measured by a UV-visible detector (4) were recorded on a dual channel recorder (6).

phase column and eluted with distilled water at 1 ml/min. The effluent was directly infused into the perfused heart. The pressure change in the coronary circulation resulting from the eluting substance was detected by the pressure transducer, and UV absorption due to the eluting substance was recorded simultaneously.

RESULTS AND DISCUSSION

Evaluation of Chemical Extraction The vasoconstrictor component of plasma extract was separated by reversed phase HPLC column. A solvent system of 10% methanol and 90% water produced the most efficient separation. A representative chromatogram obtained by the above described methodology is shown in figure 2. As shown in the figure, two major peaks are found in the early period of elution and approximately 15 peaks were detected, indicating that the crude plasma extract contains at least 15 substances. Some variability was observed in the profile of the chromatogram among different extract preparations; however, the major features of the chromatogram were consistent. If the features were substantially different from the ones shown in figure 2, that extraction sample was discarded.

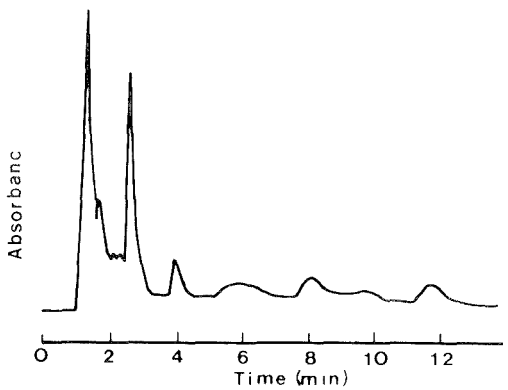

Figure 2. Chromatography of the crude plasma extract using 10% methanol and 90% water. Experimental details as described in Methods.

Although a solvent system of 10% methanol and 90% water produced the most efficient separation, because of the toxic nature of methanol this solvent system was not used for the bioassay experiment. Since a pure water solvent system produced a similar elution pattern to the methanol-water system, we used pure water for the HPLC-bioassay system.

Rat heart-HPLC Bioassay In order to determine which peaks represented the vasoactive fractions we directly infused elution solution from the reversed phase HPLC (mobile phase H_2O) to the perfusing line of the coronary circulation of the rat heart. A chromatogram and a pressure response from a typical experiment are shown in figure 3. In this particular

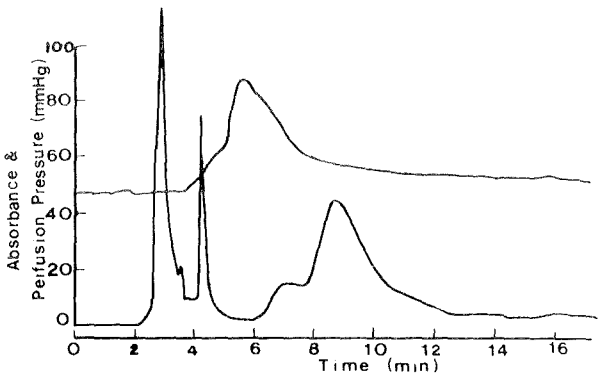

Figure 3. A typical bioassay experiment showing the pressure response (upper trace) and HPLC chromatogram (lower trace) of the crude plasma extract. Experimental details as described in Methods.

experiment, the total flow was 9 ml/min (8 ml/min of Tyrode's solution and 1 ml/min of elution solution). Although there was some possible development of ischemic conditions, the heart functioned as an assay organ throughout the course of the experiment.

Three groups of peaks were observed in this particular chromatogram when 10 µl (10 ml eq.) of plasma extract was injected. The perfusion pressure before constriction was 46 mmHg. With the elution of the various fractions, a pressure increase to a maximum of 86 mmHg was observed. This level of pressure change was sufficient to reliably locate the active component in the HPLC elution profile. Although only one peak is observed in the pressure

tracing, a close inspection of the pressure tracing shows a stepwise increase in the perfusion pressure. The stepwise increase in the pressure indicates the existence of more than one active substance in the plasma extract. Since the recorder pens for the pressure and chromatogram are one half minute out of phase, the first group of peaks on the chromatogram is related to the initial pressure increase and the second large peak to the second pressure increase. Apparently the third group of peaks yields no pressure change.

We performed six perfusion experiments using plasma extracts obtained from different extract preparations. The pressure peak was produced at 5.13 ± 0.26 min (S.D., $n = 6$). This information enabled us to determine the particular peak in the chromatogram which is related to the coronary vasoconstriction. Similarly, we could conclude that the first small pressure increase was produced by the substance which was eluted right after the void volume.

While the first and second peaks on the chromatogram eluted relatively quickly, the pressure response lasted approximately 5 minutes. The slow return toward preconstriction pressure level is pos-

sibly intrinsic in the nature of the coronary circulation system. One may note that the perfusion pressure after the constriction never returned to the preconstriction pressure. This effect may be attributed to the absence of the stabilizing influence of large-molecule substances (i.e. proteins) lacking in the perfusate. Due to edema formation in the heart, response to the plasma fraction gradually diminished. In general the heart functioned as an assay organ for approximately two hours.

If the HPLC effluent fractions were collected from the outlet of the HPLC column and assayed subsequently for biological activity, 30 to 50 runs might be required to locate the active component. Since each individual biological test by direct injection to the coronary circulation system (instead of using this on line assay system) takes 5 minutes, at least 150 minutes of testing time is required to do the task. In addition to this testing time, each fraction has to be collected, dried and redissolved into a small volume of solution for the bioassay. Because each additional step in the collection and purification process causes additional losses of the compound, a larger amount of sample has to be extracted to start with. Therefore, the

total time required to identify the active fraction by individual testing methods becomes enormous. The same work can be done on-line within 15 minutes in the system reported herein. This HPLC and rat heart bioassay system saves considerable time, effort and expense without compromising accuracy in the detection of the active fraction of plasma borne vasoconstrictor.

Finally studies to establish the true identity of the two vasoconstrictive factors are underway at present. The preliminary results suggest that the vasoactive substances are heat stable, small molecules and are not commonly known vasoactive substances; however, the characterization of the exact nature of the substance will require further research.

ACKNOWLEDGMENTS

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REFERENCES

1. Wurzel, M., On a Vasoactive Peptide in Rabbit Serum, Arch. Intern. Pharmacodyn., 143, 550 (1963).

2. Nayler, W.G., Robertson, P.G.C., Price, J.M., and Lowe, T.B., Some Properties of a Cardioactive

Substance Isolated from Human Plasma, Circ. Res., 16, 539 (1965).

3. DeWardener, H.D., and MacGregor, G.A., Dahl's Hypothesis that a Saluretic Substance may be Responsible for a Sustained Rise in Arterial Pressure: Its Possible Role in Essential Hypertension, Kidney International, 18, 1 (1980).

4. Bricker, N.S. and Light, A., Natriuretic Hormone: Biological Effects and Progress in Identification and Isolation, Hormonal Regulation of Sodium Excretion, P. 399 (1980).

5. Bohr, D.F. and Johanson, B., Contraction of Vascular Smooth Muscle in Response to Plasma Comparison with Response to Known Vasoactive Agents, Circ. Res. 19, 593 (1966).

6. Stainsby, W., Autoregulation in Skeletal Muscle, Circ. Res. Sup I 14,15, 39 (1964).

7. Bohr, D.F. and Sobieski, J., A Vasoactive Factor in Plasma, Fed. Proc., 27-6, 1396 (1968).

8. Bohr, D.F. and Verrier, R.L. and Sobieski, J., Non-neurogenic Tone Isolated Perfused Resistance Vessels, Circ. Res. Sup. I 28, 59 (1971).

9. Moretti, R.L., Abraham, S. and Echer, R.R., The Stimulation of Cardiac Prostaglandin Production by Blood Plasma and Its Relationship to the Regulation of Coronary Flow in Isolated Isovolumic Rabbit Heart, Circ. Res., 39-2, 231(1976).

10. Moretti, R.L., and Abraham, S., Stimulation of Microsomal Prostaglanding Synthesis by a Blood Plasma Constituent which Augments Autoregulation and Maintenance of Vascular Tone in Isolated Rabbit Hearts, Circ. Res. 42-3, 317 (1978).

11. Moretti, R.L. and Abraham, S., Stimulation of Microsomal Prostaglandin Synthesis by a Vasoactive Material Isolated from Blood Plasma, Prostaglandins, 15, 603 (1978).

12. Hazeyama, Y., and Moretti, R.L., Comparison of a Blood-Borne Vasoconstrictor with Other Vasoactive Agents. Fed. Proc. 39, 383 (1980).

13. Sparks, H.V. and Belloni, F.L., The Peripheral Circulation: Local Regulation. Physiol. Rev. 40, 67 (1978).

14. Shibata, S., Ho, W.K.W., Ishida, U., Possible Generation of Potent Vasoconstricting Substance From Prostaglandin Endoperoxide Analogs, Human Platelets Mixture. J. Pharm. Exp. Therap. 215, 357 (1980).

15. Gunther, S. and Cannon, P.J., Modulation of Angiotensin II Coronary Vasoconstriction by Cardiac Prostaglandin Synthesis, Am. J. Physiol. 238, H895 (1980).

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STUDIES ON STEROIDS CLXXVIII. SEPARATION OF ESTROGEN GLUCURONIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Separation of monoglucuronides of estrone, estradiol, estriol and 16-epiestriol by high-performance liquid chromatography on a reversed-phase column has been carried out. The effects of pH and salt concentration of a mobile phase on the k' value were investigated with a TSK GEL LS-410 ODS-SIL column. Isomeric monoglucuronides of estriol and 16-epiestriol were distinctly separated on this column when 0.7% disodium hydrogen phosphate (pH 3.0)/tetrahydrofuran was used as a mobile phase.

INTRODUCTION

In recent years, considerable attentions have been drawn to the metabolic significance and physiological role of estrogen conjugates in the feto-placental unit. These metabolites are determined by spectrophotometry (1), gas chromatography-mass spectrometry (2,3), or radioimmunoassay (4). Several papers have been recently published dealing with the method for quantitative determination of estrogens in biological fluids by

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high-performance liquid chromatography (HPLC) which involves prior hydrolysis and/or solvolysis of the conjugates (5-8). These methods, however, have inevitable disadvantages, the lack of reliability on analytical results and the loss of information about the conjugated form. This paper describes the separation of monoglucuronides of classical estrogens, i.e. estrone, estradiol, estriol and 16-epiestriol, by HPLC.

EXPERIMENTAL

Materials

Estrogen glucuronides were synthesized in these laboratories by the methods previously reported (9). All the reagents used were of analytical reagent grade. Solvents were purified by distillation prior to use.

Instruments

The apparatus used for this work was a Toyo Soda HLC-803A high-performance liquid chromatograph (Toyo Soda Co., Tokyo) equipped with a Model SF-770 ultraviolet (UV) detector monitoring the absorbance at 280 nm. A TSK GEL LS-410 ODS-SIL column (30 cm x 0.4 cm i.d.) (Toyo Soda Co.) was employed under ambient conditions. The pH of the mobile phase was adjusted with phosphoric acid.

RESULTS AND DISCUSSION

The separation of estrogen glucuronides by HPLC has been previously reported by several groups. However, the resolution of estriol 16-glucuronide and estriol 17-glucuronide still remains unsatisfactory (10-12). An initial effort was therefore directed to the separation of these two conjugates. Among the typical columns suitable for the polar compounds, TSK GEL LS-410 ODS-SIL was chosen for this purpose.

The effect of pH of a mobile phase on the capacity ratio (k') was investigated with the 0.7% disodium hydrogen phosphate /tetrahydrofuran system. The k' values of typical estrogen glucuronides relative to estriol 17-glucuronide were plotted against pH of the mobile phase (Figure 1). The close similarity in the chromatographic behaviors was observed between estriol 16-glucuronide and estriol 17-glucuronide in the pH range 5.0 to 7.0. The relative k' value of estriol 16-glucuronide increased with decreasing pH value from 5.0 to 3.0. A similar relation was also found between 16-epiestriol 16-glucuronide and 16-epiestriol 17-glucuronide. This phenomenon can be explained

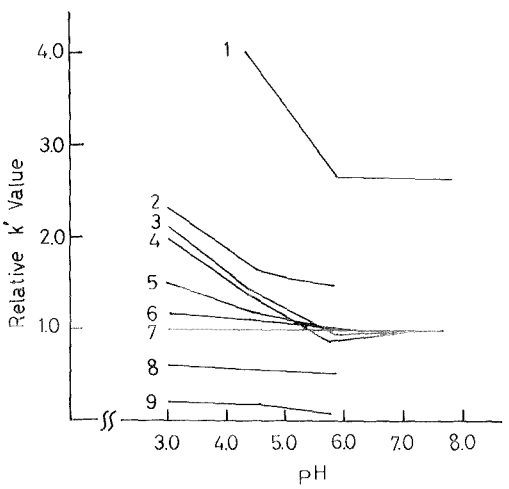

FIGURE 1 Effect of pH on Relative k' Value.
1, Estradiol 17-glucuronide; 2, 16-epiestriol 16-glucuronide; 3, estradiol 3-glucuronide; 4, estrone 3-glucuronide; 5, 16-epiestriol 17-glucuronide; 6, estriol 16-glucuronide; 7, estriol 17-glucuronide; 8, 16-epiestriol 3-glucuronide; 9, estriol 3-glucuronide. Conditions: column, TSK GEL LS-410 ODS SIL; mobile phase, 0.7% Na_2HPO_4/tetrahydrofuran (6:1), 2 ml/min; detection, UV 280 nm.

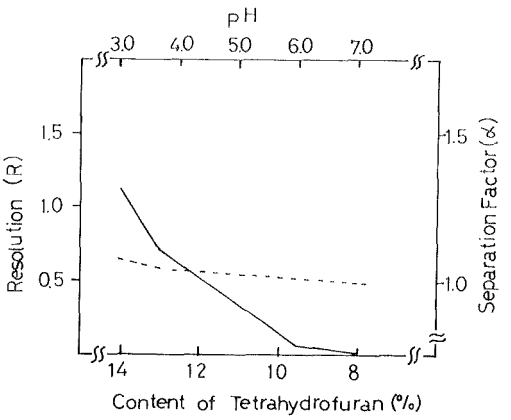

FIGURE 2 Effect of pH and Composition of Mobile Phase on the Separation of Estriol 16- and 17-Glucuronides. ——:Resolution, ---:separation factor. Conditions: mobile phase, 0.7% Na_2HPO_4/tetrahydrofuran. Other conditions were as in Fig. 1.

in terms of dissociation of steroid glucuronides having a glucuronic acid moiety (pK 3.20) in acidic medium where undissociated species are dominant. The effects of pH and composition of the mobile phase on the resolution of estriol 16-glucuronide and estriol 17-glucuronide were also observed at the constant k' value (Figure 2). The separation factor (α) was not significantly influenced, while the resolution (R) was improved with decreasing pH and increasing content of tetrahydrofuran of the mobile phase. These data suggested that the separation of these compounds is considerably dependent upon the pH value of the mobile phase.

The effect of salt concentration on the retention value was then examined by using aqueous disodium hydrogen phosphate (pH 3.0)/tetrahydrofuran as a mobile phase. The k' values of three glucuronides relative to the corresponding value of each glucuronide obtained with 0.4% disodium hydrogen phosphate (pH

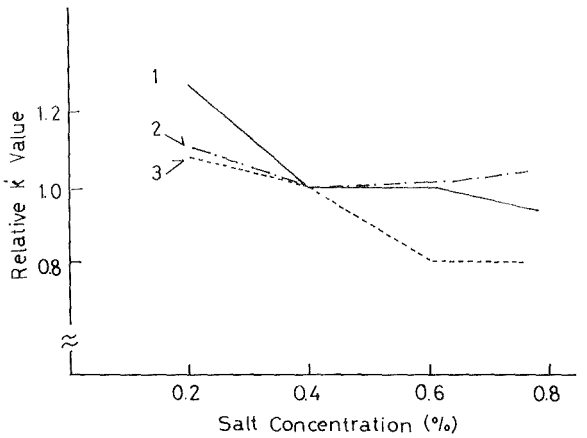

FIGURE 3 Effect of Salt Concentration of Mobile Phase on Relative k' Value of Estriol Monoglucuronides. 1, Estriol 17-glucuronide; 2, estriol 16-glucuronide, 3, estriol 3-glucuronide. Conditions: mobile phase, Na_2HPO_4 (pH 3.0)/ tetrahydrofuran (6:1). Other conditions were as in Figure 1.

3.0) were determined (Figure 3). No remarkable difference in the retention value was found between estriol 16-glucuronide and estriol 17-glucuronide in the concentration range 0.4-0.6%, but the relative k' value of estriol 17-glucuronide slightly decreased with increasing salt concentration from 0.6 to 0.8%. Also estriol 3-glucuronide showed a slight decrease in the k' value with increasing salt concentration.

Based upon these data, 0.7% disodium hydrogen phosphate (pH 3.0)/tetrahydrofuran (6:1) was chosen as a suitable mobile phase. A synthetic mixture of 3-, 16- and 17-glucuronides of estriol and 16-epiestriol were efficiently separated by using the above solvent system (Figure 4). To the best of our knowledge this is the first reported complete separation of isomeric monoglucuronides of estriol and 16-epiestriol by HPLC. Under this condition estradiol 17-glucuronide required a long

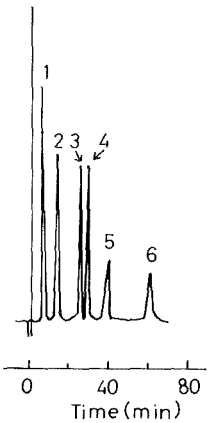

FIGURE 4 Separation of a Synthetic Mixture of Estrogen Monoglucuronides.
1, Estriol 3-glucuronide; 2, 16-epiestriol 3-glucuronide; 3, estriol 17-glucuronide; 4, estriol 16-glucuronide; 5, 16-epiestriol 17-glucuronide; 6, 16-epiestriol 16-glucuronide.
Conditions: mobile phase, 0.7% Na_2HPO_4 (pH 3.0)/tetrahydrofuran (6:1), 1.5 ml/min. Other conditions were as in Figure 1.

time for elution (Figure. 1), but this problem was overcome by using 0.4% disodium hydrogen phosphate (pH 7.5)/methanol (2:1) as a mobile phase.

The application of the present method to the separation of estriol 16-glucuronide and estriol 17-glucuronide in rat bile and human pregnancy urine (13) will be reported elsewhere in the near future.

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REFERENCES

1. Bauld, W.S.,Errors in the colorimetric estimation of estriol, estrone and estradiol by the Kober reaction, Biochem. J., 56, 426 (1954).

2. Thompson, R.M., Gas chromatographic and mass spectrometric analysis of permethylated estrogen glucuronides, J. Steroid Biochem., 7, 845 (1976).

3. Miyazaki, H., Ishibashi, M., Itoh, M., Morishita, N., Sudo, M., and Nambara, T., Analysis of estrogen glucuronides. I. Characterization of estrogen glucuronides by gas chromatography mass spectrometry, Biomed. Mass Spect., 3, 55 (1976).

4. Numazawa, M., Tanaka, T., and Nambara, T., Determination of estrogen ring D glucuronides in pregnancy plasma by direct radioimmunoassay without hydrolysis, Clin. Chim. Acta, 91, 169 (1979).

5. Huber, J.F.K., Hulsman, J.A.R.J., and Meijers, C.A.M., Quantitative analysis of trace amounts of estrogenic steroids in pregnancy urine by column liquid-liquid chromatography with ultraviolet detection, J. Chromatogr., 62, 79 (1971).

6. Dolphin, R.J., The analysis of estrogenic steroids in urine by high-speed liquid chromatography, J. Chromatogr., 83, 421 (1973).

7. Dolphin, R.J. and Pergande, P.J., Improved method for the analysis of estrogenic steroids in pregnancy urine by high-performance liquid chromatography, J. Chromatogr., 143, 267 (1977).

8. Hiroshima, O., Ikenoya, S., Ohmae, M., and Kawabe, K., Electrochemical detector for high-performance liquid chromatography III. Determination of estriol in human urine during pregnancy, Chem. Pharm. Bull. (Tokyo), 28, 2512 (1980).

9. Nambara, T., Kawarada, Y., Shibata, K., and Abe, T., Studies on steroid conjugates IX. New synthesis of estriol 16- and 17-monoglucuronides, Chem. Pharm. Bull. (Tokyo), 20, 1988 (1972).

10. Van der Wal, Sj. and Huber, J.F.K., High-pressure liquid chromatography with ion-exchange celluloses and its application to the separation of estrogen glucuronides, J. Chromatogr., 102, 353 (1974).

11. Musey, P.I., Collins, D.C., and Preedy, J.R.K., Separation of estrogen conjugates by high pressure liquid chromatography, Steroids, 31, 583 (1978).

12. Hermansson, J., Separation of steroid glucuronides by reversed-phase liquid column chromatography, J. Chromatogr., 194, 80 (1980).

13. Nambara, T. and Kawarada, Y., Biliary conjugated metabolites of estriol in the rat, Chem. Pharm. Bull. (Tokyo), 25, 942 (1977).

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A COMPUTER PROGRAM FOR THE IDENTIFICATION OF THE ELUTION ORDER OF PEAKS IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY[1]

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INTRODUCTION

High performance liquid chromatography (HPLC) is a rapidly growing separation technique. Its popularity is due to its relatively low cost, selectivity, ease of operation and the ability to separate different compounds (structurally and chemically), of large and small molecular size. The results can be quantified quickly and easily.

The most difficult aspect of HPLC is the selection of a mobile phase that gives good resolution of the components of a mixture in a reasonable time, with optimum separation. Recently, many research papers have suggested a systematic approach to mobile phase selection for the optimum separation of a mixture, by using statistical methods of analysis. Glajch et al (1) and Issaq et al (2) have used such a technique based on the work of Snee (3) which employed overlaping resolution mapping for the selection of a mobile phase that would give optimal separation. To establish such a mobile phase, the analyst should run 7-10 experiments using different solvent combinations of three

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TABLE 1

RATIO OF SOLVENT COMBINATIONS USED

Experiment #	1	2	3	4	5	6	7	8	9	10
% Solvent A	100	0	0	50	50	0	33	20	20	60
% Solvent B	0	100	0	50	0	50	33	60	20	20
% Solvent C	0	0	100	0	50	50	33	20	60	20

Note: Other solvent combinations may be used; the analyst needs 10 data points. The ratios can be different from those above.

organic solvents (Table 1). The solvents selected, based on Synder's solvent Synder's solvent selectivity triangle (4), should have different chemical properties in order to give different selectivities of the solutes. Belinky (5), on the other hand, used 17 combinations of three solvents to achieve an optimum mobile phase. Laub (6) used the window technique, which was developed for the selection of GC mixed phases, and which would require a minimum of 10 experiments to achieve an optimum mobile phase if three different solvents were used. Others (7-9) have used statistical approaches which in the end led to an isocratic mobile phase that efficiently separated the components of the mixture under study.

Finding a mobile phase that would separate the components of a mixture using statistical, or any other, technique can be time consuming because, in each of the experiments, solvents of different selectivities are used, and this can lead to a different elution orders of the components, (ref. 2 gives a good example). This means that the analyst would have to identify each eluted peak at the end of every experiment. Assuming that a mixture is composed of six components, and that they elute off the column in each of the 10 required experiments in an average of 15 min, it would take a chromatographer approximately 12-15 hours or 2 days to identity the peaks in all 10 experiments.

DuPont (10) have developed the Sentinel System whereby eluted peaks are identified, if there is no peak crossovers, by a statistical technique. The company has not released this information. If peak crossover takes place, standard solutions should be injected and elution times compared to identify the composition of the peaks. In this case, the analyst needs to know if peak crossover takes place, which is difficult to ascertain unless standards are injected and eluted. James (11) described wavelength chromatography for the identification of eluted components, which is based on a multiwavelength monitoring system employing a diode array spectrophotometer. This is undoubtedly an accurate and rapid method, which also requires the use of a computer, and that the solutes should absorb light in the 200-800nm range.

We present here a computer program (Appendix 1) which can identify the peak's elution order, taking into consideration (a) peak reversal; (b) peaks coalescing, i.e. six peaks in one run but five peaks or less in another; and (c) peak splitting, i.e. four peaks in one mobile phase and six in another. The peak elution order identification is based on one final run of the standards. The peaks are identified by the area ratio of each peak compared with the other eluted peaks in that experiment. It is assumed that the peaks are Gaussion and symmetrical, with no peak frontage or tailing. Since absorption is a function of the number of molecules present, the shape of the peak due to diffusion should not affect the area under the peaks. In HPLC the peak which elutes first is sharper than it would be if it eluted last because of diffusion. It is also possible that the extinction coefficient of a compound can change from one solvent to another. Since mixtures of solvents are used this phenomenon is minimized (Table 2).

This computer program is also suited for use with radiolabeled compounds and their metabolites, since the number of counts is directly proportional to the amount of radioactivity. A selected hypothetical example will be presented which illustrates the value of this computer program.

TABLE 2

Effect of Mobile Phase Composition on the % Peak Areas of Anthraquinone (A), Methylanthraquinone (MA), and Ethylanthraquinone (EA)

	A (%)	MA (%)	EA (%)
$CH_3CN:H_2O$ / 64:36	22.5	40.6	36.9
$CH_3OH:H_2O$ / 75:25	22.2	41.1	36.7
$THF:H_2O$ / 45:55	23.6	40.7	35.7
$CH_3OH:CH_3CN:H_2O$ / 38:32:30	22.4	40.8	36.7
$CH_3OH:THF:H_2O$ / 38:22:40	22.8	41.0	36.2
$CH_3CN:THF:H_2O$ / 32:22:36	22.7	40.8	36.5
$CH_3OH:CH_3CN:THF:H_2O$ / 25:21:15:39	23.3	40.7	36.0
$CH_3CN:THF:CH_3OH:H_2O$ / 42:7.5:12.5:38	23.2	40.0	36.8
$CH_3CN:THF:CH_3OH:H_2O$ / 11:30:12.5:46.5	23.4	41.0	35.6
$CH_3CN:THF:CH_3OH:H_2O$ / 11:7.5:50:31.5	23.1	40.6	36.3
Mean ± Standard Deviation:	22.9 ± 0.5	40.7 ± 0.3	36.3 ± 0.5

EXPERIMENTAL

Materials: Solvents were glass distilled (Burdick and Jackson). Chemicals were analytical grade (Aldrich Chemical Co.) and used without further purification.

Apparatus: A modular HPLC system consisting of Laboratory Data Control (LDC) Constametric I and II pumps attached to an LDC Gradient Master, a Chromatronix dual-channel uv absorbance detector, Rheodyne injector, and a strip-chart recorder operated at 0.2 in/min. was used.

The RP-18 reversed phase column (Merck) was 250 mm x 4.6 mm prepacked with 10 µm particle size materials. 10 µl samples were injected. Experiments were run at room temperature using a mobile phase flow rate of 1.2 ml/min. Retention times, peak areas, and peak area percent were determined with a Hewlett-Packard

1865 A/D converter connected to the UV detector output of the liquid chromatograph. The output from the data system was recorded on a 9866A thermal line printer (Hewlett-Packard).

COMPUTER PROGRAM

The PKSEP program which identifies peaks by peak area percent is written in Basic for a Hewlett Packard 3354 Lab Data System. This system uses a model 2100 CPU with 32 K of core. Currently, the program allows for as many as 12 runs with up to 15 peaks in each run, storing retention times, peak area percents and results of the analysis for each run.

Eluted peaks are identified by comparing the peak area percents of the trial run peaks to those of a standard run. Peak area percents are computed automatically by the HP 3354 Lab Data System. Currently the retention times and area percent for each peak are entered in the program, but the program can be modified to accept the retention time and area percent from the processed data files of each LC analysis.

The run with the most peaks is chosen as the standard. If there are several runs to choose from, the last one entered is used unless the user selects another. The selected run must have the maximum number of peaks.

The analysis for each trial run is as follows. For each trial peak, the standard peak or sum of two peaks which best match the trial peak area percent is chosen. Peak summing is not used at this point if the trial run has the same number of peaks as the standard run. If a standard peak is chosen which defined a previous peak, both trial peak definitions are rechecked and only the better area percent match is kept. If the trial run has the same number of peaks as the standard, standard peaks are not summed in this initial selection. If the area percent of the standard selected differs from the trial by more than 10%, a warning message is printed. Mis-matches of this size will most probably be resolved in the identification of later trial peaks. This initial selection produces no peak identification conflicts, but all trial and standard

peaks may not have been matched. All unused trial peaks are then compared against all unused standards and the best available area percent fit is used, if the areas match by 90% or more. Note that not all standard peaks are forced to match a trial peak due to this 90% requirement. All selected peaks, warning messages and re-defined standards are printed out during the analysis.

A summary report of each trial run in elution order of the standard run is printed out, showing the corresponding trial peak number, retention time and area %, or a message is given to indicate that the standard peak cannot be identified.

Finally a summary table giving the elution order for each run is printed out.

A HYPOTHETICAL EXAMPLE

A mixture containing seven components was selected as an example. The output is listed as follows:

```
ENTER RT, AREA % FOR EACH PEAK FROM RUN 1
  END WITH -1,-1
:? 1.1,6%
:? 2.7,4%
:? 11,20%
:? 21.4,15%
:? 23.7,14%
:? 34,21%
:? 37,19%
? -1,-1

ENTER RT, AREA % FOR EACH PEAK FROM RUN 2
  END WITH -1,-1
:? 1.1,20%
:? 11.2,30%
:? 22.3,10%
:? 35.7,40%
:? -1,-1

ENTER RT, AREA % FOR EACH PEAK FROM RUN 3
  END WITH -1,-1
:? 1,10%
:? 12,19.5%
:? 23.2,14.9%
:? 24.7,14.1%
:? 35.7,40%
:? -1,-1
```

```
ENTER RT, AREA % FOR EACH PEAK FROM RUN 4
  END WITH -1,-1
:? 1,5.9%
:? 2.4,4.1%
:? 11.3,30.1%
:? 23.1,14.8%
:? 27.3,14.1%
:? 35.2,20.9%
:? 37.8,19.1%
:? -1,-1

ENTER RT, AREA % FOR EACH PEAK FROM RUN 5
  END WITH -1,-1
:? 1,5.8%
:? 2.1,4.2%
:? 11.1,20.1%
:? 13.4,14.9%
:? 15.8,14.1%
:? 24.7,21.9%
:? 31.2,18.6%
:? -1,-1

ENTER RT, AREA % FOR EACH PEAK FROM RUN 6
  END WITH -1,-1
:? 1,48%
:? 2.4,6%
:? 13.2,15%
:? 21.7,14%
:? 25.7,17.4%
:? -1,-1

ENTER RT, AREA % FOR EACH PEAK FROM RUN 7
  END WITH -1,-1
:? 1,10%
:? 2.3,20%
:? 3.5,30%
:? 4.8,40%
:? -1,-1
```

These are the seven required runs to perform an optimum solvent mixture analysis using seven different mobile phases. The next step is the analysis of the areas in these seven runs to determine which area belongs to which peak/peaks.

Run # 1 is selected as the standard; it has the maximum number of peaks. The computer will identify the peaks in the remaining six runs as follows:

```
ANALYSIS FOR RUN #2
    PEAK #1     - 20     STD #3     - 20
    PEAK #2     - 30     STD #4     - 15     + #5     - 14     = 29
    PEAK #3     - 10     STD #1     - 6      + #2     - 4      = 10
    PEAK #4     - 40     STD #6     - 21     + #7     - 19     = 40
```

```
ANALYSIS FOR RUN #3
    PEAK #1      - 10      STD #1      - 6      + #2      - 4      = 10
    PEAK #2      - 19.5    STD #7      - 19
    PEAK #3      - 14.9    STD #4      - 15
    PEAK #4      - 14.1    STD #5      - 14
    PEAK #5      - 40      STD #6      - 21     + #7      - 19     = 40
   *** REDEFINITION OF STD  7      ***
    PEAK #2       MAY BE   STD #3


ANALYSIS FOR RUN #4
    PEAK #1      - 5.9     STD #1      - 6
    PEAK #2      - 4.1     STD #2      - 4
    PEAK #3      - 30.1    STD #6      - 21
   ***PEAK AREAS DIFFER BY >10% ***
    PEAK #4      - 14.8    STD #4      - 15
    PEAK #5      - 14.1    STD #5      - 14
    PEAK #6      - 20.9    STD #6      - 21
   *** REDEFINITION OF STD  6      ***
    PEAK #7      - 19.1    STD #7      - 19


ANALYSIS FOR RUN #5
    PEAK #1      - 5.8     STD #1      - 6
    PEAK #2      - 4.2     STD #2      - 4
    PEAK #3      - 20.1    STD #3      - 20
    PEAK #4      - 14.9    STD #4      - 15
    PEAK #5      - 14.1    STD #5      - 14
    PEAK #6      - 21.9    STD #6      - 21
    PEAK #7      - 18.6    STD #7      - 19


ANALYSIS FOR RUN #6
    PEAK #1      - 48      STD #3      - 20     + #6      - 21     = 41
   *** PEAK MAY BE SUM OF 3 OR MORE STDS ***
    PEAK #2      - 6       STD #1      - 6
    PEAK #3      - 15      STD #4      - 15
    PEAK #4      - 14      STD #5      - 14
    PEAK #5      - 17.4    STD #2      - 4      + #5      - 14     = 18
   *** REDEFINITION OF STD  5      ***
    PEAK #5       MAY BE   STD #7


ANALYSIS FOR RUN #7
    PEAK #1      - 10      STD #1      - 6      + #2      - 4      = 10
    PEAK #2      - 20      STD #3      - 20
    PEAK #3      - 30      STD #4      - 15     + #5      - 14     = 29
    PEAK #4      - 40      STD #6      - 21     + #7      - 19     = 40
```

SUMMARY OF RESULTS

RUN # 2

STD PEAK #	PEAK #	RT	STD AREA %	PEAK AREA %
1 -1.1	3	11	6	10
2 -2.7	3	11	4	10
3 -11	1	1.1	20	20
4 -21.4	2	2.7	15	30
5 -23.7	2	2.7	14	30
6 -34	4	21.4	21	40
7 -37	4	21.4	19	40

RUN # 3

STD PEAK #	PEAK #	RT	STD AREA %	PEAK AREA %
1 -1.1	1	1.1	6	10
2 -2.7	1	1.1	4	10
3 -11	2	2.7	20	19.5
4 -21.4	3	11	15	14.9
5 -23.7	4	21.4	14	14.1
6 -34	5	23.7	21	40
7 -37	5	23.7	19	40

RUN # 4

STD PEAK #	PEAK #	RT	STD AREA %	PEAK AREA %
1 -1.1	1	1.1	6	5.9
2 -2.7	2	2.7	4	4.1
3 -11	- PEAK NOT IDENTIFIED			
4 -21.4	4	21.4	15	14.8
5 -23.7	5	23.7	14	14.1
6 -34	6	34	21	20.9
7 -37	7	37	19	19.1

RUN # 5

STD PEAK #	PEAK #	RT	STD AREA %	PEAK AREA %
1 -1.1	1	1.1	6	5.8
2 -2.7	2	2.7	4	4.2
3 -11	3	11	20	20.1
4 -21.4	4	21.4	15	14.9
5 -23.7	5	23.7	14	14.1
6 -34	6	34	21	21.9
7 -37	7	37	19	18.6

RUN # 6

STD PEAK #	PEAK #	RT	STD AREA %	PEAK AREA %
1 -1.1	2	2.7	6	6
2 -2.7	- PEAK NOT IDENTIFIED			
3 -11	1	1.1	20	48
4 -21.4	3	11	15	15
5 -23.7	4	21.4	14	14
6 -34	1	1.1	21	48
7 -37	5	23.7	19	17.4

```
RUN # 7
STD PEAK #      PEAK #          RT          STD AREA %      PEAK AREA %
1      -1.1          1          1.1          6               10
2      -2.7          1          1.1          4               10
3      -11           2          2.7          20              20
4      -21.4         3          11           15              30
5      -23.7         3          11           14              30
6      -34           4          21.4         21              40
7      -37           4          21.4         19              40
```

Note that in the analysis of run #3, standard peak 7 was selected as a match to peak 2, then selected again as matching peak 5. Standard peak 3 was not initially selected as matching any trial peak, but was matched to peak 2 when testing all unidentified peaks. However in run #4, standard peak 6 was matched first to peak 3, then to peak 6, and standard, and trial, peak 3 are left unidentified even after testing all unmatched peaks, and is recorded as -1, which means that that peak has not been identified. In this case, the chromatographer will have to use other means to identify that peak.

Currently the program sums only two standard peaks if summing is required. The program can be modified to search for the sum of more than two peaks. If the summed area percent is 10% less than the trial peak area percent, a message such as the one in run #6 is printed, indicating that three or more standard peaks may be represented in the trial peak area.

Finally, the computer will list the summary of the above analysis, in table form, (Table 3).

TABLE 3

```
                        RUN #
STD #  1     2     3     4     5     6     7     8     9     10

1      1     3     1     1     1     2     1
2      2     3     1     2     2     -1    1
3      3     1     2     -1    3     1     2
4      4     2     3     4     4     3     3
5      5     2     4     5     5     4     3
6      6     4     5     6     6     1     4
7      7     4     5     7     7     5     4
BASIC
>
```

CONCLUSION

A computer program has been written which can identify the peak elution order according to their area percent.

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REFERENCES

1. Glajch, J.L., Kirkland, J.J. and Squire, K.M., J. Chromatogr. 199, 57 (1980).

2. Issaq, H.J., Klose, J.R., McNitt, K.L., Haky, J.E. and Muschik, G.M., J. Liquid Chromatogr. 4, 2091 (1981).

3. Snee, R.D., Chem. Tech. 9, 702 (1979).

4. Snyder, L.R., J. Chromatogr. Sc. 92, 223 (1974).

5. Belinky, B.R., Analytical Technology and Occupational Health Chemistry, ACS Symposium Series, Volume 220, pp 149-168, American Chemical Society, Washington, DC, 1980.

6. Laub, R.J., Am. Laboratory 13(3), 47 (1981).

7. Lindberg, W., Johansson, E. and Johansson, K., J. Chromatogr. 211, 201 (1981).

8. Guiochon, G. and Colin, H., Pittsburgh Conf. on Anal Chem. and Appl. Spectrosc., Atlantic City, 1981.

9. Sachok, B., Kong, R.C. and Deming, S.N., J. Chromatogr. 199, 317 (1980).

10. Sentinel System, DuPont Technical Report, USA, 1981.

11. James, G.E., Hewlett Packard, Washington, DC. Publication No. 23-5943-4792 (1981).

APPENDIX - Program Listings

```
10  DIM A[180],T[180],P[180],N[12]
20  DEF FNZ(X)=INT(100*X+.5)/100
30  PRINT
40  PRINT "PEAK IDENTIFICATION BY AREA PROGRAM - 4/82"
50  PRINT
60    FOR I=1 TO 180
70    LET P[I]=-1
80    NEXT I
90  LET M=0
100  LET I=1
110  LET N1=0
120  LET T2=0
130  PRINT
140  PRINT "ENTER RT, AREA % FOR EACH PEAK FROM RUN ";I
150  PRINT "  END WITH -1,-1"
160    FOR J=1 TO 15
170    PRINT ":";
180    INPUT T1,A1
185    LET K=(I-1)*15+J
190    IF T1+A1<0 THEN 290
210    IF T1>T2 THEN 240
220    PRINT "PEAKS OUT OF ORDER"
230    GOTO 110
240    LET T[K]=T1
250    LET T2=T1
260    LET A[K]=A1
270    LET N1=N1+1
280    NEXT J
290  IF N1<M THEN 320
300  LET M=N1
310  LET I1=I
320  IF N1<1 THEN 360
330  LET N[I]=N1
340  LET I=I+1
350  IF I<13 THEN  GOTO 110
360  LET N5=I-1
370  PRINT
```

```
 380  PRINT "RUN # ";I1;" USED AS STANDARD"
 390  PRINT "OKAY";
 400  INPUT N$
 410  IF N$[1,1]#"N" THEN 440
 420  PRINT "RUN # OF STD ";
 430  INPUT I1
 440  LET N2=N[I1]
 450  IF M=N2 THEN 480
 460  PRINT "↖ MAXIMUM # OF PEAKS IN RUN ";I1
 470  GOTO 420
>



 480  LET I9=(I1-1)*15
 490    FOR K=1 TO N2
 500    LET P[I9+K]=K
 510    NEXT K
 520  LET A[I9+N2+1]=0
 530    FOR K=1 TO N5
 540    IF K=I1 THEN 850
 550    LET K9=(K-1)*15
 560    PRINT
 570    PRINT "ANALYSIS FOR RUN #";K
 580    LET N1=N[K]
 590      FOR J=1 TO N1
 600      LET M=1000
 610      LET A2=A[K9+J]
 620        FOR I=1 TO N2
 630        LET K8=I+1
 640        IF N2=N1 THEN  LET K8=N2+1
 650          FOR I2=K8 TO N2+1
 660          GOSUB 1620
 670          NEXT I2
 680        NEXT I
 690      LET A8=A[I9+K1]
 700      LET A9=A[I9+K2]
 710      LET A1=A8+A9
 720      PRINT "    PEAK #";J;"- ";A2;TAB(25);
 730      IF K2>N2 THEN 770
 740      PRINT "STD #";K1;"- ";A8;"+ #";K2;"- ";A9;"= ";A1
 750      IF A2>1.1*A1 THEN  PRINT "   *** PEAK MAY BE SUM OF 3 OR MORE STDS ***"
 760      GOTO 790
 770      PRINT "STD #";K1;"- ";A[I9+K1]
 780      IF M/A2>.1 THEN  PRINT "   ***PEAK AREAS DIFFER BY >10% ***"
 790      GOSUB 1150
 800      IF F>0 THEN 830
 810      LET P[K9+K1]=J
 820      LET P[K9+K2]=J
 830      NEXT J
 840    GOSUB 1390
 850    NEXT K
>
```

```
  860  PRINT
  870  PRINT "SUMMARY OF RESULTS"
  880    FOR I=1 TO N5
  890    IF I=I1 THEN 1010
  900    PRINT
  910    PRINT "RUN # ";I
  920    PRINT "STD PEAK #     PEAK #           RT           STD AREA %   ";
  930    PRINT "   PEAK AREA %"
  940      FOR J=1 TO N2
  950      LET K8=(I-1)*15
  960      LET J1=P[K8+J]
  970      LET J2=I9+J
  980      IF J1<0 THEN  PRINT J;"-";FNZ(T[J2]);TAB(20);"- PEAK NOT IDENTIFIED"
  990      IF J1>0 THEN  PRINT J;"-";FNZ(T[J2]);TAB(20);J1,T[J1],A[J2],A[K8+J1]
 1000       NEXT J
 1010     NEXT I
 1020  PRINT
 1030  PRINT
 1040  PRINT "                          RUN #"
 1050  PRINT "STD #  1     2     3     4     5     6     7     8     9    10"
 1060    FOR I=1 TO N2
 1070    PRINT
 1080    PRINT I;
 1090      FOR J=1 TO N5
 1100      PRINT P[(J-1)*15+I];
 1110      NEXT J
 1120    NEXT I
 1130  STOP
>
```

```
 1140  REM **FIND REDEFINED PEAK - WHICH USE IS BETTER FIT
 1150  LET F=0
 1160  LET K8=N2+1
 1170  LET J1=P[K9+K1]
 1180  LET I2=K1
 1190  IF J1>0 THEN 1240
 1200  LET J1=P[K9+K2]
 1210  LET I2=K2
 1220  IF J1>0 AND K2 <= N2 THEN 1240
 1230  RETURN
 1240  PRINT "   *** REDEFINITION OF STD  ";I2;"***"
 1250    FOR K5=1 TO N2
 1260    IF K5=I2 THEN 1280
 1270    IF P[K9+K5]=J1 THEN  LET K8=K5
 1280    NEXT K5
 1290  LET A8=A[I9+I2]
 1300  LET A9=A[I9+K8]
 1310  LET A1=A8+A9
 1320  IF ABS(A1-A[K9+J1])<M THEN 1360
 1330  LET P[K9+I2]=-1
 1340  LET P[K9+K8]=-1
 1350  RETURN
 1360  LET F=1
 1370  RETURN
>
```

```
 1380  REM **FIND UNDEFINED PEAKS - DO ANY MATCH
 1390    FOR J=1 TO N1
 1400      FOR I=1 TO N2
 1410      IF P[K9+I]=J THEN 1590
 1420      NEXT I
 1430    LET M=1000
 1440    LET A2=A[K9+J]
 1450    LET P[K9+N2+1]=-1
 1460      FOR I=1 TO N2
 1470      IF P[K9+I]>0 THEN 1520
 1480        FOR I2=I+1 TO N2+1
 1490        IF P[K9+I2]>0 THEN 1510
 1500        GOSUB 1620
 1510        NEXT I2
 1520      NEXT I
 1530    IF M/A2>.1 THEN  RETURN
 1540    PRINT "    PEAK #";J;" MAY BE";TAB(25);
 1550    IF K2 <= N2 THEN  PRINT "STD #";K1;" + #";K2
 1560    IF K2>N2 THEN  PRINT "STD #";K1
 1570    LET P[K9+K1]=J
 1580    IF K2<N2+1 THEN  LET P[K9+K2]=J
 1590    NEXT J
 1600  RETURN
 1610  REM **DIFFERENCE IN AREA ROUTINE
 1620  LET A1=A[I9+I]+A[I9+I2]
 1630  LET D=ABS(A1-A2)
 1640  IF D>M THEN  RETURN
 1650  LET M=D
 1660  LET K1=I
 1670  LET K2=I2
 1680  RETURN
 1690  END
>
```

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(9), 1787-1799 (1982)

GEL CHROMATOGRAPHIC EVALUATION OF THE BINDING ABILITY OF LINEAR PHOSPHATE ANIONS TO MAGNESIUM IONS

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ABSTRACT

The binding ability of linear phosphate anions of various degrees of polymerization to magnesium ion has been evaluated by a zonal gel chromatographic method at pH 9.8 and 4.2. The average number of bound magnesium per one phosphate unit was determined at free magnesium concentration of 1.00×10^{-5} M in 0.1 M tetramethylammonium chloride solution at 25°C. At pH 4.2, the binding ability increased as the degrees of polymerization of the sample phosphate increased, while it decreased at pH 9.8. A gel chromatography-atomic absorption detector system was applied which allowed a rapid and continuous analysis.

INTRODUCTION

Linear phosphates (LP) of various degrees of polymerization have found widespread applications in industry because of their utility as a complexing agent. The binding characteristic of these homologous series of ligands is expected to be dependent on the numbers of phosphorus atoms which constitute the phosphate molecules. From a practical stand point, it is important to evaluate the binding abilities of various LP at a given condition.

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However, since there is a considerable difficulties in the quantitative description of the ion-binding equilibria of polyelectrolyte (1), theoretical consideration has been limited mainly to relatively short LP, such as di- or triphosphate complexation.

In this work, instead of the determination of the critical equilibrium constants, a semiquantitative parameter is obtained and is compared among the LP of various degrees of polymerization, p. A zonal gel chromatographic method (2-5) has been applied, which determines the average number of bound metal ions per one phosphate unit of an LP molecule at a specified free metal concentration.

In order to automate the chromatographic procedure, an atomic absorption detector, AAD, (6-8) was applied. The fundamental problems encountered in the use of AAD as a flow detector of gel chromatography have also been examined.

THEORY

A zonal gel chromatographic method for the investigation of the binding of metal ion, M, to high-molecular-weight ligand, L, has previously been described in detail (3,5,7). A sample solution containing M, L and their complexes is applied to a gel column which is preequilibrated with an eluent containing M of a specified concentration, $[M]_0$, and is eluted with the same eluent. L and their complexes are excluded from the gel phase, while M migrates through the column with much slower velocity. By this procedure, the zone of ligand is forced to be equilibrated with the solution of M whose concentration is the predetermined value, $[M]_0$. After this zone is sufficiently equilibrated, the distribution of chemical species in this zone is characterized by the free metal concentration, $[M]_0$.

If the sample ligand is monodisperse, the average number of bound metal ions per one ligand, $\bar{n}$, can be determined as follows;

$$\bar{n} = \frac{\sum_{1}^{n} i\beta_i [M]_0^i}{1 + \sum_{1}^{n} \beta_i [M]_0^i} \qquad (1)$$

where β_i is the overall stability constant of the complex M_iL. In case where a mixture of LP is used as a ligand, $\bar{n}$ defined in eqn. (1) cannot be calculated. Instead, the average number of bound metal ions per one phosphate unit, $\bar{m}$, has been determined. As with $\bar{n}$, $\bar{m}$ is a function of $[M]_0$. When $[M]_0$ is kept constant, constant $\bar{m}$ value is expected to be obtained. The value of $\bar{m}$ can be calculated by dividing the amount of bound metal ion, N_M by the total amount of PO_3 unit, N_P, which is applied to the column.

EXPERIMENTAL

Chemicals

Sodium triphosphate hexahydrate (9) and ammonium tetraphosphate hexahydrate (10) have been prepared according to the literatures. Sodium phosphate glasses (a mixture of $Na_{p+2}P_pO_{3p+1}$) with average degrees of polymerization, $\bar{p}$, of 6.4, 9.2, 13.7, 22.2 were prepared according to the literatures (11,12). In order to prepare samples of LP of higher $\bar{p}$ value, a sodium phosphate glass whose $\bar{p}$ value was 75.8 was fractionated by solubility fractionation using acetone. The $\bar{p}$ values of the fractions were 36, 50, 85, 101 and 127. Molecular weight distribution analysis of these sample phosphates was carried out by a Sephadex G-100 column (12). All the sample solutions of LP were standardized colorimetrically with a molybdenum (V)-molybdenum (VI) reagent. All other chemicals were of analytical grade.

Elution experiments

A Kyowa KHU-W-188 reciprocating pump was used throughout. A column of 700x8 mm I.D. Pyrex tubing was packed with Sephadex

G-15 (Pharmacia). The temperature of the column was kept at 25.0 ± 0.1°C. The column was first conditioned with an eluent containing 1.00×10^{-5} M magnesium chloride, 0.1 M tetramethylammonium chloride and buffer agent of desired concentrations. Two types of buffer agent were used. One type was 2×10^{-3} M NH_3 - 10^{-3} M NH_4Cl (pH 9.8), and another type was 2×10^{-3} M CH_3COOH - 10^{-3} M CH_3COONa (pH 4.2). The sample solution contained magnesium chloride and sample phosphate in addition to tetramethylammonium chloride and buffer agent of the same concentrations as those in the eluent. A 0.6-ml portion of a sample solution was fed onto the column with a loop injector. The elution flow rate was kept at 2.18 ml/min. The effluent was introduced into the AAD (Perkin-Elmer 403) for the automatic recording of the absorbance of magnesium at 285.2 nm. No inhibition effect of polyphosphates on the atomization of magnesium has been observed (3,7,14). Elution patterns as shown in Figs. 5 and 8 were obtained. The horizontal lines at the rear edge of the complex peak promises that the sample ligand zone was sufficiently equilibrated with magnesium solution of 1.00×10^{-5} M.

RESULTS AND DISCUSSION

Atomic Absorption Detector (AAD)

Usually the column effluent flow rate, v_c, is much less than the nebulizer aspiration rate, v_c, which is adjusted to maximize sensitivity. In order to balance the flow, water was drawn into the AAD from an open reservoir through a three-way connector at a flow rate, v_b (Fig. 1). The value of v_b varies with the variation in v_c, keeping the relation, $v_n = v_b + v_c$. This system gives a stable stream of liquid to the AAD which produces a stable flame. One disadvantage of this flow system is the fact that the effluent is always diluted by a factor of v_c/v_n (8,14).

The diluting ratio, R, has been determined without the gel column in the following way. First, water was introduced to the flow system at a flow rate, v_c, and 1.00×10^{-5} M magnesium chloride

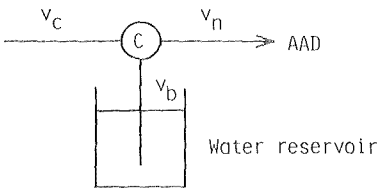

FIGURE 1.

Flow system of the AAD.

solution was drawn from the compensating reservoir (Experiment A). The absorbance of magnesium was measured at various flow rates, and was plotted against v_c (Fig. 2). Next, these two liquids were exchanged, i.e., 1.00×10^{-5} M magnesium chloride solution was introduced to the system at the flow rate of v_c, and water was drawn from the reservoir (Experiment B). The absorbance obtained was also plotted against v_c (Fig. 2). As can be seen from Fig. 2, the sum of the two absorbances at a specific v_c value was constant. This result ensures that v_n is independent of v_c, and indicates that the recorder response can be quantitatively correlated to the amount of sample introduced to the nebulizer. The value of R at a specific v_c value can be calculated by the following equation.

$$R = \frac{(Abs)_A}{(Abs)_A + (Abs)_B} \qquad (2)$$

where suffixes "A" and "B" represent the experiment "A" and "B", respectively. The calculated R value is plotted against v_c (Fig. 3). When v_c is 2.18 ml/min the column effluent is expected to be diluted by a factor of ca. 0.3.

In Fig. 4, the calibration graphs for magnesium chloride obtained at various pump flow rates are shown. It can be seen

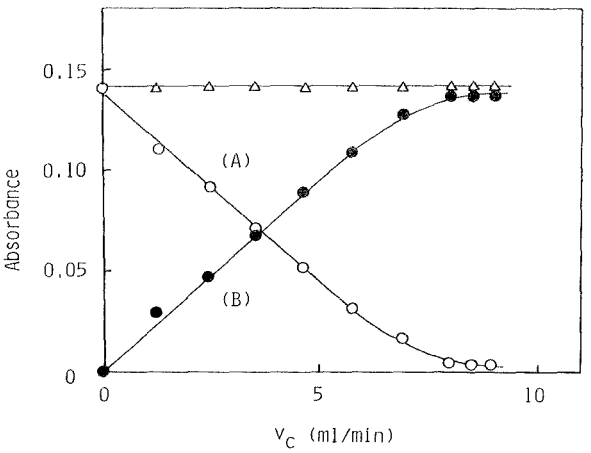

FIGURE 2.

Plots of absorbance of magnesium vs. v_c.

○ : $(Abs)_A$, ● : $(Abs)_B$, △ : $(Abs)_A + (Abs)_B$.

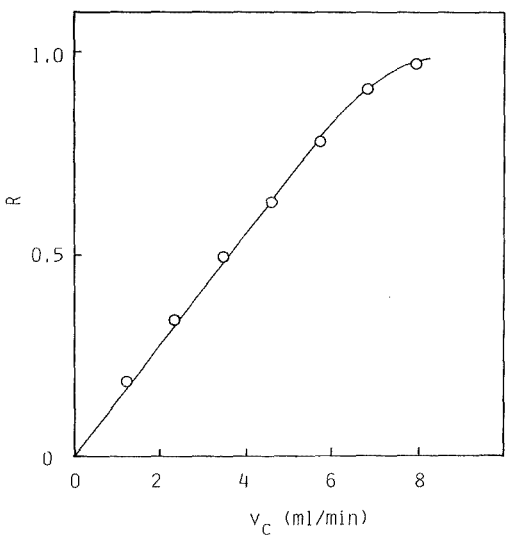

FIGURE 3.

Plots of R vs. v_c.

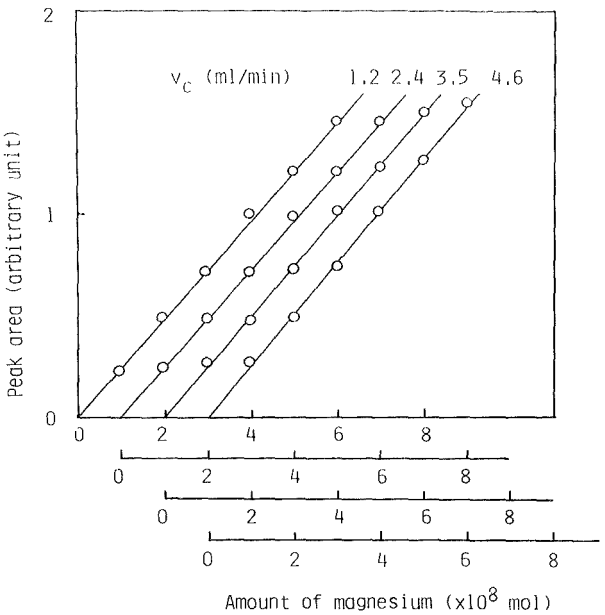

FIGURE 4.

Calibration graph for magnesium.
The value of v_c is shown in the figure.

that the plots give a good linearity. As expected, the slopes of the calibration graphs for various pump flow rates are almost consistent with each other.

Calculation of $\bar{m}$

The representative elution patterns obtained for the magnesium-triphosphate system are shown in Fig. 5. The positive peak corresponds to the complex and the negative peak reflects the deficiency of the free magnesium. The horizontal line at the rear edge of the complex peak assures that the sample ligand zone is sufficiently equilibrated. The area of the complex peak increased

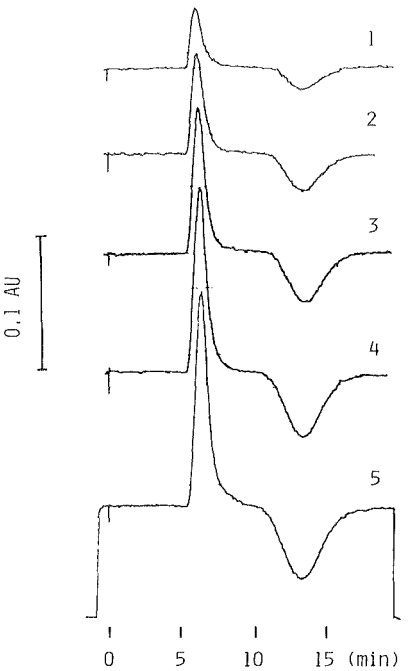

FIGURE 5.

Representative elution patterns of the Mg-P_3O_{10} system. $[M]_0 = 1.00 \times 10^{-5}$ M, pH 9.8. $N_P \times 10^9$ mol 1):0.37, 2):0.65, 3):0.90, 4):1.20, 5):1.46.

with an increase in the amount of triphosphate in the sample solution. N_M calculated from the peak area is plotted against N_P (Fig. 6). N_M increase is proportional to N_P. The slope of this straight line is the value of $\bar{m}$. As expected, a constant $\bar{m}$ value is obtained when free metal concentration is kept constant. In order to check the effect of flow rate on $\bar{m}$, the elution flow rate was varied between 1.3 and 3.5 ml/min. However, no change in $\bar{m}$ value could be detected at all.

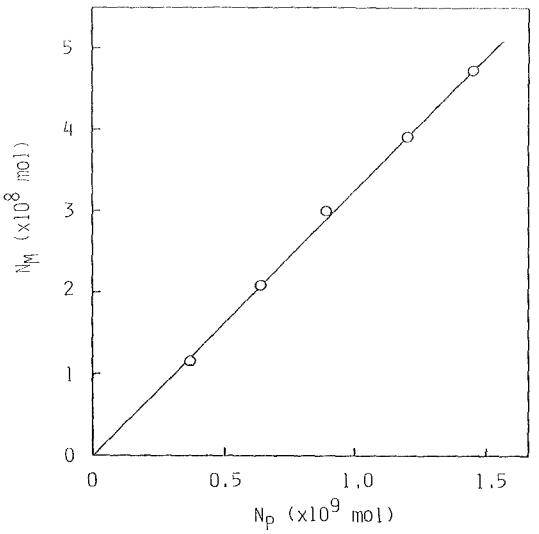

FIGURE 6.

Plots of N_M vs. N_P for the Mg-P_3O_{10} system.
$[M]_0$ = $1.00x10^{-5}$ M, pH 9.8.

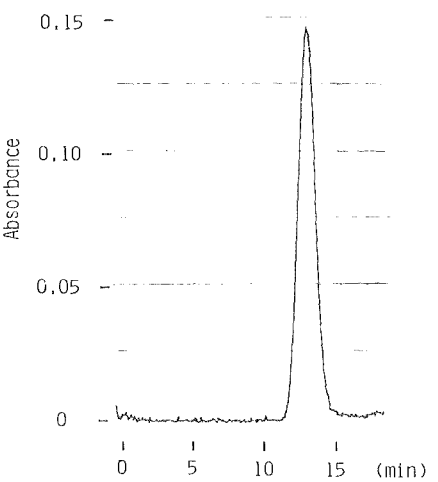

FIGURE 7.

Elution behavior of sodium ions in the Mg-P_3O_{10} system.
Absorbance of sodium at 589.0 nm was monitored by the AAD.
$[M]_0$ = $1.00x10^{-5}$ M, N_P = $1.46x10^{-9}$ mol, pH 9.8.

As source materials of LP, their sodium or ammonium salts have been used. The elution behavior of sodium ions in the magnesium-triphosphate system was monitored by the AAD (Fig. 7). Since the sodium ions are completely separated from sample ligand zone, it can be concluded that the ion-pair formation of sodium ions with phosphate anions need not to be taken into account in the calculation of $\bar{m}$. In Fig. 8, an example of the continuous analysis of magnesium-LP binding by the gel chromatography-AAD system is shown. Sample solutions could be applied to the column continuously every ten minutes.

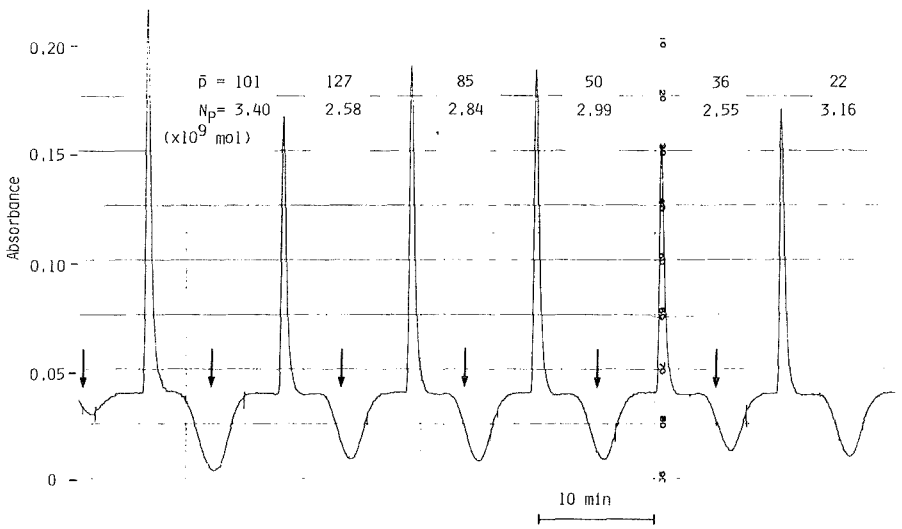

FIGURE 8.

Representative elution patterns of the Mg-LP systems (pH 4.2). The average degrees of polymerization, $\bar{p}$, and the N_P values of the samples are shown in the figure. Arrows show the time of sample injection.

Comparison of $\bar{m}$

The values of $\bar{m}$ thus obtained are plotted against the logarithms of p or $\bar{p}$ (Fig. 9). It can be seen that the binding characteristic of the LP anions is greatly dependent on the degrees of polymerization of the sample phosphate. It was found that at pH 9.8, di- or tri-phosphate has greater binding ability than the long LP, though at pH 4.2 an opposite trend is observed.

LP anions are composed of two types of phosphate units, i.e., end and middle ones which bear negative charge of two and one minus, respectively. At pH 9.8, where all the sample phosphates are considered to be almost completely dissociated, the average negative charge on one phosphate unit decreases with an increase in the degrees of polymerization. This may be the reason for the decrease in $\bar{m}$ value

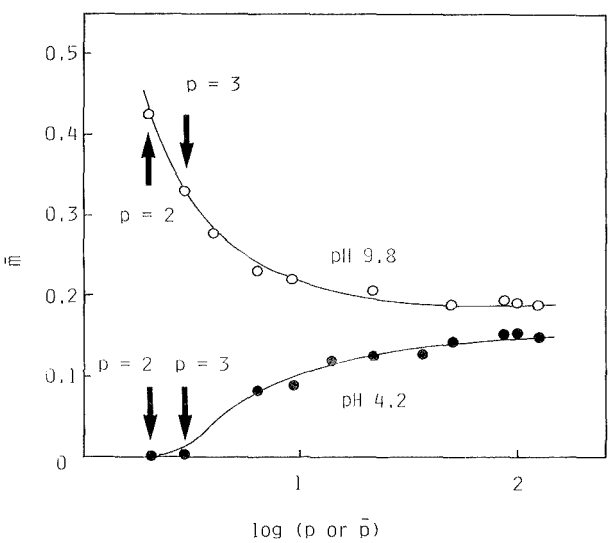

FIGURE 9.

Plots of $\bar{m}$ vs. log (p or $\bar{p}$).

with an increase in p or $\bar{p}$ at pH 9.8. However, at pH 4.2, each units of LP anions are considered to be almost monoprotonated. Under this condition, the contribution of end unit to the binding of metal ions greatly decreases, which results in the great decrease in the $\bar{m}$ values of short LP systems. Since the longer LP are composed mainly of middle units which are almost dissociated even at pH 4.2, the $\bar{m}$ values approach to those obtained at pH 9.8, when $\bar{p}$ increases.

It should be pointed out that the $\bar{m}$ values of the long LP are much greater than diphosphate at pH 4.2. At pH 4.2, diphosphate anions are considered to be in the form of $H_2P_2O_7^{2-}$ (15). Results obtained at pH 4.2 indicates that LP anion whose p value is 100 can bind magnesium much strongly than the group of fifty molecules of $H_2P_2O_7^{2-}$ can. This phenomenon should be attributed to the trapping of magnesium ions by the strong electric field on the long LP molecule formed by the crowded negative charge and/or some specific complex formation.

As has been pointed out in the section of "Theory", $\bar{m}$ value is a function of $[M]_0$. Therefore, in order to clarify the polymer-metal ion binding, it is necessary to determine $\bar{m}$ values under various free metal concentrations (16). Determination of $\bar{m}$ values at a specific free metal concentration, however, gives useful information for the comparison of the binding ability of these homologous series of ligands. The zonal gel chromatographic method is well suited for this purpose. A combination of gel chromatography with an AAD provides a rapid and continuous analysis technique for these polymer-metal ion interaction study.

ACKNOWLEDGEMENT

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REFERENCES

1. Marinsky, J. A., Coord. Chem. Rev., 19, 125(1976).
2. Hummel, J. P. and Dreyer, W. J., Biochem. Biophys. Acta, 63, 532(1962).
3. Miyajima, T. and Ohashi, S., Bull. Chem. Soc. Jpn., 51, 2543(1978).
4. Wood, G. C. and Cooper, P. F., Chromatogr. Rev., 12, 88(1970).
5. Yoza, N., J. Chem. Educ., 54, 284(1977).
6. Yoza, N. and Ohashi, S., Anal. Lett., 6, 595(1973).
7. Yoza, N., Kouchiyama, K. and Ohashi, S., At. Absorpt. Newsl., 18, 39(1979).
8. Yoza, N., Aoyagi, Y., Ohashi, S. and Tateda, A., Anal. Chim. Acta, 111, 163(1979).
9. Watters, J. I., Loughran, E. D. and Lambert, S. M., J. Am. Chem. Soc., 78, 4855(1956).
10. Griffith, E. J., J. Inorg. Nucl. Chem., 26, 1381(1964).
11. Van Wazer, J. R., J. Am. Chem. Soc., 72, 644(1950).
12. Miyajima, T., Yamauchi, K. and Ohashi, S., J. Liq. Chromatogr., 4, 1891(1981).
13. Van Wazer, J. R., J. Am. Chem. Soc., 72, 647(1950).
14. Kouchiyama, K., Yoza, N. and Ohashi, S., J. Chromatogr., 147, 271(1978).
15. McNabb, W. M., Hazel, J. F. and Baxter, R. A., J. Inorg. Nucl. Chem., 30, 1585(1968).
16. Onaka, T.,Miyajima, T. and Ohashi, S., J. Inorg. Nucl. Chem., 43, 3323(1981).

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LC CALENDAR

1982

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

SEPTEMBER 13 - 17: 14th Int'l. Symposium on Chromatography, London. Contact: Mrs. Jennifer Chalis, Chromatography Discussion Group, Trent Polytechnic, Burton Street, Nottingham NG1 4BU, U.K.

OCTOBER 10 - 13: 21st Annual Mtg. of ASTM Committee E-19 on the Practice of Chromatography, Marriott Hotel, New Orleans. Contact Mr K. Riley, ASTM Headquarters, 1916 Race Street, Philadelphia, PA, 19103, USA.

OCTOBER 12 - 14: 3rd International Symposium On Chlorinated Dioxins and Related Compounds, International Congress Center, Salzburg, Austria. Contact: Prof. O. Hutzinger, University of Amsterdam, Nieuwe Achtergracht 166, 1018WV Amsterdam, The Netherlands.

OCTOBER 14: Ninth ANACHEM Symposium, sponsored by The Association of Analytical Chemists, Dearborn Inn, Dearborn, MI, USA. Contact: J. W. Auld, Detroit Edison Co., Detroit, MI, 48226, USA.

OCTOBER 14 - 15: "New Perspectives in Racemic Coumpound Separation" sponsored by CNR-PF Chimica

Fine e Secondaria, Societa Chimica Italiana, and Universita degli Studi di Roma, in Rome, Italy. Contact: Prof. Domenico Misiti, Inst. di Chimica Organica, Via del Castro Laurenziano 9, 00161 Roma, Italy.

OCTOBER 19 - 20: short Course on LC/MS, Maison des Congres, Montreux, Switzerland. Sponsored by Int'l Assoc. of Environmental Anal. Chem. Contact: Dr. Alain Donzel, Workshop Office, Case Postal 5, CH-1052 Le Mont-sur Lausanne, Switzerland.

OCTOBER 21 - 22: 2nd Workshop On LC/MS and MS/MS, Maison des Congres, Montreux, Switzerland. Sponsored by Int'l. Assoc. of Environmental Anal. Chem. Contact: Dr. Alain Donzel, Workshop Office, Case Postale 5, CH-1052 Le Mont-sur Lausanne, Switzerland.

NOVEMBER 2 - 5: 1st Inter-American Congress in Forensic Medicine and Sciences, Pan-American Assoc. of Forensic Sci., Sacramento, CA. Contact: John D. DeHaan, Calif. Department of Justice Lab. Box 13337, Sacramento, CA 95813,USA.

NOVEMBER 11 - 14: Applied Seminar for the Association of Clincial Scientists, Chicago, IL. Contact: Dr. F. M. Sunderman, Jr., Dept. of Lab. Medicine, Univ. of Connecticut School of Medicine, 263 Farmington Avenue, Farmington, CT 06032.

NOVEMBER 16 - 18: Medical and Laboratory Instrumentation Soc. Annual Int'l. Congress and Exhibition, Sheraton-Washington Hotel, Washington, DC. Contact: John Wolf, MLIS, 11310 Palisades Court, Kensington, MD, 20895, USA.

NOVEMBER 17 - 19: Eastern Analytical Symposium, Statler-Hilton Hotel, New York. Contact: Dr. H. Issag, Frederick Cancer Res. Facility, P.O. Box B, Frederick, MD, 21701, USA, or Dr. D. Strumeyer, Rutgers University, Chem. Dept, New Brunswick, NJ 08903.

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

1983

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

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

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

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

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

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

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

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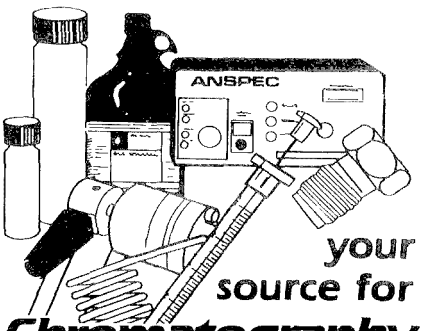

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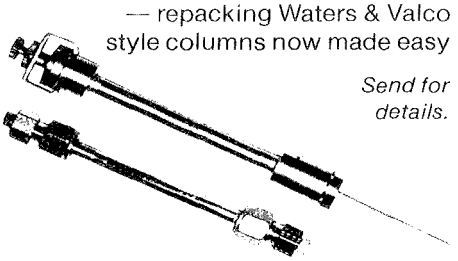

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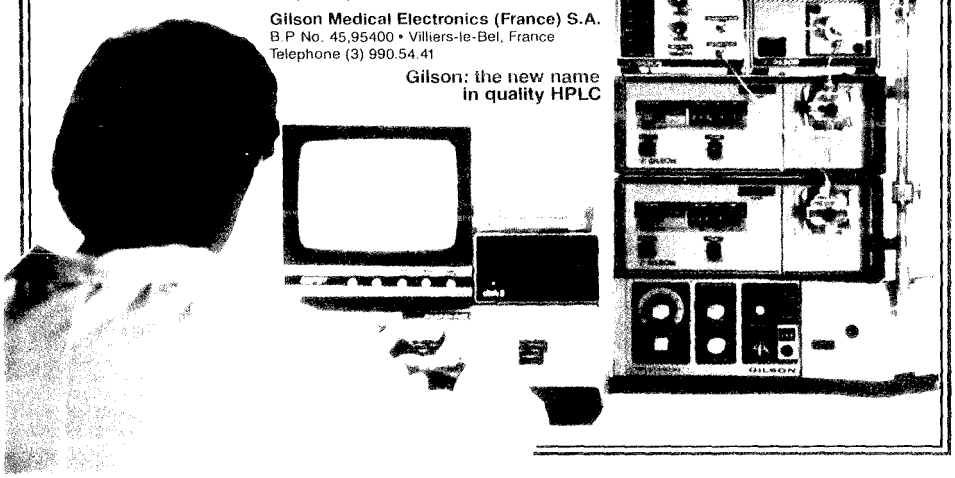

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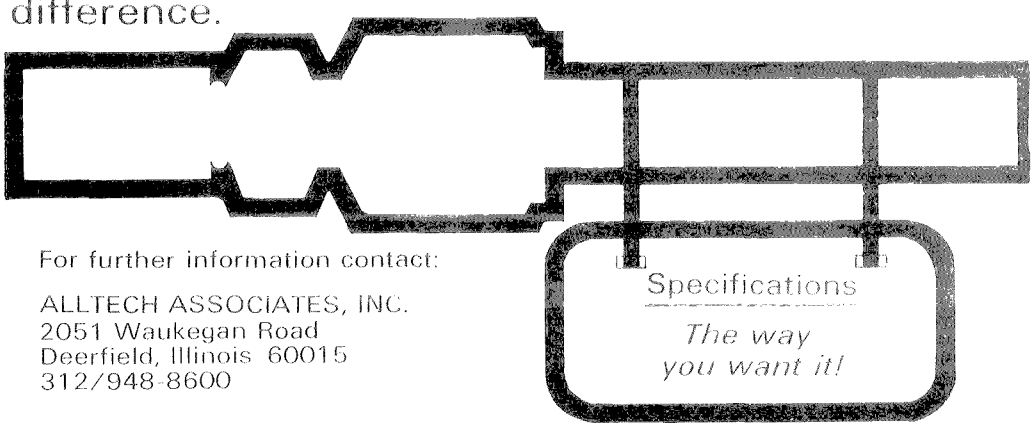

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