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JOURNAL OF LIQUID CHROMATOGRAPHY

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JOURNAL OF LIQUID CHROMATOGRAPHY

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A CALIBRATION TECHNIQUE FOR SIZE EXCLUSION CHROMATOGRAPHY

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ABSTRACT

A modified Weibull distribution function is shown to be useful in calibrating the molecular size separation capabilities of both rigid and swellable gel packing materials. Two parameters are used in this function which are related to a packing material's micropore volume distribution. The calibration curves of a set of different packing materials connected in series were predicted from the Weibull calibration functions obtained for each individual packing material.

INTRODUCTION

Quantitative size exclusion chromatography (SEC) can be accomplished only after a calibration of the system has been performed. SEC calibration relates a polymer macromolecular property, usually size or molecular weight, to the elution volume penetrated by the macromolecule. This is usually accomplished by using a calibration function which describes an SEC separation model. It is the purpose of this paper to briefly review the SEC models previously proposed and to introduce and examine a new SEC model based on a Weibull distribution function.

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SEC Calibration Models

In 1967, Grubisic, et al [1], observed that known molecular weight fractions of linear and branched polystyrene eluted at different times. The branched polymers of equal molecular weight always had a greater elution volume. From this observation, the authors concluded that the molecular hydrodynamic volume could be used as a calibration parameter.

The hydrodynamic volume of a polymer molecule in dilute solution can be obtained from the Einstein-Simha equation [2]. This equation shows that the hydrodynamic volume of a polymer molecule in dilute solution is proportional to the product of the polymer's molecular weight, M, and intrinsic viscosity $[\eta]$.

Grubisic used the logarithm of $M[\eta]$ versus elution volume for SEC calibration and also suggested that this calibration procedure was universal for any type polymer. The Grubisic calibration function is given by:

$$V_e = A + B \log (M [\eta]) \quad (1)$$

In equation (1), V_e is the elution volume of a molecule which is linearly related to the logarithm of the molecular hydrodynamic volume by the calibration constants A and B. Grubisic showed that this plot characterized SEC separation of polymers with differing structures and extents of branching [3].

The concept of hydrodynamic volume as the controlling parameter in liquid chromatography gained support in subsequent articles

by Wild and Guliana [4], Coll and Gilding [5], Boni et al. [6], and Berry [7]. In all cases, a linear correlation of hydrodynamic volume with elution volume was obtained.

Dawkins [8] suggested that the molecular root mean squared, unperturbed end-to-end distance was the controlling factor in SEC. Cassassa and Tagami [9] also expressed this same view. However, there is no basis for choosing between hydrodynamic volume and end-to-end distance as the controlling size separation parameter, since both correlate data equally well.

Giddings [10] used statistical thermodynamics to explain SEC molecular separation phenomena. Giddings modeled the packing microstructure as a network of lines. This micropore model is called a "brush pile". From this SEC model, the fraction of the micropore volume penetrated, K, by a molecule having an external length, ℓ, was related to micropore surface area, S, and volume, ν.

$$K = 1 / EXP [\ell S/2\nu] \quad (2)$$

The fraction of the micropore volume penetrated by a molecule, K, is usually called the distribution coefficient. The molecular external length was compared to the hydrodynamic volume and end-to-end distance. It was found that the external length was more effective as a calibration parameter when characterizing the separation behavior. It was also concluded that the "brush pile" model was a good approximation of the micropore structure of swellable gel packing materials.

Cassassa [9] explained SEC separation phenomena by assuming that rigid spherical molecules were in equilibrium with micropores which were modeled by: (1) spherical cavities of diameter D_s, (2) long circular cylinders of diameter D_c, and (3) cavities formed by long parallel plane surfaces separated by a distance D_p. The distribution coefficient for a spherical molecule of diameter d which is in equilibrium with each cavity type is given by:

$$K = [\, 1 - (d/D_s) \,]^3 \tag{3}$$

$$K = [\, 1 - (d/D_c) \,]^2 \tag{4}$$

$$K = [\, 1 - (d/D_p) \,]^1 \tag{5}$$

The Cassassa micropore models do not describe real SEC packing micropores; however, the models do show the general effects that pore geometry will have on size exclusion.

In all three of the Cassassa micropore models, a single size pore geometry was assumed. In a more realistic approach, Dawkins [12] modeled the micropore volume by using a Schulz-Zimm exponential distribution to describe the pore volume associated with cylindrical pores. The distribution coefficient of a molecule of diameter d in this porous medium model is given by:

$$K = \int_d^{d_o} \left\{ \frac{d}{D^2} \Big/ \text{EXP} \left[d/D \right] \right\} \partial d \tag{6a}$$

$$K = (1 + d/D) \Big/ \text{EXP} [\, d/D \,] \tag{6b}$$

In equation (6), D is the average pore diameter; and the upper integration limit, d_o, represents the diameter of the smallest large molecule which will not penetrate any of the micropore volume (i.e., at K = 0). This micropore model gave a reasonable average pore diameter for real SEC packing materials.

In an SEC separation model, which is similar to that of both Giddings and Dawkins, Hester and Mitchell [13] used a simple exponential probability function to describe the distribution of the micropore volume. This model predicted that the distribution coefficient for a molecule of diameter d was a function of the mean effective pore diameter, $\overline{D}$.

$$K = \int_{d}^{d_o} \left\{ 1 / \overline{D} \ \text{EXP} \left[d / \overline{D} \right] \right\} \partial d \tag{7a}$$

$$K = 1 / \text{EXP} \left[d / D \right] \tag{7b}$$

The mean effective pore diameter was shown to be a parameter which characterizes a packing material's molecular separation capabilities. Packing materials, having a larger mean effective pore diameter, have a larger range of molecular size separation. However, packing materials with a larger mean effective pore diameter are less sensitive in distinguishing between molecular sizes. It was shown that SEC calibration is more closely correlated by equation (7) than by the more commonly used Grubisic calibration function, equation (1).

It is evident from equations (6) and (7) that an SEC calibration function specifies a micropore volume distribution function.

An SEC calibration defines the fraction of the total micropore volume which is penetrated by a specific size polymer molecule. The total volume penetrated by a molecule is the volume sum of all the interstitial channels and micropores which have controlling entry sizes which are equal to or larger than the size of the molecule.

Calibration Using a Weibull Function

It will be shown that a modified Weibull distribution can be used to model the micropore volume distribution of both rigid and swellable gel SEC packing materials. The Weibull distribution function for a single packing material is given by:

$$\partial V/\partial d = (V_p n/d)\ (d/\overline{D})^n\ /\ EXP\ [\ (d/\overline{D})^n] \qquad (8a)$$

$$\partial V/\partial \ln d = V_p n\ (d/\overline{D})^n\ /\ EXP\ [\ (d/\overline{D})^n\ ] \qquad (8b)$$

In the above equation, ∂V, is the incremental change in the micropore volume associated with an incremental change in the micropore entry size, ∂d. The parameters, V_p, $\overline{D}$, and n, are material properties which characterize the packing material. They are the total micropore volume, the most dominant pore entry size, and micropore volume distribution shape factor, respectively.

The total micropore volume, V_p, can be experimentally determined. It is the volume difference between the total permeation volume, V_t, and the interstitial volume, V_o.

$$V_p = V_t - V_o \qquad (9)$$

V_t is the elution volume of a very small molecule and V_o is the elution volume of a very large molecule which only penetrates the interstitial volume.

For the distribution in equation (8b), the largest quantity of micropore volume will have a pore entry size equal to $\bar{D}$. An increase in the distribution shape factor, n, produces both a decrease in the width of the micropore volume distribution and a decrease in skewness to the smaller pore entry sizes. It will be shown that the distribution parameters, $\bar{D}$ and n, can be adjusted such that the function describes the micropore volume distribution of real packing materials.

Equation (8) can be used to obtain an SEC calibration function. The following relationships can be obtained from the definition of the distribution coefficient.

$$K = \int_{d}^{d_o} (\partial V/\partial d) \quad (\partial d/V_p) \tag{10a}$$

$$K = \int_{d}^{d_o} \left\{ (n/d) \ (d/\bar{D})^n \ / \ EXP \ [\ (d/\bar{D})^n \ ] \right\} \partial d \tag{10b}$$

$$K = 1 \ / \ EXP \ [\ (d/\bar{D})^n \ ] \tag{10c}$$

$$V_e = V_o + V_p \ / \ EXP \ [\ (d/\bar{D})^n \ ] \tag{10d}$$

In equation (10d), V_e is the elution volume of a molecule having a diameter which is proportional to a pore entry size of d. Work by Halasz et al. [14-16] has shown that the pore entry size, as measured by capillary condensation and mercury porosimetry, is two to

three times larger than the molecular hydrodynamic diameter. However, this relationship cannot be adequately tested at small pore sizes because porosimetry is not accurate if the pore entry size is less than 175 Å [20].

The hydrodynamic diameter of a polymer molecule is proportional to the cube root of the hydrodynamic volume [13].

$$d = k (M [\eta])^{1/3} \tag{11}$$

Puckett [17] has determined that for both linear or branched polymer molecules, the best value of the constant, k, is 4.8. This value for k in equation (11) will give molecular hydrodynamic diameters in Angstrom units, when the values for the intrinsic viscosity are in deciliters per gram.

Polymer standards of known molecular weight and intrinsic viscosity can be used to determine packing material properties, $\overline{D}$ and n. The best values for $\overline{D}$ and n can be obtained by fitting V_e vs d data to equation (10d). It will be shown that equation (10d) can be used as a calibration function for many different types of packing materials. In addition, it will be shown that equation (10d) can be used to calibrate a set of packing materials from a knowledge of the material properties of each individual packing material.

EXPERIMENTAL

The literature contains many papers on SEC calibration; however, most of these papers do not contain all the information needed to evaluate the utility of the modified Weibull calibration function,

equation (10d). From the few papers which did contain sufficient SEC information, data from Dawkins [12], Squire [19], and Van Kreveld [11] were selected because they represent the major types of SEC packing materials: swellable polystyrene gels, controlled pore glass chemically bonded with hydrophilic molecules, and silica gel, respectively. For the sake of brevity, the experimental details used to obtain this data are not included in this paper.

In addition to the SEC data taken from the literature, SEC data was experimentally obtained for controlled pore glass and silica gel packing materials [19]. This SEC data will be referred to as Hester data. This data was collected using the following conditions:

Porous glass packing was obtained from Electro-Neucleonics (Fairfax, NJ). Silica gel packing material, trade named Fractosil, was obtained from E. M. Merck (Elmsford, NY).

Three sizes of controlled pore glass packing were used, CPG 75, 24, and 350. Also, three sizes of silica gel packing were used, Fractosil 200, 500, and 2500. Material specifications are given in Table I. Three columns of each size control pore glass material were packed and connected in series, then calibrated using polystyrene solutions. Only one column of each size silica gel material was packed. Each column was individually calibrated with polystyrene solutions.

Reagent grade THF or toluene were used as a mobile phase or solvent. THF was distilled (66-67°C) over calcium hydride under a nitrogen atmosphere to remove an inhibitor.

TABLE I

PACKING MATERIAL PROPERTIES

Date Source	Type Packing	Micropore Volume, v, cm^3/g	Surface Area S, m^2/g	Ratio Volume to Surface Area, v/S, $\AA$	Most Dominant Micropore Size* $\bar{D}$,$\AA$
Hester & Mitchell	CPG 350	1.39	79	176	178
	CPG 240	1.31	118	111	169
	CPG 75	0.48	207	23	48
Hester & Mitchell	Fra 2500	0.5	8	630	785
	Fra 500	0.6	35-65	90-170	118
	Fra 200	0.6	120-170	35-50	64
Van Kreveld & Van Den Hold	Porasil D	0.912	34.8	262	252
	Porasil C	0.864	71	122	119

*From fitted Weibull functions

Polystyrene standards with low polydispersity were obtained from Pressure Chemical and Waters Associates. Each standard was dissolved in THF or toluene. The concentration of each standard solution was less than the reciprocal of its measured intrinsic viscosity. This concentration level assured that a polymer solution was dilute.

A Waters UK6 sample injector equipped with a 2 ml sample loop was used for the majority of this work. However, for some experiments, a Rhoedyne (Cotati, CA) Model 7125 injector fitted with a 2 ml loop was also used.

The SEC pump used was a Waters Associates (Milford, MA) Model 6000 A. Volumetric output was monitored by a Waters siphon volumetric counter equipped with a 5 ml siphon counter. The mobile phase flow rate through the packed columns was always regulated between 0.5 and 1.0 ml/min.

A Waters Model 440 UV absorbance detector was the primary means of detecting polystyrene in the SEC eluent. The wave length used was 254 nm.

RESULTS

The SEC data taken from the literature contained several individual data sets. However, not all of the data sets in each group were used. For example, the Dawkins data contained 23 individual sets of SEC elution data which were obtained by using combinations of two solvents, four polystyrene gel packing materials, and three different types of polymer standards. However, only four data sets were used. These four data sets were selected because they repre-

sent each size packing material and also because each data set contained a large number of data points, thereby providing the largest degree of freedom when fitting the Weibull calibration function. Similar discriminations were performed on both the Squire and Van Kreveld data.

Table II gives a summary of the data groups and the individual data sets within each group which were used to fit the Weibull calibration function. A total of 15 individual SEC data sets in five data groups were fitted to Weibull function, equation (10d), using a parametric fitting routine. The fitting routine was programmed in BASIC to run on a Hewlett-Packard HP85 microcomputer [21]. The fitting technique used a search routine to find the best fit values of the packing material parameters, $\overline{D}$ and n, which minimized the standard error between data and function. The best fit parameters for each SEC data set are listed in Table II.

Also included in Table II, is a criterion for the overall goodness of the function fit to each data set. This goodness of fit value, g, for N data points has been defined in a manner similar to that used by Samay [22]. If V_{ei} and $\hat{V}_{ei}$ are the experimental elution volume and corresponding elution volume estimated from the fitted function, respectively; then g can be expressed by:

$$g = 100 \sqrt{\frac{\sum_{i=1}^{N} (V_{ei} - \hat{V}_{ei})^2}{N - 2}} \Bigg/ \frac{\sum_{i=1}^{N} V_{ei}}{N} \qquad (12)$$

TABLE II

DATA FOR CALIBRATION PLOTS

Data Source	Figure #	Symbol	Type Packing	Type Polymer	Type Solvent	Intrinsic or Mark Houwink Equation	V_t	V_o	Fitted Weibull Parameters		Goodness of Fit Criterion
									n	$\bar{D}_o$, A	g
Dawkins & Hemming	2	x	Styragel A	PS	CL	a	10.9	9.3	1.22	500	0.59
	2	+	Styragel B	PS	CL	a	10.2	7.2	1.20	425	0.79
	2	*	Styragel C	PS	CL	a	9.4	5.6	1.08	165	1.48
	2	o	Styragel D	PS	CL	a	8.8	5.9	1.22	74	1.80
Squire	3	+	TSK Gel 4000	PEG	W	b	49.1	19.2	1.00	141	1.19
	3	o	TSK Gel 3000	PEG	W	b	47.5	18.8	1.27	59	1.07
	3	*	TSK Gel 2000	PEG	W	b	44.0	22.1	1.58	37	0.80
Van Kreveld & Van Den Hold	4	+	Porasil D	PS	THF	c	36.0	17.8	1.38	252	0.49
	4	*	Porasil C	PS	THF	c	35.3	17.0	1.40	119	0.90
Hester & Mitchell	5	+	CPG 350	PS	Tol	d	70.3	39.4	1.46	178	1.88
	5	o	CPG 240	PS	Tol	d	79.3	37.7	1.47	169	1.33
	5	*	CPG 75	PS	Tol	d	69.1	36.4	1.37	48	2.14
	7	*	CPG 75,240, 350	PS	Tol	d	218.7	113.5	-	-	2.86
Hester & Mitchell	6	*	Fractosil 2500	PS	THF	c	19.9	10.2	1.49	785	0.94
	6	o	Fractosil 500	PS	THF	c	19.9	11.4	0.95	118	0.63
	6	+	Fractosil 200	PS	THF	c	18.7	10.4	1.43	64	0.95
	8	o	Fractosil 200,500, 2500 & CPG 75	PS	THF	c	59.8	32.7	-	-	1.42

PS polystyrene
CL chloroform
PEG Polyethylene glycol
W water
Tol toluene
THF tetrahydrofuran

a) $[\eta] = 4.9 \times 10^{-5} M^{0.79}$

b) $[\eta] = 0.2 + 1.6 \times 10^{-4} M^{0.76}$

c) $[\eta] = 6.76 \times 10^{-5} M^{0.761}$

d) $[\eta] = 1.63 \times 10^{-4} M^{0.682}$

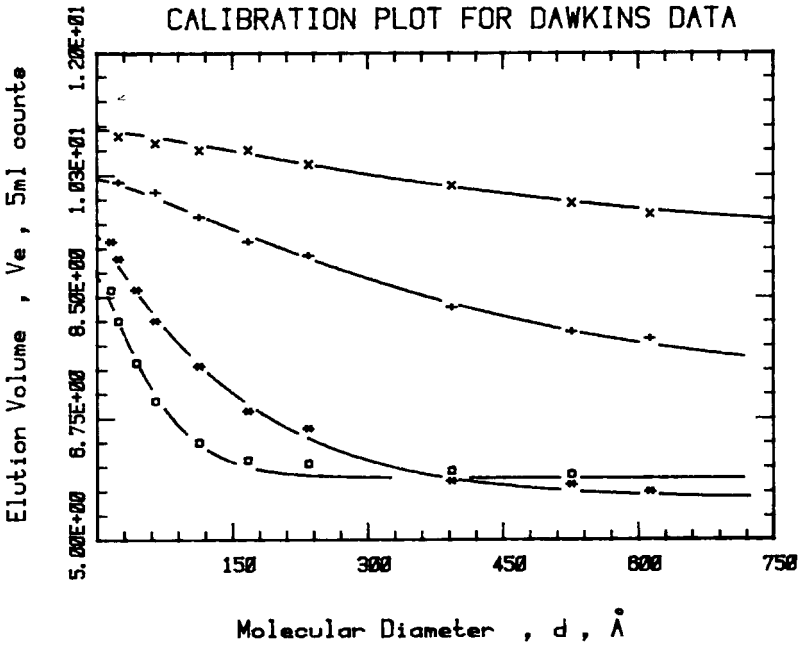

Figure 1. SEC calibration plots for styragel packing materials using polystyrene and chloroform. See Table II for details.

As shown by equation (12), the overall goodness of fit has been defined as the standard error expressed as a percentage of average experimental elution volume. A g value of zero corresponds to a perfect fit of the function to the experimental data.

The elution volume versus molecular hydrodynamic diameter has been plotted for each data set within each data group. The Dawkins, Squire, and Van Kreveld calibration plots are shown in Figures 1 through 3, respectively. The Hester data, using porous glass and silica gel, are shown in Figures 4 and 5, respectively. Also shown on the above plots are the curves representing the best fit

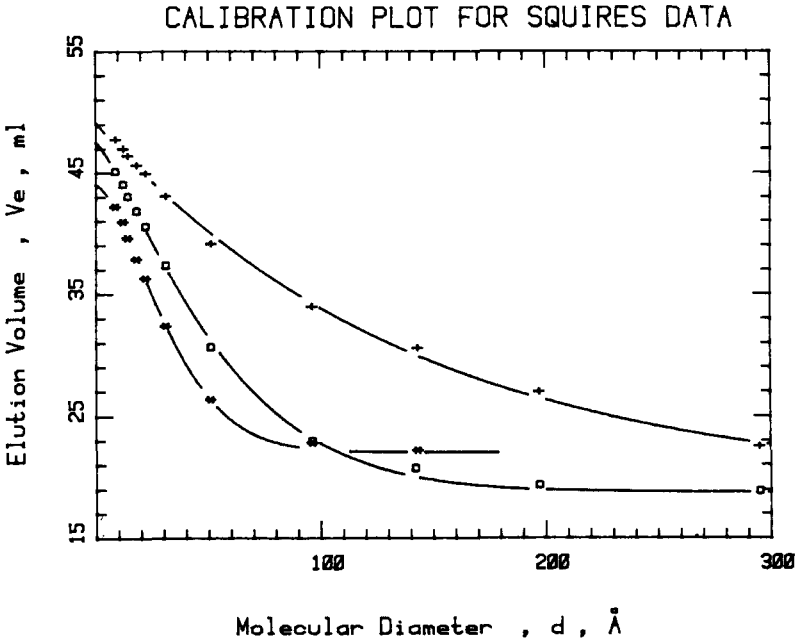

Figure 2. SEC calibration plots for TSK gel packing materials using polyethylene glycol and water. See Table II for details.

Weibull calibration function for each data set. The molecular hydrodynamic diameters used in the calibration plots were calculated from the polymer molecular weights, the appropriate Mark Houwink equation listed in Table II and equation (11). The total permeation volume, V_t and the total interstitial volume, V_o, used in fitting the Weibull functions are also listed in Table II.

DISCUSSION

SEC Calibration of Single Packing Materials

As can be observed from the calibration curves of Figures 1 through 5 and the corresponding goodness of fit values listed in

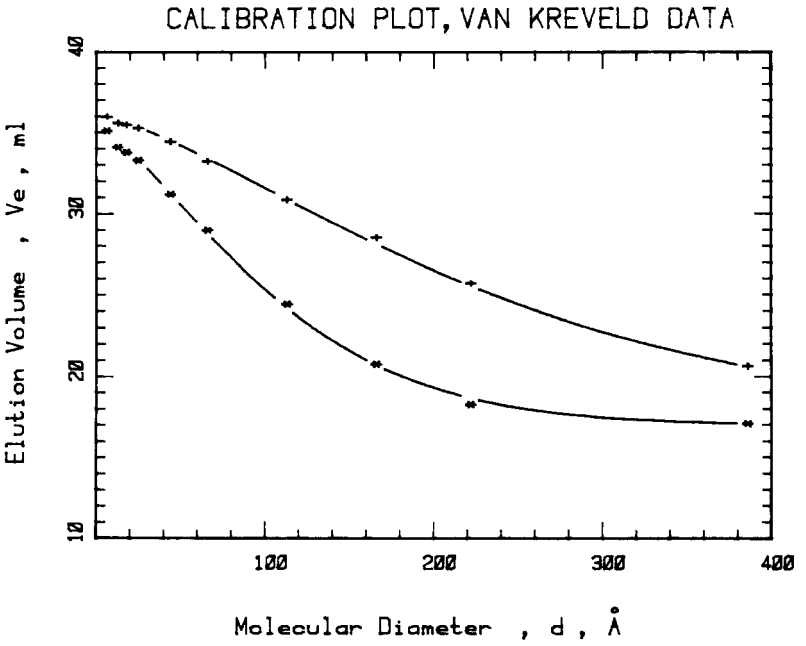

Figure 3. SEC calibration plots for Porasil packing materials using polystyrene and tetrahydrofuran. See Table II for details.

Table II, the Weibull function developed for each data set closely models the SEC separation performance regardless of the packing material. Most of the packing materials had a shape factor, n, which ranged from 1.0 to 1.5. This indicates that the pore volume distribution of packing materials are similar. This same similarity of shape was also noted by both Scott [23] and Zhdanov [24] for silica gel packing and porous glass packing, respectively.

Most of the packing materials had a most dominant pore entry size, $\overline{D}$, which was very close to that expected from the approximate pore size specified by the manufacturers. It appears that a

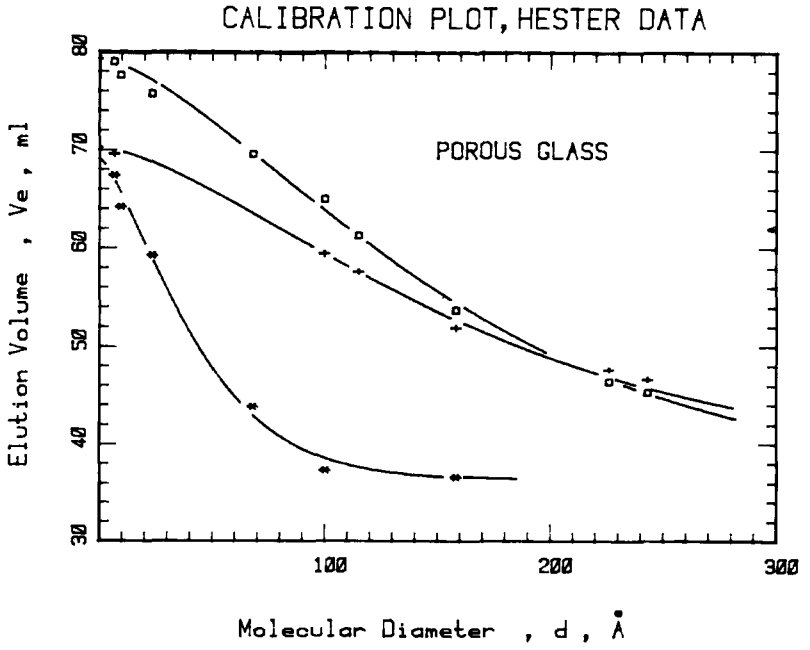

Figure 4. SEC calibration plots for Control Pore Glass packing materials using polystyrene and toluene. See Table II for details.

packing material's most dominant pore size is approximately equal to the ratio of micropore volume to surface area. This can be noted from Table I where this ratio and $\overline{D}$ can be compared. A similar observation was made by Berek [3] using polystyrene standards and silica gel packing materials. The work of Berek showed that the molecular weight, at the inflection point on an SEC elution volume versus log molecular weight calibration curve, was linearly related to the ratio of the micropore volume to surface area. It can be mathematically shown that the modified Weibull calibration function would also have an inflection point which corresponds to a molecular size equal to $\overline{D}$.

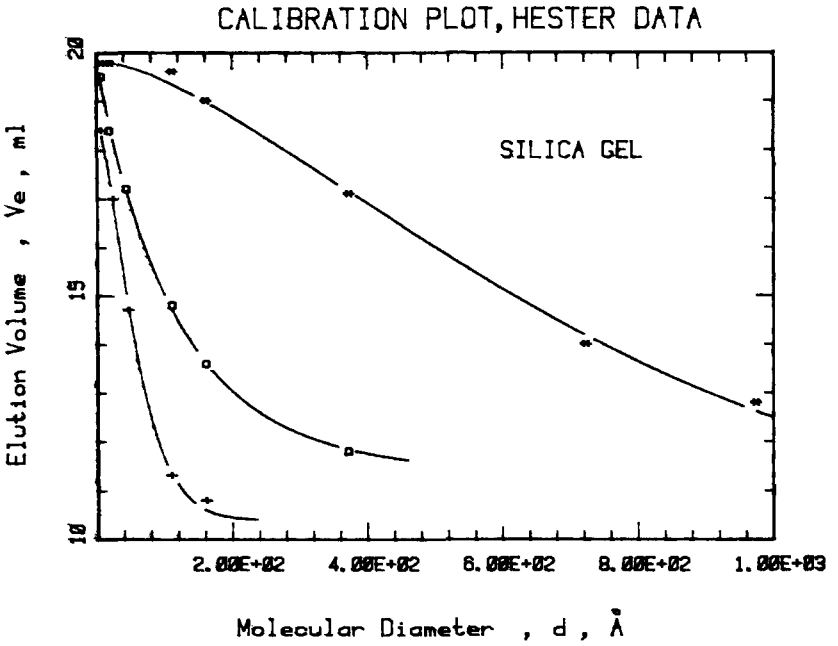

Figure 5. SEC calibration plots for Fractosil packing materials using polystyrene and tetrahydrofuran. See Table II for details.

SEC Calibration of Several Packing Materials in Series

A definite advantage of a Weibull calibration is that the calibration of a column set of individual packing materials connected in series can be determined from the individual calibrations obtained from each single packing material. This can be shown from the following argument.

For a set of packing materials operating in true size exclusion, the elution volume of a molecule is equal to the sum of the individual packing interstitial volumes added to the sum of the

individual packing micropore volumes penetrated by the molecule. Therefore, the elution volume for a set of P packing materials is given by:

$$V_e = \sum_{i=1}^{P} V_{oi} + \sum_{i=1}^{P} V_{pi} K_i \qquad (13)$$

In equation (13), the subscript i refers to an individual packing material. However, equation (10c) can be used to express each individual distribution coefficient in terms of the hydrodynamic diameter of the molecule passing through the packing set. Therefore, equation (13) can be expressed as:

$$V_e = \sum_{i=1}^{P} V_{oi} + \sum_{i=1}^{P} V_{pi}/\mathrm{EXP}\ [\ (d/\bar{D}_i)^{n_i}\ ] \qquad (14)$$

Equation (14) shows that, if the individual packing material parameters V_{oi}, V_{pi}, $\bar{D}_i$, and n_i are known, the calibration curve for the packing materials operating in series can be constructed.

Equation (14) was used to construct the calibration curves for two sets of packing materials. The first set was composed of three porous glass packing materials, CPG 75, 240, and 350. These packing materials have been individually calibrated and their material parameters are listed in Table II. The second set was composed of three silica gel packing materials, Fractosil 200, 500, and 2500, and a small quantity of CPG 75. The individual Fractosil materials have been individually calibrated and their material

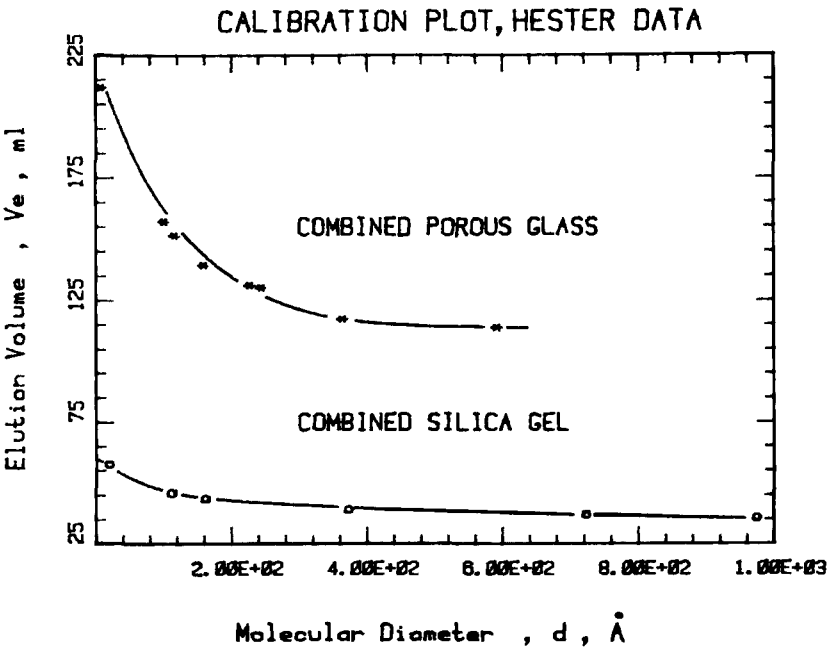

Figure 6. SEC calibration plot for the combined Control Pore Glass and combined Fractosil packing materials used in Figures 4 and 5, respectively. See Table II for details.

parameters are listed in Table II. The CPG 75 of the second set was used in a small guard column. The guard column packing material was estimated to have a V_t and V_o of 1.3 and 0.7 ml, respectively.

Polystyrene standards were analysed on these two sets of packing material. Plots of elution volume versus molecular hydrodynamic diameter are shown in Figure 6. Equation (14) and the individual packing material parameters were used to construct the curves shown in Figure 6. The calculated calibration curves very closely predict the elution volumes experimentally obtained for the

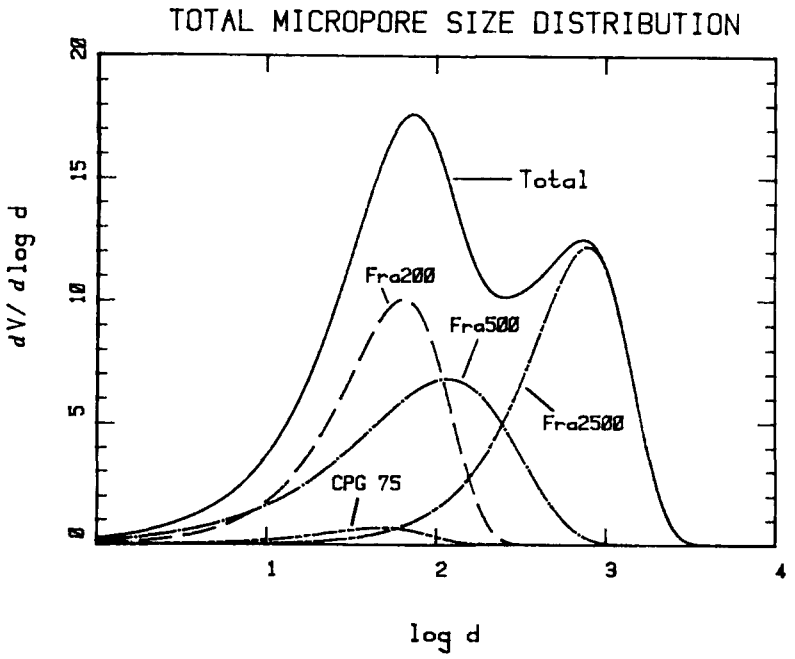

Figure 7. Combined and individual log normal size distribution curves for the Fractosil packing materials used in Figure 6.

polystyrene molecules. Thus, the validity of equation (14) appears to be experimentally confirmed.

Equation (8) can be used to construct both a micropore volume distribution curve for individual packing materials and the total micropore volume distribution curve resulting from the combination of several individual packing materials. The same arguments used to develop equation (14) can be used to show that for P packing materials, connected in series, the total micropore volume distribution function is given by equation (15).

$$\frac{\partial V}{\partial \ln d} = \sum_{i=1}^{P} V_{pi}\, n_i \left(\frac{d}{\overline{D}_i}\right)^{n_i} \Bigg/ \text{EXP}\,[(d/D_i)^{n_i}] \qquad (15)$$

Figure 7 shows each of the individual packing micropore volume distributions and the total micropore volume distribution for the set of four Fractosil packing materials associated with Figure 6. Figure 7 gives a visual description of how the total micropore volume is affected by adding the individual packing materials together. Together, equations (14) and (15) can be used to estimate the calibration curves and micropore volume distribution for any combination of packing materials. In addition, without extensive experimentation, both SEC separation sensitivity, $\partial V/\partial d$, and range of separation can be closely determined for any combination of packing materials.

CONCLUSION

Packing materials for size exclusion chromatography (SEC) can be calibrated for molecular size separation capabilities by using a four parameter modified Weibull distribution function. The four parameters are the interstitial volume, the total micropore volume, the most dominant pore entry size, and the micropore volume distribution shape factor. The last two parameters and the total micropore volume divided by weight of the packing material are intrinsic properties of rigid packing materials. The same parameters are not constant for swellable gel packing materials because the gel pore geometry and volume distribution vary with changes in

solvent. However, these packing parameters should be constant for each type solvent. The interstitial volume is not an intrinsic property for any type packing material because this volume is dependent upon the efficiency used to pack material into a column.

The most dominant pore entry size appears to be approximately equal to the ratio of micropore volume to surface area. The distribution shape factor ranges between 1.0 and 1.5 and is related to the shape of the micropore volume distribution. A larger shape factor indicates a more narrow micropore size distribution. A narrow micropore size distribution reflects that a packing material has more sensitivity in separating molecules which have a hydrodynamic size close to the most dominant micropore entry size. However, a decrease in sensitivity exists at larger and smaller pore entry sizes.

If several individual columns, each composed of a single packing material, have been calibrated using the Weibull function, then an SEC calibration can be estimated, without experimentation, for any multiple column set containing any combination of the individual packing materials. The above capabilities of the calibration technique shows that it is extremely powerful and accurate.

NOMENCLATURE

A	Constant, used in the Grubisic SEC calibration, see equation (1).
B	Constant, used in the Grubisic SEC calibration, see equation (1).
D	Average micropore diameter, used in the Dawkins SEC calibration, see equation (6).

$\bar{D}$ Most dominant micropore entry size, used in the Weibull SEC calibration, see equation (8).

D_c Cylindrical micropore cavity diameter, used in the Cassassa SEC calibration, see equation (4).

D_p Micropore cavity size defined to be the separation distance between two parallel planes, used in the Cassassa SEC calibration, see equation (5).

D_s Spherical micropore cavity diameter, used in the Cassassa SEC calibration, see equation (3).

d Molecular hydrodynamic diameter, see equation (11).

d_o Smallest molecular diameter which cannot penetrate into any of the packing micropore volume, see equation (6).

g Overall goodness of fit, defined as the standard error expressed as a percentage of the average experimental values, see equation (12).

K Distribution coefficient, the fraction of the micropore volume that is penetrated by a molecule.

k Proportionality constant, used in equation (11).

ℓ Molecular external length, used in the Giddings SEC calibration, see equation (2).

M Molecular weight.

N Number of data points, see equation (12).

n Micropore volume distribution shape factor, see equation (8).

P Number of packing materials used in an SEC column set, see equation (13).

s Micropore surface area per unit mass, see equation (2).

V_{ei} Experimental elution volume for data point i, see equation (12).

$\hat{V}_{ei}$ Elution volume of data point i which eas estimated from a function, see equation (12).

V_o Total interstitial volume, see equation (9).

V_p Total micropore volume, see equation (9).

V_t Total permeation volume, see equation (9).

$[\eta]$ Molecular intrinsic viscosity, see equation (11).

ν Micropore volume per unit mass, see equation (2).

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A METHOD OF CORRECTING FOR FLOW-RATE FLUCTUATIONS IN SIZE EXCLUSION CHROMATOGRAPHY CALCULATIONS: APPLICATIONS TO METHYLENE CHLORIDE/HEXAFLUOROISOPROPANOL SOLVENT SYSTEM

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ABSTRACT

The mixture of methylene chloride/hexafluoroisopropanol (70/30, v/v) is an excellent polyester solvent, but its low boiling point causes unstable flow when it is used for size exclusion chromatography (SEC). In high-performance SEC experiments, retention time is normally used to measure elution volume; however, unstable flow makes it difficult to calibrate an SEC column set or calculate molecular weight parameters from a chromatogram. We have devised a simple and inexpensive method to compensate for the effect of unstable flow in SEC calculations. A calibration marker injected along with each sample is used to indicate flow-rate variations. The ratio of the sample retention time to the marker retention time is invariant to flow-rate changes and is used in place of retention time as a measure of elution volume in the universal calibration technique. Calibrating a column set and analyzing chromatograms by this method result in a large improvement in the accuracy and precision of calculated molecular weight parameters.

INTRODUCTION

In an earlier report[1], the use of 70/30 (v/v) methylene chloride/hexafluoroisopropanol ($MeCl_2$/HFIP) as a size exclusion chromatography solvent for poly(ethylene terephthalate) (PET) was discussed. This solvent has one serious disadvantage; it is subject to large flow variations when used with the equipment configuration described by Overton and Browning. The flow rate must be constant

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for modern SEC equipment to be used: flow-rate fluctuations result in incorrect elution volume measurements, and thus molecular weight distributions and averages cannot be calculated correctly. Several investigators have used internal standards to compensate for flow-rate changes.[2,3] These methods are not directly applicable to high-performance SEC experiments in which the universal calibration approach is used. In this note, we describe a method applicable to the use of $MeCl_2$/HFIP to compensate for the effects of unstable flow in high-performance SEC.

A set of SEC columns is usually calibrated by measuring the elution volume of narrow-distribution polymer samples of known molecular weight. The elution volume is the amount of solvent that has passed through the chromatograph when the calibrant elutes through the column set. The molecular weight-elution volume function can be used in a universal calibration algorithm[4] to generate absolute molecular weights of polymer samples.

Modern high-performance chromatographic equipment operates with small solvent volumes. For example, a typical column set will elute six orders of magnitude of molecular weight in 6 mL of solvent volume. A stable pumping system is required as the elution volume must be measured by using the flow rate. In low-performance chromatography the solvent volume is measured with siphoning devices, but this procedure is not practical with the small range of elution volume in a high-performance machine.

Elution volume is measured in high-performance instruments with the relationship EV = RT•FR

where

EV = elution volume (mL),
RT = retention time (sec),
FR = flow rate (mL/sec).

Elution volumes of macromolecules injected at low concentrations and in small volumes are essentially independent of flow rate.[5] This means that fluctuations in the flow rate will be compensated for by an inverse change in retention time, and the product of the two variables will remain constant.

A calibrant or internal reference can be added to a polymer sample to take advantage of the constant elution volume in SEC systems. The elution volume of the marker will remain constant even if the flow rate changes between experiments and, thereby, will provide a means of compensating for changes in flow rate. The elution volume of a calibrant can be described as

$$EVc = RT'c \cdot FR'c = RTc \cdot FRc$$

where the subscript c stands for calibrant and the primed and unprimed variables describe two different experiments.

The elution volume of a sample isochromate can be expressed as

$$EVs = RT's \cdot FR's = RTs \cdot FRs$$

where the subscript s stands for sample isochromate.

The ratio of EVs and EVc is

$$\frac{EVs}{EVc} = \frac{FR's \cdot RT's}{FR'c \cdot RT'c} = \frac{FRs \cdot RTs}{FRc \cdot RTc}$$

Assuming that the flow rate is constant during each experiment (but not necessarily the same), we simplified this expression to

$$\frac{EVs}{EVc} = \frac{RT's}{RT'c} = \frac{RTs}{RTc} \qquad \text{or} \qquad EVs = \frac{RT's}{RT'c} \cdot EVc = \frac{RTs}{RTc} \cdot EVc$$

These equations show that the ratio of any isochromate retention time and the calibrant retention time is independent of the flow

rate. Therefore, the ratio is a constant of the SEC system and can be used to construct a molecular weight calibration.

A normal universal calibration is constructed by finding the functional relationship between log {[η] x MW} and EV for polystyrene standards, where [η] is the intrinsic viscosity of the standard. Replacing EV with the ratio of retention times and setting EVc equal to one, as it is a constant, results in a calibration method that is independent of flow variations.

EXPERIMENTAL

A Waters Model 244 chromatograph equipped with a Model 440 absorbance detector and Model 45 solvent delivery system was used. The column set was a bimodal Zorbax IIS manufactured by Du Pont Instruments. Samples were run at 0.1% w/v with an injection volume of 25 μL. The flow rate was set at 1 mL/min, but the apparent elution volume of the marker varied by as much as 0.6 mL, which indicated large flow-rate changes.

The data rate was fixed at 1 Hz and 10 min of data or 600 data points were acquired for each chromatogram. This data rate was adequate to properly locate the marker's peak maximum, which was used to identify the marker's RTc. Use of the points to either side of the peak maximum resulted in changes of approximately 0.01 I.V. units (dL/g) and 1,000 g/mol for the weight-average molecular weight ($\bar{M}w$) in the calculated distribution. A slower data rate would not permit the peak maximum to be located properly. Data collection and computation were done with an Intel 80/30 microprocessor.

Pressure Chemical's narrow molecular weight distribution polystyrene standards were used to generate the MW vs. retention time ratio. PET cyclic trimer was added to all polystyrene standards as the marker. A sample of melt-phase-produced PET with

0.54 dL/g I.V. was used to evaluate the correction method by measuring 10 chromatograms over several days. The naturally occurring cyclic trimer in PET served as its marker.

DISCUSSION

It is impossible to calibrate a column set when the flow rate fluctuates unless the fluctuations are considered. A calibration generated during a period of stable flow will be incorrect as soon as the flow rate is perturbed. This change in flow rate can lead to large errors in molecular weight measurements. Table 1 shows the elution volumes, measured at different times, for several standards. The table also shows the ratio of the retention times of the standards to the retention time of cyclic trimer. The measurement precision is increased by using the ratio method.

A linear model of log [(η) x MW] vs. EV is normally used to calculate molecular weight data from SEC chromatograms except when

TABLE 1

Comparison of EV and Ratio Measurements for Several Polystyrene Standards

Standard	EV, mL*	Ratio	Marker EV
860 K	6.43	0.612	10.50
860 K	6.00	0.611	9.82
498 K	6.62	0.634	10.44
498 K	6.26	0.638	9.82
37 K	8.04	0.770	10.44
37 K	7.53	0.767	9.82

*EV as measured assuming the flow rate is constant.

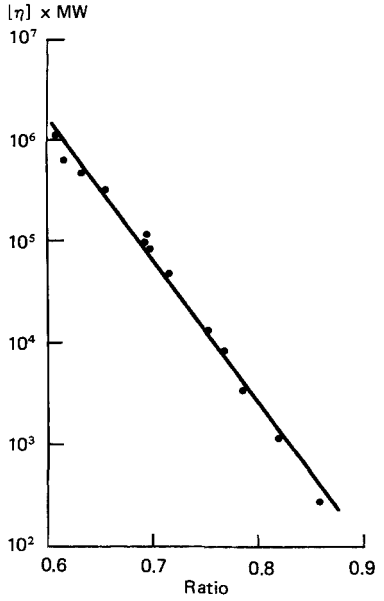

Fig. 1. SEC Calibration Curve Generated From Narrow Molecular Weight Polystyrene Standards by Using the Ratio Technique.

the linear correlation between the variables is poor. Figure 1 is the calibration curve generated by using the ratio RTs/RTc in place of EV, and its fit to a linear model is excellent. This model was used to generate the molecular weights and I.V.'s shown under the heading "Correction" in Table 2. A normal EV calibration model was constructed from data obtained over a short time when the flow was relatively constant. This model was used to calculate the molecular weights and I.V.'s shown under the heading "Normal" in Table 2. These are the normal results expected when the correction technique is not used. A figure illustrating the normal model is not presented here, but the correlation coefficient of the model is -0.996.

The correction method gave excellent results in an evaluation experiment in which PET was used as the testing sample. Table 2

TABLE 2

Evaluation Results

	Correction		Normal	
Run No.*	I.V. (dL/g)	$\bar{M}w$ (1000 g/mol)	I.V. (dL/g)	$\bar{M}w$ (1000 g/mol)
1	0.562	40.0	0.771	61.8
2	0.568	39.7	0.794	64.6
3	0.569	39.8	0.781	53.1
4	0.581	41.0	0.798	65.1
5	0.556	38.5	0.804	65.7
6	0.573	40.2	0.815	67.0
7	0.557	38.5	0.792	64.2
8	0.569	39.7	0.795	64.6
9	0.581	41.2	0.672	51.0
10	0.583	41.3	0.691	53.2

	Correction	Normal
I.V. mean	0.570	0.771
I.V. standard deviation	0.0097	0.049
Mw mean	39,886	62,032
Mw standard deviation	1,051	5,437

*The flow rate changed after the calibration samples were run and again between runs 8 and 9.

shows the I.V. and $\bar{M}w$ calculated from the chromatograms by using the correction scheme and normal calibration (described above). The universal $\bar{M}w$ of this polymer is 33,500 g/mol and the I.V. is about 0.54 dL/g. The normal calibration method shows large errors for both I.V. and $\bar{M}w$. These errors were caused by several flow-rate shifts. The flow rate changed after the calibration samples were run and again between experiments eight and nine.

Results obtained from the correction algorithm show some scatter, which is caused in part by small flow-rate fluctuations

during the SEC runs. Changes in flow rate can be seen by carefully examining the test and calibration chromatograms. The amount of fluctuation during a run limits the precision of the correction method. The data in Table 2 give a relative coefficient of variation equal to 2.6% for the $\bar{M}w$. This precision is adequate for most purposes.

Compounds other than PET cyclic trimer can be used as internal standards. We routinely use toluene, which is convenient, for polymers other than PET. The only requirement for an added standard is that it be well resolved from the polymer chromatogram. Otherwise the molecular weight calculations will be biased by the extra intensity of the standard.

We have presented a simple, inexpensive method to compensate for the effect of unstable flow in SEC calculations. The technique is useful with SEC applications that use solvent systems like $MeCl_2$/HFIP, when the high-performance pumps do not provide stable flow. The technique does not compensate for variations that may occur during a run. The degree of data correction is adequate for most applications.

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HPLC ANALYSIS OF NONIONIC SURFACTANTS – PART V; ETHOXYLATED FATTY ACIDS

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ABSTRACT

High performance liquid chromatography technique was used in order to achieve separation and identification of product composition of nonionic surfactants of ethoxylated fatty acids.

Lichrosorb SI-60 (10μm) column, under gradient elution of mixture of isopropanol, methanol and n-hexane (50°C) and UV detector at 220 nm, were used for best separation of ethylene oxide (EO) adducts of fatty acids consisting of up to 20 EO units.

No derivatization of the compounds was needed. An improved baseline, in spite of gradient elution, was achieved by adding negligible amounts of anthracene to the eluents.

Brominated ethoxylated fatty acids resulting from addition of bromine to the double bond of the hydrophobic chain were also separated without a need for change in elution conditions or derivatization.

INTRODUCTION

Ethoxylation of fatty acids with ethylene oxide or polyethylene glycols, in the presence of a variety of alkaline catalysts, yields a mixture of polyethylene glycol monoesters, polyethylene glycol diesters and free polyethylene glycols. A variety of such adducts can be obtained depending on molar ratio of EO to fatty acids used in the reaction (1-2).

Attempts to analyze the fatty acid adduct by chromatographic methods have been carried out by several authors. GLC analysis was done after chemical decomposition of the crude product by using the

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mixed acetic anhydride and p-toluene sulfonic acids. In this way the hydrophobic fatty acids composition was determined, but no information could be gathered on the internal EO adduct distribution (3). Aitzetmüller (4) was the first to report on the use of HPLC technique to analyze a commercial product of ethoxylated fatty acids. A most complicated chromatograph having typical pattern was shown but no identification of the peaks was given. Similarly, finger-prints of the product mixture were presented by Brüschweiler using its 3,5-dinitrobenzoyl chloride derivatives (5). More qualitative analysis were carried out by Nakamura and Matsumoto (6). The samples were acetylated prior to chromatography in order to decrease the materials adsorption on the column. The molar distribution of EO adducts in the range of 30 EO units was directly proportional to the reaction time. Several mobile phases were tested and their effect on the reaction time and the detector response were examined. The chromatogram was quite complex and no identification of the peaks was shown.

The following study demonstrates elution of fatty acid ethylene oxide adducts from various commercial sources without a need for either prior chemical treatment or derivatization. In addition, peaks identification was achieved by quantitative separation followed by their identification using chemical ionization mass-spectrometry.

Exthoxylated oleic acid were further brominated to obtain dibromostearic derivatives e.g. 9,10 dibromostearoyl-(9)-ethoxylated ester. These new surfactants were also easily eluted by our technique at the same conditions without any need for derivatization.

EXPERIMENTAL

Materials

Ethoxylated fatty acids with various ethylene oxide (EO) units were commercially available products from three different sources: 1. Oleic acid ester with average 4.5, 9 and 13.5 EO units (Mapeg-200, Mapeg-400 and Mapeg-600) were obtained from Mazer Chemicals, Inc. (USA); 2. Oleic acid ester with average 2 and 10 EO units (Myo-2 and Myo-10) were obtained from Nikko Chemicals Co., Ltd. (Japan); and 3. Commercial polyoxyethylene oleate without given specifications concerning the numbers of EO units (G-5507 and G-2143) were purchased

from Atlas Europol A.p.S. (Italy). The addition of bromine to the double bond of the oleic chain was carried out in our laboratory (7). The eluents were isopropanol, methanol and n-hexane HPLC grade from Bio-Lab Laboratories, Ltd. (Israel) and pure anthracene from Riedel-de Haën (Germany).

Procedure Technique

The analyses were performed on Spectra Physics HPLC chromatograph model SP-8000 equipped with a UV SP-770 variable wavelength detector (Schoffel Instrument Corp.) at 220 nm. The column was a commercially available 250 x 4.6 mm packed with Lichrosorb SI-60 (10 μm) purchased from Alltech Associates, Inc.

A gradient elution with isopropanol, methanol and n-hexane was carried out as described in Table 1, at a flow rate of 1 ml/min and a pressure of about 200 psi at 50°C.

The samples were dissolved in 7:93 volume percent (isopropanol: n-hexane) up to 5% w/w. Ten μl of solution were injected using an automatic loop injector.

Each component of the mixture eluted from the column was trapped using a fraction collector and analyzed by chemical ionization mass-spectrometry (CIMS, DuPont model 21-490 B, single focusing) equipped with a commercial double source CI/EI in an inert atmosphere of isobutane.

RESULTS AND DISCUSSION

Polyoxyethylene oleyl ester with average 4.5 EO units (Mapeg-200) was injected to the HPLC column after addition of traces of anthracene to the eluents in order to achieve best baseline and to obtain minimum destortion of the chromatogram caused by the gradient elution (8). Figure 1 demonstrates typical chromatogram recorded within 40 minutes. It can be seen that the main peaks are 4 and 5. The mass-spectra analysis confirmed that peak 4 is oleyl ester with 4 EO units (M.W. = 458) while peak 5 consists of 5 EO units. Peak 0 reveals existence of free oleic acid unethoxylated (M.W. = 282). Indeed free acid was determined either by its acid value or GLC techniques. In a similar way identification of the other peaks was

TABLE 1

The Method of Gradient Elution Used for the Separation of Ethoxylated Fatty Acids. (Continuous gradient).

Time (min.)	Isopropanol (Vol.%)	Methanol (Vol. %)	n-Hexane (Vol. %)
0.0	7.0	0.0	93.0
20.0	42.0	10.0	48.0
40.0	65.0	35.0	0.0
60.0	65.0	35.0	0.0
63.0	65.0	0.0	35.0
65.0	7.0	0.0	93.0

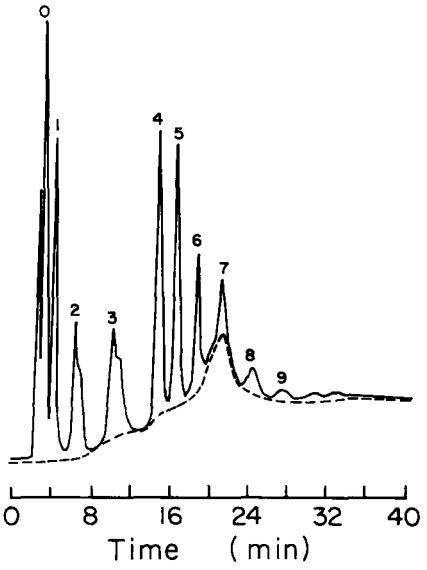

Figure 1: HPLC chromatogram of polyoxyethylene oleyl ester with 4.5 (av.) EO units (Mapeg-200). The peak number corresponds to the number of EO units on the fatty acid chain. The dashed line shows the baseline.

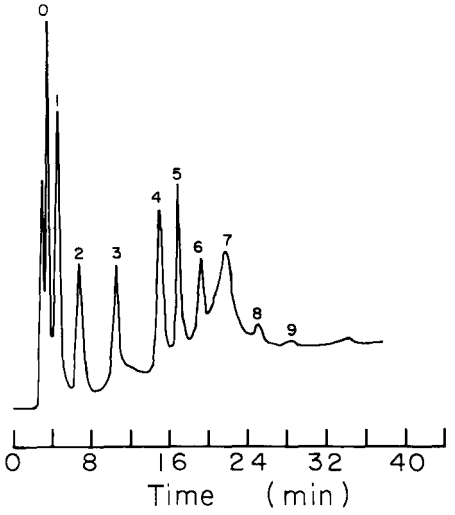

Figure 2: HPLC chromatogram of 9,10 dibromostearoyl-polyoxyethylene ester with 4.5 (av.) EO units.

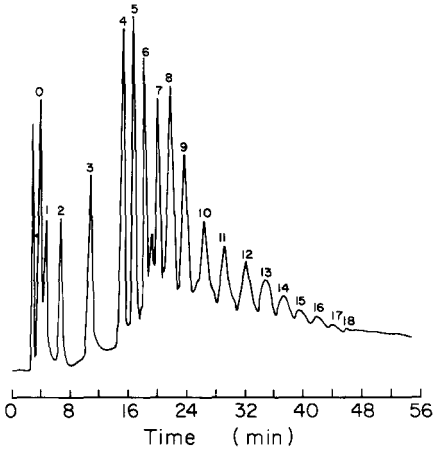

Figure 3: HPLC chromatogram of oleic acid (9) ethoxylated (Mapeg-400).

TABLE 2

Peaks Area Distribution of the Ethoxylated Oleic Acids Esters

Number of the peak in the Chromatogram	Relative area of the peaks (%)						
	Mapeg 200 (4.5 EO)	Mapeg 400 (9 EO)	Mapeg 600 (13.5 EO)	Myo 2 (2 EO)	Myo 10 (10 EO)	G 5507	G 2143
0	8.05	4.51	4.44	6.70	0.92	3.17	0.80
1	8.58	3.30		11.82	1.66	4.28	0.83
2	13.10	3.32		25.83	1.89	5.00	2.65
3	15.79	5.00		23.50	3.25	8.58	3.39
4	17.79	7.53	1.86	15.53	5.93	12.87	5.31
5	16.73	7.90	2.20	9.32	6.82	9.77	7.04
6	9.60	8.19	2.99	5.06	8.35	5.24	7.33
7	3.87	8.37	5.19	2.19	8.74	2.86	7.60

8	3.55	8.92	6.90	9.48	7.94	8.01
9	1.82	7.82	7.01	9.96	11.98	8.49
10	0.90	6.44	7.26	8.53	10.25	9.40
11		5.73	7.53	7.72	8.34	9.69
12		5.30	7.66	7.14	5.80	9.53
13		5.02	7.82	6.75	3.89	8.17
14		4.35	8.07	5.15		5.62
15		3.08	7.91	3.48		3.61
16		2.51	7.56	2.01		1.57
17		1.70	5.81	1.30		0.52
18		0.47	4.29	0.59		0.30
19			2.90	0.30		
20			1.69			
21			0.90			

obtained by chemical ionization mass-spectrometry. The numbers on top of each peak in the chromatogram stands for the number of EO units in the adduct, as confirmed by mass-spectra analysis. The dashed line in Figure 1 shows the baseline after addition of anthracene to the eluents to equalize its absorbance at 220 nm (8).

Figure 2 shows the chromatogram of Mapeg-200 after bromination in order to obtain 9,10-dibromostearoyl-polyoxyethylene ester with an average 4.5 EO units.

By doubling the number of EO units from 4.5 to 9 (molecular weight of ethoxylated chain 400;Mapeg-400) a more complicated chromatogram is obtained. Figure 3 illustrates that most isomers consist of 4 to 9 EO units. Nevertheless, one can see the appearance of all derivatives from peak 0 to 17 with excellent separation resolution.

It was impossible to identify existence of diesters probably due to their very high molecular weight. Therefore, Table 2 presents calculations of products distribution based on peak area. The diester isomers, although most probably exist in the product, were not taken under consideration. Table 2 shows that the commercial products are named according to the main isomers that have the highest accuracy in the compound. The areas were measured substracting any baseline shifts.

Evidently from Figure 4 it can be seen that the bromination does not drastically change the chromatogram fingerprints in spite the fact that the peaks are less pronounced due to the bromine addition to the chromophoric group.

Increasing the number of EO units to 13.5 caused the chromatogram to be quite difficult to interpret (see Figure 5 for Mapeg-600 and Figure 6 for the brominated Mapeg-600). Twenty one peaks were obtained. The most common isomers were 12-16 with good agreement with the manufacturer claim.

In order to evaluate the commercial materials and to find possible differences in their product distribution, two other ethoxylated oleyl esters with average 2 and 10 EO units were injected under similar conditions. As expected the product with two EO units (Myo-2; see Figure 7) will contain less EO units then the one with 4.5 EO (see Figure 1) therefore the main adducts will

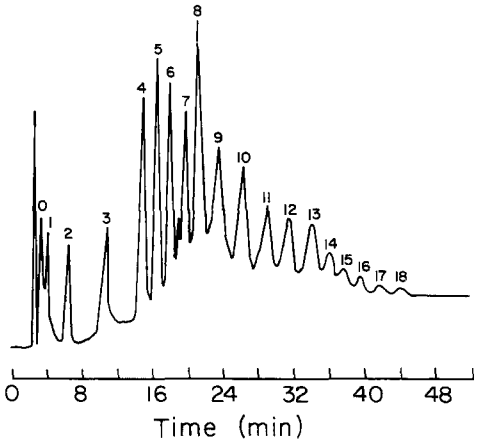

Figure 4: A typical HPLC chromatogram of 9,10 dibromostearoyl-polyoxyethylene ester with 9 (av.) EO units.

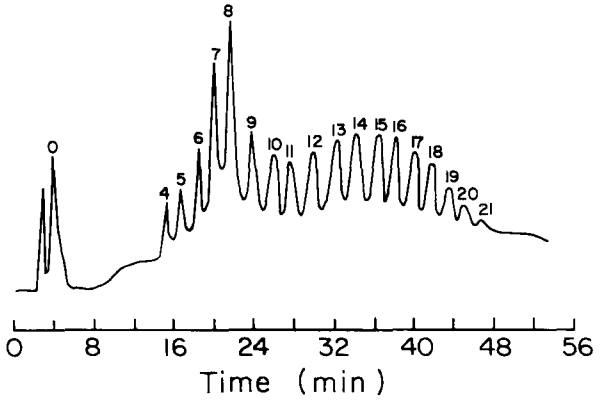

Figure 5: HPLC chromatogram of polyoxyethylene oleyl ester with 13.5 (av.) EO units (Mapeg-600).

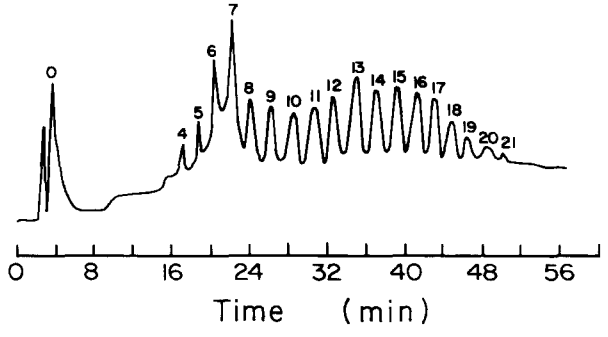

Figure 6: HPLC chromatogram of 9,10 dibromostearoyl-polyoxyethylene ester with 13.5 (av.) EO units.

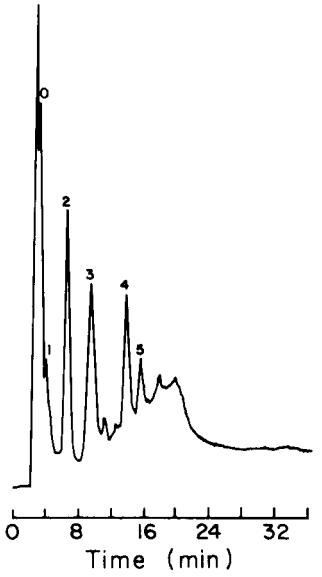

Figure 7: HPLC chromatogram of oleic acid (2) ethoxylated (Myo-2).

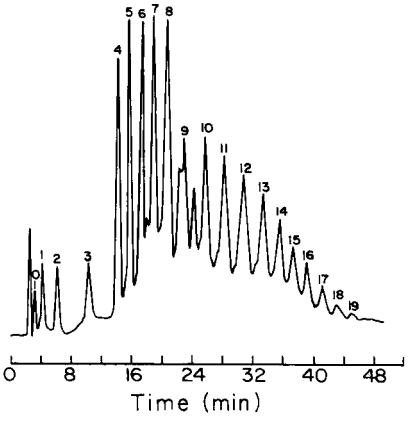

Figure 8: HPLC chromatogram of oleic acid (10) ethoxylated (Myo-10).

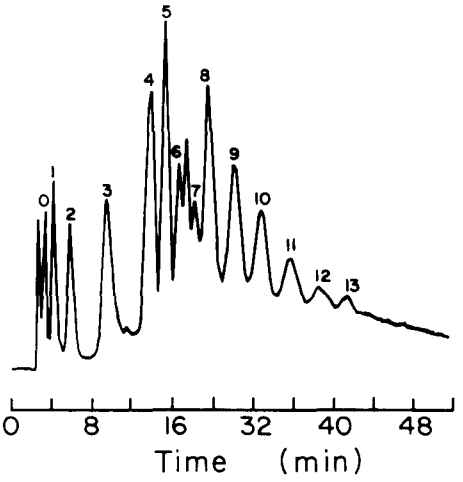

Figure 9: HPLC chromatogram of commercial polyoxyethylene oleate (G-5507).

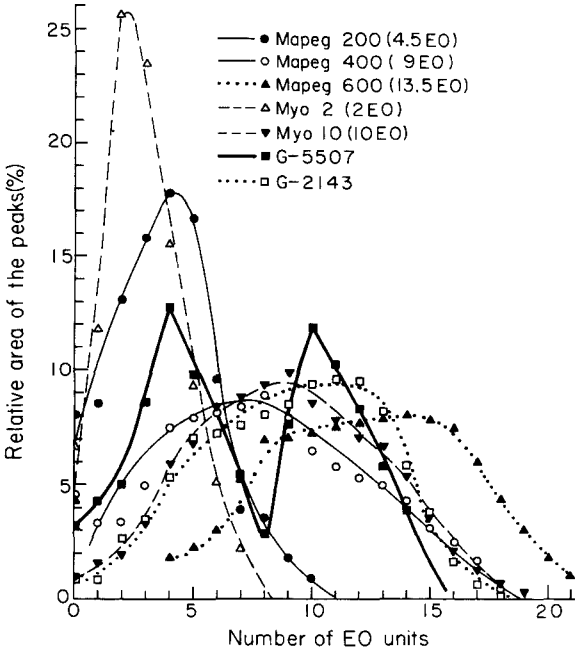

Figure 10: Illustration of the relative area distribution of the peaks versus the number of ethylene oxide units.

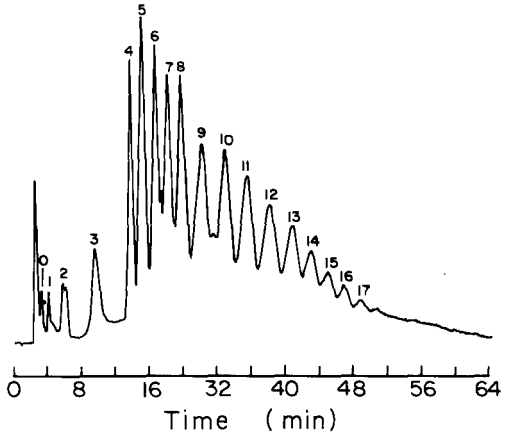

Figure 11: HPLC chromatogram of commercial polyoxyethylene oleate (G-2143).

appear under peak 2 and 3. Similarly, products containing 10 EO (Myo-10) will show quite identical chromatogram to the one with 9 EO units (Mapeg-400); (See Figure 8 and Figure 3).

When commercial surfactant identified only by its HLB (Hydrophilic Lipophilic Balance) value, without any other details or specification on a product's internal distribution one can use the HPLC method to have a full picture of its content. For example, two other commercial products from Atlas Europol (G-5507 HLB=10.4 and G-2143 HLB-12.2) were tested. A clear chromatogram was obtained (Figure 9) revealing that the first emulsifier is probably a mixture of two completely separate products. In order to distinguish between the mixture of the two surfactants and to obtain more accurate product distribution of the mixture a plot of relative area of the peaks versus number of EO units of all separated surfactants, including the G-5507, was demonstrated. It can be seen, for example, that Myo-2 has a maxima at 2 EO units with accordance to the manufacturer's claim. Similarly, all other surfactants have only one maxima revealing consistency of only one isomeric product. The G-5507 shows two peaks of 4 and 10 EO units. The other unknown surfactant G-2143 is probably a single product with average 10 EO units with a typical internal distribution (see Figures 10 and 11). While the second one (G-2143) is probably a single product

with average 10 EO units and typical internal distribution of the EO adducts (Figure 10).

CONCLUSION

The HPLC technique developed in this laboratory allows analysis, both qualitative and quantitative of any commercial ethoxylated fatty acid adducts or ethoxylated fatty alcohols. A simple injection of the crude product to the column, without the need for any pretreatment such as hydrolysis, acetylation of derivatization, is sufficient to obtain excellent separation. In addition, full identification of the adduct peaks was obtained using mass-spectrometry technique.

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THE DETERMINATION OF NON-OXIDIZABLE SPECIES USING ELECTROCHEMICAL DETECTION IN ION CHROMATOGRAPHY

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ABSTRACT

The use of an electrochemical detector for the ion chromatographic detection of non-oxidizable anions such as F^-, PO_4^{3-}, NO_3^-, and SO_4^{2-} is described. The electrochemical detector is placed in line after a fiber suppressor and responds to eluent pH changes as the dissociated acids pass through the detector. The intensity of the signal is dependent on the applied potential at the cell with 0.3V being an optimum. Minimum detection limits with a 0.10 mL sample injection volume are below 0.5 ppm for F^-, Cl^-, PO_4^{3-}, NO_3^-, and SO_4^{2-}. No adverse effects on the silver working electrode have been observed.

INTRODUCTION

Ion chromatography as originally developed by Small et al. (1) used conductimetric detection to measure the ions separated by an ion exchange column. This technique uses a second column placed after the separator column to chemically suppress the conductance of the eluent by converting the buffer solution to a solution of weakly dissociated acid. This chemically suppressed ion chromatography system is generally applicable to anions with pK_a values smaller than 7. The need for dissociation restricts the analysis of such species as iodide, sulfide, thiocyanate, etc. The

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electrochemical detector has been used to measure species which can be relatively easily oxidized but do not undergo sufficient dissociation for conductimetric decection (2). In practice it has been the custom to use both electrochemical and conductimetric detection to fully characterize the anions in a solution.

The work described here involves placing the electrochemical detector after the suppressor column. This arrangement causes the electrochemical detector to operate as a pH detector and thus respond to analyte ions that are not easily oxidizable. This phenomenon has been reported previously, but a thorough investigation was not undertaken (3).

EXPERIMENTAL

Equipment and Reagents

The equipment and general operating conditions used in this work are given in Table 1. All of the equipment was "as received" from the manufacturer. All operating conditions are standard conditions for the routine analysis of inorganic anions.

The standard anion solution was prepared by the appropriate dilution of 1000 ppm standard stock solutions made from reagent grade sodium salts. Distilled-deionized water was used to prepare all solutions which were introduced into the ion chromatograph including the ion chromatographic eluent.

Procedure

The normal procedure for the use of the ion chromatograph was followed with the exception that the electrochemical detector was placed after the fiber suppressor.

The pH of the effluent was measured in the following manner. The pH sensing assembly involved placing the combination pH electrode inside the barrel of a 6 mL disposable syringe. The outlet from the electrochemical detector was connected to the outlet of the disposable syringe with a female luer adapter. The pH of the solution was read manually from the pH meter display.

TABLE 1

Instrumental Parameters

Instrument:	Dionex Ion Chromatograph 10 Dionex Conductivity Detector Dionex Electrochemical Detector
Columns:	Dionex HPIC-AS3 Anion Analysis, 250mm Dionex AFS Fiber Suppressor
Eluent:	0.003 M $NaHCO_3$ + 0.0024 M Na_2CO_3
Injection Volume:	0.10 mL
Electrochemical:	0.00V to 0.45 V applied potential versus Ag/AgCl 5 nA to 100 nA full scale deflection
Conductivity:	30 μS full scale deflection
pH Meter:	Orion Research digital ionalyzer 501 Orion Lab Grade Combination pH electrode
Chart Speed:	0.5 cm/min
Standard Anion Solution:	4 ppm F^-, 4 ppm Cl^-, 20 ppm PO_4^{-3} 20 ppm NO_3^-, and 20 ppm SO_4^{-2}

RESULTS AND DISCUSSION

Figure 1 is an example of the conductivity and electrochemical chromatograms of the standard anion solution. The peaks in the conductivity chromatogram come later than the corresponding peaks in the electrochemical chromatogram due to the fact that the solution must travel through additional tubing between the two detectors. For all practical purposes, the two chromatograms are virtually indistinguishable.

The response of the electrochemical detector to nonoxidizable species can be explained based upon the changes in the eluent pH. As the eluent passes through the fiber suppressor, the cations are exchanged for hydronium ions. The eluent is converted into the weakly dissociated carbonic acid which does not liberate gas due to the pressures involved in ion chromatography. The cations which accompany the analyte ions are also exchanged producing the corresponding acid. Since all of the species routinely analyzed with conductivity detection in ion chromatography are reasonably

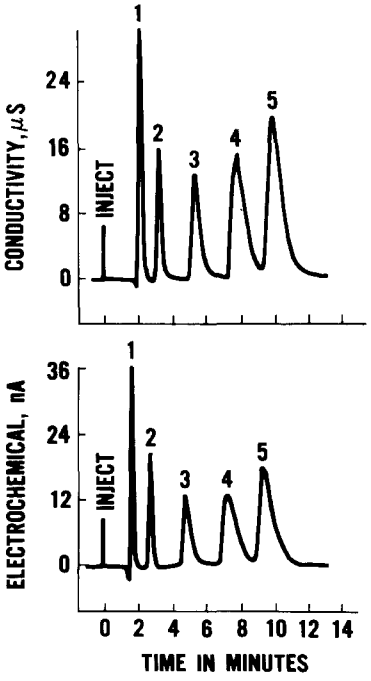

FIG. 1. Conductivity and Electrochemical Chromatograms of Standard Anion Solution. 1 = F^-, 2 = Cl^-, 3 = PO_4^{3-}, 4 = NO_3^-, and 5 = SO_4^{2-}.

well dissociated, the eluent pH will change from the background carbonic acid solution pH due to the presence of the dissociated acid. Species which are electroactive still respond to the potential applied to the cell.

The pH detector was assembled and placed in line immediately after the electrochemical detector. Figure 2 shows the electrochemical and pH chromatograms of the standard anion solution. The pH chromatogram was constructed by plotting the observed pH every 5 seconds for the 15 minutes of the chromatogram for a total of 181 data points. It is important to note that five separate pH variations are observed which correspond almost exactly to the electrochemical chromatogram.

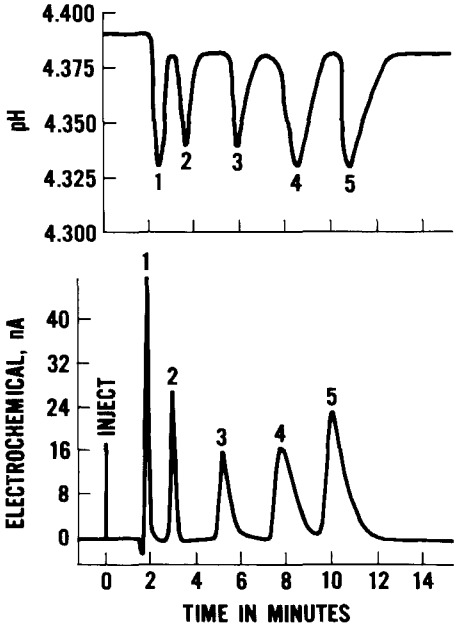

FIG. 2. Electrochemical and pH chromatograms of Standard Anion Solution. 1 = F^-, 2 = Cl^-, 3 = PO_4^{3-}, 4 = NO_3^-, and 5 = SO_4^{2-}.

The standard anion solution was chromatographed at different applied potentials. The anion peak heights were measured for five injections of the solution at each of four different applied potentials. The range of peak heights at each potential for each ion and the percent relative standard deviation (% RSD) were calculated to illustrate the reproducibility of the detector. The results from these experiments and calculations are reported in Table 2. It should be noted that the detector full scale response setting was varied to allow for peaks of sufficient height to minimize peak height measurement errors.

The following conclusions can be drawn from the data. The electrochemical detector will respond to common anions (whether oxidizable or not) at applied potentials customarily used in ion

TABLE 2

Peak Height Reproducibility*

ION		APPLIED POTENTIAL			
		0.00V	0.15V	0.30V	0.45V
F^-	range-cm % RSD	12.80-13.00 0.61%	10.55-10.70 0.58%	20.70-21.20 0.95%	9.55-10.00 2.35%
Cl^-	range-cm % RSD	10.05-10.15 0.35%	6.50-6.60 0.54%	11.70-11.90 0.70%	5.15-5.50 2.93%
PO_4^{3-}	range-cm % RSD	4.25-4.40 1.54%	3.60-3.65 0.76%	7.10-7.25 0.79%	3.30-3.55 3.36%
NO_3^-	range-cm % RSD	4.50-4.70 1.64%	3.65-3.70 1.13%	7.25-7.45 1.01%	3.35-3.55 2.60%
SO_4^{2-}	range-cm % RSD	7.35-7.45 0.60%	5.10-5.20 0.81%	10.05-10.20 0.56%	4.65-4.90 2.44%
Detector Full Scale Response		5nA	30nA	30nA	100nA

*Data is for five injections of same solution

chromatography using the silver electrode. The intensity of the signal is related to the magnitude of the applied potential indicating that higher applied potentials will produce increased sensitivities. As the potential nears the upper limit for the silver working electrode, the reproducibility begins to show a marked decrease. The optimum working potential appears to be approximately 0.30 V which will produce a relatively intense signal without compromising reproducibility.

Various dilutions of the standard anion solution were prepared to ascertain the range of concentrations amenable to detection using the electrochemical detector. The upper concentration limit is imposed by column overloading as is the case with conductimetric detection. Column overloading conditions vary with the anion but are apparent from the irregular peak shape

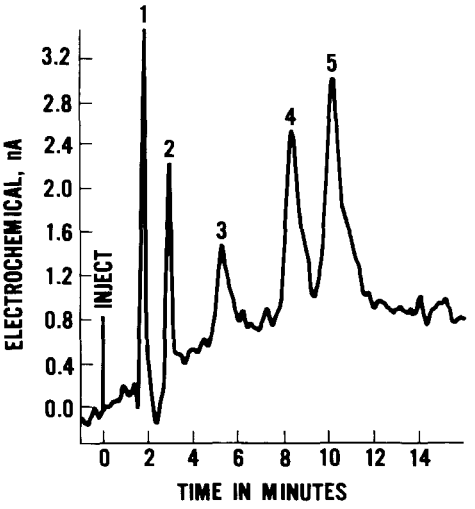

FIG. 3. Electrochemical chromatogram of dilute anion solution. Detector response 5 nA/V full scale deflection. 1 = 0.2 ppm F^-, 2 = 0.2 ppm Cl^-, 3 = 1.0 ppm PO_4^{3-}, 4 = 1.0 ppm NO_3^-, and 5 = 1.0 ppm SO_4^{2-}.

which results. This is easy to overcome simply by dilution of the sample. In general, linear response of 3 orders of magnitude can be expected depending upon column conditions and baseline stability due to pump noise. Figure 3 is the chromatogram of the diluted standard anion solution. 5.0 mL of the standard anion solution were diluted to 100.0 mL in a volumetric flask using the chromatographic eluent as the diluent. The baseline is not as stable as in Figures 1 or 2 but the peaks are still quite easily quantified. The concentrations suitable for analysis could be lowered even further with the use of either a larger injection volume or a preconcentrator column.

No adverse effects have been observed upon the silver working electrode by using this procedure. The electrode should be cleaned periodically to assist in baseline stability for solutions of low concentration, but this takes only a few minutes.

CONCLUSIONS

The use of the electrochemical detector as a pH detector provides for the use of one detector to determine the range of ionic species which normally required two detectors in the past. This should assist in simplifying ion chromatographic analysis and further extending the usefulness of this technique.

ACKNOWLEDGEMENTS

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A STUDY ON LIPID-LIPID AND LIPID-POLYPEPTIDE INTERACTIONS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Ternary systems containing phosphatidylcholine-cholesterol, phosphatidylcholine-gramicidin A or cholesterol-gramicidin A in tetrahydrofuran have been examined by high performance liquid chromatography. Preferential solvation of cholesterol and especially gramicidin A by phosphatidylcholine is observed. These results are interpreted in terms of hydrophobic interactions between membrane components.

INTRODUCTION

The basic matrix of biomembrane structure consists of a phospholipid bilayer, in which sterols, proteins and occasionally other lipids are embedded. The interaction of the various membrane components is currently a matter of study and discussion (1). The amphiphilic nature of phospholipids determines that the lamellar phase occurs only in the presence of excess water, for the naturally occurring phospholipid mixtures (2). However, phospholipids should interact with other membrane components even in solution, and the study of such interactions in organic solvents could provide a complementary view to the data obtained in aqueous suspensions. In the present paper we propose a novel

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approach to the study of lipid-lipid and lipid-protein interactions using high performance liquid chromatography (HPLC).

The method involves the equilibration of a chromatographic column with a phospholipid in organic solution; cholesterol or any other membrane component is then injected, dissolved in the solution used to equilibrate the column. Elution onto the column with the same equilibrating solution leads to the emergency of cholesterol (polypeptide, etc.) from the column, accompanied by a "trough" in the elution diagram; the trough (or vacant peak) is an indication of preferential solvation (3-7) of the second solute. When this preferential solvation is by phospholipid, it may be interpreted in terms of solute-solute interactions (8-10).

In this paper, we describe our results concerning the preferential solvation parameters in ternary systems containing tetrahydrofuran (THF)(1)/phosphatidylcholine (PC)(2)/cholesterol (CH)(3) and THF(1)/PC(2)/gramicidin A (3). Gramicidin A is used as a model of intrinsic polypeptide. Our results indicate the potential usefulness of HPLC as a tool for the study of the interactions between membrane components in non-aqueous solutions.

EXPERIMENTAL

CH was obtained from Sigma Chemical Co., St. Louis, Mo. USA. Gramicidin A was from Koch Light Lab. Egg yolk PC was purchased from Merck, purified according to Singleton et al. (11) and its purity checked by thin layer chromatography. THF was a Merck spectroscopic reagent.

A M-45 solvent delivery system, a U6K universal injector and a differential refractometer, model R 401, from Waters Assoc. were used in all the experiments. Samples were occasionally monitored with a Varian Varichrom variable wavelenght UV detector. The system was equipped with two μ-styragel columns with 10^4 and

10^2 Å nominal porosities, from Waters Assoc. The flow rate was 1.0 mL/min and the temperature, 28 °C.

Columns were equilibrated with THF/PC solutions, previously filtered through 0.45 μm Millipore filters, at concentrations ranging from 0.97×10^{-2} to 3.11×10^{-2} M. Higher concentrations could not be used because under these conditions the columns did not work adequately. For each eluent system CH or gramicidin A samples at several concentrations were prepared immediately before injection by dissolving them in the corresponding equilibrating solution. The injected volume was always 100 μL.

Before injecting any CH (or gramicidin A) sample, several 100 μL injections of binary solution containing a known excess or defect in weight fraction (Δw_i°) of any of the i components (i=1,2) at a fixed eluent composition were injected. The Δw_i° are related to the areas, A_i°, of the excess or defect peak appearing in the chromatogram. The subsequent injection of the same volume of a CH (or gramicidin A) solution, at a concentration c_3, will cause a peak (vacant peak), of area A_i, with a defect in the component preferentially adsorbed. The Δw_i value corresponding to the area A_i of the vacant peak can be deduced from the calibration Δw_i° vs. A_i°.

RESULTS AND DISCUSSION

Figure 1A shows a typical chromatogram of CH at a 30 mg/mL concentration with a 3.11×10^{-2} M solution of PC in THF as eluent. The first eluting peak appearing at an elution volume (V_e) of 13.3 mL corresponds to the vacant peak and the second one to the solvated CH (V_e=16.5 mL). As depicted in figure 1B the areas of the vacant peak are proportional to the amount of injected CH. This indicates that CH is preferentially solvated by PC.

The elution volume of the vacant peak depends on the PC concentration in the eluent, increasing as this concentration

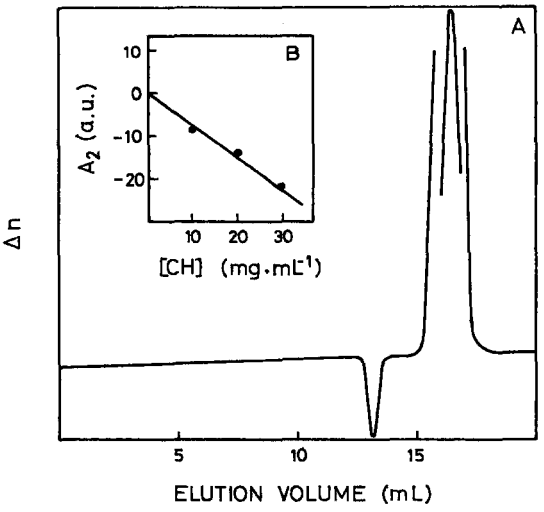

FIGURE 1. A. Chromatogram obtained by injection of 100 µL of a solution of CH (30.0 mg/mL) in the eluting solution. Eluent: 3.11 x 10^{-2} M solution of PC in THF.
B. Dependence of the vacant peak area on CH concentration.

decreases. This phenomenon is more pronounced at low PC concentrations. It seems that the retention of PC in the column is not only due to size-exclusion, and a reversible adsorption may also occur. On the other hand, we have checked that for a given concentration of injected CH, the vacant peak areas increase as PC concentration in the eluent becomes higher.

The preferential solvation coefficient λ has been classically measured by means of techniques such as dialysis equilibrium - differential refractometry (12-15), light scattering (16-18) and more recently gel permeation chromatography. The evaluation of λ by HPLC has been described by several authors (3-5) and by ourselves (6) for ternary systems solvent 1/ solvent 2/ macromolecule 3. When the eluent is composed of two liquids, λ is expressed as the excess or defect

in volume fraction of one of the solvents in the domain of the macromolecule with respect to the bulk solvent, once the thermodynamic equilibrium has been attained. However, in this case our eluent is a mixture of a liquid (THF) and a solid (PC) and it is more convenient to express the excess or defect in weight fraction instead of volume fraction, because in this way no PC density values are needed. According to this, λ may be expressed as:

$$\lambda = \frac{\Delta w_i}{c_3} \qquad (i= 1 \text{ or } 2) \qquad (1)$$

Negative values will be arbitrarily assigned to λs deduced from equation (1) when preferential solvation of component 3 (CH) by component 2 (PC) occurs. Positive λs would imply preferential solvation by component 1 (THF).

Results corresponding to the calibration curves at different eluent compositions are plotted in figure 2. In order not to overcrowd the figure, only two calibration curves have been included, but similar plots have been obtained for all the eluents used.

The results for CH samples injected at three concentrations in eluents with a PC composition ranging from 0.97×10^{-2} to 3.11×10^{-2} M are summarized in table 1; w_2 being the weight fraction of PC in the eluent.

The solutions under study correspond to PC/CH molar ratios in the injected sample between 1:1 and 1:8. A preferential solvation of CH by PC involves a PC defect, expressed as a negative increment of weight fraction for component 2, Δw_2 , in the outer binary phase in thermodynamic equilibrium with the ternary phase, and yielding a vacant peak of area A_2. From A_2 and the corresponding calibration curves, Δw_2 values can be obtained. λ values are negative for all the eluent compositions tested (see table). This indicates, as explained above, that CH is preferentially solvated by PC.

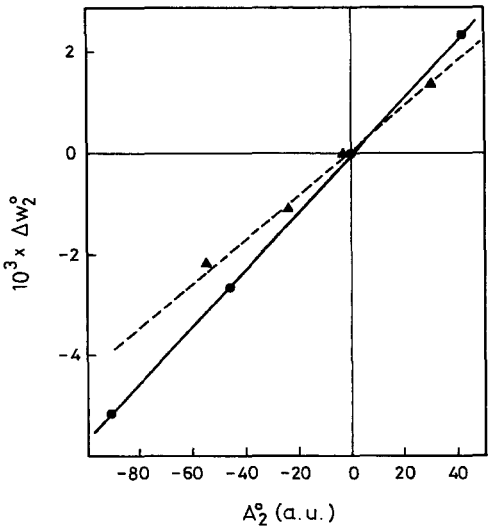

FIGURE 2. Calibration curves of excess and defect increments of weight fraction for PC (Δw_2°) vs. areas, A_2° , at two eluent concentrations. (●) 3.11 x 10^{-2} M; (▲) 1.30 x 10^{-2} M.

The variation of the preferential solvation parameter λ is plotted in figure 3 as a function of w_2; it can be observed that the absolute value of λ increases gradually as the eluent PC concentration increases. We have not found data in the literature about these systems for comparison. However λ values are of the same order of magnitude than those described for two solvents/one polymer (4-6) or one solvent/two polymers (7) systems, when a similar range of weight (or volume) fraction is used.

When gramicidin A is used as component 3 in a similar range of eluent PC concentrations varying from 0.97 x 10^{-2} to 2.59 x 10^{-2} M, a slight overlapping of the vacant peak with gramicidin A peak occurs (figure 4) in all the range of injected gramicidin A concentrations (from 0.5 to 16.0 mg/mL).

TABLE 1

Values Obtained for THF/PC/CH System from HPLC Chromatograms at Different Eluent Compositions.

Eluent PC concentration $.10^2$ (mole.L^{-1})	$w_2.10^2$	c_3 (mg.mL^{-1})	$\Delta w_2.10^4$	V_e (mL)	$\lambda^*.10^2$ (mL.g^{-1})
3.11	2.63	10.0 20.0 30.0	- 4.8 - 7.7 -12.1	13.3	-4.0±0.4
2.59	2.20	10.0 20.0 30.0	- 3.2 - 5.7 - 7.4	13.3	-2.9±0.3
2.15	1.83	10.0 20.0 30.0	- 2.4 - 4.7 - 8.3	13.5	-2.4±0.2
1.72	1.47	10.0 20.0 30.0	- 1.3 - 3.8 - 5.4	13.6	-1.8±0.3
1.30	1.11	10.0 20.0 30.0	- 1.8 - 2.8 - 4.1	13.7	-1.4±0.2
0.97	0.84	10.0 20.0 30.0	- 0.5 - 1.5 - 2.2	13.8	-0.7±0.1

* λ parameter for each eluent is the median of the values obtained for the three injected CH concentrations ± their average deviation.

The PC defect peak increases with gramicidin A concentration, the injected volume and the eluent PC concentration. Therefore, even an accurate quantitative evaluation of λ parameter being not possible in this system, a qualitative estimation of λ allows the comparison with the PC (2)/ CH (3) system. So, similarly to CH in this latter system, gramicidin A is preferentially solvated by PC, but with λ values in the gramicidin A case up to 50 fold higher than those obtained for CH.

The gramicidin A/ PC molar ratios in all the experiments with this polypeptide (from 1:1 to 1:50) are within the range

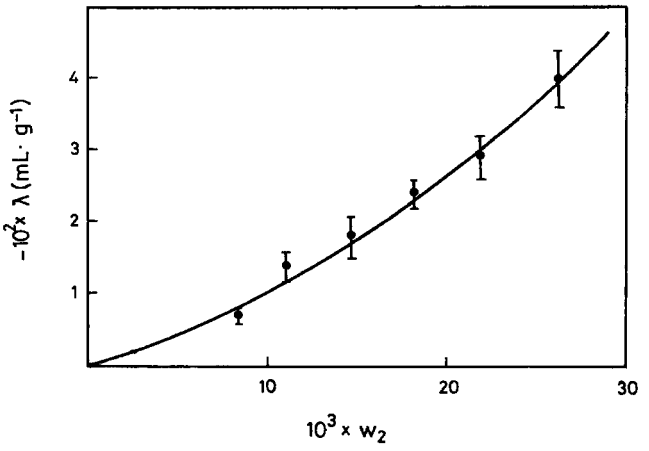

FIGURE 3. Preferential solvation parameter λ vs. weight fraction of PC in eluent, w_2 , for the THF/PC/CH system. Each point is the median value $\pm$ its average deviation.

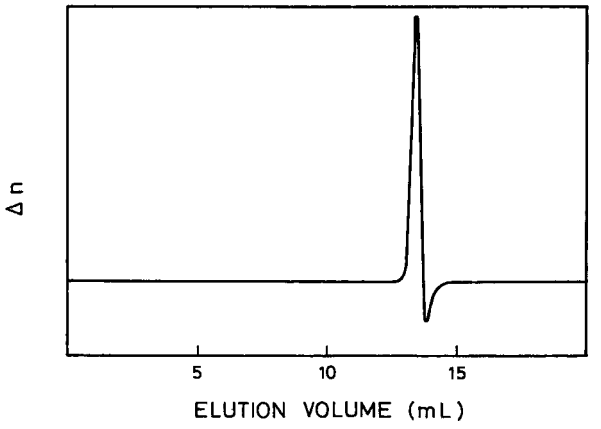

FIGURE 4. Chromatogram obtained by injection of 100 µL of a solution of gramicidin A (1.5 mg/mL) in the eluting solution. Eluent: 1.30 x 10^{-2} M solution of PC in THF corresponding to a PC/gramicidin A molar ratio of 16:1.

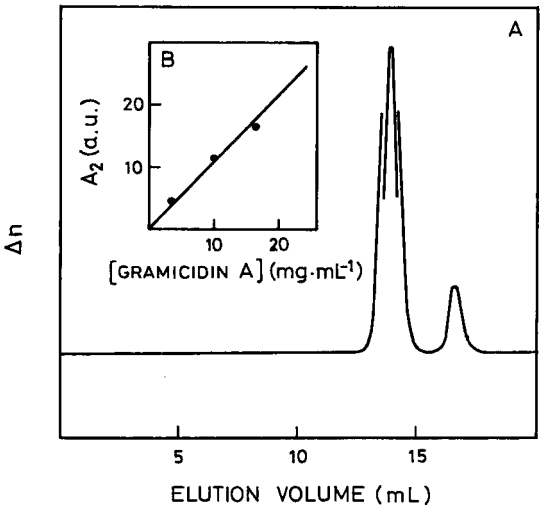

FIGURE 5. A. Chromatogram obtained by injection of 100 µL of a solution of gramicidin A (10.0 mg/mL) in the eluting solution. Eluent: 5.17 x 10^{-2} M solution of CH in THF.
B. Dependence of the peak of CH excess on gramicidin A concentration.

used in studies with liposomes, whereas for the THF(1)/PC(2)/CH(3) system it was necessary to increase the CH/PC molar ratio up to 8:1 in order to obtain significant vacant peaks.

Similar experiments with CH as component 2 and gramicidin A as component 3 have been performed in a range of eluent compositions varying from 2.32 x 10^{-2} to 5.17 x 10^{-2} M. Figure 5A shows a typical chromatogram for this system. The first peak eluting at 14.0 mL corresponds to gramicidin A and the second peak (V_e=16.5 mL) to an excess of CH. As depicted in figure 5B, the peak area of CH excess is proportional to the injected gramicidin A amount. This means, in contrast with the above results, that gramicidin A is preferentially solvated by THF.

TABLE 2

Values Obtained for THF/CH/gramicidin A System from HPLC Chromatograms at Different Eluent Compositions.

Eluent CH concentration $.10^2$ (mole.L^{-1})	$w_2.10^2$	c_3 (mg.mL^{-1})	$\Delta w_2.10^4$	V_e (mL)	$\lambda^*.10^2$ (mL.g^{-1})
5.17	2.20	3.1 10.0 16.6	1.4 4.3 6.3	16.5	4.2±0.4
4.33	1.85	3.1 10.0 16.6	0.8 2.6 3.7	16.5	2.5±0.2
3.62	1.55	3.1 10.0 16.6	0.4 1.5 1.8	16.5	1.3±0.2
2.32	1.00	3.1 10.0 16.6	0.2 0.9 1.3	16.5	0.8±0.1

* λ parameter for each eluent is the mean of the values obtained for the three injected gramicidin A concentrations ± their average deviation.

In table 2, the results corresponding to this system are summarized. Δw_2 values are obtained from the corresponding calibration curves (not shown). Although λ parameter is now positive, its absolute value remains close to that for the THF(1)/PC(2)/CH(3) system.

In summary, using THF as a solvent, gramicidin A-PC and CH-PC interactions are stronger than gramicidin A-CH ones. On the other hand, PC solvates better gramicidin A than CH. These results indicate the possibility of studying lipid-lipid and lipid-protein interactions by HPLC. Preliminary experiments using less polar

solvents, such as dichloromethane, failed to show any preferential solvation of gramicidin A or CH by PC; the requirement of a relatively polar solvent is an indication that the observed interactions are mainly hydrophobic in nature, such as they occur in the lipid matrix of biomembranes (studies using a variety of solvents are presently being carried out by us). The fact that PC interacts with both CH and gramicidin A, whereas these two do not show any interaction, suggests that the long, flexible hydrocarbon chains of the phospholipid are essential for establishing the hydrophobic bonds leading to preferential solvation of the other components. Also, the stronger PC-gramicidin A than PC-sterol interactions may be related to the different effects of proteins and CH, respectively disordering and ordering, on the static order of phospholipid chains, as observed by ^{2}H-NMR (19).

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RAPID DETERMINATION OF METHOTREXATE AND 7-HYDROXYMETHOTREXATE IN SERUM AND CEREBROSPINAL FLUID BY RADIAL COMPRESSION LIQUID CHROMATOGRAPHY

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ABSTRACT

A rapid, specific and sensitive radial compression reverse phase liquid chromatographic method for the analysis of methotrexate and 7-hydroxymethotrexate in serum and cerebrospinal fluid is reported. A mobile phase consisting of acetonitrile-methanol-pH 3 phosphate (8:15:77) at 6 ml/min flow rate was employed. The U.V. detector was set at 317 nm, and folic acid was used as an internal standard. A rapid extraction of methotrexate and 7-hydroxymethotrexate was performed using Sep-Pak cartridges with high extraction efficiency for both compounds. Patients serum and cerebrospinal fluid samples were analyzed by the described method and the concentrations of methotrexate were compared to those obtained by an enzyme immunoassay. No interference from other metabolites or anticancer drugs in the described assay was observed.

INTRODUCTION

Methotrexate, L-(+)-N-[p-[[(2,5-Diamino-6- pteridinyl) methyl] methyl-amino] benzoyl] glutamic acid, is a folic acid

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antimetabolite, highly effective in treatment of acute leukemia (1), non-Hodgkin's lymphoma (2), and trophoblastic tumors such as choriocarcinoma (3). In recent years, high doses of methotrexate with leucovorin rescue have been utilized for the treatment of various neoplasmic diseases. High plasma concentrations of 7-OH methotrexate are normally produced following this treatment. These concentrations are usually associated with nephrotoxicity due to low solubility in normal pH urine. This has generated a considerable interest in monitoring both methotrexate and its 7-OH metabolite in serum and other biological fluids. Numerous techniques have been utilized for the analysis of methotrexate in biological fluids. These include fluorometric methods (4,5), radioimmunoassays (6,9), competitive protein binding assay (10), enzyme immunoassay (11), radiochemical-ligand binding assay (12), isotachphoresis (13), enzyme assay (14) and high performance liquid chromatographic (HPLC) methods (15-22).

The use of HPLC methods eliminates the potential cross-reactivity of methotrexate with its metabolites, and makes the simultaneous analysis of this drug and its 7-OH metabolite feasible. Various modes of chromatography including anion-exchange, reverse-phase, and paired-ion have been employed for the analysis of methotrexate and other pteroylglutamate related compounds. The use of radial compression separation system for methotrexate and its 7-OH metabolite has not been previously reported. The utilization of such a system, coupled with a rapid C18 Sep-Pak sample cleanup as described in this report, results in higher sensitivity and chromatographic efficiency, and shorter analysis time.

MATERIALS

Reagents and Solvent

Methotrexate, U.S.P. grade (American Cyanamid Co.) was used as received. The purity of methotrexate was 80±4% as determined

by the U.V. absorbance of 0.01% (w/v) in neutral solution at 315 nm assuming molar absorptivity of 23,000 (23). An analytical sample of 7-OH methotrexate was kindly provided by Dr. David Johns (National Cancer Institute, Bathesda, MD). Folic acid (Eastman Kodak), leucovorin (American Cyanamid Co.), vincristine-sulfate (Eli Lilly Co.), adriamycin (Adria Lab.), bleomycin-sulfate (Bristol Lab.), etoposide (Mead Johnson Co.), cytarabine (Upjohn Co.), and prednisone (Philips Roxane Lab.) were either reagent or pharmaceutical grade. Sodium monobasic phosphate, 85% phosphoric acid, methanol, and acetonitrile, all from Fisher Scientific Co., were HPLC grade, and were employed without further purification. The HPLC purified water used was obtained by passing reverse osmosis water through a Norganic (TM) trace organic removal cartridge and 25-venting membrane filter (Millipore Co.).

Apparatus

The chromatography was performed using an HPLC system (Waters Associates) consisting of a system controller (Model 720), a ternary solvent delivery system (Models 6000 A and 45), a sample injection module (WISP), a data module (Model 730), and a radial compression separation system (Z-Module). A radial Pak uBondapak C18, 10 μ, 8 mm x 10 cm cartridge (Waters Associates) was employed.

Chromatographic Conditions

A mobile phase consisting of 0.08 M sodium monobasic phosphate-acetonitrile-methanol (77:8:15) adjusted to pH 3-3.1 with 85% phosphoric acid and filtered before use was employed. The flow rate used was 6 ml/min (pressure = 1500-1800 psi). The detector was set at 317 nm. The chromatography was performed isocratically on a Model 45 solvent delivery system after the mobile phase composition was established.

Calibration Curves Preparation

Separate stock solutions of methotrexate, 7-OH methotrexate, and folic acid in 0.01 N sodium hydroxide solution were prepared and employed for the construction of the calibration curves. Three ranges of calibration curves for methotrexate (viz. 0.0064-0.4, 0.4-8, and 8-80 µg/ml) or 7-OH methotrexate (viz. 0.01-0.5, 0.5-10, and 10-50 µg/ml) were constructed by transferring appropriate aliquots of the methotraxete or 7-OH methotrexate stock solutions to 1 ml of serum or CSF to yield serial concentrations in the above ranges. Aliquots of the folic acid stock solution equivalent to 0.14, 5, or 50 µg of folic acid were then added and the volume was brought to 1.75 ml with 0.01 N sodium hydroxide. The solution was then submitted to sample cleanup procedure.

Sample Cleanup

Sep-Pak C18 cartridges (Waters Associates) were utilized for sample cleanup and concentration. The cartridge was pretreated with 10 ml of methanol-acetonitrile (50:50) followed by 10 ml of methanol, 10 ml of sodium phosphate buffer (pH 3), and 10 ml of HPLC water, consecutively. The diluted sample was then passed slowly through the cartridge which was then treated with 15 ml of the phosphate buffer and 25 ml of HPLC water. The cartridge was vacuum dried and the compounds were eluted with 0.5 ml of methanol-water (95:5). The eluate was evaporated to dryness under gentle stream of nitrogen. The residue was then reconstituted with 75 µl of the mobile phase by sonication for 2 minutes, and transferred to an autosampler microvial. The tube was rinsed with additional 75 µl of the mobile phase (or mobile phase adjusted to pH 10 when a complete dissolution was not obained) and the rinsate was added to the microvial. The solution was then analyzed in duplicate under the above conditions. The automatic sample

injection processor was programmed to inject 40-75 µl of each microvial in duplicate. The cartridge was reused once, after washing and pretreatment as described above.

Patient Samples Analysis

Serum samples were collected at preselected time intervals from patients treated with different doses of methotrexate (Table 4). Cerebrospinal fluid specimens were also obtained from one of these patients at different intervals. To one ml of each of these samples, an aliquot of the internal standard stock solution equivalent to that used for the calibration curve was added, and the resulting solution was analyzed as described above. The samples were also analyzed for methotrexate by an enzyme immunoassay method (24) and the results of both techniques were compared.

RESULTS AND DISCUSSION

Representative chromatograms of blank, calibration, and patients serum and CSF samples are shown in Figures 1 and 2. As demonstrated in these figures, the peaks were sharp and symmetrical and all three compounds were eluted rapidly (viz. less than 6 min). High capacity factors were also preserved.

The specificity of the described assay was examined by determining the retention times of other anticancer drugs commonly used with methotrexate in combined chemotherapy (Table 1). In high dose methotrexate treatment, no other anticancer is utilized, and leucovorin rescue is usually initiated 24 hours after infusion of methotrexate. No interference in the assay from leucoverin was observed (retention time = 1.4 min). The serum endogenous folic acid was hardly detectable even at low AU detection setting (viz. 0.005). A full resolution between methotrexate and its 7-OH metabolite was obtained.

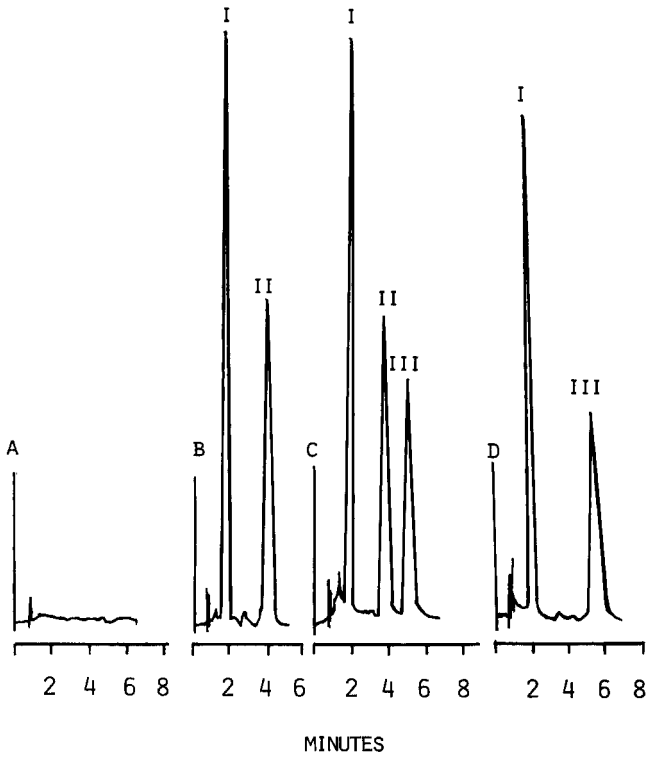

FIGURE 1

Representative chromatograms of a blank serum sample (A), a calibration serum sample containing 5 µg/ml of I and 1.6 µg/ml of II (B), a patient's serum sample collected 18 hours following the initiation of 6-hour infusion of 100 mg/kg of II and contains 5 µg/ml of I (C), and a calibration serum sample containing 5 µg/ml of I and 2.5 µg/ml of III (D),. Key internal standard (I); methotrexate (II); 7-OH methotrexate (III).

The determination of concentrations of methotrexate and 7-OH methotrexate in patient samples was performed using peak height ratio (methotrexate or 7-OH methotrexate/internal standard) vs. concentration calibration curves. Since the concentrations of methotrexate and its 7-OH metabolite following high dose methotrexate infusion extend over a wide scope, three calibration

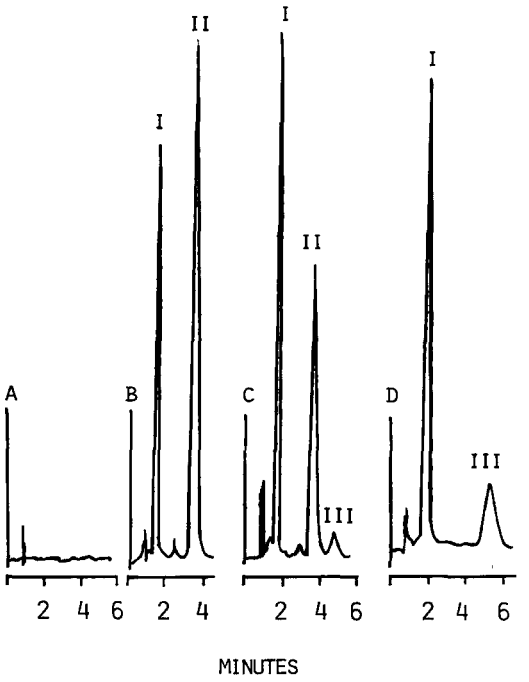

FIGURE 2
Representative chromatograms of a blank CSF sample (A), a calibration CSF sample containing 5 µg/ml of I and 4.8 µg/ml of II (B), a patient's CSF sample collected 24 hours following the initiation of 6-hour intravenous infusion of 100 mg/kg of II and contains 5 µg/ml of I (C), and a calibration CSF sample containing 5 µg/ml of I and 1 µg/ml of III (D). Key: internal standard (I); methotrexate (II); 7-OH methotrexate (III).

curves for methotrexate or 7-OH methotrexate had to be constructed to cover this range. Excellent linearity was obtained for these curves. Table 2 presents the ranges and correlation coefficients obtained for these curves.

A rapid extraction of methotrexate, 7-OH methotrexate, and folic acid from serum or CSF samples was obtained using Sep-Pak C18 cartridges under the conditions utilized. The recovery of

TABLE 1

RETENTION TIMES OF METHOTREXATE AND OTHER RELATED COMPOUNDS UNDER THE CONDITIONS EMPLOYED

Name of Drug	Retention Time (min.)
Methotrexate	3.79
7-Hydroxymethotrexate	5.06
Folic Acid	1.83
Leucovorin	1.41
Adriamycin	ND*
Vincristine	ND
Prednisone	ND
Etoposide	0.75
Bleomycin	ND
Cyclophosphamide	ND
Cytarabine	0.64

* Non-detectable in 10 minutes after injection.

methotrexate exceeded 91% at concentrations equal to 6.4 ng/ml (the sensitivity limit of the described method for methotrexate). Equally high recovery was also obtained for 7-OH methotrexate (viz. >0.95) at the sensitivity limit of the assay for this metabolite viz. 10 ng/ml. Various solvents and cartridges treatments were investigated, and the use of a small fraction (0.5 ml) of methanol-water (95:5) for elution of the compouds yielded cleaner chromatograms and substantially reduced the evaporation time.

TABLE 2

STANDARD CURVE DATA FOR METHOTREXATE AND 7-HYDROXYMETHOTREXATE IN PLASMA AND CEREBROSPINAL FLUID

Compound	Concentration Range (μg/ml)	No. of Experiments	Specimen	Correlation Coefficient
Methotrexate	0.0064-0.4	6	plasma	0.996 * (0.00693)
	0.4-8.0	6	plasma	0.9961 (0.00515)
	0.4-8.0	2	CSF	0.9998 (0.000033)
	8-80	3	plasma	0.9949 (0.011)
7-Hydroxymethotrexate	0.01-0.5	5	plasma	0.9949 (0.00311)
	0.5-10.0	5	plasma	0.9947 (0.00286)
	0.1-10.0	2	CSF	0.9952 (0.000778)
	10.0-50.0	2	plasma	0.9898 (0.0129)

* standard deviation

The accuracy of the described assay was tested by spiking blank serum samples with different amounts of methotrexate or 7-OH methotrexate and performing the analysis in duplicate as described earlier. Table 3 lists the amounts added and found of both compounds and the percent error for the samples analyzed. As illustrated in Table 3, the percent error for no sample did it exceed 7.8%, indicating excellent assay accuracy and reproducibility.

Table 4 presents the plasma and CSF concentrations of methotrexate and 7-OH methotrexate at different intervals in

TABLE 3

ACCURACY OF THE DESCRIBED HPLC METHOD

Compound	Amount Added (μg)	Amount Found (μg)	Percent Error
Metho-trexate	0.0064	0.0059	-7.81
	0.032	0.0302	-5.75
	0.064	0.0673	+5.16
	0.160	0.157	-1.88
	0.320	0.317	-0.94
	0.400	0.403	+0.75
	0.800	0.804	+0.5
	2.0	2.065	+3.25
	4.0	3.862	-3.45
	6.0	5.835	-2.75
	8.0	7.721	-3.49
	20.0	19.656	-1.72
	40.0	39.735	-0.66
	60.0	58.995	-1.68
	80.0	76.810	-3.99
7-Hydroxy-metho-trexate	0.01	0.0102	+2.0
	0.05	0.0534	+6.8
	0.125	0.118	-5.6
	0.500	0.492	-1.6
	2.5	2.489	-0.44
	5.0	5.003	+0.06
	10.0	10.587	+5.87
	20.0	20.996	+4.98
	30.0	28.584	-4.72

patients treated with different doses of methotrexate. As demonstrated in this table, the methotrexate concentrations obtained by the described method were generally smaller (= 29% less) but well correlated (r = 0.996) with those obtained according to a commonly used immunoassay (EMIT) (24). Similar differences were found between another HPLC method (18) and those acquired by a protein binding assay. These variations appear to

TABLE 4

METHOTREXATE AND 7-HYDROXYMETHOTREXATE PLASMA CONCENTRATIONS IN PATIENTS TREATED WITH METHOTREXATE

Patient No.	Time after Initiation of Infusion (hr)	specimen	Methotrexate Concentrations (μg/ml) Described Method	Methotrexate Concentrations (μg/ml) EMIT Method	7-Hydroxy-methotrexate Concentrations (μg/ml)
1[a]	3.0	serum	49.91	68.6	25.982
	6.0	serum	81.362	105.3	27.467
	12.0	serum	8.775	11.4	18.943
	18.0	serum	1.119	1.6	3.416
	27.0	serum	0.367	0.27	2.009
	39.0	serum	0.172	0.14	1.299
	51.0	serum	0.0933	<0.100	0.947
	63.0	serum	0.0756	<0.100	0.862
	3.0	CSF	2.352	1.63	ND
	6.0	CSF	4.500	5.86	ND
	12.0	CSF	2.065	3.00	0.097
	24.0	CSF	0.451	0.54	0.263
	48.0	CSF	0.251	0.14	0.0114
2[b]	0.5	serum	4.131	4.90	0.259
	8.5	serum	2.068	2.18	0.285
	22.5	serum	0.695	1.09	0.300
3[c]	3.0	serum	28.091	38.2	3.709
	6.0	serum	33.127	33.0	7.336
	12.0	serum	13.930	16.7	10.810
	18.0	serum	6.160	5.5	3.405

[a] Dose = 100 mg/kg infused over 6 hours.

[b] Dose = 5.9 mg/kg I.V. bolus followed by 11.8 mg/kg infused over 24 hours.

[c] Dose = 38.8 mg/kg infused over 6 hours.

be the result of the difference in specificity between the two techniques. While the metabolites and other structurally related compounds are completely separated from methotrexate by the described HPLC method, a 30% quantitation error is expected by EMIT due to interferences of these compounds at concentration > 1 μM (24).

In conclusion, the described HPLC assay is an accurate and specific method for the simultaneous analysis of methotrexate and

7-OH methotrexate in serum and cerebrospinal fluid. The high rapidity and sensitivity of this method make it an excellent tool for routine monitoring of this drug and its 7-OH metabolite in biological samples.

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AN HPLC METHOD FOR THE DETERMINATION OF THEOPHYLLINE AND ITS METABOLITES IN SERUM AND URINE

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ABSTRACT

A urine and a serum assay have been developed to quantitate theophylline and its major metabolites:1,3-dimethyluric acid, 3-methylxanthine and 1-methyluric acid. Reverse phase chromatography follows a serum acetone extraction procedure and a urine anion exchange clean-up procedure. Lower limits of sensitivity are 0.04 µg/ml for serum metabolites and 1 µg/ml for urine metabolites. Both assays are free of interference from endogenous substances. These assays have been tested successfully in pharmacokinetic and metabolic studies of theophylline.

INTRODUCTION

Measurement of the bronchodilator theophylline (1,3-dimethylxanthine) and its metabolites in various biological fluids has been the subject of much interest, as investigators study the pharmacokinetic characteristics and metabolic pathways of

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the drug (1-5). The major metabolic products of theophylline have been identified as 3-methylxanthine (3-mx), 1,3-dimethyluric acid (1,3-mu) and 1-methyluric acid (1-mu) (1,4). These metabolites arise from the 8-oxidation (1,3-mu), 1-demethylation (3-mx) and consecutive 3-demethylation and oxidation (1-mu) of theophylline (6). Methylation of theophylline occurs in infants (7) and is also said to occur in adults (2), with detectable serum levels of caffeine (1,3,7-trimethylxanthine) having been observed after multiple dose administration of theophylline.

To date, various assay methods have been developed for the determination of theophylline and its metabolites in plasma or serum and urine (1,4,5,8-15), but have not generally proven to be reproducible in our laboratory. Two investigators (5,8) describe a serum assay that resolved only 1 metabolite in addition to theophylline. Others (9,11,14) were able to resolve metabolites and parent drug in aqueous solution, but failed to show subsequent resolution in human sera. Tang-Liu et al (4) have developed an assay suitable for their analytical and pharmacokinetic work, which has been previously described.

Publication of urine analyses have been more abundant. However, we concur with Muir et al (10) who, in a recent comparative discussion, stated that previously reported procedures have not demonstrated sufficient selectivity and sensitivity. Published work by Tang-Lui and Riegelman (15) describes the most selective and sensitive assay to date but the procedure is time-

consuming and requires access to a pump that can automatically vary its rate of delivery of an individual solvent. In view of this, new methods have been developed in our laboratory that simply and reliably quantitate theophylline and its known metabolites in both serum and urine.

MATERIALS AND METHODS

Reagents

Reagents used were anhydrous crystalline theophylline, caffeine, ß-hydroxyethyl theophylline, tetrabutyl ammonium hydrogen sulfate and an anion exchange resin (DOWEX-1) chloride form, 200 to 400 mesh, all obtained from Sigma Chemical, St. Louis, Missouri. 3-Methylxanthine, 1,3-dimethyluric acid and 1-methyluric acid were purchased from Adams Chemical, Roundlake, Illinois. Mobile phase constituents used were methanol (HPLC grade) and water (HPLC grade) from Burdick and Jackson, Muskegon, Michigan and sodium phosphate monobasic from Fisher Scientific, Fair Lawn, New Jersey.

Aqueous stock solutions of parent drug (theophylline), metabolites (1-mu, 3-mx, 1,3-mu) and internal standard (ß-hydroxyethyl theophylline) were prepared on a daily basis. For serum assays, both theophylline and internal standard were dissolved to give concentrations of 200 µg/ml while metabolites were combined as a 20 µg/ml solution. For urine assays, theophylline and metabolites were prepared as a 50 µg/ml solution and

the internal standard as a 25 µg/ml solution. The mobile phase in both serum and urine assays consisted of methanol (solvent A) and 10 mM sodium phosphate (solvent B) adjusted to pH 4.5. The solvent system was degassed through 0.5 micron Durapore filter (Millipore, Bedford, Massachusetts).

Instrumental Conditions

The HPLC system consisted of a Perkin Elmer Series 2 liquid chromatograph pump, an LC-85 variable UV spectrophotometric detector and an LC autocontrol. The detection wavelength was set at 275 nm in order to optimize the absorption of all compounds of interest. The methylated xanthines absorb maximally at 273 nm while the methylated uric acids absorb at 290 nm. Samples were injected by means of a Perkin Elmer ISS-100 automatic injector. A Perkin Elmer Sigma 10 Data Processing System initiated the solvent program. Detector response was set at 0.02 absorbance units full scale for serum assays and 0.04 for urine assays. All determinations were performed at a solvent flow rate of 2.0 ml/minute.

Urine assays were performed using a µBondapak C18 10 micron column (3.9 mm I.D. X 30 cm) (Waters Scientific, Mississauga, Ontario). For serum analysis, the column was changed to an Ultrasphere ODS 5 micron (4.6 mm I.D. x 250 mm) (Beckman Instruments, Toronto, Ontario). This improved resolution of compounds of interest from endogenous material. Recently, this

Ultrasphere ODS 5 micron column was tried in the urine assay and was found to provide similar resolution.

Procedure

Serum assay: Three ml of acetone containing the internal standard (ß-hydroxyethyl theophylline) (65 ng/ml) were added to 200 µl of serum. Samples were acidified with 20 µl of glacial acetic acid to increase the recovery of 1 methyluric acid, vortexed for 2 minutes and placed in a centrifuge for 10 minutes. The organic layer were separated and evaporated to dryness by means of a Buchler Vortex Evaporation set at a temperature of 40°C. The residue was reconstituted with 125 µl of distilled water and 100 µl were injected onto the column. The methanol phosphate buffer solvent system was delivered in a stepwise linear gradient fashion, consisting of methanol at 6% for 8 minutes, increased from 6 to 21% in 5 minutes, retained at 21% for 7 minutes, then reset to 6% (Figure 1). The column was equilibrated for 10 minutes before the next injection. Pressure registered by the column remained between 3800 and 4200 psi.

Urine Assay: Pasteur capillary pipettes (145 mm length) (Maple Leaf) with distal ends plugged with glass wool were filled with 8 ml (0.42 g) of Dowex-1 suspended in distilled water with gentle stirring. Filled columns were washed with 6 ml deionized water. A sample volume of 200 µl was introduced onto the resin followed by 200 µl of distilled water containing the internal

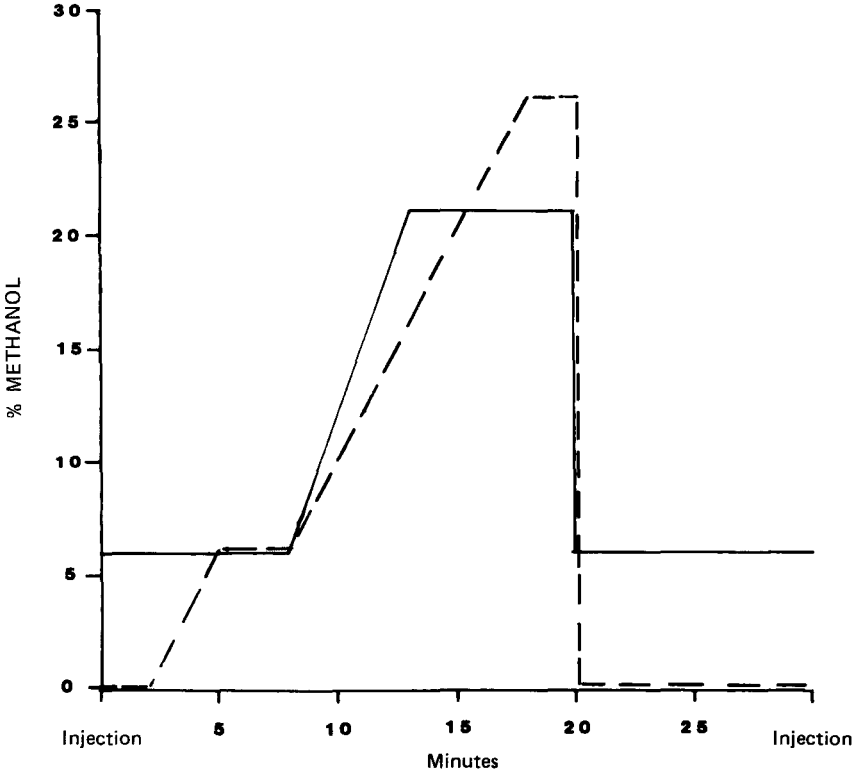

FIGURE 1: Solvent gradient program for serum (——), and urine assay (– – –). The percentage of methanol is shown as a function of elution time. Injections of sample occur every 30 minutes.

standard (25 µg/ml). Two ml of tetrabutylammonium hydrogen sulfate (TBA) 0.05 M were added to elute the desired compounds. Fifty µl of the eluant were injected onto the column. New columns were prepared for each sample analyzed. The mobile phase consisted of phosphate buffer with methanol at 0% for the first 2 minutes, increased at 2% per minute from 2 to 5 minutes and again from 8 to 18 minutes, (Figure 1). A 10-minute lagtime between

injections was used to equilibrate the column. The operating pressure was approximately 4000 psi.

RESULTS

Figure 2 shows the separation of an aqueous mixture of theophylline and its metabolites obtained with the described procedure (serum gradient program). Linearity of detector response was demonstrated by injecting onto the column, known amounts of theophylline and metabolites in aqueous solution. The resulting calibration curves were obtained by plotting the amount injected against the measured peak height. Curves were linear over a 20 fold variation in concentration, encompassing the range of serum concentration values expected from metabolites and theophylline after therapeutic administration of the latter (Figure 3). The correlation coefficient calculated for each compound was greater than 0.998.

Determinations of theophylline and metabolite concentrations in serum and urine were performed using an internal standard technique, where the peak height of compound of interest is compared to that of the internal standard. The appropriate equation for quantitation is:

$$\text{Conc} = \text{resp factor} \times \text{conc int std} \times \frac{\text{peak height unknown}}{\text{peak height int std}}$$

The relative response factor for each compound is previously determined by analyzing a sample in which the concentration of

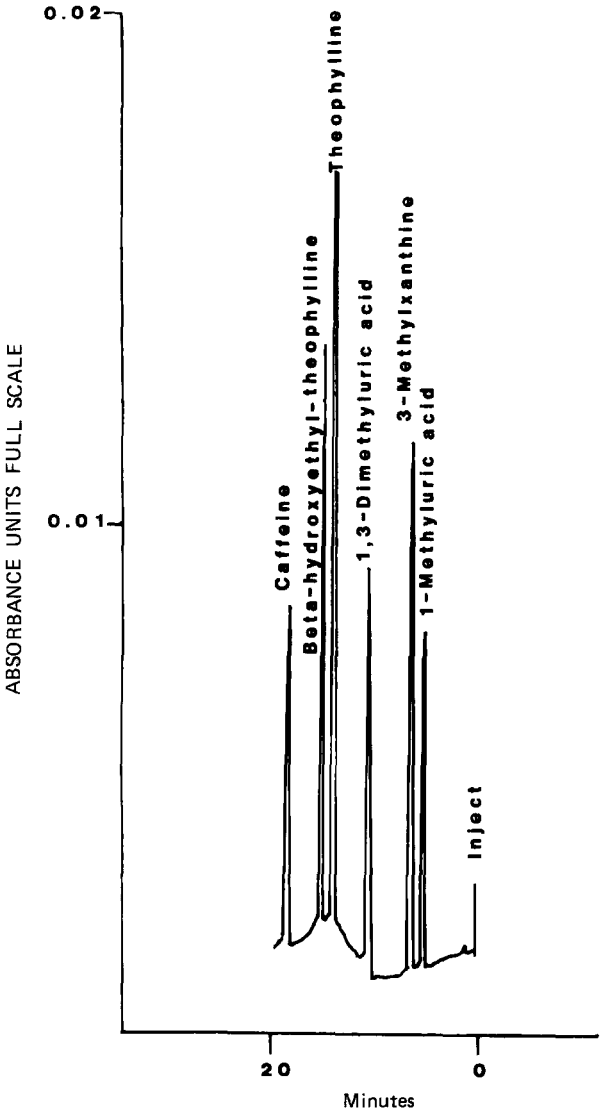

FIGURE 2: The HPLC separation of theophylline and its metabolites in aqueous mixture. Beta-hydroxyethyl theophylline serves as the internal standard.

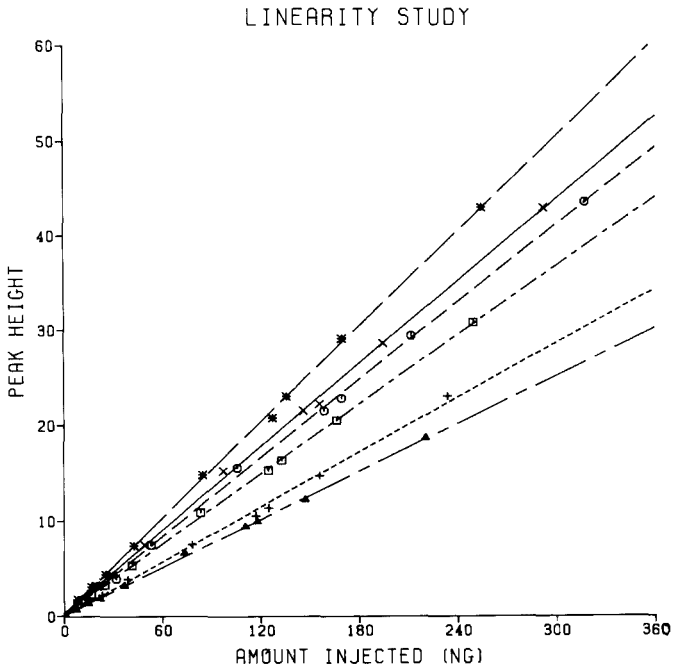

FIGURE 3: Theophylline and metabolite standard curves. Peak heights are proportional to amount injected: 1-methyluric acid (x), 3-methylxanthine (0), 1,3-dimethyluric acid (+), theophylline (*), ß-hydroxyethyl theophylline (□), caffeine (Δ).

both the component of interest and internal standard is known:

$$\text{resp factor} = \frac{\text{concentration of compound}}{\text{concentration of standard}} \times \frac{\text{peak height of standard}}{\text{peak height of compound}}$$

Table 1 presents the recovery and precision data obtained from the serum assay. The absolute recovery from serum was measured following the addition of a known concentration of compound to drug free serum and comparing the peak height obtained after extraction to that obtained after direct injection of the substance in aqueous solution. Six determinations

TABLE 1

Absolute Recovery and Retention Time of Theophylline and Metabolites in Serum (n=12)

Compound	Retention Time	Absolute Recovery	CV (%)
8-hydroxyethyl theophylline	19 min	102%	1.6%
theophylline	17 min	98%	5.6%
1,3-mu	14 min	96%	6.1%
3-mx	10.5 min	88%	8.1%
1-mu	8.5 min	66%	7.3%

were performed at two concentrations of metabolites (0.5 µg/ml, 1 µg/ml) and two concentrations of theophylline (10 µg/ml, 20 µg/ml). Similar data are presented for the urine assay (Table 2). Six determinations were performed at 20 µg/ml and 40 µg/ml.

DISCUSSION

The serum extraction procedure resulted in the immediate precipitation of serum proteins. Figure 4-A shows the chromatogram of theophylline-free serum from a volunteer who had abstained from xanthine containing food and beverages for the previous 48 hours, and again after ingestion of 10 mg/kg of oral theophylline (Figure 4-B). No endogenous substances were found to interfere with the analysis. In this assay, caffeine elutes at 20 minutes. Since all volunteers received only 1 therapeutic dose of theophylline, no quantifiable peaks of caffeine were observed at its elution time (2). The range of 24 hour serum concentrations of

TABLE 2

Absolute Recovery and Retention Time of Theophylline and Metabolites in Urine (n=12)

Compound	Retention Time	Absolute Recovery	CV (%)
8-hydroxyethyl theophylline	18 min	89%	10.2%
theophylline	17 min	70%	8.6%
1,3-mu	14 min	73%	10.3%
3-mx	11 min	78%	11.1%
1-mu	9.8 min	72%	7.3%

the major metabolites seen after one dose (6-10 mg/kg) of theophylline was from 0.04 µg/ml to 0.6 µg/ml for 1-methyluric acid and 3-methylxanthine and from 0.05 µg/ml to 1.5 µg/ml for 1,3-dimethyluric acid. The limit of sensitivity of this assay for all compounds is 0.04 µg/ml.

The urine assay involves a preliminary clean up procedure, adapted from the work of Thompson et al (8). It entails anion exchange separation where Dowex, a strongly basic anion exchange resin, binds the polar ionized compounds of interest as well as the endogenous interfering substances. The methylated xanthines, being weak bases, are not preferentially retained by the column and are eluted with the water. The polar methylated uric acids are retained by the column and will elute following the introduction of an ion with higher affinity for the resin. In the present example, the sulfate ion contributed by the TBA has a fairly high selectivity for the resin. Figure 5-A shows the efficiency of this procedure in removing endogenous interfering compounds

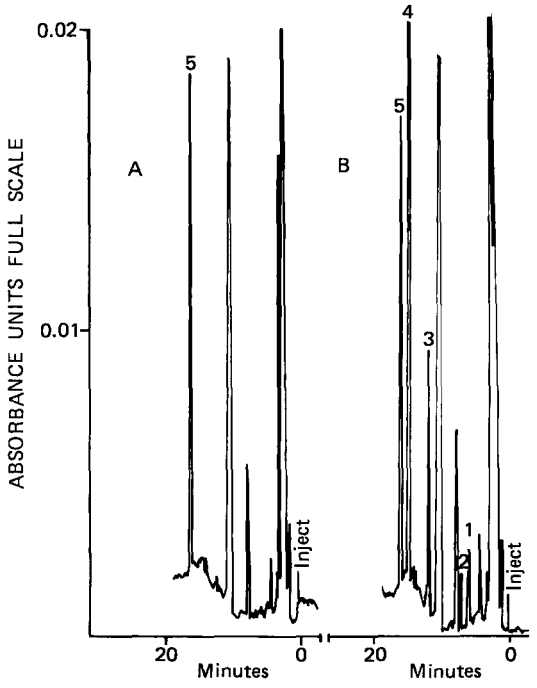

FIGURE 4: A. Chromatogram of a volunteer's theophylline-free serum after a 48 hour abstinence from dietary xanthines: 5) internal standard (ß-hydroxyethyl theophylline). B. Volunteer's serum after ingestion of 10 mg/kg theophylline: 1) 1-methyluric acid, 2) 3-methylxanthine, 3) 1,3-dimethyluric acid, 4) theophylline, 5) internal standard.

from a volunteer's drug free urine. Figure 5-B is a urine chromatogram of the same subject after the administration of 10 mg/kg of theophylline. The assay sensitivity for all compounds is 1 µg/ml.

Separation of the 3 major metabolites of theophylline from endogenous urinary xanthines and uric acids eluting from the column at similar retention times has plagued investigators in

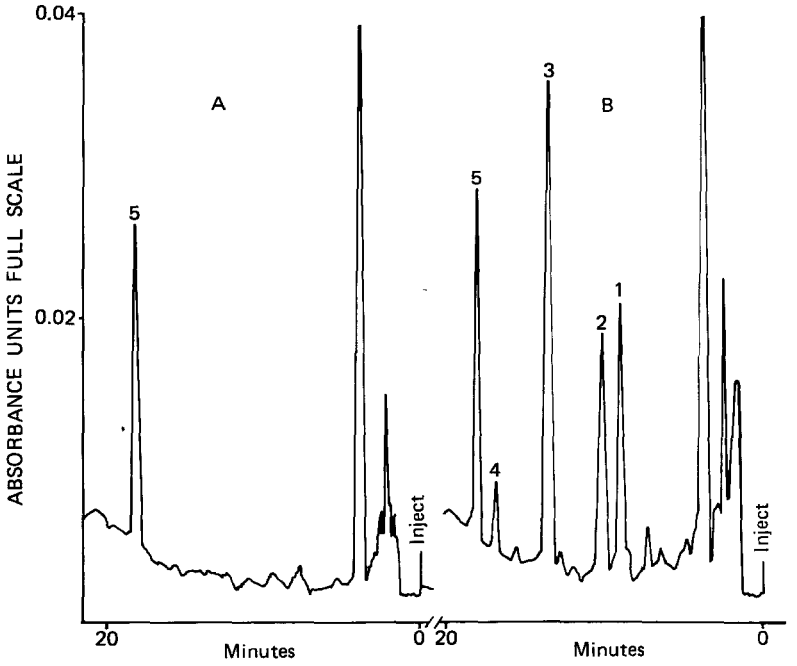

Figure 5: A. Chromatogram of volunteer's theophylline-free urine maintained on a xanthine-free diet for 48 hours: 5) internal standard (ß-hydroxyethyl theophylline). B. Volunteer's urine after ingestion of 10 mg/kg theophylline: 1) 1 methyluric acid, 2) 3-methylxanthine, 3) 1, 3-dimethyluric acid, 4) theophylline, 5) internal standard.

this area of chromatography (3). While urine composition will vary among individuals (15), usually a xanthine-free diet was found to abolish interfering peaks.

Our procedure has sufficient selectivity and precision for practical use, as demonstrated by the results obtained from the following pharmacokinetic study. A single intravenous dose of theophylline as aminophylline (363 mg) was administered to a

TABLE 3

Recovery of Theophylline and its Metabolites from the Urine of A Human Volunteer

Compound	% Recovered (Dose 1)	% Recovered (Dose 2)
theophylline	14.3%	16.8%
1,3-dimethyluric acid	39.2%	40%
1-methyluric acid	17.7%	16.33%
3-methylxanthine	11%	13.2%
Total	82.3%	86.3%
Collection period	48 hours	48 hours

normal male subject on 2 separate occasions. Total (mole per cent) urinary recovery of theophylline and its metabolites after a 48 hour urine collection was found to be 82.3% and 86.3% (Table 3).

This study was repeated in 7 additional subjects who received a dose of 6 mg/kg intravenously on 2 separate occasions. Assuming theophylline is 80% of the aminophylline dose administered, the molar percent urinary recovery of theophylline varied from as low as 73% to as high as 123% (mean 95%). Two explanations for greater than 100% recovery can be put forward: 1) even though subjects consented to xanthine-free diets for 48 hours prior to dosing, some dietary intake of xanthine containing foods might have occurred (10); 2) aminophylline USP standards have allowable limits of $\pm$ 7%. Some subjects may have received more than the labelled dose of aminophylline.

Few authors have been able to adequately describe an assay that consistently separates and quantitates all known metabolites

of theophylline in biological fluids. While a serum (4) and urine (15) assay have been reported that are both selective and sensitive, we have found the present assays simpler and more suitable for our laboratory purposes than previously published reports. These assays are being used in current pharmacokinetic studies of theophylline and its metabolites.

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LIQUID CHROMATOGRAPHIC DETERMINATION OF MECLOFENAMIC ACID IN EQUINE PLASMA

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ABSTRACT

Meclofenamic acid is extracted to dichloromethane together with the internal standard diclofenac sodium. After evaporation of the organic solvent the residue is dissolved in the chromatographic eluent and analyzed by liquid chromatography. The acids are separated on a column packed with Spherisorb ODS with methanol - phosphate buffer as the eluent and detected at 280 nm. A possible metabolite of meclofenamic acid was also detected in the chromatograms. The detection limit for meclofenamic acid in plasma was 0.361 µmol/L (0.107 µg/ml) for 1.5 ml sample size. The conditions of quantitative extraction of meclofenamic acid and diclofenac to dichloromethane are given.

INTRODUCTION

Meclofenamic acid is a potent non-steroidal anti-inflammatory agent belonging to the fenamate group [1]. The metabolism of the drug in man has been studied with tritium-labeled drug in combination with fluoro-

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metry, gas chromatography, and thin-layer chromatography [2,3]. The drug has been approved in some countries for the treatment of horses and analysis of meclofenamic acid in equine plasma has been performed with fluorometric assay [4,5].

Meclofenamic acid was included in a study of the effects of drugs on performance in the horse [6]. Quantitative analysis of the acid in equine plasma was required to correlate possible effects of the drug with the plasma concentrations. Our method involves liquid chromatographic isolation of meclofenamic acid after an initial extraction and concentration of the substance together with the internal standard, diclofenac sodium. Fluorometric detection of the chromatographic eluent was tested but photometric detection at 280 nm gave better sensitivity.

EXPERIMENTAL

Apparatus

The liquid chromatographic system consisted of a Constametric I pump (Milton Roy Company, Riviera Beach, Fla., U.S.A.), a sample injection valve with a 20 µl loop (Rheodyne, Berkeley, CA, U.S.A.), and an ultraviolet detector with variable wavelength, LDC Spectro Monitor III.

The chromatographic columns were of 316 stainless steel, 100 mm x 3.0 mm, equipped with zero volume Swagelok unions and column end fittings. Two-µm stainless steel frits from Altex were used. The columns were packed with LiChrosorb RP-18, 5 µm (Merck, Darmstadt, G.F.R.) or Spherisorb ODS, 10 µm (Phase Separations Ltd., Queensferry, United Kingdom).

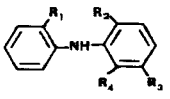
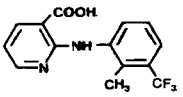
A Shimadzu spectrophotometer, UV-210 A, was used for the photometric measurements in the batch extraction experiments and the determination of pH was performed with a Metrohm 620 pH-meter.

Chemicals and Reagents

Methanol and dichloromethane were of analytical-reagent grade quality and obtained from Merck. Meclofenamic acid (mol. wt. = 296.2) and mefenamic acid were kindly supplied by Parke, Davis & Co. (Pontypool, United Kingdom), diclofenac sodium by Ciba-Geigy AG (Basle, Switzerland) and flunixin meglumine by Schering Corporation (Kenilworth, NJ, U.S.A.). Their structures are given in Table 1. Tetrabutylammonium hydrogensulfate (TBA) was obtained from Hässle AB (Mölndal, Sweden). The phosphate buffers used in the batch extraction and chromatographic experiments were prepared from sodium dihydrogen phosphate and disodium hydrogen

TABLE 1.

Structure of the Acids

Formula	Name	R_1	R_2	R_3	R_4
	Mefenamic acid	COOH	H	CH_3	CH_3
	Meclofenamic acid	COOH	Cl	CH_3	Cl
	Diclofenac sodium	CH_2COONa	Cl	H	Cl
	Flunixin				

phosphate. Citrate buffer, prepared from citric acid and sodium hydroxide, was used in the preparation of plasma samples. All buffers had an ionic strength of 0.1 and were prepared with ion-exchanged and distilled water.

Determination of Distribution Ratio

Meclofenamic acid was dissolved in 0.1 M sodium hydroxide as the acid has a limited solubility at lower pH. The solubility of meclofenamic acid at pH 7 is ca. 91 µmol/L (27 µg/ml) [3]. Diclofenac sodium was dissolved in 0.05 M disodium hydrogen phosphate. Buffer solutions with known concentrations of the acids were prepared. The excess of sodium hydroxide, used to dissolve the meclofenamic acid, was neutral-

ized with phosphoric acid. The buffer solutions containing the drugs were mechanically shaken with equal volumes of dichloromethane at 25°C in a water bath for 30 min. After phase separation, the absorbance of the aqueous phase was measured photometrically and the concentration of the acid was calculated using 278 nm, ε = 6570 for meclofenamic acid and 276 nm, ε = 10600 for diclofenac. The concentration of the acid in the organic phase was then calculated from the initial concentration. The pH of the aqueous phase was also measured after phase separation.

Chromatographic Technique

The eluents were prepared by mixing methanol and phosphate buffer pH 6.1. The solution was allowed to stand overnight and was treated in an ultrasonic bath for some minutes before use. TBA, when used, was dissolved in the phosphate buffer.

The columns were packed at 39 MPa with methanol as the driving liquid. LiChrosorb RP-18 was suspended in dichloromethane (0.1 g/ml) and the column was packed downwards [cf. 7]. Spherisorb ODS was suspended in methanol and the column was packed by the upwards slurry packing technique [8].

The void volume of the column, V_m, was determined by injecting sulfathiazole, which was not retained in

the system. This value of V_m was used to calculate the capacity ratio, k'.

Sampling

Blood samples were collected in vacuum tubes, Venoject®, containing sodium heparin. Plasma was prepared and stored frozen at -20°C until analyzed.

Pooled equine plasma from four horses that had not received any drug was used as blank plasma.

Sample Preparation

Meclofenamic acid was dissolved in 0.025 M disodium phosphate with the addition of 0.1 M sodium hydroxide. The solution was diluted with 0.025 M disodium phosphate to obtain aqueous samples containing 1.6-81 µmol/L (0.48-24 µg/ml) of meclofenamic acid. Plasma samples were spiked with 0.68-16 µmol/L (0.20-4.8 µg/ml) (50 µl/ml plasma).

Analytical Method

Plasma (1.50 ml) was mixed with 2.00 ml of citrate buffer pH 4.6, containing 0.53 µmol/L (0.15 µg/ml) of diclofenac sodium as internal standard, 5.00 ml of dichloromethane was added and the tube was rotated for 30 minutes. After centrifugation, ca. 3 ml of the organic phase was transferred to another tube and evaporated. The residue was dissolved in

200 µl of the chromatographic eluent and 20 µl was injected onto the liquid chromatographic column.

The chromatographic isolation was performed on a column containing Spherisorb ODS (10 µm) with 40 % methanol in phosphate buffer pH 6.1 as the eluent. The analysis time was 14 minutes at a flow-rate of 0.8 ml/min. UV-detection was performed at 280 nm.

All calculations of the drug concentrations were based on peak height measurements.

RESULTS AND DISCUSSION

Choice of Liquid Chromatographic System

Flunixin, diclofenac sodium, and mefenamic acid, all belonging to the group of anti-inflammatory drugs, were included in the chromatographic study as conceivable standards together with meclofenamic acid. At first LiChrosorb RP-18 was used as support with mixtures of methanol - buffer pH 6.1 as eluents. Meclofenamic acid and mefenamic acid did not separate from each other on this support. Flunixin was eluted too close to the front and consequently diclofenac was chosen as the internal standard. Figure 1A shows how the retention is influenced by the concentration of methanol in the eluent. Extracts from equine plasma

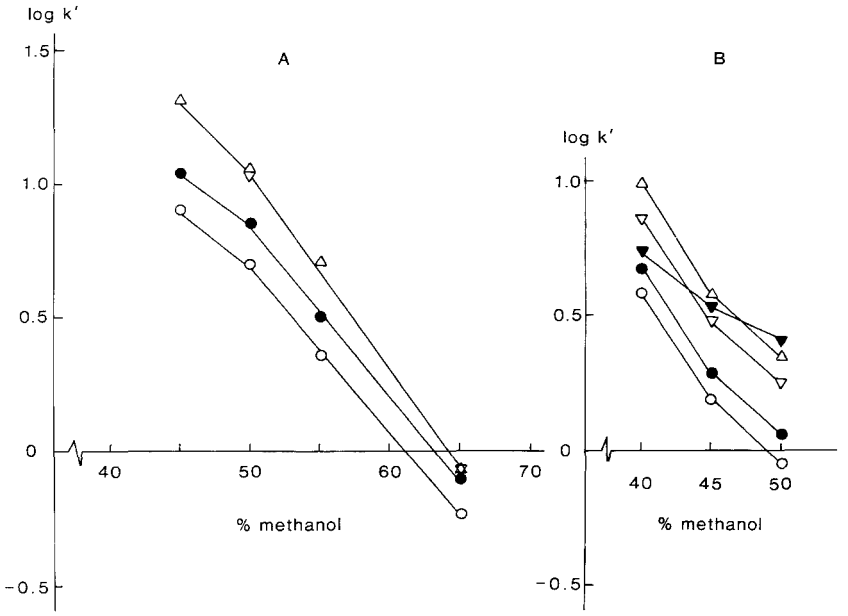


FIGURE 1. Influence of the Methanol Content in the Eluent on the Capacity Ratios.
Support: A = LiChrosorb RP-18; B = Spherisorb ODS.
Eluent: Methanol in phosphate buffer pH 6.1.
Δ = meclofenamic acid; ∇ = mefenamic acid;
● = diclofenac; o = flunixin; ▼ = interfering peak.

showed, however, a peak that interfered with diclofenac in the chromatogram. A separation could not be achieved by changing the pH or the methanol concentration of the eluent.

However, the separation was possible after the addition of TBA to an eluent containing 66 % methanol. In this system the interfering peak was eluted in the front and the acids emerged later, migrating as ion-

-pair with TBA. One disadvantage of the use of TBA was the poor column stability and another problem was the low solubility of meclofenamic acid in the eluent containing TBA.

Small differences in the selectivity between the acids were obtained by exchanging LiChrosorb RP-18 for Spherisorb ODS as the support (Figure 1B). About 10 % less methanol was needed in the eluent to get the same retention of meclofenamic acid on this support compared with LiChrosorb RP-18. Figure 1B shows the influence of methanol on the retention. The retention of the interfering peak was much less influenced than was the retention of the drugs by the concentration of methanol. A chromatogram of a standard solution of the acids is given in Figure 2.

During the investigation, it was observed that plasma extracts from only one horse contained the interfering peak in the chromatogram and that this peak might be related to some plastic caps in the storage of plasma samples. The caps were exchanged for further work. Spherisorb ODS was used for the plasma studies with 40 % methanol in phosphate buffer pH 6.1 as the eluent.

Extraction

Both meclofenamic acid and diclofenac are protolytes in aqueous solutions and can be transferred in

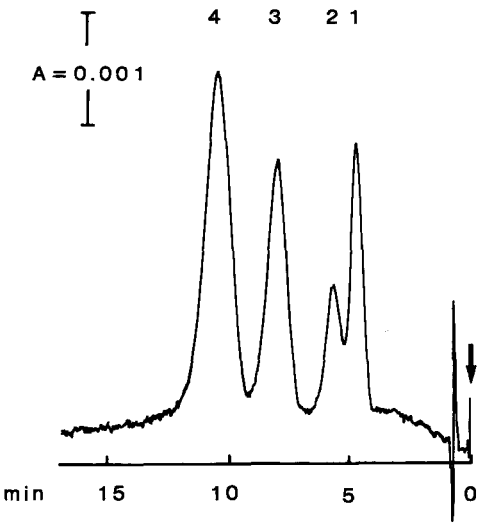


FIGURE 2. Chromatogram of Some Acidic Drugs.
Support: Spherisorb ODS, 10 µm.
Eluent: 40 % methanol in phosphate buffer pH 6.1 (0.8 ml/min, 3500 kPa).
Peaks: 1 = flunixin (25 ng); 2 = diclofenac (18 ng); 3 = mefenamic acid (50 ng); 4 = meclofenamic acid (152 ng).

uncharged forms into organic solvents. The degree of extraction will depend both on the nature of the organic solvent and the pH of the aqueous solution [9]. The extraction of an acid, HX, can be expressed by the distribution ratio, D_{HX}

$$D_{HX} = \frac{C_{HXorg}}{C_{HXaq}} = \frac{[HX]_{org}}{[HX]+[X^-]} = \frac{K_D \cdot a_{H^+}}{K'_{HX} + a_{H^+}} \qquad (1)$$

where

$$K_D = \frac{[HX]_{org}}{[HX]} = \text{distribution constant of HX} \qquad (2)$$

$$K'_{HX} = \frac{a_{H^+} \cdot [X^-]}{[HX]} = \text{apparent dissociation constant of HX} \qquad (3)$$

If the constants are known the distribution ratio can be estimated at any pH and the degree of extraction, P %, to the organic solvent calculated by use of

The Formula $$P\% = 100 \cdot \left(1 + \frac{V_{aq}}{V_{org} \cdot D_{HX}}\right)^{-1} \qquad (4)$$

where V_{org} and V_{aq} are the volumes of the organic solvent and the aqueous solution, respectively.

The distribution ratios of meclofenamic acid and diclofenac were determined between dichloromethane as organic solvent and phosphate buffers of pH 7.5-9.5. Inversion of eq. (1) gives

$$\frac{1}{D_{HX}} = \frac{1}{K_D} + \frac{K'_{HX}}{K_D \cdot a_{H^+}} \qquad (5)$$

Plots according to eq. (5) gave linear relationships and K'_{HX}/K_D was calculated from the slope. The intercept was too small to enable a calculation of K_D. The results are given in Table 2 together with the experimental conditions.

In our analytical procedure the extraction is performed at a pH of about 5.2, which for both meclofena-

TABLE 2.
Partition Coefficients
Aqueous solution: Phosphate buffer (μ = 0.1).
Organic solvent: Dichloromethane.

Acid	$C_{HXaq} \cdot 10^4$	$C_{HXorg} \cdot 10^4$	pH	$\log (K'_{HX}/K_D)$	pK'_{HX}
Meclofenamic acid	0.25-0.97	0.53-1.3	8.4-9.4	-9.11	4[a]
Diclofenac	1.4 -3.7	0.92-3.2	7.5-8.5	-7.84	4.0[b]

C_{HX} denotes the total concentration of the acid in mol/L. [a] Ref. [1], [b] Ref. [10].

mic acid and diclofenac gives a theoretical recovery of 99.8 % (eq. 4). The acids are concentrated about 4.5 times by the extraction procedure.

Buffer solutions of meclofenamic acid, 1.6-81 μmol/L (0.48-24 μg/ml), analyzed according to the method, showed a deviation from linearity at higher concentrations of meclofenamic acid. The obtained standard curve was compared with standards of meclofenamic acid - diclofenac dissolved in the eluent and not taken through the extraction step, Figure 3. The non-linearity might be due to the low solubility of meclofenamic acid in acidic aqueous solutions. The standard curve was considered linear up to 17 μmol/L (5 μg/ml). The same degree of linearity has previously been reported for the fluorometric analysis of meclofenamic acid [5].

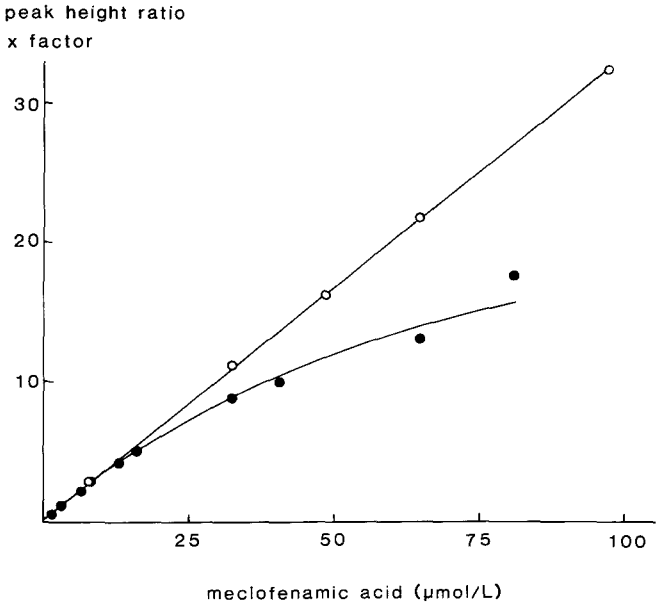


FIGURE 3. Standard Curve of Meclofenamic Acid. ● = meclofenamic acid dissolved in 0.025 M phosphate buffer pH 9.2 and extracted according to the Analytical Method; o = meclofenamic acid directly injected onto the column. Internal standard: Diclofenac. Chromatographic conditions as in Figure 2.

Determination of Meclofenamic Acid in Equine Plasma

The standard curve in Figure 4 is prepared from spiked equine plasma in the range of 0.68-16 µmol/L (0.20-4.8 µg/ml). An absolute recovery of 95 % was obtained. The recovery was not increased by doubling the extraction time.

Figure 5 shows chromatograms of two plasma samples taken before and 45 min after an oral dose of meclofe-

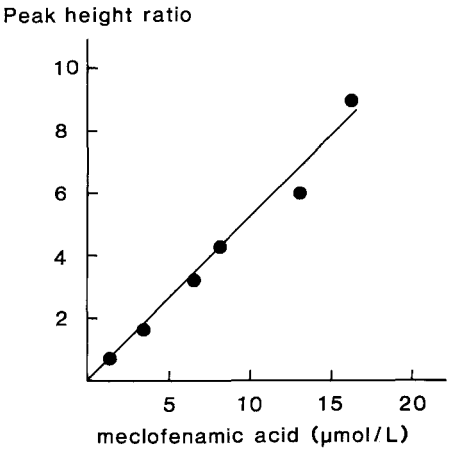


FIGURE 4. Standard Curve for the Determination of Meclofenamic Acid in Equine Plasma. Analyses performed according to the Analytical Method with diclofenac (0.53 μmol/L) as the internal standard. Chromatographic conditions as in Figure 2.

namic acid. From the chromatogram the presence of a metabolite of meclofenamic acid is strongly evident. It can be isolated and quantified with the present liquid chromatographic method after its structure elucidation. This is an improvement compared with the fluorometric assay [4,5] where probably the metabolite is codetermined with the parent drug after extraction from acidified solutions with carbon tetrachloride [3].

The simple work-up procedure, with a single extraction of the plasma sample to dichloromethane followed by evaporation of the organic solvent and redissolving in a small volume of eluent, will result in rather

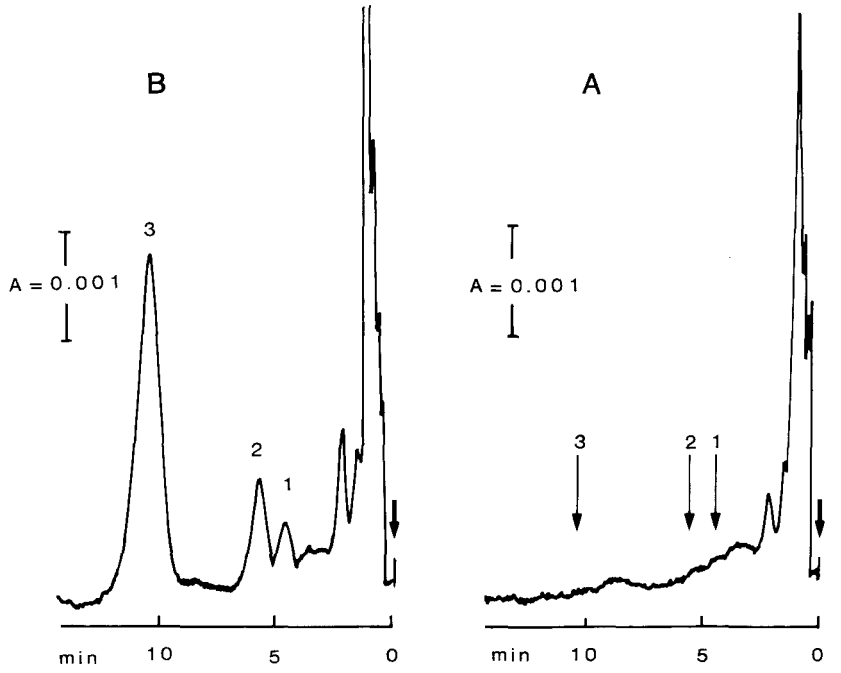


FIGURE 5. Chromatograms from Equine Plasma. Conditions as in Figure 2. Peaks: 1 = possible metabolite; 2 = diclofenac (internal standard); 3 = meclofenamic acid; A = blank plasma; B = plasma sample 45 minutes after oral administration of meclofenamic acid, 2.2 mg/kg body weight. Calculated concentration of meclofenamic acid, 7.4 µmol/L (2.2 µg/ml).

dirty samples to be injected onto the liquid chromatographic column. This causes no problem provided the column is changed after the injection of 200 plasma samples.

The recovery and precision of the analytical method, summarized in Table 3, were calculated from repeated analyses of spiked equine plasma at the con-

TABLE 3.
Recovery and Precision of the Method.
Data obtained with internal standard, diclofenac sodium 0.47 µmol/L (0.15 µg/ml). The relative standard deviation, s_r %, is calculated from repeated analyses of spiked plasma samples.

Drug added µmol/L	µg/ml	Drug found %	s_r %	n
6.50	1.92	102.3	4.95	9
1.30	0.384	96.3	12.6	5

centration levels of 6.50 µmol/L and 1.30 µmol/L. The relative standard deviation, 4.95 % and 12.6 %, respectively, can probably be reduced by the injection of a larger volume onto the column to increase the signal to noise ratio. With the present injection of a 20 µl sample, the detection limit in equine plasma, defined as the concentration which gives a signal twice the base-line noise, was 0.361 µmol/L (0.107 µg/ml) for meclofenamic acid.

Figure 6 shows the plasma concentration of meclofenamic acid obtained from one horse after oral administration of meclofenamic acid, 2.2 mg/kg body weight. With our method, meclofenamic acid can be determined in equine plasma up to 12 hours after a single oral dose. The simple sample treatment, high column stability,

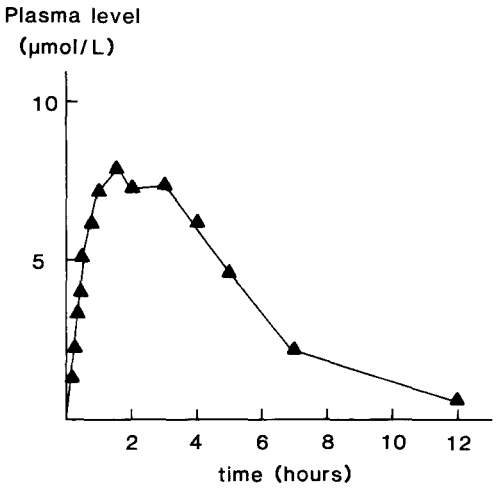


FIGURE 6. Plasma Levels of Meclofenamic Acid after Oral Administration. Administered dose: 2.2 mg/kg body weight.

rapid liquid chromatographic separation of meclofenamic acid, and the possibility of metabolite determination constitute a powerful tool for pharmacokinetic measurements (work in progress).

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ANALYSIS OF FLUORESCENT COMPOUNDS IN URINE BY LIQUID CHROMATOGRAPHY

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ABSTRACT

High speed separation of fluorescent compounds was examined. The retention time of 21 compounds was measured in two reversed phase modes and an ion-exchange mode liquid chromatography. Furthermore, urine samples of new-born babies, cancer patients and normal subjects were analyzed by the above systems. Several peaks were positively identified from the retention time, however there were many unknown fluorescent compounds. Among them, two peaks were found on the chromatograms in the reversed phase modes. These compounds were very polar and could not be identified, however the ratio of these peak height was used for classification of urine samples. Furthermore, indole-3-acetic acid and 5-hydroxyindole-3-acetic acid in urine were selectively analyzed on an ion-exchange resin with isocratic eluent after filtration.

INTRODUCTION

The liquid chromatography separation of ultraviolet absorbing constituents in urine becomes faster and will be a practical technique in a

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clinical research due to development of new packings. In an ion-exchange liquid chromatography, the separation time was drastically reduced by using a small particle macro-porous anion-exchange resin [1,2]. Nucleosides and polar compounds were analyzed by reversed phase liquid chromatography [3,4,5].

In a previous report, we have demonstrated the possibility of high-speed separation of ultraviolet absorbing constituents in urine with 50 standard compounds in both hydrophobic and ion-exchange modes on chemically bonded silica gels [6]. Further improvements of the separation and the selectivity of the detector simplifies the chromatograms. Urine samples of new-born babies and patients with cancer were analyzed with the new systems. The possibility of fingerprint analysis of the chromatograms and the simple determination of the amount of 5-hydroxyindole-3-acetic acid and indole-3-acetic acid were demonstrated.

MATERIALS AND METHODS

Equipment

A liquid chromatograph was assembled with two Waters model 6000A pumps, Waters model 660 solvent programmer (Waters Associates, Milford, MA 01757), Rheodyne model 7125 injector (Rheodyne, Berkeley, CA 94710), Altex model 153 ultraviolet absorbance monitor (UV 254 nm) (Altex Scientific Inc., Berkeley, CA 94710) and Perkin-Elmer model MPF-4 fluorescence spectrophotometer (Perkin-Elmer Corp., Normalk, CT 06856) with an Aminco model B18-63019, 20 μL flow cell (American Instrument Com., Silver Spring, MD 20910). The recorder was Linear Instrument model 915 (Linear Instrument Corp., Reno, NV 70044).

A 5 μm chemically bonded ion-exchanger, TSK 540 DEAE and 5 μm octadecyl bonded silica gel, TSK LS 410, were kindly gifted by Dr. T. Hashimoto (TOYO SODA Mtg. Co. Ltd., Tokyo, Japan) and a 5 μm anion-exchange resin, Hitachi 3013N, was kindly gifted by Dr. N. Takai (Tokyo University, Tokyo Japan).

Chemicals

Chemicals were mainly supplied from Sigma Chem. Com., St.Louis, MO 63178, and Chem. Service Inc., West Chester, PA 19380. UV grade acetonitrile

was from Burdick & Jackson Lab. Inc., Muskegon, MI 49442. Distilled water was further treated through MilliQ system (Millipore Corp., Bedford, MA 01730).

The urine samples from babies were directly injected and the other samples analyzed were after filtration through a 0.45 μm membrane filter (Gelman Sci., Ann Arbor, MI 48106).

RESULT AND DISCUSSIONS

The retention times of fluorescent compounds in different chromatographic modes are listed in Table I and some typical chromatograms of urine samples are shown in Figs. 1-5.

The urine samples of new-born babies were very pure and clear. One example of chromatograms in different separation modes of a four days old boy's urine is shown in Figs. 1-a and b. The compounds surmounted by a symbol were positively identified from the results of ion-exchange and reversed-phase chromatographies. Two unidentified interesting compounds were found with the reversed-phase system and are numbered 1 and 2. The same compounds were found several times in different samples.

The chromatograms of one and half year old boy's urine sample showed the influence of food, and the obtained fingerprint was close to that observed for adult samples. The chromatograms are shown in Figs. 2 and 3.

Examples of chromatograms of the urine of a patient with cancer are shown in Figs. 4 and 5. The patient was 79 years old, and it contained large amounts of protein related to the intake of food.

The chromatograms observed for 38 samples obtained at different times, from 26 persons, among them 5 new-born babies, 12 healthy persons whose age ranges from 1 to 50 years, 9 patients with cancer, one was 5 years old and the others were over 50 years old, revealed an interesting feature. The peak height ratio of the well resolved unknown compounds labelled Nos. 1 and 2 indicates the possibility for a classification of these urines in three

TABLE I. Retention Index of Urinary Fluorescent Compounds (Unit: mL)

Compound	Symbol	RP[1]	RP[2]	Ion-exchange[3]
Noradrenalin	NA	1.40	1.44	-
Adrenalin	AD	1.80	1.80	-
Dopamine	DA	2.60	3.08	1.34
Dopa	DP	-	4.40	-
4-Hydroxy-3-methoxymandelic acid	4H3MeOMA	6.62	8.00	24.9
5-Hydroxytryptophan	5HT	8.62	12.0	3.60
2,5-Dihydroxyphenylacetic acid	25DHPhA	9.90	11.0	25.9
bis(4-Hydroxy-3-methoxyphenylglycol)	4H3MeOPG	10.0	10.6	-
3,4-Dihydroxyphenylacetic acid	34DHPhA	14.1	15.6	24.0
Tryptophan	Try	14.7	13.4	3.00
5-Hydroxyindole-3-acetic acid	5HIAA	16.7	18.0	26.5
4-Hydroxyphenylacetic acid	4HPhA	17.2	18.5	23.1
4-Hydroxy-3-methoxybenzoic acid	4H3MeOBA	17.3	18.8	24.1
3-Methoxymandelic acid	3MeOMA	18.3	19.4	25.3
4-Hydroxy-3-methoxyphenylacetic acid	4H3MeOPhA	18.9	19.6	23.1
2-Hydroxyphenylacetic acid	2HPhA	19.3	20.7	25.3
Indolelactic acid	ILA	22.0	22.6	28.3
Indole-3-acetic acid	IAA	24.2	24.4	25.9
4-Methoxyphenylacetic acid	4MeOPhA	24.1	24.5	-
3-Methoxyphenylacetic acid	3MeOPhA	24.2	25.2	23.3
Indole-3-propionic acid	IPA	26.5	26.9	26.2

Experimental conditions: 1) The column was 15 cm long, 4.1 mm i.d. packed with TSKLS 410 (ODS silica gel). The gradient went from 0.01 M phosphoric acid to 60% acetonitrile in 0.01 M phosphoric acid. The gradient mode was No. 7 of Waters model 660 solvent programmer and the interval was 30 min. The column temperature was 24 $\pm$ 2° C. The flowrate was 1 mL/min and the chart speed was 0.5 cm/min. 2) 0.05 M phosphoric acid was used instead of 0.01 M. The other conditions were the same as for 1). 3) The column was 15 cm long, 4.1 mm i.d. packed with TSK 540 DEAE ion-exchanger. The gradient elution went from 5% acetonitrile-water to 50% acetonitrile-water with 0.5 M ammonium acetate buffer at pH 4.5. The gradient was the same as for 1. The column temperature was 40° C. The flowrate and the chart speed were 1 mL/min and 0.5 cm/min.

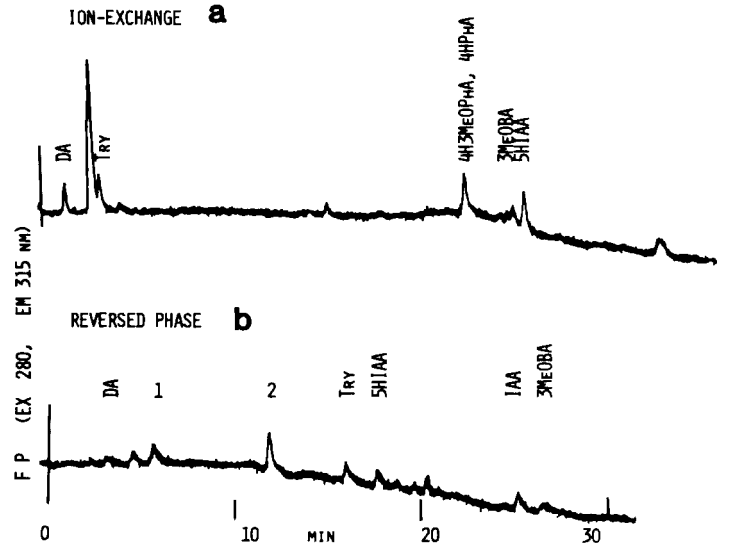

Fig. 1-a) An ion-exchange liquid chromatogram of a new-born baby's urine sample. The details of the experimental conditions are given in Table I. The sample volume was 20 µL.

b) A reversed-phase liquid chromatogram of a new-born baby's urine sample. The details of the experimental conditions are given in Table I. The sample was the same as in Fig. 1-a and the injected volume was 20 µL. The concentration of phosphoric acid in the eluent was 0.01 M.

groups: The first group includes new-born babies, whereas the second group includes the patients with cancer and the last group the healthy persons. The results of the analyses of urine of cancer patients and normal subjects were collected in Fig. 6-a, and those of new-born babies and normal subjects were summarized in Fig. 6-b. The analyses were carried out under different conditions, however the chromatograms identified by an asterisk were those of the same urine and were analyzed in two systems.

As seen in Fig. 6, the urine samples from the first two groups contain a relatively large amount of the compound under peak No. 2. This compound

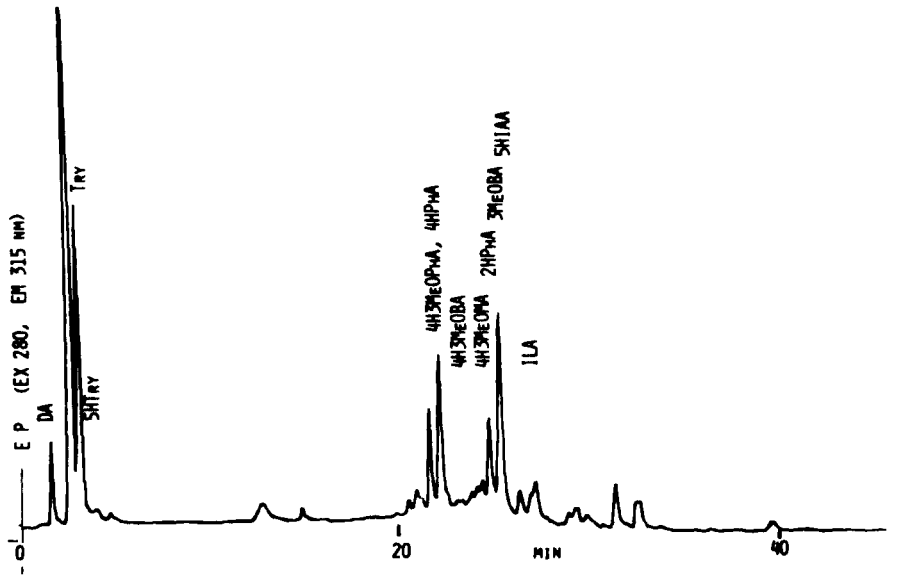

Fig. 2 An ion-exchange liquid chromatogram of a healthy boy's urine sample. The details of the experimental conditions are given in Table I. The sample volume was 20 μL.

was very polar, and unfortunately the compound could not be identified from the retention time and seems not to be an oxidation product of tryptophan. The retention time of peak No. 1 obtained with 0.05 M phosphoric acid in the eluent was close to that of 4-hydroxy-3-methoxymandelic acid, but such a large peak was not found on the chromatograms in 0.01 M phosphoric acid as the eluent.

The analysis of the fingerprint of the chromatograms, hence, the comparison of peak height ratio may help to find irregular metabolism related to health condition of people and may minimize the effect of food intake. This type of approach was applied to monitor patients [7] and used in cancer research [8]. It seems that for one person's urines whether it was collected on different days or in the morning or evening the fingerprints

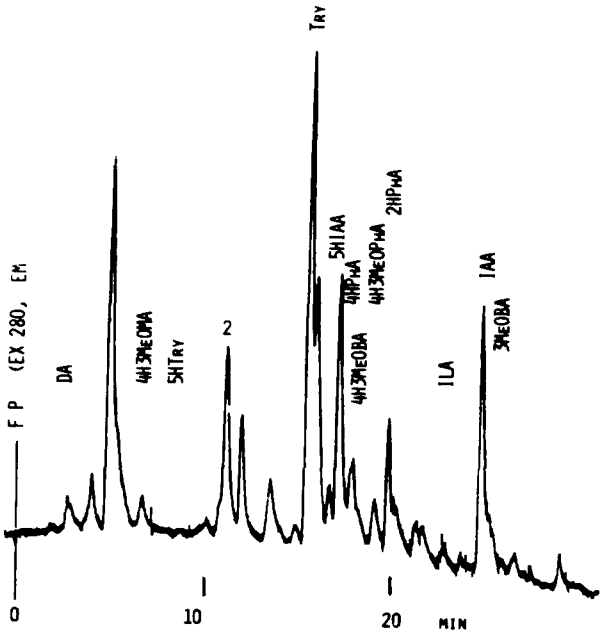

Fig. 3 A reversed-phase liquid chromatogram of a healthy boy's urine sample. The details of the experimental conditions are given in Table I. The sample was the same as in Fig. 2 and the volume was 20 µL. The concentration of phosphoric acid in the eluent was 0.01 M.

are similar. They are different compared with other persons. This means that introducing liquid chromatography in regular physical tests could help to detect irregular health conditions. The problem encountered for such applications is the long time of analysis and therefore, it may be used only in special cases.

Instead of analyzing all the components in urine, some target compounds can be analyzed in a short time with isocratic elution, for example indole-3-acetic acid and 5-hydroxyindole-3-acetic acid. 5-Hydroxyindole-3-acetic acid is the end product of the metabolism of serotonin and is recognized as an important compound related to carcinoid tumors [9-16]. The selective

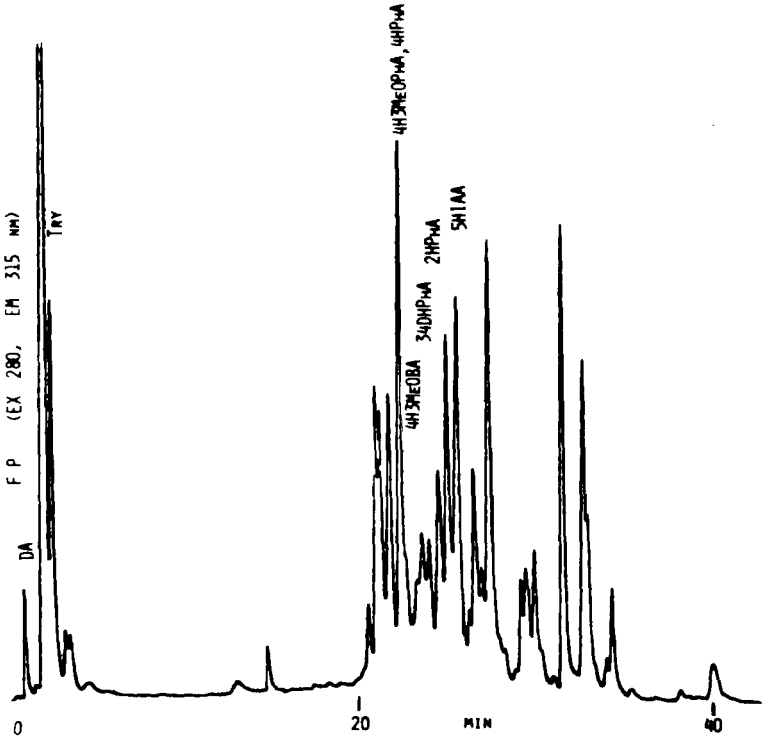

Fig. 4 An ion-exchange liquid chromatogram of a urine sample from a patient with cancer. The details of the experimental conditions are given in Table I. The sample volume was 10 μL.

separation of these compounds from urine was done on an macro-porous ion-exchange resin [17]. The column efficiency was comparable with a bonded ion-exchange silica gel, however, the stability of the packing was superior to bonded silica gels. In addition, when a urine sample was passed through alumina or Dowex 1X8 columns like for purification of 4-hydroxy-3-methoxy-mandelic acid, the fingerprint of the fluorescent compounds was not significantly changed but over 30% of the aromatic acids were lost and such a pre-treatment is not acceptable for the analysis of aromatic acids. No pressure

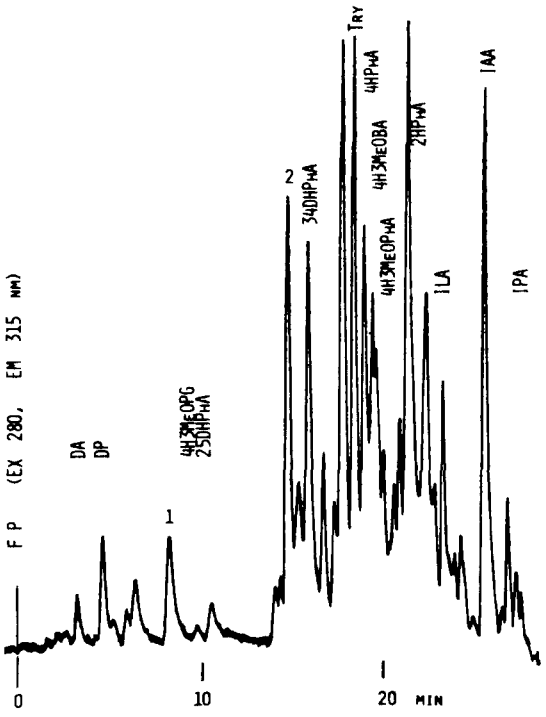

Fig. 5 A reversed-phase liquid chromatogram of a urine sample from a patient with cancer. The details of the experimental conditions are given in Table I. The sample was the same as in Fig. 4 and the volume was 10 µL. The concentration of phosphoric acid in the eluent was 0.05 M.

drop was observed in this system after analysis of over 100 injections and therefore this system was very useful to analyze urine directly after filtration. Examples of chromatograms are shown in Figs. 7-9 and the samples are the same as those in Figs. 1-5.

The peaks of 5-hydroxyindole-3-acetic acid and indole-3-acetic acid were not contaminated by indolepropionic, indoleglyoxylic, indolepyruvic and indolelactic acids. The detection limits of 5-hydroxyindole-3-acetic and

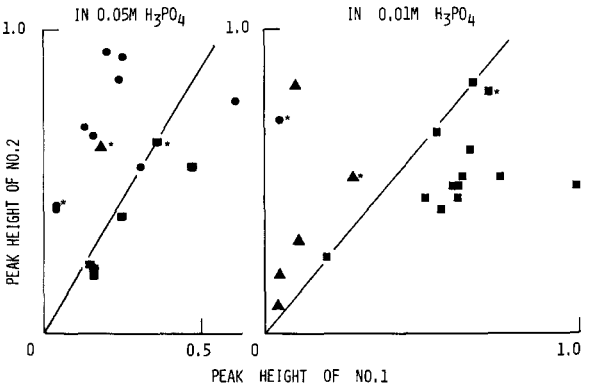

Fig. 6 Peak height ratio of Nos. 1 and 2 in reversed-phase liquid chromatography. The experimental conditions are given in Table I.

*: indicates the same samples in different chromatographic modes.

●: cancer patient's urine

▲: new-born baby's urine and

■: normal subject's urine.

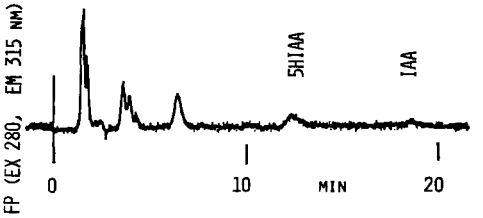

Fig. 7 Chromatogram of a new-born baby's urine sample. The sample volume was 20 μL. The column was 15 cm long, 4.1 mm i.d. and packed with Hitachi 3013N. The eluent was a 25% acetonitrile-water mixture containing 0.05 M ammonium acetate, 0.01 M octylsodiumsulfate. The flowrate was 1 mL/min. The chart speed was 0.5 cm/min and the column temperature was 40° C.

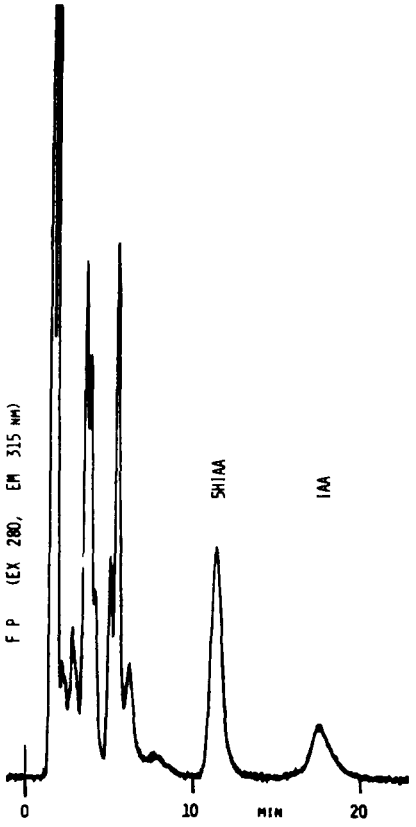

Fig. 8 Chromatogram of a one and half year old boy's urine sample. The sample volume was 20 µL. The chromatographic conditions are the same as in Fig. 7.

indole-3-acetic acids were respectively 3 and 4 ng. The distribution of these acids is summarized in Fig. 10.

The average amounts of 5-hydroxyindole-3-acetic and indole-3-acetic acids in normal subjects were 2.3 and 1.4 ppm respectively and those found for pathological subjects were 3.7 and 2.8 ppm respectively. The ratio between 5-hydroxyindole-3-acetic and indole-3-acetic acid concentrations in normal and

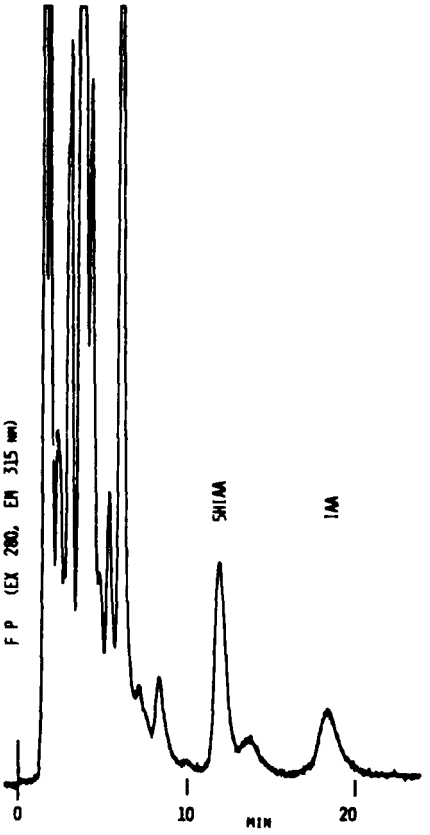

Fig. 9 Chromatogram of a urine sample from a patient with cancer. The sample volume was 10 µL. The chromatographic conditions are the same as in Fig. 7.

pathological subjects were respectively 1.7 and 1.3. There was no significant difference between these two subjects.

The reference values of indole-3-acetic acid concentration was 6.6 ppm [18], and 5-hydroxyindole-3-acetic acid concentrations were 0.6 ppm [18], 1.2 ppm [13], 0.5 - 4.0 ppm [10] and 3.7 $\pm$ 0.7 ppm [12] in normal subjects. In urine samples from patients with carcinoid tumors, stable cirrhosis and

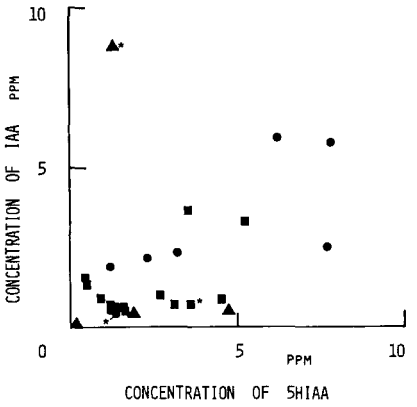

Fig. 10 Relationship between the amount of 5-hydroxyindole-3-acetic acid and indole-3-acetic acid in human urine.

*: indicates the same sample as in Fig. 6.

●: cancer patient's urine

▲: new-born baby's urine

■: normal subject's urine.

hepatic encephalopathy the concentrations were respectively 5.1 - 473 ppm [13], 4.4 ± 0.8 ppm and 4.6 ± 1.1 ppm [12].

Such small differences between normal and abnormal subjects could not be easily related to disease and it might be safer to look for metabolites instead of measuring the absolute value for one compound.

All the samples analyzed with the above systems were freshly collected and stored at -15°C until the analysis, therefore, a discussion about the concentration of each acid is not appropriate, but for pathological urine relatively large amount of proteins and the previously described acids were found.

CONCLUSION

Metabolism profiling is a very complex field of research, for a simple disease like acidurias, there are many possibilities that should be analyzed

[19]. The gas chromatography-mass spectrometer is presently the best analytical thechnique, but its power is not strong enough to solve all the chromatographyic problems related to certain diseases [18]. Liquid chromatography is a promising technique, but even for the chromatograms monitored with a fluorescent detector, the resolution and the time of analysis are presently not sufficient. One example is the analysis of indole-3-acetic and 5-hydroxyindole-3-acetic acids by a simplified system. One problem in the interpretation of urine analysis is the diet on the urine content.

ACKNOWLEDGEMENT

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A HPLC METHOD FOR THE DETERMINATION OF BUTAPERAZINE IN SOLUTIONS, TABLETS, PLASMA AND BILE

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ABSTRACT

A specific HPLC method for the determination of the antipsychotic drug butaperazine (B) in solutions, tablets, plasma and bile has been developed. The instrument used was a Waters HPLC equipped with a Model 440 Spectrometer and a μ-Bondapak-NH_2 column. The mobile phase, chloroform-methanol (100:3.5) was pumped at a rate of 1.5 ml per minute. Ultraviolet absorbance at a wave length of 280 nm was used for detection. The procedure involved the extraction of the drug from the dosage forms with chloroform and from plasma and bile with hexane-isopropanol (9:1). Hydrocortisone acetate was used as the internal standard. Retention times of 2.4 and 3.9 minutes were obtained for the internal standard and B respectively. Analytical calibration yielded a linear relationship from 0.1-25 μg per ml, with r^2 value of 0.99. The percentage recovery of B averaged 99% from the dosage forms and 94% from the biological fluids. An improvement in the method for determining B in bile and plasma was later developed. It involved the use of a different mobile phase and detecting the drug fluorometrically.

INTRODUCTION

Butaperazine is 2-butyryl-10-[3-(4-methylpiperazin-1-yl)-propyl] phenothiazine. It is a commonly used antipsychotic medication for the treatment of patients with emotional or mental disorders (1). Several methods for butaperazine determination were reported. Included among the methods are Colorimetric, Titrimetric, UV absorption, Spectrophotometric and Chromatographic procedures (2,3). The thin layer chromatographic (TLC) method (2) is not sensitive enough to measure butaperazine

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in the therapeutic range. Although the gas chromatographic (GC) method (3) was found sensitive enough to determine B in biological fluids yet, some investigators (2) observed that because of its high boiling point and low thermal stability, butaperazine was too low in volatility to be eluted quantitatively without extensive thermal decomposition. High reproducibility and sensitivity were reported for the fluorometric methods (4-6) used to determine butaperazine. In this study, a specific, simple and sensitive HPLC method for the determination of butaperazine in solutions, tablets, plasma and bile is reported.

MATERIALS

Instrumentation

The instrument used was a Waters Associates (Milford, MA) liquid chromatograph, equipped with a 6000A pump, a 440 spectrometer, a 420C fluorometer and a data module with dual pen recorder. A micro BONDAPAK-NH_2 column (3.9mm X 30cm, Waters Associates) was used for all separation.

Chemicals

The solvents used for drug extraction and in the mobile phases were chloroform, B.D.H. Chemicals Ltd. (Poole, England), methanol and hexane, Merk (Darmstadt, Germany), and isopropanol and methylene chloride, Reidel-De Haen AG (Seelze-Hannover, Germany). The mobile phases were prepared by mixing the corresponding solvents and filtering the mixture through a 0.5 µm pore size membrane filter obtained from Millipore Corporation (Bedford, Massachusetts) before degassing. Butaperazine tablets and solutions were obtained from the local market. A reference sample was also obtained from A.H. Robins Company (Richmond, Virginia, USA) for standardization. All other chemicals were of analytical grade.

METHODS

Development of HPLC Conditions

Based upon the semipolar nature of butaperazine, the μ-Bondapak NH_2 column was chosen for this study. Preliminary trials with other columns of different polarities were unsatisfactory. Mobile phase selection was based upon achieving good resolution for the drug within a reasonable retention time. Upon using chloroform-methanol mixture (100:3.5) retention time of 3.9 minutes was obtained for butaperazine. By examining the integrated areas under the peaks obtained for two different drug concentrations, proper drug resolution was confirmed. Hydrocortisone acetate obtained from Sigma Chemical Company (St. Louis, Missouri, USA) was used as the internal standard. It was found to have a well defined peak separated from that of the drug with a retention time of 2.4 minutes. Ultraviolet absorption at a wave length of 280 nm was used for detection. Solutions of different concentrations of butaperazine in the mobile phase were prepared and used to calibrate this method of analysis.

Determination of Butaperazine in Tablets and Solutions

A number of B tablets obtained from the local market was crushed. A known weight of the powder equivalent to 10 mg B was treated with 20 ml 5N solution of sodium hydroxide and mixed thoroughly in a separatory funnel. Two portions of chloroform, each 50 ml, were used to extract B from the mixture. Ten milliliters of the chloroform extract were evaporated to dryness and the residue was dissolved in 50 ml mobile phase containing 200 μg/ml of the internal standard. Exactly, 20 μl of the product were injected in the HPLC for B determination. A second injection of 30 μl was carried for confirmation. Essentially the same procedure was followed for B determination in solutions beginning with

the addition of 20 ml 5N sodium hydroxide solution to a volume containing 10 mg of B.

Determination of Butaperazine in Presence of Bile and Plasma

A stock solution of known concentration of B was prepared by dissolving 10 mg pure butaperazine maleate in 20 ml of water acidulated with dilute hydrochloride acid. To a 0.5 ml of freshly obtained bile or plasma, 0.2 ml of the stock solution of B was added. The mixture was shaken with 2 ml of 5N sodium hydroxide solution, then extracted with 10 ml of hexane-isopropanol (9:1). Two milliliters of the extract were evaporated to dryness and the residue was dissolved in 2 ml mobile phase containing the internal standard before injection into the HPLC.

Determination of Butaperazine in Bile and Blood of Rats Administering the Drug

Rats (350-400 gm) anaesthesized with urethane were injected intravenously with 2.5 ml of B solution in normal saline (100 μg/ml). Bile was collected through a cannula for about 4 hours. Before the animals were sacrificed, a blood sample was obtained from the heart from which the plasma was separated. Both plasma and bile were extracted in the same way as before except that the residue after evaporating the organic solvent was dissolved in 1 ml of a mobile phase consisting of hexane-chloroform-methylene chloride (1:3:6). This new mobile phase when used at a flow rate of 1.2 ml/min., was found to better separate B from other extracted constituents including B metabolites. The drug was detected both by the UV detector (280 nm) and fluorescence detector (280 nm activation and 530 nm fluorescence).

RESULTS AND DISCUSSION

Figure 1 shows typical chromatograms for drug formulations and a placebo mixture in which the internal standard and the butaperazine peaks appear at 2.4 and 3.9 minutes respectively. The chromatograms show no interference from the excipients in either of the solutions or tablets.

A plot of the ratio of butaperazine peak area to the internal standard peak area versus the butaperazine concentration in the solution was linear up to at least 25 µg/ml and essentially passed through the origin ($r^2 = 0.99$). Butaperazine recovery from both solutions and tablets ranged from 97.1 to 101%. This result was obtained through the addition of known amounts of B to either placebo formulations or previously assayed dosages containing the active ingredient before analysis. Replicate analysis of five individual sample preparations of different concentrations showed high precision. The relative standard deviation was found to be in the range of ± 0.4 to ± 1.5%.

Similar results were obtained when B was added to bile and plasma then extracted. Recovery of B from both bile and plasma ranged from 91-96.5%. Concentrations down to 0.5 µg/ml were detected. This procedure was found, however, to be unsatisfactory to determine B in bile and blood collected from animals administering the drug.

Two major and three minor B metabolites were previously (7) separated from biological fluids obtained from rats and dogs. It is beleived that under the conditions used in this procedure B was not properly separated from its metabolites. Through the modification in the mobile phase and the flow rate complete separation of B was achieved. The new retention time of B was 5.15 minutes. Also, by using the fluorescence detector, the sensitivity was improved by a factor of 6-7. Concentrations of B in bile and blood down to 0.1 µg/ml were determined.

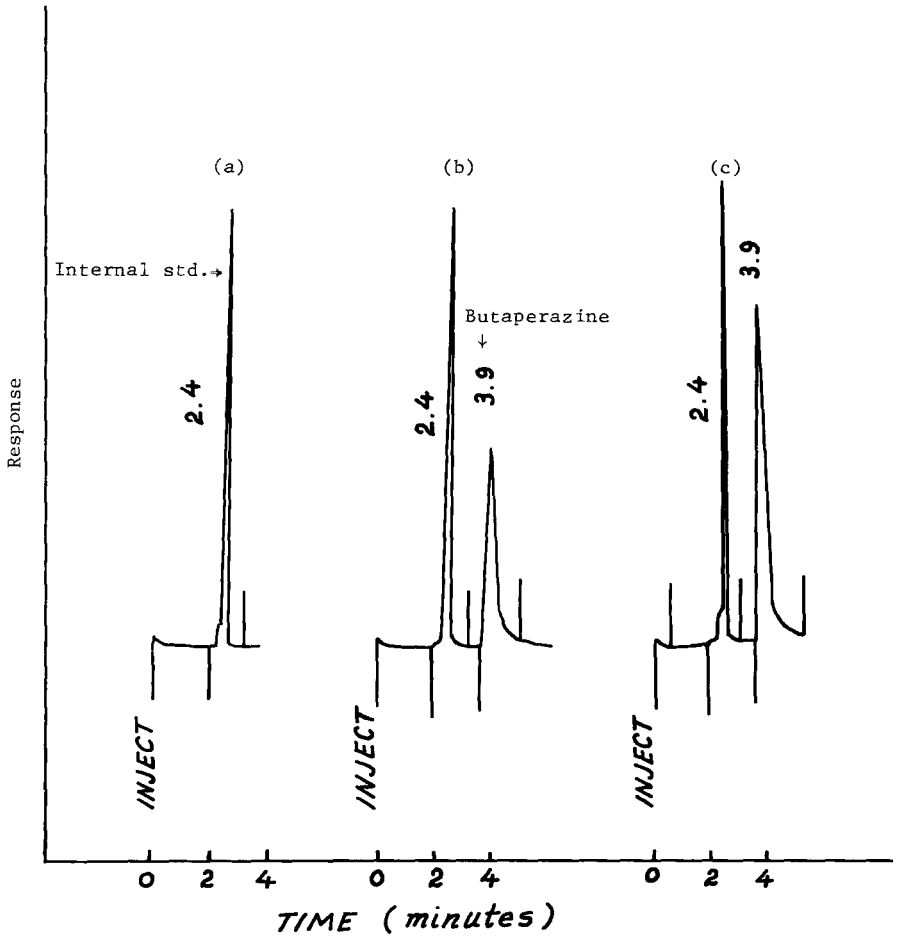

Figure 1: Typical butaperazine chromatogram with retention times, a, Internal standard; b, Butaperazine extracted from solutions; c, Butaperazine extracted from tablets.

This method of determining B has proved to be simple and accurate. A high performance liquid chromatograph equipped with a fluorescence detector would be more useful for B determination in biological fluids. The separation of B from its metabolites in biological fluids should enable the study of the drug pharmacokinetics under different physiological and nonphysiological conditions.

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SIMULTANEOUS ANALYSIS OF CIMETIDINE AND RANITIDINE IN HUMAN PLASMA BY HPLC

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ABSTRACT

A rapid method for the simultaneous quantitation of the H_2-receptor antagonist drugs cimetidine and ranitidine in human plasma by isocratic ion-pair reverse-phase HPLC is described. The method involves a simple organic extraction step of the alkalinized plasma containing added internal standard followed by back extraction of the extract with dilute acetic acid and subsequent analysis of the aqueous acidic phase on a reverse-phase (C18) column. The eluting solvent was acetonitrile-water (20:80 v/v) containing 0.005 mole/litre octanesulphonic acid and was monitored at 229 nm. The run time for the assay was 12.5 minutes, with a detection limit for cimetidine of 50 ng/ml/(0.2 μmole/1) and that for ranitidine was 20 ng/ml (0.06 umole/1).

INTRODUCTION

The H_2-receptor antagonists cimetidine and ranitidine are widely used to treat gastric and duodenal ulcers. It has been shown that certain minimal plasma drug concentrations must be attained to achieve adequate inhibition of gastric acid secretion (1). Routine plasma drug assay is not indicated for these

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compounds but is of value when compliance is in doubt or for clinical trials. Patients who fail to respond to one of the agents are commonly changed to the other and it is therefore convenient to have an assay that will detect either or both drugs. We developed a sensitive liquid chromatographic assay for a trial in which patients were changing from one H_2-receptor antagonist to the other and we describe it here. There have been several reports for analysis in biological fluids of cimetidine (2-11) and ranitidine (12-15) as individual drugs using HPLC with either normal phase or reverse phase separation. Mobile phases have varied and sample preparation in each case have varied from multistep extraction procedures employing prior salting or protein precipitation of the biological matrix to a single solvent extraction.

Whilst there are significant structural differences between cimetidine and ranitidine they are both weakly basic compounds and our approach for their simultaneous analysis was to use a single solvent extraction of the alkalinised plasma followed by back extraction into dilute aqueous acid solution. No further sample preparation was necessary except for removal of traces of volatile solvent from the aqueous extract. Two internal standards were investigated for quantitating each drug. These were SKF 92373 and AH 20480, analogues of cimetidine and ranitidine respectively. The chromatography was performed on a reverse phase C18 column using an isocratic mobile phase containing paired-ion reagent. No pH adjustment was necessary. The known metabolites of cimetidine and

ranitidine were also investigated for potential interference with analysis of the parent compounds.

EXPERIMENTAL

Materials:

All solvents and reagents used were analytical and LC grade. Cimetidine, cimetidine sulphoxide, guanyl urea cimetidine and SKF 92373 were supplied by Smith, Kline and French, Herts., England. Ranitidine hydrochloride, desmethylranitidine, ranitidine S-oxide, ranitidine N-oxide and AH 20480 were supplied by Glaxo (Australia) Pty. Ltd. Standard stock solutions of cimetidine (mol. wt. = 252) and ranitidine (mol. wt. = 351) were made in methanol at concentrations of 5 mg/100ml and 1.3 mg/100ml respectively. Similarly stock solutions of SKF 92373 and AH 20480 in methanol were made at concentrations of 50 and 40 mg/100ml respectively. All these were diluted appropriately in drug free plasma to calibrate the assay.

Extraction of Samples and Assay Calibration:

Plasma (500 µl) containing the internal standards SKF 92373 and AH 20480 at respective concentrations of 0.2 µg/ml and 0.16 µg/ml was made alkaline with 2M sodium hydroxide (50 µl). The plasma was then extracted with a mixture of ether, chloroform and isopropanol (2:1:1)(4 ml) by vortexing for 30 sec. After centrifugation at 3000 rpm for 10 min. the organic top layer was transferred to a second tube containing 100 µl of dilute acetic

acid (2% v/v) and the mixture again vortexed for 30 sec. After centrifugation the organic (top) layer was removed and discarded. A gentle stream of nitrogen was blown over the aqueous extract to remove traces of volatile solvent following which 20 µl aliquots were used for subsequent analysis. The assay was calibrated from drug free plasma containing added cimetidine over a concentration range of 0.2 to 2.0 µg/ml (0.79 to 7.9 µmole/l) and added ranitidine over a concentration range 0.05 to 0.5 g/ml (0.14 to 1.4 µmole/l). Each sample contained the internal standards as above. Recovery of each compound was calculated by measurement of peak area after extraction from plasma and compared to that of the same amount of compound from standard stock solution after chromatography.

Chromatography and Assay Conditions:

The analyses were carried out on a Varian Series 5000 liquid chromatograph coupled to a Vista CDS 401 controller. Detection was with a Waters Model 441 U/V detector operated at 229 nm (0.01 to 0.02 auf). Sample injection (20 µl) was made by Wisp Model 710B (Waters Associates) automated injector. The chromatography was performed on a 30 cm µ-Bondapak C18 (Waters Associates) reverse phase column (in line with a Bondapak C18/Corasil pre-column) using an isocratic mobile phase consisting of acetonitrile-water (20:80) containing 0.005 mole/litre octanesulphonic acid (as PIC-B8, low U/V reagent) (Waters Associates). The flow rate was 1.5 ml/min.

Quantitation was by ratio of peak areas of cimetidine and ranitidine to the internal standards.

RESULTS AND DISCUSSION

Using the chromatography procedure described the separation of cimetidine, ranitidine, internal standards and some potential metabolites is shown in Figure 1. The retention time under these conditions for cimetidine was 5.8 min. and that for ranitidine was 7.0 min. with adequate separation from the internal standards (retention times 4.3 and 9.7 min. for SKF 92374 and AH 20480 respectively). The monitoring at 229 nm gave adequate response for both parent compounds and standards (each compound has absorption maxima in that portion of the spectrum).

The chromatograms obtained following plasma extraction are shown in Figure 2. Chromatograms from drug-free plasma containing no added drug (Figure 2A) and from the same plasma containing added cimetidine and ranitidine in the amounts indicated (Figure 2B) are shown. Figure 2C and Figure 2D show sample chromatograms obtained from patients undergoing treatment with cimetidine or with ranitidine respectively. The total run time for the analysis is 12.5 min.

Specificity:

The analysis was specific for the parent compounds and known metabolites. The relative retention times (relative to cimetidine)

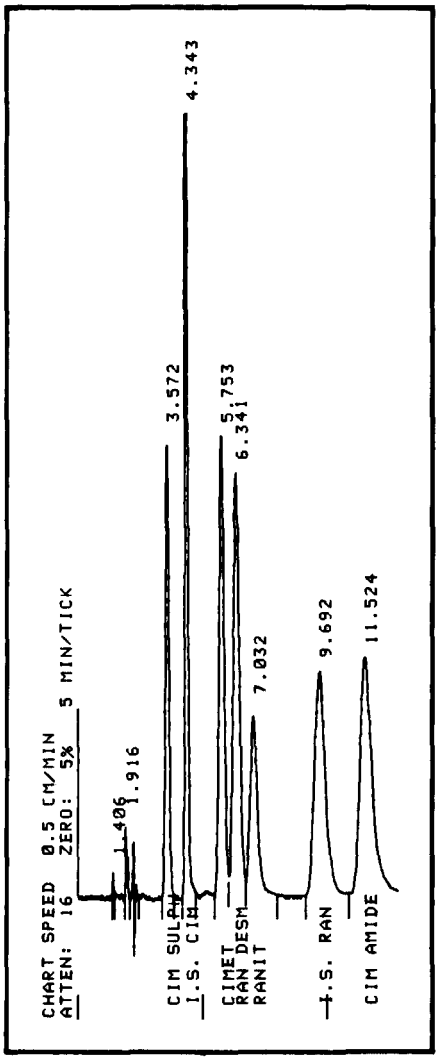

FIGURE 1. Chromatogram of standard compounds showing separation of cimetidine, ranitidine, internal standards SKF 92373 and AH 20480 and major metabolites.

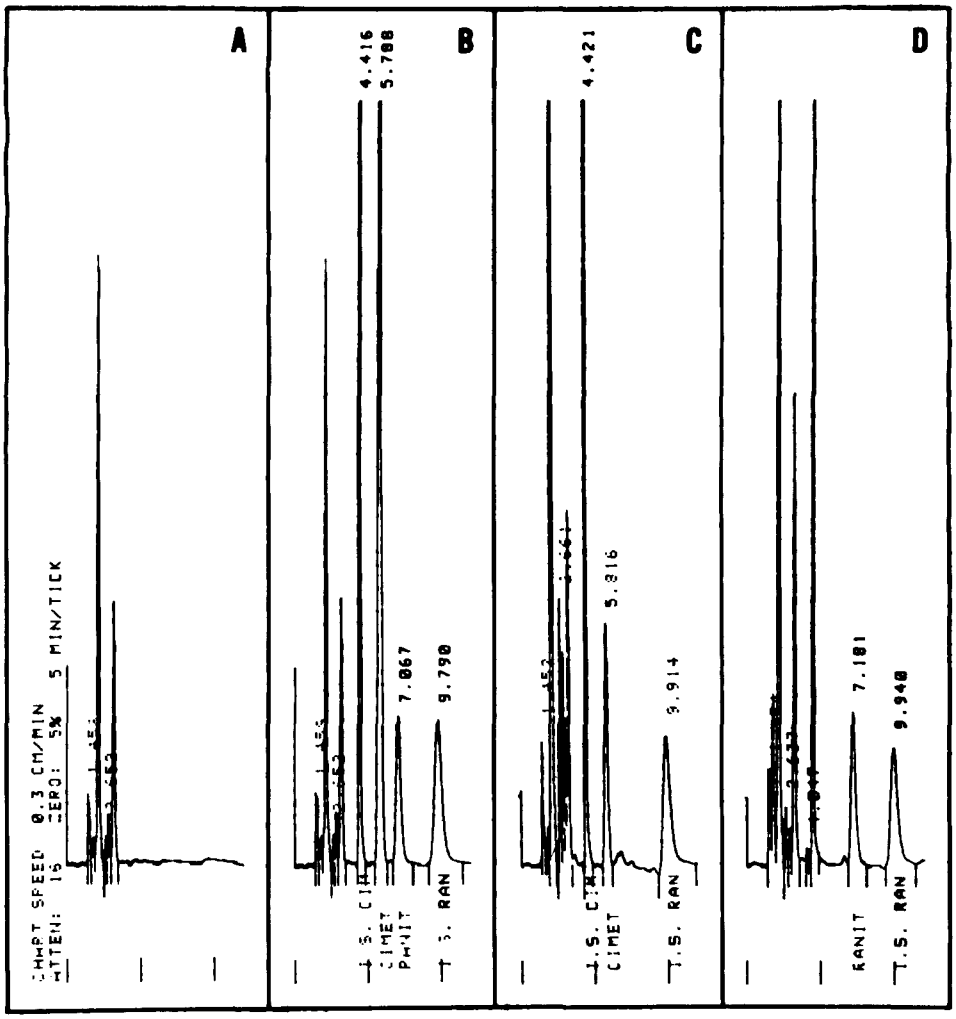

FIGURE 2. Chromatogram following plasma extraction. **A.** Drug free plasma. **B.** Drug free plasma containing added cimetidine (6.58 μmole/1), ranitidine (0.81 μmole/1) and internal standards. **C.** Plasma from patient receiving cimetidine. **D.** Plasma from patient receiving ranitidine.

TABLE 1

Relative Retention Times of Cimetidine, Ranitidine, Known Metabolites and Some Basic Drugs

Compound	Relative retention time (cimetidine = 1.0)
Cimetidine	1.0
SKF 92374	0.76
Ranitidine	1.22
AH 20480	1.69
Ranitidine N-Oxide	1.31
Ranitidine S-Oxide	0.65
Procainamide	1.16
N-Acetylprocainamide	1.29
Lignocaine	> 3
Mexiletine	> 3
Quinidine	> 3
Cimetidine sulphoxide	0.62
Desmethylranitidine	1.11
Cimetidine amide	2.02

for these compounds and other potentially administered basic drugs are shown in Table 1. There was adequate resolution for each of the compounds shown. The metabolites of cimetidine and ranitidine did not interfere and furthermore the S-oxide and the N-oxide of ranitidine when added to drug-free plasma did not extract adequately to be detected.

Linearity:

The standard curve for cimetidine and ranitidine was constructed from drug free plasma with constant amount of internal standards added per sample. Cimetidine was calibrated with SKF 92374 as the internal standard and produced a linear plot (r=0.999) over the concentration range of 0.2 to 2.0 μg/ml (0.79 to 7.9

μmole/l) (Figure 3). For ranitidine, curves were constructed with either AH 20480 or with SKF 92374 as standards. There was no significant difference as to which standard was used and all ranitidine assays were subsequently calibrated using SKF 92374 as the standard. The plot obtained was linear (r=0.999) from 0.05 to 0.5 μg/ml (0.14 to 1.4 μmole/l) (Figure 4). For both cimetidine and ranitidine the curve was still linear at values greater than twice the concentration shown for each plot. The lower limit of detection was 0.05 μg/ml (0.2 μmole/l) for cimetidine and 0.02 μg/ml (0.06 μmole/l) for ranitidine. The plasma concentrations known to cause 50% inhibition of food-stimulated gastric acid secretion are 0.37 to 0.45 μg/ml (1.5 to 1.8 μmole/l) for cimetidine and 0.07 to 0.09 μg/ml (0.20 to 0.26 μmole/l) for ranitidine (1).

Recovery:

Known amounts of cimetidine and ranitidine were added to drug free plasma to make high and low concentrations of each drug. After extraction and adjustment of the final volume of the acidic extract the peak area after chromatography of each compound was measured and compared to the peak area of the same amount of compound added directly to the equal volume of the aqueous extract. The recovery or extraction efficiencies are shown in Table 2 and ranged from 73-81%. In the same way the recovery for the internal standards were also determined at the concentrations used in the assay.

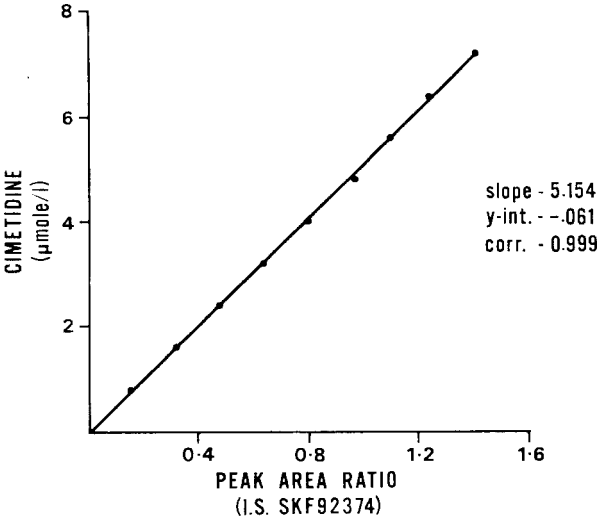

FIGURE 3. Calibration curve for cimetidine from plasma using SKF 92374 as standard.

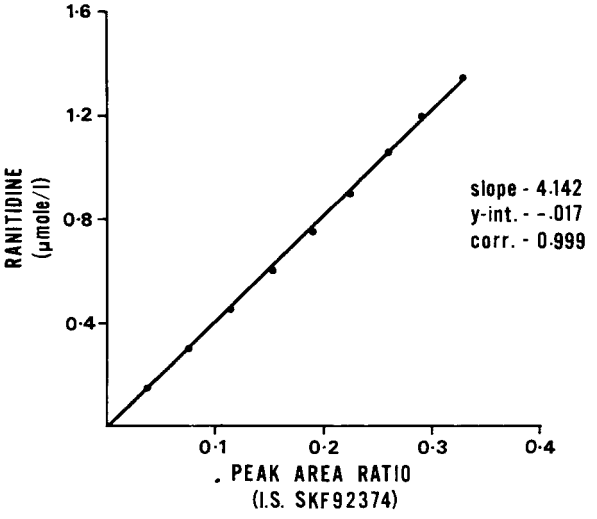

FIGURE 4. Calibration curve for ranitidine from plasma using SKF 92374 as standard.

TABLE 2

Recovery from Plasma. Extraction Efficiency was Calculated for Cimetidine and Ranitidine from High and Low Plasma concentrations for each drug. (N = 5 for each determination).

Compound	Concentration (μmole/l)	Recovery (mean % ± S.D.)
Cimetidine	1.70	74.4 ± 2.0
	6.58	81.1 ± 3.2
Ranitidine	0.21	73.2 ± 3.1
	0.81	73.7 ± 2.2

The extraction efficiency for SKF 92374 and AH 2480 was 65.2 ± 3.1% and 78.3 ± 10.6% respectively at the plasma concentrations in the assay.

TABLE 3

Assay Reproducibility. The Within-Run Precision and the Day-to-Day Precision (Calculated Over a Period of Five Days) were Calculated for Cimetidine and Ranitidine at Two Different Plasma Concentrations. (N = 5 for each determination).

	Mean (μmole/l ± S.D.)	%C.V.
WITHIN-RUN		
Cimetidine	1.70 ± 0.04	2.3
Cimetidine	6.58 ± 0.09	1.4
Ranitidine*	0.21 ± 0.019	4.4
Ranitidine*	0.81 ± 0.044	5.5
Ranitidine#	0.21 ± 0.008	3.9
Ranitidine#	0.81 ± 0.023	2.9
DAY-TO-DAY		
Cimetidine	4.21 ± 0.15	3.5
Cimetidine	8.64 ± 0.32	3.7
Ranitidine#	1.40 ± 0.06	4.3
Ranitidine#	0.36 ± 0.012	3.3

* Ranitidine measured using an AH 20480 as internal standard

Ranitidine measured using SKF 92374 as internal standard

Precision:

The reproducibility and precision of the assay following analysis of five aliquots of plasma pools at concentration shown in Table 3. For within day assays the CV's ranged from 2.3 to 5.5% and for the day-to-day precision ranged from 3.3 to 4.3%.

Background Interference:

Where possible, plasma samples from patients were tested prior to administration of either drug. With those samples and with pooled drug-free plasma processed in this way peak interference with endogenous substances at retention times corresponding to either parent drug or standards was not significant.

Stability:

Storage of plasma at $-20^{\circ}C$ for a period of at least one month did not alter the amount of either drug recovered. This enabled samples to be batched and assayed at the one time.

CONCLUSION

This assay procedure has proved successful for the repeated analysis of cimetidine and/or ranitidine in plasma samples. In our laboratory to date over 200 patient samples have been assayed by this procedure. The assay is rapid, reproducible and sufficiently sensitive. The use of the paired-ion (as a commercial reagent pack) made the preparation of the mobile phase relatively easy without the need for fine pH adjustment, giving good reproducible control over retention volumes. With the relatively simple

extraction procedure and with the use of the autosampler many samples can be batched and assayed at the one time.

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CHROMATOGRAPHIC ANALYSIS OF SUBERIMIDATE-CROSSLINKED LYSINE*

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ABSTRACT

Quantitative analysis of bislysylsuberamidine and monolysylsuberamidinic acid, which are obtained by an acid hydrolysis of protein cross-linked with dimethyl suberimidate, on an amino acid analyzer are described. Both of ninhydrin and fluorometric detection with o-phthalaldehyde were applied and less than 50 pmol of cross-linked lysine was analyzed in the latter case. The first-order rate constant for hydrolysis of amidine bond under standard conditions of acid hydrolysis of protein was found to be $3.4 \times 10^{-3} h^{-1}$.

INTRODUCTION

During the last decade, an application of cross-linking reactions to protein has become one of the most popular tools in the field of protein biochemistry. The application is not only restricted to topological analysis of protein assembly: recent studies show further possibilities of the reaction, such as the fixation of a conformation or a functional state of protein by an artificially introduced cross-links(1,2).

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Nevertheless, against so many increasing number of the reports on the use of the reaction, there has been no method for quantitative analysis of cross-linked amino acid reported, except those of the reaction products of lysine and dimethyladipimidate(3) and radioisotopic analysis of suberimidate-treated lysine(4). In the present paper, we will describe the chromatographic analysis of the lysine derivatives of dimethylsuberimidate, which is generally selected as the best choice among the bifunctional imidoesters, together with the behaviour of the compounds under the conditions of total acid hydrolysis of the modified protein.

MATERIALS AND METHODS

Preparation of Bislysylsuberamidine (I) and Monolysylsuberamidinic acid (II). Dimethylsuberimidate dihydrochloride was prepared from suberonitrile by the method of McElvain and Schroeder(5). N^{α}-benzyloxycarbonyllysine (α-Z-Lys) was prepared according to the method of Bezas and Zervas(6). α-Z-Lys (2 g/100 ml of 0.4 M Na_2CO_3, pH 10.0) was treated with dimethylsuberimidate dihydrochloride (1.8 g), which was added as a solid in three times with 30 min-intervals. After the last addition of the reagent, the reaction mixture was gently stirred for 2 h at room temperature. The reaction was quenched by the addition of concentrated HCl to 6 N, and then the reaction mixture was refluxed overnight for the removal of the benzyloxycarbonyl group from lysyl moiety. After the reflux, the reaction mixture was extracted with ether and the aqueous layer was evaporated to leave a residue containing suberimidate-modified lysines and salts. The lysine derivatives were extracted from the residue with methanol and

chromatographed on Dowex 50X2 (1.3 x 50 cm) with pyridine-acetate buffer, pH 5.5, under a linear gradient of pyridine concentration from 1 M to 2 M (total, 2,000 ml). Pure bislysylsuberamidine (I) was obtained by the chromatography. The ninhydrin-positive material which was eluted between lysine and (I) on the chromatography was treated with 6 N HCl for 3 h at room temperature to give the monolysyl derivative (II).

The desired products, (I) and (II), were obtained as an oil and were found to be pure in terms of NMR spectra and amino acid analysis, except for the presence of a small amount of ammonia. Proton NMR spectra were obtained with JEOL FT100 in D_2O and the proton chemical shifts for (I) and (II), which were represented in ppm from the methyl signal of 3-(trimethylsilyl)-propanesulfonic acid sodium salt, are as follows: for (I); 3.70 (C_α-H of lysine), 3.26 (C_ε-H of lysine), 2.44 (C_α-H of suberamidine), 1.2-1.8 (20 protons). For (II); 4.08 (C_α-H of lysine), 3.28 (C_ε-H of lysine), 2.47 (C_α-H of suberamidinic acid), 2.38 (C_ζ-H of suberamidinic acid), 1.2-1.8 (14 protons).

Amino Acid Analysis. Amino acid analysis was carried out with a 15-cm (i.d. = 0.5 cm, packed with JEOL LCR-1 resin) or a 5-cm(i.d. = 0.3 cm, Shodex HC095 resin) column. A 30-cm column (i.d. = 0.3 cm, Shodex HC095 resin) was used for the analysis of (II) under the conditions of single column methodology. A $NaBH_4$-ninhydrin system(7) or o-phthalaldehyde solution(8) was used to detect amino acids.

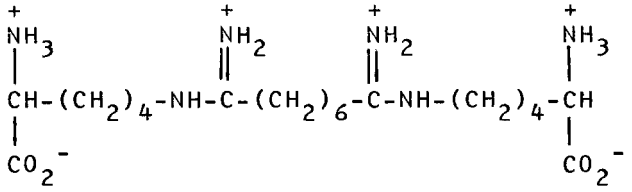
Other Procedures. Acid hydrolysis was carried out with constant-boiling HCl at 110°C in a sealed and evacuated tube. Deamidination was performed with methylamine-formate buffer (3.3 M, pH 11.5) as described by Dubois et al.(9).

RESULTS AND DISCUSSION

Analysis of (I) and (II). In this study, we prepared the authentic samples of (I) and (II) by the reaction of α-Z-Lys with dimethylsuberimidate. After the removal of the blocking group, the reaction products were chromatographed on Dowex 50 with a volatile buffer system. In the present preparation, the compound (II) was isolated as its methylester (NMR, $COOCH_3$ at 3.70 ppm), which was obtained as the result of a methanol treatment of deblocked products (see "MATERIALS AND METHODS"), and it was necessary to treat with an acid to convert it into free acid (II). The esterification of (II), which was not expected initially, facilitated the purification on Dowex 50 due to the loss of a negative charge on a carboxyl group: (II) itself was co-eluted with lysine remaining in the reaction mixture, but (II)-methylester was eluted between lysine and (I) on Dowex 50 chromatography.

Chromatographic data of (I) and (II) on an amino acid analyzer are summarized in Table 1. (I) was analyzed on a 5-cm column with citrate buffer (Na^+=1.4 M, pH 6.0, for ninhydrin analysis) or borate buffer (Na^+= 0.35M, pH 9.5, for fluorescence detection) to appear as a sharp peak with a tolerable retention time for a routine work. A 5-cm column analysis could be also applied to the analysis of bislysyladipamidine, which was eluted as a sharper peak than that observed on a 15-cm column analysis(3). Furthermore, a trislysyl derivatives of adipamidine, which was tentatively assigned and appeared with a retention time of 210 min on a 15-cm column analysis using the citrate buffer, was eluted at 40 min. The analysis of (II) was performed on a 30-cm column under the single column

(I) Bislysylsuberamidine (N,N'-bis(5-amino-5-carboxypentyl)suberamidine).



(II) Monolysylsuberamidinic acid (N-(5-amino-5-carboxypentyl)suberamidinic acid).

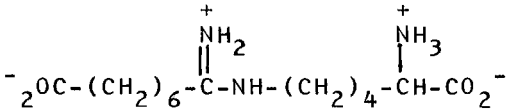


TABLE 1

Analytical conditions[a]	Retention Time (min) (I)	(II)	KWK[b]	Lys	NH_3	Arg
A	43	--[c]	21	--[c]	--[c]	--[c]
B	25	--[c]	--	--[c]	--[c]	--[c]
C	300	29	--	37	51	--
D	--	100	--	66	69	86

a) A: 5-cm column; 65°C; Na^+=1.4 M, 0.35 M citrate, pH 6.0. (For ninhydrin analysis)
B: 5-cm column; 65°C; Na^+=0.35 M, 0.1 M borate, pH 9.5. (For fluorescence detection)
C: 15-cm column; 65°C; Na^+=0.35 M, 0.12 M citrate, pH 5.6.
D: 30-cm column; first buffer, Na^+=0.2 M, 0.067 M citrate, pH 3.22 (0-12 min); second buffer, Na^+= 0.2 M, 0.067 M citrate, pH 4.25 (12-32 min); third buffer, Na^+=1.6 M, 0.2 M citrate, pH 5.00; 45°C (0-57 min), 55°C (afterward).
Buffer flow rate: 30 ml/h for A, B, and C; 12 ml/h for D.
b) KWK = bislysyladipamidine.
c) appears at the void.

methodology. (II) was eluted later than arginine and the retention time of (II) was similar to that of tryptophan under the chromatographic conditions employed. Since tryptophan is almost completely destroyed under the standard conditions of the total acid hydrolysis, the quantitative analysis of (II) was not interfered by the presence of tryptophan residue(s) in protein. When the analysis of (II) was carried out on a 15-cm column at 60°C, (II) and lysine were eluted separately but the peak of histidine overlapped with that of (II). However, when the column temperature was 45°C, the peak of (II) was very broad and overlapped with those of lysine and histidine. We could not find the appropriate conditions under which (II) was analyzed on a 15-cm column without a peak overlapping with lysine and histidine. Ninhydrin color values for (I) and (II) were determined as 1.8 and 1.0 relative to lysine, respectively, from the alkaline hydrolysis technique(3). Our most work on cross-linked protein hydrolyzates has been accomplished with fluorescence detection system employing o-phthalaldehyde(8). Approximately, 0.5-1.0 nmol of (I) was routinely analyzed. The lowest limit of detection may be below 50 pmol of (I).

Decomposition of (I) and (II) under the Standard Conditions of Acid Hydrolysis of Protein. (I) and (II) were subjected to acid hydrolysis (6 N HCl, at 110°C) for various periods up to 340 h. The acid hydrolyzate of (I) showed the presence of (I), (II), lysine, and ammonia, and no other ninhydrin-positive material was detected. Similarly, acid hydrolysis of (II) did not produce any ninhydrin-positive material except lysine and ammonia in hydrolyzates. A semi-logarithmic plot of the hydrolysis of (II) yielded a straight line,

which gave a first order rate constant $K_1 = 3.4 \times 10^{-3}$ h^{-1} for the hydrolysis of the amidine bond. The result that (I) degraded to (II) with a rate constant $6.8 \times 10^{-3} h^{-1}$, exactly twice the above k_1, indicates that two amidines of (I) were hydrolyzed independently at the same rate, as previously observed for bislysyl-adipamidine(3).

Deamidination of Suberimidate-crosslinked Lysine. Recently Dubois et al. reported that acetamidine was readily deamidinated by the treatment with methylamine buffer and the side reactions such as non-specific cleavage of peptide bonds were not observed(9). We examined the deamidination of (I) with methylamine-formate buffer. Methylamine buffer was found to induce the rapid cleavage of the suberamidine bonds; (I) completely disappeared after the incubation of (I) in the methylamine-formate buffer for 1 h at 37°C and lysine was regenerated quantitatively.

Application to the Analysis of the Suberimidate-treated Actin. Actin, one of major muscle proteins, was treated with dimethylsuberimidate(2). Amino acid analysis revealed that the acid hydrolyzate of the suberimidate-treated actin contained (I) and (II) and that the sum of lysine, (I) and (II) gave the original number of lysine in intact actin. These results indicate the validity of the analytical method described here. A removal of suberimidate-crosslink in the modified actin was accomplished by using the methylamine buffer. After the incubation of the suberimidate-treated actin in methylamine buffer for 2 h at 37°C, (I) and (II) completely disappeared on an amino acid analysis of the acid hydrolyzate of the modified protein, and the polyacrylamide gel electrophoresis in the presence of

sodium dodecylsulfate indicated that the treatment with methylamine buffer did not induce the non-specific cleavage of the peptide bonds in the protein.

Therefore, as demonstrated, the present study has established the basis to analyze the suberimidate-induced cross-links quantitatively, thus enabling us to investigate the location of the cross-links, in a strict sense of a primary structure.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF ZINC AND COPPER PHEOPHYTINS

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ABSTRACT

The zinc or copper chelates of pheophytins a and b were formed and separated on a reversed-phase C-18 column. Allomerized products were produced readily during the chelation reaction. Resolution of the allomerized compounds from the non-allomerized chelates was achieved using a gradient elution technique. Compound identification was facilitated by monitoring the column eluate at both 436 and 658 nm. The method allowed for isolation of individual pigments for further study.

INTRODUCTION

A number of porphyrin metal complexes are readily formed from chlorophyll derivatives(1). Chromatographic methods developed for the analysis of chlorophylls are numerous and have involved liquid chromatography. Review articles summarizing these techniques have been published (2,3,4).

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The formation of zinc and copper pheophytin chelates has been previously reported in canned vegetables (5,6). Recently, the formation of metal chlorophyll complexes has been used to enhance the appearance of heat processed vegetables (7). Copper pheophytin chelates are manufactured commercially as food colorants and are permitted for use in some European countries (8). Jones et al. (9) described a reversed-phase thin layer method for the detection of the zinc and copper complexes in processed foods.

High performance liquid chromatography (HPLC) has recently been used to monitor chlorophylls and their derivatives during the processing of foods (10). However, no HPLC methods are available for the determination of zinc and/or copper metal porphyrin compounds. This report investigates the use of HPLC for the separation and detection of zinc or copper pheophytins.

MATERIALS AND METHODS

Extraction of chlorophylls a and b

Chlorophylls a and b were extracted from forty grams of surface cucumber tissue by blending with 160 g of acetone for two minutes. The extract was filtered through Whatman #1 and #42 filter paper. All pigment extracts were stored at 4,C under nitrogen.

Preparation of pheophytins a and b

Pheophytins a and b were prepared from the chlorophyll extract after acidification with 250 ul of concentrated hydrochloric acid. The pigments were transferred to diethyl ether (100 ml) and the excess acid removed by three successive washings with 100 ml of water. The course of pheophytin formation was monitored by HPLC. After complete conversion (10 min), the diethyl ether layer was dried over anhydrous sodium sulfate. The diethyl ether was removed under nitrogen and the pigments dissolved in dry acetone until further use.

Preparation of copper and zinc pheophytin complexes

The copper and zinc pheophytin complexes were prepared following a procedure similar to that reported by Jones et al. (11). Copper pheophytins a and b were formed by adding 1.0 ml of 2.5 M copper (II) chloride to 4.0 ml of the acetone pheophytin extract (total pheophytin conc. ≃ 0.1 mg/ml). Zinc pheophytins a and b were prepared by adding crystalline zinc chloride (0.3 g) to 4.0 ml of the pheophytin acetone mixture. The samples were periodically mixed and the progress of chelation was monitored by HPLC. Following completion of the reaction (30 - 90 min.), the chelates were transferred to diethyl ether and the organic layer washed with water and dried over anhydrous sodium sulfate. Prior to analysis by

HPLC, the diethyl ether was evaporated under nitrogen and the pigments dissolved in acetone.

Pheophytins a and b were also separated by HPLC and isolated. Each metal chelate was then formed individually and compared to those prepared in pigment mixtures.

TABLE 1

Apparatus and Conditions for the Separation of Zinc and Copper Pheophytins by HPLC

column	μ Bondapak C_{18} (Waters Associates, Milford, MA)
pump A	Waters Associates, Model 510
pump B	Model 510, equipped with an inlet manifold assembly for gradient elution.
solvent A	75:25 CH_3OH:H_2O (v/v)
solvent B	ethyl acetate
initial condition	55% solvent A - 45% solvent B
final condition	50% solvent A - 50% solvent B
gradient	Linear gradient - curve 6 (solvent programmer, Model 680, Waters Associates) for a duration of 15 min.
flow rate	2.0 ml/min.
detector	Waters Associates Model 440, dual channel
injector	Waters Associates Model U6K
sample size	20 ul in acetone
detection wavelength	658 and/or 436 nm

Apparatus and conditions for the HPLC analysis

The HPLC apparatus and conditions used for the separation of the pheophytin metal chelates are outlined in Table 1.

Identification of zinc and copper pheophytins

The metal chelates were identified by their retention times on reversed-phase columns, their visible light absorption characteristics compared to reported literature values, and from observations made of their relative responses at the two monitored wavelengths. Visible absorption spectra were obtained after collecting selected peaks following repeated sample injections. All samples were transferred to diethyl ether for the determination of the visible spectra. Spectra were recorded using a Gilford (Oberlin, Ohio) Model 2600 spectrophotometer and plotted with a Hewlett-Packard (San Diego, CA) Model 7225B graphic plotter.

RESULTS AND DISCUSSION

HPLC chromatograms of fresh cucumber extracts showed only the presence of chlorophylls *a* and *b* when monitored at 658nm. Pheophytins *a* and *b* were formed by the addition of HCl to the chlorophyll *a* and *b* extract. Monitoring the column eluate at 658 nm allowed for selective detection of the chlorophyll compounds and derivatives without interferences from other pigments

present in the extracts. Simultaneously screening the eluate at 436 nm and 658 nm permitted more sensitive detection of the zinc and copper pheophytin *b* chelates since these compounds have a visible light absorption maxima near the monitored 436 nm wavelength. Measurements of the ratio of the two detector responses also aided in the identification of the separated compounds.

Table 2 summarizes and compares the spectral data for the separated pigments to reported literature values. Since the literature values for the pigment complexes were reported with diethyl ether as the solvent, all experimental pigments were transferred into ether before determining their light absorption characteristics. Use of the eluate mixture as solvent rather than diethyl ether resulted in a slight shift (approx. 2 nm) of the wavelength maxima toward the red region.

Figure 1 shows a representative chromatogram of the zinc pheophytin compounds formed from a mixture of pheophytins *a* and *b*. Peaks B (retention time = 8.1 min) and D (retention time = 10.9 min.) were identified as zinc pheophytins *b* and *a*, respectively (Table 2). Their order of elution on the reversed-phase column was expected. A similar elution order was observed for the chlorophyll *a* and *b* and pheophytin *a* and *b* compounds.

TABLE 2

Visible Absorption Spectra Identification Data of Zinc and Copper Chelates[a].

Peak	Peophytin Complex	Retention Time (min)[b]	Absorption max (nm) found	Absorption max (nm) reported[c]
B	Zn b	8.1	448;637	446;634
D	Zn a	10.9	425;654	423;653
B'	Cu b	11.8	441;629	438;627
D'	Cu a	16.2	422;650	421;648

[a] data reported were recorded in diethyl ether.
[b] refer to Figures 1 and 2.
[c] from Jones et al. (11).

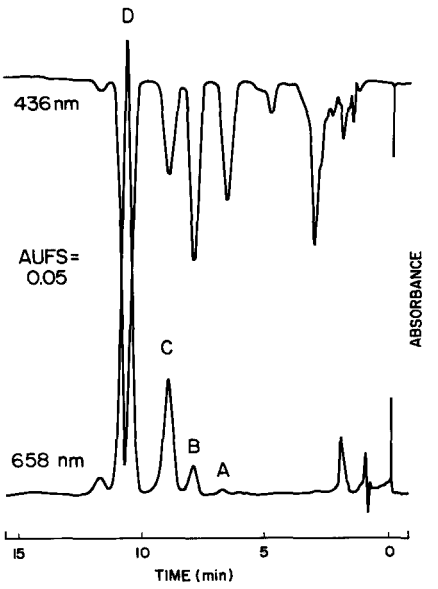

Figure 1. Chromatogram of zinc pheophytins. Peak A = allomerized zinc pheophytin b, peak B = zinc pheophytin b, peak C = allomerized zinc pheophytin a, peak D = zinc pheophytin a.

However, a shift to shorter retention times occurred upon chelation of the pheophytins with zinc.

The rate of chelation of pheophytin a with zinc was much more rapid than that of pheophytin b. Within ten minutes after the addition of zinc chlorides, the chromatogram showed a peak for the zinc pheophytin chelate and no peak for pheophytin a. Approximately one hour was required before a peak for zinc pheophytin b could be detected.

Peaks A and C are believed to be the allomerized (oxidized) zinc pheophytins b and a, respectively. These peaks form in greater concentrations after the mixture was exposed to the atmosphere and continued to increase during the chelation reaction. These findings are in agreement with those reported by Jones et al. (11). These authors found that pigment changes attributed to allomerization were readily induced during the formation of the metal complexes. The allomerized products were found to be more strongly bound (more polar) to a sugar column. The shorter retention times of peaks A and C on the reversed-phase C-18 column suggests this relationship. The visible absorption spectra of certain allomerized complexes lack a secondary peak or a plateau at a wavelength shorter than that of the major absorption peak in the blue region (11). The spectra of the allomerized compounds found in this study agree with these characteristics.

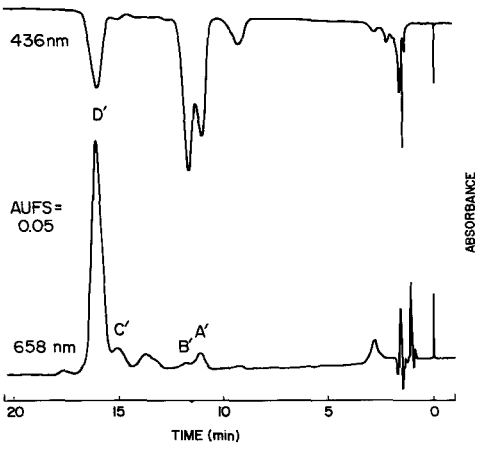

Figure 2. Chromatogram of copper pheophytins. Peak A' = allomerized copper pheophytin b, peak B' = copper pheophytin b, peak C' = allomerized copper pheophytin a, peak D' = cooper pheophytin a.

Figure 2 is a representative chromatogram of the copper pheophytin compounds formed from the reaction of pheophytin a and b with copper chloride. Peaks B' (retention time = 11.8 min) and D' (retention time = 16.2 min) were identified as copper pheophytins b and a, respectively (Table 2). In contrast to the zinc chelates, the copper a and b derivatives have longer retention times than their parent pheophytins. Allomerization compounds (peaks A' and C') were also apparent during the course of the chelation reaction. If a solution of pheophytins a and b were simultaneously injected with the above pheophytin copper ion mixture, the oxidation products were found to co-elute with

pheophytin b (Peak A') and a (Peak C'). Therefore, in order to obtain the copper allomerization compounds, it is necessary to allow sufficient time to complete the chelation reaction. As noted in the formation of the zinc complexes, the pheophytin a copper chelate formed much more rapidly than the b complex and allomerized products were noted particularly in the presence of oxygen. If the chelation reactions were performed under N_2, allomerization products were detected, but to a much lesser extent.

The HPLC method described in this study allows for the separation of either zinc or copper pheophytin complexes. Other pigments present in the sample mixture did not hinder the metal chelation reaction or interfere with the separation. An isocratic solvent system could be used to achieve a similar separation. However, the time required to complete the analysis was shortened considerably by using a gradient elution technique. The developed method also allowed for isolation of individual pigments for further study.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(8), 1685-1689 (1984)

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF IRIDOIDS IN CRUCIATA TAURICA

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ABSTRACT

The aerial and underground parts of Cruciata taurica (Pallas ex Willd.) Ehrend. s.l.(Rubiaceae) yielded three iridoids. Of these, Monotropein, Asperuloside and Aucubin. These compounds were separated and quantitated by reversed-phase HPLC.

INTRODUCTION

In the previous papers(1,2), the plant Cruciata taurica was investigated in regard to botanical properties and chemical constituents. Continuing our work on iridoids from C.taurica, we have now detected three iridoid glycosides. Of the possible methods for the analysis of iridoids(3), high-performance liquid chromatography is becoming the most widely used because it is fast, accurate, reproducible and can be used to determine both individual and total iridoids. Recently, there have been several HPLC methods developed for iridoid analysis(4-9).

In this paper, a separation and determination of iridoids of C.taurica using HPLC is reported.

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EXPERIMENTAL

The plant material of Cruciata taurica (Pallas ex Willd.) Ehrend.s.l.(Rubiaceae) used in this research were collected in May from Ahlatlıbel,Ankara,Turkey. Herbarium specimens are stored in the herbarium,Faculty of Pharmacy, University of Ankara.

The powdered aerial and underground parts of C.taurica (1 g) were extracted with methanol under reflux for 3 hr. The methanolic extract was concentrated to dryness and the residue was dissolved in bidistilled water and diluted with bidistilled water to 20 ml*. The aqueous layer was filtered through "Sep-Pak" silica cartridge. 5 Ml of filtrat were injected to HPLC column.

High-performance liquid chromatography was carried out in an M 730 Waters Liquid Chromatograph Data Module equipped with model 6000 A pump dual reciprocating piston heads model U6 K septumless injector, a 660 Solvent programmer, M 450 variable-wavelength detector and M Bondapak C_{18} column(300 x 3.9 mm). The HPLC mobile phase was methanol-water(20-80 % by volume). The mobile phase components were degassed by immersion in an ultrasonic bath and filtered through a Millipore HA(0.45 Mm) membrane filter. Flow rate was 2 ml/min. The effluent was monitored at 230 nm and detector sensitivity 0.04 aufs. Chart speed was 0.5 cm/min.

*Full details of the isolation of the iridoids are available on request to the authors.

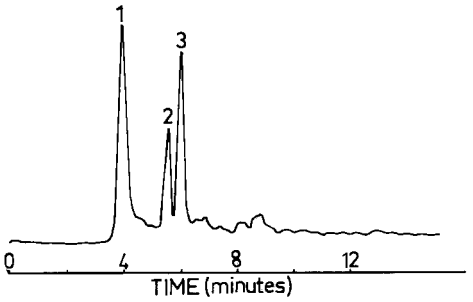

Figure 1. Isocratic analysis of iridoids derived from aerial parts of Cruciata taurica (Peak 1:Monotropein, Peak 2:Aucubin, Peak 3:Asperuloside)

RESULTS AND DISCUSSION

The following separation was achieved by using the mixed iridoids of C.taurica. An isocratic HPLC chromatograms are illustrated in figure 1 and 2.

Retention times obtained for monotropein, aucubin and asperuloside using this method were 3.95, 5.55 and 6.00 minutes, respectively. The values were also obtaimed by iridoids isolated from this plant. All isolated iridoids were identified by standart spectral data(6,10,11), as well as by authentic sample comparison.

The HPLC chromatogram of the aerial parts of C.taurica showed three distinct peaks: These are monotropein(38.1 %), asperuloside(26.3 %) and aucubin(18.6 %). The underground parts of C.taurica contained two iridoid compounds, monotropein(51.7 %) and asperuloside(4.4 %) but not aucubin.

The HPLC analysis of iridoid constituents of plants has been used extensively.

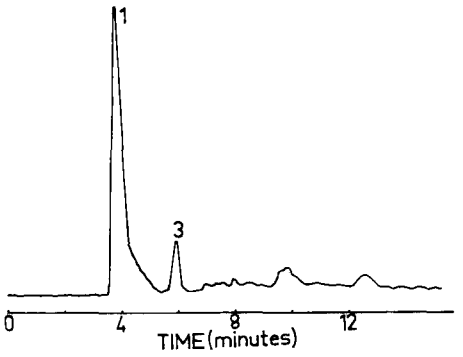

Figure 2. Isocratic analysis of iridoids derived from underground parts of Cruciata taurica.

ACKNOWLEDGEMENT

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(8), 1691-1706 (1984)

SEPARATION OF ANIONS AND CATIONS ON THORIUM TELLURITE - A NEW AMPHOTERIC ION EXCHANGER

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ABSTRACT

A new amphoteric ion exchanger, thorium tellurite has been synthesized. Its chemical stability, composition determination and potentiometric titration have been performed. K_d values for various anions and cations have been determined. The material was found useful for separation of NO_3^- from NO_2^-; MoO_4^{2-} from PO_4^{3-}, VO_3^- from MeO_4^{2-}, SO_4^{2-} from $S_2O_3^{2-}$ and BrO_3^- from Br^- and also of Cu(II) from Hg(II), Cu(II) from Ni(II), Pb(II) from Cd(II) and UO_2(I) from Hg(II).

INTRODUCTION

Separation of anions have been of much interest to various workers (1-2). But that have largely been accomplished on organic anion exchangers. Some of the synthetic inorganic ion exchangers are also known to behave both as cation as well as anion exchangers. Such an amphoteric behaviour has been found to be limited mainly to the hydroxides of Sn(IV) (3), Zr(IV) (4), Al(III) (5) and few others (6-7). Their behaviour is usually a function of pH.

In our present studies we have prepared a new inorganic ion exchanger, thorium tellurite, which works as a cation exchanger in the alkaline medium and as an anion exchanger in the acidic

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medium. Its applications towards separation of BrO_3^- from Br^-, NO_2^- from NO_3^- from the reaction products mark their analytical utility. Such exchangers should be of much use where a single mixed beds of cation and anions exchangers are used.

MATERIALS AND METHODS

Reagents: Thorium nitrate (B.D.H.) and sodium tellurite (B.D.H.) were used for precipitation of the exchanger. The other chemicals employed for separation studies were of analar grade.

Apparatus: A Metzer spectra 75 and an Elico pH meter Li-10 were employed for spectrophotometric and pH measurements. The shaking of the samples were done on a SICO shaking machine.

Synthesis: For precipitation of thorium tellurite, a 0.1M solution of thorium nitrate and a 0.1M solution of sodium tellurite were mixed in the volume shown in Table 1. The white precipitate so obtained was kept at room temperature for 48 h to ensure complete precipitation. The precipitate was then filtered, washed and dried in an oven at 40°C. It took nearly a week for the product to dry completely. On treatment with deionized water, the dried sample broke into small pieces. It was again dried at 40°C. The exchanger was then converted into desired form. For conversion into a particular anionic form an acidic solution of the anion should be taken. When desired to be taken in a cationic form, a basic cation solution, e.g. $Ca(OH)_2$ for Ca^{2+} form should be employed.

Anion exchange capacity: The anion exchange capacity was determined by column operation. One gram thorium tellurite in desired anionic form was taken on a glass wool support of a column and 1M solution of various anions were passed through it at a flow rate of 0.5 ml min^{-1}. The eluted anions were then determined in the effluents. To determine the anion exchange capacity for sulphate, the exchanger was taken in the sulphate form. The eluted sulphate ions were determined by

TABLE - 1

Conditions of preparation and properties of thorium tellurite

Sample	Conditions of synthesis				Properties	
	Molarity of		Mixing volume ratio	pH	Ion-exchange capacity (m.eq. g^{-1})	Nature of precipitation
	Thorium nitrate	Sodium tellurite				
S I	0.1	0.1	1:1	0	0.82	Thick
S II	0.1	0.1	1:2	1	1.40	Thick
S III	0.1	0.1	1:3	2	0.44	Mild
S IV	0.1	0.1	3:1	3	0.26	No precipitation
S V	0.1	0.1	2:1	1	0.60	Thick

precipitation with barium chloride and back titrating the excess of barium ions with 0.1 EDTA. For other anions the exchanger was taken in the chloride form and the eluted chloride ions were determined by Mohr's method (8).

Cation sorption capacity: One gram of thorium tellurite was taken in a glass column and 10 ml fractions of cationic solution containing predetermined amount of the cations, Cu(II), Fe(III), Ni(II), Co(II), Cd(II) and Mg(II) were passed through the column at a flow rate 0.5 ml min^{-1}. The amount of cation in the collected effluent was determined for each fraction. The process was kept continued till the amount of cation in the influent and the effluent remained the same. The amount sorbed was then calculated by substracting the amount of cation found in the effluent from the amount initially taken in each fraction.

Effect of drying temperature on anion exchange capacity: Thorium tellurite sample was heated at different temperatures in a muffle furnace for 2 hrs. The anion exchange capacity of each of the dried sample was then determined by column operation.

Chemical composition: 200 mg portion of the ion exchanger was dissolved in 30 ml of hot concentrated hydrochloric acid. The solution was then cooled to room temperature and diluted to 250 ml with deionized water. 100 ml of this solution was taken and T_e was precipitated with hydrazine hydrochloride-sulphur dioxide mixture (9). In another 100 ml portion Th(IV) was determined volumetrically with EDTA solution.

Chemical stability: A 0.5 g of the exchanger was shaken in a conical flask for four hours in the solution in which its dissolution was to be checked. The supernate was decanted and its thorium(IV) content was determined titrimetrically with EDTA. Tellurium was determined spectrophotometrically with thiourea (10).

Ion exchange potentiometric titration: Thorium tellurite being amphoteric in nature, the pH titrations were performed in both alkali and acid solutions with their respective salts following Topp and Pepper method (11). To study the cation exchange behaviour, 0.1M solution of alkali (NaOH, KOH and LiOH) and 0.1M solution of their respective salts were shaken with 0.5 g of the exchanger. The mixing ratio of the two solutions were taken in such a way that total volume remained 50 ml in all the cases. After being shaken for four hours the pH of the reaction mixture was measured. Similar experiments were performed taking HCl, H_2SO_4 and HNO_3 with their respective salts for determining the anion exchange behaviour on thorium tellurite.

K_d values: Distribution coefficient values for various anions and cations were determined by batch process. A known amount of cation or anion solution was shaken for six hours at room temperature with 0.5 g of the exchanger in a conical flask containing the solutions in which its distribution studies were desired to be made. The total volume of the equilibrating mixture was maintained at 50 ml. The liquid was drained off and its cation/anions content was determined. The K_d values were then determined by the formula

$$K_d = \frac{\text{Amount of cation/anion in the solution phase}}{\text{Amount of cation/anion in the exchanger phase}}$$

Separation: The separation of anions/cations with appreciable differences in their K_d values were tried. 2 g of thorium tellurite was taken in the glass wool support of the columns having a height 30 cm and diameter 0.69 cm . The column was washed thoroughly with deionized water and a mixture containing known amounts of anions or cations to be separated was passed through the exchanger bed at a very slow rate. Repeated recyclization of the mixture was done to allow adequate adsorption of the anions/cations. The elution was then done by the solutions in which the K_d values were lowest. In the eluted fraction the qualitative tests for both the components were performed.

RESULTS AND DISCUSSION

The exchanger, thorium tellurite is best prepared when its acid concentration was maintained at pH 1 as shown in Table. The exchanger so prepared not only gave a good yield but also better chemical stability and, therefore, sample II was taken for the detailed studies.

The results of the ion exchange capacity of thorium tellurite are shown in Tables 2-3. The anion exchange capacity varies from 0.8 $m.eq.g^{-1}$ to 1.4 $m.eq.g^{-1}$. The material possesses a high affinity for OH^- as is evident from the fact that releasing capacity for OH^- is only 0.60 $m.eq.g^{-1}$ while that for its uptake is 1.4 $m.eq.g^{-1}$. This shows that thorium tellurite acts as a weak base in OH^- form.

Although the exchanger, thorium tellurite lacked a H^+ liberation capacity it shows strong uptake for certain metal ions. Table 2 also shows the results of the sorption capacity for some of the bi and trivalent metal ions. The sorption capacity varies from 0.68 to 0.84 $m.moles\ g^{-1}$. Strong adherence of some of the metal ions to the exchanger frame work makes it an extremely useful material for

TABLE - 2

Anion exchange and cation sorption capacity for various anions

Sl. No.	Anions	Exchange capacity, $m.eq.g^{-1}$	Cations	Sorption capacity $m.moles\ g^{-1}$
1.	Cl^-	1.40	Cu^{2+}	0.84
2.	NO_3^-	1.00	Ni^{2+}	0.80
3.	SO_4^{2-}	1.24	Fe^{3+}	0.68
4.	PO_4^{3-}	0.80	Co^{2+}	0.74
5.	Br^-	1.22	Mg^{2+}	0.80
6.	OH^-	1.40 (OH^- uptake)		
		0.60 (OH^- liberation)		

TABLE - 3

Dissolution of thorium tellurite

Solvent	Amount, mg	
	Th	Te
Deionized water	0.0	0.00
HNO_3 (1M)	0.0	0.05
HNO_3 (2M)	14.2	22.62
NH_4OH (2M)	0.0	0.0
NH_4OH (4M)	1.21	1.82
HCl (1M)	0.03	0.06
HCl (2M)	12.80	21.20
CH_3OH	0.0	0.0
NaOH (1M)	8.0	10.1
NaOH (2M)	24.0	22.2
Acetone	0.0	0.0

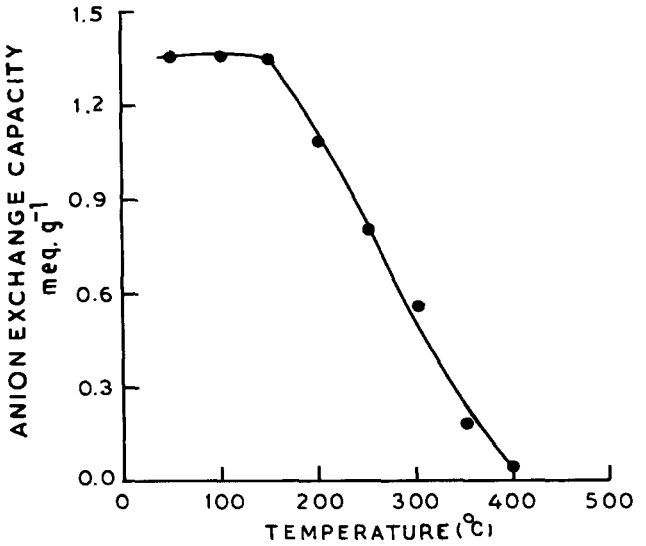

Fig. 1 - ANION EXCHANGE CAPACITY AT DIFFERENT TEMPERATURES

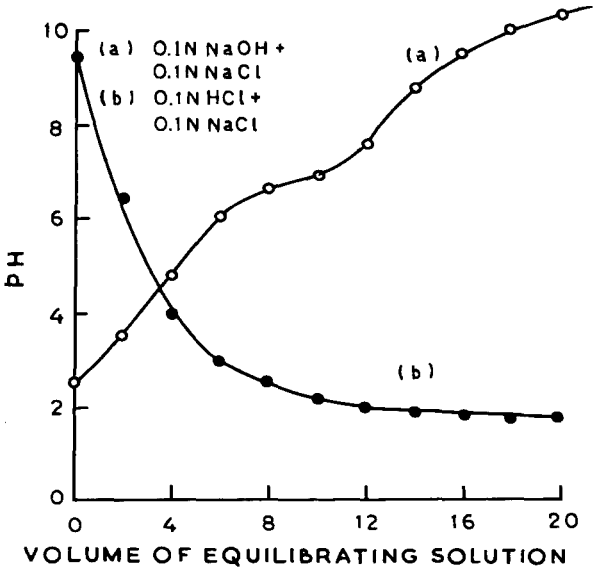

Fig. 2 - POTENTIOMETRIC TITRATIONS ON THORIUM TELLURITE

TABLE - 4

Distribution coefficient values of some anions

Anions	Method of determination	K_d ml g^{-1}				
		Deionized water	0.001M NH_4OH	0.01M NH_4OH	0.1M NH_4OH	0.1M HCl
NO_3^-	Spectr.	T.A.	2000	940	28	T.A.
NO_2^-	Spectr.	400	165	38	06	380
Cl^-	Titr.	2400	1992	728	102	2400
Br^-	Titr.	2800	2120	948	86	2394
I^-	Titr.	3100	2052	1012	495	2843
SO_3^{2-}	Titr.	1680	436	230	112	1290
SO_4^{2-}	Titr.	1440	1020	156	60	1320
AsO_3^{2-}	Iod.Titr.	2049	1150	609	349	1922
AsO_2^-	Spectr.	2296	1164	522	310	1984
IO_3^-	Iod.Titr.	1100	842	491	226	988
BrO_3^-	Iod.Titr.	156	26	06	00	180
SrO_3^{2-}	Iod.Titr.	85	10	00	00	78
SCN^-	Spectr.	720	529	321	206	688
CrO_4^{2-}	Titr.	T.A.	2811	852	224	T.A.
WO_4^{2-}	Spectr.	585	446	319	216	476
MoO_4^{2-}	Spectr.	T.A.	3242	1086	156	T.A.
VO_3^-	Spectr.	442	82	15	00	591
$Fe(CN)_6^{3-}$	Spectr.	714	512	328	164	740
PO_4^{3-}	Spectr.	340	116	42	00	290

the ligand exchange studies in ammonium hydroxide medium - one of the most widely used eluent for LEC studies.

The results of the effect of drying temperature on the ion exchange capacity of thorium tellurite is shown in Fig. 1. It reveals that the exchanger shows no deterioration in its exchange

TABLE - 5

Distribution coefficient values of some cations

Metal ions	K_d ml g^{-1}						
	Deionized water	0.001M HNO_3	0.01M HNO_3	0.1M HNO_3	0.01M NH_4OH	0.1M NH_4OH	0.01M $NaNO_3$
Cu(II)	800	468	335	93	90000	T.A.	600
Fe(III)	T.A.	340	78	4	T.A.	T.A.	T.A.
Ni(II)	21	18	13	2	59	637	782
Co(II)	435	313	268	103	4800	5688	532
Pb(II)	863	30	28	20	8421	9200	8000
Cd(II)	319	22	6	00	27	419	302
Hg(II)	165	105	63	45	185	1440	196
Ag(I)	761	252	182	74	1292	2281	820
Sr(II)	250	92	64	38	143	290	211
UO_2(I)	648	342	221	89	7952	8836	630
Mg(II)	260	150	00	00	280	300	460
Ba(II)	110	103	40	2	400	630	108
Ca(II)	342	220	119	82	480	596	390

behaviour upto 150°C, but the anion exchange capacity decreased appreciably when the drying was done above this temperature.

The results of dissolution of thorium tellurite in different concentrations of acids, base and neutral solutions are shown in Table III. It can be seen that the material is stable in aqueous ammonia upto 4M, HCl and HNO_3 upto 1M. Concentration of neutral solutions and non-aqueous solvents have no effect on the stability of the exchanger. Higher concentrations of strong acids and strong bases, however, cause appreciable dissolution of its constituents.

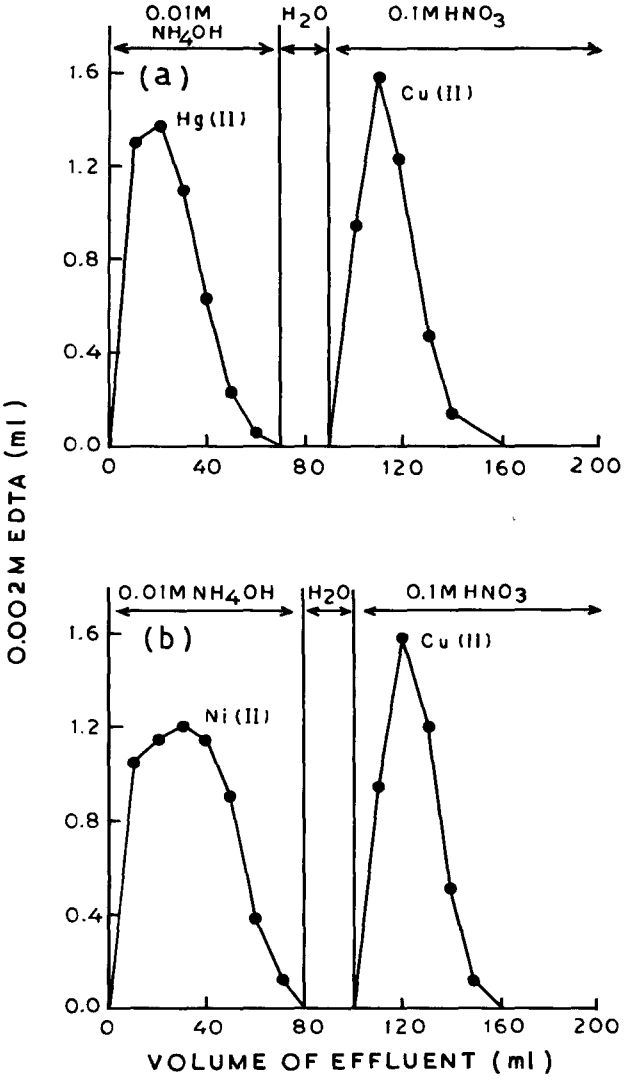

Fig. 3

A - SEPARATION OF Hg (II) FROM Cu (II)

B - SEPARATION OF Ni (II) FROM Cu (II)

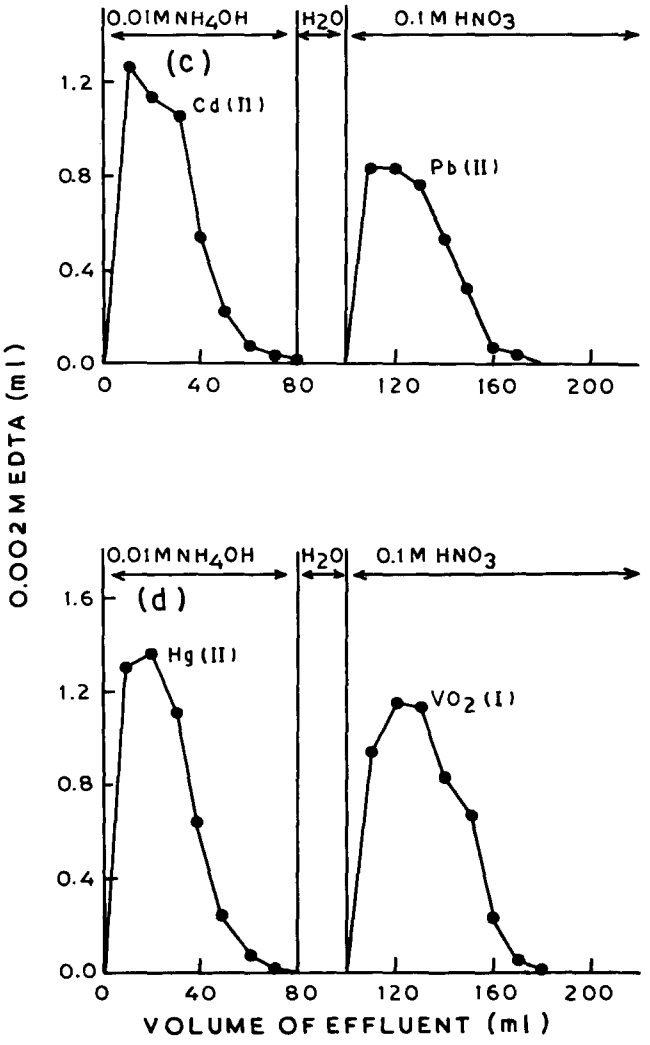

Fig. 3

C - SEPARATION OF Cd (II) FROM Pb (II)

D - SEPARATION OF Hg (II) FROM UO_2 (I)

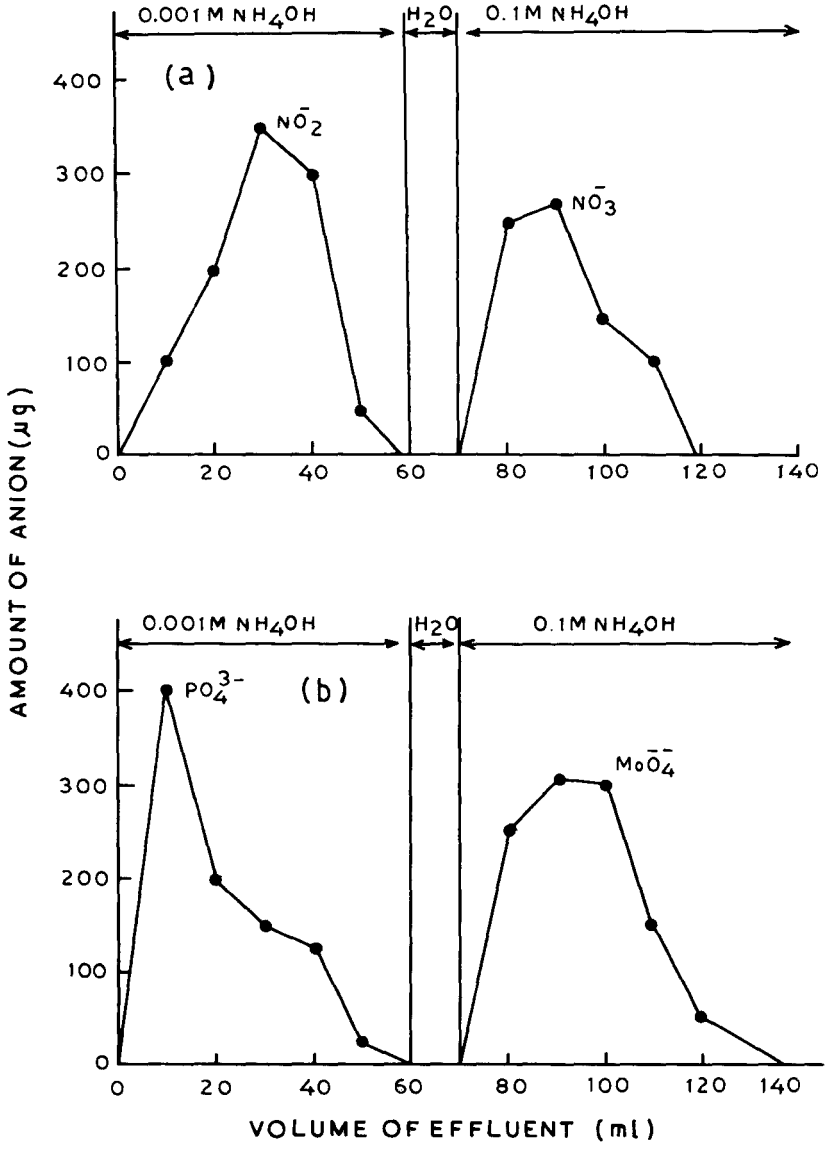

Fig. 4

A - SEPARATION OF NO_2^- FROM NO_3^-

B - SEPARATION OF PO_4^{3-} FROM MoO_4^{2-}

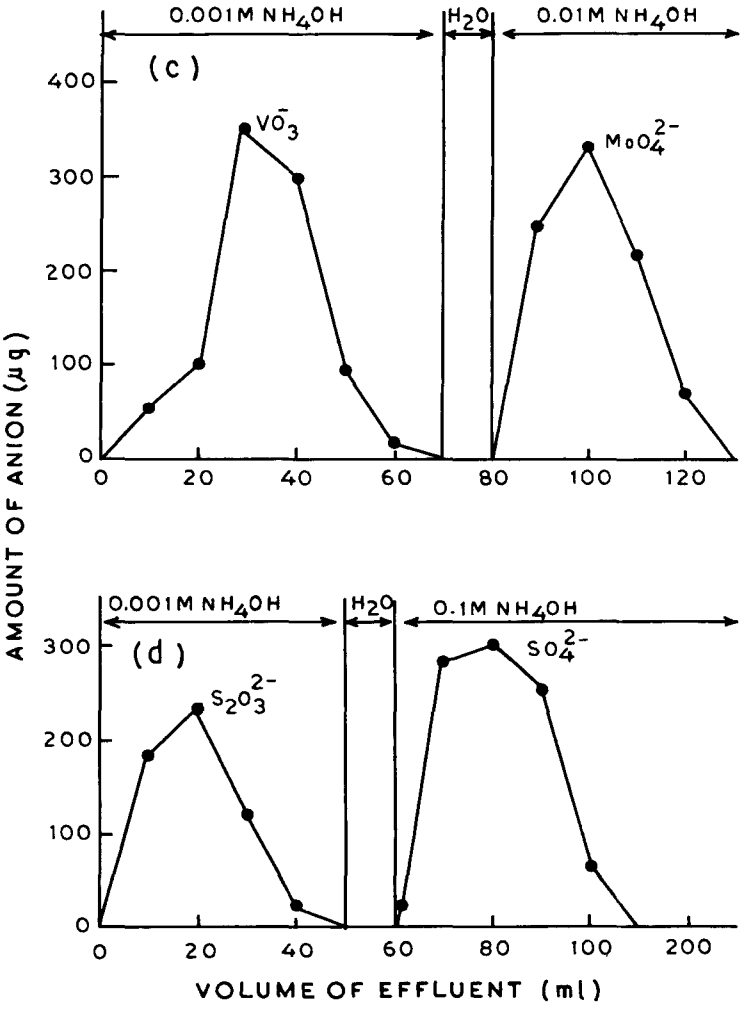

Fig. 4

C - SEPARATION OF VO_3^- FROM MoO_4^{2-}

D - SEPARATION OF $S_2O_3^{2-}$ FROM SO_4^{2-}

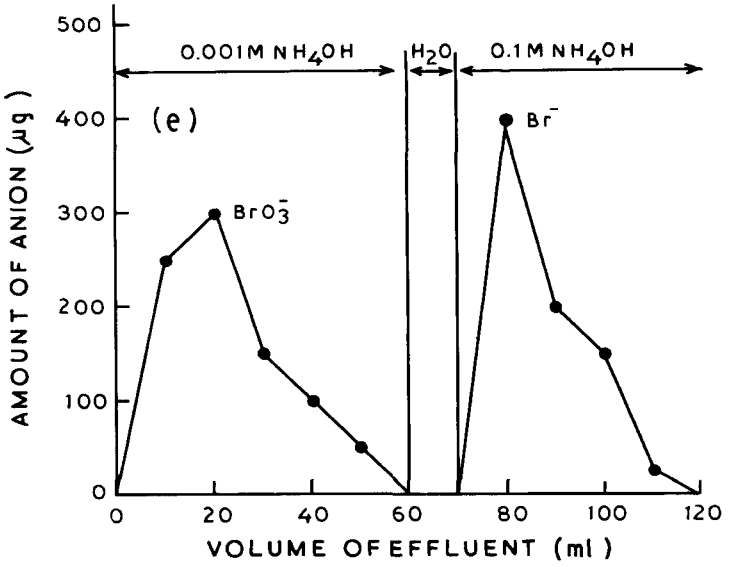

Fig. 4

E - SEPARATION OF BrO_3^- FROM Br^-

The results of the chemical composition of the exchanger showed that thorium and tellurium are present in the ratio Th.Te as 1:2. Shown in Fig. 2 are the pH titration curves for anion as well as cation exchange behaviour on thorium tellurite. A clear bifunctional cation exchange behaviour is observed in NaOH medium while its behaviour as an anion exchanger is not clear in the acidic medium.

The results of the distribution studies for various anions and cations are presented in Tables 4 and 5. It can be seen from these tables that K_d values for both anions as well as cations decrease with increase in the concentration of the equilibrating solution. K_d values were the highest in water medium. It can also be seen that almost all the anions exhibit greater adsorption in 0.1N HCl. This shows that unlike most of the other known inorganic ion exchangers, thorium tellurite, shows anion exchange behaviour in acidic medium.

The lower K_d values of various anions in aqueous ammonia is due probably to the greater preference of the exchanger for OH^- ions than for all other anions. Thorium tellurite serves as a cation exchanger in the basic medium and hence the cationic uptake in 0.01N NaOH was higher than that in water medium. With increasing concentration of acid the desorption of metal ions increased.

On the basis of large differences in K_d values various separations of anions and cations have been tried. Separation of Cu(II) from Hg(II), Cu(II) from Ni(II), Pb(II) from Cd(II) and UO_2(I) from Hg(II) have been successfully achieved and the order of elution and eluents used are shown in Figs. 3a-d. The successful separations of anions are NO_3^- from NO_2^-; MoO_4^{2-} from PO_4^{3-}; VO_3^- from MoO_4^{2-}, SO_4^{2-} from SrO_3^{2-} and BrO_3^- from Br^-. The order of separations are presented in Figs. 4a-e.

ACKNOWLEDGEMENT

Authors are thankful to Prof. W.Rahman, Chairman, Department of Chemistry, Aligarh Muslim University, Aligarh for providing research facilities. One of us (M.I.) is thankful to CSIR (New Delhi) for financial assistance.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(8), 1707-1709 (1984)

LC NEWS

HPLC INJECTOR STATION for the Laboratory Automation System combines HPLC sample introduction with automated sample preparation procedures. Samples are automatically prepared by the system and then directly introduced to the HPLC via either syringe injection or aspiration from a sample tube. Zymark, Corp., JLC/84/8, Zymark Center, Hopkinton, MA, 01748, USA.

pH GRADIENT CONTROLLER/PROGRAMMER uses a discontinuous, non-linear approach for feedback control, which is ideally suited to pH control, both batch and continuous. Varying of plug-in range resistor (potentiometer) permits stepwise or continuous programming of pH. Luft Instruments, Inc., JLC/84/8, Old Winter Street, Lincoln, MA, 01773, USA.

AUTOMATED PREPARATIVE-SCALE HPLC INSTRUMENT utilizes axial compression column technology to produce high-efficiency columns with all rigid stationary phases. The column is prepared just prior to using it by axially compressing a slurry of the packing to produce a uniforn chromatographic bed in which deleterious wall effects and diffusional band spreading are all but eliminated. User-friendly automation features include sample injection, fraction collection, recycle, column conditioning, safety alarms, step gradients, etc. Elf Aquitaine Development Corp., JLC/84/8, P. O. Box 1678-Murray Hill Station, New York, NY, 10157, USA.

IMPROVED HPLC SEPARATIONS with ion pair reagents for ionic solutes without the use of ion exchange columns. Ion pair reagents modify the mobile phase to effectively attenuate or enhance solute retention, improve peak symmetry and control selectivity. Both cationic and anionic species are available, as well as perfluoroalkanoic acids and triethylamine. Pierce Chemical Co., JLC/84/8, P. O. Box 117, Rockford, IL, 61105, USA.

TLC STICK is a solid, elongated block of silica gel designed for use in a wide variety of separations. It can be spotted with a maximum of 300mg or 20ml of sample...1,000 to 10,000 times more sample than other related methods. Recovery is achieved by slicing

the desi"ed band from the stick and extracting with an appropriate solvent. Alltech Associates, Inc., Applied Science Labs, JLC/84/8, 2051 Waukegan Rd., Waukegan, IL, 60031, USA.

HPLC COLUMN BLOCK HEATERS are available for precise control of tolumn temperature to improve sample loading, baseline stability, separation efficiency, and resolution. Phenomenex, Inc., JLC/84/8, 426 Via Corta, Bldg. 305, Palos Verdes Estates, CA, 90274, USA.

MAXIMIZING HPLC PRECISION BY SKILLFUL INJECTION is a report that can help both the beginner and the experienced chromatographer. Some very practical questions are addressed: "How much sample must be wasted to fill a sample loop?"; "When you are judging the amount of sample being loaded by reading a syringe, how much sample can you inject without impairing accuracy?" Ask for Tech Note No. 5. Rheodyne, Inc., JLC/84/8, P. O. Box 996, Cotati, CA, 94928, USA.

COMPUTING INTEGRATOR for re-integrating, re-plotting, recalculating features 32K RAM peak storage. It offers 6 external event controls, autosampler interface, choice of thermal and impact printer plotters, IEEE interface, 100 data slices per second programmable sampling rate. Applied Chromatography Systems (USA), Inc., JLC/84/8, Suite 125, 315 S. Allen Street, State College, PA, 16801, USA.

MINIATURE INERT VALVES, fittings and controllers include 2, 3, 4, 6, and 8-port variations, large or small port, ON-OFF selection, distribution, sampling, and other flow directional control applications. Hamilton Co., JLC/84/8, P. O. Box 10030, Reno, NV, 89520, USA.

HPLC DETECTOR RESOLVES EVEN HIDDEN COMPOUNDS by monitoring at two different user-selectable wavelengths. Thus, sample comparisons can be made on the basis of absorbance, absorbance ratios, sums and differences within a single sample cell. The detector uses a deuterium lamp, a single high pressure flow cell (up to 4,000 psi, variable angle concave grating, two photodiode pairs, and measures between 190 and 750 nm. Micromeritics Instrument Corp., JLC/84/8, 5680 Goshen Springs Road, Norcross, GA, 30093, USA.

ACETYLCHOLINE ANALYZER utilizes a rapid reverse phase separation of acetylcholine and choline, and a post-column enzymatic reactor to achieve detection limits of 2 pmol or better. Detection is based upon the electrochemical oxidation of hydrogen peroxide realeased in the enzymatic reaction using a platinum electrode. Bioanalytical Systems, Inc., JLC/84/8, 2701 Kent Avenue, West Lafayette, IN, 47906, USA.

PORTABLE PIPETTING AID is rechargeable and can give over two hours of continuous operation with one charge. It will accept all

standard glass and plastic pipettes, including Pasteur, serological, and transfer types. Sarstedt, Inc., JLC/84/8, P. O. Box 4090, Princeton, NJ, 08540, USA.

THE FILTER BOOK is a handy reference tool for filtration users. It contains informatoon on how to select the right filter for any labortory filtration application. Gelman Sciences, Inc., JLC/84/8, 600 S. Wagner Road, Ann Arbor, MI, 48106, USA.

VARIABLE WAVELENGTH CHROMATOGRAPHY DETECTOR has a master-blazed holographic grating which virtually eliminates stray light and provides high accuracy in absorbance measurements, with an 8 microliter cell. Pye Unican Ltd., JLC/84/8, York Street, Cambridge CB1 2PX, England.

GPC/HPLC FLOWMETER measures liquid flow rates from 0.1 to 100 cc/min with accuracies up to 0.1%. The instrument uses a thermal pulse time of flight technique that is independent of changes in the properties of the solvent or ambient temperature. Two glass-encapsulated thermistors protrude into the liquid stream about 1" apart. A heat pulse, generated by the upstream thermistor, moves downstream where it is detected by the second thermistor. The time required for the pulse to reach the downstream thermister is a function of the liquid flow rate. Molytek, Inc., JLC/84/8, 2419 Smallman Street, Pittsburgh, PA, 15222, USA.

POLYACRYLAMIDE RIGID GEL FOR SEC/GPC may be operated at high pressures and undergoes minimum swelling and shrinkage when transferred between polar eluents. Applications include separations of polysaccharides, polyphenols, and synthetic aqueous polymers. Polymer Laboratories, JLC/84/8, Essex Road, Church Stretton, Shropshire, SY6 6AX, UK.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(8), 1711-1715 (1984)

LC CALENDAR

1984

JUNE 3-5: International Symposium on LCEC and Voltammetry, Indianapolis Hyatt Regency Hotel, Indianapolis, IN. Contact: The 1984 LCEC Symposium, P. O. Box 2206, West Lafayette, IN, 47906, USA.

JUNE 10 - 13: Annual Meeting of the Institute of Food Technologists, Anaheim, CA. Contact: IFT, 221 N. LaSalle Street, Suite 2120, Chicago, IL, 60601, USA.

JUNE 10-14: 14th Northeast Regional ACS Meeting, sponsored by the Western Connecticut and New Haven Sections, at Fairfield University, Fairfield, CT. Contact:D. L. Swanson, American Cyanamid Co., Stamford, CT, USA.

JUNE 18-20: Second International Conference on Chromatography & Mass Spectrometry in Biomedical Sciences, sponsored by the Italian Group for Mass Spectrometry in Biochemistry & Medicine, Milan, Italy. Contact: Dr. A. Frigerio, via Eustachi 36, I-20129 Milan, Italy, or Dr. H. Milon, P. O. Box 88, CH-1814 La Tour-de-Peilz, Switzerland.

JUNE 18-21: Symposium on Liquid Chromatography in the Biological Sciences, Ronneby, Sweden, sponsored by The Swedish Academy of Pharmaceutical Sciences. Contact: Swedish Academy of Pharmaceutical Sciences, P. O. Box 1136, S-111 81 Stockholm, Sweden.

JUNE 19 - 23: SPI/SPE Plastics Show & Conf. East, Philadelphia, PA. Contact: SPE, 14 Fairfield Drive, Brookfield Center, CT, 06805, USA.

JUNE 21 - 22: Conference on Quantitative Characterization of Plastics & Rubber, McMaster University, Hamilton, Ont., Canada. Contact: John Vlachopoulos, Dept. of Chem. Eng., McMaster University, Hamilton, Ont., L8S 4L7, Canada.

JULY 1 - 7: 12th International Carbohydrate Symposium, Jaarbeurs Congress Centre, Utrecht, The Netherlands. Contact: J. F. G. Vliengenthart, Dept. of Bio-Organic Chem., State Univ. of Utrecht, P. O. Box 5055, NL-3502JB, Utrecht, The Netherlands.

JULY 9 - 13: 10th International Conference on Organic Coatings Science & Technol., Athens, Greece. Contact: V. Patsis, Materials Research Lab, CSB 209, State Univ. of NY, New Paltz, NY, 12561, USA.

JULY 15 - 20: International Conference on Ion Exchange, Cambridge College, UK. Contact: Conference Committee, IEX-84, Soc. Chem. Ind., 14 Belgrave Square, London, SWIX 8PS, UK.

AUGUST 21 - 24: 24th Int'l Conf on Analytical Chem. in Development, Sri Lanka. Contact: Secretary, Organizing Committee, Centre for Anal. Chem R & D, Dept. of Chem., University of Colombo, P. O. Box 1490, Colombo 3, Sri Lanka.

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

SEPTEMBER 10-14: Advances in Liquid Chromatography, including the 4th Annual American-Eastern European Symposium on LC and the Int'l Symposium on TLC with Special Emphasis on Overpressured Layer Chromatography, sponsored by the Hungarian Academy of Sciences' Chromatography Committee & Biological Research Center and the Hungarian Chemical Society, in Szeged, Hungary. Contact: Dr. H. Kalasz, Dept. of Pharmacology, Semmelweis University of Medicine, P.O.Box 370, H-1445 Budapest, Hungary, or Dr. E. Tyihak, Research Inst. for Plant Protection, P.O.Box 102, H-1525 Budapest, Hungary.

SEPTEMBER 16 - 21: Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Marriott Hotel, Philadelphia, PA. Contact: D. B. Chase, DuPont Co., Experimental Station 328, Wilmington, DE, 19898, USA.

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

OCTOBER 8 - 10: ASTM Committee E-19 on Chromatography, St. Louis Sheraton Hotel, St. Louis, MO. Contact: F. M. Rabel, Whatman, Inc., 9 Bridewell Place, Clifton, NJ, 07014, USA.

OCTOBER 24 - 26: Third Workshop/Symposium on LC/MS and MS/MS, Montreux, Switzerland. Contact: R. W. Frei, Dept. of Anal. Chem., Free University, De Boelelaan 1083, NL-1081 HV Amsterdam, The Netherlands.

OCTOBER 28 - NOVEMBER 1: 2nd International Congress on Computers in Science, Washington, DC. Contact: S. R. Heller, EPA, PM-218, Washington, DC, 20460, USA.

NOVEMBER 13 - 16: 23rd Eastern Analytical Symposium, New York Penta Hotel, New York City. Contact: S. D. Klein, Merck & Co., P. O. Box 2000/R801-106, rahway, NJ, 07065, USA.

DECEMBER 10-12: "TLC/HPTLC-84: Expanding Horizons in TLC," Sheraton-University City, Philadelphia, PA. Contact: J. C. Touchstone, University of Pennsylvania, Dept. OB-GYN, 3400 Spruce eStreet, Philadelphia, PA.

DECEMBER 16-21: International Chemical Congress of Pacific Basin Societies, Honolulu, Hawaii, sponsored by the Chemical Inst. of Canada, Chemical Soc. of Japan, and the American Chem. Soc. Contact: PAC CHEM '84, International Activities Office, American Chem. Soc., 1155 Sixteenth St., NW, Washington, DC, 20036, USA.

1985

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

FEBRUARY 25 - MARCH 1: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, New Orleans, LA. Contact: Paul E. Bauer, 1985 Pittsburgh Conference, 437 Donald Rd., Dept. FP, Pittsburgh, PA, USA.

APRIL 15 - 17: Second International Symposium on Instrumental TLC, Wurzburg, West Germany. Contact: H. M. Stahr, 1636 College Veterinary Medicine, Iowa State University, Ames, IA, 50011, USA., or Prof. S. Ebel, Institute for Pharmacies, A. M. Hubland, D-8700 Wuerzburg, West Germany.

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

JUNE 24 - 28: 59th Colloid & Surface Science Symposium, Clarkson College of Technology, Potsdam, NY. Contact: J. P. Kratohvil, Institute of Colloid & Surface Science, Clarkson College of Technology, Potsdam, NY, 13676, USA.

JULY 1-5: Ninth International Symposium on Column Liquid Chromatography, sponsored by the Chromatography Discussion Group and by the Royal Society of Chemistry's Chromatography & Electrophoresis Group, Edinburgh, Scotland. Contact: Prof. J. H. Knox, 9th ISCLC Secretariat, 26 Albany Street, Edinburgh, EH1 3QH, Great Britain.

JULY 4: 4th International Flavor Conference, Greece. Contact: C. J. Mussinan, IFF R&D, 1515 Highway 36, Union Beach, NJ, 07735, USA.

SEPTEMBER 1-6: 6th International Symposium on Bioaffinity Chromatography & Related Techniques, Prague, Czechoslovakia. Contact: Dr. J. Turkova, Institute of Organic Chemistry & Biochemistry CSAV, Flemingovo n. 2, CS-166 10 Prague 6, Czechoslovakia.

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

SEPTEMBER 29 - OCTOBER 4: Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Marriott Hotel, Philadelphia, PA. Contact: T. Rains, NBS, Center for Analytical Chemistry, Chemistry B-222, Washington, DC, 20234, USA.

1986

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

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

SEPTEMBER 21-26: XVIth International Symposium on Chromatography, Paris, France. Contact: G.A.M.S., 88,Boulevard Malesherbes, F-75008 Paris, France.

1987

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

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

1988

JUNE 5 - 11: 3rd Chemical Congress of the North Americanmn Continent, Toronto, Ont., Canada. Contact: A. T. Winstead, ACS, 1155 Sixteenth St, NW, Washington, DC, 20036, USA.

SEPTEMBER 25 - 30: 196th ACS National Meeting, Los Angeles, CA. Contact: A. T. Winstead, ACS, 1155 Sixteenth Street, NW, Washington, DC, 20036, USA.

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F. D. Pierce and H. R. Brown
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Salt Lake City, Utah 84108

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

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

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