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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(10), v (1984)

FROM THE PUBLISHER

The Journal of Liquid Chromatography has reached a milestone with the receipt of its one-thousandth contribution. The support the Journal has received from the international scientific community is a tribute to all -- the authors, the editorial board members, the publisher and, particularly Dr. Jack Cazes, the editor. Dr. Cazes has, from its inception, worked with vigor and determination to make the Journal of Liquid Chromatography the leading journal for the field of liquid chromatography. Under the capable editorship and diligence of its' editor, we expect that it will increasingly reflect the amazing developments in all phases of liquid chromatography.

We, the publisher, look to the future with great optimism and rededication to even higher standards of excellence. I personally, wish to thank everyone who has participated in making the Journal of Liquid Chromatography the leading journal that it unquestionably is today.

Sincerely,

Maurits Dekker

Maurits Dekker, Ph.D.
Chairman of the Board

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(10), 1911-1934 (1984)

AN ELUENT PRESSURE DETECTOR FOR AQUEOUS SIZE EXCLUSION CHROMATOGRAPHY

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ABSTRACT

An on-line viscometer which measures the eluent pressure drop across a long capillary was developed for use in aqueous size exclusion chromatography (SEC). Intrinsic viscosities of several polymer standards were calculated from data collected by the viscometer. These viscosities agree well with the measurements made with a Ubbelohde four-bulb shear dilution viscometer. The on-line viscometer becomes more sensitive as polymer hydrodynamic volume increases. Therefore, it can be more effective than a refractive index detector for SEC analysis of high molecular weight, water soluble polymers.

INTRODUCTION

Characterization of large, water soluble macromolecules, having high polydispersity, can be accomplished by using size exclusion chromatography (SEC). In SEC analysis, molecules are separated according to their hydrodynamic size. This separation is accomplished by using a solvent to force a polymer sample in solution through columns packed with porous particles. Smaller size polymer molecules are retained for a longer time because a greater fluid volume located within the packing particles is

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accessible to smaller molecules. After separation within the packed columns, some type of detector is used to sense the existence of polymer molecules in the eluent stream leaving the columns. A plot of detector signal versus the elution counter, which is the total mass or volume of the eluent that has passed through the column since the injection of the polymer sample, is called an SEC chromatogram. From the shape of this chromatogram, a molecular weight distribution of the polymer sample can be determined.

To assure efficient separation by the packing, the total injected mass of the polymer sample must be small. In addition, the concentration of the injected polymer should be low to assure dilute solution conditions. This requirement enables the polymer coils to occupy independent volume domains with respect to one another.

Typically, the polymer solution injected should not be greater than one-half the reciprocal of its intrinsic viscosity. Under these conditions, polymer molecules do not interact and dilute solution conditions exist. For example, if a polymer sample has an intrinsic viscosity of 5 dl/g, the maximum polymer concentration that should be injected is 0.10 g/dl. It becomes obvious that, as the molecular weight or intrinsic viscosity of the polymer increases, the sample concentration injected must decrease.

In aqueous SEC, a polymer sample may be diluted by a factor of 50 during its flow through the columns to the detectors. Thus,

if our example polymer sample has an intrinsic viscosity of 5 dl/g, the eluting concentration at the detectors is only 20 ppm. Usually in organic SEC, flourescence or ultraviolet detectors are used at these low concentrations. However, due to the lack of active chromophores in many water-soluble polymers, these detectors are not useable in aqueous SEC and a less sensitive refractive index detector (RI) must be employed.

RI detectors measure the eluent refractive index. Therefore, both temperature and solvent compositional changes in the eluent, as well as the presence of polymer solute, will be detected without discrimination. Unfortunately, small changes in solvent composition or temperature may result in a large RI response which may completely hide the signal due to the small presence of polymer. Thus, many SEC chromatograms obtained by using RI detectors are not usable due to the presence of phantom signals and/or highly unstable baselines. As an alternative to RI detectors in aqueous SEC, a continuous capillary pressure (CP) detector was developed to measure eluent viscosity. Tests with this detector have shown that it has the sensitivity to both measure the presence of polymer in the eluent and simultaneously determine the polymer sample intrinsic viscosity.

EXPERIMENTAL

The first CP detector for SEC was developed by Ouano [1,2]. More recently, other researchers have designed and reported the performance of similar CP detectors [3,4]. The CP detector

used in this study was based on the design recommendations of the above authors.

A Validyne Model DP-15 differential pressure transducer, equipped with a number 42 diaphragm, was used to measure the fluid pressure drop during flow through a 91.5 cm long, stainless steel capillary with an inside diameter of 0.023 cm. This detector and its location in the SEC system is shown schematically in Figure 1. SEC system equipment details and operating conditions are given in Table 1. SEC data acquisition and analysis was performed by a Hewlett-Packard microcomputer system which has been previously described [5].

The Hagen-Poiseuille relationship, equation (1), for steady laminar flow in a circular capillary, was used to determine the eluting viscosity, μ, from the differential pressure measure ΔP, across a capillary:

$$\mu = \frac{\pi D^4 \Delta P}{128 \ell Q} \tag{1}$$

In equation (1), ℓ is the the length of the capillary and Q is the volumetric flow rate of the eluent through the capillary.

Because the capillary length to diameter ratio is extremely large (almost 4000), no capillary entrance or exit corrections were necessary when using equation (1). In our SEC system, the eluent volumetric flow rate was always maintained at approximately 0.50 ml/min. These conditions produce laminar fluid flow in the capillary (a Reynold's number of approximately 50), and a shear rate at the wall of 6500 sec^{-1}.

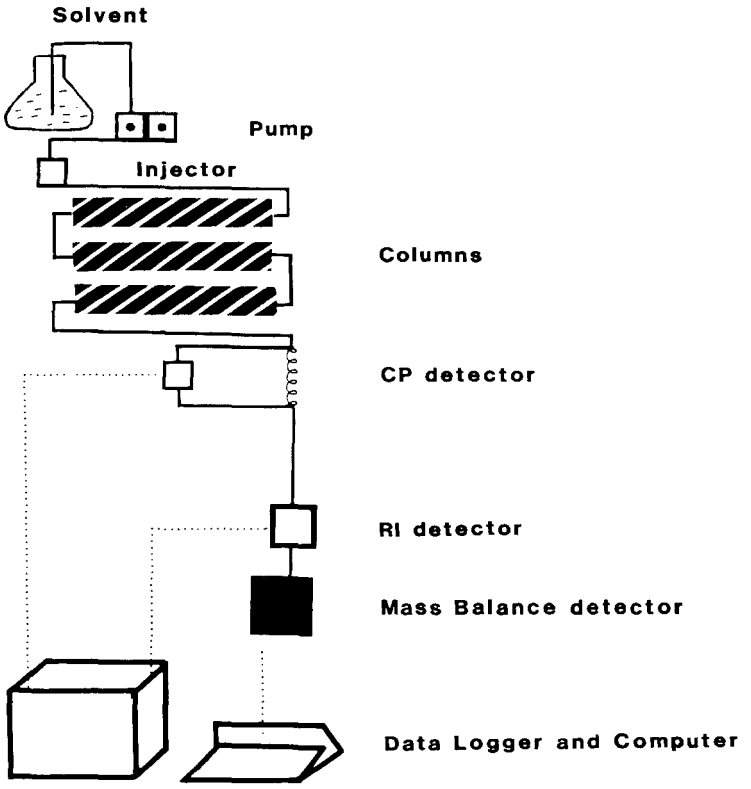


FIGURE 1. SEC System Schematic

DATA ANALYSIS

A definite advantage of a CP detector is that, unlike an RI detector, its signal is proportional only to the eluent viscosity; therefore, it is unaffected by small changes in solvent composition or temperature. Figure 2 shows both an RI and CP chromatogram taken simultaneously on a dextran polymer sample. Note that the RI detector signal has an unstable base line and has a solvent impurity signal with negative polarity convoluted into a polymer

TABLE 1

System Equipment Description and Operating Conditions

System/Equipment	Details and Conditions
Polymer/Solvent	All dextran samples were purchased from Pharmacia and prepared in deionized water with 10 ppm NaN_3 added as a biocide. Sodium polystyrene sulfonates (NaPSS) were purchased from Pressure Chemical Co., and were prepared with 0.2 M NaCl/Triton-40-5L as a surfactant. All solvents were degassed and filtered through a 0.45μ Nucleopore filter before use.
Pump	A Waters Model 6000A was used with an additional pulse dampener (Milton Roy Mark III). The nominal flow rate was maintained at 0.5 ml/min.
Injector	A Rheodyne Model 1025 injector with a 0.5 ml sample loop was used. The sample injections, typically, were 0.5 ml.
Packing Material	All columns were packed with glyceryl-controlled porous glass purchased from Electro-Nucleonics. The nominal pore size and dry weight of packing material used per column set were: (1) 3000 Å, 10.62 g; (2) 1400 Å, 6.22 g; (3) 500 Å, 6.40 g. A 0.45μ Nucleopore filter was placed in front of the column set to prevent possible pluggage. The column end fittings contained 10μ frits.
Capillary Pressure Detector	A capillary having a length of 0.91 cm and an ID of 0.023 cm was connected to the transducer at each end by capillary tubing with a length of 10 cm and ID of 0.04 cm. The pressure transducer was a Validyne Model DP-15 with a Model CD15 Sine Wave Demodulator. The diaphragms for the transducer are interchangeable with a No. 42(880-2250 cm H_2O) being used in this study.
Refractive Index Detector	A Waters Model 400 Differential Refractive Index Detector having a cell volume of 10 microliters.
Data Logger and Computer	The Data Logger is a Hewlett-Packard Model 3497A Acquisition/Control unit. The computer is a Hewlett-Packard Model 85A microcomputer.

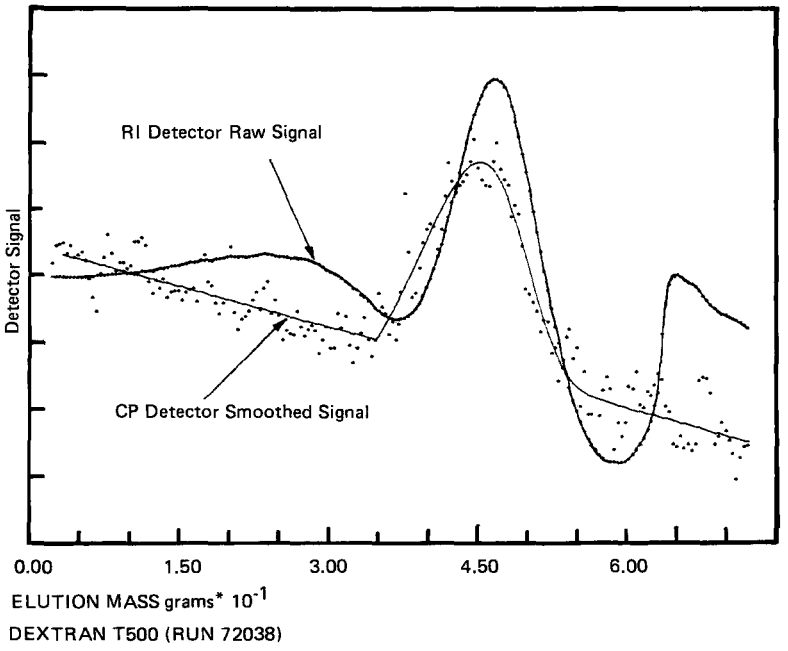


FIGURE 2. Typical Capillary Pressure (CP) and Refractive Index (RI) Chromatograms

signal with positive polarity. In contrast, the CP detector signal although noisy, has a declining but steady linear base line and shows only one peak which is due only to the presence of polymer in the eluent. Most of the noise in the CP detector is from fluid pressure pulses introduced by the SEC reciprocating pump. Other factors such as fluid temperature fluctuations and fluid leaks in the SEC system can also introduce additional noise [6].

Because the raw CP detector data has a low signal to noise ratio, a computer algorithm was developed to smooth pressure

detector data. A typical smoothed CP detector signal is shown as the continuous curve in Figure 2.

The computer smoothing algorithm was a nonlinear regression that utilized the method of Marquardt [15] to fit raw CP detector data to a smoothing function [7]. The smoothing function was the sum of two functions, a modified generalized exponential function (GEF) and a linear base line function (LBF). This smoothing function is given by equation (2):

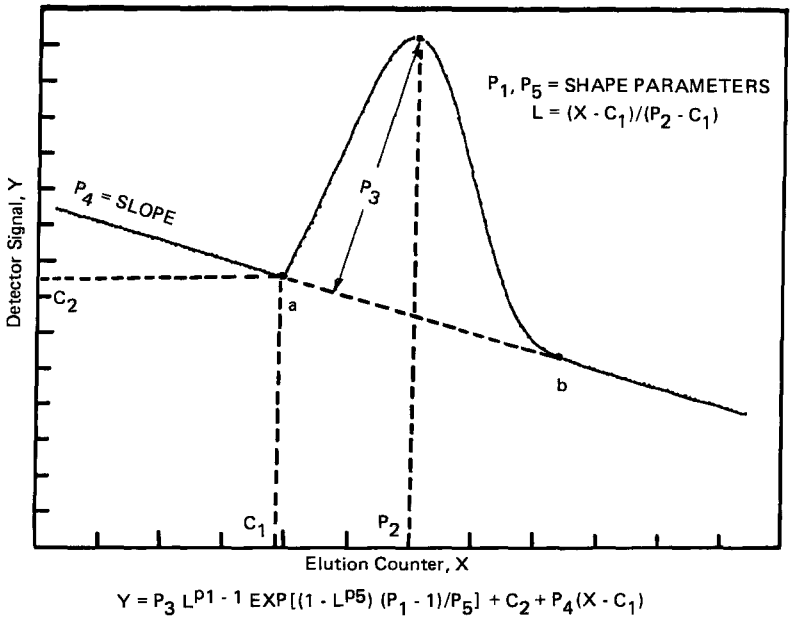
$$Y = GEF + LBF \tag{2a}$$

$$Y = P_3 L^{P_1 - 1} \, EXP[(1-L^{P_5})(P_1 - 1)/P_5] + C_2 + P_4 \, (X - C_1) \tag{2b}$$

where $$L = (X - C_1)/(P_2 - C_1) \tag{2c}$$

In equation (2), the detector signal and elution counter are Y and X, respectively. When using a CP detector, Y is equal to the eluent pressure drop across the capillary and X is equal to the elution volume. The five fitted parameters in the smoothing function are designated by P with a numeric subscript, and the two constants are designated by C with a numeric subscript. The use of the GEF function to fit chromatographic data has been previously described by Vaidya and Hester [8].

The smoothing function appears complex when it is presented in the form of equation (2). However, in this form, all parameters and constants in the smoothing function have a geometrical meaning whose value can be closely estimated from a chromatogram. This can be explained with the help of Figure 3. The constants



$$Y = P_3 L^{P1-1} EXP[(1 - L^{P5})(P_1 - 1)/P_5] + C_2 + P_4(X - C_1)$$

FIGURE 3. Chromatogram Fitting Function Description

C_1 and C_2 are the coordinates where the chromatogram signal first deviates from the linear base line (point a). The linear base line has a constant slope of P_4. The parameter P_2 is the elution counter X coordinate where the maximum polymer signal, P_3, is obtained. The parameters P_1 and P_5 determine the shape of the GEF function, and for SEC chromatograms they usually have values between 2 and 6. When $P_1 = P_5 = 3.2$, the GEF is nearly normal in shape.

The first term on the right side of equation (2b) is the GEF which represents the portion of the pressure detector signal due to the presence of polymer, ΔP_p. The last two terms on the right side of equation (2b) are the LBF portion of the chromato-

gram and represent the portion of the detector signal due to the presence of solvent, ΔP_0.

The total pressure detector signal at any point on the chromatogram, ΔP_i, is equal to:

$$\Delta P_i = \Delta P_{pi} + \Delta P_{oi} = GEF_i + LBF_i \tag{3}$$

The subscript 'i' used in equation (3) refers to the values of the GEF and LBF functions taken a point i on the chromatogram having a counter value X equal to an elution volume of V_{ei}.

Equation (3) can be rearranged to give:

$$\frac{\Delta P_i - \Delta P_{oi}}{\Delta P_{oi}} = \frac{\Delta P_{pi}}{\Delta P_{oi}} = \frac{GEF_i}{LBF_i} \tag{4}$$

The Hagan-Poiseuille equation can be used to show that the ratio of pressures is equal to the ratio of fluid viscosities.

$$\frac{\mu_i - \mu_{oi}}{\mu_{oi}} = \frac{\Delta P_i - \Delta P_{oi}}{\Delta P_{oi}} = \frac{GEF_i}{LBF_i} \tag{5}$$

where μ_i is the solution viscosity and μ_{oi} is the solvent viscosity at elution volume V_{ei} on the chromatogram. The left term of equation (5) is called the specific viscosity. Equation (5) can be used to determine the specific viscosity of the eluent at each point on an SEC chromatogram. Curves showing fluid specific viscosity versus elution volume will be called specific viscosity chromatograms. Figures 4 and 5 show the specific viscosity

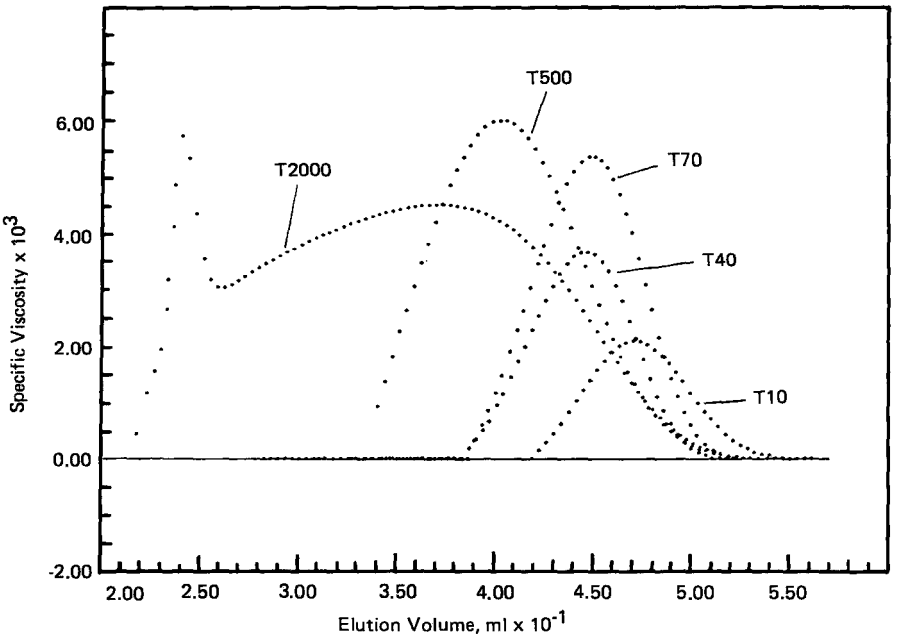


FIGURE 4. Normalized Specific Viscosity Chromatograms for Dextran Polymers

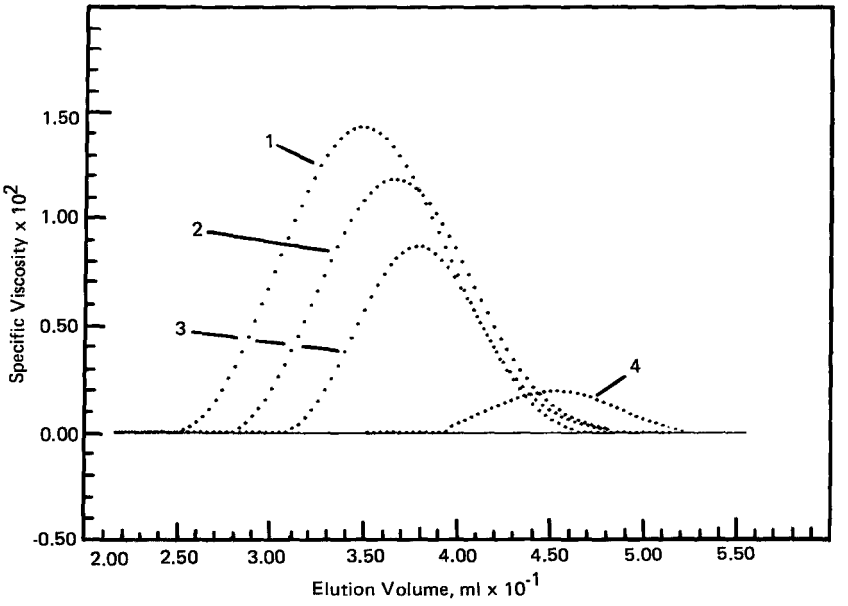


FIGURE 5. Normalized Specific Viscosity Chromatograms for Sodium Polystyrene Sulfonate Polymers

chromatograms of the dextran and sodium polystyrene sulfonate (NaPSS) polymers, respectively, which were obtained by using equation (5).

The specific viscosity chromatogram of a polymer sample can be related to the intrinsic viscosity of the polymer sample, $[\eta]$. The following rationale can be used to explain this relationship.

The intrinsic viscosity is defined as the limiting ratio of specific viscosity to polymer concentration, c, when the polymer concentration approaches zero [9].

$$[\eta] = \lim_{c \to 0} \frac{\mu - \mu_o}{c\mu_o} = \lim_{c \to 0} \frac{\Delta P - \Delta P_0}{c\Delta P_0} \tag{6}$$

For very dilute polymer concentration, such as those existing in SEC, equation (6) can be closely approximated by:

$$[\eta] \simeq \frac{\Delta P - \Delta P_0}{c\Delta P_0} \tag{7}$$

The specific viscosity for random coil polymer molecules in dilute solution can also be expressed by the Einstein relationship [10]:

$$\frac{\Delta P - \Delta P_0}{\Delta P_0} = k\Phi \tag{8}$$

In equation (8), Φ is the volume fraction of polymer existing in the solution, and k is the Einstein constant. For random

coil polymer molecules in solution which are spherically shaped, k is approximately equal to 2.5. Combination of equations (7) and (8) gives:

$$[\eta] = \frac{k\Phi}{c} \tag{9}$$

The volume fraction of polymer in all the eluent that flows through the columns, Φ, is equal to the volume due to polymer, V_p, divided by the total elution volume, V_e. The total elution volume is the fluid volume that passed through the SEC columns. This is the volume collected from point 'a' to point 'b' on the typical chromatogram shown in Figure 3.

$$\Phi = \frac{V_p}{V_e} \tag{10}$$

But, these volumes are equal to the sum of the incremental volumes taken at each point on the chromatogram starting at point 'a' and ending at point 'b'. If Φ_i and ΔV_{ei} are the average volume fraction polymer and the change in elution volume, respectively, at point i on the chromatogram, then:

$$V_p = \Sigma \Phi_i \Delta V_{ei} \tag{11a}$$

$$V_e = \Sigma \Delta V_{ei} \tag{11b}$$

Therefore equation (10) can be expressed as:

$$\Phi = \frac{\Sigma \Phi_i \Delta V_{ei}}{\Sigma \Delta V_{ei}} \tag{12}$$

Combining equations (12) and (9) gives:

$$[\eta] = \frac{k \Sigma \Phi_i \Delta V_{ei}}{c \Sigma \Delta V_{ei}} \tag{13}$$

If no polymer is lost in the packing by adsorption or entrapment, then the polymer concentration, c, is the total mass of polymer injected, m, divided by the total elution volume. Thus:

$$c = \frac{m}{\Sigma \Delta V_{ei}} \tag{14}$$

Combination of equation (13) and (14) eliminates $c\Sigma\Delta V_{ei}$ and gives:

$$[\eta] = \frac{k \Sigma \Phi_i \Delta V_{ei}}{m} \tag{15}$$

By using equation (8), the volume fraction of polymer at point i can be expressed in terms of solution and solvent pressures at point i.

$$\Phi_i = \frac{\Delta P_i - \Delta P_{oi}}{k \Delta P_{oi}} \tag{16}$$

Elimination of Φ_i and k is obtained by combining equations (15) and (16). The sample intrinsic viscosity then becomes:

$$[\eta] = \frac{\Sigma \left[(\Delta P_i - \Delta P_{oi}) \, \Delta V_{ei} / \Delta P_{oi} \right]}{m} \tag{17}$$

The summation term in equation (17) is simply the area within the specific viscosity chromatogram envelope. In addition, the specific viscosity term, $(\Delta P_i - \Delta P_{oi})/\Delta P_{oi}$, in equation (17) can be expressed in terms of the smoothing function by using equation (4):

$$[\eta] = \frac{\Sigma \left(\frac{GEF_i}{LBF_i} \right) \Delta V_{ei}}{m} \tag{18}$$

PRESSURE DETECTOR CALIBRATION

In order to quantitate data from the CP detector, a calibration must be developed which relates the detector voltage signal to the differential pressure across the capillary. It is our experience that a calibration using a static pressure head across the transducer diaphragm does not correlate with the pressures developed in a flowing system. For this reason a dynamic calibration was performed by recording the CP detector voltage at various fluid flow rates through the capillary. Plots of this data always were straight lines which indicated good instrument linearity; however, line intercepts were not at the zero voltage, zero flow rate origin. Therefore, the intercept value from a dynamic calibration was always subtracted from all pressure voltage signals prior to further analysis. This subtraction

insured a direct proportionality between voltage signal and the eluent pressure drop across the capillary. The stability of this calibration was found not to vary significantly from day to day.

RESULTS AND DISCUSSION

Data from the CP detector and equation (18) were used to determine the intrinsic viscosities of several polymer standards. These calculated intrinsics are listed in Table 2 together with the intrinsic viscosity determined by a Ubbelohde four-bulb shear dilution viscometer. Excellent agreement exists between the two measurements. A plot of intrinsic viscosities is shown in Figure 6.

Examination of equation (17) shows that the area of a specific viscosity chromatogram per unit mass of injected polymer increases as the intrinsic viscosity of the polymer increases. This is confirmed by the dextran and NaPSS chromatograms shown in Figures 4 and 5, respectively. All the chromatograms in these figures were normalized to the same mass of injected polymer, one milligram. These chromatograms were not corrected for axial dispersion; however, corrections have been developed for SEC using similar on-line viscometers [12,13].

In true size exclusion chromatography, larger molecules will elute first followed by decreasingly smaller molecules. Also, larger molecules, due to their larger intrinsic viscosities, will have a larger specific viscosity chromatogram. This is also shown by Figures 4 and 5. This detection characteristic gives an advantage to CP detectors over RI detectors when dealing with high

TABLE 2

Results of Intrinsic Viscosities for Dextrans and Sodium Polystyrene Sulfonates

Sample	Reported Weight Average Molecular Weight g/mole	Intrinsic Viscosity dl/g @ 25°C	
		Measured from Ubbelohde Viscometer[1]	Calculated from CP Detector Data[2]
DEXTRAN			
T-2000	2.00×10^6	0.78	0.91
T-500	4.80×10^5	0.55	0.55
T-70	7.00×10^4	0.34	0.35
T-40	3.90×10^4	0.23	0.23
T-10	9.90×10^3	0.07	0.13
SODIUM POLYSTYRENE SULFONATES			
NaPSS-1	1.06×10^6	1.56	1.47
NaPSS-2	6.90×10^5	1.11	1.12
NaPSS-3	3.54×10^5	0.78	0.69
NaPSS-4	3.10×10^4	0.12	0.16

[1]Values determined by a Ubbelohde 4-bulb shear dilution viscometer using Carreau Rheological Equation (11).

[2]Values determined by the on-line CP detector and equal to the area of the respective specific viscosity chromatogram shown in Figure 4 or 5.

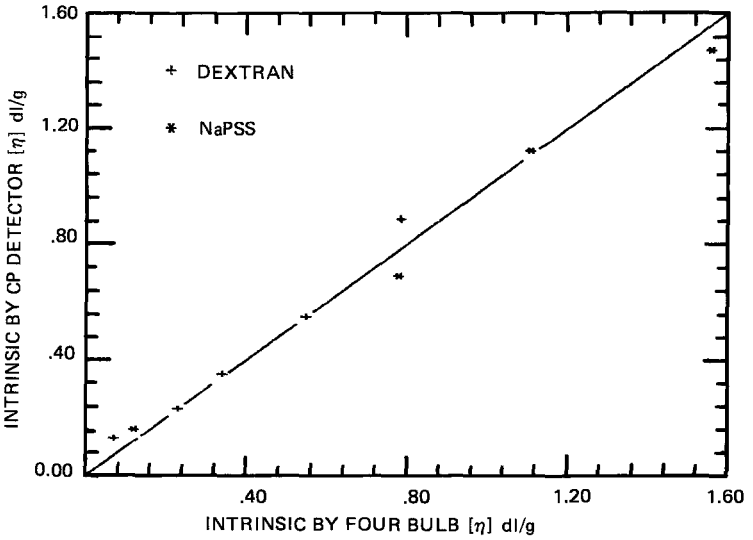


FIGURE 6. Comparison of Intrinsic Viscosities Measured by CP Detector and Ubbelohde Viscometer. See Table 2

molecular weight polymers. Because RI detector signals are proportional to the mass of injected sample and since the concentration of polymer must decrease as molecular weight increases (to insure dilute solution properties), a reduction in RI detector signal must occur as the molecular weight increases. In contrast, because the specific viscosity signal increases with molecular weight, the CP detector sensitivity increases with increasing polymer molecular weight. This accounts for different chromatogram shapes obtained for RI and CP detectors as shown by Figure 7.

No mass detector is required to determine the concentration of polymer in the eluent when using equation (18) to establish total sample intrinsic viscosity. However, similar on-line visco-

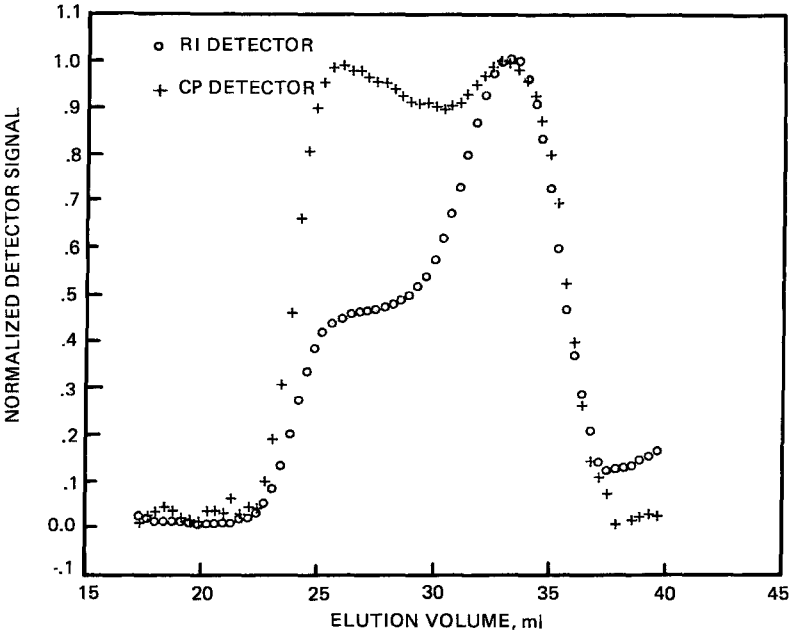


FIGURE 7. Comparison of RI and CP Chromatograms

meters have been previously used with mass detectors to determine both the concentration and the pressure drop at each point along an SEC chromatogram. This detector coupling technique can be used to determine one-point intrinsic viscosities [1-4,12,13]. The mass averaged sum of these intrinsics at each point is equal to the sample intrinsic viscosity [14].

$$[\eta] = \frac{\Sigma m_i [\eta]_i}{\Sigma m_i} \tag{19}$$

In equation (19), $[\eta]$ is the total intrinsic viscosity, m_i is the

mass of polymer at point i, and $[\eta]_i$ is the intrinsic viscosity at point i.

In order to compare our data collection and analysis techniques with equation (19), a Dextran T500 chromatogram was collected using both a CP and RI detector. The total intrinsic viscosity for this polymer was calculated using equation (18) as well as equation (19) to sum the individual intrinsic viscosities along the chromatogram. The total intrinsics determined by using equations (18) and (19) were 0.54 and 0.55 dl/g, respectively.

One advantage in calculating individual intrinsic viscosities along a chromatogram is that Mark-Houwink "k" and "a" values, if known, can be used to determine the molecular weight distribution. A typical distribution determined in this manner is shown in Figure 8 for Dextran T500.

In Figure 8, the relative number of molecules is plotted against log molecular weight and shows the expected molecular weight distribution. Molecular weights for Figure 8 were calculated by using Mark-Houwink "k" and "a" values of 9.78×10^{-5} dl/g and 0.50, respectively [16]. The calculated values for number and weight average molecular weights were 135,000 and 310,000 g/mole. The values reported by the dextran manufacturer are 165,000 and 480,000 g/mole.

A unique feature of many high molecular weight polymers is their shear thinning behavior in a flow field. It is, therefore, important to approach zero shear conditions if the intrinsic vis-

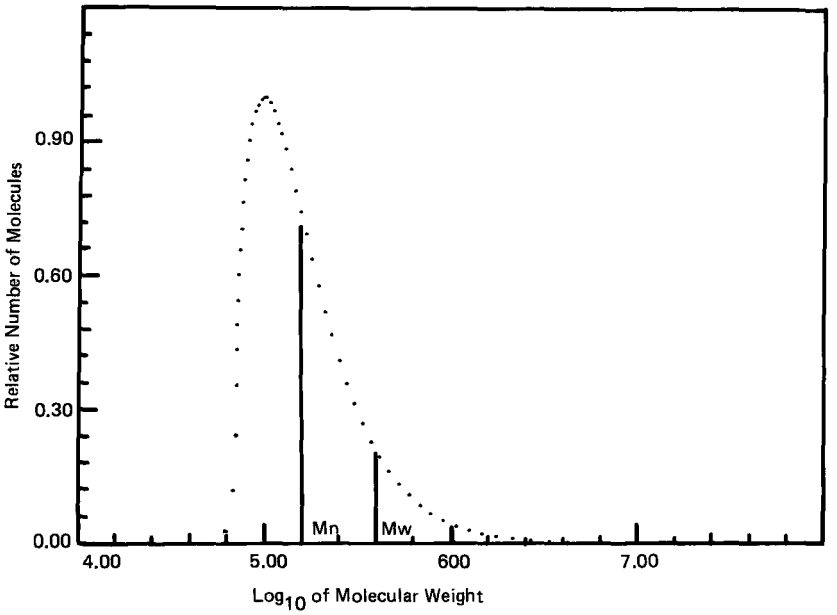


FIGURE 8. Molecular Weight Distribution of Dextran T-500 as Determined by CP Detector

cosity is to be determined under Newtonian conditions. The shear rate through the CP detector capillary was approximately 6500 sec^{-1}. This shear rate is quite high and one might normally expect shear thinning. However, no effect of shear rate on the intrinsic viscosity could be detected. This absence of shear thinning is probably due to the very low concentrations of polymer in the eluent. Shear thinning may be experienced in SEC analysis of extremely large macromolecules. Future work will deal with polymer samples having larger intrinsic viscosities. This work may show that shear thinning does influence the viscosities measurements made by the CP detector.

CONCLUSIONS

We have described the application of a viscosity detector for aqueous size exclusion chromatography. This detector senses the volume fraction of polymer present in the eluent rather than its mass concentration. Thus, it can be more sensitive to higher molecular weight species than a mass sensitive refractive index detector. Therefore, for the characterization of water-soluble polymers, where large molecules with large hydrodynamic volumes are common, a viscosity detector can be extremely useful. A data analysis technique has been developed in which sample intrinsic viscosities can be calculated using only an eluent viscosity detector.

NOMENCLATURE

a	point on the chromatogram at which polymer first starts to elute, see Figure 3
b	point on the chromatogram at which all polymer finishes eluting, see Figure 3
c	polymer concentration
C_i	constants used in the smoothing function; defines point 'a' on the chromatogram shown by Figure 3
GEF_i	value of the generalized exponential function at point 'i' on the chromatogram, see equation (2)
k	Einstein constant
ℓ	capillary length, see equation (1)
L	parameter used in the data smoothing function, see equation (2c)
LBF_i	value of the base line function at point 'i' on the chromatogram, see equation (2)
m	total mass of polymer sample injected, see equation (14)

$\overline{M}_n$ number average molecular weight

$\overline{M}_w$ weight average molecular weight

P_i fitted parameters used in the data smoothing function, see equation (2)

V_e total elution volume, defined as the total eluent fluid volume that contains polymer; elution volume from point 'a' to point 'b' of Figure 3

V_p total volume of polymer found within V_e, see equation (10)

X elution counter

Y detector signal

ΔP_i total fluid pressure drop across the detector at point 'i' on the chromatogram, see equation (3)

ΔP_{oi} fluid pressure drop across the detector at point 'i' which is due only to the presence of solvent, see equation (3)

ΔP_{pi} fluid pressure drop across the detector at point 'i' which is due only to the presence of polymer in the fluid, see equation (3)

ΔV_{ei} change in elution volume at point 'i' on the chromatogram, see equation (11)

$[\eta]$ polymer sample intrinsic viscosity

$[\eta]_i$ intrinsic viscosity at point 'i' on the chromatogram

μ_i total fluid viscosity at point 'i' on the chromatogram

μ_{oi} fluid viscosity at point 'i' which is due only to the presence of solvent

μ_{pi} fluid viscosity at point 'i' which is due only to the presence of polymer

Φ volume fraction of polymer in the total elution volume defined by equation (10)

Φ_i volume fraction of polymer in the elution volume at point 'i' on the chromatogram

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COMPARISON OF REVERSED STATIONARY PHASES FOR THE CHROMATOGRAPHIC SEPARATION OF INORGANIC ANALYTES USING HYDROPHOBIC ION MOBILE PHASE ADDITIVES

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ABSTRACT

Alkyl-modified silica (RSi) and polystyrenedivinylbenzene (PRP-1) stationary phases are compared for the chromatographic separation of inorganic analyte anions and cations using hydrophobic ions of opposite charge as mobile phase additives. Tetraalkylammonium salts were used for anion separations and alkyl sulfonate salts for cation separations. Two major equilibria influence the retention of analyte ions on PRP-1. These are: retention of the hydrophobic ion on PRP-1 and an ion exchange selectivity between the hydrophobic counterion and the analyte ion. When using RSi retention is also influenced by ion exchange at residual silanol groups, which act as weak cation exchange sites. Mobile and stationary phase variables that influence analyte retention are identified. Optimization of these provides favorable eluting conditions for the separation of inorganic ionic analytes. Of particular interest is the potential use of PRP-1 and RSi columns for the separation of inorganic cations; conditions for the separation of alkali metals and alkaline earths are discussed.

INTRODUCTION

A useful reversed phase liquid chromatographic (RPLC) technique for the separation of charged organic analytes, often called ion pair chromatography (IPC), is to add a hydrophobic ion of opposite charge to the predominately aqueous mobile phase and

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take advantage of an enhanced analyte retention. Although many different hydrophobic ions can be used, most applications employ alkylsulfonate (RSO_3^-) salts or tetraalkylammonium (R_4N^+) salts for the separation of organic analyte cations and anions, respectively. Recent studies have demonstrated that this approach can also be used for the separation of inorganic cations (1-4) and anions (1,5-9) and should compliment the inorganic ion exchange LC procedures known as ion chromatography (10).

Several views concerning the importance of the interactions between the organic analyte ion, hydrophobic ion, and the stationary and mobile phase have emerged as IPC has been developed. A recent review that focuses on R_4N^+ salts as mobile phase additives documents these different views (11). Clearly, one model does not fit all possible experimental situations. For predominately aqueous mobile phases and low concentrations of hydrophobic ions of modest hydrophobicity one major view suggests that ion pairs form between the hydrophobic ion and analyte ion prior to retention onto the stationary phase. The other suggests that the hydrophobic ion is first retained by the stationary phase and ion pairing or ion exchange takes place between the analyte ion and the charged site provided by the retained hydrophobic ion. These models, their variations, and evidence supporting them are discussed in detail elsewhere (11,12-20).

Recent studies of inorganic analyte anion and cation retention using R_4N^+ (7,19) and RSO_3^- (20) salts, respectively, and a polystyrenedivinylbenzene (PSDB) copolymeric nonpolar adsorbent as the stationary phase suggest that the enhanced analyte retention is the result of the contribution of two key equilibria. One describes retention of the hydrophobic ion onto the stationary phase surface while the second describes an ion exchange selectivity between the analyte ion and the counterion

accompanying the hydrophobic ion. This mode of interaction is similar to the ion interaction (dynamic ion exchange) model suggested to account for analyte ion retention onto an alkyl-modified silica (RSi) stationary phase from an aqueous-organic modifier mobile phase containing a hydrophobic ion additive (11,14,17). Applying this model to organic analyte ion retention must be done with caution since the hydrophobic nature of organic ions can vary widely. Conductance studies (see 7, 15, and references within) have suggested that association between hydrophobic and organic analyte ions can be appreciable and depends on the hydrophobicity of both ions. For example, association constants for the more polar catecholammonium octylsulfonate salts are reported to be about $18M^{-1}$ while for the less polar octylammonium octylsulfonate salt the constant is $500M^{-1}$ (15). Thus, at some point contribution of ion association (ion pairing) in the mobile phase must also be considered as the hydrophobicity of either or both the organic analyte ion and hydrophobic ion mobile phase additive increases. In contrast conductance data (7,21) strongly suggest that association between certain inorganic ions and hydrophobic ions of opposite charge is negligible particularly if the R groups in the hydrophobic salts are of modest hydrophobicity, hydrophobic salt concentrations are well below critical micelle formation, and mobile phase solvent mixtures are predominately aqueous.

There are several intrinsic differences between RSi and PSDB stationary phases even though both are reversed phases. This report focuses on a comparative study of these two stationary phases using R_4N^+ and RSO_3^- salts as mobile phase additives and inorganic anions and cations, respectively, as analytes. These analytes were used for two major reaons. First, equilibria involving inorganic analytes should be less complex than with organic analytes because: 1) association equilibria between inorganic analyte ions and hydrophobic ions should be

minimal; 2) for many inorganic analytes dissociation via pH is unnecessary; and 3) these analytes should not be retained by the stationary phases in the absence of the hydrophobic ions. Second, from a practical viewpoint, this single column procedure has many potential applications in the separation of inorganic ions and should be a viable alternative to ion chromatography (10).

MATERIALS AND METHODS

Materials

Analytical reagent grade inorganic salts, acids, and bases, tetrapentylammonium ($TPeA^+Br^-$) bromide and sodium octane sulfonate ($C_8SO_3^-Na^+$) were obtained from Aldrich, Eastman Kodak, or Sigma Chemical Co. TPeAF was prepared as previously described (19). $C_8SO_3^-Li^+$ was prepared by passing the Na salt through a strong acid cation exchanger in a H^+ form and subsequently titrating $C_8SO_3^-H^+$ with a standard LiOH solution. MeOH and CH_3CN were obtained as LC quality from MCB Manufacturing Co. LC quality water was prepared with a Sybron/Bronstead water purification unit.

Prepacked columns were obtained from Hamilton Co. (PRP-1) and DuPont (Zorbax C_1, C_8, C_{18}). The PRP-1 column is a PSDB, 10 μm, spherical particle while the Zorbax columns are 6 μm, spherical, alkyl-modified silica particles where the alkyl groups are methyl, octyl, or octadecyl, respectively. The columns are 150 mm x 4.6 mm i.d. except PRP-1 which is 4.2 mm i.d.

Instrumentation

A Waters 202 and Altex 421 LC were used with a Beckman 160 selectable wavelength, a Spectra Physics 770 variable wavelength, or a Wescan 213 or 213A conductivity detector.

Procedures

Procedures for column conditioning, changing of counterion form, and determination of breakthrough volumes are described elsewhere (7,19,20). HCl, NaOH, and phosphate salts, except where noted, were used to adjust mobile phase pH. Ionic strength was fixed when desired by the addition of known amounts of inorganic electrolyte. All solvent mixtures are per cent by volume and column temperature (25 ± 1°C) was ambient.

Analyte solutions, prepared by dissolving 1 to 5 mg per 5 mL of H_2O, were stored in closed containers and refrigerated when not in use. Sample aliquots were 1 to 5 µl. Column inlet pressures ranged from 500 to 3000 psi depending on the column, mobile phase, and flow rate (usually 1 or 2 mL/min). Detection was at 254 nm or by conductance. Capacity factors were calculated in the usual way where the column void volume was determined by using several samples that were known to have no retention at the mobile conditions being tested.

RESULTS AND DISCUSSION

Mobile and Stationary Phase Variables. Two equilibria which appear to be the major factors influencing the enhanced retention of inorganic analyte anions or cations on PRP-1 from a mobile phase containing a hydrophobic ion (7,20) are given by eq. 1 and 2

$$\left.\begin{aligned} A + R_4N^+ + C^- &\underset{}{\overset{K_1}{\rightleftharpoons}} A\cdots R_4N^+C^- \quad (1a)\\ A\cdots R_4N^+C^- + X^- &\underset{}{\overset{K_2}{\rightleftharpoons}} A\cdots R_4N^+X^- + C^- \quad (1b)\end{aligned}\right\} (1)$$

$$\left.\begin{aligned} A + RSO_3^- + C^+ &\underset{}{\overset{K_1}{\rightleftharpoons}} A\cdots RSO_3^-C^+ \quad (2a)\\ A\cdots RSO_3^-C^+ + X^+ &\underset{}{\overset{K_2}{\rightleftharpoons}} A\cdots RSO_3^-X^+ + C^+ \quad (2b)\end{aligned}\right\} (2)$$

where A is the stationary phase, R_4N^+ and RSO_3^- are the mobile phase additives, X is the analyte ion, and C is the counterion. Equations 1a and 2a describe the retention of the hydrophobic ion on PRP-1 while eqs. 1b and 2b describe an ion exchange selectivity between the analyte ion and any counterions that are part of the mobile phase due to the presence of hydrophobic, ionic strength, and buffer salts. Both experimental evidence and control of experimental conditions are consistent with this view and are discussed elsewhere (7,19,20).

The relationship between retention of the analyte, the mobile phase variables, and the equilibrium constants defining the equilibria (7,19,20) is given by

$$1/k'_X = \frac{1}{q\,K_o}\left[[X]_m + \frac{1}{K_1[L]_m} + \frac{[C]_m}{K_2}\right] \qquad (3)$$

where k'_X is the capacity factor for the retention of the analyte ion, q is the ratio of stationary phase volume to mobile phase volume, K_o is the sorption capacity for PRP-1, and m is the mobile phase. When a R_4N^+ salt is in the mobile phase X, L, and C, are the analyte anion, hydrophobic cation, and counteranion concentration, respectively, and K_1 and K_2 are equilibrium constants for retention of the R_4N^+ salt and for the ion exchange selectivity between the analyte anion and a given counteranion; for RSO_3^- salts X, L, and C are analyte cation, hydrophobic anion, and countercation concentrations, respectively, and K_1 and K_2 are equilibrium constants for the retention of the RSO_3^- salt and an ion exchange selectivity between the analyte cation and a given countercation, respectively. The significance of eq. 3 is that it focuses on the key equilibria and the controllable mobile phase variables that influence analyte retention. For PRP-1 and defined mobile phase conditions eq. 3 was consistent with retention data when using inorganic analyte anions and

cations and R_4N^+ (7) and RSO_3^- (20) salts as mobile phase additives, respectively.

Preliminary experiments indicated that eqs. 1-2 also apply to RSi. When analytical samples of R_4N^+ and RSO_3^- salts were used their retention on C_1, C_8, and C_{18} at controlled mobile phase conditions: 1) increased as R group hydrophobicity increased; 2) increased as the mobile phase organic modifier: water ratio decreased; 3) was greater in MeOH:H_2O over CH_3CN:H_2O at identical solvent ratios; 4) increased as ionic strength increased; and 5) was dependent on the type of counter-ion accompanying the R_4N^+ or RSO_3^- analyte salt where retention of the $R_4N^+C^-$ and $RSO_3^-C^+$ analytes for the counterion C followed the order

$$I^- > NO_3^- > Br^- > NO_2^- > Cl^- > F^- \qquad (4)$$

and

$$M^{+2} > Cs^+ > Rb^+ > K^+ > Na^+ > Li^+ \qquad (5)$$

These trends are similar to those found when using PRP-1 (7,19, 20).

A well-defined linear relationship was found on PRP-1 (7,19,20) when plotting retention, $1/k'$, of analytical samples of R_4N^+ and RSO_3^- salts versus $1/\sqrt{\mu}$, where μ is the mobile phase ionic strength. This indicates analyte retention occurs in a double layer (22,23), where the hydrophobic ion occupies the primary layer at the PRP-1 surface and the counterion occupies a diffuse secondary layer. A similar result was found when using the C_1, C_8, and C_{18} and several different RSO_3^- salts as analytes. This is illustrated in Fig. 1 for $C_8SO_3^-Na^+$ as the analyte indicating RSO_3^- salt retention occurs as a double layer. The mobile phase solvent for C_1 and PRP-1 was 1:9 CH_3CN:H_2O while for

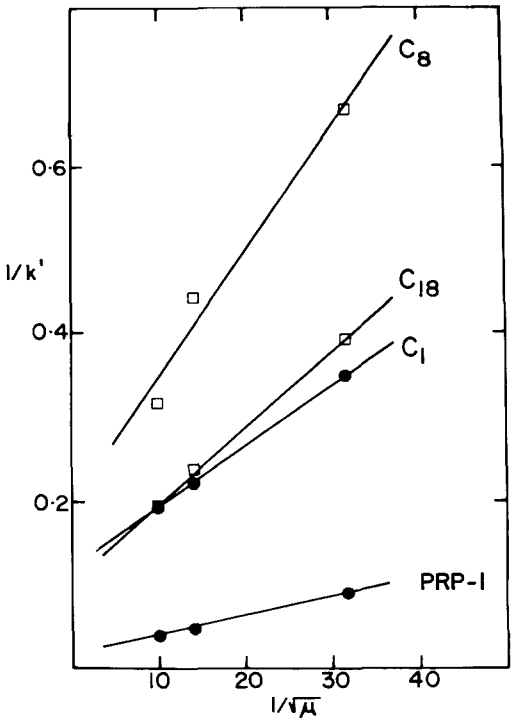


Figure 1 Relationship between analyte retention on RSi and PRP-1 and mobile phase ionic strength.

C_8 and C_{18} it was 1:1 MeOH:H_2O; the ionic strength covered the range 1.0 x 10^{-3} to 1.0 x 10^{-2}M NaCl.

Attempts to determine a similar relationship between 1/k' and $1/\sqrt{\mu}$ for retention of analytical samples of R_4N^+ salts on C_1, C_8, and C_{18} were not successful. This was not the case when PRP-1 was used (20). It appeared that the R_4N^+ salts (R > C_2) were never eluted from the column even when 100% CH_3CN, a very strong eluent if retention is hydrophobic in nature, was used. The reason for this, which became clear in subsequent experiments, is due to a second type of interaction, namely, ion ex-

change between R_4N^+ and H^+ at the free -SiOH sites within the RSi stationary phase.

An inherent difficulty in comparing PRP-1 and RSi is their intrinsic difference in their ability to sorb hydrophobic ions, eqs. 1a and 2a. For a given mobile phase composition the amount of hydrophobic ion retained (moles/column) will vary for the four columns. Thus, the number of electrostatic interactions between the retained hydrophobic ion and the analyte ion of opposite charge (see exchange equilibria in eqs. 1b and 2b) will differ between the stationary phases. If an electrostatic interaction, as the data appear to indicate, is a major retention process, then a viable comparison can be made only when the number of such interactions are normalized. This requirement was satisfied by maintaining the hydrophobic ion concentration in the mobile phase at a constant concentration and varying the mobile phase solvent composition (organic solvent:H_2O ratio) so that the amount of retained hydrophobic ion on the stationary phase was approximately the same.

Figure 2 illustrates the retention isotherms for the retention of $TPeA^+F^-$ and $C_8SO_3^-Na^+$ on PRP-1, C_1, C_8, and C_{18} as a function of hydrophobic ion concentration in the mobile phase. The amount retained was calculated from breakthrough volumes obtained by passing a mobile phase of defined concentration through the column and monitoring the column effluent for appearance of the hydrophobic ion. Since appearance time and the mobile phase concentrations are known the amount retained can be calculated. Manipulating the CH_3CN:H_2O ratio (R_4N^+ salts) or the MeOH:H_2O ratio (RSO_3^- salts) controls the amount of retained hydrophobic ion. Data for one set of solvent compositions where the isotherms are nearly equal are shown in Fig. 2. As hydrophobic ion concentration increases the amount retained (number of apparent ion exchange sites, or the

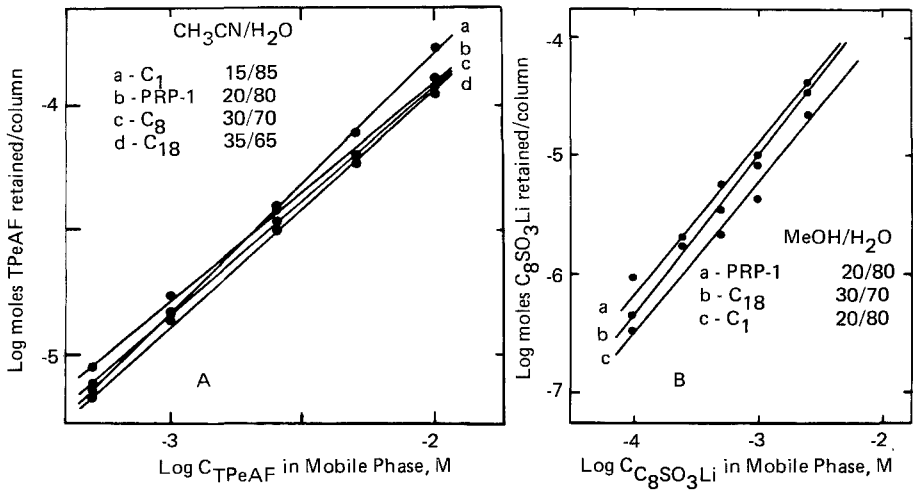


Figure 2 Sorption isotherms on RSi and PRP-1 at a solvent composition that provides the same number of retained hydrophobic ion sites.

apparent ion exchange capacity) increases. For TPeAF and the concentration range studied retention from a mobile phase containing an organic modifier follows the order $C_{18} > C_8 > PRP\text{-}1 > C_1$ while for C_8SO_3Li retention the order is $C_{18} > PRP\text{-}1 > C_8 > C_1$. Thus, organic modifier was adjusted accordingly so that the amount of hydrophobic salt retained at a given mobile phase hydrophobic salt concentration would be the same for the four columns. Increasing mobile phase R_4N^+ or RSO_3^- salt concentration or decreasing organic modifier increases the number of ion exchange sites.

According to eq. 3 analyte retention is indirectly proportional to analyte and counterion concentration and directly to hydrophobic ion concentration. These trends were followed for the retention of inorganic anion and cation analytes when using PRP-1 and R_4N^+ (7) or RSO_3^- (20) salts, respectively.

Similarly, an indirect relationship between analyte retention and analyte or co-ion concentration was found when using RSi stationary phases (24). The dependence on analyte concentration occurs only at higher concentrations. At low analyte concentration the analyte term in eq. 3 becomes negligible in comparison to the other terms, and at these conditions retention is independent of analyte concentration. Subsequent column experiments and separations were carried out, in general, at these latter conditions. When different electrolytes were used to establish that analyte retention at a fixed hydrophobic ion concentration on RSi is indirectly related to the counterion concentration, analyte retention varied with the type of counterion used. This is consistent with an ion exchange like selectivity as shown in eqs. 1b and 2b. Thus, for different ionic strength salts inorganic analyte anion or cation retention changes, just like with PRP-1 (7,20), according to the selectivity order listed in eqs. 4 and 5, respectively. That is, analyte anion and cation retention is the highest for F^- and Li^+ salt solutions, respectively, at constant hydrophobic ion concentration. However, when inorganic analyte retention was determined as a function of hydrophobic ion concentration (see Fig. 3), several differences between the PRP-1 and RSi were apparent. Since the number of ion exchange sites due to retained hydrophobic ion is approximately the same for the four columns, this factor is not responsible for the differences.

In a R_4N^+ salt mobile phase retention of inorganic analyte anions on PRP-1 increases with $TPeA^+$ salt concentration and passes through a well-defined maximum; only NO_2^- retention is shown in Fig. 3a. The maximum is less defined on RSi and appears to require a higher $TPeA^+$ salt concentration. The major difference is a reduced retention on RSi compared to PRP-1. Similar results were found when using other monovalent inorganic analytes. The maximum is consistent with an ion exchange like

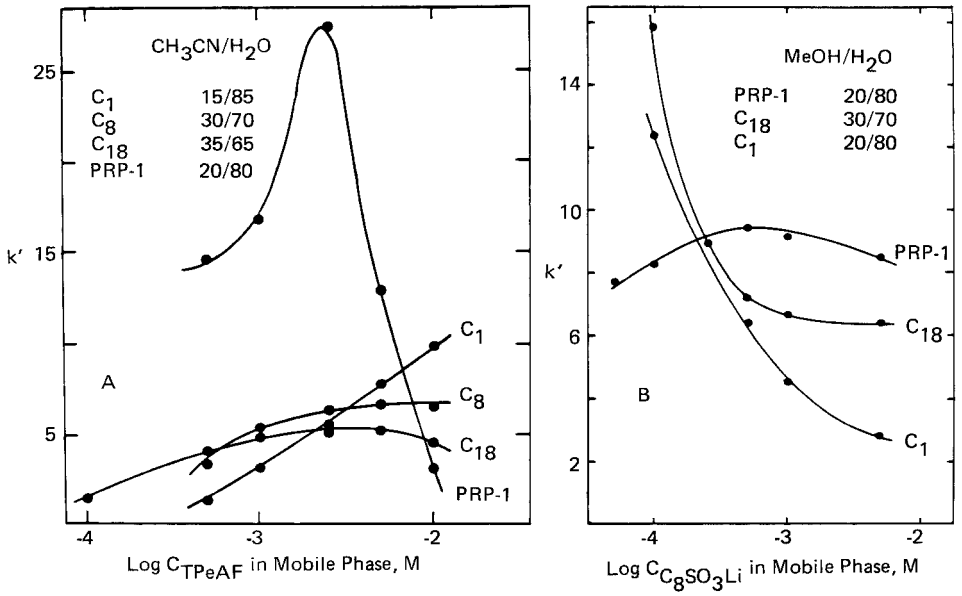


Figure 3 Retention of analyte ion on RSi and PRP-1 as a function of hydrophobic ion concentration. Analyte is NO_2^- (A) and K^+ (B); mobile phase composition is adjusted to provide the same number of retained hydrophobic ion sites in (A) and (B).

selectivity, as shown in eq. 1b. As the R_4N^+ salt concentration increases, its retention, and subsequently the number of exchange sites, becomes larger causing increased analyte retention. Since the counteranion concentration also increases, it competes with the analyte anion for the charge site and causes analyte retention to decrease (analyte retention is inversely related to counteranion concentration as shown in eq. 3) due to mass action and the ion exchange selectivity constant for the exchange between the analyte anion and the counteranion; this competition is discussed in detail elsewhere (7,19,20). For a RSO_3^- salt

and K^+ (or other inorganic cations) as the analyte (see Fig. 3B) retention on RSi differs significantly from that on PRP-1. On PRP-1 a well-defined maximum is observed and is consistent with the ion exchange like selectivity shown in eq. 2b and the influence of mass action and selectivity due to the counter-cation. In contrast, retention increases on RSi as the $RSO_3^-C^+$ concentration approaches zero and no maximum was found even when dilute $RSO_3^-C^+$ solutions were examined. The data further suggest that inorganic cations are retained by RSi in the absence of RSO_3^- and that retention differs between these cations. This was verified in subsequent experiments (see Fig. 7).

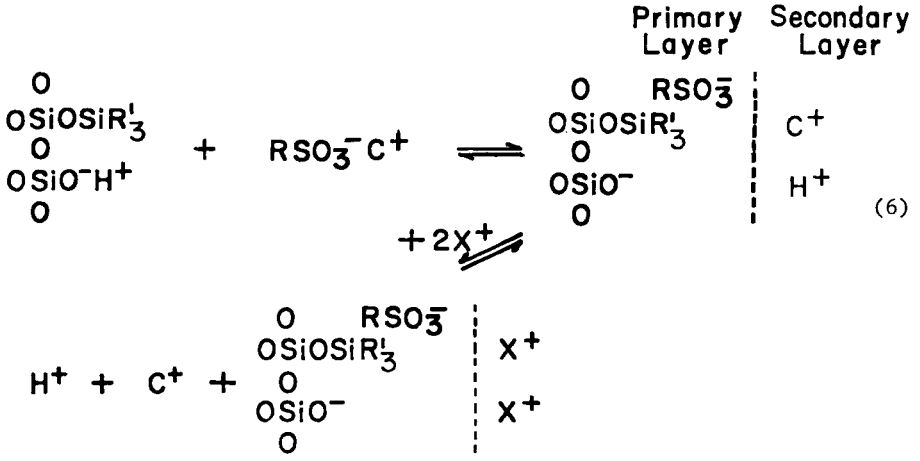
These trends on RSi are consistent with the presence of two types of cation exchange sites. One is provided by the retained hydrophobic ion while the other, we conclude, is provided by the residual -SiOH groups on the RSi stationary phase. When using a R_4N^+ salt as an analyte, its retention on the RSi, as indicated previously, is very high because of cation exchange at the -SiOH group. Similarly when R_4N^+ is used as a mobile phase additive it is partially consumed by the -SiOH cation exchange site. Thus, the available hydrophobic ion exchange sites indicated by the isotherms in Fig. 2A are less than that shown and analyte anion retention on RSi compared to PRP-1 (see Fig. 3A) is less. When RSO_3^- salts are used, the retention of K^+ and other inorganic cations on RSi is high even at low RSO_3^- salt concentration because of the availability of the -SiOH exchange sites. As the RSO_3^- salt concentration increases, the RSi coverage increases probably making the -SiOH exchange sites less accessible and causes the exchange between the $RSO_3^-C^+$ sites and the analyte cation, see eq. 2, to become the more important interaction.

The presence of -SiOH exchange sites in RSi and their chromatographic effects have been noted by many workers (25-28). Several have suggested using short chain R_4N^+ cations to mask the

-SiOH groups via cation exchange (25-27). It is also likely that RSi stationary phases obtained from different manufacturers will differ in amounts of residual -SiOH exchange sites; only Zorbax RSi stationary phases were used in this study.

A typical silica is estimated to have about 7 μmol/m^2 of reactive, free silanol groups (29). Depending on the derivatization procedure, approximately half of these are available for conversion to the -SiOR group. Thus, a maximum mono-layer phase coverage of organic material of about 3.5 μmol/m^2 is obtained; it is not unusual for commercial R-Si stationary phases to be below this value. Because of steric properties the number and accessibility of the remaining -SiOH groups should differ between C_1, C_8, and C_{18}. Any other variable which influences accessibility will therefore also influence exchange capacity. Our experiments, based on breakthrough measurements indicated a cation exchange capacity of about 4 to 17 μmole/column for the three RSi columns; capacities of 10 to 15 μmole/column have been reported for a C_8 column (30). Although the data suggested a difference between the three RSi columns an accurate determination of the exchange capacity at these low levels is difficult because -SiOH is a weak acid and it shows a high exchange selectivity for H^+. Even the higher capacities reported for silica (0.2 to 1.0 mmole/g) are difficult to determine accurately (29).

The two electrostatic interactions that appear to be responsible for the retention of inorganic cation analytes, X^+, on RSi from mobile phases containing RSO_3^- salts are schematically shown in eq. 6. The corresponding equilibria contributing to this retention are: 1) retention of the $RSO_3^-C^+$ on RSi (eq. 2b); 2) an ion exchange selectivity between the countercation accompanying the RSO_3^- salt and the inorganic analyte cation; 3) dissociation of the weak acid -SiOH group; its pK_a is estimated to be 4 to 7 (25,29); and



(6)

4) ion exchange selectivities between the countercations, C^+ and/or H^+ associated with the silanol sites, and the analyte cation.

If it is assumed that analyte cation retention at the accessible -SiOH exchange sites is electrostatic and is only by cation exchange, retention at this site, considering the cation exchange selectivities and the ionization of the -SiOH site, can be shown (24,30) to be given by

$$1/k'_{X^+} = \frac{1}{q\,K_o}\left[\frac{[H^+]_m(1 + K_a)}{K_3} + [X^+]_m + \frac{[C^+]_m}{K_4}\right] \qquad (7)$$

where k'_{X^+} is the capacity factor for the retention of X^+ by cation exchange at the -SiOH site, q is the ratio of stationary phase volume to mobile phase volume, K_o is the available exchange capacity, m is the mobile phase, K_a is the ionization constant for the -SiOH site, and K_3 and K_4 are cation exchange selectivities according to eqs. 8 and 9, respectively. Thus, inorganic cation retention at the -SiOH sites is indirectly

$$-SiO^-H^+ + X^+ \underset{}{\overset{K_3}{\rightleftharpoons}} -SiO^-X^+ + H^+ \qquad (8)$$

$$-SiO^-C^+ + X^+ \underset{}{\overset{K_4}{\rightleftharpoons}} -SiO^-X^+ + C^+ \qquad (9)$$

proportional to H^+, analyte cation (at low concentration retention becomes independent of analyte concentration), and countercation concentration, to silanol K_a, and directly to the cation exchange selectivities between the analyte cation and mobile phase cations. Combing eqs. 3 and 7 to account for cation exchange at both the retained RSO_3^- site and the -SiOH site (24) yields

$$1/k'_{X^+} = \frac{1}{q\,K'_o}\left[\frac{1}{K_1[RSO_3^-]_m} + \frac{(1+K_a)[H^+]_m}{K_3} + [X^+]_m + K_2K_4[C^+]_m\right] \qquad (10)$$

where k'_{X^+} is now the capacity factor for retention at the two sites, K'_o is total retention capacity due to the two exchange sites, K_1 is an equilibrium constant for the retention of the RSO_3^- salt, K_2 is the cation exchange selectivity at the RSO_3^- site for exchange between the analyte cation, X^+, and a mobile phase countercation, C^+, K_3 and K_4 are cation exchange selectivities for cation exchange at the -SiOH site according to eqs. 8 and 9, respectively, and K_a is the ionization constant for the -SiOH group. If the analyte cation concentration is low enough then its retention is independent of concentration and $[X^+]$ is insignificant in eq. 10. A practical consequence of eq. 10 is that it identifies the key mobile phase parameters and equilibria and how they can be manipulated in order to optimize separation of inorganic analyte cations on RSi. For example, increasing RSO_3^- salt concentration and decreasing H^+ and countercation con-

centration should increase analyte retention. However, the range over which these can be adjusted is not unlimited because of additive and/or competitive effects. Thus, using buffer salts to control pH and -SiOH ionization will also contribute to C^+ concentration. Similarly, increasing the $RSO_3^-C^+$ concentration will increase its countercation concentration. Adjustment of the type of countercation will also influence analyte cation retention because of the cation exchange selectivities and eluting power can be altered according to eq. 5. In general, this selectivity order is similar to that observed when using conventional strong acid cation exchangers (32).

Separations. Figure 4 shows the separation of a mixture of four inorganic anions on PRP-1, C_1, C_8, and C_{18} using a TPeAF mobile phase. The exchange capacity due to the retained $TPeA^+$ salt is the same for the four columns (15 $\pm$ 1 μmole/column); this was accomplished by adjusting the solvent mixture according to the isotherms in Fig. 2. The retention order is the same on the four columns and is also identical to the order found for typical strong base anion exchangers (32). Resolution at these conditions is better on PRP-1 because of a higher retention and a more favorable selectivity even though efficiency, which favors the order $C_{18} > C_8 > C_1$, is more favorable with the RSi columns (part of this is due to smaller RSi particles and lower retention times). Increasing the ionic strength or using a counteranion of greater eluting power (see eq. 4) reduces analyte retention. The reasons for using TPeAF and the concentrations listed are provided elsewhere (7,19). In general, it would appear that the high efficiency offered by C_{18} would be preferable, however, if a basic (pH > 8) mobile phase condition is required, as would be the case for anions derived from certain weak acids, only PRP-1 would be compatible with this condition.

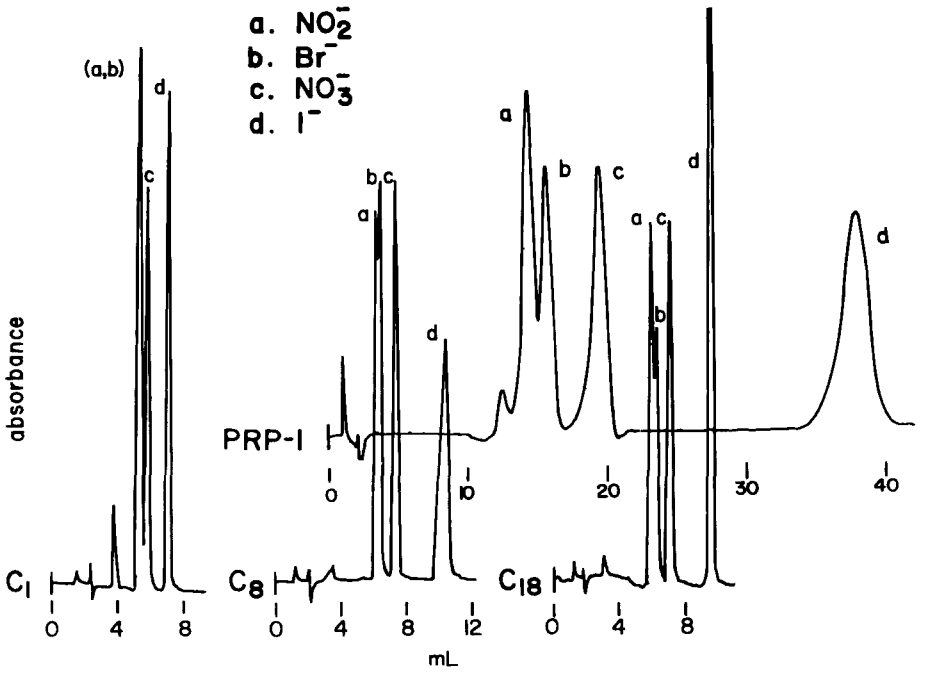


Figure 4 Separation of inorganic anions on PRP-1 and RSi columns using a $TPeA^+F^-$ mobile phase additive. PRP-1: A 22:78 $CH_3CN:H_2O$, 1.0 x 10^{-3}M $TPeA^+F^-$ mobile phase; C_1: Same except 15:85 $CH_3CN:H_2O$; C_8: Same except 30:70 $CH_3CN:H_2O$; C_{18}: Same except 35:65 CH_3CN; at 1.0 mL/min flow rate.

Figures 5 to 7 focuses on the parameters that influence inorganic cation retention. Chromatograms for the separation of alkali metals are shown in Fig. 5. In Fig. 5, the MeOH:H_2O ratio is adjusted to fix the exchange capacity of the retained $C_8SO_3^-$ salt at 30 μmole/column (see isotherms in Fig. 2B). When compared with PRP-1 with a similar number of sites (20), retention on the C_8 is higher apparently due to the contribution of the -SiOH exchange sites.

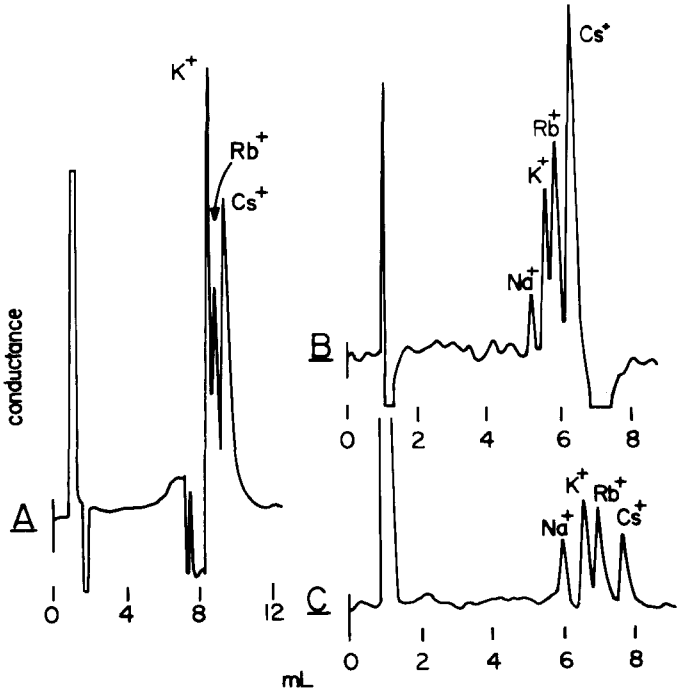


Figure 5 Separation of alkali metal cations on a Zorbax C_8 column as a function of mobile phase variables.

(A) A 27.5:72.5 MeOH:H_2O, 2.5 x 10^{-3}M $C_8SO_3^-Li^+$ mobile phase; (B) A 100% H_2O, 1.0 x 10^{-3}M $C_8SO_3^-Li^+$, 1.0 x 10^{-2}M LiCl, 1.0 x 10^{-3}M HCl (pH=2.9) mobile phase; (C) Same as B except 1.0 x 10^{-5}M HCl (pH=5.2) mobile phase; at a 1.0 mL/min flow rate.

The mobile phase solvent composition has opposing effects on retention. When the MeOH increases at low MeOH:H_2O ratios retention drops because retention of the RSO_3^- salt decreases. However, at higher MeOH ratios the MeOH influences the cation exchange selectivity. The former produces the more significant change. For example, k' for the retention of Na^+ on C_{18} is 6.10

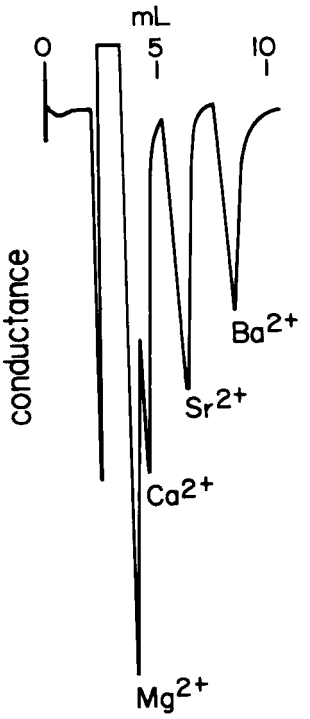


Figure 6 Separation of alkaline earth cations on a Zorbax C_8 column using a $C_8SO_3^-Li^+$ mobile phase additive. A 3:7 MeOH:H_2O, 5.0 x 10^{-4}M $C_8SO_3^-Li^+$, 5.0 x 10^{-4}M Na citrate (pH=7.0), 1.0 x 10^{-3}M LiCl mobile phase at a 1.0 mL/min flow rate.

from a 1:10 CH_3OH:H_2O, 5.0 x 10^{-3}M LiCl, 5.0 x 10^{-4}M $C_8SO_3^-Li^+$ mobile phase. If the $C_8SO_3^-Li^+$ is omitted, the k' is 0.34 while at 9:1 MeOH:H_2O, 2.5 x 10^{-3}M LiCl the k' is 1.29. These and similar data for other cation analytes suggest that the majority of the exchange sites at MeOH:H_2O ratios, where the RSO_3^- salt is retained (its k' > 3), are due to the RSO_3^- salt and not the -SiOH sites.

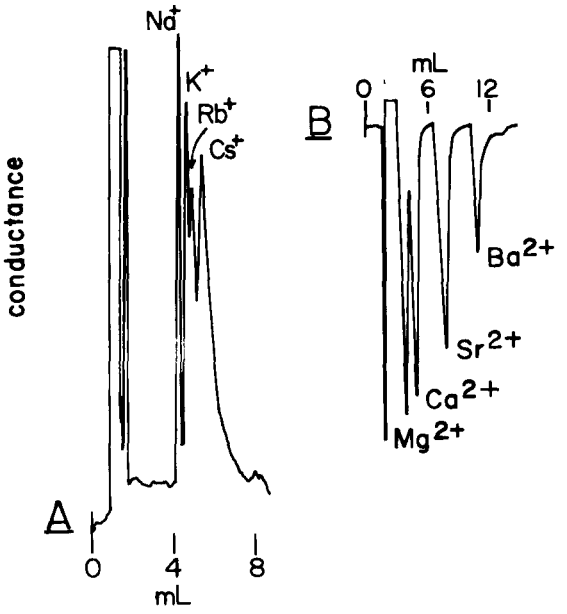


Figure 7 Separation of alkali metal (A) and alkaline earth (B) cations on a Zorbax C_8 column in the absence of a RSO_3^- salt additive.

(A) A 3:7 MeOH:H_2O, 2.5 x 10^{-3}M LiCl mobile phase and (B) A 3:7 MeOH:H_2O, 5 x 10^{-4}M Na citrate (pH=7.0) mobile phase; at 1.0 mL/min flow rate.

Increasing the pH increases inorganic analyte retention due to ionization of the -SiOH sites, however, this effect is restricted by the upper pH limit (pH = 8) of RSi. In Fig. 5B retention and resolution is less favorable than at the higher pH used in Fig. 5C where retention is almost 15% greater. Increasing the pH also decreases the cation exchange selectivity due to H^+ but adds the selectivity effects of other cations if buffer salts are used. The location of the Cs^+ peak in Figure 5B is not well-defined because of a system peak caused by the H^+ and

detected by the conductivity detector. At a higher pH the system peak has much less effect on the detector response.

Cation retention follows the order shown in eq. 5 ($NH_4^+ \simeq Rb^+$) and is similar to that found on strong acid cation exchangers (32). This is also the order for eluent strength for countercations that accompany the RSO_3^- salt or are introduced for ionic strength control or as buffer salts. Thus, Li^+ salts were usually used (Figures 5 to 7) since it provides the weakest eluting power. Switching to other countercations according to eq. 5 or increasing ionic strength will decrease retention.

Figure 6 illustrates the separation of alkaline earths on a C_8 column. Since divalent inorganic cations are more retained than monovalent ones the mobile phase eluting strength was increased to reduce analysis time. The complex mobile phase used and the effects of each component are predictable. 1) The $C_8SO_3^-$ salt provides many of the exchange sites. If its concentration is increased more sites are produced and retention is increased. However, eventually this is compensated for by increased eluting power due to higher countercation concentration. (Increased eluting power can be achieved by using a cation of greater selectivity.) 3) Since the divalent analytes are highly retained adding a ligand (citrate) sharply decreases retention due to analyte-ligand complex formation; increasing ligand concentration therefore decreases analyte retention. 4) The 3:7 MeOH:H_2O ratio influences retention of the RSO_3^- salt but has a larger effect on the formation constant for the analyte-ligand complex; increasing the MeOH decreases RSO_3^- salt retention and increases the formation constant both of which contribute to reduced retention. 5) The mobile phase at pH = 7 provides a large number of dissociated -SiOH sites, thus, increasing analyte cation retention. 6) Adding LiCl improves the eluting power because of increased countercation concentration.

Figure 7 shows that even after omitting the RSO_3^- salt from the mobile phase enough cation exchange capacity due to the residual -SiOH sites is available in the RSi column to effectively separate mixtures of alkali metal and alkaline earth cations. Since the total number of cation exchange sites is reduced due to the absence of retained RSO_3^- salt, mobile phase eluting power is decreased and its pH is adjusted to favor -SiOH ionization. The alkyl group on RSi is not necessary for the separation of inorganic cations in the absence of the RSO_3^- salt and its elimination should increase cation exchange capacity, retention times, and perhaps efficiency. Data illustrating the retention and separation of alkali and alkaline metal ions using ordinary silica columns are reported elsewhere (31).

ACKNOWLEDGEMENTS

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(10), 1961-1968 (1984)

ON CONSTRUCTING A QUANTITATIVE THEORY OF OPEN CAPILLARY CHROMATOGRAPHY

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Summary.

Electric double layers which occur at the conductive surface-electrolytic solution interface may be used as virtual stationary phases for separating macromolecules. The quantitative estimations of the efficiency of separation processes based on the features of double layers are essentially dependent not only on the values of pH, the characteristic size of a column or granules and the flow rate, but also on the externally applied potential and the current in the circuit. The specific models advanced herein are illustrated by analytical formulae which determine the effect of an increase of the field and hence the very process of separating macromolecules as that of proteins, depending on the characteristic size thereof, the space between the electrodes and the current in the circuit. The theoretical description needs no phenomenological coefficient as in theory advanced by K.Li and makes it possible to determine the conditions for open capillary chromatography more specifically, according to any advanced aim.

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The effects of an electric double layer appreciable in understanding the mechanism of separation processes by liquid chromatography have recently become the subject matter of a number of publications. The present analytical technique of separating proteins, ferments and other charged macromolecules incorporates a variety of procedures (such as electrophoresis, affine chromatography, isoelectric focusing etc.). At the same time, the use of properties of an electric double layer occurring at the interface of phases exhibites some advantages clearly disclosed in [1]. In addition, a practical use has been recently gained by methods which may be referred to open capillary chromatography, differing only in the treatment of substantial quantities of liquids. Thus, as shown in [2-4], the mechanisms of sorptive purification of blood with the use of activated carbon, which have gained a wide acceptance in clinical medicine, and the mechanisms of electrofiltration for water cleaning may also be explained by similar effects of a double layer increase in external electric fields. As a result of the practical use of such separation processes it is extremely necessary to obtain a quantitative estimation of the virtual stationary phase associated with double layers [1] under special conditions. However, such estimations are lacking in literature, since even the infrequent theoretical investigations in this direction lead to formulae with indefinitely and problematically defined parameters [1, 3] which naturally involves some difficulties in using these formulae. We guess that any of the theories should be brought up to such analytical expressions which might be easily calculated with the aim of testing the same, and the main thing here is to determine the conditions suitable for performing open capillary chromatography and detect

specific macromolecules. In some instances, such a program may be realized.

The theory of open capillary chromatography disclosed in [1] is based on the effect of a double layer as a virtual stationary phase for separating charged macromolecules. The author of the above-mentioned article has managed to determine the confinement Re /the designations hereinafter referred to are given by the author [1] / as a function of a certain dimensionless parameter λ for the most real situation in the form of

$$Re = 4/\lambda \tag{1}$$

$$\lambda = (ze\alpha V\kappa R)/(6\pi D\eta\rho) \tag{2}$$

where D is the diffusion coefficient of macromolecules to be separated, z is the number of charges per each of them, e is the elementary charge of an electron, R and ρ are the radii of a metal capillary and a macromolecule whose form is taken, for simplicity, as spherical, respectively, η is viscosity of a dispersion medium, κ is the Debye screening radius. All the values are generally known. However, it is not a simple matter to determine the parameter of α which to a great extent controls the original separation effect according to the relationship proposed in [1]:

$$\Phi = \alpha V, \tag{3}$$

where Φ is the potential of the outer Helmholtz plane for simplicity designated as ζ-potential, V is the external voltage. The parameter α is not quite definite in [1] and this presents some difficulties in using the theory of open capillary chromatography for practical calculations.

Now let us try to estimate an increase of the field of an elecric double layer more strictly. This is particularly necessary, since equation (3) is not suitable for estimating the size of area of the virtual stationary phase which increases when an external field is applied. The gain factor may be found through a simultaneous solution of the Nernst-Planck and Poisson equations. Here should be noted that the condition for an electric neutrality, which can substancially simplify such a set of equations, is unusable therewith. With the use of singular perturbation methods for a set of ordinary differential equations which disclose the passage of an electric current through a binary electrolytic solution[5], it has been found that the effective thickness of the diffuse layer S_d depends on the value of the current j passing through the system according to the law

$$S_d \simeq \frac{1}{\kappa R \sqrt{\frac{2+j}{2}}} , \qquad (4)$$

the dimensionless current j being related to the dimensional density of the current J by the following relationship

$$j = \frac{2Jh}{ND} , \qquad (5)$$

where h is the space between electrodes, $\mathcal{D}$ and N are diffusion coefficient and ion concentration, respectively. Reference [5] also deals with a model wherein the electrode surface is positively charged. Therefore, $j < 0$ and formula (4) has no sense when $j \simeq -2$ (limiting current). The last condition may be written as the following inequality defining the field of application:

$$\kappa h (2 + j) \gg 2 \qquad (6)$$

Formula (4) practically defines a thickening of the diffuse electric double layer by a factor of $\sqrt{\frac{2}{2+j}}$ as a result of a decrease in the electrolyte concentration in the vicinity of the electrode.

The effect of an increase of the virtual field may take place not only inside a metal capillary to capture macromolecules but also at an ion-exchange conductive membrane (ionite). These features are characteristic for activated carbons used, for example, as hemosorbents [2]. In this case the counter ions of ionite can move not from the membrane to the electrolyte but in the opposite direction. The charged macromolecules suspended in an electrolytic solution (with a fixed direction of the field), whose sign is coincident with that of counter ions of the membrane, will move in the same manner. In other words, here takes place the movement of particles onto the membrane surface. Therefore, we can make a conclusion that on the membrane surface close to which the effect of a field increase may be observed, there takes place a transportation of colloid particles whose sign of charge coincides with that of membrane counter ions and thus is opposite to the sign of the membrane charge. From this it follows that with an increase of the field, the first monolayer of particles on the membrane surface is formed as a result of different charges of particles and the membrane. An increase of the field is also necessary for forming polylayers of particles observed, for example, by means of chromatography.

To provide a maximum effect of the field increase and a formation of the virtual stationary phase it is necessary to use the densities of current which comply with the conditions:

$$\frac{2}{\kappa\rho}\cdot\frac{\rho}{h} < 2+j < \frac{3\rho}{h} \qquad (7)$$

The field increase in the area of the n-monolayer of paticles may be expressed as

$$m_{n+1} = \frac{1}{2n+1}\cdot\frac{h}{\rho} \qquad (8)$$

Formulae (7) and (8) are substantially based on the model proposed in [5]. For other conditions for open capillary chromatography, the formular for an increase of the field may have peculiar analytical representations.

When $h = 10^{-2}$cm, according to formula (8), $\rho = 10^{-5}$cm and $m_2 \approx 300$. Any decrease in h intensifies the transportation of particles into the area of the field maximum, but as may be seen from (8) this is followed by a decrease in the effect of the field increase.

Thus, the thickness of an effective virtual stationary phase and the coefficient of a field increase for open capillary chromatography have been found. The described approach to the problem needs no special treatment of α included into the formula of [1]. The calculation of this parameter may be performed within the limits of the proposed model but it has not been given here.

The sedimentation (separation) of charged macromolecules may take place with an increase of the field and an elongation of electric double layers not only in a conductive (metal) capillary as has been shown by K.Li [1] but also in a packed bed of ionite or activated carbon granules. In this case the effect is dependent on the space between the two adjacent granules. An illustrative practical example of realizing such a situation is the method of hemocarboperfusion [2]. As has been recently revealed by experiments, the rate of soption (sedimentation) on the sorbents

widely used in clinical practice depends to a great extent on the external potential of a certain polarity and magnitude which is applied to the system[6]. It is not difficult to note that under such conditions, which are generally speaking analogous to a chromatographical column, there also take place conditions similar to those disclosed in [1] . Under assumption of the same scheme in discussing and constructing quantitative formulae (likely to be permissable at this stage of investigations) the efficiency of the method is also dependent on the relationsip of type (1)-(8). However, the most significant for such models is the relationship between the conductivities of particles (granules) $æ'$ which should be much higher than those of substances $æ$ to be settled. The gain factor of the field may be calculated to a first approximation in considering a nonconductive medium with particles of an infinitely high conductivity (with an error of the order of $æ/æ'$) [2].
A more detailed similar investigation is given in [2].

Thus, a refinement of the conditions for an effective use of double layers as a virtual stationary phase for charged macromolecules, which is disclosed in this paper, makes it possible to relate the Li theoretical model [1] with the conditions of an experiment or a practical use thereof.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF GLOBIN CHAINS ON A LARGE-PORE C_4 COLUMN

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ABSTRACT

Excellent resolution of human and baboon globin chains may be obtained by HPLC on a Vydac large-pore C_4 column. The procedure is rapid and uses a gradient between aqueous trifluoroacetic acid and trifluoroacetic acid in acetonitrile. The common human γ chains are easily separable from each other as are some α- and β-chain variants from the normal chains and from each other.

INTRODUCTION

Because only a small sample of hemoglobin is necessary to separate globin chains rapidly by HPLC, this methodology affords an effective and sensitive way of examining hemoglobin samples for abnormalities. Three HPLC procedures which have been described for the separation of globin chains use a Waters μBondapak C_{18} column but differ in the eluting solvents and gradients. Congote and Kendall (1) develop with a gradient between TFA-water-acetonitrile and water-acetonitrile. Huisman et al. (2) employ initially an isocratic development with a phosphate-methanol-acetonitrile mixture and complete the chromatogram with a gradient between two solutions with different ratios of the compounds. Shelton et al. (3) use a gradient between different mixtures of perchlorate solution, methanol, acetonitrile, phosphoric acid, and nonylamine. Although the procedures differ markedly in the time

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required for completion of the chromatogram, they all succeed in separating globin chains with very small differences in sequence. Thus, the $^{G}\gamma$ and $^{A}\gamma$ chains which differ only by a methylene group are separable. The above references are those of the most recent description of methodology by the three groups and cite prior publications. Shelton et al. (4) have also reported experiments with packings other than that in a Waters μBondapak C_{18} column.

Recently, large pore HPLC columns with shorter hydrocarbon chains have become available commercially. The use of such a column and its advantages in separating globin chains are reported here. An application of these methods to the separation of baboon globin chains has been described (5).

MATERIALS AND METHODS

Equipment consisted of an Altex system previously described (4) or one with two Waters 6000 A solvent delivery systems, a Waters U6K universal injector, an Altex/Hitachi Model 155-10 UV-Vis Variable Wavelength Detector, an Axxiom Model 711 HPLC System Controller (Cole Scientific, Calabasas, CA), and a Watanabe Model SR6252 Single Pen Chart Recorder.

The chromatographic column which is manufactured by The Separations Group, Hesperia, CA 92345 was a Vydac large-pore (330 Å) C_4 column (Cat. #214TP54) (4.6 x 250 mm).

The developers for the chromatograms were linear gradients between mixtures of 0.1% aqueous TFA and 0.1% TFA in acetonitrile. Mixture A had 80% of aqueous TFA and Mixture B had 40%. When the Waters system was employed, the developers were degassed with helium. Mixtures may be prepared and used over a period of several days to a week.

Hemoglobin solutions were prepared as previously described (4) or were samples that had been isolated by chromatography. If it was necessary to concentrate a solution after isolation, this was done with Millipore Immersible CX Filtration Units (Millipore Corp., Bedford, MA), and the concentrated solution was dialyzed salt free.

The sample size was 0.1 to 0.2 mg. All chromatograms were made at ambient temperature. Specific conditions for development are given in the text or figure legends. Purging is not necessary after each run; after return in 2 min to the desired percentage of B for the start of the next gradient, reequilibration is done with 25 ml. At the end of 5 or 6 runs, purging is done with a 2-min gradient to 100% B and 15 min of isocratic development with 100% B. The column was stored in 100% acetonitrile.

RESULTS

Figure 1 depicts the separation of the globin chains of a normal adult and Fig. 2 that of chains from a newborn infant. On this packing, the peaks are sharp and almost symmetrical. The α peak always shows greater asymmetry than β or γ peaks and sometimes has an inflection on the trailing edge.

Not only are the major chains well separated but, in each instance, a number of minor peaks are evident. These minor peaks are common to all samples of the same type and presumably represent the chains in the minor components that may be observed on all chromatograms of hemolysates on ion exchange columns; their identity is under investigation. Although evidence of such minor peaks may be seen in chromatograms by other procedures (1,2,3), they are more obvious here because of the significantly sharper peaks.

The separation of the three γ peaks is excellent. This sample may be atypical in that, contrary to previous experience (3,6), the percentage of $^{A}\gamma^{T}$ chain is greater than that of the $^{A}\gamma^{I}$ chain. The relationship remains the same even if the minor peaks after the $^{G}\gamma$ and $^{A}\gamma$ peaks are included in the calculations, although the percentages change slightly. Presumably such a minor peak after the $^{A}\gamma^{T}$ chain is under the leading edge of the $^{G}\gamma$ peak.

It is not necessary to purge the column after each chromatogram as is done in other methods (1,2,3). The small

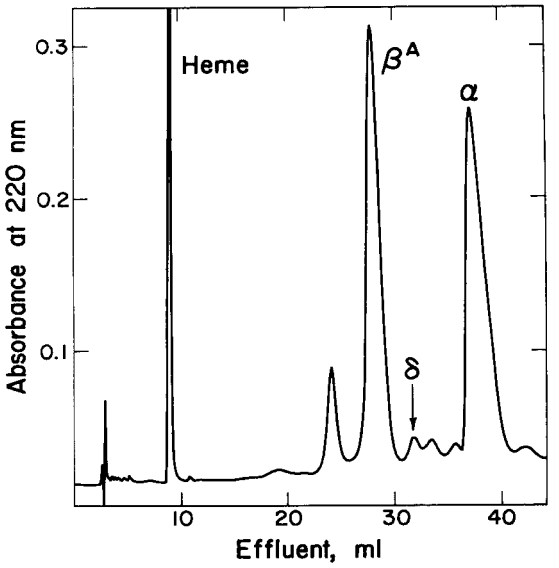

FIGURE 1 Separation of the globin chains of a normal adult. The sample was 0.12 mg, the gradient from 44 to 56.5% of Mixture B in 60 min, and the flow rate 1 ml/min.

amount of material that is labeled "purge" in Fig. 2 came from five chromatograms and amounted only to 2-3% of the total that was injected. In some experiments in which the material in a peak was isolated for amino acid analysis, approximately quantitative data indicated a recovery of at least 85%.

The procedure also is able to separate other globin chains as depicted in Fig. 3. The δ chain is well separated from the β^A chain (Fig. 3a) and in a position far from β^C, $\beta^{O\text{-Arab}}$, β^E, and probably other β chains which as hemoglobins may not be separable from Hb A_2 on ion exchangers. Thus, Hb A_2 can now be determined chromatographically in the presence of Hb E, and not only by radioimmuneassay (7). Of the examples shown, only β^A and β^S chains do not separate well (nor, or course, δ and β^S), but the difference is adequate to distinguish them. Numerous modifications of procedure did not improve the β^A-β^S separation.

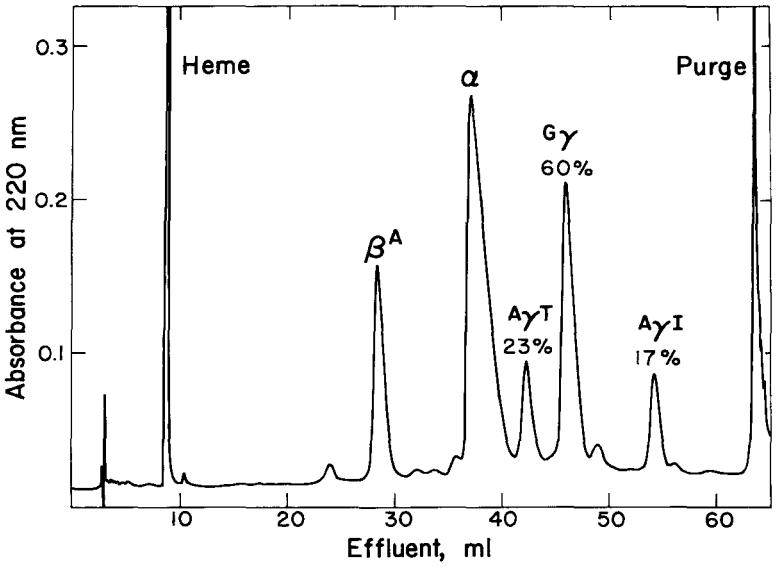

FIGURE 2 Separation of the globin chains in the cord blood of a newborn child. Sample was 0.10 mg. Same conditions as Figure 1.

When globin chains are isolated in this way, the amino acid analyses are in excellent agreement with the expected values. With an amino acid analyzer of appropriate sensitivity, the 50-100 μg of individual chain from a single chromatogram is more than adequate for an analysis.

DISCUSSION

The Vydac large-pore C_4 column which has been used in these experiments not unexpectedly has properties significantly different from those of the small-pore Waters μBondapak C_{18} column. For example, the complex developer with perchlorate-phosphate-methanol-acetonitrile-nonylamine (3) which is effective on the μBondapak column moves the $^{G}\gamma$ chain ahead of the α chain on the Vydac column but does not adequately separate the two. The

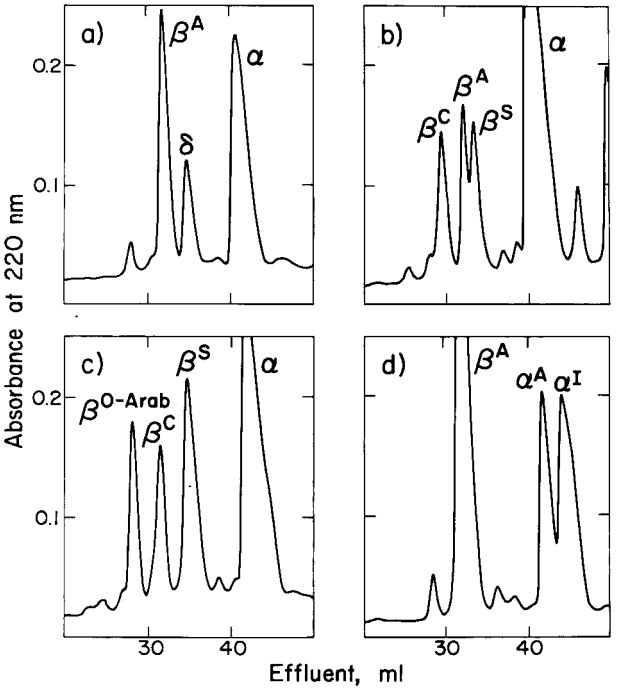

FIGURE 3 Separation of certain other globin chains. a) A 2:1 mixture of chromatographically isolated Hb A and Hb A_2; b) Mixture of hemolysates of cord blood (Figure 2) and of an SC patient; c) Mixture of hemolysates of blood from SC and SO-Arab patients; d) Hemolysate of an AI individual. Same conditions as Figure 1.

TFA-water-acetonitrile mixtures which are excellent with the Vydac column are similar to those of Congote and Kendall (1). They have the advantage not only of simplicity but also (as frequently pointed out in the literature) of volatility so that subsequent operations such as amino acid analysis, sequencing, etc. may be done after evaporation of the solvent. Because of the excellent separations which were achieved under the stated conditions, no study has been made of variations such as the concentration of TFA, a TFA gradient, flow rate, etc.

A distinct advantage of the Vydac column is the elimination of the need to purge the column after each chromatogram. After one column had been used approximately 250 times and separations had worsened, reversing the flow returned separations and peak sharpness to the original behavior.

When the experimental conditions for the separations in Figs. 1-3 are applied, there is no gradient of TFA and the actual concentration of acetonitrile changes only from 37.6 to 42.6% Because of the slight gradients if 0.1% aqueous TFA and 0.1% TFA in acetonitrile are the limiting solvents, mixtures have been used to produce a steeper and more reproducible gradient.

The separations are sensitive to changes in slope of gradient and/or to the initial percentage at which the gradient is started. This is illustrated in Fig. 4 in which the same sample was chromatographed under different conditions. In Fig. 4b (same as Fig. 2) the gradient from 44 to 56.5% B changed at 0.21% per ml. If the same slope is used but the gradient is from 47 to 59.5% B, the pattern (not shown) is virtually unchanged but translated along the abscissa so that each peak emerges about 10 ml sooner. Apparently, most of the separation occurs in the initial part of the column, and adequate results probably could be obtained with a shorter column. The chromatogram in Fig. 4a resulted from a higher initial percentage of B (47 instead of 44) and a steeper gradient (0.30 instead of 0.21% per ml). As anticipated, peaks emerge more slowly if the gradient is started at 44% but the change is only 0.15% per ml (Fig. 4c). The separations improve with little loss of quality in peak shape. Despite the marked changes in pattern, the quantitative agreement is good despite the varied experimental conditions; the differences reflect mainly the inclusion or exclusion of minor components. These samples illustrate the versatility with which conditions may be tailored to fit the requirements of a particular separation.

The large-pore packing is also excellent for the resolution of baboon globin chains, although a different gradient is

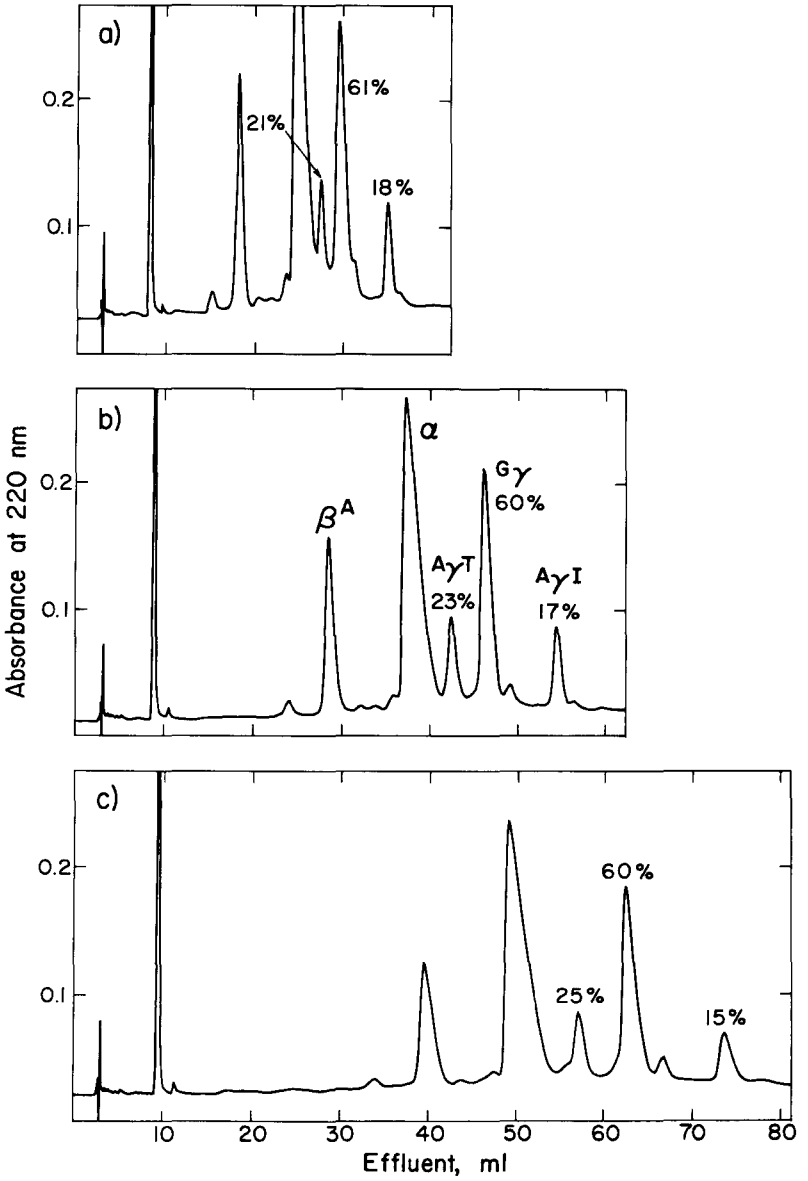

FIGURE 4 The effect of gradient changes on the separation of the components in identical samples. Figure 4b duplicates Figure 2. In these examples, a 60-ml gradient was used at 1 ml/min, and the gradients were as follows: a) 47 to 65% B, b) 44 to 56.5% B, and c) 44 to 53% B.

necessary. Illustrations of its effectiveness have been given (5). The method should be capable of separating hemoglobin chains of other species as well.

ACKNOWLEDGMENTS

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AUTOMATED OPTIMISED HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF PRE-COLUMN O-PHTHALDIALDEHYDE-AMINO ACID DERIVATIVES

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ABSTRACT

The feasibility of injecting o-phthaldialdehyde/2-mercaptoethanol reagent and amino acid mixture simultaneously, from separate injector vials in order to allow automation of HPLC pre-column amino acid derivative analysis was investigated. The modified duel injection system described proved to be successful. Reaction of amino acids and reagent takes place during flushing of the injection loop. This is immediately followed by the introduction of the formed derivatives into the main HPLC eluent stream leading to the chromatographic column. The described procedure gave reproducible results with no loss in sensitivity. Further established was the optimum pH of reagent for derivative formation and of eluents for the separation of derivatives.

INTRODUCTION

Automated amino acid analysis systems in use today are based predominately on the ninhydrin procedure.[1] This procedure requires expensive specialized equipment, analysis times of generally 2 to 3 hours, and high running costs.

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Over the past several years numerous high performance liquid chromatographic (HPLC) amino acid analysis procedures have been described. The latter is rapid and simple and the instrument can be used for other types of analyses if necessary. The amino acid HPLC methods include both pre-column and post-column derivatising. However, various factors, primarily the instability of amino acid derivatives, have made the automation of these methods (particularly pre-column derivatization) extremely difficult. Jones, Paabo et al[2] have recently described a non-automated procedure in which o-phthaldialdehyde was used successfully as a pre-column derivatizing reagent.

A mixture containing 26 of these derivatives was efficiently resolved by these workers with an analysis time of less than 35 minutes. In this report the automation of Jones' method was investigated. The optimum reagent pH and solvent pH whereunder the amino acid derivatives are formed and subsequently separated and detected were also determined with this study. The applicability of the developed automated analysis to beerwort and beer is demonstrated.

MATERIALS AND METHODS

Apparatus

The HPLC system used for this study consisted of:

(a) Micromeritics Model 750 solvent delivery system;

(b) Micromeritics Model 731 column compartment with variable temperature control from ambient to 150°C;

(c) Micromeritics Model 725 auto-injector modified to inject the contents of two sample vials simultaneously through a 5 µl injection loop;

(d) Micromeritics Model 753 ternary solvent mixer;

(e) Micromeritics Model 740 Microcomputer based module with printer.

(f) Kratos FS Fluorimat detector equipped and set up as follows: 20 µl flow cell, a FSA 403 excitatation filter and FSA 111 lamp for excitation in the 330-375 nm range. The emission was measured with a FSA 426 (418 nm cut-off) filter, sensitivity dial setting at 6.6 units and the range 0.05;

(g) Hewlett Packard Model 3390A recorder/integrator;

(h) Altex Ultrasphere ODS column (250 x 4.6 mm); particle size 5µm

and

(i) Brownlee Guard Column with a 5 µm C18 cartridge fitted between the solvent delivery system and sample injector for filtering of the eluent only;

(j) Solvent A was tetrahydrofuran : methanol : 0.05 M sodium acetate (pH 7.5) 4:95:400 and solvent B was methanol : 0.05 M sodium acetate (pH 7.5) 8:2. Solvent C was methanol. Further details of the chromatographic procedure are given in the figure legends.

Reagents and Standards

Amino acid standards, internal standard β-alanine, sodium acetate, boric acid and 2-mercaptoethanol were purchased from Merck Chemicals. O-phthaldialdehyde was obtained from Sigma Chemical Co. HPLC grade methanol used as an eluent was purchased from Waters Associates and Merck Chemicals.

Preparation of o-phthaldialdehyde/2-Mercaptoethanol reagent

200 mg of o-phthaldialdehyde was dissolved in 5 cm^3 absolute methanol. To this was added 200 µl 2-mercaptoethanol and 90 cm^3 0,4 mol dm^{-3} boric acid (pH = 10.3). The mixture was flushed with argon and stored in a refrigerator.

Preparation of Amino Acid and Internal Standard Solutions

A mixture of 19 primary amino acids (excluding cystine) and 4-amino butyric acid was made so as to match approximately the

concentration of amino acids in a standard lager beerwort (e.g. Table I). The solution was transferred to smaller bottles, 3 drops of pentachlorophenol solution (330 mg PCP/50 cm^3 ethanol) added to each bottle as a preservative and stored at -20°C. The internal standard, βeta-alanine solution (50 mg/dm^3) was prepared separately and treated in the same manner as the amino acid standard mixture.

Beerwort and Beer

Beerwort and beer samples were centrifuged at 14 000 rpm, filtered through Kieselguhr to degas and finally Millipore filtered (0,45 μm).

Preparation of samples and reagent for Chromatography

200 μl of internal standard solution was added to 200 μl aliquots of amino acid standard solution, beerwort and beer respectively. Each sample was then made up to 800 μl with 0,6% Brij 35. The 800 μl aliquots of the prepared samples were then placed in every alternate injection vial of the auto-injector. 800 μl aliquots of the o-phthaldialdehyde/2-mercaptoethanol reagent solution were placed in the open positions left between the samples.

RESULTS AND DISCUSSION

A modified version of the conditions of chromatography described by Jones, Paabo et al[2] produced the amino acid standard mixture chromatogram shown in Figure I. The optimum column temperature for chromatography was found to be 38°C. This also reduced column backpressure and would ultimately lead to increased column life. Diluting standard mixture samples with methanol enhanced fluorescence of peaks generally and specifically lysine. However, the use of the surfactant Brij-35 was continued as methanol forms a precipitate when added to beerwort.

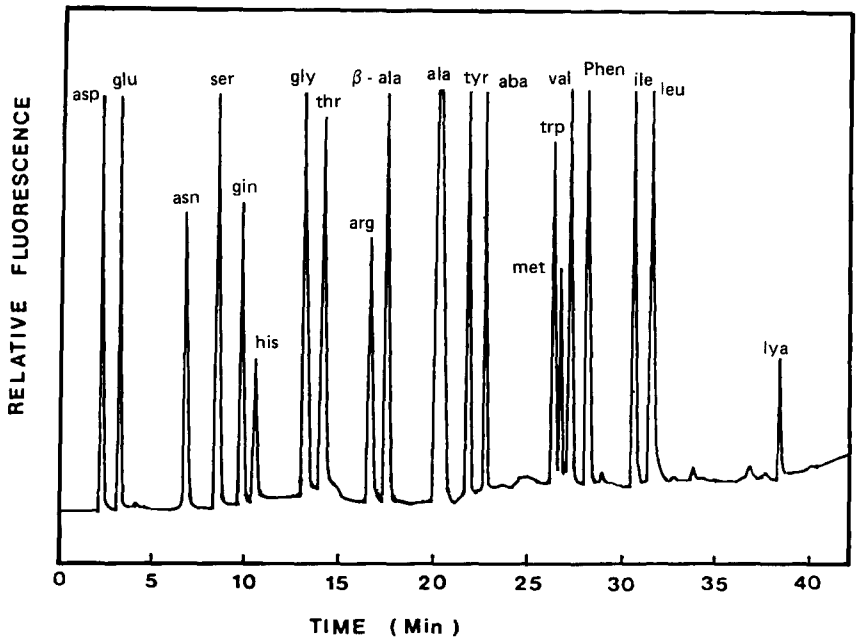

Figure I. Chromatogram of automated analysis of a standard aminoacid mixture. Conditions: Solvent A, methanol :0.05M potassium acetate (pH 7.5):tetrahydrofuran, 400:95:4; Solvent B, methanol : 0.05M potassiumacetate (pH 7.5), 8:2, Solvent C, methanol; program: 100% A isocratic step for 11 min, inject sample, 100% A to 80% A, 20% B linear step in 15 min,80% A, 20% B isocratic step for 3 min, immediate step to 41% A, 59% B, 41% A, 59% B isocratic step for 1 min., immediate step to 40% A, 60% B, 40% A, 60% B isocratic step for 3 min, linear step to 30% A, 70% B in 10 min, linear step to 100% C in 10 min, 100% C isocratic step for 3 min,return to initial conditions; flow rate of 1.7 ml min^{-1}.

Quantification of the amino acids using the method of manual pre-sample injection derivatization proved to be extremely tedious and intricate. Experimentation showed that reaction products are formed immediately after addition of o-phthaldialdehyde/ 2-mercaptoethanol reagent to the amino acid mixture. If the derivatized sample is allowed to stand for more than 2 minutes prior to analysis, a decrease in fluorescence on analysis is observed. A delay of ± 30 minutes on analysis of the derivatized sample would

Figure IIa Figure IIb

Normal Auto-injector and the Modified Version which allows for Simultaneous Injection of Reagent and Sample.

give unreliable results. This demands that the derivatized samples must be prepared immediately prior to analysis.

The feasibility of automating derivative formation by the simultaneous injection of sample and reagent from separate vials was investigated. Results showed that the 5 µl injection loop allowed sufficient mixing and reaction time for complete derivatization. Increasing the reaction period by 90 seconds by delaying the actual injection of the derivatized sample on the column did not significantly affect derivative fluorescence.

As illustrated in Figure IIA the Micromeritics Model 725 auto-injector principle of operation is simply the manual

displacement of the sample from a vial, through an inverted type syringe action. The sample is displaced through the HPLC injection loop. Sample flushing the loop is run off into waste. Once the auto-injector has displaced the full contents of the vial, the injector loop containing a fixed volume of sample, is switched into the main HPLC eluent stream. Analysis of the sample can now take place.

To allow the simultaneous injection of reagent and sample, the auto-injector had to be modified (Figure IIB). The auto-injector syringe holder was replaced with one housing two injection needles. The needle housing which has only one outlet, has an internal Tee-junction which links the two injection needles. Mixing of reagent and sample can thus take place during displacement thereof from the vials to the injector loop.

A further modification was made to the auto-injector to allow the vial tray to advance two places instead of one. Reagent and sample are loaded alternatively into the tray. With the above modifications the instrument can be safely left running unattended.

A detailed description of the design and operation of the modified auto-injector was given by J.C. Hodgin at the 1983 Pittsburgh Conference.[3]

The reactant, o-phthaldialdehyde/2-mercaptoethanol solution, proved to be stable at room temperature. As a result, the auto-injector could be loaded with reagent and sample for overnight analyses. With the internal standard, β-alanine, the automated analysis of 11 each beerwort and beer samples produced the results shown in Table I.

The optimum solvent pH (7.5) was found by obtaining amino acid standard mixture chromatograms at eluent pH ranging from 5.5 to 11.5. The pH of solvents A and B were adjusted individually after mixing of buffer, methanol and THF. Table II reflects the results of this experiment. Using peak areas as the basis of calculations, pH

TABLE I

Results of Automated Analysis of Pre-column o-Phthaldialdehyde/ 2-Mercaptoethanol Amino Acid Derivatives of 11 Beerwort and 11 Beer samples

Amino Acid	Beerwort			Beer		
	$\bar{t}_r$ (Min)	$\bar{c}$ (mg/dm^3)	s (mg/dm^3)	$\bar{t}_r$ (Min)	$\bar{c}$ (mg/dm^3)	s (mg/dm^3)
Asp	2.25	62.4	0.4	2.25	1.8	0.2
Glu	3.18	56.0	0.4	3.21	2.1	0.2
Asn	6.77	111.8	0.6	6.85	Trace	-
Ser	8.53	71.8	0.4	8.67	0.8	0.1
Gln	9.80	81.1	0.2	10.00	1.0	0.1
His	10.50	61.0	0.6	10.72	8.6	0.1
Gly	13.37	42.7	0.4	13.55	9.9	0.2
Thr	14.31	61.4	0.2	14.51	1.1	0.1
Arg	16.79	170.8	1.3	17.03	15.7	0.2
β-Ala[a]	17.76	50.0	-	17.93	50.0	-
Ala	20.42	97.5	1.0	20.90	15.4	0.3
Tyr	21.63	114.2	0.5	21.71	68.9	0.5
Trp	25.10	44.5	0.5	25.13	12.5	0.3
Met	25.36	38.7	0.2	25.41	Trace	-
Val	25.79	92.0	0.6	25.85	4.3	0.3
Phen	26.51	140.9	0.8	26.60	5.8	0.8
Ile	28.46	83.2	0.4	28.60	0.7	0.3
Leu	29.38	139.5	0.8	29.54	5.9	0.1
Lys	36.53	171.2	0.6	36.66	Trace	-

a Internal Standard
s Standard deviation of mean concentrations
$\bar{c}$ Mean concentration of amino acids
$\bar{t}_r$ Mean retention time

O-phthaldialdehyde/2-mercaptoethanol reagent was prepared with the 0,4M boric acid pH ranging from 6 to 12. With the differing pH reagents amino acid standard mixture chromatograms were now obtained. Using reagent pH 10.4 and peak areas as the basis of calculations, Table III was compiled. From the table itis clear that pH10.3 is the optimum as fluorescence intensity of formed derivatives are the highest.

Example chromatograms of beerwort and beer are shown in figures III and IV respectively.

TABLE II

Percentage Fluorescence Difference[a] of Opa-Amino Acid Derivatives with Change in Solvent pH

Amino Acid	Norm 5.9	-al[a]	SOLVENT PH 6.0	6.5	7.0	7.5[c]	8.0	10.0	10.3	11.0	11.5
Asp	-12	-	-2	15	35	52	58	80	85	74	93
Glu	-20	-	1	42	42	55	61	80	86	84	94
Asn	0	-	2	38	52	55	58	67	63	63	61
Ser	-30	-	5	50	87	94	98	102	118	112	114
His	35	-	1	*	75	259	280	438	435	445	435
Gln	-7	-	0	*	31	44	47	54	53	57	58
Gly	-21	-	10	50	80	105	104	111	121	124	124
Thr	1	-	8	56	92	179	182	206	200	214	211
Arg	-1	-	5	32	50	72	72	81	212	*	*
β-Ala[b]	-8	-	6	42	60	82	98	102	29	*	*
Ala	-4	-	4	43	42	136	148	160	116	*	*
Tyr	-2	-	2	20	66	161	186	192	172	*	*
ABA	-8	-	5	14	-26	-22	-9	-12	8	*	*
NH_4^+	-3	-	21	55	29	55	41	*	*	*	*
Trp	-10	-	33	38	116	205	287	*	*	*	*
Met	10	-	8	22	37	68	99	115	130	111	103
Val	3	-	6	13	17	25	31	26	54	53	46
Phen	2	-	13	29	70	115	137	153	201	178	185
Ile	3	-	6	17	26	41	51	57	76	67	71
Leu	10	-	17	23	46	70	90	97	140	114	128
Lys	-22	-	-3	83	57	198	284	478	537	*	471

a Sodium acetate buffer pH 5.9 (2)
b Internal Standard
c pH 7.5 Optimum. pH > 7.5 harmful to reverse phase column.
ABA, 4-aminobutyric acid present in beer wort and beer.
* Merged peaks

TABLE III

Percentage Fluorescence Difference[a] of Opa-Amino Acid Derivatives with Change in Opa-Reagent pH

Amino Acid	OPA REAGENT PH										
	7.0	8.0	9.0	9.5	10.0	10.3	10.4[a]	10.5	11.2	11.5	12.0
Asp	*	-79	-73	-54	-25	8	-	-4	-23	-40	-50
Glu	*	-64	-46	-33	-18	5	-	-3	-15	-33	-54
Asn	*	-79	-73	-54	-25	8	-	4	-23	-40	-50
Ser	*	-53	-36	-20	-8	3	-	-2	-15	-28	-16
His	*	c	c	c	-12	0	-	-8	-40	-61	-79
Gln	*	-16	-12	-4	2	6	-	0	-41	-47	-73
Gly	*	-33	-7	-7	-7	5	-	-2	-5	-5	-5
Thr	*	-54	-44	-31	-12	3	-	2	-53	-63	-92
Arg	*	-27	-15	-10	-8	7	-	-5	-10	-33	-53
β-Ala[b]	*	-47	0	0	0	0	-	0	0	0	-22
Ala	*	-50	-37	-24	-15	3	-	-6	-7	-17	-24
Tyr	*	-61	-29	-21	-12	4	-	-5	-16	-39	-47
NH_4^+	*	-70	-24	-7	-1	0	-	-11	-40	-44	-40
Trp	*	-32	-27	-16	-11	-2	-	-6	-8	-16	-52
Met	*	-30	-23	-17	-10	0	-	-7	-7	-27	-63
Val	*	-38	-30	-20	-14	3	-	-9	-10	-15	-62
Phen	*	-50	-37	-24	-15	3	-	-6	-8	-18	-24
Ile	*	-53	-44	-31	-18	2	-	-7	-10	-11	-63
Leu	*	-42	-31	-17	-9	-1	-	-3	-9	-17	-28
Lys	*	-60	-23	-10	-4	0	-	-3	-33	-52	-91

a Boric acid pH 10.4 (2)
b Internal Standard
c Peak not integrated
* Fluorescence response poor

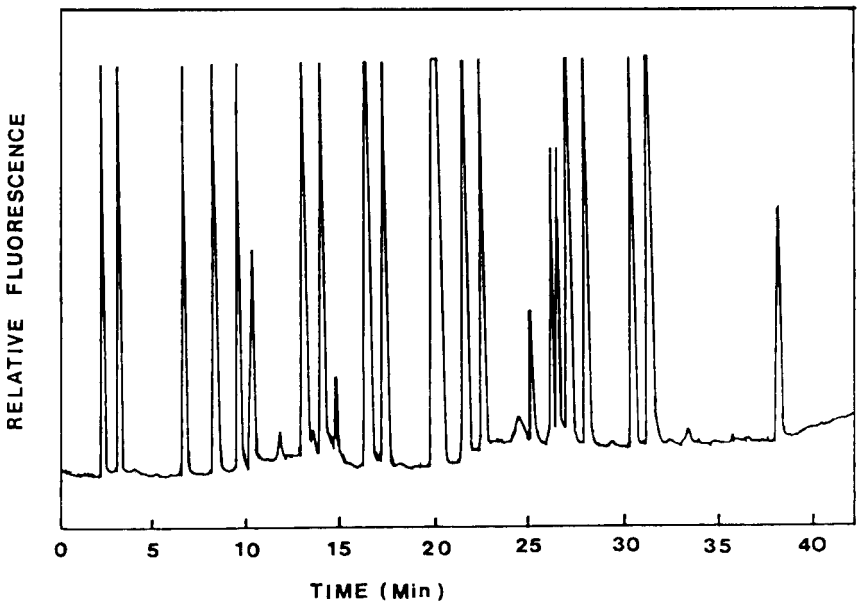

Figure III: Chromatogram of Automated Analysis of Beerwort.
Conditions: Identical to those in Figure I.

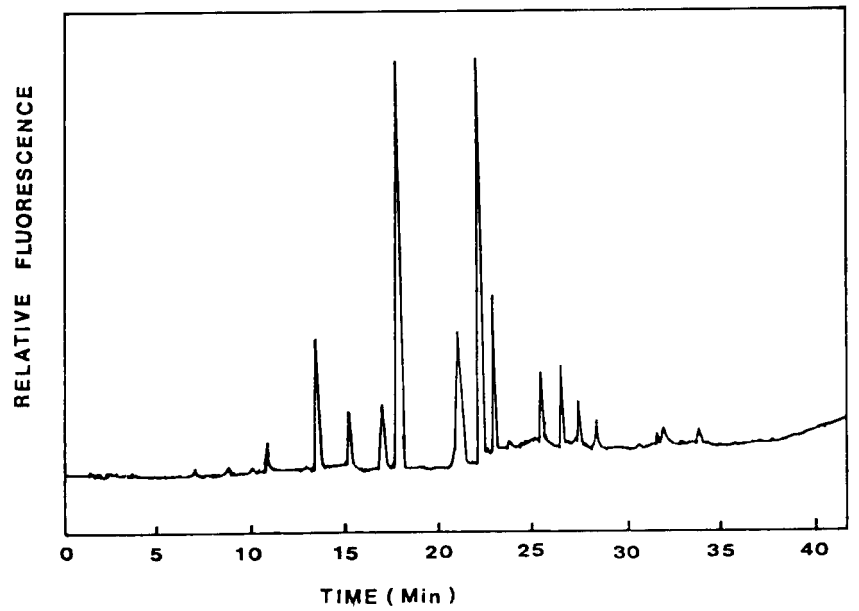

Figure IV: Chromatogram of Automated Analysis of Beer.
Conditions: Identical to those in Figure I.

7.5 was chosen as the optimum. Indications are that for pH > 8.0 fluorescent intensity generally tend to become constant followed by a decrease from pH 10.3 upwards. It is further known that solvent pH > 7.5 is harmful to the reverse phase column used.

CONCLUSIONS

The method described here has been used as a routine procedure in our laboratory. The accuracy described has been maintained over the life of the column which is generally more than 2 000 sample injections.

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ISOLATION AND DETERMINATION OF TRACE LEVELS OF D-ARABINO-2-HEXOSULOSE (D-GLUCOSONE) BY MICROCOLUMN, THIN LAYER AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Liquid chromatographic methods have been developed for the isolation and determination of D-glucosone at nanogram and sub-nanogram levels. D-glucosone is separated from complex matrices by reverse phase column chromatography (RPCC) and reacted with 2,4-dinitrophenylhydrazine to yield D-glucosone-bis-2,4-dinitrophenylhydrazone (bis-DNP). The latter compound is analyzed by normal phase thin layer chromatography (TLC) and by normal phase high performance liquid chromatography (HPLC). Visualization of bis-DNP on thin layer plates is enhanced by spraying with an alcoholic alkali reagent whereby concentrations as low as 50 ng D-glucosone can be detected. HPLC offers a highly sensitive and specific method for the quantitation of bis-DNP and concentrations as low as 500 pg D-glucosone are detected at a wavelength of 436 nm. The HPLC analysis follows the Beer-Lambert law at 436 nm with a precision (relative standard deviation) of 5.1%, 4.5%, 4.2%, 3.9% and 2.4% at 5, 10, 15, 20 and 25 ng respectively of standard D-glucosone.

INTRODUCTION

In spite of the increasing interest in D-glucosone, the key intermediate in the process of conversion of D-glucose to D-fructose, there is at present no method for its determination at trace levels. A colorimetric method involving the reaction of D-glucosone with triphenyltetrazolium chloride has been

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reported. Such method suffers from interference of D-glucose, D-gluconic acid and other reducing compounds (1). A gas chromatographic method involving the analysis of D-glucosone trimethylsilyl derivative has also been reported, but it is time consuming since rigorous removal of water from the samples is required, and qualitative at best owing to the heat lability of the formed derivative (1). A thin layer chromatographic method has been reported for the separation of hydroxycarbonyl compounds as their 2,4-dinitrophenylhydrazone derivatives from complex mixtures (2). However, such study was purely qualitative.

A high performance liquid chromatographic method has been reported for the analysis of D-glucosone (1). The authors used a u-Bondapak carbohydrate analysis column (Waters Associates, Milford, MA, U.S.A.) and a mobile phase of aqueous acetonitrile buffered with potassium phosphate (pH 6.0). Detection is achieved using both a refractive index detector (RI) and a U.V. absorbance detector at 192 nm. The minimum detectable quantity of D-glucosone is 20 ug using RI detection, and 0.1 ug using U.V. detection at 192 nm. However, the use of lower UV detectors to monitor the absorption due to the carbohydrate carbonyl groups requires that great care be taken to ensure the purity of solvents used as the mobile phase and the chance of interference from trace levels of contaminants is increased (3).

The present study was undertaken to develop rapid procedures for the routine determination of trace levels of D-glucosone in complex biological matrices. D-glucosone is isolated and purified by RPCC and converted to its bis-DNP derivative which is subsequently analyzed by TLC and HPLC. The application of both techniques allows the rapid analysis of a large number of samples for D-glucosone content.

MATERIALS AND METHODS

APPARATUS

Sample clarification kits, aqueous and organic. (Waters Associates, Milford, MA, U.S.A.)

Solvent clarification kit. (Waters Associates)

Millipore filters (organic), type FH with pore size 0.45 um. (Millipore Corporation, Bedford, MA, U.S.A.)

Reverse phase C_{18} SEP-PAK Cartridges. (Waters Associates)

U.V. - Visible Spectrophotometry - Hewlett Packard Model 8450A UV/Vis spectrophotometer moduled with Hewlett Packard Model 7225B Plotter.

TLC apparatus - 9-1/8" x 11-1/2" x 3-7/8" developing chamber with cover.

High resolution precoated TLC plates - SilG-25HR, 20 x 20 cm. (Brinkmann Instruments, Inc., Westbury, NY, U.S.A.)

Aeresol spray unit and aspirator. (Brinkmann Instruments, Inc.)

High performance liquid chromatograph (Waters Associates) equipped with Model 6000A solvent delivery system, U6K septum-less injector, dual channel Model 440 absorbance detector with filters for 436 nm and 405 nm, analytical 5u Partisil Silica Column - 4.6mm ID x 25 cm - (Whatman, Inc., Clifton, NJ, U.S.A.) and Omniscribe dual pen, 10", 10 mv recorder (Houston Instruments, Bellair, TX, U.S.A.)

For completely automated routine analysis the liquid chromatograph is equipped with a Waters WISP Model 710B automated sample injector, a Waters Model 720 System Controller and a Waters Model 730 Data Module.

REAGENTS

All reagents and solvents were of analytical reagent (AR) grade. Unless otherwise specified, the water used was double deionized.

Dichloromethane, anhydrous methanol, distilled in glass (Burdick & Jackson, Muskegon, MI, U.S.A.).

Ethyl acetate (Fisher Scientific, Fair Lawn, NJ, U.S.A.)

Ethyl alcohol, 95%.

Sodium sulfate, anhydrous.

Sodium hydroxide, pellets.

Methanolic sodium hydroxide soln. - prepared by dissolving 2 g sodium hydroxide in 10 ml water and diluting the resulting solution to a volume of 100 ml with anhydrous methanol, followed by thorough mixing.

Sulfuric acid, conc.

2,4-Dinitrophenylhydrazine-DNPH (Eastman Kodak Co., Rochester, NY, U.S.A.).

DNPH derivatizing reagent - prepared by adding 2 ml conc. sulfuric acid to 0.4 g DNPH in a 25 ml Erlenmeyer flask with swirling or stirring until solution is complete. To the resulting warm solution 10 ml of 95% ethyl alcohol are added down the sides of the flask, mixed and kept tightly covered when not in use.

D-glucosone - Synthesized by the Biochemistry Department, Nabisco Brands, Inc., Wilton, CT, U.S.A., purified by micro-column reverse phase chromatography of its aqueous solution on C_{18} SEP-PAK cartridges, dried under vacuum and kept frozen when not in use.

Authentic standard D-glucosone - bis-2,4-dinitrophenylhydrazone (bis-DNP) - is prepared by reacting purified D-glucosone with an excess of DNPH derivatizing reagent at room temperature for one hour. The resulting orange precipitate is filtered, washed with water and recrystallized from 95% ethyl alcohol to give yellow-orange crystals, m.p. 258°C (literature 257-258°C). Analytical microanalysis of the bis-DNP was performed by Galbraith Laboratories, Knoxville, TN, U.S.A. Found: C 40.22%, H 3.39%, N 20.77%; calculated for $C_{18}H_{18}N_8O_{12}$: C 40.16%, H 3.37%, N 20.81%.

Purity of the bis-DNP derivative is additionally checked by thin layer and high performance liquid chromatography of its dilute methanolic solution. The bis-configuration is confirmed by treating its dilute solution or spraying TLC chromatograms with methanolic sodium hydroxide solution whereby a violet color is obtained (4). High performance liquid chromatography using dual absorbance detection at 436 nm and 405 nm give peaks having

the absorbance ratio A_{436}/A_{405} = 1.05. This value is used in addition to retention times to confirm the identity and purity of the glucosone-bis-DNP peak in the analysis of natural isolates (5).

Standard-bis-DNP solutions - Standard solutions of bis-DNP are prepared in methanol or ethyl acetate to have a D-glucosone equivalent concentration of 1, 2, 3, 4, and 5 ug/ml for HPLC analysis and 10 ug/ml for TLC analysis.

TLC developing solvent - Dichloromethane and methanol (95 + 5) V/V. Prepare fresh daily.

HPLC mobile phase - Dichloromethane and methanol (95 + 5) V/V. Filter the dichloromethane solvent using the solvent clarification kit fitted with an organic filter. Degas the filtrate thoroughly by swirling for about 5 minutes while under vacuum. Filter the methanol solvent the same way. Using the degassed solvents, prepare a 5% solution V/V of methanol in dichloromethane. Filter and degas the resulting solution for about 2 minutes.

PROCEDURE

Isolation of D-glucosone:

Solid Samples - Weigh 1 g sample into a 100 ml glass stoppered Erlenmeyer flask. Add 50 ml methanol, secure stopper with masking tape, agitate 30 minutes on a wrist action shaker at room temperature. Filter through Whatman No. 4 filter paper, rinse the flask with 3 x 10 ml portions methanol, passing the methanol washings through filter paper. Collect methanol extract and washings in a 100 ml volumetric flask, make up to volume with methanol, stopper and mix thoroughly. Evaporate 50 ml of methanol extract to dryness under vacuum at room temperature. Redissolve residue in 1 ml water and subject to reverse phase column chromatography (RPCC).

Biological Solutions - Filter aqueous solutions to remove any undissolved constituents. Subject clear aqueous filtrates to RPCC.

RPCC - Pipette an appropriate aliquot (1-2 ml) of aqueous solution containing D-glucosone into the barrel of a 10 ml hypodermic syringe to which is attached a single or a series of C_{18} SEP-PAK cartridge(s) which are already activated by passing methanol followed by water through them. Filter the solution through the cartridge or cartridge train. Wash with additional 30-35 ml water. Any coloring material will be tightly held as a narrow band at the top of the cartridge bed. The clear solution eluting from the cartridge(s) is collected into a 50 ml volumetric flask, adjusted to volume with water and thoroughly mixed. Evaporate 25 ml to dryness under vacuum at 30°C. Redissolve the residue in 1 ml of 50% aqueous methanol. Derivatize the resulting solution by reacting it with DNPH.

Derivatization of D-glucosone - Add excess (0.5 ml) of derivatizing DNPH reagent to the aqueous methanol solution in a screw cap 4 dram vial, mix by swirling and let stand for one hour at room temperature. At the end of the reaction and depending on the level of D-glucosone in the aliquot derivatized, the reaction mixture will acquire an orange color or an orange precipitate will be formed.

Depending on the method of analysis to be applied the reaction products are treated as follows:

For TLC - Add 5 ml methanol to the reaction vial, dissolve by swirling, quantitatively transfer into a 10 ml volumetric flask, make up to volume with methanol, stopper and mix thoroughly. Subject to TLC analysis.

For HPLC - Dissolve the contents of the sample reaction vial in the minimum volume of anhydrous methanol (1-5 ml), quantitatively transfer the resulting solution into a 125 ml separatory funnel. Add 50 ml water and extract with 3 x 20 ml portions of ethyl acetate. Filter ethyl acetate extract through a 2 g bed of anhydrous sodium sulfate contained in a filtering funnel fitted with a glass wool plug. Collect the dried filtrate into a 100 ml volumetric flask. Wash the separatory funnel and the sodium sulfate bed with fresh ethyl acetate until

complete removal of yellow color. Adjust to volume with ethyl acetate, stopper and mix thoroughly. Evaporate 50 ml - under a stream of N_2 - to dryness and redissolve in 5 ml ethyl acetate, filter through a 0.45 um FH Millipore filter. Subject concentrated solution to HPLC.

Thin Layer Chromatography - Spot 50 ul of each sample bis-DNP solution alongside 5, 10 and 20 ul of standard bis-DNP solution (10 ug D-glucosone/ml), having 50, 100 and 200 ng D-glucosone respectively, on a high resolution thin layer plate. Develop the plate in an unlined unequilibrated developing chamber containing 200 ml freshly prepared TLC developing solvent. After 20 minutes the solvent will travel about 15 cm, remove plate and air dry in a hood. Spray the dried plate with methanolic sodium hydroxide solution. A violet color will develop for both standard and sample spots having an R_f value of 0.15. Compare the intensity of the violet color of sample spots with those of standard spots and estimate the level of D-glucosone in the samples.

High Performance Liquid Chromatography - The liquid chromatograph is equilibrated using the following parameters:

Column	: Whatman PXS Partisil Column, 5u (4.6mm ID x 25 cm)
Mobile phase	: Dichloromethane and methanol (95 + 5) V/V
Mobile phase flow rate	: 2 ml/minute
Chart speed	: 1 cm/minute
Temperature	: Ambient
Detector	: 440 Absorbance detector at 436 nm, 0.005 AUFS or at both 436 nm and 405 nm, 0.005 AUFS

Inject 5 ul from each bis-DNP standard solution - D-glucosone level of 1, 2, 3, 4 and 5 ug/ml - into the liquid

chromatograph. This will be equivalent to 5, 10, 15, 20 and 25 ng D-glucosone respectively. The bis-DNP peak will elute with a retention time of about 3.8 minutes (Fig. 1). Measure peak height responses. Establish a calibration curve by plotting D-glucosone concentration in ng versus corresponding responses. Establish instrument linearity and thereafter monitor detector response using only one external standard having an equivalent

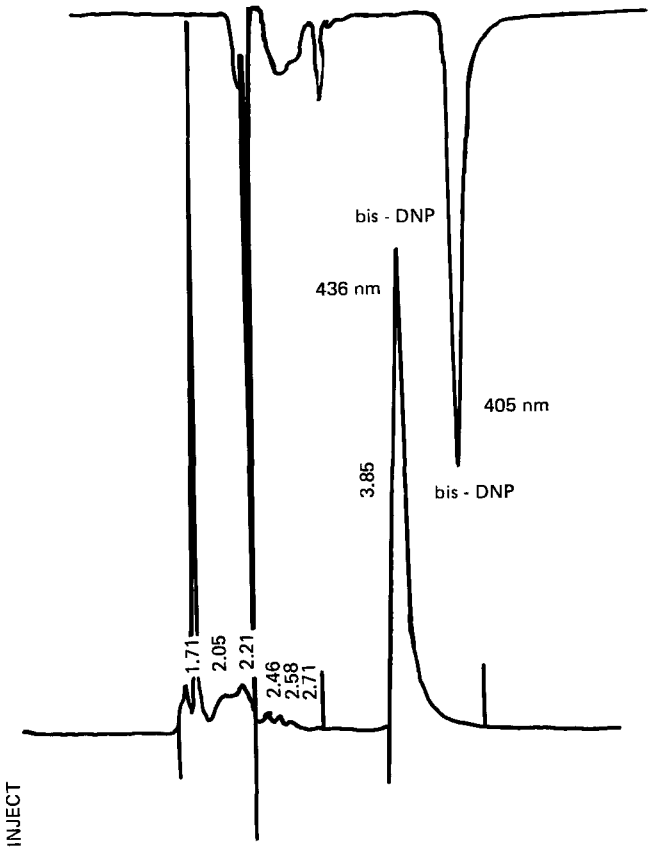


Figure 1. HPLC Chromatogram of 5 ul of bis-DNP solution (4 ug/ml) at 436 nm and 405 nm, 0.005 AUFS.

level of bis-DNP as in sample(s). Inject 5 ul from each sample bis-DNP solution in ethyl acetate into the liquid chromatograph. Measure bis-DNP peak height responses and estimate the level of D-glucosone in original samples by comparing sample peak responses with the responses of standards.

It is recommended that an aliquot of a standard solution be chromatographed between sample runs, e.g. an injection from a standard solution between 4-5 sample injections. This will monitor the chromatographic behavior and detector responses during the analysis of a large number of samples.

Aliquots of 1% aqueous solutions of potential interfering substances, e.g. D-glucose, D-fructose, galactose and a-ketogluconic acid when subjected to the specified D-glucosone-DNPH reaction conditions (1 hour at room temperature) do not yield any products that interfere with the TLC and HPLC of bis-DNP. The bis-DNP peak purity is confirmed by com-

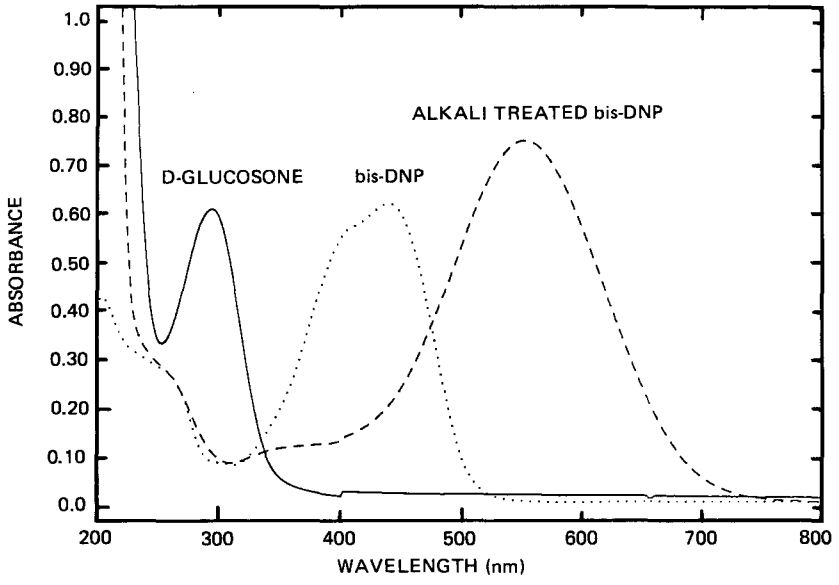
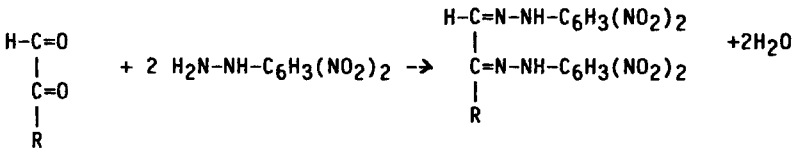


FIgure 2.

paring the peak absorbance ratio A_{436}/A_{405} for samples and authentic standard (5).

RESULTS AND DISCUSSION

D-glucosone is a 1,2-dicarbonyl polyhydroxy compound. Such 1,2-dicarbonyl compounds react with DNPH to give bis-DNP derivatives which have orange-yellow colors. When bis-DNP's are treated with solutions of alcoholic alkali, they are transformed to highly conjugated compounds that will undergo a bathochromic shift and as a result will absorb light at a higher wavelength acquiring purple-violet colors (4). Figure 2 illustrates the bathochromic shifts that take place when D-glucosone is derivatized to bis-DNP and when the latter compound is treated with alcoholic alkali.



1,2-dicarbonyl compound R=-(CHOH)$_3$ -CH_2OH in glucosone

DNPH

bis-DNP

The formation of bis-DNP from the reaction between D-glucosone and DNPH takes place at room temperature. Maximum bis-DNP yield is attained after one hour reaction time. Potential interfering compounds, e.g. D-glucose, D-fructose, galactose and a-ketogluconic acid - when subjected to the specified D-glucosone - DNPH reaction conditions - do not yield any products that interfere with the TLC and HPLC analyses of bis-DNP.

As low as 50 ng D-glucosone are detectable by TLC and 500 pg are detectable by HPLC using a 10 mv recorder. The HPLC sensitivity could be further increased to detect as low as 50 pg D-glucosone by using a 1 mv recorder.

The TLC methodology allows for the simultaneous analysis of a large number of samples in a short time. Such a cost effective technique will streamline the analysis of biological samples for D-glucosone.

An added advantage is that any further required HPLC analysis to quantitate D-glucosone could be performed on the same solutions subjected to TLC analysis.

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RELIABLE SEPARATION OF XYLITOL FROM SOME CARBOHYDRATES AND POLYOLS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

An acceptable separation of xylitol from a mixture of sucrose, glucose, fructose, mannitol and sorbitol was carried out by High Performance Liquid Chromatography. A Sugar Pak I column at 80ºC was used employing acetonitrile/water (25/75) (v/v) as the mobile phase.

INTRODUCTION

Xylitol is a polyol that has been proposed recently as a sugar substitute for oral use in diabetic foods due to its retarded release of glucose which avoids blood sugar peak (1). Moreover, it presents the advantage of its relative sweetness, approximately equal to that of sucrose, and it is the only sweetener available to the food technologist which permits a 1:1 replacement of sucrose in his formulation (2), thus making it recommendable for use in products considered cariogenics, such as chewing gums, lozenges, candies and similar sweets (3).

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The analysis of a carbohydrate mixture by conventional means is dreary and time consuming task. The high performance liquid chromatographic (HPLC) technique has greatly facilitated this endeavour, allowing not only the identification, but the quantification of these mixtures. The technique has been widely used in the field of nutrition (4-8).

The determination of soluble carbohydrates in dietetic foods for diabetic subjects poses the problem of the simultaneous determination of sugars and polyols since the latter have been used as sweeteners.

The information already published on this point is scarce. Olano has described(9) a gas liquid chromatographic (GLC) method to determine carbohydrates and polyols in wines. The GLC technique presents in our opinion, a complex picture since each sugar originates two or more peaks, thus complicating the interpretation of the chromatograms. Samarco et al. (10) separated sorbitol, mannitol and xylitol by HPLC but did not report on the presence of sugars. Dokladalova et al. (11) and Vidal-Valverde et al. (12), acceptably separated carbohydrates and polyols, but xylitol and sorbitol eluted simultaneously. We have found no previous references on the analysis of xylitol in the presence of sorbitol, mannitol, sucrose, glucose and fructose.

There are indications that xylitol will be the sweetener of choice in the near future. It is important, then, to have a method of separating and quantifiyng xylitol in the presence of other sugars and polyols which may be present in dietetic foods.

EXPERIMENTAL

High performance liquid chromatography was carried out in an ALC/GPC (Model 201) equipped with model 6000 A pump dual reciprocating piston heads, model U 6K septumless injector, Sugar Pak I column 30cm x 6.5mm i.d., with a column temperature control accessory at 75ºC, or 80ºC, pre-column filter, and model R-401 differential refractometer detector optical deflection type, maintained at 30ºC (Waters Associates, Milford, Mass. USA). The detector signal was recorded on a M 730 data module. Chart recorder speed: .25cm/min.Attenuation x16. Injection volume : 25µl.

Mobile phase: acetonitrile/water 25/75, filtered through a Millipore FH(.5µm) membrane, degassed by immersion in an ultrasonic bath. The flows rates were .4 ml/min. and .5 ml/min.

Standard solution: various amounts of sucrose, glucose, fructose, mannitol, xylitol and sorbitol (Merck) were dissolved in the mobile phase.

The use of acetonitrile/water (25/75)(v/v) as the mobile phase and the Sugar PakI column has meant a considerable improvement in the separation of mixtures containing xylitol. The operating conditions were somewhat different from the ordinary ones, but with no adverse effects on the life span of the column The operating procedure was as follows: After ten analyses (generally a day's work), the column pressure rose to 2500 psi. At this point, the column was placed tail first and bidistilled water (previously filtered and degassed) was pumped through it overnight at 90ºC. In this way, the operating pressure dropped from 2500 psi to normal values and the column was ready for a new set of analyses. Approximately once a week the

column was regenerated by passing through it, tail first, a 0.001M solution of calcium acetate during 3.5 hours at .5ml/min. flux. The column was kept at 90ºC during the process. This treatment was followed by the pass of bidistilled water (filtered and degassed) during 30 minutes at the flux previously indicated.

RESULTS AND DISCUSSION

The analysis of xylitol, in the presence of sugars and polyols, which may be present in dietetic foods, could be done by thin layer chromatography (13), but,in practice, this is not feasible since some of these compounds are present in very large amounts while some others are only present in minute amounts. HPLC technique constitutes a great improvement for this type of analyses. In this work, the aim has been to set up appropiate HPLC conditions to detect and quantify xylitol in the presence of polyols, such as mannitol and sorbitol, and carbohydrates, such as sucrose, glucose and fructose.

Fig. 1 represent two chromatograms obtained with a standard solution of sucrose, glucose, fructose, mannitol, xylitol and sorbitol, under two different set of conditions.

Table 1 lists the chromatographic constants obtained from the standard curve (Fig 1 A). The void volume was determined using the retention time of Cl_2Ca. The response factor of the detector was a rectilinear response between peak height (cm) and weight (25-200µg) of the carbohydrates and polyols. The equations y=a+bx of the six lines were:

for sucrose a=-0.15 b=0.037 r=0.999

for glucose	a=-0.15	b=0.044	r=0.999
for fructose	a=-0.10	b=0.038	r=0.999
for mannitol	a=-0.25	b=0.038	r=0.999
for xylitol	a=-0.12	b=0.033	r=0.999
for sorbitol	a=-0.07	b=0.032	r=0.999

The conditions established, both in Fig 1A and Fig. 1B, were adequate to quantify xylitol on the

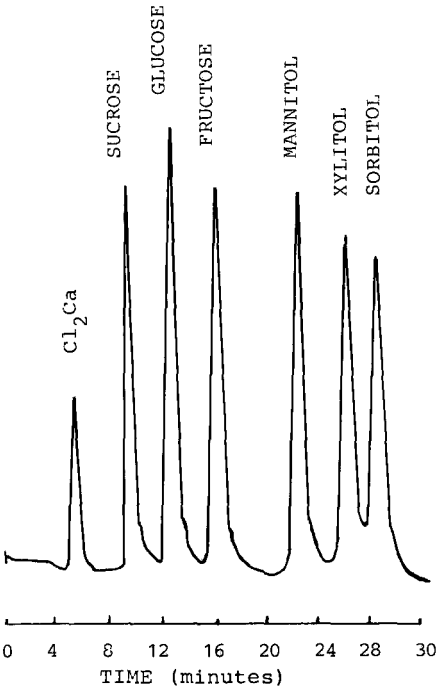

FIG. 1 A.-

Mobile phase:
acetonitrile /water, 25/75

Flow rate: 0.4 ml/min.

Temperature column: 75ºC

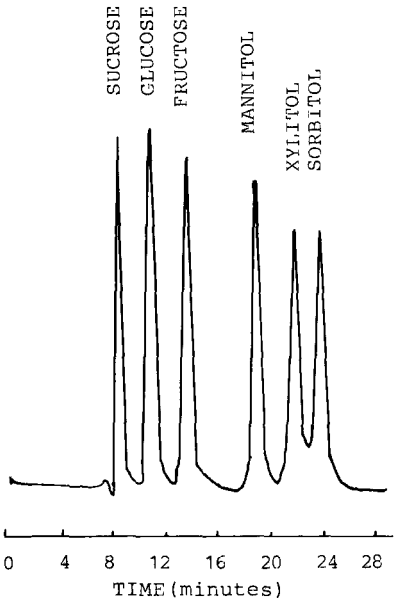

FIG. 1 B.-

mobile phase:
acetonitrile/water, 25/75

Flow rate: 0.5 ml/min.

Temperature column: 80ºC

TABLE 1

Standardization of Waters Sugar Pak I High Performance Liquid Chromatographic Column

	RT_S	Sucrose	Glucose	Fructose	Mannitol	Xylitol	Sorbitol	K'	N
		Separation factor (α)							
Sucrose	0.338	\	1.867	2.779	4.406	5.360	5.954	0.655	958
Glucose	0.454	2.056	\	1.488	2.360	2.870	3.189	1.223	1287
Fructose	0.576	3.750	1.922	\	1.586	1.929	2.142	1.820	1045
Mannitol	0.793	7.600	5.665	3.250	\	1.217	1.351	2.886	1778
Xylitol	0.921	9.187	7.361	4.895	1.905	\	1.111	3.511	1672
Sorbitol	1.000	10.438	8.611	6.020	3.089	1.125	\	3.900	1816
		Resolution (R)							

RT_S= relative retention time to sorbitol; Separation factor (α)=k'_2/k'_1;
W = peak width; Resolution (R)=$V_2-V_1/\frac{1}{2}(W_2+W_1)$; V= retention volume;
V_0= void volume; Capacity factor (K')= $V-V_0/V_0$;
Number of theoretical plates (N)= 16 $(V/W)^2$

presence of the sugars and polyols indicated above. Acetonitrile/water, 35/65 (v/v), with a flow rate of 0.5 ml/min. also separates the standard mixture but cannot be recommended as a mobile phase, since it would produce a rapid build up of the column pressure.

The other components of the mixture could also be quantified since the separation between them was acceptable and the correlation coefficients of the curves were adequate. Nonetheless, it has been observed that occasionally the sucrose peak splits in two, fact which could interfere with the quantitation of it.

It can be concluded that the chromatographic conditions established here are appropriate for the separation and quantification of a mixture of xylitol, mannitol, sorbitol, sucrose, glucose and fructose, compounds which may be present in dietetics foods.

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SERUM AND PELVIC TISSUE CONCENTRATIONS OF CEFTRIAXONE AND CEFAZOLIN AT HYSTERECTOMY

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ABSTRACT

Ceftriaxone and cefazolin concentrations were assayed by high-pressure liquid chromatography in serum and pelvic tissue. Specimens were obtained at uterine removal subsequent to a 1-g intramuscular preoperative dose given to 117 women scheduled for elective vaginal or abdominal hysterectomy. The mean serum concentration of cefazolin was 43.2 ± 13.1 and 39.8 ± 15.4 µg/ml after vaginal and abdominal hysterectomy, respectively. For ceftriaxone they were 59.2 ± 16.8 and 56.1 ± 18.3 µg/ml for vaginal and abdominal hysterectomy, respectively. Mean tissue concentrations of ceftriaxone were 22.5 ± 10.4, 17.4 ± 6.9, 27.9 ± 10.7, and 16.4 ± 6.3 µg/g for vagina, myometrium, fallopian tube, and ovary, respectively, and respective mean tissue concentrations for cefazolin were 15.8 ± 7.6, 14.4 ± 8.5, 15.6 ± 8.0, and 12.4 ± 5.8 µg/g. Pelvic tissue concentrations of cefazolin were similar, but concentrations of ceftriaxone in fallopian tube and vagina were higher than those in ovary and myometrium. Tissue to serum ratios of ceftriaxone remained constant throughout the time intervals studied, whereas cefazolin ratios increased with time.

INTRODUCTION

Ceftriaxone is a new third-generation cephalosporin antibiotic with a 6-hour half-life (1). Cefazolin, a first-generation

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cephalosporin with a 1.5-hour half-life, is effective in reducing infection after hysterectomy (2). We determined serum and pelvic tissue concentrations in specimens from elective hysterectomy patients. The serum and tissue levels were determined by high-pressure liquid chromatography (HPLC) and were compared to a microbiological assay.

MATERIALS AND METHODS

Patients and Specimens

One hundred and seventeen women scheduled for elective vaginal or abdominal hysterectomy gave informed consent, and were assigned one of the two drugs by a computer-generated randomization list. On call in the operating room, each woman received 1 g of either ceftriaxone or cefazolin by deep intramuscular injection and the time of injection was recorded. At uterine excision, the time was noted, a blood sample was drawn, and the serum was separated and frozen at -20°C until assayed for antibiotic concentration. In addition, vagina was collected after vaginal hysterectomy and myometrium, a segment of fallopian tube, and ovarian tissue were obtained from the abdominal hysterectomy patients. All tissue specimens were maintained at -20°C until processed and assayed for antibiotic concentrations.

Sample Preparation

Both serum and whole tissue specimens were processed as described previously (3, 4). There was no attempt to determine the concentration of antibiotic in extracellular tissue fluid. There were no modifications in the acetonitrile dichloromethane phase extraction procedure. All specimens were assayed in duplicate. Tissue concentrations of both antibiotics were corrected for blood contamination by the cyanomethemoglobin colorimetric method (5).

HPLC Assay

Serum and tissue specimens were assayed by a modification of the reverse-phase HPLC assay method for cefoperazone as previously

described (3). Modifications in the procedures were in the mobile phases for both ceftriaxone and cefazolin. The mobile phase for ceftriaxone consisted of 10% methanol and 90% 0.10 M sodium phosphate buffer, pH 8.0. The flow rate was 2.3 ml/min and the UV detector was operated at 254 nm and 0.1 absorbance units (AU) for serum and for tissue concentration the detector was operated at 0.01 AU.

The mobile phase for cefazolin was 12% acetonitrile and 88% 0.10 M sodium phosphate buffer, pH 6.1. The flow rate was 2.0 ml/min and the UV detector was operated at 254 nm and 0.1 AU for serum and 0.01 AU for tissue specimens.

Between-group and within-group reproducibility studies were made by injecting either ceftriaxone or cefazolin at concentrations of 50 µg/g and 10 µg/g into a pre-weighted portion of normal gynecologic tissue with a micro syringe. These tissue were then extracted with 0.1 M PO_4 buffer pH 6.1 as described previously (3). All determinations in these recovery studies were done a minimum of five times.

The components of the HPLC system consisted of a 30-cm C_{18} µ Bondapak column (Water's Associates, Milford, Mass.), a model 6000-A pump, a model 710 B Water's Intelligent Sample Processor, and a 440 UV detector (Water's Associates, Milford, Mass.). Peaks were recorded on a 10 mv chart recorder (Linear Instruments, Inc., Reno, Nevada) at a chart speed of 0.50 cm/min.

Microbiological Assay

Twenty serum and tissue specimens were assayed for ceftriaxone and cefazolin concentrations by the microbiological assay. The bioassay for ceftriaxone was done using *E. coli* ATCC 10536. For cefazolin the bioassay was done using a *Bacillus subtilis* spore suspension (Difco Laboratories, Detroit, Michigan). Each petri plate contained 10 ml of antibiotic assay agar number one (Difco Laboratories, Detroit, Michigan). Fifteen microliters of serum or tissue extracts of the specimens and spiked serum or tissue extracts were added to 6 mm sterile filter paper discs.

The plates were incubated at 35°C for 12-18 hrs. Zone diameters were measured using a dial calipers.

Statistical Analysis

Least square linear regression was used to correlate the HPLC and microbiological bioassay data. An analysis of variance followed by the Students' Newman-Keuls multiple comparison procedure was used for the statistical analysis of these data.

RESULTS

Chromatograms of cefazolin and ceftriaxone are shown in Figures 1 and 2, respectively. The HPLC assay was linear when both serum and tissue were spiked with ceftriaxone and cefazolin in concentration from zero to 200 μg/ml (g). The correlation between concentration on peak height was > .99 for both antibiotics. The correlation between the microbiological bioassay and the HPLC assay for both antibiotics was > .90. Between-group and within-group recovery in pelvic tissue was > 95% for both ceftriaxone and cefazolin (data not shown).

The women in both antibiotic groups had clinical and surgical variables that were similar; 51 women underwent abdominal hysterectomy, and 66 had vaginal hysterectomy. There was a significant difference in the interval from dose to sample time in the two surgical procedures ($P < .05$). The mean collection times were 142.9 ± 37.5 and 164.1 ± 45.4 min following preoperative dose for vaginal and abdominal hysterectomy, respectively. Although the times to specimen collection were different, serum concentrations of both antibiotics were not significantly different for the two procedures. The mean serum concentrations of ceftriaxone were 59.2 ± 16.8 and 56.1 ± 18.3 μg/ml for the vaginal and abdominal hysterectomy. Similarly, the mean serum cefazolin levels were 43.2 ± 13.1 and 39.8 ± 15.4 μg/ml. Serum concentrations of cefazolin were 71.9% of the ceftriaxone levels. The mean tissue concentrations of ceftriaxone were 22.5 ± 10.4, 17.4 ± 6.9, 27.9 ± 10.7, and 16.4 ± 6.3 μg/g for vagina, myometrium, fallopian tube,

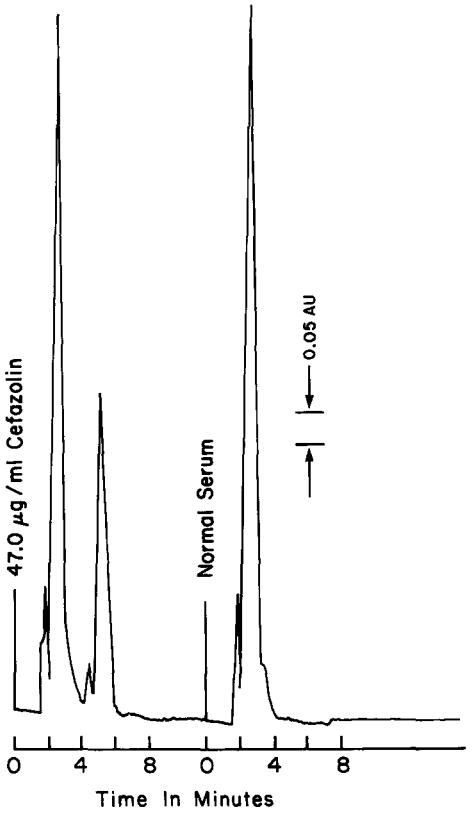

FIGURE 1

The HPLC serum assay of cefazolin. The chromatogram of cefazolin on a C_{18} µ Bondapak column at an absorption wave length of 254 nm. The mobile phase was 12% acetonitrile and 88% 0.1 M PO_4 buffer pH 6.0. The flow rate was 2.0 ml/min. The chromatograms were prepared with the sensitivity of the chromatograph at 0.10 absorbance units (AU).

and ovary, respectively. The mean tissue levels for cefazolin were 15.8 ± 7.6, 14.4 ± 8.5, 15.6 ± 8.0, and 12.4 ± 5.8 µg/g for vagina, myometrium, fallopian tube and ovary, respectively. The tissue to serum ratio of ceftriaxone remained constant throughout the time intervals studied with the initial ratio being 0.342 ± 0.12, and after 6 hours it was 0.383 ± .05. Cefazolin tissue to serum ratios appeared to increase with time. Initially the mean

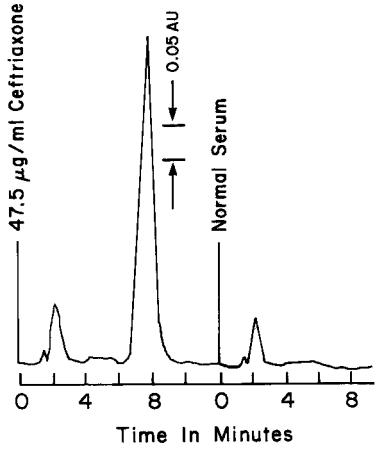

FIGURE 2

The HPLC serum assay of ceftriaxone. The chromatogram of ceftriaxone on a C_{18} μ Bondapak column at an absorption wave length of 254 nm. The mobile phase was 10% methanol in 90% 0.1 M PO_4 buffer pH 6.0. The flow rate was 2.3 ml/min. The chromatograms were prepared with the sensitivity of the chromatograph at 0.10 absorbance units (AU).

tissue ratio was 0.293 ± .06, and at > 200 min the ratio was 0.544 ± .17. Tables 1 and 2 represent a summary of serum and pelvic tissue and the ratio of tissue concentrations to simultaneous serum concentrations.

DISCUSSION

There were significant differences in the tissue concentrations of ceftriaxone, whereas they were similar for cefazolin. The fallopian tube and vagina tissue concentrations of ceftriaxone were significantly higher than myometrium and ovarian tissue concentrations ($P < .05$).

In a previous study, the concentrations of ceftriaxone in serum and gynecological tissue were studied in 31 patients following a 2-g injection (6). These investigators showed that fallopian tube concentrations were significantly higher than those found

TABLE 1

Mean (± SD) Serum and Pelvic Tissue* Concentrations of Ceftriaxone Following Hysterectomy.

Time Minutes	Specimens Per Unit Time	Concentrations				RATIO		
		Serum µg/ml	Myometrium µg/g	Fallopian Tube µg/g	Ovary µg/g	M/S	T/S	O/S
Abdominal Hysterectomy								
90 - 120	4	57.7 ± 22.6	17.3 ± 4.3	28.3 ± 12.4	11.5 ± 1.8	.300	.490	.199
120 - 160	8	58.0 ± 15.1	18.6 ± 8.4	30.1 ± 11.6	18.5 ± 6.6	.321	.519	.319
160 - 200	8	54.3 ± 22.5	15.5 ± 5.9	28.5 ± 9.9	13.8 ± 5.1	.285	.525	.254
200 - 255	3	53.7 ± 17.8	19.3 ± 10.1	20.2 ± 10.1	24.3 ± 8.2	.359	.376	.453

Time Minutes	Specimens Per Unit Time	Concentration		RATIO
		Serum µg/ml	Vaginal Tissue µg/g	V/S
Vaginal Hysterectomy				
85 - 120	10	64.2 ± 22.4	24.4 ± 12.1	.380
120 - 160	15	57.7 ± 12.2	22.1 ± 10.7	.383
160 - 240	6	52.8 ± 9.6	18.2 ± 2.8	.344

* All tissue concentrations were corrected for blood contamination
M/S - Myometrium to serum
T/S - Fallopian tube to serum
O/S - Ovary to serum
V/S - Vagina to serum

TABLE 2

Mean (± SD) Serum and Pelvic Tissue Concentrations of Cefazolin Following Hysterectomy.

Time Minutes	Specimens Per Unit Time	Concentrations				RATIO		
		Serum µg/ml	Myometrium µg/g	Fallopian Tube µg/g	Ovary µg/g	M/S	T/S	O/S
Abdominal Hysterectomy								
105 - 140	9	45.8 ± 17.9	14.1 ± 7.9	17.5 ± 10.9	11.3 ± 6.5	.305	.382	.247
140 - 180	13	41.7 ± 12.4	13.1 ± 5.1	14.7 ± 6.1	13.7 ± 5.7	.314	.353	.329
180 - 200	4	32.5 ± 5.7	24.2 ± 14.9	18.6 ± 5.0	12.3 - 6.2	.744	.527	.378
200 - 390	2	17.8 ± 10.5	11.0 ± 5.1	13.4 ± 1.9	6.8 ± 8.4	.618	.753	.382

Time Minutes	Specimens Per Unit Time	Concentration		RATIO
		Serum µg/ml	Vaginal Tissue µg/g	V/S
Vaginal Hysterectomy				
90 - 120	7	47.4 ± 14.4	11.3 ± 6.5	.238
120 - 160	14	44.3 ± 16.3	16.3 ± 7.8	.368
160 - 220	14	40.0 ± 8.4	16.9 ± 8.2	.422

* All tissue concentrations were corrected for blood contamination
M/S - Myometrium to serum
T/S - Fallopian tube to serum
O/S - Ovary to serum
V/S - Vagina to serum

in the myometrium and endometrium. They further reported that after 5 h, tissue concentrations were > 20 μg/g of tissue.

In another study, protein binding at doses > 1500 mg was thought to give higher protein-free drug levels in blister and tissue levels. They found that the blister and tissue levels were linear with the change in dose (7).

Data from the 2-g study (6) and those in our study indicate that the serum to tissue ratios for ceftriaxone do not change appreciably, and appear to be linear. Accordingly, the high proten binding (95%) as compared to cefazolin which is 80% protein bound, the unusually long elimination half-life in tissue, and the high levels make the drug interesting for study, and predictably useful clinically since even with a 1-g injection tissue concentration of > 20 μg/g were observed over 3 hours post-dose.

Ceftriaxone is the only antibiotic we have assayed in pelvic tissue that appears to have an affinity for the fallopian tube and vaginal tissue. In a previous study, we found no interregimen or intertissue differences in concentrations of cefoperazone or cefoxitin, as was true for cefazolin in the current study (3). The significance of these differences could be attributed to the longer half-life, or the higher protein binding capacity of ceftriaxone. These tissue/serum ratios for ceftriaxone are different from cefoperazone and cefoxitin as we previously reported (3).

In summary, the concentration of ceftriaxone and cefazolin were determined through about 4 hours subsequent to a 1-g intramuscular preoperative dose given to women undergoing hysterectomy. Mean cefazolin serum levels were significantly lower than serum levels of ceftriaxone ($P < .01$). Similarly, mean tissue concentrations of cefazolin were 56 to 82% of ceftriaxone tissue levels. The longer half-life of ceftriaxone is probably responsible for the higher serum and tissue concentrations of ceftriaxone.

ACKNOWLEDGEMENTS

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HPLC DETERMINATION OF IODIDE IN SERUM USING PAIRED ION CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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Abstract

HPLC utilizing paired ion chromatography with electrochemical detection is used for the determination of iodide in serum. Serum samples are prepared by precipitation of protein, centrifugation and removal of interfering substances with a bonded phase column. The resulting sample is then analyzed. The method has successfully been applied to human serum and gives data that agrees well with values obtained by other methods. The method is accurate precise, and time conservative when compared to more classical methods.

Introduction

Serum iodide determinations are done primarily to evaluate thyroid function in man and animals. The thyroid gland utilizes inorganic iodide and protein to form organic compounds with some hormonal activity. Increased thyroid activity may be associated with elevated serum iodide levels while decreased activity results in lower levels. Additionally if a subject has been exposed to any of a number of environmental or medical sources of iodide elevated serum iodide levels will result. Elevated serum iodide levels can interfere with diagnostic tests of thyroid function. Some of these agents include radiopaque dyes used for various

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diagnostic procedures and Lugol's solution. Exposure to both of these agents will result in elevated iodide levels.(1)

Existing methods for the determination of serum iodide entail the precipitation of the protein bound iodide and a digestion of the residue by dry ashing or wet digestion.(2,3) All of these methods require extensive sample preparation, specialized equipment or extended periods of time prior to the final determination. The method described in this paper involves simple sample preparation. The final HPLC determination step is rapid and accurate. An analyst can prepare and analyze about 25-30 samples in an eight hour day.

Materials and Methods

Sample preparation: Samples of serum were placed in test tubes and diluted 1:1 with distilled water and mixed for 30 seconds. Eight ml of acetonitrile was added to the test tubes and they were mixed again. After this they were placed in a centrifuge for 10 minutes at 2000 rpms, the supernatant withdrawn and placed on previously prepared columns.

Column preparation: Sample columns were purchased from Analytichem, International and consisted of an amino bonded phase packed in 2.8 ml capacity polyethylene columns. They were prepared for use by first rinsing with 3 ml of CH_3CN followed by 3 ml of distilled water.

Sample clean-up: The previously prepared samples were placed onto the clean-up columns. After all of the supernatant was allowed to pass through the columns, the iodide containing fraction was eluted with 1.0 ml of 0.2N KH_2PO_4.

HPLC: All analyses were performed on a high performance liquid chromatograph system. The solvent delivery system was a M590 pump (Waters Associates). Samples were injected using a Model 7125 Loop Injector (Rheodyne) or a Model 710B WISP (Waters Associates). The HPLC detector used was a Model LC-4B Amperometric Detector equipped with a Ag electrode (Bioanalytical Systems) operated at + 0.01 volts. The column temperature was maintained at 25°C. The HPLC column used was a 3.9 mm I.D. x 30 cm long column packed with 10μ Spherisorb ODS (HPLC Technology).

The mobile phase was previously used for the determination of iodide in milk (4) and consisted of 12.78 g of Na_2HPO_4, 3.2 g of hexadecyltrimethyl ammonium chloride, 1.4 g of Na_2EDTA added to 2.8 ℓ of HPLC grade water. This mixture was combined with 1 ℓof HPLC grade acetonitrile, mixed and thoroughly degassed.

The pH of the resulting mobile phase was then adjusted to 6.8 (± 0.1) with 85% H_3PO_4. The mobile phase was flowing at 2.0 ml/min. The iodide standard was ultrapure potassium iodide that was prepared as a stock solution containing 1.0 ng of iodide per µl.(Alfa).

Analysis

Fifty microliter quantities of a diluted iodide standard (.04 ng/µl) were injected in duplicate at an instrument setting of 2 nafs (nanoamps full scale). Varying portions of sample ranging from 5 to 100 µl were injected depending on the concentration of iodide in the sample. The peak height of samples were compared to peak height of standards to arrive at the final iodide concentration.

Results

The methodology described in this communication was evaluated through the use of accuracy and precision studies. The dilute iodide standard (.04 ng/µl) was injected ten times and a pooled serum extract was also injected ten times to arrive at precision data. These data are summarized in Table 1.

Measured amounts of iodide standard were added to a pooled serum sample. This standard was added prior to any sample cleanup and therefore, was subjected to the same conditions as were the samples. These data are summarized in Table 2.

Table 1

Iodide Precision Study

Matrix	Conc.	%Cv
Iodide Standard	2 ng	0.7
Serum Extract	4.3 µg/dl	1.4

Table 2

n = 3

Recovery of Added Iodide to Pooled Serum

Amt. Iodide Added (µg/dl)	Amt. Iodide Recovered (µg/dl)	% Recovery
0	4.30	-
0.5	4.77	94.0
1.0	5.21	91.0
1.5	572	94.4

The results indicate an average recovery of 93.1% for triplicate recovery studies. Based on twice the S/N ratio with all other conditions remaining the same this method has a lower limit detection of 0.4 µg/dl.

Varying concentrations of iodide were injected in duplicate over a 200 fold range from 500 pg to 100 ng in increments ranging from 1-10 ng. Data points correlated well linearly with a regression coefficient of 0.988.

To arrive at an optimum applied Eo for use in this study, the same concentration of iodide was injected at Eo (applied) ranging from 0 volts to + 0.150 volts. The results indicate that as applied voltage increases peak height decreases. The applied Eo of 0 volts gave a maximum peak height but the noise was excessive therefore, +0.010 volts was used as a compromise between noise and detector response. Over the range from 0 to +0.150 volts a decrease in peak height of about 30% was seen.

A particular advantage of the method results from the combination of the Ag electrode and the applied voltage which allows the detector to be selective for iodide and ignore possible anions. Figure 1 shows several anions using UV detection at 210 nm while Figure 2 shows the same anions using the Ag electrode. Figure 3 shows the chromatogram for a human serum extract.

Finally, Table 3 outlines some data obtained from the serum of six subjects.

Pooled serum samples were run everyday for a two week period to also evaluate the method's precision. These data indicated an average concentration of 4.51 µg/dl with a variance of 0.17 µg/dl and a coefficient of variation of 3.6%

These serum iodide values show good agreement with the literature values from several sources which indicate the values for serum iodide range from 3 to 8 µg/dl.(5)

Discussion

All of the current clinical procedures involve the determination of Protein Bound Iodine (PBI). It is therefore necessary to convert the iodine to its inorganic form by either wet or dry ashing with the final determination based on the catalytic activity of iodide (Kotthoff-Sandell reaction). This is a redox reaction between cerium (IV) and arsenic (III) which is catalyzed by iodide. If all parameters are kept constant, the concentration of iodide is proportional to the change in absorbance at 420 nm. This assay is not without problems due to its

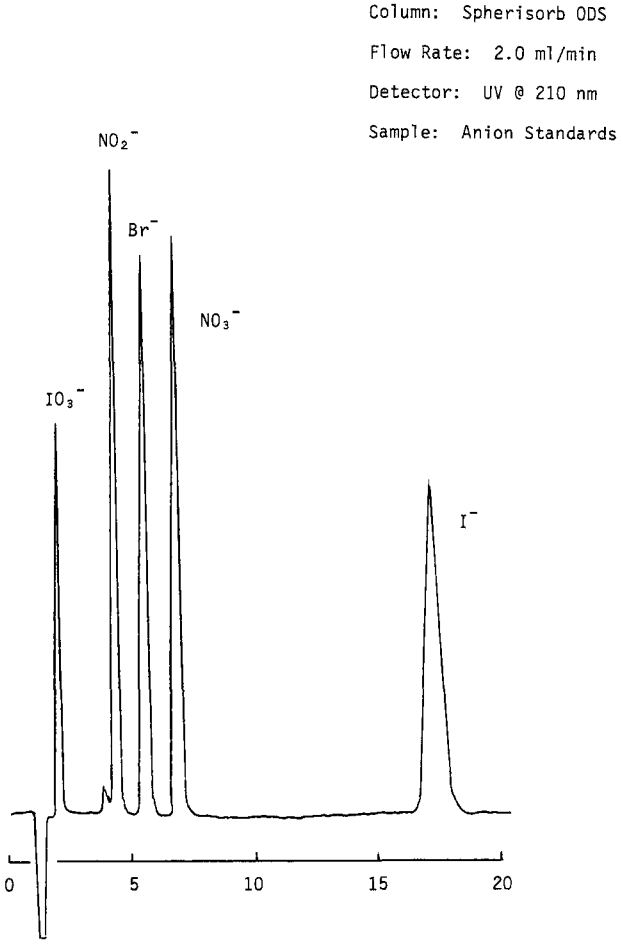

Figure 1
Chromatogram of Anion Standards with UV Detection

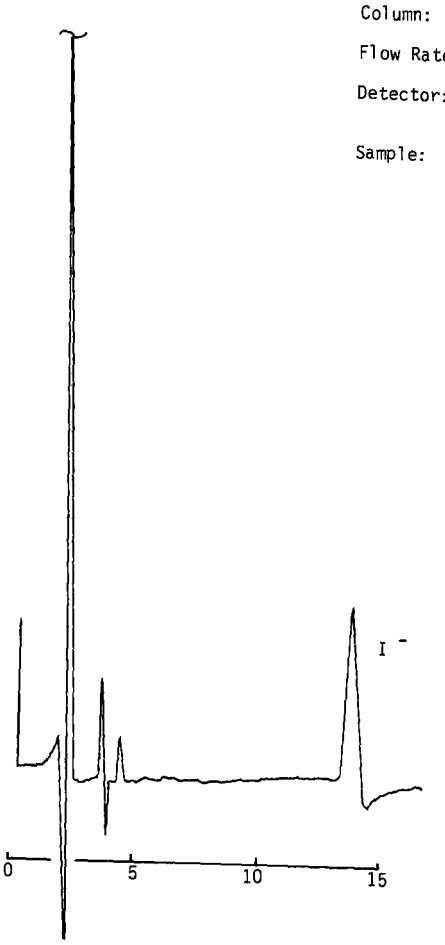

Figure 2

Chromatogram of Standards with Electrochemical Detection

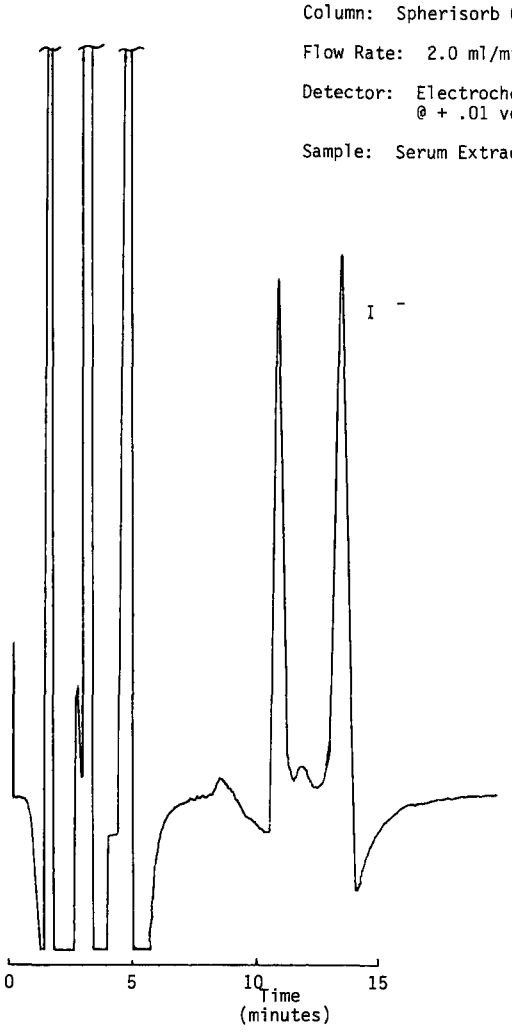

Figure 3

Chromatogram of Serum Extract

Table 3

Serum Iodide Concentration of Six Subjects

Subject	Iodide Conc. (µg/dl
1	10.41
2	8.30
3	9.42
4	11.72
5	7.10
6	6.54

catalytic nature. All parameters must be kept constant or erroneous data can result. Additionally, the entire detection system cannot come in contact with metals since any contamination will reduce the cerium (IV) and cause a decolorization of the solution.(6)

In some cases, it is not desirable to analyze serum for total iodide but to analyze for butanol-extractable iodine (BEI) which eliminates interferences. This procedure involves the extraction of an acidified serum with butanol and a back extraction of the butanol with Blau reagent to eliminate interfering substances.(3,4) The butanol fraction is removed by evaporation and the extract is then digested prior to its final determination. Preliminary studies in our laboratory indicate that this assay can be accomplished by extraction of the serum with butanol, removal of the butanol followed by the addition of buffer prior to final HPLC determination.

The concept of ion chromatography using a variety of detectors, mobile phases and supports has been well documented.(7,8) The present study demonstrates that the use of a silver electrode provides a selective and sensitive surface for the detection of iodide and for the determination of serum iodide. The results indicate that there is little contamination of the electrode surface after long periods of use. In our laboratory the electrode was used for over 600 injections of standard and sample before cleaning. Even at that point, the cleaning consisted of repolishing the electrode with alumina and washing with water and methanol. The electrode was then reassembled and used. A precolumn was used prior to the

analytical column and was packed with large particle C_{18}.(9) This seems to add to column and electrode longevity. The column was used for the same number of injections and showed no deterioration using either peak height or column plates. The disodium EDTA was added to the mobile phase to passivate the system and is recommended by the manufacture of the detector.(10) Reduction in the baseline noise before and after the addition of this compound supports this observation. Additional noise reduction was accomplished by temperature control.

The procedure described provides an attractive alternative for the determination of iodide in serum and was successfully applied to determination of serum iodide in other species. These data will be published elsewhere.

The method described eliminates many of the problems associated with this assay. It seems as if the addition of water and acetonitrile serves to liberate the iodide and reinforce the 1948 study of Taurog and Charkoff who reported that iodide in plasma is not stably bound to protein.(11)

The data presented indicate that the method described provides a useful alternative to the current methods for the determination of serum iodide. It is accurate and precise with excellent recovery of iodide. Iodide determinations are routinely not done by most laboratories due to the complexity of the assay. This assay would permit serum iodide determinations to become feasible in laboratories with HPLC equipment.

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ENHANCEMENT OF LCEC RESPONSE BY USE OF ELECTROCHEMICALLY PRETREATED GLASSY CARBON ELECTRODES

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ABSTRACT

A simple electrochemical pretreatment procedure has been shown to increase the selectivity of LCEC detection by lowering the required operating potential compared to that needed at conventional untreated glassy carbon. The effect of a conditioning procedure consisting of sequential application of brief positive and negative potentials to a polished glassy carbon surface was examined for a group of model LCEC analytes including p-hydroquinone, dopamine, dihydronicotinamide adenine dinucleotide (NADH), ascorbic acid, and hydrazine. The lowering of the potential required for detection of these species was greatest for the irreversibly oxidized systems which exhibit the largest overvoltage at untreated glassy carbon--namely, NADH, ascorbic acid, and hydrazine. For these species at pretreated electrodes, comparable detector response was achieved using potentials 0.2 to 0.5 V less than that otherwise needed for optimum response. The improved performance of the electrochemically pretreated electrodes was maintained over a wide range of pH conditions and mobile phase compositions and dramatically facilitated the determination of the latter set of compounds in physiological matrices.

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INTRODUCTION

In recent years, electrochemical measurement techniques have been shown to offer a useful approach for the detection of numerous easily oxidized or reduced compounds following high performance liquid chromatography (1,2). In practice, of course, the success with which such liquid chromatographic/electrochemical detection (LCEC) methods can be applied is determined primarily by the electrode potential required to effect oxidation or reduction of the compound of interest. Thus, LCEC has found its principal applications for the determination of easily oxidized species such as catechols and aromatic amines which require the use of modest positive potentials where the typical background current is low and few electrolyzable interferences are likely to be encountered. Unfortunately, many other compounds of considerable analytical interest undergo electrolysis only at activation-controlled rates and, as a result, at potentials drastically exceeding their thermodynamic redox potentials. For these compounds, detection by LCEC cannot provide optimum levels of sensitivity and selectivity and, in extreme cases, can provide no useful quantitation at all.

Consequently, considerable effort has been directed toward the development of electrode systems which exhibit enhanced response towards these activation-controlled systems. In this regard, both chemical modification of the native electrode surface by attachment of catalytic mediator molecules (3,4) and electrode conditioning by chemical (5), thermal (6), and electrochemical (7-13) treatment have been utilized. Of these possibilities, the electrochemical pretreatment procedures, most of which consist of the imposition of alternating oxidizing and reducing potentials at carbon electrodes, represent the simplest and most widely applicable approach. In this laboratory, such an electrochemical conditioning sequence has been shown to make LCEC detection of simple hydrazines attractive, lowering the potential required for their detection by 0.5 to 1.0 V and yet permitting detection limits two to three orders of magnitude lower than previously achieved at conventional untreated

electrodes (14). However, aside from this one instance, the potential of electrochemical pretreatment procedures to improve LCEC detection has not been seriously considered. Accordingly, in this paper, we will investigate the general utility of electrochemical pretreatment for use in LCEC. The effect of electrode pretreatment on the LCEC determination of a series of standard compounds including dopamine, p-hydroquinone, ascorbic acid, hydrazine, and dihydro-nicotinamide adenine dinucleotide (NADH) will be characterized; and it will be shown that this simple procedure can significantly decrease the potential required for detector operation. The effect is most pronounced for electrochemically irreversible analytes such as ascorbic acid, hydrazine, and NADH which exhibit a high overvoltage at the ordinary electrode surface. As a result, LCEC quantitation of these species in real sample matrices containing a complex spectrum of possible interferents can be significantly simplified.

MATERIALS AND METHODS

Chemicals

NADH (Grade III), ascorbic acid, and dopamine hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO). Hydroquinone was obtained from Fisher Scientific Co. Practical grade n-octylamine was obtained from Eastman Kodak Co. (Rochester, NY). All were used as received without any further purification. Deionized water was used for all solution preparation.

The buffer medium used during electrode pretreatment usually consisted of a solution of 0.1 M KNO_3 and 0.01 M Na_2HPO_4 with the pH adjusted to 7 with 0.015 M HNO_3. When other buffers were used for pretreatment or for cyclic voltammetry (CV), they were prepared from KH_2PO_4 and Na_2HPO_4 according to reference 15.

To inhibit sample degradation, ascorbic acid, dopamine, and hydroquinone sample solutions always contained 0.05 M $HClO_4$. In addition, fresh standards were made each day. Urine samples were

filtered through a 0.27-μ Gelman glass filter and, for ascorbic acid determinations, were diluted with an equal volume of 0.05 M $HClO_4$ prior to chromatographic analysis.

Instrumentation

CV experiments and electrode pretreatment were performed with a Bioanalytical Systems (West Lafayette, IN) Model CV-1B potentiostat and Model MF-2012 glassy carbon working electrode. A three-electrode cell was employed with an Ag/AgCl reference and a Pt counter electrode. The pretreatment procedure used was similar to that reported by Engstrom (12). First, the electrode was polished three times with a 5-μ alumina slurry for one minute and rinsed thoroughly each time with deionized water. To eliminate the possibility of Al_2O_3 catalysis as reported recently by Kuwana (16), the electrode was then thoroughly sonicated in deionized water. The electrochemical pretreatment itself consisted of holding the electrode first at +1.75 V vs. SCE for five minutes and then at -1.2 V for ten seconds. Unless otherwise indicated, the electrode was immersed in pH 7 phosphate buffer during this potential sequence.

The liquid chromatograph employed consisted of a Waters Associates Model M-600 or a Perkin-Elmer Model LC-10 pump, a Rheodyne Model 7125 injector, a Waters Associates Model 440 UV detector, and an IBM Model EC-230 electrochemical detector with a Bioanalytical TL-5 thin layer glassy carbon electrode assembly. All chromatography was performed on an octadecylsilane column of 10-μ, irregular particles (Alltech Associates, Deerfield, IL). A home-made silica (Adsorbosil, Mesh 200/425, Alltech Associates) presaturation column was placed between the pump and the injector in order to prevent the neutral mobile phase from attacking the analytical column. A octadecylsilane guard column (Brownlee Labs, Santa Clara, CA) was used when urine samples were injected. Unless otherwise specified, the flow rate used in all chromatographic experiments was 1.0 ml/min.

RESULTS AND DISCUSSION

Five compounds were selected for use as test systems to evaluate the effectiveness of electrode pretreatment to enhance the analytical performance of LCEC: dopamine, p-hydroquinone, ascorbic acid, hydrazine, and NADH. All of these species can be oxidized at glassy carbon electrodes and have previously been the objects of LCEC analysis. Of this group, dopamine and hydroquinone are oxidized quasi-reversibly at low positive potentials and thus represent ideal candidates for LCEC. The other three systems are electrochemically irreversible; and, although LCEC has been employed with some success for each, significant improvements might be expected to result from prior electrochemical conditioning of the sensing electrode.

Cyclic voltammetry (CV) was employed to survey the effect of electrochemical pretreatment on all five systems. The results obtained for CV performed in pH 7 buffer both before and after pretreatment of the glassy carbon electrodes are summarized in Table 1. In general, this data is consistent with results previously obtained at pretreated glassy carbon electrodes by Engstrom (12) and in our own laboratory (14). All of the oxidations were shifted to lower potentials; and, in general, the CVs exhibited sharper peaks which resemble more closely the shapes expected for ideal reversible electrode processes. The shifts in peak potential (ΔE_p's) were more pronounced for the irreversible systems displaying the highest overvoltage at the untreated electrode. Thus, the hydrazine oxidation changed from a broad, poorly defined wave at +0.85 V vs. Ag/AgCl at untreated glassy carbon to a sharp, well-shaped peak at +0.30 V at the pretreated electrode. It was these consistent and sometimes dramatic shifts to lower potentials which initially attracted our attention to the possible advantages of employing electrochemical pretreatment procedures in LCEC.

If the pretreated electrodes are to be employed in practical chromatographic applications, it is necessary that the performance

TABLE 1

Effect of Electrode Pretreatment on Cyclic Voltammetry Response

Analyte	Ep (Oxidation), V vs. Ag/AgCl		ΔE_p,mV
	Before Pretreatment	After Pretreatment	
Hydroquinone	+0.18	+0.11	70
Dopamine	+0.21	+0.19	20
Ascorbic Acid	+0.30	+0.00	300
NADH	+0.56	+0.41	150
Hydrazine	+0.85	+0.30	550

enhancements of Table 1 are retained under a variety of solution (or mobile phase) conditions. It has already been demonstrated that the effects of pretreatment are unaltered by the presence of significant proportions of organic solvents such as methanol and acetonitrile in the electrolysis medium (14). However, as all previous work with the pretreated electrodes has been carried out only in neutral solutions, their performance in solutions of various pH also needed to be examined. Accordingly, the voltammetric behavior of several of the test systems at electrodes pretreated as above in pH 7 buffer was further characterized by obtaining the CVs in solutions of different pH and comparing the results with the behavior under the same conditions at untreated electrodes. The behavior at electrochemically conditioned electrodes, summarized for dopamine and ascorbic acid in Table 2, was again directly analogous to the well-known redox behavior of these compounds at the untreated surfaces. In all cases examined, pH-dependent shifts in the peak potentials observed for the oxidations at the untreated electrodes were paralleled by shifts in the same direction and of roughly the same magnitude at the pretreated surfaces. At the same time, the enhancement resulting from the pretreatment procedure occurred uniformly over the entire pH range employed as evidenced by the roughly constant ΔE_p's observed at each pH. Thus, for example, ascorbic acid oxidation at both electrode surfaces showed little or no change in potential between pH 7 and 4 but shifted, as expected, to more positive

Table 2

Effect of Solution pH on Treated and Untreated Electrode Response

pH	DOPAMINE[a]		ASCORBIC ACID	
	E_p Before Pretreatment, V vs. Ag/AgCl	E_p After Pretreatment, V vs. Ag/AgCl	E_p Before Pretreatment, V vs. Ag/AgCl	E_p After Pretreatment, V vs. Ag/AgCl
7.0	+0.21	+0.19	+0.30	+0.00
6.1	+0.30	+0.25	+0.26	-0.05
5.0	+0.42	+0.34	+0.30	+0.06
4.3	+0.46	+0.38	+0.31	+0.07
3.1	+0.54	+0.44	+0.38	+0.13

[a]Data is given here for the oxidation only.

potentials at lower pH values. But, over the entire pH range, ΔE_p for the oxidation between the two surfaces remained relatively constant (240 to 300 mV). For hydrazine at untreated glassy carbon, the hydroxide-catalyzed oxidation shifted rapidly to more positive potentials as the solution was made more acidic. The effect was so great that, at pH values less than 5, the hydrazine oxidation consisted only of an extremely broad and greatly reduced wave whose plateau occurred at potentials well in excess of +1.0 V but whose "peak" position could not be clearly resolved. At the pretreated electrode, pH decreases likewise resulted both in severe broadening of the hydrazine oxidation and in shifts to higher potentials. However, even at pH 4.1, the plateau position occurred at a potential of only +0.61 V.

In view of the encouraging performance of electrochemically pretreated glassy carbon in decreasing the overvoltages observed in CV for the test analytes, the identical electrode conditioning operations were subsequently applied in LCEC and evaluated for the same set of compounds. The mobile phase composition employed for each compound was selected individually on the basis of previously reported chromatographic analyses (14,17-19) and is summarized in Table 3. No attempt was made to utilize identical chromatographic conditions for all systems because the direct application to a variety of already established procedures was deemed to provide

TABLE 3

LCEC Response at Treated and Untreated Electrodes

Analyte	Mobile Phase	$E_{plateau}$, V vs. Ag/AgCl		Detection Limit (ng injected)	
		Before Pretreatment	After Pretreatment	Before Pretreatment	After Pretreatment
Hydroquinone	pH 7 buffer/ 20% methanol	+0.60 V	+0.50 V	0.30	0.30
Dopamine	pH 3.8 phosphate-citric acid buffer/ 5% acetonitrile	+0.60 V	+0.45 V	0.20	0.20
Ascorbic Acid	pH 5 acetate buffer, 1 mM octylamine, 200 mg/l EDTA	+0.60 V	+0.35 V	0.12	0.06
NADH	pH 7 buffer/ 5-20% methanol	+0.80 V	+0.60 V	1.2	0.70
Hydrazine	pH 7 buffer	>+1.0 V	+0.50 V	--	0.01

a better test of the practical utility of the electrode pretreatment approach as a general means of providing enhanced LCEC response. Typical responses obtained for NADH and ascorbic acid are shown respectively in Figures 1 and 2. Each of the chromatograms was recorded using a detector potential of +0.30 V vs. Ag/AgCl both for the polished but untreated and the electrochemically conditioned glassy carbon surfaces. With both analytes, greatly increased current levels were observed for the surfaces which had been subjected to the pretreatment process. Further, the response enhancement was quite long-lived as was evidenced by the chromatograms obtained for ascorbic acid at the same pretreated surface over the 5-hour observation period employed (Figure 2).

The LCEC data presented in Table 3 match closely the behavior expected for the pretreated electrodes on the basis of the CV data of Table 1. Again, lower potentials were required for detection of each of the test analytes--with the decrease greatest for the irreversible systems showing the largest overvoltage at the untreated electrode. As shown in Figure 3, hydrodynamic voltammograms (HDVs) observed upon pretreatment for dopamine and hydroquinone showed only comparatively small shifts which were of relatively minor importance since both compounds are already detectable before pretreatment at modest detector potentials. On the other hand, the shifts observed for the other systems were sufficiently large that significant reductions in the potentials required for optimum LCEC analysis resulted. The most dramatic example was, of course, hydrazine for which electrode pretreatment has been shown to decrease the potential required from +1.0 V vs. Ag/AgCl to +0.5 V (14).

In general applications, we expect that the most important advantage resulting from the use of pretreated electrodes for LCEC analysis will be increased selectivity due to the greatly decreased number of interferents at the lower values of applied potential made possible by the pretreatment procedure. For hydrazine, this selectivity improvement was accompanied by a

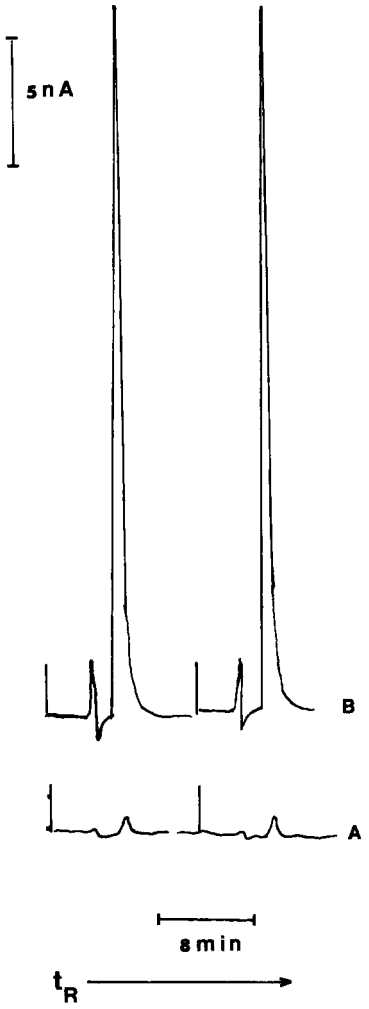

FIGURE 1. Chromatograms obtained at +0.30 V vs. Ag/AgCl for repeated injection of 30 ppm solution of NADH at (A) untreated and (B) electrochemically pretreated glassy carbon electrodes. Injection volume: 20 µl; flow rate: 2.0 ml/min.

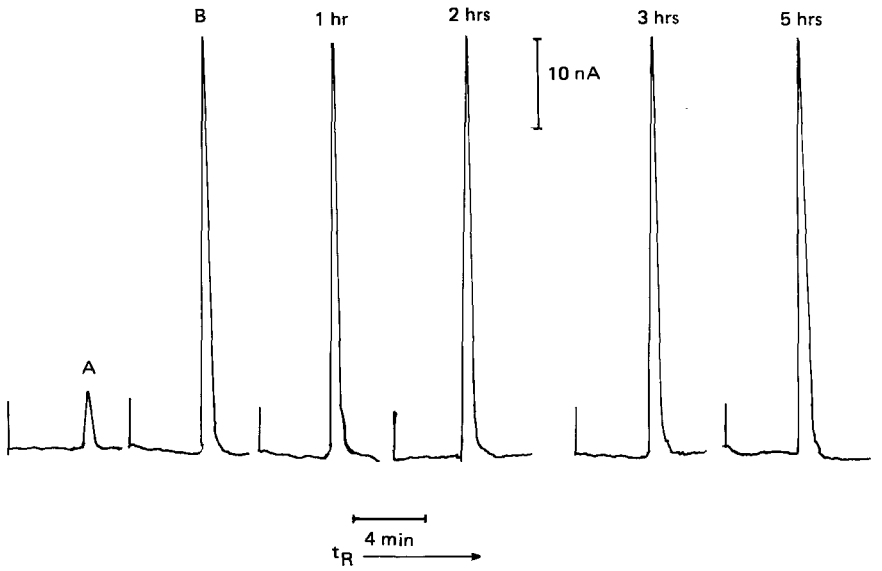

FIGURE 2. Chromatograms obtained a +0.30 V vs. Ag/AgCl for repeated injections of 10 ppm ascorbic acid solution at (A) untreated electrode and (B) electrochemically pretreated electrode. Injection volume: 6 μl.

decrease in the detection limit by a factor of more than 100 (14). However, as expected from the similar current levels attained at the HDV plateaus for both the pretreated and untreated surfaces in Figure 3 and shown by the actual detection limits reported in Table 3, absolute sensitivities for the other analytes were enhanced by a factor of 2 at best by the electrochemical conditioning. Additional examples demonstrating the increased selectivity for the detection of NADH and ascorbic acid are provided in Figures 4 and 5. In the first example, NADH was added at the 50 ppm level to the urine of a healthy volunteer; and chromatograms were recorded under a variety of detector conditions. The only sample treatment performed prior to injection consisted of filtration through a 0.27-μ glass filter. Curves 4B and 4D illustrate the results obtained at both untreated and electrochemically pretreated

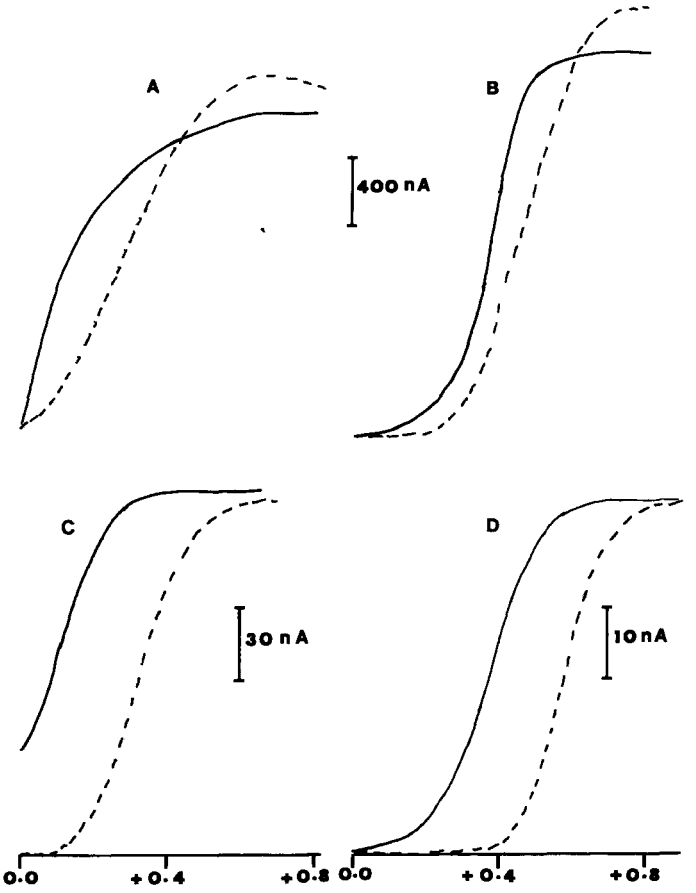

FIGURE 3. Hydrodynamic voltammograms for (A) hydroquinone (60 ppm), (B) dopamine (100 ppm), (C) ascorbic acid (10 ppm), and (D) NADH (20 ppm) at untreated (---) and electrochemically pretreated (——) glassy carbon electrodes. See Table 3 for chromatographic conditions.

electrodes at +0.80 V vs. Ag/AgCl, the potential ordinarily prescribed for optimum detection of NADH by LCEC (19). In both cases, the quantitation of the NADH was impractical as the NADH signal (retention time of 16 minutes) was severely obscured by the large background currents due to the numerous early eluting sample components oxidizable at this relatively high potential. When the background signals were reduced to a more manageable level by lowering the applied potential to +0.30 V, no NADH peak at all could be observed at the untreated surface (curve 4A). At the pretreated electrode, however, a smaller but nevertheless quite usable peak, now largely resolved from the background, was still in evidence.

In the second example, the chromatographic response upon injection of a urine sample doped with 10 ppm ascorbic acid is shown for several LCEC situations (Figure 5). In all cases shown, the chromatographic background was greatly reduced compared to that seen in Figure 4 because this urine sample had been obtained from a volunteer who had been fasting for the preceding 24-hour period. The chromatographic conditions employed were the same as those suggested by Kissinger (18) and detailed for ascorbic acid in Table 3, and the only sample treatment consisted of filtration and dilution with 0.05 M $HClO_4$. The chromatograms in Figures 5A and 5B represent the results obtained using a standard LCEC approach employing an untreated glassy carbon electrode held respectively at +0.30 V and +0.60 V vs. Ag/AgCl. The ascorbic acid peak, occurring at a retention time of about five minutes, was observed at both potentials but, as expected from the HDV provided earlier for the compound in Figure 3, was severely reduced at the lower potential. A second, more slowly eluting peak, observed only at the higher potential, was identified as being due to uric acid (which undergoes oxidation at about +0.4 V). Since the ascorbic acid and uric acid peaks fortuitously were well resolved under these chromatographic conditions, this potential could in fact have been employed successfully for ascorbic acid quantitation. However, had the two species possessed more similar retention properties, a

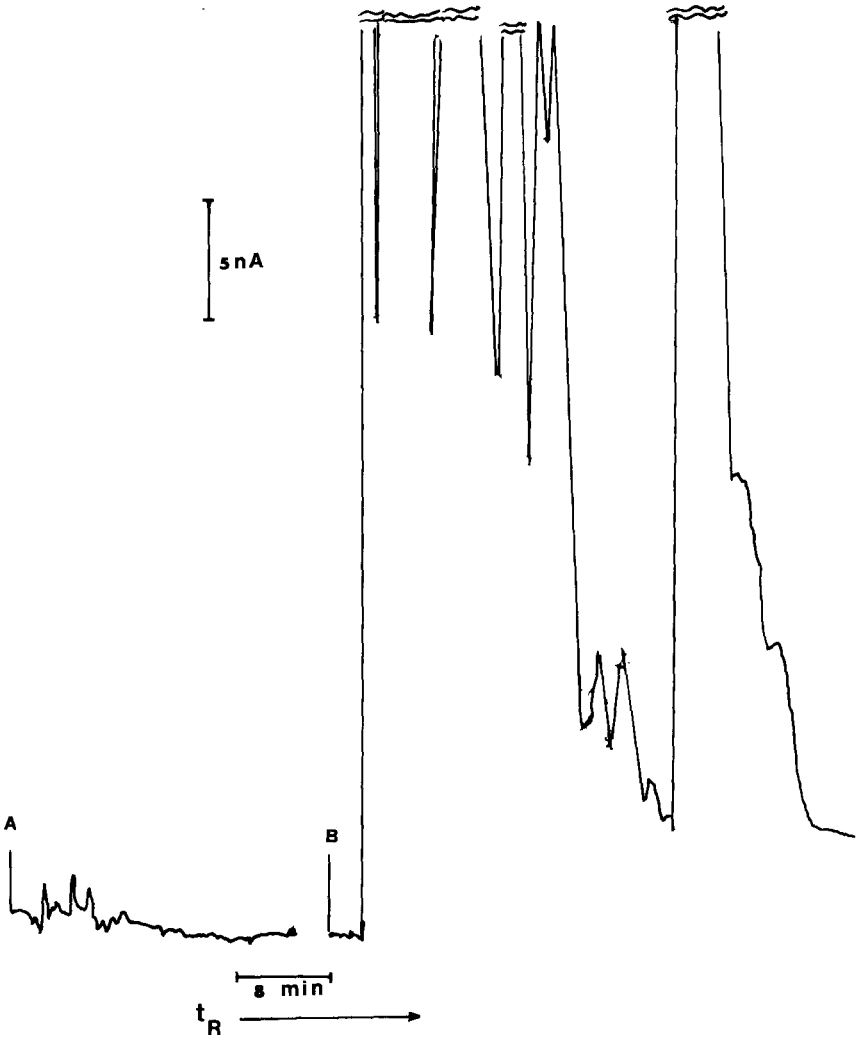

FIGURE 4. Effect of electrochemical pretreatment on LCEC response for a urine sample doped with 50 ppm NADH. (A) untreated electrode at +0.30 V; (B) untreated electrode at +0.80 V; (C) pretreated electrode at +0.30 V; (D) pretreated electrode at +0.80 V. All potentials are reported vs. Ag/AgCl. Injection volume: 20 µl.

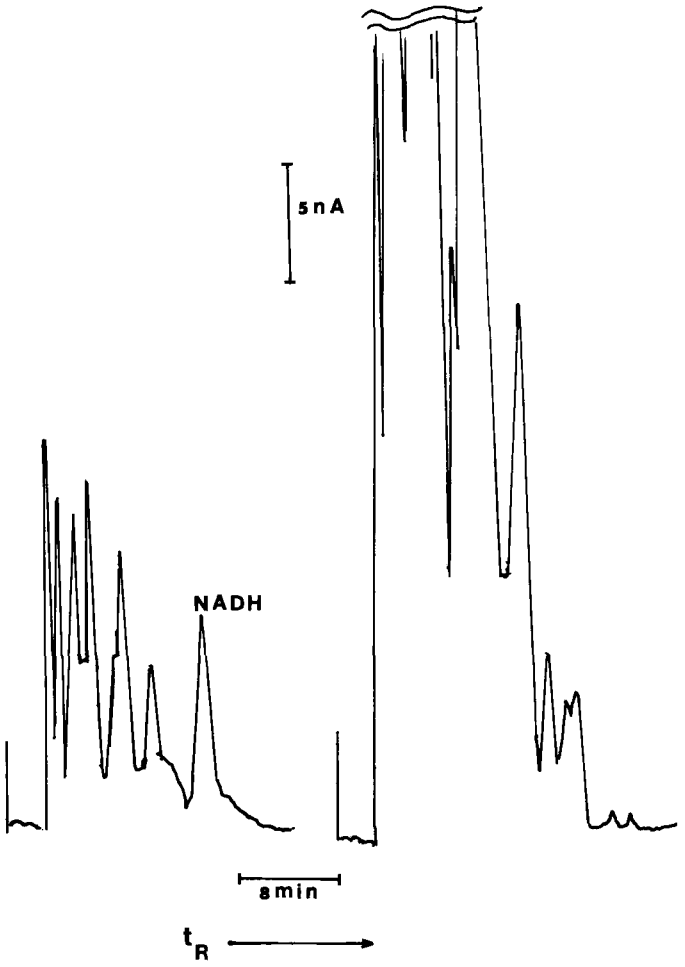

FIGURE 4 (continued)

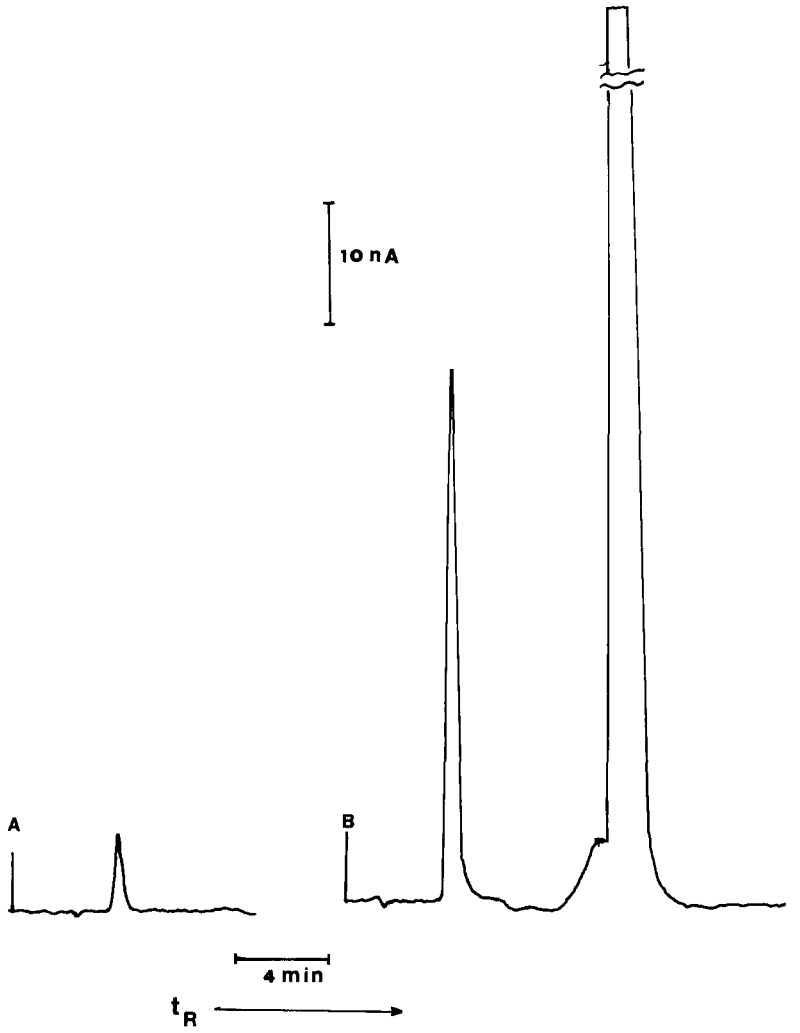

FIGURE 5. Effect of electrochemical pretreatment on LCEC response for a urine sample doped with 10 ppm ascorbic acid. (A) untreated electrode operated at +0.30 V; (B) untreated electrode at +0.60 V; (C) pretreated electrode at +0.30 V; (D) pretreated electrode at +0.60 V. All potentials are reported vs. Ag/AgCl. Injection volume: 6 µl.

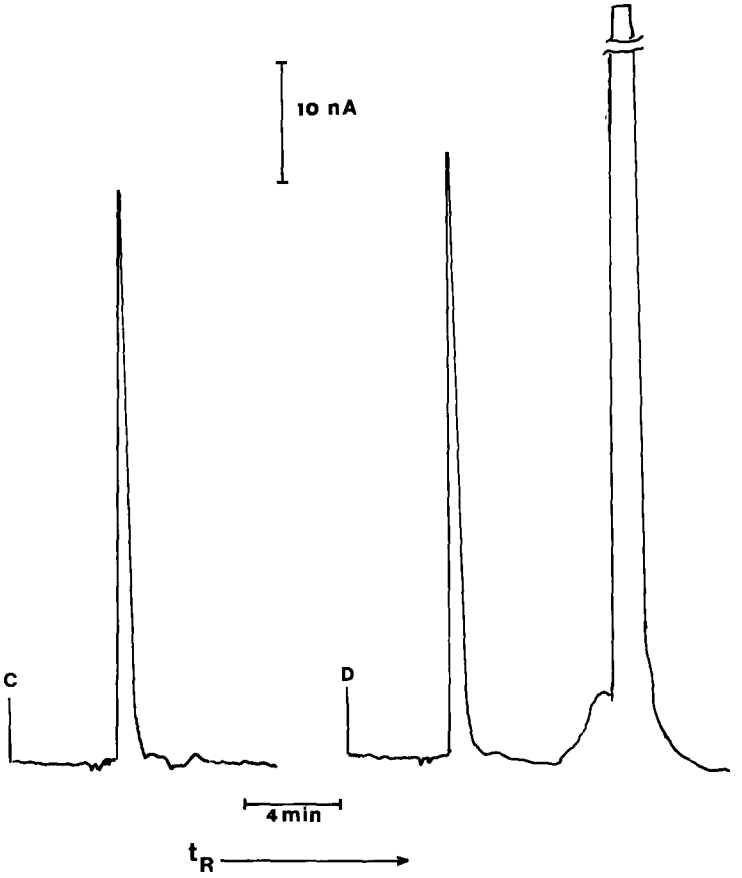

FIGURE 5 (continued)

serious interference would have resulted. Figures 5C and 5D show equivalent chromatograms obtained at electrochemically pretreated glassy carbon. The response at +0.60 V was virtually the same as that seen in curve 5B for the conventional electrode. But the chromatogram taken at +0.30 V (which corresponds to the HDV plateau for ascorbic acid at the pretreated electrode) not only showed no response for the uric acid but also exhibited nearly as much current response for ascorbic acid as was observed for the

untreated electrode at +0.60 V. Thus, quantitation of ascorbic acid could be accomplished at the pretreated surface with no significant loss of sensitivity without concern over the presence or retention behavior of the uric acid. In fact, the ascorbic acid peak observed even at +0.10 V was reduced by only approximately 30%. Operation at this extremely low potential thus offers a unique route to analysis which is both acceptably sensitive and, at the same time, tremendously selective.

An additional advantage of the electrochemical pretreatment approach is the ease with which the electrode modification involved can be performed in practice. As described in the experimental section, the pretreatment process itself required only about five minutes to carry out and could even be performed in situ while the electrode was simply set up as usual for LCEC and exposed to the chromatographic mobile phase. Further, the performance enhancements which resulted were particularly long-lived as the glassy carbon electrodes, once conditioned, generally showed no observable decrease in detector current even over 8 to 10 hours of continuous chromatography. In addition, the process was quite versatile, showing little sensitivity to the particular medium in which the pretreatment was conducted. Although all of the examples cited previously were performed with electrodes pretreated in pH 7 phosphate buffer, virtually no limitations on the pretreatment medium were observed in our work. Identical enhancement in electrode performance was obtained regardless of whether the electrode was immersed in solutions ranging from pH 3 to 7.

CONCLUSION

Relatively simple electrochemical pretreatment has been shown to comprise a generally applicable approach to achieve enhanced analytical response in LCEC determinations employing glassy carbon working electrodes. The primary effect produced by such pretreatment procedures consisted of a shift of the electrode processes examined to less extreme potentials than were otherwise required. Although the resulting increases in selectivity and

sensitivity depended greatly on the specific nature of the analyte system under consideration, significant improvements were found for systems which exhibited extremely irreversible behavior at the conventional untreated electrode surface. In addition, the enhancements resulting from electrode pretreatment occurred over a wide range of pH and solution conditions similar to those usually employed in reverse-phase HPLC.

This study is not meant to suggest that electrochemical pretreatment can be expected to produce dramatic improvements in LCEC response for all species currently determined by this technique. Rather for well-behaved electrochemical systems such as dopamine and hydroquinone which are oxidized at relatively low potentials at untreated glassy carbon, only modest improvements at most are to be anticipated. However, considering the practically insignificant investments in time, equipment, and experimental convenience required by the electrode conditioning procedures employed here, we believe that anyone involved in glassy carbon LCEC should at least consider their use.

ACKNOWLEDGMENT

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USE OF AN INTERNAL STANDARD TO ASSAY 6 β-HYDROXYCORTISOL IN URINE

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ABSTRACT

Currently 6 β-hydroxycortisol is assayed by radioimmunoassay or high performance liquid chromatography techniques. We have developed an HPLC method, utilizing gradient elution and an internal standard (Δ 4-pregnenetetrol-3-one). In this way, accuracy and sensitivity of the assay were greatly improved and allowed the application of this modified method for monitoring the time-course of hepatic microsomal enzyme activity.

INTRODUCTION

A polar metabolite of cortisol, 6 β-hydroxycortisol (6 β-OHF) is formed in the liver during the phase I of biotransformation reactions. The binding of this metabolite to plasma proteins is low and it is excreted in the unconjugated form in urines. Measurement of 24h urinary 6 β-OHF excretion is a non invasive method and would appear to be one of the best endogenous index of hepatic oxidation of drugs in vivo (1). It is particularly useful to assess the influence of inducers (barbiturates, rifampicine, antiepileptic drugs) or inhibitors (cimetidine) of drug metabolism on the enzyme activity of the microsomal system.

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Therefore it was of great interest to develop an accurate and easy method for quantitative determination of urinary 6 β-OHF.

EXPERIMENTAL

Reagents. All reagents were of analytical grade and were used without any further purification.

6 β-hydroxycortisol (Δ 4-pregnene-6β, 11β, 17α, 21 tetrol-3,20 dione) was supplied by Steraloids Inc. (Pawling, NY, USA) and the internal standard (Δ 4-pregnene-11β, 17α, 20α, 21 tetrol-3-one) by Sigma (St-Louis, MO, USA) abbreviated as Δ-4). 16 α-hydroxy hydrocortisone was kindly supplied by Roussel Uclaf (Romainville, France). Stock solutions of 6 β-OHF and the internal standard were prepared in absolute ethanol and when stored at +4°C, were stable for at least 4 months.

Equipment. The HPLC system was from Dupont Instruments with a pump module 841, a variable-wavelength UV/VIS detector (837 model) operating at 243 nm, and a programmable gradient 838 model (Wilmington, DE, USA). The detector was connected to a Shimadzu C-R1A Chromatopac integrator (Shimadzu Corporation, Kyoto, Japan). The column was a Rsil C 18 HL (10 µm or 5 µm), 25cm x 4.6mm i.d. stainless steel (RSL, Eke, Belgium). The injection valve was a Rheodyne 7010 model fitted with a 200 µl sample loop.

Sample preparation. Urines were stored frozen at -20°C prior to analysis. Extraction of 6 β-OHF was performed as described previously by Frantz and al. (1), from 10 ml portions of urine after addition of 200 µl of the internal standard (5 mg Δ-4 in 50 ml of absolute ethanol).

Calibration curve. The calibration curve was established as follows : 10 ml portions of distilled water, instead of urine, containing increasing concentrations of 6 β-OHF were subjected to the extraction procedure des-

cribed for urine and the data were plotted on the calibration curve (peak area ratio method). The curve obtained by the least-squares was linear over the range used (1 µg to 15 µg per 10 ml). The linear regression value was : r = 0.995 (n = 6).

Analysis. The mobile phase consisted of a solution of KH_2PO_4 0.01 M which was freshly prepared in distilled water and to which 0.05 % trichloroacetic acid 1.7 N was added. Subsequently acetonitrile (Baker UV grade) was added to the prepared solution in the proportions : 9/1 buffer/acetonitrile (v/v) for reservoir A and 7,5/2,5 buffer/acetonitrile (v/v) for reservoir B (Eluent A and B respectively).

The analysis was performed by using a linear elution gradient flow over a period of 10 minutes. The gradient flow was started 3 minutes after the injection of the sample.

The flow-rate was about 1.5 ml min^{-1}, corresponding to a pressure of 2000 psi. After addition of 500 µl of eluent A to the dry extract, 200 µl were injected through the sample loop of the HPLC system. The concentration of the urinary 6 β-OHF was calculated from the peak area ratio compared to the calibration curve.
All procedures were carried out at room temperature.

RESULTS

Our first results were obtained with a 10 µm particles column packing. The retention times of 6 β-OHF and of the internal standard (Δ-4) were 10.0 min and 18.5 min respectively.

Sharper peaks but longer retention times were obtained if the column was packed with 5 µm particles (15.7 and 28.5 min for 6 β-OHF and the internal standard respectively). A typical chromatogram of a urine sample obtained with this last column is shown in the Fig. 1.

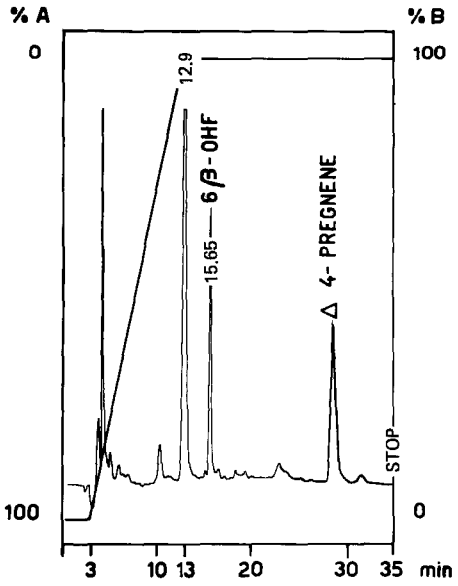


FIGURE 1. Typical chromatogram of a urine sample containing the internal standard (Δ 4), with illustration of the time-course crossing from eluent A to eluent B

Assuming the magnitude of the signal to noise ratio to be at least of the order of three, the detection limit of 6 β-OHF corresponds to a concentration of 10 ng ml^{-1}. This limit could be increased still further by carrying out the extraction step on larger volumes of urine.

The reproducibility of the method was investigated with aliquots of urine containing low, moderate and high concentrations of 6 β-OHF (Table 1). When urines were kept frozen, no significant decrease in 6 β-OHF values was observed after a period of 6 months.

Recovery of the internal standard (Δ-4) from urine was found to be above 95 %. In addition to Δ-4, the use of 16 α-hydroxycortisol as an internal standard was investigated.

Table 1. Reproducibility and accuracy of the method expressed as the coefficient of variation on 6 samples (for high values, the samples were different) ; 6 β-OHF concentrations were given in μg per 10 ml of urine.

Concentration range of β-OHF	Intra-assay	C.V. %	Inter-assay	C.V. %
Low	2.74 ± 0.09	7	2.73 ± 0.07	5
Moderate	5.38 ± 0.11	6	5.29 ± 0.24	9
High	13.13 ± 0.43	6	9.87 ± 0.19	5

A blank and a spiked urine containing both standards are shown in Fig. 2.

DISCUSSION

For clinical purposes, measurements of urinary 6 β-hydroxycortisol from a 24h collection require a simple, rapid and reproducible method. Others have described methods utilizing radioimmunoassay (2,3,4) and high performance liquid chromatography (5,6). A comparison of the two methods was done by Gerber-Taras and al. (7). Both gave identical values and reliable intra-assay variation. Those authors who utilized HPLC technique measured the concentrations of urinary 6 β-OHF by comparison with a standard solution run daily or with spiked urine samples.

To our knowledge, a true internal standard in order to eliminate the losses during the processing of the urines has not yet been described as so far.

We have improved the HPLC method for quantitative determination of urinary 6 β-OHF by including Δ4-pregnene-tetrol-3-one in the samples analyzed.

The use of a gradient elution allowed a fairly good separation of 6 β-OHF and to elute two internal standards

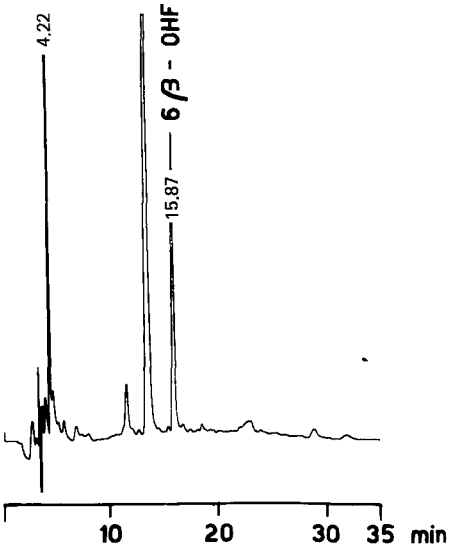
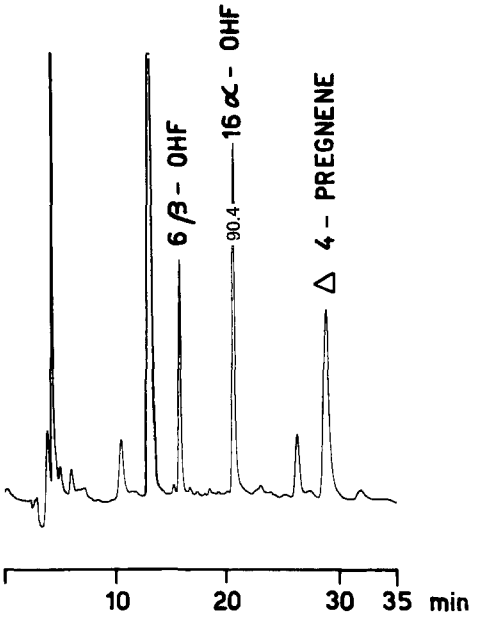


FIGURE 2a) Chromatogram of a blank urine



2b) Chromatogram of a urine spiked with 16 β-OH cortisol and Δ4-pregnene

from the corticosteroids family which are normally absent from urines. Unfortunately, 16 α-OHcortisol was unstable in ethanol solution (evidenced by the additional peak eluting just before Δ4-pregnene, in Fig. 2b).

There is little hope to speed-up the analysis with the 5 μm particles column packing unless we added a higher proportion of acetonitrile to eluent B, but increasing the risk to contaminate the 6 β-OHF peak.

Mean values of urinary 6 β-OHF in 23 healthy volunteers of both sexes aged 18 to 25 years were 199 μg ± 17/24h (M ± SEM). There was no significant difference between male and female volunteers.

ACKNOWLEDGMENTS

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DEUTERATED SOLVENTS AS MOBILE PHASE IN MICRO-HPLC

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ABSTRACT

The chromatographic performance of deuterated solvents, heavy water and deuteriobenzene, has been investigated in reversed phase and normal phase micro-HPLC. The performance of heavy water in separation was comparable or superior to that of light water in reversed phase mode. A slight improvement was also observed in catecholamines separation with heavy water. The performance of deuteriobenzene was observed as complicated in metal complexes separation, but a little difference was present. From those results, the combinations of micro HPLC and IR via flow-cell technique, proton-NMR, and ICP could be accomplished free from absorption or signals from mobile phase solvents.

INTRODUCTION

The selection of satisfactory separation conditions is still a major problem in liquid chromatography; two approaches are adapted. One is the control of column temperature, and a number of publications concerned therewith have recently appeared (1-6). The other is the control of mobile phase compositions. Several rules (7-9) have been presented for ascertaining a suitable mobile phase solvent for a specific chromatographic purpose.

In a case of higher selectivity and resolution being required, it becomes necessary to reconsider mobile phase systems. Novotny has demonstrated in his recent article (10) that micro-HPLC technique can

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provide the oppotunity to use "exotic solvents" as a mobile phase system because of an advantageous feature as smaller flow-rates as like on the order of microliters per minute.

Therefore the authors previously reported the chromatographic performance of deuterated solvents with micro-HPLC (11) such as CD_3OD and CD_3OD/D_2O in reversed phase mode, although some works on the use of deuterated solvents as mobile phase on conventional-HPLC have been already published (12-14). However, only very scant details of this subject were given there.

In this contribution, we will describe the more advanced discussion on the chromatographic performance of deuterated solvents in normal and reversed phase micro-HPLC.

EXPERIMENTAL

A micro-HPLC system consisted of a microfeeder MF-2(Azuma Electric, Co.Ltd., Tokyo, Japan) as a pump and a Jasco(Tokyo, Japan) Uvidec-100II UV spectrophotometer as a detector. The infrared detection was performed by JEOL(Tokyo, Japan) JIR-40X Fourier transform infrared spectrometer. The micro-HPLC columns used were as follows;

a) silica column: PTFE tubing (0.5mm i.d. x 15 cm length) packed with Jasco FineSIL-5(5 μm).

b) C 18 column : fused silica capillary (0.35 mm i.d. x 30 cm length) packed with Chemcosorb ODS/H(7 μm, Chemco, Osaka, Japan), PTFE tubing (0.5 mm i.d. x 12 cm length) packed with Jasco FineSIL C-18(10 μm) and PTFE tubing (0.5 mm i.d. x 15 cm length) packed with Jasco SC-01 (5 μm).

c) C-2 column : PTFE tubing (0.5 mm i.d. x 10 cm length) packed with Jasco FineSIL C-2(10 μm).

d) C-8 column : PTFE tubing (0.5 mm i.d. x 13 cm length) packed with Jasco FineSIL C-2(10 μm).

e) column for IR : PTFE tubing (0.65 mm i.d. x 44 cm length) packed with Jasco FineGEL SC-220(11.7 μm) attached with on-column flow-cell (15).

Those columns were prepared by the slurry technique (16).

Mobile phase solvents in reversed phase mode were HPLC grade methanol and acetonitrile, 99.75 % heavy water and purified water. Benzene and 99.73 % deuteriobenzene were used in normal phase separation. All of test substances except few metal complexes were commercially available products as received. Metal complexes of Co-,Cr-and Fe-diethyldithiocarbamates were synthesized in normal ways.

The temperature control of each column was performed by the thermostat of Komatsu DW-620 (Tokyo, Japan).

The standard samples were injected into columns as a few hundred ppm concentration of methanol or acetonitrile solution. Sodium nitrite for reversed phase mode (17,18) and FC-78(N-trifluoromethylperfluoro-morpholine, marketed from 3M Company, U.S.A.) (19) for normal phase mode were used as t_0-materials, respectively. The capacity factor, k', was calculated from the retention time of the eluate, t_R, according to the equation of $k' = (t_R - t_0)/t_0$.

All measurements were made in at least triplicate. The average reproducibility of each run was better than about 1 % relative.

RESULTS AND DISCUSSION

To examine the chromatographic performance of heavy water, separations of alkylbenzenes were performed on various columns. Table-1 shows the capacity factors of toluene, ethylbenzene and n-propylbenzene with

Table-1 Capacity factors of toluene, ethylbenzene and n-propylbenzene with light and heavy water-acetonitrile aqueous mobile phase systems.

acetonitrile concentration (%)	capacity factor, k' toluene H_2O	toluene D_2O	ethylbenzene D_2O	n-propylbenzene D_2O
30	12.0	14.8	28.4	64.5
40	4.90	6.32	10.3	18.5
50	2.58	3.10	4.42	6.64
60	1.45	1.64	2.12	2.90
70	0.66	0.76	0.94	1.24

Chromatographic conditions: column; 0.5 mm i.d. x 12 cm, C-18(FineSIL)
flow-rate; 20 µL/min
column temperature; 25 °C

various concentration of heavy water and light water in acetonitrile aqueous mobile phase. The slight increase of the capacity factors caused from the use of former as a mobile phase component is clearly observed in the results. The difference of the capacity factors of toluene between heavy water and light water is about 20 % relative. This is only small increment in retention, but the difference of both systems is apparently present. The results shown in Table-2 were obtained at the concentration of acetonitrile in mobile phases where toluene eluted at the same capacity factor. The retention behavior of alkylbenzenes in both systems is completely the same to the general reversed phase separation mechanism.

The retention factors of alkylbenzenes in acetonitrile and methanol aqueous mobile phase systems are tabulated in Table-3, in which (A) shows the result on C-2 column, (B) shows for C-8 and (C) shows for C-18, respectively. For all the systems, the capacity factors in using heavy

Table-2 Capacity factors of alkylbenzenes with light and heavy waters in acetonitrile aqueous mobile phase system.

compound	capacity factor, k' CH_3CN/H_2O=80/20	CH_3CN/D_2O=82/18
toluene	0.68	0.68
ethylbenzene	0.96	0.95
propylbenzene	1.42	1.39
n-butylbenzene	2.09	2.04
n-amylbenzene	3.13	3.00
n-hexylbenzene	4.71	4.77

Chromatographic conditions: column; 0.35 mm i.d. x 30 cm, Chemcosorb.
flow-rate: 2 μL/min
column temperature; 25°C

water are larger than those with light water. No improvements with the former are observed in separation factors for acetonitrile mobile phase, where α is consistent with each other in ± 1.0 % relative, while small increases in α are found in methanol system. The separation performance depending on heavy water seems to relate with the kind of column packing materials, and their order is approximately depending on the length of bonded carbon chains.

It is clear from above results that the separation factors of alkylbenzenes are slightly improved with the use of heavy water instead of light water in methanol aqueous system and these effects are influenced from some characteristics of the stationary phase.

The second example to show the performance of heavy water is the separation of catecholamines. In the separation of catecholamines water is used as a major solvent in mobile phase, and therefore it is expected that the different retention performance would be observed by the use of heavy water as a mobile phase solvent.

Table-3 Retention data for alkylbenzenes with C-2, C-8 and C-18 columns.

(A) C-2 column.

sample	CH_3OH/D_2O k'	α	CH_3OH/H_2O k'	α	CH_3CN/D_2O k'	α	CH_3CN/H_2O k'	α
benzene	0.84	-	0.79	-	1.69	-	1.48	-
toluene	1.15	1.37	1.03	1.30	2.51	1.49	2.12	1.44
ethylbenzene	1.52	1.80	1.38	1.74	3.53	2.09	3.03	2.05
n-propylbenzene	2.18	2.60	2.00	2.53	5.14	3.04	4.53	3.07
n-butylbenzene	3.12	3.72	2.86	3.62	7.41	4.41	6.64	4.50
n-amylbenzene	4.64	5.52	4.26	5.40	11.1	6.57	9.79	6.64
composition	7/3				1/1			

(B) C-8 column.

benzene	1.07	-	1.04	-	1.16	-	1.05	-
toluene	1.44	1.35	1.38	1.33	1.49	1.29	1.34	1.27
ethylbenzene	1.91	1.79	1.82	1.75	1.91	1.64	1.72	1.63
n-propylbenzene	2.77	2.59	2.58	2.48	2.53	2.18	2.27	2.15
n-butylbenzene	4.14	3.87	3.79	3.64	3.31	2.86	3.02	2.86
n-amylbenzene	6.27	5.86	5.62	5.40	4.39	3.78	3.97	3.76
composition	3/2				1/1			

(C) C-18 column.

benzene	1.05	-	1.05	-	1.22	-	1.12	-
toluene	1.67	1.59	1.55	1.48	1.64	1.34	1.45	1.30
ethylbenzene	2.25	2.14	2.14	2.05	2.12	1.73	1.89	1.69
n-propylbenzene	3.48	3.31	3.18	3.04	2.90	2.37	2.63	2.35
n-butylbenzene	5.32	5.06	4.84	4.63	3.98	3.25	3.66	3.27
n-amylbenzene	8.30	7.89	7.46	7.14	5.55	4.53	5.16	4.61
composition	7/3				3/2			

α is referred to benzene.

Column; 0.5 mm i.d. x 12 cm, C-18(FineSIL)

Chromatographic conditions: flow-rate; 8 μL/min

column temperature; 25°C.

In Table-4, the capacity factors and the separation factors of noradrenalin, adrenalin and dopamine are listed. It is apparent that the retention factors with heavy water seems to be rather agreeable. Although preferable separation was attained with heavy water, the same or similar effect could have been obtained by changing the chromatographic conditions, e.g., column temperature and dimension, packing materi-

Table 4 Retention data for catecholamines with heavy water and light water as mobile phase.

sample	retention factor			
	heavy water		light water	
	k'	α	k'	α
noradrenalin	0.85	—	0.57	—
adrenalin	1.81	2.13	1.19	2.09
dopamine	3.20	3.77	2.05	3.61

Chromatographic conditions:
column; 0.5 mm i.d. x 12 cm length, SC-01
flow-rate; 16 μL/min, 0.1 M acetic acid buffered.
column temperature; 25°C
α is referred to noradrenalin.

als, flow-rate of mobile phase, etc.. So these behaviors of heavy water may be of little real benefit.

A chloroform solution of the test mixture of four diethyldithiocarbamates was injected in the silica column. The chromatograms were separately measured with benzene and deuteriobenzene as the mobile phase. Then capacity factors for each metal complexes were calculated in the normal way using FC-78 as non retained substance.

The results are shown in Table 5. The capacity factors with deuteriobenzene mobile phase are about 10 % smaller than those with benzene, although the theoretical mean of this fact is not cleared yet. The separation performance of deuteriobenzene is inferior to that of benzene. However, it is apparent that the slightly faster separation would be possible by using deuterated solvent as mobile phase.

The observed isotope effects on chromatographic performance are considered to be caused from the reasons as follows;
deuterated solvents such as heavy water and deuteriobenzene have the same electronic structures as light water and benzene, but the higher

Table 5 Retention data for metal complexes with benzene and deuteriobenzene as mobile phase.

sample	retention factor			
	benzene		deuteriobenzene	
	k'	α	k'	α
Cu-complex	0.39	—	0.41	—
Cr-complex	0.95	2.45	1.05	2.56
Co-complex	1.31	3.38	1.47	3.55
Fe-complex	1.57	4.07	1.73	4.20

Chromatographic conditions:
column; 0.5 mm i.d. x 15 cm, silica.
flow rate; 8 μL/min
column temperature; 25°C
α is referred to Cu-complex.

mass of deuterium restricts their nuclear motions relative to hydrogen, making deuterated molecules more structured. Therefore it is expected that deuterated solvents behave as chromatographically more "polar" in nature when compared with the normal substances, since the more structured deuterated molecules would give still tighter packing on the basis of hydrophobic effects associated with tight packing of normal molecules around the solute. This explanation has been confirmed in many publications (20-23), in which separations of normal and deuterated substances were carried out in reversed phase mode and deuterated solutes eluted faster than normal solutes, because tight packing of water molecules about the solute and of specific solvation of C-H bonds (24,25) might explain the more restricted motion in the aqueous phase relative to the hydrophobic phase that is indicated by the isotope effects, which relatively favor deuterium over protium in the aqueous phase.

The potential of the use of deuterated solvents as mobile phase is expected in combination of liquid chromatography and infrared spcetrometry (IR).

Although the two detectors based on the UV absorption and refractive index (RI) are the most often used, they provide little structural information on the separated components. IR is undoubtedly one of the most useful tools providing quantitative information. The large number of absorption bands present in an IR spectrum offer specific information of functional groups, possible structure assignments and confirmation of postulated structure.

Many efforts have so far been made to combine HPLC with IR spectrometry on-line (26-28). One of them is a simple IR photometer designed for use a HPLC/IR detector with a flow-cell manufactured by Foxboro/Wilks (29). The use of this kind of IR detector is however limited to the mobile phases that are transparent at the wavelength of interest. In most cases at least 30 % transmission of the solvent with the cell enough required on the pathlength is needed for satisfactory application. Unfortunately, most of practical HPLC solvents have strong bands in mid-IR regions. The experimental results, in which deuterated solvents affect the solute selectivity only marginally, indicate that HPLC/IR combination via direct flow-cell technique can be easily accomplished free from mobile phase absorption interference. For instance, Figure-1 shows the infrared spectrum of benzene and deuteriobenzene measured by FT-IR. It is appeared that the absorption of C-H stretching frequencies of any solute can be detected because of the shift of $\nu_{CH} - \nu_{CD}$. The second example in Figure-2 is the IR spectrum of light water, heavy water and PTFE on-column flow-cell (15). The detection of components which have O-H bonds in their structures can be attained without any change of chromatographic conditions by the use of heavy water as mobile phase instead of light water, since the isotope shift of the $\nu_{OH} - \nu_{OD}$ eliminates the O-H absorption of aqueous mobile phase. As shown in

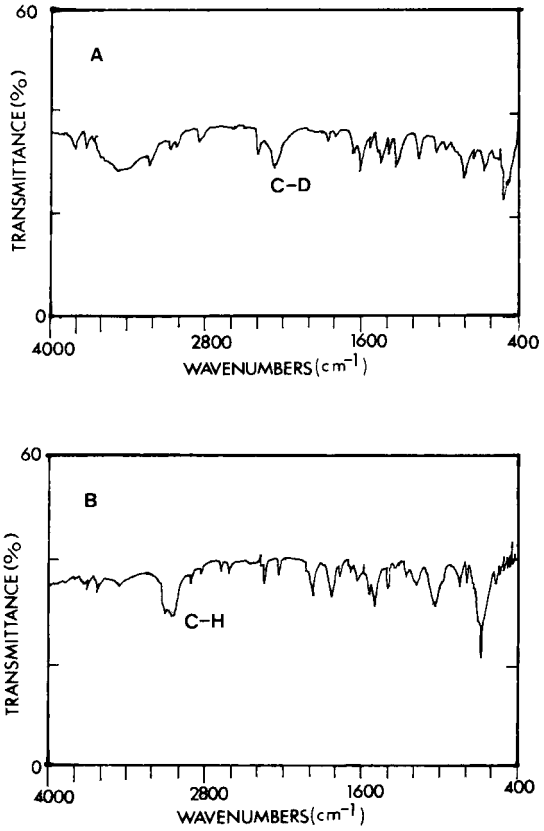

Figure 1 Infrared Spectra of Benzene and Deuteriobenzene.

A: Benzene
B: Deuteriobenzene
FT-IR conditions: 1 drop on a KBr disc.
resolution; 4 cm^{-1}.
accumulation; 10 times.
detector; TGS.

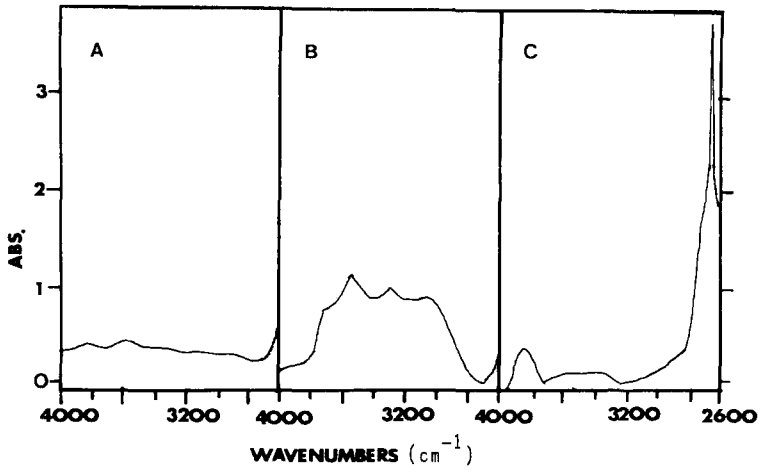

Figure 2 Infrared Spectra of (A) PTFE on-column flow-cell, (B) light water and (C) heavy water.
FT-IR conditions: on-column flow-cell with a beam condensor.
resolution; 16 cm^{-1}
accumulation; 10 times.
detector; MCT.

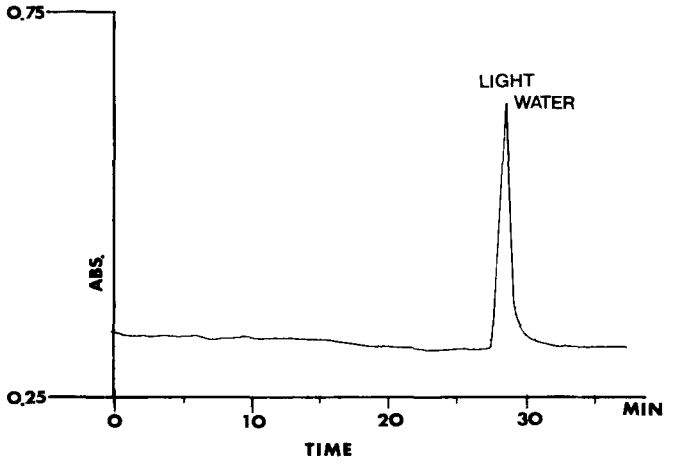

Figure 3 FT-IR Chromatogram of Water by Size Exclusion Mode.

column: FineGEL SC-220, PTFE tubing of 0.65 mm i.d. x 44 cm.
mobile phase: heavy water.
flow-rate: 4 µL/min.
sample amount: 0.5 µL.
FT-IR conditions: resolution; 16 cm^{-1}.
accumulation; 10 times.
detector; MCT.

Figure-3, this approach clearly demonstrates that trace water in heavy water could be measured with FT-IR on monitoring at 2950 cm^{-1} chromatographically.

This concept can be also applied to the combination of micro-HPLC and proton-NMR free from interference of protons belongs to mobile phase solvents.

In the combination of micro-HPLC and Inductively Coupled Plasma Atomic Emission Spectrometry (ICP) (30-32), deuterated solvents will promise the possibility of monitoring of the emission of hydrogen atom contained in solutes, because the emission line of deuterium atom is measured at 656.1 nm, while that of hydrogen atom is 656.3 nm. The use of high resolution monochromator can resolve both lines as separated.

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REVERSED-PHASE CHROMATOGRAPHIC SYSTEM AS A MODEL FOR CHARACTERIZING THE OFFSET RATE OF ACTION OF AZIDOMORPHINES IN GUINEA-PIG ILEUM

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ABSTRACT

The relationship between the offset rate of action and physicochemical properties of azidomorphines was investigated. The offset rate of action of 12 compounds was measured in isolated preparations of guinea-pig ileum. Physicochemical properties of azidomorphines were characterized by their hydrophobicity parameters, determined by reversed-

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phase high-performance liquid chromatography and by their activity in lowering surface tension.

The offset rate of action of azidomorphine derivatives in guinea-pig ileum proved to be inversely related to their hydrophobicity, as shown by correlation analysis.

A reversed-phase chromatographic system was developed which appears to be suitable for the estimation of the offset rate of action of azidomorphines.

INTRODUCTION

The investigation of quantitative relationships between biological activity and physicochemical properties of drugs /1/, /2/ can help in the understanding of the mechanism of pharmacological action.

The azidomorphine derivatives synthetized by Bognár and Makleit /3/ have advantageous pharmacological effects in certain tests /4/, /5/. The relationship between the chemical structure and pharmacological activity of the derivatives has already been studied /6/. It has been shown by Friedmann and knoll /7/ that 3-ethoxy-N-cyclopropylmethyl-azidomorphine exhibits an extremely slow offset of inhibitory action in the isolated myenteric

plexus-longitudinal muscle strip of the guinea-pig ileum.

Kosterlitz et al. /8/ demonstrated the role of hydrophobicity in the rate of offset of the action of different narcotic analgesics. Herz and Teschemacher /9/ and Wüster and Herz /10/, /11/ demonstrated the importance of surface tension-lowering activity of loperamid, an opiate-like antidiarrheal agent, in its extremely long lasting action on the guinea-pig ileum.

The question arose whether the offset rate of action of the investigated azidomorphines could be explained solely by their hydrophobic character and surface activity or other specific interactions at the receptor should also be invoked.

In the present study the rate of offset of the inhibitory action was measured in the isolated preparation of guinea-pig ileum. Hydrophobicity of the selected azidomorphine derivatives was caracterized by reversed-phase high-performance liquid chromatographic (RP-HPLC) retention data as they are often used in the literature (/12/, /13/, /14/) according to eq. 1

$$\log K = a \log k' + b \qquad (1)$$

where K is the partition coefficient of a compound, k' is the capacity ratio measured in a given RP-HPLC system, and a and b are constants.

The surface tension-lowering effects, $\Delta\sigma$, of the compounds were characterized by the difference of the surface tension of the pure buffer and the drug solution.

The relationship between the biological and physicochemical data was tested by correlation analysis.

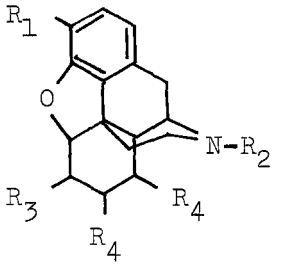
MATERIALS AND METHODS

The chemical structures of the compounds studied are shown in Table 1.

The rate of offset of inhibitory action was measured on myenteric plexus-longitudinal muscle prepared according to Paton and Vizi /15/. The muscle strip was suspended in a 5 ml organ bath (Krebs solution) and was field stimulated by 0.1 Hz supramaximal square wave pulses of 1 msec duration voltage. The Krebs solution had the following composition (mM): NaCl 113, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.5, $MgSO_4$ 1.2.

TABLE 1.

CHEMICAL STRUCTURE OF SOME AZIDOMORPHINE DERIVATIVES



Serial number	Name	R_1	R_2	R_3	R_4
1.	Azidomorphine	OH	CH_3	N_3	H
2.	Azidocodeine	OCH_3	CH_3	N_3	H
3.	N-cyclopropylmethyl-azidomorphine	OH	CH_2-cyclopropyl	N_3	H
4.	Azidoethylmorphine	OC_2H_5	CH_3	N_3	H
5.	N-phenylethyl-azido-ethylmorphine	OC_2H_5	CH_2CH_2-Ph	N_3	H
6.	N-phenylethyl-azido-morphine	OH	CH_2CH_2-Ph	N_3	H
7.	Acetylazidomorphine	$OCOCH_3$	CH_3	N_3	H
8.	Norazidoethylmorphine	OC_2H_5	H	N_3	H
9.	N-cyclopropylmethyl-azidoethylmorphine	OC_2H_5	CH_2-cyclopropyl	N_3	H
10.	Norazidomorphine	OH	H	N_3	H
11.	Normorphine	CH	H	OH	double bound
12.	Morphine	OH	CH_3	OH	"

All the investigated compounds exhibited opiate agonist activity. The offset rate of action was characterized by the $t_{1/2}$ value, which indicates the time needed for the 65-75 percent inhibition caused by the compound to decrease to one-half after washout with 100 ml Krebs solution. Pairs of compounds (one of them always azidomorphine) were tested on the preparation. The logarithm of the $t_{1/2}$ values relative to azidomorphine were used in correlation analysis ($\log t_{1/2}^{*}$).

The chromatographic retention data were measured on a LiChrosorb RP-18 (250 mm ID 4.6 mm dp 10 µm, Chromatronix) column. The parts of the HPLC equipment are listed in Table 2.
Detection was carried out at 280 nm. The temperature was 22 °C ± 2 °C. The flow rate of the eluent was 1.51 ml/min. Pressure was 3 - 5 x 10^5 Nm^{-2}.

The retention time values of compounds and the dead times were measured at least at three different concentrations of acetonitrile in the eluent. Sodium nitrate was used to determine the dead time. The eluent consisted of 5 to 80% acetonitrile and 0.05M phosphate buffer, pH 2. At this pH value compounds were in cationic form. Sodium-butylsulphonate (Aldrich Milwauke Wisc. USA) was applied as counter

TABLE 2.

HPLC EQUIPMENT

Injector: Rheodyne Model 7010 Sample Injection Valve
Detector: ISCO Model 226 Absorbance Monitor
Pump: Labormim Liquopump Model 312
Integrator: Chinoin Digint Model 24
Recorder: Endim Model 621.01
Calculations: Apple II+ microcomputer

ion in 0.005 M concentration in the eluent. Under such circumstances symmetric peaks were obtained after 20 µl injection of about 0.01% solutions of the drugs in the eluent. The logarithm of the capacity ratios (log k') was calculated at different concentrations of acetonitrile in the eluent (OP%). The slope and intercept ($\log k'_o$) of the log k' vs. OP% straight lines were also considered as characteristic of hydrophobic properties of drugs on the basis of an earlier study /16/, which showed good correlation between the 1-octanol/water partition coefficient and RP-HPLC retention data for different types of compounds.

The regression analysis yielded the slope and the intercept ($\log k'_o$) of the following equation:

$$\log k' = \text{'slope'}\ OP\% + \log k'_o \qquad (2)$$

To measure surface tension, compounds were dissolved at 10^{-3} M concentration in 0.067 M phosphate buffer, pH 7.4, prepared from KH_2PO_4 and NaOH. The surface tension of the buffer and the drug solutions was measured in a Traube stalagmometer. The mass of 60 drops of drug solution (m_x) was measured and compared to the mass of 60 drops of distilled water (m_w). The surface tension (σ_x) of the given solution was calculated according to eq. 3:

$$\sigma_x = \frac{m_x}{m_w}\,\sigma_w \qquad (3)$$

where the σ_w is the surface tension of distilled water (72.57 mN/m). Measurements were carried out at 24.6 °C.

The surface tension-lowering activity ($\Delta\sigma_{pH=7.4}$) of compounds was expressed as the difference in σ_x values of the drug and the buffer solutions.

Several compounds were insoluble in the buffer at 10^{-3} M concentration, therefore surface tension-lowering activity of the compounds was also measured in 0.05 M KH_2PO_4 (pH=4.6) solutions, in which all investigated compounds dissolved. The differ-

ences of the surface tension ($\Delta\sigma_{pH=4.6}$) for the drug and the KH_2PO_4 solutions were also used in the correlation analysis.

All other chemicals were reagent grade preparations obtained from REANAL (Hungary). Acetonitrile was purified by us for chromatographic purposes.

RESULTS AND DISCUSSION

The biological and physicochemical data obtained for the 12 compounds are listed in Table 3.

Table 4 shows the correlation coefficient for each pair of variables.

Since washing of the ileum preparation failed to abolish the inhibitory action of compounds 5 and 9 ($t_{1/2} > 120$ min), these two compounds were omitted from the correlation analysis.

Our finding that the action of drugs 5 and 9 was completely antagonized by naloxone excluded the possibility that the extremely long-lasting effect of the two compounds was due to covalent binding to opiate receptor sites. The fact that subsequent washing of the preparation (removal of naloxone) restored inhibition showed that the drugs were still present in the preparation near the receptor.

TABLE 3.

THE BIOLOGICAL AND PHYSICOCHEMICAL DATA OF AZIDOMORPHINE DERIVATIVES

Ser. num.	log $t^{*}_{1/2}$	'slope'	log k'_o	log k'_{40}	$\Delta\sigma$ * pH=4.6	$\Delta\sigma$ * pH=7.4
1.	0.000	-0.0311	0.975	-0.259	0.38	-0.19
2.	0.294	-0.0064	0.499	0.188	0.95	1.75
3.	0.468	-0.0056	0.358	0.078	1.17	-§
4.	0.642	-0.0088	0.862	0.501	1.65	3.02
5.	>1	-0.0250	2.164	>0.6	0.79	-§
6.	0.734	-0.0150	1.124	0.527	1.97	-§
7.	0.060	-0.0080	0.594	0.272	2.19	0.93
8.	0.641	-0.0106	0.880	0.454	0.86	1.12
9.	>1	-0.0194	1.644	>0.6	0.32	5.32
10.	0.009	-0.0271	0.851	-0.202	1.46	-§
11.	-0.523	-0.0277	0.344	-0.703	-0.30	-§
12.	-0.229	-0.0298	0.467	-0.653	0.91	0.38

* $\Delta\sigma$ values are given in mN/m.

§ $\Delta\sigma_{pH=7.4}$ values could not be measured for these compounds because they were insoluble in the buffer (pH=7.4) at 10^{-3} M concentration.

TABLE 4.

CORRELATION COEFFICIENTS OF PAIRS OF VARIABLES

	'slope'	log k'_o	log k'_{40}	$\Delta G_{pH=4.6}$
log $t^{*}_{1/2}$	0.711	0.537	0.925	0.582
'slope'		-0.086	0.802	0.530
log k'_o			0.524	0.382
log k'_{40}				0.693

It can be seen from Table 4 that the values of log $t^{*}_{1/2}$ show good correlation with the logarithm of the capacity ratios measured at 40% acetonitrile in the eluent (log k'_{40}) as described by eq. 4:

$$\log t^{*}_{1/2} = 0.843 \log k'_{40} + 0.192 \qquad (4)$$

$n=10 \quad R=0.925 \quad s=0.168 \quad F=47.3 \quad F_{(1,8\ p=0.95)}=5.3$

where n is the number of compounds considered in the calculations, R is the correlation coefficient, s is the standard error of the estimate, and F is the F-test value.

The offset rate of acetylazidomorphine (compound 7) was nearly the same as that of azidomor-

phine (number 1), although their hydrophobic characters differed markedly. Consequently, there were great differences between the measured and calculated (by eq. 4) log $t^{*}_{1/2}$ values for acetylazidomorphine. An explanation of this phenomenon may be that acetylazidomorphine was hydrolyzed to azidomorphine by the tissue's cholinesterases.

Omission of its data from the calculations in eq. 4 gave significantly better correlations (Fig. 1.).

$$\log t^{*}_{1/2} = 0.900 \log k'_{40} + 0.230 \qquad (5)$$

n=9 s=0.103 R=0.976 F=137.7 $F_{(1,7\ p=0.95)}=5.6$

The log k' values of compounds measured on reversed-phase chromatographic columns are usually taken to be proportional to the logarith of partition coefficients of the compound in two immiscible solvents /12/, /13/, /14/. Riley et al. /13/ underlined the relevance of liquid-liquid distribution phenomena of solute retention in ion-pair liquid chromatographic systems as well. Thus, the significant relationship between log $t^{*}_{1/2}$ and log k'_{40} suggests that the half-time of the offset of the investigated compounds in guinea-pig ileum depends on their hydrophobic character.

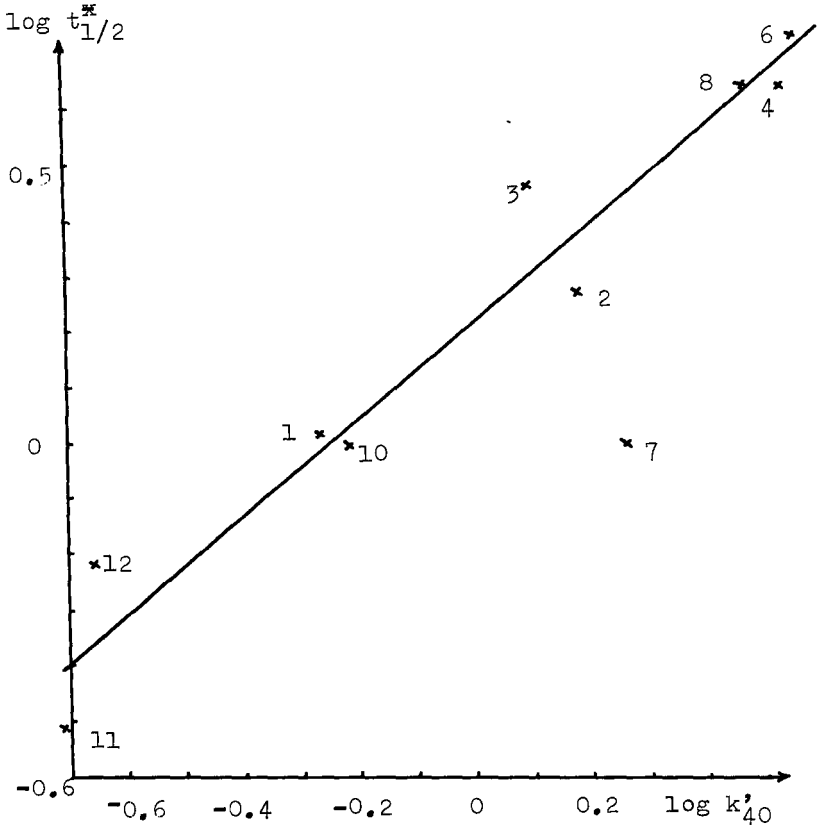


FIGURE 1.

THE RELATION OF log k' VALUES TO THE log $t^{*}_{1/2}$ VALUES ACCORDING TO EQ. 5.

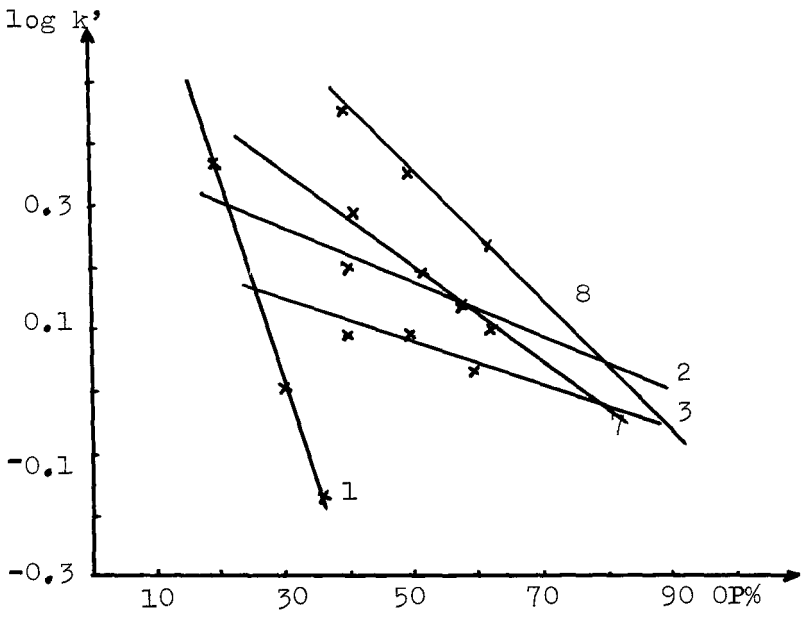
The whole set of compounds could be analyzed only when 40 - 50% acetonitrile was used in the eluent. At lower concentrations of acetonitrile hydrophobic compounds (No. 5, 6, 4, 8 and 9) could not be eluted, whereas at higher acetonitrile con-

centration more hydrophilic compounds (No. 12, 11, 1) had no retention on the column. At 40% acetonitrile concentration only two compounds could not be eluted, No. 5 and 9, the ones that could not be washed out from the ileum preparation.

The relationships between log $t^{*}_{1/2}$ and log k'_{40} values (eq. 4 and 5) are significant according to the F-test. These results indicate that a well-defined reversed-phase chromatographic system can provide a useful means to estimate the rate of offset of azidomorphine derivatives in the isolated guinea-pig ileum.

However, one may question the use of log k' values as the measure of hydrophobicity when log k' vs. OP% straight lines are not parallel for all compounds, rather they intersect each other. In such cases different ranks of log k' values can be obtained at different percentages of acetonitrile in the eluent. It was pointed out earlier /16/ that in such instances the hydrophobic properties of compounds can be characterized by the slope and the intercept (log k'_o) of the log k' vs. OP% straight line.

In case of the compounds investigated the OP% vs. log k' straight lines intersected each other as can be seen in Fig. 2.



Numbers refer to the compounds in Table 1.

FIGURE 2.

THE OP% vs. log k' STRAIGHT LINES FOR FIVE AZIDOMORPHINE DERIVATIVES

Therefore, the statistical parameters of the relation of hydrophobicity to the offset rate were also calculated from the 'slope' and log k'_o values of compounds:

$$\log t^{*}_{1/2} = 29.906\,\text{'slope'} + 0.908 \log k'_o + 0.087 \quad (6)$$

n= 10 R=0.931 s=0.172 F=22.8 $F_{(2,7\ p=0.95)}=4.7$

The calculated and measured log $t^{*}_{1/2}$ values of acetylazidomorphine were again different. Omitting

this compound from eq. 6 we obtained eq. 7:

$$\log t^{*}_{1/2} = 33.390\text{'slope'} + 0.862 \log k'_{o} + 0.219 \quad (7)$$

$$n=9 \quad s=0.112 \quad R=0.975 \quad F=57.9 \quad F_{(2,6\ p=0.95)}=5.1$$

Considering eq. 1 and 2, it can be derived /16/ that the quotient of the regression coefficient referring to the 'slope' and log k'_{o} values in eq. 7 give the concentration of acetonitrile in the eluent (OP%) at which the biological partition system can be best modelled. As it equals 38.7%, it is conceivable why log k' values referring to 40% acetonitrile concentration showed good correlation with log $t^{*}_{1/2}$ values.

Wüster and Herz /10/, /12/ have pointed out that surface activity of opiates can be an important factor in their accumulation in isolated organs. Therefore, we have determined the surface tension-lowering activity of azidomorphines at two different pH values. At near-physiological hydrogen-ion concentration none of the compounds showed significant surface tension-lowering activity. The highest value was only 5.32 (see Table 3) for compond 9. As this compound exhibited also a very slow offset rate of action, some tendency for the ΔG- vs. log $t^{*}_{1/2}$ relationship cannot be completely excluded.

The ΔG values of every compound could be measured at pH 4.6, but these values did not show correlation either with log $t^{*}_{1/2}$ or with log k' values. The ΔG values measured in the two buffers did not correlate either, which suggests that the degree of protonation of the compounds markedly influences their surface activity.

In conclusion, it can be stated that the hydrophobic properties of azidomorphines play an important role in the half-time of offset of action on longitudinal muscle of guinea-pig ileum. On this basis a well-defined chromatographic system was established by which the offset of action in guinea-pig ileum can be estimated.

ACKNOWLEDGEMENTS

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF MDL-035 IN THE PLASMA OF RATS, DOGS AND HUMANS.

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ABSTRACT

A sensitive and reproducible high performance liquid chromatographic method was set up for the assay of MDL-035, a new non-steroidal, nonacidic analgesic antiinflammatory agent, in the plasma of rats, dogs and humans. Plasma samples (0.5 ml) containing flurazepam as the internal standard, were diluted and extracted with ethyl ether. After centrifugation, the organic phase was taken to dryness, the residue was redissolved and injected into an RP-2 column. The elution was made in isocratic conditions using a CH_3CN/phosphate buffer solution as mobile phase. The UV detection was made at 320 nm. The method was validated for the concentration range from 0.05 to 10 µg/ml, and applied to pharmacokinetic studies. A typical plasma concentration-time profile in the rat after an oral administration is here presented.

INTRODUCTION

3-(1-methylethyl)-2-(4-methoxyphenyl)-3H-naphth-[1,2-d]-imidazole, MDL-035, is a new non-steroidal nonacidic analgesic antiinflammatory agent (1) currently being studied as anti-rheumatic agent.

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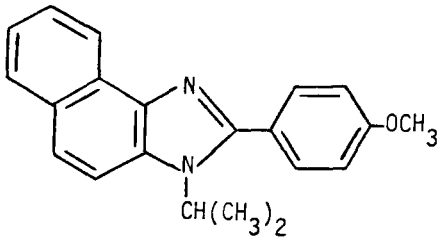

$C_{21}H_{20}N_2O$

M.W. 316.4

MDL-035, when tried in the pharmacological tests (1, 2), showed a high and prolonged activity. Remarkably, the compound resulted to be devoid of acute toxicity (LD_{50}= 15 g/kg, rat) and gastric lesivity (ED_{50} 1.05 g/kg, rat).

The aim of this work was to set up a sensitive and reproducible analytical method to determine the plasma levels of MDL-035 in preclinical and clinical pharmacokinetic studies and in toxicological investigations. The present HPLC method was validated for the concentration range from 0.05 to 10 µg/ml, providing for a sensitivity 8 times higher than that reported for the TLC method already available (3).

MATERIAL AND METHODS

The procedure involves the extraction with ethyl ether of the sample containing the internal standard. After evaporation of the organic solvent, the residue is redissolved and analyzed by HPLC using an RP-2 column as stationary phase and acetonitrile/phosphate buffer solution as eluant. The monitoring is made by UV detection at 320 nm.

Chemicals

MDL-035, Lepetit working standard of appropriate high purity.

Flurazepam, "Fabbrica Italiana Sintetici" (Milano, Italy), used as the internal standard.

Solvents and reagents high purity grade, Merck (Darmstadt, G.F.R.).

Distilled water, filtered through the Millipore Mille-Q system.

Plasma, from sprague-dawley rats, beagle dogs, and healthy volunteers.

Apparatus

The chromatographic determinations were made with a Waters Associates liquid chromatograph equipped with Model 6000A flow pumps, a Waters Model 620 solvent programmer, an LDC variable wavelength detector Spectromonitor III, a W.I.S.P. Mod. 710A automatic sampler, and a Tarkan 600 W+W recorder connected to a HP 3357 Data System, or a Hewlett-Packard Mod.3380A integrator for the quantitative determination of MDL-035 with reference to the internal standard. An RP-2 column, Brownlee Labs (Santa Clara, CA, USA), 25 cm x 4.6 mm, packed with 10 µ particles was used as stationary phase.

Standard solutions

Internal standard: 6 mg of flurazepam was dissolved in 10 ml of acetonitrile/2-propanol (1:1, v/v) solution.

MDL-035 and internal standard: 3 mg of MDL-035 and 6 mg of flurazepam were dissolved in 10 ml of acetonitrile/2-propanol (1:1 v/v) solution.

Extraction procedure

0.5 ml of rat, dog or human plasma was pipetted into a screw-cap tube containing 10 µl of the internal standard solution. The sample was then diluted with 0.5 ml of 1M Na_2HPO_4 and extracted with 10 ml of ethyl ether shaking for 10 min at 300 inversions per minute. After centrifugation at 2500 g for 5 min, 9 ml of the organic phase was transferred to a conical tube and taken to dryness at 37°C under a stream of nitrogen.

Chromatography

The residue was reconstituted with 20-25 µl of acetonitrile/2-propanol (1:1, v/v) with the aid of a Bransonic 12.

A suitable volume of this solution was finally put in the microvial of the HPLC automatic sampler. Ten microliters of the sample was injected into an RP-2 column and the isocratic elution was made at a flow-rate of 2 ml/min using a 45% B in A mixture as mobile phase, where A was a 0.05 M NaH_2PO_4/Na_2HPO_4 pH 7.8 aqueous solution and B was acetonitrile/water (9:1, v/v).

The UV detection of MDL-035 and the internal standard was made at 320 nm.

RESULTS AND DISCUSSION

Chromatographic separation

Figures 1-3 show that the method described affords a selective determination of MDL-035 and flurazepam in rat, dog and human plasma samples and a good chromatographic resolution of the peaks. The tracings are devoid of any peaks which could

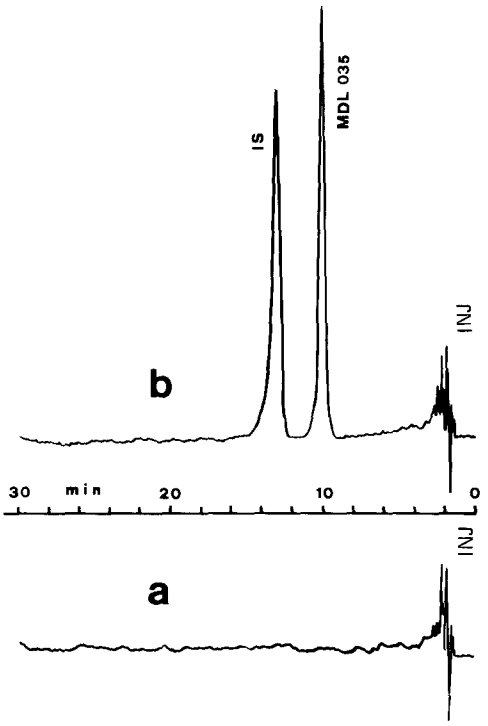

FIGURE 1 . Chromatograms of rat plasma samples (untreated animal)
a) no addition
b) plus MDL-035 (1 µg/ml) and the internal standard.

produce interference for the identification or the quantitative measurements of MDL-035 and the internal standard.

Validation of the method

In order to test the recovery, the precision (repeatability), the accuracy and the linearity of the method, plasma samples of untreated rats, dogs and humans were spiked with MDL-035 and flurazepam (internal standard) by adding known volumes of the standard solution (see Material and Methods).

Four concentration levels were established, based on the amounts of MDL-035 expected to be in the plasma: 0.05, 0.1, 1 and

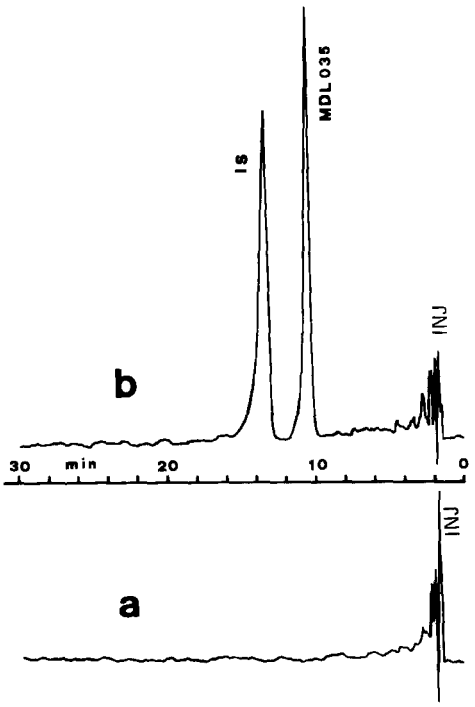

FIGURE 2. Chromatograms of dog plasma samples (untreated animal)
a) no addition
b) plus MDL-035 (1 µg/ml) and the internal standard.

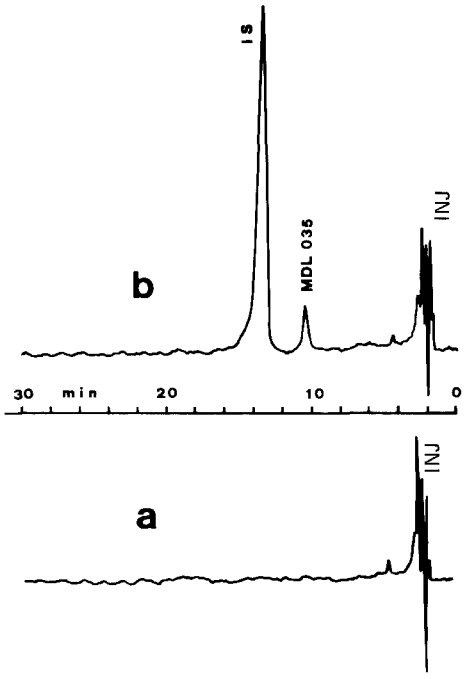

FIGURE 3. Chromatograms of human plasma samples (untreated subject)
a) no addition
b) plus MDL-035 (0.1 µg/ml) and the internal standard.

10 µg/ml . Five samples were prepared for each concentration level, extracted and analyzed as indicated above.

The average recovery of MDL-035 over the range tested was 106.7-117.7% (rat), 100.0-111.4% (dog), 101.9-106.7% (man), indicating that the extraction procedure yields a good recovery of both MDL-035 and flurazepam and the composition of plasma samples obtained from these three different species does not influence the recovery of the compounds (Table 1).

In Table 1 the mean recovery % (R %) and the coefficients of variation (C.V.%) of MDL-035 from rat, dog and human plasma samples are reported. C.V. = S.D./$\bar{X}$, where S.D. is the standard deviation and $\bar{X}$ is the mean of five analyses.

The precision of the method estimated by the coefficients of variation was satisfactory. The values of C.V.% calculated

TABLE 1

PLASMA CONCENTRATION (ng/ml)	RAT		DOG		MAN	
	R %	C.V.(%)	R %	C.V.(%)	R %	C.V.(%)
10000	108.4	6.0	111.4	2.7	101.9	1.4
1000	106.7	2.4	110.8	1.9	106.7	4.5
100	108.2	11.1	109.4	7.4	104.8	7.0
50	117.7	13.1	100.0	12.9	101.9	11.4

over the concentration levels tested ranged between 2.4-13.1 (rat), 1.9-12.9 (dog), 1,4-11.4 (man).

The accuracy was computed over the 20 points of each calibration curve and a mean recovery R of 110.3%, C.V. 9.5.% was found for rat plasma samples, R 108.3% C.V. 7.4% and R 103.7%, C.V. 6.9% for dog and human plasma samples, respectively.

The linear regression analysis provided for the equations:

$y = -0.0039 + 1.0848\,x \qquad r = 0.9979$ rat
$y = -0.0049 + 1.1146\,x \qquad r = 0.9996$ dog
$y = 0.0141 + 1.0183\,x \qquad r = 0.9999$ man

where y represents the amount of MDL-035 found, x the amount added and r the correlation coefficient.

The true value of the MDL-035 concentration in a plasma sample can be found by the equations above reported.

Operating wavelength

The operating wavelength was selected on the basis of the UV spectrum of MDL-035 in water-methanol 1:1 at different pH values. At pH 8.4 (next to the pH of the buffer used to make mobile phase) the UV spectrum exhibited 5 bands in the interval 220-350 nm, one of them with λ max 320 nm. At this operating wavelength the HPLC tracing resulted to be devoid of interferring peaks related to endogenous substances extracted from plasma.

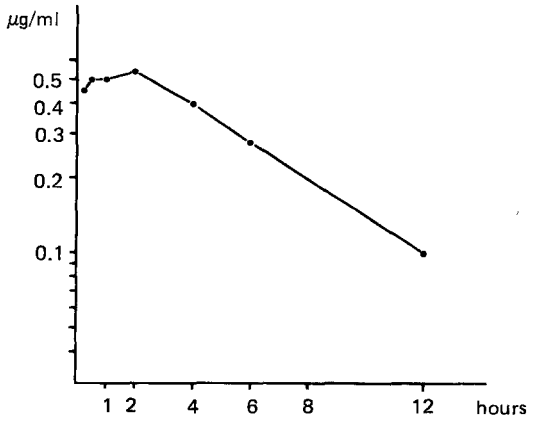

FIGURE 4. Mean plasma concentration-time curve of unchanged MDL-035 in rats treated with a 5 mg/kg oral dose.

Pharmacokinetic study

Some recent results of a pharmacokinetic study in the rat (4) are here reported. Peak plasma levels of unchanged MDL-035 in male rats were attained in 0.25-2 h (plateau) after the administration of a 5 mg/kg oral dose, reaching values of about 0.5 µg/ml. The compound was undetectable 24 h after treatment, while a mean concentration of 0.1 µg/ml was measured at the 12^{th}h (Fig.4). The elimination half life of the parent compound from plasma estimated after the 2^{nd}h, resulted to be about 4 h.

The method proved to be reliable and suitable for routine analysis.

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2. P.Schiatti, D.Selva, G.Galliani, A.Diena, E.Baldoli, A.Glasser, Antiinflammatory activity and other pharmacological properties of 3-(1-methylethyl)-2-(4-methoxyphenyl)-3H-naphth-[1,2-d]-

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4. A.Assandri, A.Bernareggi, T.Cristina, E.Toja,
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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(10), 2103-2104 (1984)

BOOK REVIEW

"HPLC of Biological Compounds - A Laboratory Guide", by William S. Hancock & James T. Sparrow, (Volume 26 of the Chromatographic Science Series, Jack Cazes, Editor), Marcel Dekker, Inc., New York, NY, 1984, 361 pp., $39.75 (US).

William S. Hancock and James T. Sparrow have, in their book, HPLC Analysis of Biological Compounds, provided the biologist with a thorough, clear and useful explanation of the basis of HPLC separations. The book, part of a series of monographs on Chromatographic Science, has five chapters: What is the "heart" of a HPLC system?; The Column - the "vitals of a HPLC separation; The mobile phase -"the circulatory system" of HPLC; The practical details or the "guts" of a HPLC separation; and Separation examples - insights into the "minds" of chromatographers. Although all of the chapters are useful, the chapters on the column, the mobile phase and the practical details of HPLC separation are the most valuable. I would recommend that any biological researcher read these chapters before embarking in the fast-moving arena of HPLC. The basis of separations is covered in a clear, practical way and many mistakes can be avoided by understanding this material. The chapter on Separation examples, which takes up about 40% of the book, provides useful examples of separations of amino acids, peptides, proteins, nucleotides, carbohydrates and lipids. The examples are well chosen and representative of either useful techniques or points of departure.

The field is moving so fast that the only way to get the "best" methods is, in many cases, to go to the current literature. There is a list of suppliers appended. The writing is generally clear and concise, making a readable book, but there are the usual number of distracting typographical errors seen in photo-offset publication. These are generally minor in nature. The book is well referenced and

has a valuable index. Although it is subtitled, " A Laboratory Guide," it is not a "cookbook" but more useful for the preparation and design of separations. This book is highly recommended for any biologist doing HPLC or considering doing HPLC.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(10), 2105-2107 (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/84/10, 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/84/10, 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 uniform 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/84/10, 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/84/10, 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 desired band from the stick and extracting with an appropriate solvent. Alltech Associates, Inc., Applied Science Labs, JLC/84/10, 2051 Waukegan Rd., Waukegan, IL, 60031, 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/84/10, P. O. Box 996, Cotati, CA, 94928, 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/84/10, 600 S. Wagner Road, Ann Arbor, MI, 48106, 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/84/10, Essex Road, Church Stretton, Shropshire, SY6 6AX, UK.

LC CAPABILITY BOOK contains 56 pages of illustrations and information including "Choosing the System", that helps potential users determine the most appropriate equipment for their needs. Pye Unicam, Ltd., JLC/84/10, York Street, Cambridge CB1 2PX, UK.

CHROMATOGRAPHY AUDIO COURSES are available covering Basic Gas Chromatography; Column Selection in Gas Chromatography; Modern Liquid Chromatography; Modern Liquid Chromatography, Special Topics. American Chemical Society, JLC/84/10, 1155 Sixteenth Street, NW, Washington, DC, 20036, USA.

"NEW FOR OLD" HPLC COLUMNS is a service that includes cleaning out the hardware, changing the frits or meshes, and repacking with new material. Repacked columns are claimed to have the same or higher efficiencies that those of other manufacturers' new ones. Phenomenex, JLC/84/10, 426 Via Corta, Bldg 305, Palos Verdes Estates, CA, 90274, USA.

DUAL PISTON HPLC PUMP includes an electronically controlled motor, two sapphire pistons, two cartridge-type ruby ball check valves, and an ingenious cam arrangement to maintain the flow rate of the mobile phase. The cam achieves the correct precompression and pressure feedback compensates electronically for liquid compressibility. Sonntek, Inc., JLC/84/10, P.O.Box 8731, Woodcliffe Lake, NJ, 07675, USA.

ACETYLCHOLINE ANALYZER utilizes a rapid reverse phase separation of acetylcholine and choline and a post-column enzymatic reactor module to achieve detection limits of 2 pmol or better. Detection is based upon electrochemical oxidation of hydrogen peroxide, released in the enzymatic reaction using a platinum electrode. Bioanalytical Systems, Inc., JLC/84/10, 2701 Kent Avenue, West Lafayette, IN, 47906, USA.

RESOLUTION OF EVEN HIDDEN COMPOUNDS is achieved through use of two user-selected wavelengths to analyze compounds of like or similar chemical structure. Overlapping peaks are resolved without changing analysis conditions or resorting to exotic and expensive detection methods. Micromeritics Instrument Co., JLC/84/10, 5680 Goshen Springs Road, Norcross, GA, 30093, USA.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(10), 2109-2112 (1984)

LC CALENDAR

1984

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.

NOVEMBER 19-24: Expoquimia: International Chemical Forum, Barcelona, Spain. Contact: Expoquimia, Feria de Barcelona, Barcelona, Spain.

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 10-12: Fourth International Symposium on HPLC of Proteins, Peptides and Polynucleotides, Baltimore, MD. Contact: Shirley E. Schlessinger, Symposium Manager, 400 East Randolph, Chicago, IL, USA.

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 10-15: Symposium on the Interface Between Theory and Experiment, Canberra, Australia. Contact: Leo Radom, Research School of Chemistry, Australian National University, Canberra, ACT 2601, Australia.

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: Linda Briggs, 437 Donald Road, Pittsburg, PA, 15235, USA.

MARCH 23-24: Conference on Creativity & Science, Honolulu, Hawaii. Contact: D. DeLuca, Scientists and Humanities Conf., Winward Community College, University of Hawaii, 45-720 Keaahala Rd., Kaneohe, Hawaii, 96744, 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 15-18: Materials Research Society Spring Meeting, San Francisco, CA. Contact: Susan Kalso, Xerox Palo Alto Res. Center, 3333 Coyote Hill Road, Palo Alto, CA, 94304, USA.

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

MAY 19: Middle Atlantic Regional ACS Meeting, Sponsored by ACS Monmouth County Section. Contact: M. Parker, Dept. of Chem., Monmouth College, West Long Branch, NJ, USA.

JUNE 9-15: ACHEMA 85, Frankfurt, West Germany. Contact: DECHEMA, Organization ACHEMA, P.O.Box 97 01 46, D-6000 Frankfurt, 97, West Germany.

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.

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

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(10), 2113 (1984)

ERRATUM

L. H. Fleming and N. C. Reynolds, Jr., J. Liquid Chrom., 7(4), 793-808 (1984), "LC-EC of Endorphins".

The legend given for Figure 1 erroneously presented the abscissa equal to 15 minutes. It should read:

".....and Abscissa = 5 minutes."

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

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

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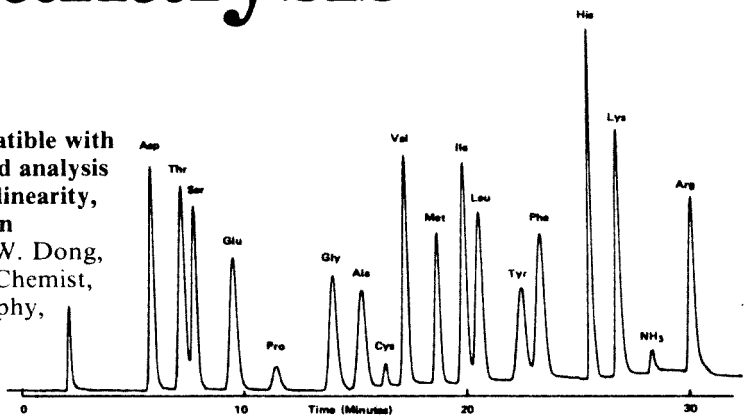

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