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A SIMPLE METHOD OF EVALUATING PARTICLE SIZE DISTRIBUTIONS AND SETTLING RATES FOR SILICA BASED CHROMATOGRAPHIC SUPPORTS

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ABSTRACT

A simple and inexpensive method of evaluating particle settling rates and the particle size distribution of chromatographic supports using common laboratory equipment is reported. The time dependence of the turbidity of a solution of silica support particles suspended in an appropriate liquid is measured on a spectrometer. Digital acquisition allows data manipulation using simple algorithms to yield the distribution of particle sizes based on the Stoke's diameter. This data can easily be transformed into the most useful form for presentation, e.g. number average, weight average, cumulative distribution, etc.

INTRODUCTION

Knowledge of the average particle size and the particle size distribution is useful in the evaluation of the efficiency of chromatographic columns. Measurement of the reduced plate height (h=H/d) and velocity (v=ud/D) require estimates of the particle size (d). It is not clear what the best measure of particle size

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should be, e.g. number average, cumulative weight average, etc. Differences in the values of the weight and number averages, however, often vary as much as 20% for small (3 and 5 μm) particles. In addition, because commercial packing materials are not perfectly monodisperse, a knowledge of the distribution is useful since column efficiency will tend to reflect the largest particles present while net pressure drops will be most influenced by the smallest particles [1]. In this context alternative efficiency calculations suggested by Bristow [2] and Halasz [3] which are based on the column pressure drop, permeability, or flow resistance parameter weight the smallest particles most heavily in the determination of an effective particle diameter.

The particle size and distribution will vary widely not only between different manufacturers and types of supports, but also between lots of the same silica. In addition, the manufacturer's provided particle size is often not measured directly for a particular lot, or the method of analysis is not stated. Because the actual size can often vary significantly from the nominal particle size, direct comparison of different gels is often difficult. In addition, post manufacturing handling and/or various derivitization schemes employed prior to column packing may alter the particle size distribution profile. The common proce-

dure of extended ultrasonication during out-gassing is one such procedure which can fracture particles and alter the distribution [3]. Finally, changes in the surface properties of derivitized silicas may alter the aggregation properties of small support particles, and aggregate size analysis may allow comparison of behavior in various solvent systems.

We have investigated the use of time-dependent turbidimetry during the settling of silica gel particles as a means of estimating the size distribution of chromatographic supports. While commercial instrumentation is available for particle sizing, for our purposes this alternative was cost prohibitive, less flexible and does not give the desired information on settling kinetics as a function of solvent. The method exploited here is similar to that suggested by Gumprecht and Sliepcevich [4] but uses a simple spectrometer as a measuring device. Digital data acquisition with a microcomputer was used to manipulate data to yield the various particle size distributions reported.

THEORY

The scattering of light by particles which are large relative to the wavelength of the light falls in the realm of Mie scattering. The apparent absorbance (related by the turbidity) of a solution of non-absorbing monodisperse particles in a non-absorbing medium

is proportional to 1) the pathlength (b), 2) the concentration of scattering particles of size d in the light beam (C(d)), 3) the square of the particle size (d^2), and 4) the scattering coefficient, Q, which is itself a function of particle size, the wavelength of light used, and the ratio of refractive indices of the particle and the medium (m).

$$A = (\text{const})[b\ C(d)\ d^2\ Q(d,\lambda,m)] \qquad (1)$$

Equation 1 is often represented in terms of a dimensionless size parameter, α, which relates the diameter and the wavelength so that at a given wavelength

$$A = (\text{const})[b\ C(\alpha)\ \ \alpha^2 Q(\alpha,m)] \qquad (2)$$

where

$$\alpha \equiv \pi d/\lambda \qquad (3)$$

Implicit in the above is the assumption that only transmitted light (i.e., not scattered light) is measured at the detector. In Mie scattering the greatest scattering intensity is directed nearly along the axis of transmission [5] so that proper measurement of exact turbiditimetric data requires precise and narrow detector geometries. In practice some scattered light will be measured and the scattering coefficient Q should be replaced in equation 1 with an effective scattering coefficient Q_{eff}, which is also a function of the optical geometry. While formal evaluation of the scattering coefficient involves the solution of Maxwell's

equations, approximate solutions valid at high and low values of α are more tractable. For silica particles in water, and using 300 nm radiation, the values of the dimensionless size (α) are approximately 10 times the particle diameter in microns. Thus for particles above about 2.5 microns the approximate solutions of Van de Hulst and of Walstra [6] can be used to calculate values of the real and effective scattering coefficients as a function of α, the refractive index ratio, and the angle ω, which may be viewed as the finite angle of acceptance of the spectrometer. As shown in Figure 1, the effective scattering coefficient, Q_{eff}, rapidly approaches a limiting value of unity as either the particle size (i.e. α) or the angle ω increases. For simple spectrometers the value of ω will be large. In our case the value was estimated to be significantly larger than 5° [6]. The result of this is that to a first approximation, the scattering coefficient term may be taken as a constant independent of particle size, and the absorbance (turbidity) is approximated as being proportional to the square of α, the concentration, and the path length.

$$A = (\text{constant})[b\ C(\alpha)\ \alpha^2] \qquad (4)$$

For a polydisperse suspension the total absorbance will then be the integral over all particles of the absorbances of each particle size, or

$$A_{tot} = \int_0^\infty A(\alpha)\ d\alpha = (\text{constant}) \int_0^\infty C(\alpha)\ \alpha^2\, d\alpha \qquad (5)$$

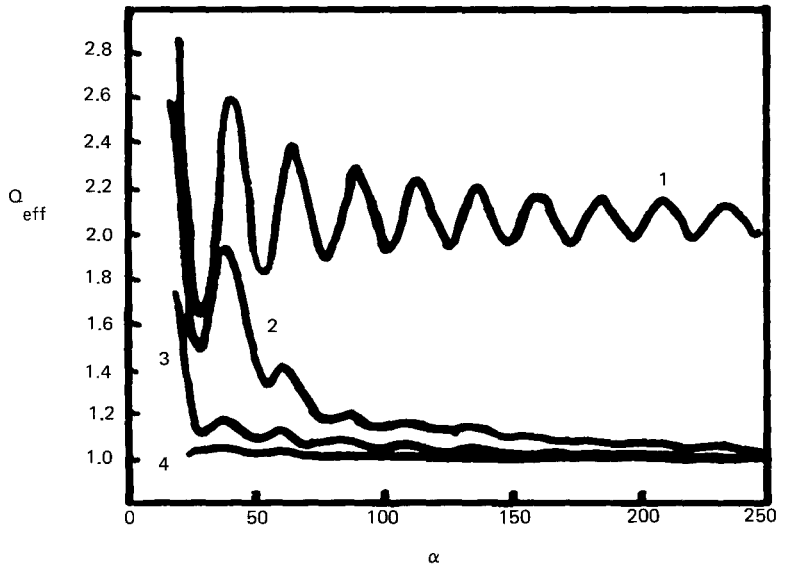

Figure 1. Scattering coefficient ($Q(\alpha,m)$) and the effective scattering coefficient ($Q_{eff}(\alpha,m,\omega)$) for various values of ω. Plots calculated for the approximate forms of Van de Hulst and of Walstra as taken from reference 6. Curves 1-4 denote ω values of 0, 2, 5, and 10 degrees, respectively.

A suspension of particles will eventually settle out under the influence of gravity. However, for spherical particles with density greater than that of the suspending medium the particles will settle out at a terminal velocity (v(d),cm/s) given by Stoke's law

$$v(d) = d\ g\ (\rho_p-\rho_s)/18\ \eta \qquad (6)$$

where g is the gravitational constant (980 cm/s^2), ρ_p the particle's density, ρ_s the solvent density (gm/cm^3), and η the solvent viscosity (in poise). While Stoke's law is strictly valid only for spherical

particles, it may be used to describe the settling of irregular particles with diameter ratios of up to approximately four with little error [7]. In such cases the so-called Stoke's diameter, or the diameter of a spherical particle with the same volume, is measured. In addition, for porous particles the density of the particle will be decreased relative to that of the solvent due to the inclusion of solvent within the pores of the particle. It is also implicit that the particles are settling in a quiescent liquid and that the particles are large enough that thermal (Brownian) agitation of the particles is negligible.

For an initially homogeneous suspension, as particles of a given size settle from an observation plane (light path) they will be replaced from above by an equal number of particles of the same size until such time as all the particles of that size have settled to a point below the level of the plane. Thus for a defined observation plane at time t there will be a critical particle diameter given by

$$d_{crit}(t) = [18\eta x/tg(\rho_p-\rho_s)]^{1/2} \qquad (7)$$

below which all particle sizes are present at their initial concentrations and above which size the concentration is zero. In the above equation x is the distance measured from the top level of liquid to the observation plane. Therefore the total concentration

of particles at the observation plane at a given time will be the integral of the initial concentrations of all particles over all particle sizes up to the critical diameter.

Re-expressing equation 7 in terms of dimensionless diameters (α) the dependence of the absorbance on time is

$$A(t) = \int_0^{\alpha_{crit}(t)} A(\alpha)d\alpha = (\text{constant}) \int_0^{\alpha_{crit}(t)} C(\alpha)\alpha^2 d\alpha \quad (8)$$

which when differentiated with respect to alpha and divided by the square of alpha critical yields

$$C(\alpha) = (dA/d\alpha)/(\alpha^2) \quad (9)$$

This is easily transformed into the size distribution.

EXPERIMENTAL

All absorption measurements were made on a Bausch and Lomb Spectronic 88 spectrophotometer. For convenience, the standard cuvette holder was used in place of the universal test tube cuvette holder supplied. No modification of the detector or optics were made in contrast to most true turbidimetry analyses [6] with the exception of masking the cuvette to define the plane of observation. Data was taken at the 2V outputs, digitized, and stored on an Apple II Plus computer (48K) via an ADALAB (Interactive Microware, Inc., State College, PA) ADC. Calculations and data manipulation were carried out via BASIC programs on the Apple computer.

Silica gels analyzed included samples of various batches of LiChrosorb Si60 (Merck, GFR) of 5 and 10 micron nominal particle sizes. Two of these samples were donated by Micromeritics, Inc. (Norcross, GA) and had been analyzed previously with respect to particle size on their Model 5000 SediGraph analyzer. Other samples included LiChrosorb Si60 and Si100 DIOL phases, as well as LiChrosorb Si60 derivatized by various methods with the affinity ligand Cibacron Blue 3G-A. Reverse-phase supports Hypersil ODS 5 micron (Shandon, UK) and Nucleosil 7 C_{18} (Macherey-Nagel, GFR) were also studied. Solvents used in this work included doubly deionized water, methanol (Mallinkrodt, HPLC grade), ethanol (University of Minnesota, technical grade), isopropanol (MCB, HPLC grade), toluene (Fisher, scintillation grade), and cyclohexane (Eastman, reagent grade). All solvents were filtered through 0.45 micron filters before use.

The procedure involved weighing out 10-20 (typically 15) mg portions of silica gels and suspending each in 3 ml of solvent. The suspensions of silica gel in solvent were thoroughly outgassed under vacuum with ultrasonication for a short time. To assure disaggregation, samples were also vigorously agitated on a vortex mixer for 1 minute immediately prior to measurement. Timing of the settling commenced with pouring

the suspension into a cuvette which was then placed in the spectrometer. Solvent temperature was recorded at the onset of each experiment and no significant thermal heating of the solution during the settling experiment itself was observed.

In order to define the plane of observation for the scattering, the cuvette was masked off with opaque plastic tape, leaving a 1 mm plane exposed approximately 1 cm from the base of the cuvette. Knowledge of the distance between this plane and the top level of solution in the cuvette is necessary to measure the size distribution (x in equation 7). Minimum data acquisition intervals varied with solvent and mean size of the silica gel. It was found that 1000 points at a 10 sec. interval yield more than sufficient data density to define the 5 μm support in water. Shorter times can be used for larger particles or solvents with lower viscosities. An example of the typical raw data (absorbance vs. time) is shown in Figure 2. Data acquisition was continued until the absorbance leveled off after a steep decline.

Construction of the size distribution from absorbance-time data was achieved using a BASIC program. Values for the solvent density and viscosity, acquisition time increment, and settling distance (x) are required as well as an estimate of the net particle

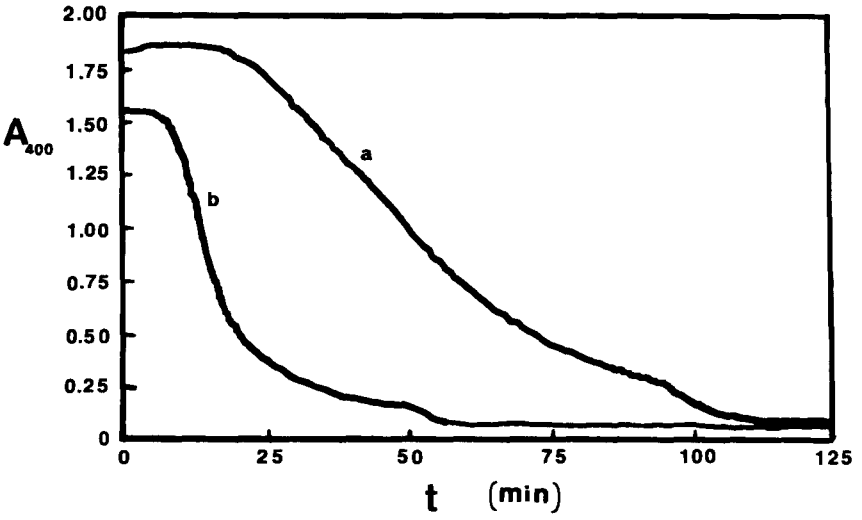

Figure 2. Absorbance-time raw data for a settling experiment. Upper (a) curve shows LiChrosorb Si60 5 micron (nominal size) silica, lower (b) curve is LiChrosorb Si60 10 micron silica. Solvent (both curves) is water, 30°C, x=2.00 cm, λ=400nm.

density. Values of the density and viscosity for non-aqueous solvents and mixtures at the experimental temperatures were obtained in standard references or were estimated using the procedures in [8]. We have estimated the net particle density by assuming a percent porosity of the particle and calculating a density based on a mean silica density of 2.36 gm/cm^3 [9] and the percent volumes of the silica and the solvent. Silica densities of 2.30 and 2.46 gm/cm^3 were used for LiChrosorb Si60 and LiChrosphere Si500 supports respectively [9]. Percent porosities for different gels were

estimated on the basis of previous experience with porosities of columns packed with the packing material or on the basis of relative packing densities and pore volumes. In this manner we used a value of 1.43 gm/cm^3 for the solvated LiChrosorb Si60 particle in water based on a porosity of 67%. It should be noted that the effect of inaccuracies in the estimation of the density is decreased due to the dependence of the particle diameter on the square root of the density. An option to skip over a set number of points was included in the computer program in order to reduce the data density in the differentiation step, if necessary, since high density at low particle sizes increases the scatter in this step.

A 9 point differentiating Savitsky and Golay smooth [10] was used to differentiate the raw data with respect to time. The critical particle sizes and α values for each time were then calculated and the corrections for differentiation with respect to α and the α squared term were performed. The size distributions in terms of number and weight concentrations (arbitrary units) as well as cumulative number and weight distributions versus the critical diameter were automatically computed and plotted on the video screen.

RESULTS AND DISCUSSION

Results of the determination of replicate runs of 5 and 10 micron LiChrosorb Si60 supports are shown in

Figures 3 and 4 respectively. In 3a and 4a the number and weight distributions are shown as a function of particle size. The integrated cumulative distributions are shown in the 3b and 4b. Two replicate runs are shown in each case. The open circles in 3b and 4b represent values provided by Merck on the labels for the cumulative weight percent, while the open squares represent the corrected data obtained from the Sedigraph analyzer. The Sedigraph data was corrected in order to normalize for a different value of the particle density used in the analysis at Micromeritics [11]. The agreement between our cumulative weights with those provided by Merck for these lots is excellent while the agreement of our distributions with those provided by Micromeritics is still quite reasonable. It should be noted that due to the algorithm used in plotting, the areas of different plots have not been conserved.

At very high data densities, for small particles the scatter in the data increases significantly due to the numerical process of differentiation of data evenly spaced in time. As the particles become very small the difference in critical diameters between successive times becomes very small, thereby greatly expanding the scatter due to noise. Time increments of 10 to 100 seconds can be used to cover the range from approximately 3 to 20 micron silica particles. In general we

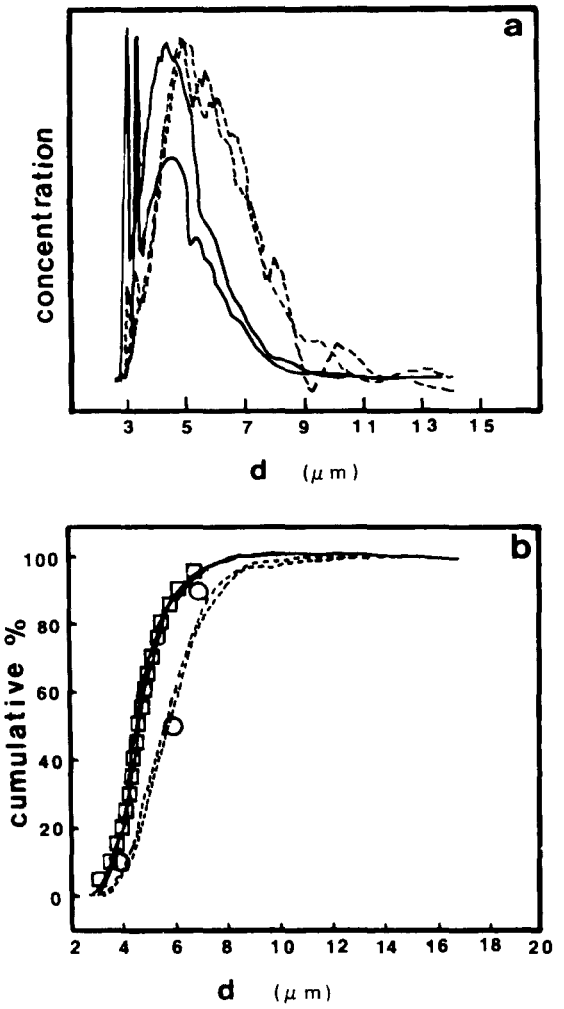

Figure 3. Size distributions for LiChrosorb Si60 silica gel (5 μm). Solid lines depict number distributions while dashed lines depict weight distributions. Replicate runs are shown for each curve. Left plot (a) shows the actual distributions of the data in (a). Squares indicate values obtained by corrected SediGraph analysis while circles indicate Merck provided data.

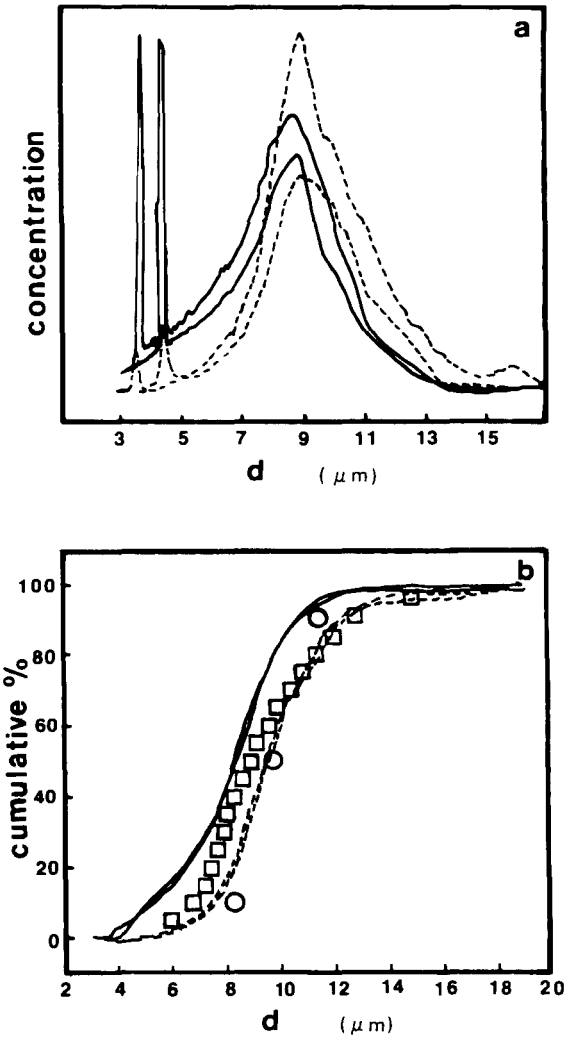

Figure 4. Size distributions for LiChrosorb silica gel (10 μm). Solid lines depict number distributions while dashed lines depict weight distributions. Replicate runs are shown for each curve. Left plot (a) shows the actual distributions while the right plot (b) shows cumulative distributions of the data in (a). Squares indicate values obtained by corrected Sedigraph analysis while circles indicate Merck provided data.

found 500 points spaced at 20 second intervals provided a reasonable compromise between data density at large diameters and data scatter in the small diameter region. We have obtained reasonable plots using as few as 100 points.

In all the data sets involving underivatized LiChrosorb 5 and 10 μm particles a significant change in the absorbance curve occurred at times corresponding to silica particles of approximately 3 and 4 microns respectively. We are uncertain of the origin of this phenomenon which manifests itself as the brief region of a high concentration (highly scattered) of particles at the very lower extreme of the distribution. Identical results were obtained at both 400 and 800 nm, indicating that this is not an anomaly of the particular scattering conditions. We speculate the existence of fine particles of much higher density for which the settling rate is the same as that for approximately 4 μm support particles. The algorithm would interpret these co-settling fine particles as a higher concentration of the larger support particles. In such a case, the high scatter in this region on the distribution plots would not signify an actual deviation in the shape of the tail of the distribution of the support particles. In any case the total effect on the cumulative number distribution is small and is negligible on

the cumulative weight distribution. This phenomenon was not observed with the same LiChrosorb gels after treatment to derivitize the particles with a DIOL functionality. In addition, the similarity of results at 400 and 800 nm wavelengths is strong support of our assumption that the effective scattering coefficient is virtually independent of α for our spectrometer, since changing the wavelength will alter the α value (see eqn. 3). If the scattering coefficient was changing significantly with α the greatest differences between 400 and 800 nm experiments should be noted at small particle sizes where our agreement is still good.

While the settling time for very small particles can be quite long, use of a solvent with lower density and/or viscosity will decrease the required time. In this case, however, changes in the surface wetting characteristics due to variation in other physical properties, e.g. the surface tension, may alter particle agglomeration properties and change the distribution. We examined LiChrosorb DIOL supports in various solvent systems and found that the DIOL settling behavior in water, methanol, and ethanol varied systematically and quantitatively as expected from equation 6 with changes in solvent density and viscosity. For these solvents the cumulative weight average particle sizes (d_{50}) were calculated to be between 10.7 and 11.8

microns. Drastically different behavior was observed in isopropanol, toluene, and cyclohexane, which can be attributed to particle agglomeration in these solvents. The values for the measured apparent d_{50} values in these solvents were 13.8, 23.1, and 29.0 microns, respectively. The distributions of the particles in methanol, isopropanol, toluene, and cyclohexane are shown in Figure 5. Similar results were observed for both commercial and in house preparations of the DIOL phase. The shift in the measured distribution to particles of larger (apparent) size also suggests that particle agglomeration is occurring. The actual size of the larger particles may not be accurately reflected by the abcissa value as the agglomerate net density probably varies from the assumed value for single particles due to a probable change in the effective porosity of the agglomerate. Because the algorithm assumes a single density value for all particles, increased porosity in agglomerates, due to the interparticle volume, would actually result in a larger particle size than shown.

The data from the DIOL phases agrees well with the expected results based upon the assumption that highly polar particles will agglomerate in very non-polar solvent to minimize the exposed surface area. The results in isopropanol are nonetheless surprising. The solvent order in which agglomeration apparently occurs

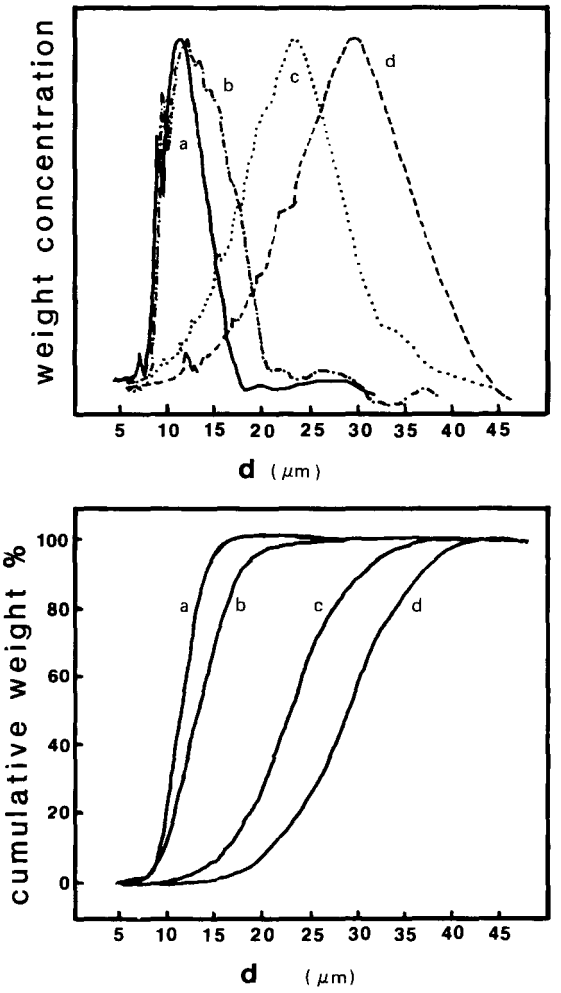

Figure 5. Particle weight distributions of LiChrosorb Si60 DIOL (10 m)samples in the solvents (a) methanol, (b) isopropanol, (c) and toluene, and (d) cyclohexane. The cumulative weight distributions are shown in the lower plot.

is consistent with the solvent polarity as measured by Snyder's polarity (P') scale [12] in which the order of solvents decreases in the sequence as water > methanol > ethanol > isopropanol > toluene > cyclohexane.

Less solvent dependent results were obtained using the reverse-phase octadecylsilyl derivitized support Hypersil ODS (5μm) in various non-aqueous solvents. Results for Hypersil in methanol, ethanol, isopropanol, toluene, and cyclohexane indicate no significant agglomeration occurs for the reverse-phase support in these solvents. Weight distributions for the Hypersil ODS in methanol, isopropanol, and cyclohexane shown in Figure 6 illustrate this point. The dry silica density of 2.36 gm/cm^3 and a net porosity of 45% was used in calculations for this material. Water was not tested due to its inability to wet the support, so in order to test the effect of increased solvent polarity on the settling behavior of the Hypersil ODS supports, an electrolyte was added to methanol. The use of 12 millimolar hydrochloric acid in methanol as solvent resulted in doubling the average particle size relative to the value in pure methanol. When the solvent was comprised of 16 millimolar ammonium hydroxide, however, a slight decrease in the average particle size relative to pure methanol was noted. These results can be understood both in terms of agglomera-

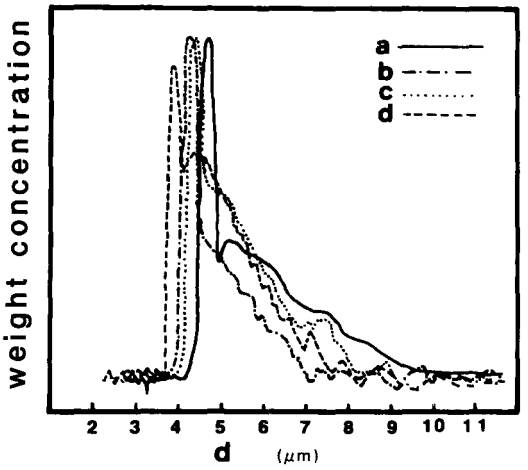

Figure 6. Particle weight distributions of Hypersil (5 μm) ODS samples in the solvents (a) methanol, (b) isopropanol, (c) toluene, and (d) cyclohexane.

tion of a non-polar support in the polar solvent and in terms of the existence of an electrostatic barrier to agglomeration created by residual surface charge. In the former case the addition of hydrochloric acid to the solvent should result in a decrease of the net surface charge due to the increased degree of protonation of residual surface silanols, thereby reducing the electrostatic barrier to agglomeration. In the case of added ammonia, the degree of silanol ionization should increase, creating a net negative charge on the particle [1] for which the electrostatic repulsion opposes agglomeration and offsets the effect of the increased solvent polarity.

A strong solvent dependence of the settling behavior was also observed for DIOL phases derivitized with the triazine dye Cibacron Blue 3G-A. Figure 7 shows size distributions for LiChrosphere Si500 DIOL, derivitized with Cibacron Blue by the procedures of Mosbach et al. [13], in various solvents. The anionic triazinyl dye is a polysulfonated aromatic ring system covalently bound to the DIOL phase via a 1,6-hexane diamine spacer arm at very low surface concentrations (ca. 10 nmole/m^2). While the equations derived earlier do not strictly hold in the case in absorbing scatterers (Cibacron Blue absorbs somewhat throughout the visible spectrum) the above approach should be able to differentiate changes in the shape of the size distribution between solvents. This is seen in Figure 7 in which the differences in the size distributions in methanol, isopropanol, toluene, and cyclohexane again suggest that agglomeration is occurring. In comparison with their precursor DIOL phases, the Cibacron modified phases display a similar pattern but appear to be slightly more hydrophillic, which is consistent with the existence of several highly polar and ionic functional groups on the dye molecule. While a detailed knowledge of the interactions between solvent and Cibacron supports is unknown at this time the data suggests that at least the non-polar solvents interact with the

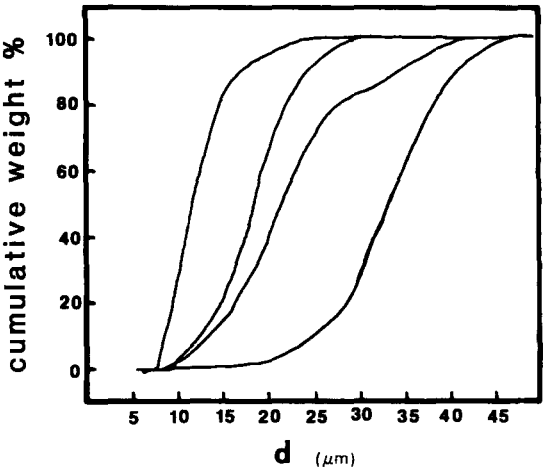

Figure 7. Cumulative weight distributions of samples of Cibacron Blue 3G-A modified LiChrosphere Si500 DIOL in the solvents (a) methanol, (b) isopropanol, toluene, and (d) cyclohexane.

support in a very different manner than do polar solvents.

Figure 8 summarizes the results of experiments on five different silica supports in various solvent systems. The abscissa in Figure 8 represents the measured value of the cumulative weight average particle size (d_{50}) for the given silica gel in the specified solvent system. Silica gel 1 is the underivitized 5 and 10 micron LiChrosorb Si60 while silica 2 is the data from the 5 micron Hypersil ODS support. Silica 3 is LiChrosorb Si60 DIOL and silicas 4 and 5 are Cibacron modified phases. Silica 4 is lightly loaded with

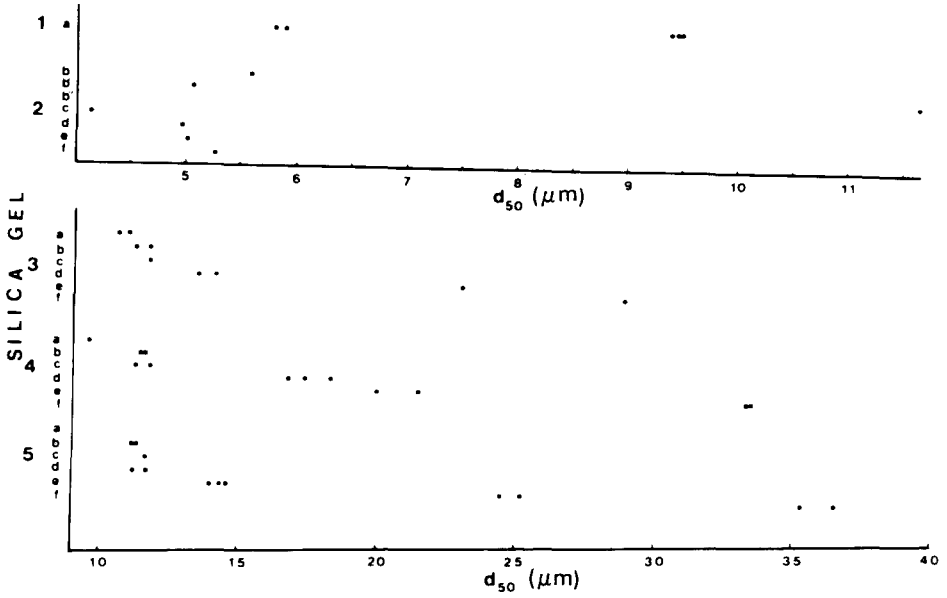

Figure 8. Summary of the dependence of the observed 50% cumulative weight diameter (d_{50}) on the solvent for a variety of silica gels: (1) underivitized LiChrosorb Si60 5 and 10 μm particles, (2) Hypersil (5 μm) ODS, (3) LiChrosorb Si60 DIOL, (4) Cibacron modified (Mosbach prep) LiChrosphere Si500 DIOL and (5) Cibacron modified (directly coupled) LiChrosorb Si60 DIOL. Solvents are designated as (a) water (b) methanol, (b') 16 nM NH_4OH in methanol, (b'') 12 nM HCL in methol, (c) ethanol, (d) isopropanol, (e) toluene, and (f) cyclohexane.

ligand immobilized via the hexanediamine spacer arm as described above, while silica 5 is a much heavier loading of directly bonded (no spacer arm) dye. The solvent symbols are given in the figure legend.

CONCLUSIONS

The results obtained by this method of studying the particle size distribution are comparable to those

of other methods for silica chromatographic supports. Because the method fundamentally estimates the number distribution most other widely used definitions of the distribution can be readily calculated. The method uses only common laboratory experiment. The parameters necessary involve an estimation of the density of the particles in the solvent, a measurement of the settling distance, and values of the solvent density and viscosity. Of these the particle density can be obtained based on an approximation of the porosity of the particle. In addition to determination of the size distribution for the purposes of column efficiencies, the method can be useful in the evaluation of solvent effects on particle agglomeration. By evaluating the size distribution of a support matrix in various solvent systems and comparing the results, significant information can be inferred as to the nature of the particle surface and solvent wetting under the experimental conditions. By means of an example, settling rates and size distribution data for Cibacron Blue affinity chromatographic supports suggest substantial particle agglomeration occurs in non-polar solvents, implying a strongly polar character to the surface. Knowledge of the particle settling rates and agglomeration properties can be useful in the optimization of solvent conditions during column packing.

ACKNOWLEDGEMENTS

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COLUMN LIQUID CHROMATOGRAPHY OF REDUCING CARBOHYDRATES BY FLUOROMETRIC REACTION DETECTION WITH A PRESSURIZED REACTOR OUTLET*

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ABSTRACT

The use of reaction detection in column liquid chromatography is not always precluded by the limitation of the reaction time to one or two minutes. A closed pressurized reactor allows heating of the reaction mixture to temperatures that exceed the boiling point of the mixture to enhance reaction speed.

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* Part of this paper has been presented at the Seventh International Symposium on Column Liquid Chromatography, Baden-Baden, G.F.R., 1983.

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INTRODUCTION

The translation of classical analytical procedures in which chromophoric or fluorophoric products are formed to flow systems is often encountered in modern liquid chromatography and flow injection analysis literature. Reaction detection considerably extends the application potential of column liquid chromatography (1). In reviews and comparative studies (1-4) on the subject of the performance of post-column reactors in column liquid chromatography, the residence time of the components in the reactor determines the applicability of different types of reactors. For fast reactions (residence time 1 minute), most of the reactors investigated meet the requirements set by state-of-the-art column liquid chromatography. However, for reaction times exceeding 5 minutes the performance of all reactor types falls short of the demands of modern LC, with the exception of gas-segmented flow reactors without a debubbler, which are not commercially available yet. However, the packed reactor and the tubular reactor have distinct advantages. The latter is commercially available and widely used as post-column reactor. It should be emphasized that the limitation of the reaction time to one or two minutes precludes the use of inefficient reaction detectors. Slow reactions can often be accelerated considerably by heating. When tubular or packed reactors are used a reactor with a pressurized outlet allows the reaction mixture be heated to above its boiling point.

This is demonstrated in the present study for the determination of reducing carbohydrates by the reaction with

2-cyanoacetamide. 2-Cyanocetamide reacts with carbohydrates in a slightly alkaline medium to yield intensely fluorescent products (5).

EXPERIMENTAL

Equipment

The chromatographic system, including separation column, tubular reactor and cooler, is shown in Figure 1. High pressure pumps from various manufacturers (type HP1081B, Hewlett Packard, Waldbronn, G.F.R.; type SP 740B, Spectra Physics, Santa Clara, CA, U.S.A.; type M6000A, Waters Associates, Milford, MA, U.S.A.) were used as solvent and reagent delivery systems. Sampling valves (type 7010 and 7410, Rheodyne, Berkeley, CA, U.S.A.) with 5 and 20 µl loops and a 1 µl internal loop respectively, were used for injecting inert tracers or reactants. A filter fluorometer (type 420C/E, Waters Associates) equipped with a low pressure mercury lamp (type F4T5/BL) covered with a smooth layer of crystalline phosphorus and further equipped with a bandpass filter at 337 mnm as excitation filter and a cut-off filter at 375 nm as emission filter was employed as detector. Later, for comparison a refractive index detector (type 79877A, Hewlett Packard) was used instead of the fluorometric reaction detector.

Capillary tubes (316 SS) of 20 m and 15 m (ID 0.25 mm) and 2 m (ID 0.10 mm) all with an OD of 1/16" were purchased from Handy & Harman (Norristown, PA, U.S.A.) and wound to coils with a diameter

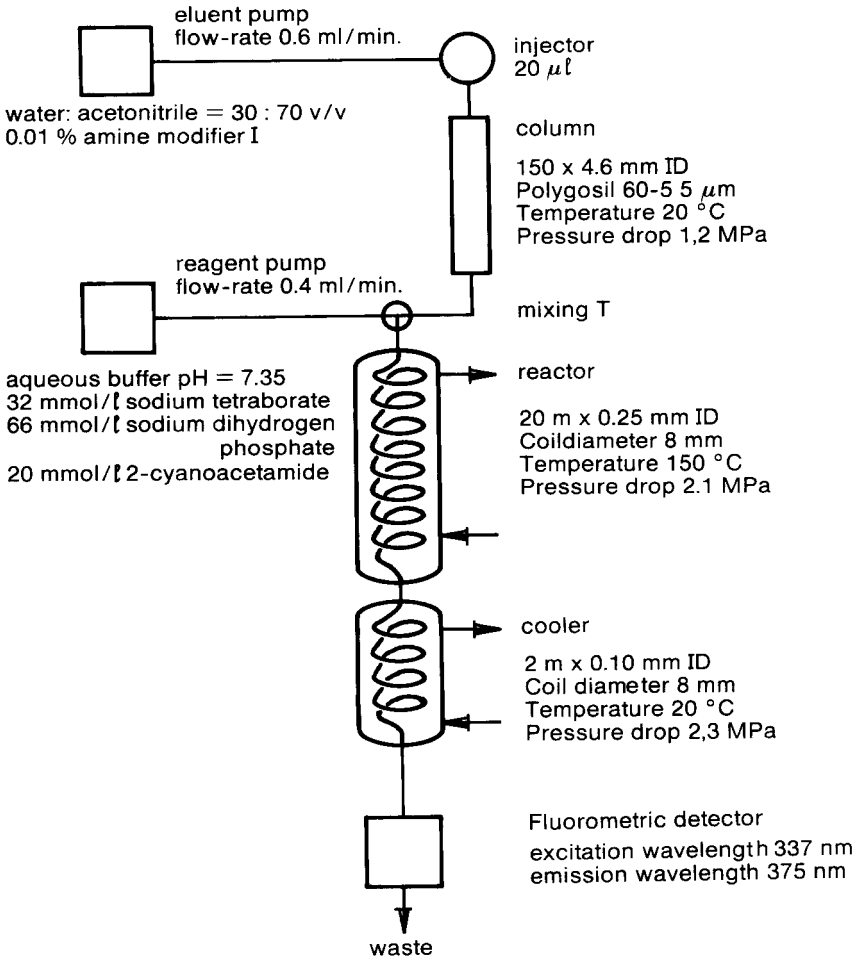

FIGURE 1

Schematic diagram of the liquid chromatographic equipment with post-column detection system.

of 8 mm. The tubular reactor was held at constant temperature by silicone oil, which was circulated by a thermostat (type U3 S15/12, Lauda, Königshofen, G.R.F.) with an accuracy of 0.5 °C. The reactor was mounted in a SS vessel. The cooler is also held at constant temperature (20 °C) by means of a thermostat of the same type. The estimated back pressure required to keep the effluent from the reactor in the liquid phase is 0.6 MPa. For safety reasons the cooler was designed to produce a back pressure of 2.3 MPa. A 150 mm long 316 SS liquid chromatographic column (type Lichroma, Handy & Harman) with an ID of 4.6 mm was filled with silica (type Polygosil 60-5, mean particle size 5 μm, Macherey & Nagel, Düren, G.F.R.) by means of a slurry technique. The column was thermostatted by means of an oven at 20 °C in the HP 1081 B liquid chromatograph.

The detector signal was digitized by means of a digital voltmeter (type HP3473A, Hewlett Packard) and the peaks were reconstructed by batchwise processing of the data on a desk computer (type HP 85, Hewlett Packard). The same procedure for data collection was used, when calculation of second moments and deconvolution according to Yau et al (6) were required. Peak area measurements and data handling were done with a chromatographic datasystem type SP 4000, Spectra Physics).

Chemicals

All reagents and pure analytical reference chemicals were of pro analyse grade and had been purchased from Merck (Darmstadt,

G.F.R.), unless stated otherwise. The liquid chromatographic mobile phase consisted of double quartzglass distilled water and acetonitrile (Lichrosolv grade, Merck) in the amounts of 30 and 70 vol.% resp.. To the water 0.01 % amine modifier I (Natec, Hamburg, G.F.R.), which is 2,4 N-di(2-ethylamino)diaminobutane, $NH_2(CH_2)_2NH(CH_2)_4NH\ (CH_2)_2NH_2$, was added. The 2-cyanoacetamide reagent was prepared in a buffer solution of pH = 7.35 containing 32 mmole l^{-1} sodium tetraborate and 66 mmole l^{-1} sodium dihydrogen phosphate. To this buffer 20 mmole l^{-1} 2-cyanoacetamide was added. The pH of the buffer was varied by adding different amounts of the sodium tetraborate and sodium dihydrogen phosphate.

RESULTS AND DISCUSSION

The separation of carbohydrates was performed on a silicagel column physically modified with a polyfunctional amine (7), water acetonitrile mixtures being used as mobile phases. A recent review on the analysis of carbohydrates by column liquid chromatography is that by Verhaar and Kuster (8). The reaction was carried out in a coiled tubular reactor because this type of reactor can be used in an alkaline medium at elevated temperatures, while a packed bed reactor filled with glass beads may cause difficulties at pH values higher than 7.0.

The reaction time was chosen to be 60 s. A short capillary tube was used to cool the reaction mixture before it entered the fluorometric flow cell and to prevent the mixture from boiling.

This capillary tube thus acted as a cooler and as a back pressure device. The low temperature enhances fluorometric sensitivity; at elevated temperatures the signal is strongly quenched.

Optimization of the reaction conditions

The yield of the reaction can be increased considerably by heating the reaction mixture (9). Figure 2 shows the fluorescence intensity of the reaction product of 2-cyanoacetamide and glucose as a function of the reactor temperature at a constant residence time in the reactor. The gain is about a factor of 10 if 150 °C is applied instead of 100 °C, as was done by Honda et al (5,10). Even higher temperatures can be applied but as for the sake of easy handling 150 °C was chosen. As shown by Honda et al (5) the pH dependence of the reaction is of importance for optimization purpose. Figure 3 illustrates the pH influence on the reaction yield and thus on the fluorescence response for glucose at 150 °C. The pH of the reagent solution was measured at room temperature and the optimum was found at the value of 7.35 for a tetraborate/phosphate buffer. For a borate/boric acid buffer the optimum conditions are found to be pH = 9.30. These conditions are less favourable because of solubility problems, although the same sensitivity is obtained.

Band broadening

In a properly designed post-column reactor the additional band broadening should be as low as possible to reduce the inevi-

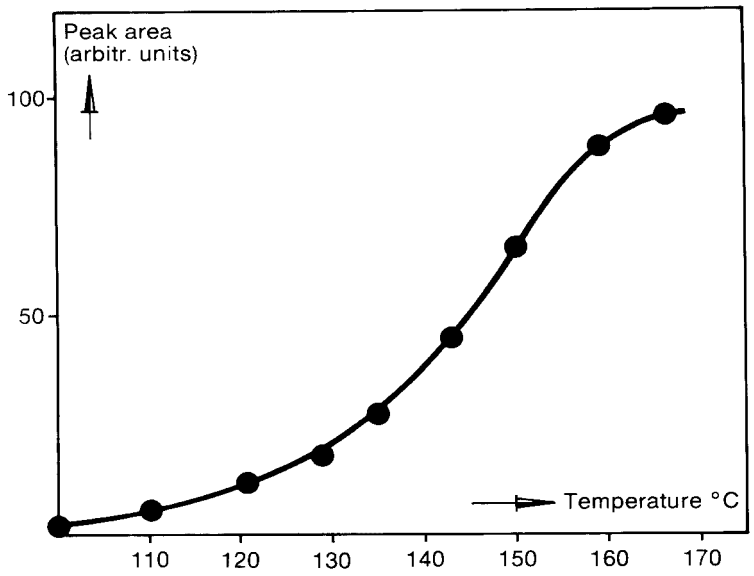

FIGURE 2

Influence of the temperature on the reaction measured by the fluorescence intensity of the reaction product of 2-cyanocetamide and glucose. The residence time is 60 s and the pH of the reaction mixture at room temperature is 7.35.

table loss in resolution (11). In this study we paid attention to this subject also. The variance of the chromatographic peaks was measured and calculated by an interactive procedure (6) by which the second order central moment is determined from the zeroth and first moments. The additional band broadening contribution of each individual part of the liquid chromatographic equipment was measured: in constructing the system, one component was added at a time and the increase in total variance due to the addition of each component was measured. This was done by injecting the reac-

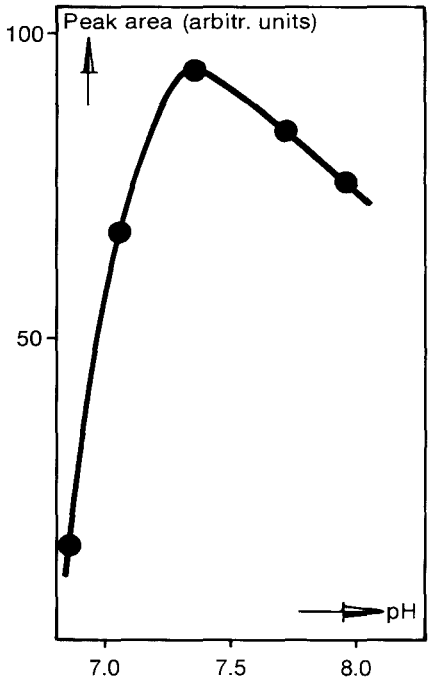

FIGURE 3

Influence of pH of the reaction medium on the reaction yield determined by measuring fluorescence intensity of the reaction product of 2-cyanoacetamide and glucose. Reactor temperature: 150 °C.

tion product of 2-cyanoacetamide and glucose, which had been prepared separately by refluxing 120 ml of reagent solution and 50 mg of glucose for 20 hours, into the system, from which the separation column had been deleted. The results of these experiments are summarized in Table 1. It is clear that the contribution of the reactor is small in comparison to the 15 m long cooler. This can be explained by the difference in diffusion coefficient of the

TABLE 1

Experimental determination of the band broadening in the reaction detector for carbohydrates.

	σ_t^2, s^2
injector + fluorometer	3.4
reactor 20 m x 0.25 mm ID, 150 °C	2.0
cooler 15 m x 0.25 mm ID, 20 °C	13.9
cooler 2 m x 0.10 mm ID, 20 °C	1.0

carbohydrate or the product formed (1) in the reactor effluent at 150 and 20 °C. The miniaturized cooler of 2 m in length and 0.10 mm ID is to be preferred for its lower level of additional band broadening. The total variance in time units of the reaction detection system becomes 6.3 s^2 when the 2 m long cooler is used, which is only a minor contribution to the overall variance (including the separation column) of 40 s^2. Resolution of the components and overall efficiency (the HETP value of fructose is 26 μm) are only little influenced by this extra-column band broadening. The separation of a test mixture is illustrated in Figure 4. Although a reaction detection system was used the resolution of fructose and glucose is good and even better than with several separation systems on polystyrene-divinylbenzene packing materials as presented in the literature.

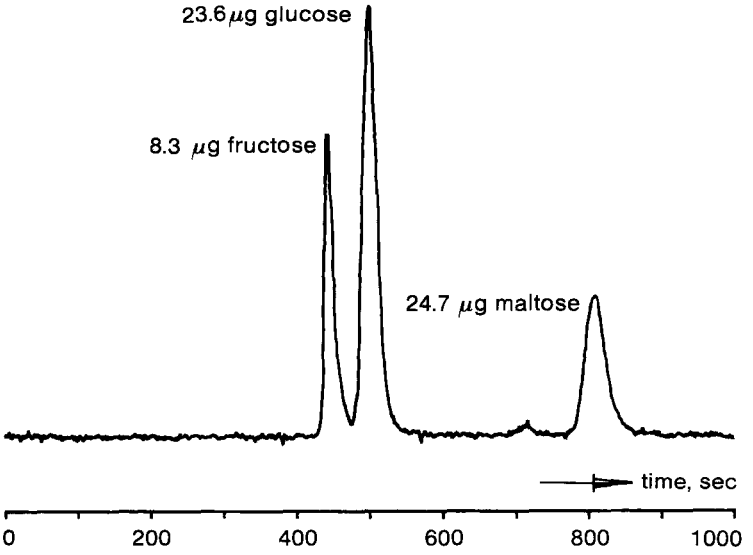

FIGURE 4

Chromatogram of a test mixture of fructose, glucose and maltose.

Quantitative analysis

Quantitative evaluation of the chromatographic data is generally done by the external standard method. In order to find the linear dynamic range of a method and the detection limits, calibration curves are measured. Calibration curves of the chromatographic method using reaction detection are shown in Figure 5. For glucose and fructose a linear relationship is obtained; all datapoints are average values of duplicate measurements.
The precision of the analytical method is determined by at least 5 consecutive injections of standard test mixtures at several

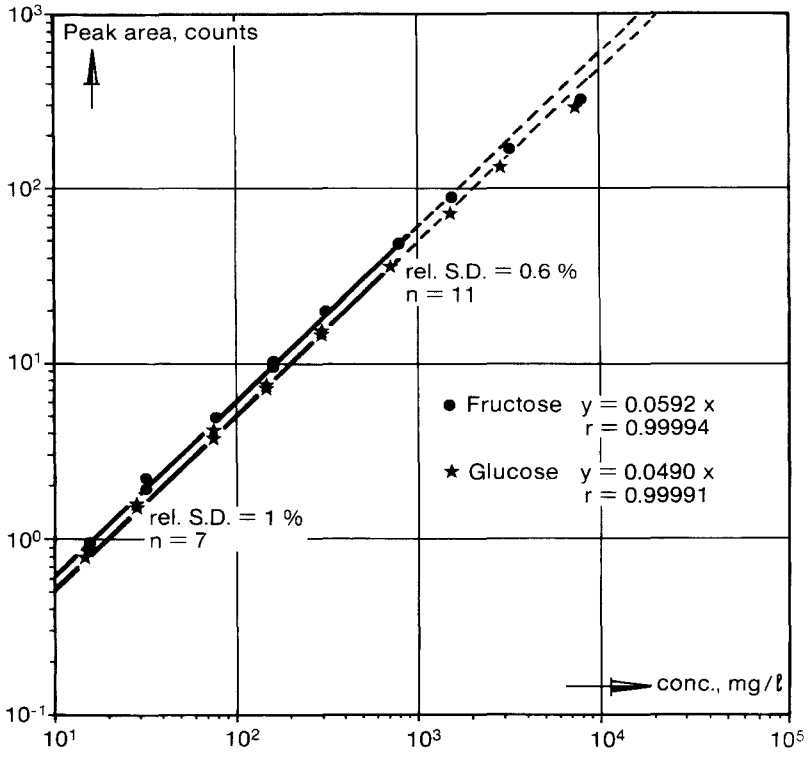

FIGURE 5

Calibration curves for glucose and fructose using fluorometric reaction detection with 2-cyanoacetamide.

concentration levels and calculation of the relative standard deviation (rel. S.D.) of the response factors. As indicated in Figure 5 a relative standard deviation better than 1 % can be achieved even for a reaction detection system as described. This is confirmed by earlier studies (12). For comparison reasons the calibration curves of fructose and glucose are determined with a refractive index detector too (Figure 6). A good linearity over

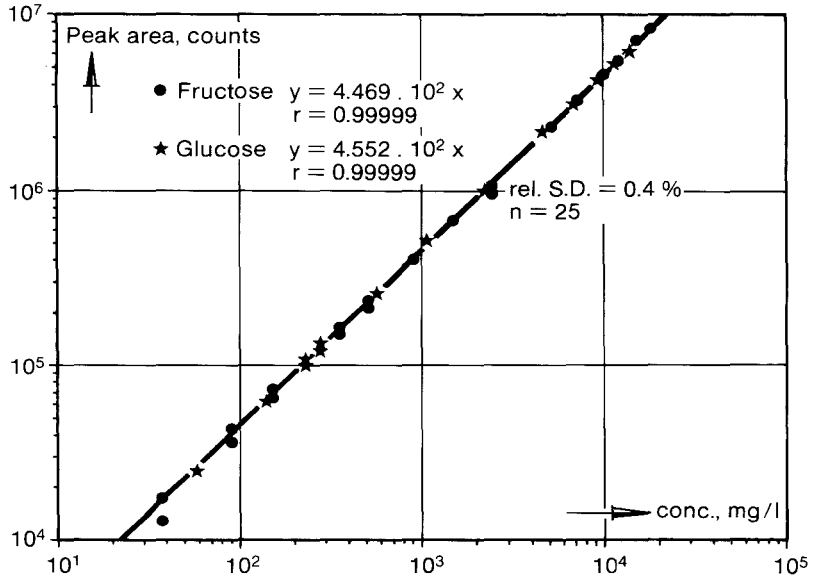

FIGURE 6

Calibration curves for glucose and fructose using differential refractive index detection.

3 decades was obtained. The refractive index detector has the same detection limit (10 mg l^{-1} for fructose) as the reaction detection fluorometry and a wider linear dynamic range. The use of a excitation as well as emission variable wavelength fluorometer would be preferable in order to use at the maximum excitation and emission wavelengths of 331 nm and 383 nm respectively. Moreover no cut-off filters and bandpass filters, which reduce light intensity, need to be used. Besides the concentration of 2-cyanoacetamide is restricted because of limited solubility in water/acetonitrile mixtures. On the other hand the fluorometric reaction detection

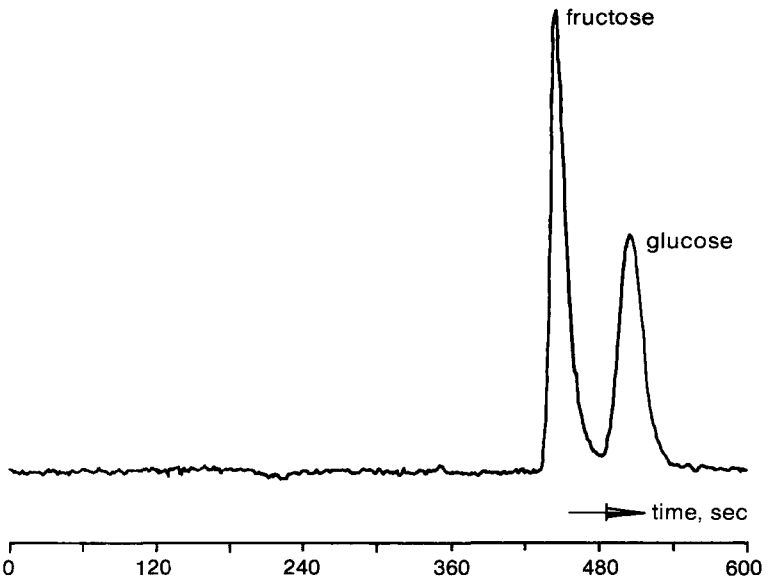

FIGURE 7

Chromatogram of honey containing 41 wt.% fructose and 41 wt.% glucose (a solution of 0.09 wt.% honey in water was injected).

method shows a higher detection selectivity, which is illustrated by the chromatogram of honey in Figure 7. Of the numerous constituents of honey, only fructose and glucose are detected.

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VISCOSITY DETECTION WITH A PULSELESS PUMP FOR LIQUID CHROMATOGRAPHY

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ABSTRACT

The performance of a differential pressure transducer as a viscosity detector in conjunction with a pulseless high pressure syringe pump designed for the purpose is described. The detector, although relatively insensitive, is inexpensive and extremely rugged. Because it is non-destructive and introduces very little dispersion, serial coupling ahead of other detectors is possible. Response behavior is demonstrated.

INTRODUCTION

The differential refractometer is the only commonly used universal detector in the current practices of HPLC (1). Although optical absorption detectors dominate analytical HPLC, overloading of such detectors (especially fixed wavelength types) are frequent in preparative applications and therefore necessitate unusual designs. Viscosity is a bulk property, analogous

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to refractive index. The viscosity of a chromatographic effluent changes accompanying solute elution. Viscosity of a chromatographic effluent is most easily monitored by measuring the pressure drop across a capillary as the column effluent is made to flow through it as a constant rate. The pressure drop P across a capillary of radius r and length L is given by the Hagen-Poiseuille equation (2):

$$\Delta P = 8FnL/\pi r^4$$

where a fluid of viscosity n is flowing through the capillary at a constant flow rate F. Since F, L and r are constants for a given experimental condition, the pressure drop is related directly to the effluent viscosity. Ouano (3-5) first conceived the design and application of a flow through continuous viscometer, based on a pressure transducer, as a detector in HPLC. Such detectors are of particular utility in size exclusion chromatography, both because macromolecules cause significant changes in effluent viscosity and also because intrinsic viscosity values can be used for molecular weight determinations. By using a differential refractometer in tandem, the sample concentration may be independently evaluated and be used in conjunction with the viscosity detector output to calculate the intrinsic viscosity and thence the MW (3-7).

As the Hagen-Poiseuille equation indicates, a capillary pressure drop type viscosity detector is sensitive to flow fluctuations and as demonstrated by Lesec et al. (7), may in fact be used as a highly sensitive flow meter. The applications of a viscosity

detector have thus far been limited to size exclusion chromatography. To minimize flow pulsations common to high pressure reciprocating pumps, various types of pulse dampening systems have been used.

In this communication, the design and construction of a simple pulseless high pressure syringe pump, of particular utility with a capillary pressure drop based viscosity detector, is described. The response behavior of small solutes with such a detector is demonstrated.

MATERIALS AND METHODS

The Detector was based on a variable reluctance type differential pressure transducer (model DP 45) coupled to a carrier demodulator (model CD 15) both from Validyne Engineering Corporation, Northridge, CA. This transducer translates the pressure induced mechanical displacement of a flexible diaphragm into an electrical signal. The particular transducer-demodulator combination used in this work produced a 0-10V adjustable output with a differential pressure input equal to 1 inch of water column (0.035 psi). The voltage output is linearly related to the differential pressure input and could be offset up to 15 psi in either direction.

The detector was used generally in the true differential mode, as in the configuration depicted in Figure 1a. The capillary tube (316 stainless steel, 5-15 cm long, 0.05-0.015 cm internal diameter) was connected to the transducer inputs via zero dead volume tees (Valco) and universal couplings (Alltech). The latter fittings are not essential, but facilitate rapid

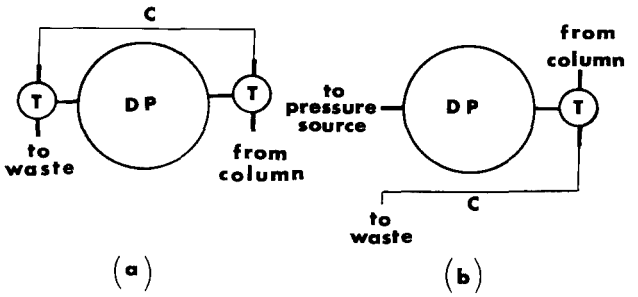

Figure 1. Two modes of detector configuration. T : zero dead volume tee; C : capillary; DP : differential pressure transducer.

exchange of different capillaries for experimental purposes. In this configuration the output can be offset only to the extent allowed by the detector electronics and thus sets a limit on the length of the capillary and/or flow rate (which together govern the pressure drop across the transducer) if baseline signal is to be adjusted to zero.

The alternative configuration depicted in Figure 1b utilizes the capillary connected to one side of the transducer, the capillary effluent being allowed to drain into a waste beaker. The other side is pressurized with a suitable source (e.g. cylinder gas with two-stage regulator) to obtain a zero or near zero differential pressure with the eluent flowing through the capillary at the desired rate. In this configuration, there are no restrictions on the capillary bore/length or flow rate, up to the maximum absolute pressure limit of the transducer.

Initial experiments with this detector used in a chromatographic system with a single piston

reciprocating pump equipped with a hydraulic capacitor type pulse dampener (Altex model 110 A) revealed that the attainable sensitivity is limited by the baseline noise (sawtooth type) induced by the pump pulsation. A high pressure syringe pump therefore was designed and built.

A stainless steel (type 316) cylinder, 15 inches in length, 1.5 inch in diameter and 0.25 inch wall thickness was inside polished to a mirror-finish (3-4 rms, Miether Machine Works, Odessa TX). If non-corrosive eluents are used, the hydraulic shock-absorber housing of a truck is an inexpensive alternative for this purpose. A stainless steel end cap, provided with a 1.5 mm diameter 0.75 mm bore stainless steel outlet tube was welded onto one end of the cylinder. Piston movements in this cylinder was accomplished via a worm-driven screw jack of 2 ton capacity (screw diameter 1 inch, screw lift 15 inches, model no. NKM-1802-15, Duff-Norton Company, Charlotte, NC). The lifting screw of this jack is keyed such that the lifting screw does not rotate during ascent/descent. The gear ratio produces 1 inch translational movement of the screw for 100 turns of the worm. The worm shaft was coupled to a 1/8 H.P. shunt wound D.C. bidirectional motor with 40:1 gear reduction (drive model 589 with type 916 speed control unit, Bodine Electric Company, Chicago, IL). Except for replacing the single turn potentiometer used for speed control by a 10-turn vernier readout potentiometer, no changes were made. The worm rotation speed could be reproducibly adjusted from 1-56 rpm.

The jack and the motor were firmly mounted on a 1.5 inch thick wooden base with the housing for the lifting screw protruding beneath the base through a suitably accomodating hole. The piston is attached to the top of the lifting screw (described in more detail below) and fits within the cylinder. The cylinder is held in place by two 0.75 inch thick aluminum plates containing holes of appropriate size and are aligned in position by four all-thread (0.5 inch diameter, 13 threads to the inch) steel supports. The pump may be used in the horizontal or vertical position. The latter position is preferable since it occupies little bench space and in this case, C-clamps are recommended for securely clamping the pump to the benchtop. The pump assembly is shown in Figure 2.

The piston was fabricated from stainless steel; a 3 inch length was machined to closely fit inside the cylinder. Approximately 1.5 inch length of this piece was further shaved to a diameter of 1 inch and threaded with 20 threads/inch. The threaded portion accomodated a set of cup shaped nylon V-rings in position. Neither the stainless steel body of the piston, nor the teflon disc contacts the cylinder wall during operation; the sealing is provided only by the rings. The top 0.75 inch poriton of the threaded lifting screw was shaved to a diameter of 0.75 inch. The stainless steel piston piece was machined to accomodate this portion of the screw at the end opposite from the teflon disc. With the lifting screw head tightly butting against the piston body, a recessed and somewhat loosely fitting retaining screw was put in to attach the two pieces. The retaining screw plays a role only during the withdrawal of the piston. The piston design is shown in Figure 3.

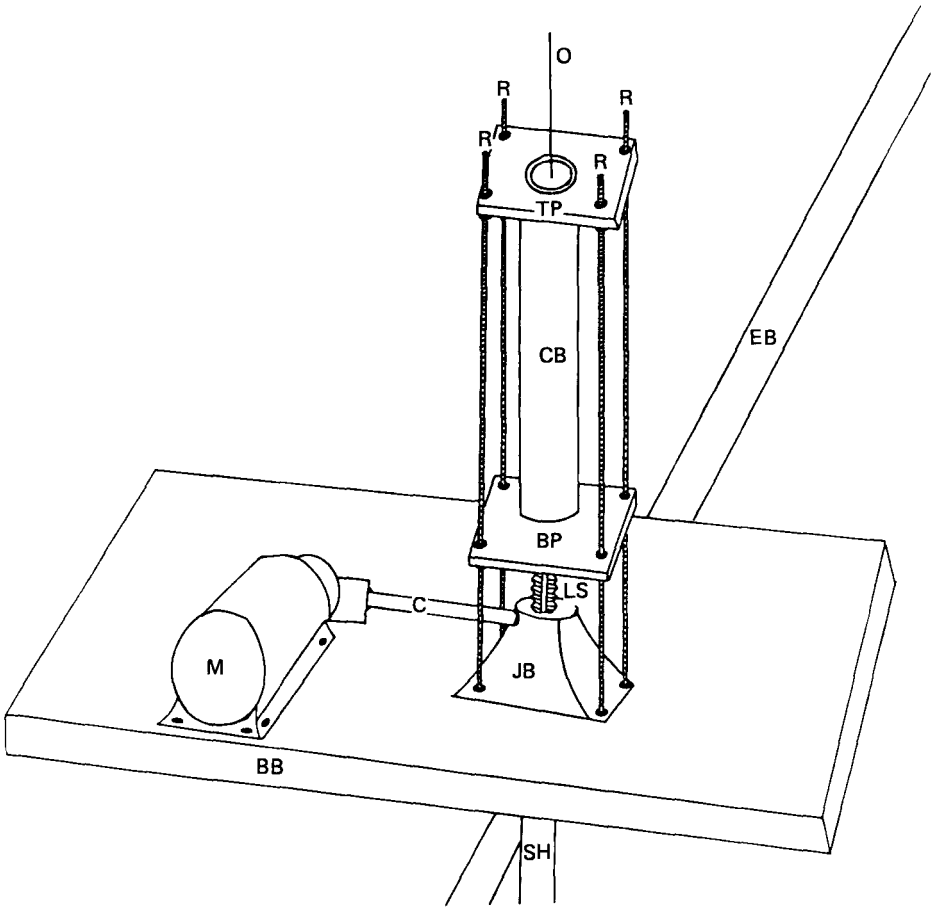

Figure 2. High pressure syringe pump. M : motor; C : coupling, motor shaft to worm shaft; JB : jack body; LS : lifting screw; SH : lifting screw housing; BB : baseboard; EB : edge of workbench; CP : syringe barrel; BP : bottom retaining plates; TP : top retaining plate; R : threaded support rods; O : pump outlet.

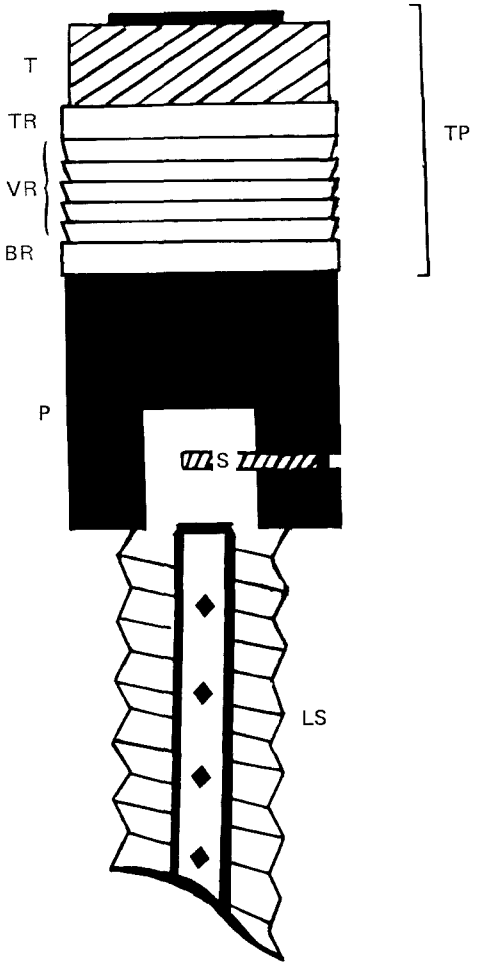

Figure 3. Details of the piston. T : teflon disc; TR : top, V-ring set; VR : v-rings (5); BR : bottom, V-rings set; P : stainless steel body of piston; TP : threaded portion of P: LS : lifting screw; S : retaining screw attaching lifting screw to piston. Diamonds in the lifting screw key slot indicate where numerals are engraved.

The pump outlet is connected via a 0.5 μm line filter (SSI) to a 3-way high pressure valve (SSI) which connects it either to an injection valve (Rheodyne type 7000) or a solvent reservoir. These connections are made with 0.75 mm bore tubing, to avoid cavitation during pump filling. To fill the pump (capacity 350 mL), the motor is run maximum speed in reverse with the inlet/outlet connected to the solvent reservoir, which should ideally be placed a level above the pump head to avoid cavitation. Less than 25 min are required for complete filling. Since the piston itself is not visible, it is necessary to provide a means to judge the position of the piston at any time. To accomplish this, numerals were engraved every 0.5 inch on the keying slot of the lifting screw (See Figure 3) and could be inspected between the cylinder bottom plate and the top of the jack housing. This allows one to avoid driving the piston beyond either of its intended limits. As a matter of convenience and further safeguard, especially to permit unattended filling and operation, two pairs of photoelectric sensors (infrared emitter-detector pair, Radio Shack 276-142), coupled to requisite electronics, may be mounted in the lifting screw housing which protrudes beneath the pump base. Obstruction of the light path in the bottom pair by the descending screw indicates complete filling of the pump and is configured to prevent further reverse movement of the motor. Lack of obstruction of the light path in the top pair by the ascending screw indicates the piston is at the end of its travel and is configured to prevent further forward movement of the motor. The control circuitry is shown in Figure 4.

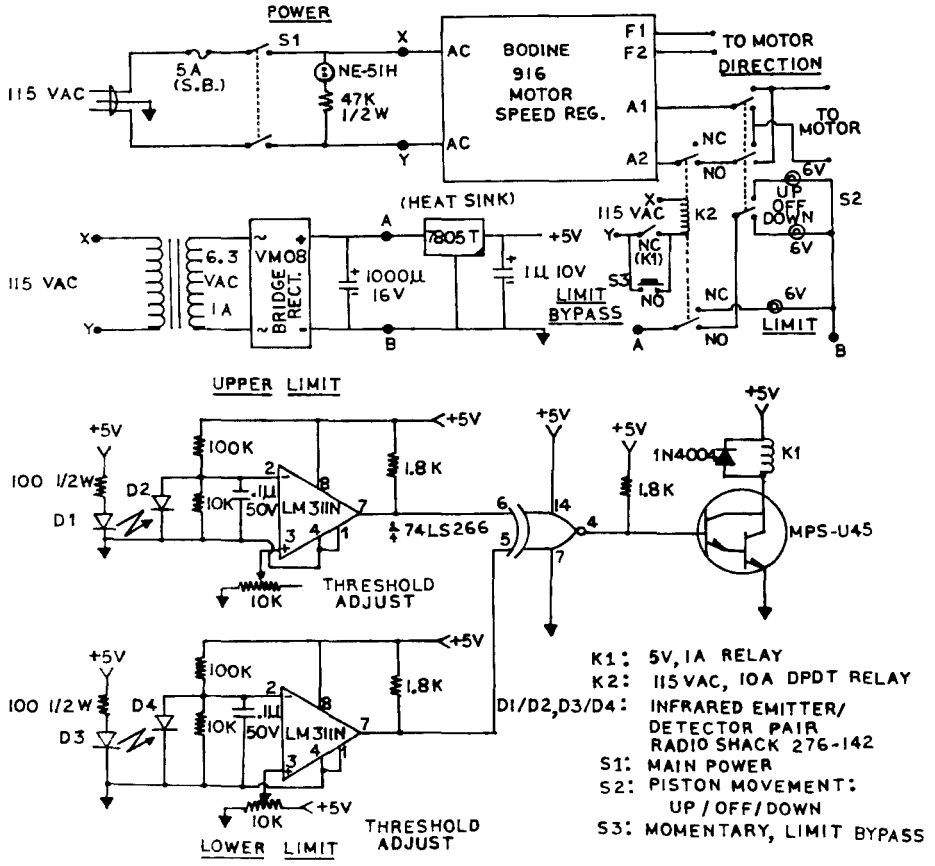

Figure 4. Control circuitry for pump. All resistors are 1/4 W, 5% unless otherwise specified.

RESULTS AND DISCUSSION

Pump Performance. The attainable flow rate of the pump is 0.4-16 mL/min and was primarily designed for conventional analytical and preparative work. Smaller flow rates may be obtained with a slower revolving motor although fill time will increase proportionally. A simple scaling down of the cylinder size and choosing a

slower motor easily permits a pump that delivers pulseless flow at flow rates down to 1 μL/min. Unattended pump filling is easily achieved by a suitable turn-off timer whether or not photocontrols are incorporated. The design pressure for this pump was 3,000 psi, primary limit being the rating of the jack. The pump has, however been used routinely at 3,500 psi without any observable ill effects. On a number of occasions, a partially blocked column had inadvertently led to the pump operating at 5,000-5,500 psi head pressure which caused leakage at the injection valve but was seemingly without effect on the pump itself. A pressure relief valve (Rheodyne 7037) set at 5,000 psi was subsequently incorporated ahead of the injector for safety measures. The long term effect of operating at pressures higher than 3,500 psi is not known however. No detectable pulsation or any other flow aberration was noticeable when a rotameter was connected directly to the pump output for flow measurements. Baseline noise levels, when directly coupled to a Schoeffel 770 optical detector set at 190 nm were two orders of magnitude below that obtained with an Altex 110 A reciprocating pump and its associated pulse dampener while reagent grade methanol was pumped through either system. At all but the maximum operating speed (e.g., during filling) the opertion of the pump is inaudible. The pump was built at a cost under $1,200.

Detector Performance. The directionality of a signal on such a detector is not predictable a priori. This is unlike refractive index detectors where a knowledge of the R. I. of the pure solute (or that of a

concentrated solution) and that of the eluent generally permits prediction of signal directionality. Viscosity of a mixture of two substances A and B may often be greater than that of A or B, such as for methanol and water. Because in a chromatographic situation a solute elutes from the column as a solution in the eluent and the peak concentration in that effluent band is determined by amount/concentration of the solute injected as well as the column efficiency and other chromatographic parameters, the viscosity behavior (or the detector response) is impossible to predict based solely on viscosity data of the eluent and pure solute (or that for one specific concentration of the solute in the eluent). Further, for the same solute-solvent combination, the viscosity of the solution may change from a value less than that of the solvent to a value greater than that of the solvent (or vice-versa) as a function of the solute concentration. With water/methanol or water/acetonitrile eluent systems in conjunction with conventional reverse phase (C-18) silica columns and small organic molecules as solutes, I find for most solutes that at higher concentrations, the peak response is indicative of a viscosity lower than that of the eluent. At low concentrations however, the eluted solutes generally produce a viscosity greater than that of the eluent. Because concentrations of the solute in the chromatographic band changes continuously as the solute elutes, the injection of a high concentration solute generally elicits not only a peak (decreasing viscosity) but is also commonly followed by a dip (increasing viscosity) formed by the low

concentration tailing portion of the solute band, before the signal returns to baseline. That the phenomenon is not a detector induced artifact is confirmed by the fact that the peak/dip ratio continually decreases with decreasing solute concentration injected until the peak disappears altogether. This behavior is demonstrated in Figure 5 with toluene as the test solute and 80/20 acetonitrile/water as eluent. The lack of a dip preceeding the peak, as may be expected for an eluted band of Gaussian symmetry, is not presently understood.

The response, whether measured in peak height, dip height, peak to dip height, peak and/or dip areas is not a linear function of injected concentration/amount, as may be expected. The departure from linear behavior increases with increasing solute concentration. Also, detection by viscosity measurement is relatively insensitive. A separation of benzene (5 mg) and Toluene (5 mg) on an analytical column (5 μ-ultrasphere-ODS, 250 x 4.6 mm) is demonstrated in Figure 6. Benzene produces a peak and dip, while the toluene produces a dip only. At higher acetonitrile/water ratios, which causes faster elution (and thus less dilution), the same mixture yields two peaks.

When the detector is operated in the experimental arrangement depicted in Figure 1b, a greater pressure drop across the capillary is feasible by using a longer or narrower capillary. Consequently, detection sensitivity is substantially increased. A long term drift or slow oscillatory behavior of the baseline under these conditions has however been noted. This may be due to thermally induced viscosity changes or gas

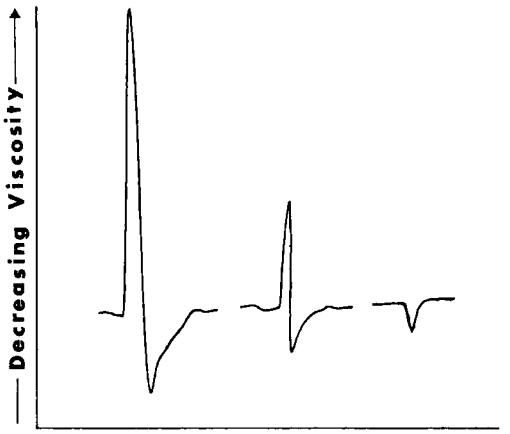

Figure 5. Detector response to toluene. The second and third sample are four- and sixteenfold dilutions of the first sample, respectively. Eluent 80/20 acetonitrile/water, 1 mL/min.

sorption/desorption on the gas pressurized side of the transducer. The results reported in this paper pertain to work carried out at room temperature with the experimental setup insulated by styrofoam blocks. Under these conditions, the experimental arrangement in Figure 1a produces a baseline noise equal to 2×10^{-4} times the total pressure drop experienced by the capillary. More elaborate thermostatic control will be necessary to attain lower baseline noise levels.

Bearing in mind that the transducer used in this work is not optimum for this application, the sensitivity can be potentially much better. Remarkable advances have been made in recent years towards fabricating miniature pressure transducers on silicon wafers. The accuracy and low cost of such devices open

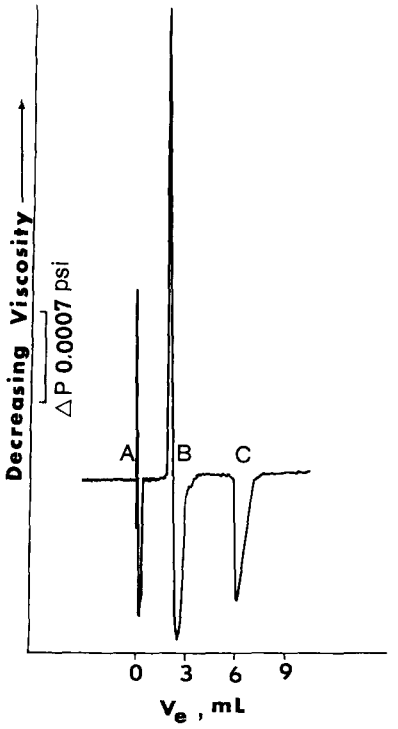

Figure 6. Separation of benzene and toluene. A : this is caused by actuation of the injection valve. Depending on the exact technique of actuating the valve, this spike can be smaller or greater. B : benzene peak and dip. C : toluene dip. Eluent 60/40 acetonitrile/water, 1 mL/min. Capillary : 0.25 mm bore, 10 cm length. Detector sensitivity : 1 Volt/0.0035 pounds/sq. in.

up new vistas for viscosity detectors in HPLC. Transducers that are both higher in sensitivity as well as tolerate a higher absolute pressure are commercially available with approximate cost less than $1,000. While such detectors are not likely to be attractive for the more common analytical applications, certain advantages

are worthy of consideration. Low costs, extreme ruggedness, lack of overloading at high concentrations, all make them good candidates for process chromatography. The fact that the detector is nondestructive and can be configured to introduce essentially no dispersion, makes it possible to use it in series with other detectors following it. For gradient applications, signal processing (e.g., differentiation) will be necessary however.

ACKNOWLEDGEMENT

This work would not have been completed without the assistance and machining expertise of J. R. Hall. I thank D. R. Speed and Ellis Loree for valuable suggestions in improving the pump design. The loan of the differential pressure transducer by Scientific Marketing (Georgetown, TX) is gratefully acknowledged.

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ROUTINE PREPARATIVE HPLC OF AROMATIC SILYL COMPOUNDS ON CAFFEINE COATED ON SILICA GEL

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ABSTRACT

A liquid chromatographic method using caffeine coated on silica gel, for separation and purification of acenaphthenylsilyl compounds is described. This has been achieved on a preparative scale.

INTRODUCTION

The rapid and facile separation of a few grams compounds for synthesis or analytic identification have always been the hope of the bench chemist.

When the purifications are not possible by distillation or fractionated crystallization, the used of an HPLC method is a solution which can help the chemist.

A mixture of polyaromatic hydrocarbons is easily separated on an analytic scale by reversed-phase chromatography (C_8 or C_{18}). But the transposition of the separation on a preparative scale (a few grams) is not possible because of the poor solubility of the polyaromatic hydrocarbons in the reversed phase eluents. The introduction of a trimethylsilyl group to the

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aromatic hydrocarbons as a substituent increase its insolubility. For a mixture of trimethylsilyl-1 acenaphthene and acenaphthene, our experiments show that 300 mg is the highest quantity that we can separate (1). It would not be an interesting way.

In fact in this example we have to separate two aromatic compounds so that a donor-acceptor chromatography appeared to be the best way to resolve the problem because the eluents (hexane) are solvents for acenaphthenyl compounds.

For the separation of polyaromatic hydrocarbons Lam used caffeine as acceptor (2-4) and Krasnec pointed out that the caffeine forms one of the strongest donor-acceptor complexes (5). On an other hand in T.L.C. caffeine is totaly retained with dry hexane as eluent, whereas acenaphthenyl compounds are eluted. So we used caffeine coated on silicagel to separate our compounds.

EXPERIMENTAL

Apparatus

a) Préparative LC : the apparatus consisted of a Chromatospac Prep 10, Jobin-Yvon system equipped with a Gilson holochrom H.M. The following samples were injected directly on the column by means of a syringe.

b) General equipment : The NMR spectra were recorded on a Perkin-Elmer R 24 B spectrometer. Results are reported in the δ scale in parts per million (ppm) with methylene chloride as internal standard (5,15 ppm down field from TMS).

The gas chromatographic analyses were performed on an Intersmat I.G. C 120 with a flame -ionization detector equipped with a

1,5 meters x 1/8 inch SE 30 10 % on chromosorb PAW column. Gas carrier was nitrogen with a flow rate of 25 ml/mn.

Reagents

Caffeine, acenaphthene, acenaphthylene were purchased from Aldrich chemical, caffeine and acenaphthene were used without further purification.

Acenaphthylene was recrystallizated from methanol.

Aceton and hexane (Aldrich Chemical) were dried on molecular sieves.

The silyl acenaphthene compounds were synthetized by procedure published earlier (6).

Silica gel is Lichroprep Si 60 (5 - 20μ) from Merck.

PROCEDURES

50 g of caffeine were dissolved in one liter of dry acetone After dissolution 250 g of silica gel were added and mixed. The solvent was evaporated with a rotary evaporator and caffeine silica gel (20 % in weight) was activated at 80°C during 12 hours.

A 200 g amount of caffeine-silica gel were mixed with 400 ml of dry hexane and they were packed into the column (40 mm-I.D.) until the excess of hexane get out. The height of the bed was 270 mm.

Results and Discussion

a) Tests of the column

In a first time we have studied the chromatographic caracteristics of the column to know its maximum of efficiency. So we have to determinate on optimun packing pressure (pressure on

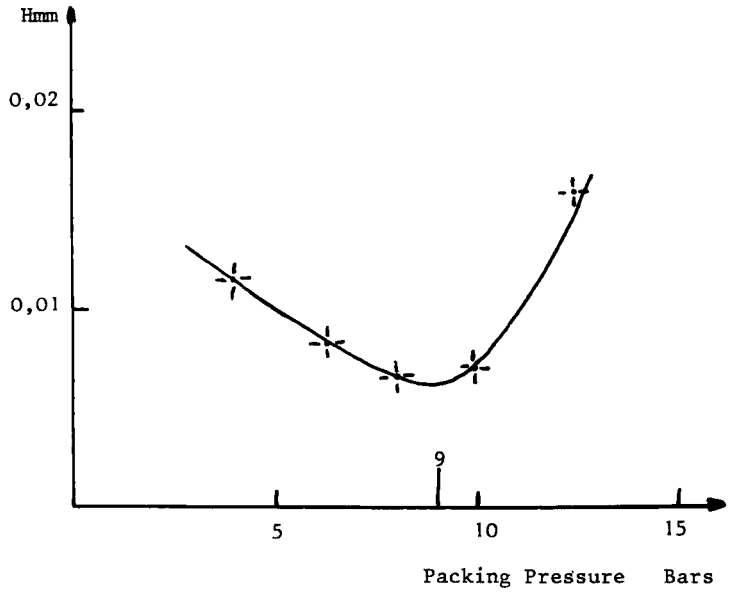

Figure 1. Variation of HETP vs packing pressure
Flow rate 30 ml/mn

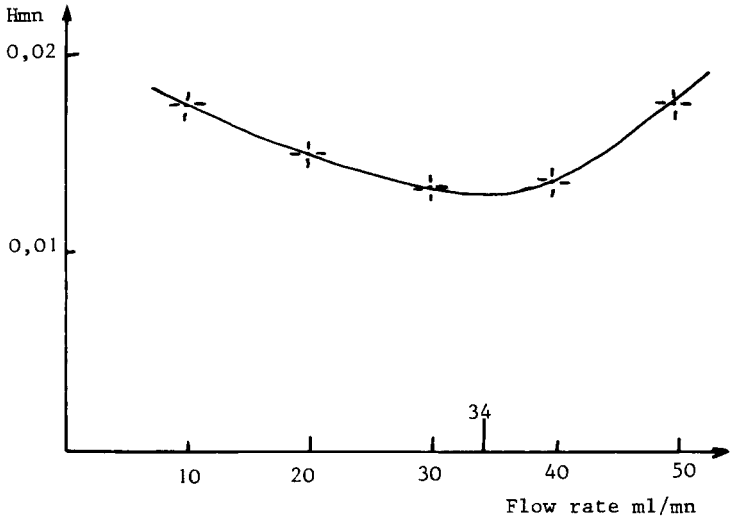

Figure 2. Variation of HETP vs flow rate
packing pressure 8.5 bars.

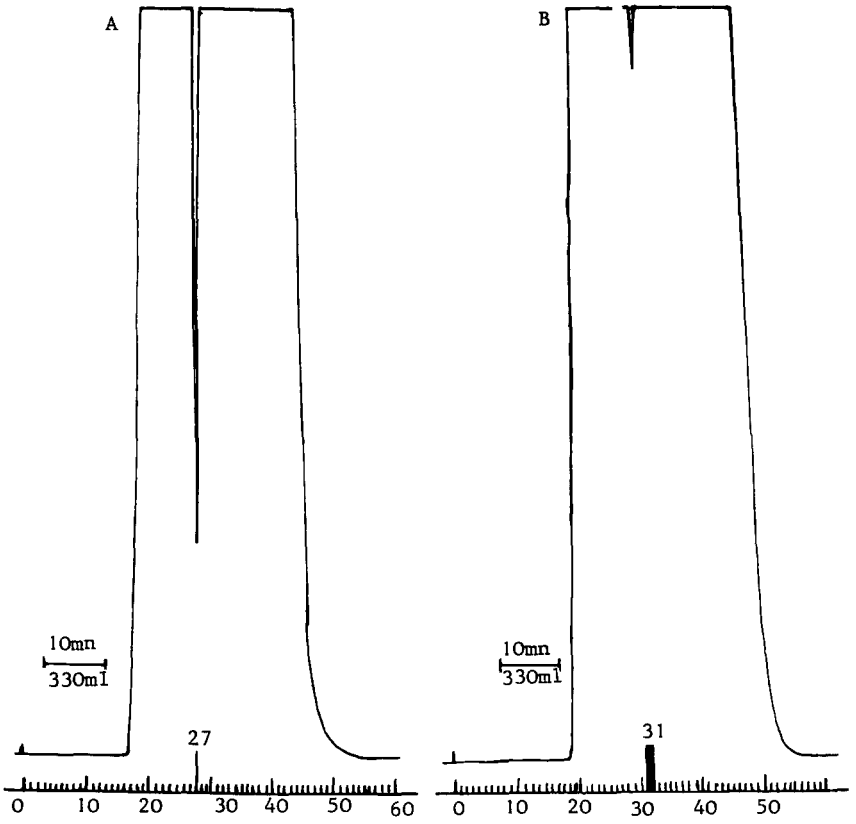

Figure 3. Separation of acenaphthene and acenaphthylene eluent dry hexane. λ = 254 nm.
(A) 2 g injected in 20 cc of dry hexane.
(B) 2,5 g injected in 25 cc of dry hexane.

the piston) and the best flow rate. These tests were made with acenaphthene (1 ml of a solution of 100 mg in 10 cc of hexane). The results are given on figures 1 and 2 : packing pressure : 9 bars, flow rate : 34 ml/mn respectively. The figure 1 shows that it is preferable to have a pressure on the piston who

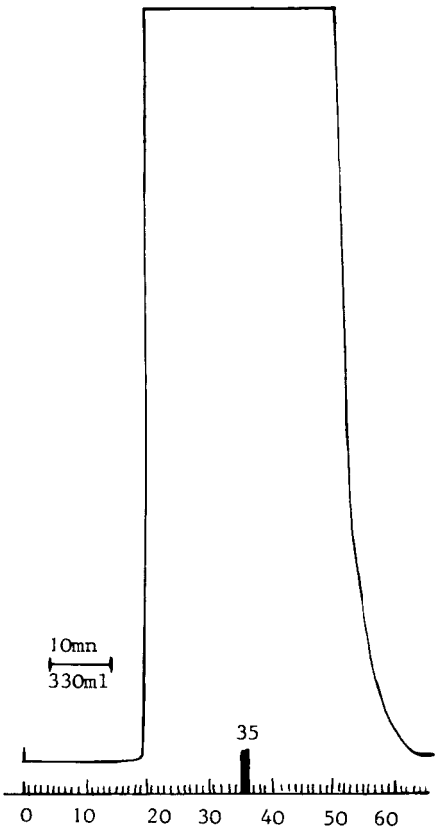

Figure 4. Separation of acenaphthene and acenaphthylene : eluent dry hexane λ = 254 nm
3 g injected in 30 cc of dry hexane

stay in the first part of the curve. So we selecte 8.5 bars. For the flow rate we choose a value of about 34 ml/mn.

Secondly we studied the capacity of the column. For that we choose to separate a synthetic mixture of acenaphthene and acenaphthylene (50/50 W/W) they are respectively white and yellow and it is easy to see observe its separations,

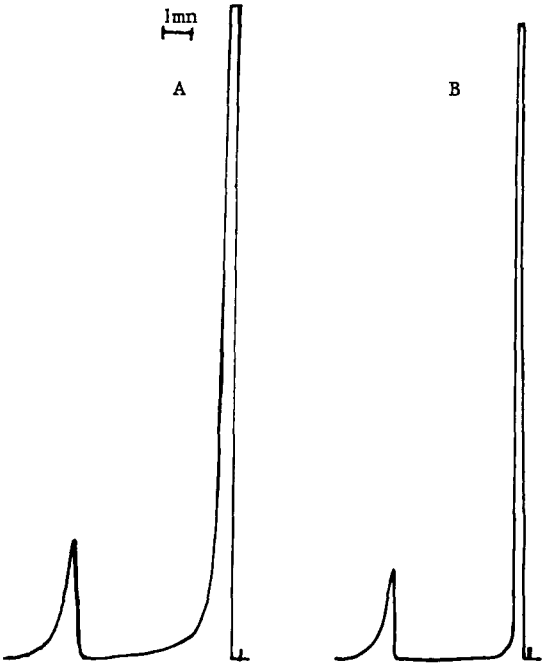

Figure 5. GC analysis: 2 g injected Temp 190°C
(A) fraction 26 acenaphthene
(B) fraction 27 acenaphthylene

in particular when the UV spectrometer is overgained (7). So that we did not oblige to analyse a lot of fractions.

Figure 3 shows that for 2 g : pure products are obtained (GC fig 5) ; for 2,5 g fraction 31 is mixed and contains less than 0,05 g of mixture (GC fig 6). The maximum of mixture that we can separate on this column is 3 g (fig 4). Only fraction 35 is mixed and contains 0,1 g of mixture (fig 7). The combined fractions 20 to 34 are acenaphthene (1,40 g) and 36 to 40 are acenaphthylene (1,35 g). The difference of 0,15 g

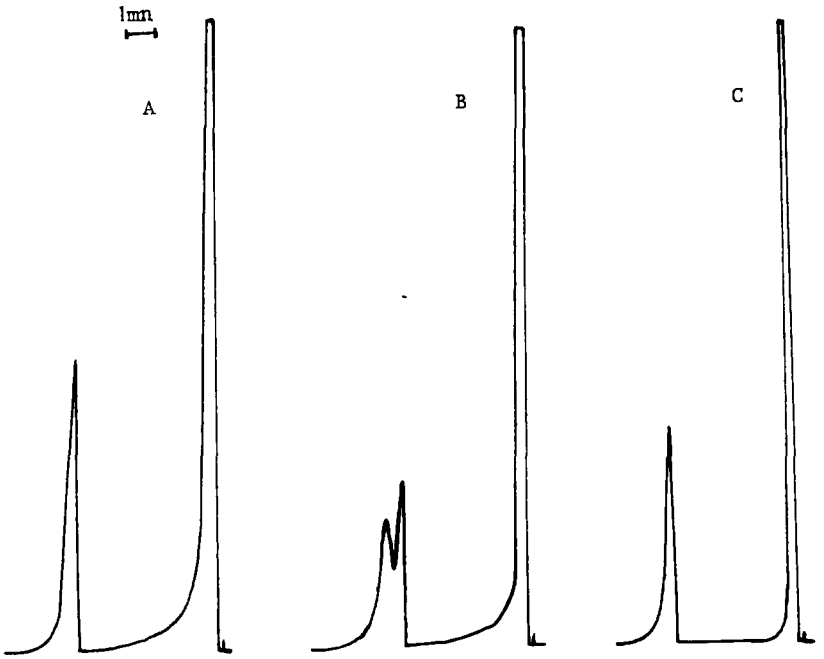

Figure 6. GC analysis 2,5 g injected Temp 190°C
(A) fraction 30
(B) fraction 31
(C) fraction 32

is due to losses in the injection and oxydatives species in acenaphthylene which stay at the top of the column.

In any fraction we did not find caffeine.

b) Purification of silylacenaphthene compounds

These studies were followed in this manner:

1) the GC analysis of the mixture, 2) the preparative chromatogram of the mixture and 3) the GG analysis of the fractions which contain the silyl compound and its NMR analysis.

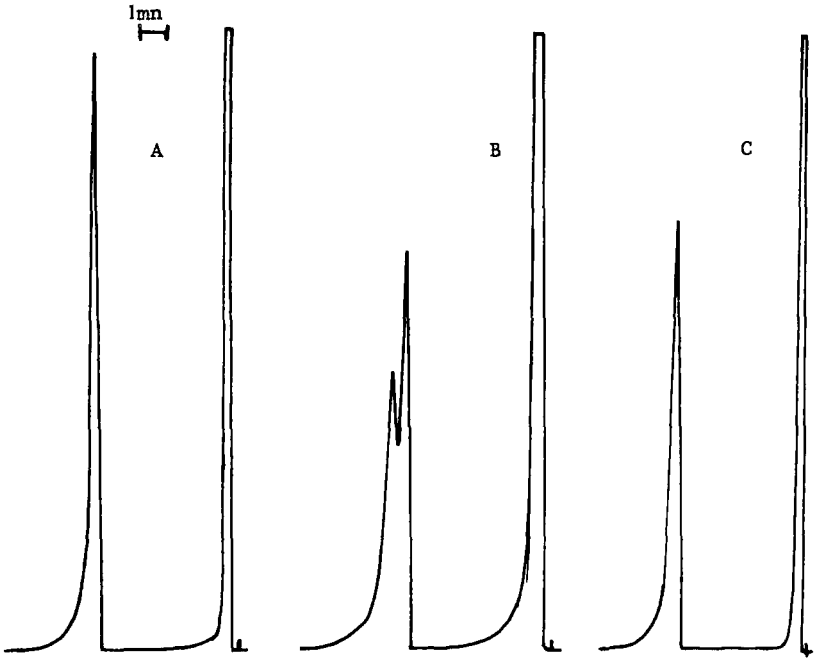

Figure 7. GC analysis 3 g injected Temp 190°C
(A) fraction 34
(B) fraction 35
(C) fraction 36

1-(trimethylsilyl) acenaphthene : this compounds is a mixture which contents about 10 % of impurities (acenaphthene, acenaphthylene and bis(acenaphthenyl-1) tetramethyldisiloxane (fig.8). The preparative chromatogram of 2,5 g is showed on figure 9.

The first fraction (14-15) contains the siloxane. The two acenaphthenyl groups of this compound are not able to form good charge - transfer with caffeine. The siloxane chain gives to the molecule a polarity less strong than the $SiMe_3$ group. So it was eluted

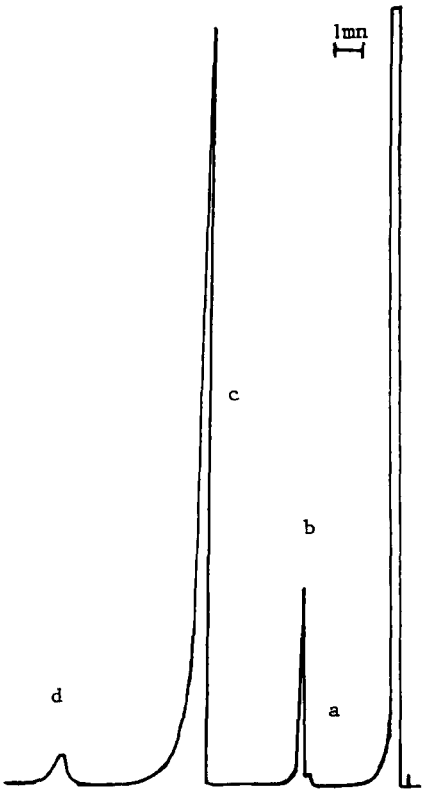

Figure 8. GC analysis of crude 1-(trimethylsilyl) acenaphthene temp 190°C
a) acenaphthylene b) acenaphthene c) 1-(trimethylsilyl)acenaphthylene d) bis(acenaphthenyl-1) tetramethyldisiloxane.

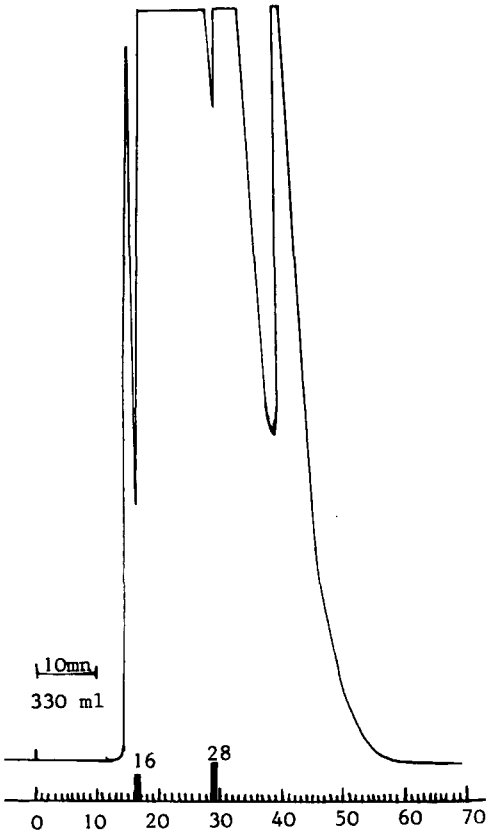

Figure 9. HPLC preparative chromatogram of crude 1-(trimethylsilyl) acenaphthene separation. λ : 254 nm.

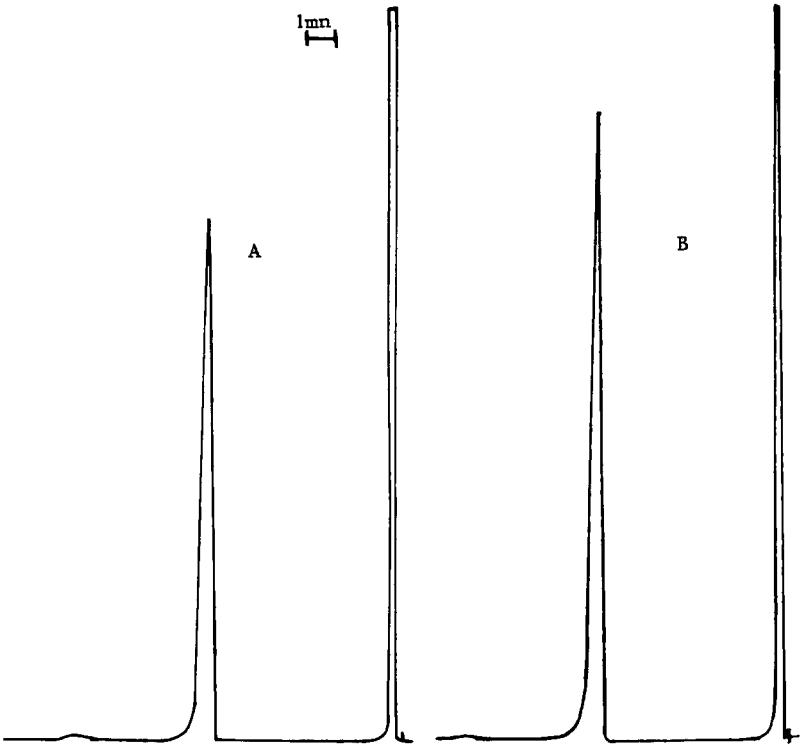

Figure 10. GC analysis of fractions of the separation of 1-(trimethylsilyl) acenaphthene Temp 190°C
(A) fraction 16
(B) fraction 17

first. The second product is the trimethylsilylacenaphthene. The figures 10 and 11 show the purity of the differents fractions examined by GC. If we take out fractions from 17 to 29 we obtained 2,3 g of pure product (NMR fig. 12). Fractions 29-40 contain acenaphthene and fractions 40-55 acenaphtylene respectively.

1-(dimethylsilyl)acenaphthene : the purity of this mixture is better than 95 % (fig. 13). The same impurities were found by GC

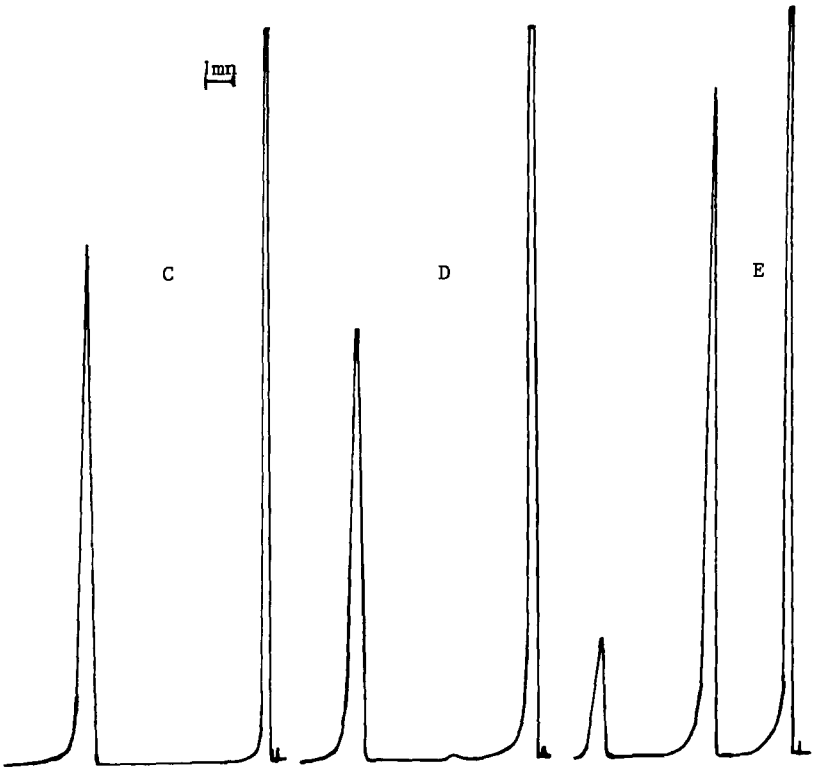

Figure 11. GC analysis of fractions after HPLC separation of 1-(trimethylsilyl) acenaphthene Temp 190°C

(C) combined fractions 18-25
(D) fraction 27
(E) fraction 28

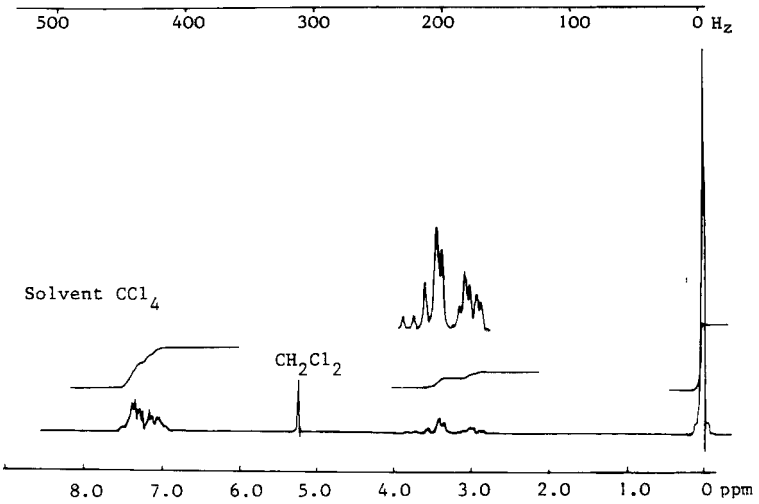

Figure 12. NMR of combined fractions 18-25. 1-(trimethylsilyl) acenaphthene

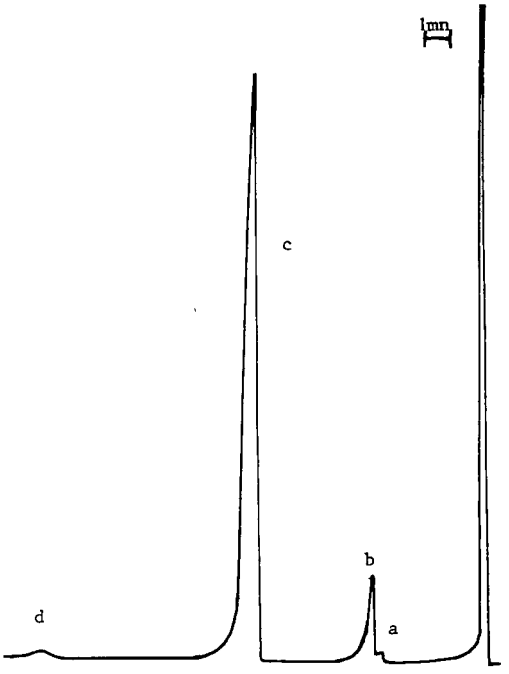

figure 13. GC analysis of crude 1-(dimethylsilyl)acenaphthene
Temp 170°C
a) acenaphthylene, b) acenaphthene, c) 1-(dimethylsilyl) acenaphthene d) bis(acenaphthenyl-1)tetramethyldisiloxane.

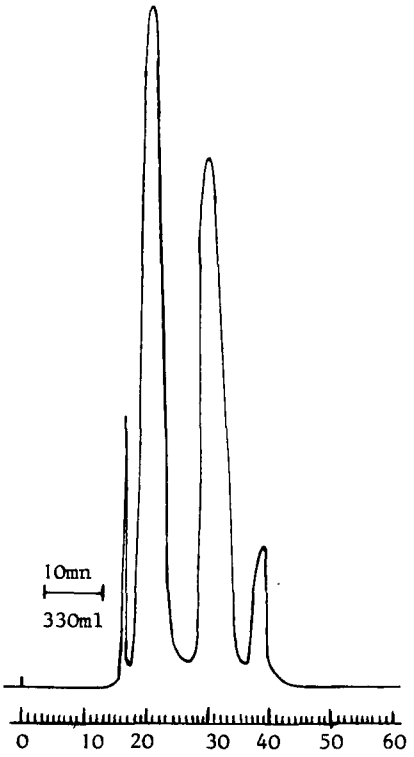

Figure 14. HPLC preparative chromatogram of crude 1-(dimethylsilyl)acenaphthene separation. λ = 320 nm

as for 1-(trimethylsilyl)acenaphthene. The quantity injected was 1 g ; so we obtained one chromatogram directly analysable (fig 14). Fractions 15-16 are the siloxane, fractions 18 to 25 contain 0,9 g of the silane which is obtained with an excellent purity (fig. 15). Fractions 28-35 are acenaphthene, and fractions 37-42 acenaphthylene.

The NMR spectrum (fig. 16) shows that the methyl groups in the Me_2HSi moiety are not in the same environnement. One of

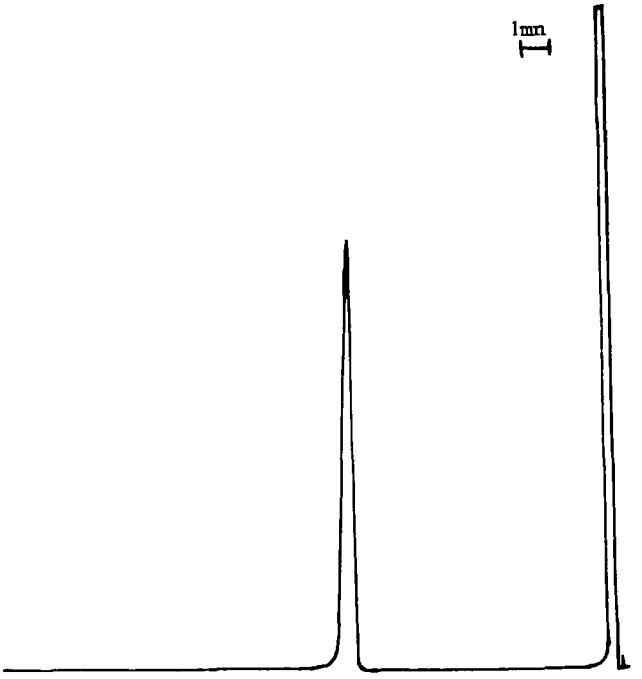

Figure 15. GC analysis of combined fractions 18-25 of 1-(dimethylsilyl)acenaphthene
Temp 170°C

them is in the shelding aera of the cycle, the other is out: so we have four signals.

In our studies we didn't find caffeine in any fraction if we used dry solvents. If we used solvents saturated with water we have been able to eluete caffeine. We found about 100 mg of caffeine by gram of injected product. In the case it is preferable to use 7-(2,3 dihydroxypropyl)theophylline (8) than caffeine.

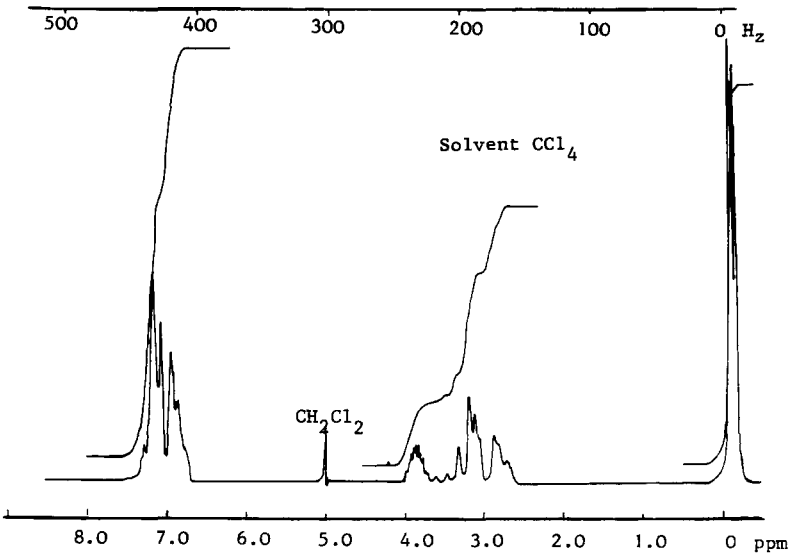

Figure 16. NMR of combined fractions 18-25 of 1-(dimethylsilyl)acenaphthene

Conclusion

Preparative HPLC on silica gel coated with caffeine has been found to be a good method for the separation of silylacenaphthenyl mixtures resulting in high yield of the silyl compounds having excellent purity.

References

1. For the separation : 200 g Lichroprep RP8 15-25 µ; Packing Pressure 10 bars ; eluents Methanol/water, 80/20 ; flow rate 30 ml/mn ; quantity injected : 300 mg in 30 ml of methanol.

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DETERMINATION OF PIRENZEPINE IN DOSAGE FORMS AND IN BIOLOGICAL FLUIDS

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ABSTRACT

A simple sensitive method for the determination of Pirenzepine in its dosage form and biological fluids by High-Performance Liquid Chromatography with U.V. detector has been developed. A tablet of 25 mg drug was gound, suspended in 10 ml of water, shaken and then filtered. A known volume of the filtrate is adjusted to appropriate concentration. Twenty µl of this solution was injected. Plasma or urine samples were made alkaline with ammonia before extraction with chloroform which was evaporated and the residue was dissolved in the mobile phase, 20 µl of this solution was injected. The determination limit for quantitation was about 1 µg/ml of pirenzepine. Complete separation of the drug was achieved in about 5.4 min. under the present chromatographic conditions.

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INTRODUCTION

Pirenzepine a [5,11 Dihydro-11-[(4 methyl-1-Piperazinyl) acetyl] -6H-Pyrido-[2,3-b][1,4] benzodiazepin-6-one is a selective muscarinic receptor antagonist blocking the acetylcholine receptors of the parietal cells of the stomach and inhibits gastric secretion (1) is a recently developed drug claimed to provide safe and unproblematic treatment for pepticl ulcer (2).

The only analytical techniques reported for the determination of pirenzepine are radioimmunassay (3) and HPLC (4). The purpose of this study was to develop a simple high performance liquid chromatographic (HPLC) procedure that will provide a method for the determination of pirenzepine in both dosage forms and biological fluids.

EXPERIMENTAL

Apparatus

HPLC was carried out using a Waters Associates System (Milford, Massachusettes, U.S.A.). The system was fitted with a model 6000A solvent delivery system, model 481 LC detector at 285 nm and model 710B WISP automatic injector. Chromatograms were recorded on Waters Data Module Model M730.

Chromatographic System

A 30 x 3.9 cm ID commercially available stainless steel C_{18} column (Waters Associates) was used. Mobile phase consisted of acetonitrile, methanol and 5% acetic acid (70:40:15). The mixture was degassed for 5 minutes by filtration: Flow rate 2 ml/min. and detector range 0.02 (AUFS).

Reagents

Standard solutions were made by dissolving Pirenzepine in the mobile phase. Acetonitrile, methanol and acetic acid (spectral grade)

were obtained from Merck (61 Darmstadt, Germany). Authentic sample (99.2 w/w) of Pirenzepine was obtained from Boehringer, Ingelheim Co., West Germany.

Standard Curve

Ten mg of the drug was dissolved in 100 ml of distilled water. From this stock solution a series of dilutions were made to cover a range of 2.5 to 20 µg/ml. Twenty µl of these solutions were injected onto the column in triplicate, the peak area was recorded and were plotted versus the concentration injected. The results are shown in Figure 1.

Determination of Pirenzepine in Tablets

Twenty tablets of 25 mg content were weighed accurately and the average weight of each tablet was calculated. The tablets were then ground and an accurate weight equivalent to about 20 mg of the active material was taken. The powder was then transformed quantitatively into 100 ml volumetric flask with aid of distilled water. The suspension was shaken for 10 minutes and the volume was then adjusted to the mark with distilled water. The suspension was then filtered through 0.22 µ millipore filter. From the filtrate a series of dilutions were made to cover concentration ranging from 2.5 - 20 µg/ml. From each solution, 20 µl was injected onto the column in triplicate. The average area under the peak was calculated for each sample. The drug concentration was then calculated from Figure 1.

Extraction from Urine and Plasma

Urine and plasma are collected from healthy adult male. In each run various amount of the stock solution of the drug were added to 2 ml or urine or 1 ml of plasma giving final concentration ranging from 2.5 to 20 µg/ml. Extraction was performed by adding 3 drops of concentrated

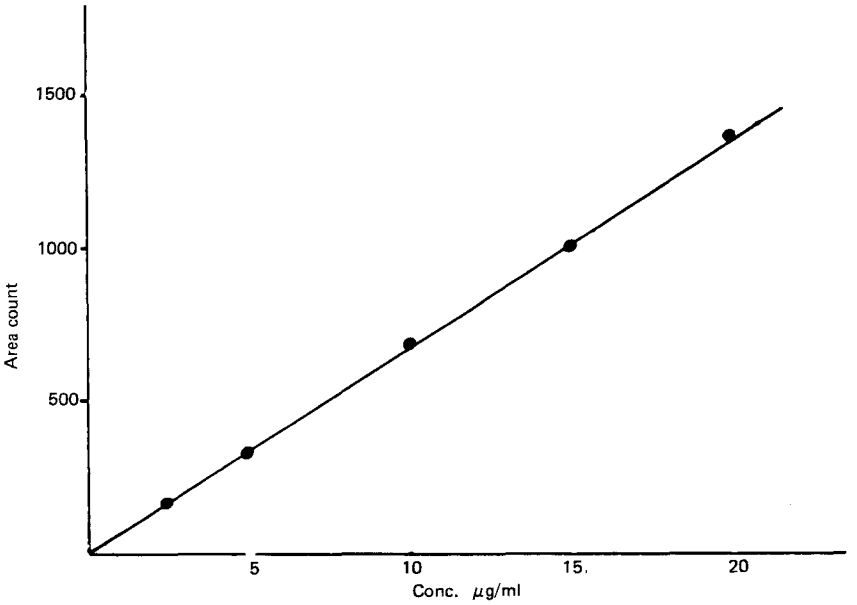

Figure 1. Calibration curve for Pirenzepine in solution under the present chromatographic conditions.

ammonia solution and the drug was then extracted with two portions of chloroform 10 ml each. The sample was centrifuged for 10 minutes at 2000 r.p.m. The combined chloroform layer was then evaporated at 60^{o} using rotavaporator. The residue was dissolved in 1 ml of the mobile phase and 20 µl of the resulted solution was injected in triplicate.

RESULT AND DISCUSSION

An HPLC method for the determination of Pirenzepine in dosage form and in biological fluids was developed. A typical graphs of the results when the peak area was plotted versus concentration from urine and plasma are shown in Figures 2 and 3. Typical chromatograms using this

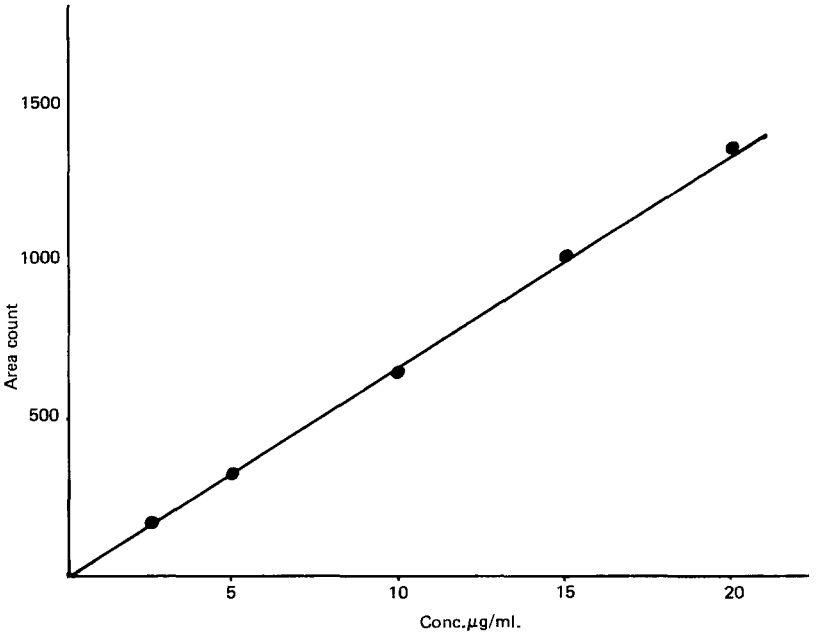

Figure 2. Calibration curve for Pirenzepine from urine under the present chromatographic conditions.

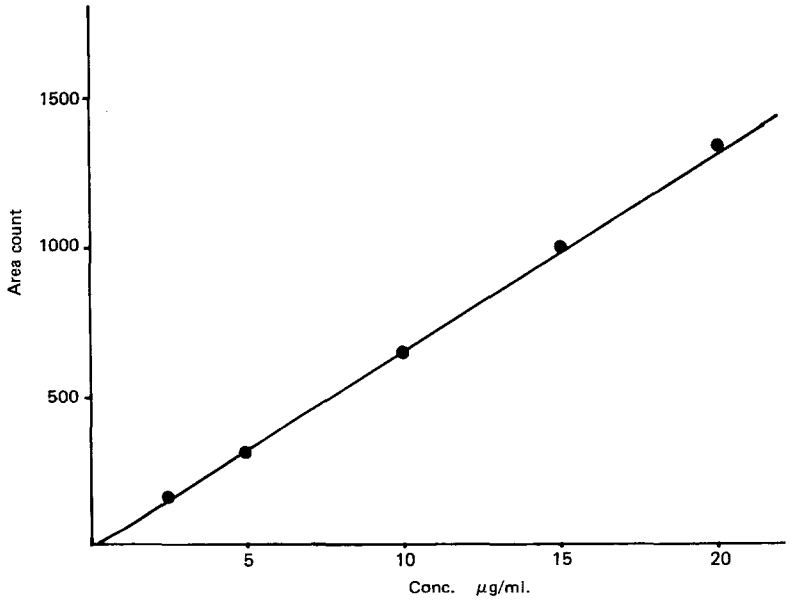

Figure 3. Calibration curve for Pirenzepine from plasma under the present chromatographic conditions.

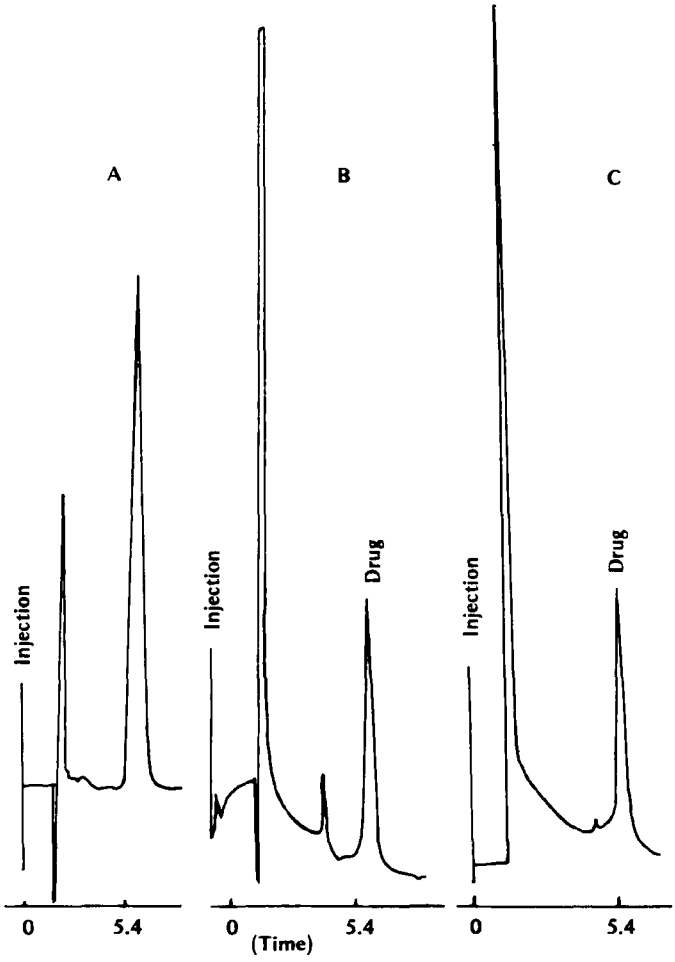

Figure 4. Typical chromatograms obtained using the procedure given. (A) Drug-free plasma, (B) Drug extracted from plasma, (C) Drug extracted from urine.

Figure 5. A chromatogram of 1μg/ml drug extracted from plasma.

method are shown in Figure 4 with a retention time of 5.4 minutes. The method is relatively sensitive and concentration as low as 1 µg/ml (which covers the range found in clinical studies) could be detected (S/N > 2) Figure 5.

Peak areas were linear to the drug concentration in the range used with corelation coefficients from water, urine and plasma of 0.996, 0.9936, 0.9944 and intercepts of -1.09, 0.27, - .27 respectively. Interference from other constituents of urine and plasma were minimal with a recovery of 95-98% and a standard error of ± 0.7 calculated from the triplicate injections using this method of extraction.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR PROSTAGLANDIN E_2 DETERMINATION IN HUMAN GASTRIC JUICE WITHOUT DERIVATIZATION

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ABSTRACT

A rapid and practical method for the separation and quantitation of PGE_2 in human gastric juice by high performance liquid chromatography is described. Separation on a reversed-phase column and UV detection allows quantities as little as 20 nanograms of PGE_2 to be detected. Specificity, sensitivity, high yield and reproducibility make this method particularly suitable for prostaglandin determination in human gastric juice.

INTRODUCTION

Many prostaglandins (PGs) and their metabolites are present in gastric juice, indicating the relevant role that these substances play in gastric pathophysiology, such as modulation of acid secretion, cytoprotection and motility control.

Up till the present, various methods for specifically measuring PGs in the gastric juice and in other biological fluids have been reported; bio-assay (1), radioimmunoassay (RIA) (1-4) and gas chromatography - mass spectometry (GC/MS) (5) are the most frequently used.

Recently, high performance liquid chromatography (HPLC) was introduced to separate PGs (1,3,6,7) which were then quantified by RIA (2,3) or GC/MS (5,8).

Derivatization of PGs has also been carried out for fluorescence and UV detection by HPLC (9,10,11), but derivative proce-

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dures may have problems of specificity and recovery; in fact it is not clear if PGs give a single derivative under the conditions of analysis used and if the derivatization is completely carried out.

Terragno et al. recently used HPLC for the separation and quantitation of PGs; eleven standard PGs and metabolites were evaluated and a sensitivity of as little as 30 ng was obtained (12).

We therefore used HPLC to quantitate PGE_2 in human gastric juice without derivatization and aliquots as little as 20 ng were detected.

MATERIALS AND METHODS

Apparatus

A HPLC system was used (Perkin-Elmer) consisting of: a Model Series 4 solvent delivery system equipped with a Rheodyne 7120 injection valve with a 20 µl injection loop, a Model LC-85 variable wavelenght UV detector (with a 2.4 µl flowcell) operating at 192.5 nm (12) and a Sigma 15 Chromatography Data station. A reverse phase C-8 column (Perkin-Elmer, 10 cm x 4.6 mm i.d., 5 µm particle size) was used.

The mobil phase consisted of 17 mM orthophosphoric acid-acetonitrile (67.2: 32.8, v/v). The system was constantly kept at pH 3.5. Flow rate was 1.7 ml/min (12); column temperature 37°C.

Reagents and Chemicals

PGE_2 was purchased from Upjohn Co., Kalamazoo, Mi., U.S.A., PGB_1 from Sigma, Saint Louis, Mo. U.S.A. and Cortisone from Makor, Jerusalem, Israel. ^3H-PGE_2, specific activity 160 Ci/mmol and ^3H-PGB_1, specific activity 60 Ci/mmol, were purchased from New England Nuclear, Boston, Mass., U.S.A.

Acetonitrile LiChrosolv, methanol,chloroform, isopropanol, formic acid and absolute ethanol of HPLC grade were obtained from Merck, Darmstadt,F.R.G. Water for Chromatography was distilled three times and passed through a 0.20 µm pore size filter

(Sartorius). Solvents and other chemicals were of analytical grade.

Preparation of Biological Samples

Gastric juice was obtained from 12 healthy donors (6 males and 6 females) after a fasting period of 12 hours, at the same hour of the morning in order to avoid diurnal variations in PG levels.

The total volume (51±5.8 ml, mean value ± SE) of gastric juice collected during a single period of 30 minutes in basal conditions, was used for analysis. 10 µCi ^{3}H-PGB_1 were immediately added to each sample as an internal standard (IS) for total recovery evaluation of the method.

pH was controlled and eventually adjusted between 1.5 and 3 with 1N HCl.

All the samples were immediately centrifuged (2500 g) at 4 °C for 20 minutes and the supernatants extracted with ethyl acetate (5:1 ;v/v).

The organic phase , after filtration through a 0.20 µm pore size filter (Sartorius) was evaporated to dryness in a nitrogen atmosphere at 37 °C.

^{3}H-PGB_1 was chosen as IS for the total yield because: 1) it is not present in gastric juice (4,13); 2) it is stable and not metabolized in the gastric pH (14); 3) its HPLC retention time is very different from that of PGE_2. Unlabelled PGB_1 was used for collecting ^{3}H-PGB_1 from the column.

We did not use ^{3}H-PGE_2 as IS because PGE_2 is in part converted to PGA_2 in the gastric juice pH (13).

Thin Layer Chromatography (TLC)

Silica gel plates (Merck, 60; F 254, 20 x 20 cm) were used for ascending TLC in closed glass chambers. The following solvent system was employed: chloroform-isopropanol-ethanol-formic acid (45:5:0.5:0.3, v/v) (15). Dried samples were redissolved with 100 µl of methanol and applied on the silica gel plates as a single

spot using a Hamilton syringe. The plates were allowed to run at room temperature for 75 minutes. Aliquots of standard PGE_2 and PGB_1 were also spotted as markers at one corner of the plates and submitted to the same TLC development conditions. The entire TLC plate, except the standard PG channel, was covered with a glass plate and then exposed to iodine vapour in order to visualize standard spots. The areas of the plate containing biological samples, corresponding to the marker spots, were scraped off and eluted with methanol (4,16). Samples were then filtered (Sartorius, 0.20 µm pore size) and evaporated to dryness in nitrogen atmosphere.

HPLC Calibration Curve

Standard solutions of PGE_2 ranging from 40 µg/ml to 1.0 µg/ml were prepared in absolute ethanol containing 200 µg/ml of Cortisone used as HPLC external standard (ES) (7). 20 µl of each solution were injected into the column.

The calibration curve was obtained by calculating the ratio between the peak areas of standard PGE_2 and ES. These ratios were plotted against the concentrations. The curve was used to convert peak area ratios of unknown samples to PGE_2 concentrations.

HPLC Analysis

Dried samples were redissolved with 20 µl of absolute ethanol containing ES and injected into the chromatograph.

The sample preparation procedure is shown schematically in table 1.

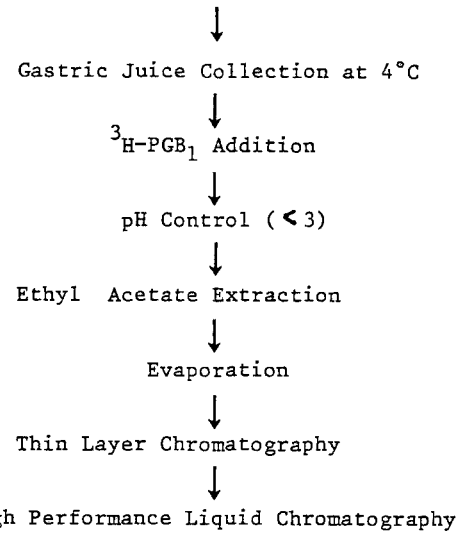
RESULTS

Separation of PGs

A typical chromatogram of biological samples processed by the method described is shown in Fig.1. A good separation of cortisone, PGE_2 and PGB_1 was obtained. Retention times of these compounds were 3.12, 5.62 and 18.06 minutes respectively.

TABLE 1

Scheme of the Sample Preparation Procedure



PG peaks were identified on the basis of their absolute and relative retention times and by adding known amounts of standard PGs to the samples.

Furthermore, in some sets of experiments, ^3H-PGE_2 was added to the samples, at various concentrations, immediately before injecting them into the chromatograph. A linear correlation($r=0.997$) was obtained between the single amounts of ^3H-PGE_2, added to the samples and the radioactivity found in the fractions corresponding to the PGE_2 collected from the column.

In order to avoid the possibility of other compounds interfering with chromatographic separation of PGE_2, spectral scans of the fractions corresponding to PGE_2 peak of biological samples eluted from the column were performed in some sets of experiments by using a variable wavelength UV scanning spectrophotometer (Perkin-Elmer-Hitachi, mod.200). Spectral scans of the fractions eluted overlapped those of the corresponding standard preparations

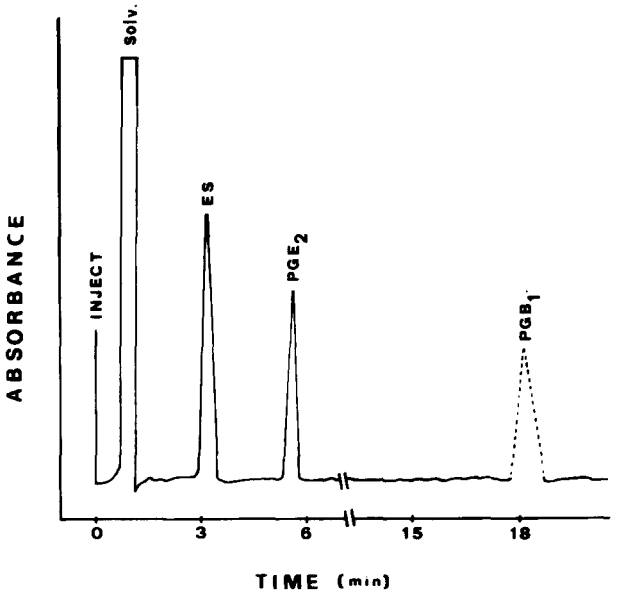


Figure 1. Representative HPLC chromatogram of a biological sample. Solv = Solvent; ES = Cortisone; PGB_1 = Area for collecting ^3H-PGB_1.

of PGE_2 at a maximum absorbance of wavelength between 192 and 193 nm.

Calibration Curve and Sensitivity

A linear calibration curve was obtained at concentrations of standard PGE_2 in the range used (y = 0.00051940x - 0.003;r=0.999).

The minimum detectable aliquot of PGE_2 in our samples was 20 ngin 20 µl injected into the chromatograph (S/N ratio=5).

Recovery

Total recovery of the method, obtained by evaluating the final cpm of ^3H-PGB_1 collected from the column in the biological samples, was 75 ± 2.08% (n=12). No radioactivity was recovered in the fra-

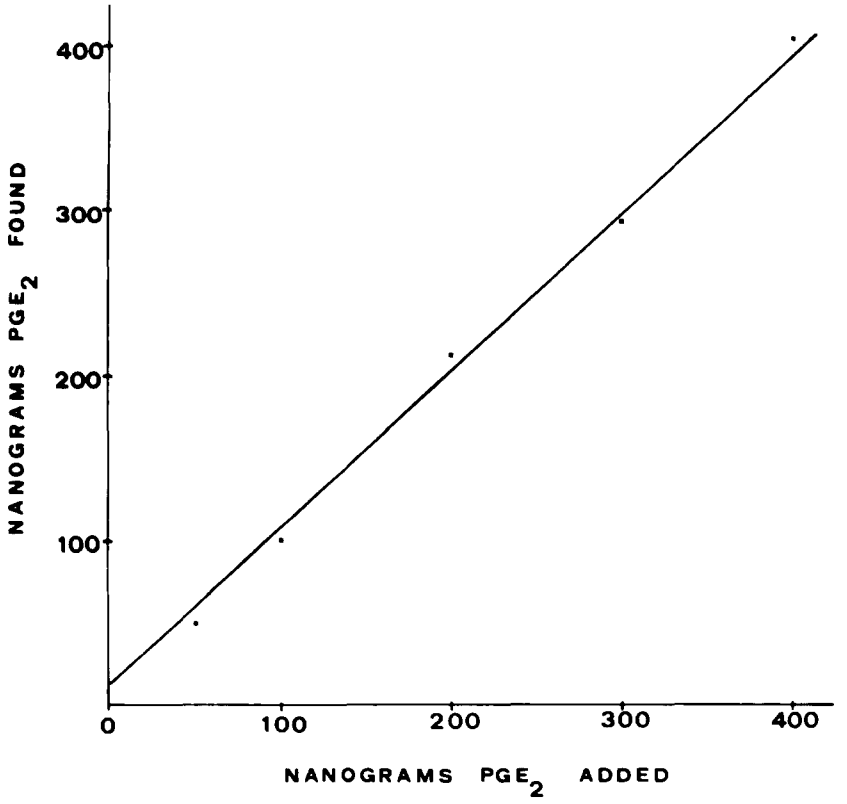


Figure 2. Recovery of PGE_2 added to gastric juice samples. All values are corrected for a recovery of 75% of PGE_2.

ctions collected before and after the one corresponding to the PGB_1 peak.

Accuracy

The accuracy of the method was checked by adding increasing amounts of PGE_2 (ranging from 25 ng to 400 ng) to 10 ml aliquots of gastric juice immediately before extraction (13). Figure 2 shows that the amounts of PGE_2 added can be reliably recovered from human gastric juice by the method described.

TABLE 2

Day-To-Day Precision Over Eight Weeks

PGE_2 Concentration	CV%
ng	
1600	0.36
400	1.26
200	2.78
100	4.50
50	0.35
25	1.50

n = 15

CV = Coefficient of variation

n = Number of assay

ng = Quantity injected

Precision

Day-to-day precision was evaluated by adding different amounts of standard PGE_2 to the same biological samples and processing them several times by the same method during a period of eight weeks. It was expressed as coefficient of variation (table 2).

PGE_2 Gastric Juice Concentration

The healthy donors studied showed a gastric juice basal secretion of 51±5.8 ml(mean value ± SE) during a period of 30 minutes. According to the HPLC determination, gastric juice concentration of PGE_2 was 504 ± 21 pg/ml (mean value ± SE).

DISCUSSION

The use of HPLC seems to be a very rapid and practical method for PG determination in the gastric juice. The specificity of the method is based on: 1) absolute and relative retention ti-

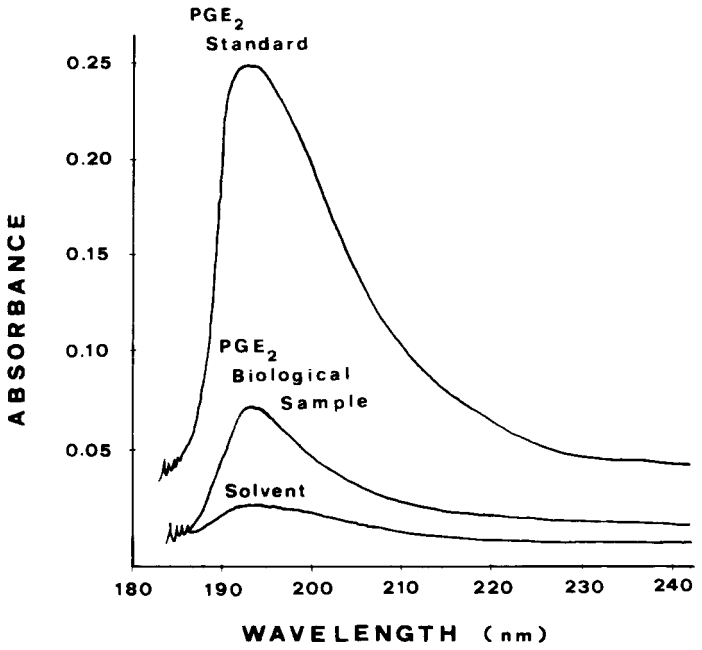


Figure 3. UV spectral scans of the fractions corresponding to PGE_2 peak of a biological sample and of a standard solution (800 ng/20 µl) eluted from the HPLC column. Solvents: 17mM H_3PO_4:CH_3CN(67.2:32.8,v/v).

mes of PGE_2; 2) enrichment of biological samples by standard preparation of PGE_2 and ^3H-PGE_2 immediately before the injection into the chromatograph; 3) performance of spectral scans of the eluted fractions corresponding to the PGE_2 (Fig.3).

Because of the very low UV wavelength (192.5 nm) used for detecting PGE_2 without derivatization, TLC pretreatment of the extracted samples provides a helpful procedure to reduce interference in the HPLC determination.

The sensitivity, although lower than that obtained by RIA and fluorescent derivative HPLC determination, is sufficient for detection of PGE_2 in the gastric juice. On the other hand, our procedure

offers a high specificity and the possibility of performing simultaneous analyses of PG mixtures.

The values reported in the study are similar to those obtained by Peskar at al. with RIA (4).

The high yield, accuracy and speed of chromatographic separation (less than 20 minutes) make this method particularly suitable and reliable for PGE_2 determination in the gastric juice in normal and pathological conditions.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF CEFOPERAZONE IN SERUM AND URINE

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ABSTRACT

A high performance liquid chromatographic method was developed for the quantitative analysis of cefoperazone in serum and urine. Standard curves were linear over the range of concentrations 2-30 µg/ml and a good correlation was established between the amount of cefoperazone injected and peak height. The mean percentage analytical recovery of cefoperazone was 96.3% and the mean within day coefficient of variation in serum was 2.8%. Serum and urine components, as well as several beta-lactam antibiotics, did not interfere with the measurement of cefoperazone. This is a rapid, reproducible, and sensitive assay suitable for use in pharmacokinetic studies.

INTRODUCTION

Cefoperazone is a third-generation semisynthetic parenteral cephalosporin (Figure 1) with marked antipseudomonal activity, particularly against Pseudomonas aeruginosa (1). The major route of elimination appears to be biliary secretion with about 15-30% of a dose excreted unchanged in the urine. Less than 1% of an administered dose of the drug is identified as metabolites in the urine (2).

Most published pharmacokinetic data on cefoperazone have been determined using microbiological assay techniques. While bioassays

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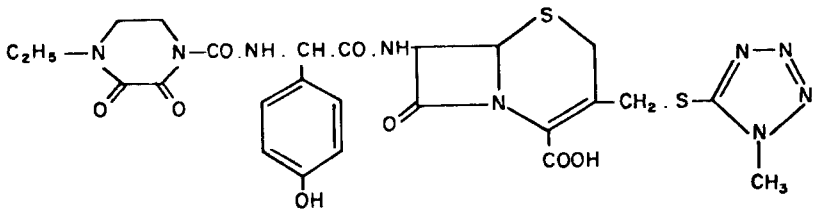

FIGURE 1 Chemical structure of cefoperazone.

offer the advantage of short analysis time and high capacity for analysing a large number of samples, they have the associated disadvantages of lacking specificity and reproducibility. We have developed a rapid, reproducible, and sensitive high performance liquid chromatography (HPLC) assay in order to determine concentrations of cefoperazone in serum and urine. Previously published HPLC assays have cumbersome extraction procedures and/or more complex chromatographic methods of analysis (3,4,5,6). Our assay is simple and its suitability for use in pharmacokinetic analysis has been demonstrated in a study of the disposition of cefoperazone in cystic fibrosis patients and healthy control volunteers (7) (Figure 2).

MATERIALS AND METHODS

(i) Chemicals:

All reagents and solvents used were HPLC grade. Cefoperazone and Cephalothin were supplied by Pfizer Canada Inc. (Pointe-Claire, Quebec) and Eli Lilly and Co. (Indianapolis, Ind.) respectively. Acetonitrile was obtained from Burdick and Jackson Laboratories, Inc., (Muskegon, Michigan), methanol from Caledon Laboratories, (Georgetown, Ontario), and sodium phosphate monobasic from Fisher Scientific (Fair Lawn, New Jersey).

(ii) Preparation of Buffer and Stock Solutions:

For both the serum and urine assay, 10 mM sodium phosphate monobasic buffer (pH 4.5) was prepared by dissolving 2.7598 g of the buffer in 200 ml of distilled water and bringing the solution to a final vol-

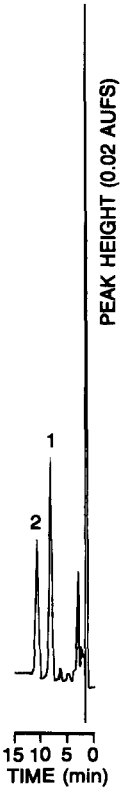

FIGURE 2 Representative chromatogram of cefoperazone (1) and cephalothin (2) in the serum of a cystic fibrosis patient 1 minute following a 1 g/m^2 dose infused over 20 minutes.

ume of 2 L. The pH was then adjusted to 4.5. Stock solutions of cefoperazone and cephalothin were prepared fresh daily. The working cefoperazone standard solution was prepared by dissolving 10 mg of cefoperazone in 10 ml of distilled water and bringing the solution to a final volume of 25 ml. To prepare the working internal standard solution, 10 mg of cephalothin was dissolved in 5 ml of distilled water and an aliquot from this solution was added to a 25 ml volumetric flask containing methanol to reach the desired final concentration of 15 µg/ml.

(iii) Handling of Samples:

Serum: 200 µl of methanol containing the internal standard cephalothin was added to 100 µl of the serum sample to precipitate any soluble proteins. Each sample was vortexed for 1 min and then centrifuged for 3 min in an Eppendorf 5412 centrifuge. Twenty µl of the resultant supernatant was manually injected directly onto the column.

Urine: No extraction procedure was necessary for the urine and thus 20 µl of the urine sample was injected directly into the HPLC. Appropriate dilutions were prepared to fall within the linear range of the standard curves for both serum and urine using blank serum and a 1:4 ratio of urine to distilled water as diluents.

(iv) Apparatus:

Chromatographic analysis was performed using a Perkin-Elmer series 2 liquid chromatograph, LC-75 variable wavelength detector, and a 023 chart recorder. Separation was achieved using a reverse phase C-18 µ Bondapak column (3.9 mm x 20 cm, 10 µ pore size, Waters Scientific, Milford, MA). The mobile phase consisted of acetonitrile (CH_3CN) and 10 mM sodium phosphate monobasic buffer at pH 4.5 in a ratio of 15%:85% and 12%:88% for the serum and urine respectively. Before use, each mobile phase was degassed using a 0.5 µM Durapore filter system (Millipore Corporation, Bedford, MA).

(v) Conditions of Analysis:

All serum and urine samples were stored at -20°C prior to analysis. Optimal conditions for the analysis of cefoperazone serum and urine samples included a wavelength of 228 nm based on the absorbance characteristics of the drug. The detector output was attenuated at 0.02 absorbance unit full scale (AUFS) and the mobile phase was delivered at a flow rate of 2.5 ml/min. All analyses were performed at room temperature.

(vi) Calculations:

Serum cefoperazone concentrations were determined using an internal standard technique with cephalothin being the internal standard.

Standard curves were drawn up on each day of analysis based on five standard concentrations ranging from 2-30 µg/ml. A response factor (RF) was then calculated using the following equation:

$$RF = \frac{\text{Peak Height Cephalothin}}{\text{Peak Height Cefoperazone}} \times \frac{\text{Conc. of Cefoperazone}}{\text{Conc. of Cephalothin}}$$

The mean of the response factors of each of the 5 standard concentrations was then used to calculate the serum cefoperazone concentration in the following equation:

$$\text{Conc. of Cefoperazone} = \frac{\text{RF} \times \text{Peak Height Cefoperazone} \times \text{Conc. of Cephalothin}}{\text{Peak Height Cephalothin}}$$

Urine cefoperazone concentrations were determined using an external standard technique. The equation for the line of best fit through 5 urine standard concentrations was calculated by linear least squares regression. The equation was then used to calculate the urine cefoperazone concentrations by substituting in the values for the dependent variable, peak height, to determine the corresponding value for the independent variable, cefoperazone urine concentrations.

RESULTS AND DISCUSSION

Chromatograms of cefoperazone and cephalothin in serum and cefoperazone in urine are shown in Figures 3 and 4. Endogenous components in the plasma and urine did not interfere with the identification of cefoperazone. Other beta-lactam antibiotics, cefaclor, cephalexin, cefamandole, ticarcillin, ampicillin, and cloxacillin were found to have elution times that differed from those for cefoperazone and cephalothin. In addition, the aminoglycosides, tobramycin and gentamicin did not appear in chromatograms or alter assay results.

Linearity of the standard curve was established by plotting peak height versus cefoperazone concentration at 5 standard concentrations ranging from 2-30 µg/ml ($r^2 = 0.998$, Figure 5). The detection limit for both the serum and urine assay was approximately 2 µg/ml.

The within day precision of the assay was measured by using pooled sera with cefoperazone to produce three different concentrations,

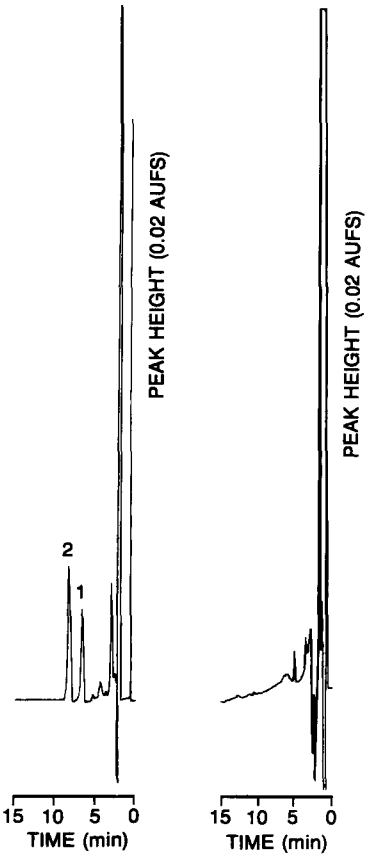

FIGURE 3 (i) Representative chromatogram of cefoperazone (1) and cephalothin (2) in serum. (ii) Representative chromatogram of a blank serum sample.

4.65 µg/ml, 13.95 µg/ml, and 29.45 µg/ml. Six replicates were made for each of the three concentrations involving three sets of six independent extractions. Coefficients of variation were 0.7%, 4.7%, and 3.0% respectively. The percent absolute recovery at low, medium, and high concentrations was 103.2%, 91.8%, and 94.0% with the mean percent recovery being 96.3%.

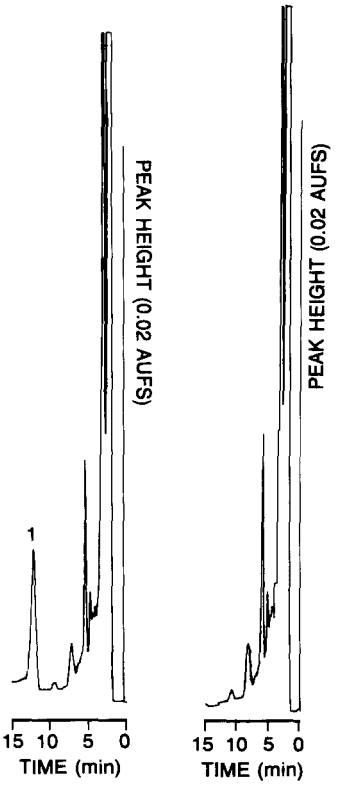

FIGURE 4 (i) Representative chromatogram of cefoperazone (1) in urine. (ii) Representative chromatogram of a blank urine sample.

Some of the previously described HPLC assays use an external standard technique to quantify serum cefoperazone concentrations (3,5). This method of quantification, although acceptable, is generally not preferred if a suitable internal standard exists. Our current method of quantification, using an internal standard, results in reduced variability since quantitation is independent of the volume injected.

The practical application of certain HPLC assays for cefoperazone to pharmacokinetic analysis may not be suitable since extraction procedures are tedious and time consuming (3,4,5). Furthermore the range of cefoperazone serum concentrations over which the standard

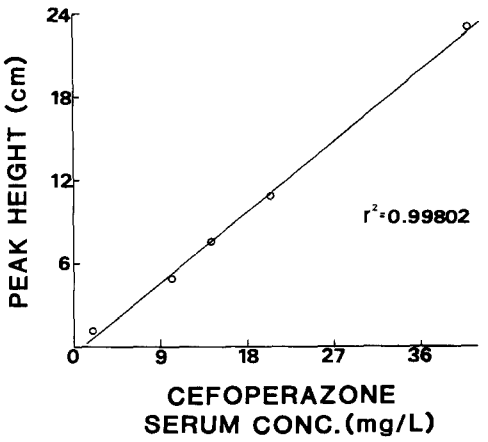

FIGURE 5 Standard curve for cefoperazone over the range of concentrations from 2 - 30 mcg/mL.

curves are linear is too small to make precise extrapolations for concentrations normally found in the clinical setting. Our assay overcomes these problems and is suitable for use in pharmacokinetic analysis as previously demonstrated (7).

This assay is rapid, reproducible, sensitive, and precise and offers an alternate means of determining cefoperazone concentrations in serum and urine with advantages over traditionally used bioassay techniques.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF ESTRADIOL-17α AND -17β IN BIOLOGICAL FLUIDS; APPLICATION TO PLASMA, MILK AND URINE OF COWS

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ABSTRACT

A rapid HPLC technique was developed to separate estradiol epimers. In order to improve the sensitivity of the detection, a radioimmunoassay was used.

Estrone, estradiol-17α and estradiol-17β were separated within 20 min using 10 ml of chloroform: acetone (90:10), as the mobile phase. The efficiency of the technique was assessed with ^{3}H steroids and the assay of collected fractions with antisera specific to each estrogen. Using a non-specific radioimmunoassay, profiles of endogenous estrogens in different biological fluids (blood plasma, milk, urine) were obtained.

The efficiency of HPLC as a separation method and the high sensitivity of radioimmunoassay as a detector allows us to obtain profiles of estrogens from biological samples where steroid concentration is below 100pg/ml.

INTRODUCTION

Estrone (E_1), estradiol-17α ($E_{2\alpha}$) and estradiol-17β ($E_{2\beta}$) are known to be present in various biological fluids as free or conjugated steroids. $E_{2\alpha}$ is the major metabolite of biologically active estrogen $E_{2\beta}$ in farm animals (1); it has been measured in high concentration in blood plasma, milk and urine (2, 3). Despite the fact that it has no biological activity, $E_{2\alpha}$ can be of

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clinical interest as changes in its concentration are related to folliculogenesis (4) and development of pregnancy (3).

The major problem with the assay of $E_{2\alpha}$ is the separation of two epimers of estradiol. Previous published methods are inconvenient, and particularly in HPLC, have utilised large elution volume which increases the time (5, 6). These methods are not applicable for large number of samples as required for physiological studies. Some new methods are quicker but were evaluate only with pure steroids (7, 8). When used for biological samples, other difficulties come from the low concentration of estrogens (few pg/ml) which require efficient purification prior to quantification and high sensitivity of detection which can be reach only by radioimmunoassay.

The present paper describes an high performance liquid chromatographic separation of picogramms of estradiol epimers with subsequent quantification of purified steroid fraction by radioimmunoassay.

MATERIALS AND METHODS

Radioactive steroids 2,4,6,7-^{3}H estrone (98 curies/mmole) and 2,4, 6,7,16,17,^{3}H estradiol-17β (145 curies/mmole) were obtained from Radiochemical Centre, Amersham, Bucks, United Kingdom. 2,4,6,7-^{3}H estradiol-17α was prepared by incubation of labelled estrone with cow's erythrocytes (9). After incubation, the reaction mixture was diluted with equal volume of distilled water. 54% radioactivity was extracted with diethyl ether and the residue was delipidated with methanol: water (70:30) (10). The methanol extract was evaporated under nitrogen and the residue was chromatographed on HPLC. A ratio of ^{3}H-estrone to ^{3}H-estradiol-17α 1:12 was recovered and the synthesized radioactive estradiol-17α was further checked for its binding with specific antiserum.

All the reagents were of analytical grade. The solvents were dehydrated using a molecular sieve and were filtered through membrana filters (Membra fils 47 mm) obtained from Nucleopore Corporation, 7035 Commence Circle, Pleasanton, CA 94566 USA.

A Varian model 5010 was employed for the separation of estrogens. The model contains within a single unit the component of an isocratic high performance liquid chromatography. Elution was performed using commercial pre-packed S_1-10 silice column (30 cm x4.0 mm I.D.). In order to prevent the damage of column, a small pre-column (5.0 cm x 4.0 mm) of the same material was connected before the column. Fractions were collected on fraction collector Model 568 (ISCO, Lincoln, Nebrasca USA). Radioactivity was counted in Packard Tri-Carb Model N°3385 Liquid Scintillation Spectrometer. Evaluation of RIA data was performed on Hewlett Packard Computer.

High Performance Liquid Chromatography

Radioactive standard estrogens or samples were dissolved in the eluent. Sample (0.2 ml) was applied on Valco automatic injector and was injected directly on the column. Fractions were collected in glass tubes, dried and the radioactivity was counted in 2 ml of scintillation fluid.

Biological samples preparation

All the samples were collected around estrus from Friesian cows. Some animals were superovulated with gonadotrophin-prostaglandin $F_{2\alpha}$ treatment (11).

Blood plasma: Free estrogens were extracted thrice from blood plasma with dietyl ether. The conjugated estrogens present in the remaining plasma were enzymatically hydrolysed (12) by Helix Pomatia juice obtained from Pharmindustry, Departement Reactifs (35, avenue Jean Jaurès, 92390 VILLENEUVE LA GARENNE, France). The liberated estrogens were extracted with dietyl ether. The etherial extract was evaporated under nitrogen and the residue was delipidated using 2 ml of methanol: water

(70:30). The mixture was incubated at 40°C for 1 h and at -20°C for overnight. The lipids were compacted by centrifugation at 1500g for 30 min at -20°C and the supernatant containing estrogens was decanted and evaporated under nitrogen. The residue was dissolved in the eluent and subsequently chromatographed on HPLC.

Milk: Whole milk was centrifuged twice and the upper cream layer was removed. The defatted milk was extracted with diethyl ether for free estrogens. The conjugated estrogens present in the remaining milk were hydrolysed in the same manner as for blood plasma.

Urine: Mostly estrogens are present as conjugated steroids in the urine (13). It is, therefore, conjugated estrogens were enzymatically hydrolysed in the same manner as for blood plasma. Then, total estrogens were extracted with diethyl ether and the extract was evaporated under nitrogen. The residue was dissolved in IN NaOH and was extracted with toluene. The organic phase was discarded and the aqueous phase was mixed with equal volume of 1N HCl. The estrogens from the mixture were extracted with dichloromethane and the organic phase was dried under nitrogen. The residue was dissolved in the eluent and subsequently chromatographed.

Radioimmunoassay: Estrogens were measured using non specific antiserum by minor modifications in earlier published procedure from this laboratory (14). Estradiol-17β and ^{3}H-estradiol-17β were used to assay the various fractions of HPLC from the extract of plasma, milk and urine. The antiserum was raised against estrone 17-hydrazobenzoyl coupled with bovine serum albumin in rabbits. Using ^{3}H-estradiol-17β as labelled antigen, the antiserum showed almost equal cross reaction with estrone, estradiol-17α and estradiol-17β . The radioimmunoassay procedure in brief is as follows: HPLC fractions were dissolved in 0.6 ml phosphate buffered saline (0.1 M phosphate pH 7.0

containing 0.15 M sodium chloride, 0.015 M sodium azide and 0.1% gelatin in glass distilled water) by keeping at 40°C for 30 min and vortexing for 30 sec. For the assay, 0.2 ml was pipetted in duplicate in polypropylene tubes and was mixed with 0.1 ml ^{3}H-estradiol-17 and antiserum. The tubes were mixed and incubated at 37°C for 30 min. The bound and unbound hormones were separated by double antibody immunoprecipitation. The precipitate was washed twice with 1.5 ml of polyethylene glycol solution (6%) and finally 0.05 ml 5N HCl was added. The radioactivity was counted directly after addition of 2 ml of scintillation fluid.

The specific antiserum of estrone was raised in rabbits against estrone-6-(O-carboxy) methyloxine conjugated to bovine serum albumin. The antibodies cross-reacted at a level of 0.2% with estradiol-17α and 0.95% with estradiol-17β. The antiserum to estradiol-17α was raised against estradiol-17α-6-O-Carboxy methoxine coupled with BSA, in rabbits. The antiserum cross reacted 1% with estrone and negligible with estradiol-17β (15). The antiserum against estradiol-17β was raised in rabbits against estradiol-17β -6 (O-Carboxy) methyloxine bovine serum albumin and cross reacted 0.7% with estrone and 0.88% with estradiol-17α (16).

RESULTS

As there were variations in the retention time of estrogens due to the moisture present in the solvents (17), therefore, it was felt necessary to dehydrate the solvents with a molecular sieve just before using into HPLC system. In order to obtain a suitable mobile phase for separation of $E_{2\alpha}$ and $E_{2\beta}$, a number of solvent systems known to be efficient in TLC (18) were tested (Table 1). Two mobile phases containing chloroform resolved the estradiol epimers with appreciable difference in the retention time.

TABLE 1
Chromatographic Elution of Various Radiolabelled Estrogens Using Various Mobile Phases. Flow Rate 0.5 ml/min; Temperature 30°C; Fraction Volumne 0.5ml.

Mobile phase	Retention time (min)		
	E_1	$E_{2\alpha}$	$E_{2\beta}$
Ethyl Acetate: Cyclohexane (50:50)	5	6	6.5
Chloroform: Ether (75:25)	5	7.5	8.5
Dichloromethane: Acetone (75:25)	4	5	5.5
Chloroform: Acetone (80:20)	4	6.0	7.0

As the system chloroform: acetone (80:20) was rapid as compared to chloroform: ether (75:25), therefore, it was further tested using various percentages of acetone in chloroform (Table. 2). There was increase in the difference of retention time between various estrogens with the decreasing percentage of acetone in chloroform. When the flow rate was further decreased, there was increase in the retention time of estrogens with improved resolution (Table. 3).

The HPLC profiling of radioactive estrogens using chloroform: acetone (90:10) has been shown in Fig. 1-4A. The estrogens were separated in 20 min using 10 ml of mobile phase. Recoveries of ^{3}H-estrone, ^{3}H-estradiol-17α and ^{3}H-estradiol-17β from HPLC were 81.6%, 85.2% and 74.8% respectively. The pattern of radiolabelled estrogens on HPLC was not affected using plasma (Fig 2A), milk (Fig. 3A) and urine (Fig. 4A) extracts. The

TABLE 2
Effect of Decreasing Percentage of Acetone in Solvent System Chloroform: Acetone on Separation of Estrogens. Parameters as in Table 1.

Percentage of Acetone	Retention time (min)		
	E_1	$E_{2\alpha}$	$E_{2\beta}$
50	4	4	4.5
30	4	5	5.5
20	4	6	7
10	5	7.5	9
5	6	10	11

TABLE 3
Effect of Flow Rate on the Separation of Estrogens Using Chloroform: Acetone (90:10). Parameters as in Table 1.

Flow rate ml/min	Retention time (min)		
	E_1	$E_{2\alpha}$	$E_{2\beta}$
1.5	3.5	5.0	6.0
1.0	5.0	7.5	9.0
0.5	10.0	14.5	16.5

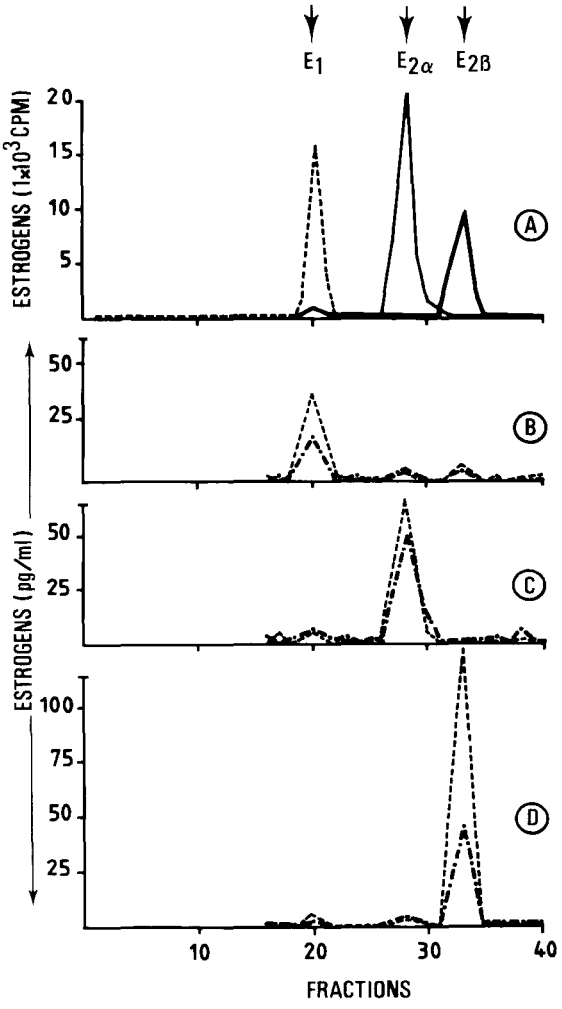

Figure 1: HPLC prolifing of estrogens. Mobile phase, chloroform: acetone (90:10); flow rate, 0.5 ml/min; temperature 30°C, pressure, 23 atmosphere; fraction volume, 0.25 ml. ^{3}H-radiolabelled (·----) E_1, (——) $E_{2\alpha}$ and (——) $E_{2\beta}$ (A). Immunoreactive profile of various estrogens in plasma of two superovulated animals (— — —) and (—·—·—) using specific antiserum against estrone (B), estradiol-17 α (C) and estradiol-17β (D) across the tritiated hormones using the entire elute.

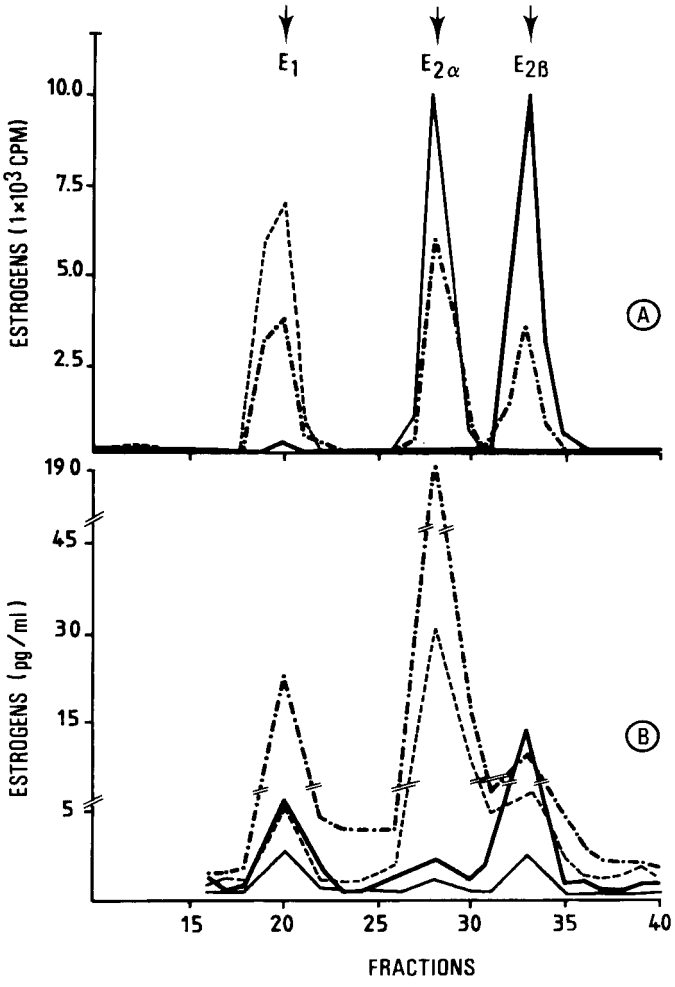

Figure 2: Chromatogram of estrogens. Parameters as in Fig. 1.
A: Radioactive profile (-----) E_1, (——) $E_{2\alpha}$, (——) $E_{2\beta}$ and (—·—·—) mixture of estrogens extracted from plasma.
B: Plasma immunoreactive estrogens in normal animal (——) free and (- - -) conjugated; superovulated animal (——) free and (—·—·—) conjugated.

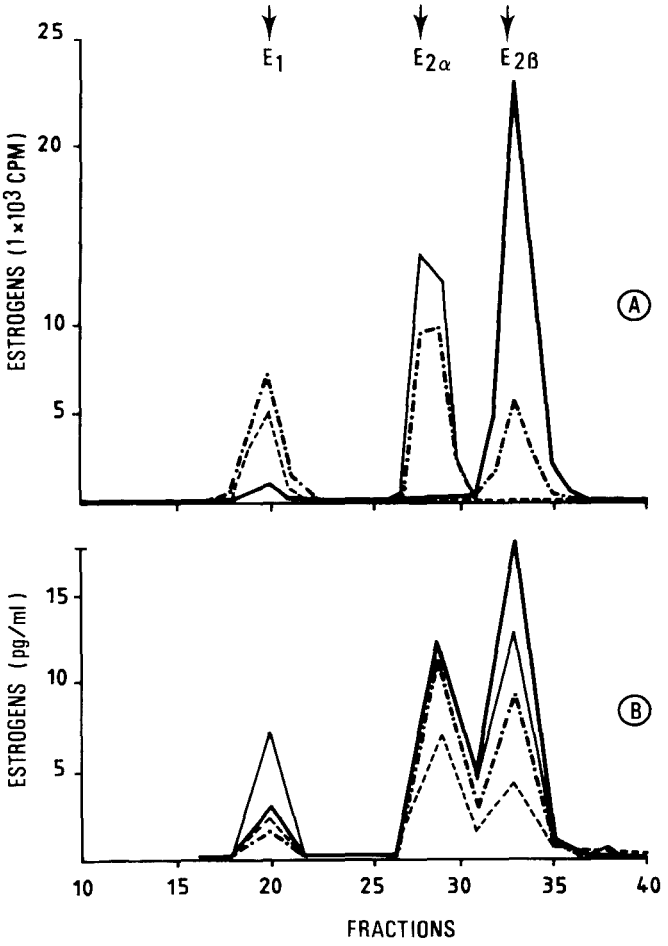

Figure 3: Chromatogram of estrogens. Parameters as in Fig. 1.
A: Profile of radioactive estrogens as in Fig. 2 and (—·—·) mixture of estrogens extracted from milk.
B: Milk immunoreactive conjugated estrogens in (— — —) normal animal; (——), (— -) and (—·—·) superovulated animals.

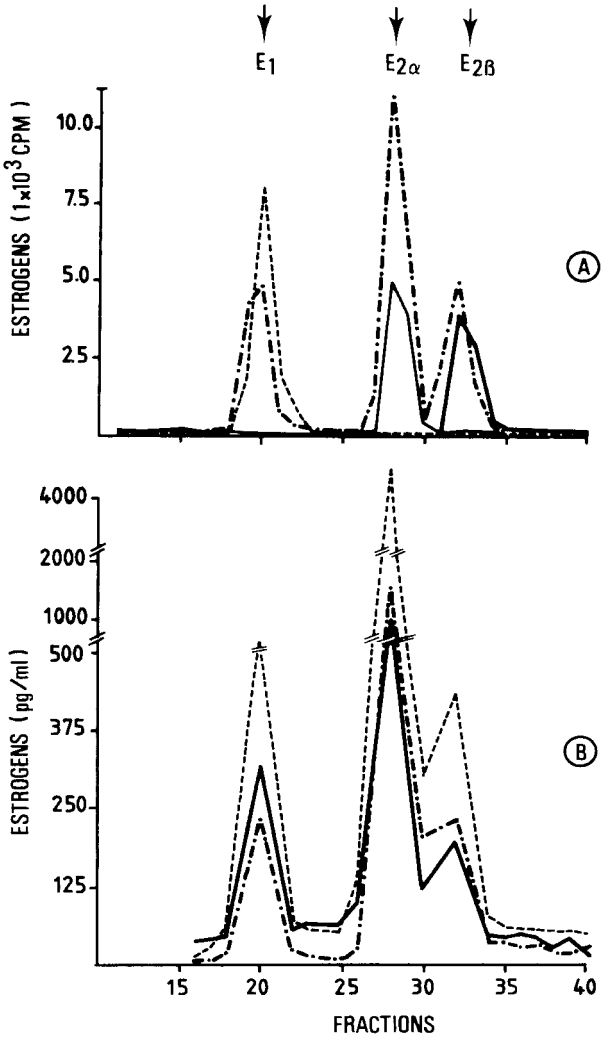

Figure 4: Chromatogram of estrogens. Parameters as in Fig. 1.
A: Profile of radioactive estrogens as in Fig. 2 and (·—·—·) mixture of estrogens extracted from urine.
B: Total immunoreactive estrogens of urine in (·—·—·) normal animal, (- - - -) superovulated animal and (——) non-responsed superovulated animal.

elution profile of endogenous estrogens across radioactive estrogens were confirmed by RIA using specific antiserum against estrone (Fig. 1B), estradiol-17α (Fig. 1C) and estradiol-17β (Fig. 1D).

The effectiveness of the developed HPLC technique was assessed by its application to various biological samples. The separated estrogens on HPLC were measured by RIA using non specific antiserum in blood plasma (Fig. 2B), milk (Fig. 3B) and urine (Fig. 4B).

DISCUSSION

The present high performance liquid chromatographic system that we have described for the separation of estradiol epimers is rapid and more convenient and sensitive than earlier available methods (5, 6, 7, 8). The separation is completed within 20 min using small volume (approximately 10 ml) of mobile phase over the earlier methods which requires long column and/or large elution volume. The method is more convenient than indirect method (12) where $E_{2\alpha}$ was separated from $E_{2\beta}$ by enzymatic oxidation of estradiol-17β to estrone.

The sequence of elution of E_1, $E_{2\alpha}$ and $E_{2\beta}$ is in accordance with their polarities. By creating a change in the polarity in the mobile phase, the resolution of the two epimers were improved. The changing in the polarity in chloroform: acetone, though increased the retention time, but the separation was better. The decrease in flow rate also increased the retention time, but the elution volume was decreased with improved resolution.

The estrogens concentrations in blood plasma of normal animal are in agreement with earlier report from this laboratory (11) and other workers (3, 19, 20, 21). Estradiol-17β concentrations in milk of cyclic cows are in the range of those

reported previously (22, 23); moreover, the conjugated estrogenic activity partly in the ketonic and mainly in non-ketonic fraction of defatted colostrum was also reported by previous worker (24). Similarly, all the 3 estrogens were present in urine of normal animal with the predominance of $E_{2\alpha}$ which agrees with other workers (2, 25).

It is, therefore, concluded that the present HPLC method can be utilised for the simultaneous quantification of free and conjugated E_1, $E_{2\alpha}$ and $E_{2\beta}$. The rapid and precise separation of estradiol epimers makes it an invaluable technique in the clinical diagnosis.

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A LIQUID CHROMATOGRAPHIC ELECTROCHEMICAL ASSAY FOR S-2-(3-AMINOPROPYLAMINO)ETHYLPHOSPHOROTHIOATE (WR2721) IN HUMAN PLASMA

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ABSTRACT

A liquid chromatographic electrochemical method for the determination of the radioprotective drug WR2721 in human plasma has been developed. This method includes the use of a Hg/Au electrochemical detector for the direct measurement of WR2721 concentration. An analog of WR2721, S-3-(4-aminobutylamino) propylphosphorothioate (WR80855) is the internal standard. The retention times for WR2721 and WR80855 are approximately 4.5 and 9 minutes, respectively. WR1065, the free sulfhydryl metabolite of WR2721, is retained on the column under the described chromatographic conditions and therefore does not interefere with the determination of the parent drug. With modification of the mobile phase WR1065 is eluted from the column at a retention time of approximately 20 minutes. This method has good linearity, precision and accuracy, and is free from interference from endogenous plasma substances. Preliminary results showing the applicability of this method to human pharmacokinetic studies and to investigating the enzymatic hydrolysis of WR2721 are presented.

INTRODUCTION

A number of studies in animals have shown that WR2721 provides significant protection of normal tissues from radiation injury (1,2). Recent observations suggest that this experimental

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drug will also protect normal tissues from alkylating agent toxicity (3). On the other hand, the radiosensitivity of a number of solid animal tumors is not altered by the administration of WR2721. The recent observation of facilitated uptake of WR2721 by normal cells compared to passive absorption by solid tumors has been proposed to account for its differential protective effect (4). The protective effect of WR2721 is presumed to result from the appearance, at a very rapid rate, of its dephosphorylated free sulfhydryl metabolite, WR1065, within cells (5). WR2721 is now in clinical trials in the United States and Japan (6,7,8).

In order to pursue pharmacological and pharmacokinetic studies of WR2721 in man an assay for WR2721 is required which is reliable, fast, not subject to interference from endogenous substances and that could readily be adapted to the investigation of metabolites. We describe here our HPLC method. The direct electrochemical detection of WR2721 in the column effluent is based on the finding that it is oxidized at a potential of +0.15 volts at the surface of a mercury/gold amalgam electrode.

MATERIALS AND METHODS

Apparatus

A Bioanalytical Systems LC-154 liquid chromatograph including a dual piston pump operated at 3,000 psi and a single mercury/gold detector was used as recently described by Allison and Shoup (9). Column temperature was maintained at 25°C with a temperature jacket. All teflon tubing was replaced with stainless steel to exclude oxygen. The column used for these studies was the BAS Biophase ODS 5μ (4.6 x 250 mm). The mobile phase was continuously purged with nitrogen to remove dissolved oxygen.

Chemicals

S-2-(3-aminopropylamino)ethylphosphorothioate (WR2721), S-2-(3-aminopropylamino)ethanethiol (WR1065) and S-(3-aminobutyl-

amino)propylphosphorothioate (WR80855) were supplied to us by Dr. Lawrence Fleckenstein of the United States Army Medical Research and Development Command at Walter Reed Army Institute of Research. Acetonitrile and methanol were obtained from Fisher Scientific (King of Prussia, PA) and sodium octyl sulfate was from Eastman Kodak Co. (Rochester, NY). Acid phosphatase isoenzyme 2A prepared from human seminal fluid was a gift from Dr. Norman Yang, isoenzyme 5 prepared from human spleen was a gift from Dr. Bill Lam. Human liver alkaline phosphatase was a gift from Dr. Claude Petitclerq and the calf intestine enzyme (Type XXX-TA) was obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents used were of the highest analytical grade available.

Sample Preparation and Chromatography

Plasma specimens were prepared, at 4 °C, immediately from blood drawn into EDTA vacutainer tubes which had been stored in an ice bath. To a 1 mL aliquot of each plasma sample was added 0.1 mL of a 1 mmol/L aqueous solution of the internal standard WR80855 and the samples were then stored at -70 °C until they were analyzed. Just prior to analysis the plasma specimens were thawed and maintained at 0 °C in an ice bath. In order to remove plasma proteins prior to liquid chromatographic analysis an equal volume aliquot of ice-cold acetonitrile was added to an aliquot of each thawed plasma specimen. After mixing, the samples were spun in a refrigerated centrifuge at 4 °C. Twenty microliter aliquots of the supernatants were injected onto a Biophase 5μ octadecylsilane column (250 x 4.6 mm) that was maintained at 25 °C with a constant temperature jacket. Elution of WR2721 and the internal standard WR80855 was achieved isocratically using an aqueous mobile phase containing 0.1 mol/L monochloroacetic acid and 1.5 mmol/L sodium octylsulfate, pH 3.0, at a flow rate of 2.0 mL/min.

Pharmacokinetic Study

In order to evaluate the application of the HPLC method for determining WR2721 in human plasma, a patient was given a single 300 mg dose (3.4 mg/kg) as a 10 second intravenous bolus. Blood samples were obtained prior to and after completion of the infusion of WR2721 at 2, 2.5, 3, 4, 8, 12, 20, 30, 45 minutes and 1, 2, 4, 8 and 24 hours. The WR2721 plasma concentration versus time data obtained on the patient were analyzed using the MK MODEL II PLUS version of the Extended Least Squares Nonlinear Regression Program, ELSNLR (10).

Acid and Alkaline Phosphatase Assays

Acid phosphatase catalytic assay conditions are essentially those described by Kachmar and Moss (11). The reaction mixture contained in final concentrations, p-nitrophenyl phosphate, 5 mmol/L; sodium citrate, 100 mmol/L, pH 5.0; and 0.1 mL of enzyme solution in a total volume of 1.0 mL. Incubation was for 30 minutes at 37 °C. The reaction was stopped with 0.1N NaOH and absorbance measured at 405 nm. In testing for the possible hydrolysis of WR2721 by acid phosphatase, the latter was substituted for p-nitrophenyl phosphate at a final concentration of 0.2 mmol/L and the incubation at 37 °C conducted for 30 minutes. A control reaction mixture consisted of all constituents except the acid phosphatase. After 30 minutes the reaction tubes were placed in ice and then 20 μL aliquots were injected onto the HPLC column for WR2721 analysis.

Using p-nitrophenyl phosphate as substrate, alkaline phosphatase activity was measured. The reaction mixture contained in final concentrations: p-nitrophenylphosphate, 16 mmol/L; tris (hydroxymethyl)aminomethane, 50 mmol/L, at pH values of 7.4, 8.0, 8.6, 9.2 and 10.0; magnesium acetate, 2 mmol/L; and 0.02 mL of enzyme in a total reaction mixture volume of 1.0 mL. Incubation was for 15 minutes at 37 °C. Absorbance was measured at 405 nm

after the addition of 0.1N NaOH. In order to determine the rate of hydrolysis of WR2721 by alkaline phosphatase the latter substrate was substituted for p-nitrophenylphosphate at a final concentration of 0.2 mmol/L and the incubation at 37 °C conducted for 15 minutes. Control reaction mixtures consisted of all constituents except for alkaline phosphatase at each of the above pH values. At the completion of the incubation period the reaction tubes were placed in ice and the 20 μL aliquots were injected onto the HPLC column for WR2721 analysis. In a separate chromatographic run these reaction mixtures were analyzed for WR1065.

RESULTS AND DISCUSSION

Detection

A differential pulse voltammogram of a solution of WR2721 in the HPLC mobile phase, (an aqueous solution of 0.1 mol/L monochloroacetic acid and 1.5 mmol/L sodium octylsulfate, pH 3.0), is displayed in Figure 1. From this experimental data it is clear that WR2721 would be oxidized on the surface of a Hg/Au electrode set at an operating potential of +0.15 volts. Thus the HPLC column effluent was monitored with a single Hg/Au working electrode at an operating potential of +0.15 volts. A typical chromatogram showing detector response versus elution time for WR2721 and the internal standard WR80855 is displayed in Figure 2.

It is important to emphasize the fact that in establishing experimental conditions for this HPLC assay for WR2721 considerable precautions were taken to minimize hydrolysis. Since WR2721 is reported to undergo nonenzymatic hydrolysis with increasing rates as pH is lowered, conditions in this method minimize the possibility of any loss of WR2721 due to hydrolysis: plasma specimens (standards and patient specimens) are stored at -70 °C until analyzed; use of a neutral polar organic solvent (acetoni-

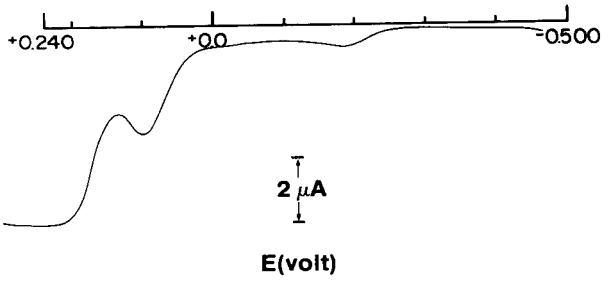

FIGURE 1. A differential pulse voltammogram of 4.5 mmol/L WR2721 in an aqueous solution of 0.1 mol/L monochloroacetic acid and 1.5 mmol/L sodium octylsulfate, pH 3, using a Hg/Au electrode.

trile) instead of an acid, such as perchloric acid, for protein precipitation; a relatively short chromatography time and, therefore, short time in the pH 3.0 mobile phase. Swynnerton, et al. (12) have measured the rate of nonenzymatic hydrolysis of WR2721 as a function of pH at 37 °C. Using a value for k_{obs} of 0.007 min^{-1} and the standard kinetic equation for a first order reaction, it is predicted that in 5 minutes at 37 °C, pH 3.0, 3.44% of WR2721 would be hydrolyzed. Since the retention time for WR2721 is less than 5 minutes and the operating temperature of the HPLC column is 25 °C our experimental conditions should not produce significant losses of WR2721 due to hydrolysis.

Linearity, Recovery, Precision and Accuracy

As shown in Figure 3 the response of the Hg/Au detector was linear over the WR2721 concentration range of 1 to 1000 μ mol/L (R^2 = 0.998). The absolute recovery of WR2721 was determined using ^{14}C-WR2721 added to normal plasma (Table 1). 98.4% (68,688/69,774 x 100) of ^{14}C-WR2721 added to normal plasma was recovered in the acetonitrile supernatant. Eighty-nine percent (61,112/68,688 x 100) of the labelled WR2721 in the supernatant aliquot applied to the HPLC column was recovered (a total of sixty 0.4 mL fractions were collected and counted).

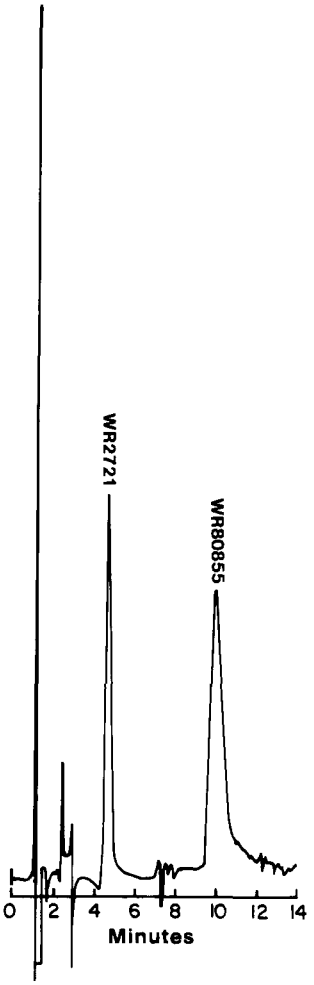

FIGURE 2. Chromatogram of 500 μmol/L WR2721 and 100 μmol/L WR80855 in normal human plasma. Sensitivity is 50 nA full scale through 7.3 min at which point it was changed to 10 nA. The acetonitrile extraction step and other experimental details are described in Materials and Methods.

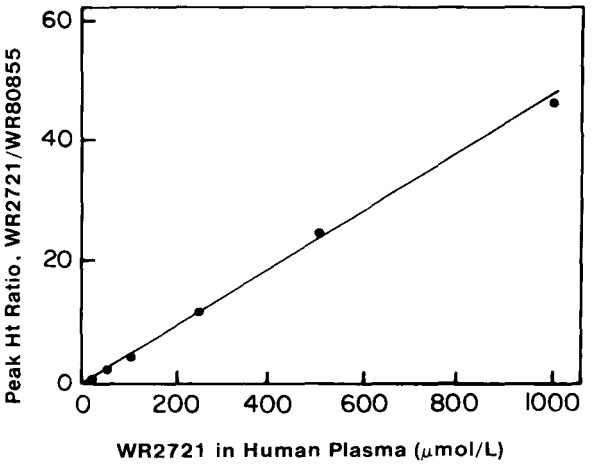

FIGURE 3. The average of duplicate peak height ratios of WR2721 to WR80855 over the range of WR2721 concentrations in normal human plasma of 1 to 1000 μmol/L are plotted against WR2721 concentration.

TABLE 1

Recovery of ^{14}C-WR2721[a] from Spiked Normal Human Plasma[b]

	Counts per min. per 0.02 mL
Plasma containing ^{14}C-WR2721 and unlabelled WR2721 (100 μmol/L)	69,774
Acetonitrile supernatant[c]	68,688
Total counts recovered from chromatograph[c]	61,112
Total counts recovered in WR2721[c] peak	53,388

[a]S-2-(3-aminopropylamino)ethyl-1,2-^{14}C-phosphorothioic acid.

[b]Human plasma from a healthy drug-free subject was collected in EDTA vacutainer tubes. 0.02 mL of a freshly prepared ice-cold solution, in 10 mmol/L phosphate buffer, pH 7.4, of 2.5 mmol/L WR2721 and 1.87 X 10^6 DPM ^{14}C-WR2721 was added to 0.48 mL of the ice-cold plasma pool.

[c]Values corrected for dilution of plasma by an equal volume of acetonitrile.

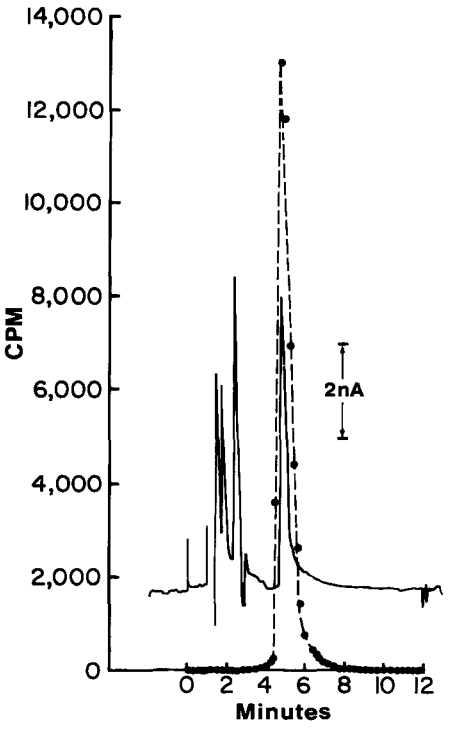

FIGURE 4. Chromatogram of 100 μmol/L WR2721 in human plasma to which ^{14}C-WR2721 had been added. A plot of the ^{14}C cpm for each of sixty collected effluent fractions is displayed as a function of time after the sample was injected. Other experimental details are described in Table 1.

Examination of the chromatogram obtained for this experiment (Figure 4) shows that no radioactivity peak other than that corresponding to the WR2721 chromatographic peak could be detected. Of the total ^{14}C counts recovered from the column, 87% (53,388/61,112 x 100) was recovered in the WR2721 peak. The net recovery of WR2721 carried through all of the steps in the procedure is therefore 76.5% (53,388/69,774). These recovery data compare favorably to those obtained by Swynnerton, et al. (12)

TABLE 2

Determination of the Precision and Accuracy of the HPLC Method

Spiked-in WR2721 Conc. µmol/L	Mean Measured Conc.[a]	Precision (SD)CV[b]	Percent Deviation (D)[c]
2	1.74	(0.11)6.5%	-13.0
5	5.3	(0.36)6.5%	6.0
40	35.8	(1.22)3.4%	-10.5
200	211	(7.38)3.5%	5.5
800	785	(10.1)1.2%	-1.9

Average % deviation = 7.4 (The average of the sum of the absolute values of D)

[a]The mean measured concentrations obtained with our HPLC method for each of four plasma samples at each WR2721 concentration using individually spiked specimens. The internal standard was 100 µmol/L WR80855.

[b](SD)CV, the standard deviation and coefficient of variation of the quadruplicate determinations.

[c](D) is the percent deviation of the mean measured concentration from the spiked-in concentration.

with their recently described fluorescamine derivatization HPLC method.

With WR80855 as an internal standard we obtained the precision and accuracy data summarized in Table 2. Using aliquots of a normal human plasma pool to which WR2721 was added to final concentrations ranging from 2 to 800 µmol/L we obtained an average coefficient of variation of 4.4% and an average deviation from the spiked-in concentration value of 7.4%.

Chromatography of S-2-(3-Aminopropylamino)ethanethiol (WR1065)

WR1065, the free sulfhydryl metabolite of WR2721, does not interfere with the HPLC assay for WR2721. In control experiments we tested for the possible appearance of this compound in our

chromatograms. WR1065 did not elute from the column during 20 minutes after injection of 20 µL of 0.1 mmol/L and 4 mmol/L solutions of this compound in 10 mmol/L tris(hydroxymethyl)aminomethane, pH 7.4, or of 20 µL of 0.1 mmol/L and 4 mmol/L of WR1065 in 10 mmol/L phosphate buffer, pH 7.4.

By modifying the mobile phase used for WR2721 chromatography to include 30% methanol it was possible to elute WR1065 from the Biophase ODS 5µ column in 20 minutes at a flow rate of 1 mL/min (Figure 5).

Human Pharmacokinetic Study

A pharmacokinetic study was performed on a patient treated with 300 mg (3.4 mg/kg) of WR2721 administered as a 10 second intravenous bolus dose. Blood samples were obtained prior to and after completion of WR2721 administration at 2, 2.5, 3, 4, 8, 12, 20, 30, 45 minutes and 1, 2, 4, 8 and 24 hours. A semilog plot of WR2721 concentration versus time is displayed in Figure 6 and a typical chromatogram of this patients' plasma compared to the pre-dose plasma is shown in Figure 7. Examination of the data in Figure 6 shows that the majority of the drug was cleared from plasma within about 5 minutes. Consistent with this conclusion is the very short distribution half-life, $T_{1/2}$, of 0.84 minute and the rapid clearance from the central compartment, Cl, of 0.977 L/hr/kg calculated from the data points with the extended least squares nonlinear regression (ELSNLR) program (10).

Enzymatic Hydrolysis of WR2721

It has been shown in experiments with mice that within 15 minutes after administration of an intravenous dose of WR2721 the principal metabolite in most tissues is WR1065 (5). Previous studies with mammalian tissues have raised the possibility that WR2721, or phosphorothioates with similar structure such as cysteamine S-phosphate, are hydrolyzed by either acid phosphatase

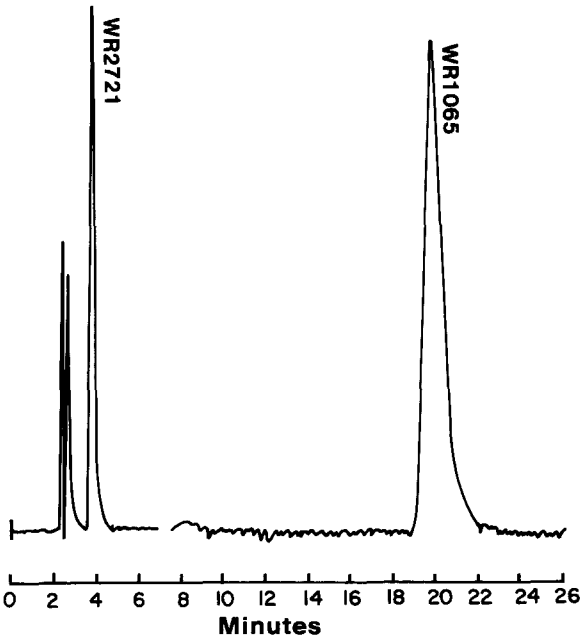

FIGURE 5. Chromatogram of an aqueous solution of 200 μmol/L WR2721 and 200 μmol/L WR1065 in 2.7 mmol/L EDTA and 10 mmol/L tris(hydroxymethyl)aminomethane, pH 7.4. The mobile phase is 30% methanol, by volume, in water. Monochloroacetic acid and sodium octylsulfate, pH 3.0, are in the mobile phase at final concentrations of 0.1 mol/L and 1.5 mmol/L, respectively. The mobile phase flow rate is 1.0 mL/min. Sensitivity was changed from 500 nA full scale to 100 nA full scale at 7 min.

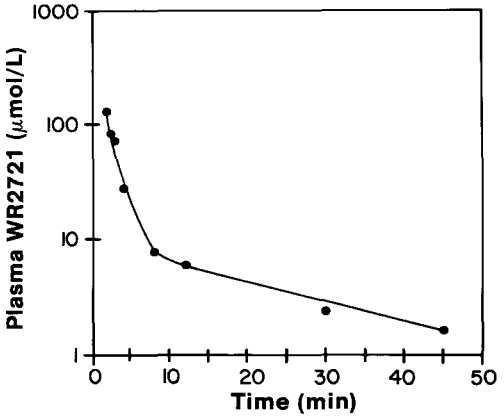

FIGURE 6. A semilog plot of WR2721 plasma concentration versus time. Each point is the average of duplicate determinations. The line fitting the points is the least squares best fit line determined with the ELSNLR program (10).

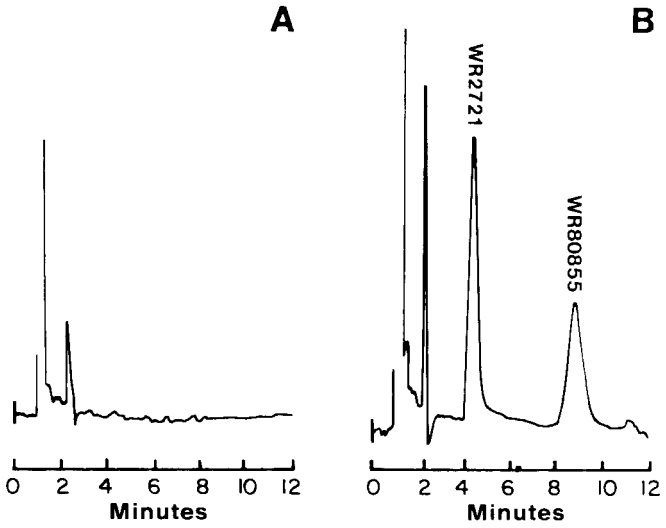

FIGURE 7. Chromatograms of acetonitrile-plasma supernatants prepared from A, plasma obtained from a patient immediately before a 300 mg intravenous bolus dose and B, plasma obtained from the patient two minutes after the dose. The sensitivity is 50 nA full scale.

(13,14) or alkaline phosphatase (15,16,17) to produce the corresponding free sulfhydryl metabolite. As shown in Table 3, WR2721 is not hydrolyzed by either human acid phosphatase isoenzyme 2A prepared from prostatic fluid as previously described (18) or isoenzyme 5 from human spleen (19). On the other hand, human liver alkaline phosphatase, prepared as described by Daigle (20) and calf intestine alkaline phosphatase do catalyze the hydrolysis of WR2721 (Table 3). The fact that the rate of WR2721 hydrolysis was higher than that achieved with PNPP as substrate using the human liver enzyme, but lower using the calf intestine enzyme may result from kinetic differences between the alkaline phosphatase isoenzymes. More detailed kinetic studies will be required to characterize this difference.

TABLE 3

Rates of Hydrolysis of p-Nitrophenylphosphate and WR2721 by Acid and Alkaline Phosphatases

	Hydrolysis Rates[a] (nmol/min/mL reaction mixture)	
	WR2721	p-Nitrophenylphosphate
Acid Phosphatase		
I. Isoenzyme 2A[b]	0.0002	2.75
II. Isoenzyme 5[c]	0	2.07
Alkaline Phosphatase		
I. Human liver[d]	11.49	6.56
II. Calf intestine[e]	11.25	508

[a]Each activity value is the average of duplicate determinations as described in Materials and Methods.

[b]Each reaction mixture contained 2.75 mU of isoenzyme 2A acid phosphatase per mL with p-nitrophenylphosphate as substrate. In testing for possible WR2721 hydrolysis each reaction mixture contained 44,000 mU of isoenzyme 2A acid phosphatase per mL.

[c]Each reaction mixture contained 2.07 mU of isoenzyme 5 acid phosphatase per mL with p-nitrophenylphosphate as substrate. In testing for possible WR2721 hydrolysis each reaction mixture contained 83 mU of isoenzyme 5 acid phosphatase per mL.

[d]Each mL of reaction mixture contained 6.6 mU of human liver alkaline phosphatase.

[e]Each mL of reaction mixture contained 508 mU of calf intestine alkaline phosphatase. All alkaline phosphatase reaction mixtures were incubated at pH 8.6.

The rate of WR2721 hydrolysis as a function of pH using human liver alkaline phosphatase is shown in Figure 8. The maximal rate of hydrolysis catalyzed by human liver alkaline phosphatase was obtained at pH 8.6. In contrast the optimal pH for the synthetic substrate, p-nitrophenylphosphate, is much higher than 8.6 (Figure 8). The highest activity was at pH 10 (the highest pH value tested). Previous studies have obtained pH optima, with p-nitrophenylphosphate as substrate, of 10.2 for alkaline phosphatase extracted from human liver cells grown in tissue culture

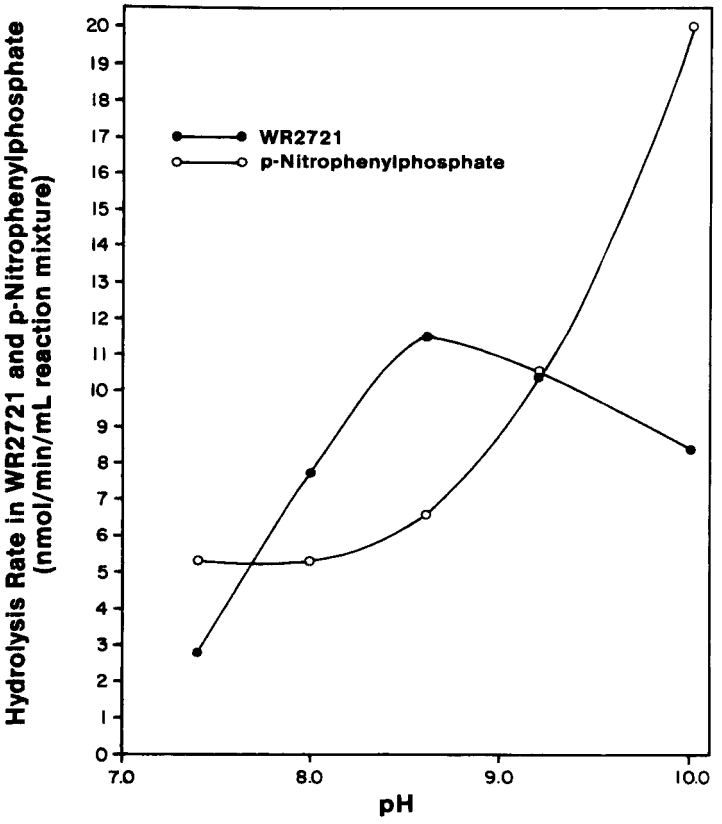

FIGURE 8. A plot of the rates of hydrolysis of WR2721 and p-nitrophenylphosphate by human liver alkaline phosphatase as a function of pH. Each ml of reaction mixture contained 6.6 mU of enzyme.

(21) and 10.4 for alkaline phosphatase purified from human liver obtained at autopsy (22). Our finding of different pH optima for these two alkaline phosphatase substrates is consistent with previous observations of the dependence of the pH optimum for alkaline phosphatase on both the chemical nature of the substrate as well as on the substrate concentration (23).

CONCLUSIONS

The HPLC electrochemical detection method reported here for the measurement of WR2721 has the following advantages: a) it is rapid, since each chromatographic cycle is 15 minutes; b) it is a direct method which does not require additional derivatization steps; c) it is both precise and accurate; d) it eliminates plasma proteins prior to chromatography using a neutral polar organic solvent; e) neither the free sulfhydryl metabolite of WR2721, WR1065, nor endogenous substances from patients' plasma co-elute with either WR2721 or WR80855; f) it is readily applicable to the study of WR2721 pharmacokinetics in humans and to the investigation of its metabolism by mammalian alkaline phosphatases.

ACKNOWLEDGMENTS

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SIMULTANEOUS DETERMINATION OF HEAVY METALS IN BOVINE LIVER AND OYSTER TISSUE BY SOLVENT EXTRACTION-REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

High performance liquid chromatographic method for the simultaneous determination of ppm levels of cadmium, nickel, lead, zinc, cobalt, copper and bismuth in biological samples has been developed. Each 250 mg of Bovine Liver(NBS 1577) or Oyster Tissue(NBS 1566) was ashed in a muffle furnace over night at 500 to 550°C. Then the ash was treated with 1.5 ml of 2 N hydrochloric acid, and the solution was put into a separatory funnel. The dissolved heavy metals were extracted into chloroform as hexamethylenedithiocarbamato chelates. The metal chelates were separated on a reversed phase column(5 μm, ODS, 4.6 × 150 mm), and determined by measuring the peak height of each metal chelate. Cd, Ni, Pb, Zn and Cu were determined accurately over the concentration range of 0.5-850 ppm with standard deviation ca. 7%.

INTRODUCTION

High performance liquid chromatography(HPLC) has been used mainely for the separation of various organic materials, but in recent years, the separation and determination of inorganic materials has been reported(1).

Dithiocarbamates(2-9) and other chelating agents(10,11) have been used for the determination of heavy metals in water(5,6) and alloy(6). Edward-Inatimi(9) determined Cu, Ni, Pb and Mn in industrial effluents, standard kale and standard fish meal as diethyldithiocarbamato(DDTC) chelates. Unfortunately the separation of

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metals as chelates is not complete because the Zn chelate peak overlaps with Cu and Ni chelates. Thus the presence of Zn precludes determination of Cu and Ni.

A simple HPLC method for the simultaneous determination of heavy metals in water(12,13) and Orchard Leaves(NBS 1571) using dithiocarbamates as chelating agents has also been reported. In this paper, the authors reported a simple and accurate method for the simultaneous determination of Cd, Ni, Pb, Zn, Co, Cu and Bi in Bovine Liver and Oyster Tissue by reversed phase HPLC using hexamethyleneammonium hexamethylenedithiocarbamate(HMA-HMDC) as chelating agent.

MATERIALS

Reagents

All chemicals were analytical grade unless otherwise stated.

Standard reference materials(Bovine Liver and Oyster Tissue) were obtained from National Bereau of Standard(NBS, U.S.A.).

Two molar ammonium chloride-ammonia buffer solution(pH 7.5) was prepared as follows. 107 g(2 moles) of ammonium chloride was dissolved in ca. 900 ml of water which was then adjusted to pH 7.5 with 25% ammonia water(super special grade) and diluted with water to 1000 ml. Heavy metals were removed from this solution by extraction. Fifty ml of 0.1% diphenylthiocarbazone(dithizone)-chloroform solution was added and stirred vigorously by use of a magnetic stirrer. The aqueous phase was then transferred to another 1 l beaker and stirred with 50 ml of chloroform. This procedure was repeated until the chloroform phase did not give green color.

One molar ammonium citrate(pH 9.3) was prepared by a procedure similar that described above.

HMA-HMDC was synthesized by a modified method of Busev et al. (15). Two moles(124 ml) of hexamethyleneimine(Aldrich) and ca. 400 ml of benzene were added to a 1 l beaker placed in an ice bath. One mole (60.1 ml) of carbon disulfide was then added from a separatory funnel over 30 min under a nitrogen gas stream. Stirring was accomplished by a glass bar, and benzene was added when formation of

HMA-HMDC made stirring difficult. The white crystals formed were filtered through a glass filter, and washed 2 times on the filter with ethyl ether. Methyl alcohol was then added to the filtered crystals until ca. 80% of the crystals were dissolved. This mixture was filtered and the filtrate put into a 2 l beaker. Ethyl ether(1/3 volume of the filtrate) was added to the filtrate. Recrystallization was accomplished by standing over night in a refrigerator. The white flocculent crystals formed were filtered through a glass filter and washed 3 times on the filter with ethyl ether, then dried in a desiccator(silica gel). The dried crystals were ground to a powder to facilitate dissolution in water. The purity and structure of the crystals(HMA-HMDC) were confirmed by titration with a copper standard solution, melting point, elemental analysis, mass spectrometry and N.M.R.

The mixed metal standard solution for the analysis of Bovine Liver(A) and Oyster Tissue(B) were prepared as follows. The 500 ml of metal standard solution(A) was prepared, which contained 0.1 M hydrochloric acid(pH 1), 13.5 ng/ml(ppb) of Cd(II), 17 ppb of Pb(II), 6.5 μg/ml(ppm) of Zn(II), 9.65 ppm of Cu(II), 9.0 ppb of Co(II) and 0.8 ppb of Hg(II). The metal standard solution(B) for Oyster Tissue contained 175 ppb of Cd(II), 51.5 ppb of Ni(II), 24 ppb of Pb(II), 42.6 ppm of Zn(II), 20 ppb of Co(II) and 3.15 ppm of Cu(II). Five ml of each standard solution containes the same amount of the metals as 250 mg of Bovine Liver or Oyster Tissue samples as calculated from their certified values.

Apparatus

A liquid chromatograph consisted of a Model 6000 A pump(Waters Assoc.), a U6K universal injector(Waters), UVIDEC 100-III spectrophotometer(Japan Spectroscopic Co., Tokyo, Japan) and a Model 056 recorder(Hitachi Ltd., Tokyo, Japan) were employed. The detector was fitted with a 10 μl flow-cell volume of 10 mm pathlength and samples monitored at 260 nm.

A 4.6 × 150 mm Cosmosil 5 C_{18} packed column(5 μm, ODS, Nakarai Chemicals, Kyoto, Japan) was used. This column was immersed in a

water thermostated bath consisting of a Model 150 L cool pipe and a Model Ace 80 thermominder(Taiyo Scientific Industrial Co. Ltd., Tokyo, Japan).

A Hitachi Model 200-20 spectrophotometer was employed for the measurement of UV and visible spectra of metal-HMDC chelates and determination of their molar extinction coefficients.

A Model SA-31 auto shaker (Yamato Scientific Co. Ltd., Tokyo, Japan) was used for extraction of HMDC chelates.

METHODS

Extraction Conditions

In our previous work(13), 50 ml of aqueous sample of heavy metals were extracted into 1 ml of chloroform as the metal-HMDC chelate. When the dry ash method was employed for a biological sample, 0.1-1 g of the sample were frequently collected. Sample and chloroform volumes were modified to 25 ml and 0.5 ml, respectively in order to concentrate the metal-HMDC chelates in the chloroform phase. This modification in preveous work(13) required some investigation for optimization of the extraction conditions. Optimums of HMA-HMDC concentration and shaking time were investigated for the simultaneous quantitative extraction of Cd(II), Ni(II), Pb(II), Zn(II), Co(II), Cu(II), Hg(II) and Bi(III). Peak height were measured at various volume of HMA-HMDC solution and shaking time with metal concentrations in the range of 10-1000 ppb.

Stability of Some Dithiocarbamates

Both dithiocarbamates and some of their chelates are unstable in reversed phase mobile phases such as acetonitrile/water and methyl alcohol/water under ambient conditions. However, it was found that addition of the chelating agent to mobile phases suppressed the decomposition of the some dithiocarbamato chelates such as those of cadmium, lead and zinc. Because the chelating agent added is also unstable in the mobile phase, decomposition of Na-DDTC, ammonium pyrrolidinedithiocarbamate(APDC) was investigated according to the procedure in Figure 1. The time course of the decomposition of the three dithiocarbamates was also examined.

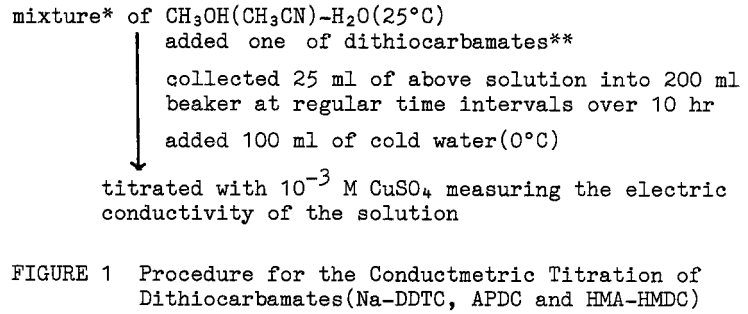

FIGURE 1 Procedure for the Conductmetric Titration of Dithiocarbamates(Na-DDTC, APDC and HMA-HMDC)

* : methyl alcohol(acetonitrile)/water=70/30
** : Concentration of each dithiocarbamate was 8×10^{-3} M in the mixture

UV and Visible Spectra of Metal-HMDC Chelates

UV and visible spectra of eight metal-HMDC chelates were measured by following the procedure shown in Figure 2.

Molar extinction coefficient at the maximum absorption wavelength was also measured. The reference(blank) solution was fleshly prepared because its absorbance increased slowly with time.

HPLC Separation of Eight Metal-HMDC Chelates

In our earlier work(12), Ni(II), Pb(II), Co(II), Cu(II), Hg(II) and Bi(III) have been separated as APDC chelates. Cd(II), Ni(II), Co(II), Cu(II), Hg(II) and Bi(III) have been also separated as HMDC chelates(13), but Pb(II) and Zn(II) chelates could not be separated from Ni(II) chelate.

In order to separate eight metal-HMDC chelates described above, an ODS column(Cosmosil 5 C_{18}, 4.6 × 150 mm) was employed. Various solvents such as diethyl ether, ethyl acetate, ethyl alcohol, acetonitrile, water and buffer solutions were examined as mobile phases.

Addition of 1.0×10^{-3} M HMA-HMDC to the mobile phase was needed to obtaine reproducible chromatograms of HMDC chelates. Peak heights of the HMDC chelates were measured at various HMA-HMDC concentration in order to determine the optimum concentration of HMA-HMDC in the mobile phase.

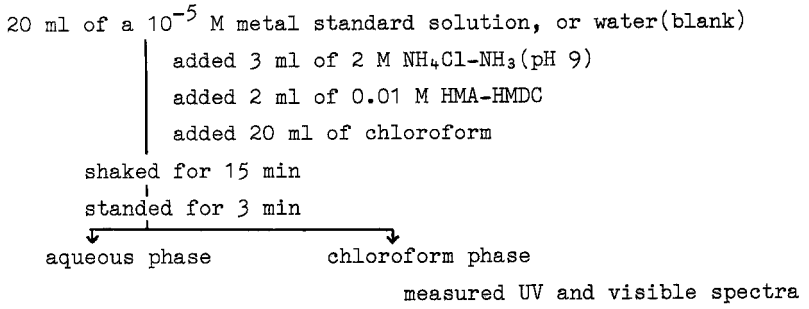

FIGURE 2 Extraction Procedure for Measuring UV and Visible Spectra of the Metal-HMDC chelates

Effect of column temperature on the separation of HMDC chelates was also investigated over the range of 15-50°C.

Working Curves

It was found that the working curves for studied heavy metals HMDC chelates have good linearity over the concentration range of 0.3-2000 ppb(13). When heavy metals in 250 mg of a biological sample were dissolved in 25 ml of aqueous phase, this concentration range corresponds in the range of 0.03-200 ppm in a original sample.

The working curves for Bovine Liver and Oyster Tissue were made as follows; 5 ml of mixed metal standard solution(A) or (B)(see Reagents) and 20 ml or 25 ml of water(blank) were first put into a separatory funnel. After extraction, 5 µl of the chloroform extract was injected into the HPLC column. Each working curve was made with only the two plots(standard and blank).

Ashing Method for Bovine Liver and Oyster Tissue

The dry ash method was employed for the two biological samples because it is simple and has no reagent blank except a mineral acid required for dissolution of the metals in the ash. Ashing temperature was investigated over the range of 350-600°C. Dry ashing was carried out over night for convenience.

RESULTS AND DISCUSSION

Extraction Procedure

Effect of the amount of HMA-HMDC on extraction of Cd(II), Ni(II), Pb(II), Zn(II), Co(II), Cu(II), Hg(II) and Bi(III) is summarized in Figure 3. It was found that 1.5-4.0 ml of 0.01 M HMA-HMDC gave maximum and constant peak heights of the eight metal chelates in the concentration range of 10-1000 ppb. The effect of shaking time is given in Figure 4. Quantitative extraction of the eight metals in the concentration range of 10-1000 ppb was accomplished by shaking for 10-20 min.

Based on the above results, the following procedure is recommended(Fig. 5) for the simultaneous extraction of Cd(II), Ni(II), Pb(II), Zn(II), Co(II), Cu(II), Hg(II) and Bi(III).

Evaluation of HMA-HMDC as Chelating Agent for HPLC Analysis

The measured UV and visible spectra are shown in Figure 6. The molar extinction coefficients of the chelates are given in table 1 with DDTC and APDC chelates measured by similar methods as shown in Figure 1. Based on the above results, 260 nm was selected for the detection of HMDC chelates. Molar extinction coefficients of the HMDC chelates were larger than those of APDC and DDTC chelates, and apparent decomposition rate of HMA-HMDC in water or in mobile phase (CH_3OH-H_2O or CH_3CN-H_2O) is the smallest in the three dithiocarbamates(Fig. 7).

In view of above fact it is reasonable to conclude that HMA-HMDC is more sensitive and stable chelating agent than APDC or DDTC.

HPLC Separation of Eight Metal HMDC Chelates

Reproducible chromatograms were obtained when 10% or more 10^{-3} M HMA-HMDC and 2.4% or more 2 M NH_4Cl-NH_3 buffer solution were added to the mobile phase. Optimum column temperature for separation of the eight HMDC chelates was 30-50°C. Higher temperatures were not examined.

Best separations were obtained when a mixture of ethyl alcohol/methyl alcohol/water/10^{-3} M HMA-HMDC/2 M NH_4Cl-NH_3(pH 7.5) (=40/32/

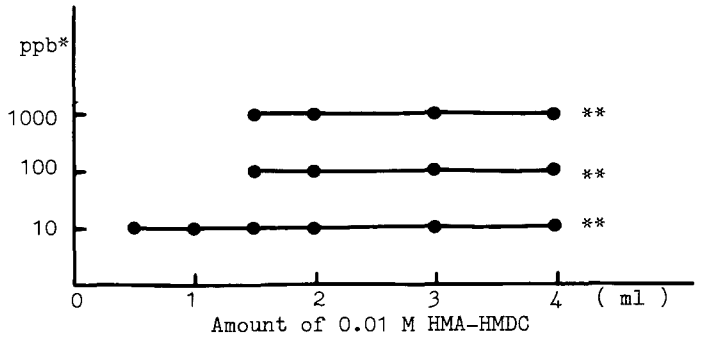

FIGURE 3 Effect of HMA-HMDC Amount on the Extraction of the Eight Heavy Metals

* : concentration of each heavy metals in 25 ml of aqueous phase
** : These lines indicate maximum and constant peak heights range for the all eight heavy metals.
shaking time : 15 min

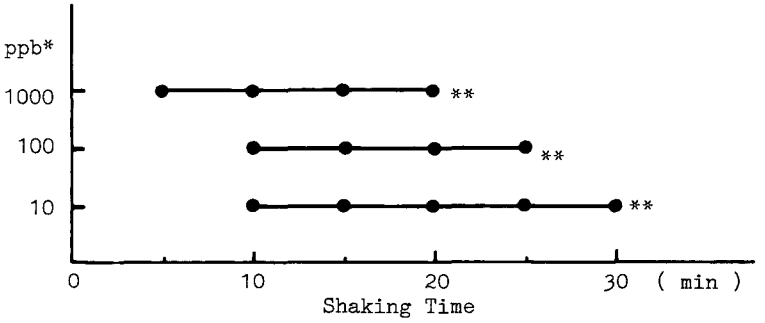

FIGURE 4 Effect of Shaking Time on the Extraction of Eight Heavy Metals

Amount of 0.01 M HMA-HMDC was 2 ml.
* : concentration of each metal in 25 ml of aqueous solution
** : These lines indicate maximum and constant peak heights range for the all eight heavy metals.

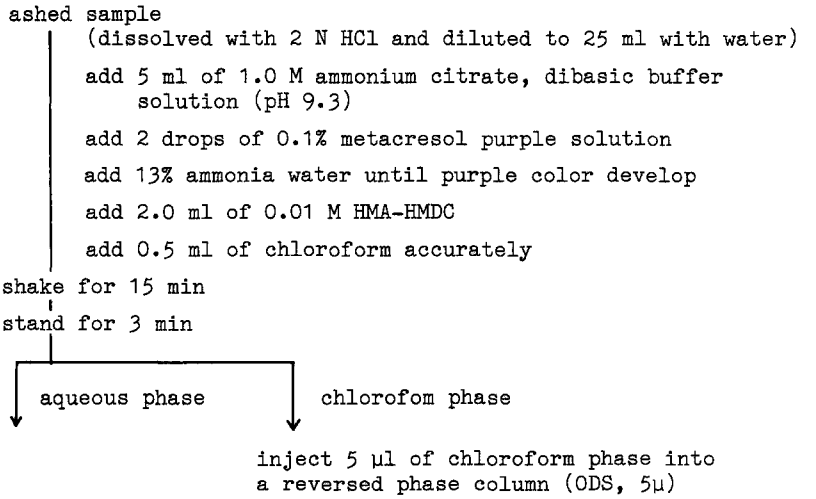

FIGURE 5 Recommended Extraction Procedure for the Determination of Cd, Ni, Pb, Zn, Cu, Co, Hg and Bi in Biological Samples

13/12/3) was used as the mobile phase. A typical chromatogram is shown in Figure 8. Although Mn(II), As(III) and Sn(II) were also extracted by the recommended procedure(Fig. 5), they did not appeared on the chromatogram. This is probably due to their low stability constants. Fe(III) was not extracted by the extraction procedure because the added citrate masked Fe(III) ion.

Determination of Heavy Metals in Bovine Liver and Oyster Tissue

When the dry ash method was employed, 1 g or more of sample was difficult to ash completely. Therefore, 250 mg of samples were used. High temperature(600°C) resulted in low analytical results for Zn and Pb. At low temperatures(350°C and 400°C) ashing was not accomplished completely. The optimum temperature for Bovine Liver and Oyster Tissue samples was 550°C and 500°C, respectively.

Porcelain crucibles and quartz boats were used for the ashing of the Oyster Tissue and Bovine Liver, respectively. It was felt

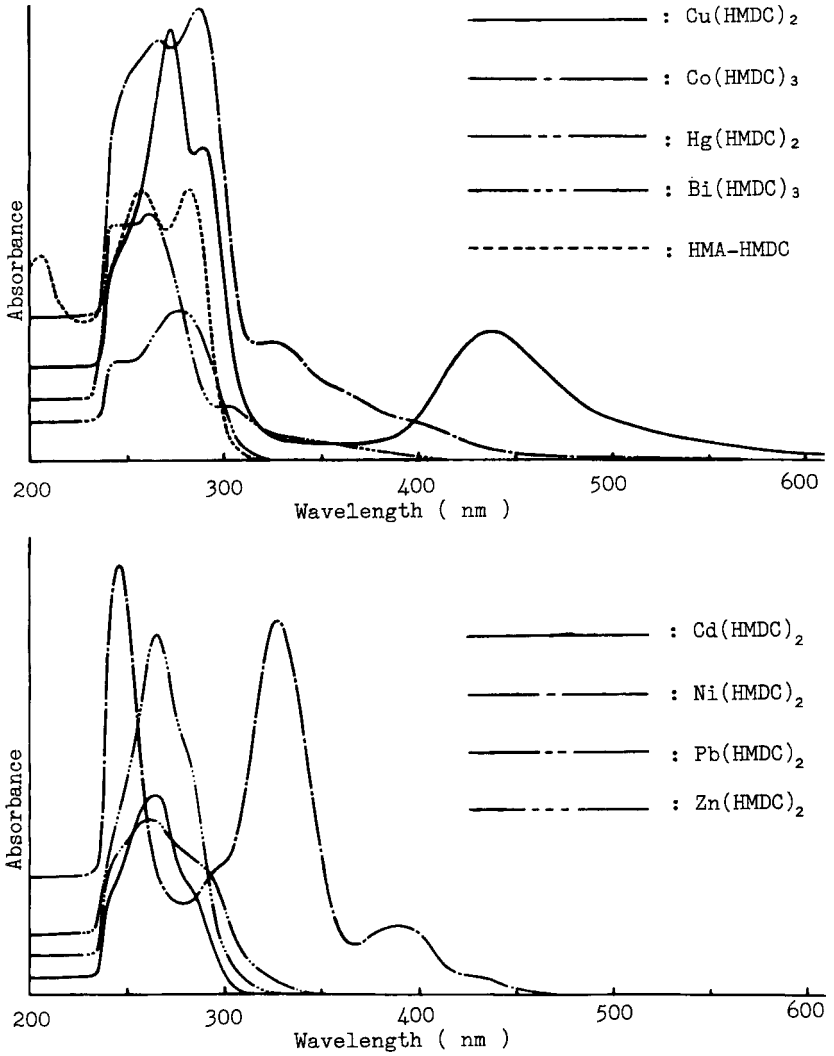

FIGURE 6 UV and Visible Spectra of Metal-HMDC Chelates

The mole ratio of above chelates was determined by the mole ratio method.

TABLE 1 Molar Extinction Coefficients of Metal Dithiocarbamates

Metal	Molar Extiction Coefficient Na-DDTC(λ max)	APDC(λ max)	HMA-HMDC(λ max)
Cd	37000 (264 nm)	35000 (261 nm)	50000 (266 nm)
Ni	37000 (247 nm)	38000 (246 nm)	43000 (246 nm)
Pb	37000 (263 nm)	43000 (260 nm)	65000 (262 nm)
Zn	33000 (264 nm)	36000 (260 nm)	36000 (264 nm)
Co	36000 (272 nm)	38000 (269 nm)	84000 (266 nm)
Cu	34000 (272 nm)	33000 (269 nm)	52000 (273 nm)
Hg	39000 (277 nm)	40000 (272 nm)	73000 (278 nm)
Bi	77000 (261 nm)	84000 (260 nm)	108000 (262 nm)

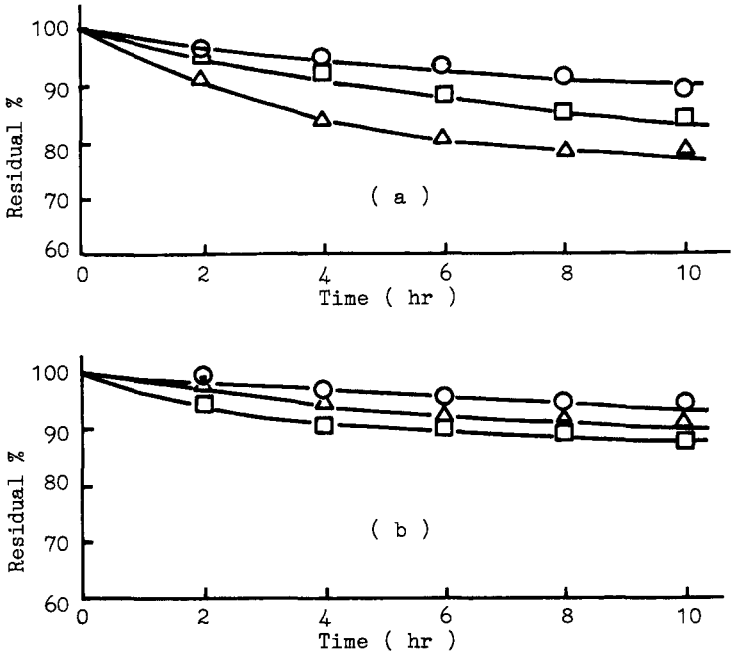

FIGURE 7 Time Courses for Na-DDTC, APDC and HMA-HMDC in Eluent

○ : HMA-HMDC, △ : APDC, □ : Na-DDTC

Experimental Conditions

(a) : CH_3CN/H_2O = 70/30

(b) : CH_3OH/H_2O = 70/30

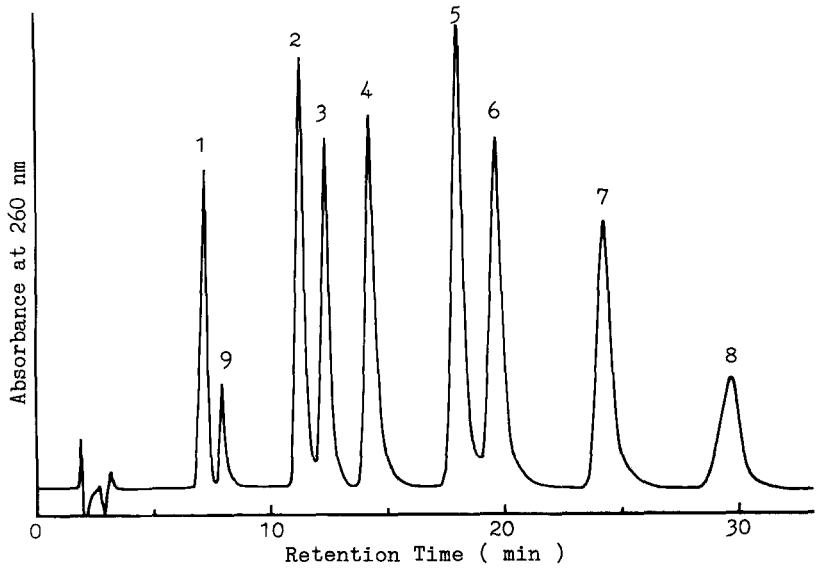

FIGURE 8 Typical Chromatogram of Metal-HMDC Chelates

1 : Cd(62.5 ng), 2 : Ni(125 ng), 3 : Pb(125 ng)
4 : Zn(62.5 ng), 5 : Cu(125 ng), 6 : Co(62.5 ng)
7 : Hg(250 ng), 8 : Bi(125 ng),
9 : decompositon product of HMA-HMDC

HPLC Conditions
column : Cosmosil 5 C_{18}, 4.6×150 mm, 35.0±0.1°C
mobile phase : ethyl alcohol/methyl alcohol/water/ 10^{-3} M HMA-HMDC/2 M NH_4Cl-NH_3(pH 7.5) =40/32/13/12/3
flow rate : 1.0 ml/min, sample size : 5 µl
The mobile phase reservoir was cooled in an ice bath

that quartz was not necessary at 550°C or below. A boat is, however, convenient to spread a sample thinly.

The analytical results are shown in Table 2 and Figure 9. In the case of Oyster Tissue, Zn content is larger than that of Pb by a factor of about 2000. However, the separation of the chelates was good and analytical results showed good agreement with the certified values. Cobalt and bismuth were not detected because the contents are too low. Mercury probably evaporated at the ashing step.

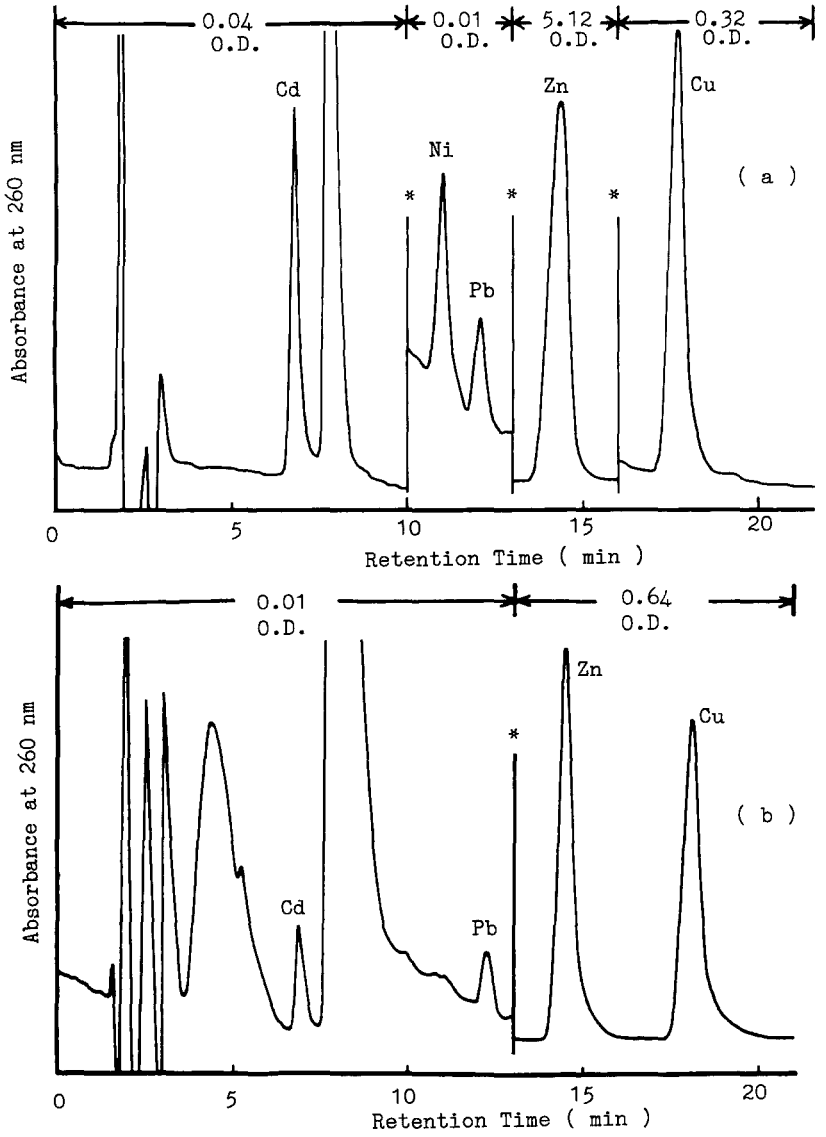

FIGURE 9 Analytical Chromatograms of Oyster Tissue(a) and Bovine Liver

* : change point of detector response
HPLC conditions were same as Fig. 8.

TABLE 2 Analytical Results of Heavy Metals in Biological Samples by Presented HPLC Method

Sample	Metal	Certified Value(ppm)	Found* (ppm)	Detector Range(O.D.)
Oyster** Tissue (NBS 1566)	Cd(II)	3.5 ± 0.4	3.2 ± 0.2	0.04
	Ni(II)	1.03 ± 0.19	0.80 ± 0.1	0.01
	Pb(II)	0.48 ± 0.04	0.44 ± 0.06	0.01
	Zn(II)	852 ± 14	848 ± 17	5.12
	Cu(II)	63.0 ± 3.5	64.0 ± 4.6	0.32
Bovine*** Liver (NBS 1577)	Cd(II)	0.27 ± 0.04	0.29 ± 0.02	0.01
	Pb(II)	0.34 ± 0.08	0.29 ± 0.03	0.01
	Zn(II)	130 ± 13	133 ± 6	0.64
	Cu(II)	193 ± 10	191 ± 7	0.64

* : average value ± standard deviation
** : N=4, ash ; 500°C-13 hr (porcelain crucible)
*** : N=8, ash ; 550°C-13 hr (quartz boat)

CONCLUSION

Heavy metals in Bovine Liver and Oyster Tissue were determined easily and accurately. Determination of heavy metals by HPLC is very simple in comparison with AAS. When AAS is employed for the determination of several metals, a lamp change is required and the working curves must be made for individual metals in the optimum concentration range. The sensitivities of flameless AAS is exellent, but it requires significant skill for reproducible results and the apparatus is more expensive than in HPLC. HPLC requires no specific skill and enables simultaneous determination of heavy metals in environmental samples as reported in this paper and previous work(13). Determination of ppm levels of heavy metals could be accomplished with preconcentration(solvent extraction).

Simultaneous determination of ppm levels of the heavy metals in plant samples is in progress.

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HIGH-PRESSURE LIQUID CHROMATOGRAPHIC ASSAY OF CEFOTAXIME AND DESACETYLCEFOTAXIME IN HUMAN MYOMETRIUM

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ABSTRACT

An analytic high-pressure liquid chromatographic (HPLC) procedure for the assay of desacetylcefotaxime and cefotaxime in gynecologic tissue was developed. Normal individuals undergoing elective hysterectomy were subjects in this study. Blood and myometrium were removed up to four hours after a 1-g intramuscular dose of cefotaxime. Since cefotaxime is unstable in homogenized tissue at room temperature, the specimens must be maintained at 4°C during homogenization and extraction. Mean serum desacetylcefotaxime and cefotaxime levels were 3.2 ± 2.0 µg/ml and 6.8 ± 4.4 µg/ml, respectively. The mean myometrium concentrations of desacetylcefotaxime and cefotaxime were 8.4 ± 10.0 µg/g and 6.3 ± 8.9 µg/g, respectively. The cefotaxime to desacetylcefotaxime ratios in serum and tissue were 2.12 and 0.75, respectively. Our results suggest that in antimicrobial synergistic studies evaluating serum and tissue levels, the optimal ratio of one part cefotaxime to at least one part desacetylcefotaxime.

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INTRODUCTION

The pharmacokinetics of cefotaxime and desacetylcefotaxime in serum have been well documented (1, 2). The serum concentration of both antimicrobials is easily measured in serum using high-pressure liquid chromatography (HPLC) (3, 4). Direct tissue and bile concentrations of desacetylcefotaxime have not been determined because of body metabolites which interfere with the assay (5). The rapid elution of desacetylcefotaxime from reverse-phase HPLC columns also contributed to the problem. Desacetylcefotaxime has been quantified in bile by transformation into a lactone, and then determined by HPLC (1). The HPLC assays of desacetylcefotaxime in bile was 1.43 times higher than cefotaxime after 90 minutes. At 240 minutes the desacetylcefotaxime was 2.2 times higher than the cefotaxime levels (6).

There are other problems associated with the assay of cefotaxime and desacetylcefotaxime, related to hemolysis of red cells and tissue destruction. Both processes result in the release of enzymes that promote the rapid deacetylation of cefotaxime to the desacetylcefotaxime metabolite (4). This study examines the metabolism of cefotaxime and desacetylcefotaxime subsequent to tissue homogenization, and the development of a direct tissue (myometrium) assay for desacetylcefotaxime.

MATERIALS AND METHODS

To determine the fate of cefotaxime in tissue, normal myometrium was collected, maintained at 4°C, and spiked with 50 μg/g of cefotaxime (cefotaxime and desacetylcefotaxime were obtained from Hoechst-Roussel Pharmaceuticals, Inc., Somerville, New Jersey). Additional specimens were spiked with cefotaxime and 10 mM sodium p-hydroxymecuribenzoate (PHMB) (Sigma Chemical Co., St. Louis, Missouri), an enzyme inhibitor. These specimens were maintained at 4°C and homogenized with a Polytron (Brinkman Instruments, Inc., Westbury, New York). They were then centrifuged at 3,000 X g for five minutes at 4°C and the supernatant divided into

2.0 ml aliquots and incubated at 4°C, 25°C, and 37°C for two hours. At the end of the incubation period, the reactions were stopped by processing through the Sep-pak C_{18} column (Water's Associates, Milford, Massachusetts) extraction procedure.

The Sep-pak cartridges were attached to the barrel of a 10 cc disposable syringe which serves as a solvent reservoir. The plunger of the syringe is used to force the specimen through the Sep-pak cartridges. To prepare the cartridges for the specimens, the cartridges were rinsed with 3.0 ml of HPLC grade methanol followed by 5.0 ml of 0.1 M sodium phosphate buffer pH 6.1. The flow rate through the cartridge was about 5 ml/minute.

To each of the cartridges was added 1.0 ml of the standards or tissue extract. The specimens were pushed through the Sep-pak C_{18} column with the syringe plunger. One ml of 0.1 M sodium phosphate buffer was added to rinse all the specimen into the column and pushed through as before. An additional 5.0 ml of the 0.1 M sodium phosphate buffer was added and pushed through the column. The cefotaxime and desacetylcefotaxime were then eluted through the column with 1.0 ml of 50% methanol in water (v/v).

The most rapid HPLC assay of cefotaxime in serum and tissue may be obtained by using the previously described acetonitrile dichloromethane extraction methodology with modification of only the mobile phase (7). The HPLC mobile phase consisted of 92% 0.10 M sodium phosphate pH 6.1 and 8% acetonitrile. The flow rate was 2.5 ml/minute. The mobile phase for the assay of desacetylcefotaxime in tissue was 95% 0.1 M sodium phosphate pH 6.1 and 5% acetonitrile. For cefotaxime the 8% acetonitrile mobile phase was used subsequent to the Sep-pak extraction. There was no interference between the assay of cefotaxime and desacetylcefotaxime.

Samples from 15 women undergoing elective hysterectomy were assayed. A 1-g IM dose of cefotaxime was given on call to the operating room, and the injection time was recorded. At the time of uterine removal, a blood sample was obtained, normal appearing myometrium was removed and the time recorded. The serum and uterine tissue specimens were maintained at -20°C until assayed.

The sera from these women were assayed for cefotaxime and desacetylcefotaxime as described above. The standard curve for serum assays consisted of normal serum which was spiked with 0-100 μg/ml of both cefotaxime and desacetylcefotaxime. Similarly, tissue or tissue extracts were spiked with 0-25 μg/g for tissue assay controls. The tissue was extracted by the addition of five parts 0.1 M sodium phosphate buffer pH 6.1 to one part tissue. The tissues were then disrupted with the Polytron and processed through the Sep-pak C_{18} column as described earlier. Between- and within-batch recoveries were made on both the Sep-pak and the acetonitrile dichloromethane phase extraction procedures. Linear least squares regression analysis was used to analyze the between- and within-batch data.

RESULTS AND DISCUSSION

The chromatograms of cefotaxime and desacetylcefotaxime in serum using the phase extraction system are shown in Figure 1. This method is extremely sensitive for both antibiotics in serum, and there was no interference from body metabolites. Between- and within-batch recoveries of the HPLC extraction procedures for both were > .95 (data not shown).

The Sep-pak C_{18} tissue extraction eliminated interference from body metabolites, and the standard curve from 0 to 100 μg/g was linear and had a > .98 correlation with peak height. Chromatograms of desacetylcefotaxime from spiked myometrium are shown in Figures 2 and 3. Cefotaxime resolves late in the 5% acetonitrile mobile phase, and should be assayed using the 8% acetonitrile mobile phase to increase sensitivity and decrease column retention time. Desacetylcefotaxime may occasionally be resolved by the 8% acetonitrile mobile phase, but was completely resolved with the 5% acetonitrile mobile phase.

The results of incubating spiked tissue extract at various temperatures and with PHMB are shown in Figure 4. There is a linear relationship between temperature and the amount of

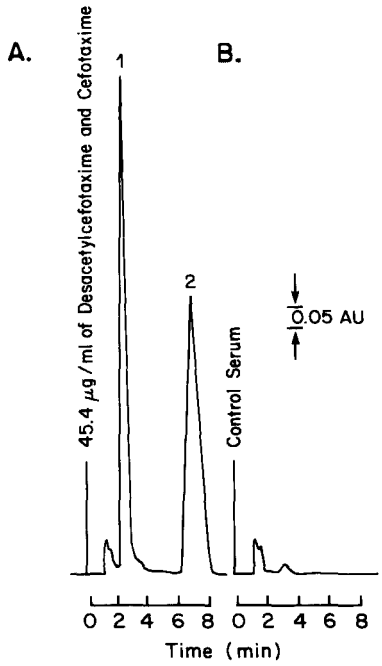

Figure 1. Chromatograms of desacetylcefotaxime and cefotaxime in serum subsequent to a 1-g IM dose of cefotaxime. The serum was extracted with acetonitrile and dichloromethane. The retention times were 3.0 minutes and 7.0 minutes for desacetylcefotaxime and cefotaxime, respectively. AU: Absorbance units. (1) desacetylcefotaxime and (2) cefotaxime. (A) Control serum spiked with desacetylcefotaxime and cefotaxime.

desacetylcefotaxime formed. Approximately 8.0 µg/g of desacetylcefotaxime was formed after the incubation of 50 µg/g of cefotaxime at 4°C for two hours with a 50% reduction after the addition of PHMB. At 25°C PHMB reduced the formation of desacetylcefotaxime by 84.4%, from 24 µg/g to 4.2 µg/g. At 37°C, 34.5 µg/ml of desacetylcefotaxime was formed after two hours incubation. It is therefore essential that all tissue specimens be maintained at 4°C after collection and during extraction for the accurate determination of the desacetylcefotaxime concentrations

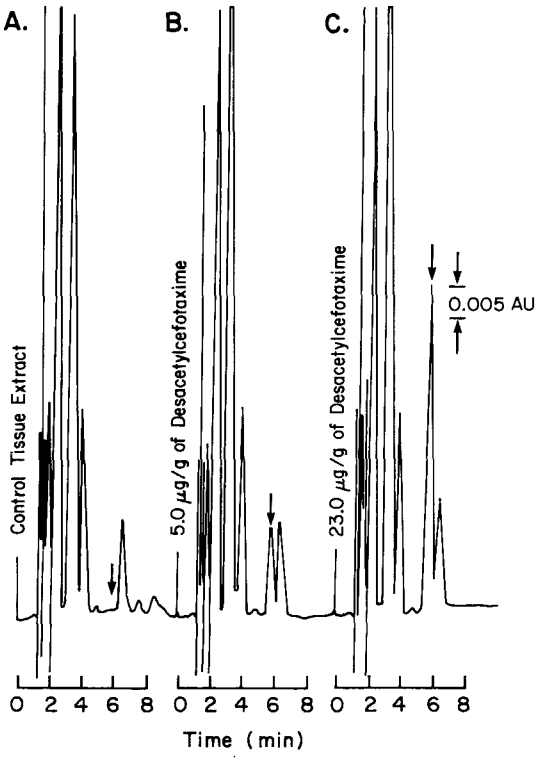

Figure 2. Chromatograms of desacetylcefotaxime as extracted from myometrium using the Sep-pak C_{18} extraction column. The HPLC mobile phase was 5% acetonitrile and 95% 0.10 M sodium phosphate buffer pH 6.1. Retention time was 5.8 minutes. AU: Absorbance units (A) myometrium spiked with no desacetylcefotaxime. (B) tissue extract with 5.0 µg/g desacetylcefotaxime added, and (C) tissue extract with 23 µg/g of desacetylcefotaxime added. Arrows indicate desacetylcefotaxime peak.

in tissue. As long as this temperature is maintained, little desacetylcefotaxime is formed and the addition of PHMB is not necessary. Enzymes capable of deacetylation such as those found here in myometrium tissue may also be present in lysed blood (4). The serum and tissue concentrations of cefotaxime and desacetylcefotaxime levels are shown in Table 1. The mean serum concen-

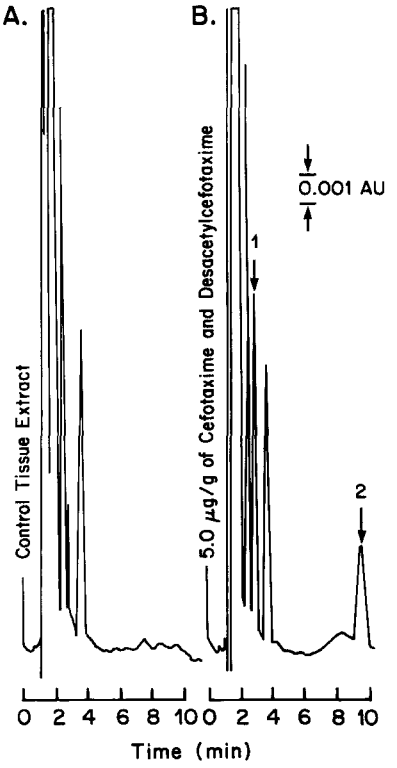

Figure 3. Chromatograms of desacetylcefotaxime and cefotaxime as extracted from myometrium using Sep-pak C_{18} extraction column. The HPLC mobile phase was 8% acetonitrile and 92% 0.10 M sodium phosphate buffer pH 6.1. Retention time was 3.0 minutes for desacetylcefotaxime and 9.6 minutes for cefotaxime. AU: Absorbance units (A) Control tissue extract and (B) 5 µg/g of desacetylcefotaxime and cefotaxime added to myometrium. (1) desacetylcefotaxime and (2) cefotaxime.

trations of cefotaxime and desacetylcefotaxime were 6.8 ± 4.4 and 3.2 ± 2.0, respectively. The mean tissue concentrations were 6.3 ± 8.9 µg/g and 8.4 ± 10.0 µg/g for cefotaxime and desacetylcefotaxime, respectively. The ratio of serum cefotaxime to serum desacetylcefotaxime was 2.12, and for myometrium the ratio was .75. The ratio of serum cefotaxime and desacetylcefotaxime is

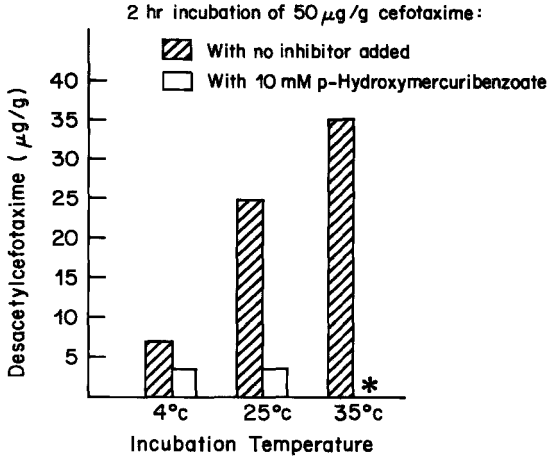

Figure 4. Formation of desacetylcefotaxime from 50 μg/g of cefotaxime added to tissue by the enzymes of myometrium. *PHMB was not added to specimens at 35°C.

TABLE 1

Concentration of cefotaxime and desacetylcefotaxime in serum and myometrium from 15 women.

	Serum μg/ml		Tissue μg/g	
†Time Collected	Cefotaxime	Desacetyl-cefotaxime	Cefotaxime	Desacetyl-cefotaxime
2:15	7.4	3.0	0.8	2.6
2:05	7.2	4.4	5.4	0.7
3:05	2.3	1.5	2.8	2.2
3:00	12.7	2.8	3.9	5.5
1:25	10.8	1.2	31.4	3.3
1:25	12.0	1.5	4.6	4.3
6:10	2.2	2.0	0.7	4.6
2:10	2.4	7.4	1.1	5.1
2:30	2.5	2.4	4.1	3.0
1:57	9.8	7.8	23.1	4.3
2:40	1.8	1.8	1.5	9.4
1:50	9.5	3.5	2.6	18.0
1:25	14.1	2.1	9.2	38.0
2:10	3.9	3.5	2.6	21.4
5:00	3.0	2.7	1.3	3.1
	6.8 ± 4.4	3.2 ± 2.0	6.3 ± 8.9	8.4 ± 10.0

* Standard deviation

† Time in hours and minutes subsequent to a 1-g IM dose

essentially the same as reported previously (8). The ratio of cefotaxime to desacetylcefotaxime in myometrium is also very similar to the ratio reported in human bile (6). Since there is reported antibacterial synergy of cefotaxime and desacetylcefotaxime, and the tissue ratio of these antibiotics is about one part cefotaxime to at least one part desacetylcefotaxime, we suggest that in vitro antimicrobial synergy between cefotaxime and desacetylcefotaxime studies be done at a minimum of a 1:1 ratio. It will be interesting to determine if these ratios are similar in other tissues.

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HPLC STUDY OF SOME BIOLOGICALLY ACTIVE QUINAZOLINES

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ABSTRACT

Experimental log $\overline{k}$ (R_M) values were determined for a series of pharmacologically active quinazolines. Statistically significant linear relationships were found between log k' and log P, log $\overline{k}$ and Hansch's parameter. The retention indices (RI) of these compounds were measured, and a statistically significant linear relationship was also found between log P and RI. The resolution factors and selectivity were determined for some pairs of the tested compounds. The relationship between the structure and Δ log $\overline{k}$ was interpreted. The best linear relationship was found at 60% methanol and 40% water for C_{18} and C_8 columns.

INTRODUCTION

High-performance liquid chromatography (HPLC) has proved to be a valuable technique for the pharmaceutical chemist. It plays important roles in pharmaceutical analysis and in drug research and development. As far as the application of HPLC for research purposes is concerned, study of the relationship between retention

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and other physical and chromatographic parameters has received the main emphasis (1,2). Experiments have been carried out to verify the existence of a linear correlation between the HPLC capacity factor and the chemical structure or structure-coherent physical properties for a given type of potencial drug compund (3-5). The main purpose of the present study is to seek such relationships between HPLC data (log $\bar{k}$) and log P and π parameter for newly synthetized quinazoline derivatives (Table 1).

The selectivity and efficiency of some chromatographic systems (with stationary-phase C_8 and C_{18} columns) have also been studied.

EXPERIMENTAL

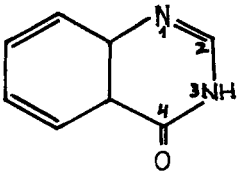
Materials: All the model substances were synthetized in our laboratory (6): Their identification and quality control were achieved by melting point determination and chromatography.

All chemicals and solvents were of analytical grade (Merck) and were used without further purification.

Apparatus, chromatography: The HPLC apparatus was a LIQUOCHROM Model 2010 (LABOR MIM, Budapest, Hungary). A variable-wavelength detector was used, and the column effluent was monitored at different wavelengths between 270 and 330 nm.

Table 1

Structure of model substances



No.	C_2	C_3
1	H	H
2	CH_3	H
3	H	CH_3
4	CH_3	CH_3
5	CH_3	C_2H_5
6	CH_3	C_3H_7
7	CH_3	C_4H_9
8	C_2H_5	CH_3
9	C_2H_5	C_2H_5
10	C_2H_5	C_3H_7
11	C_2H_5	C_4H_9

The reversed-phase C_{18} and C_8 columns were 250 mm x 4.6 mm, prepacked with materials with a particle size of 5 um (Beckman).

25 ul of sample solutions (0.1 mg/ml in methanol) was injected. Mobile phase: methanol-water mixtures containing 80%, 70%, 60% methanol.

The flow rate was 0.7 m l/min.

All experiments were run at room temperature i.e. 25 °C.

Equations used

a: For retention indices (7):

$$I = 100 \frac{\log k_D - \log k_N}{\log k_{N+1} - \log k_N} + 100\,N \qquad \ldots 1$$

where

k_D = the capacity factor of the drug;

k_N = the capacity factor for 2-ketoalkane eluting just before the test compound;

k_{N+1} = the capacity factor for 2-ketoalkane eluting just after the test compound.

b: For Hansch's parameter:

$$\pi = \log P_X - \log P_H \qquad \ldots 2$$

π = Hansch's parameter;

P_X = the partition coefficient of a substututed compound;

Table 2

The log k and log P values of qzinazoline compounds

No	C_{18} column			C_8 column			log P
	Methanol-water %			Methanol-water %			
	80:20	70:30	60:40	80:20	70:30	60:40	
1	-0.553	-0.398	-0.2518	-0.7533	-0.5314	-0.3853	0.9121
2	-0.5229	-0.2518	-0.1427	-0.8325	-0.3853	-0.2527	1.1008
3	-0.3873	-0.237	-0.1249	-0.7533	-0.4175	-0.2762	0.924
4	-0.319	-0.1612	-0.032	-0.6863	-0.2762	-0.1512	1.1115
5	-0.2366	-0.066	0.155	-0.4900	-0.1512	0.0595	1.5759
6.	-0.066	-0.127	0.384	-0.3273	-0.0843	0.2678	2.0648
7.	0.0531	0.323	0.6395	-0.1512	0.2009	0.5216	2.5722
8.	-0.1427	0.0354	0.2380	-0.45229	-0.05435	0.1119	1.6369
9.	-0.04095	0.2069	0.439	-0,2762	0.1119	0.3318	2.1361
10.	0.111	0.373	0.668	-0.1512	0.2609	0.5548	2.5560
11.	0.2695	0.5961	0.925	0.0483	0.4597	0.7907	3.0206

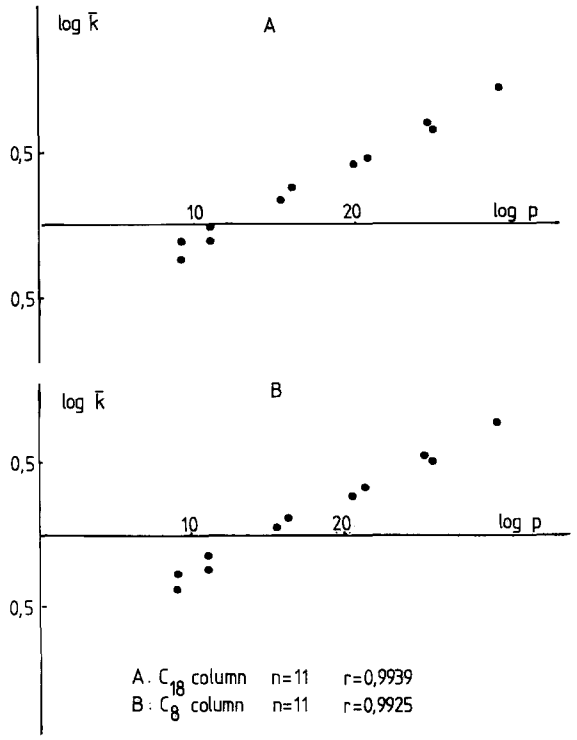


Figure 1. Correlation between log k values and log P values.

P_H = the partition coefficient of the corresponding unsubstituted compound.

RESULTS AND DISCUSSION

The log $\bar{k}$ values of the 11 compounds are shown in Table 2. The differences in $\bar{k}$ values due to the different hydrophobicities of the stationary phases may be observed. A good linear relationship was found between

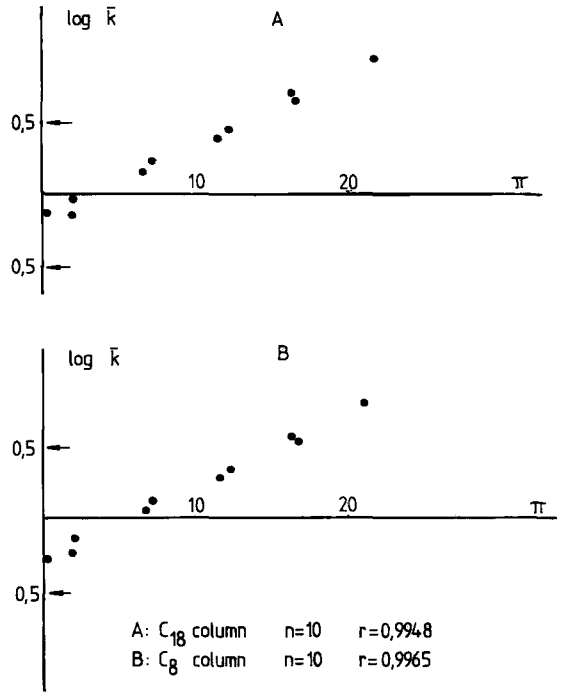


Figure 2. Correlation between log k values and Hansch's π parameter.

log $\bar{k}$ (at 60% methanol and 40% water) and log P (see Fig. 1); analogous correlations were obtained for C_{18} and C_8 columns (Table 2 and Fig. 1). This also holds for Hansch's parameter (π) calculated according to Eq. (2) (see Fig. 2).

The effect of hydrophobic properties can be seen in Table 3 where R_s is reported for 16 pairs of test compounds. The selectivity is shown in Fig. 3.

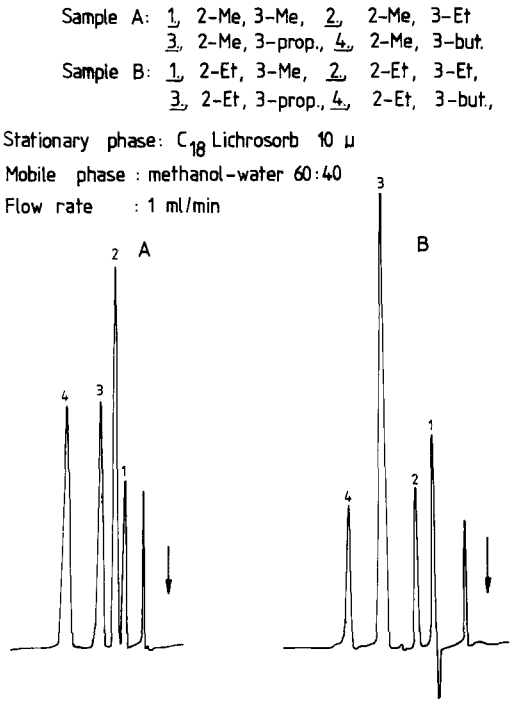


Figure 3. Separation of quinazoline homologues.

It may be seen that the separation is much better for a C_8 than for a C_{18} column. Table 3 also gives the selectivity (relative retention): the results were nearly the same for the C_{18} column and the C_8 column. The effect of hydrophobic properties can also be seen in Table 4, where N and H were calculated for our tested compounds. The results again indicate the higher efficiency of the C_8 column. For the same test compounds,

Table 3

Resolution- and selectivity factors of some pair of compounds

Mixture	C_{18} column		C_8 column	
	R_s	selectivity α	R_s	selectivity α
1/ 2	0.67	0.77	0.93	0.74
1/ 3	0.83	0.75	1.15	0.78
2/ 3	0.128	0.96	2.04	0.95
2/ 4	0.85	0.77	0.97	0.79
2/ 5	2.48	0.50	3.75	0.49
2/ 6	5.02	0.30	7.58	0.30
2/ 7	9.13	0.17	12.05	0.17
4/ 9	5.43	0.34	6.13	0.33
8/ 9	2.9	0.63	3.45	0.60
8/10	6.98	0.37	9.07	0.36
8/11	12.3	0.21	17.37	0.21
7/11	6.55	0.52	8.88	0.54
6/10	4.75	0.52	7.56	0.52
5/ 9	3.8	0.52	4.45	0.53
8/ 4	2.67	0.53	2.8	0.55
10/11	5.93	0.55	8.28	0.58

Table 4

The efficiency data of C_8 and C_{18} columns of quinazoline compounds

No.	C_{18}		C_8	
	N	H	N	H
1	1058	0,236	1888	0,132
2	936	0,267	1984	0,126
3	1077	0,232	1445	0,173
4	1002	0,249	1612	0,155
5	1313	0,190	3869	0,065
6	1471	0,169	5791	0,043
7	2325	0,107	4788	0,05
8	1688	0,148	2334	0,107
9	2162	0,115	2997	0,08
10	2687	0,093	5851	0,04
11	3304	0,075	10068	0,025

Table 5

Retention index data of quinazoline compounds in four different systems

No.	RI			
	C_{18}		C_8	
	70%	60%	70%	60%
1	382	391	346	355
2	461	433	457	444
3	469	440	445	436
4	506	476	457	477
5	553	552	565	557
6	646	642	647	636
7	737	759	732	727
8	605	588	614	577
9	682	687	691	659
10	760	741	759	712
11	859	833	855	823

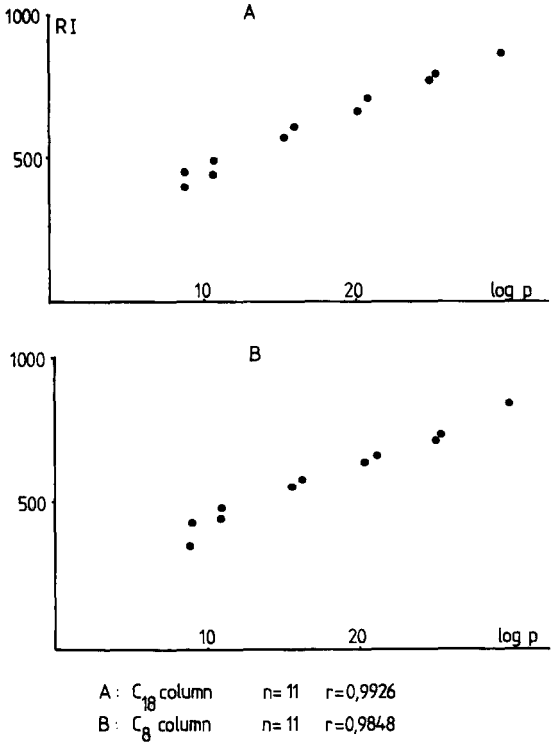


Figure 4. Correlation between RI values and log P values.

retention index (RI) was calculated via Eq. 1 (see Table 5). To some extent there are similarities between the RI values obtained at 60% and 70% methanol for the C_{18} and C_8 columns. The RI data obtained at 60% methanol on both C_{18} columns. The RI data obtained at 60% methanol on both C_{18} and C_8 columns were correlated with log P (see Fig. 4). The results show that HPLC-RI can be used in the prediction of log P and also in other fields of SAR research.

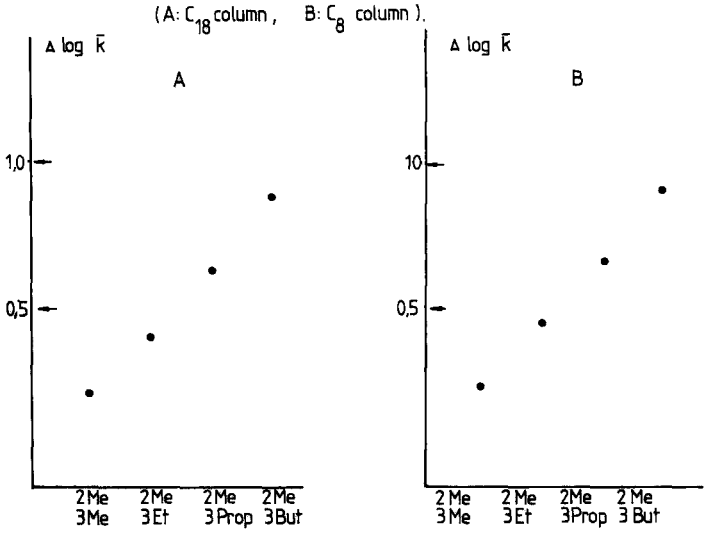


Figure 5. Relationship between log k and structure of some tested compounds.

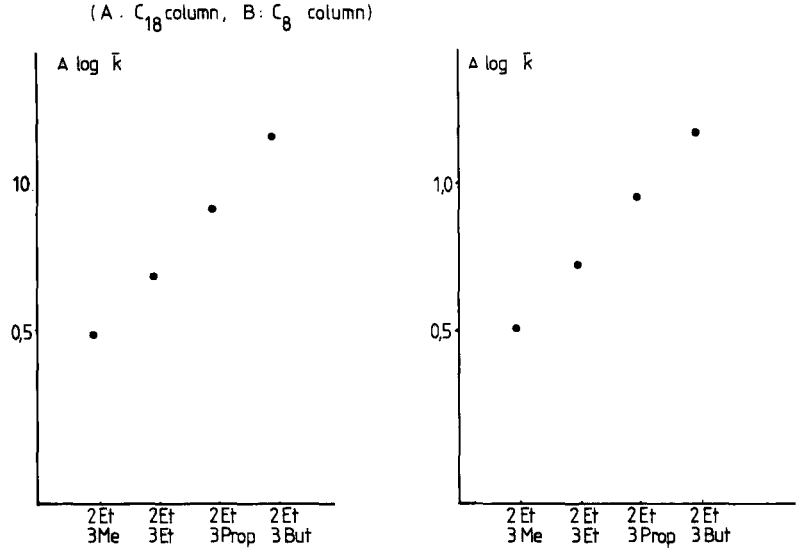


Figure 6. Relationship between log k and structure of some tested compounds.

Figures 5 and 6 illustrate the linearity between log $\overline{k}$ and the carbon atom number at positions C_2 and C_3 for the same compounds using C_{18} and C_8 columns. The average values for log k are: log $k_{C_2-CH_3}$: 0.22 (C_8), 0.23 (C_{18}), log $k_{N_3-CH_3}$: 0.27 (C_8), 0.28 (C_{18}).

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LC NEWS

HPLC COLUMNS AND ACCESSORIES catalog features normal and reverse phase pacings including fluorinated, adsorption, ion exchange, and molecular size exclusion packings. ES Industries, Inc., JLC/84/12, 8 S. Maple Ave., Marlton, NJ, 08053, UsA.

CHROMATOGRAPHY REFERENCE GUIDE contains many clear illustrations and sample chromatograms to help one select the right product for specific separation needs. It includes easy-to-read charts. Whatman, Inc., JLC/84/12, 9 Bridewell Place, Clifton, NJ, 07014, USA.

BIOCHEMISTRY AND BIOLOGY LAB AUTOMATION USING ROBOTICS includes binding assays, e.g., RIA, EIA, ELISA and EMIT; kinetic and endpoint enzyme assays; and chemical assays, e.g., protein assays and analytical chemical techniques including spectroscopy and chromatography. Zymark, Inc., JLC/84/12, Zymark Center, Hopkinton, MA, 01748, USA.

HPLC COLUMN KITS contain selections of columns to solve methods development problems. They are available in 100mm, 150mm, and 250mm lengths and are made with polished 4.6mm i.d. tubing and inverse compression fittings. Shandon Southern Instruments, JLC/84/12, 515 Broad Street, Drawer 43, Sewickley, PA, 15143, USA.

REVERSE PHASE COLUMN FOR HPLC contains five micron spherical c18 bonded silica particles, endcapped, and packed into a precision polished tube. Burdick & Jackson Laboratories, JLC/84/12, 1953 S. Harvey Street, Muskegon, MI, 49442, USA.

MICRODISPLACEMENT PUMP/HPLC GRADIENT PROGRAMMING SYSTEM features flow range from .01 ml to 10 ml per minute with choice of 19 gradient forms, hold/run/reset, gradient duration up to 999 minutes. Applied Chromatography Systems, Inc., JLC/84/12, Suite 125, 315 S. Allen Street, State College, PA, 16801, 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/84/12, 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/84/12, 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/84/12, P. O. Box 117, Rockford, IL, 61105, 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/84/12, 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/84/12, York Street, Cambridge CB1 2PX, UK.

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/84/12, P.O.Box 8731, Woodcliffe Lake, NJ, 07675, 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/84/12, 5680 Goshen Springs Road, Norcross, GA, 30093, USA.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(12), 2509-2512 (1984)

LC CALENDAR

1984

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.

OCToBER 28 - NOVEMBER 2: 98th Annual AOAC International Meeting - Centennial Meeting, Shoreham Hotel, Washington, DC. Contact: Margaret Ridgell, AOAC, 1111 N. 19th Street, Suite 210, Arlington, VA, 22209, 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 e Street, 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 8 - 11: 10th Annual AOAC Spring Training Worshop, Sheraton Hotel, Dallas, Texas. Contact: M. V. Gibson, USFDA, 332 Bryan, Dallas, TX, 75204, 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. qinstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

APRIL 29 - MAY 2: Symposium on Analytical Metods in Forensic Chemistry & Toxicology, Miami, Florida; in conjunction with 189th

ACS Nat'l Meeting. Contact: Dr. M. H. Ho, Dept. of Chem., University of Alabama, Birmingham, AL, 35294, USA.

MAY 13 - 15: Infant Formula Conference, Sheraton Hotel, Virginia Beach, VA. Contact: Dr. James Tanner, USFDA-Hff-266, 200 C Street, SW, Washington, DC, 20204, 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.

OCTOBER 27 - 30: 99th Annual AOAC Meeting, Shoreham Hotel, Washington, DC. Contact:Margaret Ridgell, AOAC, 1111 N. 19th Street, Suite 210, Arlington, VA, 22209, 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.

OCTOBER 12 - 16: 100th Annual AOAC International Meeting, Shoreham Hotel, Washington, DC. Contact: Margaret Ridgell, AOAC, 1111 N. 19th Street, Suite 210, Arlington, VA, 22209, USA.

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.

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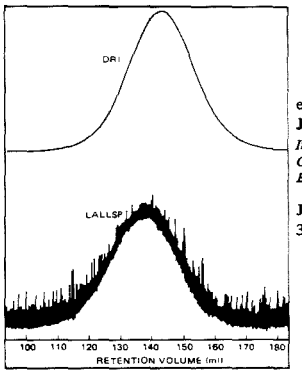

edited by
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January, 1984
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