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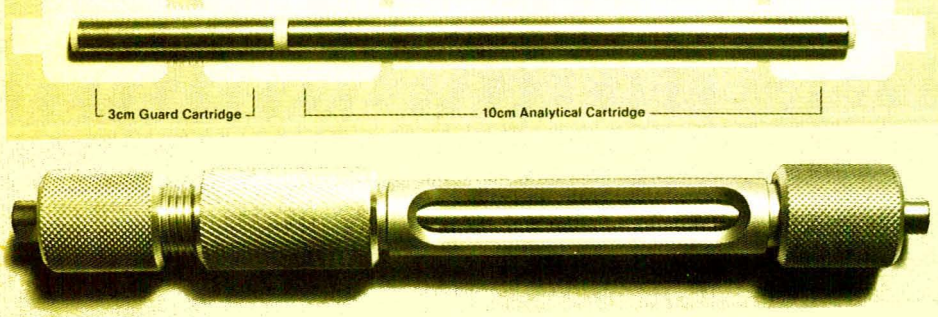

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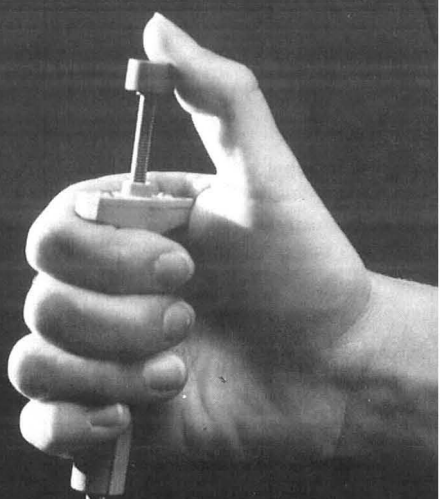

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CHARACTERIZATION OF INORGANIC LONG-CHAIN POLYPHOSPHATE BY A SEPHADEX G-100 COLUMN COMBINED WITH AN AUTOANALYZER DETECTOR

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ABSTRACT

A Sephadex G-100 column with an AutoAnalyzer detector was used in order to characterize a long-chain polyphosphate mixture. This technique enabled us to obtain the molecular weight distribution profile of the mixture within 2 h. A linear relationship between distribution coefficient values and the logarithms of the average chain lengths was obtained.

INTRODUCTION

Sodium phosphate glasses consist mainly of a mixture of long-chain polyphosphate molecules which are known to form complex ions with alkaline earth and other metal ions. They have found widespread industrial applications such as the softening of water (1). In order to characterize the molecular weight distribution of chain phosphates in sodium phosphate glasses, solubility fractionation (2) combined with chain length determination by pH titration (3) has been employed. However, this method is too tedious and cautions should always be taken to prevent hydrolysis of these polyphosphate molecules. A rapid and automatic procedure has been desired.

Ion-exchange chromatography combined with an AutoAnalyzer detector (4-6) developed in our laboratory has been applied to the

analysis of relatively short-chain polyphosphates. Gel chromatography on a cross-linked dextran gel, Sephadex (7,8) has been successfully applied to characterize inorganic polyphosphates. In this work, a Sephadex G-100 column combined with an AutoAnalyzer detector was applied to the routine analysis of the mixture of long-chain polyphosphates. This system has been proved to provide a molecular weight distribution profile of the mixture with a good reproducibility within 2 h.

EXPERIMENTAL

Preparation of sodium phosphate glasses (1)

Appropriate quantities of anhydrous mono- and disodium orthophosphate were mixed and heated in platinum dish at 1000°C. After the melts were heated for 2 h at 1000°C, they were quenched by pouring on a slab and quickly pressing another copper slab on top. By this procedure sodium phosphate glasses with various average chain lengths could be obtained (Table 1).

Determination of average chain length (3)

To a portion of the sample polyphosphate solution, hydrochloric acid was added to lower the pH of the solution to ca. 3. Then 0.1 M carbonate free sodium hydroxide solution was added and the pH of the solution was recorded. This titration was carried out automatically with a KEM Potentiometric Automatic Titrator Recorder model ATR-107 (Kyotodenshi Kogyo Ltd., Japan). From this titration, the amount of end phosphates, N_e, was calculated. The total amount of phosphate monomers, N_t, was determined colorimetrically using Mo(V)-Mo(VI) reagent (9,10). The average chain length, $\bar{n}$, was calculated according to eqn.(1).

$$\bar{n} = 2N_t/N_e \qquad (1)$$

Solubility fractionation with acetone (2)

14 g of phosphate glass (Sample 10 in Table 1) was dissolved in 140 ml of water, then 14 ml acetone was added. To this original

TABLE 1.

Data for the Sodium Phosphate Glasses

Sample	1	2	3	4	5	6	7	8	9	10
$(\bar{n})_{nom.}$*	4.7	6.4	9.2	13.7	22.2	28.7	40.0	50.4	55.8	75.8

* Not corrected for cyclic phosphates.

solution an appropriate volume of acetone was added to cause it appear cloudy. This suspension was stirred for ca. 10 min and then centrifuged at ca. 2000 rpm for 15 min. An oily substance could be found below the solution which had become clear. After decantation the oily substance was dissolved into water. Seven fractions were taken and the amount of acetone used for each was gradually increased as fractionation proceeded. Data for the fractionation experiments are tabulated in Table 2.

Gel chromatography with AutoAnalyzer as a detector

Sephadex G-100 column (15x300 mm) was used. Eluent contained 0.1 M sodium chloride, 0.01 M sodium acetate, and 5×10^{-4} M acetic acid (pH 6). Sample solutions contained sodium chloride and buffer agent of the same concentrations as the eluent and sample phosphates of the concentration of ca. 10^{-3} M (as phosphate monomer). A 0.5 ml portion of the sample solution was applied to the column with a loop injector. The elution flow rate was kept to be 0.52 ml/min because the higher flow rate caused the shrinkage of the gel bed.

A portion of an effluent from the column was introduced to the AutoAnalyzer detector (Technicon). The details of the experimental conditions of the AutoAnalyzer detector have been described previously (5). For about 18 min the sample had to remain in the reaction coil to permit the hydrolysis of polyphosphate and a color reaction of the resultant orthophosphate with a molybdenum reagent.

TABLE 2.

Data for the Acetone Fractionation

Fraction	1	2	3	4	5	6	7*
Volume of acetone added (ml)	6	8	8	8	16	32	100
$\bar{n}$	-**	127	101	85	50	36	-

* Solid precipitate which may consist of cyclic phosphates was obtained.

** Since the quantity of the sample obtained was too small for titration, $\bar{n}$ could not be determined.

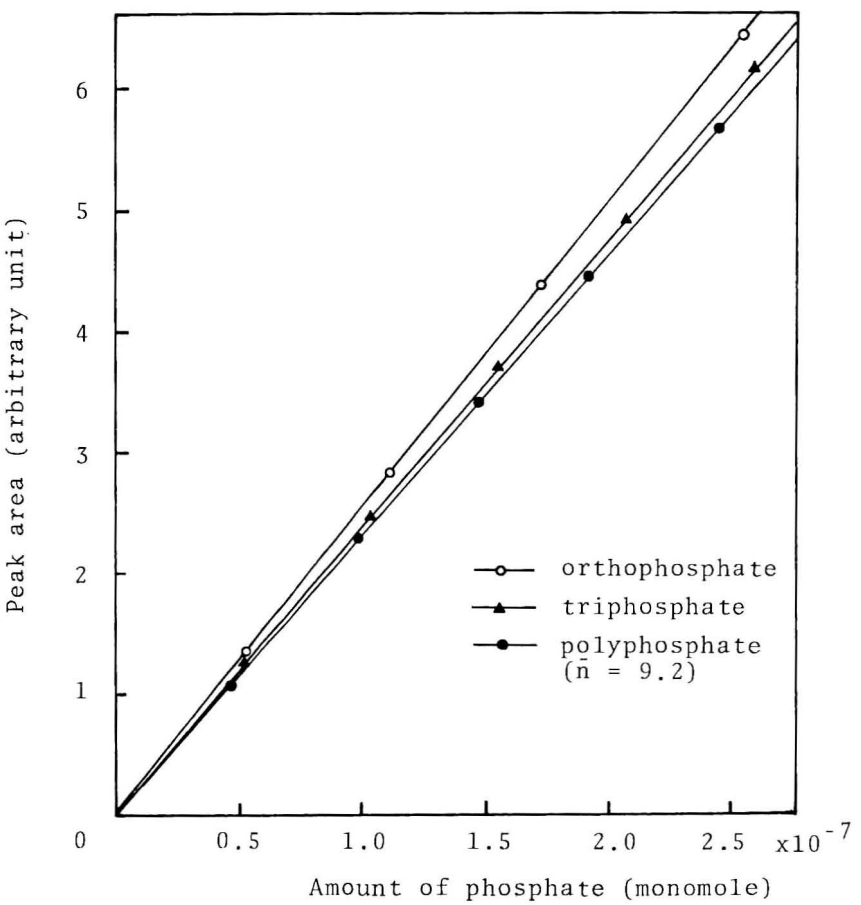

FIGURE 1.

Peak area vs. the amount of phosphate plots for ortho- and polyphosphates.

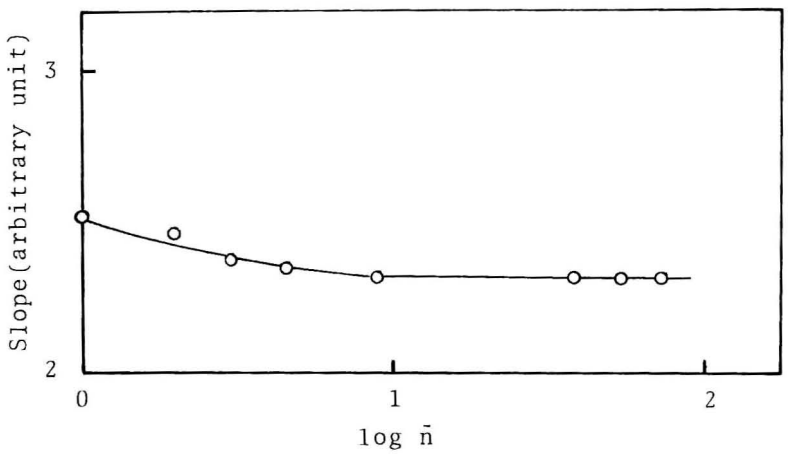

FIGURE 2.

Effect of degrees of polymerization of polyphosphates on the slope of the calibration curve.

A good linearity was obtained for the amount of polyphosphate and the peak area for samples of various degrees of polymerization (Fig. 1). Tough the sample was mixed with sulfuric acid and heated at 98°C, polyphosphates could not completely be hydrolyzed. The slope of the calibration curve was plotted against the chain length of the polyphosphate sample (Fig. 2). Since the slope did not change when the chain length is more than 10, no correction was made for the chromatograms obtained in this work.

RESULTS AND DISCUSSION

In the previous work (11-13), it has been expected that an eluting agent greatly affects the elution behavior of long-chain polyphosphates. In this work, therefore, 0.1 M sodium chloride solution (pH 6) was used throughout. In Fig. 3, the elution profiles are shown for various sodium phosphate glass samples. It can be seen that as the value of $(\bar{n})_{nom}$ increased the resulting peak position shifted forward, and that these glasses have a wide-

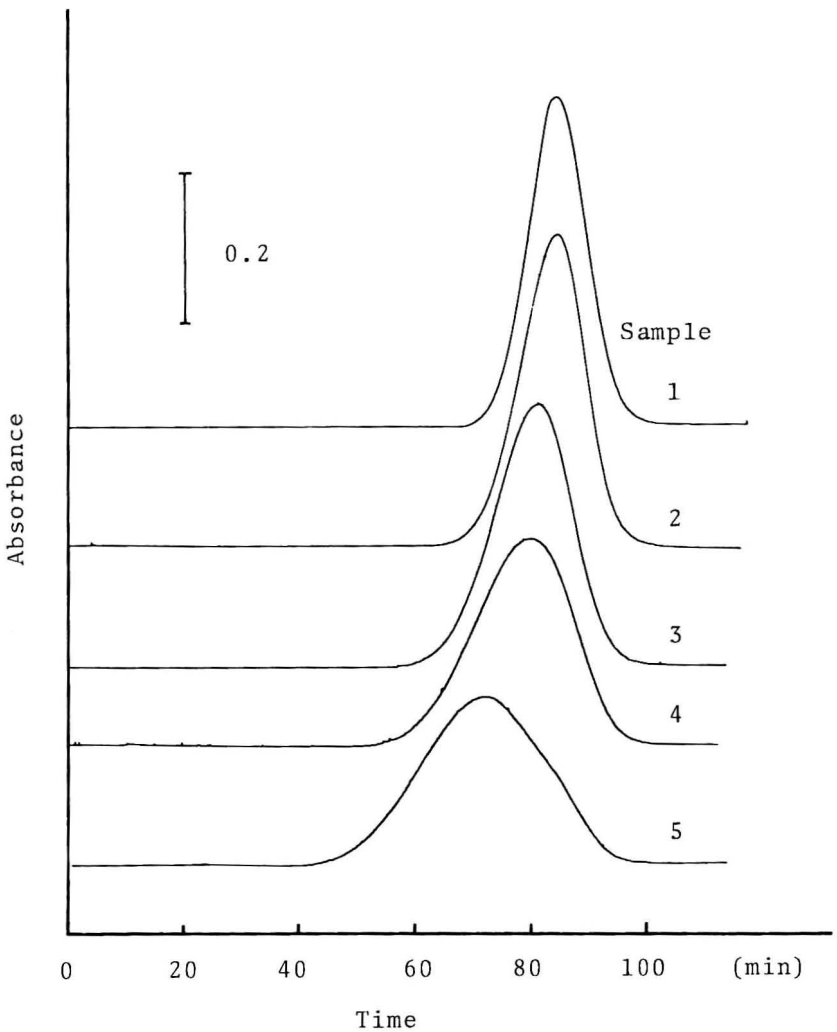

FIGURE 3-1.

Elution profiles of sodium phosphate glasses. Sample numbers are the same as those in Table 1.

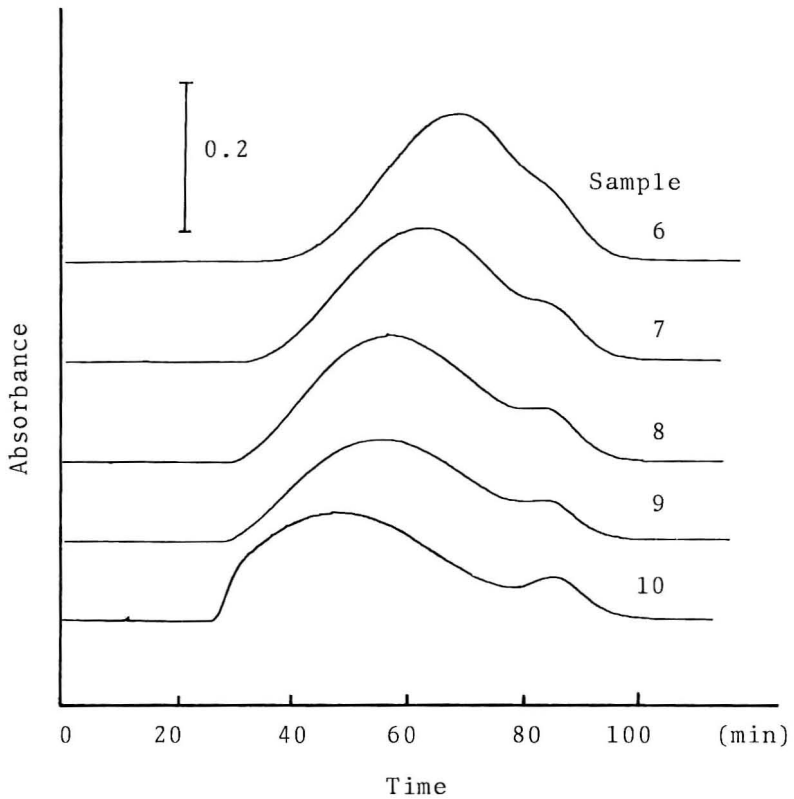

FIGURE 3-2.

Elution profiles of sodium phosphate glasses. Sample numbers are the same as those in Table 1.

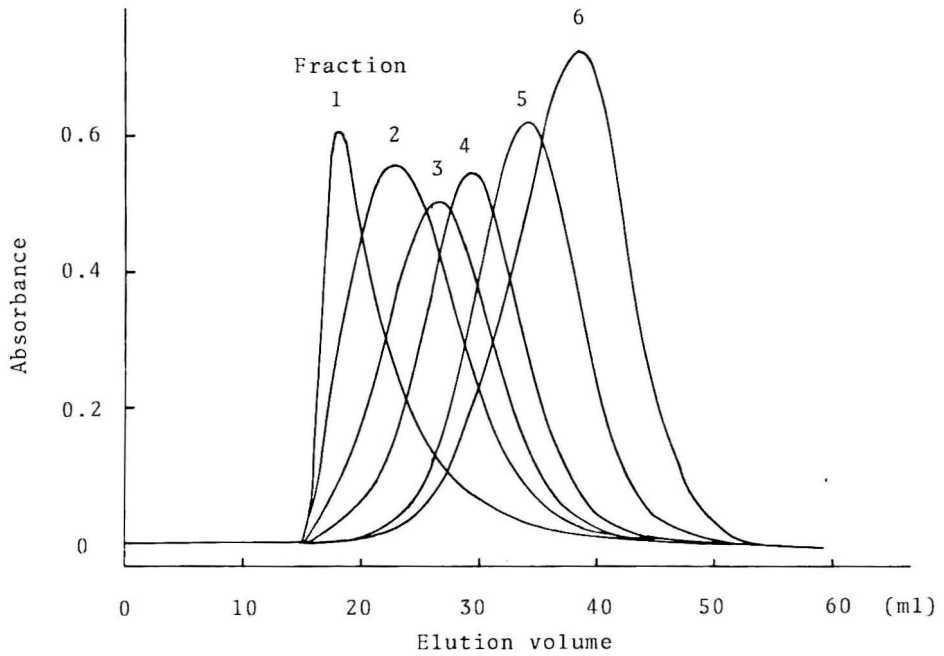

FIGURE 4.

Elution profiles of the samples obtained by acetone fractionation. Fraction numbers are the same as those in Table 2.

spread molecular weight distribution. Small peak whose retention time is approximately 85 min can be observed. These peaks correspond to cyclic phosphates, mainly trimeta- and tetrametaphosphate (2).

In Fig. 4, the elution profiles of the long-chain polyphosphate samples obtained by acetone fractionation are shown. It is evident that the sample prepared by the acetone fractionation shows much narrower dispersion than the original phosphate glass (Sample 10 in Fig. 3). Cyclic phosphates were completely removed off for fractions 1-6. Elution volumes, V_e, for these samples were deter-

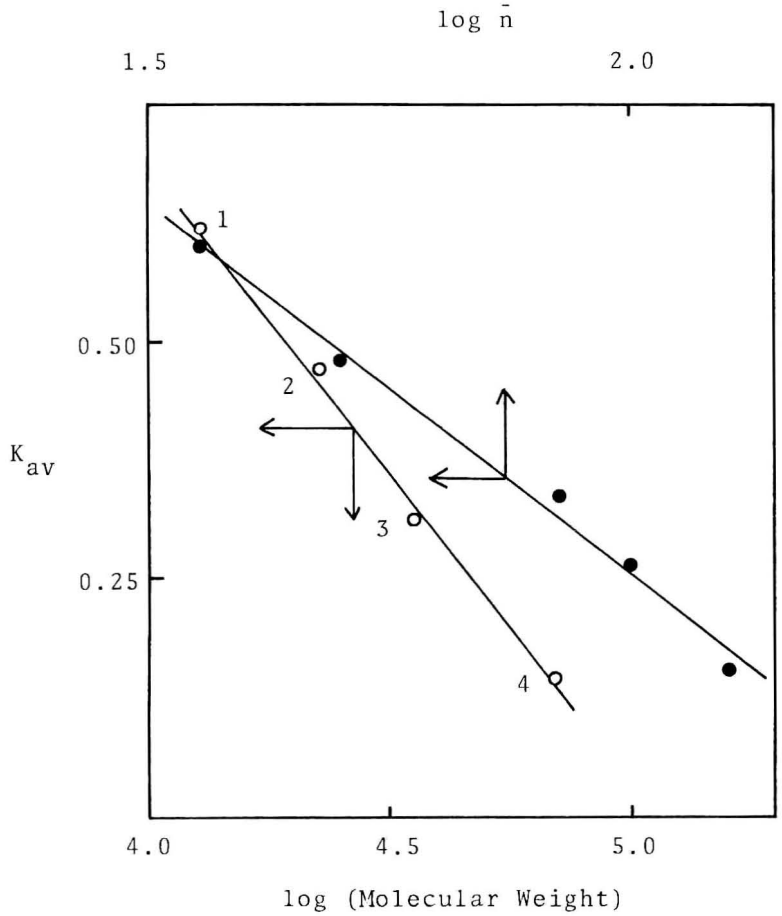

FIGURE 5.

Plots of distribution coefficient (K_{av}) vs. log $\bar{n}$ or log (Molecular Weight) for linear phosphates or proteins.
V_0 was determined by Blue Dextran 2000. 20 mg of protein sample was dissolved into 10 ml of the eluent. A 0.5 ml portion of the sample solution was applied to the column.
1) Cytochrome C, 2) α-Chymotrypsin, 3) Pepsin, 4) Bovine serum albumin

mined from the peak positions of these samples. Distribution coefficients, K_{av}, were calculated according to eqn.(2).

$$K_{av} = (V_e - V_0)/(V_t - V_0) \quad (2)$$

where V_0 is the void volume and V_t is the total bed volume.

In order to calibrate this column, several proteins were chromatographed with 0.1 M sodium chloride solution (pH 6). A UV detector (228 nm) was used in order to monitor these proteins. As has been pointed out by Whitaker (14), a linear relationship was obtained between the K_{av} values and the logarithms of the molecular weights of the proteins (Fig. 5). For long-chain polyphosphate samples, the values of K_{av} were also plotted against the logarithms of the average chain lengths (Fig. 5). As with the proteins system, a linear relationship was obtained. The relationship may be useful for calculating the distribution of the chain length of sodium phosphate glasses.

As can be seen from Figs. 3 and 4, a rough evaluation of the molecular weight distribution profile of the long-chain polyphosphate mixture could be made within 2 h by the gel chromatography-AutoAnalyzer system. This system is quite useful for the characterization of long-chain polyphosphates.

ACKNOWLEDGMENTS

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OPTIMIZATION OF PEAK SEPARATION AND BROADENING IN AQUEOUS GEL PERMEATION CHROMATOGRAPHY (GPC) - POLY (SODIUM STYRENE SULFONATES)

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ABSTRACT

An experimental study of the gel permeation chromatography of a series of poly (sodium styrene sulfonates) in aqueous media has been undertaken. The objective of this study was to develop a set of operating conditions that would maximize peak separation and minimize peak broadening. Using CPG-10 porous glass packing, it has been found optimal to work at pH near 6 and ionic strength near 0.05. Under those conditions, ion exclusion is eliminated and the optimum balance between resolution and reproducibility is obtained. Addition of a few ppm neutral surfactant to the mobile phase prevents adsorption to the packing surface, and improves resolution.

INTRODUCTION

The objective of the present paper, as for previous papers in the series (1,2), was to develop an experimentally optimal system for aqueous GPC analysis of a particular class of polymer - in this case, a series of anionic poly(sodium styrene sulfonates). Phenomena that would interfere with a purely size exclusion mechanism in such analyses are adsorption to the surface of the packing material, and ion exclusion due to charge repulsion. The latter is expected to be a particular problem with polyelectrolytes. In order to approach a system in which peak separation and

broadening are optimal, such phenomena must first of all be eliminated or minimized. At that point, it is possible to choose a column system with a combination of pore sizes that gives adequate peak separation without excessive peak broadening. As for previous work, we have chosen CPG-10 porous glass as a suitable packing material since it is available in a wide range of narrowly distributed pore dimensions and particle size. It is thus possible to choose a pore size combination to match the size range of the polymers of interest.

EXPERIMENTAL

The polymers investigated were a series of six sulfonated polystyrenes in the MW range 31,000 to 1.06×10^6 obtained from Pressure Chemical Company (Pittsburgh). These are prepared from polystyrenes of low polydispersity by sulfonation to a level of approximately one sulfonate group per styrene residue. Since sulfonic acids are strong acids, these materials would be expected to show polyanion behaviour over a wide range of pH. Molecular weight data supplied by Pressure Chemical Company are shown in Table I.

TABLE 1
Poly (sodium styrene sulfonate) Standards - M.W. Data of Supplier (Pressure Chemical Co.)

Designation	$\bar{M}_w$	$\bar{M}_w/\bar{M}_n$
Na PSS 31	31,000	$\sim$ 1.10
Na PSS 88	88,000	$\sim$ 1.10
Na PSS 195	195,000	$\sim$ 1.10
Na PSS 354	354,000	$\sim$ 1.10
Na PSS 690	690,000	$\sim$ 1.10
Na PSS 1060	1,060,000	$\sim$ 1.10

Chromatography was carried out using a Waters Associates Model ALC/GPC 300, with a differential refractometer. Polymer concentrations were in the range 0.01 to 0.1 wt % and mobile phase flowrates varied between 4 and 8 ml min^{-1}. Columns were dry-packed with CPG-10 glass packing (Electronucleonics, Fairfield, NJ). Experimental conditions such as flowrate, additive concentrations and pH are specified below where the results are presented. The neutral surfactant Tergitol NPX (Union Carbide), an alkylphenoxypolyoxyethylene, was used in some experiments in an attempt to minimize adsorption.

Intrinsic viscosities were measured at room temperature in various aqueous media over a range of pH and ionic strength using Cannon Ubbelohde dilution viscometers. Viscosity data were obtained to give information on the dimensions of polymer molecules in solution under differing conditions of pH, ionic strength, and concentration of various additives. Such information, is of assistance in the interpretation of GPC behaviour.

RESULTS AND DISCUSSION

Effect of pH and Ionic Strength on Polymer Dimensions in Aqueous Solution

A summary of intrinsic viscosity data in the pH range above 3.0 is shown in Fig. 1. For a given polymer, it is seen that [η] decreases sharply with increasing ionic strength. This would be expected on the basis of charge screening by the added electrolyte. For the lower MW polymers, the coil dimensions appear reasonably constant at ionic strength above 0.1, whereas [η] does not level off for higher MW polymers. These data suggest that SEC behaviour, regardless of any other effects, would be expected to depend on ionic strength at values less than about 0.3. Viscosity data showing the effect of ionic strength and presented in the form of the Mark-Houwink equation are shown in Fig. 2. The effect of charge is again clear from these data. Thus, while the slopes

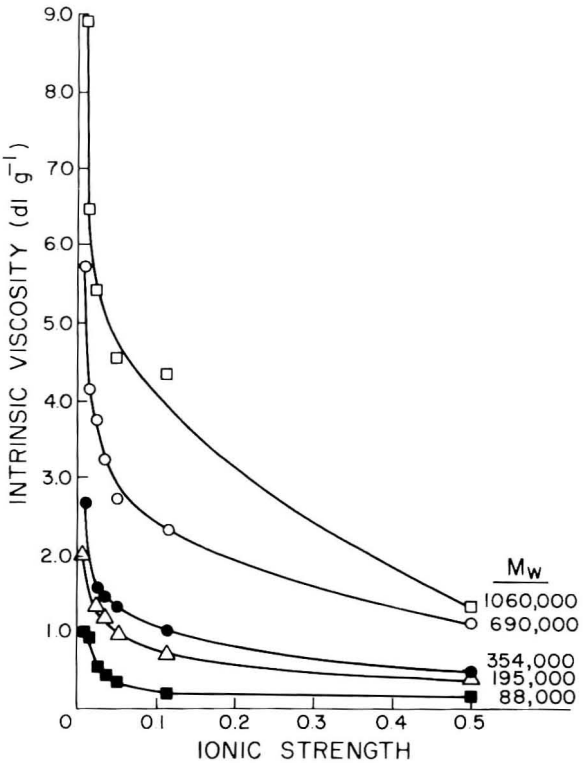

Figure 1 Effect of ionic strength on intrinsic viscosity of poly (sodium styrene sulfonates) (pH > 3, 25°C).

of the plots are similar, the intercepts vary, increasing with decreasing ionic strength and increasing pH. These data again suggest that the polymer coil dimensions depend on solution conditions insofar as these influence the charge characteristics of the chains. The data also suggest that GPC should be conducted at moderate ionic strength for these polymers. Thus it is clear that at ionic strength greater than 0.1 the coils of the lower MW polymers are essentially "collapsed", a situation that would result in poor GPC resolution. At ionic strength less than 0.1 the coil size is larger but variable so that peak positions would

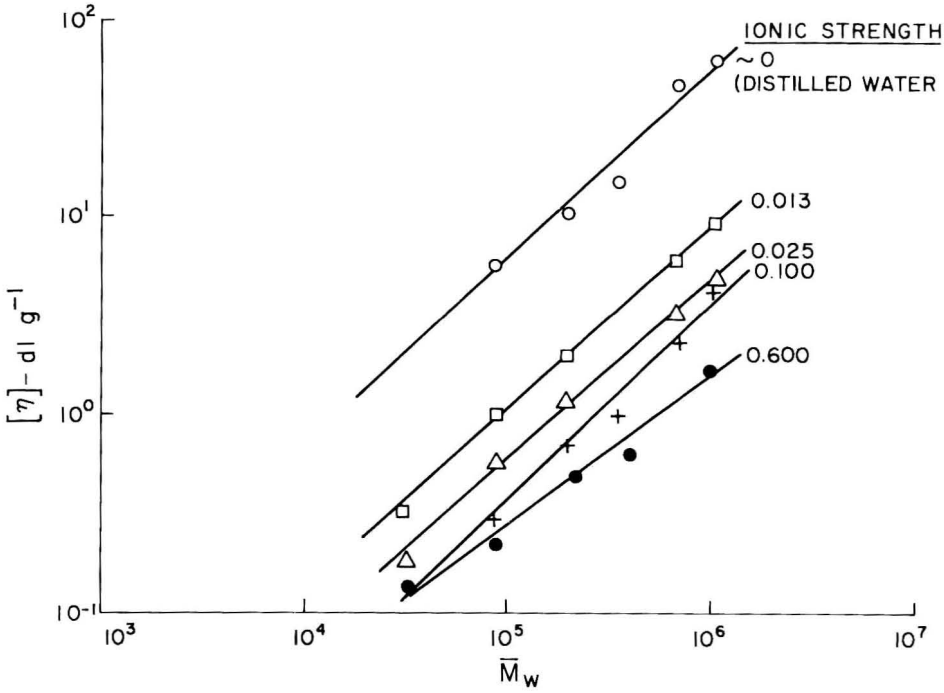

Figure 2 Intrinsic viscosity versus MW for poly (sodium styrene sulfonates), 25°C.

be highly sensitive to ionic strength and reproducibility might suffer. Clearly a balance between these two effects must be sought.

Effect of Ionic Strength on Elution Volumes

To determine the effect of various experimental variables (such as ionic strength and pH and presence or absence of surfactants) on chromatographic performance, molecular weight calibration curves were obtained under different conditions, using a single column. This column had dimensions of 4' x 3/8" and contained CPG-10 packing of 2000 Å pore size.

The effect of neutral salts is demonstrated in Fig. 3. In these experiments, the pH varied between 7.5 and 5.3, a range within which the ionization of sulfonic acid groups would be expected to be complete. Such pH variation should thus not affect the charge characteristics of the polymers. As can be seen from Fig. 3, all the polymers are essentially excluded from the pores in distilled water. Such exclusion could conceivably be a result of size in the case of the high MW polymers, but since even the

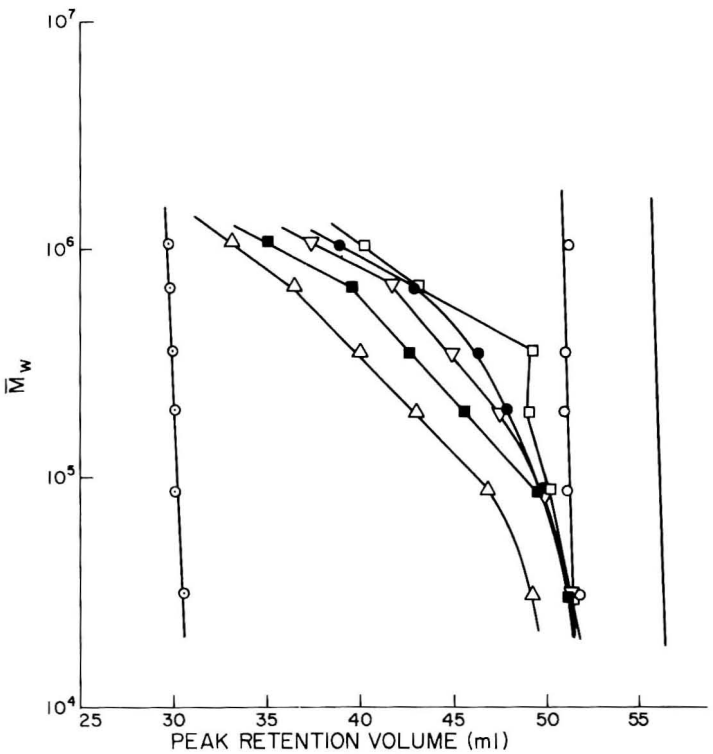

Figure 3 Effect of ionic strength on MW calibration curves of poly (sodium styrene sulfonates) on CPG-10. Column length 4 ft, pore size 2000 Å, flowrate 4.2 ml min^{-1}. Ionic strength: ⊙, zero; △, 0.01; ■, 0.025; ▽, 0.05; ●, 0.10; □, 0.30; ○, 0.5; ——, NaCl peak position.

lowest MW polymers are excluded, it is more likely that the effect derives from charge repulsion. The addition of small amounts of neutral salts (e.g. 0.01 M NaCl) allows pore permeation even for the high MW polymers and while such addition has been shown to decrease the polymer dimensions, it is again most probable that the salts are acting by a charge screening mechanism. At high ionic strength (0.5 M NaCl), all the polymers show total permeation of the column. Such behaviour cannot be explained by a size effect alone since, as the viscosity data show, the size of the polymers does not change significantly. It seems likely that these effects can be explained by the onset of adsorption at higher ionic strength. It can also be seen from Fig. 3 that the apparent total permeated volume suggested by the elution volumes of the polymers is less than that observed for NaCl. This effect was also noted for polyacrylamides and dextrans (1,2) and may reflect the existence of a bimodal pore size distribution in the column packing.

Effect of pH on SEC Elution Volumes

The pH of the mobile phase would be expected to have an effect on GPC behaviour through its influence on polymer and/or surface charge. Glass is generally believed to acquire a negative charge at intermediate and higher pH via dissociation of silanol groups. Hair and Hertl (3) have reported a pK value of the order of about 7. The sulfonic acid bearing polymers would be expected to be charged at pH above about 3. Thus, ion exclusion effects would be expected only at high pH, whereas at low pH, charge effects should be absent. The curves in Fig. 4 are in accord with these general precepts. At a pH of 1.6, there is almost total permeation of the packing by polymers of MW less than 200,000. Some adsorption is probably occurring under these conditions. Again, there appears to be a gap between the total volume available to polymers compared to small species such as NaCl. The particular pore size represented by this packing is clearly providing the best resolution for polymers in the MW range 10^5 to 10^6.

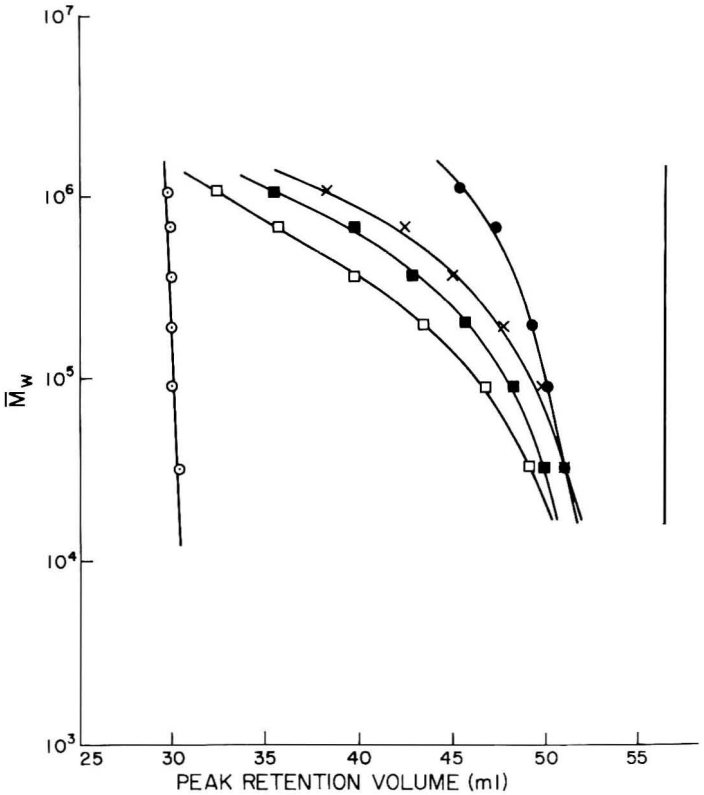

Figure 4 Effect of pH on MW calibration curves of poly (sodium styrene sulfonates) on CPG-10. Column length 4 ft, pore size, 2000 Å, flowrate 4.2 ml min^{-1}. pH values: ⊙, 7.0; □, 5.19; ■, 2.28; x, 1.98, ●, 1.62; ——, NaCℓ peak position.

Effect of Neutral Surfactant on SEC Elution Volumes

Use of neutral surfactants in SEC has frequently been advocated as a means of minimizing adsorption (4), presumably by a pre-emptive adsorption of the surfactant itself. It was found in the present work that adsorption occurred at high ionic strength, especially on the smaller pore size packing. In line with previous investigations in this series it was also found that such

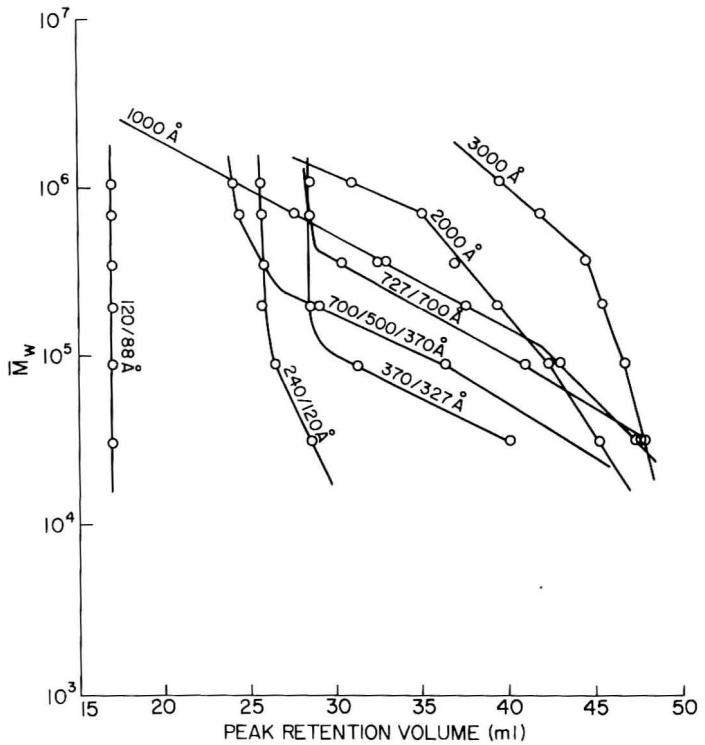

Figure 5 MW calibration curves of poly (sodium styrene sulfonates) for single columns of various pore sizes, pH 6.6, ionic strength 0.053, flowrate 4.2 ml min^{-1}, column lengths, 4 ft.

adsorption could be eliminated by addition of a few ppm of the nonionic surfactant Tergitol to the mobile phase (data not shown). It should be pointed out that even in the presence of Tergitol at high ionic strength (> 0.2) the polystyrene sulfonates were strongly adsorbed. It seems possible in this connection that the solubility of the polymers may be reduced in these media, perhaps due to a salting-out tendency.

In terms of the mobile phase, the optimal conditions appear to be at moderate ionic strength (~ 0.1) and pH in the

neighbourhood of 6 in presence of ~ 40 ppm Tergitol. Under these conditions, both charge and adsorption effects are minimal.

Choice of Range of Pore Size (Column Combination)

The above discussion relating to mobile phase optimization was based on experiments with a single column packing with a pore size of 2000 Å. As was pointed out, good resolution of polymers above 10^5 MW is obtained but the lower MW materials are not well resolved. Clearly, if good resolution is to be obtained over a wider range of MW, it is necessary to use a multi-column system containing appropriate pore sizes.

Fig. 5 shows calibration curves using a number of single column systems covering a wide range of pore sizes. The mobile phase was selected based on the results presented above and had the following composition: ionic strength 0.055, 0.04 g/l Tergitol, pH 6.6. This mobile phase, in addition to eliminating charge and adsorption, was also found to resolve the lowest MW polymer (31,000) well from the added salt, Na_2SO_4 (which is also present in the as-received polymers).

Based on these data, several column combinations were studied in order to provide an optimal SEC system for these polymers. The MW averages and polydispersities calculated by integration of the experimental chromatograms using the appropriate calibration curve were used as the criteria of optimal conditions. Table 2 shows a selection of data for several column combinations. In terms of minimum polydispersity over the whole range of MW's, the system employing a 28.7 foot column containing pore sizes 2000, 1000, 729, 700, 500, 370 and 327 Å, operating at pH 6.6 and ionic strength 0.053 gave the best results (system #1). The calibration curve for this system is shown in Fig. 6. At higher ionic strength the resolution (data not shown) was considerably reduced.

The results of this study may be compared to those of Spatorico and Beyer (5) who also investigated polystyrene sulfonates as standards for aqueous SEC. These workers prepared their own standards by sulfonation of narrow polystyrene fractions

TABLE 2

Measured MW Averages and Polydispersities of NaPSS Standards using Selected GPC Systems

System #	1			2			3			4		
Calibration Curve	$M(v) = 0.339 \times 10^{9} \exp(-0.151v)$			$M(v) = 0.636 \times 10^{9} \exp(-0.17v)$			$M(v) = 0.347 \times 10^{10} \exp(0.283v)$			$M(v) = 0.131 \times 10^{10} \exp(-0.227v)$		
Column Length (ft)	28.7			28.7			21.4			20.8		
pH	6.6			6.8			3.4			3.25		
Ionic Strength	0.053			0.031			0.025			0.013		
	$\bar{M}_w$ $\times 10^{-3}$	$\bar{M}_n$ $\times 10^{-3}$	$\bar{M}_w/\bar{M}_n$	$\bar{M}_w$ $\times 10^{-3}$	$\bar{M}_n$ $\times 10^{-3}$	$\bar{M}_w/\bar{M}_n$	$\bar{M}_w$ $\times 10^{-3}$	$\bar{M}_n$ $\times 10^{-3}$	$\bar{M}_w/\bar{M}_n$	$\bar{M}_w$ $\times 10^{-3}$	$\bar{M}_n$ $\times 10^{-3}$	$\bar{M}_w/\bar{M}_n$
	32.60	27.90	1.17	33.40	28.90	1.16	31.80	26.90	1.18	52.10	47.00	1.11
	81.50	73.30	1.13	81.30	70.80	1.15	89.00	73.20	1.22	97.40	86.80	1.12
	194.00	171.00	1.14	204.00	178.00	1.14	231.00	195.00	1.19	199.00	172.00	1.16
	366.00	327.00	1.12	392.00	342.00	1.14	383.00	317.00	1.21	360.00	299.00	1.21
	606.00	470.00	1.29	605.00	453.00	1.34	602.00	447.00	1.35	582.00	389.00	1.50
	965.00	812.00	1.19	889.00	698.00	1.27	819.00	561.00	1.46	904.00	572.00	1.58

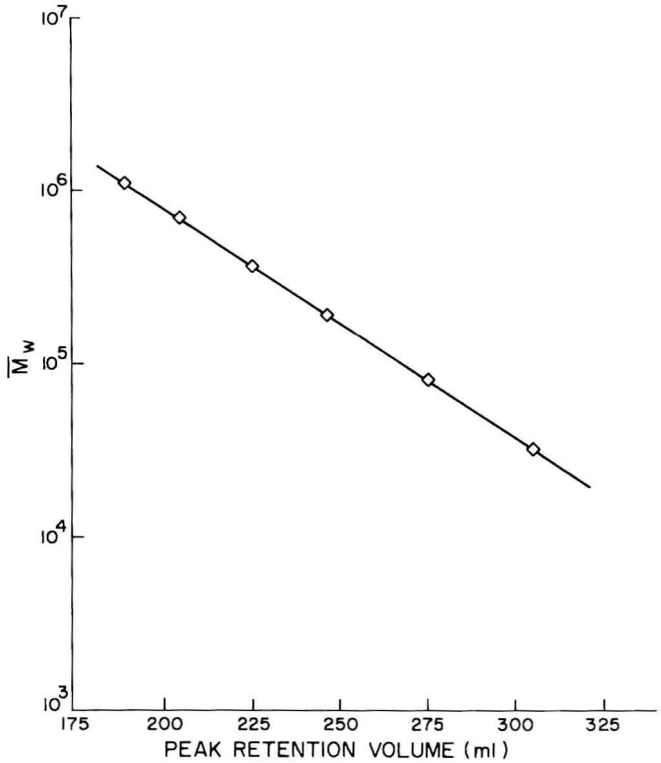

Figure 6 MW calibration curve for poly (sodium styrene sulfonates) for optimally chosen mobile phase and pore size combination. Column length 28.7 ft; pore diameters: 2000, 1000, 729, 700, 500, 370 and 327 Å; pH 6.6; ionic strength 0.053; flowrate 4.2 ml min^{-1}.

in the MW range 8,000 to 800,000. They obtained resolution comparable to that reported here using CPG-10 packing (pore sizes 1250 to 75 Å) with a mobile phase of 0.2 M Na_2SO_4 (ionic strength = 0.6). The pH, although not specified was presumably in the neutral range. A comparison of the two studies can be made in terms of the most obvious differences between them, namely the level of ionic strength and the analysis time, both of which are greater in the work of Spatorico and Beyer.

As indicated previously our intrinsic viscosity results suggest that a compromise is required between loss of resolution at high ionic strength and poor reproducibility at lower ionic strength (but still high enough to eliminate ion exclusion). More specifically our experience suggests that the mobile phase used by Spatorico and Beyer, with an ionic strength of 0.6, would give less than optimal resolution since the polymer coils appear to be effectively collapsed. It is possible that this has been compensated by inclusion of very small pore sizes in the system (5). In this connection it should be noted (Fig. 6) that using our mobile phase conditions of moderate ionic strength the polymers were completely excluded from 120/88 Å pores and were not well resolved at pore sizes less than 300Å.

With regard to analysis time this too is presumably influenced by the use of very small pores. The column lengths in the two studies are about the same while the column diameters are respectively 0.375" (this study) and 0.17" (5), leading to a system volume ratio of about 5. Since the respective flowrates were 4.2 (this study) and 0.5 ml min^{-1} (5) the analysis time is less in the present study by a factor of about 2.

No attempt has been made in the present series of studies (1,2) to test the applicability of the universal calibration concept. Indeed we have found that each water soluble polymer system requires a somewhat different mobile phase for optimal resolution so that the universal calibration approach simply is not appropriate. Studies touching on this point are those of Spatorico and Beyer (5) and Rollings et al (6). Spatorico and Beyer found that for four different polymer types (including NaPSS) a common universal-type calibration curve was obtained in 0.2 M Na_2SO_4. Furthermore, the curve was independent of Na_2SO_4 concentration in the range 0.2 to 0.8 M. The recent work of Rollings et al (6) showed that a common universal-type calibration curve could not be obtained for any of a variety of mobile phases. However when an excluded volume parameter was included in the hydrodynamic volume term a common curve was obtained. In agree-

ment with Spatorico and Beyer these authors obtained a common curve only at high ionic strength (0.1 M or greater, NaCl). As has already been pointed out, resolution can be improved for each individual polymer system by careful choice of conditions. Thus although the universal calibration may or may not be applicable for a given set of conditions, it would seem preferable where possible to operate under "customised" optimal conditions for the polymer system under consideration.

ACKNOWLEDGEMENT

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EFFECT OF ALKYL CHAIN LENGTH OF BONDED SILICA PHASES ON SEPARATION, RESOLUTION AND EFFICIENCY IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A comparative study of alkyl bonded phases was carried out under optimum solvent conditions for each phase. Three columns, RP-2, RP-8 and RP-18, were tested for their efficiency and resolving power using three groups of compounds in three binary organic-water mobile phases. The organic solvents were acetonitrile, methanol and tetrahydrofuran which are widely used as solvent modifiers.

The results indicate that each of the three factors, i.e. solvent, solute and bonded alkyl chain length, play an important role, with the solvent being the most significant. When tetrahydrofuran-water was used as the mobile phase, the ratio of THF/H_2O did not vary by much when an RP-2, RP-8 or RP-18 column was used to separate naphthalene from biphenyl, dimethylphthalate from diethylphthalate or anthraquinone from methyl anthraquinone and ethyl anthraquinone. When acetonitrile-water and methanol-water were used the ratio of organic modifier to water changed so as to accomodate the hydrophobic properties of the columns. The efficiency of the columns, expressed as theoretical plates per meter (TPM) was highest when acetonitrile-water was used as the mobile phase. Although there were variations in TPM and resolution from column to column, the three columns gave good separation of the components of the three groups of compounds.

INTRODUCTION

Modification of the adsorbent is an important aspect of liquid chromatography which enables the chromatographer to achieve separations that are not otherwise possible. Reverse-phase (RP) adsorbents for both high performance liquid chromatography (HPLC) and thin-layer chromatography are both good

examples of this. In RP today chemically bonded phases are used in which an organic moiety is bonded to silica. Such moieties may include an alkyl chain varying in length from C_2 to C_{22}, a phenyl or other organic groups (cyano, amine, etc.). Currently, the most popular RP is the C_{18} bonded phase, although other phases are commercially available.

Majors and Hopper (1) and Korpi and Janicki (2) have studied the effect of alkyl chain length using C_6, C_8 and C_{18} packing materials. The results indicated an increase in selectivity and longer retention times with increase in chain length, when the columns were compared using the same mobile phase.

Hemetsberger et al (3) studied the effect of chain length (C_8-C_{22}) of bonded organic phases. They reported that selectivity depended on the chain length of the bonded phase and the molecular structure of the solute. They also found that the utilization of long chain phases made it possible to reduce the water content of the water:methanol mobile phase, which increases efficiency and the loading capacity. Hemetsberger et al (4,5) studied the behavior, and effect of structure, of bonded phases. The effect of ligand structure and geometry on selectivity in RP was also investigated (6). Kikta and Grushka (7) studied the retention behavior on alkyl bonded phases as a function of chain length, surface coverage, solute type, mobile phase composition and temperature. Conditions during the experiments were kept constant. Colin and Guiochon (8) compared some packings for RPHPLC. In this study the alkyl chain lengths were between C_6 and C_{22}. Also, the experimental conditions, and especially the mobile phase composition, were kept the same. No attempt was made to optimize separation by changing the ratios of the binary mobile phase to fit the hydrophobicity of the bonded phase. As a result, the shorter length alkyl chain columns generally gave the worst resolution and efficiency.

The objectives of the present study are (a) to compare the separations achieved on commercially available alkyl bonded phases C_2, C_8 and C_{18}, (referred to as RP-2, RP-8 and RP-18, respectively), and (b) to compare the efficiency of each column under its optimum mobile phase conditions. Three different mobile phases (acetonitrile/water, methanol/water, and tetrahydro-

furan/water) and three groups of solutes [anthraquinone (A), 1-methyl anthraquinone (MA) and 1-ethyl anthraquinone (EA); naphthalene (N) and biphenyl (BP); and dimethylphthalate (MP) and diethylphthalate (EP)] with varying chemical properties were used.

EXPERIMENTAL

Materials: All solvents were glass distilled (Burdick and Jackson). The chemicals were analytical grade (Aldrich Chemical Co.) and used without further purification.

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

The columns were all 250 mm X 4.6 mm pre packed with 10 μm particle size materials (Merck). The reverse phase materials were RP-2, RP-8 and RP-18 10 μl sample solution were injected. Experiments were run at room temperature using a mobile phase flow rate of 1.2 ml/min. Retention times, peak widths at half height($W_{1/2}$), theoretical plates/meter (TPM) and resolution (R) were determined by a 3352A Laboratory Data System (Hewlett-Packard) linked through a Hewlett-Packard 1865 A/D converter to the UV detector output of the liquid chromatograph. The output from the data system was recorded on a 9866A thermal line printer (Hewlett-Packard).

RESULTS AND DISCUSSION

Since RP-2, RP-8 and RP-18 columns have different hydrophobic properties it seemed logical to optimize the mobile phase ratio and composition for each column and solute mixture separately. It was felt that a comparison of the three columns under the same mobile phase conditions may, or may not, be the correct approach depending on the solute tested and the organic modifier used. Figure (1) shows the separation of anthraquinone, 1-methyl anthraquinone and 1-ethyl anthraquinone (referred to as the quinone mixture) using the mobile

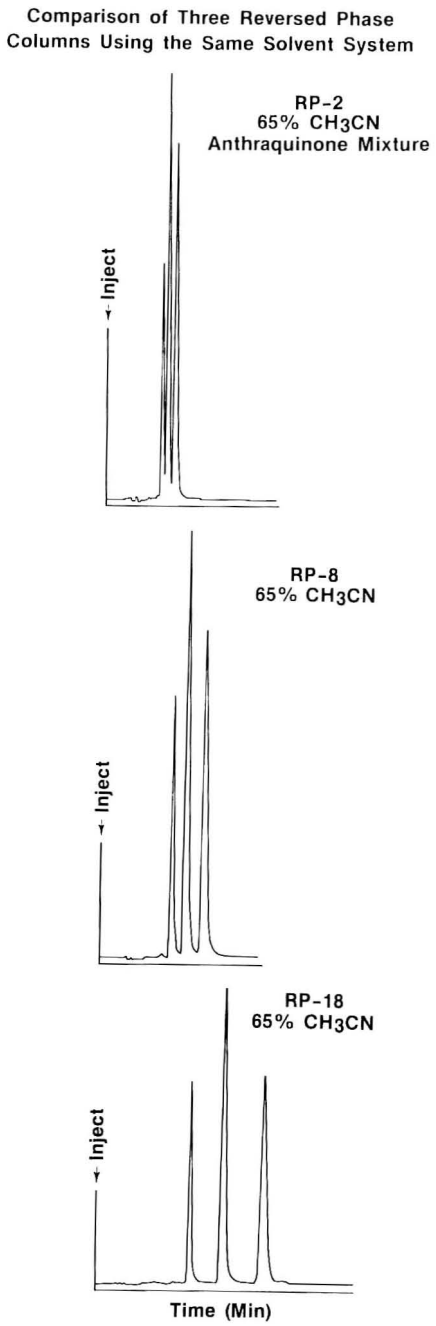
Comparison of Three Reversed Phase
Columns Using the Same Solvent System
RP-2
65% CH3CN
Anthraquinone Mixture
Inject
RP-8
65% CH3CN
Inject
RP-18
65% CH3CN
Inject
Time (Min)

FIGURE 1

phase 65% acetonitrile:water. As expected, the resolution of the quinone mixture on each column is different, because the experimental conditions did not take into consideration the lower retaining power of the shorter chain length columns (RP-2 vs RP-8 vs RP-18). As a result, the best resolution was obtained with the RP-18 column. On the other hand, when the binary mobile phase ratios were altered for each column to accomodate the hydrophobic properties of each, resolution of the quinone mixture was comparable with RP-2, RP-8 and RP-18 (Figure 2). The same was true, when optimum binary solvent mixtures were used for the separation of naphthalene and biphenyl on the three reverse phase columns (Figure 3). An optimum binary mixture is that mobile phase which gives baseline separation in the minimum amount of time. Comparable results were obtained for dimethyl and diethyl phthalates.

Table 1 compares the retention time, width of peak at half height, theoretical plates per meter and resolution of the quinone mixture on RP-2, RP-8 and RP-18 using the solvent system 65% acetonitrile:water. The table clearly shows that the shorter the alkyl chain of the bonded phase the worse the resolution. When the mobile phase ratios were adjusted for each column (Table 2) better resolution of the quinones was obtained, but the RP-18 column still gave the best resolution if the retention times on each column were comparable. Table 2 also shows that when acetonitrile:water was used as the mobile phase the percentage of acetonitrile dropped from 70% (RP-18) to 55% (RP-2) in order to obtain comparable retention times. The same was true when methanol:water was used, Table 3. Also note that the resolution between methyl- and ethyl anthraquinones is better with RP-2 than with the other two columns. When tetrahydrofuran (THF):water was used as the mobile phase, the THF percentage variation for the separation of the quinone mixture using RP-2, RP-8 and RP-18 was only ±4% giving comparable resolution Table 4. The same trend of mobile phase composition was observed for the resolution of the naphthalene and biphenyl mixture using the three solvent systems, Tables 5-7. Again, it was found that the variation in mobile phase composition was smallest when THF: water was used.

Separation of Anthraquinone, 1-Methyl-anthraquinone and 1-Ethylanthraquinone at Optimum Solvent Mixture for Each Column

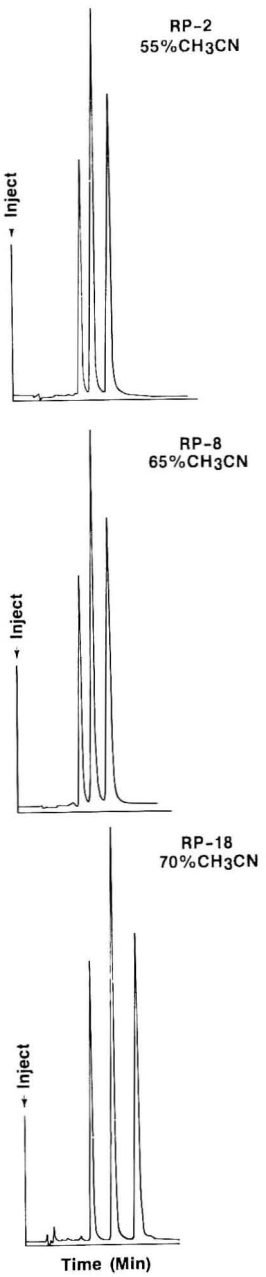

FIGURE 2

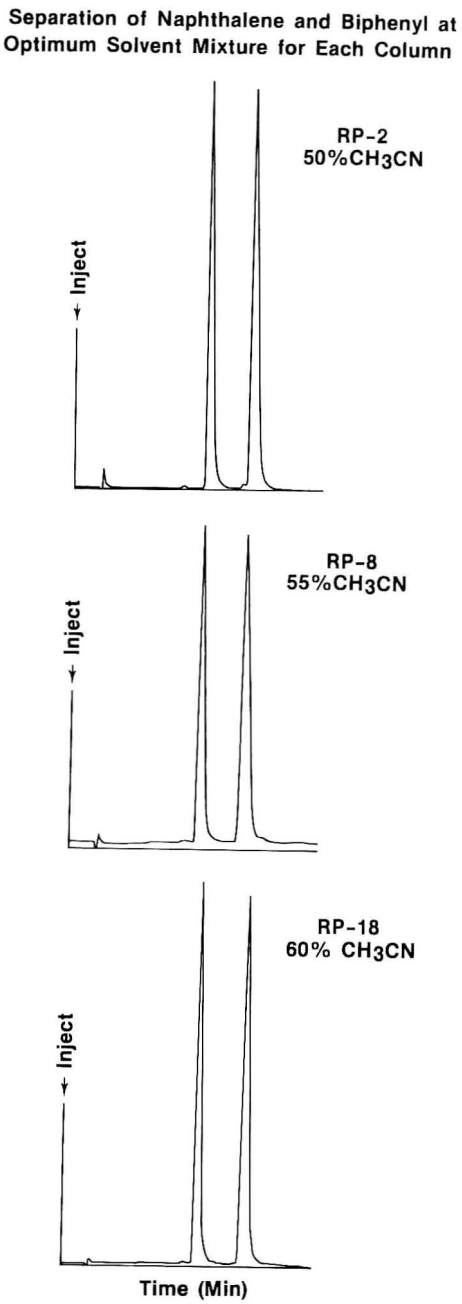
Separation of Naphthalene and Biphenyl at
Optimum Solvent Mixture for Each Column
RP-2
50%CH3CN
Inject
RP-8
55%CH3CN
Inject
RP-18
60% CH3CN
Inject
Time (Min)

FIGURE 3

TABLE 1

COMPARATIVE SEPARATION OF THE QUINONE MIXTURE ON RP-2, RP-8 AND RP-18 USING 65% ACETONITRILE/WATER

COLUMN	MOBILE PHASE	COMPOUND	R_t	$W_{1/2}$	TPM	R
RP-2	65% CH_3CN	AQ	4.61	0.206	11112	-
		MAQ	5.08	0.225	11249	2.19
		EAQ	5.72	0.245	12021	2.70
RP-8	65% CH_3CN	AQ	5.98	0.282	9926	-
		MAQ	7.14	0.332	10209	3.76
		EAQ	8.65	0.397	10529	4.15
RP-18	65% CH_3CN	AQ	7.18	0.292	15756	-
		MAQ	10.65	0.386	16786	8.34
		EAQ	14.08	0.502	17402	7.71

TABLE 2

COMPARATIVE SEPARATION OF THE QUINONE MIXTURE ON RP-2, RP-8 AND RP-18 USING ACETONITRILE/WATER

COLUMN	MOBILE PHASE	COMPOUND	R_t	$W_{1/2}$	TPM	R
RP-2	55% CH_3CN	AQ	6.47	0.266	13063	-
		MAQ	7.56	0.304	13630	3.79
		EAQ	9.14	0.355	14639	4.79
RP-8	65% CH_3CN	AQ	5.98	0.282	9926	-
		MAQ	7.14	0.332	10209	3.76
		EAQ	8.65	0.397	10529	4.15
RP-18	70% CH_3CN	AQ	6.36	0.233	16417	-
		MAQ	8.41	0.293	18170	7.76
		EAQ	10.71	0.377	17896	6.85

TABLE 3

COMPARATIVE SEPARATION OF THE QUINONE MIXTURE ON RP-2, RP-8 AND RP-18 USING METHANOL/WATER

COLUMN	MOBILE PHASE	COMPOUND	R_t	$W_{1/2}$	TPM	R
RP-2	60% MeOH	AQ	8.22	0.401	9273	-
		MAQ	10.77	0.502	10176	5.64
		EAQ	14.56	0.650	11121	6.57
RP-8	64% MeOH	AQ	8.76	0.427	9335	-
		MAQ	11.81	0.555	10013	6.21
		EAQ	15.18	0.692	10675	5.4
RP-18	76% MeOH	AQ	8.42	0.385	10571	-
		MAQ	11.94	0.532	11162	7.66
		EAQ	15.24	0.674	11305	5.45

TABLE 4

COMPARATIVE SEPARATION OF THE QUINONE MIXTURE ON RP-2, RP-8 AND RP-18 USING TETRAHYDROFURAN/WATER

COLUMN	MOBILE PHASE	COMPOUND	R_t	$W_{1/2}$	TPM	R
RP-2	44% THF	AQ	10.36	0.458	11481	-
		MAQ	12.38	0.532	11985	4.1
		EAQ	16.36	0.659	13632	6.66
RP-8	40% THF	AQ	11.15	0.554	9303	-
		MAQ	13.86	0.654	9966	4.51
		EAQ	19.06	0.877	10460	6.78
RP-18	44% THF	AQ	9.64	0.436	10815	-
		MAQ	12.10	0.534	11346	5.05
		EAQ	16.23	0.698	11978	6.70

TABLE 5

COMPARATIVE SEPARATION OF NAPHTHALENE AND BIPHENYL ON RP-2, RP-8 AND RP-18 USING ACETONITRILE/WATER

COLUMN	MOBILE PHASE	COMPOUND	R_t	$W_{1/2}$	TPM	R
RP-2	50% CH_3CN	N	10.43	0.374	17364	-
		BP	13.9	0.491	17961	8.07
RP-8	55% CH_3CN	N	10.04	0.387	14866	-
		BP	13.41	0.511	15245	7.49
RP-18	60% CH_3CN	N	10.58	0.349	20307	-
		BP	14.33	0.471	20489	9.14

TABLE 6

COMPARATIVE SEPARATION OF NAPHTHALENE AND BIPHENYL ON RP-2, RP-8 AND RP-18 USING METHANOL/WATER

COLUMN	MOBILE PHASE	COMPOUND	R_t	$W_{1/2}$	TPM	R
RP-2	64% MeOH	N	6.25	0.326	8109	-
		BP	8.21	0.401	9268	5.37
RP-8	75% MeOH	N	6.13	0.315	8403	-
		BP	7.86	0.383	9314	4.93
RP-18	80% MeOH	N	6.43	0.261	13375	-
		BP	8.22	0.329	13795	6.07

TABLE 7

COMPARATIVE SEPARATION OF NAPHTHALENE AND BIPHENYL ON RP-2, RP-8 AND RP-18 USING TETRAHYDROFURAN/WATER

COLUMN	MOBILE PHASE	COMPOUND	R_t	$W_{1/2}$	TPM	R
PR-2	44% THF	N	14.03	0.560	13902	-
		BP	19.32	0.750	14703	8.07
RP-8	44% THF	N	11.17	0.500	11056	-
		BP	14.92	0.661	11289	7.83
RP-18	44% THF	N	13.88	0.567	13180	-
		BP	19.03	0.770	13512	7.69

The separation of dimethyl and diethyl phthalates on RP-2, RP-8 and RP-18 using acetonitrile:water, methanol:water and THF:water was also achieved, Tables 8-10. A comparison of Tables 2-10 indicates that when THF is used as the modifier of the mobile phase there are two results: (a) the percentage of THF is lower than any of the other two organic components of the mobile phase; and (b) the percentage of composition of THF in the mobile phase does not change appreciably (∓4%) from column to column (RP-2, RP-8 or RP-18), or from one sample mixture to the other, quinones, phthalates or naphthalene and biphenyl (see Tables 4, 7 and 10).

It is also interesting to note that, in the case of the quinone mixture, with acetonitrile as the mobile phase, the highest TPM and lowest $W_{1/2}$ are obtained, while methanol and THF give lower TPM and higher $W_{1/2}$ values, Tables 2-4. The same is true in the case of naphthalene and biphenyl; however TPM, $W_{1/2}$ and R values for phthalates when THF was used were comparable for both RP-2 and RP-18, Table 7. Again when the phthalate mixture was analyzed

TABLE 8

COMPARATIVE SEPARATION OF DIMETHYL AND DIETHYLPHTHALATES ON RP-2, RP-8 and RP-18 USING ACETONITRILE/WATER

COLUMN	MOBILE PHASE	COMPOUND	R_t	$W_{1/2}$	TPM	R
RP-2	50% CH_3CN	MP	5.56	0.235	12306	-
		EP	8.08	0.317	14398	9.1
RP-8	55% CH_3CN	MP	4.83	0.218	10885	-
		EP	7.12	0.291	13240	8.96
RP-18	60% CH_3CN	MP	4.17	0.178	12131	-
		EP	6.12	0.239	14424	9.29

TABLE 9

COMPARATIVE SEPARATION OF DIMETHYL AND DIETHYLPHTHALATES ON RP-2, RP-8 AND RP-18 USING METHANOL/WATER

COLUMN	MOBILE PHASE	COMPOUND	R_t	$W_{1/2}$	TPM	R
RP-2	56% MeOH	MP	5.59	0.293	8035	-
		EP	8.73	0.418	9671	8.82
RP-8	56% MeOH	MP	4.82	0.279	6624	-
		EP	7.98	0.402	8727	9.27
RP-18	64% MeOH	MP	5.03	0.253	8739	-
		EP	9.39	0.424	10835	12.84

TABLE 10

COMPARATIVE SEPARATION OF DIMETHYL AND DIETHYLPHTHALATES ON RP-2, RP-8 and RP-18 USING TETRADYDROFURAN/WATER

COLUMN	MOBILE PHASE	COMPOUND	R_t	$W_{1/2}$	TPM	R
RP-2	48% THF	MP	4.64	0.284	5909	-
		EP	6.57	0.346	7985	6.12
RP-8	44% THF	MP	4.43	0.272	5900	-
		EP	6.35	0.338	7800	6.26
RP-18	48% THF	MP	3.82	0.225	6406	-
		EP	5.29	0.276	8094	5.83

Comparison of RP-2 and Silica Columns Using the Same Solvent System

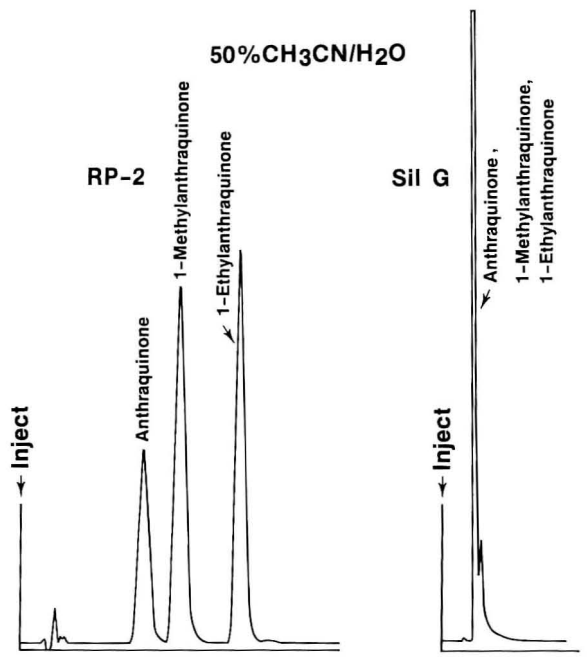

FIGURE 4

using the three columns and the three organic modifiers, the highest values for $W_{1/2}$ and TPM were obtained when acetonitrile was used, Table 8. Note that R, $W_{1/2}$ and TPM values are almost identical using RP-2 or RP-8, Table 8. In the case of THF, Table 10 the retention times are lower and that may account for low TPM values.

To test its reverse-phase properties, an RP-2 column was compared with a silica gel column. In Figure (4) the results obtained on both columns are shown. It is clear that RP-2 gives better resolution under the same experimental conditions. The order of elution of the quinones, phthalates, naphthalene and biphenyl on RP-2 was the same as when RP-8, and RP-18 columns were used. These results agree with those reported by Van Der Wal and Huber (9) for the separation of estrogen conjugates.

CONCLUSION

It is clear from the results that acetonitrile/water gives better TPM values for the compounds tested, and that 40-50% THF/water is an adequate mobile phase for the resolution of the three tested mixtures on either RP-2, RP-8 or RP-18 columns.

It was surprising that the RP-2 column gave such good results. It is also obvious, except in the case of THF, that the comparison of reverse phase columns of various alkyl chain length under the same mobile phase conditions, can result in erroneous chromatographic values, R, $W_{1/2}$ and TPM. In certain cases altering the ratios of the binary mobile phase can give base line separation of a mixture.

The results also show, that TPM is a function of the column, the solvent, and the sample used if other parameters are kept constant.

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TRACE ENRICHMENT ON PRE-COLUMNS IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY. THE ADSORPTION CAPACITY OF A REVERSED PHASE COLUMN

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ABSTRACT

The use of break-through experiments in investigations of the adsorption step in pre-column trace enrichment has been demonstrated.

The adsorption capacity of LiChrosorb RP-8 for phenol, 2,4-dichlorophenoxyacetic acid, ethylbenzene and pentachlorophenol has been determined as a function of the pH, ionic strength and flow-rate of the sample solution and as a function of the concentration of test substance in the sample.

The slope of the break-through curves has been found to show very little variation with the experimental conditions.

INTRODUCTION

During the last five years the trace enrichment technique has received some attention for the analysis of organic micropollutants in water. While the first reports *(e.g.* 1,2) were often limited to showing examples of the use of trace enrichment, a number of recent papers are also concerned with the efficiency of the technique. Determination of the recovery of selected test substances using one fixed sample volume has been much used (3-8). Another approach has been to compare the peak heights

produced by known quantities of test substances, loaded on to the column in increasing sample volumes (8-12). Plots of peak height as a function of sample volume at fixed concentrations of test substance have also been used to determine the maximum permissible sample volume (8,13).

The present paper is concerned mainly with the adsorption step of trace enrichment. The effect of a number of variables on the adsorption capacity of a pre-column is shown using break-through experiments.

MATERIALS AND METHODS

Chemicals

2,4-Dichlorophenoxyacetic acid (Ega, Steinheim) was recrystallized from benzene.

All other chemicals and solvents were of reagent grade quality and used as received.

Apparatus

The liquid chromatograph (see Figure 1) consisted of Waters model 6000A pumps, of which one solvent selection valve was used as valve 1, a Rheodyne model 7120 injection valve (valve 2), a Waters model 440 UV-detector (254 nm) and a Waters model 660 solvent programmer. Break-through curves were recorded using an Omniscribe model 5117-2 (Houston Instruments, Texas) recorder.

Chromatography

The adsorbent used was LiChrosorb RP-8, 10 μm particles (Merck, Darmstadt). It was packed in stainless

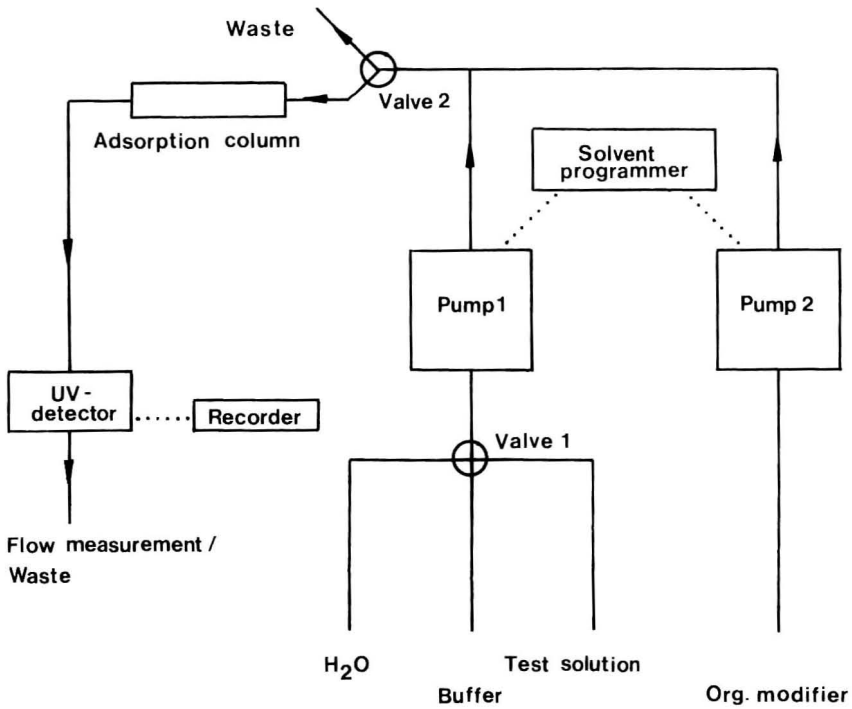

FIGURE 1

Schematic of the apparatus used for the break-through experiments.

steel columns (50 x 4.6 mm i.d.) according to a previously described procedure (14). Fittings were modified Swagelok unions. The efficiency of the columns was 950-1050 plates for phenetole (k' = 7.0) measured with methanol/water (1+1) as the mobile phase.

Procedure for Break-Through Measurements

The configuration of the liquid chromatograph used in the break-through experiments is shown in Figure 1.

Immediately before each break-through experiment the adsorption column was cleaned by running a gradient from 100 % water to 100 % methanol. After cleaning, the gradient was reversed and when 100 % water was reached valve 1 was changed to a buffer of the desired pH and ionic strength. The adsorption column was brought into equilibrium with this buffer at the flow-rate to be used in the break-through experiment after which valve 1 was switched to the test solution while valve 2 was switched to waste. Pump 1 and the tubing before valve 2 was flushed with test solution and the break-through experiment was then started by switching valve 2 back to the adsorption column.

Calculations

An example of a break-through curve is shown in Figure 2.

The area shown cross-hatched in Figure 2 corresponds to the amount of test substance adsorbed by the column, In Ref. 15 this area is integrated and the adsorption capacity calculated therefrom. If, however, the break-through curve is assumed to be symmetrical this procedure may be simplified somewhat. For a symmetrical S-curve the area under the break-through curve before the inflection point, shown by horizontal hatching in Figure 2, is identical to the area above the curve after the inflection point. This means, that the area corresponding to the amount adsorbed can be described by a rectangle, eliminating the need for integration of the S-shaped curve.

The equilibrium adsorption capacity, S (µg/g), may then be calculated from the equation:

$$S = \frac{(V_{50} - V_m)\ c_i}{W} ,$$

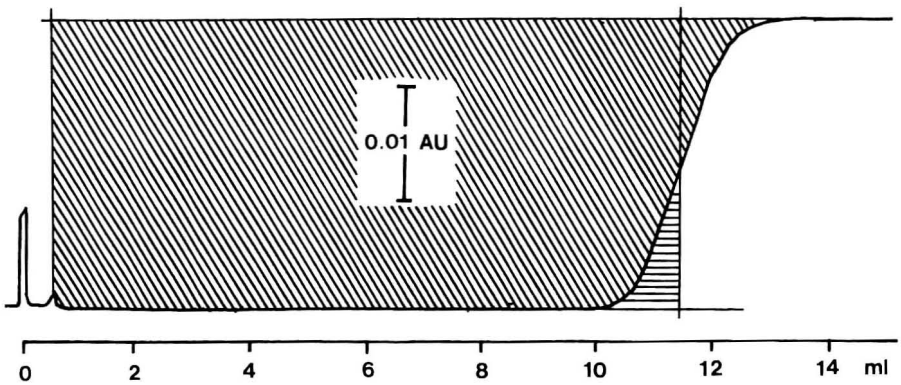

FIGURE 2

Break-through curve for phenol.
Test solution: Phenol 5.5 µg/ml in sodium phosphate buffer pH 8.0, µ 0.1. Flow-rate 5.0 ml/min.

where V_{50} (ml) is the 50 % break-through volume, V_m (ml) the void volume, c_i (µg/ml) the concentration of test substance in the test solution and W (g) the weight of adsorbent in the column.

This equation is similar to equations derived from statistical (16) and physico-chemical (17) considerations.

Another quantity of interest is the slope of the break-through curve. This may be found from mathematical descriptions of break-through curves *(e.g.* 17*)* but in the present investigation a simplified measure of the slope is used:

$$V_{10,rel} = \frac{V_{10}}{V_{50}} ,$$

that is, the 10 % break-through volume is measured relative to the 50 % break-through volume.

RESULTS AND DISCUSSION

Break-through experiments were conducted on LiChrosorb RP-8 for some selected test substances, *viz.* phenol, pentachlorophenol, ethylbenzene and 2,4-dichlorophenoxyacetic acid, at three concentration levels. The effects of flow-rate, pH and ionic strength of the test solution were examined.

Slope of Break-Through Curve

The influence of flow-rate (1-10 ml/min) on the relative 10 % break-through volume for phenol was examined and proved to be non-significant. Also the type of test substance had little effect on the slope, although the relative 10 % break-through volume for phenol (mean: 0.92) was slightly smaller compared to the other compounds (mean: 0.94). Phenol has the smallest absolute break-through volume and the difference observed is therefore not surprising, using evidence from elution liquid chromatography. Here it is known that the efficiency of the system increases with increasing capacity ratio in the beginning of a chromatogram, while the effect levels off at greater capacity ratios.

The age of the adsorption column, defined as the number of experiments performed on the column, proved to have the largest effect on the relative 10 % break-through volume, as shown in Figure 3. Experimental conditions other than the age of the adsorption column have not been kept constant in the figure. This procedure is justified by the fact, that these conditions have very little influence on the slope. But even the effect of adsorption column age is small, and when the column is used for a limited number of experiments, *e.g.* fifteen, the effect is negligible.

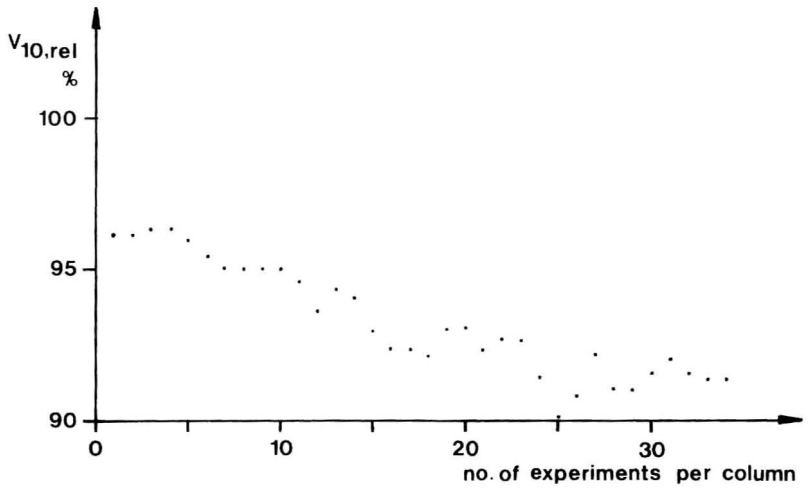

FIGURE 3

Influence of the age of the adsorption column, defined as the number of experiments performed per column, on the relative 10 % break-through volume. Points shown are mean values for seven columns.

The conclusion is, that the slope of the break-through curve under the conditions tested is nearly constant as long as the adsorption columns are not used more than fifteen times.

Adsorption Capacity

The adsorption isotherms for the four test substances at pH 8 are shown in Figure 4.

Isotherms for phenol and ethylbenzene are linear in this concentration range, the isotherm for 2,4-dichlorophenoxyacetic acid is curved, while the isotherm for pentachlorophenol shows a marked curvature. Pentachlorophenol and 2,4-dichlorophenoxyacetic acid are most pro-

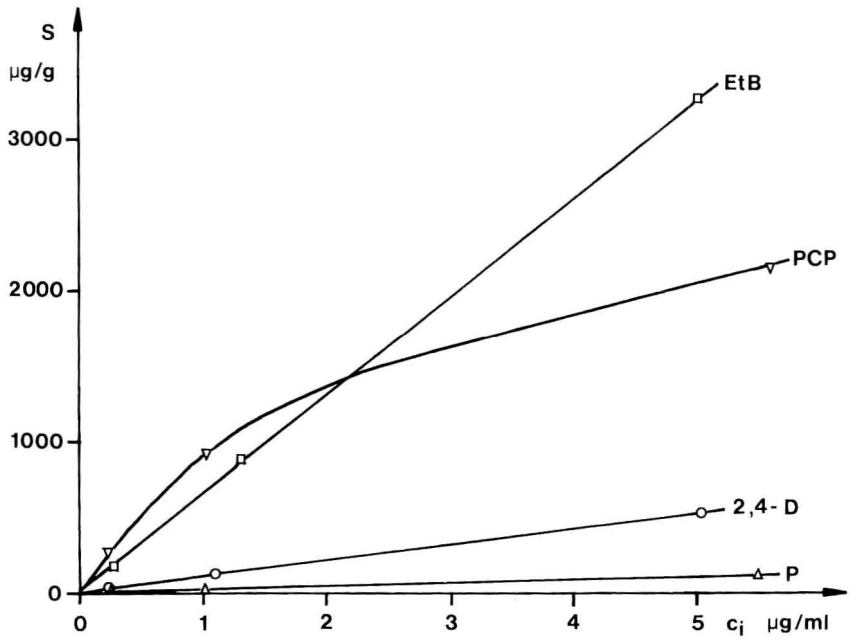

FIGURE 4

Adsorption isotherms at pH 8, µ 0.1.
Substances: Ethylbenzene (EtB), Pentachlorophenol (PCP), 2,4-Dichlorophenoxyacetic acid (2,4-D), Phenol (P).

bably retained as ion-pairs with sodium from the buffer as the counter ion, since they are both fully ionized at this pH. The linear isotherms mean, that the 50 % break-through volumes for phenol and ethylbenzene are independent of the concentration of test substance, while the 50 % break-through volumes for the two substances with convex adsorption isotherms give decreasing 50 % break-through volumes with increasing concentration of test substance.

The flow-rate of the test solution had no effect on the adsorption capacity for phenol in the range of flow-rates examined (1 - 10 ml/min). Assuming the same to be the case for the more retained test substances the rest

TABLE 1

50 % Break-through Volumes (ml) of Phenol (P), 2,4-Dichlorophenoxyacetic acid (2,4-D), Ethylbenzene (ETB) and Pentachlorophenol (PCP) at Concentrations of 5 +/- 0.5 µg/ml. Numbers Given in Parentheses are Standard deviations (n = 4).

		P	2,4-D	ETB	PCP
pH 8	µ 0.01	11 (0.1)	20 (0.3)	310 (10)	120 (4)
	µ 0.1	11 (0.3)	50 (0.3)	330 (6)	190 (11)
pH 6	µ 0.01		30 (1)		380 (48)
	µ 0.1		60 (2)		570 (60)
pH 4	µ 0.01	11 (0.5)	60 (1)	300 (2)	>5000
	µ 0.1	12 (0.3)	80 (3)	330 (8)	

of the experiments were conducted at a fixed flow-rate of 5 ml/min.

The effects of pH and buffer concentration of the test solution are seen in Tabel 1.

It is evident, that for phenol which is unionized at the pH-values in question and for ethylbenzene which is non-ionic, no measurable effect of pH on the break-through volumes is found. The buffer concentration has a small influence, which is only measurable for ethylbenzene, in that "salting out" is observed as the buffer concentration increases.

For pentachlorophenol and 2,4-dichlorophenoxyacetic acid, which have pK_a-values of 5.3 and 2.9, respectively, the break-through volumes increase as the degree of ionization decreases. For these compounds the buffer concentration has a larger influence on the break-through volumes, supporting the theory that the compounds in the ionized state are adsorbed as ion-pairs with the buffer cations. This is further supported by the fact that for 2,4-dichlorophenoxyacetic acid the influence

of the buffer concentration decreases with decreasing pH-value.

Figure 4 shows, that the adsorption isotherms for pentachlorophenol and 2,4-dichlorophenoxyacetic acid are more or less curved at pH 8. Decreasing the pH-value improved the linearity of the adsorption isotherms for these two compounds. As an example the isotherms for 2,4-dichlorophenoxyacetic acid at pH 4, 6, and 8 are shown in Figure 5.

The isotherms are slightly convex at pH 6 and 8, but at pH 4, where the substance is no longer fully ionized, the isotherm is linear. Pentachlorophenol

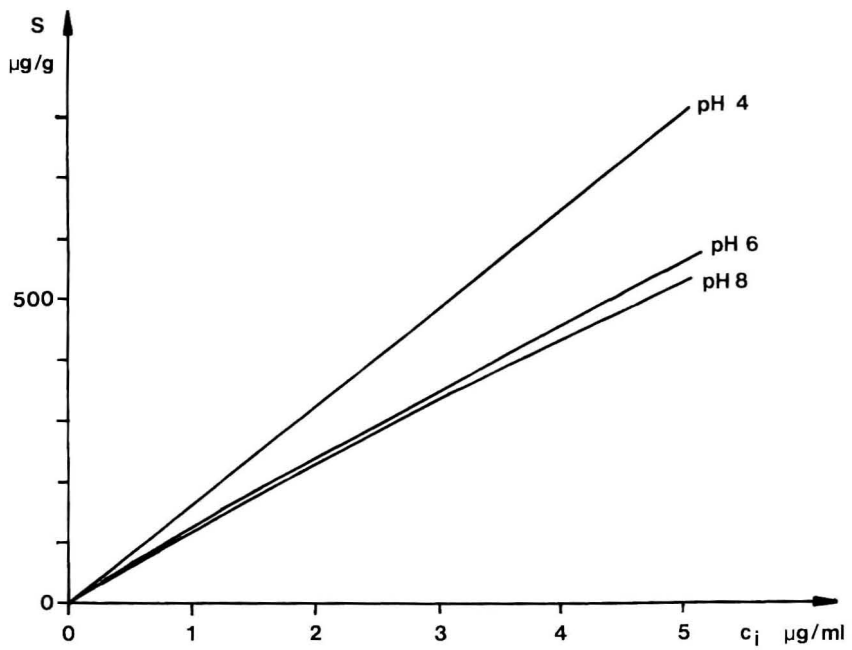

FIGURE 5

Adsorption isotherms for 2,4-Dichlorophenoxyacetic acid at three pH-values.

gave isotherms that at pH 6 were still curved, but less so than at pH 8. These findings agree well with experience from bonded-phase chromatography where many compounds have the best chromatographic properties at pH-values where the compounds are unionized (18).

CONCLUSION

The results found show, that the adsorption isotherms for the unionized and non-ionic test substances are linear and that the linearity of the isotherms for the ionized, anionic substances improves as the degree of ionization decreases. Trace enrichment of solutes in the unionized state is to be preferred, because the adsorption capacity of LiChrosorb RP-8 is largest for unionized solutes and because the degree of loading of the adsorption column is more easily controlled when the concentration of test substance has little influence on the break-through volume. In the range of buffer concentrations used very little "salting out effect" is observed.

The slope of the break-through curves show very little variation under the conditions tested. Loading of the adsorption columns up to 80-90 % of their adsorption capacities can therefore be used with very little risk of loss of the test substance in the effluent.

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RAPID CHARACTERIZATION BY THIN-LAYER CHROMATOGRAPHY OF AMINO ACID AND PEPTIDE DERIVATIVES ENZYMICALLY PREPARED DURING PROTEASE-MEDIATED PEPTIDE SYNTHESIS.

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ABSTRACT

Thin-layer chromatographic position matching combined with color matching appeared to be a convenient method for characterization of the products of individual synthetic reactions during the protease-catalyzed synthesis of Leu- and Met-enkephalin.

INTRODUCTION

Recently, we have reported two alternative pathways to the protease-catalyzed synthesis of Leu- and Met-enkephalin (1). In the course of this study, several promising pathways had to be discarded or modified. The specificity of the proteases used as catalysts was often not sharp enough to permit predictions whether and to what extent the desired compounds were going to be formed (2). These imponderables required permanent supervision of the progress of enzymic reactions and a criti-

cal evaluation of the nature of the resulting products.

Therefore, the outcome of each enzymic reaction was routinely monitored by thin-layer chromatography. The chromatograms were developed in two solvent systems, which usually provided for sufficient resolving power to ensure rapid discrimination of the resulting products. In several cases, however, products derived from enzymic reactions moved to positions on the chromatoplates difficult to be distinguished from those of the reactants. The overlapping of the spots thus formed prevented an unambiguous assignment by position matching of the enzymically prepared compounds to their chemically synthetized analogs, which served as reference substances.

In this work a method has been developed, which allows a valid assignment of the products in question by a combination of position and color matching. The procedure is based on groupspecific chromogenic reagents and on the capacity of ninhydrin to develop distinct transient colors when reacted with various amino acid and peptide derivatives.

MATERIALS AND METHODS

All solvents used in this work were reagent grade. Ninhydrin and sodium nitroprusside were purchased from

Merck, Darmstadt, F.R.G., 1-naphthol and bromine were obtained from Fluka, Switzerland. Amino acid analyses of acidic hydrolysates were performed on a Biotronik analyzer LC 6000 E. Elemental analyses were carried out by Fa. Beller, Göttingen, F.R.G.

Ninhydrin spray was prepared by dissolving 1.0 g of ninhydrin in 25 ml of acetone-pyridine-acetic acid (485:15:10) (v/v/v).

1-naphthol - hypobromite (Sakaguchi-reagent) (3):

Solution 1: 50 ml of a 16% urea solution were combined with 10 ml of a 0.2% ethanolic 1-naphthol solution.

Solution 2: Bromine (0.5 ml) was added to 50 ml of a 5% sodium hydroxide solution.

To visualize the spots the plates were first sprayed with solution 1, then dried for 1 h at 40°C and subsequently sprayed with solution 2. Sakaguchi-positive substances gave pink-colored spots.

Sodium nitroprusside-reaction (3):

The plates were sprayed with a 2% aqueous sodium nitroprusside solution. Bluish-grey spots were formed in the presence of reactive compounds on exposure to ammonia vapor. Dilution series of chemically prepared amino acid and peptide derivatives ranging from 1.0 to 10.0 µg were applied in 1.5 µl quantities of methanolic solution to precoated Silia Gel 60 plates (layer

thickness 0.25 mm)(Merck) and were run alongside the samples to be analyzed for purposes of quantitative and qualitative comparison. The plates were developed both in the solvent system chloroform-methanol (3:1)(v/v)(A) and in the system chloroform-methanol-acetic acid (45:4:1)(v/v/v)(B). The chromatograms were allowed to run in a saturated tank, the walls of which were covered with filter paper, until the solvent front had advanced a sufficient distance (18 cm), and were then air-dried to remove the solvents. In the case of ninhydrin treatment, the plates were heated for 20 min at 110°C to deacylate the α-amino group of the protected amino acids or peptides and immediately sprayed while still hot. An atomizer connected to a container filled with propellant gas (Pierce Chem.Co., Rockfort, Ill., U.S.A.) at a distant of 30 cm from the plate was used to achieve even dispersion of the freshly prepared spray.

RESULTS AND DISCUSSION

As a consequence of the abovementioned uncertainties in the outcome of protease-catalyzed peptide synthesis it is rather difficult to design a synthetic pathway. One is therefore often confined to a mere trial and error procedure. As a matter of course, it

is favorable in this situation to quickly establish whether or not a desired compound is formed under the prevailing conditions. Thin-layer chromatography was found to be a valuable tool to rapidly facilitate a provisional but nevertheless valid estimation of the progress and the results of the individual enzymic reaction. The solvent system chloroform-methanol (3:1) enabled a rapid and distinct separation of fully protected products from partially deprotected reactants. Due to their low adsorptive affinity to the polar adsorbent (silica gel) and their considerable solubility in the eluotropic solvent, the hydrophobic products displayed high R_F-values whereas the educts, the free α-carboxyl or α-amino groups of which contributed to their more hydrophilic character, exhibited medium R_F-values.

This was not the case when N^{α}-acylated amino acid or peptide ethyl esters served as carboxyl components in enzymic reactions. The ethyl ester derivatives were used, because they were shown to be excellent donor-substrates for α-chymotrypsin-mediated peptide bond formation (4). But they moved to positions on the chromatograms so close to those occupied by the resultant products that there were serious possibilities of confusion. The solvent system chloroform-methanol-acetic acid (45:4:1) indeed enabled satisfactory resolution

with regard to products and esterified reactants, but it did not always separate the products from the degradation products resulting from enzymic ester hydrolysis of the donor-substrates. Therefore this solvent system was also of only limited use for monitoring α-chymotrypsin-catalyzed reactions. However, it was useful to control the homogeneity of purified compounds.

The phenylhydrazide protection of the amino components was originally chosen, because it promotes peptide bond formation by strengthening the nucleophilicity of the α-amino group and by decreasing the solubility of the resulting products, which were to be precipitated (1,2). In addition, this protecting group now opened up a suitable means to distinguish between the newly formed N^{α}-acylated peptide phenylhydrazides and the esterified reactants. The finding, that phenylhydrazide derivatives gave color reactions both with hypobromite - 1-naphthol (Sakaguchi-reagent) and with sodium nitroprusside whereas ethyl ester derivatives remained unaffected, enabled a rapid, convenient supervision of the following α-chymotrypsin-catalyzed reactions (details are given in Table 1):

Boc-Phe-OEt (5) + H-Leu-N_2H_2Ph

⟶ Boc-Phe-Leu-N_2H_2Ph

Boc-Gly-Phe-OEt + H-Leu-N_2H_2Ph

⟶ Boc-Gly-Phe-Leu-N_2H_2Ph

Boc-Gly-Phe-OEt + H-Met-N_2H_2Ph

⟶ Boc-Gly-Phe-Met-N_2H_2Ph

Boc-Tyr(Bzl)-Gly-Gly-Phe-OEt + H-Leu-N_2H_2Ph

⟶ Boc-Tyr(Bzl)-Gly-Gly-Phe-Leu-N_2H_2Ph

The partially deprotected amino components could be easily separated owing to their differing R_F-values.

Visually comparing the color development resulting from ninhydrin-treatment provided another approach to distinguish ethyl ester from phenylhydrazide derivatives. The latter initially produced faintly yellow spots which gradually darkened whereas the former compounds quickly displayed dull colors (Table 1).

The usefulness of color matching could also be demonstrated by the following examples:

Reacting N^{α}-acylated amino acid or peptide phenylhydrazides with N-bromosuccinimide, followed by treatment of the resulting diimides with anhydrous ethanol (6) produced the corresponding ethyl ester derivatives required for α-chymotrypsin-catalysis. For the same reason as described above identification of the products and reactants by position matching failed with respect to the following reactions:

Boc-Tyr-N_2H_2Ph ⟶ Boc-Tyr-OEt

Boc-Phe-N_2H_2Ph ⟶ Boc-Phe-OEt

Boc-Gly-Phe-N_2H_2Ph ⟶ Boc-Gly-Phe-OEt

Assignment of educts and products could be achieved according to procedures reported above for the α-chymo-

Table 1: Characterization of amino acid and peptide derivatives

Compound	R_F A[a]	R_F B[b]	Color reactions [d]	Color reactions [e]	Color with ninhydrin[c]	Elemental analyses	Found	Calcd.	Amino acid analyses	Found	Calcd.
Boc-Phe-N_2H_2Ph	0.85	0.85	+	+	reddish-brown (26)[f]	C	67.65	67.59			
						H	7.08	7.09			
						N	11.77	11.82			
Boc-Phe-OEt	0.87	0.89	−	−	violet (11)	C	65.54	65.51			
						H	7.80	7.90			
						N	4.51	4.77			
Boc-Phe-Leu-N_2H_2Ph	0.87	0.84	+	+	brown (23)	C	67.15	66.66			
						H	7.69	7.74	Leu	1.00	1.00
						N	12.06	11.96	Phe	0.96	1.00
Boc-Gly-Phe-N_2H_2Ph	0.84	0.68	+	+	yellowish-brown (22)	C	64.04	64.07			
						H	6.62	6.84	Gly	1.00	1.00
						N	13.73	13.59	Phe	1.02	1.00
Boc-Gly-Phe-OEt	0.85	0.85	−	−	reddish-brown (26)	C	61.86	61.71			
						H	7.18	7.48	Gly	1.00	1.00
						N	7.80	8.00	Phe	0.97	1.00
Boc-Gly-Phe-Leu-N_2H_2Ph	0.86	0.70	+	+	yellow (2)	C	64.40	63.98	Gly	1.00	1.00
						H	6.91	7.48	Leu	0.95	1.00
						N	13.42	13.32	Phe	0.98	1.00
Boc-Gly-Phe-Met-N_2H_2Ph	0.86	0.68	+	+	yellow (2)	C	59.84	59.56			
						H	6.93	6.86	Gly	1.00	1.00
						N	13.08	12.88	Met	0.94	1.00
						S	5.83	5.90	Phe	1.01	1.00
Boc-Gly-Leu-N_2H_2Ph	0.84	0.69	+	+	yellowish-orange (4)	C	60.23	60.30			
						H	7.96	7.99	Gly	1.00	1.00
						N	14.78	14.80	Leu	0.96	1.00

Boc-Tyr-N_2H_2Ph	0.84	0.67	+	+	brown (25)	C H N	64.32 6.84 11.52	64.64 6.78 11.36		
Boc-Tyr-OEt	0.84	0.77	—	—	reddish-violet (10)	C H N	61.75 7.39 4.49	62.13 7.49 4.53		
Boc-Tyr-Gly-N_2H_2Ph	0.77	0.49	+	+	orange (5)	C H N	61.43 6.49 13.16	61.68 6.59 13.08	Gly 1.00 Tyr 0.91	1.00 1.00
Boc-Tyr(Bzl)-Gly-Gly-Phe-OEt	0.89	0.70	—	—	reddish-violet (10)	C H N	65.68 7.00 8.52	65.44 6.71 8.48	Gly 2.00 Tyr 0.91 Phe 0.98	2.00 1.00 1.00
Boc-Tyr(Bzl)-Gly-Gly-Phe-Leu-N_2H_2Ph	0.90	0.62	+	+	bluish-violet (12)	C H N	65.96 6.82 11.88	66.09 6.87 11.73	Gly 2.00 Leu 0.94 Tyr 0.89 Phe 0.95	2.00 1.00 1.00 1.00

[a]Solvent system A: chloroform-methanol(3:1); [b]Solvent system B: chloroform-methanol-acetic acid(45:4:1); [c]Colors have been determined 30 min after ninhydrin treatment by use of a hue indication chart; [d]Reaction with Sakaguchi-reagent; [e]Reaction with sodium nitroprusside; [f]Arabic numerals given in brackets refer to hue differences illustrated in the chart, which forms part of a color index (7).

trypsin-mediated syntheses. The results are depicted in Table 1.

As the outcome of protease-catalyzed peptide syntheses is sometimes unpredictable the nature of the resulting products requires critical evaluation. This may be explained by the following reaction: the tripeptide Boc-Gly-Phe-Leu-N_2H_2Ph was an essential intermediate in the preparation of Leu-enkephalin(2). It could be synthetized via α-chymotrypsin-catalysis (vide supra). Nevertheless, it was attempted to prepare it by papain-mediated coupling of Boc-Gly-Phe-OH and H-Leu-N_2H_2Ph as well. Monitoring of the papain-catalyzed reaction by means of thin-layer chromatography using the solvent systems A and B revealed a ninhydrin-positive spot, which had moved to a position hardly distinguishable in both systems from that occupied by the abovementioned tripeptide. Spraying with Sakaguchi-reagent and sodium nitroprusside indicated the presence of a phenylhydrazide derivative. However, visual comparison of the two spots after ninhydrin-treatment suggested non-identity of the two products (Table 1). This assumption could be confirmed by both amino acid and elemental analysis, which proved the substance in question to be the dipeptide Boc-Gly-Leu-N_2H_2Ph (Table 1). Obviously papain had at the very beginning hydrolyzed the dipeptide Boc-Gly-Phe-OH to furnish H-Phe-OH, which could be

traced by thin-layer chromatography, and Boc-Gly-OH, which subsequently reacted with H-Leu-N_2H_2Ph via papain-catalysis to give Boc-Gly-Leu-N_2H_2Ph.

The attempt to synthetize Boc-Tyr-Gly-N_2H_2Ph by papain-mediated reaction between Boc-Tyr-OH and H-Gly-N_2H_2Ph resulted in a product, which could be shown to be a phenylhydrazide compound according to hypobromite - 1-naphthol and sodium nitroprusside treatment. Notwithstanding these results, the corresponding R_F-values provided strong evidence, that the product in question was not identical with the desired dipeptide. Color matching using ninhydrin suggested the compound to be Boc-Tyr-N_2H_2Ph. This characterization could be confirmed by elemental analysis (Table 1).

The varied colors observed for ethyl esters and the corresponding phenylhydrazide derivatives may be explained in terms of the oxidizing power of ninhydrin, which presumably transforms the latter into their phenyldiimide analogs. These azo compounds displayed a faintly yellow color on thin-layer plates already in the absence of ninhydrin. The above reasoning is supported by the finding that a substance resulting from ninhydrin-treatment of H-Phe-N_2H_2Ph (20 min, 110°C) exhibited the same behavior as phenylalanine diimide with regard to both color and position when run in the solvent systems 1-butanol-pyridine-acetic acetic-water (20:10:3:10) and chloroform-methanol (3:1).

It is taken for granted, that the reaction of amino acids and peptides with ninhydrin proceeds via Schiff bases (8,9) an integral part of which are the distinct amino acid side chains. As the formation of differing Schiff bases is responsible for the varied color developments (8), the divers color reactions of Boc-Gly-Leu-N_2H_2Ph and Boc-Gly-Phe-Leu-N_2H_2Ph on the one hand and of Boc-Tyr-Gly-N_2H_2Ph and Boc-Tyr-N_2H_2Ph on the other hand seems explicable if one assumes that they are influenced not only by the first but also by the second amino acid side chain of the peptides.

The Sakaguchi-reagent is commonly used in peptide chemistry to identify arginine residues (10). During this work it proved also to be a suitable agent to detect phenylhydrazide protected amino acids and peptides. The color reaction presumably implies the oxidation by sodium hypobromite of the phenylhydrazide moiety to give the corresponding phenyldiimide group, which is subsequently removed upon reaction with 1-naphthol to form 4-phenylazo-1-naphthol.

The preliminary characterization of each of the compounds described above could be verified by elemental analysis. As far as peptides were concerned amino acid compositions were determined as well (Table 1).

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REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH CETRIMIDE CONTAINING ELUENTS

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ABSTRACT

A radially compressed column, packed with micro particle, reversed phase (C 18) material, was used to study HPLC with cetrimide containing eluents. The amount of cetrimide adsorbed onto the stationary phase was measured; not the number of available adsorption sites, but rather the presence of micelles in the eluent appears to be the limiting factor for the uptake of cetrimide from the eluent.

The capacity factors, k', of several - mainly acidic - compounds were determined in this system, with varying pH and cetrimide concentration of the eluent (methanol-water, 50% w/w). The results obtained upon changing the pH of the eluent can not all be explained with an ion-exchange (or ion-pair) model.

Upon increasing the cetrimide concentration, maximum values in k' are reached at about the critical micelle concentration (cmc) of cetrimide in the eluent. The results of conductimetric experiments suggested, that the decrease of k' at cetrimide concentrations above this CMC, observed for most of the compounds, can be explained by partitioning of the solutes between the micelles and the bulk of the mobile phase. From these experiments it was also clear that ion-pair formation between cetrimide and solutes is possible.

INTRODUCTION

Recently, chromatography on non-polar phases with ion-pair reagents, with or without marked surface activ-

ity, added to the eluent has been the subject of many studies. Different theories have been developed to explain the retention behaviour of solutes in these systems. Most of these theories have been discussed recently by Bidlingmeyer (1) and by Knox and Hartwick (2). One of the points which have not been settled yet is, how to explain a maximum in the capacity factor, k', when k' is plotted against the pairing-ion concentration in the eluent.

Three theories have been proposed to explain this phenomenon which was observed by many authors (2-10);

a) "The decrease in k' at higher counter ion concentrations may be due to the combined effect of a decrease in the adsorption capacity of the retaining phase and an ion-pair formation ....." (7);

b) Above a critical concentration a number of surface active ions are able to form micelles; solutes are supposed to partition between the micelles and the bulk of the mobile phase (2-4, 10);

c) Hung and Taylor (9) explain the decrease in k' by assuming a decrease of the surface which is available for hydrophobic interaction, caused by the larger amount of pairing-ion adsorbed onto the surface.

Secondly, according to these authors, the increased concentration of the counter ions accompanying the pairing ions results in less possibilities for ion-exchange of the solute ions with the adsorbed pairing-ions.

In this investigation the retention behaviour of various compounds, with different acidic and basic properties, were studied at different cetrimide (the "pairing-ion") concentrations. Possible interactions between solutes and cetrimide above as well as below the critical micelle concentration (CMC) were studied by conductimetry.

The factors which influence the adsorption of cetrimide onto the stationary phase are also of interest. Different authors studied the adsorption of cetrimide as a function of the cetrimide concentration in the eluent (3, 4, 9, 10); little is known about the influence of the pH on the adsorption phenomena (4).

In the present study the influence of pH on the chromatographic behaviour of mainly acidic compounds is described. However, the chosen pH range is higher than usual, so the effect of pH on k' could be studied under conditions where for instance carboxylic acids are completely deprotonated.

MATERIALS

Apparatus

The chromatographic system consisted of a 6000 A solvent delivery system, a U6K injection system and a Radial-Pak A cartridge, 10 cm x 8 mm I.D., in combination with an RCM-100 Radial Compression Module (all from Waters Assoc., Milford, Mass., USA). The column was in-

stalled between the U6K injection system and two on-line detectors, a model 440 differential UV absorbance detector, operated at 254 nm and an R401 refractive index detector (both from Waters Assoc.). By means of a Valco rotary six port 7000 psig valve (Chrompack, Middelburg, The Netherlands), connected between the column and the detectors, a bypass could be used to flush the entire system except the column.

Retention times were obtained by means of an SP4000 (Spectra-Physics, Santa Clara, CA, USA) data system.

For measuring pH values a Radiometer (Copenhagen, Denmark) PHM 64 pH meter was used.

The conductance was measured with a Consort (Turnhout, Belgium) K620 conductometer in combination with a type EA 645 titration cell (Metrohm, Herisau, Switzerland).

Conductance and chromatographic experiments were carried out at 25.0 ±0.1° C and 25.0 ±0.2° C, respectively.

Chemicals

Methanol, potassium bromide and sodium hydroxyde were of analytical reagent grade and were obtained from Merck (Darmstadt, G.F.R.). Cetyl trimethylammonium bromide (cetrimide) was purchased from B.D.H. (Poole, England). Boric acid, "analyzed" reagent, was obtained from Baker (Deventer, The Netherlands). 2-Methoxy methyl benzoate was prepared by methylation of methyl salicylate

with methyl iodide, in an analogous manner as was reported for the propylation of nalidixic acid with propyl iodide (11). The other compounds under investigation were commercially available products and were used without further purification.

Cetyl trimethyl ammonium (CTA) containing eluents were prepared in water-methanol mixtures (final ratio in the eluent, 1:1 w/w) with 0.1% w/w up to 1.6% w/w (= 2.74×10^{-3} - 4.39×10^{-2} mol·kg^{-1}) cetrimide; potassium bromide was added to a final bromide concentration of 0.1 mol·kg^{-1}; the eluents were buffered with boric acid buffers, 0.025 mol·kg^{-1} eluent and adjusted to the desired pH with sodium hydroxide, measured against water-methanol buffers (12). The pH values in the water-methanol system are indicated by pH*.

METHODS

The amount of cetrimide adsorbed onto the column was studied as a function of pH and of the cetrimide concentration in the eluent.

First the chromatographic system was equilibrated with the eluent of the desired pH without the cetrimide; after that the cetrimide containing eluent with the same pH was pumped through the column. The amount of cetrimide adsorbed would then be $(V_r - V_o)$. C, where V_r = breakthrough volume of cetrimide as indicated by the refrac-

tive index detector, V_o= void volume and C= concentration of cetrimide in the eluent.

After the breakthrough volume of cetrimide was registered more solvent was pumped through the column, until the pH of the eluate had become exactly the same as the pH of the eluent (12). Cetrimide was removed from the column with the same eluent without cetrimide. This stripping process was followed with the refractive index detector. In order to check whether all of the adsorbed cetrimide had been removed, the retention volume of salicylic acid was determined, because this volume was found to be strongly influenced by the presence of even small amounts of cetrimide.

The pKa values of some of the compounds under investigation in water-methanol, 1:1 w/w, were determined according to the method described by Hulshoff and Perrin (14); these pKa values are indicated by pKa*.

Critical micelle concentrations of cetrimide in the different eluents were measured conductimetrically as described before (10). Other conductimetric measurements were carried out to study possible interactions between some of the compounds under investigations and cetrimide. In these measurements 6.25×10^{-6} mol of these compounds were added to 40 g water-methanol mixtures, containing varying concentrations of cetrimide (below and above the CMC).

RESULTS AND DISCUSSION

Adsorption of cetrimide onto the column packing material

We reported earlier on the breakthrough patterns of cetrimide containing eluents and on the pH-shifts which can be observed during these processes (13). The amount of cetrimide adsorbed onto the column is shown as a funciton of pH* in Fig. 1a and in Fig. 1b as a function of the cetrimide concentration in the eluent. The phenomenon of increasing amounts of cetrimide adsorbed onto the column at increasing pH we have discussed before (13). Knox and Laird (3) found, that the adsorption isotherm of cetrimide on SAS silica obeyed a Freundlich type equation.

Hung and Taylor (9) state that neither a Freundlich nor a Langmuir type of equation can be used to describe the adsorption of quaternary nitrogen compounds. Assuming that the carbon chains lie flat on the surface, they made the contradictory observation that the larger the alkyl groups of the quaternary nitrogen compounds, the more surface area seemed to be available. They therefore introduced the wellknown Hansch theory to describe the adsorption as a function of the hydrophobicity of the longest alkyl group of the pairing ion. However, we suppose that the carbon chains of the pairing ions are situated between the C_{18} brushes of the stationary phase. In principle for each type of reagent molecule an equal number of sites of adsorption will then be

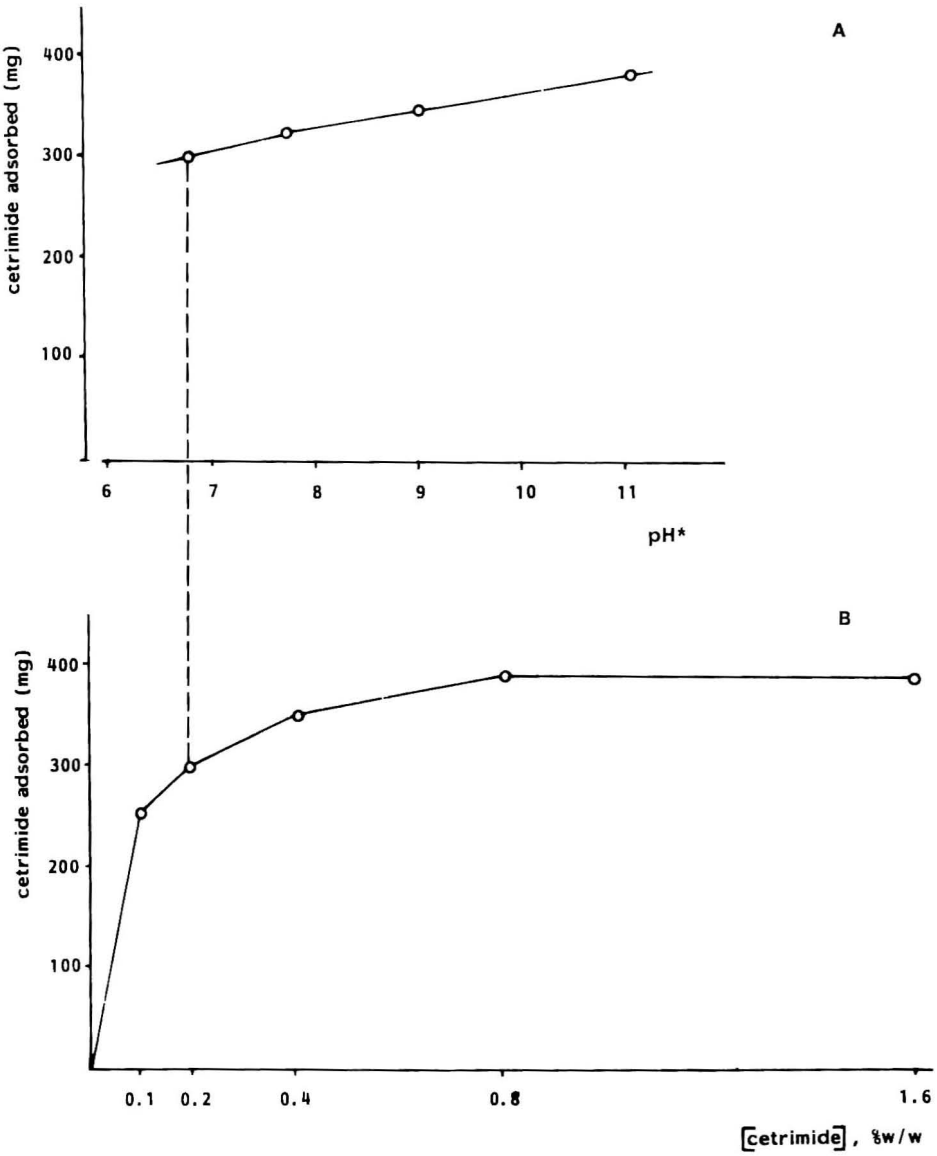


FIGURE 1 Adsorption of cetrimide onto the column, (A) as a function of the pH* of the eluent; cetrimide concentration, 0.2% w/w, and (B) as a function of the cetrimide concentration in the eluent at pH* 6.8. For other conditions, see Materials.

available and for each of these reagents an adsorption isotherm can be used. The hydrophobic interaction increases with the length of the carbon chain, resulting in a higher level of maximal adsorption for the reagents with longer alkyl groups. The adsorption isotherms found in our experiments, particularly with respect to the data at the higher cetrimide concentrations, did not fit quite well to the empirical Freundlich type equation. The reason for this is simply the presence of cetrimide micelles above 0.7% w/w cetrimide in the eluent. The CMC values, as determined in the eluents and in different methanol-water mixtures, are summarized in Table I.

At increasing cetrimide concentration above the CMC the cetrimide monomer concentration will only slightly increase (dependent on the hydrophobicity of the eluent). So the limiting factor for the uptake of cetrimide out of the mobile phase is not necessarily the surface coverage of the stationary phase, but more likely the cetrimide monomer concentration in the eluent. As is evident from Figure 1b, the maximal adsorption is indeed reached near the CMC of cetrimide.

Although our results fit quite well with a Langmuir type of equation, in fact neither a Freundlich nor a Langmuir isotherm can therefore be used when concentrations above the CMC are included in the data.

TABLE I

Critical Micelle Concentrations of Cetrimide

Solvent		CMC ± SD (% w/w)
eluent[1)]	pH* 6.8	0.70 ± 0.03
	pH* 9.0	0.70 ± 0.03
	pH* 11.0	0.85 ± 0.04
water		0.036 ± 0.001[2)]
methanol/water	10% w/w	0.045 ± 0.001
	20% w/w	0.090 ± 0.004
	30% w/w	0.21 ± 0.01
	40% w/w	0.47 ± 0.04
	50% w/w	0.95 ± 0.04

1) see MATERIALS, *Chemicals*

2) the same value has been reported in REFERENCE (25)

Recently Knox and Hartwick (2) published adsorption isotherms of some anionic surfactants onto Hypersil ODS. They estimated the CMC values of these surfactants in the eluents from data reported in the literature. Their results show that the adsorption of these reagents increases even above these CMC values, so we suppose that their estimations are not accurate enough. The adsorption curve for cetrimide, as reported by Hung and Taylor (9) reaches a maximum close to the CMC of cetrimide in water (which is the eluent they used). The consequences of this for their hypothesis concerning the retention mechanism will be considered further below.

Influence of pH on the retention

In Figure 2, k' values for the different compounds are plotted versus the pH* of the eluent. Some unexpected results were obtained. 2-Methoxy-methyl benzoate, a neutral compound, shows a marked decrease in k' with increasing pH*. The conclusion has to be that at higher

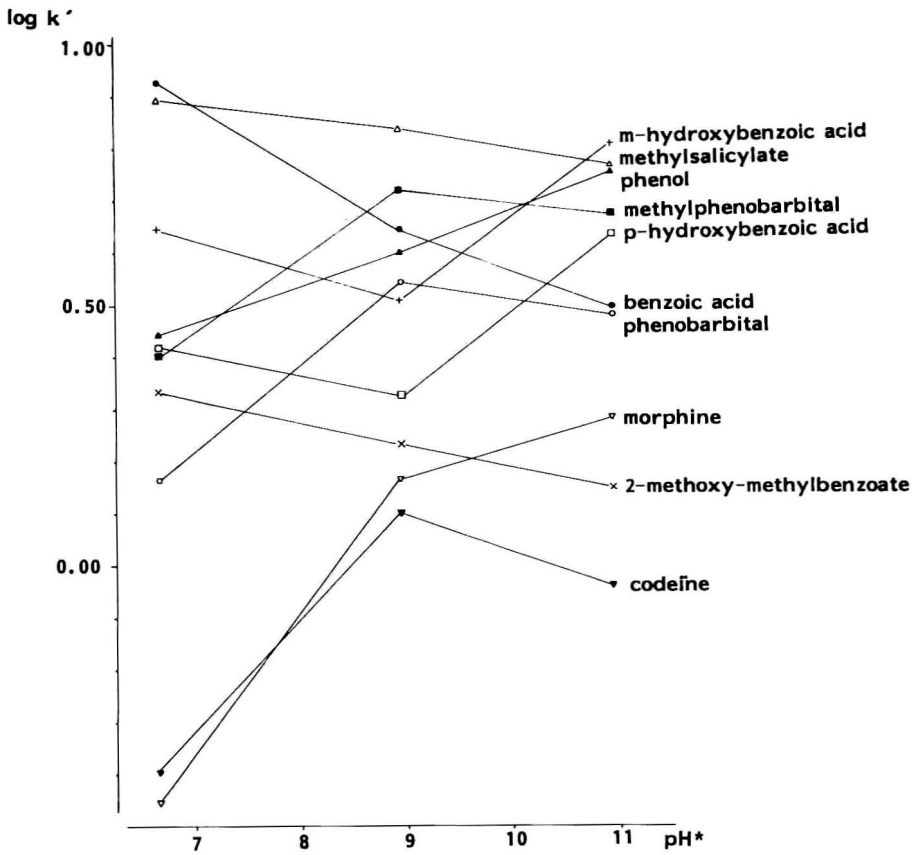


FIGURE 2 Influence of the pH* of the eluent on log k'; cetrimide concentration, 0.2% w/w; for other conditions, see Materials.

pH values the available area for hydrophobic interactions is decreased. A possible explanation can be found in the work of Hung and Taylor (9), who stated that the more ion-pairing reagent is adsorbed onto the surface, the less sites are available for desolvation of solutes during chromatography.

We observed that more cetrimide was bound onto the column at higher pH (Fig. 1a); therefore their hypothesis might help to explain this phenomenon of decreasing k' with increasing pH. With benzoic acid (pKa* = 5.2), present as anion in the entire pH range, the same behaviour was observed as the neutral compound.

Although it was expected that k' would increase, because the increased amount of adsorbed cetrimide should give more possibilities for ion-exchange chromatography, we have to conclude that another (unknown) mechanism is working in the opposite direction.

On introducing a phenolic function into the benzoic acid molecule, k' increases between pH* 9 and 11 (See data for m-hydroxybenzoic acid and p-hydroxybenzoic acid). This is in agreement with an increase in retention due to anion-exchange, for in this pH range the phenolic functions are getting deprotonated, thus doubling the negative charge of the ions.

Phenol itself shows the same behaviour in the pH range (9-11). Of the other phenolic compounds only

methyl salicylate is an exception; it shows a decrease in k'.

The intramolecular hydrogen bonding, which is known to exist with this type of compound, is probably responsible for this different behaviour.

For phenobarbital (pKa* = 8.2) and mephobarbital (pKa* = 8.4) k' increases between pH* 7 and 9, this is also in good agreement with the ion-exchange theory. However, at pH* 11, k' for both compounds is decreased. Possibly the same mechanism is responsible as the mechanism causing the decrease in k' for e.g. benzoic acid and 2-methoxy-methyl benzoate.

For codeine (pKa* = 7.6) and morphine (pKa* = 7.7) k' increases from pH* 7 to 9; in this range the nitrogen groups are getting deprotonated, so the possibilities for hydrophobic interaction are increased. Above pH* = 9, the phenolic function of morphine causes an increase in k'.

Similar patterns of log k' versus pH* were obtained at 1.6% w/w cetrimide (above the CMC) in the eluent (Results not shown).

Influence of the cetrimide concentration on k'

In figure 3, k' is plotted versus the cetrimide concentration in the eluent at pH* 9. At this pH the CMC is 0.70% w/w (see Table I). For all weak and strong acids there is a sharp increase in k' when cetrimide is added to the eluent, till a maximum value is reached.

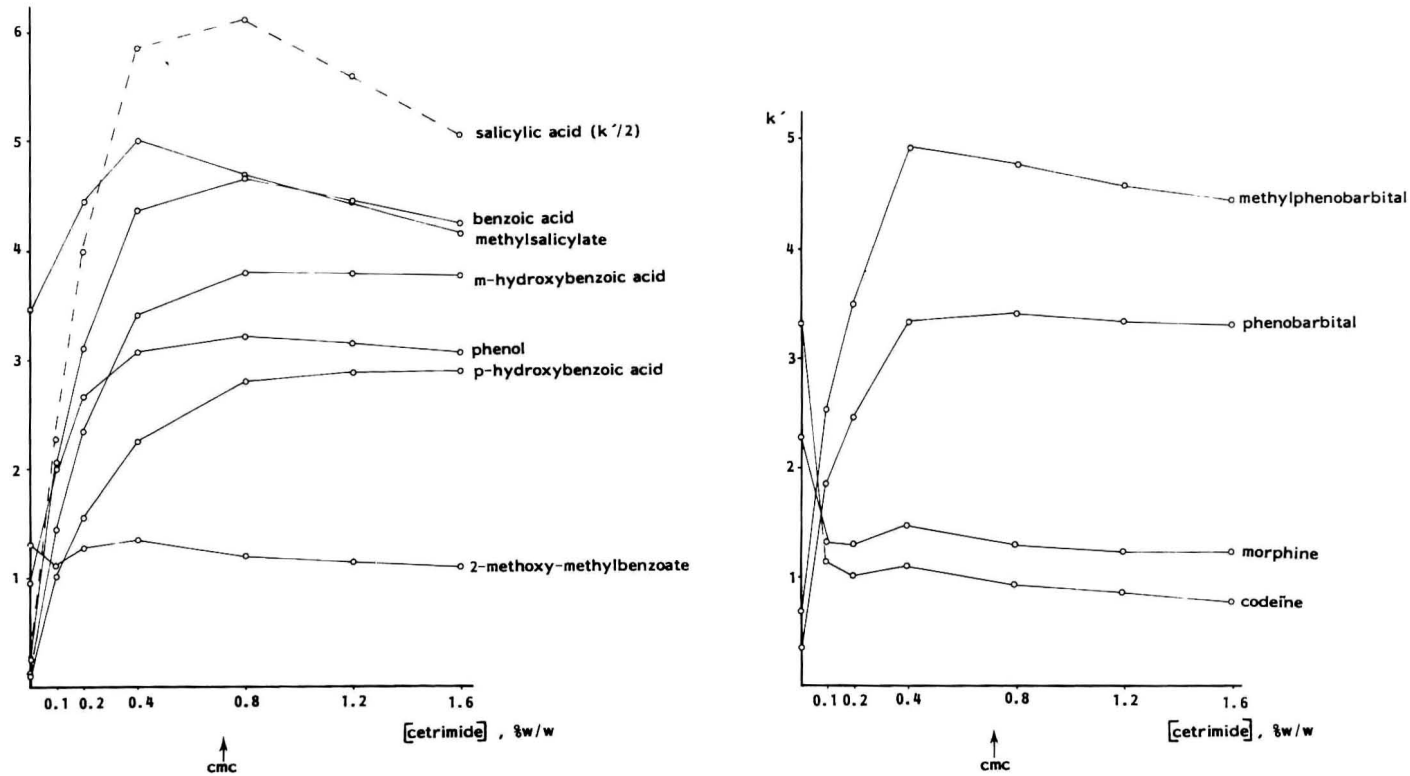


FIGURE 3 Influence of the cetrimide concentration in the eluent on k' at pH* 9. For other conditions, see Materials.

These first parts of the different plots correspond very well with the adsorption isotherm of cetrimide (Fig. 1b). The ion-exchange mechanism, therefore, seems to be an important retention mechanism for these compounds. This is in agreement with the results found by several investigators (2,4, 9, 15-19). The maximum in k' for the acidic compounds coincides rather well with the CMC of cetrimide in the eluent.

At cetrimide concentrations above the CMC, for some compounds, k' decreases (e.g. salicylic acid) or k' does not change anymore (e.g. m-hydroxybenzoic acid). For the two basic compounds, codeine and morphine, the addition of cetrimide to the eluent results in a decrease of k', caused by the loss of adsorption sites onto the silica matrix, as was apparent from the improved peak symmetry. Furthermore, k' of morphine is larger than k' of codeine in the presence of cetrimide; this is caused by the anion-exchange possibilities for morphine due to its phenolic function.

Hung and Taylor (9) claim that the decrease in k' at higher pairing-ion concentrations has nothing to do with the occurrence of micelles, because they also found a decrease in k' with non-surfactants as ion-pair reagents (tetramethyl ammonium, tetraethyl ammonium and tetrabutyl ammonium). This general decrease in k' is ascribed by them to two factors: a) a decrease in the C_{18} surface available for desolvation of the solutes, and

b) the increasing counterion concentration at higher concentrations of the ion-pair reagents, which will lead to a decrease in retention due to ion-exchange processes.

However, in the present investigation, the surface area available for desolvation will not change anymore above the CMC (as discussed before). Secondly, the counterion concentration was kept constant, so we have to conclude that another mechanism is responsible for the decrease in k'.

In fact the same conclusions can be drawn for their (9) own results with cetrimide. Because the eluent used by Hung and Taylor was water, they also have to deal with micelles above 0.036% cetrimide.

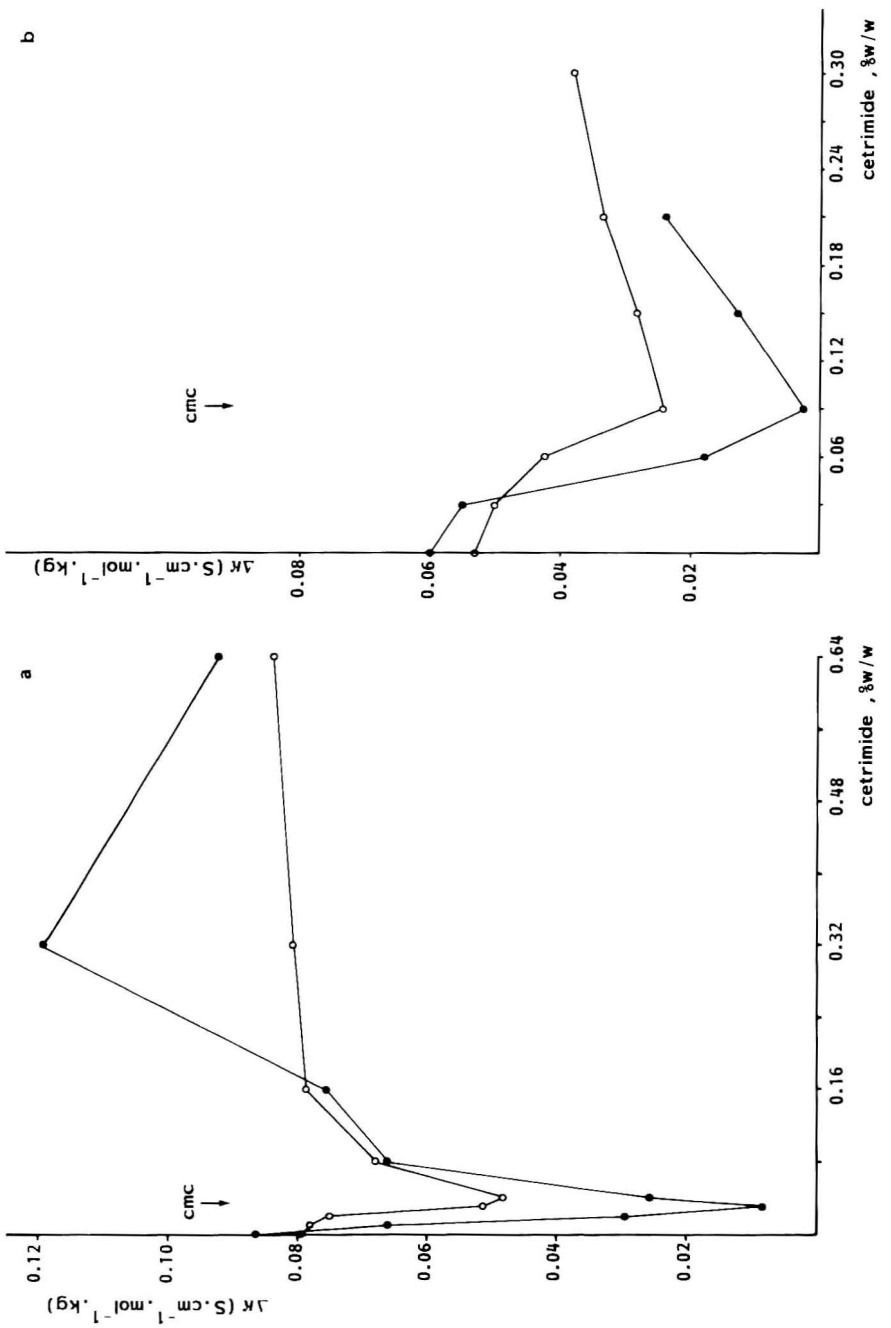
Once the CMC has been reached, the addition of more cetrimide to the eluent will only result in an increase of the number and size of the micelles. The monomer concentration, and therefore also the amount of adsorbed cetrimide and the free counterion concentration will remain practically constant above the CMC. The sharp decrease in k' values observed by Hung and Taylor in cetrimide containing eluents must therefore be attributed to another mechanism than those proposed by them. However, for the other pairing ions in their investigation, their theory is possibly correct, because indeed the counterion concentration increases at increasing pairing ion concentration.

Interaction between cetrimide and solute anions

Possible interactions between some of the anions under investigation and cetrimide were studied by conductimetric experiments. Salicylicate and m-hydroxy benzoate were chosen; the first anion, because it shows a strong decrease in k' above the CMC of cetrimide, while for the second anion k' seems to be independent of the cetrimide concentration above the CMC (Fig. 3). Both compounds were added as their sodium salts to solutions of different concentrations of cetrimide (below and above the CMC). With the offset of the conductometer the contribution of the cetrimide itself to the conductance was corrected for.

The eluents used in this study, contain comparatively high concentrations of ionic substances; this would prevent the measurement of small changes in conductivity, so pure water-methanol mixtures were used.

Figure 4 shows the *increase* in specific conductivity, Δk expressed in $S \cdot cm^{-1} \cdot mol^{-1} \cdot kg$, of different cetrimide containing solutions after the addition of sodium salicylate or sodium m-hydroxy benzoate. It is apparent from these figures that in solvents containing up to 30% methanol a minimum value for Δk exists, which coincides with the CMC of cetrimide, determined at each of these methanol concentrations. There is obviously an interaction between salicylate (and m-hydro-


a
b
cmc
cmc
Δκ (S.cm⁻¹.mol⁻¹.kg)
Δκ (S.cm⁻¹.mol⁻¹.kg)
cetrimide , %w/w
cetrimide , %w/w
0.02
0.04
0.06
0.08
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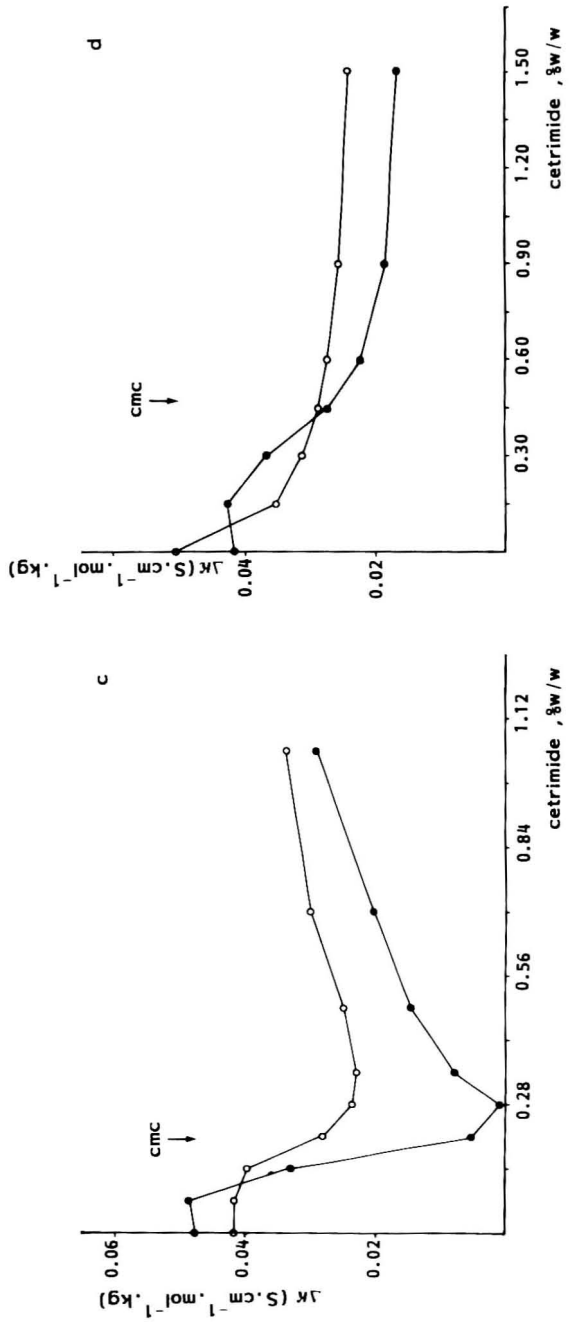

FIGURE 4 The increase in the specific conductivity, ΔK, of cetrimide containing solutions after the addition of 6.25×10^{-6} mol sodium salicylate (●—●) or sodium m-hydroxy benzoate (○—○); a, water; b, 20% w/w methanol; c, 30% w/w methanol; d, 40% w/w methanol.

xy benzoate) and cetrimide below the CMC, for otherwise Δk should be the same value at each cetrimide concentration, resulting in a horizontal straight line.

Bidlingmeyer et al. (20) also using conductimetric experiments, found no interactions between octane sulphonate and octyl amine (and some drugs) in water-methanol mixtures. However, Melander et al. (21), using the data of Bidlingmeyer et al. (20), concluded that ion-pair formation can not be excluded.

In our study the values for Δk were found to increase above the CMC, probably by partitioning of the solutes between the micelles and the bulk solution, resulting in a bromide (micelle counter-ion) release. From the literature (22) it is known that anions are able to release bromide from cetrimide micelles. The observed increase in Δk is more pronounced for salicylate than for m-hydroxy benzoate, so this phenomenon offers a possible explanation for the decrease of k' of salicylate as compared with m-hydroxy benzoate.

Assuming that below the CMC of cetrimide ion-pair formation occurs, theoretically the ion-association constant, K_{ip}, can be calculated from the measurements in the following way:

For ion-pair formation the following equation can be written,

$$CTA^+ + S^- \rightleftarrows CTAS$$

and $$K_{ip} = \frac{[CTAS]}{[CTA^+][S^-]} = \frac{C_{ip}}{C \cdot C^1} \quad (1)$$

where $[CTA^+] = C$ is the cetrimide cation concentration, $[S^-] = C^1$ is the solute anion concentration and $[CTAS] = C_{ip}$ is the concentration of the ion-pair (all in $mol \cdot kg^{-1}$).

For the specific conductivity, k, the following equation can be written, when no ion association occurs,

$$k \times 1000 = \Lambda_{CTABr} \cdot C + \Lambda_{NaS} \cdot C^1 \quad (2)$$

where Λ_{CTABr} and Λ_{NaS} are the equivalent conductivities of CTABr and NaS respectively.

The following equation for K_{ip} can be derived, in an analogous way as was done by Tomlinson and Davis (23), using Kohlrausch's law of the independent migration of ions and the Law of Mass Action.

$$K_{ip} = \frac{\frac{1000 \cdot \Delta k}{\lambda_{CTA^+} + \lambda_{S^-}}}{\left(C - \frac{1000 \cdot \Delta k}{\lambda_{CTA^+} + \lambda_{S^-}}\right)\left(C^1 - \frac{1000 \cdot \Delta k}{\lambda_{CTA^+} + \lambda_{S^-}}\right)} \quad (3)$$

where λ is the equivalent conductivity for each ion.

The value Δk can be calculated from Λ_{CTABr} and Λ_{NaS} which are determined separately; from the sum of these two values the measured value of k, obtained after addition of NaS to the cetrimide solution, is subtracted. The values λ_{CTA^+} and λ_{S^-} can be obtained by

subtracting the equivalent conductivities of bromide and sodium ions (24) from Λ_{CTABr} and Λ_{NaS}, respectively.

However, with the available equipment the standard deviations obtained for measured values were such, that the comparatively small Δk values lacked sufficient precision to allow the determination of reliable K_{ip} values. This was also concluded by Melander et al. (21) for the octyl amine - octyl sulfonate system. For instance in water the K_{ip} for cetrimide salicylate was found to be 2×10^3 kg·mol^{-1} at 0.01% cetrimide and 2×10^4 kg·mol^{-1} at 0.02% cetrimide.

The conclusions from these conductimetric experiments are therefore qualitative in nature. Below the CMC interaction between cetrimide and the solute anions in the mobile phase seems likely, particularly at lower methanol concentrations.

Above the CMC of cetrimide the results suggest the partitioning of solutes between micelles and bulk phase; for salicylate the interaction with the micelles is more pronounced than for m-hydroxy benzoate, the decrease in k' of salicylate relative to m-hydroxy benzoate could be explained in this way.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF TOLMETIN, INDOMETHACIN AND SULINDAC IN PLASMA

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ABSTRACT

Isocratic and gradient reversed phase high-performance liquid chromatographic (HPLC) methods for the quantitation of tolmetin, indomethacin, and sulindac and their respective metabolites in plasma were developed. Only the determination of the parent drugs was possible using the isocratic technique. Specific, simultaneous determination of each drug and its respective metabolites was achieved using the gradient technique. The effect of pH and ionic concentration of the mobile phase on retention time was studied. Statistical analysis demonstrated excellent precision and linearity over the following ranges: 1-40, 0.1-3, and 0.1-3 ug/ml plasma for tolmetin, indomethacin, and sulindac respectively. Both methods have been applied to the analysis of patient samples.

INTRODUCTION

The nonsteroidal, anti-inflammatory drugs are used in the treatment of arthritis and for the relief of mild to moderate pain (1). Many adverse reactions are common to all these drugs. Almost every patient experiences some symptoms of gastrointestinal toxicity, but even when such symptoms are absent, GI lesions are often present (2,3). Reduction of renal function has also been reported (4). Severe and even life threatening adverse reactions have been reported (5,6).

Direct spectrophotometric (7-10) and spectrofluorometric (8,11,12) determination of these drugs have been reported. These methods lack specificity due to interference from metabolites and salicylic acid. A TLC separation with quantitation by a TLC scanning spectrophotometer has been described (13). Several GLC procedures have been reported which require derivatization (7,14-22) and/or special detectors such as electron capture (15-20) or mass spectroscopy (21-23). Also described in the literature are radioimmunoassay methods (24,25). Quantitation has been performed by high-performance liquid chromatography (HPLC). The reported methods involve precipitation of plasma proteins followed by injection of the supernatent (26-28) or have not been applied to the analysis of patient samples (29,30). An HPLC method for the screening of solid dosage forms using dual UV detectors has been reported recently (31).

This paper describes a rapid, specific method for the determination of indomethacin, sulindac and tolmetin by isocratic elution. Also described are alternate methods using gradient elution making it possible to determine these drugs and their metabolites. Both methods are by HPLC using a reversed-phase octadecylsilane column and a 254 nm detector. These methods are applicable to the direct determination of plasma levels even in the presence of other drugs. The applicability

was demonstrated by the analysis of plasma from patients taking oral indomethacin, sulindac or tolmetin.

MATERIAL AND METHODS

Instrumentation - Isocratic - A Waters Associates Model 202 liquid chromatograph was equipped as follows: an M6000 pump, a U6K Universal injector, a Zorbax ODS column (25.0 cm x 4.6 mm), a Schoeffel Model SF770 variable wavelength UV detector and a Perkin-Elmer Model 56 recorder. The detector was set at a wavelength of 254 nm. The degassed mobile phase was pumped through the column at 1.5 ml/min (22.0-23.4 MPa) at ambient temperature until a stable baseline was obtained.

Gradient - A Hewlett-Packard Model 1084B liquid chromatograph with a variable wavelength UV detector was equipped as follows: A Model 79850B LC terminal, a Zorbax ODS column, and an automatic injector. The wavelength was set at 254 nm. The degassed mobile phase set at the proportions used initially was pumped through the column at 1.5 ml/min (20.7-25.5 MPa) at 40°C until a stable baseline was obtained.

Chemicals and Reagents - Reagent grade sodium hydroxide, sodium acetate, sodium phosphate, acetic acid, phosphoric acid, sulfuric acid, citric acid, methylene chloride, chloroform, 2-propanol, hexane, ethyl acetate, diethylether, diphenylacetic acid and p-phenylphenol were

used. HPLC grade methanol (Fisher) was used. Indomethacin, sulindac, and tolmetin and their respective metabolites were obtained courtesy of the manufacturer.

Mobile Phases - Sodium acetate buffers of pH 4 and 5 (0.01,0.02,0.03,0.04,0.06,0.08,0.10,0.12 and 0.15M) and acetic acid solutions (0.01,0.02 and 0.03M) were prepared and an appropriate amount was added to methanol. All mobile phases were degassed under vacuum. The effect of ionic concentration and pH on retention time was studied.

Drug Solutions - Methanolic solutions of each drug and metabolite were prepared at the appropriate concentrations to be used for the preparation of plasma standards.

Internal Standard Stock Solution - A solution of 3 mg p-phenylphenol in 100 ml methanol was prepared and kept refrigerated.

Extraction Solutions - A. (0.12 ug/5ml) - A 0.2 ml aliquot of the p-phenylphenol stock solution was diluted to 250 ml with methylene chloride.

B. (0.3ug/5ml) - A 0.5 ml aliquot of the p-phenylphenol stock solution was transferred to a 250 ml volumetric flask and brought to volume with methylene chloride.

C. (8.5ug/5ml) - A solution containing 1.7 mg diphenylacetic acid in 1 liter methylene chloride was prepared.

D. (50ug/5ml) - A solution was prepared containing 5 mg diphenylacetic acid in 500 ml methylene chloride.

Analytical Procedure - To a known volume of heparinized plasma in 15 ml screw capped centrifuge tube, an aliquot of the methanolic solution of the drug (and its metabolite(s) if gradient elution is used) was added. The plasma was acidified with 1.0M sulfuric acid and 5 ml extraction solution containing the appropriate internal standard was added. The tubes were vortexed for 10 sec and centrifuged for 5 min at 500xg. A 4 ml volume of the organic phase was transferred to a Concentratube^{R} (32) and evaporated to dryness at ambient temperature under a gentle stream of nitrogen.

The residue was dissolved in 100 ul methanol and an aliquot was injected (Table 1).

HPLC Separation - Isocratic Analysis - Ambient temperature was maintained during all assays. The mobile phases used were as follows: for indomethacin, 60:40 methanol: 0.10M acetate buffer, pH5; for sulindac, 60:40 methanol:0.04M acetate buffer, pH4; and for tolmetin, 60:40 methanol: 0.10M acetate buffer, pH5.

Gradient Analysis - All Analyses were performed at an oven temperature of 40^{o}C. Indomethacin and its metabolites - The initial mobile phase consisted of 45% methanol and 55% 0.10M acetate buffer, pH5. This proportion was maintained for 3 min followed by a linear gradient

TABLE 1

Sample Preparation Parameters

Parameters	Drug		
	Indomethacin	Sulindac	Tolmetin
Plasma volume in ml	0.5	0.5	0.25
Concentration of Standards, μg/ml plasma	0.1-3.0	0.1-3.0	1-40
Volume of 1.0M H_2SO_4 in ml	0.25	0.25	0.125
Extraction Solution			
For Isocratic	B	B	D
For Gradient	A	C	D
Injection Volume in μl			
For Isocratic	20-30	20-30	5-8
For Gradient	20	20	10

to 62% methanol in 2 min. This percentage was continued for 10 additional min.

Sulindac and its metabolites - For 10 min a mobile phase consisting of 51% methanol and 49% 0.10M acetate buffer, Ph5, was used. Using a linear gradient, the methanol was increased to 80% in 3 min. This ratio was maintained for an additional 6 min.

Tolmetin and its metabolite - The initial composition of the mobile phase was 22% methanol and 78% 0.10M acetate buffer, pH5. This proportion was used for 5 min, then the methanol percentage was increased to 53% in 2 min and was continued as such for 8 additional min.

Quantitation - A standard curve was constructed by injecting plasma extracts simulating concentrations of the drug (and metabolite(s) if gradient elution). The chromatograms were recorded at a chart speed of 0.5 cm/min. The ratios of peak heights (drug or metabolite to internal standard) were calculated and plotted against the concentration in micrograms per milliliter plasma.

Interferences - The possible interference of normal plasma constituents was tested by the analysis of blank plasma samples. The interference of other drugs was tested by direct injection of methanolic drug solutions or by the analysis of extracts of plasma samples containing therapeutic concentrations.

Recovery - For the recovery study, plasma standards were prepared as described under Analytical Procedure. After evaporation, the residue was dissolved in methanol containing a known concentration of the drug or the metabolite being studied. An aliquot was injected onto the column.

Patient Sample Preparation and Assay - Heparinized plasma samples from patients receiving oral indomethacin, sulindac or tolmetin were processed in duplicate as described under Analytical Procedure. The amount of drug was calculated by comparison with standards prepared daily.

RESULTS AND DISCUSSION

Both the isocratic and gradient procedures require an extraction step. In both cases methylene chloride extracts of plasma acidified with 1.0M sulfuric acid were selected based on optimal recovery of drugs and metabolites (Table 2) with minimum interference from plasma constituents (Figures 1 & 2).

To determine the optimum chromatographic conditions for each drug and its metabolite(s), the effects of pH and molarity of mobile phase buffers or acids on the capacity factor was studied. It can be concluded that:

1. As acidity increases the capacity factor increases and the peaks become broader. Generally a

TABLE 2

Recovery of Drugs and Metabolites

Drug or Metabolite	Recovery,% ± Standard Error
Indomethacin	66.41 ± 0.71
des(chlorobenzoyl) metabolite	100.43 ± 4.99
desmethyl metabolite	97.63 ± 5.66
Sulindac	69.04 + 1.13
Sulfide metabolite	77.61 ± 1.70
Sulfone metabolite	100.03 ± 4.14
Tolmetin	73.06 ± 2.65
Dicarboxylic acid metabolite	93.83 ± 2.88

mobile phase of pH 5 afforded adequate resolution and a reasonable assay time (Figure 3); however, a pH 4 mobile phase was required to resolve sulindac from its sulfone metabolite (Figure 4).

2. Increasing the molarity affected the capacity factor in a complex manner (Figure 5).

3. Elution order can be altered by changing pH (Figure 6) or molarity (Figure 7).

Excellent separation with sharp peaks is possible with the use of reversed-phase chromatography on an octadecylsilane column. Isocratic elution with mobile phases of methanol and acetate buffers is used for the analysis of the parent drugs indomethacin, sulindac and tolmetin (Figure 8).

Isocratic elution also has the advantage of short assay time; however, it is not possible to deter-

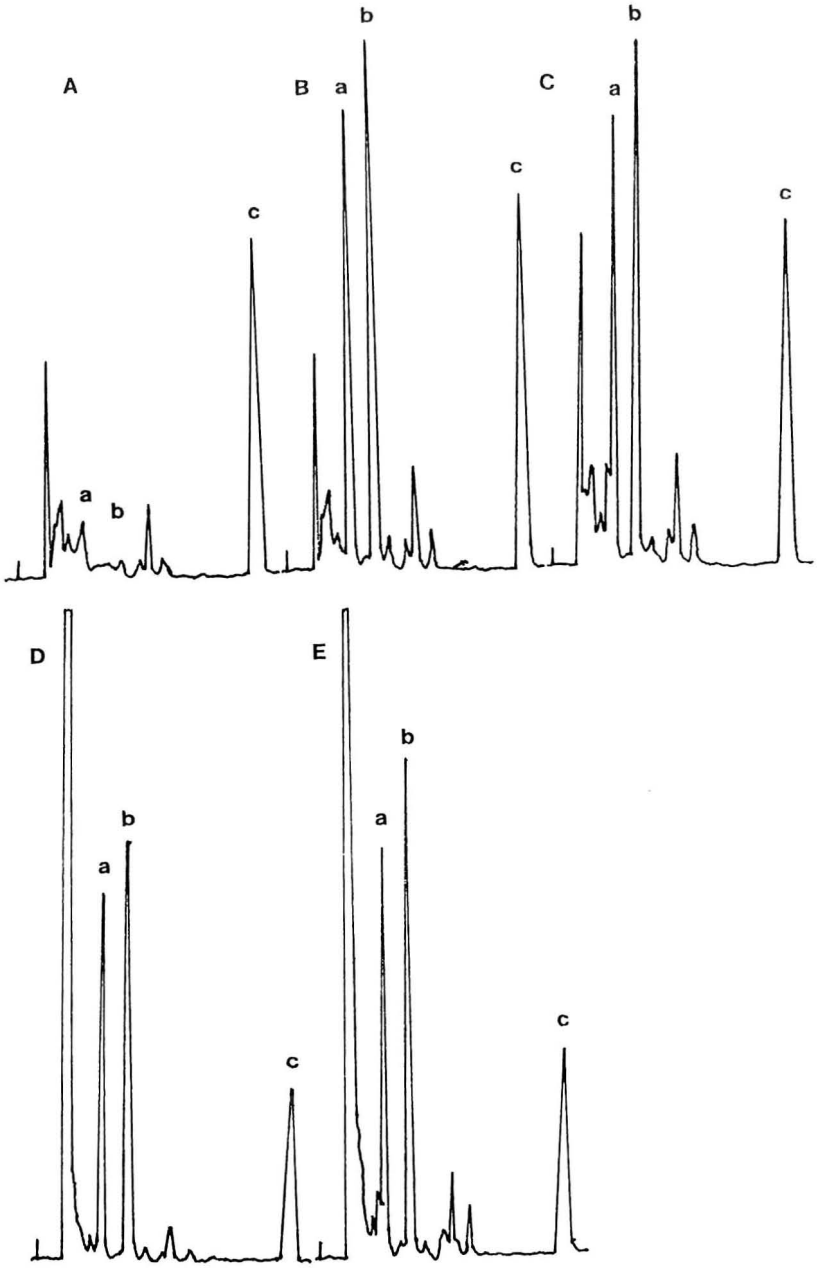


FIGURE 1 Chromatograms of plasma extracts containing tolmetin (3 µg/ml) (a), sulindac (3 µg/ml) (b) and indomethacin (3 µg/ml) (c) using the following solvents for extraction: A, Hexane; B, methylene chloride; C, chloroform containing 5% 2-propanol; D, diethylether, and E, ethyl acetate.

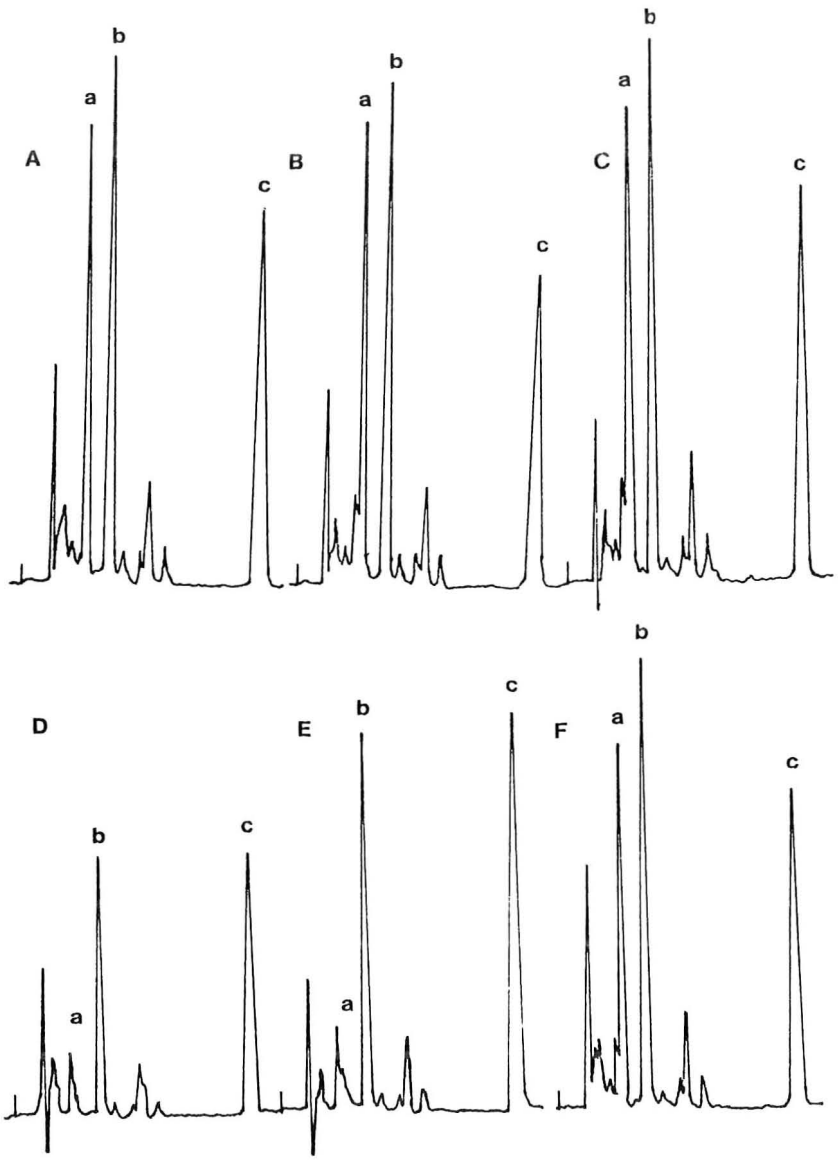
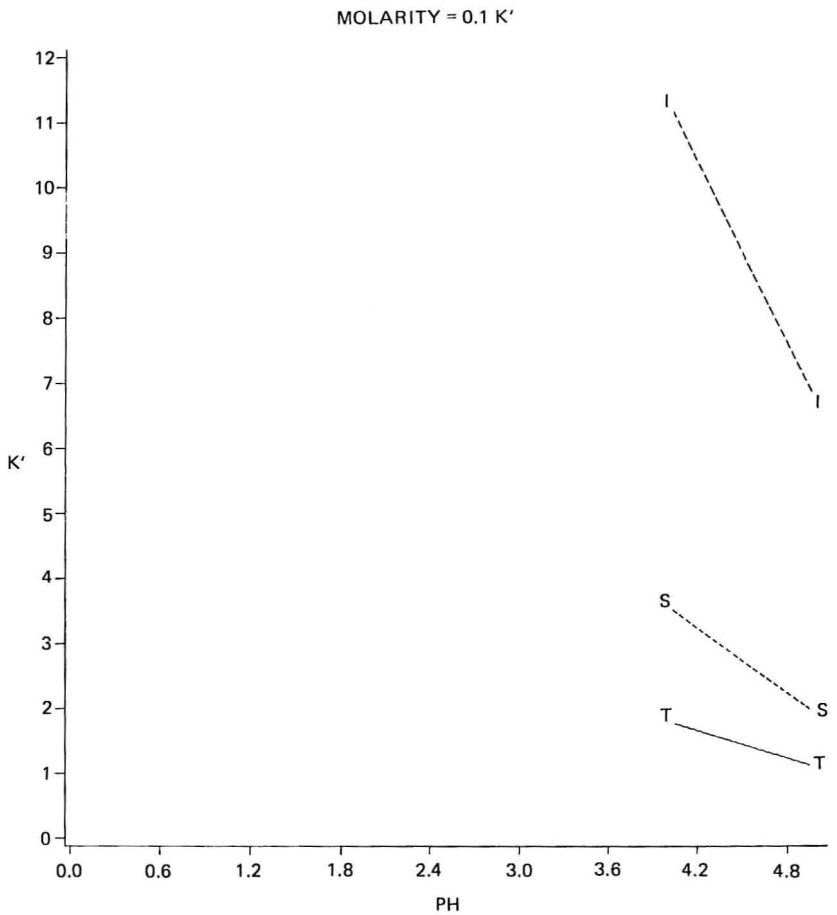
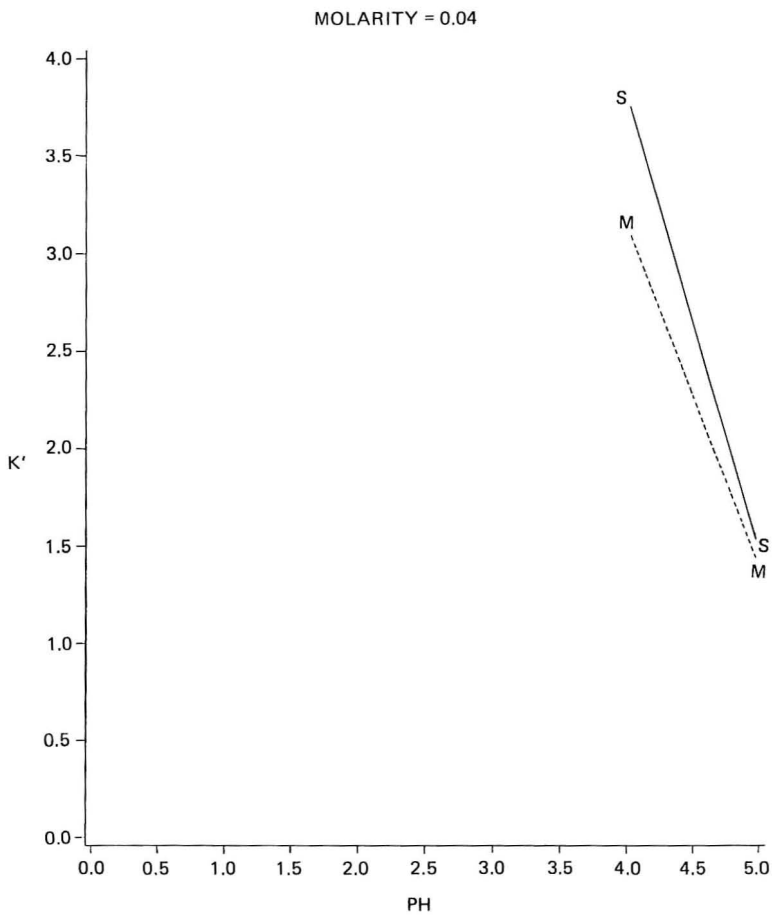


FIGURE 2 Chromatograms of plasma extracts containing tolmetin (3 µg/ml) (a), sulindac (3 µg/ml) (b) and indomethacin (3 µg/ml) (c) using methylene chloride for extraction. Plasma acidified with the following: A, 1.0M sulfuric acid: B, 1.0M phosphoric acid; C, 0.1M acetate buffer (pH4); D, 0.1M phosphate buffer (pH3); E, 0.1M phosphate buffer (pH4); and F, 0.1M citrate buffer (pH 2.5).



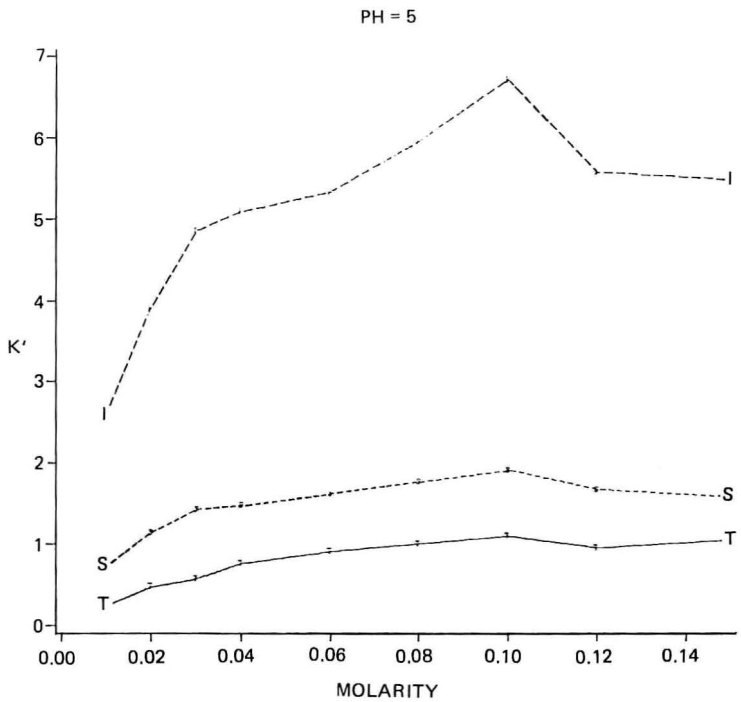
KEY: I = K' FOR INDOMETHACIN S = K' FOR SULINDAC
T = K' FOR TOLMETIN

FIGURE 3 Effect of Ph on K'



KEY: S = K' FOR SULINDAC
M = K' FOR SULFONE METABOLITE

FIGURE 4 Effect of Ph on K'

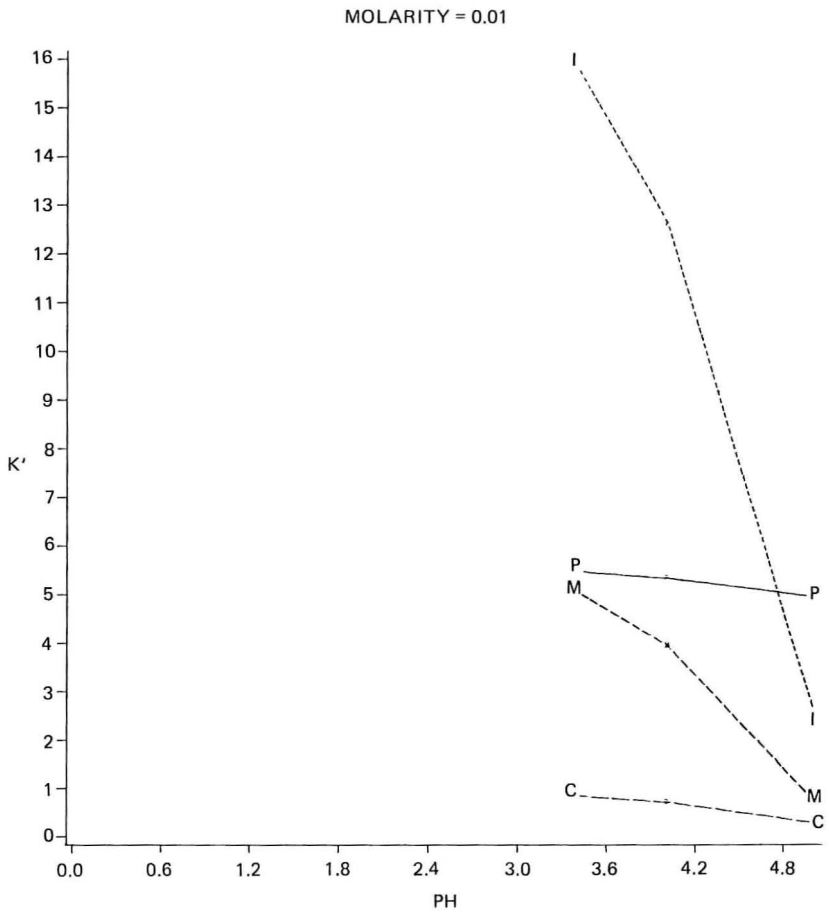


KEY: I = K' FOR INDOMETHACIN S = K' FOR SULINDAC
T = K' FOR TOLMETIN

FIGURE 5 Effect of Molarity on K'

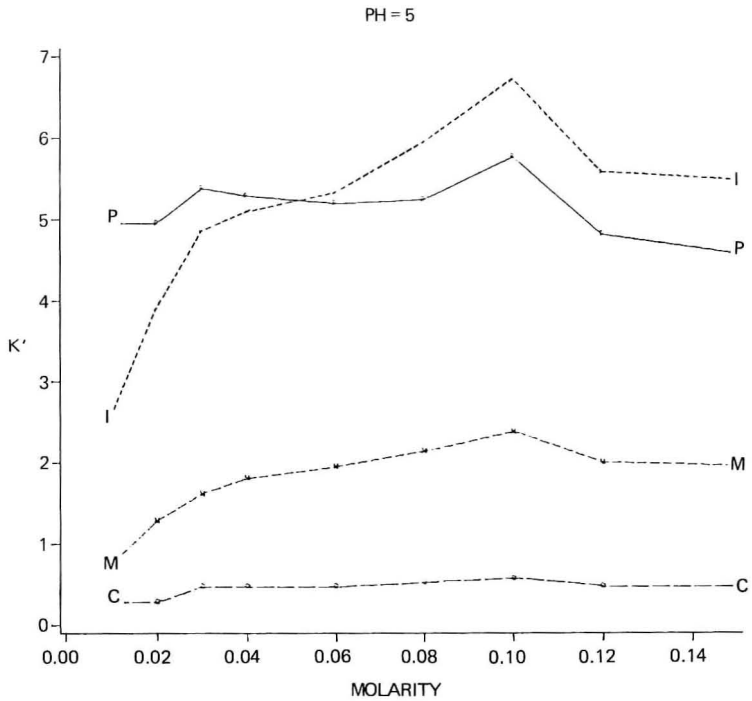
mine all the metabolites for the drugs considered here unless gradient elution is used (Figure 9).

The ratio of peak height of drug or metabolite to the peak height of the internal standard was calculated and plotted versus concentration. Statistical analysis by linear regression indicated excellent linearity (Table 3) and reproducibility (Tables 4,5).



KEY: I = K′ FOR INDOMETHACIN
C = K′ FOR (DESCHLOROBENZOYL) INDOMETHACIN
M = K′ FOR DESMETHYLINDOMETHACIN
P = K′ FOR P - PHENYLPHENOL

FIGURE 6 Effect of Ph on K'



KEY: I = K' FOR INDOMETHACIN
C = K' FOR (DESCHLOROBENZOYL) INDOMETHACIN
M = K' FOR DESMETHYLINDOMETHACIN
P = K' FOR P-PHENYLPHENOL

FIGURE 7 Effect of Molarity on K'

The retention times of the drugs, metabolites and internal standards used in this study under all the assay conditions are reported (Table 6). The possibility of interference due to other commonly used drugs was also studied (Table 7).

Patient samples were analyzed using the methods described here. A patient receiving oral tolmetin 400

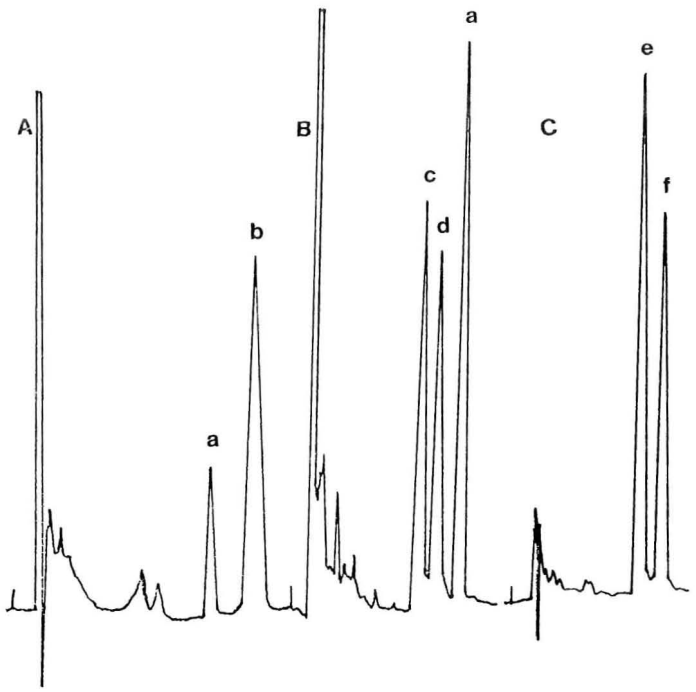


FIGURE 8 Isocratic analysis of antiarthritic drugs from plasma extracts. A, indomethacin (3 μg/ml); B, sulindac (2 μg/ml); and C, tolmetin (20 μg/ml). Key: a, p-phenylphenol (internal standard); b, indomethacin; c, sulfone metabolite; d, sulindac; e, tolmetin; and f, diphenylacetic acid (internal standard).

mg three times daily had a plasma level of 12.23 ± 1.40 μg/ml tolmetin and 7.62 ± 0.43 μg/ml dicarboxylic acid metabolite. On analyzing samples from 3 patients receiving oral indomethacin 25 mg three times daily no detectable levels of the des(chlorobenzoyl) metabolite were observed. Indomethacin was present at a level

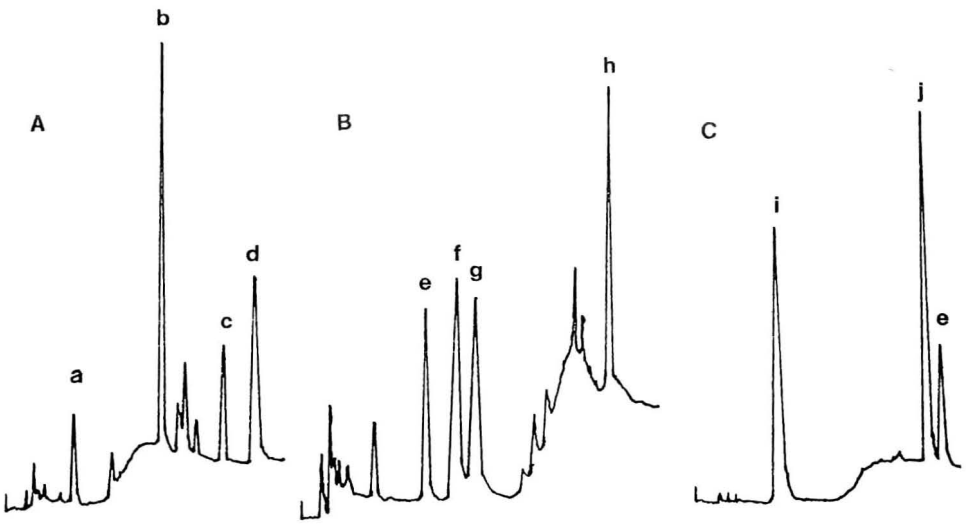


FIGURE 9 Gradient analysis of antiarthritic drugs from plasma extracts. A, indomethacin and metabolites (2 μg/ml); B, sulindac and metabolites (2 μg/ml); and C, tolmetin and metabolite (30 μg/ml). Key: a, des(chlorobenzoyl) metabolite; b, desmethyl metabolite; c, p-phenylphenol (internal standard); d, indomethacin; e, diphenylacetic acid (internal standard); f, sulfone metabolite; g, sulindac, h, sulfide metabolite; i. dicarboxylic acid metabolite; and j, tolmetin.

of 0.10-0.21 μg/ml and the desmethyl metabolite was determined to be 0.26-0.32 μg/ml. The range of plasma levels in 3 patients receiving oral sulindac 150 mg twice daily was 0.39-1.16 μg/ml sulindac, 0.35-1.57 μg/ml sulfide metabolite, an 0.41-1.28 μg/ml sulfone metabolite.

Major advantages of the proposed methods are their simplicity and rapidity. Both the parent drug

Table 3

Statistical Analysis by Linear Regression

Drug or Metabolite	Range of Standards (μg/ml plasma)	Correlation Coefficient, r	Slope	Intercept
Isocratic:				
Indomethacin	0.1-3.0	0.9957	0.7724 ± 0.0170	0.0425 ± 0.0270
Sulindac	0.1-3.0	0.9985	0.3419 ± 0.0043	-0.0170 ± 0.0067
Tolmetin	1-30	0.9985	0.0660 ± 0.0001	0.0027 ± 0.0134
Gradient:				
Indomethacin	0.5-2.0	0.9911	0.7931 ± 0.0376	-0.1278 ± 0.0522
des(chloro)	0.5-2.0	0.9956	0.3910 ± 0.0151	-0.0413 ± 0.0206
desmethyl	0.5-2.0	0.9932	1.7157 ± 0.0712	-0.2289 ± 0.0988
Sulindac	0.5-2.0	0.9945	0.5489 ± 0.0219	-0.0648 ± 0.0319
Sulfide	0.5-2.0	0.9913	0.6318 ± 0.0317	-0.1817 ± 0.0461
Sulfone	0.5-2.0	0.9793	1.8351 ± 0.1433	-0.7979 ± 0.2082
Tolmetin	5-30	0.9996	0.0911 ± 0.0010	0.0527 ± 0.0208
dicarboxylic acid	5-30	0.9985	0.0765 ± 0.0014	-0.0876 ± 0.0267

TABLE 4

Assay Precision of Isocratic Technique

	Theoretical µg/ml	Experimental[a] µg/ml	Standard Error
Indomethacin	0.1	0.092	0.039
	0.3	0.328	0.016
	0.6	0.559	0.057
	1.0	0.966	0.034
	1.5	1.487	0.068
	2.0	2.125	0.008
	3.0	2.940	0.067
Sulindac	0.1	0.146	0.005
	0.3	0.318	0.004
	0.6	0.569	0.040
	1.0	0.986	0.017
	1.5	1.460	0.038
	2.0	1.983	0.038
	3.0	3.039	0.033
Tolmetin	1	1.16	0.023
	3	3.07	0.087
	5	5.15	0.231
	10	9.93	0.191
	15	14.46	0.647
	20	20.11	0.554
	30	30.07	0.237
	35	35.53	0.309
	40	40.02	0.622

[a]Mean of 4 Determinations

and its metabolites can be determined with no interference from many commonly used drugs in a single assay using a standard single wavelength UV detector. The methods described here can be recommended for routine patient monitoring or for pharmacokinetic studies.

TABLE 5

Assay Precision of Gradient Technique

	Theoretical µg/ml	Experimental[a] µg/ml	Standard Error
Indomethacin	0.5	0.47	0.01
	1.0	1.07	0.02
	1.5	1.48	0.02
	2.0	2.00	0.09
Des(chloro) Metabolite	0.5	0.50	0.05
	1.0	1.03	0.02
	1.5	1.44	0.02
	2.0	2.03	0.05
Desmethyl Metabolite	0.5	0.51	0.02
	1.0	1.02	0.03
	1.5	1.44	0.04
	2.0	2.02	0.07
Sulindac	0.5	0.55	0.02
	1.0	0.96	0.01
	1.5	1.42	0.02
	2.0	2.05	0.02
Sulfide Metabolite	0.5	0.60	0.02
	1.0	0.98	0.00
	1.5	1.38	0.05
	2.0	2.09	0.03
Sulfone Metabolite	0.5	0.53	0.01
	1.0	0.94	0.01
	1.5	1.48	0.06
	2.0	2.06	0.00
Tolmetin	5	4.75	0.00
	10	10.08	0.01
	20	20.46	0.09
	30	29.80	0.13
Metabolite	5	5.15	0.23
	10	9.85	0.26
	20	19.91	0.17
	30	30.07	0.64

[a]Mean of 3 Determinations

TABLE 6

Retention Time of the Nonsteroidal Anti-Inflammatory Drugs, Metabolites and the Internal Standards Used in this Study.

Drug, Metabolite or Internal Standard	Retention Time in Minutes					
	Isocratic Elution of			Gradient Elution of		
	Indomethacin	Sulindac	Tolmetin	Indomethacin	Sulindac	Tolmetin
Diphenyl acetic acid*	4.7	9.6	9.7	7.95	6.97	13.93
Indomethacin	14.0	>20	>20	13.83	15.44	>15
des(chlorobenzoyl)	3.3	3.7	3.7	3.98	3.15	10.37
desmethyl	7.1	9.9	16.9	8.89	10.92	>15
p-Phenylphenol*	11.6	9.9	>20	12.43	15.02	>15
Sulindac	6.1	8.5	15.8	8.70	9.52	>15
Sulfide	>20	>20	>20	>20	17.42	>15
Sulfone	5.8	7.6	14.8	8.38	8.47	>15
Tolmetin	4.4	6.0	7.4	7.46	5.90	13.11
Dicarboxylic Acid	S	S	S	S	S	4.65

*Internal Standard

S = Solvent Front

TABLE 7

Drugs Studied for Possible Interference Under the Assay Conditions

	Retention Time in Minutes					
	Isocratic Elution of			Gradient Elution of		
Drug	Indomethacin	Sulindac	Tolmetin	Indomethacin	Sulindac	Tolmetin
Acetaminophen	S	S	S	S	S	4.09
Caffeine	3.4	3.8	4.5	5.11	4.02	10.96
Carbamazepine	7.2	11.1	16.5	9.95	12.51	18.20
Ethosuximide	S	2.8	3.0	3.35	2.69	9.00
Fenoprofen	10.1	15	20	11.82	14.79	> 15
Ibuprofen	>20	>15	> 20	> 20	16.20	> 15
Naproxen	6.6	13.2	16.2	9.45	11.37	> 15
Metabolite	3.2	4.6	4.4	5.96	3.42	10.80
Phenobarbital	3.2	4.0	5.1	5.93	4.24	11.38
Phenytoin	4.6	6.8	9.8	8.08	7.31	14.40
Primidone	2.0	3.5	4.3	4.57	3.49	10.44
Quinidine	S	S	S	2.15	2.02	4.12
Salicylic Acid	S	S	S	S	S	4.06
Theophylline	S	2.6	2.9	2.91	2.54	8.21
Valproic Acid	NA	NA	NA	NA	NA	NA

S = Solvent Front

NA = No Absorbance

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ASSAY OF THE ANTI-INFLAMMATORY COMPOUND CGS 5391B IN BLOOD PLASMA BY AUTOMATED HPLC

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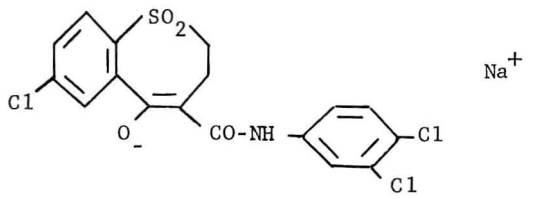
ABSTRACT

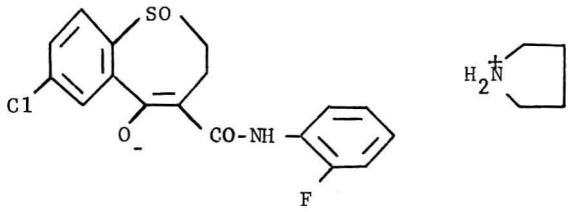
An automated HPLC method for the quantitative determination of the anti-inflammatory compound CGS 5391B in blood plasma was devised and tested. The method provides quantitation in the concentration range of 1 to 200 µg/ml of drug in plasma, with an average recovery of 96.9 ± 6.0%.

INTRODUCTION

CGS 5391B is the sodium salt of 7-chloro-N-(3,4-dichlorophenyl)-2,3,4,5-tetrahydro-5-oxo-1-benzothiepin-4-carboxamide-1,1-dioxide. The structural formula is shown in Figure 1. CGS 5391B is presently being tested for the treatment of arthritis. Reversed phase HPLC proved to be an effective means for quantitative determination of the compound in blood plasma.



CGS 5391B



CGS 5089A (Internal Standard)

FIGURE 1: Structural formulas.

MATERIALS

Chemicals and Reagents

Solutions of CGS 5391B (0.2 and 2.0 mg/ml) and of the internal standard CGS 5089A (0.25 mg/ml) were prepared in absolute ethanol and stored in a refrigerator. The pH 5 buffer used for extractions was freshly prepared each week by mixing 0.1 M citric acid and 0.1 M Na_2HPO_4 solutions in the volume ratio of 1.00 to 1.06. CGS 5089A solution was added to the extraction buffer to provide a concentration of 10 µg/ml of the internal standard. The buffer used for chromatography was 7.0 g of KH_2PO_4 per liter of water, adjusted to pH 2.9 with 85% H_3PO_4. This buffer was

mixed with glass distilled acetonitrile (Burdick and Jackson Laboratories Inc.) in the volume ratio of 35:65 and passed through an 0.5 μ filter (Millipore FH) which was prewetted with acetonitrile. Glass distilled chloroform (Burdick and Jackson Laboratories Inc.), HPLC grade methanol (Fisher Scientific Co.) and house distilled water were used.

Chromatograph

The chromatography system consisted of a Waters Associates Model 710A Intelligent Sample Processor, Model 6000A Solvent Delivery System, Model 440, 254 mμ Absorbance Detector, and a Hewlett-Packard Model 3385A Automation System for plotting and integration. A Whatman pre-column filled with Du Pont Permaphase packing and a Du Pont 250 x 4.6 mm Zorbax 10 μ C8 column were used. Instrument paramenters were: sample processor - 15 μl injection volume, run time 6 minutes; pump - flow rate 2.0 ml/min; detector - sensitivity 0.005 AUFS; integrator - chart speed 1.00 cm/min, zero 10% of scale, attenuation 2 to the third power, slope sensitivity zero, area reject 99980 changed to 10 at 1.8 min, stop at 7.0 min.

METHODS

An aliquot (0.5 ml) of each plasma for analysis and six to eight aliquots of control plasma for calibration standards were pipetted into 40 ml screw-cap centrifuge tubes. CGS 5391B stock solutions were added to the calibration standard tubes. One ml of extraction buffer containing internal standard was added to each plasma, followed by 10 ml of chloroform. The tubes were

closed with teflon-lined screw caps and rotated 15 minutes at 15 RPM. The phases were separated by centrifugation for 10 minutes at 1300-1400 RPM.

The upper, aqueous layer and associated solids were removed by aspiration. The chloroform extracts (ca 9 ml) were transferred into 12 ml centrifuge tubes and evaporated to dryness under nitrogen, with a bath temperature of 45-55° C. To each tube was added 2.0 ml of acetonitrile and the tubes were vortexed for 5 seconds. To allow complete dissolution of the drug, residues from human and monkey plasma required storage for 1.5 hours at room temperature, or overnight in a freezer before further processing. A different procedure was necessary for rat plasma. Residues from rat plasma dissolved readily but the accompanying CGS 5391B was subject to decomposition. The reasons for the observed differences are not known. Reconstituted residues from rat plasma were either chromatographed immediately or stored in a freezer and then chromatographed with a maximum interval of two hours at room temperature.

The ratio of peak areas for CGS 5391B to internal standard were calculated and linear least squares curve fitting was carried out with the calibration data. Concentration values for the unknowns were then calculated from the slope of the regression line.

The composition of the HPLC peak corresponding to CGS 5391B in the plasma of dosed patients was examined. A Waters Associates C-18 Sep-Pak Cartridge was washed by passing through in succession 3 x 5 ml of acetonitrile, 3 x 5 ml of methanol and 3 x 5 ml of water. The cartridge was allowed to drain between portions. Approximately 100 ml of chromatograph eluate containing about 10 µg

of apparent CGS 5391B was obtained by repeated collections. The eluate was diluted three fold with water and passed through the washed Sep-Pak cartridge, which was then rinsed with 3 x 5 ml of water. Retained materials were then eluted with 2 ml of methanol. The Sep-Pak eluate was compared with authentic drug by HPLC in three chromatographic systems: (1) methanol/water (60:40) on Applied Science Laboratories Inc. Lichrosorb C-18; (2) methanol/water/acetic acid (80:20:1) on Waters Associates μ Bondapak Phenyl; and (3) methylene chloride on E. Merck Lichrosorb Si 60.

RESULTS AND DISCUSSION

CGS 5391B and the internal standard CGS 5089A are chemically related (Figure 1) but are sufficiently different in structure to permit good resolution by reversed phase HPLC. A representative chromatogram showing distinct, well-resolved peaks for the two compounds is shown in Figure 2. Extracts of blank plasma showed no significant interference, and chromatograms of samples from dosed humans and animals were similar to that shown in Figure 2.

Six to eight calibration standards were prepared on each analysis day to bracket and thoroughly cover the expected range of unknowns (Figure 3). The slopes of regression lines obtained on different occasions were in good agreement, intercept values were insignificant and the correlation coefficients demonstrated good definition of the calibration curves (Table 1).

The assay procedure was further tested by blind analysis of human plasma samples to which 1.2 to 75.0 μg/ml of CGS 5391B had been added. Spiked plasma of each concentration was divided into

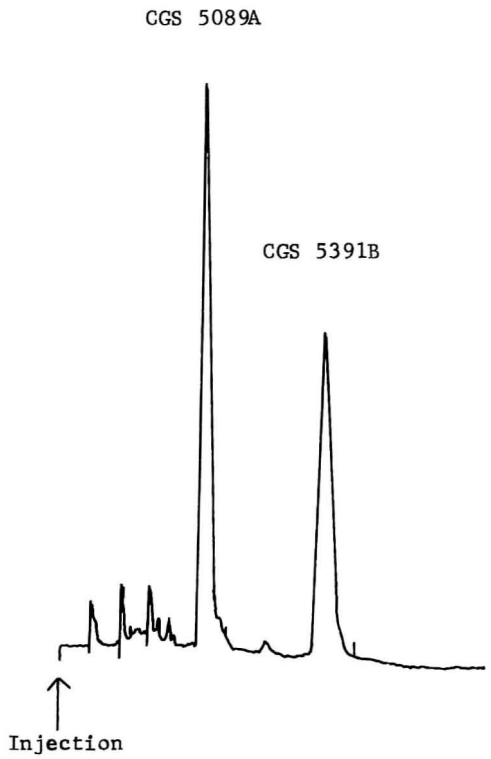


FIGURE 2: Chromatogram of an extract of monkey plasma containing 10 μg/ml of CGS 5391B.

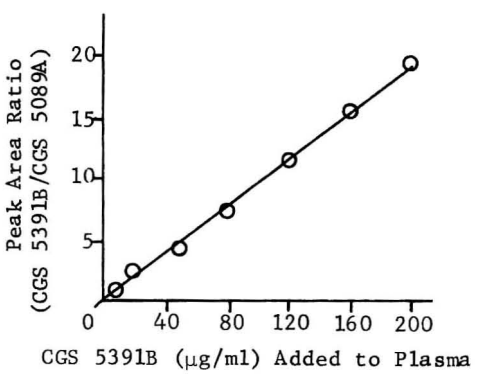


FIGURE 3: A typical standard curve for the assay of CGS 5391B in human plasma.

TABLE 1

CGS 5391B Assay Calibration Data

Plasma	Number of Analysis Occasions	Average ± Relative Standard Deviation		
		Slope	Intercept	Correlation Coefficient
Human	13	0.0946 ± 9	-0.08 ± 86	0.9988 ± 0.1
Rat	10	0.0934 ± 4	-0.12 ± 69	0.9986 ± 0.1
Cynomolgus monkey	4	0.0817 ± 6	0.06 ± 41	0.9994 ± 0.0

aliquots for replicate analyses to allow assessment of precision as well as accuracy. The resulting samples were number coded in random sequence and frozen to await analysis. The concentrations were made known to the analyst only after the assay results were recorded. Similar experiments were done on three occasions and the results demonstrated good accuracy and good precision (Table 2).

The identity and homogeneity of the CGS 5391B HPLC peak from plasma of dosed patients was established by comparing the collected peak material to authentic compound by HPLC in three systems of diverse nature, as described previously. The recovered material gave a single peak corresponding in retention to authentic CGS 5391B in all systems.

The validation studies showed that the assay provides sufficient accuracy and precision for pharmacokinetic studies of CGS 5391B in plasma. The blind validation data indicated satisfactory performance in the 1 to 75 μg/ml concentration range, while the

TABLE 2

Percent Recoveries for Blind Analysis of Human Plasma Spiked with CGS 5391B

CGS 5391B Added (μg/ml)	Number of Analyses	Average Percent Recovery ± Relative Standard Deviation
1.2	3	102.7 ± 4.5
1.8	3	100.0 ± 6.0
3.0	3	99.0 ± 5.6
4.4	3	95.0 ± 3.8
5.0	3	94.7 ± 3.2
6.0	9	92.6 ± 5.1
17.5	3	87.3 ± 2.4
18.0	3	99.7 ± 6.4
55.0	3	91.3 ± 1.3
75.0	5	107.0 ± 1.9
	38	96.9 ± 6.0

linearity and precision of calibration curves showed the method performed well up to 200 μg/ml. The use of an automatic sample injector and electronic integrator proved very convenient because the chromatography required only infrequent attention, and the preparation of additional samples could be done concurrently with operation of the chromatograph.

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IMPROVED HIGH-PRESSURE LIQUID CHROMATOGRAPHIC ASSAY OF SERUM 25-HYDROXYCHOLECALCIFEROL AND 25-HYDROXYERGOCALCIFEROL AFTER REVERSE-PHASE SEP-PAK C_{18} CARTRIDGE PREPARATION OF SAMPLE

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ABSTRACT

A simplified method is described for extracting and purifying 25-hydroxycholecalciferol and 25-hydroxyergocalciferol from serum for quantitation by high-pressure liquid chromatography. The method involves extracting and purifying these metabolites from serum (1-10 ml) with a reverse-phase octadecylsilane bonded silica cartridge (Sep-Pak C_{18}). This method is faster than a previously described method involving extraction with dichloromethane and purification by Sephadex LH-20 chromatography. The correlation between the two methods was excellent ($r^2 = 0.96$, $p<0.0001$). The coefficient of variation for the new method is 4.3%. The new method allows measurement of 25-hydroxyergocalciferol from human serum since both 25-hydroxycholecalciferol and 25-hydroxyergocalciferol are extracted equally. This allows the use of [^{3}H]25-hydroxycholecalciferol to monitor the recovery of both the D_2 and the D_3 forms of the metabolite.

INTRODUCTION

High-pressure liquid chromatography (HPLC) (1) has been shown to resolve 25(OH)D_3 and 25(OH)D_2 from each other and from other

metabolites of the vitamin D_2 and D_3 family (2,3). It has been used to measure 25(OH)D_2 and 25(OH)D_3 in bovine (4) and human (5-10) sera. HPLC methods are more precise and expedient than previously reported competitive protein-binding (CPB) methods for assay of 25-hydroxyvitamin D (5-10). CPB methods do not distinguish the D_2 from the D_3 forms of 25-hydroxyvitamin D, whereas HPLC methods can. Quantitation by HPLC requires extraction and purification of 25(OH)D_3 from serum to a degree that analytical resolution of the sample becomes possible. The published HPLC methods are based upon lipid extraction and some purification of the extract via column chromatography with Sephadex LH-20 (5,6,8), Sephadex LH-20 and Lipidex 5000 (10), or silica gel (9) prior to analytical chromatography. These methods suffer in that the measurement of 25(OH)D_2 relies upon the assumption that the fractional recoveries of 25(OH)D_2 and 25(OH)D_3 through sample extraction and purification are the same. This assumption allows the use of [^{3}H]25(OH)D_3 to monitor overall recovery of both 25(OH)D_2 and 25(OH)D_3. The assumption is hazardous and can lead to error in measurement of 25(OH)D_2 as has been pointed out by Stryd and Gilbertson (11). One group (5) has synthesized and used [^{3}H]25(OH)D_2 to validate this assumption for their preparative procedure. However, [^{3}H]25(OH)D_2 is not commercially available and the routine use of [^{3}H]25(OH)D_3 to monitor 25(OH)D_2 recovery is not valid without demonstration of equal recovery of both congeners. An improved method for extracting and purifying 25(OH)D_2 and 25(OH)D_3 from human serum is presented in this report. It involves precipitation of serum

proteins with methanol and rapid extraction and purification of 25(OH)D_2 and 25(OH)D_3 with a reverse-phase octadecylsilane bonded silica cartridge (Sep-Pak C_{18}). This gives results for 25(OH)D_3 indistinguishable from those obtained by one of the published procedures (8), which employed Sephadex LH-20 chromatographic purification. The time and labor per assay are reduced substantially. Since the Sep-Pak C_{18} preparative method reported herein extracts 25(OH)D_2 and 25(OH)D_3 equally, recovery of both metabolites can be monitored by use of [^{3}H]25(OH)D_3 added initially to serum.

MATERIALS

Reagent grade methanol, isopropyl alcohol, chloroform, and dichloromethane were redistilled in glass. Hexanes (Mallinckrodt, Inc., St. Louis, Mo., certified), toluene, and tetrahydrofuran (reagent) were used as received. Chromatography columns of Sephadex LH-20 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) were used as previously reported (8). Reverse-phase cartridges (1.2 x 0.9 cm) with an octadecylsilane bonded silica adsorbent (Sep-Pak C_{18}, Waters Associates, Inc., Milford, Mass.) were washed with 10 ml of tetrahydrofuran followed by 10 ml of glass-distilled water. High-pressure liquid chromatography was effected with two μPorasil columns (0.4 by 25 cm, Waters Associates) connected in series. [^{3}H]25(OH)D_3 (17 Ci/mmole, New England Nuclear, Boston, Mass.) was used after chromatographic purification through two

μPorasil columns. Concentrations of solutions of purified $25(OH)D_2$ and $25(OH)D_3$ were determined by uv absorbance at 265 nm (Beckman Model 24 Spectrophotometer); ε_{265} of 18,000 was used (8). [^{3}H]Vitamin D_3 (22 Ci/mmol, New England Nuclear, Boston, Mass.) and [^{3}H]$1,25(OH)_2D_3$ (110 Ci/mmol, New England Nuclear, Boston, Mass.) were used as received. Both $25(OH)D_2$ and $25(OH)D_3$ were gifts from the Upjohn Co., Kalamazoo, Mich. Serum obtained from subjects (informed consent obtained) was stored at -20°C until use.

METHODS

High-pressure liquid chromatography of purified serum extracts was performed as previously described (8) except that isopropyl alcohol:hexanes (4:96, v:v) was used as eluent. Chromatography was conducted at room temperature with a solvent flow rate of 1.0 ml/min (800 psi). Under these conditions $25(OH)D_2$ and $25(OH)D_3$ were eluted 16 and 18 minutes following injection, respectively. For each analysis the fraction corresponding to $25(OH)D_3$ (17.5 to 20.0 ml) was collected. The recovery of [^{3}H]$25(OH)D_3$ was then determined by liquid scintillation counting with correction for quenching. The areas of the $25(OH)D_2$ and $25(OH)D_3$ peaks were determined by triangulation (peak absorbance at 254 nm x peak width at half-height in ml); ε_{254} of 16,900 was used (8). Extraction and purification of $25(OH)D_3$ from serum on Sephadex LH-20 chromatography was performed as previously reported

(8). Extraction and purification of 25(OH)D_2 and 25(OH)D_3 from serum with the Sep-Pak C_{18} cartridges were performed as follows. All steps were carried out at room temperature. Each serum sample (1-10 ml) was labeled with 5000 dpm of [^{3}H]25(OH)D_3 (in 0.02 ml of isopropyl alcohol) to monitor overall recovery of 25(OH)D_3. The sample was added dropwise to a volume of methanol two times the volume of serum with vortexing to precipitate serum proteins. The concentration of methanol was adjusted to 50% by addition of glass-distilled water, and the precipitated proteins were removed by centrifugation at 1000 x g for 10 min. After separation of pellet and supernatant fractions, the pellet was washed with a volume of methanol:water (1:1, v:v) two times the volume of serum. The supernatant fraction from this washing was combined with the initial supernatant fraction and both were decanted into a glass syringe which was coupled to a Sep-Pak C_{18} cartridge. The supernatant fraction was passed through the cartridge with gentle pressure on the plunger. The cartridge was washed with 10 ml of methanol:water (7:3, v:v). Then, 25(OH)D_2 and 25(OH)D_3 were eluted with 10 ml of methanol:water (85:15, v:v) and concentrated to dryness in a stream of nitrogen. The residue was dissolved in 0.25 ml of isopropyl alcohol:hexanes (4:96, v:v), and this solution was analyzed by HPLC (8).

RESULTS

Application of [^{3}H]25(OH)D_3 in methanol:water (1:1, v:v) to the Sep-Pak C_{18} cartridge resulted in almost quantitative

retention of tritium. Washing the Sep-Pak C_{18} cartridge with 10 ml of methanol:water (7:3, v:v) resulted in recovery of 6% of total activity in the effluent. Washing of the cartridge with 10 ml of methanol:water (85:15, v:v) eluted 93% of the total activity. [^{3}H]Vitamin D_3 and [^{3}H]1,25$(OH)_2D_3$ were also retained quantitatively when applied with methanol:water (1:1, v:v). [^{3}H]1,25$(OH)_2D_3$ was eluted along with [^{3}H]25$(OH)D_3$. Only ten percent of the [^{3}H]vitamin D_3 was eluted by methanol:water (85:15, v:v). Virtually 100% was eluted by methanol. HPLC of serum extracts prepared with the Sep-Pak C_{18} method yielded chromatograms with clear resolution of 25$(OH)D_2$ and 25$(OH)D_3$ peaks upon a substantially flat baseline (Figure 1).

The extraction and purification procedure for serum 25$(OH)D_3$ by the Sep-Pak C_{18} cartridges was compared with that by Sephadex LH-20 (8). Both methods were used to assay sera (1 ml sample volume) obtained from 15 healthy adults. Recovery of tritium activity averaged 52.5% for the Sep-Pak C_{18} purification as compared with 31% for Sephadex LH-20 purification. The range of concentrations of 25$(OH)D_3$ in the 15 serum samples was 14-57 ng/ml. Linear regression analysis of the results (Figure 2) demonstrated no statistically significant difference in the assay of sera prepared by these two methods (intercept = -0.2, slope = 1.006, $p<0.0001$, $r^2 = 0.96$). The coefficient of variation (C.V.) for seven separate assays of a single serum (mean 25$(OH)D_3$ of 37 ng/ml) purified by the Sep-Pak C_{18} method was 4.3% compared to the C.V. of 5.1% for the Sephadex LH-20 method (8).

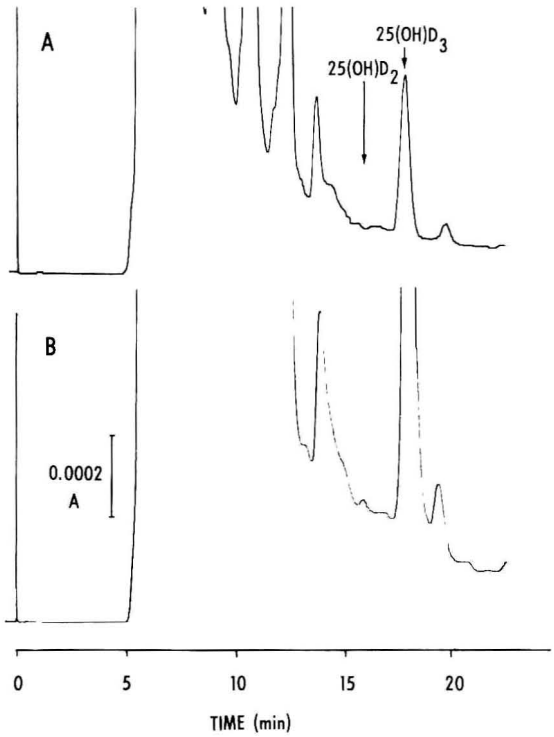

FIGURE 1. HPLC chromatograms of 2 aliquots of the same human serum prepared by the Sep-Pak C_{18} method. A, 1-ml aliquot (25(OH)D_2 not detected; 25(OH)D_3=14 ng; recovery=55%. B, 10-ml aliquot (25(OH)D_2 peak area far less than 3 ng; 25(OH)D_3=120 ng; recovery=48%). See Methods Section for conditions of chromatography.

To test the hypothesis that 25(OH)D_2 and 25(OH)D_3 are recovered equally from serum by the present method, assays were performed on a serum to which known amounts of these metabolites were added. These metabolites were initially undetectable in assay of 1 ml of the serum used in this experiment. The serum was obtained from a patient with primary biliary cirrhosis of greater

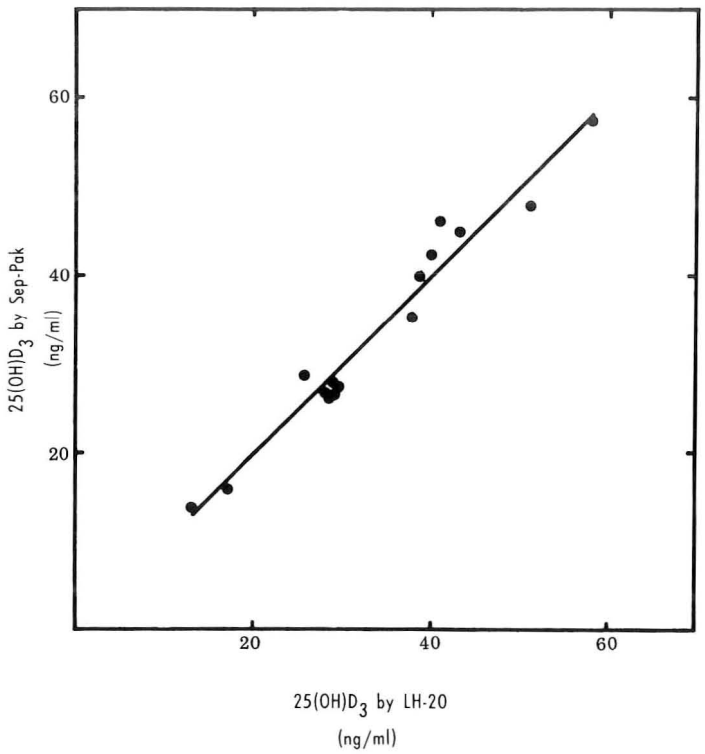

FIGURE 2. Concentration of $25(OH)D_3$ in serum determined by HPLC analysis. Data obtained by Sephadex LH-20 purification (X) are plotted along the abscissa. Data obtained by Sep-Pak C_{18} purification (Y) are plotted along the ordinate. ($Y = -0.2 + 1.006\ X$, $r^2 = 0.960$, $p<0.0001$).

than 3 years' duration. Marked depression of serum 25-hydroxy-vitamin D is a reported consequence of this illness (12). To 1-ml aliquots of this serum were added purified $25(OH)D_2$ and $25(OH)D_3$ in known but varying amounts (Table 1). These samples were assayed and the ratio of $25(OH)D_2$ to $25(OH)D_3$ recovered was determined for each sample. The ratios of $25(OH)D_2$ to $25(OH)D_3$ added to serum and recovered from serum were not statistically

TABLE 1

Recovery of $25(OH)D_2$ and $25(OH)D_3$ Added to Serum (see text for experimental design).

$25(OH)D_2$ (ng added)	$25(OH)D_3$ (ng added)	$\frac{25(OH)D_2}{25(OH)D_3}$ added*	$\frac{25(OH)D_2}{25(OH)D_3}$ recovered*
57.0	13.8	4.13	4.26
57.0	13.8	4.13	3.87
42.8	27.5	1.56	1.69
42.8	27.5	1.56	1.53
28.5	55.0	0.52	0.59
28.5	55.0	0.52	0.48
14.3	82.6	0.17	0.18
14.3	82.6	0.17	0.20
14.3	110	0.13	0.13
14.3	110	0.13	0.15

*Linear regression of ($\frac{25(OH)D_2}{25(OH)D_3}$ recovered upon $\frac{25(OH)D_3}{25(OH)D_3}$ added): slope = 0.981, intercept = 0.03, r^2 = 0.995.

different. Since the Sep-Pak C_{18} preparative method recovers $25(OH)D_2$ and $25(OH)D_3$ equally, $[^3H]25(OH)D_3$ can be used to determine the recovery of $25(OH)D_2$.

To test the hypothesis that as much as 10 ml of serum could be extracted for assay with the present method, 1-ml and 10-ml aliquots were prepared from 3 different sera and assayed for $25(OH)D_3$ concentration. Essentially the same results were obtained for the 3 sera (25.4 and 24.9 ng/ml, 28.6 and 29.1 ng/ml, 38.7 and 36.6 ng/ml for the 1-ml and 10-ml aliquots, respectively). This demonstrates that the performance of the Sep-Pak C_{18} cartridge is independent of sample volume to at least 10 ml.

If 10 ml of serum are prepared by this technique, as little as 0.6 ng/ml of either $25(OH)D_2$ or $25(OH)D_3$ should be reliably

measured by HPLC and uv detection (on the assumption of 50% recovery and of a lower limit of detector sensitivity of 3 ng) (8). To test this lower limit of sensitivity, serum with a very low concentration of $25(OH)D_2$ was assayed. A 10-ml aliquot of this serum had no measurable $25(OH)D_2$ (Figure 1). To another 10-ml aliquot of this serum was added 7.3 ng of purified $25(OH)D_2$. Results from the assay of this sample were 3.6 ng with 48% recovery. Total amount assayed was 7.5 ng, which is within 3% of the expected result.

The Sep Pak C_{18} preparative method and HPLC assay for $25(OH)D_2$ and $25(OH)D_3$ were applied to 1-ml samples of 49 sera from healthy individuals (22-66 years of age) residing in San Antonio and the surrounding area. Blood was sampled in April, August, and November. Samples were obtained from many of the individuals during all three periods (Table 2). Serum 25(OH)D concentration (the sum of $25(OH)D_2$ and $25(OH)D_3$) was lower in April than in either August or November ($p<0.001$). There was no significant difference between the August and November values. The majority of individuals had no measurable $25(OH)D_2$, regardless of season. When $25(OH)D_2$ was found to be present, it contributed little to the total 25(OH)D concentration. The highest concentration of $25(OH)D_2$ was 11.3 ng/ml, which was 36% of that subject's total 25(OH)D concentration.

All of the sera sampled in August and November were analyzed for differences in 25(OH)D concentration as a function of sex. The mean 25(OH)D concentration was 27.1 ng/ml $\pm$ 1.6 (SEM) in men (n=27) and 31.9 ng/ml $\pm$ 1.6 in women (n=18). These are

TABLE 2

Total 25(OH)D and 25(OH)D_3 Concentrations(ng/ml) in Normal Adults*

Month	Total 25(OH)D	25(OH)D_3	Sera with measurable 25(OH)D_2**
April			
mean ± sem (n=20)	21.4 ± 1.5	19.6	5
range	12.8 - 36.4		
August			
mean ± sem (n=18)	28.0 ± 2.0	26.7	3
range	14.8 - 42.2		
November			
mean ± sem (n=30)	29.4 ± 1.5	29.4	1
range	12.1 - 47.7		

* Assay of 1 ml serum from 49 healthy adults living in or near San Antonio, Texas.

** Measurable if at least 3 ng eluted. Another 7 sera had 25(OH)D_2 peaks smaller than 3 ng.

statistically different with $p<0.05$. Linear regression analysis of 25(OH)D concentration versus age showed no correlation.

DISCUSSION

Extraction and purification of serum 25(OH)D_3 by the Sep-Pak C_{18} method in preparation for HPLC is an improvement over the previously-reported Sephadex LH-20 method (8). Sephadex LH-20 chromatography imposes a limitation upon the rate at which sera can be assayed. Sephadex LH-20 purification involves reuse of the columns and requires stripping with one solvent and re-equili-

brating with yet another solvent. This is the major inconvenience in time and labor encountered in the routine use of these columns. Although an assay can proceed from serum to HPLC within half a workday (with 6 prepared columns, 6 sera require 4 to 5 hours), preparation for reuse of the Sephadex LH-20 columns requires an additional 11 to 12 hours.

The Sep-Pak C_{18} purification method requires minimal preparation of the cartridges. Sera in groups of 6 to 10 can be processed through purification in about 3 hours, a rate faster than the rate at which HPLC analysis can be performed upon the purified samples. Therefore, the rate at which sera can be assayed is not limited by extraction and purification when the Sep-Pak C_{18} method is used.

Another advantage offered by the Sep-Pak C_{18} preparative method is that both $25(OH)D_2$ and $25(OH)D_3$ recoveries can be monitored with $[^3H]25(OH)D_3$, which is commercially available.

It has been demonstrated in this laboratory that at least 3 ng of $25(OH)D_3$ must be eluted to insure reproducibility and linearity of the uv detector response (8). With the Sep-Pak C_{18} preparative method the average recovery of $25(OH)D_2$ and $25(OH)D_3$ from serum is 52%. The lower limit of concentration for each metabolite in serum that can be measured is dependent upon the volume of serum extracted. For a 1-ml serum sample this lower limit of sensitivity is 6 ng/ml. For a 10-ml serum sample this lower limit is decreased to 0.6 ng/ml. The concentration of 25(OH)D in serum of normal individuals has been reported by various groups in North America, Europe and Japan (5,6,8-10,12-

22). The concentration of 25(OH)D has been found to be lower in Europe than in North America, and lower in winter and spring than in summer and autumn (Table 3). The lower limits of 25(OH)D concentration were less than 6 ng/ml in only 3 of the 17 cited studies. The lowest reported concentration for a normal subject was 3 ng/ml (in England during the winter) (17). No lower limit of "normal" in North American populations studied was less than 6 ng/ml. For practical purposes a 1-ml sample of serum prepared and assayed by the procedure reported herein would be sufficient to determine whether 25(OH)D concentration is depressed. With a

TABLE 3

Series Reporting 25(OH)D Concentrations in Various Populations.

Location	Season (if given)	25(OH)D range* (ng/ml)	reference**
Madison, Wis.	Summer	16 - 42	5
	Winter	19.6 - 41.9	10
Rochester, Minn.	Summer	21.2 - 29.6	6
San Antonio, Tx.	---	11 - 52	8
Toronto	Winter	8.2 - 23.8	9
Chicago	---	17.6 - 40	12
St. Louis	Spring	11 - 55	13
	---	8.2 - 29	16
Boston	---	18 - 36	14
Tuscon, Ariz.	---	25 - 40	20
France	---	6.6 - 23.4	15
Belgium	Spring	5.2 - 21.6	21
United Kingdom	Winter	3 - 22	17
	Spring	6.6 - 25.2	18
	Autumn	10.8 - 41.8	18
	---	4 - 26.4	19
Japan	---	13 - 33	22

* Range observed, if reported, otherwise ± 2 SD from mean.

** First 5 series employed HPLC methods, the others employed CPB methods.

10-ml serum sample, concentrations of $25(OH)D_2$ or $25(OH)D_3$ as low as 0.6 ng/ml can be reliably determined, a sensitivity approaching that reported for many of the CPB assays (13-15,17,20).

ACKNOWLEDGMENTS

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1. Abbreviations used:
   HPLC, high-pressure liquid chromatography;
   CPB, competitive protein-binding;
   $25(OH)D_3$, 25-hydroxycholecalciferol;
   $[^3H]25(OH)D_3$, 25-hydroxy[26(27)-methyl-^3H]cholecalciferol;
   $25(OH)D_2$, 25-hydroxyergocalciferol;
   $[^3H]$vitamin D_3, [1,2-^3H]cholecalciferol;
   $[^3H]1,25(OH)_2D_3$, 1α,25-[26,27-^3H]-dihydroxycholecalciferol.

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LARGE VOLUME INJECTION OF BIOLOGICAL SAMPLES DISSOLVED IN A NON-ELUTING SOLVENT : A WAY TO INCREASE SENSITIVITY AND A MEANS OF AUTOMATING DRUG DETERMINATION USING HPLC

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ABSTRACT

The injected volume of a sample dissolved in the mobile phase of an HPLC system must be maintained as small as possible so as to minimize the loss in efficiency. Generally this requirement limits the sensitivity of HPLC methods devoted to trace quantity determinations of drugs in biological fluids. In order to avoid this limitation and to increase the effective sensitivity of HPLC methods for determination of drugs such as antrafenine, nifuroxazide and cipropride, the samples were dissolved in a non-eluting solvent and a large volume (> 100 µl) was injected on to the chromatographic column.

The above-mentioned compounds and their internal standards were dissolved in a series of eluting and non-eluting solvents and increasing volumes (5 to 1000 µl) were injected. Peaks corresponding to injections made in an eluting solvent showed retention times independent of the injection volume but their variances increased with the volume injected. In contrast, peaks corresponding to injections made in a non-eluting solvent, similar to the mobile phase, had a variance independent of the injection volume but their retention times increased linearly with the injection volume. The repeated injection of such non-eluting solvents had no influence on chromatographic behaviour. Peaks corresponding to compounds injected in a non-eluting solvent made with components different from those of the mobile phase had a variance independent of the injection volume but their retention times varied both with the injection volume and with the interval between injection.

The application of non-eluting solvents has been defined theoretically and it has been demonstrated that solutions composed

of 25 % of the mobile phase diluted with the least eluting of its components act as non-eluting solvents and can be injected in large volume without loss in efficiency. This feature could be used to inject all the samples volume or only part of it, manually or automatically, since any automatic injector can be used with large volumes.

Thus, using the relatively simple procedure of making injections with a non-eluting solvent it is possible to increase both sensitivity and the rate of sample analysis.

INTRODUCTION

The interest of high pressure liquid chromatography (HPLC) as a useful tool for drug determination in biological samples is demonstrated by the very large number of papers published monthly. However, despite this apparent success, HPLC is sometimes limited by lack of sensitivity and the rate of routine analysis.

In most of the HPLC methods described, part of the lack of sensitivity is due to the method used to introduce the sample on the column. At the end of the purification and concentration step, generally an extraction by an organic solvent, the sample is evaporated to dryness. Then, in order to inject the extract on the column, it is dissolved in an organic solvent or in the mobile phase (which are both eluents for the column). Although some authors use less, at least 50 to 100 µl of liquid is needed to easily obtain a complete and reproducible dissolution of the extracted sample. Apart from a small number of drugs which normally have a high concentration in biological samples with high U.V. absorbance or high fluorescence, the size of the injection volume is a problem. The injection of all the diluted sample will involve a loss of efficiency, this is because if it is injected in an eluent, the sample will begin to elute before the end of the injection. This extra column effect creates a loss in efficiency (then in selectivity) and a relative decrease in sensitivity (1 to 5). The injection of an aliquot small enough to maintain the efficiency (and selectivity) will involve a loss of sensitivity. In front of this dilema, the chromatographer generally uses a compromise in order to obtain the least loss of efficiency.

In order to solve these problems of sensitivity and rate of analysis, an easy alternative was investigated during the development of automated sensitive analytical HPLC methods for a series of drugs in biological fluids (6, 7). The main idea was to dissolve the extracted sample in a large volume of a "non-eluting" solvent and to inject all this volume manually or part of it by means of an automatic injector.

In this study the concept of "non-eluting" solvent is further defined and its experimental consequences are considered. For this purpose, the investigation was divided into three parts :

A) Influence of the injected volume of eluting or "non-eluting" solvent on retention volume and peak broadening.

B) Influence of repetitive injection of non-eluting solvents upon the chromatographic behaviour.

C) Definition of a non-eluting solvent as close as possible to the mobile phase.

EQUIPMENT, SOLVENTS AND STANDARDS

The analyses were carried out either on a Micromeritics 7000 B liquid chromatograph with manual injection valve (volume of loop 1000 µl), U.V.visible spectrophotometer and Perkin Elmer 56 recorder or on a laboratory-built automated liquid chromatograph comprising the following system : Micromeritics 725 Automatic Injector (with a 500 µl loop), LDC Constametrics II G pump, Micromeritics 785 or LDC Monitor III U.V. spectrophometer and Perkin Elmer Sigma 10 Chromatography Data Station.

The columns, used in this study, were all identical stainless steel tubing (L = 15 cm, int. Ø = 4.6 mm) and ZDV reducers. They were all packed in the laboratory with Spherisorb ODS 5 µ. In order to measure the variations only due to the injection process, the columns were thermostatically controlled by a water jacket (Touzard et Matignon, Vitry, France) and a circulating water bath FE2 (Haake, Karlsruhe, West Germany).

Analytical grade orthophosphoric acid (H_3PO_4), sodium acetate (AcONa) and "Lichrosolv" acetonitrile used for the mobile phases were all purchased from Merck (Darmstadt, West Germany).

Apart from methylmercadone which was kindly supplied by Fumouze S.A. (France), all the pure compounds used in this study were synthesized in the laboratories of Synthélabo.

CHROMATOGRAPHIC CONDITIONS

The chromatographic conditions developed to analyse the drugs in biological extracts were the following :

. Antrafenine :

Mobile phase : MeCN/AcONa 0.1 M (72.5:27.5 v/v)

Flow rate : 2.00 to 1.25 ml.min^{-1} depending on "the age of the column" (6)

U.V. detection : 353 nm Internal standard : EFQB.

. Nifuroxazide :

Mobile phase : MeCN/H_3PO_4 pH 2.5 (30:70 v/v)

Flow rate : 1 ml.min^{-1}

U.V. detection : 362 nm Internal standard : methylmercadone.

. Cipropride :

Mobile phase : MeCN/AcONa 0.1 M (62:38 v/v)

Flow rate : 1 ml.min^{-1}

U.V. detection : 230 nm Internal standard : flubepride.

The chemical structure of these drugs and their internal standards are shown in Figure 1 and a typical chromatograph of each pair of compound is displayed in Figure 2.

A- INJECTION OF SAMPLES DISSOLVED IN INCREASING VOLUMES OF ELUTING AND NON-ELUTING SOLVENTS

Standard solutions of nifuroxazide and methylmercadone were prepared in the following solvents :

a) pure acetonitrile

b) mobile phase

c) non acidified mobile phase : MeCN/H_2O (30:70 v/v)

d) MeCN/H_3PO_4 pH 2.5 (10:90 v/v)

e) MeCN/H_2O (10:90 v/v)

which corresponded to the different ways to dissolving the dry extract :

(i) - small volume of a very good solvent (which is often a very good eluent, in reverse phase HPLC) : pure acetonitrile.

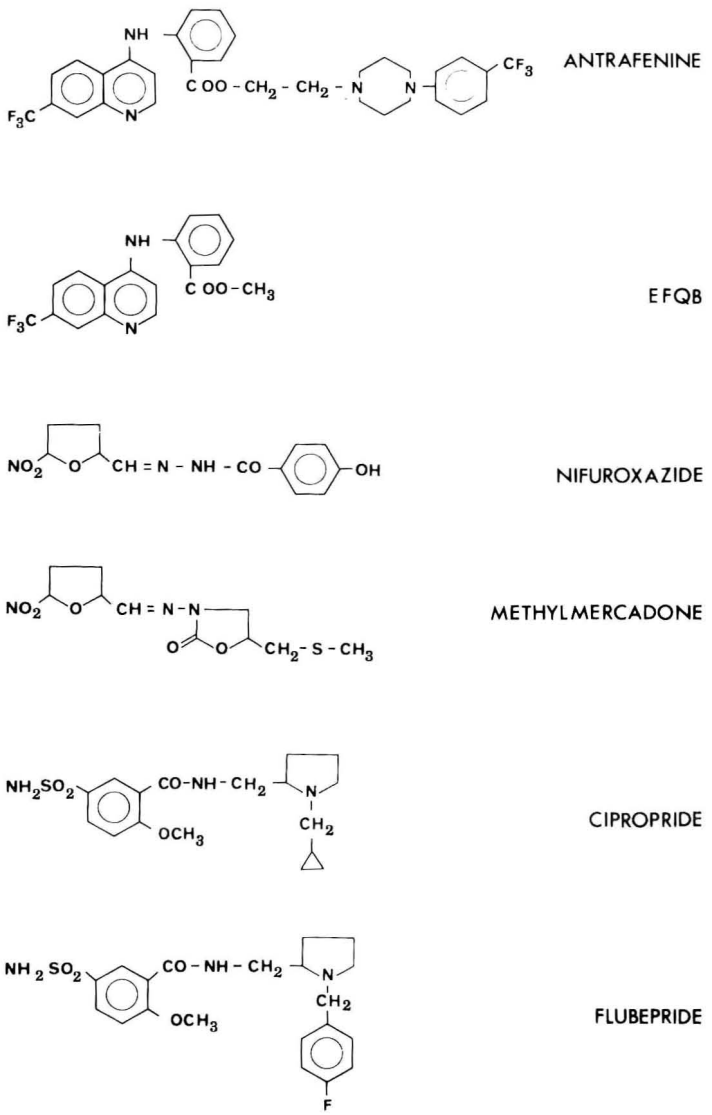

Figure 1: Chemical structures of Compounds used in the study.

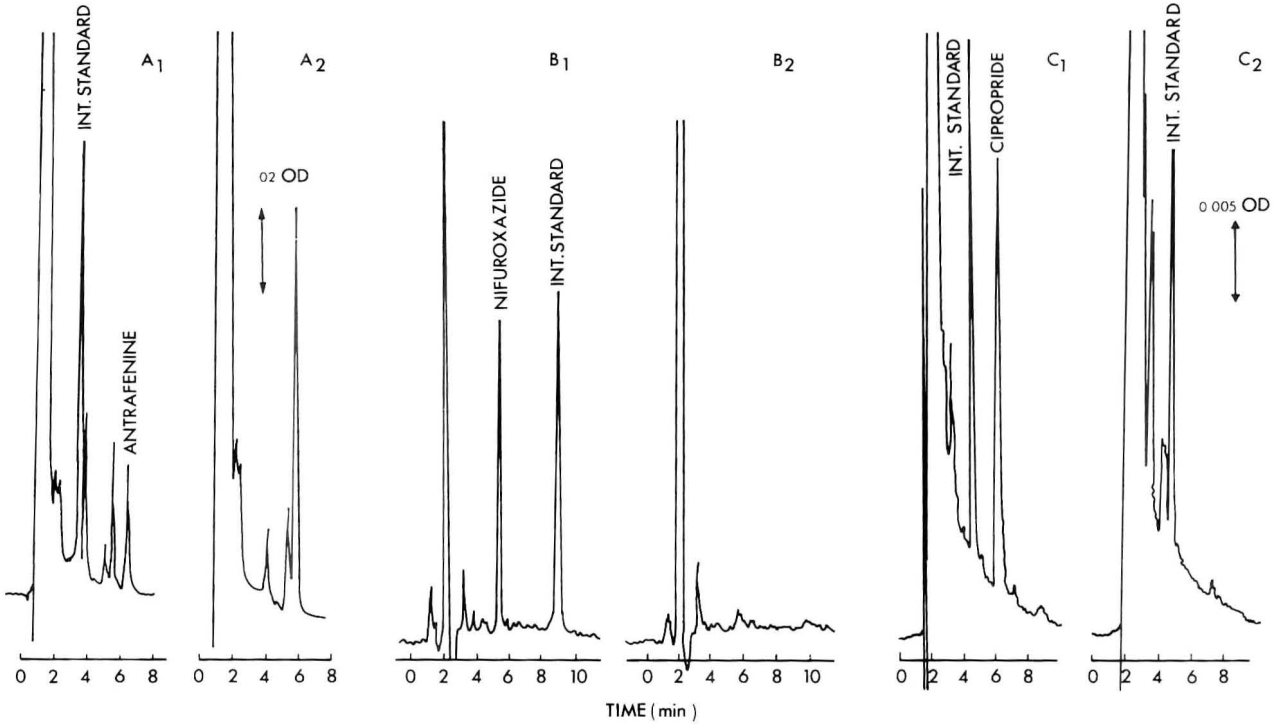

Figure 2: Chromatograms of plasma extracts for determination of drugs in biological samples:

a_1) spiked plasma — a_2) blank plasma for antrafenine

b_1) " " — b_2) " " for nifuroxazide

c_1) " " — c_2) " " for cipropide

Conditions: see the text.

(ii) - larger volume (50 to 200 µl) of the mobile phase or of a similar solvent (which is obviously an eluent) : MeCN 30 % (b and c).

(iii) - larger volume (100 to 1000 µl) of a solvent considerably less eluting than the mobile phase : MeCN 10 % (d and e).

Increasing volumes (10 to 1000 µl) of each solution were injected on the column.

Results and Discussion

The values of retention times of nifuroxazide and methylmercadone are reported in Table 1. The variation of $b_{0.5}$ (width at 1/2 height of peak) and reduced H.E.T.P. are displayed in Figures 3 and 4, respectively.

For standards injected in MeCN, shoulders on the peaks profiles appeared for a 50 µl injection. The retention times (t_R) did not vary between 5 to 10 µl but decreased rapidly when 25 µl of MeCN standard solutions was injected. The value of $b_{0.5}$ increased with the injected volume from 5 to 25µl. These reversed variations of t_R and $b_{0.5}$ explain the very rapid increase of reduced H.E.T.P. with the volume of MeCN injected. Similar results were noticed for antrafenine and EFQB injected in MeCN. The efficiencies were maintained on the column only when the injected volume was equal to or lower than 5 µl. With such a small volume, it is not reasonable to expect a total and reproducible dissolution of any dry extract and the use of an automatic injector is impossible. In addition, care must be taken in the choice of such solvents : Wu and Wittick (8) noticed a large tailing in the peaks of vitamins B_{12} and D_2 when injected in 5 µl of methanol, ethanol or acetonitrile. As a consequence, the use of a small volume of eluting liquid as a solvent for the dry extract cannot be a worthwile way of improving sensitivity and increasing the rate of analysis.

For standards injected in mobile phase or in MeCN/H_2O 30:70, the retention times seemed to be independent of the injected volume from 1 to 500 µl. An increase was noticed for injection of 1,000 µl of MeCN/H_2O 30:70. The values of $b_{0.5}$ were almost constant from 1 to 100 µl of sample injected but increased rapidly after

TABLE 1

Variation of Retention Times with the Injected Volume : Influence of the Solvents

INJECTED VOLUME (µl)	NIFUROXAZIDE					METHYLMERCADONE				
	INJECTION LIQUID					INJECTION LIQUID				
	a	b	c	d	e	a	b	c	d	e
1	-	-	4.8	-	4.8	-	-	8.4	-	8.4
5	4.6	4.8	4.7	4.6	4.7	8.2	8.4	8.3	8.0	8.3
10	4.7	4.7	4.7	4.6	4.7	8.3	8.4	8.3	8.0	8.3
25	3.7	4.7	4.7	4.6	4.7	6.4	8.2	8.3	8.0	8.3
50	-	4.7	4.7	4.6	4.8	-	8.3	8.3	8.0	8.3
100	-	4.7	4.8	4.6	4.7	-	8.2	8.4	8.0	8.3
200	-	4.8	4.8	4.7	4.8	-	8.3	8.3	8.0	8.3
300	-	4.8	4.8	4.8	5.0	-	8.3	8.3	8.2	8.5
400	-	4.8	4.8	5.2	5.0	-	8.3	8.3	8.8	8.5
500	-	5.0	4.8	5.2	5.3	-	8.4	8.3	8.8	8.8
1000	-	-	5.3	5.7	5.7	-	-	8.6	9.2	9.2

100 µl and explained the variations of reduced H.E.T.P. The samples injected in MeCN/H_2O 30:70 differed from those injected in the mobile phase only by the pH. However for equal injected volume, the $b_{0.5}$'s related to MeCN/H_2O were a littler smaller than those related to the mobile phase, whereas the retention times remained similar. This difference could be explained by a decrease in the eluting character due to the variation of pH. Nevertheless it is not sufficient to maintain the efficiency of the column at its best. As a consequence, even if the dissolution of the dry extract were total and reproducible in 100 and 200 µl of such solutions, even if some automatic injectors could inject the largest part of these volumes, the best sensitivity and the best efficiencies are

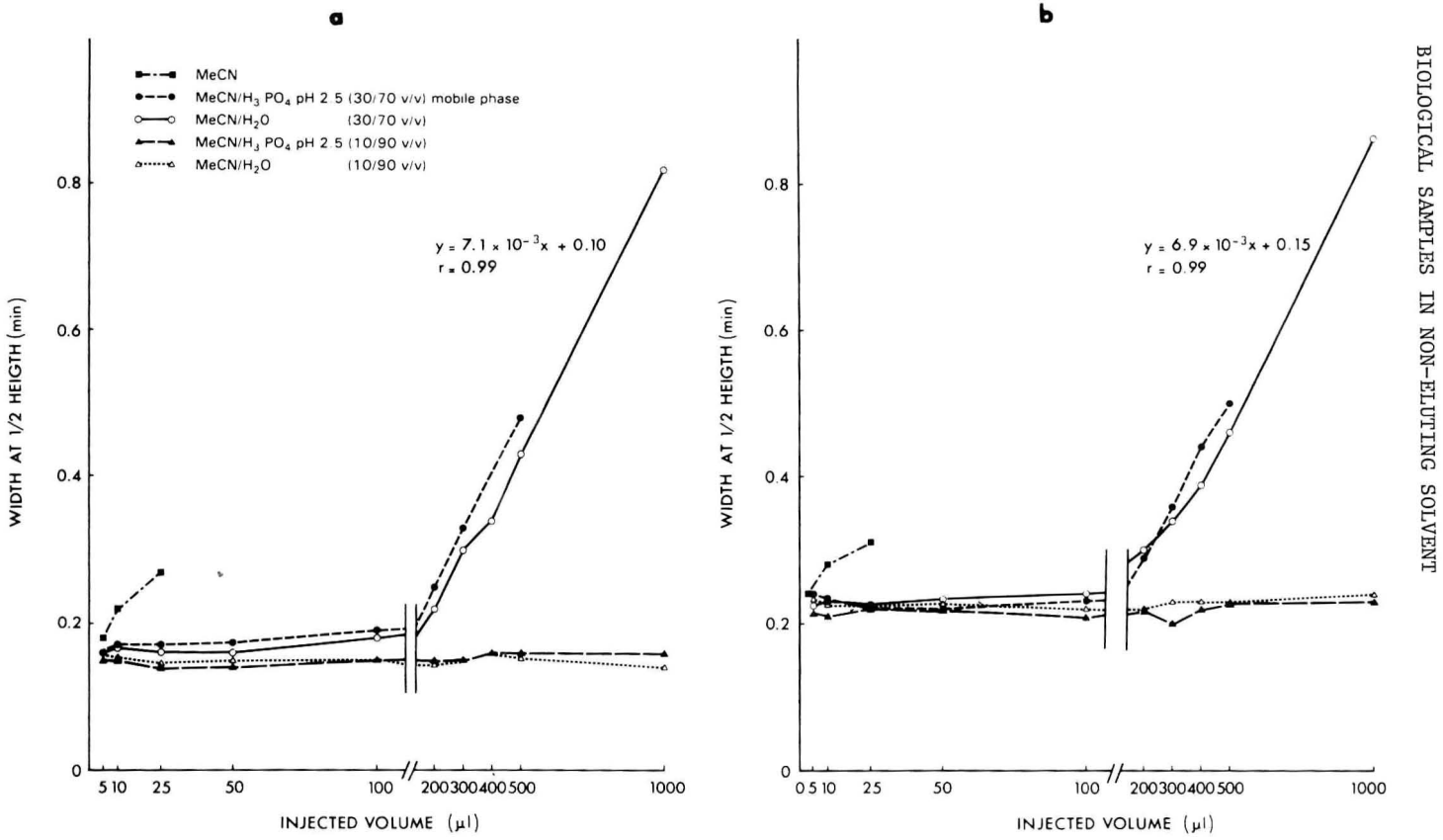

Figure 3: Variation of $b_{0.5}$ of nifuroxazide (a) and methylmercadone (b) with the injected volume: influence of the composition of the injected liquid.

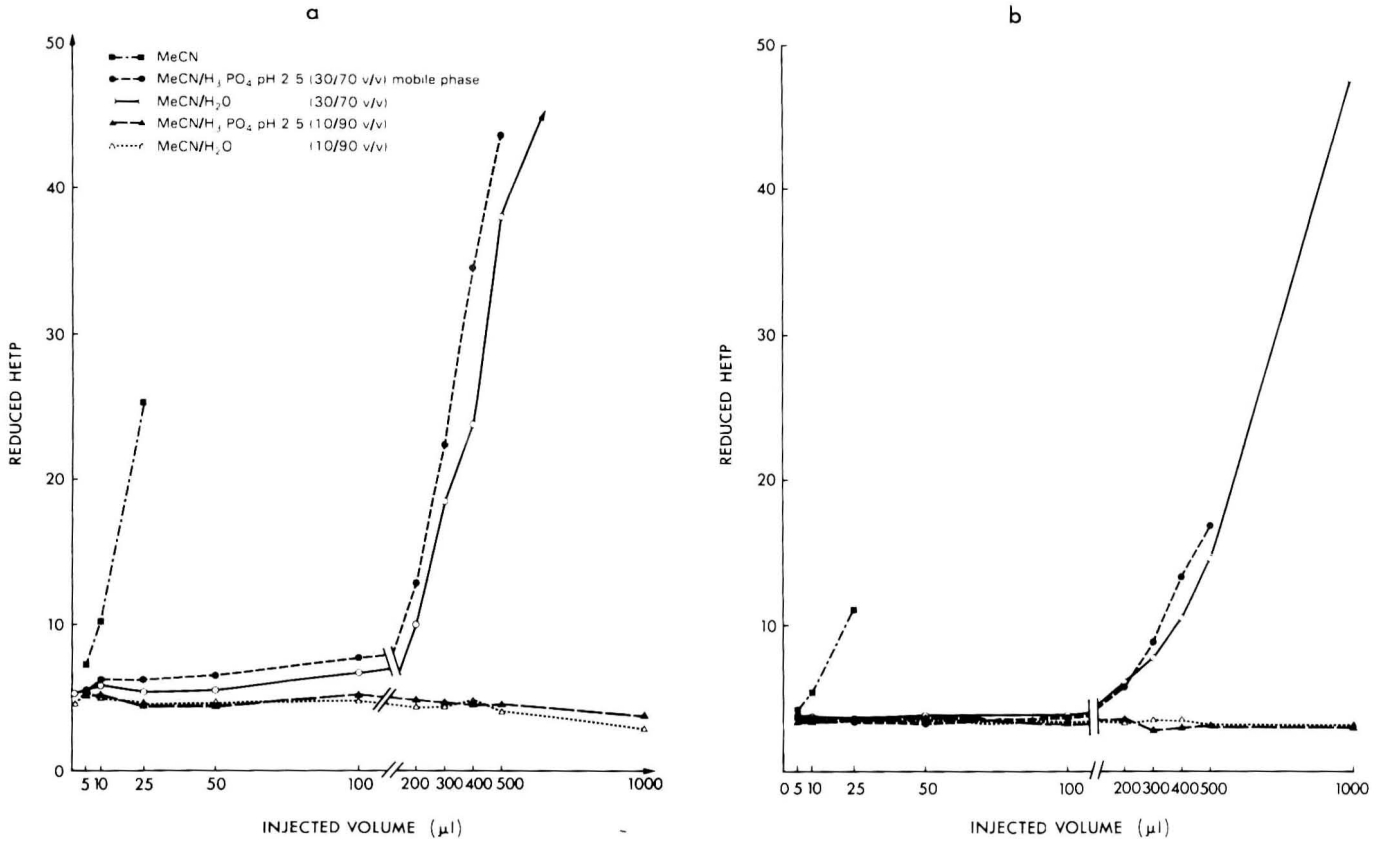

Figure 4: Variation of the reduced H.E.T.P. of nifuroxazide (a) and methylmercadone (b) with the injected volume: influence of the composition of the injected liquid.

not reached when the dry extract is dissolved in the mobile phase or in a similar solvent.

The values of $b_{0.5}$ for standards injected in solvent containing only 10 % of MeCN were quite independent of the injected volumes and of the pH. The retention times were also independent of the pH but increased linearly with the injected volume (Figure 5). These variations of t_R explained the small improvment of reduced H.E.T.P. observed with samples injected in 10 % MeCN (Figure 4). Similar results were noticed for Antrafenine and EFQB injected in MeCN/AcONa 25:75. As $b_{0.5}$ was independent from the injected volume, V_{inj}, the dry extract could be dissolved in a large volume and totally injected on the column, leading to the best sensitivity which can be expected. As V_{inj} can be large, most of the automatic samplers commercially available become usable to introduce the sample on the column. The rate of routine analysis increases as the analyses can be performed without human intervention.

Aside from their advantages in increasing the possibilities of HPLC for drug determination in biological samples, some results obtained during this study must be considered at a more general level. During these last five years, several theoretical or pratical papers have been published, dealing with extra column effects in high performance liquid chromatography (1 to 4). It is usually assumed that the variance of the chromatographic peak, σ^2_{tot} is equal to the sum of the variance of the chromatographic process itself and of the variances of all the parameters contribution to broadening, in particular that of the injection process σ^2_{inj}. Therefore, the total variance can be written as :

$$\sigma^2_{tot} = \sigma^2_{inj} + \Sigma\sigma^2 \qquad (1)$$

where $\Sigma\sigma^2$ reprensents the variance of all the other parameters.

The variance of a plug sample injection, expressed in volume, is given by :

$$\sigma^2_{inj} = \frac{V^2_{inj}}{K^2} \qquad (2)$$

where V_{inj} is the injected volume and K a constant depending on the solute and on the injected technique. Then,

$$\sigma^2_{tot} = \frac{V^2_{inj}}{K^2} + \Sigma\sigma^2 \quad (3)$$

or in term of $b_{0.5}$ expressed in volume :

$$b^2_{0.5} = 8 \text{ Log2 } (\frac{V^2_{inj}}{K^2} + \Sigma\sigma^2) \quad (4)$$

In a study similar to that described above, Westerlund et al. (9) reported a linear relationship between $b_{0.5}$ and V_{inj}. In a first approximation, the values of $b_{0.5}$ measured in the present investigation confirmed the observation of Westerlund ; linear correlations were found between $b_{0.5}$ and V_{inj} when nifuroxazide and methylmercadone were injected in the mobile phase and in a similar solvent (Figure 3). The correlation coefficients were equal to 0.99 in both cases. However, no reason could be found to explain the observed correlations between 100 and 1,000 µl but not below 100 µl.

Assuming that all the contributions to band broadening, except that due to V_{inj}, were constant, and assuming that the contributions due to small V_{inj} (< 25 µl) were negligible compared with the sum of all the others, $\{b^2_{0.5} (V_{inj} < 25 \text{ µl}) \simeq 8 \text{ Log2 } \Sigma\sigma^2 = b^2_{0.5}(0)\}$, $b^2_{0.5} - b^2_{0.5}(0)$ was computed versus V^2_{inj} according to equation (4).

The experimental correlations were found to be as follows :

- for injection made in mobile phase :

  $b^2_{0.5} - b^2_{0.5}(0) = 0.207\ V^2_{inj} + 0.068$ (r = 0.995) for methylmercadone

  $b^2_{0.5} - b^2_{0.5}(0) = 0.206\ V^2_{inj} + 0.037$ (r = 0.998) for nifuroxazide

- and for injection made in $MeCN/H_2O$ (30:70) :

  $b^2_{0.5} - b^2_{0.5}(0) = 0.172\ V^2_{inj} + 0.019$ (r = 0.999) for methylmercadone

  $b^2_{0.5} - b^2_{0.5}(0) = 0.162\ V^2_{inj} - 0.061$ (r = 0.999) for nifuroxazide

whatever the values of V_{inj} between 25 and 1,000 µl.

All these considerations concerned injections of sample diluted in mobile phase or a similar solvent.

The experimental data concerning injections made in (MeCN 10 %) solutions did not satisfy equation (2). $b_{0.5}$ was independent of V_{inj}. Therefore, it is realistic to assume that σ_{inj} is a constant, or

$$\sigma^2_{inj} = C^{ste} \quad (5)$$

The parameters which were related to V_{inj} were the retention volumes. Considering the linear relationship between t_R and V_{inj} (Figure 5), the same variation of 1 minute was measured in the retention time of both standards injected in both solvents when injection volumes varied from 1 to 1,000 µl. As the injections were made through a loop at a flow rate of 1 ml.min^{-1} (see chromatographic conditions), the retention times increased with the duration of the injection, t_{inj}, (assuming a square wave plug injection). This can be translated by the equation :

$$t_R = t_{Ro} + t_{inj} \quad (6)$$

or in terms of retention volumes :

$$V_R = V_{Ro} + V_{inj} \quad (7)$$

where t_R and V_R are the retention time and the retention volume corresponding to an injection of volume V_{inj}, and t_{Ro} and V_{Ro} the retention time and the retention volume corresponding to a zero injection volume.

Equations (5) and (7) define the theoretical aspect of the injection of samples dissolved in a non-eluting solvent and at the same time the concept of non-eluting solvent.

Whatever the injected volume is, its contribution to band broadening is effectively zero. All the sample is concentrated at the top of the column during the time of the injection. With the general trend towards short times of analysis on short columns packed with microparticules (5 µ or less) this result is particularly important as it avoids band broadening due to the injection volume.

B - INFLUENCE OF REPETITIVE INJECTIONS OF LARGE VOLUMES OF NON-ELUTING SOLVENT ON THE CHROMATOGRAPHIC BEHAVIOUR

The repeated injection of a large volume of solvent different from the mobile phase could disturb the equilibria existing inside

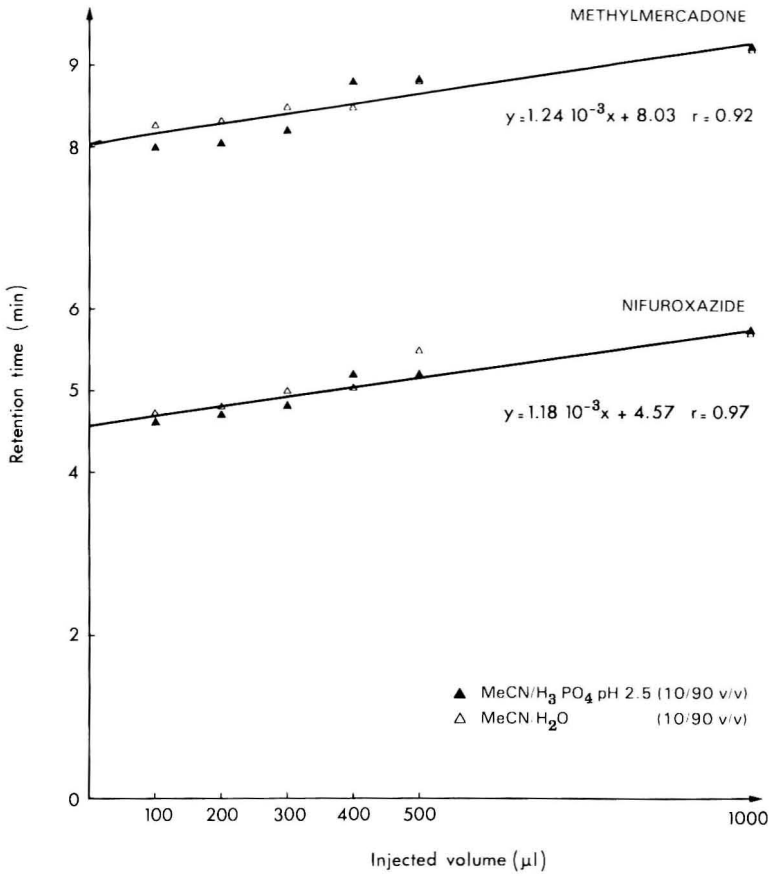

Figure 5: Injection of solutes dissolved in a non-eluting solvent: variation of retention times with the injected volume.

the column between the stationary and mobile phases and the solute, leading to erratic retention time. The stability of retention times had to be checked before developing a repetitive analytical method involving such injections either with a manual valve or with an automatic injector.

Experimental

Repeated injections of 500 µl of standard dissolved in non-eluting solvent were made following the general schedule described below :

- n_1 injections were made each time interval 1 ΔT ; n_2 injections, each 2 ΔT ; n_3 injections, each 3 ΔT ; n_4 injections, each 4 ΔT; n'_1 injections, each 1 ΔT. ΔT was dependent on the standards and on the chromatographic conditions used. In each series, the values of ΔT was set on the automatic injector as the time between two consecutive injections to make each injection ΔT. In order to make the injections at intervals of 2, 3 and 4 ΔT, 1, 2 and 3 vials filled with mobile phase were placed on the rack between two vials filled with standard solutions.

The standard solutions were prepared as described in Table 2. The value of ΔT and n_i for each series of standards are given in Table 3. Two standards solutions of nifuroxazide and methylmercadone were prepared in MeCN/H_3PO_4 pH 2.5 and in MeCN/H_2O in order to explore the effect of pH.

Standards of cipropride and flubepride were prepared either in MeCN/AcONa 0.1 M and in HCl 0.01 M in order to investigate the solvent totally different from the mobile phase.

TABLE 2

Influence of Repeated Injections on Chromatographic Behaviour : Composition of Standard Solutions

MAIN COMPOUND	CONCENTRATION ($\mu g.ml^{-1}$)		NON-ELUTING SOLVENT
ANTRAFENINE	ANTRAFENINE	0.92	MeCN/AcONa 0.1 M 50/50
	EFQB	0.57	
NIFUROXAZIDE	NIFUROXAZIDE	0.06	MeCN/H_3PO_4 pH 2.5 10/90
	METHYLMERCADONE	0.17	MeCN/H_2O 10/90
CIPROPRIDE	CIPROPRIDE	0.29	MeCN/AcONa 0.1 M 10/90
	FLUBEPRIDE	0.29	HCl 0.01 M

TABLE 3

Influence of Repeated Injections on Chromatographic Behaviour : Experimental Parameters and Mean Retention Times

ΔT (min)	Θ (o_c)	n ΔT	n_i	MEAN RETENTION TIME ± S.D. (C.V.) (min)	
Antrafenine				Antrafenine	E F Q B
8.2		1	12	5.977 ± 0.008 (0.13 %)	3.263 ± 0.005 (14 %)
		2	5	5.976 ± 0.014 (0.25 %)	3.256 ± 0.009 (0.3 %)
		3	5	5.968 ± 0.222 (0.4 %)	3.258 ± 0.004 (0.14%)
		4	5	5.962 ± 0.018 (0.3 %)	3.244 ± 0.005 (0.17%)
		1	7	5.941 ± 0.013 (0.23 %)	3.247 ± 0.013 (0.4 %)
Nifuroxazide in H_3PO_4				Nifuroxazide	Methylmercadone
10.2	2.9	1	13	4.872 ± 0.004 (0.08 %)	8.067 ± 0.006 (0.08%)
		2	5	4.874 ± 0.005 (0.1 %)	8.076 ± 0.005 (0.07%)
		3	4	4.878 ± 0.005 (0.1 %)	8.088 ± 0.005 (0.06%)
		4	5	4.882 ± 0.004 (0.09 %)	8.096 ± 0.009 (0.1 %)
		1	13	4.892 ± 0.004 (0.09 %)	8.107 ± 0.009 (0.1 %)
Nifuroxazide in H_2O				Nifuroxazide	Methylmercadone
9.6	28	1	10	4.816 ± 0.005 (0.1 %)	7.946 ± 0.008 (0.1 %)
		2	5	4.826 ± 0.005 (0.1 %)	7.956 ± 0.005 (0.06%)
		3	5	4.824 ± 0.005 (0.1 %)	7.954 ± 0.005 (0.07%)
		4	5	4.826 ± 0.005 (0.1 %)	7.960 ± 0.000 -
		1	13	4.828 ± 0.008 (0.2 %)	7.963 ± 0.003 (0.09%)
Cipropride in MeCN				Flubepride	Cipropride
8.8	28.5	1	7	3.633 ± 0.012 (0.3 %)	6.274 ± 0.022 (0.3 %)
		2	5	3.606 ± 0.005 (0.15 %)	6.228 ± 0.008 (0.13%)
		3	5	3.602 ± 0.016 (0.5 %)	6.202 ± 0.023 (0.4 %)
		4	6	3.610 ± 0.013 (0.35 %)	6.220 ± 0.015 (0.25%)
		1	12	3.608 ± 0.009 (0.26 %)	6.230 ± (2 measures)
Cipropride in dilute HCl				Flubepride	Cipropride
9.2	28	1	10	4.563 ± 0.005 (0.1 %)	6.219 ± 0.009 (0.14%)
		2	5	4.32 ± 0	6.413 ± 0.010 (0.15%)
		3	5	4.230 ± 0.012 (0.3 %)	6.434 ± 0.015 (0.20%)
		4	7	4.183 ± 0.008 (0.18 %)	6.439 ± 0.007 (0.11%)
		1	12	4.588 ± 0.008 (0.18 %)	6.301 ± 0.006 (0.09 %)

Results and discussion

The mean values of retention times of each compound are shown in Table 3 apart from cipropride and flubepride injected in HCl 0.01 M. The reported values correspond in this case to mean values of retention times after equilibration. For all the other experiments, the repeated injections of a large volume of a non-eluting solvent similar to the mobile phase did not disturb the chromatographic behaviour : the retention times remained rigorously constant whatever the interval between injections. In the case of nifuroxazide and methylmercadone, the difference in pH between the two standard solutions did not appear to have an influence on the retention times or on chromatographic behaviour. The very small difference noticed between the retention times (< 0.06 min.) could also be due to the difference in temperature (1°C) between the two experiments.

The variation in retention times of cipropride and flubepride injected in HCl 0.01 M versus time are displayed in Figure 6.

These results indicated that the chromatographic behaviour was disturbed by the injection of large volume of solvent totally different from the mobile phase. If the injections are repeated at a constant interval, the chromatographic equilibria are modified until a different stabilization occurring after some injections : at this stage, the retention times remain constant as long as the interval between two injections remains constant. Modification of chromatographic equilibria can have an opposite influence on compounds as close together as cipropride and flubepride : the retention time of flubepride increased while that of cipropride decreased. In a preliminary study, carried out on a column packed with a different batch of Spherisorb ODS 5 µ, an inversion of retention time between cipropride and flubepride occurred during the "saturation period".

As a consequence of these results, the repeated injection of large volume of eluting solvent can be used to inject diluted samples either manually or automatically for routine analysis. If the injected solvent is similar to the mobile phase, no precaution

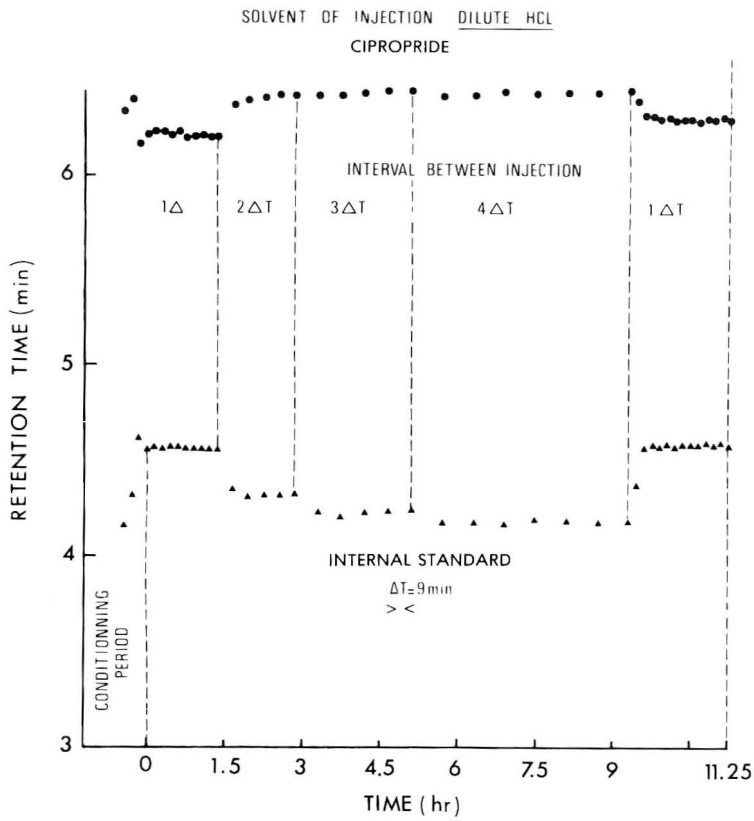

Figure 6: Injection of solutes dissolved in a non-eluting solvent different from the mobile phase: effect of the repeated injection on retention times.

is needed ; if the solvent is different, the first sample must be injected after a "saturation period" made with some injections of the solvent used for the following analysis. The interval between two consecutive injections must remain constant.

C - EXPERIMENTAL DETERMINATION OF A NON-ELUTING SOLVENT SIMILAR TO THE MOBILE PHASE

The non-eluting character of the injection solvent is obviously dependent from the composition of the mobile phase. It could be

TABLE 4

Injection of Samples Dissolved in a Large Volume of Non-eluting Solvent : Influence of the Composition of the Solvent on Retention Time and $b_{0.5}$

X %	ANTRAFENINE		E F Q B		NIFUROXAZIDE		METHYLMERCADONE		FLUBEPRIDE		CIPROPRIDE	
	t_R	$b_{0.5}$	t_R	$b_{0.5}$	t_R	$b_{0.5}$	t_R	$b_{0.5}$	t_R	$b_{0.5}$	t_R	$b_{0.5}$
0					5.43	0.15	9.17	0.23	3.65	0.12	6.31	0.26
2.1	6.06	0.19	3.37	0.10								
10.0									3.65	0.12	6.27	0.26
16.6					5.43	0.14	9.14	0.25	3.65	0.12	6.29	0.26
21.0	6.06	0.19	3.39	0.10								
33.3					5.36	0.15	9.07	0.26	3.61	0.12	6.24	0.28
34	6.09	0.20	3.37	0.10								
48	6.04	0.20	3.35	0.10								
50									3.55	0.12	6.21	0.30
60	6.00	0.20	3.29	0.10								
66.6					5.33	0.19	9.06	0.29	3.51	0.16	6.22	0.34
69	5.95	0.20	3.27	0.12								
76	5.92	0.21	3.23	0.15								
83.3	5.89	0.23	3.20	0.18	5.24	0.29	8.97	0.41	3.43	0.26	6.22	0.46
90	5.86	0.36	3.15	0.23								
97	5.76	6.34	3.09	0.32					3.34	0.40	6.21	0.56
100					5.03	0.50	8.35	0.53				

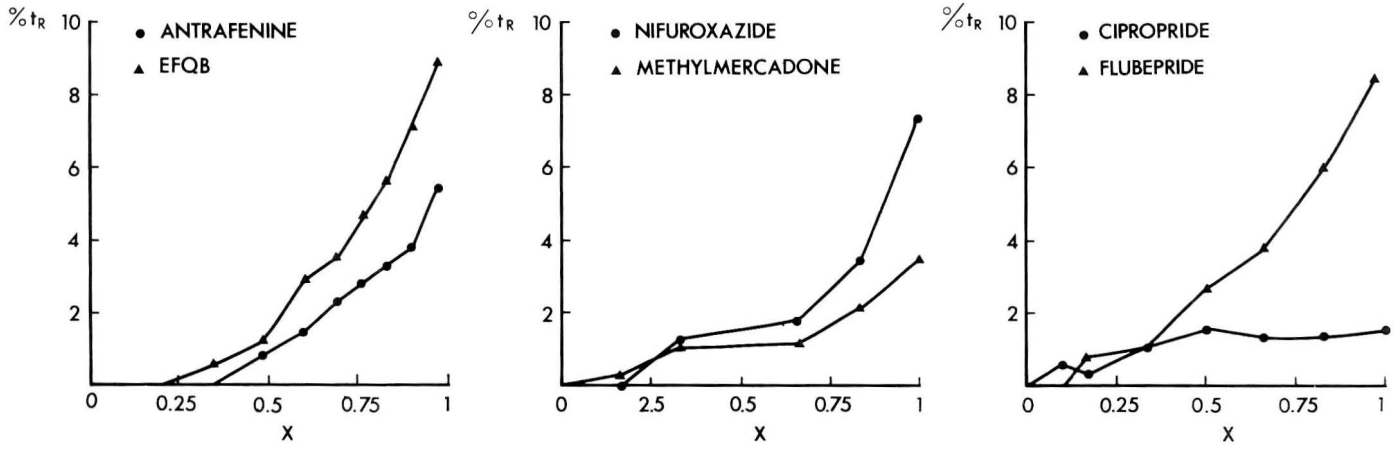

Figure 7: Injection of solutes dissolved in a non-eluting solvent: influence of the composition of the solvent on retention time.

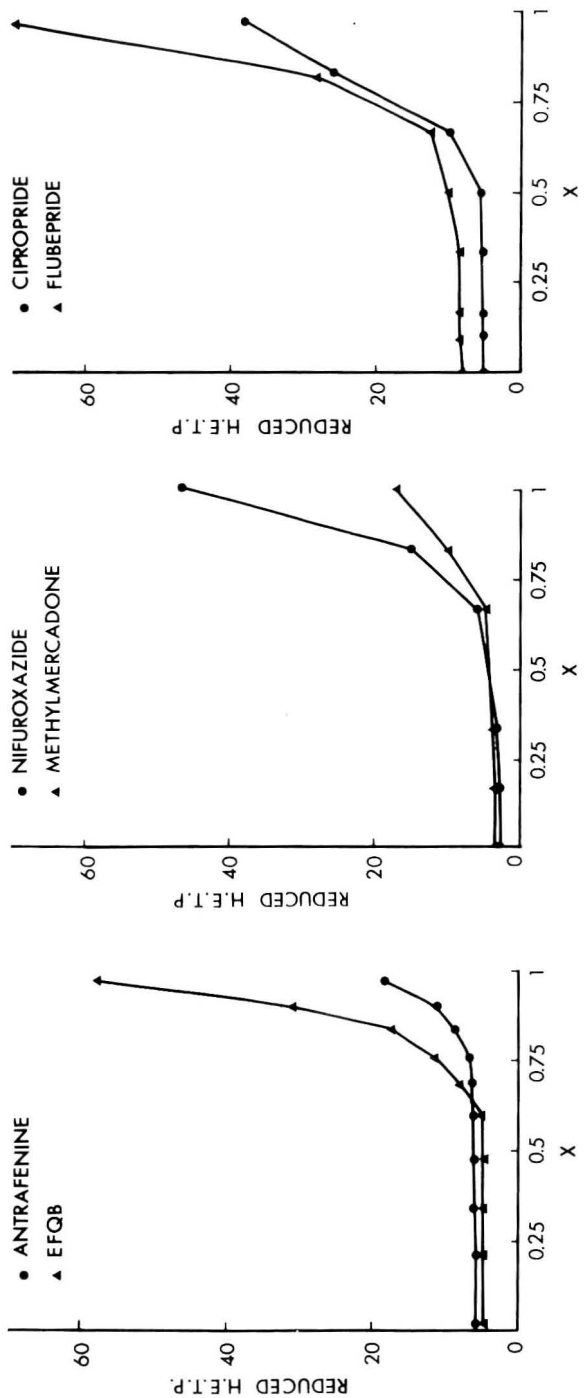

Figure 8: Injection of solutes dissolved in a non-eluting solvent: influence of the composition of the solvent on reduced H.E.T.P.

determined independently by considering the fraction of mobile phase X diluted in its weakest component to make the injection solvent. A non-eluting solvent can be determined experimently by measuring the range of X for which equations 5 and 7 remained true.

Experimental

For each pair of compounds, standard solutions were prepared in a series of solvents similar to the mobile phase and containing increasing amounts of MeCN. 500 µl of these solutions were injected on the column and eluted using the chromatographic conditions related to each compound.

Results and discussion

The retention times and the corresponding $b_{0.5}$ are reported in Table 4. The variations of retention time, expressed in per cent of t_R (corresponding to the lowest value of X) displayed in Figure 7, showed that in all the cases, the variations of t_R remained lower than 1 % for X lower than 0.25. Apart from cipropride, t_R of which remained constant for X varying between 0.5 and 1,% t_R increased with X : the largest variations were observed for the lowest retention times. Apart from methylmercadone $b_{0.5}$ appeared to be independent of X for X values lower than 0.16 to 0.20. For higher values of X, $b_{0.5}$ increased with X, the largest variations being due to the least retained compound. The variation of the reduced H.E.T.P. with X, displayed in Figure 8, remained negligible for values of X between 0 and 0.5 but increased rapidly with larger values of X ; the largest variations were noticed for the lowest retention time. Finally, for values of X lower than 0.25, equations (5) and (7) were satisfied in all cases, allowing the determination of the non-eluting solvent composition limit which may give the maximum efficiency and selectivity.

CONCLUSION

The injection of samples dissolved in a non-eluting solvent has been demonstrated as an easy and powerful way to minimize the losses of efficiency related to the injected volume. As a consequence, the sensitivity and rate of analysis of HPLC methods for trace amounts determination are considerably improved. This

injection method has already been successfully used in several automatic HPLC methods for drug determination in biological samples where concentrations as small as 5 $ng.ml^{-1}$ were routinely assayed.

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RAPID, HIGH-YIELD PURIFICATION OF RAT LIVER GLUTATHIONE PEROXIDASE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Glutathione peroxidase (GSH:H_2O_2 oxidoreductase, EC 1.11.1.9) was purified 3500-fold from rat liver with a yield of 42% using high performance liquid chromatography. The crucial purification step was size-exclusion chromatography on a Spherogel TSK-3000SW column, and the purified enzyme eluted as a single peak. The enzyme stained as a single band following SDS-gel electrophoresis. The molecular weight of the enzyme was estimated to be 105,000, and the subunit molecular weight determined by SDS-gel electrophoresis was 25,000. Polyacrylamide gel electrophoresis indicated five bands of protein with a broad band of enzymatic activity. Isoelectric focusing resulted in a peak of enzymatic activity at pH 6.9 with a shoulder at pH 7.3. The specific activity of the purified enzyme was 1,100 μmol of NADPH oxidized per minute per milligram of protein.

INTRODUCTION

Glutathione peroxidase (glutathione hydrogen peroxide oxidoreductase, EC 1.11.1.9) was first reported by Mills (1,2) to catalyze the breakdown of hydrogen peroxide in bovine erythrocytes using glutathione as the reducing agent. Since that time, glutathione peroxidase from several mammalian and avian species has been studied. The enzyme has been obtained in highly purified form from bovine erythrocytes (3), ovine erythrocytes (4), human erythrocytes (5), bovine lens (6), and rat liver (7). In all

these studies, the yields of enzymatic activity were low. Low yields necessitate large quantities of starting material to obtain sufficient glutathione peroxidase to use in other experiments. Only Flohé et al. (8) and Little et al. (9) reported specific activities greater than 400 units/mg of protein when the enzyme assays were done by the method described by Paglia and Valentine (10). Stults et al. (11) reported obtaining a specific activity of 278 units/mg of protein, and Stults (12) indicated that it was possible to achieve a specific activity of 800 units/mg of protein from rat liver. Nakumura et al. (7) reported a final specific activity for purified rat liver glutathione peroxidase of 35.2 units/mg of protein; this low value probably reflects the loss of enzymatic activity as a result of the purification procedures used. This paper reports the relatively high yield purification of rat liver glutathione peroxidase with high specific activity. High yield and high specific activities are both required for studies of physical properties of the enzyme or for the use of the enzyme as a biochemical tool.

MATERIALS AND METHODS

Sephadex G-100 and DEAE-Sephacel were obtained from Pharmacia Fine Chemicals; reduced glutathione, glutathione reductase, NADPH, α-amylase from B. subtilis, and bovine erythrocyte carbonic anhydrase from Sigma Chemical Co.; cumene hydroperoxide from Bio-Polymers, Inc.; SDS-gel electrophoresis standards from Bio-Rad Laboratories; and [^{75}Se]selenite (9 to 35 Ci/mmol) from New England Nuclear.

Fifteen male Sprague-Dawley rats (350-400 g) purchased from Simonsen Laboratories, Inc. were fed Ralston Purina Rat Chow. The rats were fasted for 24 hr prior to killing them by decapitation. The livers were removed and placed in 0.25 M sucrose at 4°C. All subsequent procedures were done at 4°C unless otherwise specified. Three of the fifteen rats were injected intraperitoneally with 50 μCi of [^{75}Se]selenite 3 days before they were killed. The livers from all fifteen rats were minced, washed twice with 0.25 M

sucrose, and homogenized 1:4 in 0.25 M sucrose, 11 mM glutathione for 30 sec in a Waring blendor. The homogenate was centrifuged at 13,000 X g for 30 min. The supernatant portion was adjusted to pH 7.6 with 100 mM Tris base, heated at 50°C for 45 min and cooled to 4°C. The cooled sample was made 52% acetone (-20°C) and centrifuged at 13,000 X g for 20 min. The acetone precipitate was resuspended in 10 mM Tris-HCl, 0.1 mM EDTA, 5 mM reduced glutathione (pH 7.6) and centrifuged at 27,000 X g for 30 min. The supernatant portion was chromatographed on a Sephadex G-100 column (5 X 100 cm) equilibrated with 10 mM Tris-HCl, 0.1 mM EDTA (pH 7.6). The fractions that contained active glutathione peroxidase were pooled and made 7.1 mM in 2-mercaptoethanol. After maintaining the samples for 20 min at 4°C, 10-ml aliquots of DEAE-Sephacel equilibrated with 10 mM Tris-HCl, 0.1 mM EDTA (pH 7.6) were added until there was no further change in the absorbance at 280 nm as monitored on small filtered aliquots. The resultant mixture was filtered and the DEAE-Sephacel was washed with 20 ml of buffer. The filtrate and the wash solution were pooled, concentrated in an Amicon concentrator using a PM-10 filter, and applied to a DEAE-Sephacel column (2.5 X 100 cm) equilibrated with 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.6. The sample was eluted isocratically with the same buffer used to equilibrate the column, and the fractions that contained ^{75}Se were pooled. The pooled fractions were concentrated to a volume of approximately 1 ml using an Amicon concentrator with a PM-10 filter. The sample was injected as four 250-µl aliquots onto a Beckman HPLC model 322 with a Spherogel TSK-3000SW size-exclusion column (7.5 X 600 mm) equilibrated with 10 mM sodium phosphate, 0.1 mM EDTA, pH 7.0. After each set of four 250-µl injections, the fractions that contained ^{75}Se were pooled, concentrated in an Amicon concentrator, and rechromatographed. This procedure was repeated twice.

Glutathione peroxidase was assayed by a modification of the method of Paglia and Valentine (10) as described by Stults et al. (11). Protein in all samples was determined by a modified Lowry technique (13), except the fractions obtained in the final purifi-

cation steps on HPLC were assayed by measurement of $A_{280}-A_{260}$ (14) and $A_{224}-A_{214}$ (15,16). ^{75}Se was measured using a Packard model 5110 Auto-gamma counter with 30.6% efficiency.

Purified glutathione peroxidase obtained after the third chromatography on the HPLC was subjected to disc-gel electrophoresis according to the procedure of Davis (17). Samples of 80 µg of protein were applied to 7.5% gels that were stacked at pH 8.3 and electrophoresed at pH 9.5. SDS disc-gel electrophoresis was performed according to the method of Weber and Osborn (18) using 10% gels at pH 7.0. Gel isoelectric focusing was done by the method outlined in a Bio-Rad Technical Bulletin 1030. Coomassie blue stain was used to detect protein in the analytical and SDS gels, both of which were scanned at 540 nm with a Beckman DU spectrophotometer model 2400. The gels were frozen, sliced and counted for ^{75}Se radioactivity. An unstained analytical gel and an isoelectric focusing gel were frozen, sliced and assayed for enzymatic activity.

RESULTS

The data obtained from the purification of rat liver glutathione peroxidase are shown in Table 1. Sephadex G-100 chromatography resulted in an aggregated form of glutathione peroxidase. An aggregated form of the enzyme was first described by Stults et al. (11). Addition of 2-mercaptoethanol at 7 mM followed by a DEAE-Sephacel titration resulted in complete dissociation of the aggregated enzyme. This step resulted in a 5-fold purification of the enzyme and minimal loss of activity. Use of DEAE-Sephacel chromatography resulted in retardation of the enzyme by the gel and subsequent elution after elution of the major nonbound protein peak. Use of the 100-cm DEAE-Sephacel column resulted in a much better purification of the enzyme than previously reported (11). The fractionations to this point have been done a number of times with good reproducibility. For the four previous fractionations through the DEAE-Sephacel chromatography step, the specific activities were 52, 48, 50 and 54, and the corresponding yields

TABLE 1
Purification of Glutathione Peroxidase

Fraction	Total protein (mg)	Total activity (units X 10^{-3})[a]	Specific activity[b]	Yield (%)	Purification (-fold)
Homogenate	17 000	5.35	0.32	100	1
Supernatant 13,000 X g	6 853	5.31	0.78	99.3	2.4
Heat-treated 50°C	5 966	4.78	0.80	90.0	2.5
Acetone (52%) precipitate	3 075	4.40	1.4	82.2	4.4
Sephadex G-100	752	3.51	4.7	65.6	15
DEAE-titration	147	3.44	23	64.3	72
DEAE-Sephacel	54	2.87	54	53.7	170
TSK-HPLC[c]	2.0	2.24	1 100	41.8	3 560

[a] µmol of NADPH oxidized per minute.

[b] µmol of NADPH oxidized per minute per milligram of protein.

[c] Following the third chromatography.

were 62, 45, 53 and 51%, respectively. The use of a second DEAE-Sephacel column resulted in a 20-30% yield of glutathione peroxidase that was 30-50% pure. Use of three consecutive chromatographies on a TSK-3000 HPLC column (Fig. 1) separated glutathione peroxidase from other proteins to the limit of detection. Calibration of the TSK column was accomplished using five molecular weight standards. Chromatography of the enzyme on the calibrated column gave an apparent molecular weight for the enzyme of 105,000 $\pm$ 4000 (Fig. 2).

Analytical disc-gel electrophoresis resulted in five equally-spaced bands of protein with the middle band being predominant. Enzymatic activity was found in a wide band encompassing the 5 bands of protein. SDS disc-gel electrophoresis of the purified glutathione peroxidase resulted in a single coincident band of protein and ^{75}Se radioactivity. The subunit molecular weight of the enzyme based on comparison with six molecular-weight stand-

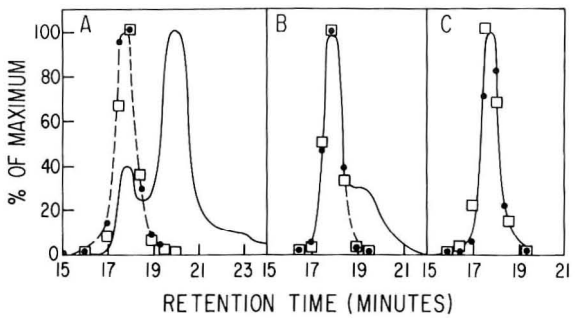

FIGURE 1. TSK-3000 HPLC size-exclusion chromatography of rat liver glutathione peroxidase. Concentrated glutathione peroxidase from DEAE-Sephacel chromatography was applied to a TSK-3000 size-exclusion column equilibrated with 10 mM sodium phosphate, 0.1 mM EDTA (pH 7.0) and resolved at 1.0 ml/min and 400 p.s.i. After the first chromatography (A), the active fractions were pooled and concentrated. Chromatography on the TSK-3000 column was repeated twice as shown in (B) and (C). ^{75}Se (□), enzymatic activity (●) and absorbance at 280 nm (—).

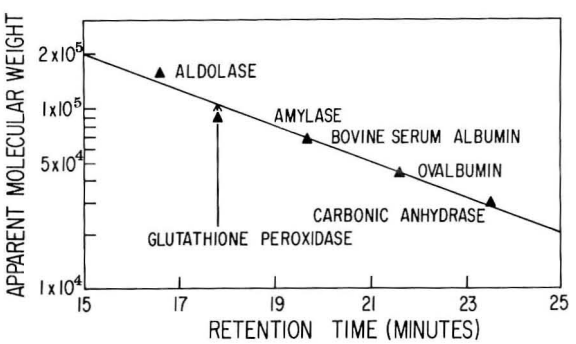

FIGURE 2. Estimation of the molecular weight of glutathione peroxidase by size-exclusion chromatography on a TSK-3000 HPLC column. Aliquots of 100 µg of each protein were injected in 50 µl of 10 mM sodium phosphate, 0.1 mM EDTA, pH 7.0.

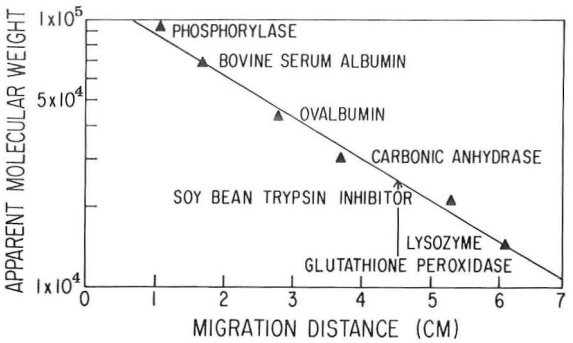

FIGURE 3. Estimation of the subunit molecular weight of glutathione peroxidase by SDS-polyacrylamide disc-gel electrophoresis. A solution that contained 2.5 μg of each standard and 80 μg of glutathione peroxidase was applied to a gel (5 X 100 mm).

ards (Bio-Rad low molecular weight standards) was 25,000 ± 2000 (Fig. 3). Disc-gel isoelectric focusing gave a coincidence of enzyme activity and ^{75}Se radioactivity in a single peak at pH 6.9 and a shoulder at pH 7.3.

DISCUSSION

The development of the purification procedure described started with that reported by Stults et al. (11). The choice of techniques used through the DEAE Sephacel chromatography step and the chosen sequence of the individual steps are based on results obtained by many purifications. The outlined procedure resulted in the purification of glutathione peroxidase with a yield higher than any yet reported and with a high specific activity surpassed only by that reported by Little et al. (9). The described HPLC procedure eliminates the problem of aggregated enzyme (11), and it further demonstrates the usefulness of HPLC for protein separation. The purification of the enzyme can be completed in three days, thus it can be used to provide a routine source of glutathione peroxidase.

The molecular weight of the rat liver glutathione peroxidase as estimated by size-exclusion chromatography is 105,000 ± 4000, which correlates well with a tetramer having an SDS-gel electrophoresis monomer size of 25,000 ± 2000. The observed molecular weight is significantly different from that reported for the rat liver enzyme (7,11), and it more closely matches the 96,000 molecular weight reported for the human erythrocyte enzyme (5).

The protein pattern obtained with analytical disc-gel electrophoresis and the multiple charge forms of glutathione peroxidase observed upon isoelectric focusing suggest the possibility that there are two isozymic forms of the monomer of glutathione peroxidase in rat liver. The two possible isozymes of glutathione peroxidase, "acidic" (pI = 6.9) and "basic" (pI = 7.3), could upon free hybridization, give five mixed enzymes. This possibility is consistent with the protein pattern seen on disc-gel electrophoresis.

ACKNOWLEDGMENT

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JOURNAL OF LIQUID CHROMATOGRAPHY, 4(11), 2073-2079 (1981)

PAPER CHROMATOGRAPHIC SEPARATIONS OF METAL IONS ON COLLIDINIUM TUNGSTOARSENATE PAPERS.

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ABSTRACT

A systematic study of the chromatography of metal ions on collidinium tungstoarsenate papers has been performed using seven different mixed solvent systems. R_f values of 30 metal ions have been determined by ascending technique and are discussed. The study demonstrates that specific extraction of both Sn(ii) and Sn(iv) is possible in 0.1 mol dm^{-3} HNO_3 in 80 % (v/v) 1-propanol and their mutual separation can be carried out in solvent system 1 mol dm^{-3} HCl in 33 % (v/v) 1-propanol. In addition some binary and ternary separation of metal ions have also been achieved on these papers. For a comparison R_f values on plain papers have also been determined in all the solvent systems.

INTRODUCTION

Ion exchange is the only technique by which separation of very similar ionic species, as different valency states of a particular ion, is possible. In the last two decades inorganic exchangers have been extensively investigated regarding their use as impregnating and column material in the separation of metal ions on papers, thin layers and columns(1-5). Of the various inorganic exchangers, the heteropolyacid salts, being more selective in their exchange properties have been more extensively used for these purposes. Ammonium molybdophosphate (6) was the first exchanger to be used as impregnating material for paper chromatographic separation of alkali metal ions.

Recently(7-11) we have reported on the usefulness of heteropoly tungstoarsenates substituted with different cations for the above purposes. It has been observed that by substituting

cations of different valency and ionic size in tungstoarsenate matrix, the structure of resulting compounds and their ion exchange selectivities are changed.

Present studies deal with the chromatographic separation of metal ions on papers impregnated with collidinium tungstoarsenate (CTA) which has been found to possess promising exchange properties (11).

MATERIAL

Reagents

All metal salts and acids were of analytical grade collidinium nitrate was prepared by mixing nitric acid and distilled 2,4,6- collidine in equimolecular quantities. The solvent propanol was purified by distillation.

Metal Ions Studied

The oxidation numbers of the metals used were Ag(i), Tl(i), Mg(ii), Sr(ii), Ba(ii), Fe(ii), Co(ii), Ni(ii), Cu(ii) Zn(ii), Pd(ii), Cd(ii), Sn(ii), Hg(ii), Pb(ii), Al(iii), Fe(iii), La(iii), Sb(iii), Bi(iii), Ce(iii), Pt(iv), Sn(iv), Ti(iv), Th(iv), Ce(iv), VO(iv), Se(iv), Mo(vi), and UO_2(vi).

Apparatus

Chromatography was performed on Whatmann No.3 chromatographic paper strips (15x5.5 cm^2), using 20x10x28 cm^3 glass jars.

Preparation of Ion Exchange Papers

Ion exchange papers were prepared by impregnating paper strips with collidinium tungstoarsenate. The paper strips were first dipped in a freshly prepared solution of sodium 12-tungstoarsenate for ~10 seconds. The solution was prepared by mixing aqueous solution of 3.5 g of sodium tungstate with 0.15 g of arsenic pentoxide in aqueous sodium hydroxide and adding concentrated hydrochloric acid until the mixture is strongly acidic. Total volume of the solution was kept 100 cm^3. The excess solution on paper strips was blotted out and these were dried at room temperature for 15 minutes. The strips were again dipped in 0.1 mol dm^{-3} collidinium nitrate solution for ~10 seconds. The excess solution was drained off and the strips were dried at room temperature.

Composition of Impregnated Papers

Impregnated paper strips were treated with~0.1 mol dm^{-3} sodium hydroxide solution for some time in order to decompse collidinium tungstoarsenate formed on papers. The strips were then washed with water and the solution was made up to 100 cm^3 in a measuring flask. Collidine was estimated spectrophotometrically at 263 nm and tungsten was determined by thiocyanate method at 398 nm.

The analysis of the ion exchange papers shows that collidine/tungsten molar ratio is same as was found for collidinium tungstoarsenate exchanger, $(C_8H_{11}NH)_3MO_{12}ASO_{40})$, used for column separation (11).

Test Solutions and Detectors

Test solutions of chlorides, nitrates or sulphates of most of the metal cations (0.1 mol dm^{-3}) were prepared in 0.1 mol dm^{-3}corresponding acids as reported earlier (8,9). Detection of metal ions was carried out by standards methods (12).

Procedure

Test solutions were spotted with thin glass capillaries and the papers were developed by the ascending technique in chromatographic chambers conditioned with the developers. It took 1.5 - 2 hours for 11 cm ascent of the solvent. After detecting the spots, the R_f values were measured. In case of spreading, the front (R_L) and rear (R_T) limits of the spots have been mentioned. For a comparison, R_f values on plain paper strips were also determined.

RESULTS AND DISCUSSION

Thirty metal ions were chromatographed on impregnated as well as on plain papers in seven different solvent systems, i.e.,

(a) 1 mol dm^{-3} nitric acid in 30 percent (v/v)1-propanol,
(b) 1 mol dm^{-3} nitric acid in 50 percent(v/v)1-propanol,
(c) 0.1 mol dm^{-3} nitric acid in 50 percent(v/v)1-propanol,
(d) 0.1 mol dm^{-3} nitric acid in 80 percent(v/v)1-propanol,
(e) 1 mol dm^{-3} hydrochloric acid in 33 percent (v/v)1-propanol,
(f) 1 mol dm^{-3} hydrochloric acid in 50 percent (v/v)1-propanol and
(g) 0.1 mol dm^{-3} hydrochloric acid in 50 percent(v/v)1-propanol.

TABLE 1

Experimentally achieved Separations on CTA Papers

Solvent System	Separations
(a)	Th(iv) (0.40-0.58) - Pd(ii) (0.75-0.91) Ce(iv) (0.54-0.68) - Sn(iv) (0.81-0.94) Ag(i) (0.00-0.00) - Tl(i) (0.20-0.56) - Ni(ii) (0.69-0.91)
(b)	Bi(iii)(0.17-0.56) - Sn(iv) (0.71-0.95) Bi(iii)(0.19-0.55) - Hg(ii) (0.90-1.00) Ag(i)(0.00-0.00) - Cu(ii) (0.61-0.76) - Hg(ii) (0.91-1.00) Ag(i)(0.00-0.00) - Ba(ii) (0.33-0.54) - Th(iv) (0.65-0.85) Ba(ii)(0.35-0.54) - Sr(ii) (0.55-0.65) - Mg(ii) (0.69-0.85)
(c)	UO_2(vi)(0.28-0.55) - Zn(ii) (0.85-0.95) Fe(iii)(0.25-0.67) - Al(iii)(0.75-0.95) Sr(ii)(0.37-0.55) - Mg(ii) (0.65-0.82) Cd(ii)(0.50-0.80) - Hg(ii) (0.90-1.00) Al(iii)(0.75-0.82) - Zn(ii) (0.88-0.98)
(d)	Pb(ii) (0.00-0.20) - Sn(ii) (0.74-0.85) Zn(ii) (0.02-0.18) - Hg(ii) (0.60-0.80) Ti(iv) (0.00-0.21) - Se(iv) (0.35-0.63) Sn(iv) (0.73-0.87)
(e)	Sn(iv) (0.50-0.75) - Sn(ii) (0.80-0.91) Pb(ii) (0.00-0.10) - Sb(iii)(0.40-0.85) Th(iv) (0.45-0.58) - VO(iv) (0.76-0.86) Th(iv) (0.44-0.60) - UO_2(vi)(0.75-0.95) Th(iv) (0.44-0.56) - La(iii)(0.65-0.88) Pb(ii) (0.00-0.09) - Th (iv)(0.44-0.60) - Bi(iii)(0.77-0.96)
(f)	Ag(i)(0.00-0.00) - Cu(ii) (0.60-0.74) Ag(i)(0.00-0.00) - Pd(ii) (0.72-0.94) Ba(ii)(0.30-0.47) - La(iii)(0.52-0.70) Ba(ii)(0.31-0.47) - Mg(ii) (0.60-0.82)

The R_f on impregnated papers and R_i(R_f on plain papers - R_f on impregnated papers) values for various metal ions on these papers were determined in all the seven solvent systems. Some important and difficult separations, seemed possible on the basis of R_f values, achieved on these papers and are listed in Table 1.

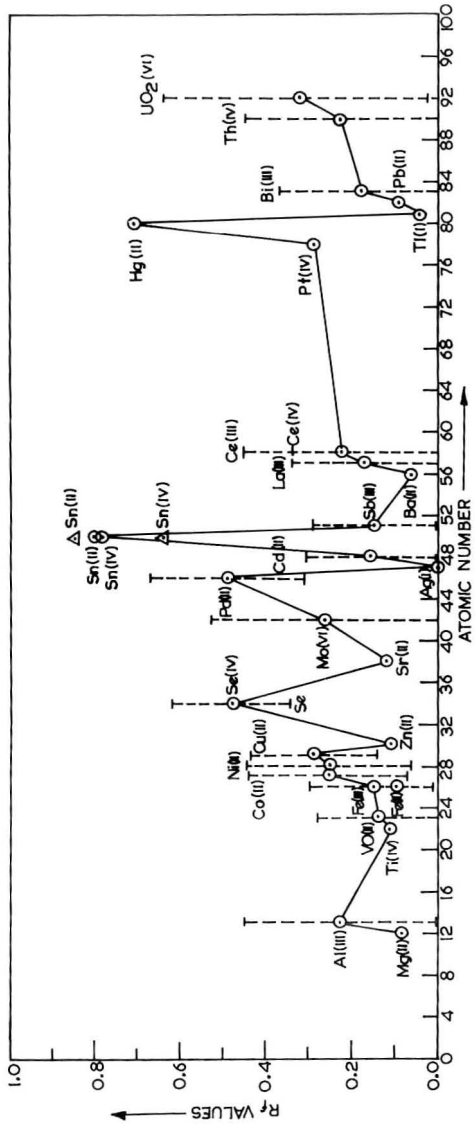

FIGURE 1. Plot of R_f values of metal ions vs. their atomic number in solvent system 0.1 mol dm^{-3} nitric acid in 80 percent v/v 1- propanol, O—O. R_f values of Sn(ii) and Sn(iv) in solvent system 1 mol dm^{-3} hydrochloric acid in 33 percent v/v 1-propanol, Δ—Δ.

The R_f value for Ag(i) is zero in all the solvent systems on impregnated as well as on plain papers. In hydrochloric acid-propanol mixtures, Tl(i) and Pb(ii) show tailing from the point of application (due to their insoluble chlorides) but nitric acid-propanol mixtures move them and can, therefore, be used to separate them from Ag(i). The fact that silver ion is also retained in nitric acid-propanol mixture as well as on plain papers, indicates some sort of metal ion matrix interaction in addition to the formation of AgCl. Sorption studies carried out on this sorbent (static)further confirmed this fact, as Ag(i) is not adsorbed at all from dilute aqueous solutions(11). Apart from Ag(i), Tl(i) and Pb(ii), specific selectivities (low R_f values) in some solvent systems, i.e., Ba(ii) in solvent system(b) and (f); UO_2(vi) and Sr(ii) in solvent system (c); and Th(iv) in solvent system (e) are also observed on these papers. In solvent system (d) R_f values are decreased significantly for almost all the metal ions. In this solvent system Sn(ii), Sn(iv) and Hg(ii) are not retained (showed high R_f) values) and can be separated from other metal ions. The decrease in R_f values in this solvent system on impregnated as well as on plain papers may be due to a check on mobility of ions in higher amount of propanol. This solvant system can, therefore, be used for almost specific extractions of Sn(ii) or Sn(iv) (Fig.1). Separation of Sn(ii) and Sn(iv) can be achieved in solvent system (e).

A comparison of R_f values on impregnated and plain papers showed almost similar pattern for most of the metal ions except that impregnated papers gave more compact spots, i.e., showed less spreading. Therefore, the separations achieved on impregnated papers are cleaner as compared to plain papers. In addition to this, some important separations which are not possible on plain papers can be carried out on impregnated papers. These separations, of course, are due to the selectivity of the impregnating material. Some of these possible separations of one metal ion from numerous others on CTA papers are: Tl(i) from 26 cations in solvent system(e), interferences are Ag(i), Pb(ii), Mo(vi); Pb(ii) from 26 cations in solvent system (e), interferences are Ag(i), Tl(i), Mo(vi); Th(iv) from 20 cations in solvent system (e), interferences are Mo (vi) , Ti(iv), Ce(iv), Ce(iii), Sb(iii), Mg(ii),

Ba(ii), Sn(iv), Se(iv); Ba(ii) from 19 cations in solvent system (f), interferences are Tl(i), Sr(ii), Fe(ii), Pb(ii), Fe(iii), Sb(iii), Ce(iii), Ti(iv), Ce(iv), Mo(vi).

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BOOK REVIEW

"Theory and Mathematics of Chromatography,"
A. S. Said; published by Dr. Alfred Huthig Verlag,
Heidelberg; 1981; 210 pp.; $38.00.

Chromatography is an analytical technique where experimental methodology has preceeded the development of an exact mathematical treatment. This volume first presents the basics of the mathematical tools needed to deal with chromatographic theory. It superficially treats such subjects as solution of algebraic equations, the properties of logarithms, differentiation, integration, series, concentration distributions, and functions, apparently as a review in preparation for the chromatographic theory. Special emphasis is given to Normal, Poisson, and Binomial distribution functions.

The theory of chromatography is mathematically developed in the second half. Subjectis covered include plate theory, variance additivity in chromatographic columns, peak shapes in gradient elution, retention relationships, composite columns, resolution, peak capacity, non-linear chromatography, temperature programming, capillary columns, and rate theory of chomatography.

This book is, by no means, light reading. It is, by its nature, highly mathematical and each section must be carefully studied more than once to be completely absorbed. It is very well written and is required reading for all who want a thorough understanding of chromatography.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 4(11), 2083 (1981)

BOOK REVIEW

"Liquid Chromatography in Clinical Analysis,"
P. M. Kabra and L. J. Marton, editors,
The Humana Press, Clifton, NJ, 1981,
466 pp., $55.00 (US)

Interest in the application of LC to clinical analysis has intensified during the past two to three years. There is a definite need for an introductory monograph on this subject, and Kabra and Marton have brought together an impessive group of experts to write it.

Included, are chapters dealing with a basic introduction to HPLC, therapeutic drug monitoring and toxicology, and analysis of endogenous human biochemicals. There is a particularly good chapter on electrochemical detection of tyrosine and tryptophan and their metabolites.

Emphasis is on methodology throughout the book, with many practical examples given. This book is recommended for those who are directly involved in clinical analysis and for scientists involved in biochemical research.

JZ

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(11), 2085-2086 (1981)

LC NEWS

HPLC COLUMN PROTECTOR SYSTEM utilizes a presaturator column and a guard column upstream of the analytical column. The packing in the presaturator column gradually dissolves, but is easily replaced by tap-filling with inexpensive bulk material. The guard column uses pre-packed replaceable cartridges containing 5 micron totally porous particles to maximize resolution. Rheodyne, Inc., JLC/81/11, P. O. Box 996, Cotati, CA, 94928, USA.

ACCESSORIES FOR LC range from a new flow reaction system to extend detection sensitivity to a modified carousel for an autosampler that permits automatic analysis of 125 samples. Several new gel permeation chromatographic columns have also been added to the company's product line. Pye Unicam, Ltd., JLC/81/11, York Street, Cambridge CB1 2PX, England.

TROUBLESHOOTING THE LIQUID CHROMATOGRAPH is a 3-part audio-visual program that provides practical instruction enabling technicians to diagnose and correct problems and to obtain good performance from their instruments, increased confidence in their data and savings in time and maintenance expenses. F. I. Scott & Associates, JLC/81/11, P. O. Box 86, Check, VA, 24072, USA.

POST COLUMN REACTION SYSTEM FOR HPLC is ideal for o-phthaldehyde reactions. It is a completely self-contained unit that is designed for procedures requiring addition of a single reagent at room temperature. It incorporates a flow-induced mixing chamber designed to provide uniform sample/reagent mixing in a minimum volume. Kratos, Inc., JLC/81/11, 24 Booker Street, Westwood, NJ, 07675, USA.

PERISTALTIC PUMP is a newly designed, compact, single channel unit for use in LC and other applications where accurately controlled flows are required. Flow rates from 0.6 to 500 ml/hr are possible. A maximum flow switch is provided to flush out tubing without changing settings. The pressure plate is designed to result in extremely low pulsation. Pharmacia Fine Chemicals, Inc., JLC/81/11, 800 Centennial Avenue, Piscataway, NJ, 08854, USA.

CARBOHYDRATE ANALYSIS BY LC utilizes a new kit which includes a prepacked analytical column, a solvent pre-conditioning column designed to prevent silica dissolution and bed subsidence, and a recomended mobile phase. Separations may be made of mixtures containing mono-, di-, and oligosaccharides. Whatman, Inc., JLC/81/11, 9 Bridewell Place, Clifton, NJ, 07014, USA.

DUAL CHANNEL COMPUTING INTEGRATOR FOR HPLC permits the user to select from a wide variety of computing routines using ROM-resident BASIC programming language. Data files may be stored on magnetic tape for later recall. Processing routines include area %, normalization, automatic recalibration, automatic baseline drift correction, and others. LDC Corp., JLC/81/11, P. O. Box 10235, Riviera Beach, FL, 33404, USA.

REACTION VIALS are used in the Reacti-Therm System for sample concentration and iso;ation prior to analysis. The system includes a nine-port evaporator for multi-sample work. Pierce Chemical Co., P. O. Box 117, Rockford, IL, 61105, USA.

THE ANALYTICAL SPECIALIST is a newsletter that describes, in detail, solutions to analytical problems by chromatographic means. It also includes useful product information. The Anspec CO., JLC/81/11, 122 Enterprise Drive, P. O. Box 7730, Ann Arbor, MI, 48107, USA.

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(11), 2087-2089 (1981)

LC CALENDAR

1981

September 28 - October 1	"Chromatography-81: Int'l. Symposium on Advances in Chromatography," Barcelona, Spain. Contact: Dr. A. Zlatkis, Chem. Dept., University of Houston, TX, 77004, USA.
September 30 - October 2	"Introduction to Liquid Chromatography," Santa Clara, CA, USA. Contact: G. Gilfillan, Hewlett-Packard Co., 1501 Page Mill Road, Palo Alto, CA, 94304, USA.
October 1 - 2	"Japan Conference on Chromatography," Palace Hotel, Tokyo, Japan. Contact: V.M. Bhatnagar, Alena Enterprises of Canada, P.O. Box 1779, Cornwall, Ontario, K6H 5V7, Canada.
October 4 - 9	"Symposium on Novel Separation Processes," at the 2nd World Congress of Chemical Engineering, Montreal, Canada. Contact: Congress Secretariat, 151 Slater Street, Suite 906, Ottawa, Ontario, Canada, K1P 5H3.
October 4 - 7	ASTM-E19 Committee Meeting, Bar Harbor Hotel, San Diego, CA, USA. Contact: ASTM, 1916 Race St., Philadelphia, PA, 19103, USA.
October 12 - 15	"EXPOCHEM '81," Houston, TX. Contact: Dr. A. Zlatkis, Chem. Dept., University of Houston, Houston, TX, 77004, USA.
October 22 - 23	"LC/MS Workshop," sponsored by International Assoc. of Environmental Analytical Chem., Palais des Congres, Montreux, Switzerland. Contact: Prof. R.W. Frei, Free University, De Boelelaan 1083, 1018WV Amsterdam, The Netherlands.
October 27 - 29	"Petroanalysis-'81," Cumberland Hotel, Marble Arch, London, England. Contact: Miss I.A. McCann, Inst. of Petroleum, 61 New Cavendish St., London, W1M 8AR, England.
November 16 - 17	"International Symposuim on HPLC of Proteins & Peptides," Washintgon, D.C. Contact: S.E. Schlessinger, Int'l. Symp. on HPLC of Proteins & Peptides, 400 E. Randolph, Chicago, IL, 60601, USA.

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

November 23 - 25 "2nd International Congress on Analytical Techniques in Environmental Chemistry," Barcelona, Spain. Contact: Expoquimia, Plaza de Espana, Barcelona-4, Spain.

November 26 - 17 Workshop: "Chem. & Anal. of Hydrocarbons in the Environment," Barcelona, Spain. Contact: J. Albaiges, Expochimia, Plaza de Espana, Barcelona-4, Spain.

1982

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

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

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

July 12 - 16 "2nd Int'l Symposium on Macromolecules," - IUPAC, University of Massachusetts, Amherst, MA, 01003, USA.

July 19 - 22 23rd Prague Microsymposium on Macromolecules: "Selective Polymeric Sorbents" - IUPAC, Institute of Macromolecular Chemistry, Prague, Czechoslovakia. Contact: P.M.M. Secretariat, Institute of Macromolecular Chemistry, 162-06 Prague 616, Czechoslovakia.

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

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

1983

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

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

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