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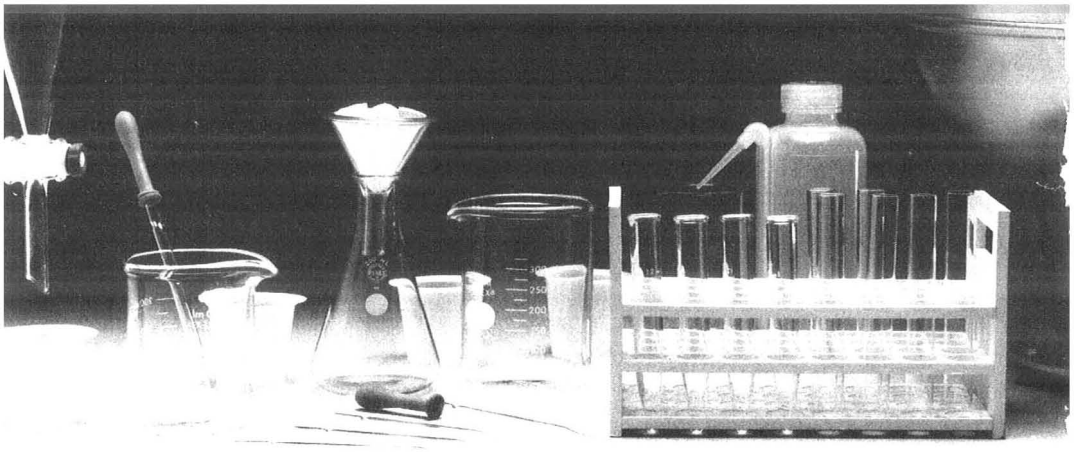

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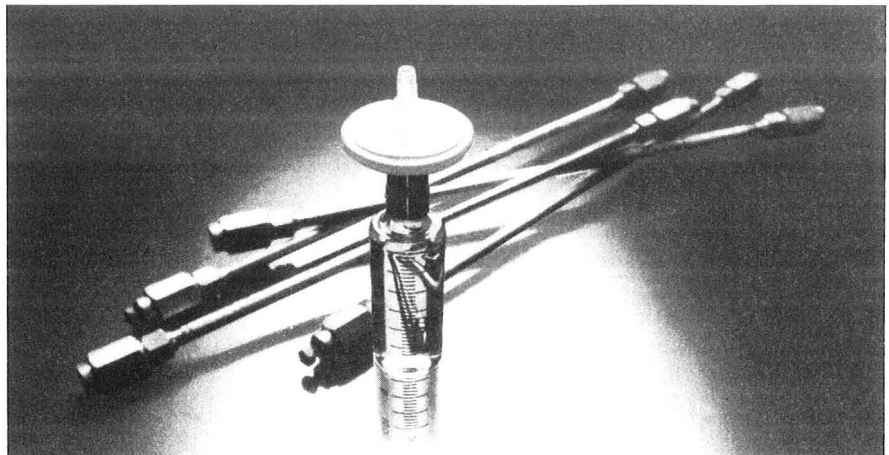

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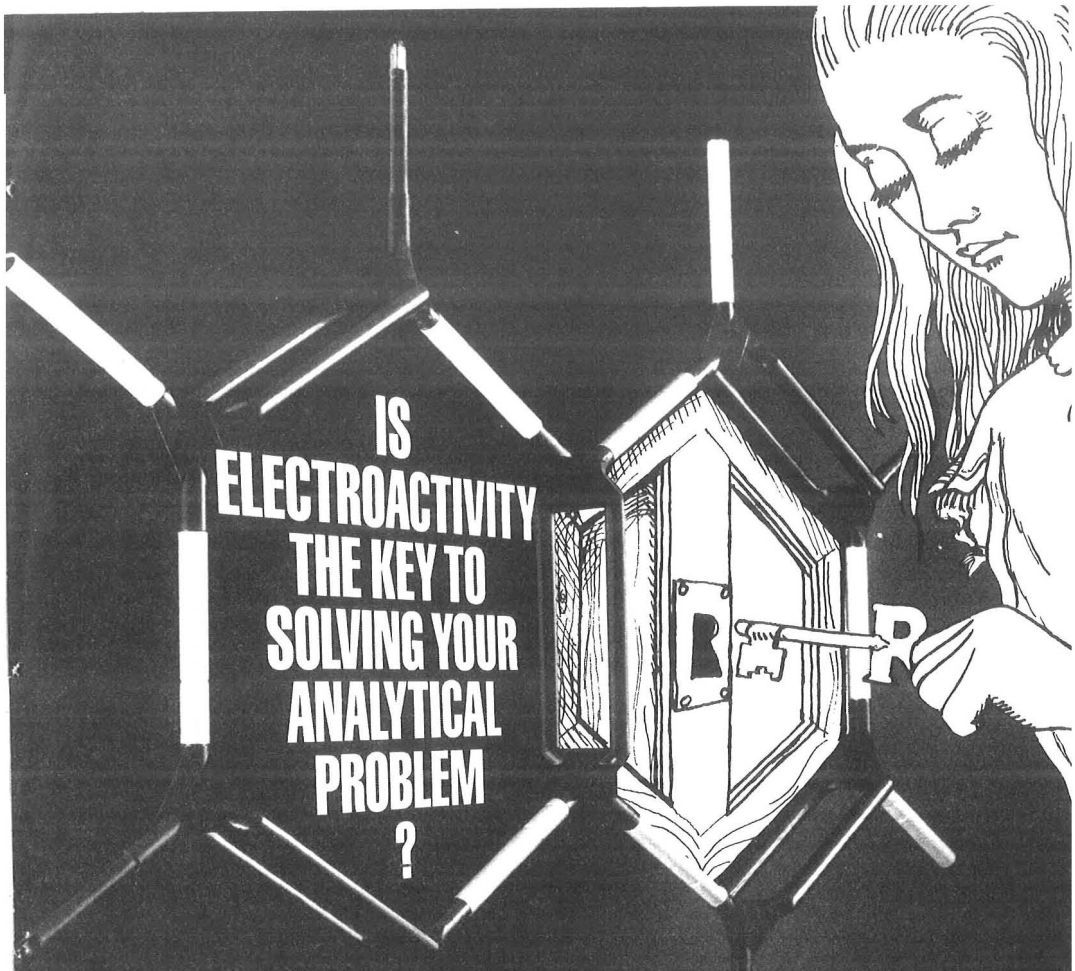

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LIQUID CHROMATOGRAPHIC DETERMINATION OF AZIDE AS THE 3,5-DINITROBENZOYL DERIVATIVE

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ABSTRACT

A method for the determination of azide after conversion to 3,5-dinitrobenzoyl azide has been developed. The derivatization reaction is fast (3 min.), quantitative, and yields a product with strong ultraviolet absorption. The derivatization reaction mixture is separated by high performance liquid chromatography so that the azide derivative can be easily quantitated. The detection limit of the method is 10 ng NaN_3/mL. The total analysis time is 20 minutes per sample.

INTRODUCTION

Sodium azide has been used in pesticides, herbicides, soil fumigants, wood preservatives, and antihypertensive drugs. More recently, it has been proposed for use as a gas generant (N_2) in inflatable systems for vehicle occupant restraint in collisions. For the latter use, interest has been focused on formulations in which all azide is decomposed, to minimize any health effects of azide. Thus, a method was needed for determining small quantities of azide in the presence of several inorganic and organic reaction products. The products were both solids and gases, collected in vehicles and in specially designed chambers (tanks).

Spectrophotometric and titrimetric methods for determining sodium azide or azide ions in aqueous solutions are quite numerous (1-3), but not satisfactory for the determination of microgram amounts, either alone or in the presence of other substances. Recently, a method for the determination of azide by ion chromatography with microgram sensitivity has been published (4). Unfortunately, this method is subject to interferences by bromide and adipate ions (present in some inflatable restraint systems), and the sensitivity is limited to about 0.1 μg NaN_3/ml.

This paper describes a rapid, sensitive method for determining azide based on its reaction in slightly acidic aqueous solution with 3,5-dinitrobenzoyl chloride to form 3,5-dinitrobenzoyl azide. An aliquot of the reaction mixture is injected onto a high performance liquid chromatograph (HPLC) where the products are separated on a reversed-phase column, and the azide derivative is detected with an ultraviolet (UV) detector at concentrations as low as 10 ng NaN_3/ml of sample. Total analysis time is 20 minutes per sample.

EXPERIMENTAL

Apparatus. A duPont Model 830 HPLC (duPont Instruments, Wilmington, DE) with a fixed wavelength (254 nm) UV detector was used. A duPont Model 833 flow controller was used to control the mobile phase flow rate at 1.0 ml/min. A pneumatically-actuated (Valco) valve (Houston, TX) with a 27-μl loop was used for sample injection.

Reagents. The mobile phase was 50 % acetonitrile and 50 % water (v/v) prepared using "distilled-in-glass" grade acetonitrile from Burdick and Jackson (Muskegon, MI) and deionized water. The 3,5-dinitrobenzoyl chloride was obtained from Aldrich Chemical (Milwaukee, WI) and was used as received. The chromatographic column was 25 cm x 4.6 mm Zorbax ODS (duPont Instruments) preceeded by a 4 cm x 3.2 mm RP—18 microparticulate precolumn (Altex Div., Beckman Instruments, Berkeley, CA).

Confirmation of the Derivative Identity. A few milligrams of 3,5-dinitrobenzoyl azide was prepared using the procedure of Munch-Peterson (5). The identity of the 3,5-dinitrobenzoyl azide in samples was confirmed by comparing the retention time to that of this known standard. In addition, in one sample, the constituent associated with the 3,5-dinitrobenzoyl azide peak was collected, the solvent was evaporated, and the residue was analyzed by high resolution mass spectrometery using an AEI MS-30 mass spectrometer. The characteristic fragmentation pattern matched that of the standard and positively identified the peak as 3,5-dinitrobenzoyl azide.

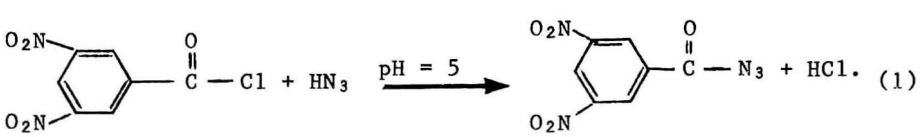
Procedure. Samples from several sources were obtained during the testing of experimental inflatable restraint systems. Air samples were collected by passing known volumes of air through midget impingers containing 0.02 N Na_2CO_3 or 0.04 N KOH solutions. The washings of tank tests (0.04 N or 0.1 N KOH) were used as received. Known quantities of solid samples were extracted at room temperature using known volumes of 0.04 N KOH to dissolve the soluble azide. Whatman filter papers were wetted with 0.02 N Na_2CO_3, used for wipe tests, and then placed in beakers containing sufficient 0.02 N Na_2CO_3 to cover the paper. Animal body fluid samples, diluted with normal saline solution, were used as received.

A 5-ml volume of one of the above prepared solutions was pipeted into a graduated, 12-ml test tube (Kontes K-569300, Vineland, NJ). (If a sample volume of less than 5.0 ml was used, sufficient 0.02 N Na_2CO_3 was added to bring the volume to 5.0 ml.) Two ml of acetonitrile and 5 drops of Bromthymol Blue indicator (0.1 g in 3.2 ml of 0.2 N NaOH diluted to 100 ml) were added. After adding 0.2 N HCl dropwise until the indicator undergoes the color change from blue to yellow, one more drop of acid was added (pH = 5). Then 50 µl of a solution of 1 g 3,5-dinitrobenzoyl chloride in 10 ml acetonitrile was added, the test tube was stoppered, shaken for

several seconds, and allowed to sit for three minutes. The final volume of the test tube was noted, and an aliquot was withdrawn for injection on the liquid chromatograph. Quantitation was accomplished by comparing the peak height for the unknown to that obtained for standards. Correction was made for the total volume in the test tube due to different amount of 0.2 N HCl being required to neutralize the sample solution and the standards. (Alternatively, all of the preparations can be brought to the same final volume by the addition of acetonitrile.)

RESULTS AND DISCUSSION

One reagent that forms derivatives that strongly absorb UV radiation is 3,5-dinitrobenzoyl chloride, which reacts in basic solution with alcohols and amines to form the 3,5-dinitrobenzoates and amides, respectively, (6). However, the nucleophilicity of the azide ion is also quite strong, and it can react with 3,5-dinitrobenzoyl chloride,



(1)

We have utilized this reaction to form a UV-absorbing derivative of azide prior to the determination by HPLC.

The chromatographic separation of the derivatization reaction mixture is accomplished without prior clean-up using reversed-phase liquid chromatography on Zorbax-ODS with 50/50 acetonitrile/water as mobile phase at a flow rate of 1.0 ml/mm. A typical chromatogram is shown in Fig. 1. As noted there, the first product that elutes is 3,5-dinitrobenzoic acid, which is the product of hydrolysis of the excess reagent. Three other peaks in the chromatogram are due to impurities in the reagent and do not interfere with 3,5-

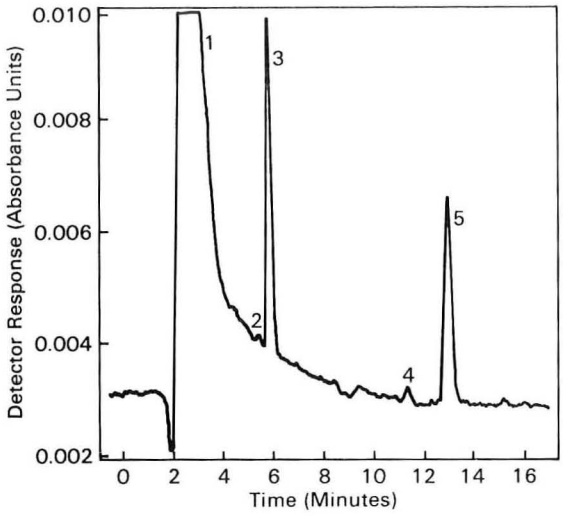


Figure 1. Chromatogram of reaction product of 0.31 μg NaN_3/ml (in impinger solution) with 3,5-dinitrobenzoyl chloride. 27-μl injection. UV detector x 0.01 absorbance units full scale. Peak 1--3,5-dinitrobenzoic acid. Peaks 2,3,4--impurities in reagent. Peak 5--3,5-dinitrobenzoyl azide.

dinitrobenzoyl azide, which elutes in about 13 minutes under these conditions.

Since hydroxide ion is a stronger nucleophile than azide, the derivatization reaction is sensitive to pH. Our results indicate that at pH $\geq$ 6.5 the azide derivative forms, but is subject to basic hydrolysis so that the amount of derivative decreases (half-life of about 2 hours at pH7). In acid solutions, pH < 3.0, the yield of azide is not quantitative, probably due to acid hydrolysis. At pH between 3.0 and 5.5 the azide derivative forms quantitatively and is quite stable, exhibiting only a 20 % loss in peak height after 10 hours. In our procedure samples are chromatographed immediately after reaction, so slow hydrolysis is not a problem. The use of Bromthymol Blue as an indicator for adjustment of the pH before the

addition of reagent is a unique and convenient way to handle samples of different pH. The indicator elutes from the HPLC column in the void volume and thus does not interfere.

The possibility of interference by other strong nucleophiles has also been investigated. Two types of interference are possible: positive interference in which the interfering specie reacts with the derivatizing reagent to yield a specie with the same retention time as the azide derivative, and a negative interference in which the interfering specie reacts with the derivatizing reagent to yield a specie with a retention time different from the azide derivative, but consumes sufficient reagent so that the reaction with azide is incomplete. The results of our study of potential interferences are shown in Table 1.

When the potential interference (except aniline) is present at eight times the azide concentration, the recovery of azide is 100 ± 3 %, which is within the precision level of the technique. It appears that thiocyanate, iodide, and methanol, although they are strong nucleophiles, do not react under the pH conditions that we have developed for the determination of azide. Acetate and ferric

TABLE 1. Effect of Potential Interferences on the Determination of Azide

Sample	Potetential Interference	Amount	Recovery of Azide, %
2.5 µg NaN_3	SCN^-	20 µg	100.0
"	I^-	20 µg	98.5
"	CH_3OH	2000 µg	100.3
"	CH_3COO^-	20 µg	100.8
"	Fe^{+3}	20 µg	101.2
"	$C_6H_5NH_2$	2 µg	97.3
"	"	20 µg	79.2
"	"	200 µg	22.0
"	"	2000 µg	5.6

TABLE 2. Typical Results for the Determination of Sodium Azide in Various Sample Matrices

Sample	Source	µg NaN_3/ml Added	µg NaN_3/ml Found	Spike Recovered (%)
1	Impinger Solution	---	< 0.01	
1A	Impinger Solution	0.30	0.30	100
2	Impinger Solution	--	5.66	
2A	Impinger Solution	3.0	8.63	99
3	Tank Wash	--	0.50	
3A	Tank Wash	0.67	1.18	101
4	Tank Wash	--	0.28	
4A	Tank Wash	0.67	0.94	99
5	Extracted Solid	--	0.51	
5A	Extracted Solid	0.30	0.80	97
6	Extracted Solid	--	2.18	
6A	Extracted Solid	1.00	3.20	102
7	Wipe Test	--	0.12	
7A	Wipe Test	0.30	0.42	100
8	Wipe Test	--	1.08	
8A	Wipe Test	3.0	4.04	99
9	Rabbit Blood Plasma	--	< 0.01	
9A	Rabbit Blood Plasma	0.30	0.31	103
10	Rabbit Blood Plasma	--	< 0.01	
10A	Rabbit Blood Plasma	5.0	4.85	97

ions, which interfere with the spectrophotometric methods for azide, do not interfere using the HPLC technique. However, aniline does exhibit negative interference (as might be expected from Eq. 1) by consuming reagent and yielding the 3,5-dinitrobenzoyl amide with a retention time of 15 minutes. At large excesses of aniline, insufficient derivatizing reagent is left to react completely with azide. Although amines do interfere, we have not noted any problems with the method, even with body fluid samples where amines may be present. Presumably their concentration is sufficiently low or they are less reactive than aniline, so they are not a problem.

Some typical results obtained with this method are shown in Table 2. The suffix "A" has been used to designate a spike sample, e.g., sample 1A was taken from the same source as sample 1 - an impinger solution used to collect air samples - but 0.3 μg NaN_3/ml was added to the impinger solution. Since no other methods are available for the determination of azide at this low level in this variety of matrices, this standard addition technique has been used to check the validity of the method.

The method has been in use for two years with no apparent problems. The reproducibility of the method is ± 3 % for repeat analyses of the same solution at the 0.3 μg NaN_3/ml level. The detection limit of the method, defined as the quantity of sodium azide originally present to give a 3,5-dinitrobenzoyl azide peak height twice the noise level, is 10 ng NaN_3/ml. This limit could be improved by the use of a larger injection loop or the use of a variable wavelength UV detector so that a wavelength closer to the maximum absorbance of the derivative (240 nm) could be utilized.

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REFERENCES

1. R. G. Clem and E. H. Huffman, Anal. Chem., 37, 366 (1965).

2. W. Selig, Mikrochimica Acta., 1971, 46 (1971).

3. E. K. Dukes and R. M. Wallace, Anal. Chem., 33, 242 (1961).

4. L. C. Westwood and E. L. Stokes, in J. D. Mulik and E. Sawicki (Editors), Ion Chromatographic Analysis of Environmental Pollutants, Vol. 2, Ann Arbor Science, Ann Arbor, MI, 1979, p. 141.

5. J. Munch-Peterson, in N. Rabjohn (Editor), Organic Synthesis, Vol. 4, Wiley, New York, 1973, p. 715.

6. J. F. Lawrence and R. W. Frei, Chemical Derivatization in Liquid Chromatography, Elsevier, New York, 1976, p. 151.

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FUEL OIL CLASSIFICATION BY GEL PERMEATION CHROMATOGRAPHY

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ABSTRACT

The use of the gel permeation chromatography (GPC) in the petrochemical laboratory is demonstrated by experimental optimization of the separation, detection and results calculation for fuel oil samples. A chromatographic response based on the fuel oil GPC separation and detection is compared with results of Conradson carbon residue (CCR) determination - which is one of the standard petrochemical tests for residue fuel. The linear relationship between the results of both tests was confirmed. The coincidence of the chromatographic method with the CCR determination is better than ±1.5% in the range 7.5 - 18.5% CCR. Comparison of refractive and UV detection for fuel oil classification is presented.

INTRODUCTION

GPC is considered as general method in petroleum analyses (1). The goal of the GPC methods development in petrochemical laboratory is to obtain molecular distribution due to a column separation with consequent detection of the components. Residual fuel contains residues from various processing units that can

vary from different refineries and frequently these residues are blended with low viscosity components to meet the requirements of the market. The identification of chemical individuals, even not groups of hydrocarbons in fuel oil is not therefore the reason of the investigation. The chromatographic results presented in form of the petrochemical parameters are more significant for the testing of the fuel quality than the separation and identification of the chemical components. Existing approaches to the fuel oil testing use measures of distillations and pyrolyses residues as described in the D 189 ASTM method - the Conradson carbon residue determination (2).

The essence of the chromatographic information is in molecular weight/size distribution. The high molecular part of a fuel oil is considered as a reason for carbon and coke deposits formed under the combustion. The direct correlation between carbon and coke deposits presence and cylinder wear of the diesel engines was confirmed (3).

Here is reported a GPC method for routine classification of fuel oils. The chromatographic system used in this study comprises an isocratic elution by tetrahydrofurane as mobile phase and styrene-divinylbenzene gel as stationary phase. The correlation between chromatographic results and results of the CCR determination are described.

EXPERIMENTAL

Apparatus

The apparatus consisted of Perkin Elmer models: serie 2/1 Liquid Chromatograph, 7010 septumless syringe injector (6 µl sample loop) and 65 variable wavelength UV detector. The

refractive index detector was Showa Denko SE 11 model. A computing integrator system Supergrator (CSI, Austin, TX, U.S.A.) was used for cumulative area measurement and data treatment.

Shodex A 803 S (25cm x 8.0 mm i.d.) analytical column (Showa Denko K.K., Tokyo, Japan) was used in this study.

Standards, reagents and samples

The individual standard solutions were prepared from the Perkin Elmer Standard kit of monodispersed polystyrenes by dissolving in mobile phase. The standards used for the calibration and testing of the column had molecular weights 600: 800: 2200: 4000: 9000 and 1 450 000.

Tetrahydrofurane and acetone, of HPLC grade, were obtained from Rathburn Chemicals Ltd., U.K.

The samples used in this study come from the Veritas fuel quality testing program.

RESULTS & DISCUSSION

Refractive index (RI) and UV detection were attempted to define optimal condition for the correlation between the CCR determination and the results of the GPC separation.

Fig. 1 shows the elution profile detected after elution by tetrahydrofurane (THF) with RI detector. It is noteworthy that compounds of highest/largest molecular weight/size have RI lower than THF and they are detected as an inversion maximum on the chromatogram (peak A-Fig. 1). The area of the inversion peak

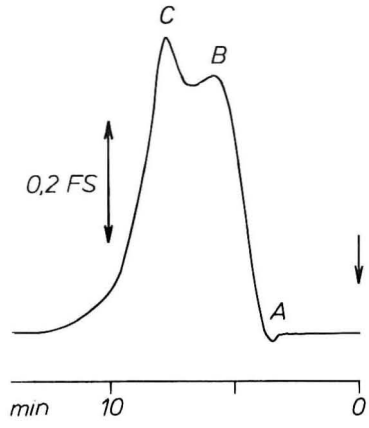

FIGURE 1

Chromatogram of the fuel oil sample, CCR = 14.5%. RI detector: 2 x 10^{-5} RI/FS, room temp. Eluent THF: flow rate 1.0 ml/min. The sample diluted 1:10 by THF(w/w).

is very close to linear function of the CCR. The inversion peak has, unfortunately, very high detection limit and for fuel oils the CCR under 11% is not clearly detectable.

Fig. 2 shows the elution profile with mobile phase consisting of THF-acetone (9:1: v/v). In this experiment the response for the compounds of high/large molecular weight/size on the positive side of the record was obtained. The maximum A (Fig. 2) is eluated with higher retention time and even the response is well detectable, the correlation between this response and the CCR is not linear. The maximum B and C monitored by RI detector are eluated for both presented chromatographic systems with the same retention time.

Table 1 illustrates the role of different wavelengths (220-400 nm) on the detection in UV region. The highest absorp-

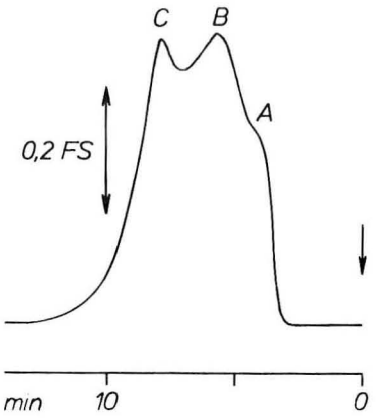

FIGURE 2

Chromatogram of the fuel oil sample (see Fig. 1). RI detector: 2 x 10^{-5} RI/FS, room temp. Eluent 9:1, THF: acetone (v/v), flow rate 1.0 ml/min. The sample diluted 1:10 by mobile phase(w/w).

tion for the high/large molecular weight/size compounds of fuel oils is obtained using the detection at 220 m.

Fig. 3 shows the chromatogram of the fuel oil sample eluated with THF and detected at 220 nm. 50 fuel oil samples were analyzed by this procedure. A linear relationship $y = a + bx + ix$ ($a = 1.3$: $b = 10.2$: $i = 0.4$) of the cumulative area (area for $t_R < 5.5$ min corresponds to molecular weight over 2500 following polystyrenes standards calibration on the CCR determination exists for the fuel oils with 7.5 - 18.5% CCR. The precision expressed as coincidence of both methods is in range 7.5 - 14.5% CCR $\pm$1.5%. The coefficient of variation for chromatographic procedure is 7% (7.5 - 13.0% CCR) and 5% (13.0 - 18.5% CCR).

In conclusion, the GPC procedure was found to be a valuable method for analyzing and classifying residual fuel oils. Using

Table 1

Absorbance at retention time corresponds to molecular weights (MW) 4000: 2500 and 2000 for the fuel oils (n=5) with CCR = 14.2% ±0.1.

λ	Absorbance at MW		
nm	4000	2500	2000
220	.307	.533	.737
230	.256	.451	.645
240	.287	.471	.626
250	.256	.405	.533
254	.297	.502	.645
260	.205	.405	.522
280	.338	.471	.579
300	.266	.445	.543
350	.133	.220	.261
400	.072	.135	.149

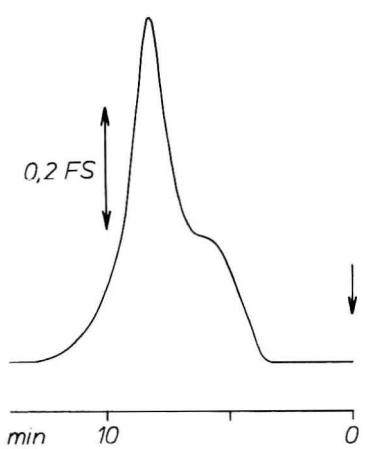

FIGURE 3

Chromatogram of the fuel oil sample (see Fig. 1) monitored by UV variable wavelength detector at 220 nm: 1: 256 AUFS, room temp., eluent THF, flow rate 1.0 ml/min. The sample diluted 1:50 by THF (w/w).

UV absorption at 220 nm rather than RI detection gave a reliable correlation of chromatographic results with CCR determination for the 50 fuel samples tested. The described method is especially attractive since it can be made fully automatic and the time required is very short, 12 minutes, compared with the CCR test which takes 40 minutes.

ACKNOWLEDGEMENTS

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REFERENCES

1. Altgelt, K.H., Gel Permeation Chromatography, in Chromatography in Petroleum Analysis. Altgelt, K.H. and Gouw, T.H., eds., Marcel Dekker, New York, p. 287 (1979).

2. Conradson carbon residue of petroleum products, standard test method D 189, in Annual book of ASTM standards, Am. Soc. Test. Mats., part 23, Philadelphia, pp. 123-129 (1980).

3. Wiborg, T.C., Wear problems - experience with how fuel quality parameters influence wear and malfunction in diesel engines, Technical report No. 81 P005, Jan. 1981, Det norske Veritas, N-1322 Høvik, Norway.

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DETERMINATION OF UV ABSORBING POLYOLEFIN ADDITIVES BY GRADIENT AND ISOCRATIC NORMAL-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A method previously developed for the rapid extraction of BHT, Irganox 1076, and Irganox 1010 from polyethylene pellets was extended to include other ultraviolet absorbing additives in polyethylene and polypropylene matrices. These were Santonox R, Ethyl 330, Goodrite 3114, and Topanol CA. Polyolefin pellets were dissolved in decalin at 110°C followed by cooling to precipitate the polymer. The concentrations of the additives present were determined by normal-phase high-performance liquid chromatography of a portion of filtered extract. The HPLC stationary phase was μ-Porasil and the mobile phase was a heptane to methylene chloride gradient. The separation of some additives strongly retained with the gradient system was studied also using an isocratic methylene chloride mobile phase.

INTRODUCTION

Recently a rapid extraction and analysis method for the three most common polyolefin additives, BHT, Irganox 1076, and Irganox

1010 was described by Schabron and Fenska (1). A hot decalin extraction procedure was followed by high-performance liquid chromatography (HPLC) on μ-Porasil with a heptane to methylene chloride gradient. The gradient was used since BHT, Irganox 1076, and Irganox 1010 have significantly different polarities, and were not well separated by isocratic HPLC systems. With this type of a gradient system compounds with a wide range of polarity can be separated. Such a system is potentially useful not only for the analysis of various additives, but also for the rapid screening of unknown or competitors products for several ultraviolet absorbing additives with a single injection. Recently, Huber and Feher (2) showed that, in general, gradient elution is superior compared to optimized isocratic elution only in the separation of not too complex mixtures of widely different components and for pilot work to find a suitable mobile phase. Thus, for a separation of one or two components of similar polarity, an isocratic system should offer better precision and simplicity.

In the present work, the rapid extraction and analysis method for BHT, Irganox 1010, and Irganox 1076 (1) was studied with four additional UV absorbing hindred phenol type additives - Santonox R (bis-(2-methyl-4-hydroxy-5-tert-butylphenyl) thioether), Ethyl 330 (1,3,5-trimethyl-2, 4,6-tris [3,5-tert-butyl-4-hydroxybenzyl] benzene), Goodrite 3114 (tris (3,5-di-tert-butyl-4-hydroxybenzyl) isocyanurate), and Topanol CA (3:1 condensate of 3-methyl-6-tert-butylphenol with crotonaldehyde). Quantitative data from both the previously described HPLC system (1) and an isocratic HPLC system were obtained and compared.

MATERIALS AND METHODS

Instrumentation

The liquid chromatograph used in this study was a Waters Model 204 liquid chromatograph equipped with two model 6000A pumps

and a Model 660 solvent programmer. The Injector was a Valco 6000 psi injector with a 25 µL sample loop. Elution was monitored with a Waters Model 450 variable wavelength detector set at 280 nm and a 10 mV strip chart recorder. The column used was a 3.9 mm i.d. x 30 cm µ-Porasil column packed with 10 micron porous silica obtained from Waters Associates, Milford, Mass. To prevent clogging the analytical column with low molecular weight polymeric material when polypropylene extracts were injected, a 4mm i.d. x 3 cm guard column packed with 37-µm C_{18} Corasil was placed in line just prior to the analytical column (1). Thermolyne Type 1000 stir plates or Lab-Line Pyro-Magnestir No. 1268 six beaker stir plate were obtained from VWR Scientific.

The sample filtering apparatus is illustrated in Reference 1. A Waters 20-30 µm stainless steel solvent reservoir filter was connected to about a 5 inch length of 3-mm i.d. Teflon tubing. The other end of the Teflon tubing was connected to a 1 1/2 inch long blunt 16-gauge Luer-Lok needle with a 1/16 inch stainless steel nut and ferrule at the end of the needle. The needle was connected to a Hamilton No. 1010 W gastight 10-mL syringe with Teflon plunger.

Reagents

Heptane was distilled in glass obtained from Burdick and Jackson, Muskegon, Mich. Chloroform was Mallinkrodt AR grade from Scientific Products. Methylene chloride was Burdick and Jackson distilled in glass. The above mobile phase solvents were all filtered through Millipore Type F-H 0.5 µm filters prior to use. Eastman decalin from Sargent-Welch was purified prior to use by passing 500 g decalin through 120 g acidic aluminum oxide activity I (Fisher Scientific) in a 30 cm x 4 cm i.d. glass column with ground glass frit. This was necessary since significant amounts of polar aromatic impurities recently have been present in

decalins from various commercial sources. Recently purified decalin has been made available by special order from Burdick and Jackson.

Naugard BHT was obtained from Uniroyal Chemical, Naugatuck, Conn. Irganox 1076 and Irganox 1010 were obtained from Ciba-Geigy, Ardsley, N. Y. Santonox R was obtained from Monsanto, St. Louis, Mo. Ethyl 330 was obtained from Ethyl Corporation, Baton Rouge, La. Goodrite 3114 was obtained from B. F. Goodrich, Cleveland, Ohio. Topanol CA was obtained from ICI, Wilmington, Del. All additives were used without further purification.

Procedure

A 50-mL portion of a standard solution containing about 0.03 mg/mL each of Ethyl 330, Santonox R, Goodrite 3114 and Topanol CA was pipetted into a 100-mL beaker. A stirring bar was added and the solution was heated to 110°C with gentle stirring for 30 min. The solution was transferred to a cool stirrer and cooled to room temperature. This heated and cooled standard solution was used to obtain quantitative data on the sample extract solutions.

About 2g polyethylene or polypropylene pellets was weighed into a 100-mL beaker. A 50-mL portion of decalin was pipetted into the beaker and the mixture was heated to 110°C on a hot plate with gentle stirring for about 30 min. or until dissolution was complete. Usually polypropylene required about 45 min. The beaker was then transferred to a cool stirrer and cooled to room temperature with stirring to precipitate the polymer.

The precipitated polymer from the above extraction was pushed aside with a microspatula. The porous metal filter portion of the filter apparatus was inserted into the solution and about 5-10 mL of solution was drawn into the syringe. The Teflon tube was removed from the ferrule on the needle and the filtered solution was dispensed into a small vial. The filter apparatus was rinsed with acetone and dried between samples. After extensive use, the

metal filter became partially clogged and was regenerated by placing it in hot decalin and stirring.

For gradient runs the Model 660 solvent programmer was set at Program 6 (linear) going from 100% heptane to 100% methylene chloride in 5 min. The mobile phase gradient was started at the point of injection. For isocratic runs the mobile phase was methylene chloride. For both gradient and isocratic systems the total flow rate was 2mL/min. The UV detector was set at 0.2 or 0.4 absorbance unit sensitivity and the recorder chart speed was 1 cm/min. Duplicate injections of each of the standard and sample solutions were made. The retention volumes in the gradient system for BHT, Ethyl 330, Irganox 1076, Samtonox R, Goodrite 3114, Irganox 1010 and Topanol CA were 8.3, 9.8, 13.2, 14.4, 17.0, 21.4 and 24.9 mL, respectively. These are not the same as those previously reported (1) since a loop injector without 2 mL dead volume was used in this study. In the isocratic system the retention volumes for Santonox R, Goodrite 3114 and Topanol CA were 4.8, 5.7 and 10.8 mL, respectively. The amount of each additive was determined from each sample injection by comparing peak heights for samples and standards. A blank decalin injection was made to determine from what points on the baseline, peak heights should be measured. For the gradient runs, gradient reset was instantaneous, from 100% methylene chloride to 100% heptane. Sample injection could be made anytime after the appearance of a refractive index peak from the UV detector, signifying the emergence of heptane from the column.

RESULTS AND DISCUSSION

A gradient chromatogram of the four additives studied in this report and the three additives previously studied (1) is presented in Figure 1. All seven additives are completely separated. This illustrates the utility of this technique for separating a wide variety of UV absorbing polymer additives.

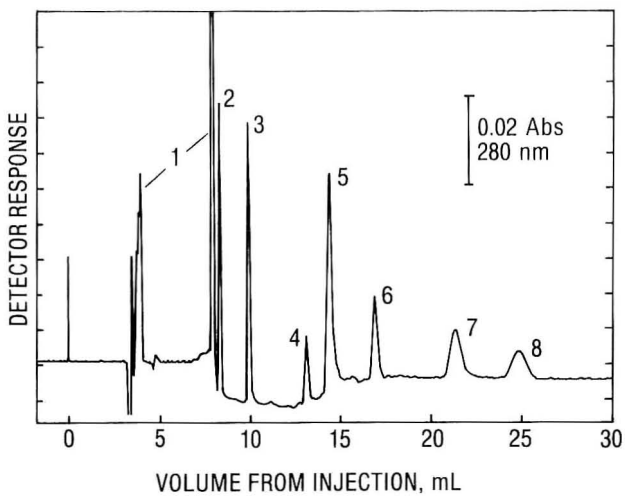

Figure 1. Separation of polyolefin additives: 1, in blank decalin; 2, 0.59 µg BHT; 3, 0.75 µg Ethyl 330; 4, 0.61 µg Irganox 1076; 5, 0.84 µg Santonox R; 6, 0.74 µg Goodrite 3114; 7, 0.64 µg Iragnox 1010; 8, 0.65 µg Topanol CA.

Accuracy

Spiking experiments were performed by dissolving polyolefin samples containing none of the additives under study in decalin containing known amounts of additives. The results of spiking 2 g portions of polyethylene and polypropylene are listed in Table I. The gradient HPLC separation was used. The results show good recovery of the additives Ethyl 330, Santonox R, Goodrite 3114, and Topanol CA at levels corresponding to 0.05% and 0.1% of each additive in the polymer. These good recoveries indicate that the additives were evenly distributed in the decalin both inside and outside the polymer "sponges" resulting from the extraction. Similar results were observed for BHT, Irganox 1076 and Irganox 1010 (1).

Sample Size

A polyethylene and a polypropylene sample, each containing the four additives, were each analyzed in duplicate at sample

TABLE I

RECOVERIES OF ETHYL 330, SANTONOX R, GOODRITE 3114 AND TOPANOL CA

Sample	Amount, g	Amount Added, mg				Amount Found, mg				Percent Recovered			
		Ethyl 330	Santonox R	Goodrite 3114	Topanol CA	Ethyl 330	Santonox R	Goodrite 3114	Topanol CA	Ethyl 330	Santonox R	Goodrite 3114	Topanol CA
PE	1.92	1.22	--	1.25	1.20	1.24	--	1.40	1.18	101	--	108	97
PE	1.86	1.22	--	1.25	1.20	1.24	--	1.38	1.22	101	--	106	101
PE	1.90	2.44	--	2.59	2.41	2.46	--	2.84	2.41	101	--	110	100
PE	2.13	2.44	--	2.59	2.41	2.44	--	2.82	2.40	100	--	109	99
PE	1.95	--	1.32	--	--	--	1.31	--	--	--	99	--	--
PE	1.98	--	1.32	--	--	--	1.30	--	--	--	98	--	--
PE	2.02	--	2.65	--	--	--	2.68	--	--	--	101	--	--
PE	2.28	--	2.65	--	--	--	2.54	--	--	--	96	--	--
PP	1.89	1.22	--	1.30	1.20	1.26	--	1.38	1.10	103	--	106	91
PP	2.20	1.22	--	1.30	1.20	1.24	--	1.34	1.12	102	--	104	93
PP	2.39	2.44	--	2.59	2.41	2.65	--	2.79	2.51	108	--	108	104
PP	2.02	2.44	--	2.59	2.41	2.58	--	2.86	2.28	106	--	110	99

amounts of about 1, 2 and 4 g, respectively. The results are listed in Table II. These data show the absence of significant constant error.

Precision

A polyethylene sample containing all four additives was analyzed in six replicate runs. The results are listed in Table III. These results show good precision for the method. A polypropylene sample containing the four additives, also was analyzed in six replicate runs. These results are listed in Table IV. They also show a good precision for the method.

The precision was slightly improved when the six polyethylene extracts (Table III) were injected using the isocratic HPLC system. These results are listed in Table V. Ethyl 330 could not be determined with a methylene chloride mobile phase because it elutes with the solvent front.

During the course of these precision studies, data comparing the precision for duplicate sample injections with the precision

TABLE II

SAMPLE SIZE VARIATION RESULTS WITH POLYETHYLENE AND POLYPROPYLENE

		Amount Found, wt %			
Sample	Amount, g	Ethyl 330	Santonox R	Goodrite 3114	Topanol CA
PE	1.01	0.074	0.063	0.090	0.086
PE	1.04	0.079	0.072	0.092	0.096
PE	2.16	0.080	0.078	0.100	0.095
PE	2.01	0.072	0.067	0.084	0.099
PE	4.01	0.072	0.070	0.091	0.082
PE	4.01	0.075	0.073	0.091	0.084
PP	1.00	0.070	0.051	0.091	0.094
PP	1.07	0.074	0.051	0.094	0.099
PP	2.02	0.076	0.058	0.097	0.096
PP	2.02	0.077	0.051	0.100	0.094
PP	4.01	0.072	0.059	0.093	0.090

TABLE III

RESULTS OF SIX REPLICATE DETERMINATIONS FOR A POLYETHYLENE SAMPLE, USING THE GRADIENT HPLC SYSTEM

Sample Amount,g	Amount Found, wt % Ethyl 330	Santonox R	Goodrite 3114	Topanol CA
1.97	0.082	0.073	0.100	0.093
2.09	0.079	0.074	0.094	0.086
2.01	0.078	0.072	0.092	0.088
2.00	0.074	0.071	0.088	0.086
2.01	0.072	0.068	0.084	0.091
2.06	0.080	0.076	0.096	0.090
$\bar{x}$[a] =	0.078	0.072	0.093	0.089
S =	0.0038	0.0029	0.0059	0.0030
95% Confidence	±0.0040	±0.0030	±0.0062	±0.0031
$\bar{x}$[b] =	0.078	0.074	0.092	0.087
S =	0.0040	0.0035	0.0063	0.0035
95% Confidence	±0.0042	±0.0037	±0.0066	±0.0037

a. Duplicate injection results.
b. Single injection results.

for single sample injections were obtained. These data are listed in Tables III, IV and V for polyethylene extracts separated by gradient HPLC, polypropylene extracts separated by gradient HPLC, and polyethylene extracts separated by isocratic HPLC, respectively. Generally, the precision drops somewhat when only single injections are made. The data show this precision drop to be slight and thus it should be possible to make single sample extract injection in cases when a relatively large number samples must be analyzed in a short period of time.

TABLE IV

RESULTS OF SIX REPLICATE DETERMINATIONS FOR A POLYPROPYLENE SAMPLE, USING THE GRADIENT HPLC SYSTEM

Sample Amount,g	Amount Found, wt % Ethyl 330	Santonox R	Goodrite 3114	Topanol CA
2.02	0.076	0.062	0.097	0.094
2.02	0.079	0.058	0.101	0.094
2.10	0.076	0.064	0.098	0.097
2.02	0.076	0.057	0.099	0.090
2.01	0.075	0.066	0.104	0.099
2.01	0.076	0.064	0.099	0.097
$\bar{X}$[a] =	0.076	0.062	0.099	0.095
S =	0.0014	0.0035	0.0025	0.0032
95% Confidence	±0.0015	±0.0037	±0.0026	±0.0034
$\bar{X}$[b] =	0.077	0.063	0.099	0.092
S =	0.0019	0.0031	0.0032	0.0033
95% Confidence	±0.0020	±0.0033	±0.0034	±0.0035

a. Duplicate injection results.
b. Single injection results.

Limits of Detection

The limits of detection for the additives were calculated based on 2-mm peak heights at 0.2 Abs. This corresponds to a S/N ratio of about 2. The limits of detection for a 25 µL extract from a 2 g polymer sample separated with the gradient HPLC system are 0.038 mg or 0.001% Ethyl 330, 0.058 mg or 0.002% Santonox R, 0.13 mg or 0.006% Goodrite 3114, and 0.32 mg or 0.016% Topanol CA. These limits are quite sufficient for the analysis of typical additive levels of about 0.05% or greater.

TABLE V

RESULTS OF SIX REPLICATE DETERMINATIONS FOR A POLYETHYLENE SAMPLE, USING THE ISOCRATIC HPLC SYSTEM

Sample Amount,g	Amount Found, wt % Santonox R	Goodrite 3114	Topanol CA
1.97	0.084	0.10	0.098
2.01	0.079	0.094	0.088
2.10	0.079	0.095	0.087
2.00	0.078	0.091	0.087
2.16	0.081	0.095	0.090
2.01	0.077	0.088	0.084
$\bar{x}^a$ =	0.080	0.094	0.089
S =	0.0025	0.0041	0.0048
95% Confidence	±0.0026	±0.0041	±0.0048
$\bar{x}^b$ =	0.080	0.093	0.089
S =	0.0027	0.0045	0.0052
95% Confidence	±0.0028	±0.0047	±0.0055

a. Duplicate injection results.
b. Single injection results.

CONCLUSION

The method described in this report, when combined with the method previously reported (1) for determining UV absorbing polymer additives by normal-phase HPLC on μ-Porasil following decalin extraction, provides a useful tool for quality assurance or lot certification analyses. The seven additives: BHT, Ethyl 330, Irganox 1076, Santonox R, Goodrite 3114, Irganox 1010 and Topanol CA can be determined individually or in any combination with a single HPLC system. Slight variations in the HPLC system also allow for the analysis of Tinuvin 144 (3) in polypropylene following decalin extraction. Other additives ammenable to the decalin extraction followed by separation on other normal-phase

HPLC systems are Irganox 1024 and UV 531. Work on these latter two additives is currently underway.

The decalin extraction followed by gradient normal-phase HPLC described in this report should provide a useful starting point for future analytical methods development work for other new UV absorbing additives, which may be used in polymer formulations.

REFERENCES

1. Schabron, J. F. ; Fenska, L. E. Anal. Chem. 1980, 52, 1411.

2. Huber J. F. K.; Feher, I. "Isocratic Versus Binary Gradient Elution in Column Liquid Chromatography", Fourth International Symposium on Column Liquid Chromatography, Boston, Ma., May 7-10, 1979.

3. Schabron, J. F.; Bradfield, D. Z. J. Appl. Polym. Sci. 1981, 26, 2479.

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THE EFFECT OF BINARY SOLVENT COMPOSITION AND POLARITY ON SEPARATIONS IN REVERSED PHASE THIN LAYER AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY[1,2]

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ABSTRACT

The effect of the solvent's composition and polarity on separation in reversed phase thin layer and high performance liquid chromatography is discussed. These results show that retention times cannot be predicted merely from the polarity of the binary mobile phase. Although organic modifiers with the same physico-chemical properties and from the same solvent group were used, the retention times obtained using binary mobile phases having the same polarity, were different. It was also observed that normal chain carbon alcohols gave retention times different from those with a branched chain (n-propanol vs. iso-propanol), and the longer the alcohol chain the higher the R_f value. The results also show that not only the organic modifier used is important but the solute mixture used.

INTRODUCTION

It is not easy to select the best solvent system for adsorption, partition or ion exchange chromatography. Important considerations are the nature of the material being separated (polar, non polar or ionic), and the solid

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[2]Presented in part at the 1981 Pittsburgh Conference on Anal. Chem. and Appld. Spectrosc., Atlantic City, NJ, Paper #439.

phase (silica gel, alumina, cellulose, ion exchange, or reverse phase C_2, C_8 or C_{18}). The appropriate solvent can only be selected when these two factors have been decided. When binary solvent mixtures are used, the analyst must consider the solubility of the solute in the solvent, the effect of solvent demixing, solvent strength (polarity) and hydrogen bonding. In ion exchange, the buffer used, pH and ionic strength are important. Selection of a binary solvent mixture and prediction of the elution times for the components of a mixture may not be that simple. In reverse, and normal phase liquid chromatography elution times of the solute are a function of the properties of the stationary and mobile phase.

Synder (1) gave the following equation for calculating the polarity of a binary solvent mixture used in reversed phase liquid chromatography:

$$P' = \phi_1 P_1 + \phi_2 P_2 \qquad (I)$$

P' is the polarity of the mixture, ϕ_1 and ϕ_2 are the volume fractions of the two solvents and P_1 and P_2 are the polarity of the pure solvents. This relation does not apply to normal phases in which the calculations are more complex (1).

The relation between retention time (R_t) and capacity factor (K') is described by the following equation:

$$K' = (R_t - R_{to})/R_{to} \qquad (II)$$

Where R_{to} is equal to the retention time of unretained solute.

Our objectives were to determine (a) the effect of the binary mobile phase polarity and composition on separation; and (b) how the solvent affects the bonded alkyl chain and, thus, the separation in reversed phase thin layer (TLC) and high performance liquid chromatography (HPLC).

EXPERIMENTAL

Materials: Solvents were glass distilled (Burdick and Jackson). Chemicals were analytical grade (Aldrich Chemical Co.) and used without further purification. Reversed phase (RP-18) and silica gel TLC plates were purchased from Whatman, Inc. Standard TLC tanks and equipment were used. Plates were spotted

with 5 μl disposable micropipettes. The mobile phase was a binary alcohol/water, and alcohol/alcohol mixture. The alcohols were methanol (MeOH), ethanol (EtOH), n-propanol (n-PrOH), iso -propanol (i-PrOH), tert-butanol (t-BuOH) and iso-pentanol (i-POH). Results with other binary solvent mixtures were also examined.

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

The RP-18 reversed phase column (Merck) and μ-porasil column (Waters Associates) were 250 mm X 4.6 mm prepacked with 10 μm particle size materials. 10 μl samples were injected. Experiments were run at room temperature using a mobile phase flow rate of 1.2 ml/min. Retention times, peak widths (W) and resolution (R_S) were determined with a Packard 1865 A/D converter connected to the UV detector output of the liquid chromatograph. The output from the data system was recorded on a 9866A thermal line printer (Hewlett-Packard).

RESULTS AND DISCUSSION

Table 1 shows the separation of benzo(e)pyrene (BeP) and anthracene spotted on reversed phase C_{18} (TLC) plates and developed in binary alcohol/water mixtures having the same polarity (5.60), calculated according to eq. (I). Note that solvent demixing occurred when n-BuOH/H_2O, and i-PenOH/H_2O were used and, as a result, high R_f values were obtained and the results had to be discarded. However, when mixtures of MeOH/H_2O, EtOH/H_2O and n-PrOH/H_2O were used the R_f values for BeP and anthracene were different. Also, note that the solvent mixtures n-PrOH/H_2O and i-PrOH/H_2O gave different elution times, with i-PrOH/H_2O giving lower R_f values for both BeP and anthracene. When the R_f values were compared using i-PrOH/H_2O and t-BuOH/H_2O, the longer carbon chain alcohols gave higher R_f values, but the separation factor α remained the same.

Since solvent demixing occurred with i-PrOH/H_2O and n-BuOH/H_2O pairs, it was decided to (a) use a lower polarity value (4.3) and to substitute methanol

TABLE I

SEPARATION OF BENZO[E]PYRENE AND ANTHRACENE ON RP-18 TLC PLATES USING A BINARY ALCOHOL/WATER MIXTURES HAVING THE SAME POLARITY (5.60)

SOLVENT	RATIO (v/v)	R_fX100 BeP	R_fX100 ANTHRACENE	α
MeOH/H_2O	90.2/9.8	18	33	1.83
EtOH/H_2O	78/22	23	38	1.65
n-PrOH/H_2O	74.2/25.8	35	51	1.46
i-PrOH/H_2O	73/27	24	38	1.58
t-BuOH/H_2O	75.4/24.6	28	44	1.57
i-POH/H_2O	70.8/29.2	68*	77*	-
n-BuOH/H_2O	73/27	63*	71*	-

*Solvent Demixing

α = R_f anthracene/R_f BeP

or acetone (AC=0) for water (Table II). The table shows that although the nine binary solvent mixtures have the same polarity, different R_f and α values were obtained for both BeP, and anthracene in each solvent mixture. R_f X 100 values ranged from 49-80 for BeP, and from 61-85 for anthracene. Note that, for example, n-BuOH/H_2O, n-BuOH/MeOH, and n-BuOH/AC=0 all gave different R_f values for both BeP and anthracene. This is because water, methanol and acetone each belong to a different solvent group (1). Differences in retention times of BeP and anthracene were observed when the plate was spotted and developed in the following pairs of binary solvent mixtures, n-BuOH/MeOH and t-BuOH/MeOH; and n-PrOH/MeOH and i-PrOH/MeOH, with the normal chain solvent giving the higher R_f values.

In another experiment, the volume of alcohol was kept constant at 90 ml (Table III) and 95 ml (Table IV), while the volume of water was calculated according to the following eq:

$$V_1 P_1 + V_{H_2O} \times P_{H_2O} = V_2 P_2 + V_{xH_2O} \times P_{H_2O} \qquad \text{(III)}$$

TABLE II

EFFECT OF BINARY SOLVENT COMPOSITION ON R_f AND α VALUES IN REVERSED PHASE TLC (P' = 4.3)

SOLVENT	RATIO (v/v)	R_fX100 BeP	R_fX100 ANTHRACENE	α
EtOH	neat	63	73	1.16
n-PrOH/H_2O	95.2/4.8	60	70	1.17
n-PrOH/MeOH	72.7/27.3	71	81	1.14
n-BuOH/H_2O	93.6/6.4	60	76	1.27
n-BuOH/MeOH	66.7/33.3	72	78	1.08
i-PrOH/MeOH	66.7/33.3	56	68	1.21
t-BuOH/MeOH	80/20	49	61	1.24
n-BuOH/AC=O	66.7/33.3	80	85	1.06
n-PrOH/AC=O	72.7/27.3	75	81	1.08

TABLE III

EFFECT OF BINARY MOBILE PHASE COMPOSITION ON R_f AND α VALUES OF BENZO[E]PYRENE AND ANTHRACENE USING CONSTANT ALCOHOL BUT VARIABLE WATER VOLUME

SOLVENT	RATIO (v/v)	P'	R_fX100 BeP	R_fX100 ANTHRACENE	α
MeOH/H_2O	90/10	5.6	18	33	1.83
EtOH/H_2O	90/17	5.2	29	44	1.52
n-PrOH/H_2O	90/19.7	5.1	38	51	1.34
i-PrOH/H_2O	90/20.6	5.1	29	44	1.52
n-BuOH/H_2O	90/20.6	5.1	53	63	1.19
t-BuOH/H_2O	90/18.8	5.2	36	52	1.44
i-POH/H_2O	90/22.4	5.0	65	73	1.12

Where V_1 and V_2 are volumes of pure alcohols 1 and 2, P_1 and P_2 are the polarity of pure alcohols 1 and 2, V_{H_2O} is the known volume of water added and $V_{x(H_2O)}$ is calculated volume of water. V_1 and V_2 were kept constant at 90 ml. V_{H_2O} was 10 ml. The experiment was performed to see the effect the water in the mobile

phase had on (a) separation and (b) the C_{18} alkyl chain. The binary solvent polarity values of the mixture were not constant and varied by ± 0.1 units, except for MeOH/H_2O (Tables III and IV). The tables indicate that, although the amount of alcohol is constant, the R_f values of both BeP and anthracene, increased with increasing amount of water for both the normal, and the branched chain alcohols, with the latter giving lower R_f values. A few conclusions can be drawn from the data in Tables III and IV. (a) The R_f X 100 values ranged from 33-73 for anthracene, and from 18-65 for BeP in 90ml alcohol/water (Table III) while they ranged from 47-79 for anthracene and from 27-72 for BeP in 95ml alcohol/water (Table IV); (b) better separation factors were found with the branched chain alcohols; and (c) the same R_f values were obtained for both compounds in EtOH/ H_2O, and i-PrOH/ H_2O, although there was more water in the i-PrOH/H_2O binary mixture, which may indicate that the solutes are more soluble in i-PrOH than in methanol.

The results (Tables I-IV) indicate that R_f values were higher as the amount of water in the mobile phase was increased, which may indicate a lack of interaction between the solute and the solid phase, and in turn collapse of the C_{18} alkyl chain in the solvent as the volume of water increased. It also could indicate a solute-solvent interaction. Tables III and IV show that, although

TABLE IV

FFFFCT OF BINARY MOBILE PHASE COMPOSITION ON R_f AND α VALUES OF BENZO[E]PYRENE AND ANTHRACENE USING CONSTANT ALCOHOL BUT VARIABLE WATER VOLUME

SOLVENT	RATIO (v/v)	P'	R_fX100		α
			BeP	ANTHRACENE	
MeOH/H_2O	95/5	5.4	27	47	1.74
EtOH/H_2O	95/12.5	5.0	30	46	1.53
n-PrOH/H_2O	95/15.2	4.9	43	57	1.33
i-PrOH/H_2O	95/16.2	4.8	33	47	1.42
n-BuOH/H_2O	95/16.2	4.8	72	79	1.10
t-BuOH/H_2O	95/14.3	4.9	46	63	1.37
i-POH/H_2O	95/18.0	4.7	70	78	1.11

the same volume of water was used in the i-PrOH and n-BuOH mixtures, different R_f and α values were obtained, which suggests that the alcohol used plays an important role in the separation process.

In view of these results another experiment was undertaken in which three different groups of compounds with different chemical properties were spotted on C_{18} reversed phase TLC plates and developed under the following conditions (a) pure alcohols: (b) constant alcohol/water ratio (90/10); (c) constant water but variable alcohol ratio; (d) constant alcohol but variable water ratio; and (e) constant polarity (5.1) using methanol/alcohol and water/alcohol. The three group of compounds selected for this study were: (1) anthraquinones [anthraquinone (A), methyl- (M) and ethylanthraquinone (E)]; (2) naphthalene (N) and biphenyl (B); and (3) dimethyl- (MP) and diethyl- (EP) phthalates.

Note that, since we are studying the interaction of the solvent-solute-bonded chain, we are ignoring the optimization of the separation factor(α). Table V lists R_f values for the three groups of compounds in pure alcohols. It can be seen that R_f values did not increase with increasing chain length of the alcohol (except methanol), which indicates that solute-solid phase interaction, due to the solubility of the C_{18} alkyl chain in the alcohols studied, is not

TABLE V

SEPARATION OF ANTHRAQUINONE, METHYL- AND ETHYL ANTHRAQUINONE, NAPTHALENE, BIPHENYL, DIMETHYL- AND DIETHYLPTHALATES IN PURE ALCOHOLS USING RP-18 TLC PLATES

	R_fX100		
SOLVENT	ANTHRAQUINONES	N+B	PHTHALATES
MeOH	53, 48, 48	58	69, 66
EtOH	72	78	88
PrOH	75	80	88
i-PrOH	71	76	83
n-BuOH	70	75	78
i-BuOH	73	79	85
t-BuOH	76	80	88

Note: When one number is reported it means that no separation was achieved in that solvent.

affected by the alcohol used. When constant ratios of alcohol/water v/v were used, the R_f values obtained for each group of compounds in the binary solvent mixture were a function of the solute. The R_f values for naphthalene and biphenyl and the anthraquinones increased with the increase in normal alcohol chain length. The phthalates gave comparable R_f values in MeOH/H_2O, EtOH/H_2O, n-PrOH/H_2O and n-BuOH/H_2O. When the branched chain alcohol/water mixtures were used (Table VI), the R_f values for each group of compounds remained approximately constant (±0.03 Units).

When the results in pure alcohol (Table V) were compared with those in (90/10) alcohol/water (Table VI), differences in R_f values were observed. It was also observed that MeOH/H_2O (90/10) resolved the components of all the three groups of compounds. The addition of 10% water to MeOH and EtOH (Table VI) resulted in lower R_f values for the anthraquinones than when developed in the pure alcohol. No differences in R_f values were observed when water was added to n-PrOH, i-PrOH, i-BuOH or t-BuOH. However, higher R_f values were obtained when water was added to n-BuOH. Almost the same effect was observed for naphthalene, biphenyl and the phthalates (Table VI).

TABLE VI

SEPARATION OF ANTHRAQUINONE, METYL ANTHRAQUINONE, ETHYL ANTHRAQUINONE, NAPTHALENE, BIPHENYL, DIMETHYL- AND DIETHYLPHTHALATES IN CONSTANT ALCOHOL/WATER RATIO (90/10) USING RP-18 TLC PLATES

	R_fX100		
MOBILE PHASE	ANTHRAQUINONES	N+B	PHTHALATES
MeOH-H_2O	47, 40, 36	53, 47	73, 66
EtOH-H_2O	45, 50, 50	52	70, 67
PrOH-H_2O	75	75	89
i-PrOH-H_2O	70	71	87
n-BuOH-H_2O	80	81	92
i-BuOH-H_2O	76	76	79
t-BuOH-H_2O	76	80	88
i-POH-H_2O	76	76	90

Table VII gives the R_f values of the three groups of compounds developed in alcohol/water. The volume of water was kept constant (10 ml), while that of methanol (90 ml) was replaced by a volume of an alcohol having the same polarity as that of methanol (0.90 X 5.1 = 4.59). In this experiment we wanted to see the effect of keeping the polarity of the alcohol and the volume of the water constant. Note that the polarity of each binary solvent mixture is not constant according to equation (I). The results show (Table VII) that resolution of the mixture in each group is achieved only when MeOH/H_2O and EtOH/H_2O were used, but no separation was achieved using an alcohol with a chain longer than C_2, although the three groups of compounds tested have different chemical properties. This indicates that solvent-solute or solute-solid phase (C_{18}) interaction was eliminated and no separation was achieved using C-3 or higher chain alcohols. Table VII also shows that, although the R_f values increased from MeOH to n-PrOH, they were very similar to those obtained with the other alcohols (n-ProH to t-BuOH).

Table VIII lists the R_f values obtained when the volume of alcohol was kept constant (90 ml) but the volume of water was changed to compensate for the polarity of the alcohols according to equation (III). The results indicate that better resolutions were obtained for the anthraquinones and phthalates but not

TABLE VII

SEPARATION OF ANTHRAQUINONE, METHYL- AND ETHYL ANTHRAQUINONE, NAPHTHALENE, BIPHENYL, DIMETHYL- AND DIETHYLPHTHALATES USING CONSTANT ALCOHOL AND CONSTANT WATER POLARITY MOBILE PHASES ON RP-18 TLC PLATES

SOLVENT	RATIO (v/v)	R_fX100		
		ANTHRAQUINONES	N+B	PHTHALATES
MeOH/H_2O	(90/10)	47, 40, 35	53, 57	75, 68
EtOH/H_2O	(107/10)	67, 64, 61	66	85, 81
n-PrOH/H_2O	(115/10)	73	74	88
n-BuOH/H_2O	(118/10)	73	74	83
i-PrOH/H_2O	(118/10)	71	71	86
t-BuOH/H_2O	(112/10)	75	74	87

TABLE VIII

SEPARATION OF ANTHRAQUINONE, METHYL-, AND ETHYL ANTHRAQUINONE, NAPHTHALENE, BIPHENYL, DIMETHYL- AND DIETHYLPHTHALATES USING CONSTANT ALCOHOL BUT VARIABLE WATER VOLUME ON RP-18 TLC PLATES

		R_fX100		
SOLVENT	RATIO (v/v)	ANTHRAQUINONES	N+B	PHTHALATES
MeOH/H_2O	(90/10)	47, 40, 35	53, 47	75, 78
EtOH/H_2O	(90/18)	63, 57, 53	56	85, 79
n-PrOH/H_2O	(90/20)	65, 61, 61	59	77, 77
n-BuOH/H_2O	(90/21)*	-	-	-
i-PrOH/H_2O	(90/21)	64, 60, 58	58	81, 78
t-BuOH/H_2O	(90/18.8)	76, 76, 76	73	92, 92

*Solvent demixing

the naphthalene or the biphenyl, which means that that the results are affected by the solute used (3).

Table IX shows the effect of replacing water with methanol to adjust the polarity of the binary solvent mixture to 5.1. The results indicate an increase in R_f values with increase in the alcohol chain length. However, the resolution of the solutes within a group was poor. This indicates that solvent-solute interactions are the predominant factor, since separation is a function of the solute distribution coefficient. Table X gives the R_f values in a binary alcohol/water (P' = 5.1) solvent mixture. Except for naphthalene-biphenyl mixture, the results indicate better resolution with alcohol/ water than with alcohol/methanol solvent mixtures (Table IX), although the R_f values obtained for each compound are comparable, (Tables IX and X). This is because the addition of water to the mobile phase decreases the solubility of the solutes (4).

Table XI gives the retention times and K' values for the three groups of compounds using a C_{18} column and binary alcohol/water mixtures having the same P' value of 6.12 calculated according to Equation (I). Note that different K' values were obtained for each compound in each mobile phase. These results agree with those obtained using C_{18} reverse phase TLC plates.

TABLE IX

SEPARATION OF ANTHRAQUINONE, METHYL- AND ETHYL ANTHRAQUINONE, NAPTHALENE, BIPHENYL, DIMETHYL- AND DIETHYLPHTHALATES IN ALCOHOL/METHANOL BINARY SOLVENT MIXTURES HAVING A CONSTANT POLARITY OF 5.1 ON RP-18 TLC PLATES

SOLVENT	RATIO (v/v)	R_fX100 ANTHRAQUINONES	N+B	PHTHALATES
MeOH	100%	58, 52, 52	63	78, 73
EtOH/MeOH	86.4/13.6	61	68	75
PrOH/MeOH	82.3/17.7	66	72	78
i-PrOH/MeOH	81 / 19	67	71	80
BuOH/MeOH	81 / 19	79	83	89
t-BuOH/MeOH	83.6/16.4	68	73	83

TABLE X

SEPARATION OF ANTHRAQUINONE, METHYL- AND ETHYL ANTHRAQUINONE, NAPHTHALENE, BIPHENYL, DIMETHYL- AND DIETHYLPHTHALATES IN ALCOHOL/WATER BINARY SOLVENT MIXTURES HAVING A CONSTANT POLARITY OF 5.1 ON RP-18 TLC PLATES

SOLVENT	RATIO (v/v)	R_fX100 ANTHRAQUINONES	N+B	PHTHALATES
MeOH	100%	56, 51, 50	60	72, 69
EtOH/H_2O	86.4/13.6	56, 52, 49	52	73, 69
n-PrOH/H_2O	82.3/17.7	74, 70, 68	68	88, 84
BuOH/H_2O*	81.0/19.0	-	-	-
i-PrOH/H_2O	81.0/19.0	69, 66, 63	63	87, 83
t-BuOH/H_2O	83.6/16.4	65, 65, 65	64	82, 82

*Solvent demixing

When the binary solvent mixture was composed of solvents from different solvent groups (1), the retention times were different on both silica gel and C_{18} reversed phase silica gel plates (Table XII), although they have the same P' value. It is interesting to note that the retention times of naphthalene on silica gel plates were approximately the same no matter what binary mobile phase

TABLE XI

SEPARATION OF ANTHRAQUINONE, METHYL- AND ETHYL ANTHRAQUINONE, NAPHTHALENE, BIPHENYL, DIMETHYL- AND DIETHYLPHTHALATES USING C_{18} HPLC COLUMN AND BINARY ALCOHOL/WATER MOBILE PHASES HAVING THE SAME POLARITY (6.12)

SOLVENT	RATIO (v/v)	R_{to}(min.)	R_t/k'	A	M	E	N	B	MP	EP
MeOH/H_2O	80/20	2.55	R_t	6.5	7.7	9.0	5.5	6.5	3.5	4.5
			K'	1.55	2.02	2.53	1.16	1.55	0.37	0.61
EtOH/H_2O	69.4/30.6	2.55	R_t	5.1	6.6	6.6	5.8	6.6	3.2	3.7
			K'	1.00	1.59	1.59	1.28	1.59	0.26	0.45
n-PrOH/H_2O	65.8/34.2	2.95	R_t	4.5	5.1	5.1	5.4	5.4	3.8	3.8
			K'	0.53	0.73	0.73	0.83	0.83	0.29	0.29
i-PrOH/H_2O	64.8/35.2	3.20	R_t	5.5	5.5	6.5	6.5	6.9	3.9	4.3
			K'	0.72	0.72	1.03	1.03	1.16	0.22	0.34

TABLE XII

SEPARATION OF A MIXTURE OF ANTHRAQUINONE, NAPHTHALENE AND DIMETHYL PHTHALATE ON REVERSED-PHASE AND SILICA GEL PLATES USING DIFFERENT SOLVENT MIXTURES HAVING THE SAME POLARITY*

			R_fX100		
SOLVENT	RATIO (v/v)	PLATE	ANTHRAQUINONE	NAPHTHALENE	Me-PHTHALATE
MeOH/H_2O	45/55	RP-18	0	0	13
CH_3CN/H_2O	52/48	RP-18	35	23	43
THF/H_2O	37/63	RP-18	8	5	21
Ethyl ether/ Hexane	52/48	Silica gel	92	99	79
$CHCl_3$/Hexane	35/65	Silica gel	50	99	25
CH_2Cl_2/Hexane	47/53	Silica gel	39	94	18

*From Reference 2.

was used. This was not the case when a polycyclic aromatic hydrocarbon mixture was separated on a μ-Porasil column using 2% ethyl acetate/hexane and 2.15% chloroform/hexane (Fig. 1).

Figures 2 and 3 show the effect of the organic modifier on the separation. Although the polarity of the mobile phases are the same, replacing acetonitrile by methanol gave different retention times, α and K' values, under the same experimental conditions.

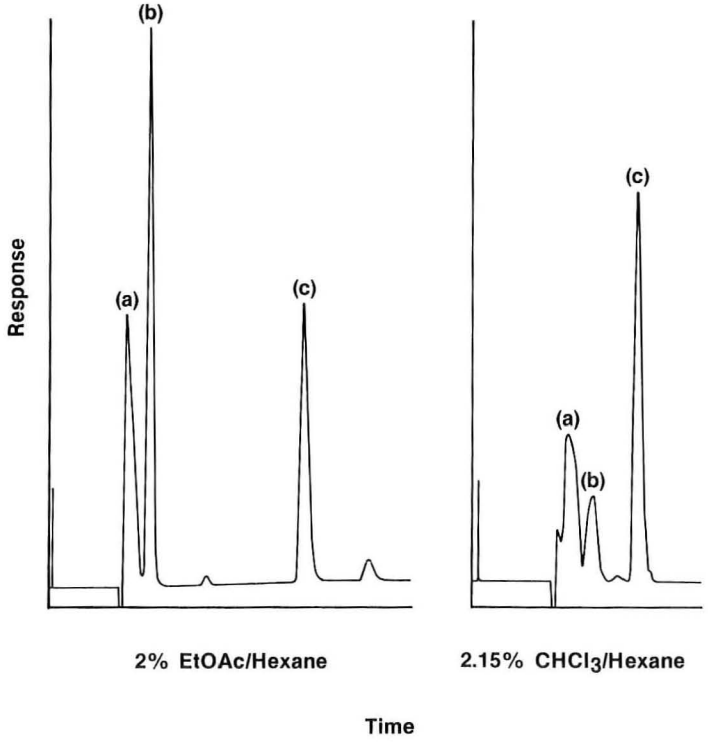

Figure 1. Comparative HPLC separation of benzo[a]pyrene (a), naphthacene (b) and anthracene (c) using a μ-porasil column and two different mobile phases having the same polarity; 2% ethyl acetate/hexane (left) and 2.15% chloroform/hexane (right).

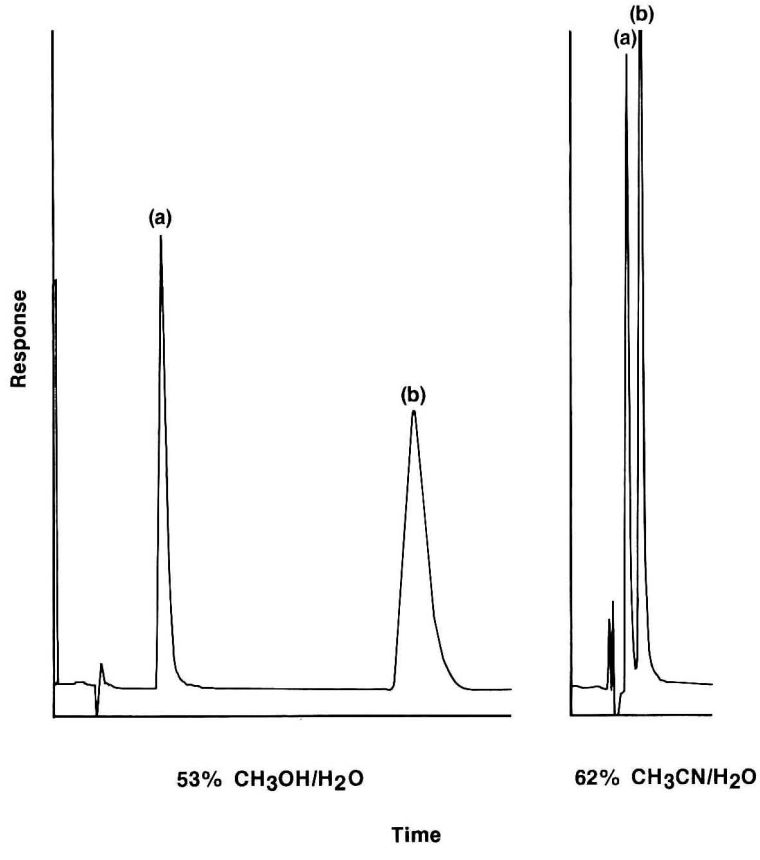

Figure 2. Comparative HPLC separation of dimethylphthalate (a) and diethylphthalate using a reversed phase C_{18} column in two different mobile phases having the same polarity; 53% methanol/water and 62% acetonitrile/water at a flow rate of 1.2 ml/min.

If the 10 ml of water in acetonitrile/water (90/10) is replaced by 20 ml of methanol, both having the same polarity, the effect on R_t, α and K' values are quite evident (Fig 4). This is due to solvent-solute and solute-solid phase interactions.

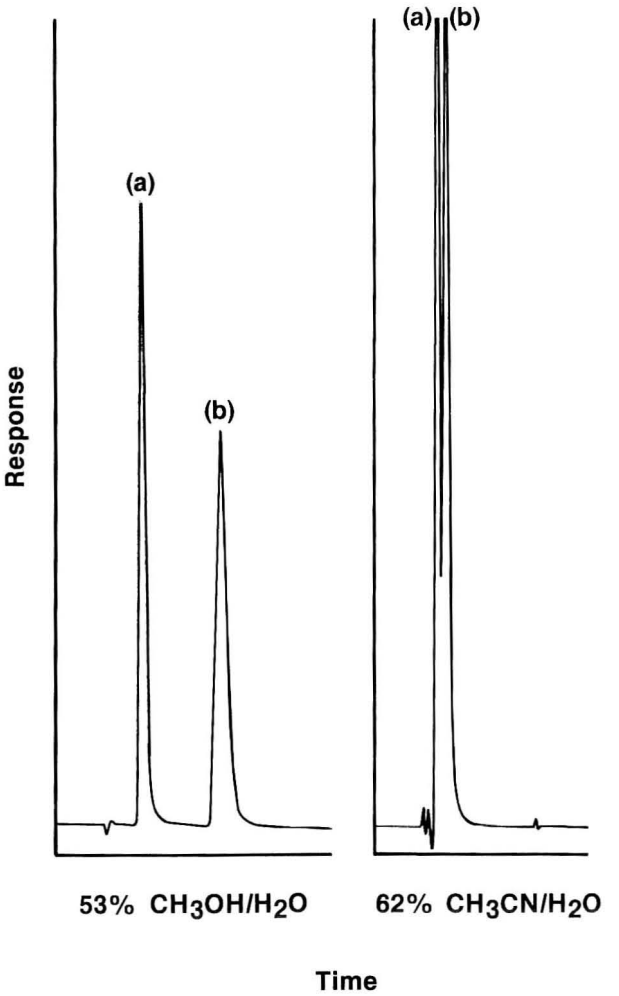

Figure 3. Comparative HPLC separation of benzene (a) and naphthalene (b) using a reversed phase C_{18} column and two different mobile phases having the same polarity; 53% methanol/water (left) and 62% acetonitrile/water (right), at a flow rate of 1.2 ml/min.

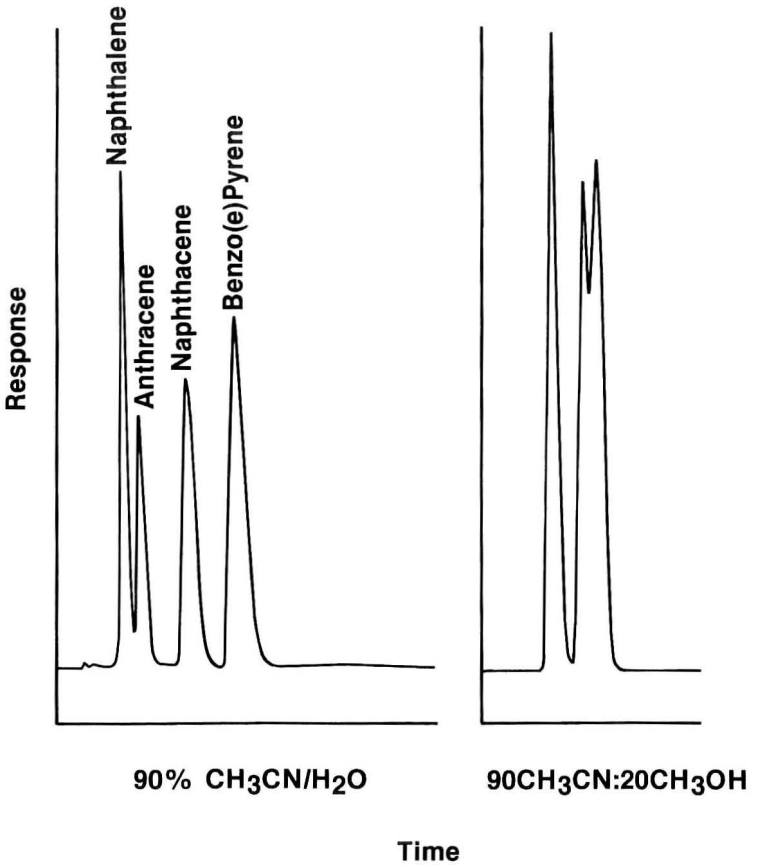

Figure 4. Comparative HPLC separation of naphthalene, anthracene, naphthacene and benzo[e]pyrene using reversed phase C_{18} column, and two different mobile phases acetonitrile/water (90/10) and acetonitrile/methanol (90/20) at a flow rate of 1.2 ml/min.

The data presented here clearly indicates that the polarity of the mobile phase is not as important as other parameters in liquid chromatography, such as solvent-solute, and solvent-solid phase interactions.

REFERENCES

1. L.R. Snyder. J. Chromatogr. Sc. 16, 223 (1978).

2. J.L. Glach, J.J. Kirkland and K.M. Squire. J. Chromatogr. 199, 57 (1980).

3. P.J. Schoenmakers, H.A. Billet and L. DeGalan. J. Chromatogr. 218, 261 (1981).

4. H.J. Issaq, unpublished results.

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HIGH PERFORMANCE SIZE EXCLUSION CHROMATOGRAPHY WITH MICROPARTICULATE POROUS SILICA SPHERES. INFLUENCE OF FLOW RATE, SOLUTE MASS TRANSFER AND POLYDISPERSITY OF SAMPLES

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ABSTRACT

The contributions to peak broadening in Size Exclusion Chromatography with microparticles of porous silica spheres having narrow size distributions have been determined by measuring the plate height dependence on flow rate for toluene and for polystyrene standards covering a wide range of molecular weights. From these contributions, the diffusion coefficients of the macromolecules in the pore matrix and the polydispersities of the samples could be evaluated.It is shown that for permeating polymers the band broadening is determined by the eddy diffusion in the mobile phase, by the slow mass transfer of the solute in the stationary phase and by the polydispersity of the standards. In properly packed columns the eddy diffusion term is of minor importance compared to the other effects, whereas the solute mass transfer, which is a velocity dependent

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process, can be minimized only at extremely low flow rates.

INTRODUCTION

Rigid inorganic packings are widely used in modern Size Exclusion Chromatography (SEC) because of their superior mechanical and thermal stability and their capability of withstanding the high pressures involved in high speed liquid chromatography. Since the efficiency of the SEC columns greatly increases by decreasing particle size (1), packing materials with particle average size of about 10 μm or less are now produced: 25 -30 cm long columns, packed with these microparticulate materials, show efficiencies several times higher than those of conventional SEC columns (2, 3).

Frequently, if not exclusively, the inorganic materials used in high performance SEC are porous silica gels. The determination of the molecular weights of polymers generally improves by decreasing the eluent flow rate: the situation is less critical when polymer samples of relatively broad molecular weight distribution are analyzed (accurate molecular weights have been obtained (4) with a flow rate of 0.5 cm^3 min^{-1}), whereas with more narrowly distributed polymer samples one has to resort either to extremely slow flow rates (5), or to a suitable correction for peak spreading (4).

The flow rate dependence of the efficiency in SEC with short columns filled by silica microspheres of narrow size distribution and mean diameter 20 μm and

8 μm was investigated by Dawkins and Yeadon (6, 7), who were succesful in separating the different contri_ butions which are operative in broadening the chromato_ graphic peaks. Similar results were obtained with a dif_ ferent procedure by Knox and McLennan (8) who pointed out that in polymer size exclusion analysis with high performance columns the peak broadening is determined by the polymolecularity of the sample, which is the pre_ vailing factor, and by a kinetic, flow rate dependent, contribution.

In the present paper the procedure suggested by Dawkins (6) to assess the importance of the different contributions to peak broadening and to determine the optimum working conditions is applied to packings of LiChrospher, a porous silica gel produced by E. Merck, Darmstadt, Germany, which has been found valuable in giving high resolutions in SEC, and which has already been the object of an investigation of one of us (4) on the use of it in molecular weight determinations by SEC. The present study has been performed on microparticles (about 10 μm) of different mean pore size, using poly_ mer samples covering a wide range of molecular weights.

PEAK BROADENING IN SEC

In a properly arranged SEC system in which extraco_ lumn effects, deriving from injection, capillary connec_ tions, detector, are minimized the peak broadening pri_ marily depends on processes occurring in the column, and is a direct consequence of the slow rate of achie_

vement of solute equilibrium between the mobile and the stagnant phase. For macromolecules the time necessary to reach this equilibrium can be very long, so that local departures from equilibrium give raise to broadening and asymmetry of the peaks.

Peak broadening in chromatography is adequately expressed in term of the number N of plates of the column, and of the plate height H which can be defined as the peak variance per unit length of the column (9):

$$H = \sigma^2 / L \qquad (1)$$

where σ is the peak standard deviation and L is the column length. For an experimental chromatogram the plate height is calculated from the relations:

$$H = L / N \qquad (2)$$

$$N = 5.54 \, (V_e / w_{\frac{1}{2}})^2 \qquad (3)$$

where V_e is the elution volume at the maximum of the peak and $w_{\frac{1}{2}}$ is the peak width at half height.

The theory of chromatography gives relationships in which the plate height is expressed in termsof sums of different contributions (10). From this general treatment the theory of peak dispersion in SEC was formulated by Giddings and Mallik (11) and applied to some experimental data (12), showing the reliability of the classical chromatographic approach to investigate even in the SEC case the peak broadening factors.

The dependence of plate height on the solute dispersion mechanism in SEC was subsequently investigated by Dawkins and Yeadon (5,6), who took into account the contribution from polydispersity in the elution of permeating polymers. In this case, the experimental value of H is given by

$$H = A + \frac{B}{u} + Cu + (\sigma_M^2 / L) \qquad (4)$$

where u is the eluent flow rate, A, B and C are coefficients related to different diffusion processes of the solute molecules, and σ_M^2 / L is the contribution of the solute polydispersity (σ_M^2 is the peak variance depending on the molecular weight distribution). When standard polymer samples with low dispersity are eluted, assuming that their molecular weight distribution may be represented by a logarithmic normal one, the polydispersity term of eq. (4) takes the form:

$$\frac{\sigma_M^2}{L} = L \frac{\ln (M_w/M_n)}{D_2^2 \; V_e^2} \qquad (5)$$

where D_2 is the slope of the SEC calibration curve, obtained by plotting ln M vs. V_e.

According to Giddings (10), the coefficients A, B and C of eq. (4) take the form:

$$A = 2 \lambda d_p \qquad (6)$$

(where λ is a constant characteristic of the packing and d_p is the particle diameter),

$$B = 2 \gamma D_m \tag{7}$$

(where D_m is the diffusion coefficient of the solute in the mobile phase, corrected by the obstruction factor γ),

$$C = q\ R(1-R)\ \frac{d_p^2}{D_s} \tag{8}$$

(where q is a configuration factor depending on the particles' geometry, R is the retention ratio V_o/V_e between the interstitial volume of the column and the elution volume, and D_s is the solute diffusion coefficient in the pores).

By introducing eqs. (5), (6), (7) and (8) into eq. (4) one obtains:

$$H = 2 \lambda d_p + 2 \gamma \frac{D_m}{u} + q\ R(1-R)\ \frac{d_p^2}{D_s}\ u + L\ \frac{\ln(M_w/M_n)}{D_2^2\ V_e^2} \tag{9}$$

which expresses the plate height as a sum of terms, the fourth of which depending upon the sample polydispersity, and the other three representing true chromatographic contributions: the first term (term A) comes from the eddy diffusion of the solute in the mobile phase, that is the fact that different solute molecules run

along different paths between the gel particles; the second term (term B) results from the longitudinal diffusion of the solute in the mobile phase; and the third (term C) comes from the mass transfer in the stationary phase associated with the slow diffusion of the solute in the packing pores.

By performing measurements with polymer samples differently excluded at different flow rates it is then possible, by applying eq. (9), to know how H varies and which factor is prevailing to change the efficiency of a given packing; the reliability of the packing procedure can also be checked together with the permeability characteristics of the porous material. Finally, by evaluating the last term of eq.(9), the polydispersity index M_w/M_n of the polymer standards can be calculated.

EXPERIMENTAL

LiChrospher packings with nominal particle size of 10 μm were supplied by E. Merck (Darmstadt, Germany). Four different silica gels were used, SI 100 (batch no. YE604), SI 500 (batch no. YE91), SI 1000 (batch no. YE645) and SI 4000 (batch no. YE445), having different mean pore size. The microparticles, examined by optical microscopy, appeared to have a regular spherical shape. The densities of the different silica gels were determined with a picnometer.

The gels were dispersed in methanol, stirred in an ultrasonic bath, and the slurries were packed according

to the upward technique proposed by Bristow et al. (13) into stainless steel columns (length: 25 cm; internal diameter: 0.46 cm) at a pressure of about 10 MPa (1 MPa= 10 bar).

High performance SEC was executed with a dual-piston diaphragm pump (Orlita DMP-SK 15/3), and an ultraviolet detector (Zeiss, PM2 DLC, cell volume 8 μl, 260 nm). An injection valve (Rheodyne 7010) with a 10 μl loop was used to introduce the samples into the columns; all the connections between injector and column and between column and detector were made of short low dead-volume capillary tubing (0.025 cm i.d.).

The mobile phase was tetrahydrofuran (Carlo Erba, Milano, Italy) stored over KOH for 12 h, refluxed over CaH_2 and distilled. The samples injected were toluene (Carlo Erba) and polystyrene standards (ArRo Laboratories, Joliet, Illinois, and Polysciences, Warrington, Pennsylvania) dissolved in tetrahydrofuran at concentrations low enough to avoid concentration effects (14) on the elution volumes. For toluene and for PS standards up to about $M = 7 \cdot 10^5$ the sample concentration was 0.1% (w/v), whereas for the highest molecular weight standards it was 0.05% (w/v). The stream flow rate was detected in each analysis by measuring the time for collecting a given volume of the eluent in a burette connected with the output of the detector. With this system no short term flow rate variations were detected. The flow rates investigated varied from 0.15 to 2.5 $cm^3\ min^{-1}$, corresponding to linear velocities ranging from about 0.03

to 0.6 cm sec^{-1}. Lower steady flow rates could not be obtained with the pumping system adopted.

The elution volumes of the injected samples were practically independent of the flow rate, except in a few cases at the highest velocities with the very high molecular weight standards. The reproducibility of the measurements was better than 1%. All the results for V_e and H are the average of at least three injections.

The characteristics of the packed columns were determined by direct calibration with the PS standards. For the column packed with SI 4000, however, the interstitial volume V_o could not be determined with an excluded polymer because even a standard of molecular weight as high as $2.6 \cdot 10^7$ showed some permeation behaviour. Therefore the porosity of the SI 4000 gel was measured by titration, according to the method of Mottlau and Fischer (15). About 1 g of dry gel was weighed in a 50 cm^3 Erlenmeyer flask in which the titrating solvent (benzene) was slowly introduced by means of a plunger microburette; the gel was continously stirred and the flask was connected to a benzene reservoir in order to eliminate solvent evaporation during the titration. The end point was taken when a small increment of liquid added to the gel, forming a very thin layer on the external surface of the particles, made them stick together to form a coherent caked mass. The specific porosity $V_{p,sp}$ of SI 4000 could then be calculated, and by multiplying it with the quantity of packing in the column, the pore volume V_p was obtained; from the latter and from the

elution volume of a totally permeating molecule, V_t, the interstitial volume $V_o = V_t - V_p$ was calculated. The titration method was checked, for our silica gels, by measuring the V_p of SI 500 and comparing it with the value obtained by SEC, with very good agreement (see Table 2).

RESULTS AND DISCUSSION

Characterization and calibration

From the particle size distributions as determined at intervals of 2 μm on the micrographs, the number average and weight average diameters reported in Table 1 were calculated according to the relations given by Dawkins et al. (16).

The results show small differences in the average particle size dimensions and narrow size distributions, particularly for SI 100 and SI 500. A narrow size distribution of particles is needed for high efficiency columns (16).

TABLE 1. Number Average and Weight Average Particle Diameters d_n and d_w and Polydispersity of Silica Particles.

Silica	d_n (μm)	d_w (μm)	d_w/d_n
SI 100	9.90	10.91	1.10
SI 500	8.20	9.30	1.13
SI 1000	8.14	9.67	1.19
SI 4000	7.66	9.10	1.19

The calibration curves, log M vs V_e, of the single columns packed with the different silica gels were determined at an eluent flow rate of about 0.5 cm^3 min^{-1} and are shown in Fig. 1. Toluene was the totally permeating solute. It can be seen from the figure that SI 500 and SI 1000 gels have quite similar pore dimensions, and that the SI 4000 column shows a lower pore volume

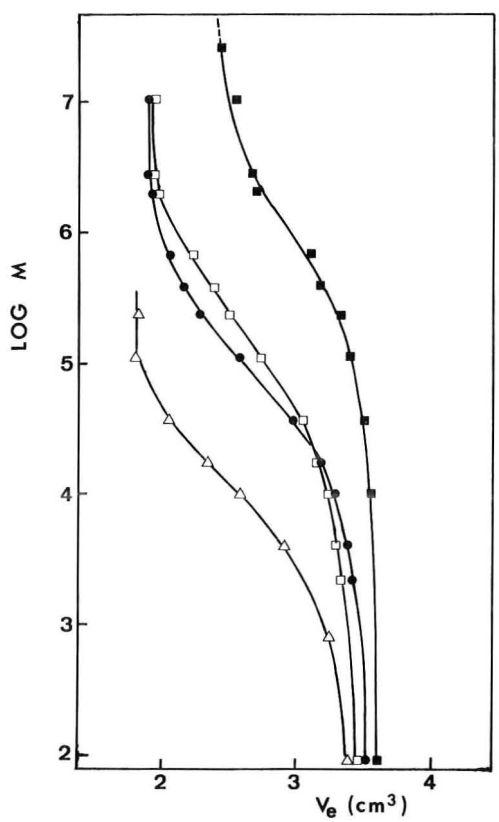

FIGURE 1. SEC calibration curves for polystyrene standards; △, SI 100 silica; ●, SI 500 silica; □, SI 1000 silica; ■, SI 4000 silica.

than the others. From the calibration data the quantities of interest in SEC can be extracted: V_o and V_t from the elution volumes of excluded and totally permeating solutes respectively, V_p from the difference $V_t - V_o$, and V_s, the volume of solid silica in the column, from the relation $V_s = V_{col} - (V_o + V_p)$, where the volume of the empty column, V_{col}, was 4.155 cm^3. With the V_s values and the measured densities of the solid part of the microspheres the quantity of silica in the different packed columns was calculated. (For the SI 4000 column V_p was obtained from the specific pore volume determined by titration).

In Table 2 are reported all the characteristics data of the packed columns. The SI 4000 column has a significantly lower quantity of gel than the other columns and this fact brings about the low value of V_p, together with the high value of V_o. It appears that the smaller

TABLE 2. Density Values of the Silica Particles and Characteristics of the Packed Columns.

Silica	Density $g\ cm^{-3}$	Grams of Packing	V_o	V_p	V_t	V_s
				cm^3		
SI 100	2.03	1.54	1.79	1.60	3.39	0.76
SI 500	2.11	1.33	1.89	1.64 1.65*	3.53	0.63
SI 1000	2.27	1.57	1.92	1.55	3.47	0.69
SI 4000	2.22	1.24	2.36*	1.24*	3.60	0.56

* by pore titration

dimensions of these particles make it more difficult to obtain a superior quality packing. For all the columns the efficiency determined according to eq. (3) from the toluene peak gave values higher than 20000 plates m^{-1}.

Dependence of H on flow rate

In Fig. 2 are reported the dependences of H on eluent flow rate for different solutes eluted in the Li Chrospher SI 100 column. Toluene is a totally permeating molecule, PS 10000 and PS 37000 are partially permeating PS standards, and PS 111000 and PS 233000 are two excluded PS standards.

It can be seen that for toluene there is a very low decrease of the H values with decreasing flow rate,

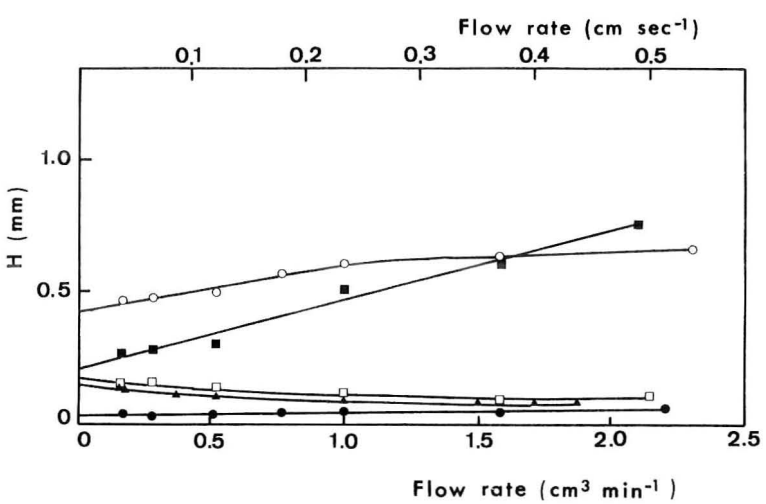

FIGURE 2. Dependence of plate height on flow rate for SI 100 silica: ●, toluene; ○, PS 10000; ■, PS 37000; □, PS 111000; ▲, PS 233000.

without any tendency of H to increase at the lowest flow rates investigated. In terms of eq. (9) this means that the contribution of the longitudinal diffusion in the mobile phase (term B) can be neglected; the polydisperity term is non operative for toluene, and the small dependence of H on flow rate indicates that the term for solute mass transfer in the stationary phase (term C) is not very important for molecules having high values of D_s. Therefore the main contribution to H for a small molecule comes from the eddy diffusion in the mobile phase (term A). Also the excluded polymers show a low variation of H with flow rate. For these solutes term C vanishes (1-R = 0), and the polydispersity term is not to be taken into account because there is no permeation into the pores. The slight increase of H at the lowest flow rates cannot be attributed to term B because, for the very low values of D_m of these molecules the longitudinal diffusion is negligible at the flow rates employed; the effect shown in Fig. 2 is similar to that observed by Knox and McLennan (8) on solute dispersion of excluded polymers, and attributed to a partial penetration of the macromolecular coils into the largest pores of the packing, which becomes evident at low flow rates. Such an interpretation is in agreement with the higher values of H for PS 111000 than for PS 233000, and with the finding of highly tailed chromatographic peaks at the low flow rates.

For the two permeating polymers term C and the fourth term of eq. (9) are important in determining the

values of H and their dependence on flow rate. The increase of H with increasing flow rates depends on the ratio $R(1 - R)/D_s$ of term C, whereas the vertical displacement of the curves with respect to the H values of toluene results from the polydispersity of the samples. The flattening experimentally found in the curve of PS 10000 at $u > 1$ cm^3 min^{-1} is not accounted by eq. (9); a decrease of the slope of these curves with increasing eluent velocities was reported by van Kreveld and van den Hoed (17) to occur at linear velocities higher than ours: such an effect increased with increasing molecular weight of the solute. It has been suggested that at high flow rates flow into the pores could facilitate the mass transfer between the mobile and the stationary phase, but no rigorous interpretation of this phenomenon is available; a better description of mass transfer effects within the pores should be supported by a detailed knowledge of the pore structure.

The curves showing the dependence of H on eluent flow rates for the SI 500, SI 1000 and SI 4000 columns are reported in Figs. 3-5. In these columns excluded polymers, when available, were not analysed at different flow rates because the very high molecular weight standards gave broad and distorted peaks which were difficult to measure. For the other solutes the behaviour was analogous to that of SI 100. The elution of toluene at the different flow rates gave practically no variation of H. With SI 4000 the peaks of toluene were somewhat less symmetrical than for the other columns,

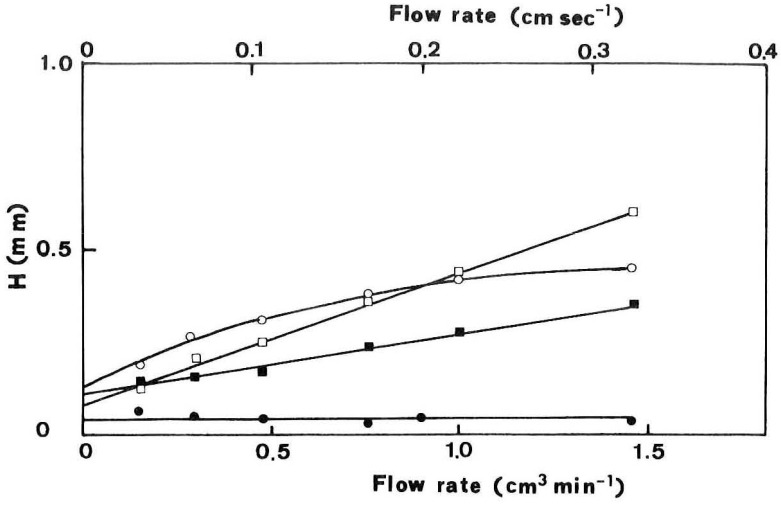

FIGURE 3. Dependence of plate height on flow rate for SI 500 silica: ●, toluene; ■, PS 37000; □, PS 111000; ○, PS 390000.

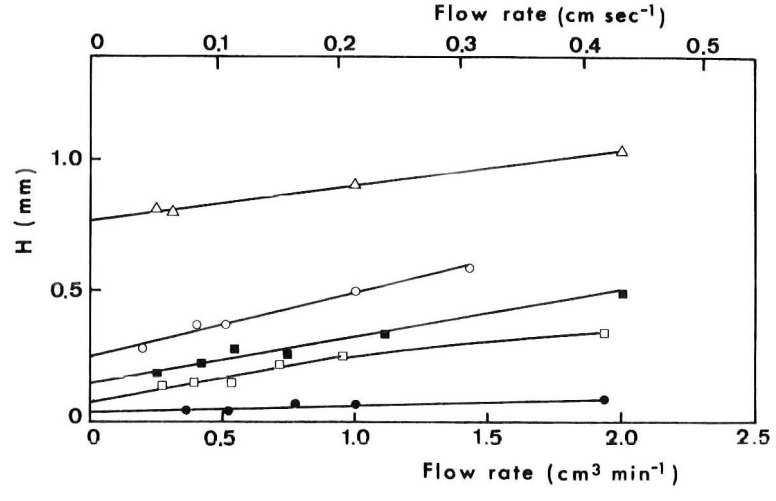

FIGURE 4. Dependence of plate height on flow rate for SI 1000 silica: ●, toluene; □, PS 37000; ■, PS 111000; ○, PS 390000; △, PS 2050000.

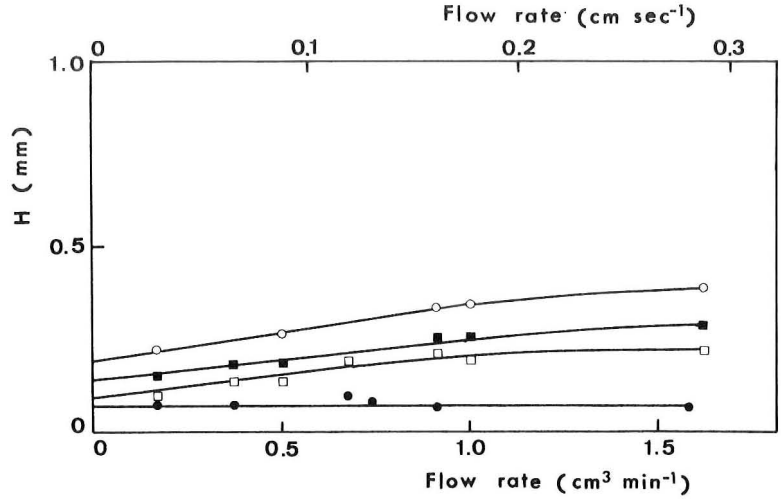

FIGURE 5. Dependence of plate height on flow rate for SI 4000 silica: ●, toluene; □, PS 111000; ■, PS 390000; ○, PS 670000.

and this might be attributed to the less homogeneous packing bed obtained with this gel. The permeating polymers show higher H values than for toluene, increasing with flow rate because of the mass transfer of the solute in the stationary phase. The expected dependence of H on flow rate is also evident in the SI 1000 column for the elution of PS 2050000, which permeates only a small portion of pores (see Fig. 1). The strong vertical displacement of the curve for this standard with respect to the other curves clearly indicates that the polydispersity of the polymer is not very low.

Diffusion coefficients of polystyrenes in the pores

From the slope of the linear part of the H vs linear velocity curves of Figs. 2-5 the term of solute disper-

sion resulting from mass transfer of the permeating samples in the stationary phase can be obtained. When the packing material is constituted by a collection of spherical particles with a size distribution, the value of the configuration factor q is 1/30 (12), and the correct mean particle diameter $\bar{d}_p$ must be introduced in term C of eq. (9). The $\bar{d}_p$ which is to be applied is the volume-weighted average value (12), which for particles of the same material is equal to the weight average diameter d_w. Therefore term C of eq. (9) becomes

$$\frac{R(1 - R)\, d_w^2}{30\, D_s}$$

and the values of the diffusion coefficients D_s of polystyrenes in the pore structure are easily calculated from the experimental slopes of Figs. 2-5. The D_s values can be compared with the diffusion coefficients at infinite dilution, D_m, of the polymers in free solution, and the ratio D_s/D_m, representing the hindrance to the molecule diffusion in the pores, is obtained. The values evaluated for the four silica gels investigated are reported in Table 3. The D_m values for the PS standards were calculated according to the relationship

$$D_m\ (cm^2\ sec^{-1}) = 3.45 \cdot 10^{-4}\ M_w^{-0.564}$$

valid in tetrahydrofuran at 24 °C for PS samples in the molecular weight range up to about $2 \cdot 10^6$ (18).

From the data of Table 3 one can see that for the same molecule eluting in different silica gels the D_s value increases with increasing mean pore size, as it is to be expected, but all the D_s values are much lower than the D_m values, indicating that the diffusion of the macromolecules in the pores is severely hindered. The results obtained in this study are completely comparable with those reported in the Literature for dif_

TABLE 3. Diffusion Coefficients of Polystyrene Standards Evaluated on LiChrospher Silicas with Mean Particle Dimensions d_w.

Standard	Column	d_w (μm)	$D_s \cdot 10^8$ ($cm^2 sec^{-1}$)	$D_m \cdot 10^8$ ($cm^2 sec^{-1}$)	D_s/D_m
PS 10000	SI 100	10.91	11.23	190.00	0.06
PS 37000			4.24	92.00	0.05
PS 37000			8.84	92.00	0.10
PS 111000	SI 500	9.30	3.52	49.70	0.07
PS 390000			1.81	24.00	0.07
PS 37000			8.89	92.00	0.10
PS 111000	SI 1000	9.67	8.46	49.70	0.17
PS 2050000			2.51	9.70	0.26
PS 111000			12.30	49.70	0.25
PS 390000	SI 4000	9.10	9.97	24.00	0.41
PS 670000			5.84	18.00	0.32

ferent packings: Giddings et al. (12) reported a value D_s/D_m=0.17 for low molecular weight polymers in porous glass ($\bar{d}_p$=59 μm); Dawkins and Yeadon (19, 7) have determined on silica microspheres D_s/D_m values in the range 0.11-0.19 ($\bar{d}_p \approx 20$ μm) and 0.08 - 0.15 ($\bar{d}_p \approx 8$ μm), and similar results were obtained by Knox and McLennan (8), who reported D_s/D_m values in the range 0.06 - 0.17 ($\bar{d}_p \approx$ 7.5 μm), and by van Kreveld and van den Hoed (17) who calculated on Porasil silica ($\bar{d}_p$ =75 - 125 μm) values decreasing from 0.312 to 0.119 by increasing the molecular weights of PS standards from 20000 to 160000. The D_s/D_m values of Table 3 vary from 0.05 to 0.41 in the different column systems, and no definite trend is shown by changing the molecular weights in the same silica gel. It can be suggested that the restriction ratio D_s/D_m varies not only with the packing porosity and with the molecular weight of the eluted samples but also with differences in the internal pore structure or in the pore size distribution. Therefore to interpret D_s/D_m variations a more detailed knowledge of the real pore structures would be needed. In any case, it can be concluded that, independently of the particle size dimensions, the transfer of solute molecules from mobile to stationary phase in the pororus particles used in SEC is very slow.

Polydispersities of the polystyrene standards

From the linear part of the curves of permeating polymers in Figs. 2-5 it is possible to extrapolate the intercept value H_{PS}^{int}. By assuming that this value appro-

ximates the plate height H^{*}_{PS} at very low eluent flow rates, but where the term B of eq. (9) would not be large and considering that at these low flow rates the contribution to H of term C is negligible with respect to the eddy diffusion term, one obtains from eq. (9):

$$H^{int}_{PS} \approx H^{*}_{PS} = 2 \lambda d_p + (L \ln \frac{M_w}{M_n} / D_2^2 V_e^2) \qquad (10)$$

Therefore the plate height H^{int}_{PS} of eq. (10) is the sum of an eddy diffusion term and a term deriving from the sample polydispersity. The results shown above indicate that the eddy diffusion term can be well approximated by the plate height value of toluene H^{int}_{t} calculated at u = 0; this value can be substituted for $2 \lambda d_p$, and the second term on the right hand side of eq. (10) can be evaluated. From this term the values M_w/M_n of the dispersities of the permeating polymers have been calculated in the different column systems and the results are shown in Table 4.

The values of M_w/M_n calculated from plate height data are quite reasonable, according to the characteristics of these "monodisperse" standards; the relatively high dispersity found for PS 2050000 is also in agreement with the fractionation data given by the supplier, and with the general higher dispersity of these high molecular weight standards. The results of Table 4 compare well with those obtained by other Authors on different PS standards, prepared as well by living anionic polymerization (6 - 8). However, having investiga-

TABLE 4. Polydispersity of Polystyrene Standards Calculated from Eq. (10).

Polymer	Column	H_{PS}^{int} (mm)	H_{t}^{int} (mm)	M_w/M_n
PS 10000	SI 100	0.430	0.030	1.073
PS 37000	SI 100	0.210	0.030	1.020
	SI 500	0.110	0.040	1.017
	SI 1000	0.080	0.040	1.019
PS 111000	SI 500	0.088	0.040	1.009
	SI 1000	0.150	0.040	1.042
	SI 4000	0.100	0.070	1.040
PS 390000	SI 500	0.130	0.040	1.043
	SI 4000	0.140	0.070	1.038
PS 670000	SI 4000	0.190	0.070	1.062
PS 2050000	SI 1000	0.770	0.040	1.160

ted the performance of silica particles with different pore size distributions, the present evaluation of the polydispersities of PS standards covers a broader molecular weight range. The agreement obtained in the results for the same polymer in different columns is relevant and further supports the applicability of this chromatographic procedure for evaluating band broadening

in high performance SEC, in spite of the approximations involved in the calculations.

CONCLUSIONS

The application of classical chromatographic equations, adequately modified, to high performance SEC has proved to be a useful tool in the investigation of the relative importance of the different processes which determine the band broadening, and in the evaluation of the permeability characteristics of the porous packings. In high performance SEC the main dispersion process occurring in the mobile phase is the eddy diffusion of solute molecules. Its contribution to plate height is minimized by using packings made of small spherical particles with narrow size distribution. For high molecular weight polymers, permeating the porous material, the band broadening is also determined, in addition to the size fractionation due to the polydispersity of the solute, by the mass transfer of the polymer molecules in the stationary phase, which is a slow and velocity dependent process. It follows that also at even lower linear velocities the plate height contribution from this term is by no means negligible, at least for polymers having low dispersities. On the other hand, for polymers broadly distributed the contribution from polydisperdity will quickly predominate over the dispersion phenomena occurring in the mobile phase, and with the values of the mass transfer coefficients generally found in the SEC packings, it should be possible to obtain re-

liable molecular weight distribution data working at about 0.1 cm sec^{-1} without broadening corrections.

REFERENCES

(1) Vivilecchia R.V., Cotter R.L., Limpert R.J., Thimot N.Z. and Little J.N., J. Chromatogr. 99, 407 (1974).

(2) Kirkland J.J., J. Chromatogr. 122, 231 (1976).

(3) Yau W.W., Kirkland J.J., Bly D.D. and Stoklosa H.J., J. Chromatogr. 125, 219 (1976).

(4) Chiantore O., Pokorny S., Bleha M. and Janca J., Angew. Makromol. Chem., in press.

(5) Dawkins J.V. and Yeadon G., Polymer 20, 981 (1979).

(6) Dawkins J.V. and Yeadon G., J. Chromatogr. 188, 333 (1980).

(7) Dawkins J.V. and Yeadon G., J. Chromatogr. 206, 215 (1981).

(8) Knox J.H. and McLennan F., J. Chromatogr. 185, 289 (1979).

(9) Yau W.W., Kirkland J.J. and Bly D.D., Modern Size Exclusion Chromatography, Wiley, New York (1979).

(10) Giddings J.C., Dynamics of Chromatography. Part 1. Principles and Theory, Dekker, New York (1965).

(11) Giddings J.C. and Mallik K.K., Anal. Chem. 38, 997 (1966).

(12) Giddings J.C., Bowman L.M. and Myers M.N., Macromolecules 10, 443 (1977).

(13) Bristow P.A., Brittain P.N., Riley C.M. and Williamson B.F., J. Chromatogr. 131, 57 (1977).

(14) Bleha T., Bakos D. and Berek D., Polymer 18, 897(1977).

(15) Mottlau A.Y. and Fischer N.E., Anal. Chem. 34, 714 (1962).

(16) Dawkins J.V., Stone T. and Yeadon G., Polymer 18, 1179 (1977).

(17) van Kreveld M.E. and van den Hoed N., J. Chromatogr. 149, 71 (1978).

(18) Mandema W. and Zeldenrust H., Polymer 18, 835 (1977).

(19) Dawkins J.V. and Yeadon G., Polymer Preprints 21, 89 (1980).

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DETERMINATION OF CARBONYL 2,4-DINITROPHENYLHYDRAZONES BY LIQUID CHROMATOGRAPHY/ELECTROCHEMISTRY

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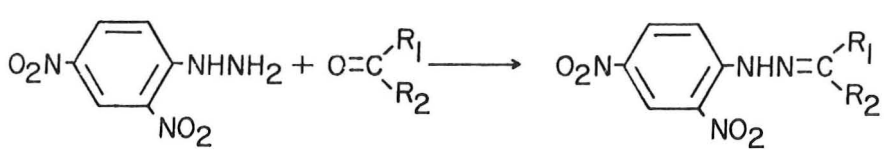
ABSTRACT

Reductive mode liquid chromatography/electrochemistry was evaluated for use in quantitating 2,4-dinitrophenylhydrazine derivatives of carbonyls. Optimum performance was achieved at an operating potential of -0.75V vs Ag/AgCl. Detection limits for DNPH-formaldehyde, acetaldehyde, acetone, and acrolein were 54, 80, 76, and 99 pg respectively. These detection limits were approximately 20 times lower than obtainable with UV absorbance at 254 nm.

INTRODUCTION

The reaction of aldehydes and ketones with 2,4-dinitrophenylhydrazine (DNPH; equation 1) has long formed the basis of



colorimetric methods for determining carbonyl content (1). More recently the use of liquid chromatography to resolve the individual derivatives followed by absorbance detection has become very popular (2-8). This approach is particularly advantageous in allowing pre-chromatographic concentration of airborne carbonyls by trapping DNPH derivatives, thereby facilitating detection at low concentrations. Selectivity is also enhanced since the reaction is quite specific for the carbonyl functionality. A further benefit is that the derivatization stabilizes otherwise volatile and often reactive compounds.

The potential utility of nitroaromatics as derivatization labels for liquid chromatography/electrochemistry (LCEC) has been discussed previously (9,10). Such an approach has already been shown to be applicable to the determination of amino acids in brain tissue (11). The purpose of this communication is to demonstrate the utility of LCEC for carbonyl determinations using DNPH derivatization. The superior sensitivity of EC detection is expected to provide advantages, including decreased detection limits and/or less restrictive sampling requirements.

EXPERIMENTAL

Chemicals: All chemicals were ACS Reagent grade or better. Tetrahydrofuran (stabilized) and n-propanol were from Mallinckrodt (St. Louis, MO). Acetonitrile was from J. T. Baker (Phillipsburg, NJ). Ammonium Acetate, perchloric acid and dimethylsulfoxide were from Fisher Scientific (Pittsburg, PA). Acetone, acrolein, acetaldehyde, and formaldehyde (37 wt. % solution) were all from Aldrich Chemical Co. (Milwaukee, WI).

Derivative Formation: Standard derivatives were prepared by precipitation from DNPH/Perchlorate (1.2g DNPH in 100 mL of 30% Perchloric acid) and purified by repeated recrystallization from 95% ethanol.

Liquid Chromatography: All separations were carried out using a Bioanalytical Systems (West Lafayette, IN) LC-304 liquid chromatograph modified to allow continuous deoxygenation of the mobile phase, and of samples prior to injection (12). The amperometric transducer was a model TL-5A glassy carbon cell (BAS). The system was equipped with a Biophase octyl column, 0.46 x 25 cm, 5 μm particle size (BAS). The mobile phase consisted of (by volume) 20% acetonitrile, 20% tetrahydrofuran, 10% n-propanol, and 50% 0.1M ammonium acetate, pH 5.0. Derivatives were dissolved in DMSO for injection onto the chromatograph.

For LCUV experiments, an LC-6 254 nm fixed wavelength UV detector was used (BAS).

RESULTS AND DISCUSSION

Voltammetry of DNPH-Derivatives

Hydrodynamic voltammograms (HDV's) of DNPH-formaldehyde, acetaldehyde, and acetone are shown in Figure 1. The derivative

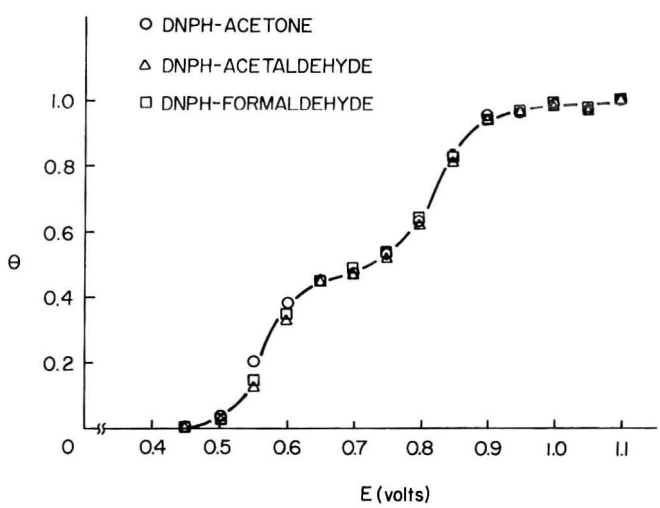


FIGURE 1. Hydrodynamic Voltammograms (HDV's) of Selected Carbonyl Derivatives. Θ is the normalized response, $\Theta_E = i_E/i_{1.1}$

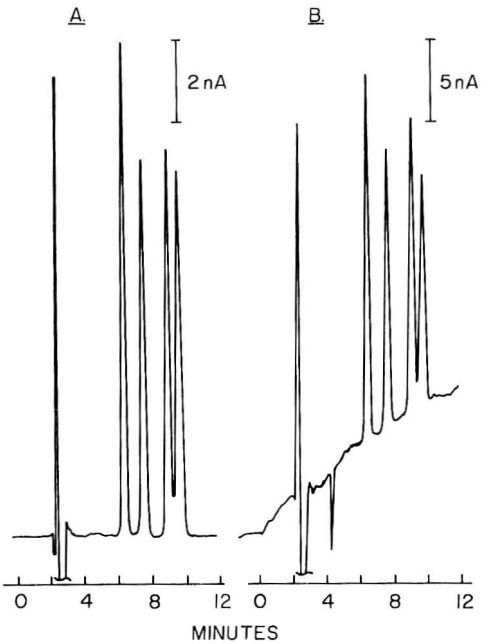


FIGURE 2. Chromatograms of Carbonyl Derivatives at A. -0.75V and B. -1.00V vs Ag/AgCl. Peaks: 1. DNPH-formaldehyde 2. Acetaldehyde 3. Acetone 4. Acrolein Approximately 2 ng of each derivative.

HDV's reflect the favorable electrochemical properties of these compounds. The voltammetric behavior of all derivatives is virtually identical, each exhibiting two equivalent reductive waves. The observed waves are presumed to be due to successive four electron reductions of the nitro groups to hydroxylamines (equations 2 and 3). This would be in agreement with the

$$R\text{-}(NO_2)_2 \xrightarrow{+4e^-,\ 4H^+} R\text{-}NO_2(NHOH) \qquad (2)$$

$$R\text{-}NO_2(NHOH) \xrightarrow{+4e^-,\ 4H^+} R\text{-}(NHOH)_2 \qquad (3)$$

classically observed electrochemistry of nitroaromatics (13).

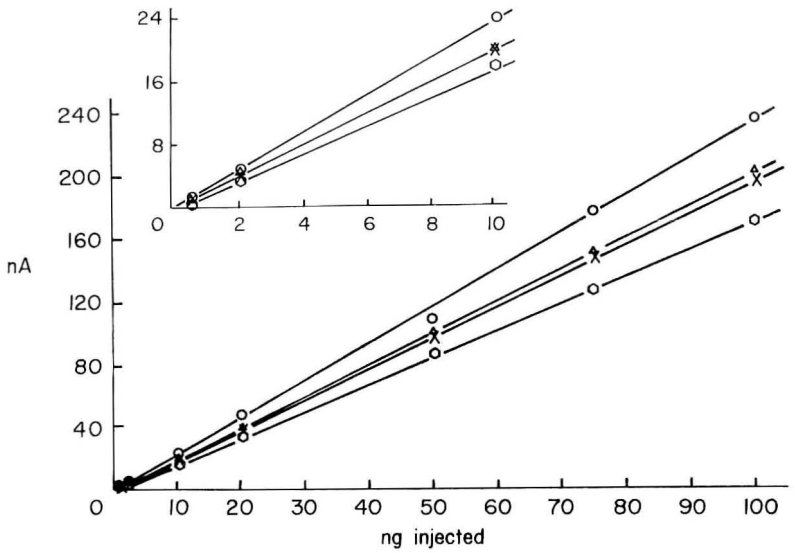


FIGURE 3. Calibration curves for Carbonyl Derivatives
⬡ DNPH - Acrolein, r = 0.999
X DNPH - Acetaldehyde, r = 0.999
Δ DNPH - Acetone, r = 0.999
O DNPH - Formaldehyde, r = 1.000

Selection of Applied Potential for LCEC

In selecting the optimum detector potential, it is desirable to maximize current response to analytes of interest while minimizing background noise. In the case of the DNPH carbonyls an applied potential of -0.75 volts (on wave 1, n = 4) was found to be superior to -1.00 volts (on wave 2, n = 8) even though the absolute current response was smaller. Increased noise and baseline drift at the higher potential more than offset the gain in absolute response (Figure 2).

Detector Linearity and Sensitivity

Typical calibration curves for DNPH-formaldehyde, acetaldehyde, acetone, and acrolein are shown in Figure 3.

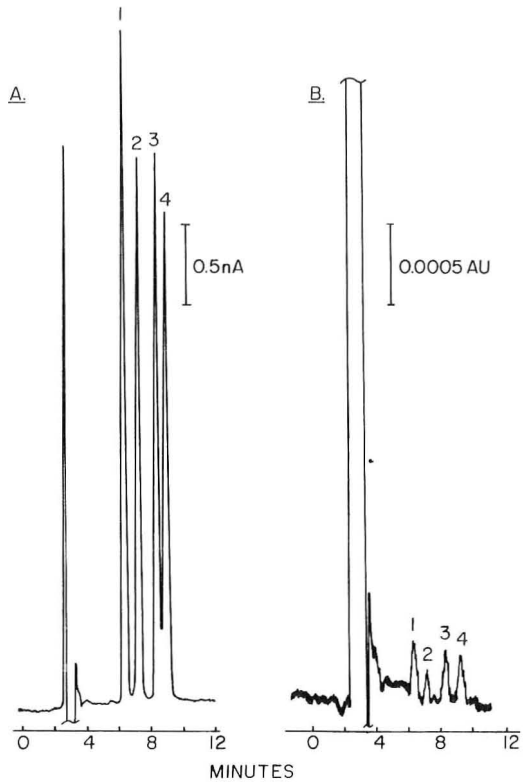


FIGURE 4. Chromatograms using A. LCEC at -0.75V and B. LCUV at 254 nm.

TABLE 1

Detection Limits for Carbonyl 2,4-Dinitrophenylhydrazones

	LCEC*	LCUV‡
Formaldehyde	54pg	10ng(5), 1.1ng(4), 1.3ng[1]
Acetaldehyde	80pg	10ng(5), 2.0ng(4), 2.0ng[1]
Acetone	76pg	10ng(5), 1.4ng[1]
Acrolein	88pg	10ng(5), 1.5ng[1]

* S/N = 4

‡ references in parentheses

[1] this work S/N = 4

Excellent linearity was obtained in the range from 100 pg to 100 ng injected for each of the derivatives. Corresponding detection limits are given in Table 1. Detection limits reported by LCUV are also listed for comparison. Electrochemistry is at least 20-30 times more sensitive than absorbance in all cases. This is clearly illustrated in Figure 4 which shows chromatograms obtained in this laboratory using EC and UV detectors in series (UV first) for 2 ng of each derivative.

As shown here, LCEC provides a viable alternative to LCUV for quantitating carbonyl 2,4-dinitrophenylhydrazones. The significant gain in sensitivity using LCEC results in substantially lowered detection limits. This should allow improvement of existing methods which utilize DNPH derivatization. In particular, the use of electrochemical detection should be useful for determinations of low level airborne carbonyls where long sampling times (typ 1-2 hrs) are currently required.

REFERENCES

1. P.E. Loren and B.J. Heinrich, Anal. Chem., 27 (1955) 1986.

2. M.A. Carey and H.E. Persinger, J. Chromatogr. Sci., 10 (1972) 537.

3. J.G. Farrelly, Cancer Res., 40 (1980) 3241.

4. K. Fung and D. Frosjean, Anal. Chem., 53 (1981) 168.

5. M.P. Maskarinec, D.L. Mamning and P. Oldham, J. Liq. Chromatogr., 4 (1981) 31.

6. D.C. Lowe, U. Schmidt, D.H. Ehhalt, C.G.B. Frischkorn and J.W. Nurnberg, Env. Sci. Tech., 15 (1981) 819.

7. R.K. Beasley, C.E. Hoffman, M.L. Rueppel and J.W. Worley, Anal. Chem., 52 (1980) 1110.

8. J.F. Sayegh and P. Vestergaard, J. Chromatogr. Sci., 19 (1981) 315.

9. P.T. Kissinger, K. Bratin, G.C. Davis and L.A. Pachla, J. Chromatogr. Sci., 17 (1979) 137.

10. W.A. Jacobs and P.T. Kissinger, paper 557, Pittsburgh Conference on analytical chemistry and applied spectroscopy (1981).

11. W.L. Caudill, G.P. Houck, R.M. Wightman, J. Chromatogr., in press.

12. K. Bratin, C.S. Bruntlett and P.T. Kissinger, J. Liq. Chromatogr., 4 (1981) 1777.

13. A.P. Tomilov, S.G. Mairanovskii, M. Ya. Fioshin and V.A. Smirnov, Electrochemistry of Organic Compounds, Halsted Press, Jerusalem (1972).

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EFFECTS OF COUNTER IONS IN ION-PAIR LIQUID CHROMATOGRAPHY OF HYDROPHOBIC AMINES ON NON-POLAR BONDED PHASES

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ABSTRACT

The retention behaviour of alprenolol and related hydrophobic amines in ion-pair adsorption systems has been examined with particular emphasis on the influence of different mono- and divalent counter ions (dihydrogenphosphate, bromide, perchlorate, dimethylcyclohexyl sulphate, sulphate and ethylenediaminetetraacetate). N,N-dimethyloctylamine (DMOA) and 1-pentanol were used as modifiers in the aqueous eluent and LiChrosorb RP-8 as stationary phase.

The retention is evaluated according to a two-site adsorption model and equilibrium constants are given for ion pair adsorption of DMOA. The retention of alprenolol has been evaluated in terms of ion exchange with DMOA and the ion-exchange constants are shown to be of the same magnitude and independent of the nature of the counter ion used. The ion-pair adsorption and the ion exchange approaches are analogous expressions for the distribution process governing the retention.

INTRODUCTION

Hydrophobic amines can be separated on non-polar bonded silica phases by use of aqueous eluents containing suitable modifiers and ion-pairing agents (1-3). Recent studies (3-6) have shown that the retention of the solutes follows a model which comprises adsorption of modifiers and solutes to two different

types of sites on the stationary phase. It has been postulated by others (7) that the chemically bonded phase constitutes one type of site with a high binding capacity, and that the other type of site is due to unsilanized hydroxyl groups on the surface of the silica. These silanol groups have a high affinity to hydrophobic amines but a low capacity, leading to overloading of solutes which, on the chromatograms, gives rise to tailing peaks and concentration-dependent capacity ratios.

The chromatographic behaviour can be improved by reducing the influence of the silanol groups by the presence of a suitable modifier in the eluent. The modifier competes with the solutes and reduces their adsorption on to the solid phase. Long chain aliphatic ammonium compounds, e.g. dimethyloctylamine (DMOA), have proved to be suitable as modifiers (1). Their effect may be due to interaction both with the carbon chains of the bonded phase and with unreacted silanol groups. Little attention has been paid to the possibilities of regulating the retention in such systems through the nature of the counter ion and its concentration in the eluent.

In the present paper mono- and divalent anions were examined as ion-pairing agents for hydrophobic amines and retention data were analysed according to the two-site adsorption model. It was found that the nature and concentration of the counter ions determine the extent of adsorption of DMOA on to the solid phase. The retention seems to be only indirectly dependent on the nature of the anion and can be treated either as ion-pair adsorption or as ion exchange between DMOA and the solutes, cf. (8-10). The ion-exchange approach seems to be particular suited for divalent anions as ion-paring agents in the eluent.

EXPERIMENTAL

Chemicals and Reagents

N,N-Dimethyloctylamine (DMOA) was purchased from ICN Pharmaceuticals (Plainview, NY, U.S.A.) and distilled before use.

Alprenolol and the other amines used as solutes (3) were supplied by the Department of Organic Chemistry, AB Hässle, and so was 3,5-dimethylcyclohexylsulphate (DMCHS) used as counter ion (potassium salt). 1-Pentanol was of Fisher Scientific A.C.S. quality and all other chemicals were of analytical-reagent grade and used without further purification.

Liquid Chromatographic System

The liquid chromatograph consisted of an Altex 110 A pump, a Rheodyne Model 70-10 sampling valve with a sample loop of 20 µl and an LDC spectroMonitor III spectrophotometer operated at 270 nm. Chromatographic columns (150 x 4.0 mm) were packed with Li-Chrosorb RP-8, 5 µm, from E. Merck and operated at 1.0 ml/min at 23 ± 1°C.

The eluents were aqueous buffers prepared from inorganic or organic acids, DMOA and sodium hydroxide, in which 1-pentanol, 0,115 moles/L, and appropriate amounts of other counter ions (DMCHS or KBr) were dissolved. The columns were conditioned with methanol before equilibriation with the eluent. As a rule, test solutions injected contained 3×10^{-4} moles/L of each of the amines.

RESULTS AND DISCUSSION

Symbols

A detailed symbol list and derivation of the equilibrium equations have been presented earlier (ref. (3)).

$[A]_s$	Available adsorption sites expressed in moles per gram of solid phase.
$[QXA]_s$, $[HBXA]_s$, $[Pe_nA]_s$	Adsorbed species in moles per gram of solid phase.
$K_{QX} = \dfrac{[QXA]_s}{[Q^+]_m [X^-]_m [A]_s}$	Equilibrium constant for adsorption of Q^+ as ion pair with X^- on site A.

$$K_{HBX} = \frac{[HBXA]_s}{[HB^+]_m [X^-]_m [A]_s}$$ Equilibrium constant for adsorption of HB^+ as ion pair with X^- on site A.

$$K_{Pe} = \frac{[Pe_nA]_s}{[Pe]_m^n [A]_s}$$ Equilibrium constant for adsorption of 1-pentanol on site A.

$$K_{Q_2Y} = \frac{[Q_2YA]_s}{[Q^+]_m^2 [Y^{-2}]_m [A]_s}$$ Equilibrium constant for adsorption of Q^+ as ion pair with Y^{-2} on site A.

$K_o = [A]_s + [QXA]_s + [HBXA]_s + [Pe_nA]_s$ Adsorption capacity of site A.

Corresponding symbols are used for a second adsorption site A^x.

$q = W_s/V_m$ Ratio of amount of solid phase (g) to volume of eluent in the column (L).

$$k'_{HB} = \frac{q\,[HBXA]_s}{[HB^+]_m}$$ Capacity ratio of the amine HB^+ retained as ion pair with X^-.

Retention Principles

The retention of ionic solutes on a non-polar solid phase can be regulated by the kind and concentration of ionic modifiers present in the aqueous eluent. The modifiers are adsorbed as ion pairs on the stationary phase in an equilibrium which can be expressed quantitatively by combining the expressions for K_o and K_{QX} given above. Ionic and neutral modifiers will compete with the solute for the adsorption capacity of the solid phase. The effect of this competition is summarized in the following expression for the capacity ratio of a solute, HB^+, migrating in a system with Q^+, X^- and 1-pentanol in the eluent (cf. (3)).

$$k'_{HB} = \frac{q\,K_o\,K_{HBX}\,[X^-]_m}{1 + K_{Pe}\,[Pe]_m^n + K_{QX}\,[Q^+]_m\,[X^-]_m} \qquad (1)$$

The equation is valid under the assumption that the adsorbed solute is a negligible fraction of K_o. The retention can, under these conditions, just as well be considered as an ion exchange process.

$$QXA_s + HB^+_m = HBXA_s + Q^+_m \qquad (2)$$

with the ion-exchange constant, K_{ie}, defined by

$$K_{ie} = \frac{[HBXA]_s \, [Q^+]_m}{[QXA]_s \, [HB^+]_m} \qquad (3)$$

A substitution of the expressions for the equilibrium constants, K_{QX} and K_{HBX} into eq. (3) gives

$$K_{HBX} = K_{ie} \, K_{QX} \qquad (4)$$

It should be emphasized that ion-pair adsorption and ion exchange are analogous expressions for the distribution processes behind the retention under the conditions given above.

Two-site Adsorption

The retention models used in earlier studies (3-6) are based on assumptions of adsorption to two kinds of adsorption sites with different binding ability. The retention can then be expressed by

$$k'_{HB} = k'_{HBA} + k'_{HBA^x} \qquad (5)$$

where k'_{HBA} and $k'_{IIBA}x$ express the retention on sites A and A^x, respectively.

The binding to A, which is the stronger of the sites, is determined by the constants K_o, K_{HBX}, K_{QX} and K_{Pe}, while the binding to the weaker site A^x can be expressed by K^x_o, K^x_{HBX}, K^x_{QX} and K^x_{Pe}. The constants for binding to A are determined by measurements

of k'_{HB} at low concentrations of Q^+ and X^-, where the influence of site A^x is minor and can be compensated for (3). The estimate of the constants is made by slope analysis based on eq. (6) obtained by inversion of eq. (1) after substitution of eq. (4).

$$\frac{1}{k'_{HB}} = \frac{1 + K_{Pe} [Pe]_m^n}{q K_o K_{ie} K_{QX} [X^-]_m} + \frac{[Q^+]_m}{q K_o K_{ie}} \qquad (6)$$

The constants for binding to site A^x are estimated at such high concentrations of Q^+ and X^- that site A is completely covered. The retention on site A, k'_{HBA}, is then constant, which gives the expression

$$k'_{HB} - k'_{HBA} = \frac{q K_o^x K_{ie}^x K_{QX}^x [X^-]_m}{1 + K_{Pe}^x [Pe]_m^n + K_{QX}^x [Q^+]_m [X^-]_m} \qquad (7)$$

The constants are determined by slope analysis after estimation of k'_{HBA}, as shown in a previous paper (3).

Monovalent Counter Ions

Hydrophilic counter ions are often used to promote suitable retention by chromatography of hydrophobic amines. Tables 1 and 2 give constants for dihydrogenphosphate, with alprenolol as solute in a system with LiChrosorb RP-8 as adsorbent, and an eluent of dimethyloctylamine (DMOA) in a phosphate buffer pH 2.2 containing 1-pentanol 0.115 M. It has not been possible to perform the measurements at constant ionic strength, but the variations have in most cases not exceeded the range 0.06 - 0.12.

The constants obtained with variation of the concentration of dihydrogenphosphate show good agreement with those obtained with variation of DMOA (Q^+). The calculation of the constants in

TABLE 1

Equilibrium Constants from Retention of Alprenolol on Site A as Ion Pair with Monovalent Counter Ions

X^-	$[X^-]_m$	$[Q^+]_m$	$K_{QX}.10^{-6}$	$K_o.K_{ie}.10^5$
$H_2PO_4^-$	0.005-0.1	0.001	1.1	1.2
$H_2PO_4^-$	0.057	0.001-0.005	0.70	2.1
ClO_4^-	0.002-0.025	0.001	8.4	1.9
Br^-	0.1	0.001-0.005	0.21	4.7
DMCHS	0.0005	0.001-0.005	24	11

Q^+ = DMOA, DMCHS = dimethylcyclohexyl sulphate. The constants for Br^- and DMCHS determined with 0.0076 M dihydrogenphosphate in the eluent. Estimates made with $K_{Pe}.[Pe]_m^n = 34.6$ and $q = 760$.

TABLE 2

Equilibrium Constants from Retention of Alprenolol on Site A^x as Ion Pair with Monovalent Counter Ions

X^-	$[X^-]_m$	$[Q^+]_m$	$K^x_{QX}.10^{-3}$	$K^x_o.K^x_{ie}.10^4$
$H_2PO_4^-$	0.01-0.07	0.05	3.5	2.7
$H_2PO_4^-$	0.057	0.01-0.05	4.8	2.2
ClO_4^-	0.02-0.13	0.02	16	3.3
Br^-	0.1	0.01-0.05	0.78	4.5
DMCHS	0.002	0.01-0.04	260	3.1

The constants for Br^- and DMCHS determined with 0.057 M dihydrogenphosphate in the eluent. Estimates made with $K^x_{Pe}.[Pe]_m^n = 9.71$ and $q = 760$.

the Tables has been made by use of values for K_{Pe} and K^x_{Pe} given in ref. (3).

The values of $K_o.K_{ie}$ and $K^x_o.K^x_{ie}$ are almost constant and seem to be independent of the counter ion used. $K^x_o.K^x_{ie}/K_o.K_{ie}$ is about 15. Previous studies (3) have shown that K^x_o/K_o is about 7, which

indicates that K^{x}_{ie}/K_{ie} is about 2, i.e. of about the same magnitude.

The effect of changing the concentration of the ions in the eluent on the capacity ratio can be illustrated graphically by a diagram constructed according to principles given in the Appendix. The influence of $[H_2PO_4^-]_m$ and $[DMOA]_m$ on the retention of alprenolol is illustrated in Fig. 1. At low concentration of DMOA (Fig. 1 a), the adsorption to the stronger site dominates and k'_{HB} can be varied within a much wider range than at the higher concentration of DMOA (Fig. 1 b), where the adsorption to the stronger site is almost constant.

Two or more counter ions are often present in the eluent and involved in the ion-pair distribution, one of them being added to regulate the retention and the others to provide pH buffering. The retention of a cationic solute, HB^+, in an eluent containing

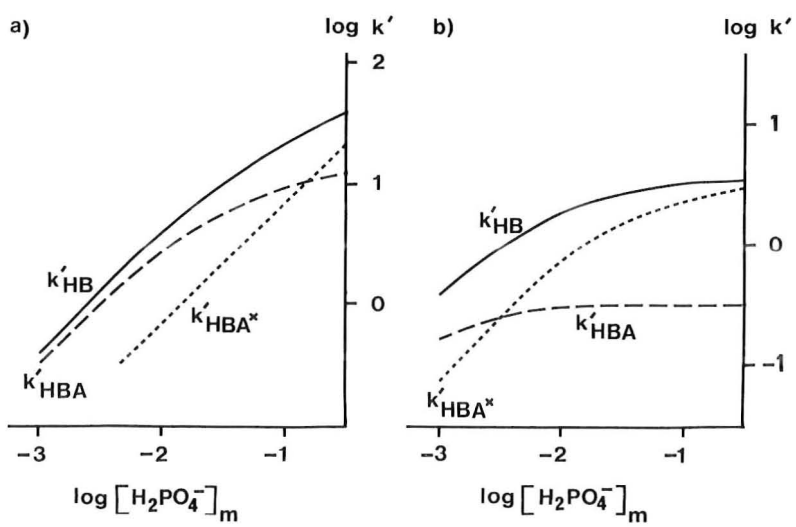

FIGURE 1. Retention of alprenolol as ion pair with dihydrogenphosphate. Eluent: DMOA and 0.115 M 1-pentanol in phosphate buffer pH 2.2. k'_{HB}, k'_{HBA} and k'_{HBA^x} are the overall capacity ratio and the capacity ratios for site A and A^x respectively. a) $[DMOA]_m$ = 0.001 M, b) $[DMOA]_m$ = 0.05 M.

Q^+, X^- and Z^-, can be derived analogously to eq. (1). If the magnitude of the ion-exchange constant is assumed to be independent of the anion, the following relationship is valid

$$K_{ie} = K_{HBX}/K_{QX} = K_{HBZ}/K_{QZ} \tag{8}$$

The expression for the capacity ratio of HB^+ then has the following form

$$k'_{HB} = \frac{q\, K_o\, K_{ie}\, (K_{QX}\, [X^-]_m + K_{QZ}\, [Z^-]_m)}{1 + K_{Pe}\, [Pe]_m^n + [Q^+]_m (K_{QX}\, [X^-]_m + K_{QZ}\, [Z^-]_m)} \tag{9}$$

The equilibrium constant of a counter ion, X^-, can be estimated in the presence of another anion, if the equilibrium constant of the latter, K_{QZ}, is known. The estimate can be made by slope analysis based on eq. (9) in inversed form.

$$\frac{1}{k'_{HB}} = \frac{1 + K_{Pe}\, [Pe]_m^n}{q\, K_o\, K_{ie}\, (K_{QX}\, [X^-]_m + K_{QZ}\, [Z^-]_m)} + \frac{[Q^+]_m}{q\, K_o\, K_{ie}} \tag{10}$$

In order to enable a good estimate, the condition used should give

$$K_{QX}\, [X^-]_m \geqslant K_{QZ}\, [Z^-]_m \tag{11}$$

This approach had to be adapted to the two-site theory in accordance with the principles given above (cf. eq. (7)).

The constants of bromide and dimethylcyclohexyl sulphate (DMCHS) are given in Tables 1 and 2. $H_2PO_4^-$ was present as buffering component in the mobile phase and the constants $K_{QZ} = 0.7 \times 10^6$ and $K_{QZ}^x = 4.8 \times 10^3$, taken from the Tables, were used to compensate for the adsorption of $H_2PO_4^-$ as ion pair. The K_{ie} found for DMCHS on site A deviates significantly from the values obtained with the inorganic counter ions. If this is due to the differences in size between the organic and the inorganic ions is at present not possible to elucidate.

The constants in Tables 1 and 2 show that the differences in extracting ability between the inorganic ions are rather small

in the chromatographic system in comparison with liquid-liquid systems (11-13). $H_2PO_4^-$ shows a remarkably high constant compared to Br^- and ClO_4^-.

The regulation of the retention of a hydrophobic amine such as alprenolol can as a rule be made by changing the concentration of $H_2PO_4^-$, which then has the double function of counter ion and buffering component in the eluent. The influence of the counter ion on the retention of alprenolol when DMOA (Q^+) is present in the eluent is demonstrated in Fig. 2. The construction of the diagram is based on values of the ion-exchange constants from Tables 1 and 2. The Figure shows that a more hydrophilic counter ion such as $H_2PO_4^-$ gives a considerably larger range of variation of k'_{HB} than the more hydrophobic ions ClO_4^- and DMCHS.

A combination of two counter ions with different extracting ability can in certain cases have advantages. An example is given in Fig. 3, which shows a comparison of the retention when the

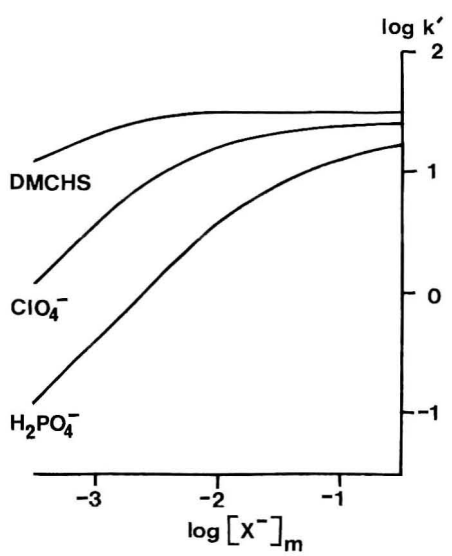

FIGURE 2. Regulation of the retention of alprenolol by the concentration of dihydrogenphosphate, perchlorate and dimethylcyclohexyl sulphate. Eluent: Counter ion, 0.01 M DMOA and 0.115 M 1-pentanol in aqueous solution of pH 2.

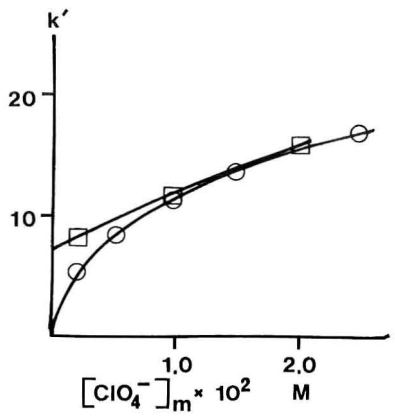

FIGURE 3. Regulation of the retention of alprenolol with perchlorate. Eluent: 0.001 M DMOA, 0.115 M 1-pentanol and sodium perchlorate with □ 0.01 M dihydrogenphosphate or ○ perchloric acid (pH ≃ 2.2) as buffering agent.

counter ions are perchlorate alone and a combination of perchlorate and dihydrogenphosphate. The relationship between k'_{HB} and $[ClO_4^-]_m$ is non-linear when perchlorate is the only counter ion, while an almost linear curve is obtained when $H_2PO_4^-$ is present in constant concentration in the eluent. An exact regulation of the retention is much easier to perform in the latter system.

Divalent Counter Ions

Sulphuric acid and ethylenediaminetetraacetic acid (EDTA) were tested as counter ions in pH ranges, where they are present mainly in divalent form. The chromatographic performance of the hydrophobic solutes in this system was as good as with monovalent counter ions. If the retention follows an ion-exchange model analogous to that applied to systems with monovalent counter ions, the following equilibrium should be valid

$$Q_2YA_s + HB_m^+ = QHBYA_s + Q_m^+ \qquad (12)$$

If the equilibrium constant of this process is K_{ie}, the capacity

ratio of HB^+ can be expressed by the following equation derived in analogy with the corresponding equations for monovalent counter ions.

$$k'_{HB} = \frac{q\ K_o\ K_{ie}\ K_{Q_2Y}\ [Q^+]_m\ [Y^{-2}]_m}{1 + K_{Pe}\ [Pe]_m^n + K_{Q_2Y}\ [Q^+]_m^2\ [Y^{-2}]_m} \qquad (13)$$

Estimates of constants according to a two-site model based on eq. (13) in inversed form gave the results presented in Tables 3 and 4.

The ion-exchange constant, K_{ie}, has the same value as obtained for monovalent counter ions (cf. Table 1), which confirms the validity of the ion-exchange model used.

TABLE 3

Equilibrium Constants from Retention of Alprenolol on site A as Ion Pair with Divalent Counter Ions

Y^{-2}	$[Y^{-2}]_m$	$[Q^+]_m$	$K_{Q_2Y}.10^{-10}$	$K_o.K_{ie}.10^5$
$EDTA^{-2}$	0.002-0.01	0.001	3.6	1.9
SO_4^{-2}	0.002-0.05	0.001	3.3	1.8

Q^+ = DMOA. Estimates made with $K_{Pe}.[Pe]_m^n = 34.6$ and $q = 760$.

TABLE 4

Equilibrium Constants from Retention of Alprenolol on site A^x as Ion Pair with Divalent Counter Ions

Y^{-2}	$[Y^{-2}]_m$	$[Q^+]_m$	$K^x_{Q_2Y}.10^{-6}$	$K^x_o.K^x_{ie}.10^4$
$EDTA^{-2}$	0.01-0.05	0.02	8.0	1.8
SO_4^{-2}	0.02-0.2	0.02	3.8	2.3

Estimates made with $K^x_{Pe}.[Pe]_m^n = 9.71$ and $q = 760$.

CONCLUSIONS

Ion-pair systems containing DMOA as modifier provide liquid chromatographic separations of hydrophobic amines with good performance. DMOA is also a major tool for regulating retention.

The nature and concentration of the counter ion are means to regulate retention. This effect levels off at high surface coverage of DMOA, i.e. at high content in the mobile phase or with hydrophobic counter ions.

The thermodynamics of the retention of the solute amines can be treated as ion-pair adsorption or as ion exchange, the two models being equivalent.

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REFERENCES

1. Wahlund, K.-G. and Sokolowski, A., Reversed-phase ion-pair chromatography of drugs and related organic compounds, J. Chromatogr., 151, 299, 1978.
2. Melander, W.R., Stoveken, J. and Horváth, C., Mobile phase effects in reversed-phase chromatography, II. Acidic amine phosphate buffers as eluents, J. Chromatogr. 185, 111, 1979.
3. Jansson, S.O., Andersson, I. and Persson, B.A., Solute-solvent interactions in ion-pair liquid chromatography of amines on non-polar bonded phases using 1-pentanol and N,N-dimethyloctylamine as organic modifiers, J. Chromatogr., 203, 93, 1981.
4. Tilly-Melin, A., Ljungcrantz, M. and Schill, G., Reversed-phase ion-pair chromatography with an adsorbing stationary phase and a hydrophobic quaternary ammonium ion in the mobile phase, J. Chromatogr., 185, 225, 1979.
5. Sokolowski, A. and Wahlund, K.-G., Peak tailing and retention behaviour of tricyclic antidepressant amines and related hydrophobic ammonium compounds in reversed-phase ion-pair liquid chromatography on alkyl-bonded phases, J. Chromatogr., 189, 299, 1980.

6. Johansson, I.M., Retention in reversed-phase ion-pair chromatography of amines on alkyl-bonded phases, J. Liq. Chromatogr., in press.

7. Nahum, A. and Horváth, C., Surface silanols in silica-bonded hydrocarbonaceous stationary phases, I. Dual retention mechanism in reversed-phase chromatography, J. Chromatogr., 203, 53, 1981.

8. Terweij-Groen, C.P., Heemstra, S. and Kraak, J.C., Distribution mechanism of ionizable substances in dynamic anion-exchange systems using cationic surfactants in high performance liquid chromatography, J. Chromatogr. 161, 69, 1978.

9. Knox, J.H. and Hartwick, R.A., Mechanism of ion-pair liquid chromatography of amines, neutrals, zwitterions and acids using anionic hetaerons, J. Chromatogr., 204, 3, 1981.

10. Karger, B.L., LePage, J.N. and Tanaka, N., High-performance liquid chromatography, advances and perspectives, vol. 1, Horváth, C. (editor), Academic Press, New York, London, Toronto, Sydney, 1980, p. 179-189.

11. Tilly, A., Extraction of ion pairs of dihydrogenphosphate, sulphate, hydrogensulphate and hydrogencarbonate with hydrogen-bonding organic solvents, Acta Pharm. Suecica 10, 111, 1973.

12. Nordgren, T. and Modin, R. Quantitative determinations by ion-pair extraction, Part 12, Extraction of amino acids with quaternary ammonium ions, Acta Pharm. Suecica 12, 407, 1975.

13. Persson, B.A. and Eksborg, S., Extraction of amines as complexes with inorganic anions, Part 6, Extraction of imipramine and desipramine ion pairs with alkyl chloride solvents, Acta Pharm. Suecica, 7, 353, 1970.

APPENDIX

The change of the capacity ratio of a cationic solute, HB^+, with the concentration of the mobile phase components X^- and Q^+ can be illustrated by a diagram. It is based on expressions analogous to eq. (1) and separate constructions are made for each of the sites.

Eq. (1) can, after combination with eq. (4) be written in the following form:

$$k'_{HBA} = \frac{a\,[X^-]_m}{[Q^+]_m\,([X^-]_m + b\,K_{QX}^{-1}\,[Q^+]_m^{-1})} \qquad (14)$$

where $a = q\,K_o\,K_{ie}$ and $b = 1 + K_{Pe}\,[Pe]_m^n$.

The diagram gives the relationship between log k'_{HB} and log $[X^-]_m$. The construction is based on the following limiting cases

$$\text{I.} \quad [X^-]_m >> b\, K_{QX}^{-1}\, [Q^+]_m^{-1} \tag{15}$$

gives

$$\log k'_{HBA} = \log a\, [Q^+]_m^{-1} \tag{16}$$

which is a horizontal line with the intercept log $a\, [Q^+]_m^{-1}$.

$$\text{II.} \quad [X^-]_m << b\, K_{QX}^{-1}\, [Q^+]_m^{-1} \tag{17}$$

gives

$$\log k'_{HBA} = \log a\, K_{QX}\, b^{-1} + \log [X^-]_m \tag{18}$$

which is a line with a slope of +1 going from the construction point ($\log k'_{HBA} = 0$; $\log [X^-]_m = -\log a\, K_{QX}\, b^{-1}$).

The final line will follow the lowest of the two lines in the diagram, passing 0.3 log units below the intersection point. The capacity ratio of HB^+, k'_{HB}, is the sum of k'_{HBA} and $k'_{HBA}x$.

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METAL CATION SPECIATION VIA EXTRACTION REVERSED PHASE HPLC WITH REFRACTIVE INDEX AND/OR INDUCTIVELY COUPLED PLASMA EMISSION DETECTION METHODS (HPLC-RI-ICP)

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ABSTRACT

Conventional high performance liquid chromatography (HPLC) instrumentation and packing materials can be inexpensively and rapidly utilized for the qualitative and quantitative analysis of various metal cations. The final approaches utilize reversed phase HPLC in the form of extraction chromatography. The detection of individually eluted, fully resolved metal cations is then possible via conventional refractive index (RI) and/or inductively coupled plasma (ICP) emission spectroscopic detection. Final data presentation can be in the form of conventional, continuous RI or ICP chromatograms, via pulsed data ICP presentations, and/or via tabular ICP data presentation.

INTRODUCTION (33)

Inorganic metal toxicity has long been an area of intense biological, toxicological, and medical interests. The apparent toxic properties of most, if not all, metals have been elucidated and extensively described for several

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decades (1-4). Unfortunately, the vast majority of published metal toxicity studies have never involved appropriate and complete analytical methodology. Wherein analytical methods have been used as part of the overall biological studies, these have provided total metal concentrations or absolute levels, rather than the more specific and desirable metal speciation profiles for the particular biological, toxicological, or medical samples of interest (5-14). Within recent years it has become apparent to most scientists and some decision makers that partial or complete metal speciation of environmental, biological, toxicological, and/or medical type samples must be undertaken on a regular basis. That is, only when all or most metal species present in any sample are accurately known, can we then appropriately ascribe biological, medical, or toxicological properties to that particular mixture of metals and/or metallic compounds or ions. Many metals can exist as the free metal, various cationic or anionic oxidations states, organically bound chelates/complexes, and/or organometals. Obviously, the final speciation of metals in a complex sample matrix, often in the presence of other metals and their species, must involve some sort of an initial separation process. Within the past few years, a tremendous interest and effort has emerged towards inorganic metal cation/anion analysis, especially in the use of ion chromatography (IC), and/or high performance liquid chromatography using ion exchange packings, together with conductivity and/or electrochemical detection (12, 15-24). It has become obvious that IC, especially via the use of commercially available instrumentation, has become quite popular, very useful, and can be readily utilized in trace environmental and/or toxicological studies. Unfortunately, such commercial instrumentation is often quite expensive ($15,000-$25,000/unit), and must be totally dedicated to the performance of inorganic/organic cation or anion type separations. It does not lend itself to the performance of conventional HPLC separations via liquid-solid, liquid-liquid, reversed phase, paired-ion, and/or gel permeation techniques.

Unfortunately, relatively little non-IC or non-ion-exchange type work has been reported in recent years, especially with regard to using any commercial type HPLC instrumentation and packing materials for performing inorganic metal cation/anion type analyses. Quite obviously, this situation should be remedied, and the work described here, in part, has been designed with this goal in mind. All of this work has utilized conventional reversed phase type packing materials, C_{18}, with extraction reagents in the mobile phase, viz, tributylphosphate (TBP).

Extraction chromatography was developed extensively in the early-mid 1970s, and the work of Horwitz and co-workers is especially relevant (25-27).

The use of organic phosphates for the separation of various radiochemical metal species was quickly extended to a large number of metal cations, but this work almost always involved the use of polymeric or silica gel type packing materials. The usefulness of extraction chromatography with modern, bonded phase packings, especially C_{18}, has not been discussed in depth.

We describe here some initial results in the separation and speciation of various metal cations utilizing extraction chromatography together with reversed phase (RP) type packings, wherein this has now been interfaced with RI and/or ICP detection methods. These methods allow for the successful resolution and speciation of various metal cations, especially when the HPLC methods are combined with ICP detection. Reproducibility studies have been performed using these approaches, both on an intra-day and inter-day basis. In addition, some attempts have been made to compare direct-ICP with HPLC-ICP for cadmium ion limits of detection.

EXPERIMENTAL

Reagents

Inorganic salts, reagent grade, were obtained as Baker analyzed reagent (VWR Scientific, Inc., Boston, Mass.), Fisher ACS certified (Fisher Scientific, Inc., Medford, Mass.), and/or Alfa/Ventron Inorganics, Inc. (Danvers, Mass.). Tributyl phosphate was purchased from Alfa/Ventron, Inc. The mobile phase water was purchased from J.T. Baker Chemical Co. (Phillipsburg, N.J.), or used directly from a Corning Mega-Pure still (Corning Corp., Corning, N.Y.).

Apparatus

We have utilized a number of HPLC instrumentation arrangements for the present work. However, for the extraction chromatography, not all columns proved equally satisfactory. A typical HPLC arrangement consisted of a Laboratory Data Control (LDC) (Riviera Beach, Fla.) Model 711 solvent delivery system, modified with a special pulse dampener, or a newer LDC Constametric III pump, a Rheodyne Model 7125 syringe injection valve (Rheodyne Corp., Cotati, Calif.), an Altex/Beckman variable wavelength UV-VIS detector (Altex/Beckman Corp., Irvine, Calif.), a Waters Model 401 RI detector (Waters Assocs., Milford, Mass.), a modified Instrumentation Laboratory Model Plasma-100 inductively coupled plasma emission spectrometer (Instrumentation Laboratory, Inc., Wilmington, Mass.), and a Linear Corp. (Irvine, Calif.) or

Honeywell Corp. (Minn., Minn.) dual pen recorder. The RI/ICP data was obtained via a dual pen recorder, and/or a separate ICP print-out from the Plasma-100 system. Often, both the recorder ICP chromatogram and the tabular data format from the ICP were obtained at the same time. At other times, the tabular data presentation could be manually used to reconstruct a pulsed type or continuous type HPLC-ICP chromatogram. This was done knowing the timed integration sequence of the tabular data presentation. In later runs, the Plasma-100 system was operated to present both pulsed type chromatograms, as below, and the simultaneous tabular data presentations. This provided additional confirmation of the ICP results for the final metal speciation.

In the work described, the HPLC columns were all of the C_{18} type and usually obtained commercially, as follows: 1) Hibar II RP-18 pre-packed column (4.6mm x 25cm)(MCB Chemicals, Inc., Cinc., Ohio); 1) Alltech C-18 (4.6mm x 25cm)(Alltech Assocs., Inc., Deerfield, Ill.); 3) Altex/Beckman Ultrasphere ODS (4.6mm x 15cm)(Altex/Beckman Corp.); 4) slurry packed in-house columns using Lichrosorb RP-18 (4.6mm x 25cm)(MCB Chemicals, Inc.).

Methods

In extraction chromatography the mobile phase consisted of varying molarities of either NaCl or LiCl, saturated with TBP. The saturation was performed by shaking the salt solution with excess TBP, standing in the presence of the excess TBP overnight, and then separating the aqueous layer from the organic layer in the morning. In some initial studies, inorganic phosphate buffers, 0.1M sodium dihydrogen phosphate/0.1M disodium hydrogen phosphate, were added to the mobile phase, producing a final pH = 3.9-4.2. In the absence of such buffering salts, the final pH was about 6.0. These differences in the pH of the mobile phase did not appear to affect any capacity factors for the metal cations studied. The aqueous mobile phase was de-gassed under vacuum, filtered, and used as quickly as feasible. It appears important that such mobile phases are prepared just prior to actual use, otherwise final retention times can differ greatly. Omission of the TBP altogether, results in all metal cations eluting in the solvent front.

The RP-HPLC columns are equilibrated with the mobile phase for about 1-2 hrs at ambient temperatures, and once the RI or ICP baselines are stable, analyses can begin. It is possible to accelerate the equilibration process by using a special equilibrating solution of TBP in MeOH:HOH (1:1), containing about 20% v/v TBP. This is then used to saturate a brand new C_{18} column. In this way, daily equilibrations with the fresh mobile phase took less time than with the first procedure above. In order to prolong column and component lifetimes, the columns were washed free of all inorganic salts with distilled

water at the end of each day's run. They were then left standing in the presence of the same distilled water. Washing with MeOH or 1:1 MeOH:HOH removed all organic TBP from the packing material, and thus required a much longer equilibration period the next working day. Individual columns used and prepared in this manner have lasted as long as six months before any signs of column degradation or component corrosion began to appear. A mobile phase saturation pre-column, consisting of large particle size silica gel, was used on-line and just before the injection valve. This was designed to extend column lifetime by preventing dissolution of the analytical packing material.

In comparing the sensitivities for direct-ICP analysis vs. HPLC-ICP interfacing for the same metal cation, viz. Cd^{+2}, this was done by injecting a number of identical samples via HPLC-ICP (20ug/20ul), and at the same time determining the ICP response on that same day for a direct-ICP nebulizer uptake analysis of the identical solution. Comparisons of both peak height and peak area responses were then made for the direct-ICP and HPLC-ICP results.

RESULTS AND DISCUSSION

We have now determined retention times and capacity factors (k') for at least three metal cations, all divalent species, of cadmium (Cd^{+2}), zinc (Zn^{+2}), and mercury (Hg^{+2}). Obviously, many other divalent and monovalent cations could be separated using these or similar chromatographic techniques. Initial separation conditions were determined using both RI and ultra-violet (UV) detection, where possible, or RI alone. Two inorganic salts, NaCl and LiCl, were separately added to the mobile phase preparations, in order to "salt-out" the metal cation species and increase or modify retention times. Table I summarizes the overall results obtained in this manner. Changing the NaCl concentrations from 1.0M to 2.0M resulted in a complete reversal of the elution order for Hg and Zn cations. Thus, as others have previously noted in extraction chromatography, retention times and capacity factors, as well as elution orders, can be easily manipulated by varying the salt concentrations and holding all other variables constant. In the case of Cd^{+2}, no NaCl concentration was able to increase the retention time beyond that of the solvent front. However, by going to the use of LiCl, at the 2.0M level, it was possible to obtain a small, but real, retention for the Cd^{+2} ion. These results with 2.0M LiCl are shown in Figure 1, wherein RI alone was used for the detection, and low ug amounts of all three metal cations were injected as a mixture, as well as separately. The total elution time for these three species is under six minutes, with baseline resolution obtained for all cations

TABLE I

TRIBUTYL PHOSPHATE REVERSED PHASE EXTRACTION CHROMATOGRAPHY IN HPLC[a]

RP-HPLC CONDITIONS	METAL CATION	t_r (mins)	k' (capacity factor)
1.0M NaCl[c]	Hg^{+2}	4.8	0.68
	Zn^{+2}	4.4	0.54
	Cd^{+2}	s.f.[b]	s.f.[b]
2.0M NaCl[c]	Hg^{+2}	4.2	0.45
	Zn^{+2}	6.3	1.17
	Cd^{+2}	s.f.	s.f.
1.0M LiCl[d]	Hg^{+2}	4.5	1.3
	Zn^{+2}	2.9	0.51
	Cd^{+2}	s.f.	s.f.
2.0M LiCl[d]	Hg^{+2}	7.6	2.0
	Zn^{+2}	5.7	1.3
	Cd^{+2}	3.3	0.32

a. HPLC conditions: Hibar-II RP-18 column (4.6mm x 25cm)(MCB Chemicals, Inc.), aqueous mobile phase saturated with TBP containing NaCl or LiCl, pH = 4.6-6.0, flow rates 0.65-1.0ml/min, RI/UV detection.
b. s.f. = solvent front; c. mobile phase flow rate = 0.65ml/min; d. mobile phase flow rate = 1.0 ml/min.

present. These same HPLC conditions, Figure 1, were then utilized for subsequent HPLC-RI-ICP studies, as well as for the determination of relative sensitivities for direct-ICP vs. HPLC-ICP.

Figure 2 is a typical HPLC-RI-ICP chromatogram for a single divalent cation, Cd^{+2}, using the same HPLC conditions as in Figure 1, but now with a 50:50 split of the total column eluent to both detectors. In this particular example, Cd^{+2} was injected at a concentration of 36.6ug/20ul, so that about 18ug is reaching each detector. Specific detector operating conditions are indicated in Figure 2. It is apparent from the RI trace that the Cd^{+2} ion is just separated from the solvent front. Also of interest is the fact that the ICP is readily able to provide continuous chromatographic profiles of each peak as these elute from the column. That is, it can function just like any more conventional HPLC detector, be this UV, RI, EC, etc.

Whenever HPLC is interfaced with an initially designed off-line detector, such as the ICP or graphite furnace atomic absorption spectrometer (GFAA), one must expect a certain loss of detection limits and overall sensitivity (28-30). The reasons for this overall loss of detection limits (MDLs) have been

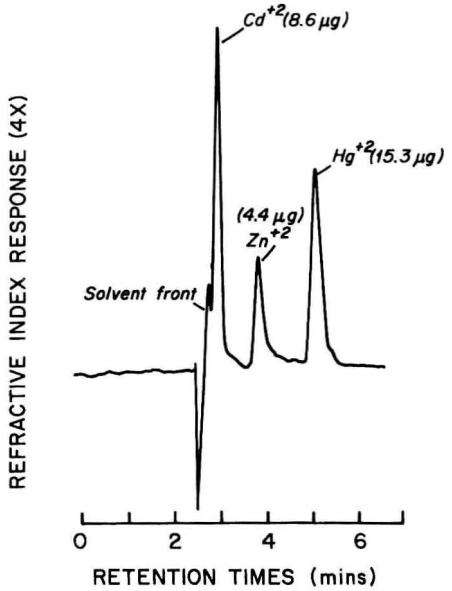

Figure 1. Extraction chromatography of a cation mixture using a 2.0M LiCl-TBP mobile phase at 1.0 ml/min flow rate with RI detection alone.

discussed at length by several workers, but it is a totally general phenomenon whenever HPLC is interfaced with an off-line type detector. We have now tried to determine experimentally the actual differences in overall minimum detection limits (MDLs) for the HPLC system of Figure 2. In reality, we are not actually comparing MDLs nor determining MDLs here, but we are comparing relative responses or sensitivities for these two approaches. These results are summarized in Table II, which compares the ICP peak heights and ICP peak areas for both direct-ICP and HPLC-ICP analyses of a known Cd^{+2} concentration. The direct-ICP values for peak height and peak area were obtained by continuously nebulizing, with a conventional cross-flow type nebulizer, a 1 ppt (parts-per-thousand) solution of Cd^{+2} into the ICP. Peak heights were then measured over several minutes, and an average reading was taken from these peak heights. In the case of direct-ICP peak area measurements, this was done by determining the total emission intensity for Cd^{+2}, via direct nebulization of a 1ppt solution, over a time span representing 20ug total amount (mass) of metal cation. Thus, we can arrive at two methods for directly comparing sensitivities via direct-ICP and HPLC-ICP for Cd^{+2} under these particular HPLC and ICP operating conditions.

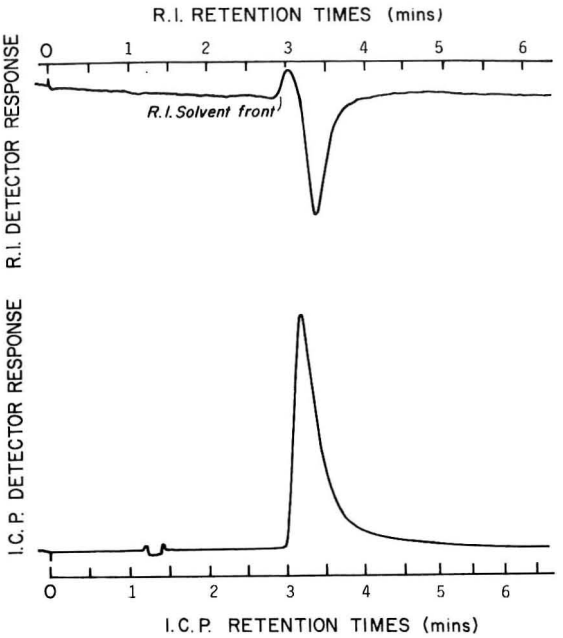

Figure 2. Extraction RP-HPLC of Cd^{+2} (36.6ug/20ul) split 50:50 between RI and ICP detectors. HPLC mobile phase of 2.0M LiCl-TBP at 1.0 ml/min. ICP set at 214.44nm, RI set at 0.10×10^{-3} units.

TABLE II

COMPARISON OF SENSITIVITIES FOR DIRECT-ICP AND HPLC-ICP INTERFACING

METHOD OF COMPARISON	DIRECT-ICP[a]	HPLC-ICP[a,d]	DIRECT-ICP/ HPLC-ICP
ICP PEAK HEIGHTS	10.2, 11.4cm[b]	0.57 ± 0.02cm	18.0
ICP PEAK AREAS	1,037,577-1,198,310[b]	435,587 ± 12,300[c]	2.6

a. Determined by injecting a 1ppt solution (20ug/20ul) of Cd^{+2} onto HPLC-ICP or using a 1ppt solution for direct nebulization into ICP. Peak heights and peak areas were determined from these analyses.
b. Numbers represent two separate runs on same day, average of these results used for final calculations of direct-ICP/HPLC-ICP.
c. Numbers represent the average ± standard deviation for three separate runs on the same day (n=3).
d. HPLC-ICP used extraction RP-HPLC with 2.0M LiCl-TBP mobile phase at 0.5ml/min, no split after column, 20ul injections of 20ug Cd(+2).

In using the ICP peak height measurements alone, the ratio of direct-ICP/HPLC-ICP turns out to be about 18.0. That is, the sensitivity via HPLC-ICP approaches is about 18 times worse than via direct-ICP methods where peak heights are used. Using a comparison of peak area intensities on the ICP, the ratio of direct-ICP/HPLC-ICP comes out to be about 2.6, Table II. Different HPLC conditions, with longer retention times and more peak spreading for Cd^{+2} would tend to produce even larger differences in these overall results. These results suggest that the ICP is acting as a mass sensitive detector in the HPLC mode, rather than as a concentration sensitive type detector. Thus, when the total mass in terms of peak areas is measured and compared for direct-ICP vs. HPLC-ICP, their relative differences tend to cancel out and the ratio approaches a value of 1.0. In fact, in other systems and with other metal cations, we have obtained relative ratios which are between 1.0-1.5 using peak area measurements (31). Using peak area measurements, as opposed to peak heights, the differences in the direct-ICP vs. HPLC-ICP responses for the same mass of cation approach zero (ratio = 1.0). From these measurements and comparisons, one might initially assume that absolute limits of detection via HPLC-ICP could be similar to direct-ICP, and that even if one used peak heights for such measurements, final MDLs might be 15-25 times worse via HPLC-ICP. In fact, wherein we have measured MDLs directly, the HPLC-ICP results are often 2-3 orders of magnitude worse than for the direct mode (31). This is discussed at much greater length elsewhere (32).

In any study involving the interfacing of HPLC with an off-line type detector, it is of interest to determine the overall system reproducibility within any given day, as well as the comparable reproducibilities between days. These overall results are summarized in Table III, for two separate days, wherein we have indicated retention times for Cd^{+2} by ICP and RI detection, ICP maximum intensity less background (tabular print-out), ICP peak heights from chromatograms, and the number of separate analyses performed. Overall retention times by both RI and ICP measurements are extremely reproducible, both intra- and inter-day, with a standard deviation of less than 5%. The ICP peak intensity and peak heights also exhibit a standard deviation of less than 5% within each day, and the inter-day comparisons are also quite close, again less than a 5% overall difference. Thus, from these initial reproducibility studies, it would appear that extraction chromatography can indeed provide a high degree of chromatographic reproducibility both inter- and intra-day. In addition, the ICP interfaced to HPLC seems to provide a high degree of overall reproducibility in the responses obtained, again both intra- and inter-day. The amounts of Cd^{+2} used in these reproducibility studies are quite high,

about 170ug/20ul injections. There is a possibility that with lower amounts and concentrations used, the degree of reproducibility evidenced in Table III may not be fully realized.

One of the major goals of any program which involves the use of ICP as a metal selective detector for HPLC is to demonstrate its true capabilities and potentials. We have therefore applied the above HPLC approaches for the three metal species involved, Cd^{+2}, Zn^{+2}, and Hg^{+2}, using extraction chromatography, Figure 1, for a direct interfacing of both RI and ICP. These overall results are depicted in Figure 3, which is a dual RI/ICP type chromatogram for a split eluent from the extraction chromatographic separation. Indicated in Figure 3 are the specific peak shapes for each metal cation by both RI and ICP, retention times for each cation, specific wavelengths used *via* ICP, and the absolute amounts of each metal species going to the RI or ICP. The apparent chromatographic peak shapes *via* ICP are comparable to those observed *via* RI detection, with an increased band broadening being observed for those metals with longer retention times. We have obtained baseline resolution for each of the three metal cations, as in Figure 1, but we have not yet optimized the final detection limits for each cation on the ICP. Thus, the results given here are not to be construed as MDLs for these species. Work remains to be done with regard to optimizing HPLC peak shapes, emission wavelengths used, nebulizer/interface arrangements and effectiveness, as well as several other parameters of interest in the overall HPLC-ICP system. Nevertheless, these initial results forthe direct interfacing of extraction HPLC with RI and ICP detection appear quite useful and of practical interest/application.

This is not to imply that there are no practical, technical problems with the long term use of extraction HPLC for HPLC-ICP metal cation speciation.

TABLE III

REPRODUCIBILITY STUDIES FOR HPLC-ICP ANALYSIS OF Cd^{+2} VIA EXTRACTION HPLC[a]

PARAMETER MEASURED	DAY ONE[b]	DAY TWO[b]
RETENTION TIME (t_r) BY ICP	3.10 $\pm$ 0.00min	3.03 $\pm$ 0.02min
RETENTION TIME (t_r) BY RI	3.15 $\pm$ 0.01min	3.15 $\pm$ 0.00min
ICP MAXIMUM INTENSITY-BKGRD.	301,410 $\pm$ 11,730	324,451 $\pm$ 9,737
ICP PEAK HEIGHT ON CHROMATOGRAM	15.8 $\pm$ 0.3cm	15.5 $\pm$ 0.3cm
NUMBER OF SEPARATE ANALYSES	4	3

a. Determined using extraction RP-HPLC with a 2.0M LiCl-TBP mobile phase at 1.0ml/min flow rate, eluent split 50:50 to RI:ICP, 20ul injections of 170ug Cd(+2).
b. Numbers represent the averages $\pm$ standard deviations

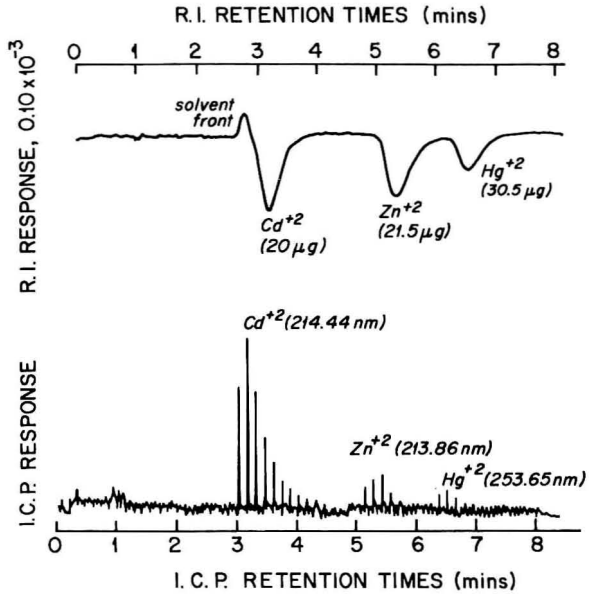

Figure 3. Extraction chromatography of a cation mixture using a 2.0M LiCl-TBP mobile phase. Detection is by RI and ICP simultaneously with a 50:50 eluent split ratio. Amounts indicated are going to each detector.

We have not, as yet, investigated the retention properties of the analogous monovalent cations, and there remains a slight possibility that some metal species may exhibit incomplete resolution of their various cationic valence species. The ICP cannot, by itself, differentiate between two different valences for the same metal species, it must depend on an initial separation prior to their introduction into the ICP system. The separation of one metal cation is apparently easy to realize; however, we have not as yet determined the extraction chromatography separations for different oxidation states of the same metal species via extraction chromatography. In general, we would not expect, for example, Hg^{+1} to interfere with the elution of Hg^{+2}, as described in Figures 1 and 3, but this has not yet been demonstrated.

The use of 1.0-2.0M salt solutions does not appear to create any major problem in the long-term use of the ICP as an HPLC detector, although there is very little in the literature describing such mobile phases in HPLC-ICP. There is no apparent build-up of residual salt onto the plasma tip or surroundings,

and there is no apparent problem of quenching of metal responses or spectral interferences. Thus, at least the ICP itself appears compatible with salt solutions containing 1.0-2.0M NaCl or LiCl. There remains the distinct problem of metal corrosion within the HPLC arrangement, and this is perhaps the greatest concern that one must face in this type of an HPLC-ICP approach. We have not yet attempted to prevent all salt corrosion, such as via the use of glass lined HPLC columns, glass lined connecting lines, Teflon surfaced injection valves, and related hardware modifications, but this is obviously a very desirable goal. The biggest problem that we have faced is that due to rust/corrosion formation in all metal parts contacting the mobile phase, but this is especially a problem within the HPLC columns. This can be somewhat alleviated or prevented using methods described already (Experimental), but eventually corrosion build-up reaches a level that causes severe back pressure in the column, rust eluting with column eluents, and ancillary deterioration of the overall HPLC system. There are known methods of preventing such salt induced problems, and it may develop that such already described approaches would make extraction chromatography an extremely useful and practical approach for performing metal cation/anion type analyses.

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REFERENCES

1. Hammond, B.P. and Beliles, R.P., in Toxicology - The Basic Science of Poisons, Second Edition, Doull, J., Klaassen, C.D., and Amdur, M.O., eds., MacMillan Publishing Co., Inc., New York, 1980, p. 468.

2. Berman, E., Toxic Metals and Their Analysis, Heyden & Sons, Ltd., London, 1980.

3. Sigel, H., ed., Metal Ions in Biological Systems. Carcinogenicity and Metal Ions, Vol. 10, Marcel Dekker, New York, 1980.

4. Some Metals and Metallic Compounds. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Volume 23. International Agency for Research on Cancer, Lyon, 1980.

5. Scheutzle, D., ed., Monitoring Toxic Substances, ACS Symposium Series No. 94, American Chemical Society, Washington, D.C., 1979.

6. Van Loon, J.C., Anal. Chem., 51, 1139A (1979).

7. Van Loon, J.C., American Laboratory, 47 (May, 1981).

8. Parks, E.J., Brinckman, F.E., and Blair, W.R., J. Chrom., 185, 563 (1979).

9. Brinckman, F.E., Jewett, K.L., Iverson, W.P., Irgolic, K.J., Ehrhardt, K.C., and Stockton, R.A., J. Chrom., 191, 31 (1980).

10. Jewett, K.L. and Brinckman, F.E., J. Chrom. Sci., in press (1981).

11. Ricci, G.R., Shepard, L.S., Colovos, G., and Hester, N.E., Anal. Chem., 53, 610 (1981).

12. MacCrehan, W.A., Anal. Chem., 53, 74 (1981).

13. Krull, I.S. and Jordan, S., American Laboratory, 21 (October, 1980).

14. Miyazaki, A. and Barnes, R.M., Anal. Chem., 53, 364 (1981).

15. Schwedt, G., Chromatographic Methods in Inorganic Analysis. Separation Methods in Inorganic Analysis. Separation Methods and Systems, Detection Methods, Applications in Inorganic Analysis, A.H. Verlag, Publ., Heidelberg, 1981.

16. Schwedt, G., Topics in Current Chemistry, Boschke, F.L., ed., Vol. 85, Springer-Verlag Publ., Berlin, 1981, p. 160.

17. Cassidy, R.M., in Trace Analysis, Lawrence, F.J., ed., Academic Press, New York, in press (1981).

18. Mulik, J.D. and Sawicki, E., eds., Ion Chromatographic Analysis of Environmental Pollutants, Ann Arbor Science, Inc., Ann Arbor, Mich., 1979.

19. Girard, J.E., Anal. Chem., 51, 836 (1979).

20. MacDonald, J.C., American Laboratory, 45 (January, 1979).

21. Wetzel, R.A., Anderson, C.L., Schleicher, H., and Crook, G.D., Anal. Chem., 51, 1532 (1979).

22. Smith, F.D., Jr. and Chang, R.C., CRC Critical Reviews in Anal. Chem., 197 (August, 1980).

23. Pohl, C.A. and Johnson, E.L., J. Chrom. Sci., 18, 442 (1980).

24. Gjerde, D.T., Schmuckler, G., and Fritz, J.S., J. Chrom., 187, 35 (1980).

25. Horwitz, E.P., Bloomquist, C.A.A., and Delphin, W.H., J. Chrom. Sci., 15, 41 (1977).

26. Horwitz, E.P. and Bloomquist, C.A.A., J. Inorg. Nucl. Chem., 37, 425 (1975).

27. Vandegrift, G.F. and Horwitz, E.P., J. Inorg. Nucl. Chem., 39, 1425 (1977).

28. Scott, R.P.W., Liquid Chromatography Detectors, Elsevier Sci. Publ. Co., Amsterdam, 1977.

29. Ettre, L.S., J. Chrom. Sci., 16, 396 (1978).

30. McKinley, W.A., Popovich, D.J., and Layne, T., American Laboratory, 37 (August, 1980).

31. Savage, R.N., Smith, S.B., Jr., Bushee, D., and Krull, I.S. Paper presented at the 1981 Federation of Analytical Chemistry and Spectroscopy Society Meeting (FACSS VIII), Phila., Penna, September, 1981, abstract no. 433.

32. Krull, I.S., Bushee, D., Savage, R.N., Schleicher, R.G., and Smith, S.B., Jr., Anal. Letters, submitted for publication (1981).

33. Abbreviations used: HPLC = high performance liquid chromatography; RI = refractive index detection; ICP = inductively coupled plasma emission detection; UV = ultraviolet detection; TBP = tributyl phosphate; RP = reversed phase; IC = ion chromatography; MeOH = methanol; HOH = water.

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(4), 707-714 (1982)

HIGH PRESSURE LIQUID CHROMATOGRAPHIC DETERMINATION OF ISONIAZID AND ACETYLISONIAZID IN HUMAN PLASMA

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ABSTRACT

A quantitative high pressure liquid chromatographic (HPLC) assay has been developed for the determination of isoniazid (INH) and acetylisoniazid (ACINH) in human plasma. Plasma samples were taken from a patient after oral administration of INH (with proven tuberculosis infection). A C_{18} reversed phase radial compression column was used to separate INH and ACINH from other plasma components. The analysis takes 10 minutes per sample and the lower limit of detection for each compound is 0.10 ug/ml plasma.

INTRODUCTION

Tuberculosis (TB) is a prevalent disease in the United States with a reported incidence in 1979 of 12.6 per 100,000 population (27,669 newly diagnosed cases in one year) (1). It is estimated that there are more

than 50,000 patients with TB at any one time. Isoniazid (INH), isonicotinic acid hydrazide, is an effective drug used in the treatment of TB. The minimal tuberculostatic concentration is 0.025 to 0.050 ug INH/ml plasma (2). An enzyme in the liver, N-acetyltransferase, is responsible for the production of the major metabolic product of ACINH (3). There is a bimodal distribution of the activity of this enzyme in the population which divides the population into rapid and slow acetylators of INH (4,5). The ACINH metabolite does not have antituberculostatic activity.

Very few HPLC procedures have been developed for analysis of INH and ACINH. Stewart et al (6) measured INH and pyrodoxime from pharmaceutical preparations and INH has also been analyzed in tablets by the method of Bailey and Abdou (7). Ion-paired chromatography was used for separation of INH and ACINH in spiked plasma and urine samples by the procedure of Saxena et al (8).

In this procedure INH and ACINH were analyzed from human plasma following oral dosing of the drug. Ion-pairing was accomplished with dioctyl sulfosuccinate as the reagent. A Waters Associates C_{18} reversed phase radial compression column allowed rapid separation of drug and metabolite at high flow rates.

EXPERIMENTAL

Materials

Isoniazid was obtained from Aldrich Chemical Co. (Milwaukee, WI), and dioctyl sulfosuccinate was purchased from Sigma Chemical Co. (St. Louis, MO). Acetylisoniazid and 1-benzoyl-2-isonicotinoylhydrazine (internal standard) were synthesized by the method of Mitchell *et al* (9) and Kerr *et al* (10), respectively.

Instrumentation

A Waters Associates 202 HPLC with a 6UK injector and a radial compression column unit was used with a 11.5 cm by 0.8 cm 5 um C_{18} uBondapak column. The guard column contained 10 um C_{18} uBondapak reversed phase packing (DuPont, Wilmington, DE).

Operating Procedures and Conditions

The mobile phase consisted of 0.001 M dioctyl sulfosuccinate in distilled water/ethanol (55/45%) adjusted to pH 2.50 and degassed for 30 minutes. The column was at ambient temperature (approximately 22°C) and the flow rate set at 4.0 ml/min (2000 psi). Sample volumes of 250 ul were injected per analysis and the detector set at 254 nm.

Sample Collection and Preparation

Informed consent was obtained from a patient who exhibited proven infection with tubercle bacillus but in otherwise good clinical status. The patient was not taking other medications at the time of the study. A heparin lock was placed in a superfical arm vein for a 12 hour sampling period. INH at a dose of 300 mg (as tablets) was given daily and 6 ml of blood withdrawn at 0, 1, 2, 4, 6, and 12 hours after dosing. Plasma was obtained by centrifugation for 10 minutes at 500 x g and 2 ml of plasma from each sampling period was diluted with 100 ul water containing 3 ug of internal standard. After addition of 2 g of ammonium sulfate and 40 ml of water saturated n-butanol-chloroform (30/70%) the samples were shaken for 10 minutes and centrifuged for 10 minutes at 500 x g. The organic layer (35 ml) was transferred into a clean tube (50 ml) and 1 ml of 0.50 N sulfuric acid was added and shaken for 10 minutes (the drug, metabolite, and internal standard were extracted into this aqueous acidic layer). The solution was then centrifuged for 10 minutes at 500 x g and 250 ul of the upper aqueous layer was injected into the HPLC.

The recovery for both compounds was 90%.

Calculations

Peak height ratios were calculated by dividing the peak height of INH or ACINH by the internal standard

peak height. Calibration curves were constructed by plotting peak height ratios (INH/internal standard or ACINH/internal standard) versus the concentration of the INH or ACINH standard (ng/ml plasma). The concentration of the drug or its metabolite was calculated from the peak height ratio using the slope and intercept obtained by a linear regression analysis of the calibration curve.

RESULTS AND DISCUSSION

Figure 1 shows a chromatogram of INH, ACINH, and internal standard isolated from human plasma 4 hours following oral dosing. The retention times for ACINH,

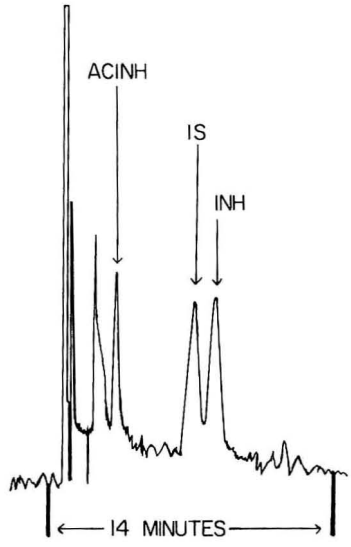

Figure 1. HPLC chromatogram of ACINH, internal standard and INH with retention times of 205, 535 and 640 seconds, respectively. Extracted from human plasma 4 hours following oral dosing with INH.

internal standard, and INH are 205, 535, and 640 seconds, respectively. Positive identification was achieved by peak superimposition, i.e., by addition of ACINH and INH standards (500 ng) to previously extracted patient samples and observing increased peak height at the corresponding retention times.

The limit of detection (2/1 signal/noise) of this procedure for INH and ACINH is 0.10 ug/ml plasma. Repetitive injections of standards gave good reproducibility of retention times (standard deviation $\pm$ 1.5%) and peak heights (standard deviation $\pm$ 2.2%). Standard curves were linear in the range of 0.10 to 10 ug for INH and ACINH and the day to day reproducibility varied less than 2.7% (standard deviation).

The standard solutions for the three compounds were stable at least one week when stored at 4°C. Samples extracted from plasma decompose within 24 hours and refrigeration at 4°C or -15°C does not slow this process; therefore, all samples were analyzed within 4 hours following plasma extraction.

In Table 1 are given the levels (ug/ml plasma) of INH and ACINH after an oral dose of 300 mg INH. The drug peaks 1 to 2 hours after administration and the metabolite, in this patient, peaks within 3 to 4 hours. INH is still detectable 12 hours after dosing from a 2 ml plasma sample taken at the specified time.

TABLE 1

Levels of Isoniazid (INH) and Acetyl-isoniazid (ACINH) in Human Plasma

Time* (Hours)	INH**	ACINH**
0	----	----
1	1.71 ± 0.51	0.30 ± 0.12
2	2.92 ± 0.72	1.41 ± 0.40
4	1.50 ± 0.50	2.09 ± 0.68
6	0.97 ± 0.43	0.61 ± 0.34
9	0.52 ± 0.33	----
12	0.25 ± 0.11	----

* Time after an oral dose of 300 mg INH.

** Levels expressed as ug drug per ml plasma (average of three determinations) ± standard deviation.

In conclusion, the HPLC method described is sufficiently specific and sensitive for determination of INH and ACINH in human plasma following oral dosing of the drug.

ACKNOWLEDGMENTS

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REFERENCES

1. DHEW Publication No. (CDC) 81-8249, 1979 Tuberculosis Statistics: States and Cities, USDHEW, CDC, TB Control Division, Atlanta, GA (1979).

2. Robinson, J. M. and Sullivan, F. M., Pharmacol. Rev., 15, 169 (1963).

3. Hearse, D. J. and Weber, W. W., Biochem. J., 132, 519 (1973).

4. Ellard, G. A., Clin. Pharmacol. Therap., 19, 610 (1976).

5. Weber, W. W. and Hein, D. H., Clin. Pharmacol., 4 401 (1979).

6. Stewart, J. T., Honigberg, I. L., Brant, J. P., Murry, W. A., Webb, J. L., and Smith, J. B., J. Pharm. Sci., 65, 1536 (1976).

7. Bailey, L. C. and Abdou, H., J. Pharm. Sci., 66 564 (1977).

8. Saxena, S. J., Stewart, J. T., Honigberg, I. L., Washington, J. G., and Keene, G. R., J. Pharm. Sci., 66, 813 (1977).

9. Mitchell, J. R., Thorgiersson, U. P., Black, M., Timbell, J. A., Snodgrass, J. A., Porter, W. R., Potter, W. Z., Jollow, D. J., and Keisner, H. R., Clin. Pharmacol. Therap., 18, 70 (1975).

10. Kerr, V. N., Ott, D. G., and Hayes, F. N., J. Am. Chem. Soc., 82, 1885 (1960).

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A RAPID ISOLATION OF HUMAN CHORIONIC GONADOTROPIN AND ITS SUBUNITS BY REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A rapid isolation of human chorionic gonadotropin and its subunits from a commercially available concentrate of human urine has been achieved using reversed-phase high performance liquid chromatography. With μBondapak C_{18} columns and a gradient employing aqueous trifluoroacetic acid as one solvent and dilute trifluoroacetic acid in acetonitrile as the other, complete separation can be accomplished in one day whereas standard column chromatographic procedures take about two weeks. Specific radioimmunoassays, polyacrylamide gel electrophoresis, and amino acid analyses were used to identify and characterize chromatographic peaks.

INTRODUCTION

Human chorionic gonadotropin (hCG) (1) is a trophic hormone composed of two dissimilar noncovalently attached glycopeptide

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subunits, termed α and β. The amino acid sequences (2-4) as well as the carbohydrate sequences of both subunits have been determined (5-6). The α subunit contains 90-92 amino acid residues while the β subunit contains 145. Each subunit is extensively crosslinked by intramolecular disulfide bonds and contains about 30% carbohydrate which causes hCG to elute from gel filtration columns earlier than expected and facilitates its aggregation.

The hormone hCG is normally synthesized by trophoblastic cells of the placenta. Levels of hCG are usually highest in the urine of women in their first trimester of pregnancy. The hormone and/or its subunits are also synthesized by malignant trophoblastic cells as well as by other human cancer cells, including carcinomas, lymphomas and melanomas (7, 8).

A previous paper from this laboratory (9) described a lengthy procedure for isolating hCG and its subunits from commercially available crude material. This procedure included dialysis, ion-exchange chromatography and gel filtration and took about two weeks. Below we describe the use of reversed-phase high performance liquid chromatography (HPLC) to rapidly obtain highly purified subunits of hCG.

EXPERIMENTAL

Chemicals

HPLC grade water was obtained by passing our standard laboratory grade water through a Critical Applications Adsorption Column (Hydro Services and Supplies, Inc., Durham, NC) which contains highly purified, activated charcoal and has a 0.2 μm polycarbonate filter (Nucleopore Corp., Pleasanton, CA) attached to its outlet. Trifluoroacetic acid (TFA) and pentafluoropropionic acid (PFPA), both Sequanal Grade, obtained from Pierce Chemical Co. (Rockford, IL), and acetonitrile purchased

from Burdick and Jackson Laboratories (Muskegon, MI), were used without further purification. Crude hCG isolated from the urine of pregnant women, was purchased from Organon, Inc. (Oss, the Netherlands). (Met) and (Leu) Enkephalins were obtained from Calbiochem-Behring Corp. (La Jolla, CA). Ribonuclease A and lysozyme were purchased from Worthington Biochemical Co. (Freehold, NJ) and insulin was obtained from Sigma Chemical Co. (St. Louis, MO).

Apparatus

HPLC was performed with an Altex Model 312 MP (Altex Instruments, Berkeley, CA) chromatographic system which consisted of an Altex Model 420 microprocessor controller/programmer, two Altex Model 110A pumps, an Altex gradient mixing chamber, and a Rheodyne (Berkley, CA) Model 7120 syringe-loading sample injection valve equipped with a 3.5 ml sample loop. Separations were performed with 0.39 x 30 cm and 0.78 x 30 cm μBondapak C_{18} columns (Waters Associates, Milford, MA). The particle size of the column packings was 10 microns. Runs were monitored at 206 nm with a Model 970A variable wavelength detector from Tracor Instruments (Austin, TX) and an Altex Model 385 recorder. A back pressure of 150 lb/in^2 was placed on the outlet tubing with a pinch clamp to prevent solvent outgassing and maintain a stable baseline (10, 11).

Solvents

A stock solution obtained by mixing 10 ml of TFA with 100 ml of purified water was used to prepare the chromatographic solvents. Solvent A was prepared by diluting 5 ml of the stock solution to 1 liter with water and solvent B was prepared by diluting 4.25 ml of the stock solution to 1 liter with acetonitrile.

Procedure

Our abbreviated program consisted of running isocratically at 0% B for 5 min and then employing a linear gradient from 0% B to 60% B in 60 min. An extended program consisted of running isocratically at 0% B for 4 min and then using a linear step gradient from 0% B to 20% B in 15 min, then 20% B to 45% B in 75 min, and finally from 45% B to 60% B in 15 min. Thus, the overall time for this program was 109 min. With both programs a flow rate of 1.0 ml/min was used. Samples to be rerun on HPLC were first placed under a stream of purified nitrogen which evaporated the acetonitrile. Aliquots were removed for identification and characterization as described below and the remaining sample was re-injected into the HPLC system.

Other methods

The hormone hCG and its subunits were identified and peaks were characterized by means of specific RIAs, SDS-polyacrylamide gel electrophoresis and amino acid analysis as previously described (9).

RESULTS AND DISCUSSION

Standards

In earlier work (9) hCG was dissociated into its subunits by incubation in 1 M propionic acid, a procedure which did not apparently disturb immunological specificity or damage the polypeptide chains. Thus, the recently reported solvent systems for reversed-phase HPLC which contain dilute aqueous TFA as the more polar solvent (10-12) seemed ideally suited for the isolation of hCG subunits. To evaluate the effectiveness of our HPLC system, a mixture of model peptides and proteins employed by others (11) was analyzed with the abbreviated program (EXPERIMENTAL) and excellent results were obtained (Figure 1).

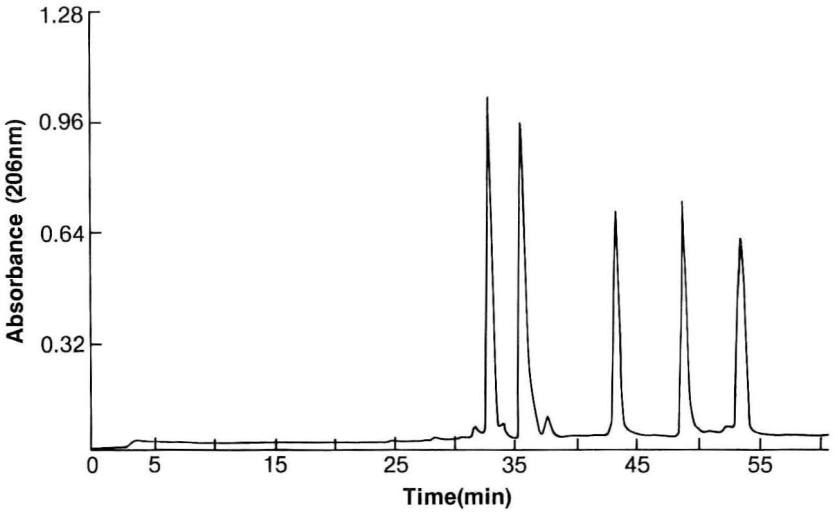

FIGURE 1: Reversed-phase HPLC chromatogram of model peptides and proteins using the abbreviated program. The order of elution is: (Met) Enkephalin, (Leu) Enkephalin, ribonuclease A, insulin, and lysozyme. Sample size: 20 µg of each component.

To determine the location of hCG and its subunits on HPLC, we used, as a standard, a mixture of hCG and β subunit obtained by chromatographing highly purified, acid-dissociated hCG on Sephadex G-100 as previously described. (Figure 3S of reference 9 illustrates that such a mixture results from the incomplete separation of hCG from the β subunit). This mixture, having been extensively lyophilized, was incubated at 37°C in solvent A for 1 hr. Immediately after incubation the mixture was analyzed by HPLC using the abbreviated program (EXPERIMENTAL). Resolution was adequate enough to distinguish peaks; these were subsequently identified by specific RIAs for the α and β subunits and by amino acid analyses. Resolution was further enhanced by using the extended program (EXPERIMENTAL).

HPLC analysis of crude hCG

A 2 mg batch of crude hCG was incubated for 1 hr in TFA and analyzed by HPLC using the extended program (EXPERIMENTAL). The

contents of each of the peaks noted in Figure 2 were subsequently rerun using the extended program (EXPERIMENTAL). Again, specific RIAs and amino acid analyses clearly identified the peaks but SDS-polyacrylamide gel electrophoresis revealed that each subunit was contaminated to about 5% with the other subunit. Changing the conditions for dissociating the crude material or using PFPA in place of TFA did little to improve the resolution of the subunits. An alternate approach for isolating the subunits consisted of trying to dissociate a partially purified sample of hCG for subsequent analysis by HPLC. This partially purified hCG was obtained by dissolving 2 mg of crude hCG in solvent A and immediately running the mixture on HPLC. The profile shown in Figure 3 indicates that omitting the incubation step results in the appearance of a new peak of aggregated hCG (underlined in the figure). The contents of this peak were freed of acetonitrile as described above (EXPERIMENTAL) and the mixture was incubated at 37°C for 1 hr. Immediately following incubation the mixture was run on HPLC using the extended program (EXPERIMENTAL) and the results shown in Figure 4 were obtained. SDS-polyacrylamide gel electrophoresis of each peak indicated no cross-contamination of the subunits (Figure 5), and specific RIAs of each subunit allowed their recoveries to be estimated. From 2 mg of crude hCG, 55 µg of α subunit and 90 µg of β subunit were obtained, which on a percentage basis indicates an improvement over standard liquid chromatographic procedures (9, 13, 14). This result is not surprising since our HPLC approach entails less sample manipulation with less opportunity for sample loss.

The profiles obtained in Figures 1-4 were quite reproducible when samples were repeatedly run on the same µBondapak C_{18} column or on other µBondapak C_{18} columns. The ability of reversed-phase columns containing other bonded packings or C_{18}

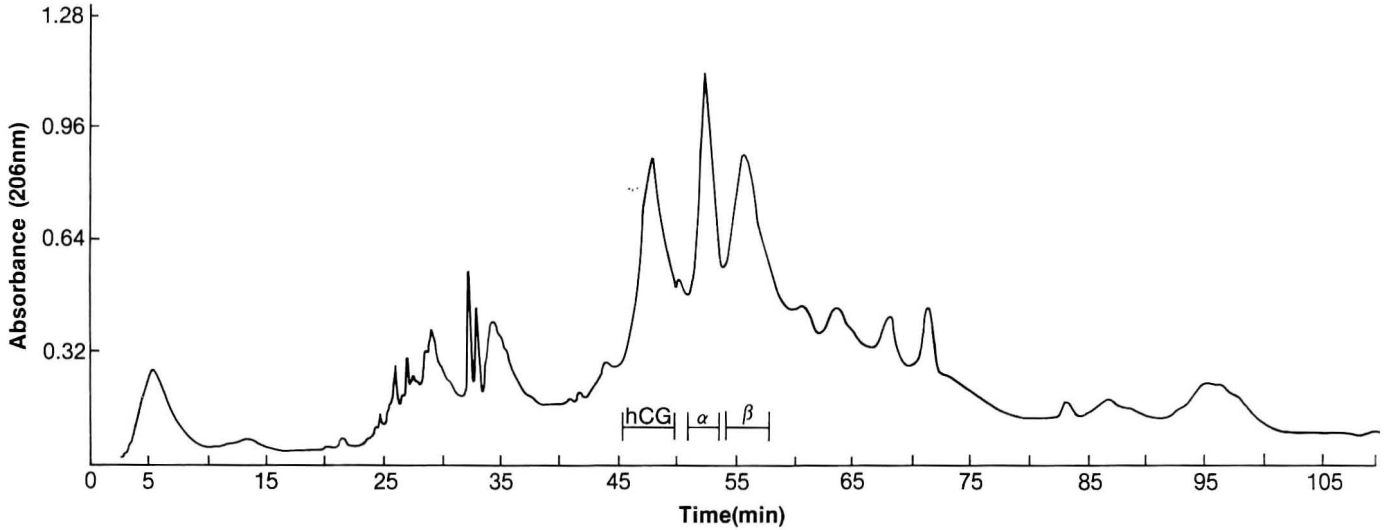

FIGURE 2: Reversed-phase HPLC chromatogram of crude hCG (2 mg) preincubated in solvent A for 1 hour at 37°C and run using the extended program.

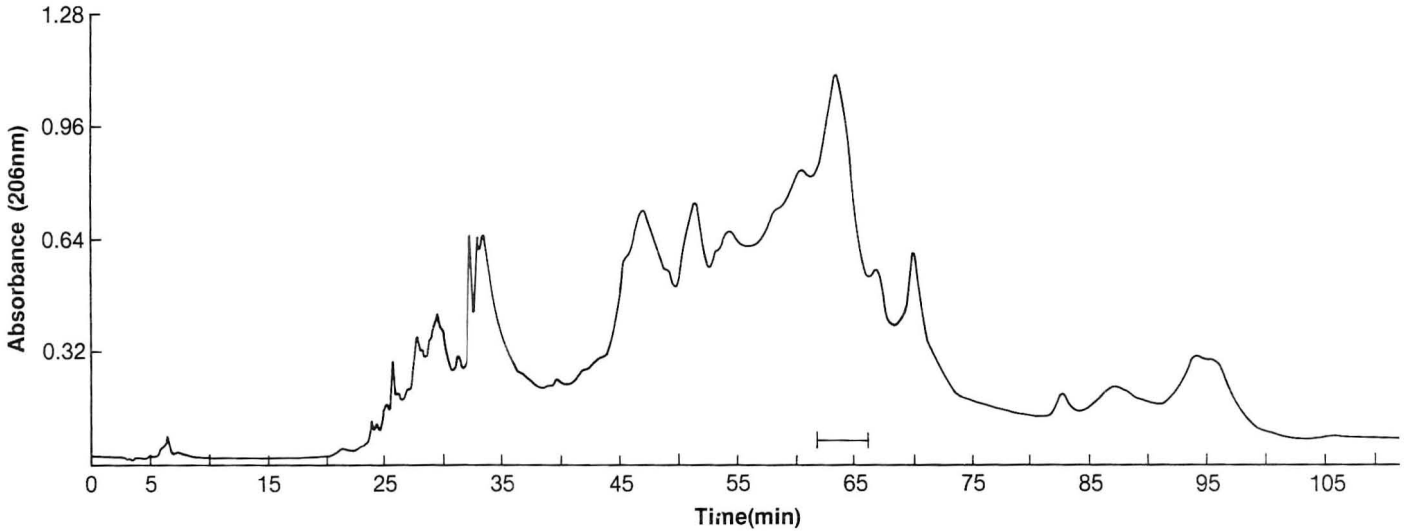

FIGURE 3: Reversed-phase HPLC chromatogram of crude hCG (2 mg) run without preincubation using the extended program. The peak underlined in the figure was subsequently incubated and rerun as described in the text.

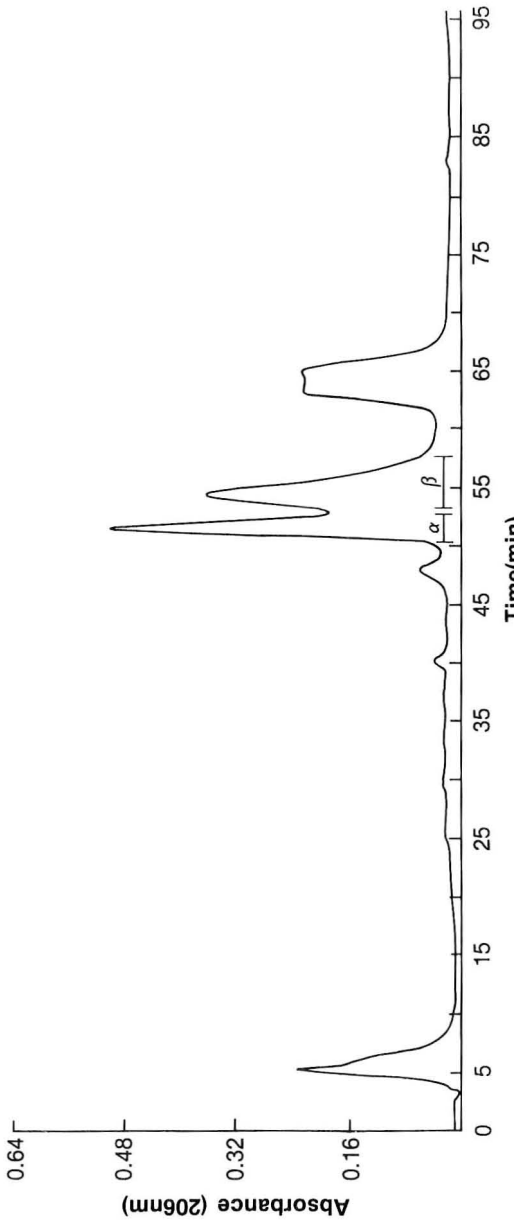

FIGURE 4: Reversed-phase HPLC chromatogram of the peak underlined in Figure 3. Sample was preincubated in Solvent A for 1 hour at 37°C and run using the extended program.

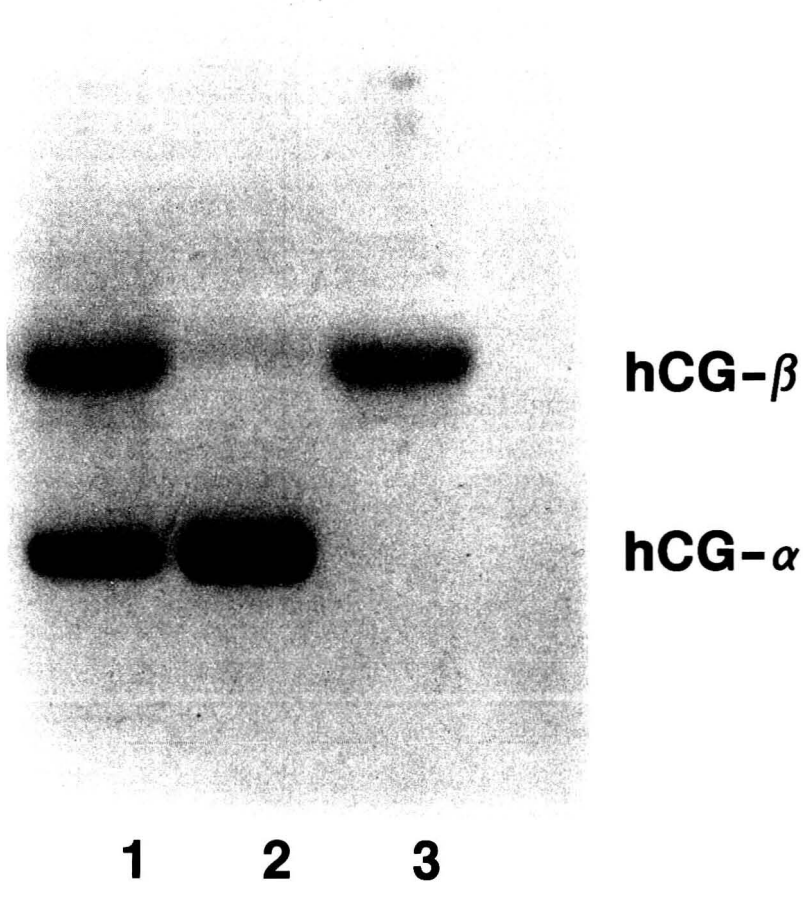

FIGURE 5: SDS-polyacrylamide gel electrophoresis of samples shown in Figures 3 and 4. Electrophoresis was performed with a 3% stacking gel and 13.5% running gel. Staining was with Coomassie blue. Lane 1: aliquot from peak underlined in Figure 3. Lanes 2 and 3: α and β subunits obtained as shown in Figure 4.

columns prepared by other manufacturers to separate hCG and its subunits remains to be determined.

Although this report has described the analysis of only 2 mg of crude hCG on 0.39 x 30 cm columns, similar or even better results have been obtained by running 5 mg of crude hCG on a 0.78 x 30 cm column of μBondapak C_{18}. The only change made in our programs in converting to the larger column consisted of doubling the flow rate to 2 ml/min. With the larger column the profile obtained for the sample without incubation indicates some subunit dissociation (probably due to the slower flow rate per column cross-sectional area). Peaks corresponding to free α and free β were observed, with α shown to be essentially pure and β slightly contaminated with α (Figure 6). This free α subunit can be combined with the α obtained following incubation and HPLC of the aggregated hCG. The free β subunit fraction can be mixed with the aggregate prior to acid dissociation and HPLC thereby giving even better yields than were obtained with the smaller column.

In addition to using HPLC to obtain subunits of hCG originating in human urine, we have also employed this technique to isolate the subunits from the media of JAR choriocarcinoma cells pulsed with [^{35}S]methionine (9). Preliminary results suggest that HPLC may be as effective as immuno-precipitation techniques in studying the synthesis, processing, and secretion of these subunits by cultured tumor cells (R. W. Ruddon, R. Hartle, M. B. Spear and G. J. Putterman, personal communication).

In conclusion, our results have demonstrated the effectiveness of reversed-phase HPLC in isolating small batches of hCG and its subunits. These products were characterized by comparing their retention times with products obtained by more "classical" approaches (9), by specific radioimmunoassays with well characterized antisera (9), by SDS-polyacrylamide gel electrophoresis

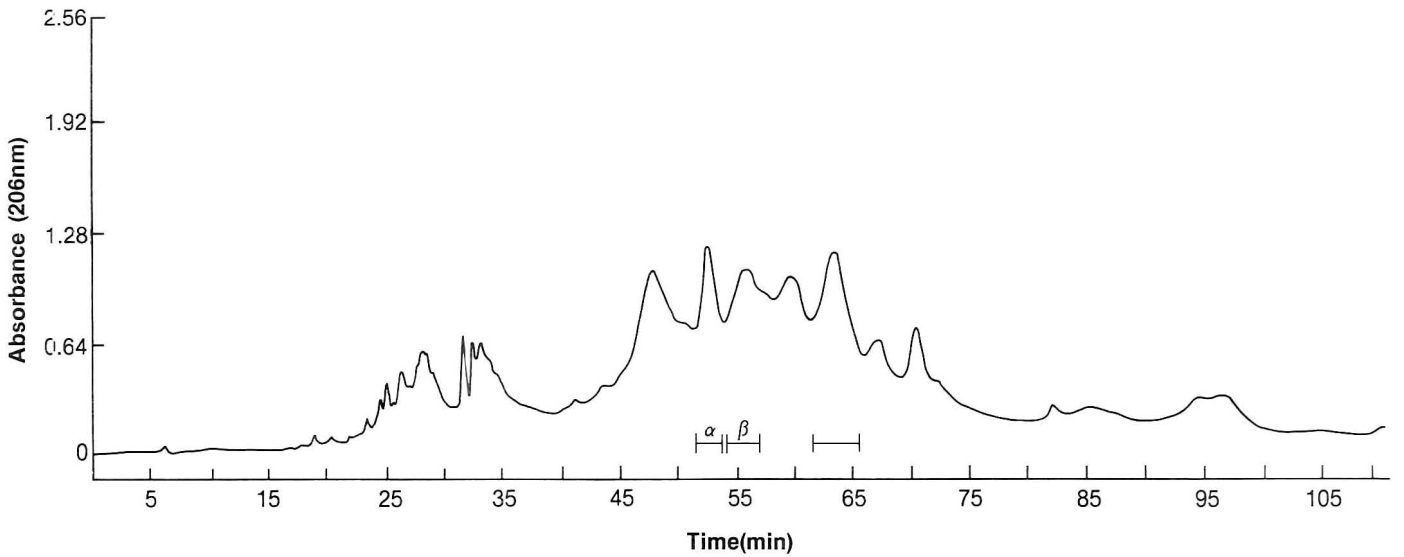

FIGURE 6: Reversed-phase HPLC chromatogram of crude hCG (5 mg) using a 0.78 x 30 cm column and extended program modified as described in the text.

and by amino acid analyses. Although no attempt has been made to subject the isolated hCG or hCG prepared by combining the isolated subunits to bioassay, the results described above regarding the isolation of the hormone and its subunits and the success observed in effectively increasing the sample load by means of a somewhat larger column encourage the use of preparative HPLC apparatus and extensive characterization of the biological properties of the isolated products. Meanwhile, analytical HPLC appears to be a rapid and convenient method of obtaining fresh hCG and its subunits to be used in immunological assays of these medically important molecules.

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REFERENCES

1. Abbreviations: hCG, human chorionic gonadotropin; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; RIA, radioimmunoassay; SDS, sodium dodecyl sulfate; PFPA, pentafluoropropionic acid.

2. Morgan, F. J., Birken, S. and Canfield, R. E., The Amino Acid Sequence of Human Chorionic Gonadotropin: The α Subunit and β Subunit, J. Biol. Chem. 250, 5247, 1975.

3. Birken, S. and Canfield, R. E., Isolation and Amino Acid Sequence of COOH-terminal Fragments from the β Subunit of Human Chorionic Gonadotropin, J. Biol. Chem. 252, 5386, 1977.

4. Keutmann, H. T. and Williams, R. M., Human Chorionic Gonadotropin. Amino Acid Sequence of the Hormone-Specific COOH-Terminal Region, J. Biol. Chem. 252, 5393, 1977.

5. Kessler, M. J., Reddy, M. S., Shah, R. H. and Bahl, O. P., Structures of N-Glycosidic Carbohydrate Units of Human Chorionic Gonadotropin, J. Biol. Chem. 254, 7901, 1979.

6. Kessler, M. J., Mise, T., Ghai, R. D. and Bahl, O. P., Structure and Location of the O-Glycosidic Carbohydrate Units of Human Chorionic Gonadotropin, J. Biol. Chem. 254, 7909, 1979.

7. Vaitukaitis, J. L., Ross, G. T., Braunstein, G. D. and Rayford, P. L., Gonadotropins and Their Subunits: Basic and Clinical Studies, Recent Prog. Horm. Res. 32, 289, 1976.

8. Ruddon, R. W., Anderson, C., Meade, K. S., Aldenderfer, P. H., and Neuwald, P. D., Content of Gonadotropins in Cultured Human Malignant Cells and Effects of Sodium Butyrate Treatment on Gonadotropin Secretion by HeLa Cells, Cancer Res. 39, 3885, 1979.

9. Ruddon, R. W., Hanson, C. A., Bryan, A. H., Putterman, G. J., White, E. L., Perini, F., Meade, K. S., and Aldenderfer, P. H., Synthesis and Secretion of Human Chorionic Gonadotropin Subunits by Cultured Human Malignant Cells, J. Biol. Chem. 255, 1000, 1980.

10. Mahoney, W. C. and Hermodson, M. A., Separation of Large Denatured Peptides by Reverse-Phase High Performance Liquid Chromatography. Trifluoroacetic Acid as a Peptide Solvent. J. Biol. Chem. 255, 11199, 1980.

11. Henderson, L. E., Sowder, R. and Oroszlan S., in Proceedings of Int. Conf. on Chemical Synthesis and Sequencing of Peptides and Proteins, Liu, D. T., Schechter, A. N., and Heinrickson, R., eds., Elsevier, Amsterdam, in press, 1981.

12. Bennett, H. P. J., Browne, C. A., and Goltzman, D., in Proceedings 6th American Peptide Symposium, Gross, E. and Meienhofer, J., eds., Pierce Chemical Company, Rockford, IL, 1979, p. 121.

13. Canfield, R. E. and Morgan, F. J., in Methods in Investigative Endocrinology, Berson, S. A. and Yalow, R. S., eds., North Holland Press, Amsterdam, 1973, Vol. 2B, p. 727.

14. Morgan, F. J., Canfield, R. E., Vaitukaitis, J. L. and Ross, G. T., in Methods in Investigative Endocrinology, Berson, S. A. and Yalow, R. S., eds., North Holland Press, Amsterdam, 1973, Vol. 2B, p. 733.

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SEMI-PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF POTATO GLYCOALKALOIDS

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ABSTRACT

A semi-preparative high-performance liquid chromatographic method has been developed to separate crude mixtures of the potato glycoalkaloids α-chaconine, α-solanine, commersonine and demissine. Milligram quantities of each substance can be obtained within an 8 hour period. A Zorbax semi-preparative NH_2 column and a solvent system of tetrahydrofuran-water-acetonitrile (55:20:25) were employed for the separation. The flow rate was 1.0 ml/min. Glycoalkaloid separations were monitored using both refractive index and ultraviolet detection (215 nm). Further analyses of these glycoalkaloids were done using analytical high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) to check compound purity and identity.

INTRODUCTION

Potato glycoalkaloids are steroidal alkaloids that are comprised of either a spirosolane or solanidine type aglycone to

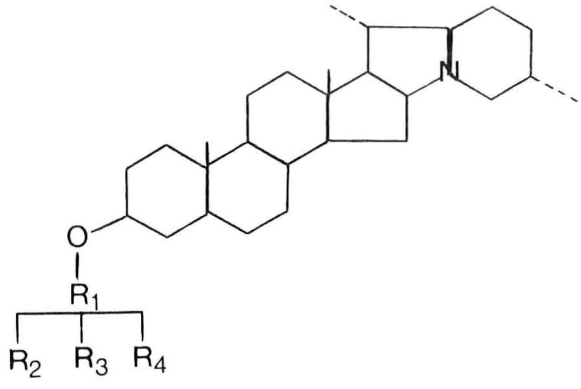
which one to four carbohydrate molecules are attached. Widespread interest has developed in these natural occurring substances because of their toxicity and insecticidal activity. Several cases of glycoalkaloid poisoning have been reported in animals (1) and man (1-4) and, in a few instances, death has occurred from ingestion of large quantities of potato glycoalkaloids (5). Futhermore there is some evidence implicating these compounds as teratogens (6-8). Their possible use as an insecticide, because of their feeding deterrent activity toward the Colorado potato beetle (9-12) and potato leafhopper (13,14), has also created much interest.

Presently, the methods used to purify milligram quantities of potato glycoalkaloids consist of preparative TLC (15) and open column chromatography techniques (16). Both procedures are very time consuming and the glycoalkaloids obtained are not analytically pure. In this paper we describe a method to separate four potato glycoalkaloids (Figure 1) -- commersonine (A), demissine (B), α-chaconine (C) and α-solanine (D) -- using semi-preparative HPLC. This technique is rapid and yields milligram quantities of analytically pure glycoalkaloids.

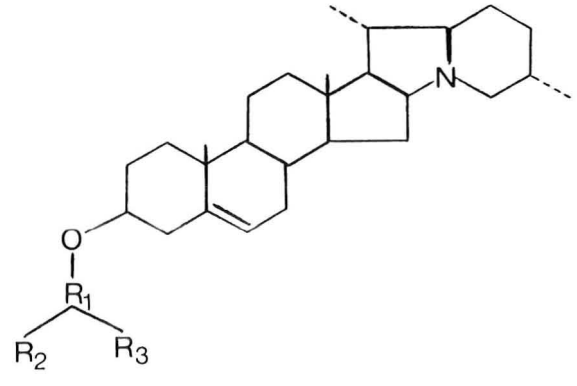
EXPERIMENTAL

Materials

Solvents used for the semi-preparative and analytical high-performance liquid chromatographic separations of glycoalkaloids were HPLC grade (Fisher Scientific Co. Fair Lawn, NJ) while the



	R_1	R_2	R_3	R_4
A	D-GAL	D-GLU	D-GLU	D-GLU
B	D-GAL	D-GLU	D-GLU	D-XYL



	R_1	R_2	R_3
C	D-GLU	L-RHAM	L-RHAM
D	D-GAL	D-GLU	L-RHAM

Figure 1. Potato Glycoalkaloids: Commersonine (A), Demissine (B), α-Chaconine (C) and α-Solanine (D).

solvents employed in the mass extraction of the glycoalkaloids from potato blossoms and thin-layer chromatography were ACS grade (Fisher Scientific Co.).

Crude glycoalkaloid mixtures, used to obtain the individual compounds by semi-preparative HPLC, were isolated using the procedure of Bushway et al. (17). α-Chaconine and α-solanine were extracted from Katahdin potato blossoms and a mixture of commersonine and demissine were obtained from blossoms of Solanum demissum Lindl.

Potato glycoalkaloid standards were a gift from Eugene A. Talley, Eastern Regional Research Center, USDA, Philadelphia, PA and Steve L. Sinden, Beltsville Agricultural Research Center, USDA, Beltsville, MD.

The TLC plates employed in this study were HP-KF high-performance silica gel plates 10x10 cm, 200 μ thickness (Whatman Inc., Clifton, NJ).

Apparatus

The HPLC system consisted of a Waters Assoc. 600C A pump, a U6K injector, a differential refractometer R 401, a Schoeffel variable-wavelength UV detector (Westwood, NJ) and a Houston Instruments dual pen recorder (Austin, TX). The semi-preparative column (25 cm x 9.4 mm I.D.) was a Zorbax NH_2 (DuPont de Nemours and Co., Wilmington. DE). Operating conditions were: mobile phase, tetrahydrofuran-water-acetonitrile (55:20:25); flow rate,

1.0 ml/min; column temperature, ambient; wavelength, 215 nm; refractive index setting, 16X; attenuation, 0.4 a.u.f.s.; and chart speed, 0.4 in/min.

The analytical column (30 cm x 4 mm I.D.) was a Carbohydrate analysis column (Waters Assoc.). Operating conditions were: mobile phase, tetrahydrofuran-water-acetonitrile (53:17:30); flow rate, 1.5 ml/min; column temperature, ambient; wavelength, 215 nm; attenuation, 0.04 a.u.f.s.; and chart speed, 0.4 in/min.

Methods

Semi-preparative HPLC separations: Two glycoalkaloid solutions were prepared using the mixtures obtained from the mass extraction of the potato blossoms. One solution contained primarily α-chaconine and α-solanine at a total glycoalkaloid concentration of 8 mg/ml, while the other consisted mostly of commersonine and demissine at a total concentration of 1.5 mg/ml. Tetrahydrofuran-water-acetonitrile (50:30:20) was used to dissolve the crude mixtures of glycoalkaloids. Before injecting 500 µl of the mixtures, the solutions were filtered through a 0.45 µm Millipore organic filter (Waters Assoc.). The individual glycoalkaloids were collected and concentrated using rotary evaporation.

Analytical HPLC separations: Analytical HPLC separations were performed using the procedure of Bushway et al. (18).

TLC separations: The thin-layer method employed was that of McCollum and Sinden (19).

RESULTS AND DISCUSSION

The chromatograms of the semi-preparative separations of the potato glycoalkaloids are shown in Figures 2 (α-chaconine and α-solanine) and 3 (commersonine and demissine). Total elution time when all compounds are considered in the crude mixtures varies from 24 min. for the α-chaconine and α-solanine mixture to 30 min. for commersonine and demissine. If one includes only α-chaconine and α-solanine in the mixture than the elution time is 20 min. compared to 24 for only commmersonine and demissine (Figures

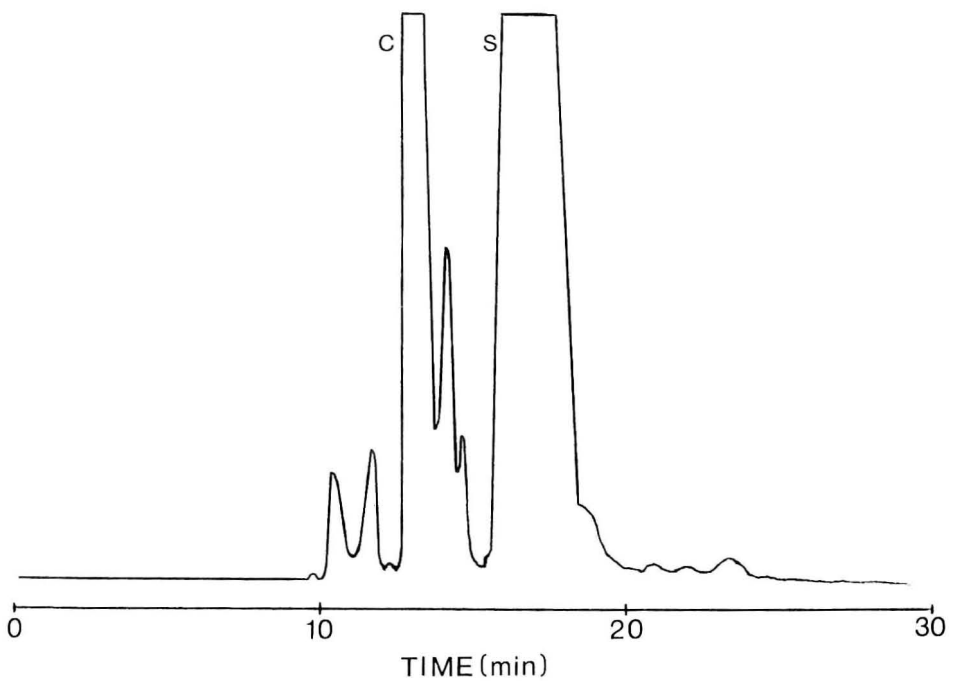


Figure 2. Semi-preparative HPLC Chromatogram of α-Chaconine (C) and α-Solanine (S). Obtained by Mass Extraction of Katahdin Blossoms.

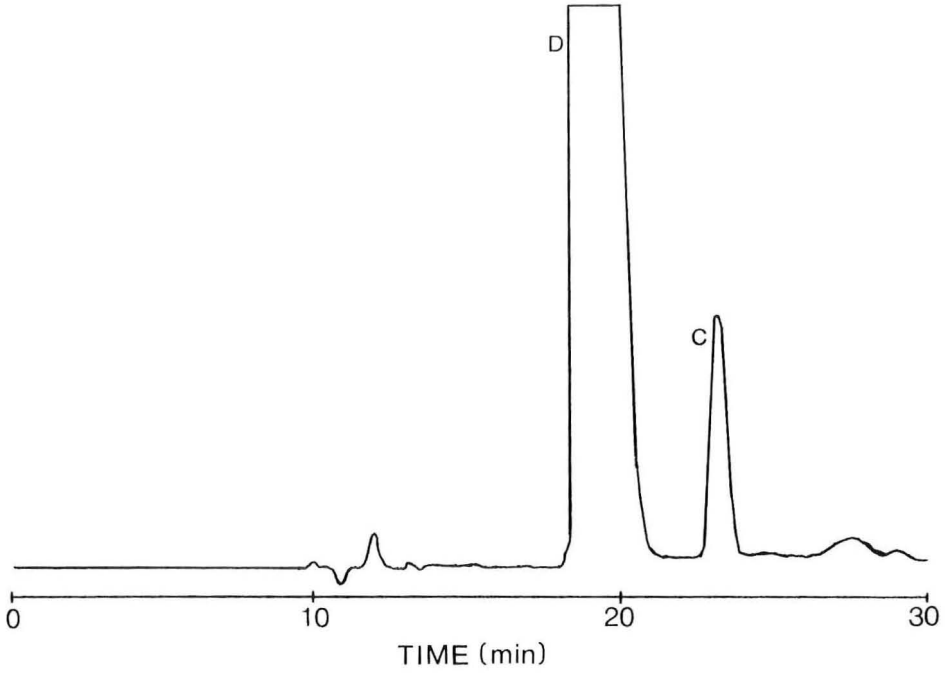


Figure 3. Semi-preparative HPLC Chromatogram of Demissine (D) and Commersonine (C). Obtained by Mass Extraction of Solanum demissum Blossoms.

2 and 3). By staggering injections, it is possible to collect 30 -60 mg each of α-chaconine and α-solanine in an 8 hour period. As for demissine and commersonine, one can obtain approximately 13 mg of demissine and 3 mg of commersonine. Of course the amount of each glycoalkaloid collected will depend upon the quantity of each in the starting crude mixtures. This can vary with the species of plant used and the number of crystallization steps since each glycoalkaloid has a different solubility product. As can be seen (Figures 2 and 3), there are other compounds in these mixtures es-

pecially in the α-chaconine and α-solanine fraction. We are presently trying to identify these substances which are most likely glycoalkaloids.

In order to ascertain the purity and to check the identity of each glycoalkaloid isolated, analytical HPLC and TLC were employed. The results are presented in Figure 4 for the analysis by TLC and Figures 5 and 6 for the evaluation of each by analytical HPLC. A trace amount of α-chaconine was shown to be present by TLC (Figure 4) in the semi-preparative fraction of α-solanine and vice versa (Figure 4). Also the commersonine fraction contained

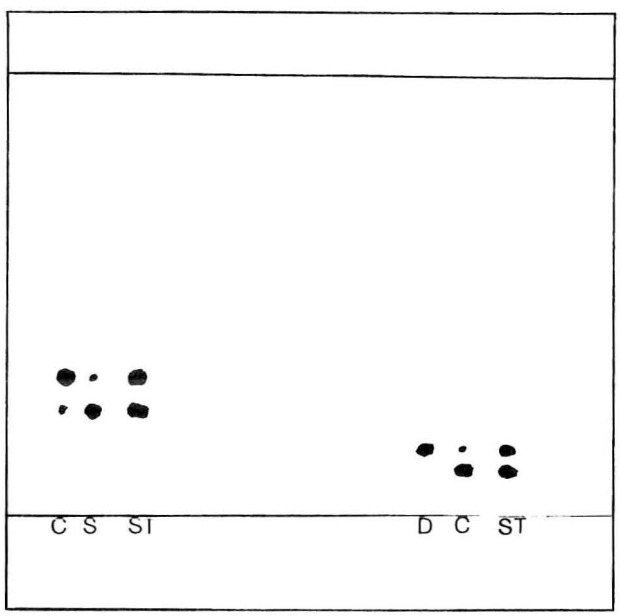


Figure 4. TLC Chromatogram of α-Chaconine (C), α-Solanine (S), Demissine (D) and Commersonine (C). Fractions Collected From the Semi-preparative Separations. ST, Standards.

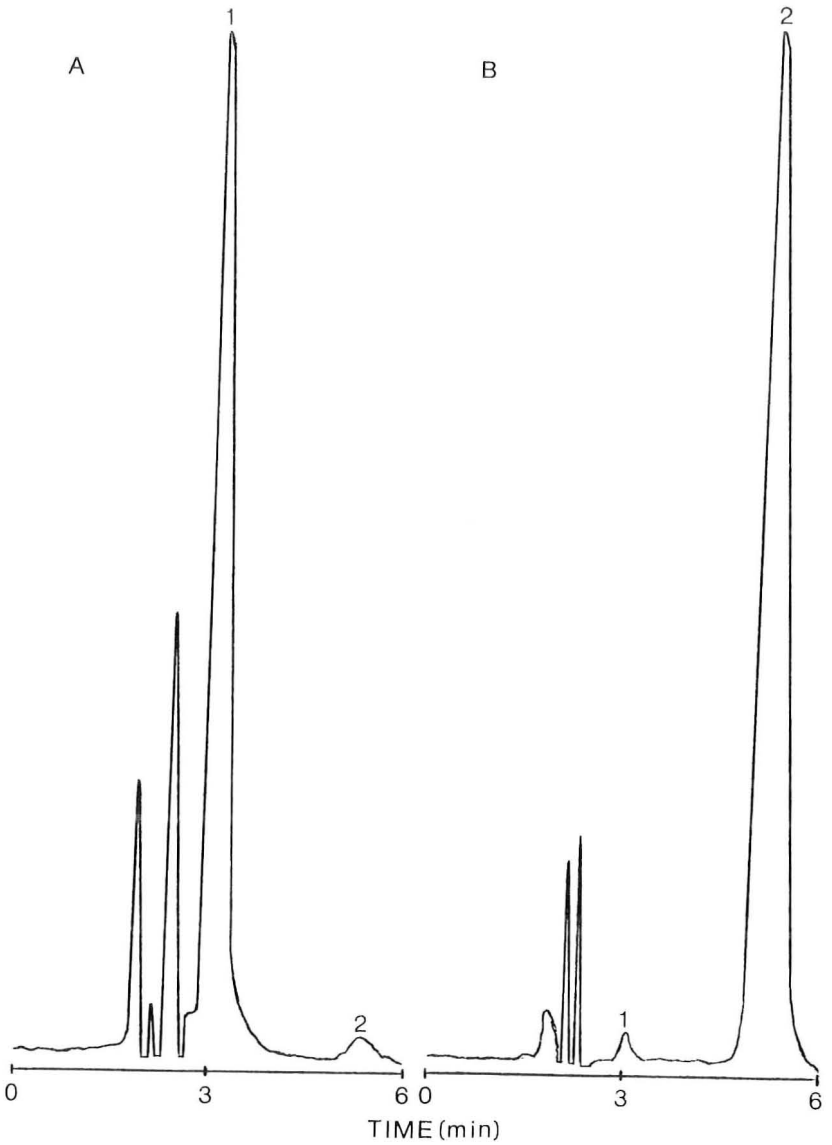


Figure 5. Analytical HPLC Chromatogram of α-Chaconine (A) and α-Solanine (B). Fractions Collected From the Semi-preparative Separations. Peak 1, α-Chaconine; Peak 2, α-Solanine.

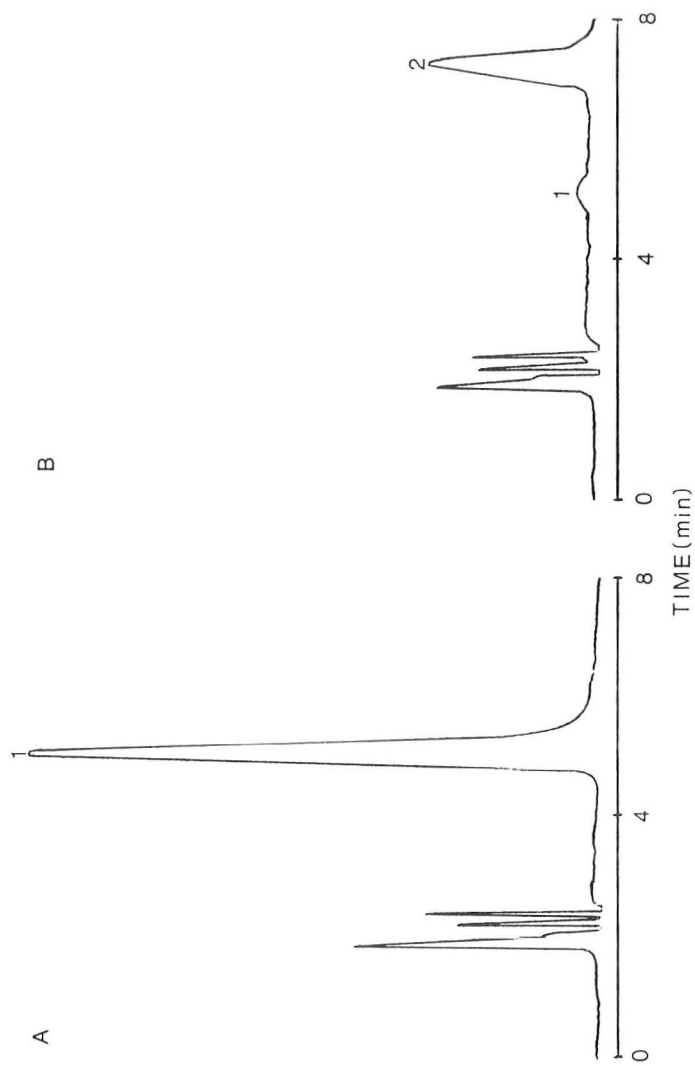


Figure 6. Analytical HPLC Chromatogram of Demissine (A) and Commersonine (B). Fractions Collected From the Semi-preparative Separations. Peak 1, Demissine; Peak 2, Commersonine.

a small amount of demissine (Figure 4). To quantify these contaminants, analytical HPLC was used (Figures 5 and 6). The trace amounts of α-solanine and α-chaconine observed in each fraction were 1.3% and 1.2%, respectively, while demissine was at a level of 1.0% in the commersonine fraction. Cross contamination can be alleviated by fine tuning the technique for collecting fractions.

This semi-preparative HPLC method offers a rapid means of obtaining potato glycoalkaloids that are analytically pure (98.7%-99%) and in sufficient quantity to do biochemical and toxicological research.

ACKNOWLEDGEMENT

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REFERENCES

(1) R. Jellema, E. T. Elema and Th. M. Malingre, J. Chromatogr., 210, 121, 1981.

(2) S. G. Willimott, Analyst, 58, 431, 1933.

(3) G. S. Wilson, Mon. Bull. Minist. Health Public Health Lab. Serv. Med. Res. Counc., 18, 207, 1959.

(4) M. McMillan and J. C. Thompson, Quart. J. Med., 48, 227, 1979.

(5) A. A. Hanson, Science, 61, 340, 1925.

(6) A. M. Mun, E. S. Barden, J. M. Wilson and J. M. Hogan, Teratology, 11, 73, 1975.

(7) R. F. Keeler, D. Brown, D. R. Douglas, G. F. Stallnecht and S. Young, Bull. Environ. Contam. Toxicol., 15, 522, 1976.

(8) R. F. Keeler, D. R. Douglas and G. F. Stallknecht, Am. Potato J., 52, 125, 1975.

(9) T. H. Hsiao, Entomol. Exp. Appl., 12, 777, 1969.

(10) T. H. Hsiao and G. Fraenkel, Ann. Entomol. Soc. Amer., 61, 485, 1968.

(11) S. L. Sinden, L. L. Sanford and S. F. Osman, Am. Potato J., 57, 331, 1980.

(12) B. Struckow and I. Low, Entomol. Exp. Appl., 4, 133, 1961.

(13) K. V. Raman, W. M. Tingey and P. Gregory, J. Econ. Entomol., 72, 337, 1979.

(14) W. M. Tingey, J. D. MacKenzie and P. Gregory, Am Potato J., 55, 577, 1978.

(15) S. O. Alozie, R. P. Sharma and D. K. Salunkhe, J. Food Safety, 1, 257, 1979.

(16) N. A. Paseshnichenko and A. R. Guseva, Biochem. (USSR), 21, 606, 1956.

(17) R. J. Bushway, A. M. Wilson and A. A. Bushway, Am. Potato J. , 57, 561, 1980.

(18) R. J. Bushway, E. S. Barden, A. W. Bushway and A. A. Bushway , J. Chromatogr., 178, 533, 1979.

(19) G. D. McCollum and S. L. Sinden, Am. Potato J., 56, 95, 1979.

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ENZYMATIC DETECTION OF URINARY STEROID-17β-GLUCURONIDES AFTER GEL FILTRATION

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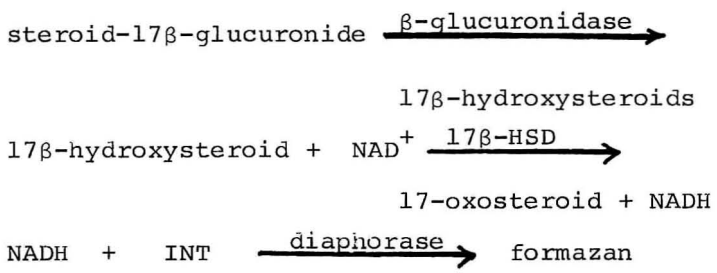
ABSTRACT

An enzymatic detection of urinary steroid-17β-glucuronides is described. The principle of the method is as follows; after gel filtration with Sephadex G-25 β-glucuronidase is added to each effluent fraction and incubated for 20 h at 37 °C. After hydrolysis, 3β,17β-hydroxysteroid dehydrogenase is added and incubated for 20 min at 37 °C. An absorbance at 500 nm is read aginst sample of first fraction effluent.

INTRODUCTION

An enzymatic detection of steroid-3α-glucuronide, steroid-3α-sulfate, and steroid-3β-sulfate in urine after gel filtration has been described previouly(1). In this paper, an enzymatic detection of steroid-17β-glucuronide after gel filtration is described. The principle of the method is as follows;



MATERIALS

All reagents were of analytical grade obtained from commercial sources and used without futher purification. 3β, 17β-hydroxysteroid dehydrogenase (3β, 17β-HSD) from P. testosteroni (EC 1.1.1.51), 2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium chloride (INT), and diaphorase (EC 1.6.99.2, from C. kluyveri) were purchased from Sigma chemical Co.

Sulfatase/β-glucuronidase from H. pomatia (EC 3.1.6.1) was prepared of 200 units as sulfatase/ml in 0.05 M acetate buffer (pH 5.0). β-Glucuronidase from E. coli (EC 3.2.1.31) was prepared of 500 Fishman units/ ml of phosphate buffer (0.1 M, pH 6.8).

Reagent for color development for 17β-hydroxysteroid was described previouly (2,3): 25 U of 3β,17 β-HSD, 30 mg of NAD^+,100 U of diaphorase and 15 mg of INT were dissolved in 0.2 M K_2HPO_4 (pH 9.0) of 100 ml.

METHODS

Sephadex Gel filtration (1,4)

Sephadex G-25 was swollen by heating a suspension of the particles in acetate buffer (0.05 M, pH 5.0) for 4 h at 90 °C under constant stirring. The fines were removed by several decantations and the slurry was poured directly into the column (1 x 20 cm) which was then washed for 3 h with acetate buffer.

Urine sample, a part of 24 h urine, was centrifuged for 3 min at 3000 rpm and supernatant of 1 to 2 ml was applied. One fraction of effluent contains 1.3 ml and 30 fractions were collected.

Procedure for detection of steroid-17β-glucuronide

To 0.6 ml of effluent of each fraction is added 0.1 ml of β-glucuronidase solution and incubated for 20 h

at 37 °C. After hydrolysis of steroid-17β-glucuronide, 1 ml of color development reagent for 17β-hydroxysteroid is added and incubated for 20 min at 37 °C. Absorbance at 500 nm was read against the sample of first fraction.

RESULTS AND DISCUSSION

A chromatogram for androsterone-glucuronide, dehydroepiandrosterone-sulfate, estrone-sulfate and estriol-16-glucuronide of standard compounds was shown in FIGURE 1. Hydrolysis with β-glucuronidase from E. coli and sulfatase/β-glucuronidase from H. pomatia was compared for steroid-17β-glucuronides detection using sample from patients of adrenal tumor. As shown in FIGURE 2 , only

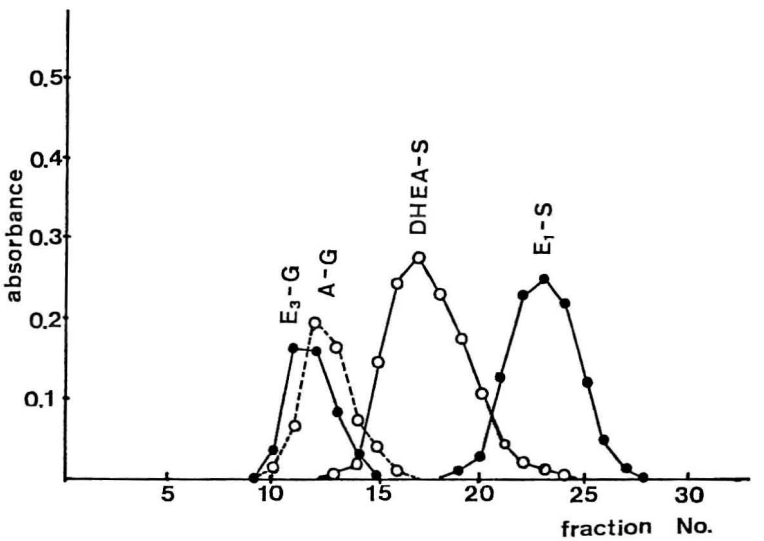


Fig. 1. Gel filtration of standard steroid conjugate.(1) Methods for each conjugated steroids were performed by enzymatic detection method described previously.

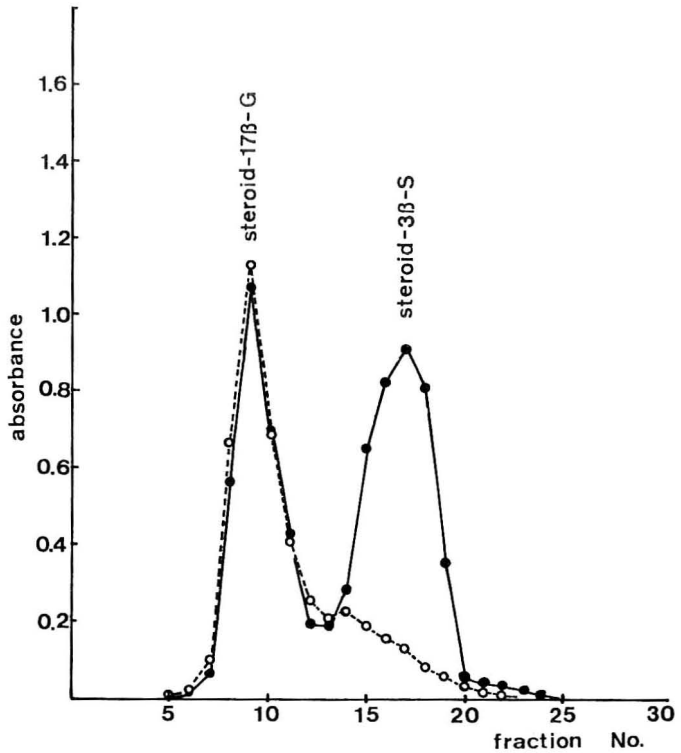


Fig. 2.Gel filtration of steroid-17β-glucuronide and steroid-3β-sulfate in urine of patient of adrenal tumor. o------o, hydrolysis with β-glucuronidase and ●——● , hydrolysis with sulfatase/β-glucuronidase.

17β-glucuronides was detected by hydrolysis with β-glucuronidase from E. coli and 3β-sulfate and 17β-glucuronide were detected by hydrolysis with sulfatase/β-glucuronidase from H. pomatia. From this chromatogram, most of the 17β-hydroxy group is conjugated with glucuronic acid. As a reference of other steroid conjugates excretion,

steroid-3α-glucuronide, steroid-3α-sulfate and steroid-3β-sulfate of the same sample which is detected by previously described method were shown in FIGURE 3. (1) The direct enzymatic detection method for steroid-17β-glucuronide can be detected for a sample of elevated excretion of 17β-hydroxysteroids, at least 5 mg/litter

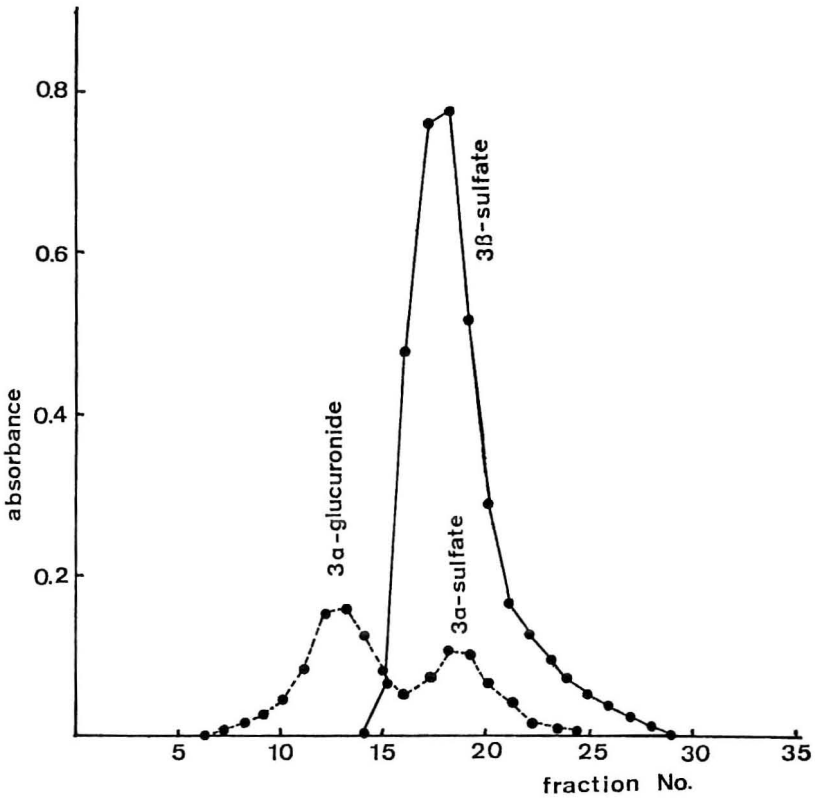


Fig. 3. Gel filtration of steroid-3α-glucuronide, steroid-3α-sulfate, and steroid-3β-sulfate in urine of patient of adrenal tumor. Each steroid conjugate was detected by previously described methods (1).

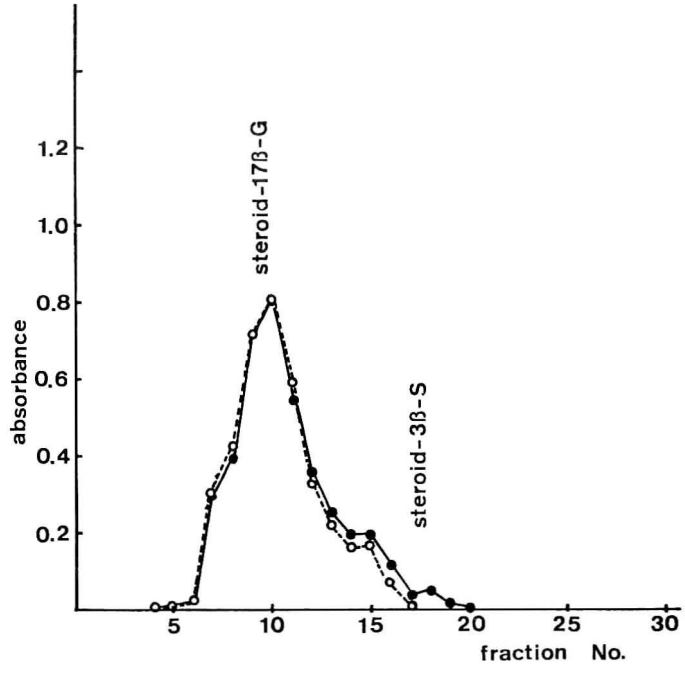


Fig. 4. Gel filtration of steroid-17β-glucuronide and steroid-3β-sulfate in urine of patient of breast tumor. o-----o, hydrolysis with β-glucuronidase and ●——●, hydrolysis with sulfatase/β-glucuronidase.

as total 17β-hydroxysteroids determined by the previously described method (2) such as breast tumor shown in FIGURE 4

An enzymatic detection of nonconjugated steroid has been described so far (2,3,4,5,6,7,8), but extraction by organic solvent is required in a procedure so that automated analysis can not be performed. This enzymic method for determination of steroid-17β-glucuronide has a possiblity for automated analysis using

high performance liquid chromatograhy and flow reaction systems.

REFERENCES

Non-standard abreviations; DHEA-S; dehydroepiandrosterone sulfate, A-G; androsterone glucuronide, E_1-S; estrone-sulfate, E_3-G; estriol-16-glucuronide:

1. Yamaguchi, Y. , J. Chromatogr. , submitted.
2. Yamaguchi, Y. and Hayashi, C. Anal. Letter, 14,433-438 (1981).
3. Yamaguchi, Y., J. Chromatogr., in press (1982).
4. Yamaguchi, Y., J. Chromatogr., in press (1982).
5. Yamaguchi, Y., J. Chromatogr., 163,253-258 (1979).
6. Yamaguchi, Y., Tateishi, K., and Hayashi, C., Clin. Chim. Acta, 66, 449-452 (1976).
7. Yamaguchi, Y., Hayashi, C., and Miyai, K., J. Chromatogr. 182, 430-434 (1980).
8. Yamaguchi, Y., Hayashi, C. and Miyai, K., J. Chromatogr. 224, 111-115 (1981).

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AN IMPROVED METHOD FOR THE PURIFICATION OF HEPATIC PROLIFERATION INHIBITOR BY ANION-EXCHANGE HPLC

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ABSTRACT

An improved method for the purification of hepatic proliferation inhibitor from rat liver by means of anion-exchange HPLC has been developed. The inhibitor can be purified on an anion exchange HPLC column by using a linear sodium phosphate gradient. The HPLC method allows repeated use of one column and is both rapid and reproducible. The hepatic proliferation inhibitor isolated by this method retains all of its biological activity and is homogeneous as revealed by reverse-phase HPLC.

INTRODUCTION

The search for endogenous factors which control the proliferation of mammalian cells has been the subject of extensive investigation for decades (1,2,3). However, the lack of purity of most of the preparations used has made it difficult to delineate their actual biological role. We have recently purified a rat liver protein which inhibits the proliferation of nonmalignant rat liver epithelial cells (4). Using DEAE-cellulose chromatography we have shown that this hepatic proliferation inhibitor (HPI) can be effectively purified. Since this procedure is both tedious and

time consuming, a simple and fast method was developed for the isolation of HPI using anion-exchange HPLC which offers a degree of resolution similar to that of electrophoretic methods (5).

MATERIAL AND METHODS

The starting material used in this study was prepared as described earlier (4,6). Prior to anion-exchange HPLC, the material was dialyzed first against 0.05% acetic acid for 12 hrs and then against 0.005M sodium phosphate, pH 6.0 for an additional 12 hrs. Anion-exchange HPLC separations were performed on a 250 x 4.1 mm ID SynChropak AX 300 column (SynChrom, Inc., Linden, IN). A Waters high-pressure liquid chromatography system which included a Model 450 variable wave length detector (Schoeffel Instrument Corp., Westwood, NJ); a M-660 solvent programmer; a U6K sample injector and two M-6000 solvent delivery pumps was used. For anion-exchange HPLC 0.5 ml samples consisting of 10 mg of protein in 0.005M sodium phosphate, pH 6.0 were injected and were eluted isocratically for 10 min in the same buffer at a flow rate of 0.5 ml per min. At this time, the samples were eluted with a 20 min linear gradient to 0.092 M sodium phosphate buffer, pH6.0, followed by isocratic elution using the same buffer. The uv absorbance of the column effluent was monitored at 260 nm. A reequilibration time for repeat injections of 20 min was used routinely. Reverse-phase HPLC separations were performed on a 250 x 4.6 mm ID Altex Ultrashere ODS column (Alltech Associates, Arlington Heights, IL) according to the method of Henderson et al.(7).

RESULTS

Fig. 1 shows the profile of the starting material chromatographed by anion-exchange HPLC. The technique resolved the sample into a number of components. The major portion of material eluted during the 10 min isocratic step. Application of the

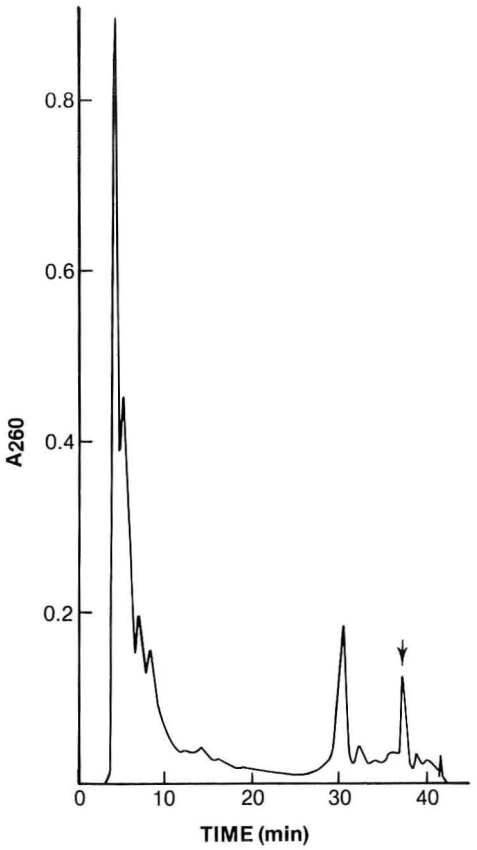

Fig. 1 Anion-exchange HPLC of starting material from rat liver. Sample volume was 0.5 ml consisting of 10 mg of protein in 0.005 M sodium phosphate, pH 6.0. The column was eluted isocratically for 10 min in the same buffer followed by elution with a linear 20 min gradient to 0.092 M sodium phosphate, pH 6.0 at a flow rate of 0.5 ml/min and isocratic elution with the final buffer. Arrow indicates the active inhibitory peak.

linear sodium phosphate gradient resolved the retained material into several peaks. The inhibitory activity resided in the peak which eluted 37 min after sample injection. When this peak fraction was collected and rechromatographed under identical conditions a single peak again eluted 37 min after sample application (Fig. 2). The active peak fraction was analyzed by reverse-

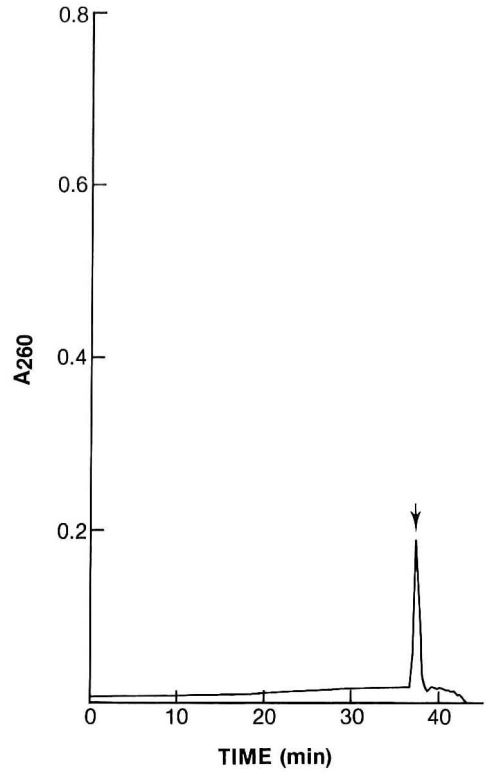

Fig. 2 Anion-exchange HPLC of HPI purified from the starting material by anion-exchange HPLC. Sample volume was 0.5 ml consisting of approximately 100 μg of protein. Chromatographic conditions identical to Figure 1.

phase HPLC and the purified inhibitor eluted from the column as a single sharp peak at 8% acetonitrile (Fig. 3).

DISCUSSION

In a previous report (4) we showed that HPI can be purified to apparent homogeneity by DEAE-cellulose chromatography. Although this method proved to be satisfactory, it was very time consuming, since the column had to be washed extensively before HPI could be effectively isolated by salt elution. In contrast, this is

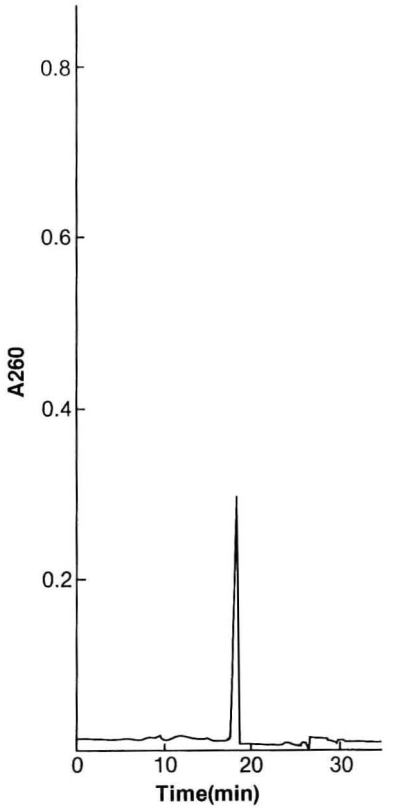

Fig. 3 Reverse-phase HPLC of HPI purified by anion-exchange HPLC. Sample volume was 0.5 ml consisting of approximately 100 μg of protein. Solvent A: 0.05% trifluoroacetic acid in water; solvent B: 0.05% trifluoroacetic acid in acetonitrile; Gradient 0% solvent B to 20% solvent B over 30 min at a flow rate of 1 ml per min.

not the case with anion-exchange HPLC where most of the contaminated material is removed from the column in less than 10 min (Fig. 1). The ability to use the HPLC column repeatedly and the short reequilibration time (20 min) are also obvious advantages. This new procedure, while saving considerable time, in no way altered the properties of the purified HPI, since its chromato-

graphic profile (Fig. 1) and biological activity (data not shown) were similar to that obtained with the HPI purified with DEAE-cellulose (4). Furthermore, the reverse-phase HPLC profile of the material isolated by anion-exchange HPLC (Fig. 3) is in good agreement with that previously published for the HPI isolated by DEAE-cellulose chromatography (4).

This demonstrates that the purification of HPI can be achieved readily with anion-exchange HPLC. This technicque is useful, not only as a preparative method where relatively large amounts (10 mg) of the starting material can be effectively separated on a column of the size and type described, but also can be modified for use as an analytical method.

ACKNOWLEDGEMENTS

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REFERENCES

1. Bullough, W.S., Cancer Res., 25, 1683, 1965.

2. Houch, J.C., and Hennings, H., FEBS Lett., 32, 1, 1973.

3. Nadal, C., Arch. Toxicol. Suppl., 2, 131, 1979.

4. McMahon, J.B., Farrelly, J.G., and Iype, P.T., Proc. Natl. Acad. Sci., 1981, in press.

5. Schlabach, T.D., Aipert, A.J., and Regnier, F.E., Clin. Chem., 24, 1351, 1978.

6. McMahon, J.B., and Iype, P.T., Cancer Res., 40, 1249, 1980.

7. Hederson, L.E., Sowder, R., and Oroszlan, S., Chemical Synthesis and sequencing of peptides and proteins, Liu, T-Y., Schechter, A.K., Heinrikson, R.L., and Condliffe, P.G., ed., Elsevier, Amsterdam, 1981, p. 251.

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PURIFICATION OF RADIO-IODINATED CHOLECYSTOKININ PEPTIDES BY REVERSE PHASE HPLC

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ABSTRACT

The purification of the iodinated tracers of CCK peptides using Sphadex G50 chromatography does not allow for a good separation between non-modified peptides and labelled peptides. We present, in this paper a simple and rapid purification method using reverse phase HPLC with a C-18 column for four of these tracers. The biological characteristics of the molecules obtained demonstrate their strong specific radioactivity and their high degree of purity.

INTRODUCTION

The use of radio-iodinated peptides as tracers in radioimmunoassay (RIA) and receptor binding studies has gained wide acceptance due to the ease of preparation and the high specific activities obtainable (1). In the particular case of peptides belonging to the cholecystokinin family (CCK), the Bolon-Hunter method for iodination reaction under conditions avoiding oxidation,

is the only one which permits the use of a tracer in radio-immunoassay that is capable of biological activity.

Up to now, the purification of these tracers has been achieve on sephadex which is not totally satisfactory. In addition, this method does not give good chromatographic differentiation of the iodinated peptide from the non-iodinated peptide, the latter being added in excess.

We propose, in this paper, a simple and rapid method for the purification of four iodinated polypeptides belonging to the CCK-family.

EXPERIMENTAL

Peptides

Highly purified CCK_{1-39} was a gift of Professeur V. Mutt (Karolinska Institutet, Stockholm, Sweden) ; the C-terminal decapeptide (CCK_{30-39}) and a derivative of the C-terminal nonapeptide namely (Thr_{34}, N Leu_{37})-CCK_{31-39} were synthetized by Prof. E. Wünsch from Max Planck Institute für Biochemie, Muncheen, West Germ. The C-terminal tetrapeptide CCK_{36-39} was purchased from Interchim (Montluçon, France). Peptides used in this study were homogenous in reverse phase HPLC. They were rechromatographied before labelling if and when necessary.

Iodination precedure

CCK peptides were radio-iodinated by conjugation of the hormone to ^{125}I - Bolton-Hunter reagent [N-succinimidyl 3-(4-hydroxy,

5 -(^{125}I) iodophenyl) propionate] of specific activity ≃ 2 Ci/ µmol. (Amersham France) according to Rehfeld (2). About five micrograms of peptide in 15 µl of 0.1 M sodium borate, pH ≃ 8.5 were added to 0.5 mCi of dried Bolton-Hunter (BH^{125}I) reagent. After a 45 min stirring in an ice bath, 500 µl of 0.2 M glycine in 0.1 M Sodium borate pH = 8.5 was added to the reaction mini-vial.

HPLC

Purification of CCK-BH-^{125}I tracers was performed on a µ-Bondapak C-18 column (3.9 x 30 cm). The mobil phase was composed of a triethylammonium phosphate (TEAP) buffer 0.25 N, pH = 3.5 combined with acetonitril. Triethylamin and acetonitril were supplied by Fluka-Lab. Water was deionized and then distilled in glass. The waters associates liquid chromatograph model 204 consisted of a U6K injector, 6000 A pump, Schoeffel model 770 multiwave lenght detector and omniscribe chart recorder.

HPLC was run isocratically in a TEAP/acetonitril (74/26) buffer for at least 15 minutes, then elution of the radiolabelled peptides was performed by increasing the acetonitril ratio of the mobil phase to 50 %. Flow rate and back pressure were respectively 2 ml/min and 1500 PSI. Non-labelled peptides in the eluant were monitored by absorption at 210 nm, fractions of 1 ml were collected and ratio-activity was determined in a Packard PGD auto gamma counter.

Biological studies

The purity of the CCK radio-iodinated peptides was tested by the study of their binding characteristics to isolated pancreatic

acini. Acini were prepared, as previously reported, from guinea-pigs (5). About 50 000 cpm of radioactive fractions were incubated alone or in combination with caerulein (Farmitalia, Milan) for 40 min at 37°C in a 24.5 mM Hepes [4-(2-hydroxyethyl)-1-piperazine ethane salfonic acid] buffer pH = 7.4 containing 1.5 % (w/v) bovine ablumin, 0.1 % (W/V) bacitracin, 50 microg/ml streptomycin sulfate, 100 U/ml potassium penicillin G and 0.01 % soybean trypsin inhibitor. After incubation, an aliquot of the suspension was removed, washed with medium plus 12 % albumin at 4°C and centrifuged. Radioactivity associated with cells was then measured. Specific binding was defined as the excess binding over that in blanks containing 10^{-6}M caerulein and I_{50} as the concentration of cerulein that inhibited 50 % of specific binding.

RESULTS

Figure 1a and 1b illustrate the elution profils of the Bolton-Hunter reagent and the iodinated product of glycine. The BH-^{125}I reagent injected in solution in benzene is not eluted with the 26 % acetonitril buffer in less than 15 minutes. However with a change to 50 % acetonitril a total elution of the reagent takes place.

Under isocratic conditions (26 % acetonitril), the radioactivity of the labelled glycine product is eluted as a major peak with t_R = 4 min ; this corresponds to gly-BH-^{125}I, and to a secondary peak with a t_R = 7.5 min, which is most likely a hydrolysis product of BH-^{125}I. It was observed that after a 26 %

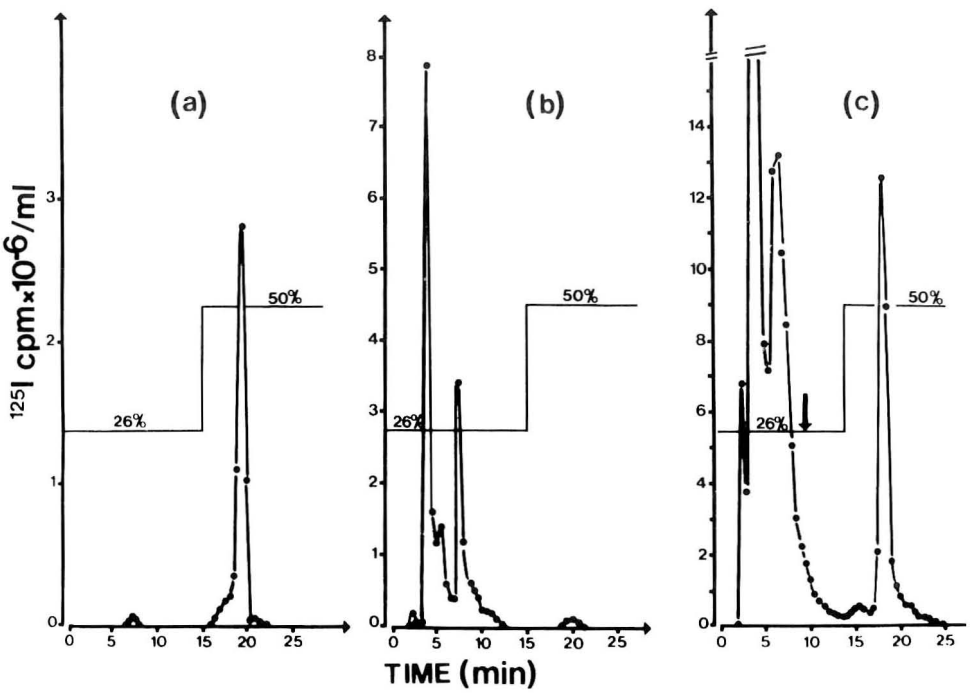

FIGURE 1. Radioactive profiles of the eluted fractions from HPLC chromatography using C-18 column.
Solutions injected :
a) 5.5 10^6 cpm of Bolton-Hunter-^{125}I reagent (BH-^{125}I)
b) 18.10^6 cpm of the iodinated product of glycine
c) 260.10^6 cpm of the iodinated solution of CCK_{30-39}
Step gradient with triethylamin phosphate buffer 0.25 N pH = 3.5 /acetonitril (74/26) during 15 min and then triethylamin phosphate buffer/acetonitril (50/50). Flow rate : 2 ml/min ; Back pressure: 1500 PSI ; volume of each eluted fraction : 1 ml
↓ : t_R of CCK_{30-39}

chromatographic run, the quasi-total radioactivity injected is eluted, and no observable residual peak is eluted when going to a 50 % acetonitril buffer (fig. 1b).

The radioactivity profiles of collected fractions from the HPLC of an aliquot of the CCK_{30-39} labelled solution used is

shown in figure 1c. Four peaks are resolved ; one peak close to Vo whose identity is not known ; another very dominant peak at 4 min, which corresponds to gly - BH - ^{125}I ; a peak at 7.5 min probably containing a hydrolysis product of BH-^{125}I and a last peak eluted with the 50 % acetonitril buffer. This last peak is the only one that contains molecules exclusively with characteristics of the CCK_{30-39} -BH-^{125}I tracers, since these molecules bind specifically to the high affinity receptors of CCK in the pancreatic acini.

All the results dealing with the tracer HPLC runs are summarized in table 1. Isocratic runs using 26 % acetonitril buffer permit the differentiation and elution of the unlabelled peptides. Using the same buffer system, the retention of the labelled peptides produces a total separation of the tracers from non-labelled peptide moiety. The choice of the 50 % acetonitril buffer within the 15 min period under isocratic conditions results in a compromise between two requisites : 1) the certainty of the total elution of the non-iodinated species and 2) the assurance of a good tracer recovery. The limiting values of the percent radioactivity incorporated in the species demonstrating a specific affinity with pancreatic acini, are reported in table 1 as well. In addition, during the course of the experiments, an average chromatographic recovery calculated on the basis of the percent radioactivity eluted within 30 minutes between 85.0 $\pm$ 3.4 % was obtained.

When CCK_{1-39}-BH^{125}I is placed in the presence of isolated pancreatic acini, a total binding of 5.7 $\pm$ 0.9 % is found. The

TABLE I

Chromatographic results of four labelled peptides belonging to CCK-family. Column - Bondapak C-18. Step gradient with triethylamin phosphate buffer 0.25N pH : 3.5 (acetonitril : 74/26, during 15 min and then, triethylamin phosphate buffer/acetonitril : 50/50 ; flow rate : 2 ml/min ; back pressure 1500 PSI ; volume of each eluted fraction : 1 ml.

Peptides	CCK_{1-39}	CCK_{30-39}	$(Thr_{34}, NLeu_{37})$ - CCK_{31-39}	CCK_{36-39}
t_R (min) un-modified peptides	7.10	9.60	8.25	3.20
t_R (min) radio-labelled peptides	19.0	19.0	19.5	20.0
% of total radioactivity recovered in biologically active fractions (limiting values)	12-30	10	4-6	16-30
% of total radioactivity eluted at 30 min	85.0 ± 3.4 ($\pm$ SEM)			

non-specific binding and the I_{50} are respectively 0.6 $\pm$ 0.2 % and 0.1 nM (6). With CCK_{30-39} BH-^{125}I and (Thr_{34}, $NLeu_{37}$) CCK_{31-39} - BH-^{125}I tracers a slightly weaker specific binding took place with I_{50} values comparable to that of CCK_{1-39} - BH-^{125}I. Regardless of which tracer used, non-specific binding values of about 10 % of the total binding were obtained. Only CCK_{36-39} BH-^{125}I, alone does not bind to pancreatic acini.

DISCUSSION

HPLC results of BH-^{125}I and gly-BH-^{125}I reveal a strong hydrophobic ability on the part of the Bolton-Hunter reagent. Its combination with peptides of CCK lead to the formation of molecules which are highly retained on the C-18 phase of the column. It is this characteristic property which led us to use a step gradient of 26 % to 50 % acetonitril.

Application of the least step gradient is sufficient enough for elution of the labelled peptides with, however a drop in the chromatographic yield as well as for the elution of a larger volume.

Our objective was not optimisation of the percent incorporation of iodine ^{125}I in peptides ; nevertheless the values were obtained are compatible with those found by other authors (2, 3).

Our method has the distinct advantage of producing a good separation of tracers from the unsubstituted peptide, the latter being perfectly eluted in the 26 % acetonitril buffer ; tracers of high specific activity could thus be anticipated.

The results of the first studies on the binding of these tracers with pancreatic acini proove their strong specific binding affinity as well as to their high degree of purity. In fact, a greater percentage of the specific binding of CCK_{1-39} BH-^{125}I than that obtained from CCK_{4-39} - BH-^{125}I prepared from chromatography using Sephadex (4,7) and a weaker non-specific binding (< 10 %) were observed. Absence of CCK_{36-39} BH-^{125}I binding is compatible

with the weak affinity of this peptide fragment for the CCK receptors of pancreatic acini (8).

All our methodology employed limits the risk of oxidation as is shown by the binding ability of recovered molecules. The time with which the separation is carried out (less than 30 min) most likely avoidssuch problems. Finally, the possible identification of oxidized components during non-iodinated peptide run, gives this method an added advantage (9, 10). All of the data give above show, in addition, that with this chromatographic method the fractions containing a maximum radioactivity are those which present the best biological characteristics, contrary to what is normally found in conventional chromatography (2). Furthermore, these tracers are recognised by antibodies reacting with C-terminal portion of CCK (Prof. J.A. Chayvialle, personal communication). They could be used in radio-immunoassay. $(Thr_{34}, NLeu_{33})$-CCK_{31-39} - BH-^{125}I seems best adapted to this end due to the substitution of oxidisable methonine residues.

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We thank Professors E. WÜNSCH and L. MOROODER who synthetised CCK_{30-39} and $(Thr_{34}, NLeu_{37})$ - CCK_{31-39} in their laboratory.

REFERENCES

1. Hunter, W.H. Greenwood, F.C. Preparation of iodine-131 labelled human growth hormone of high specific activity. Nature (London) 194, 495, 1962.

2. Rehfeld, J.F. Immunochemical studies on cholecystokinin. J. Biol. Chem. 253, (11), 4016, 1978.

3. Sankaran, H., Deveney, C.W., Goldfine, I.D., Williams, J.A. Preparation of biologically active radioiodinated cholecystokinin for radioreceptor assay and radioimmunoassay. J. Biol. Chem., 254, 9349, 1979.

4. Sankaran, H., Goldfine, I.D., Deveney, C.W., Wong K.Y., and Williams, J.A. Binding of cholecystokinin to high affinity receptors on isolated pancreatic acini. J. Biol. Chem. 255 (5), 1849, 1980.

5. Amsterdam, A. and Jamieson J.D. Sequential dissociation of the exocrine pancreas into lobules, acini and individual cells. J. Cell. Biol. 20, 301, 1978.

6. Esteve, J.P., Vaysse, N., Susini, C., Kunsch, J.M., Fourmy, D., Wunsch, E., Pradayrol, L., and Ribet, A. Action of somatostatin 28 on acini from guinea pig pancreas (submitted to Gastroenterology).

7. Jensen, R.M., Lemp, G.F., and Gardner, J.D. Interaction of cholecystokinin with specific membrane receptors on pancreatic acinar cell. Proc. Natl. Acad. Sci. USA 77 (4) 2079, 1980.

8. Robberecht, P., Deschodt-Lanckman, M., Morgat, J.L., and Christophe, J. The interaction of caerulein with the rat pancreas. Eur. J. Biochem. 91, 39, 1978.

9. Beinfeld, M.C., Jensen, R.T., and Brownstein, M.J. HPLC separation of cholecystokinin peptides. Two systems. J. Liquid. Chromatogr. 3 (9), 1367, 1980.

10. Pradayrol, L., Vaysse, N., Cassigneul, J., and Ribet, A. Use of reverse-phase high performance liquid chromatography for purification and purity control of somatostatin and CCK-PZ family peptides. In Hormone Receptors in Digestion and Nutrition G. Rosselin, P. Fromageot and S. Bonfils editors Elsevier/North Holland Biomedical Press 1979, p 95.

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DIFFERENTIAL BINDING OF SUGARS AND POLYHYDRIC ALCOHOLS TO ION EXCHANGE RESINS: INAPPROPRIATENESS FOR QUANTITATIVE HPLC

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ABSTRACT

The use of hydroxyl exchange resins (Dowex AG 1-X8 and AG 501-X8) as a component in the preparative clean up of biological samples for HPLC sugar and polyhydric alcohol analysis is inappropriate. On a weight specific basis, these resins bind 95-100% of monosaccharides (fructose and glucose), 45-85% of disaccharides (sucrose and trehalose) and 15-50% of polyhydric alcohols (glycerol and adonitol) present in sample solutions.

INTRODUCTION

Strongly basic ion exchange resins such as Dowex-1X8(OH^-) have been utilized for the selective binding of monosaccharides and presumed separation of alditols (1,2,3). Recently, it was demonstrated (4) that aldi-

tols are also selectively bound by this and other cationic exchange resins. Attempts to completely elute glucose and alditols by effecting the partitioning characteristics of the column with distilled water (up to 20 column volumes) were only partially successful.

Both the qualitative and quantitative analysis of these saccharides are facilitated by HPLC (5). However, the use of HPLC for carbohydrate analysis of biological extracts (plasma and animal/plant tissue extracts) is severely compromised by competitive binding of proteinaceous, lipid and divalent inorganic ion contaminants. Even trace levels of these contaminants may result in appreciable loss in either reverse phase (carbohydrate analysis column-Waters) or ion exchange columns (Bio Rad HPX-87) (column life= 15-50 injections). Sample preparation of biologics must therefore ensure the removal of each category of contaminant. Lipid extraction and partial deproteinization can be routinely accomplished with both solvent (i.e., chloroform:methanol) and heat (60-100^{o}C) treatment. Complete deproteinization however requires the addition of Zn^{++} and Ba^{++} salts. Therefore, the levels of divalent cations are increased over already high endogenous levels (i.e., plasma: Ca^{++}= 9-11 mg/dl and Mg^{++}= 1-3 mg/dl). Removal of disruptively high levels of cations can be simply and efficiently accomplished by sample pretreatment with an anionic exchanger [Dowex -1X8 (H^{+})]. To avoid complexing pH changes, mixed bed resins [Dowex AG -501-X8 (50% OH^{-}:50% H^{+})] are routinely used in the purification of biological samples (6).

This paper describes the degree of differential binding by a strongly basic cation exchanger (Dowex

AG-1X8 (OH^-) of polyhydric alcohols, mono- and disaccharides as effected by time and resin volume.

MATERIALS & METHODS

A Waters Model 6000A pump, Model 710B WISP automated injection system and Model R401 differential refractometer were used in conjunction with a Waters Model 730 Date Module. A Radial pak silica cartridge (10cm x 8mm ID) (10u particle size) was employed in a RCM-100 radial compression module to effect carbohydrate separation. The cartridge was initially conditioned by pumping 50 ml acetonitrile: water (70:30) containing 0.1% (v/v) tetraethylenepentamine (TEPA) (pH 9.2) at 2.0 ml/min. Following conditioning, a recirculating eluent of acetonitrile: water (81:19) containing 0.02% TEPA (pH 8.9) was introduced to the cartridge and stabilization allowed to proceed overnight. Carbohydrate and polyhydric alcohol standards were dissolved in distilled water (5mg/ml) and injected (50ul) at 26°C. A flow rate of 2.0 ml/min generated a back pressure of 300-400 psi. Acetonitrile was Fisher HPLC grade (Pittsburg, PA). Technical grade TEPA was obtained from Eastman Chemicals (Rochester, N.Y.), and carbohydrates and polyhydric alcohols from Sigma Chemical (St. Louis, Mo.). The water phase of the eluent was prepared by deionization and glass distillation. Eluents were degassed and filtered through Millipore 0.22um filter (GSWP) (Bedford, Ma.). Ion exchange resins are listed in Table I.

A 0.65 ml sample of the seven component standard (Table 2) was exposed to each resin (0-300 mg) for

TABLE I

Ion Exchange Resins

Nomenclature	Type	Source
AG 1-X8 (OH^-)	anion exchanger (20-50 mesh)	Bio Rad
AG 50-X8 (H^+, OH^-)	mixed bed exchanger (20-50 mesh)	Bio Rad
Dowex 1-X8-400 (Cl^-)	anion exchanger (200-400 mesh)	Sigma
Dowex 50-X8-400 (Cl^-)	cation exchanger (200-400 mesh)	Sigma
CX/Corasil Bondapak	cation exchanger	Waters
AX/Corasil Bondapak	anion exchanger	Waters

TABLE II

Retention Time of Standards

Order	Component	Retention Time (min)
1	Water	1.95
2	Ethylene Glycol	2.83
3	Glycerol	3.75
4	Adonitol (Ribitol)	6.33
5	Fructose	7.29
6	Glucose	9.29
7	Sucrose	14.70
8	Trehalose	20.37

periods of 3-120 min, with or without vortexing (15 sec). Samples were then filtered (0.45um) (HAWP), degassed in a vacuum for 5 min and injected. All samples were run in duplicate with mean values plotted. Reproducibility of duplicate samples was less than $\pm$2%.

RESULTS & DISCUSSIONS

Separation of polyhydric alcohol-carbohydrate mixtures at pH 8.9 is illustrated in Figure 1. Elution times ranged between 2.8 and 20.4 min and separation appeared related to carbon number (Table 2). Addition of 75 mg AG 1-X8 (OH^-) for 10 min resulted in the illustrated decreased amplitude (concentration) of each component. Attempts to elute the components by successive distilled water washes did not result in uniform recovery (4).

The time dependent binding of simple carbohydrates and polyhydric alcohols to the hydroxyl resin AG 1-X8 is illustrated in Figure 2. Polyols were not as effectively bound (~15-25%) as monosaccharides (75-85%). Disaccharides demonstrated a wide range of binding. Trehalose, a 1-a-D-glucopyranosyl-a-D-glucopyranoside, and sucrose, a 1-a-D-glucopyranosyl-B-D-fructofuranoside, are both non-reducing sugars and lack the properties characteristic of the free sugar group. The similarities in overall reactiveness between these two sugars would suggest similar binding capacities. This however was not the case. After 2 min exposure to the resin, nearly 70% of the sucrose was bound but only 25% of the trehalose. The binding

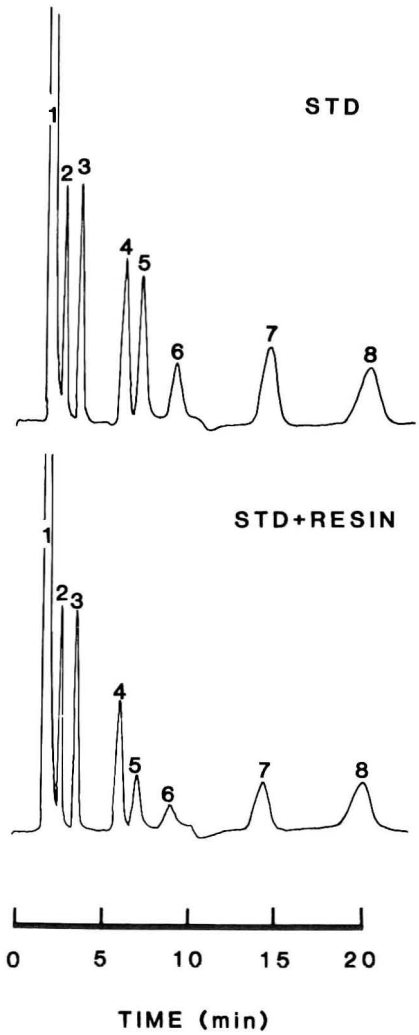

FIGURE 1. Effect of treatment with AG1-X8 Anion Exchange Resin (hydroxyl form) on polyol/saccharide recovery. Chromatogram represent polyol/saccharide separation on a hydrostatically compressed 10μ Radial-pak silica cartridge (10cm X 8mm I.D.). Elution solvent: Acetonitrile-water (81:19), pH 9.2, modified with 0.02% TEPA, flow rate = 2.0m/min., 300-450 psi. Top: Untreated standard solution. Bottom: 0.65ml of standard solution treated with 75mg of resin for 10 min. 1 = water; 2 = ethylene glycol; 3 = glycerol; 4 = adonitol; 5 = fructose; 6 = glucose; 7 = sucrose; 8 = trehalose.

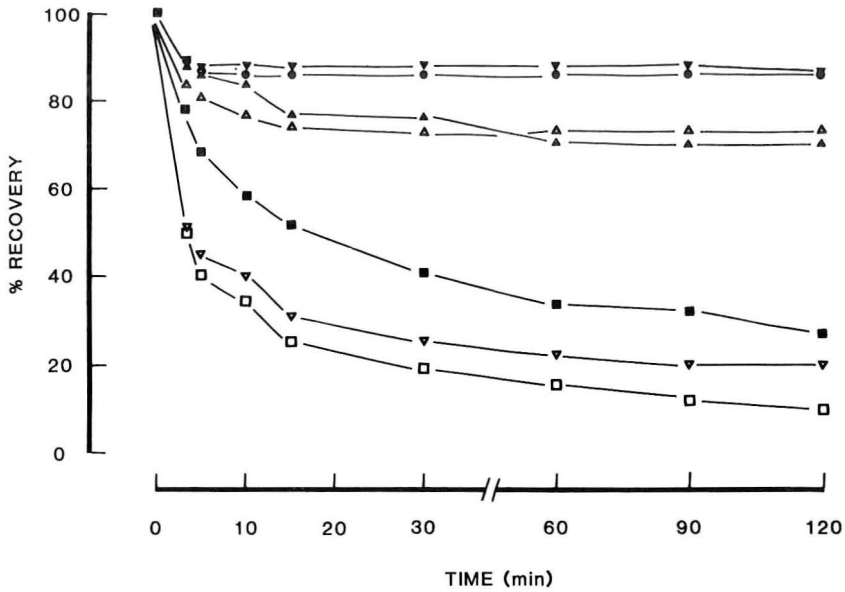

FIGURE 2. Effect of exposure time in the presence of AG1-X8 Anion Exchange Resin (hydroxyl form) on the recovery of polyol/saccharide standard mixture. ▼ = ethylene glycol; ● = glycerol; △ = adonitol; □ = fructose; ▽ glucose; ■ = sucrose; ▲ = trehalose.

characteristics of trehalose were similar to those of the polyols and sucrose to those of the monosaccharides.

Vortexing enhanced sample removal from the test solution. Rates of binding of saccharides and polyols were maximized within 1-5 minutes following brief vortexing (0.25 or 1.0 min.) (Figure 3). Equilibrium binding occurred more rapidly than would be predicted by simple probabilistic considerations.

Per cent recoveries were also lower for all components. The integrity of the binding differential observed between the non-reducing disaccharides (Δ= 42-52%) and trehalose:ethylene glycol (Δ=18-20%) suggests that

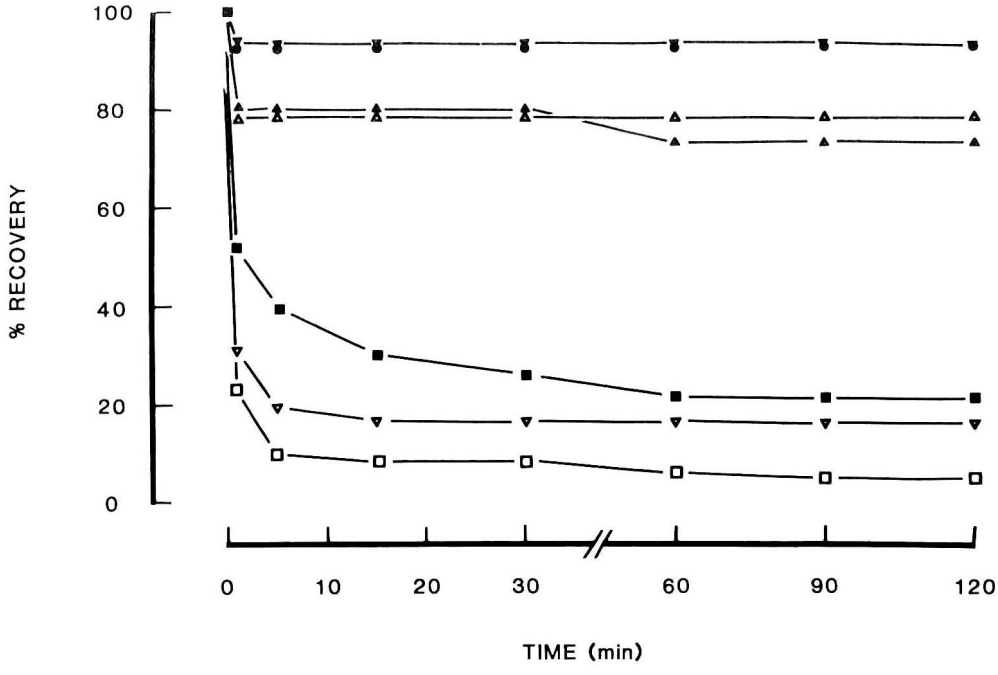

FIGURE 3. Effect of variable shaking time (240 cycles/min.) on recovery of polyols and saccharides treated with 75 mg of AGI-X8 Anion Exchange Resin (hydroxyl form). ▼= ethylene glycol; ●= glycerol; △ = adonitol; □= fructose; ▽= glucose; ■= sucrose; ▲= trehalose.

molecular density, solubility or exchange site spacing are not critical determinants of binding.

Incorporation of a cationic exchanger (H^+) with an anionic exchanger (OH^-) (Dowex AG 501-X8) (150mg) did not result in a modification of sample recovery as compared to the use of the anion exchanger only (Figure 2). Also, the use of a cationic exchanger only (Dowex 50W) (H^+) resulted in 100% sample recovery. Accordingly, only the anionic species participated in binding.

In addition to time, sample binding is dependent on resin weight (Figure 4). Interestingly, however, 100% binding (0% recovery) was encountered only for glucose in the shaken samples. Shaking for 10 minutes augmented the separation on a weight specific basis (Figure 5). However, complete binding was not observed except for glucose, fructose and sucrose. Since only 75% of trehalose was bound as compared to sucrose, binding site saturation does not appear to be the explanation for this observation. Differential binding appears due to affinity relationships

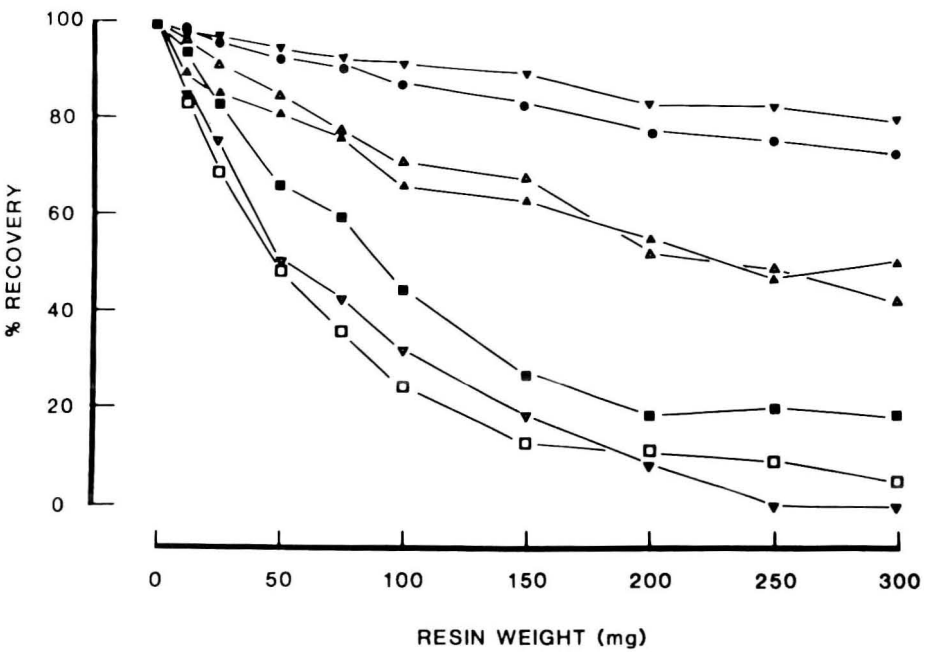

FIGURE 4. Effect of treatment with varying weights of AG1-X8 Anion Exchange Resin (hydroxyl form) on the recovery of polyol/saccharide standard mixture. ▼ = ethylene glycol; ● = glycerol; ▲ = adonitol; □ = fructose; ▽ = glucose; ■ = sucrose; △ = trehalose.

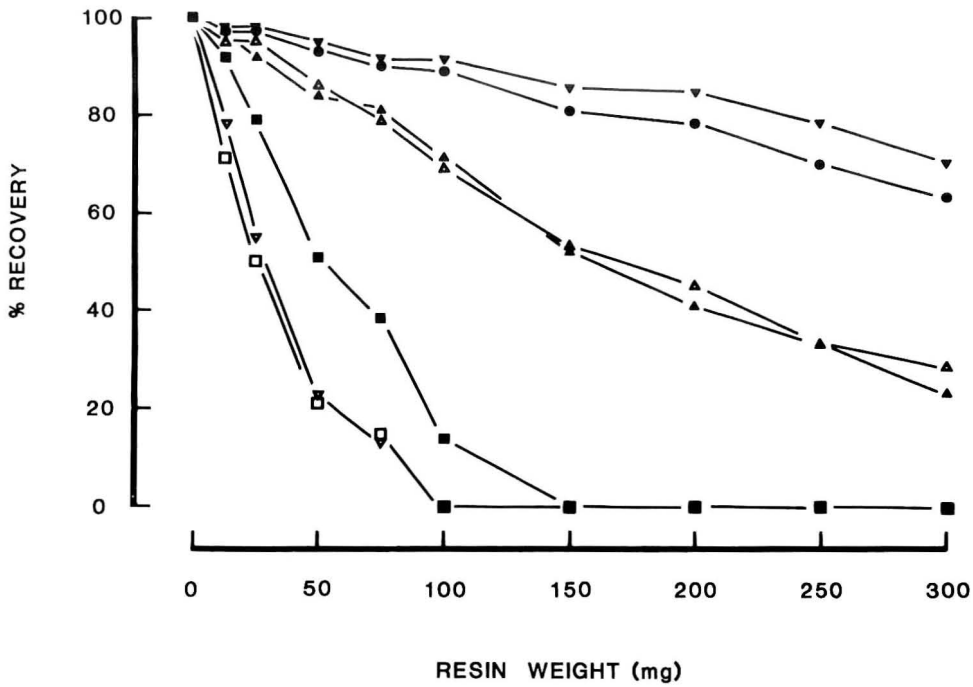

FIGURE 5. Effect of treatment with varying weights of AG1-X8 Anion Exchange Resin (hydroxyl form) on the recovery of polyol/saccharide standard mixture after shaking (240 cycles/min.) for 10 min. ▼ = ethylene glycol; ● = glycerol; △ = adonitol; □ = fructose; ▽ = glucose; ■ = sucrose; ▲ = trehalose.

between resin and carbohydrate. The comparatively high recovery of glycerol ("1/2 glucose") when considered with respect to the overall binding profile further supports this statement. Simple saturation kinetics would suggest that at resin concentrations that completely bind glucose (150mg), 50% of the glycerol ought to be bound and at a resin concentration of 300mg, 100% of the glycerol would be bound. At

these concentrations, only 19% and 37% of the glycerol was bound.

The possibility of differential binding being matrix dependent was considered. Figure 6 illustrates that recovery from a seven component test standard was solute specific and not dependent on matrix effects.

The use of hydroxyl ion exchangers is not recommended during the preparative phase of sample clean up for HPLC analysis of polyhydroxy compounds due to the pronounced differential binding. In addition, sample handling is made more complex by the

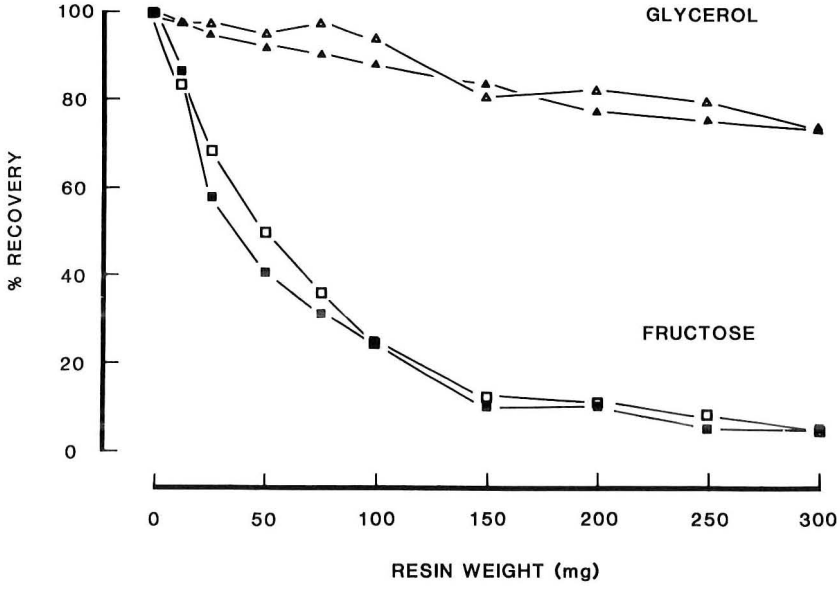

FIGURE 6. Matrix effects: Recovery of glycerol and fructose in single versus multi-solute solutions after treatment with AG1-X8 Anion Exchange Resin (hydroxyl form). Open symbol for single solute solutions; solid symbol for multi-solute solutions.

specific time/weight dependencies (exposures). The use of cationic exchangers or chloride ion anion exchangers (Table) did not effect the recovery of carbohydrates or polyhydric alcohols.

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REFERENCES

1. Yamaguchi, H., Namura, S.I., and Makino, K., Studies on a microchemical method for the determination of the degree of polymerization of neutral oligo- and polysaccharides. J. Biochem. (Tokyo), 79, 299 (1976).

2. Yamaguchi, H. and Makino, K., Studies on a microchemical method for determination of the degree of polymerization of neutral oligo- and polysaccharides. J. Biochem. (Tokyo), 81, 563 (1977).

3. Yamaguchi, H. and Okamoto, K., Structural studies on the reducing -end sugar residues of polysaccharides by GLC and mass spectrometry. J. Biochem. (Tokyo), 82, 511 (1977).

4. Tanaka, M., Binding of alditols to the hydroxyl form of Dowex-1X8, a strongly basic ion exchange resin: an improved method for estimation of the degree of polymerization of neutral oligo- saccharides and polysaccharides and polysaccharides. Carboh. Res., 88, 1 (1981).

5. Hendrix, D.L., Lee, Jr., R.E., Baust, J.G. and James, H., Separation of carbohydrates and polyols by radially compressed high-performance liquid chromatographic silica column modified with tetraethylene pentamine. J. Chromat., 210, 45 (1981).

6. Baust, J.G., and Edwards, J.S., Mechanisms of freezing tolerance in an antarctic midge, Belgica antarctica. Physiol. Entomol., 4, 1 (1979).

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AMBERLITE XAD-4 AS A STATIONARY PHASE FOR PREPARATIVE LIQUID CHROMATOGRAPHY IN A RADIALLY COMPRESSED COLUMN

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ABSTRACT

Amberlite XAD-4, a nonpolar adsorbent with a particle size of 62 to 177 μm, was packed into 58 mm i.d. x 294 mm columns and radially compressed in the Waters Prep 500 preparative liquid chromatograph. Efficiency,loading capacity (multigram samples), resolution, recovery, and types of mobile phases were major parameters studied. Since XAD-4 is chemically inert mixed solvent and pH control from 1 to 13 can be successfully used in the mobile phase. Separations illustrating these advantages and the scope of the 58 mm i.d. column are described.

INTRODUCTION

Preparative high performance liquid chromatography (PLC) constitutes a wide range of column loadings extending from a few mgs to multigram samples. The scope of PLC applications extends to almost all scientific fields. Typical applications are purifications in synthetic chemistry, purification of standards, and characterization of trace compounds in physiological, environmental, plant, etc. type samples. Even commercial applications are

feasible. Details of these and other applications are reviewed elsewhere (1-3).

Commercial LC suppliers have responded to this interest and prepacked columns of 1/4 to 1 inch diameters containing either silica or alkyl-modified silica as the stationary phase are available. Larger diameter columns, which permit greater loading, are also available. Usually these are dedicated to a specific type of instrumentation. One such example is the Waters Prep 500, which employs silica or alkyl-modified silica packed in 58 mm i.d. x 294 mm plastic columns that are radially compressed.

Amberlite XAD-4 is a macroporous polystryene-divinylbenzene nonpolar adsorbent with a large surface area and porosity. It functions as a reversed stationary phase like the alkyl-modified silica and has been used in PLC 8 mm and 20.5 mm i.d. columns (4-6) and in analytical high performance liquid chromatographic (ALC) columns (7-8). Since XAD-4 is stable throughout the entire pH range of 1 to 13, strongly acidic and basic eluting agents, which cannot be used with the alkyl-modified silica, are readily used with XAD-4 columns without loss in efficiency or changes in chromatographic behavior. Other advantages and properties of XAD-4 including favorable mass loading capabilities are described elsewhere (4).

This report describes PLC experiments using XAD-4 in a 58 mm i.d. x 294 mm plastic column which is radially compressed in the Waters Prep 500. Mass and volume overload, efficiency, resolution, recovery, and mixed solvent, acidic, and basic mobile phases are evaluated.

MATERIALS

Reagents

The chemicals used were obtained from Curtin Matheson Scientific, Eastman Kodak, and Sigma Chemical Company and used as

received. Water was purified by passing distilled water through a mixed bed ion exchange column, a charcoal column and 0.2 μm Millipore filter disks. All solvent composition is expressed as percent by volume.

Amberlite XAD-4 (20 to 50 mesh) was purchased from Mallinckrodt Chemical Works. Cleaning, crushing and sizing procedures are described elsewhere (4). Irregular shaped particles (62 to 177 μm) were used to pack plastic columns of 58 mm i.d. x 294 mm (Waters Associates) equipped with either 15 μm porous stainless steel or polyethylene column end fitting disks (Waters Associates).

Instrumentation

A Waters Prep 500 liquid chromatograph (Waters Associates) was used and the column effluent was monitored by a refractive index detector (Prep 500) and an Altex Model 153 fixed wavelength (254 nm) detector equipped with a 2 μl, 0.5 mm cell. Sample was introduced with 10 or 30 ml syringes designed to fit the Prep 500 injector. ALC separations were performed on an Altex Model 332 LC using a 4.1 mm i.d. x 150 mm, 10 μm, Hamilton PRP-1 column.

Procedures

The exit frit was inserted into the plastic column and the column was wrapped with cloth duct tape up to but not under the sealing rings. Approximately 450 g of dry XAD-4 with a size distribution of 430 g of 74 to 149 μm, 10 g of 62 to 74 μm, and 10 g of 149 to 177 μm was carefully poured into the column in small amounts (about 30 ml) and pressed into a tight bed with a plexiglass rod. This was continued until the column was full. The last two or three inches of the column which could not be pressed with the rod was tightly packed by hammering a frit (this served as the column outlet) into place using a rubber stopper to protect the frit. If the lower part of the column was not packed tight enough

a second frit was hammered in (served as column inlet). The packed column was then inserted in the Prep 500, radially compressed, and 1:5 95% EtOH:H_2O pumped through the column at 50 ml/min for approximately 1 to 2 hrs. Since the XAD-4 particles will swell as the % EtOH increases a change in % EtOH in either direction must be gradual to prevent channeling or bursting of the column. At 50 ml/min and 1:5 95% EtOH:H_2O inlet column pressures were about 15 atm. The radial compression was about 34 to 40 atm.

After the LC system stabilized in the appropriate mobile phase the sample, which was in water, ethanol, their mixture, in acidic, or basic solution, was introduced by syringe. In general, sample solvents were weaker eluting agents than that used for the separation. Effluent fractions, when desired, were collected manually.

RESULTS AND DISCUSSION

Column Properties

The LC properties of the 58 mm i.d. XAD-4 column which varied from column to column was largely due to the inability to pack the columns in an uniform manner. However, with packing practice the differences were small. The properties also change with usage; this is the largest when the column is exposed to widely different mobile phases. The properties cited here are average ones observed prior to exposing the column to widely different mobile phases.

The 58 mm i.d. x 294 mm, 62 to 177 μm XAD-4 column had an average void volume of about 260 ml determined for a mobile phase that was in the range of 5 to 10% ethanol. This corresponds to approximately 55-60% stationary phase.

The column permeability changes with solvent composition. As the organic solvent concentration in the mobile phase increases, the XAD-4 particles swell, and the column inlet pressure required to maintain the flow rate increases. For a 5 to 10% ethanol mobile

phase at a flow rate of 50 ml/min inlet pressure was about 20 atm. Inlet pressures did not change appreciably when dilute EtOH mobile phases contained 0.1M HCl or 0.1M NaOH. Because of pressure limits separations were done at either 50 or 100 ml/min.

The plastic columns were taped to increase their wall strength and to prevent the stationary phase particles from entering the instrument compression chamber if the column splits. With use wrinkles appear in the column; the more tightly the column is packed the smaller the wrinkles are. Since the column inlet end is the most difficult to pack tightly, wrinkling was the largest at this end and column splitting, if it occurred, was usually at the inlet end. In general, longer column life and reproducibility was favored by tightly packed columns and by minimal changes in the EtOH:H_2O ratio of the mobile phase. However, column and retention properties were reasonably reproducible even when the column was subjected to modest change in the solvent composition with and without acid (up to 0.1M HCl) or base (up to 0.1M NaOH) providing the transition from one mobile phase to another was a gradual one.

Column Performance

Figure 1 shows how plate number, N (number of plates for the 294 mm column), for the radially compressed XAD-4 column changes with sample loading. The arylsulfonic acid and tetraalkylammonium salt samples (k' values of 2.47 and 1.88, respectively) were used because of their large solubility in water. Thus, they could be introduced as small volumes of concentrated solution (20 and 17 ml in Figure 1, respectively, or about 5 to 7% of the void volume) without exceeding the volume overload. Greater loads were not examined because these could only be attained by a large increase in sample volume. Although not shown, k' decreased as sample loading increases which is consistent with previous results (4).

The mass overload limit, which by definition is the load that produces a 10% change in efficiency (4), occurs at about 2 to 2.5 g.

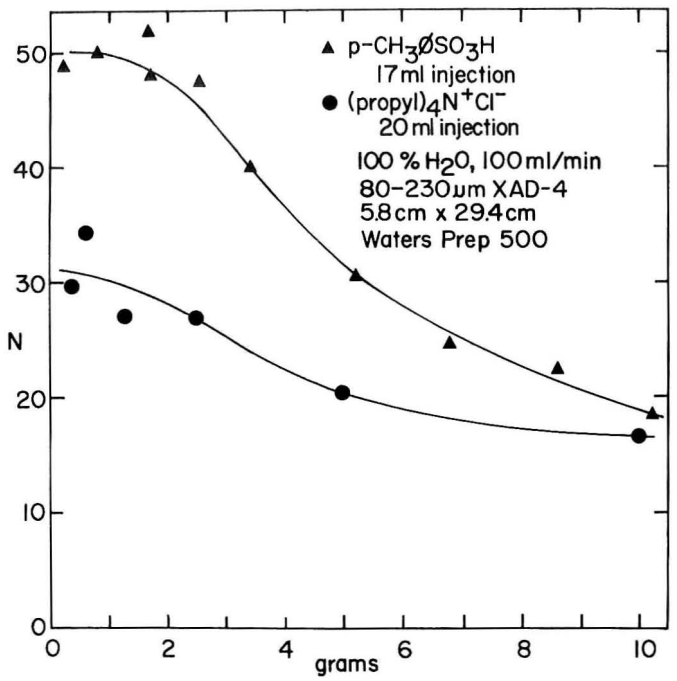

FIGURE 1

The Effect of Mass Loading on the XAD-4 Column

A 58 mm x 294 mm, 62 to 177 µm XAD-4 column was used with 100% H_2O at a flow rate of 100 ml/min.

However, even at a column load in excess of the mass overload limit many plates are still available. For example, at 4 to 5 times the mass overload limit (a 10 g load) approximately 20 plates are still obtained. Furthermore, a symmetrical peak shape was retained throughout the major part of this load range and only at higher loads does tailing begin to occur. Improvement in column efficiency would be realized by reducing both the XAD-4 particle size and its range. This has been shown in both XAD analytical and smaller diameter prep columns (4,9).

Similar loading experiments were done with pyridine and picoline samples using an aqueous 0.1M HCl mobile phase and with o-chlorophenol using a 1:5 95% EtOH:H_2O, 0.1M NaOH mobile phase. The mass overload limit in the acidic and basic mobile phases were consistent with the results found in Figure 1.

On the average for the separations reported here, the number of plates developed on the radially compressed XAD-4 column when operated below the mass overload limit was about 40 to 60 plates. In comparison, the number of plates typically found for the commercially available silica and C_{18}-modified silica columns that can be radially compressed are in the order of 100 to 130 plates (10, 11). Contributing to this difference is the fact that the commercially available silica and C_{18}-modified silica are smaller particles than the XAD-4 and that the samples and eluting conditions used in the latter studies are not the same as that used on the XAD-4.

The injection system on the Prep-500 requires the injected sample to replace liquid through and against the pressure of the column-detector. This prevents an accurate determination of the volume overload. Within experimental error no significant effect on efficiency was found up to a 50 ml injection (20% of the column void volume). This was shown by determining plate number for the elution of 1.88 g of p-methylbenzene sulfonic acid as a function of injection volume at a flow rate of 100 ml/min. Even at a 100 ml injection (40% of the column void volume) a volume overload effect was small.

Resolution

Figure 2 illustrates the preparative separation of two benzenesulfonic acids at loading levels above the mass overload limit relative to each component. At low loading the k' values of the p-methyl- and 2,5-dichloro- derivatives are 1.3 and 2.7, respectively. For a single pass a resolution of 0.93 is obtained

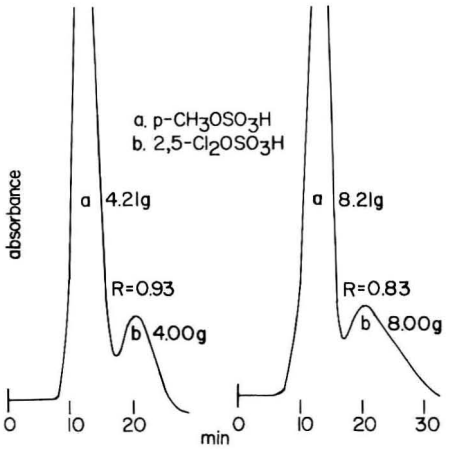

FIGURE 2

Separation of a Mixture of Benzenesulfonic Acids at a Mass Overload

A 58 mm x 294 mm, 62 to 177 μm XAD-4 column was used with 1:4 95% EtOH:H_2O at 43 ml/min. Injection volume was 9 ml.

at a total load of 8 g (see Figure 2). At lower loadings resolution is more favorable. For example, at 1.6 g total load, a resolution of 1.0 was found. As loading increases, efficiency and subsequently resolution decreases. Thus, at 16 g total load (Figure 2) resolution drops to 0.83. Two major factors which contribute to this are an increase in peak broadening and a decrease in retention with increased loading. However, even with these effects enough plates are still available to permit isolation of a significant amount of each component free of the other in a single column pass even though the column is grossly overloaded.

Mobile Phase Versatility and Sample Recovery

Retention order is the same as found on analytical XAD-4 columns and these data can be used to predict elution order and

optimum mobile phase (7-9). Thus, retention is reduced when the analyte is in a charged form, retention increases as the percent organic solvent in the mobile phase is reduced, and the eluting power changes in the order CH_3CN > EtOH > MeOH. Because of XAD-4 swelling and the flexibility of the plastic columns the better column performance is achieved when the mobile phase composition is only modestly changed during packing, conditioning and elution and when H_2O is the major mobile phase component.

A mixture of p-methyl-and 2,5-dichlorobenzenesulfonic acids were separated at a total load of about 3.7 g where the two acids are in the weight ratio of 40/1, 1/1, and 1/40. The 1/1 mixture represents the isolation of two major components while the other two are examples of separation of a minor (2.5% of the sample) and a major (97.5% of the sample) component. These chromatograms are shown in Figure 3. Verification of the separation was done by

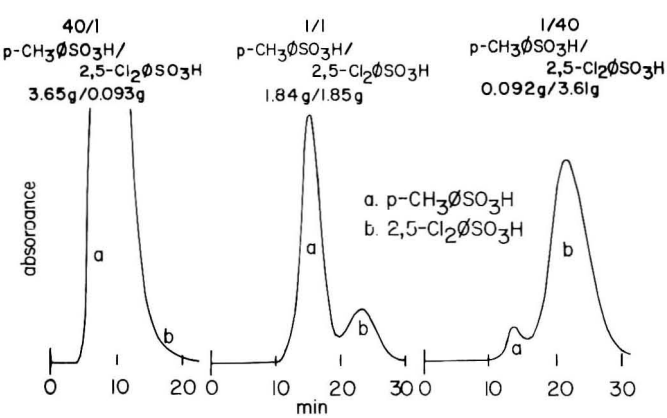

FIGURE 3

Separation of a Mixture of Benzenesulfonic Acids

The conditions and column described in Figure 2 were used. Injection volume was 5 ml.

collecting 2 min (86 ml) fractions and analyzing each fraction by ALC. The fraction analysis for each chromatogram is shown in Figure 4.

In the 40/1 mixture the p-methyl derivative swamps the detector cell because of its large concentration and masks the 2,5-dichloro peak. Fraction analysis (Figure 4) verifies the separation and shows the minor component at the tail of the peak. For a single pass and with careful fraction collecting 80 to 90% of the major component can be isolated at high purity level. The minor component could be isolated in mg quantities at a high purity level by collecting the peak tail, or a large amount of it could be collected where its enriched and purified via a second pass through the column. For the 1/1 mixture the chromatogram (Figure 3) and fraction analysis (Figure 4) indicate that a significant amount (up to 70 to 80%) of each component can be isolated at a high purity level by just a single column pass. In the 1/40 mixture, Figures 3 and 4, about 80 to 90% of the second or major component can be isolated in mg quantities of high purity by collecting the beginning of the first peak. Or, larger quantities of an enriched mixture can be isolated and purified by a second pass through the column.

Silica and C-18 modified silica are the only stationary phases that have been used in radially compressed columns in the Waters Prep-500. Although they are readily used in mixed solvents, their applications are limited to a pH range of 2 to 8. This is not the case for XAD-4 which is chemically stable throughout the pH range of 1 to 13.

The application of an acidic and basic mobile phase in PLC offers several advantages. 1. Organic acids, bases, and ampholytes can be separated as charged species rather than neutral ones. 2. Retention of charged species is greatly reduced which broadens the useful range of eluting conditions. 3. The aqueous solubility of the ionized sample is increased which allows smaller volumes of concentrated sample solution to be

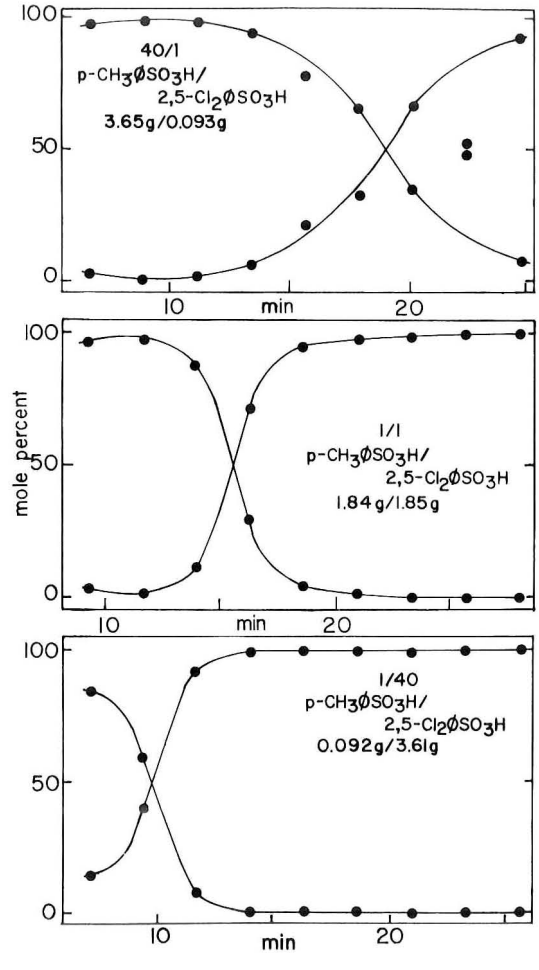

FIGURE 4

Fraction Analysis for the
Chromatogram in Figure 3

ALC was done on a 4.1 mm x 150 mm, 10 μm, Hamilton PRP-1 column using a 8.5:91.5 $CH_3CN:H_2O$ pH=6.0 (phosphate) mobile phase at a flow rate of 1 ml/min.

injected. 4. Adjustment of pH relative to the K_a values for the analytes can often improve the selectivity. 5. Addition of electrolyte to the mobile phase often reduces band width and improves selectivity when using XAD-4. Perhaps the major disadvantage of using acids, bases, or buffer salts in PLC is that the separated components contain these electrolytes. By adjustment of mobile phase conditions so that the sample is converted back to the neutral form the same or a second XAD-4 column can be used to separate the sample from the salts.

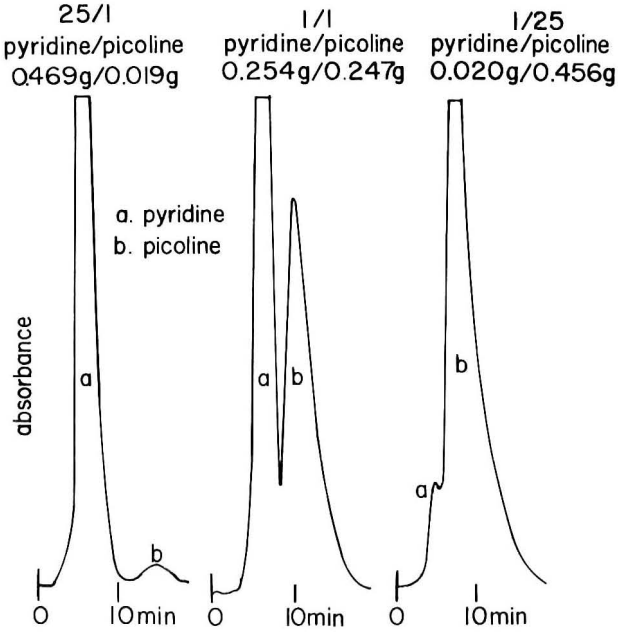

FIGURE 5

Separation of a Pyridine/Picoline Mixture with a Strongly Acidic Mobile Phase

A 58 mm x 294 mm, 62 to 177 μm XAD-4 column was used with 0.1M HCl at 102 ml/min. Injection volume was 5 ml.

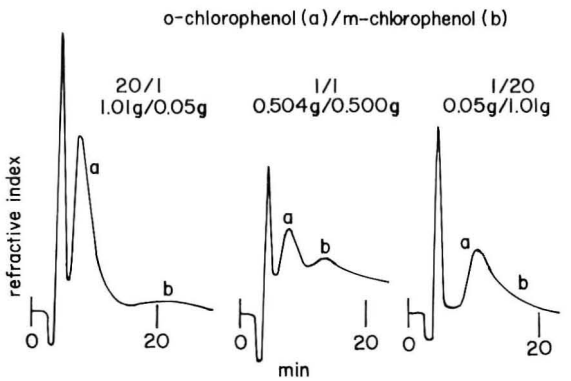

FIGURE 6

Separation of a o-Chlorophenol/m-Chlorophenol Mixture with a Strongly Basic Mobile Phase

A 58 mm x 294 mm, 62 to 177 μm XAD-4 column was used with 3:7 95% EtOH:H_2O, 0.1M NaOH at 100 ml/min. Injection volume was 10 ml.

Two separations were carried out on the XAD-4 column to demonstrate the feasibility of using an acidic and basic mobile phase. Figure 5 illustrates the separation of a pyridine/picoline mixture at a 25/1, 1/1, and 1/25 ratio using an aqueous 0.1M HCl mobile phase while Figure 6 shows the separation of a o-chloro/m-chlorophenol mixture at a 20/1, 1/1, and 1/20 ratio using 1:5 95% EtOH:H_2O, 0.1M NaOH mobile phase. No undesirable chromatographic properties are introduced into the separation as a result of using the strongly acidic or basic eluent other than the presence of HCl or NaOH in the collected fractions.

For the pyridine/picoline mixture, where the k' values are 1.6 and 4.5, respectively, chromatographic peaks are found for both components in all three mixtures. This was verified by fraction analysis. Only for the 1/25 mixture is the resolution low and this occurs because the picoline k' decreases with the increased picoline loading. Thus, the minor component in the

25/1 and 1/25 mixture can be isolated in significant amounts at a high purity level. Similarly, the major component in all three mixtures can be isolated in large quantities at a high purity level. The pyridine/picoline mixture could be separated in mixed solvent in the absence of acid, however, the EtOH concentration in the mobile phase must be significantly increased since the pyridine/picoline are now retained as neutral species. Also, in the absence of the acid the peaks become much broader. The conclusions regarding the o-chloro/m-chlorophenol separation are similar. Since the k' values in a basic mobile phase (retention as anions) are favorable a baseline separation is obtained except for the 1/20 mixture. In the absence of HCl (retention as neutral species) the EtOH would have to be about 40 to 50% to affect the separation; also, peaks would be broader.

ACKNOWLEDGEMENTS

We thank J. D. Stodola and D. L. Bakken for their help in column packing and Waters Associates for their advice and for supplying the empty column cartridges. This investigation was partly supported by Grant CA 18555 awarded by The National Cancer Institute, DHEW, and partly by Grant CHE 79-13203 awarded by the National Science Foundation. Initial experiments were reported at the Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Atlantic City, N.J., March, 1981 as part of paper Number 582.

REFERENCES

1. Verzele, M. and Geeraert, E., Preparative Liquid Chromatography, J. Chromatog. Sci. 18, 559-570 (1980).

2. Snyder, L. R. and Kirkland, J. J., Introduction to Modern Liquid Chromatography, Chapter 15, 2nd Edition, John Wiley and Sons, New York, 1979.

3. Wehrli, A., Preparative High Pressure Liquid Chromatography, Z. Anal. Chem. 277, 289-302 (1975).

4. Pietrzyk, D. J. and Stodola, J. D., Characterization and Applications of Amberlite XAD-4 in Preparative Liquid Chromatography, Anal. Chem. 53, 1822-1828 (1981).

5. Pietrzyk, D. J. and Stodola, J. D., Preparative Liquid Chromatographic Separation of Amino Acids and Peptides on Amberlite XAD-4, J. Liq. Chromatogr.

6. Pietrzyk, D. J. and Stodola, J. D., Preparative Liquid Chromatography of 1:1 Adducts Derived from the Reaction of Malondialdehyde with Amino Acids, Anal. Biochem. 117, 245-249 (1981).

7. Kroeff, E. P. and Pietrzyk, D. J., Investigation of the Retention and Separation of Amino Acids, Peptides, and Derivatives on Porous Copolymers by High Performance Liquid Chromatography, Anal. Chem. 50, 502-511 (1978).

8. Pietrzyk, D. J. and Chu, C. H., Separation of Organic Acids on Amberlite XAD Copolymers by Reversed Phase High Pressure Liquid Chromatography, Anal. Chem. 49, 860-867 (1977).

9. Baum, R. G., Saetre, R., and Cantwell, F. F., Liquid Chromatography Columns of Microparticle Amberlite XAD-2, Anal. Chem. 52, 15-19 (1980).

10. Bishop, C. A., Harding, D. R. K., Meyer, L. J., Hancock,W. D., and Hearn, M. T. W., High Performance Liquid Chromatography of Amino Acids, Peptides, and Proteins, J. Chromatogr. 192, 222-227 (1980).

11. Little, J. N., Cotter, R. L., Prendergast, J. A., and McDonald, P. D., Preparative Liquid Chromatography Using Radially Compressed Columns, J. Chromatogr. 126, 439-445 (1976).

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LC CALENDAR

1982

MARCH 8 - 12: Pittsburgh Conf. on Anal. Chem. and Appl. Spectroscopy, Atlantic City, NJ, USA. Contact: Pittsburgh Conf., Inc., P. O. Box 7780-1223, Philadelphia, PA, 19182, USA.

MARCH 28 - APRIL 2: National Amer. Chem. Soc. Meeting, Las Vegas, NV, USA. Contact: A. T. Winstead, Am. Chem. Soc., 1155 Sixteenth St., NW, Washington, DC, 20036, USA.

APRIL 14 - 16: 12TH annual Symposium on the Anal. Chem. of Pollutants, Amsterdam, The Netherlands. Contact: Prof. R. W. Frei, Congress Office, The Free University, P. O. Box 7161, 1007-MC Amsterdam, The Netherlands.

APRIL 18 - 21: 66th Annual Meeting, Federation of Amer. Soc. for Exp't'l. Biol. (FASEB), Louisiana Superdome, New Orleans, LA, USA. Contact: FASEB, 9650 Rockville Pike, Bethesda, MD, 20014, USA.

MAY 2 - 6: American Oil Chemists Soc., 73rd Annual Meeting, Sheraton Center, Toronto, Ont., Canada. Contact: J. Lyon, AOCS, 508 South sixth St., Champaign, IL, 61802, USA.

MAY 2 - 6: 2nd Int'l. Symp. on Instrumental TLC (HPTLC), Interlaken, Switzerland. Contact: Dr. R. E. Kaiser, Inst. for Chromatography, P. O. Box 1141, D-6702, Bad Durkheim, West Germany.

MAY 3 - 4: Minnesota Chromatography Forum, 4th Annual Spring Symp., Minneapolis, MN. Contact:

Meeting Management, Inc., Suite 50, 1421 E. Wayzata Blvd., Wayzata, MN, 55391, USA.

MAY 16 - 18: LCEC Symposium: Biomedical Applications of LCEC and Voltammetry, Indianapolis Hyatt Regency. Contact: K Klippel, LCEC Symposium, P. O. Box 2206, W. Lafayette, IN, 47906, USA.

MAY 24 - 27: 9th Cellulose Conf., Syracuse, NY. contact: A. Sarko, Cellulose Research Inst., State Univ. of NY, Syracuse, NY, 13210, USA.

JUNE: Symposium on Adv. in TLC & HPLC, Szeged, Hungary. Contact: H. J. Issaq, Frederick Cancer Res. Center, P. O. Box B, Frederick, MD, 21701, USA.

JUNE 2 - 3: Midwest Regional AOAC Meeting, Starlight Village Motel, Ames, IA. Contact: H. M. Stahr, 1636 College of Veterinary Medicine, Iowa State University, Ames, IA, 50011, USA.

JUNE 6 - 12: ACHEMA-20th Chemical Engineering Meeting & Conf., Frankfurt, West Germany. Contact: R. J. Sykstus, Nat'l. Chem. Expo., 59 E. Van Buren St., Rm. 1300, Chicago, IL, 60605, USA.

JUNE 7 - 11: 6th Internat'l. Symposium on Column Liquid Chromatography, Cherry Hill Inn, Cherry Hill, NJ. Contact: R. A. Barford, ERRC-SEA, USDA, 600 E. Mermaid Lane, Philadelphia, PA, 19118, USA.

JUNE 16: Workshop on Chrom. Data Reduction, Miami, Univ., Oxford, OH. Contact: F.D.Hileman, Monsanto Research Corp., 1515 Nicholas Rd., Dayton, OH, 45418, USA.

JUNE 17 - 18: 3rd World Spectroscopy/5th World Chromatog. Conf., Nice, France. Contact: V. Bhatnagar, Alena Enterprises, Box 1779, Cornwall, Ont., Canada.

JUNE 17 - 18: Chromatography Symposium, College Corner, Ohio. Contact: F. D. Hileman, Monsanto Research Corp., 1515 Nicholas Rd., Dayton, OH, 45418, USA.

JUNE 20 - 23: Int'l. Conf. on Chromatography And Mass Spectrometry in Biomed. Sci., Bordighera, Italy. Contact: Dr. Alberto Frigerio, Italian Group for Mass Spectrometry in Biochem. & Med., Via Eritrea 62, 20157 Milano, Italy.

JUNE 28 - 30: ACS Analytical Summer Symposium, Michigan State University, East Lansing, MI, USA. Contact: A.I.Popov, Chem. Dept., Michigan State University, East Lansing, MI, 48824, USA.

JULY 12 - 16: 2nd Int'l. Symposium on Macromolecules-IUPAC, University of Massachusetts, Amherst, MA, USA. Contact: J. C. W. Chien, Dept. of Polymer Science & Engineering, Univ. of Mass., Amherst, MA, 01003, USA.

JULY 12 - 16: 8th Int'l. Conf. on Organic Coatings Science & Technology, Athens, Greece. Contact: A. V. Patsos, Science Bldg., SUNY, New Paltz, NY, 12561, USA.

JULY 19 - 22: 23rd Prague Microsymposium on Macromolecules: Selective Polymeric Sorbents - IUPAC, inst. of Macromolecular Chem., Prague, Czechoslovakia. Contact: P. M. M. Secretariat, Inst. of Macromolecular Chem., 162-06 Prague, Czechoslovakia.

AUGUST 2 - 5: Int'l. Conf. on Ion Chrom., at the 24th Rocky Mountain Conf., Denver, CO, USA. Contact: Dionex Corp., 1228 Titan Way, Sunnyvale, CA, 94086, USA.

AUGUST 8 - 13: 34th Annual AACC Nat'l. Meeting, Anaheim, CA. Contact: M. Tuttle, Amer. Assoc. of Clinical Chemists, 1725 K Street, NW, Suite 903, Washington, DC, 20006, USA.

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

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

SEPT 13 - 17: 14th Int'l. Symp. on Chromatography, London. Contact: Executive Secretary, Chromatography Discussion Group, Trent Polytechnic, Burton Street, Nottingham NG1 4BU, U.K.

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

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

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

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

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- introduces TLC for use as an aid in solving basic and applied problems for scientists considering this technique
- serves as an aid for the introduction of TLC into the classroom, presenting various detailed experiments which utilize inexpensive, readily available materials

Thin-Layer Chromatography: Techniques and Applications is the primary source of information for analytical chemists, biochemists, clinical chemists, and laboratory and medical technicians requiring a greater knowledge of this technique. Zoologists and botanists interested in the separation sciences will find it a valuable reference; and it will serve college teachers of biology and chemistry equally well.

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