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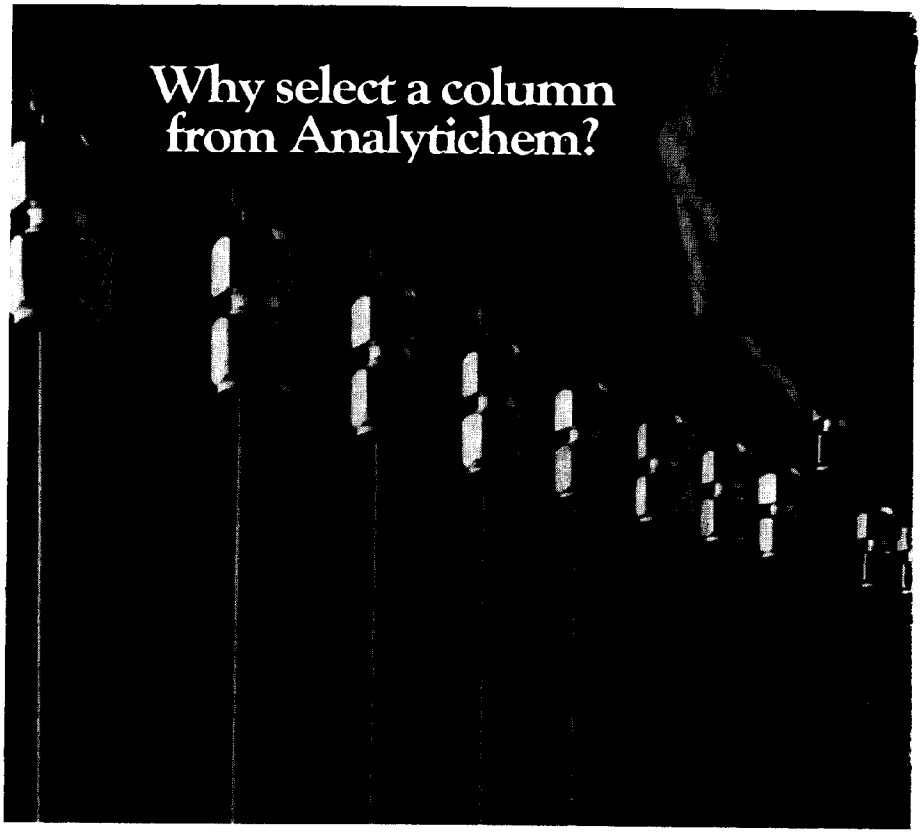

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Special Section on Thin-Layer Chromatography

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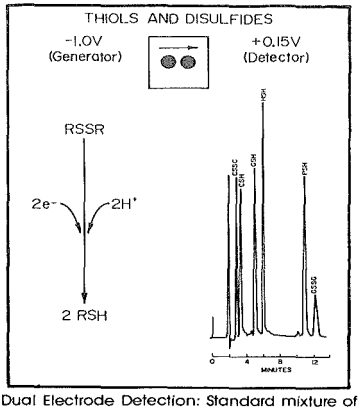

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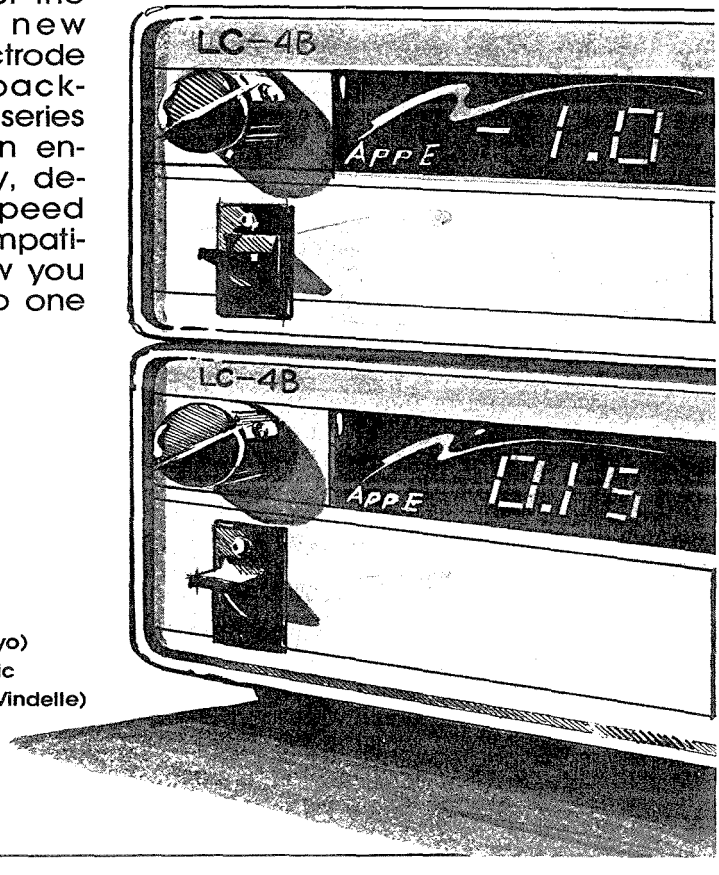

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JOURNAL OF LIQUID CHROMATOGRAPHY, 6(14), 2621-2634 (1983)

FRACTIONATION INTO COMPONENTS OF A MIXTURE OF ACIDIC NINHYDRIN-POSITIVE COMPOUNDS OF MOUSE BRAIN EXTRACTS WITH THIN-LAYER AND ION-EXCHANGE CHROMATOGRAPHY

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ABSTRACT

A mixture of more than ten acidic or highly ionized ninhydrin-positive compounds of mouse brain extracts, occurring in ion-exchange chromatography from the beginning up to aspartic acid, was analyzed using ion-exchange and thin-layer chromatography standardized with known substances. These formed four peak groups in the chromatogram of an automatic amino acid analyzer. The first group contained cysteic acid, cysteinesulfinic acid and phosphoserine, which could be separated from each others only by thin-layer chromatography. Only phosphoserine could be identified in the brain extract (about 0.14 mmol/kg brain wet weight), however. Taurine (4.1 mmol/kg) and phosphoethanolamine (1.05 mmol/kg) in the second group could be satisfactorily separated from each others after the hydrolysis of glycerylphosphoethanolamine (about 0.6 mmol/kg) and certain acidic peptides with 6 mol/l HCl. Hypotaurine (0.03 mmol/kg) and urea (6.6 mmol/kg) were completely overlapped in the third peak, but urea was decomposed in the hydrolysis with 6 mol/l HCl. The fourth group consisted of aspartic acid (2.1 mmol/kg). A number of low-molecular weight peptides also appeared in the chromatograms, above all in the phosphoserine and taurine peaks, but they were eliminated by the hydrolysis. They contained, however, some of the above-mentioned critical amino acids (phosphoserine, taurine and aspartic acid).

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INTRODUCTION

Tissue extracts, and brain extracts in particular, contain a large number of acidic amino acids and certain other ninhydrin-positive compounds, which in the ion-exchange chromatography (e.g. in the automatic amino acid analyzers) can not be separated satisfactorily well from each others, but appear in two or three peaks in the chromatograms (1-6). This fraction (often called "taurine fraction") is a complex mixture of more than ten (see Ref. 1) acidic or highly ionized compounds of physiological importance, including taurine, phosphoserine, phosphoethanolamine, glycerylphosphoethanolamine, hypotaurine, cysteic and cysteinesulfinic acids, carbamylphosphate, urea and glucosaminephosphate. Moreover, the presence of a number of low-molecular weight peptides, including taurine and phosphoserine peptides, which are soluble in sulfosalicylic, trichloroacetic and perchloric acids used in the preparation of the tissue samples, has been reported (7,8).

Taurine (2-aminoethanesulfonic acid) is often the main constituent in the free amino acid pool, but how real are its concentration values calculated from the mixture of the most acidic amino compounds eluting in the ion-exchange chromatography just before aspartic and glutamic acids (5,7,9,10) ? For their strong ionization or having phosphoric acid groups or other extra acidic moieties, the amino acids of the "taurine fraction" as well as the peptides containing taurine and phosphoserine (7,8,11-15) behave similarly in the chromatography, in the ion-exchange chromatography in particular, and overlap each others. The first eluting acidic ninhydrin-positive compounds may thus distort seriously each others' quantitative determination. A good attempt

to clarify the chromatographic determination of the components of the taurine peak has recently been made by Kontro et al. (5) and Marnela (10).

Simple hydrolytic and chromatographic means, which for their part bring facilitation to the exact quantitative determination of the individual components of the "taurine fraction" are given here. The methods were standardized with pure amino acids and some peptides as well as with certain taurine and phosphoserine peptides prepared from calf brain synaptosomes (7). The results, although not yet definitive, will show that serious attention must be paid to the interpretation of the chromatograms regarding compounds eluting before aspartic acid.

MATERIAL AND METHODS

Whole brains of NMRI mice were used, but the subcellular fractions were prepared from calf brains. Newly removed brains were rinsed free of external blood clots, and the cortical layer from calf brains separated with a knife. The synaptosomes were prepared from the homogenates in cold 0.32 mol/l sucrose by the gradient centrifugation method of Whittaker et al. (16).

Tissue samples were homogenized in 5 % trichloroacetic acid (TCA), the precipitated proteins removed by centrifugation, and the TCA then removed by shaking with diethyl ether. The residual solution was lyophilized and the amino acids and peptides then dissolved in water. When 15 % perchloric acid (PCA) was used, the precipitable proteins were first removed by centrifugation and the supernatant then neutralized with KOH and the potassium perchlorate centrifuged. The neutral, protein-free extracts were lyophilized and the residue taken up in acidified water and used for chromatogra-

phic analysis. The extracts from the synaptosomes were prepared in a similar manner.

The amino acid samples were quantitatively analyzed in an automatic amino acid analyzer (Kontron Liquimat III), equipped with a two-channel peak integrator. Ion-exchange chromatography in separate short columns according to Garvin (17) also was used. Thin-layer chromatography was performed on silica gel (Kieselgel G) plates of thickness 0.25 mm. Two-dimensional chromatograms were developed with 70 % ethanol in water and 75 % phenol in water. The dried plates were sprayed with ninhydrin (0.5 % solution in butanol:acetic acid, 20:1, pH 3.3). The spots were developed for 10 min at 105 ^{o}C.

Two unknown hydrolyzable spots on the TLC plates from calf brain synaptosomes (7,9) were projected to the corresponding unsprayed plates, the silica gel scraped up and the peptides extracted with water and lyophilized. They were analyzed for the amino acid composition after the hydrolysis with 6 mol/l HCl, and used as such for studying the chromatographic mobility in an amino acid analyzer.

Commecial pure amino acids (E. Merck, Darmstadt and Fluka AG, Buchs, Basel, Switzerland) and γ-glutamyl-taurine (Chinoin Pharmaceuticals, Budapest) were used as standards.

RESULTS

As shown in Figure 1, the initial part of a chromatogram of the PCA extract of mouse brain, obtained with a modern "high resolution" automatic amino acid analyzer, contained more than ten different ninhydrin-positive compounds, which could not be separated satisfactorily well from each others. Using known amino acid

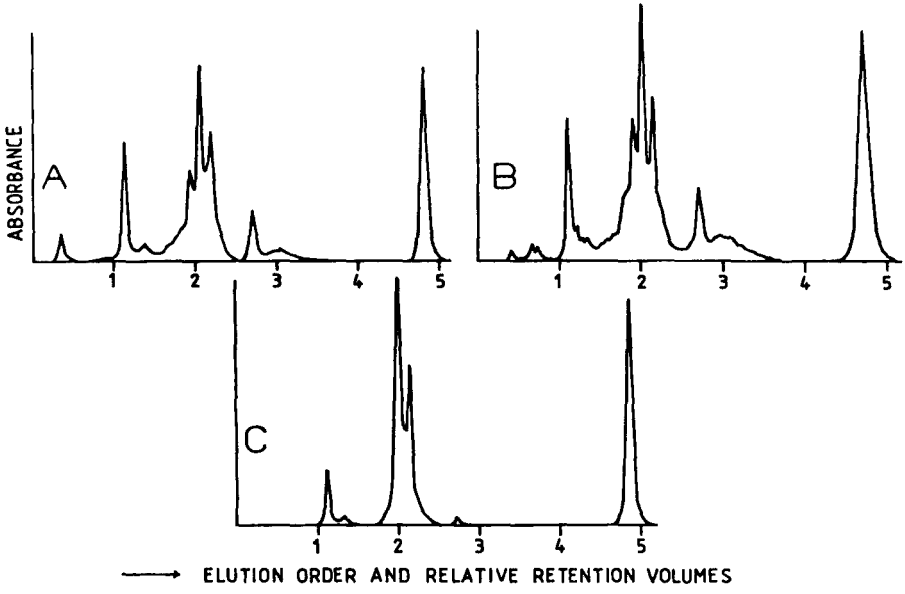

Figure 1. Initial parts of the ion-exchange chromatograms of the amino compounds of mouse brain extracts before (A) and after the hydrolysis with 2 mol/l (B) or 6 mol/l (C) HCl from a "high resolution" automatic amino acid analyzer (Kontron Liquimat III). For identification, see Figure 2.

or peptide samples, the retention volumes of 14 different compounds were determined (Figure 2). These formed four peak groups, which could be separated quite well from each others, but inside the groups the peaks of different compounds had almost the same retention volumes. The first group contained carbamylphosphate, cysteic acid, cysteinesulfinic acid, phosphoserine, γ-glutamyltaurine and certain other taurine peptides (see Ref. 8). The second group contained taurine, phosphoethanolamine, glycerylphosphoethanolamine and certain phosphoserine and other acidic peptides (see Ref. 8), and the third

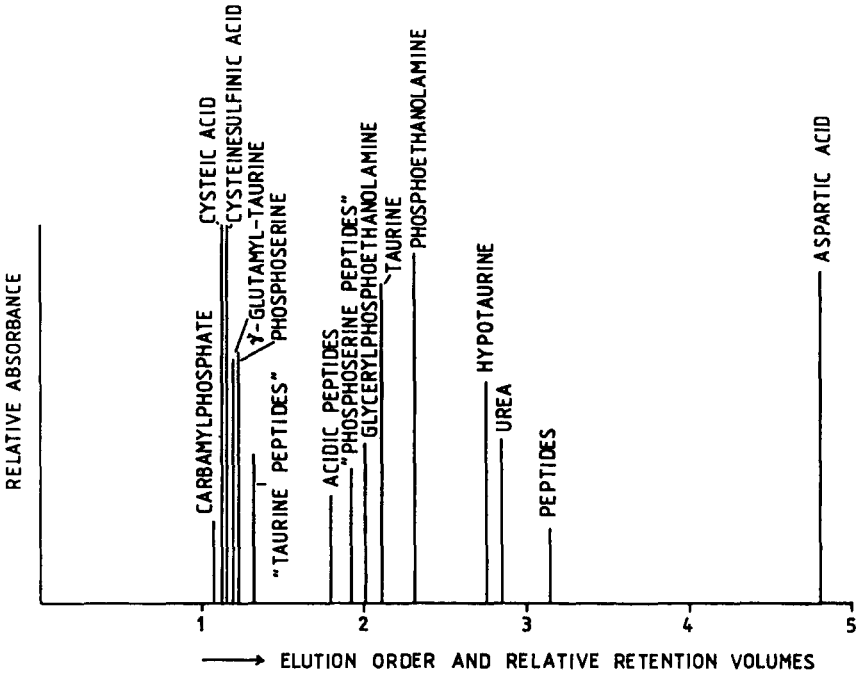

Figure 2. Retention volumes of 14 different acidic ninhydrin positive compounds analyzed with an automatic amino acid analyzer (Kontron Liquimat III) from standard amino acids, γ-glutamyl-taurine and certain other peptides prepared from calf brain synaptosomes. The hight of the absorbance lines depicts approximate molar sensitivity of the ninhydrin reaction.

group hypotaurine, urea and again some synaptosomal peptides. The fourth group consisted of aspartic acid.

The concentrations of the amino compounds of the PCA extract of mouse brain, calculated from the chromatograms of Figure 1, are given in Table 1 before and after the hydrolysis with 2 and 6 mol/l HCl. Most of the peaks or peak groups of the brain extract could be identified with the standard samples (Figure 2), but very low concentrations of unknown compounds still ap-

TABLE I

Concentrations of the acidic ninhydrin-positive compounds of mouse brain after extraction with perchloric acid and after the hydrolysis with 2 and 6 mol/l HCl.

Compound	Concentrations mmol/kg wet weight as such	2 M HCl	6 M HCl
unknown	0.01	-	-
unknown	-	0.02	-
Cysteic acid Cysteinesulfinic acid Phosphoserine	0.25	0.18	0.14
unknown	0.01	0.01	0.01
Peptides (ninhydrin-positive)	0.02	0.02	-
"Taurine peptides" (ninhydrin-negative)	-	0.27	-
Peptides (ninhydrin-positive)	0.55	0.50	-
"Phosphoserine peptides" (ninhydrin-negative)	-	0.21	-
Glycerylphosphoethanolamine	0.72	0.55	-
Taurine	4.10	4.90	4.88
Phosphoethanolamine	1.70	1.04	1.65
Hypotaurine Urea	6.86	6.59	0.03 -
Aspartic acid	2.06	6.87	7.50

Results are means of 5-6 experiments, S.D. being about 10 %. The order of substances refer to Figure 1. The concentrations of the peptides and unknown compounds were calculated using alanine as a standard.

peared both before and after the hydrolysis. Certain peptides or peptide groups appeared between the single amino acids. A part of these became visible not until after the hydrolysis with 2 mol/l HCl (for 30 min in a boiling water bath), which is known to hydrolyze the acyl groups from the amino group. This procedure converts the originally N-acetylated peptides ninhydrin-positive. All the peptides disappeared in the hydrolysis with 6 mol/l HCl. The amount of aspartic acid increased 3 fold in the hydrolysis with 2 mol/l HCl, and was derived apparently from the hydrolysis of N-acetylaspar-

tate present in brain tissue (18), and still a little in the hydrolysis with 6 mol/l HCl, this aspartate being derived from the hydrolyzable peptides (4,9). The peaks of hypotaurine and urea were completely overlapped (Figure 2), but urea disappeared in the HCl hydrolysis, and a small amount of hypotaurine remained after the hydrolysis. As satisfied with the HCl treatments of the standards, hypotaurine, taurine, phosphoethanolamine, phosphoserine, cysteic acid and cysteinesulfinic acid did not be decomposed either in the 2 or 6 mol/l HCl solution.

The peaks of taurine, phosphoethanolamine and glycerylphosphoethanolamine appeared very close together (see also Ref. 5). A small decrease in the concentration of phosphoethanolamine due the hydrolysis with 2 mol/l HCl was observed, indicating that its original peak contained some hydrolyzable, ninhydrin-positive material. At the same time the amount of taurine increased slightly, but it is not known, what this increment in the taurine peak arises from, but probably from the large number of different acidic N-acetylaspartyl peptides (8), which are converted ninhydrin-positive by the hydrolysis with 2 mol/l HCl. Glycerylphosphoethanolamine was completely hydrolyzed to phosphoethanolamine, after which the determination of taurine and phosphoethanolamine was more reliable.

Cysteic and cysteinesulfinic acids and phosphoserine could not be separated from each others, but their common amount decreased significantly after the acid hydrolysis, indicating again some hydrolyzable material present in their original peak. In two-dimensional thin-layer chromatography, using 70 % ethanol and 75 % phenol in water, these critical amino acids could be separated very well from each others (Figure 3). Phosphoserine was

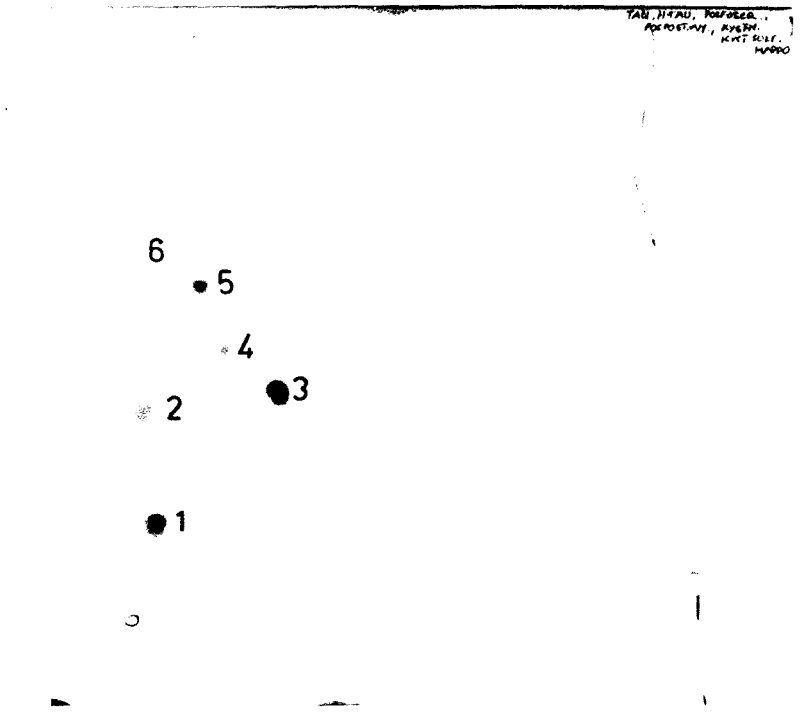

Figure 3. A two-dimensional thin-layer chromatogram of certain acidic amino acids, analyzed from a standard sample on 0.25 mm thick silica gel plates. The R_f values in brackets are $R_{fI}:R_{fII}$ = 70 % ethanol: 75 % phenol in water. 1 = phosphoethanolamine (0.17:0.03), 2 = phosphoserine (0.40:0.01), 3 = hypotaurine (0.50:0.27), 4 = taurine (0.57:0.17), 5 = cysteinesulfinic acid (0.71:0.12), 6 = cysteic acid (0.73:0.07).

identified in the mouse brain extract (Figure 4), but no traces of cysteic or cysteinesulfinic acid were observed. The first peak group (phosphoserine-cysteic acid group, Figure 1-2) contained thus apparently only phosphoserine after the hydrolysis with 6 mol/l HCl.

The separation of taurine and phosphoethanolamine from each others was unsuccesful in the ion-exchange

Figure 4. A two-dimensional thin-layer chromatogram of mouse brain extract, treated with 2 mol/l HCl for 30 min and run under identical conditions to that of Figure 3. 1 = probably phosphoethanolamine, 2 = probably phosphoserine.

chromatography by the method of Garvin (17, Table 2). Several other acidic amino acids came partially in the same fraction with these, but phosphoserine not. This old method is thus unsatisfactory for the determination of taurine, since it is contaminated by other acidic amino acids. More useful might be the o-phthalaldehyde-

TABLE II

Recovery percentages of taurine and certain other acidic compounds in ion-exchange chromatography performed according to the method of Garvin (17).

Compound	Recovery %
Taurine	100.0
Hypotaurine	26.2 ± 5.0
γ-Glutamyl-taurine	27.4 ± 15.2
Cysteic acid	22.6 ± 18.1
Phosphoethanolamine	115.2 ± 9.8
Phosphoserine	8.2 ± 2.8

Results (means ± S.D.) are from 3 determinations. The recovery value of taurine was taken as 100 and others expressed relative to this.

urea reaction of Gaitonde and Short (19), since it is more specific for taurine, even though performed after the passage of the tissue extract through the ion exchange resins, when there again is a possibility of contamination.

The acidic low-molecular weight peptides occurring in the chromatograms between the amino acids have been studied elsewhere (4, 7-9). They contained 5-6 different amino acids among which acidic amino acids, aspartic and glutamic acids, phosphoserine and taurine have a main role.

DISCUSSION AND CONCLUSIONS

The complicated composition of the fraction of the acidic ninhydrin-positive compounds in tissue extracts has been known for several years; at least 10-15 different amino acids or their derivatives have been reported in the ion-exchange chromatograms in two or three peak grouos before aspartic and glutamic acids (see e.g. Ref. 1, p. 50-51). Numerous attempts have been made to divide

these mixtures of compounds into fractions of individual substances, but the problem is still largely the same: the most acidic compounds, being eluted in the volume of cysteic acid and taurine in partucular, are completely overlapped.

The strongly acidic functional groups in the compounds dealed here make their pI values low. For instance the pK_a values of taurine are 1.5 and 8.74, giving the pI value of 5.12 for it (20). The situation is the same with most of the above-mentioned amino acids and peptides, which contain extra acidic groups. The free acidic groups in them seem to be the main chromatographic determinant in the ion-exchange chromatography, and for this reason several different compounds fall into the same fraction.

The present study led us to the conclusion that the direct analysis of a brain extract does not give correct results about the concentrations of the acidic amino acids. Better quantitative results can be obtained by comparing the analysis results after the hydrolysis of the sample with 2 and 6 mol/l HCl. In some cases qualitative thin-layer chromatography is essential for the identification of the amino acids.

ACKNOWLEDGEMENTS

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ANALYTICAL SEPARATION OF REDUCED AND OXIDIZED FORMS OF GLUTATHIONE FROM AMINO ACID MIXTURES BY OVERPRESSURED THIN-LAYER CHROMATOGRAPHY

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ABSTRACT

A new method has been developed for the separation of reduced and oxidized forms of glutathione from amino acid mixtures. The samples were spotted on Kieselgel plates and developed in phenol-water = 7:3 containing SDS. The separation was performed in pressurized ultramicro chamber. The running time was shorter than that in a normal chamber, decreasing the possibility of diffusion. In addition to the two forms of glutathione, nine different kinds of amino acids could be separated. The method affords a possibility for rapid analysis of two forms of glutathione both in the clinical and industrial practice.

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INTRODUCTION

The tripeptide glutathione, γ-glutamylcysteinylglycine, has an important role in biochemical processes, such as the maintenance of the sulfhydryl state of cysteinyl side-chains of proteins, regulation of enzyme activity by disulphide interchange, detoxifying processes, removal of peroxides generated in the metabolism (1). Glutathione is present mainly in its reduced form in the tissues and the oxidized one is a lower concentration.

There are several chromatographic methods for determination of glutathione. It has been separated with two-dimensional paper chromatography using phenol and collidine as solvents, or one-dimensional chromatography using propanol-water mixture after blocking of the sulfhydryl groups with N-ethylmaleimid (2). Glutathione and its derivatives could be separated from other thiols and ninhydrin-positive compounds by HPLC analysis (3-4) or in amino acid analyser (5).

Murata and his cowerkers (6) have published a procedure for the continuous production of glutathione from its constituent amino acids using immobilized whole cells of Saccharomyces cerevisiae entrapped in poliacrylamide gel. The cell membrane has become permeable for glutathione releasing it continually from the cells. An intensive amino acid synthesis accompanying the tripeptide production has been found.

The aim of the present study was the separation and determination of reduced (GSH) and oxidized glutathione (GSSG) in a biological mixture. The overpressured thin-layer chromatographic technique (OPTLC) reported by Thihák et al. (7-8) has been adopted. The method proved to be suitable for the separation of two forms of glutathione in phenol--water solvent system which has high viscosity.

MATERIALS AND METHODS

Chemicals for general use were analytical grade and purchased from Reanal Factory of Laboratorial Chemicals (Budapest, Hungary). Amino acid calibration mixture was purchased from BIO-RAD Laboratories (Richmond, California, USA), phenol was obtained from Loba Chemie (Wien, Austria) and it was vacuum destilled from magnesium. The Kieselgel 60 F_{254} and HPTLC chromatoplates were purchased from Merck (Darmstadt, FRG).

The development of sheets was performed in Crompres[10] pressurized ultramicro chamber obtained from Labor MIM (Budapest, Hungary). The concentration of glutathione was measured with Telechrom OE-974 videodensitometer, Chinoin Pharmaceutical Work, Ltd. (Budapest, Hungary).

EXPERIMENTS

Saccharomyces cerevisiae IFO 2044 cells were entrapped in polyacrylamide gel according to the method of Murata et al. (6).

For the production of glutathione the immobilized cells were incubated in a mixture containing 0.5 M glucose, 0.01 M $MgCl_2$, 0.02 M L-glutamate, 0.02 M L-cysteine, 0.02 M glycine and 0.1 M potassium phosphate buffer (pH 7.0), at 303 K.

The amino acids and glutathione were dissolved in 0.1 M potassium phosphate buffer (pH 5.5), their concentration varied from 1.7 to 2.3 mg/ml, each spot represents about 3-4 µg amino acid.

The chromatoplates were developed in phenol-water 7:3 (w/w) solvent system containing sodium dodecyl sulfate (SDS).

The concentration of SDS was varied between zero and one percentage. The chromatography was performed in Chrompres[10], in linear arrangement, at 303 ^{o}k. The development required about 50 minutes with a longer migration distance, setting a filter paper stripe (20x13 cm) on the top of chromatoplates. The development was conducted until the solvent saturated the filter paper. The membrane pressur was 1.2 MPa. Flow rate was varied from 3.08×10^{-3} cm^3/sec to 2.75×10^{-3} cm^3/sec, depending on the concentration of SDS in the solvent.

To remove the phenol from the layers, the plates were dried at 413 K, in -0.9 kp/cm^2 vacuum for 30 minutes.

The identification of amino acids happened with ninhydrin reagent sensitivised with polychromatic γ-collidine. The composition of staining reagent was the following:

50 ml solution A + 50 ml solution B + 2 ml γ-collidine. Solution A contained 2 g of ninhydrin in 100 ml acetone and solution B 1g of cupper acetate dissolved in 245 ml of deionized water, to which were added 5 ml of glacial acetic acid and 250 ml of acetone.

RESULTS AND DISCUSSION

The migrations of glutathione and different amino acids were investigated in phenol-water = 7:3 solvent system containing SDS in different concentrations, on Kieselgel 60 F_{254} plates. Without SDS the GSSG has been separated from the neighbour amino acids, however the GSH has migrated close to the threonine and asparagine. The relative migration distances of glutathione and amino acids are shown in the Table I. The relative migration distances are refered to valine because

Table I.

Effect of SDS on the relative migration distances of glutathione and amino acids referred to valine in phenol-water = = 7:3 solvent system containing SDS in different concentration.

	Relative migration distance		
	Phenol-water = = 7:3	Phenol-0.5% SDS = 7:3	Phenol-1% SDS = 7:3
Lys	0.036	0.119	0.245
Arg	0.094	0.238	0.570
GSSG	0.144	0.081	0.070
His	0.194	0.238	0.290
Asp	0.244	0.163	0.115
Cys_2	0.244	0.325	-
Glu	0.300	0.238	0.200
Cys	0.344	0.263	0.245
Ser	0.344	0.344	0.355
Gly	0.444	0.431	0.475
Thr	0.544	0.538	0.570
Asn	0.569	-	0.570
GSH	0.575	0.519	0.410
Ala	0.650	0.656	0.675
Gln	0.781	0.794	0.810
Val	1.000	1.000	1.000
Tyr	1.081	1.088	1.110
Met	1.156	1.163	1.150
Leu	1.156	1.163	1.150
Ile	1.156	1.163	1.150
Met	1.156	1.163	1.150
Pro	1.206	1.250	1.150
Trp	1.256	1.250	1.200
Phe	1.256	1.313	1.200

of the longer migration distance. The following amino acids showed well defined spots: Lys, Arg, His, Glu, Gly, Ala, Gln, Val, Tyr and Pro.

The order of several components have changed in phenol -0.5% SDS = 7:3 solvent system. The migrations of GSH and GSSG, Asp, Glu and Cys have decreased and those of Lys, Arg, His have increased. His, Glu and Arg run with the same velocity. The relative migration distances are presented in the Table I. The oxidized form of glutathione could be separated again, but the reduced form migrated near the threonine.

In order to separate and quantify the GSH, the concentration of SDS has been enhanced. Developing the plates in phenol-1% SDS = 7:3, the two forms of glutathione have been separated from amino acids, the position of GSSG has not changed. The GSH was localized as a well separated spot under the glycine. The relative migration distances of the components are shown in the Table I. Beside the two forms of glutathione the following amino acids were separated: Asp, Glu, His, Ser, Gly, Ala, Gln, Val and Tyr. The localization of glutathione forms and amino acids both from standard mixture and biological samples are shown in Fig. 1. Lys and Cys migrated with the same velocity, and Asn, Thr and Arg could not be separated from each other in this solvent system.

Fig. 2 shows the densitometric calibration curve for the quantitative evaluation of GSH and GSSG. The most preferable range for the determination is about 1-10 µg GSH and 1-5 µg GSSG in a spot.

In biological samples the reduced glutathione is easily converted into its oxidized form in solution or during the development. To avoid this effect, the samples were bubbled

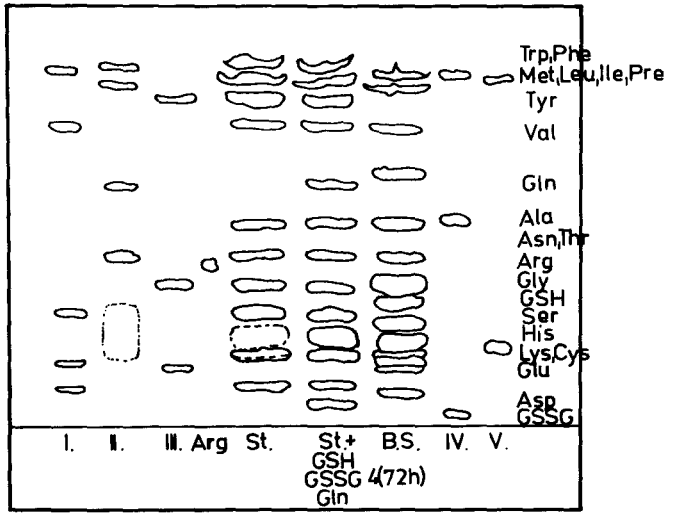

Fig. 1. Separation of reduced (GSH) and oxidized (GSSG) glutathione from amino acid mixture and biological sample on Merck 5548 HPTLC plate, in phenol-1% SDS= =7:3 solvent system. Flow rate: 2.92×10^{-3} cm^3/sec.

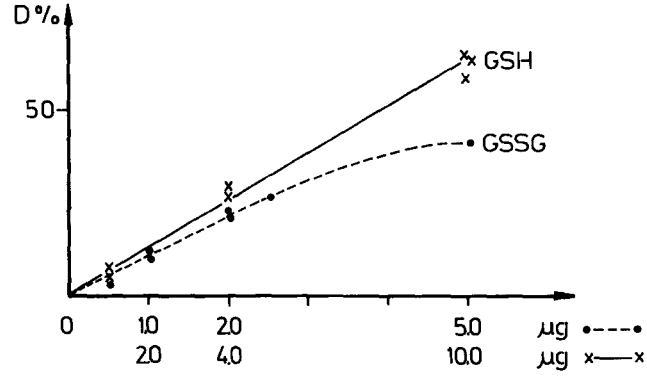

Fig. 2 Calibration curve for reduced and oxidized form of glutathione evaluated in videodensitometer.

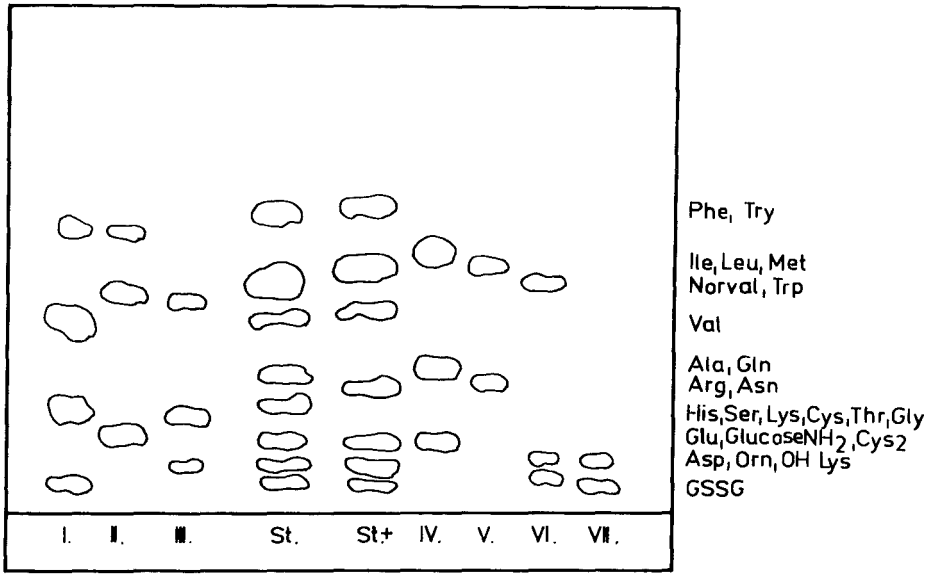

Fig. 3 Development of glutathione and amino acids in normal chamber on Merck 5554 Kieselgel 60 F_{254} plate, in phenol-1% SDS solvent system. Running time: 9 hours.

with oxigen. By this way the total glutathione content might be determined quantitatively by videodensitometer.

These findings suggest that the two forms of glutathione can be separated from amino acid mixtures in phenol-1% SDS = = 7:3 solvent system by OPTLC method. Comparing the development of samples in pressurized ultra-micro chamber with normal chamber (Fig. 3) it can be stated, that the previous method is suitable especially for solvent systems having a high viscosity. The slow migration can be reduced from 8-9 hours to 50 minutes in the case of our solvent system, decreasing the possibility of diffusion of components.

Development in phenol-water = 7:3 solvent system the reduced form of glutathione can not be separated from asparagine. The SDS changes the selectivity of the original solvent and the relative position of several components has changed. It is true especially for basic amino acids and GSH. Ahrland et al. (9) found that the titration curve of silica gel resembles that of a weakly acidic ion exchanger. A suppose is, that the detergent depletes the ion exchange capacity of layer and its partitional feature has been achieved. By this way the separation depends first of all on the polarity and size of different components.

According to our results a modified phenol-water solvent system containing SDS proved to be suitable for the separation of two forms of glutathione, especially for the oxidized form. The later showed a low migration velocity and could be localised as a well separated spot. On the basis of this phenomenon the application of OPTLC method might afford a possibility for rapid estimation of GSSG. By this way several amino acids, namely Asp, Glu, His, Ser, Gly, Ala, Gln, Val and Tyr could be separated from the glutathione forms and quantified, too.

The time of development was decreased from 9 hours to 50 minutes by application of OPTLC method, comparing it with the development in normal chamber. Reducing the running time, the possibility of diffusion was strongly limited. The oxidation of GSH in the biological samples prior to application might take possible the quantitative determination to escape the oxidation during the development. This method can be applied for rapid analysis of glutathione and amino acids in clinical and industrial practice.

Acknowledgement. We wish to thank Dr. H. Kalász for many helpful comments and suggestions.

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ISOLATION OF GLYCOLIPIDS FROM BLOOD ELEMENTS

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ABSTRACT

Several chromatographic e.g. HPTLC, HPLC, etc. methods have been published in the literature for the separation, after sufficient pretreatment, of derivatized or non-derivatized glycolipid samples.

Our task is the extraction, isolation and separation of the glycolipids from different blood elements, followed by suitable fractionation methods, giving the lipid classes in sufficient purity and quantity for HPLC, HPTLC and OPTLC measurements and possibly further biochemical use.

We show the differences between the procedures commonly used and that developed in our laboratory.

The advantage of our method, which employs 3 cm long Brownlee Labs HPLC cartridges, is that it can be automated, it gives class fractionation of the lipid samples and as it is hardware compatible with HPLC equipment it can be used directly in a coupled column system for on line separation in the individual class. The development of this column coupling method for the fractionation of a given lipid class from the total lipid extract on an analytical column is under development.

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INTRODUCTION

The sample preparation for most instrumental analytical methods is a time and labour consuming process. This is really true in the case of biological samples and HPLC, as the costly HPLC columns have to be protected from the various irreversibly or near irreversibly retained components of the biological sample. The use of short preparative columns for sample preparation is getting very common and is facilitated by the numerous commercial products available (Sep-Pak, Prep Elute, Bond Elute, Extrelute from Waters, Hamilton, Analytichem, Merck - to name a few - respectively). The problem with these is that they are suitable only for manual work (1,2), as they can not be installed in a chromatograph. We decided to develop a method suitable later for column coupling. The possibility of column coupling is necessary for our analysis, as the preparation of a very small sample can be performed with less wastage in that way.

The object of our investigations, the glycolipids of blood elements, are minor components of the cell membrane. The majority of glycosphingolipids are assumed to be present at the outer leaflet of plasma membranes and play very important biological roles. The functions of glycolipids (3,4) has been increasingly examined parallel with the methodological developments (5,6,7,8) of identification during the last few years.

At first only derivatized glycolipids were separated by HPLC (9) with UV detection, later non-derivatized glycolipids without on line detection (10) monitored by TLC runs. We presented non-derivatized glycolipid separation with refractive index (RI) detection (11), on line detection of these compounds was accomplished by Handa and Kushi (8) at 206 nm.

The importance of the separation of glycosphingolipids without derivatization is that the separated components can then be directly used for further biochemical, immunological and structural investigations.

EXPERIMENTAL

Reagents and materials. All the solvents used were HPLC grade purchased from E. Merck (Darmstadt, F.R.G.). The water emplo-

yed was prepared according to Gurkin's method (12), from water double distilled from glass after ion-exchange. DEAE Sephadex A-25 was obtained from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Staining reagents were prepared from analytical grade chemicals. ODS-GU and SIL-GU cartridges were purchased from Brownlee Labs Inc. (Santa Clara, Calif., USA). The HPTLC plates used for monitoring HPLC fractions and purity checking were bought from E. Merck (Darmstadt, F.R.G.).
Total lipid extract was prepared from human lymphocytes.

Apparatus. Centrifuge: Janetzki K70 MLW (Leipzig, G.D.R.). Incubator: LP 507/1 Labor MIM (Esztergom, Hungary). Chromatographs: HP 1084 B with HP 79875 variable wavelength UV detector, Hewlett Packard (Palo Alto, Calif., USA), Beckman 112 solvent delivery module, Altex 156 differential refractometer, Beckman Instruments Inc. (Berkely, Calif., USA). Fraction collector: LKB Minirac 1700, LKB Produkter AB (Bromma, Sweden). Data system: HP 3354 Laboratory Automation System, Hewlett Packard (Avondale, Calif., USA).

Methods.

Total Lipid Extract (TLE) preparation. Lipid extraction was carried out similarly to the methods published previously (13, 14). The TLE was extracted from the liophilized cells at room temperature with mild sonication in chloroform : methanol mixtures in the ratios 2:1, 1:1, 1:2. The supernatant were collected together and dried in vacuo. The dried TLE was dissolved in chloroform : methanol : water = 30 : 60 : 8 mixture (100 ml) and the solution used for the isolation of the lipid classes described below.
Isolation of Acidic and Neutral Lipids (AcL, NL). The separation was performed on a ϕ12 x 70 mm DEAE Sephadex A-25 column in the acetate form. The solution of TLE (100 ml) was applied to the column, was washed with 100 ml of the same solvent, then with 50 ml of methanol. The 250 ml solution issuing from the column was used after solvent evaporation (water traces were removed with benzene), for the fractionation of neutral lipids, dissolved in chloroform, as given later.
Acidic lipids were eluted with 50 ml of 0.25 M methanolic sodium acetate. This portion of the eluent was dried in vacuo

and incubated in 15 ml 0.1 M methanolic sodium hydroxide at 37°C for 2 hours to destroy the alcali labile phospholipids (PL). We have developed a two step chromatographic method for the recovery and isolation of acidic glycolipids. The first step is similar to the method using Sep-Pak C18 cartridges (2). After incubation the sample was dried in vacuo and the residue was dissolved in cold (4°C) HPLC grade water, neutralized very carefully to $4<pH<5$ with 0.5 M HCl. The salt concentration was finally adjusted to 0.1 M by adding water. This sample solution was passed through an ODS-GU cartridge at a flow rate of 1.5 ml/min, the acidic glycolipids being collected on the column. After washing the cartridge with 30-50 ml of water, the glycolipids were eluted by 50 ml chloroform : methanol = 1 : 2 mixture.

Fractionation of Neutral Lipids. The neutral lipid portion derived from 2-8 x 10^{10} lymphocyte cells were taken up in 3 ml of dry chloroform and 5 - 30 µl aliquots were injected. Detection was at 254 : 600 nm, sample : reference wavelengths, attenuation 0.9 AUFS for the UV detector and 1 V attenuation FS for the RI detector. We employed the HP 1084 B LC equiped with UV and RI detectors coupled in series, with a fraction collector after the last detector.

Numerous elution profiles, given later, were tried, of which the selected chromatographic conditions were: flow 1 ml/min, gradient elution, solvent A was chloroform, B was methanol. The gradient started with 1 min. isocratic 100% A, followed by a linear increase to 100% B in 9 minutes, then a linear decrease to 100% A in 5 minutes. The run ended after further 5 minutes isocratic 100% A. This gradient, No.5, gave optimal results for our purposes. The separation was conducted at ambient temperature. In addition to on line detection the fractions collected during the run were also monitored by parallel TLC runs on silica plates. The empty fractions were discarded and those containing the same components (same subclass) united. Of the two chromatograms one was stained with 1 : 1 sulfuric acid : water, to visualize all of the components, giving also a specific red colour for cholesterol during heating. The other plate was stained with orcinol reagent, the neutral glycolipids appearing as purple spots.

RESULTS AND DISCUSSION

There are several methods for the separation of the total lipids, derived from biological sources such as cell membranes, in the literature. To have a comparable picture the commonly used methodologies have been summarized in flowcharts, shown in Figure 1. Comparing flowcharts 1, 2 and 3, it can be clearly seen that all these methods require a great amount of work, time and materials, especially high purity solvents. The trend of analysis of biological samples points to the importance of pretreatment and sample preparation (15). Because of the high cost of HPLC grade chemicals and analytical columns the use of guard and/or precolumns and rigorous sample pretreatment became more and more necessary, while decreasing the time and material requirements of the analysis was also needed.

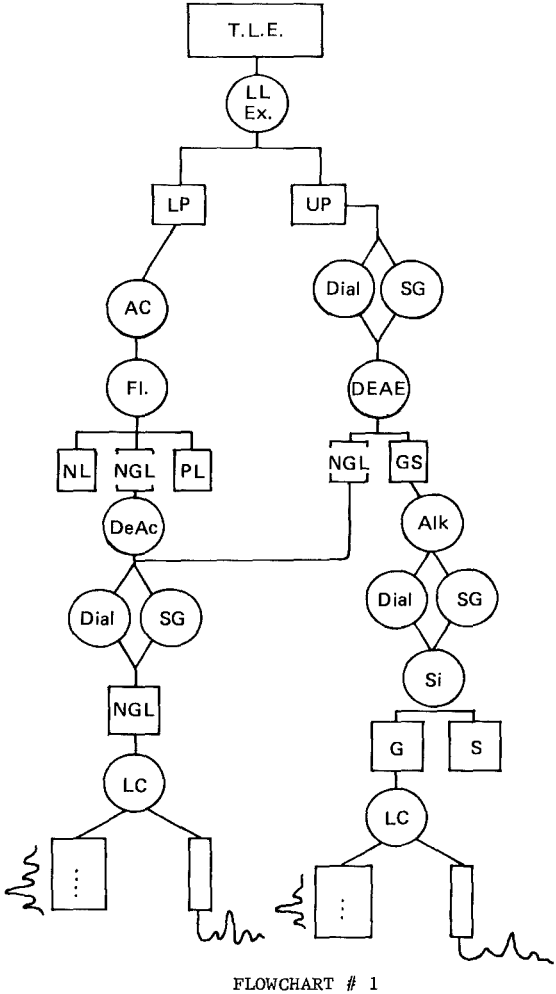
A further requirement can be inherent in the samples, like in our case. The sample can be extremely small amount and unrepeatable (for example a lymphocyte sample of a leuchemic patient before chemotherapy). For this reason the whole analytical process has to be precise, reproducible and rugged.

The method developed in our laboratory for the separation of TLE into lipid classes in purity, adequate for further fractionation into separate lipid types, has the advantage of speed, automatebility and the possibility of development into an on line complete HPLC fractionation.

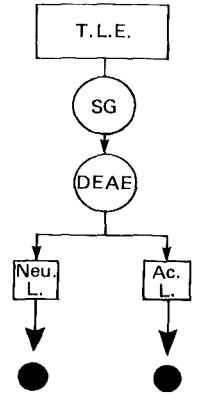
The first step in our method of TLE fractionation is the known DEAE Sephadex column chromatography for the isolation of neutral and acidic lipids. For the following steps of the separation we applied Brownlee Labs SIL-GU and ODS-GU cartridges.

Comparing the flowcharts 1, 2, 3 with 4 it can be seen (Figure 1) that the use of small cartridge columns for the class fractionation after the separation of acidic and neutral lipids is much simpler and more economic in terms of labour, solvents and time.

The acidic lipid purification with the ODS-GU cartridge described in the experimental part was checked for purity of the ganglioside class with HPTLC as shown in Figure 2. As can be seen on the chromatogram, only resorcinol positive compounds are present.



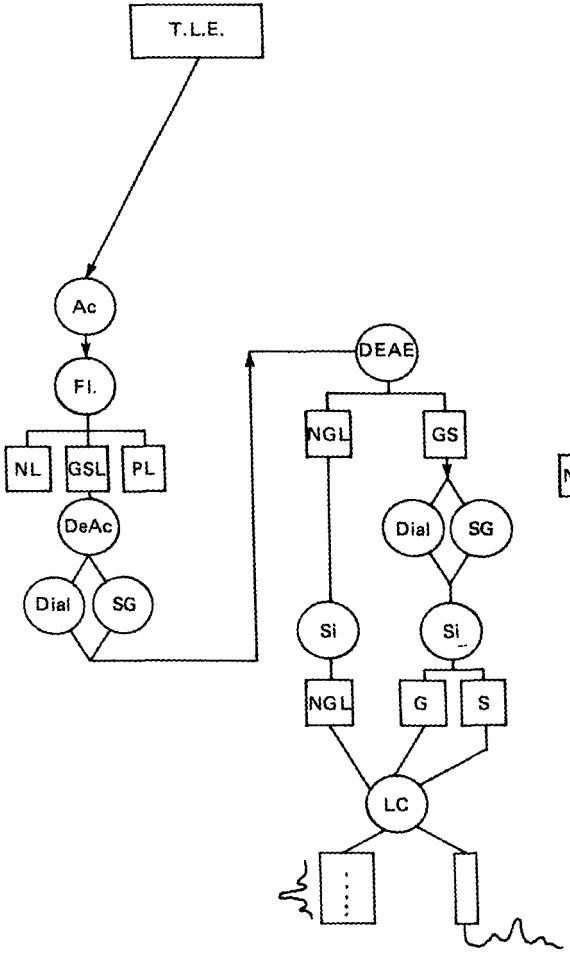
FLOWCHART # 1



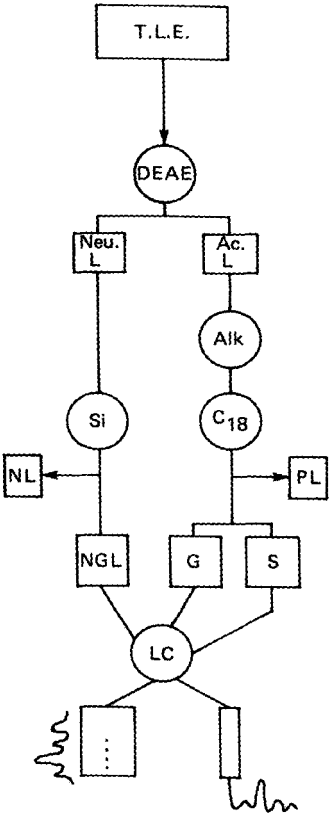
From here on same as #1

FLOWCHART #2

Figure 1. Possible work up routes of TLE.
Flowcharts #1 - 3 from literature data, #4 showing our method.
Symbols used: LL EX.: liquid-liquid extraction, LP: lower phase, UP: upper phase, Ac: acetylation, Fl: Florisil col.chrom., DeAc: deacetylation, Dial: dialysis, SG: Sephadex G col.chrom., G: gangliosides, S: sulphatides, Alk: alcalization, Si: silicic acid col.chrom., SI: SIL-GU col.chrom., C_{18}: ODS-GU col. chrom.



FLOWCHART #3



FLOWCHART #4

Figure 1 - continued

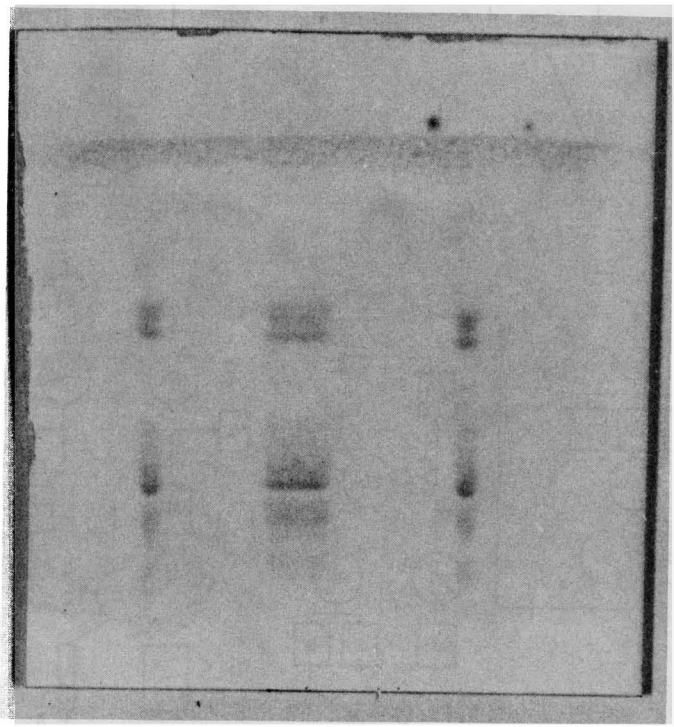
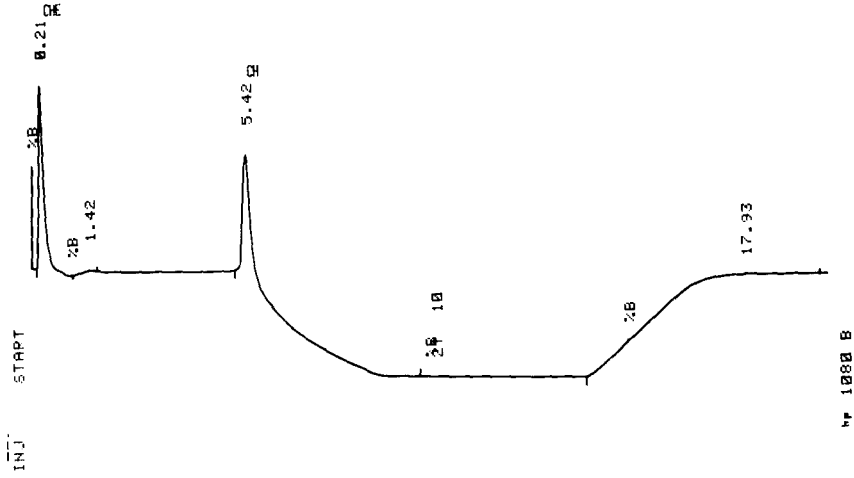


Figure 2. HPTLC separation of lymphocyte gangliosides to show their purity. Sample applications of 0.5, 1.5, 1.0 µl of ganglioside solution of 8 x 10^9 lymphocytes in 1.0 ml chloroform : methanol = 1 : 2 .
Plate: 5 x 5 cm Merck HPTLC Si 60, developing system: $CHCl_3$: CH_3OH : 0.025M KCl/H_2O = 60 : 40 : 9 , staining with resorcinol-HCl, 100°C, 20 min.



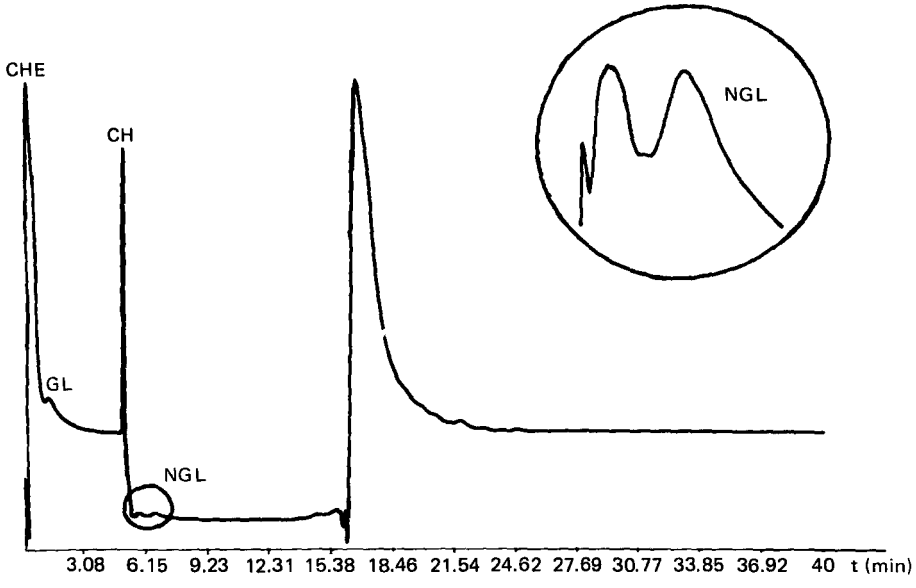
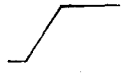


Figure 3. Chromatograms of lymphocyte neutral lipid fraction with elution profile No. 5.
1. UV detector signal, 2. RI detector signal.
CHE: cholesterol esthers, CH: cholesterol, NGL: neutral glycolipids.

Table 1. Elution profiles tested for the separation of neutral lipid classes on SIL-GU column, eluents; A:chloroform, B:methanol.

Gradient		SEGMENTS		%B at start %B at end duration min.			Flow	No. of
No.	profile	1	2	3	4	5	ml/min	fractions
1		0 0 1	0 100 10	100 100 15	-	-	1.0	26
2		0 10 10	10 100 5	100 100 1	100 0 4	0 0 20	0.5	60
3		0 0 3	0 100 9	100 100 3	100 0 4	0 0 20	0.5	20
4		10 10 20	100 100 10	10 10 10	-	-	0.5	20
5		0 0 1	0 100 9	100 0 5	0 0 25	-	0.5	5
6		7 7 1	7 100 9	100 7 5	7 7 20	-	0.5	4
7		30 30 40	-	-	-	-	0.5	6

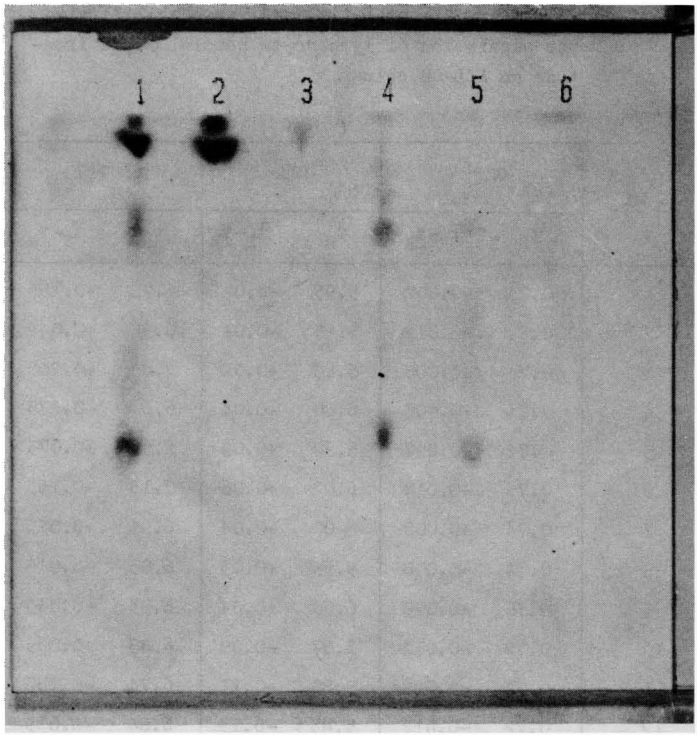


Figure 4. General monitoring plate of the separation of lymphocyte neutral lipid fraction on SIL-GU column.
Plate: 5 x 5 cm Merck HPTLC Si 60, developing system: $CHCl_3$: CH_3OH : H_2O = 65 : 25 : 4 , staining with H_2SO_4: H_2O = 1 : 1 , 100°C, 2 min.
Lanes: 1. TLE, 2., 3. neutral lipids (CHE, CH, glycerides etc.) 4.,5. neutral glycolipids, 6. background of solvents evaporated.

Table 2. Retention time stability and reproducibility of the separation of lymphocyte neutral lipid fraction on SIL-CU column.

Run No.	CHE		CH		NGL	
	t_R	$\bar{t}_R - t_R$	t_R	$\bar{t}_R - t_R$	t_R	$\bar{t}_R - t_R$
1	0.73	-0.002	5.95	-0.01	6.72	-0.155
2	0.72	-0.012	5.95	-0.01	6.92	+0.045
3	0.74	+0.008	5.98	+0.02	7.08	+0.205
4	0.74	+0.008	6.00	+0.04	6.60	-0.275
5	0.74	+0.008	5.99	+0.03	6.95	+0.075
6	0.75	+0.018	6.02	+0.06	7.13	+0.155
7	0.74	+0.008	6.00	+0.04	6.95	+0.075
8	0.74	+0.008	5.99	+0.03	6.83	-0.045
9	0.76	+0.028	6.00	+0.04	6.83	-0.045
10	0.72	-0.012	5.87	-0.09	6.88	+0.005
11	0.71	-0.022	5.85	-0.11	6.74	-0.135
12	0.72	-0.012	5.83	-0.13	6.84	-0.035
13	0.72	-0.012	5.98	+0.02	6.79	-0.085
14	0.72	-0.012	5.99	+0.03	6.90	+0.025
15	0.76	+0.028	6.00	+0.04	6.97	+0.095
16	0.71	-0.022	6.01	+0.05	6.87	-0.005
$\bar{t}_R$	0.732		5.96		6.875	
SD	0.016		0.06		0.130	

The neutral lipid class fractionation was carried out on a SIL-GU column. The dried sample was dissolved in chloroform, as the use of chloroform-methanol mixtures for sample introduction interfered with the separation of cholesterol esthers and cholesterol, although it dissolves the sample much better. After isocratic exploratory experiments, gradient runs were tried. The elution profiles tested and relevant data are summarized in Table 1. Of the several gradients tested for neutral lipid class fractionation, No.5, the best, was selected in the end. The corresponding chromatograms are shown in Figure 3. The aim was sufficient separation between classes and if possible, negligable separation in the classes themselves. This was to make possible the collection of small fractions for total recovery, or column coupling with the heart-cut method for analytical separation of the lipids of a selected class. As shown on a general monitoring plate (Figure 4) the class separation is adequate.

The stability and reproducibility of the method can be seen from Table 2, giving the retention times, their average values and standard deviation from 16 runs. It can also be seen from the table, that column performance is stable, there was no unidirectional drift in the $\bar{t}_R - t_R$ data. Cholesterol esthers and cholesterol retention times are from the UV signal, those for neutral glycolipids from the RI signal. The retention times are corrected for the delay caused by the connecting tubing between the detectors.

As shown by the standard deviations the reproducibility is very good. Numerous samples can be analysed on a small cartridge without any significant change in system behaviour, as can be seen in the random character of the variation of $\bar{t}_R - t_R$ values.

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IDENTIFICATION OF SOME POTENTIAL ANTIDIABETIC COMPOUNDS ON IMPREGNATED SILICA GEL G PLATEA AS THEIR π-COMPLEXES

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ABSTRACT

A rapid and convenient procedure for the detection of potential antidiabetics as π complexes with aromatic amines is described. The method could be of immense value even in places where normal laboratory facilities are not available.

INTRODUCTION

The field of charge transfer complexes has been investigated recently by several chromatographic techniques. Unsaturated lipids have been resolved on columns of solica gel impregnated with silver nitrate (1). Thin-layer chromatography has been employed in the investigations of the complexes of silver with terpenes (2), glycerides (3) and silylic-propenylic isomers (4). The separation of the π - complexes of m-dinitrobenzene, 2, 4-dinitrotoluene, 2,4,5-trinitrotoluene and 2,4,6-trinitroanisole with some hydrocarbons on TLC plates has been carried out (5). Nitroaromatic compounds form π - complexes with aromatic donors (6) due to polarization of the nitro group. These π complexes involve hybrid structures with bedding of the filled Sp hybridized orbital on the nitrogen atom in the amine compound with the vacant p orbital on the nitrogen of the nitro group, e.g.

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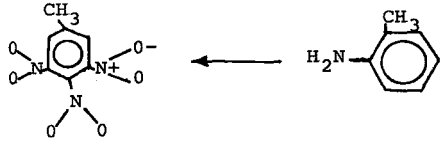
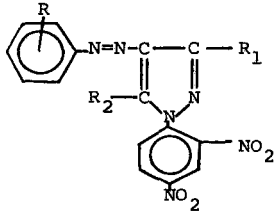


More recently it has been suggested that during the formation of 1:1 π complexes of the m-dinitrobenzene with various aminobenzoic acids only one nitro group plays role (7). The majority of the complexes are unstable. Solvent and adsorption forces, steric hindrance, temperature etc., weaken the charge transfer linkages and make their study difficult.

The present papers describes a simple and convenient method for the identification of trace amounts of 1-(2,4-dinitrophenyl)-3,5-dimethyl/diphenyl-4-arylazopyrazoles(A),which are well known as potential antidiabetic agents, as charge-transfer complexes with aromatic amines employing TLC technique. The resolved π complexes being highly coloured could be located easily on the chromatoplates. It was possible to identify as little as 1-2 μg of these compounds as their π complexes.



(A)

where R represents various substituents, R_1=R_2+CH_3 or C_6H_5

EXPERIMENTAL

The TLC plates (thickness 0.5 mm) where prepared by spraying a slurry of a mixture of 50g of silica gel G+1 g of aromatic amine in 100 ml of distilled water and drying for 24 hrs at a constant temperature of 60°C. The compounds (I-XX)

TABLE - 1

Sl No.	R	R_1	R_2	Rf x 100 A Plain Gel	Rf x 100 B Silica G	Rf x 100 A Silica Gen Toludine	Rf x 100 B C+p-	Detection limit (µg)
I	H	CH_3	CH_3	44	52	36	46	2.0
II	2-CH_3	CH_3	CH_3	55	49	29	40	1.5
III	3-CH_3	CH_3	CH_3	37	50	32	32	2.0
IV	4-CH_3	CH_3	CH_3	31	54	24	36	3.5
V	2-CI	CH_3	CH_3	21	28	20	29	2.0
VI	3-CI	CH_3	CH_3	22	34	21	23	1.5
VII	4-CI	CH_3	CH_3	25	35	16	19	1.5
VIII	2-OCH_3	CH_3	CH_3	39	42	41	52	2.5
IX	3-OCH_3	CH_3	CH_3	45	48	45	58	2.5
X	4-OCH_3	CH_3	CH_3	46	51	50	54	1.5
XI	H	C_6H_5	C_6H_5	30	46	28	42	1.0
XII	2-CH_3	C_6H_5	C_6H_5	27	40	23	38	1.5
XIII	3-CH_3	C_6H_5	C_6H_5	29	38	20	28	2.0
IXV	4-CH_3	C_6H_5	C_6H_5	32	32	18	31	2.0
XV	2-CI	C_6H_5	C_6H_5	09	16	07	19	1.5
XVI	3-CI	C_6H_5	C_6H_5	13	17	11	23	1.5
XVII	4-CI	C_6H_5	C_6H_5	15	20	13	16	2.0
XVIII	2-OCH_3	C_6H_5	C_6H_5	26	23	31	45	1.5
XIX	3-OCH_3	C_6H_5	C_6H_5	28	28	26	54	2.0
XX	4-OCH_3	C_6H_5	C_6H_5	33	30	35	51	2.0

* For p-toluidine impregnated plates
a - Average of two identical runs

Solvant Systems: (A) Xylene (60/): Chloroform (30/):Acetone (10/).
(B) Benzene (55/):Chloroform(40/): Ethyl methyl ketone(5/)

Rate of Development: 15 cm in 40 min for plain silica gel plates and 15 cm i
50 min for p-toluidine impregnated silica gel plates.

in acetone (0.05/ w/v) were applied to the chromatographic plates using glass capillary and the chromatograms were eluted with various solvents systems but the best resolution was obtained with solvent systems A and B (Table-1).

After thorough research it was found that a mixture of p-toludine and silica gel gave the most satisfactory separation. The results are presented in Table-1. For comparison the values on plain silica gel plates under similar conditions are also given. It can be seen that out of 20 pyrazoles only 12 can be separated on plain silica gel G plates. Further, it may be pointed out that the Rf-values are changed when a mixture of pryazoles was applied.

RESULTS AND DISCUSSION

The resolution of π complexes was found to be governed by three factors (i) adsorbent (ii) nature of the π donor (aromatic amine) and (iii) irrigating solvent. In general the migration of these compounds were in relation to the effect of electron attracting or repelling groups present in them.

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THE ROLE OF THE SPECIFIC SURFACE AREA OF AN ADSORBENT IN THE OPTIMIZATION OF MIXTURE SEPARATION CONDITIONS IN THIN - LAYER CHROMATOGRAPHY

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ABSTRACT

The very important aspect of effect of the magnitude of specific surface area on R_M values obtained by using TLC method is presented. In experiments were performed on 4 adsorbents of different specific surface areas 50 - 500 m^2/g and with mixed binary mobile phases.

It is shown that R_M values of chromatographed substances aromatic hydrocarbons are lineary dependente upon the speciiic surface area of adsorbents for each composition of mobile phase. This relationship can be described by a straight line, with the parameters a and b that can be tabularized. These lines can be used to calculate the R_M values of chromatographed substances for any adsorbent if

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is specific surface area is known. The illustrational comparison of experimentally obtained and theoretically predicted R_M values for different chromatographic system are presented.

INTRODUCTION

Adsorbents of difierent microporous structure (1-5) have been used for a long time in thin-layer chromatography for the separation of mixtures of various substances. The microporous structure of adsorbents is an important affacting optimization of the chromatographic process. The significance of the microporous structure of the adsorbent in the process of thin-layer chromatography was studied by Geiss (6) and Snyder (7) . Practical possibilities of controlling the process of thin-layer chromatography were also indicated by Różyło (8,9). Attemps were also explain the physical-chemical significance of the value of the adsorbent specific surface area in thin-layer chromatography (10). Recent considerable interest in the role of the specific surface area of the adsorbent in thin-layer chromatography is due to growing importance of this method as a pilot technique for determining the optimum conditions of mixture separation on both analytical and preparative scales. The latter is widely applied for obtaining and purifying of natural origin in research laboratories, as well as in pharmaceutical and food industries, and also in the nature enviroment protection (11).

Linear relations between R_M values of substances for pure components of a mixed e.g. binary mobile phase $R_{M1.2}$

and the specific surface area of the adsorbent, as has already been observed - are not favourable enough to be applied in a routine process of optimization of separation conditions. Therefore, the present paper analyses the relations between the R_M values of substances and the specific surface area of the adsorbent. The values were investigated for identical concentrations of the mobile phase on adsorbents of different values of the specific surface area.

EXPERIMENTAL

Measurements were taken of the R_F (R_M) values of model substances obtained in the process of adsorption thin-layer chromatography on silica gels of different microporous structure, produced by MERCK (12):

pore diameter Å	specific surface area m^2/g
60	500
100	400
200	150
500	50

The chromatographed substances were certain polycyclic aromatic hydrocarbons showing neither electrodonor nor electroacceptor properties: naphthalene, β-methylnaphthalene, pyrene, chrysene, fluoranthene, diphenyl . This selection of model chromatographed substances aimed at eliminating possible additional intermolecular actions with the adsorbent surface and the components of the mobile phase which was composed of four mixed binary solvents: hexane - -benzene, methylcyclohexane - benzene, benzene - methanol

andbenzene - ethanol. The procedure of conducting the chromatographic progess and substance detection were described in several earlier publications (1-4, 8, 13).

RESULTS AND DISCUSSION

It is apparent from the results presented in figures 1-4 that on silica gels of different specific surface area the R_M values of the chromatographed substance change in a regular manner. It turned out that there is a linear dependence of the R_M value on the specific surface of the adsorbent. This dependence can be presented from of a linear equation:

$$R_M = as + b \qquad (1)$$

where s is the specific surface area of the adsorbent, and values "a" and "b" - parameters of the straight line.

As appears from the graphs, the R_M values obtained from the experiment are laid on a straight line drawn so as get the smallest deviation. Table 1-4 shows the $R_M = f(s)$ straight line parameters which were calculated and tabulated for particular concentrations of the mobile phase and the chromatographed substance.

Tables 1-4 show the $R_M = f(s)$ straight lines parameters, whih were calculated and tabulated for some chosen concentrations of the mobile phase and the chromatographed substance. Tables 1 and 2 show the parameter "a" of the straight line whereas tables 3,4 - the parameters "b".

In the systems methylcycloheksane - benzene and he - benzene inactive mobile phase the "a" values decrease

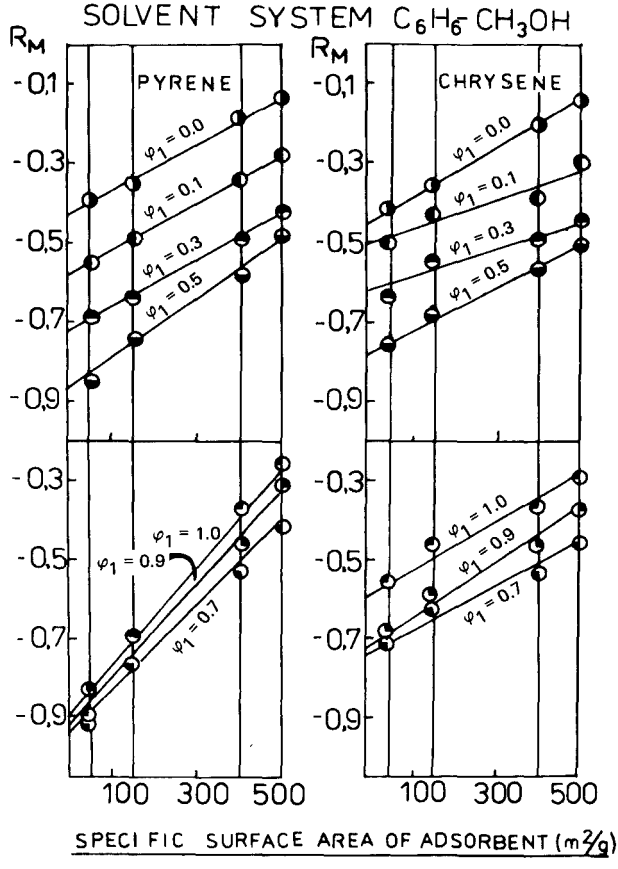

Fig. 1.

Linear relationship between R_M values and specific surface area of adsorbent. Solvent system: benzene--methanol. Points - experimental data, straight line - theoretical data, calculated from eq. 1.

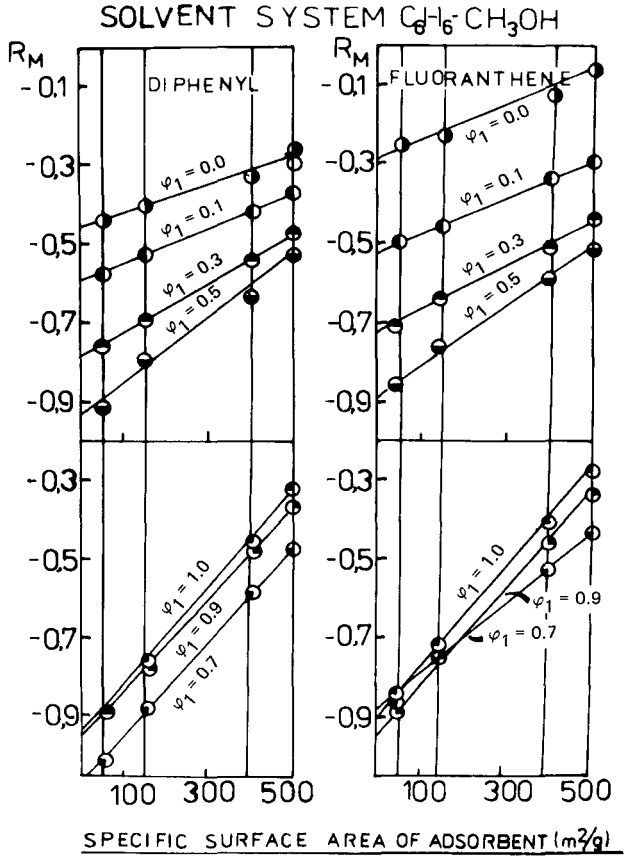

Fig. 2.

Linear relationship between R_M values and specific surface area of adsorbents. Points - experimental data, straight line - theoretical data calculated from eq.1.

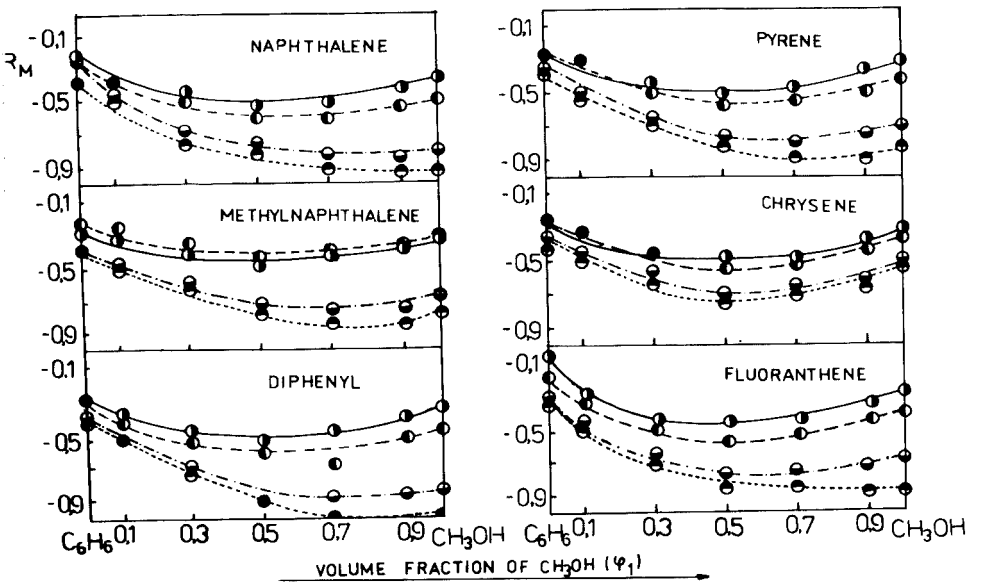

Fig.3. Relationship $R_M = f(\varphi_1)$. Points - experimental data, lines - theoretical data.

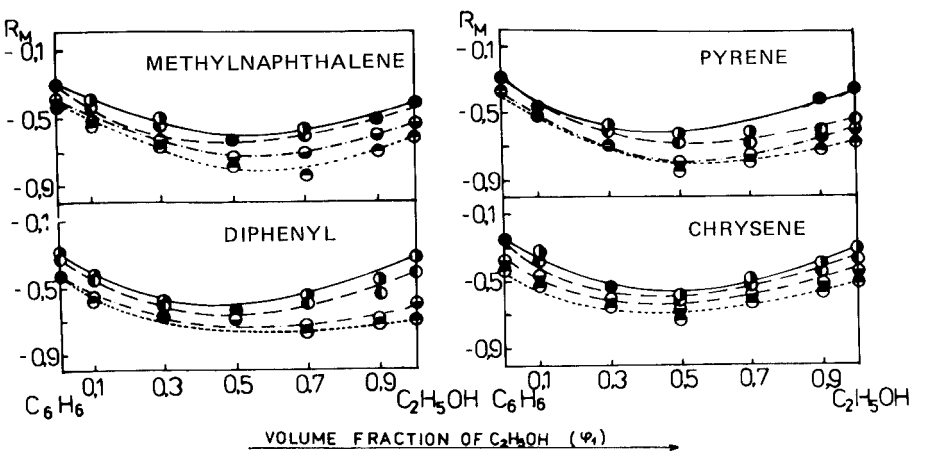

Fig.4. Relationship $R_M = f(\varphi_1)$. ◑ —— - silica gel 60, ◐ ···· silica gel 100, ◒ ··· ··· - silica gel 200, ◓ ····· silica gel 500.

Table 1

Values of parameter "a" for inactive mobile phase.

Substance	concentration of benzene						
	mobile phase: methylcycloheksane - benzene						
	0.0	0.1	0.3	0.5	0.7	0.9	1.0
diphenyl	0.14	0.09	0.09	0.08	0.07	0.03	0.03
naphthalene	0.09	0.09	0.07	0.04	0.02	.0.02	0.02
2,6-dimethyl-naphthalene	0.10	0.10	0.08	0.08	0.07	0.07	0.04
fluoranthene	0.09	0.07	0.05	0.03	0.02	0.02	0.03
anthracene	0.12	0.08	0.07	0.05	0.05	0.05	0.04
chrysene	0.11	0.10	0.09	0.08	0.08	0.07	0.03
pyrene	0.15	0.11	0.10	0.08	0.08	0.07	0.06
	mobile phase : heksane - benzene						
diphenyl	0.18	0.15	0.12	0.12	0.09	0.07	0.03
naphthalene	0.12	0.09	0.08	0.05	0.02	0.01	0.02
2,6-dimethyl-naphthalene	0.15	0.12	0.10	0.05	0.05	0.04	0.04
fluoranthene	0.12	0.12	0.11	0.05	0.04	0.04	0.03
anthracene	0.17	0.14	0.11	0.05	0.04	0.08	0.04
chrysene	0.16	0.15	0.12	0.06	0.08	0.06	0.03
pyrene	0.18	0.13	0.12	0.11	0.10	0.08	0.06

[x] Paramiter "a" values are presented as $ax10^{-2}$

with the increase in the concentration of benzene which is the more active component of the mobile phase.

In the systems where the second component most active has the ability to formation of hydrogen bonds (benzene--methanol, benzene - ethanol) the slope of the straight line parameter "a" increase with the increasing concentration of alcohol. It is possible that this behaviour of the parameter "a" is to some extent influenced by the association of alcohol that occurs in such systems.

Table 2

Values of parameter "a" for active mobile phase

Substance	concentration of alcohol						
	mobile phase: benzene - Methanol						
	0.0	0.1	0.3	0.5	0.7	0.9	1.0
naphthalene	0.02	0.03	0.07	0.07	0.09	0.11	0.13
methylnaphthalene	0.02	0.05	0.05	0.07	0.10	0.11	0.12
chrysene	0.03	0.04	0.04	0.06	0.08	0.08	0.06
pyrene	0.06	0.06	0.06	0.07	0.11	0.11	0.12
diphenyl	0.03	0.04	0.06	0.09	0.15	0.16	0.15
fluoranthene	0.03	0.05	0.06	0.08	0.12	0.12	0.13
	mobile phase: benzene - ethanol						
methylnaphthalene	0.02	0.05	0.04	0.03	0.06	0.06	0.05
chrysene	0.03	0.05	0.02	0.05	0.03	0.04	0.04
pyrene	0.06	0.04	0.03	0.05	0.04	0.07	0.07
diphenyl	0.03	0.04	0.02	0.02	0.04	0.04	0.08

x parameter "a" values are presented as $ax10^{-2}$

Tables 3 and 4 cantain the parameter "b" for considered systems. The parameter "b" may give information about the molecular action between mobile phase and chromatographed substance for the surface s = 0.

The straight lines described can be used for the calculation of the R_M values of substances on any adsorbent with a specific surface area. A series of arduous measurements can be avoided in this way. It is enough to take two measurements of possibly extreme R_M values in order to draw straight lines $R_M = f(s)$. When parameters "a" and "b" are known for two different substances, the separability of such mixture can be calculated with good approximation, with the use of the given mobile phase.

Table 3

Values of parameter "b" for nanactive mobile phase

Substance	concentration of benzene						
	mobile phase: methylcycloheksane - benzene						
	0.0	0.1	0.3	0.5	0.7	0.9	1.0
diphenyl	-0.16	-0.22	-0.36	-0.45	-0.49	-0.48	-0.48
naphthalene	-0.04	-0.14	-0.27	-0.32	-0.33	-0.35	-0.35
2,6-dimethyl-naphthalene	-0.11	-0.22	-0.36	-0.42	-0.43	-0.47	-0.45
fluoranthene	0.07	-0.05	-0.17	-0.31	-0.36	-0.38	-0.38
anthracene	0.05	-0.07	-0.11	-0.30	-0.34	-0.38	-0.38
chrysene	0.02	-0.28	-0.35	-0.42	-0.47	-0.48	-0.48
pyrene	-0.16	-0.10	-0.37	-0.41	-0.43	-0.45	-0.47
	mobile phase: heksane - benzene						
diphenyl	-0.14	-0.30	-0.43	-0.59	-0.58	-0.56	-0.48
naphthalene	-0.15	-0.20	-0.28	-0.37	-0.36	-0.37	-0.37
2,6-dimethyl-naphthalene	-0.15	-0.27	-0.41	-0.40	-0.45	-0.45	-0.45
anthracene	0.01	-0.13	-0.30	-0.37	-0.39	-0.40	-0.40
fluoranthene	0.02	-0.13	-0.30	-0.37	-0.39	-0.40	-0.40
chrysene	-0.15	-0.17	-0.21	-0.42	-0.47	-0.47	-0.48
pyrene	-0.07	-0.26	-0.40	-0.55	-0.50	-0.47	-0.47

It results from the graphs 3 and 4 of dependencies $R_{M1.2} = f(\varphi_1)$ presented in figures 3 and 4, that and theoretically on the basis of the equation 1 are within the range of a permissible experimental error, i.e. that those theoretical R_M values are adequate representations of real R_M values.

The obtained results seem to suggest thst it is possible to tabulate values "a" and "b" in order to calculate the dependence of $R_M = f(s)$. It has also been stated that there is a general dependence of the parameters of the straight

Table 4

Values of parameter "b" for active mobile phase

Substance	concentration of alcohol						
	mobile phase: benzene - methanol						
	0.0	0.1	0.3	0.5	0.7	0.9	1.0
naphthalene	-0.35	-0.53	-0.65	-0.81	-0.90	-0.94	-1.12
-methyl-naphthalene	-0.41	-0.53	-0.79	-0.86	-0.98	-0.99	-1.03
chrysene	-0.48	-0.51	-0.62	-0.79	-0.73	-0.72	-0.58
pyrene	-0.47	-0.57	-0.72	-0.85	-0.85	-0.87	-0.84
diphenyl	-0.48	-0.59	-0.79	-0.94	-1.06	-0.05	-1.13
fluoranthene	-0.38	-0.44	-0.64	-0.90	-0.89	-0.96	-0.92
	mobile phase: benzene - ethanol						
-methyl-naphthalene	-0.41	-0.63	-0.68	-0.80	-0.71	-0.71	-0.62
chrysene	-0.48	-0.53	-0.64	-0.73	-0.53	-0.58	-0.52
pyrene	-0.47	-0.52	-0.74	-0.86	-0.76	-0.76	-0.72
diphenyl	-0.48	-0.61	-0.79	-0.71	-0.75	-0.75	-0.73

line $R_M = as + b$ on the difference of the elution strenght of the components of the mobile phase, the surface on the adsorbent, occupied by the chromatographed substance and the molecular of the components of the mobile phase. This affords a wider range of possibilities of optimizing the thin-layer chromatography process.

The dependencies described above can undergo changes in the case of sieve effect leading to a non-linear from of equation 1. Consequently, the applicability of the obtained dependencies should be limited to the values of the specific surface 50 - 500 m^2/g (14).

It seems that investigations presented above can be of great importance in transforming the chromatographic data

of thin-layer chromatography to the conditions of liquid column chromatography, due to frequent differences in the properties of the stationary phase in both methods, resulting from their nature.

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DETERMINATION OF BIPHENYL IN CITRUS FRUITS BY QUANTITATIVE THIN-LAYER CHROMATOGRAPHY

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ABSTRACT

Residues of the fungicide biphenyl in citrus fruits have been determined by direct scanning of spots on phosphor-impregnated high performance silica gel TLC plates under UV light. Biphenyl was separated from fruit tissue by steam liquid-liquid extraction. Recoveries from spiked samples ranged from 92-99% at 100, 50, and 10 ppm levels. The precision of the TLC determination and overall procedure are shown to be adequate for residue analysis.

INTRODUCTION

The official AOAC method for determination of residues of the fungicide biphenyl in citrus fruits is based on steam distillation, preparative thin layer chromatography (TLC), scraping and elution of biphenyl zones from the thin layer plate, and solution UV spectrometry at 218 nm (1,2). This paper reports a more rapid, simplified method for this analysis based on the direct scanning of the UV absorbance of biphenyl spots on phosphor-impregnated high performance silica gel layers. Precision (reproducibility) and accuracy (recovery) are at least comparable to the more laborious official method, and the specificity offered by the thin layer separation is retained.

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EXPERIMENTAL

The preparation of citrus samples and biphenyl extraction using the SGA Scientific, Inc., No. JM-8590 lighter-than-water volatile oil trap were carried out as described in the official method (1,2) with the following exceptions. Extraction was performed for 3 hours at the two highest spike levels and for 4.5 hours at the lowest level. The solution was boiled vigorously during extraction and a rapid cooling-water flow was used in the condenser to prevent loss of biphenyl out of the top of the apparatus. The final heptane solution was freed from water by passing through Whatman phase separating paper (1PS) rather than a column of anhydrous Na_2SO_4.

Samples were fortified by adding 1.00 ml of ethanolic spiking solution to 100 g of blended, peeled orange or lemon fruit or ground peel in the one liter round bottom flask. The spiking solution contained 1.00 g biphenyl per 100 ml for preparation of the 100 ppm sample, 0.500 g per 100 ml for the 50.0 ppm sample, and 0.100 g per 100 ml for the 10.0 ppm sample.

TLC was carried out on 20 x 10 cm Whatman HP-KDF high performance silica gel plates. These plates contained a fluorescent phosphor that was activated by 254 nm UV light, and were divided into nineteen lanes of 8 mm width. Plates were cleaned by predevelopment with methanol-chloroform (1:1 v/v) and dried in a fume hood before use.

Biphenyl standard solutions were prepared in n-heptane at concentrations of 0.125, 0.250, 0.500, 1.00, 1.50, and 2.00 µg/µl. Standards and samples were applied to separate lanes, 2 cm up from the bottom of the plate, using disposable 4.00 µl Drummond microcap micropipets. After air drying, the layer was developed with n-heptane in a filter paper lined glass, rectangular HPTLC tank (Fotodyne) that had been pre-equilibrated with solvent for at least 10 minutes before inserting the plate.

The chromatogram was air dried in a hood and biphenyl spots were measured by scanning with a Kontes Model 800 fiber optics

densitometer equipped with a Hewlett Packard Model 3390A calculating integrator/recorder. Scanning was done in the single beam, transmission mode using the 254 nm shortwave-UV cobalt glass filter.

Precentage recovery was calculated by comparing the area of the sample zone to the area of the standard zone on the same plate representing the theoretical amount for 100% recovery. The final sample solutions were collected in 10.0 ml volumetric flasks. Sample volumes spotted and the theoretical weights representing 100% recovery were as follows for the three fortification levels: 100 ppm sample, 4.00 µl, 4.00 µg (4.00 µl of the 1.00 µg/µl standard); 50.0 ppm sample, 4.00 µl, 2.00 µg (4.00 µl of the 0.500 µg/µl standard); 10.0 ppm sample, 20.0 µl, 2.00 µg. Samples and standards were applied on adjacent lanes in duplicate, and the average area of the standard spots was compared to the two individual sample spot areas.

RESULTS AND DISCUSSION

Development with heptane porvided tight, circular zones of biphenyl with an R_F value of ca. 0.5, which is within the optimum range for quantification by densitometry (3). Approximately 12 minutes was required for a 6 cm development distance. The spots were detected as dark, absorbing zones against a bright, fluorescent background (fluorescence quenching) when the plate was viewed under shortwave UV light. The zones were measured by scanning using the 254 nm filter over the densitometer light source.

To determine that analyses were being conducted within a linear calibration region, 0.5-8 µg amounts of standard biphenyl were spotted in 4 µl volumes, and scanned. Plots of peak area vs. weight had an average correlation coefficient (R) of 0.982 and a range of 0.991-0.967 (9 replicates). Recovery values were calculated by comparison of samples to single standard zones (4 µg and 2 µg), which were within the linear calibration range.

Reproducibility of the TLC determination was measured by spotting seven 2 µg (4 µl) spots in adjacent lanes and scanning

TABLE 1

Biphenyl Recovery from Orange Fruit

Trial	Average Recovery
100 ppm Spike	
1	97.9
2	95.1
3	94.9
50.0 ppm Spike	
1	93.5
2	96.5
3	92.4
4*	95.0
10.0 ppm Spike	
1	97.9
2**	97.9
3	97.7

* orange peel

** lemon fruit

the developed chromatogram. The relative standard deviation (coefficient of variation) of the peak areas was 2.76%, which is excellent precision considering the possible combined inconsistencies resulting from plate production, sample application, mobile-phase development, and scanning.

The recovery values obtained at the same three concentrations as used in the collaborative study (1) of the official method are summarized in Table 1. Only a limited number of

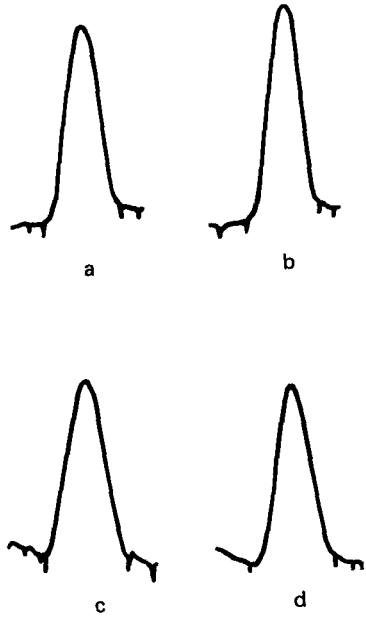

Figure 1. Densitometer scans of spots from duplicate 2.00 µg (4.00 µl) standards (a and b) and duplicate 20.0 µl extracts of the 10.0 ppm fortified lemon sample (c and d). Peak c represents 98.8% recovery and peak d 97.0%, compared to the average area of the standards.

trials were performed on three sample types because the purpose of this research was not to confirm the applicability of the sample-preparation method but to demonstrate the efficacy of the TLC determination. All of the results in the table are for fortified peeled orange fruit, except the one value for orange peel and one for lemon fruit. The average recovery results range from 92.4 to 97.0%. The somewhat higher levels of recovery at the lowest level suggest that the longer extraction time may also be beneficial at the higher levels. The percentage difference between the two sample spots for the nine experiments averaged 2.81%, which is another indication of the satisfactory precision of the TLC determination. The agreement among the

trials within each concentration level illustrate the precision of the overall procedure. The results compare favorably both in accuracy and precision with those obtained in the collaborative study (1) of the official method. Recoveries above 100% at 100 ppm were not obtained, nor were recoveries proportional to the spiking level, as in this study (1).

A blank extraction of both fruit types was carried out, and chromatograms of these extracts contained no detectable spot at the R_F value of biphenyl. Therefore, correction of the data was not necessary. Figure 1 shows typical densitometer scans of duplicate sample and standard spots used to calculate the results in Table 1.

High performance layers were chosen after determining that biphenyl zones were less diffuse and darker than on either conventional silica gel or on C_{18} chemically bonded reversed phase layers. The latter were developed with methanol-water (85:15 v/v) to provide an R_F value of 0.26 for biphenyl. Preadsorbent layers could not be used because biphenyl spotted on the preadsorbent was not consistently detected after development, indicating either loss by volatilization or irreversible sorption in the spotting area.

CONCLUSION

The above results illustrate that the official AOAC analytical method for biphenyl residues in fruit can be improved by replacing the scraping and elution of TLC zones and solution UV spectrometry by *in situ* measurement of zones directly on the thin layer plate. The changes, along with the ability to analyze multiple samples at the same time under identical conditions and to process standards in parallel, result in greater convenience and saving of time without loss of accuracy or precision. The revised method is applicable to any samples that can be successfully analyzed by the AOAC method.

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USE OF SOME SILVER SALTS OF AROMATIC SULFONIC ACIDS IN THIN-LAYER CHROMATOGRAPHY

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SUMMARY

Some silver salts of aromatic sulfonic acids were used for thin-layer impregnation. The chromatographic properties of these salts were examined by separation of fatty acid methyl esters with different degrees of saturation as well as of their cis- and trans-isomers. Benzene sulfonic acid silver salt was more thoroughly investigated. An attempt was made to prove the effect of the anion of the different silver salts on the R_f values of a model mixture of fatty acid methyl esters.

INTRODUCTION

Silver nitrate has long been used for thin-layer separation of compounds according to the degree of saturation (1, 2, 3,

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4, 5). Silver perchlorate and fluoroborate (6) as well as silver oxide (7), thallous nitrate (8) and silver sulfamate (9) have also been suggested for this purpose. Silver sulfamate has proven to be very suitable for separation of fatty acid cholesteryl esters. The metallic ion (Ag, Pt, Pd, Tl) is thought to form a π-complex with compounds having double bonds. The stability of this complex depends on the number, type, geometry and position of the double bonds in the molecule of the compound. All this is used in certain chromatographic methods for separation of compounds according to the degree of saturation.

This paper deals with the possibility of use silver salts of aromatic sulfonic acids in thin-layer chromatography.

EXPERIMENTAL

Silver salts of the following aromatic acids were prepared:

Table 1

1. Benzenesulfonic acid (HO_3SAr)
2. 4-Methylbenzenesulfonic acid
3. 2,5-Dimethylbenzenesulfonic acid
4. 1-Naphtalenesulfonic acid
5. 2-Naphtalenesulfonic acid
6. 4-Hydroxybenzenesulfonic acid
7. 3-Carboxy-4-hydroxybenzenesulfonic acid
8. Sulfamic acid

Silver nitrate

All salts were prepared in a similar manner. The aqueous solution of the corresponding acid was neutralized with freshly precipitated silver oxide at room temperature. The silver salt crystallized out either on storing at room temperature or

on evaporation under vacuum. The concentration of the stock solutions was chosen according to the solubility of the silver salt to be obtained. For example, 6 g of benzenesulfonic acid was dissolved in 100 ml water and freshly precipitated silver oxide was added to the solution until complete neutralization. The consumed silver oxide was equivalent to about 7 g silver nitrate. The yield of silver benzenesulfonate was close to the theoretical one (97%). Silver benzenesulfonate represents leaf-like crystalls with a melting point of more of 250 C which are very soluble in water and ethanol. Since some of the silver salts of the aromatic sulfonic acids are more slightly soluble in water, water and acetonitrile (4:1 v/v) were used as a solvent.

The silver salt of benzenesulfonic acid was first synthetized by Freund (1861) but by a different procedure.

Chromatographic System: 0,4 g (about 1,5 mmoles) of benzenesulfonic acid silver salt was dissolved in 5,5-6,0 ml distilled water. A mixture of 1,0 g Silica Gel G and 1,0 g Silica Gel HR (Merck) were slurried with this solution. Five glass plates (19 x 4 cm) were uniformly coated by means of spreader. Thickness is about 0,25 to 0,30 mm. The chromatographic plates were allowed to air-dry and kept in dry and dark until use. The model mixture of fatty acid methyl esters was prepared using lipid standard for chromatography (no 189-1, Sigma, St. Louis, Mo, USA) to which trans-oleic acid methyl ester and trans, trans-linoleic acid methyl ester were added. The concentration of the fatty acid methyl esters was about 2 mg/ml except for the trans, trans-linoleic acid methyl ester which was 1,0 to 1,3 mg/ml. Hexane was used as a solvent. From this solution 2 µl dropwise or 20-25 µl in a way of band was applied on the start.

Solvent System: Hexane-pentane-diethylether-acetic acid (100:30:6:3 v/v). Development time - about 30 min. Length of run - about

12 cm. Chamber volume/mobile phase volume ratio was 30:1. The development was carried out in a closed chamber at room temperature. Separation improved if a precooled plate was placed in the chamber.

After development of the chromatogramms and evaporation of the solvent the plates were sprayed with 20% solution of sulfuric acid in water-methanol (3:2 v/v) and dried in an oven at 120 C for 10 min. The chromatogramms were then placed over a heated (200 C) metallic plate for 10 min. Unsaturated fatty acids appeared as black spots on a white background. To obtain more intensive darkening of the spots of the saturated acids they were sprayed again with the sulfuric acid solution and the chromatigramms were reheated. The spots could be successfully measured by densitometry.

RESULTS AND DISCUSSION

Fig.1 shows two chromatogramms developed on a silver nitrate- and silver benzenesulfonate-impregnated layer. The silver ion concentration and all other conditions of development of the chromatogramms were the same. The chromatogramms demonstrate that silver benzenesulfonate allows for the separation of the critical pair cis-oleic acid/trans, trans-linoleic acid, which could not be achieved with silver nitrate only. The limit of detection was of the order of 0,8 to 1,0 μg for unsaturated acids and about 5,0 μg for saturated acids. The chromatographic plates impregnated with silver benzenesulfonate were more resistant to light and to mechanical damage as compared to those with silver nitrate. The Table 2 summarizes the R_f values obtained on a thin-layer impregnated with $AgNO_3$ and AgO_3SAr:

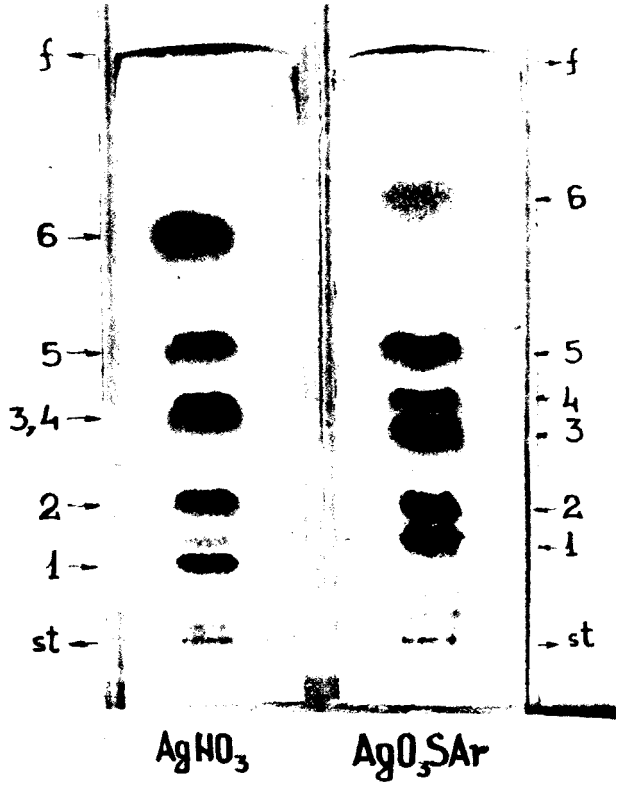

Fig. 1. Chromatography of the fatty acid methyl esters on silver nitrate and silver benzenesulfonate (AgO_3SAr) impregnated silica-gel layers:

1. all cis-trienoic esters
2. all cis-dienoic esters
3. cis-monoenoic esters
4. all trans-dienoic esters
5. trans-monoenoic esters
6. saturated esters

Table 2

R_fx100 values of some fatty acid methyl esters (mean ± SD)

Impregnated layer with	all cis-trienoic esters	all cis-dienoic esters	cis-monoenoic esters	trans-dienoic esters	trans-monoenoic esters	saturated
$AgNO_3$	17±5	25±5	37±6	-	44±8	58±4
AgO_3SAr	21±3	28±5	36±6	40±5	47±6	67±4

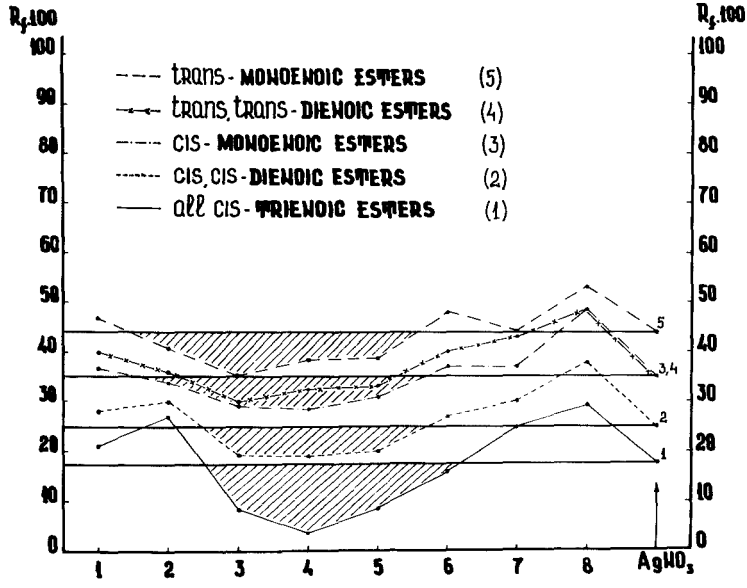

Fig. 2. R_fx100 values of 5 different fatty acid methyl esters. For numbers 1-8 see Table 1. The solid horizontal lines correspond to the R_f values obtained on $AgNO_3$-layer. The shaded areas express a greater stability of the π-complex, which can be attributed to the different chemical nature of silver salt anion.

Here again the silver ion concentration (about 1,5 mmoles) in the layer and the conditions of development of the chromatigramms were the same. From Fig.2 it is clear that the stability of the π-complex depends not only on the number and geometry of the double bonds but also on the corresponding acid resudue (anion) of the silver salt. This suggests that besides silver nitrate other silver salts could also be used for thin-layer separation of fatty acid methyl esters.

CONCLUSION

The proposed silver benzenesulfonate proved to be very suitable for separation of fatty acids with different degree of saturation as well as of their cis- and trans-isomers on thin-layer chromatographic plates. This chromatographic procedure could be used as a rapid and easy-to-perform method for control of the process of producing hydrogenated oils or for control of foods containing partially hydrogenated oils. The use of densitometry allows for the quantitative evaluation of the results.

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CHARACTERIZATION OF DERIVATIVES OF SOME CLOSELY RELATED 1-PICOLINOYL-3-METHYL-4-(SUBSTITUTED) PYRAZOLINE-5-ONES BY THIN LAYER CHROMATOGRAPHY

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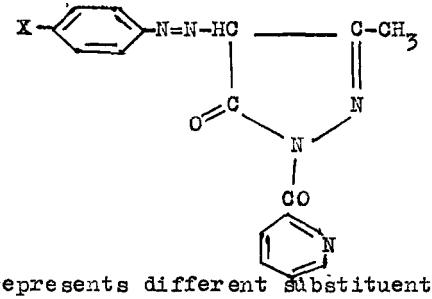
INTRODUCTION

The antitubercular activity associated with pyrazoline-5-ones (1) , picolinic acid hydrazide (2) and the antibacterial activity of sulphonamides (3) prompted us to undertake the synthesis of 1-picolinoyl-3-methyl-4-(substituted)-pyrazoline-5-ones (see formula) as possible new biological active agents. In view of their medicinal importance, it is thought worthwhile to carry out a TLC separation of these compounds.



where X-represents different substituents (Table 1)

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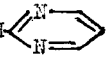
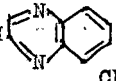
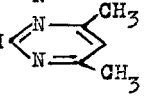
Table - 1

Sl. No.	X	R_f x100	
		A	B
1	$-So_2NH_2$	37	36
2	$-So_2NH.C(=NH)NH_2$	49	-
3	$-So_2NH$- 	59	-
4	$-So_2NH$- 	74	83
5	$-So_2NH.Co.CH_3$	76	77
6	$-So_2NH$- 	68	60
7	$-So_2NH$- 	71	73
8	$-So_2NH$- 	40	71
9	2-Cl	12	14
10	3-Cl	24	23
11	4-Cl	27	32
12	$2-OCH_3$	20	17
13	$3-OCH_3$	61	60
14	$4-OCH_3$	13	25
15	4-Br	43	77
	Rate of development for 15 cms in min	45	45

A = Chlorofarm:methanol (90:10)

B = Benzene :ethyl acetate (60:40)

EXPERIMENTAL

All the derivatives of pyrazoline-5-one were synthesized in this laboratory by usual methods (4). TLC plates (0.5 m.m. thick) were prepared from silica gel G (Sisco) by means of Stahl's applicator. The plates were activated at 60°C for 24 hrs. All the compounds were recrystallized prior to their use. The pure organic compounds were dissolved in hot glacial acetic acid and were applied to the chromatoplates with the help of a glass capillary and the spots were allowed to air dry. The chromatograms were developed at constant temperature (30 ± 2^{o}) with different solvent systems. The chamber used for the development was saturated with vapours of the solvent mixture before carrying out the separation. A mixture of chloroform:methanol (90:10) and benzene:ethyl acetate (60:40) was found to give the best results. As these compounds gave coloured spots, no visualiser was therefore employed. The R_f values of the compounds were determined and recorded in table 1. The results were found fairly reproducible within the limits of experimental error.

ACKNOWLEDGEMENT

One of us (R.K.A.) is grateful to U.G.C., India for financial assistance.

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PHOSPHOLIPID COMPOSITION AND DIFFERENTIATION OF METHANOTROPHIC BACTERIA

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ABSTRACT

A combination of TLC and extraction of native cells on the layer of sorbent was used for analysis of phospholipid composition of bacteria. The method is suitable for rapid routine differentiation of genera and species of obligate methanotrophic bacteria.

INTRODUCTION

During the past decade bacterial taxonomy has undergone essential changes which are reflected in the 8th edition of Bergey's Manual of Determinative Bacteriology (1). It was largely due to the use of modern chemotaxonomic techniques which become currently accepted in systematics of bacteria (2-3).

All organisms contain polar lipids, and their analyses often enable one to elaborate classification systems of microorganisms (3). Presently,

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researchers pay a special attention to rapid and qualitative methods of analysis which allow the study of a great number of strains and their rapid preliminary identification (express-diagnostics).

Methanotrophic bacteria attract an increasing attention due to their practical importance. However, the taxonomy of the organisms still needs further investigation. So far the questions of methanotroph classification have been tackled with help of conventional methods of bacterial taxonomy. The elaboration of progressive chemotaxonomical approaches in the taxonomy of methanotrophic bacteria is an urgent task.

Previously it was shown (4) that methanotroph types (groups) differ in their phospholipid and fatty acid composition. A more detailed analysis of fatty acids of a large group of methanotrophic bacteria (68 strains) allowed the identification of these organisms to genera and species (5). Such an analysis revealed also the differences in the phospholipid composition of different genera and types of methanotrophs (6).

In the present work the possibility of the express-diagnostics of obligate methanotrophic bacteria based on thin-layer chromatography of their phospholipids.

MATERIALS AND METHODS

Materials

Thin-layer glass plates, 20 x 20 cm (DC-Fertigplatten Kieselgel 60 F 254 from E.Merk, Darmstadt, FRG), were used. Phosphatidic acid (PA), phosphatidyl glycerol (PG), diphosphatidyl glycerol (DPG), phosphatidyl choline (PC), phosphatidyl ethanolamine

(PE), monomethyl- (PMME) and dimethylphosphatidyl ethanolamine (PDME) were obtained from Supelco (USA). Agar was purchased from Difco (USA). Solvents and mineral salts were from Khimreaktiv (USSR).

Methods

Methanotrophic bacterial cultures were grown on mineral salts medium containing (g/l distilled water): KNO_3, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.02; $Na_2HPO_4 \cdot 5H_2O$, 1.5; KH_2PO_4, 0.7; (mg/l): EDTA, 5.0; $FeSO_4 \cdot 7H_2O$, 2.0; $ZnSO_4 \cdot 7H_2O$, 0.1; $MnCl_2 \cdot 4H_2O$, 0.03; $CoCl_2 \cdot 6H_2O$, 0.2; $CuCl_2 \cdot 5H_2O$, 0.1; $NiCl_2 \cdot 6H_2O$, 0.02; Na_2MoO_4, 0.03; pH 6.9 $\pm$ 0.2. The medium was sterilized at 121°C (1 atm) for 1 h. Phosphates were sterilized separately, cooled and added to the other components. The medium was solidified with agar (2%).

The cultures were grown in test tubes with solid medium or in 0.7 l - flasks with 100 ml liquid medium. Flasks were sealed with rubber stoppers. A mixture of methane and air (1:1) was added through two glass tubes in the stoppers with cotton filters. For better aeration the flasks were shaken on a rotor shaker (140 ppm). In case of a solid medium the bacteria were grown in vacuum dissicators filled with a methane - air mixture (1:1).

Use was made of pure cultures of obligate methanotrophs of the culture collection of IBPhM, USSR Academy of Sciences. Strains OB3b and 5 were courteously provided by Prof. Wittenbury (University of Warwich, Coventry, UK); and strain Texas, by Prof. Quayle (University of Sheffield, Sheffield, UK).

For thin-layer chromatography the cell suspension was washed with saline (0.15 M NaCl) to a final concentration of 5 to 15 μg/ml of dry weight. 10 μl of the cell suspension was applied onto a silica gel

layer to obtain an evenly coated region of 5 x 5 mm, Then the plate was dried under nitrogen stream, and the procedure was repeated. The cells were extracted on the layer by a chloroform - methanol mixture (1:2). To do so, the plates were developed in the solvent mixture, the start line being 2-3 cm above the line of application of the cell suspension. This was coupled with the extraction of lipids from the cells and their movement along the layer. After this procedure was 5-6 times repeated the extracted lipids were concentrated in the form of a narrow strip at the front line of the extractant. Between two subsequent extractions the solvent was removed by drying under nitrogen stream. Then the silica gel layer with the extracted cells was removed by a scalpel.

The phospholipids were separated in corresponding solvents. For unidimensional chromatography the solvent system containing chloroform, methanol, acetone, acetic acid a water (10:3:8:2:1) was used. Two-dimensional chromatograms were obtained using the solvent systems of chloroform-methanol - 26% NH_4OH solution (65:25:4 the first separation) and chloroform-acetone-methanol-acetic acid-water (8:2:8:2:1, the second separation). To visualize the lipid spots, the plates after separation were sprayed with 10% $CuSO_4$ solution in 8% orthophosphate acid and heated for 10 min at 175-200°C. (7).

RESULTS AND DISCUSSION

Fig. 1A and 1B present the unidimensional chromatograms of phospholipids of methanotrophic bacteria of different genera and types. The chromatogram 1B was developed once in the solvent system to a length of 12 cm, whereas the chromatogram 1A was primarily

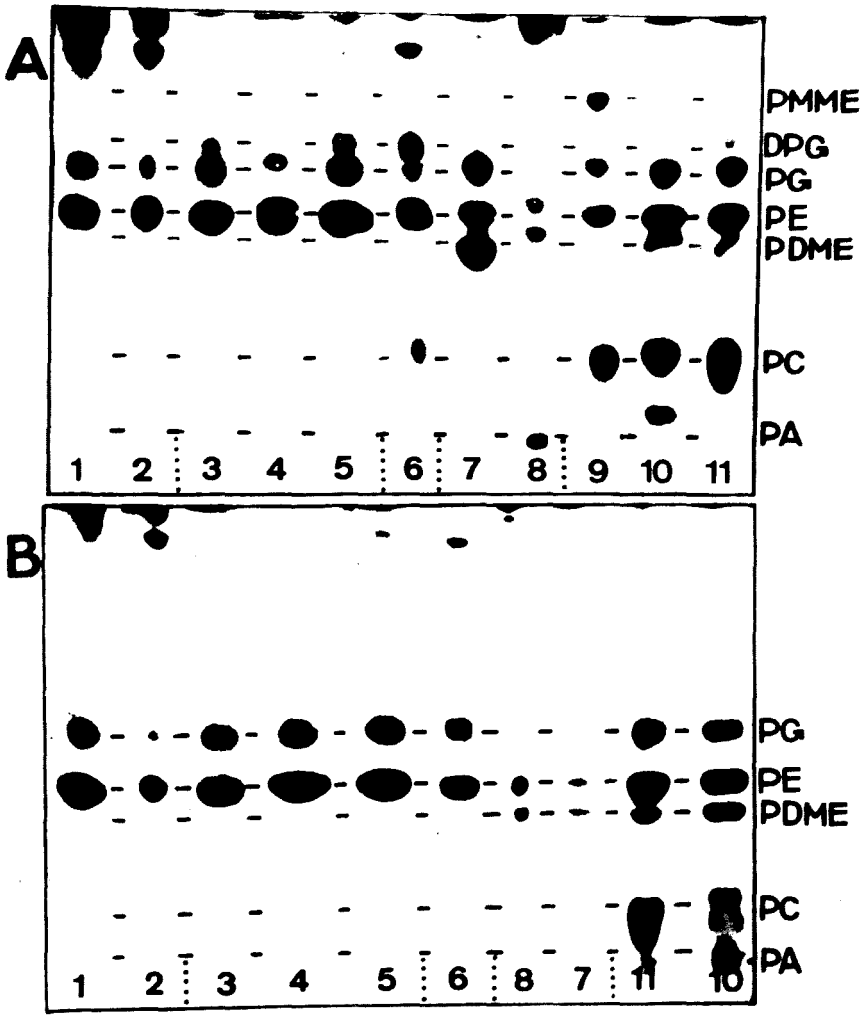

FIGURE 1. One-dimentional chromatograms of phospholipids of obligate methanotrophic bacteria grown in liquid media (A) and on solid media (B).
(1) Methylomonas methanica 12; (2) Mm.albus BG8 ;(3) Methylobacter vinelandii 87; (4) Mb.bovis 89; (5) Mb.chroococcum 90; (6) Methylococcus capsulatus "Texas"; (7) Methylosinus trichosporium OB3b; (8) Ms.sporium 5; (9) Methylocystis minimus 82; (10) Mcs.parvus OBBP; (11) Mcs.echinoides 2.

developed to a length of 6 cm and then to 12 cm in the same solvent system.

To obtain accurate results, each bacterial cultures was three times analyzed at different cell concentrations, since it was taken into consideration that the treatment with a mixture of chloroform and methanol may result in the release of some substances, e.g. polyoxybutyrate which dissolve phospholipids and thus affect their motility. Such a process looks like a reverse-phase TLC variant. Fig. 1A illustrates such a distorted chromatogram (sample 10). As is seen in the case with Methylocystis parvus OBBP this effect results in the flattening of the phosphatidyl choline zone. After the second development of the chromatogram in an alkaline solvent system this component moves upward and distorts the dimethyl phosphatidyl ethanolamine zone form (Fig. 1A, sample 10). However, it does not influence the qualitative analysis.

Comparing the phospholipid composition of bacteria grown on solid and in liquid media, one can see that in the latter case the chromatograms are far more informative. Nevertheless, in both cases the methanotrophs may be divided into two groups.

All tested strains of methanotrophic bacteria using a serine pathway (II type) contain the methylated phosphatidyl ethanolamine derivatives, monomethyl phosphatidyl ethanolamine or, as in the case of Methylocystis minimus 82, dimethyl phosphatidyl ethanolamine, and phosphatidyl choline. The said phospholipids are not contained in the lipid pool of methanotrophs with ribulose monophosphate cycle (I type).

Methylococcus capsulatus is an exception to this rule. As is seen on Figures 1B and 2C, the bacteria of this species have small amounts of phospha-

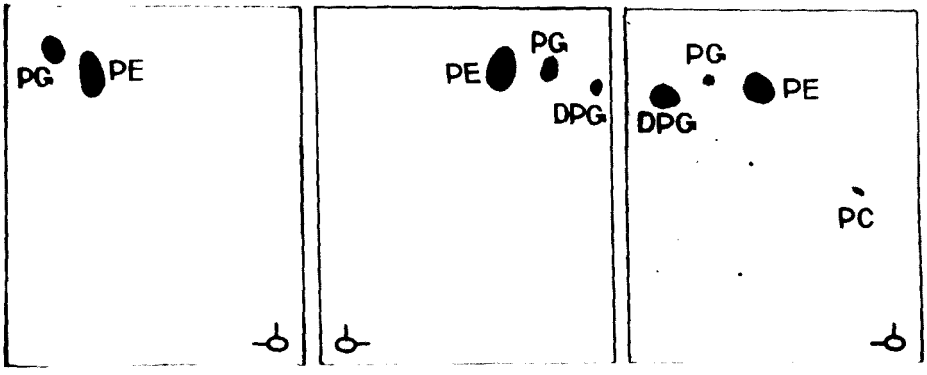

FIGURE 2. Two-dimensional chromatograms of phospholipids of obligate methanotrophic bacteria. (A) Mm.methanica 12; (B) Mb.vinelandii 87; (C) Mc.capsulatus "Texas".

tidyl choline. Methylococcus capsulatus is a I type methylotroph and uses a ribulose monophosphate partway of methane carbon assimilation. But as it was shown (8) this organism displays some characteristics of II type methanotrophs (e.g. possesses some activities of key enzymes of the serine cycle of methane carbon assimilation). This must have somehow affected its phospholipid composition as well: like the II type methanotrophs, Methylococcus capsulatus has phosphatidyl choline.

Based on the differences in the phospholipid spectra the methanotrophs within the said groups (types) can be differentiated to genera. For example, Methylomonas differs from Methylobacter by the absence of diphosphatidyl glycerol whose content in the latter in substantial. Methylobacter can easily be differentiated from Methylococcus by the ratio of diphosphatidyl glycerol and phosphatidyl glycerol. In

the former this proportion is far less than 1, whereas in Methylococcus capsulatus the content of phosphatidyl glycerol is lower than of diphosphatidyl glycerol and their ratio varies from 1.5 to 2.0 (Fig.1A). The differences in diphosphatidyl glycerol and phosphatidyl glycerol proportions are clearly seen on the two-dimensional chromatograms (Fig.2).

In the I type methanotrophs grown on a solid medium no difference in the phospholipid contens are observed. On the chromatograms only two phospholipids are seen: phosphatidyl glycerol and phosphatidyl ethanolamine (Fig.1B).

The phospholipid composition permits also to differentiate the representatives of the II type methanotrophs. Methylosinus bacteria contain small amounts of phosphatidyl choline, which is one of the major components in Methylocystis.

When using the phospholipid composition for differentiation of methanotrophic bacteria, purity of cultures is a must, because even slight contamination, e.g. with molds, results in the unproportional increase of the phosphatidyl choline content.

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REGULAR CONTRIBUTED PAPERS

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(14), 2709-2721 (1983)

MODIFICATION OF SELECTIVITY IN REVERSED-PHASE LIQUID CHROMATOGRAPHY OF POLYCYCLIC AROMATIC HYDROCARBONS USING MIXED STATIONARY PHASES

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ABSTRACT

Monomeric and polymeric C_{18} materials provide significantly different selectivities for polycyclic aromatic hydrocarbons (PAH) in reversed-phase liquid chromatography. Selectivity factors vary in a regular manner with respect to surface concentration of C_{18} groups on different C_{18} columns. In this study, we investigated the feasibility of "customizing" a C_{18} column to provide an intermediate selectivity by mixing 5-μm polymeric C_{18} material from two different lots with high and low C_{18} surface concentrations. Polymeric C_{18} materials from different production lots were mixed in ratios of 30/70, 50/50, and 70/30 (w/w). Selectivity factors for these columns were found to be similar to those predicted by the linear addition of the selectivities of the two individual phases. The PAH selectivities on these mixed columns were also found to be comparable to data obtained from coupled short columns of appropriate lengths each containing one of these different C_{18} materials. These studies indicate that columns of specific selectivity can be prepared by either mixing two different C_{18} materials or by coupling columns containing each of these different phases. The use of mixed phase columns is illustrated for the analysis of a fraction containing five condensed ring PAH isomers (molecular weight 278) isolated from an air particulate sample.

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INTRODUCTION

Monomeric and polymeric C_{18} materials provide significantly different selectivities for polycyclic aromatic hydrocarbons (PAH) in reversed-phase liquid chromatography (1,2). Polymeric C_{18} phases generally provide larger selectivity factors (α) than monomeric C_{18} phases for most planar PAH solutes. One polymeric C_{18} material investigated has a very low surface area ($\sim$90 m^2/g for the underivatized silica) which provides a high C_{18} surface coverage and a unique selectivity for PAH separations when compared to monomeric C_{18} phases and to other polymeric C_{18} phases prepared on higher surface area silica (300-400 m^2/g). Recently, we reported that selectivity differences for PAH on different C_{18} columns from various manufacturers were related to the monomeric or polymeric nature of the chemically bonded layer and to the surface concentration of the C_{18} layer (2). In addition, several different lots of a polymeric C_{18} phase from one manufacturer were studied with respect to C_{18} surface coverage and selectivity factors for PAH. In this study (2) the selectivity factors for selected PAH (relative to benzo[*a*]pyrene) on the different polymeric columns were found to vary linearly as the surface concentration of the C_{18} groups varied. As an extention of these studies, we investigated the feasibility of "customizing" a C_{18} column to provide an intermediate selectivity by physically mixing two polymeric C_{18} materials from different production lots (i.e., lots with high and low C_{18} surface coverage). The selectivities of these "mixed" phase columns were compared to that obtained by coupling two short columns (in appropriate lengths) each containing one of these same C_{18} materials.

EXPERIMENTAL

A liquid chromatograph with a fixed wavelength UV detector at 254 nm and an autosampler were used for all liquid chromatographic measurements. HPLC grade acetonitrile and water were used as the mobile phase. PAH standards were obtained from several sources as previously reported (2). Columns and bulk packing material ($\sim$2g)

from two different bonding production lots (lots 11 and 15) of polymeric C_{18} material (5-μm) and one lot (lot 17) of monomeric C_{18} material (5-μm) were obtained from the manufacturer. The lot numbers were arbitrarily assigned by the authors and correspond to those in the previous study (2). The manufacturer indicated that lot 11 had a low carbon loading, whereas lot 15 had a high carbon loading. Both the polymeric and monomeric C_{18} materials used in this study utilized the same low surface area silica support (90 m^2/g).

The columns (25 cm x 4.6 mm i.d.) containing the mixtures of lots 11 and 15 [70/30, 50/50, and 30/70 (w/w) lot 11/lot 15] were prepared by the manufacturer. Short columns (12.5 cm x 4.6 mm i.d.) containing packing from lots 11 and 15 were also prepared by the manufacturer.

Specific surface area and percent carbon measurements on the chemically modified C_{18} materials were determined and used to calculate the surface concentrations of the C_{18} ligands on the silica as described previously (2).

The mobile phase mixtures were pre-mixed and allowed to equilibrate to room temperature prior to use. All retention data were obtained under isocratic and isothermal conditions (27 °C). Retention times were obtained from injections of acetonitrile solutions of each compound. Capacity factors, k', were determined from $k' = \frac{(t-t_o)}{t_o}$, where t is the retention time of the solute and t_o is the unretained time which was determined from an injection of acetone in a mobile phase of acetonitrile. Selectivity factors (α) for each compound were calculated from $\alpha = k'/k'_{BaP}$, where k'_{BaP} is the capacity factor for benzo[a]pyrene.

RESULTS AND DISCUSSION

In gas chromatography Laub *et al.* (3) and Kong *et al.* (4) have reported the use of mixed liquid phases to optimize the separation of selected PAH solutes. In liquid chromatography Glajch and

Kirkland (5) recently reported mixing LC packing materials with different chemically bonded functionalities (i.e., C_8, CN, and phenyl) to optimize the separation of phenylthiohydantoin derivatives of 20 amino acids. Ogan and Katz (6) evaluated the retention and selectivity characteristics of several PAH on columns with different proportions of C_2 bonded material mixed with C_{18} materials and found the contribution of the individual phases to be additive.

In a recent study (2) seven different production lots of a polymeric 5-μm C_{18} material were found to have C_{18} surface concentrations varying from 4.3 — 8.2 μmoles/m^2. The selectivity factors for PAH solutes on these columns varied linearly as the C_{18} surface concentration increased. Two lots of material from this previous study were selected to evaluate the feasibility of mixing C_{18} materials with high and low surface concentrations to obtain columns with intermediate selectivities. The physical characteristics of these two lots of polymeric C_{18} material are summarized in Table 1. The materials from lots 11 and 15 were rejected by the manufacturer's normal criteria for selectivity in the separation of PAH, i.e., insufficient resolution of benz[*a*]anthracene and chrysene on the low coverage material and insufficient resolution of dibenz-

TABLE 1. Physical Characteristics of Different C_{18} Materials

Column Lot[a]	Surface Area[b] (m^2/g)	Percent Carbon[c] (wt% ± 1s)	Surface Concentration[d] (μmol/m^2)	k'_{BaP}[e]
11 (polymeric)	61.0	7.5 ± 0.2	5.7 ± 0.2	3.1
15 (polymeric)	52.4	9.3 ± 0.1	8.2 ± 0.1	4.9
17 (monomeric)	53.4	4.0 ± 0.1	3.1 ± 0.1	0.9

[a]Lot numbers were arbitrarily assigned by authors as in ref. 2.

[b]Determined by BET.

[c]Four samples, uncertainty is ±1σ.

[d]Calculated from equation 2 in ref. 2.

[e]85/15 acetonitrile/water (v/v) as mobile phase.

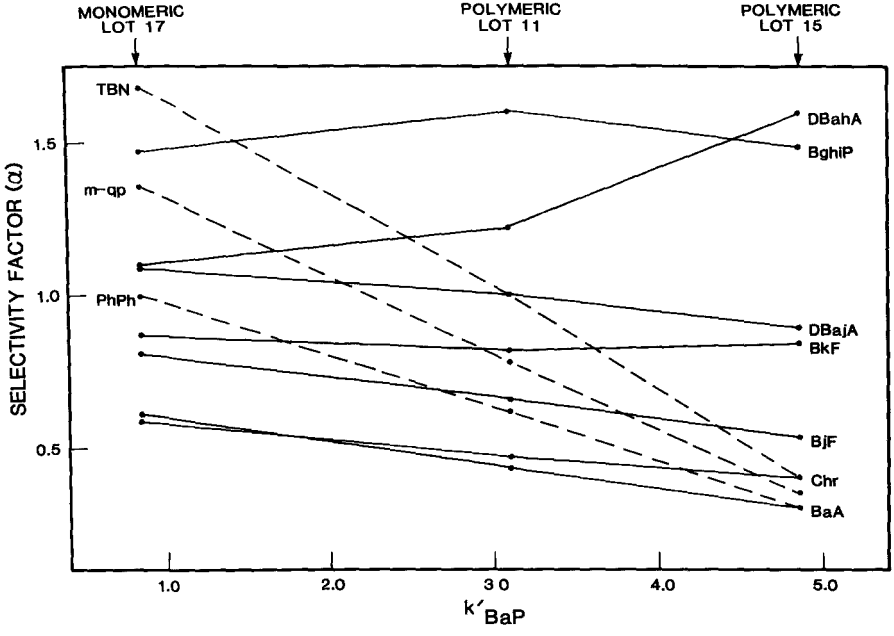

FIGURE 1: Selectivity factors (α), relative to benzo[*a*]pyrene, for selected PAH on three columns containing a monomeric C_{18} material (lot 17) and two polymeric C_{18} materials (lots 11 and 15). BaA = benz[*a*]anthracene, Chr = chrysene, BjF = benzo[*j*]fluoranthene, BkF = benzo[*k*]fluoranthene, DBajA = dibenz[*a,j*]anthracene, BghiP = benzo[*ghi*]-perylene, DBahA = dibenz[*a,h*]anthracene, TBN = 1,2:3,4:5,6:7,8-tetrabenzonaphthalene, PhPh = phenanthro-[3,4-*c*]phenanthrene, and m-qp = *m*-quinquephenyl.

[*a,h*]anthracene and benzo[*ghi*]perylene on the high coverage material. Data are also included in Table 1 for a monomeric C_{18} phase prepared on the same low surface area silica (90 m^2/g) as the two polymeric C_{18} materials.

Selectivity factors for several PAH solutes on these three C_{18} columns are plotted in Figure 1 as a function of k'_{BaP}. As noted previously (2) and as shown in Table 1, k'_{BaP} values are related to the C_{18} surface concentrations. This linear relationship

of k'_{BaP} and surface coverage is valid, however, only when C_{18} phases prepared on silica of the same surface area are compared. In the previous study (2) data for columns from five different lots of the polymeric material and from a monomeric column were plotted in a similar manner as in Figure 1. However, the monomeric column in the previous study was prepared on a high surface area silica (~300 m^2/g) with 13% carbon resulting in a k'_{BaP} value of ~5.5. The monomeric column in the present study was prepared on a low surface area silica (90 m^2/g) with a 4% carbon loading resulting in a k'_{BaP} value of 0.9. As shown in Figure 1, when the selectivity factors are plotted as a function of k'_{BaP}, the linear trends observed for the polymeric columns can generally be extrapolated back to the monomeric column. This behavior suggests that for some types of PAH solutes (particularly nonplanar solutes such as TBN and PhPh in Figure 1) selectivity is dependent only on the extent of C_{18} surface coverage (when phases prepared on silicas with similar surface areas are compared) rather than the monomeric or polymeric nature of the C_{18} phase.

Mixed phase columns were prepared by combining material from lots 11 and 15 in proportions of 70/30, 50/50, and 30/70 (w/w) lot 11/lot 15. The selectivity factors (α) for several PAH are plotted in Figure 2 as a function of k'_{BaP} values obtained for columns from lots 11 and 15. The values on the abscissa for the mixed phase columns (70/30, 50/50, and 30/70 lots 11/15) were determined from the α values of the PAH. The data in Figure 2 indicate that columns of intermediate selectivities can be prepared by mixing the two phases of different selectivity. The selectivities of these mixed phase columns were found, however, to be slightly different from those predicted by the actual mixture proportions, i.e., predicted k'_{BaP} values of 3.62, 3.97, and 4.32 compared to actual values of 3.84, 4.10, and 4.38 for the 70/30, 50/50, 30/70 lot 11/15 mixtures, respectively. The actual and predicted values are in better agreement as the proportion of lot 15 (high coverage) increases.

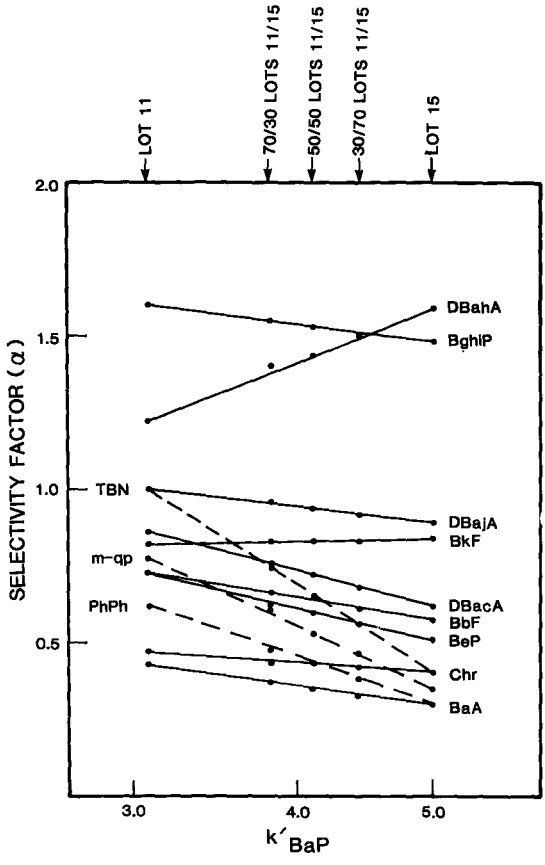

FIGURE 2: Selectivity factors (α), relative to benzo[*a*]pyrene, for selected PAH on polymeric C_{18} columns from mixtures of two different lots. BeP = benzo[*e*]pyrene, BbF = benzo[*b*]fluoranthene, DBacA = dibenz[*a*,*c*]anthracene. See Figure 1 for additional compound identification.

Differences in column selectivity for seven PAH solutes are illustrated in Figure 3. Six columns were compared: the monomeric phase, the high and low coverage polymeric phases, and three columns consisting of mixtures of the two polymeric phases. Phenanthro[3,4-*c*]phenanthrene (no. 2) and 1,2:3,4:5,6:7,8-tetrabenzonaphthalene (no. 4) are nonplanar solutes for which the selectivity factors vary significantly as the C_{18} surface concentration varies (see Figure 2). These differences in selectivity are discussed in detail elsewhere (2). On the monomeric column the phenanthro[3,4-*c*]phenanthrene and the 1,2:3,4:5,6:7,8-tetrabenzonaphthalene have longer retention times relative to the planar PAH than on the polymeric columns. Since the selectivity factors for these two solutes are very sensitive to changes in the C_{18} surface coverage, they have been used as an empirical test to evaluate the monomeric or polymeric characteristics of C_{18} columns by comparing their selectivity factors relative to a planar PAH solute (7). For the selected solutes, the columns from lots 11 and 15 did not separate all seven solutes. However, a mixed phase column of 70% lot 11 and 30% lot 15 provided the appropriate selectivity to achieve separation of all of these solutes. These studies indicate that columns of specific selectivity can be prepared by mixing C_{18} phases of different selectivities.

The selectivity factors obtained for the 50/50 lot 11/lot 15 mixed column were compared with data obtained by coupling two short columns (12.5 cm each), one containing material from lot 11 and one containing material from lot 15. The results shown in Table 2 indicate that short columns of appropriate lengths packed with materials from different lots can be coupled to achieve selectivities similar to those obtained by physically mixing the different packing materials. Thus, the chromatographer could have a collection of short C_{18} columns of different selectivities which could be coupled together in various combinations to achieve the necessary selectivity for a particular separation.

To illustrate the potential use of mixed phase columns of different selectivities, a fraction containing five condensed ring

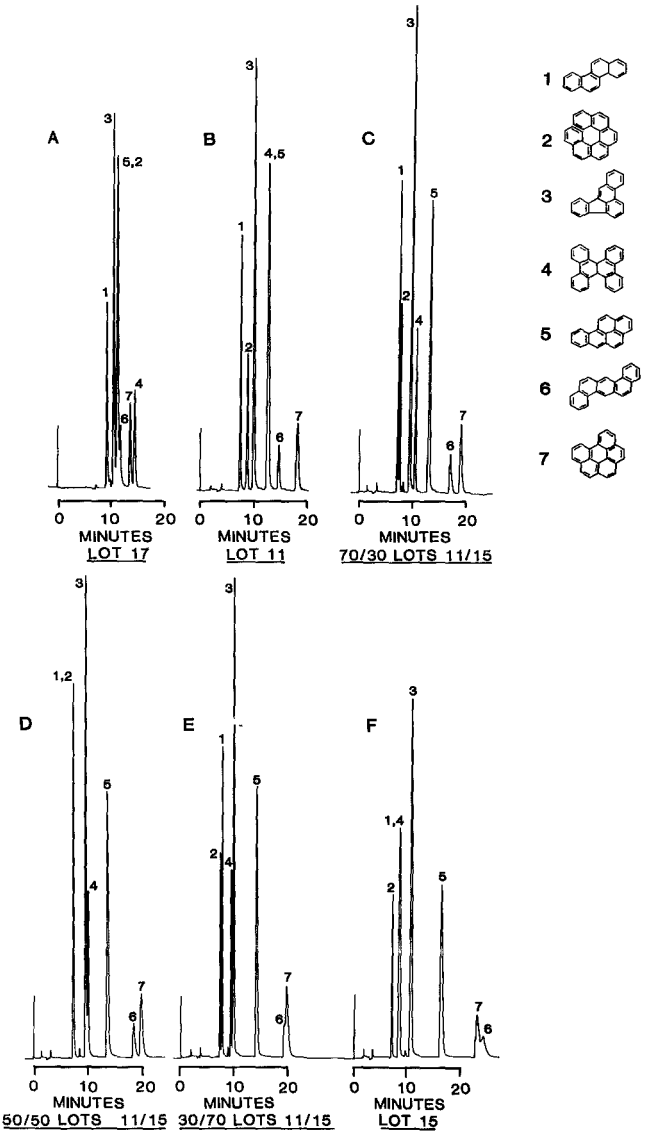

FIGURE 3: Reversed-phase LC separation of selected PAH on a monomeric C_{18} column from lot 17 (A) and on polymeric C_{18} columns from lot 11 (B) and lot 15 (F) and mixtures of these two lots [70/30 (C), 50/50 (D), and 30/70 lot 11/15 (E)]. Compound identification: (1) chrysene, (2) phenanthro[3,4-*c*]phenanthrene, (3) benzo[*b*]fluoranthene, (4) 1,2:3,4:5,6:7,8-tetrabenzonaphthalene, (5) benzo[*a*]pyrene, (6) dibenz[*a*,*h*]anthracene, and (7) benzo[*ghi*]perylene.

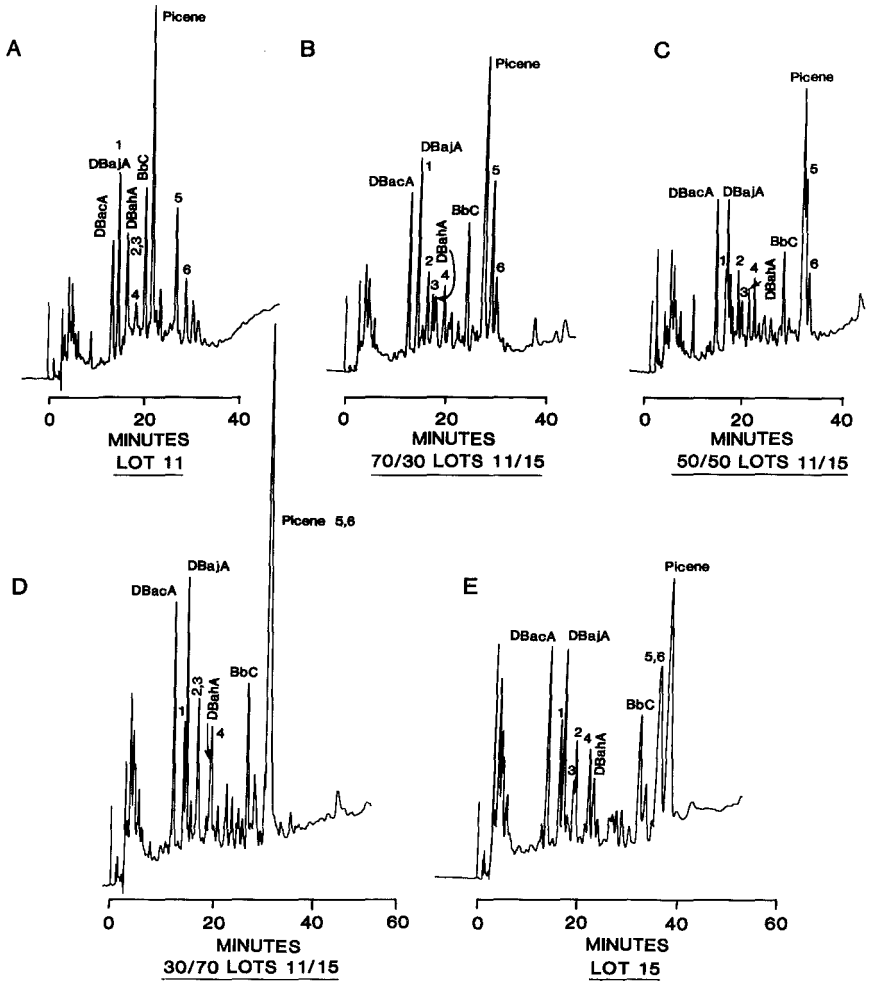

FIGURE 4: Reversed-phase LC separation of five condensed ring PAH fraction (isolated from an air particulate extract) on C_{18} columns of different selectivities (A) lot 11, (B) 70/30 lot 11/15, (C) 50/50 lot 11/15, (D) 30/70 lot 11/15, and (E) lot 15. Peak identification: DBacA = dibenz[*a*,*c*]anthracene, DBajA = dibenz[*a*,*j*]anthracene, DBahA = dibenz[*a*,*h*]anthracene, BbC = benzo[*b*]chrysene, and no. 6 = coronene. Peaks 1-5 are unknown.

TABLE 2. Selectivity Factors (α) for Selected PAH on Mixed vs. Coupled Columns

	50/50 Lot 11/15	
	Mixed	Coupled
m-Tetraphenyl	0.288	0.291
Phenanthro[3,4-*c*]phenanthrene	0.436	0.438
9,10-Diphenylanthracene	0.454	0.459
m-Quinquephenyl	0.557	0.564
Tetrabenzonaphthalene	0.676	0.676
Dibenz[*a*,*c*]anthracene	0.728	0.730
Dibenz[*a*,*j*]anthracene	0.939	0.941
Dibenz[*a*,*h*]anthracene	1.42	1.43

PAH (molecular weight of 278) and coronene, which was isolated from an air particulate sample as described previously (8), was separated on columns from lots 11 and 15 and on the three mixed phase columns. This fraction contains coronene and five PAH isomers of molecular weight 278 which were identified by gas chromatography-mass spectrometry (GC-MS) and LC with fluorescence detection as dibenz[*a*,*c*]anthracene, dibenz[*a*,*j*]anthracene, dibenz-[*a*,*h*]anthracene, benzo[*b*]chrysene, and picene. In addition, at least one unidentified PAH isomer of molecular weight 278 was confirmed by GC-MS.

The LC analyses of this fraction on the five different columns are shown in Figure 4. All of these chromatograms were obtained under the same chromatographic conditions. The five identified PAH isomers of molecular weight 278 were separated on each of the five columns. However, the unknown peaks (nos. 1-5) and coronene coelute with each other and/or with the identified peaks on some of the columns. These chromatograms illustrate the advantage of having several columns of differing selectivities for the analysis of complex environmental PAH mixtures.

CONCLUSIONS

The complexity of PAH mixtures often necessitates the modification of column selectivity to obtain the separation of particular constitutents. Two methods for achieving a particular selectivity for PAH in reversed-phase LC have been described in this paper, i.e., physically mixed C_{18} sorbents of different selectivities and coupled columns each containing a different C_{18} sorbent. Both of these methods provide a selectivity which is intermediate to that of the individual C_{18} materials and which is related to the proportions of each material. These two approaches allow the chromatographer to "customize" a column with an optimized selectivity for the separation of the components of interest. In practice the coupled column approach provides the most flexibility for preparing columns of a specific selectivity. With the availability of several short columns containing C_{18} materials with greatly differing selectivities, a particular intermediate selectivity can be achieved readily by coupling the appropriate columns.

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AGING OF PRESSURE SENSITIVE ADHESIVES. II: USE OF MULTIDETECTOR SEC

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ABSTRACT

The use of multiple detector size exclusion chromatography is described for the study of aging of pressure sensitive adhesive films based on styrene-isoprene-styrene block copolymers. In this preliminary investigation of thin films, room temperature oxidation resulted in the formation of an easily detected carbonyl chromophore. While the chromophore concentration was monitored with a UV detector on the chromatograph, the molecular weight distribution was measured with a differential refractometer. Several important general implications of this combination of detectors in SEC are described.

INTRODUCTION

The first paper in this series (1) described initial efforts to elucidate the nature of aging changes in the commercially important styrene-isoprene-styrene (SIS) block copolymers, especi-

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ally as they are incorporated in pressure sensitive adhesives (PSA). The use of multi-detector size exclusion chromatography (SEC) has proven extremely valuable in this study, and several aspects of its application will be described herein.

The use of SEC for the analysis of polymers has been widely reviewed (2-5). Since SEC is primarily used to determine the molecular weight distribution (MWD) of polymers, it has found extensive use in study of polymer degradation, many such applications of which have been discussed by Abbås (6). Relative changes in MWD are very readily observed, and with extreme care useful information can also be obtained on the kinetics of oxidation chain scission.

The usefulness of SEC for the analysis of random and block copolymers has been increased on occasion by using two or more detectors (7,8,9,10). In these studies, typically one detector (usually ultraviolet or infrared) is used to monitor the concentration of the comonomers, while a second detector (refractive index or light scattering) monitors molecular weight. Harmon and Folt (7) used such a technique to measure the comonomer ratio in SBR. In a similar study, Stojanez et al. (8) investigated both random and block styrene/butadiene copolymers. In the latter, the copolymer composition was determined by SEC, infrared spectroscopy and nuclear magnetic resonance spectroscopy, and although results for the block copolymers were in very good agreement, the random SBR results varied somewhat. This was attributed to a change in the UV extinction coefficient of styrene, depending upon the sequence length in the random copolymers.

Dual detector (UV/RI) SEC has also been used to determine the distribution of olefinic linkages in elastomers (11). In some elastomers, small amounts of unsaturated monomers are incorporated for crosslinking, and to estimate the concentration of these external double bonds, Anderson reacted double bonds in the elastomer with 2,4-dinitro-benzene sulfonyl chloride, thus forming a chromaphore with a high extinction coefficient at 254 nm. While the SEC separated the "stained" elastomer by molecular size, the

RI detector measured the total concentration of polymer and the UV detector provided the concentration of external double bonds. Thus, the distribution of external double bonds as a function of MW was determined.

In the oxidative chain scission of polyisoprene, a similar effect is obtained. According to Shelton et. al. (12), the end group residues after the chain scission of polyisoprene are typically aldehydes and ketones. However, approximately 50% of these residues are α,β-unsaturated carbonyls, which are known to have an extremely high extinction coefficient at about 237 nm (13). It is expected that the polyisoprene mid-block in SIS will behave in an anaglogous manner. It is the use of a combination of UV and RI detectors in SEC for studying aging in SIS formulated PSA's which is investigated in this study.

It should be pointed out that multiple detector SEC has received some criticism by Bressau (14), who contends that the connecting tubing and fittings between detectors not only changes the elution volume but also alters the shape of the chromatogram. In his experiments, 20-50 cm capillary tubing and high dead volume fittings were used. By shortening tubing lengths and using only low dead volume fittings, these problems can be minimized.

EXPERIMENTAL

The SEC used in these experiments consisted of a Waters 6000A solvent delivery system with a model U-6 sample injector, 4 μ-Styragel columns (10^5, 10^4, 10^3, and 500Å), a Varian model UV-50 variable wavelength UV detector, and a Waters Model R401 RI detector. These detectors were connected in series, with the RI following the UV detector. Valco low dead volume fittings were used in all connections. The tubing length between the UV and RI detectors was ~25 cm and the capillary ID was .05 cm. The UV flow through cell was cylindrical (1mm x 6mm) to minimize mixing. The solvent was freshly distilled unstablized THF, and the flow rate was 1.5 cm^3/min. The instrument was calibrated with narrow MWD

polystyrene standards (Pressure Chemical), but since the Mark-Houwink constants were not known for the SIS block-copolymers used in these experiments, data are reported as a function of elution volume.

Rubber solutions (20% SIS by weight) were mixed for 24 hours in the dark on a gentle wrist action shaker, using nitrogen-degassed toluene (reagent grade) as the solvent. SIS was from a commercial lot of Kraton 1107 (Shell Chemical Company). The resulting solutions were stored in the dark under argon until used.

Solutions were coated with a Gardner Ultra Film Applicator on 25-μm polyethyleneterephthalate films taped to glass plates. The coatings were first dried in the dark at 20-25°C for one hour under a continuous, slow nitrogen purge. They were then heated to 40°C in a 3300-Pa vacuum for 4 hours. During the heating cycle, a small nitrogen leak was provided to the drying system to sweep away any residual toluene or oxygen. It is likely that most of the butylated hydroxytoluene (BHT) incorporated in the rubber manufacturing process will be removed in this vacuum drying step.

UV spectra of polymers in unstabilized THF were recorded with a Beckman Model 25 spectrophotometer.

RESULTS

In order to determine the optimum wavelength for the UV detector, UV spectra of both polystyrene and polyisoprene in THF were recorded. While the polyisoprene can only be detected below 232 nm, the polystyrene can be detected below 232 nm, and also between 250 and 270 nm. Since it was found that the RI detector responded well to solutions of either polystyrene or polyisoprene, the UV detector was operated at 259 nm, the absorption maximum for polystyrene in that region of its spectrum. It should be noted that UV spectra of the polymer solutions cannot be recorded below ∿224 nm due to a maximum at ∿220 for THF. Further considerations of detector wavelength will be discussed later.

It was found that when solutions taken from thin SIS films were analyzed on this SEC, the UV and RI results were quite

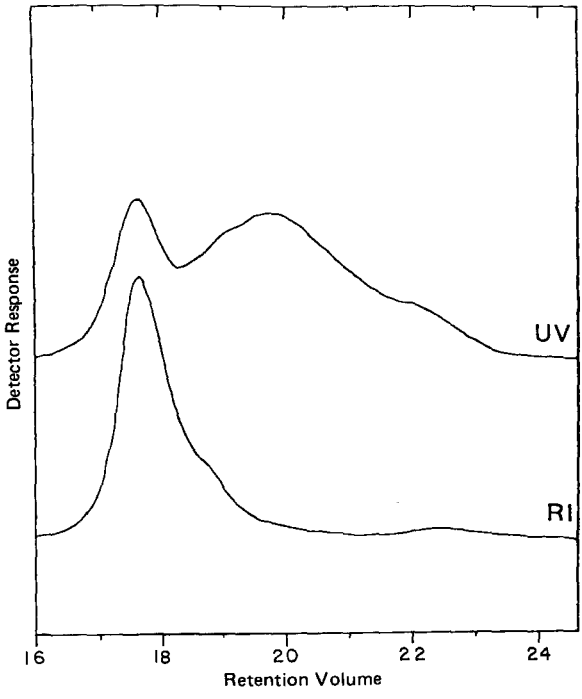

FIGURE 1: SEC chromatograms of unaged 4 μm SIS Films. The UV detector wavelength was fixed at 259 nm and the retention volume was recorded in ml.

different. As seen in Figure 1, the RI detector indicates that very little degradation has occurred to this 0.16 mil unaged SIS film. This molecular weight distribution, is typical of unaged SIS block copolymers. The large peak at 17.8 min ($\sim 2.5 \times 10^5$ amu) represents the SIS tri-block molecules, while the shoulder at 18.8 min ($\sim 1.3 \times 10^5$ amu) results from SI diblock fragments which did not couple in synthesis. The small peak at 22.5 min ($\sim 10^4$ amu) is polystyrene (PS) homopolymer which terminated prior to anionic polymerization with isoprene. For a general discussion of the synthesis of these block copolymers see Dreyfuss, et al. (15).

On the other hand the UV detector response in Figure 1 appears to indicate that substantial degradation of tri-block SIS

TABLE 1

SEC Fraction Location

Fraction	Retention Volume (ml)
1	16.0 - 17.8
2	17.8 - 18.5
3	18.5 - 19.2
4	19.2 - 20.8
5	20.8 - 21.5
6	21.5 - 24.0

molecules to di-block SI fragments has occurred, if one views the response in the usual way as an indication of MWD. Since the RI response is unmistakably MWD, it would appear that very little degradation to the SIS film has actually occurred, and that the small amount of degraded material must contain a chromophore whose molar extinction coefficient (ε) is substantially greater than that of polystyrene at 259 nm, thus yielding a large UV response in this region.

In order to determine the chemical moiety responsible for the very large absorbance at 259 nm, a 3.8μm thick film of SIS was dissolved in THF and fractionated in the SEC. Six fractions were collected, two from the SIS region, one from between the SIS and the SI peaks and three from the SI to the PS region (Table 1). UV spectra (220-290nm) were recorded for each of these fractions and the results are summarized in Figure 2. It was observed that fractions 1 and 2 are composed largely of polystyrene and polyisoprene, as expected. Fraction 3 shows some traces of polystyrene as well as a broad shoulder centered at 240 nm on the large polystyrene/polyisoprene absorption. In fractions 4, 5, and 6, very little polystyrene is observed at 260 nm while the shoulder observed in fraction 3 is now resolved into a peak which has its maximum at approximately 237 nm.

The Woodward-Fieser rules for carbonyl compounds (13), predict that a substituted α,β-unsaturated carbonyl will strongly

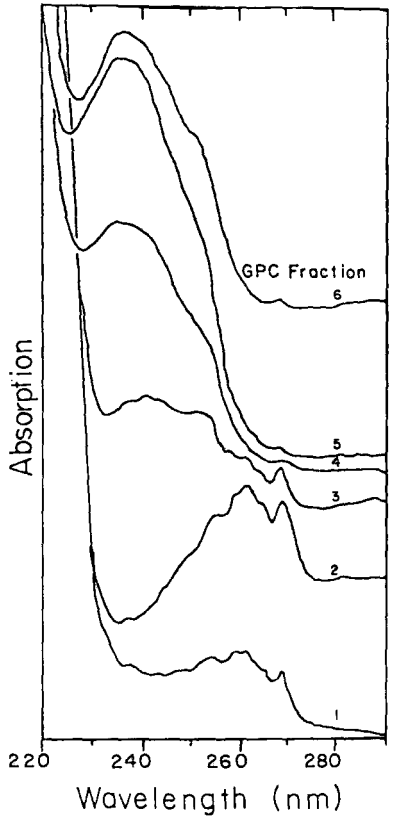

FIGURE 2: Ultraviolet spectra of SEC fractions in THF. The retention volume range of each fraction is shown in Table 1.

absorb between 220 and 250 nm. It has been shown by Shelton et al. (12) that such compounds are formed in the oxidative chain scission of polyisoprene. While low MW compounds like 2-butenone are a volatile product of this oxidative chain scission, other α,β-unsaturated carbonyls may exist as end groups on the isoprene chain after scission.

In addition to this maximum of 237 nm, a strongly absorbing shoulder at ~250 nm is also observed in fraction 3-6. This is probably a similar residue with a slightly different structure. This shoulder extends well beyond 259 nm and thus is responsible for the interference observed in the UV chromatogram (Figure 1), since its extinction coefficient may be as much as an order of magnitude greater than that of PS at this wavelength. Thus, while the RI detector shows very little material in the SI to polystyrene region of the chromatogram, a small amount of material with a high extinction coefficient produces a large UV detector response at 259 nm.

As a result of this finding, SEC chromatograms of 2.5-μm SIS films were recorded with the variable wavelength UV detector at 237 nm. A comparison of UV chromatograms at 237 nm, 226 nm and 259 nm is shown in Figure 3. At 226 nm both polystyrene and polyisoprene absorb strongly, while the α,β-unsaturated carbonyl residue absorbs only moderately. The SI and SIS peaks thus resemble those found in the RI response, and are strongly related to MWD in the polymer; there is however some interference from the strongly-absorbing carbonyl group, as seen by comparison with the RI chromatogram in Figure 1. The SEC chromatogram run at 237 nm is very sensitive to both polystyrene and PI. The chromatogram recorded at 259 nm is less sensitive to the carbonyl group, and moderately sensitive to the polystyrene, however, the carbonyl group still dominates.

These results point out a potential problem from using a fixed wavelength UV detector alone to study MWD in polymers susceptible to oxidation. If only the UV detector at 254 nm had been used in this study, as an example, the results would have

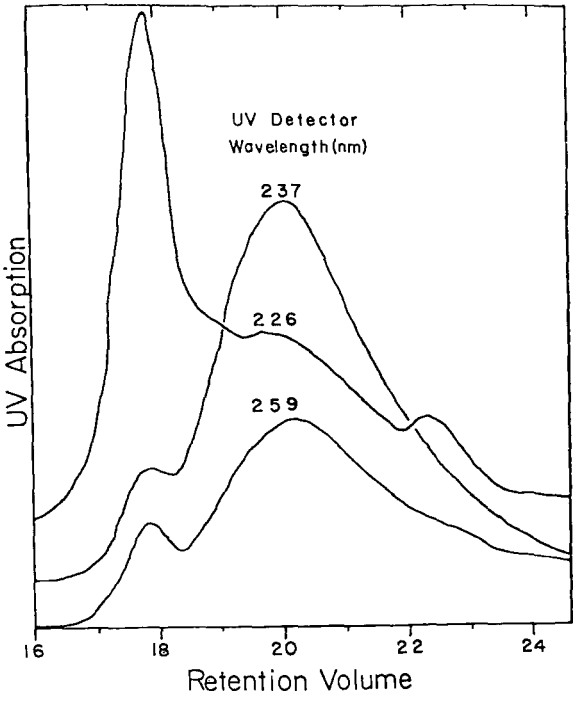

FIGURE 3: SEC chromatograms of 2.5 μm SIS films using a variable wavelength UV detector at 259, 237 and 256 nm.

been very misleading. Data collected at 254 nm would lead one to believe that extensive degradation of SIS tri-block to SI diblock had occurred, while in fact very little occurred, and the small amount of degradation which did occur was accompanied by formation of a product with a very large extinction coefficient. Information obtained simultaneously from UV (226 nm) and RI detectors prevent this misinterpretation. Thus, whenever possible, it is desirable to use both detectors simultaneously when studying unsaturated polymers under conditions of degradation.

From this information it seems that this is a very sensitive method of detecting carbonyl groups that result from the oxidative chain scission of polyisoprene. However, in order for this tech-

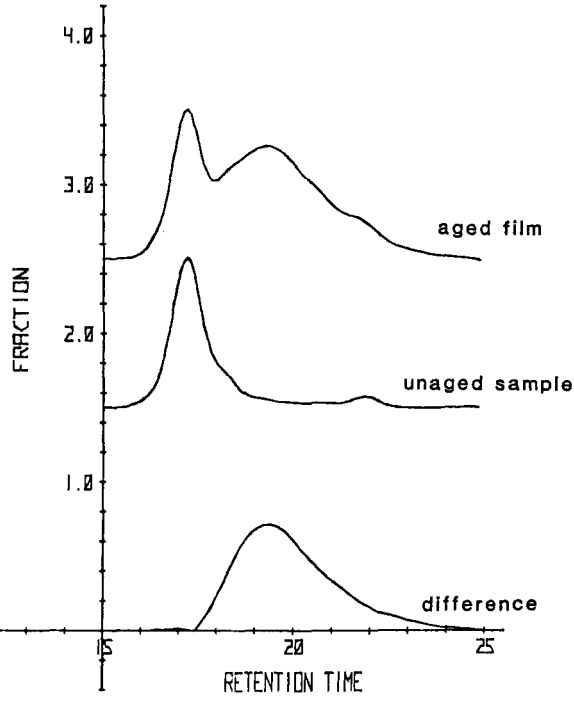

FIGURE 4: SEC difference chromatograms using an UV detector fixed at 237 nm. Normalization of the chromatograms was achieved through dividing the detector response by the response at the SIS peak (16.8 ml). The unaged sample was taken from a bulk SIS lot. The aged film was coated from the same lot and had a dry thickness of 8.6 μm. It was aged in air for seven hours at 80°C.

nique to be at least semi-quantitative, a method of normalization must be designed. Using the information obtained from both RI and UV detectors, we have found that the concentration of SIS molecules in a given sample is essentially constant, for short aging times at temperatures no greater than about 80°C. Also, since this anionically polymerized polymer has a very narrow MWD, one can achieve normalization by dividing each point on the chromatogram by the SIS peak height.

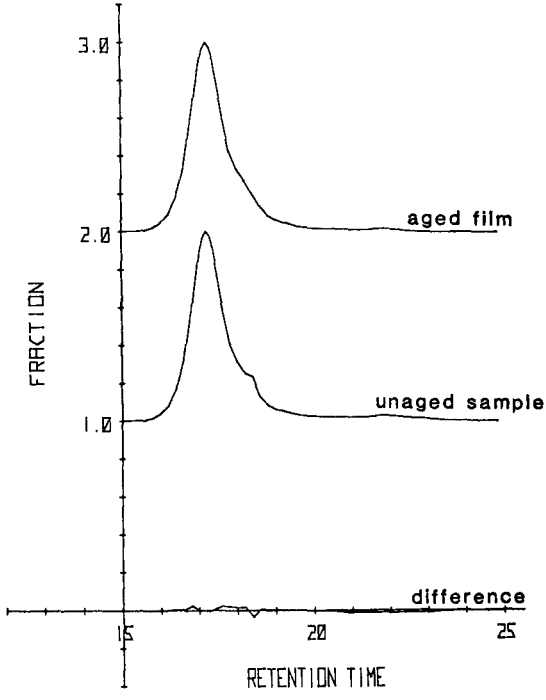

FIGURE 5: SEC difference chromatograms using a RI detector. Normalization of the chromatograms was achieved through dividing the detector response by the response at the SIS peak (16.8 ml). The unaged sample was taken from a bulk SIS lot. The aged film was coated from the same lot and had a dry thickness of 8.6 μm. It was aged in air for seven hr. @ 80°C.

Once the data have been normalized, changes in the carbonyl content can be followed with aging time. In order to facilitate such an experiment, a difference chromatogram is determined by subtracting the chromatogram of an uncoated and unaged sample from one of a coated and/or aged sample, so long as each chromatogram was previously normalized to the SIS peak height. The area under the resulting, difference chromatogram represent the carbonyl content in the sample being investigated. If the film volumes are standardized to 1cm x 1cm x the film thickness, the relative

concentration of carbonyl groups in a film can be calculated from the area under the difference chromatogram. Using such a standard sampling volume, the relative concentration of carbonyl groups in films of varying thickness can be compared.

Figure 4 illustrates the subtraction procedure used. In this case, the chromatogram of SIS bulk polymer is subtracted away from one of an aged (7 hr, 80°C, Air) 8.6 μm SIS film. The area under the difference chromatogram is then calculated and used as a measure of the carbonyl content in the specimen. To illustrate the soundness of this technique, figure 5 shows the subtraction of the RI chromatogram of SIS bulk from one of SIS film aged seven hours at 80°C in air. The difference chromatogram shows that the two films are essentially equivalent, with no appreciable mass change of SIS to SI having occurred.

DISCUSSION

Although SEC is well suited for studies of polymer degradation, detector choice is crucial. In the case of thin SIS block copolymers films a fixed UV detector at 254 nm can give misleading results due to interference from carbonyl bands. By using both a variable wavelength UV detector and an RI detector, the extent of oxidation is easily followed. Further, if the UV detector is set to a wavelength at which the degradation product absorbs, substantial information on the degradation process can be obtained. Such a method has been developed for studying the oxidation of thin SIS block copolymers films used in PSA's. The method measures the formation and/or deterioration of carbonyl groups which absorb at 237 nm. Such groups are typically found as chain end residues after oxidative scission (12).

Further improvement of such experiments could be made by using a UV spectrometer which is capable of quickly scanning (1-2 sec.) the flowing eluent from the SEC (16). This would yield a separate UV spectrum for each segment of the chromatogram. Thus typical components could easily be identified.

In the SIS films examined in this study, the presence of small amounts of oxidative chain scission products had a dramatic effect on the ultraviolet detection of these materials. It is believed that surface oxidation of these films was responsible for this effect. It has been shown by Chang et al. (17) that polybutadiene (PB) films exposed for short periods to room temperature air showed a dramatic increase in adhesive bond strength to other identical films. It was believed that surface oxidation was responsible for this effect. Polyisoprene also undergoes such surface oxidation. However, since chain scission predominates over crosslinking, no covalent bonds are formed across the adhesive interface, as in the case of PB, and no increase in adhesive strength is realized.

The films studied in these experiments were very thin (~2.5 μm) and the surface to bulk ratio was sufficiently high to allow the detection of these materials. Other methods such as multiple internal reflectance infrared spectroscopy (MIR/IR) proved too insensitive to detect the surface oxidation in these films. However, in SIS films aged for several hours at 95°C MIR/IR was found to be very useful. (1) The results of those experiments were found to be in agreement with the FTIR studies of Shelton (12).

Future studies will further characterize surface oxidation in SIS films. These data will then be used to develop a model of the influence of surface oxidation on the aging of SIS based PSA's.

ACKNOWLEDGMENTS

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DETERMINATION OF URIC ACID IN HUMAN SERUM: REVERSED-PHASE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

A method for the simultaneous determination of uric acid in human serum by reversed-phase high-performance liquid chromatography with electrochemical detection has been developed. Human serum (0.5 ml) was mixed with 0.5 ml of 0.2 N perchloric acid solution and the mixture was centrifuged at 3,000 g for 20 min. An aliquot (10 µl) of the supernatant (deproteinized human serum) was injected into the chromatographic system employed in this study. The assay limit for quantitation was about 10 pg for uric acid. Complete separation of uric acid was achieved in about 8 min under the present chromatographic conditions.

INTRODUCTION

As described elsewhere (1), high-performance liquid chromatography (HPLC) may be an obvious candidate as a reference method for the determination of serum uric acid. Different separation principles and detectors have been used for determining uric acid (UA) in serum by aid of HPLC. Ion-exchange columns have been

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used, coupled with ultraviolet detection (2) or electrochemical detection (3). Reversed-phase high-performance liquid chromatography (RPHPLC) has been also used with ultraviolet detection (4). Recently, we have developed a method for the determination of UA and catecholamines in rat serum and brain by RPHPLC with electrochemical detection (5).

The object of this paper is to report a simple, rapid, selective and highly sensitive method for the determination of UA in human serum by RPHPLC with electrochemical detection (ECD).

MATERIALS AND METHODS

UA was purchased from Wako Pure Chemicals, Tokyo, Japan. All other chemicals used in this study were the same ones as used in our previous report (6). All buffers and aqueous solutions were prepared with glass-distilled deionized water.

Serum samples prepared from healthy adult men were stored at $-80^{\circ}C$ until use. Standards were prepared by appropriately diluting a stock UA solution (1mg/ml) with water. The stock solution was prepared as follows. An aliquot (0.05 ml) of 0.05 N NaOH was dropped into 10 mg of UA for dissolving it completely because of its low solubility in pure water. A 0.1 N HCl (9.95 ml) was added into the alkaline UA solution.

Samples were analyzed using a reversed-phase partition mode of HPLC. A JASCO-HPLC, TRIROTAR III, was used throughout this

work. The instrument was fitted with an electrochemical detector (Model ECP-1, Kotaki Inc., Funabashi, Chiba, Japan). A Finepak SIL C18 column (ø 4.6 x 250 mm, JASCO, Tokyo, Japan) was used for the separation of UA. The column temperature was always kept at 35°C. The mobile phase was 0.2 M phosphate buffer (KH_2PO_4-H_3PO_4, pH 2). The flow rate was 0.5 ml/min. Ten microliters of each sample was injected into the RPHPLC-ECD system. The electrochemical detector was set at +800 mV vs. the silver/silver chloride reference electrode, as Pachela et al. (2) reported that the onset potential of UA was about +330 mV vs. the silver/silver chloride reference electrode under their electrochemical conditions and also that an electrochemical detector was set at +800 mV. Fig. 1 shows the standard curve for high-performance liquid chromatographic determination of UA under the present chromatographic conditions. As shown here, the minimum detectable quantity is about 10 pg for UA. UA was quantitated by comparing the peak height in the respective chromatogram with value from a standard curve.

RESULTS AND DISCUSSION

Recently, we have found (5) that alumina can adsorb UA as well as catecholamines and also that the adsorption of UA onto alumina is not always quantitative. These findings suggest that the so-called alumina treatment procedure recently optimized (6) is not useful for extraction and preliminary purification of UA in biological materials. Therefore, we aimed to develop a pretreatment in

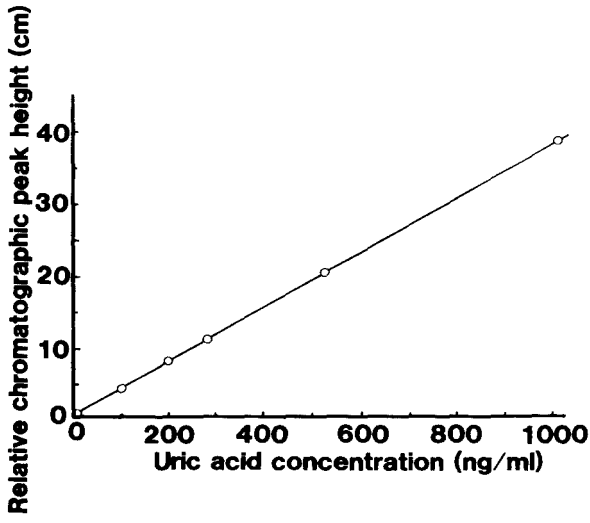

Figure 1. Calibration curve for uric acid under the present chromatographic conditions.

extracting procedure for UA of biological materials. In addition, we have found (5) that concentrations of UA in rat serum and brain are greater than those of any other electrochemically active components in both biological materials. From the above observation, we assumed that simply deproteinized serum might be able to be injected into the RPHPLC-ECD system for the determination of UA in serum without any interferences.

Deproteinization of human serum was achieved as follows: mix vigorously 0.5 ml of human serum with an equal volume of 0.2 M perchloric acid solution, and then centrifuge at 3,000 g for 20 min. An aliquot (10 μl) of the simply deproteinized serum was injected into the RPHPLC-ECD system.

Figure 2 shows such a reversed-phase high-performance liquid chromatogram. Peak X in the chromatogram has been found to be

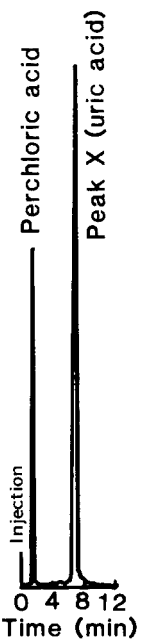

Figure 2. The typical reversed-phase high-performance liquid chromatogram, obtained by injecting 10 μl of the deproteinized human serum into the RPHPLC-ECD system. Ten microliters of the serum was injected into a column (Finepak SIL C18, JASCO) by using a microsyringe. The eluent was 0.2 M phosphate buffer (KH_2PO_4-H_3PO_4, pH 2.0). The column temperature was maintained at 35°C. The flow rate was 0.5 ml/min. Eluate from RPHPLC was electrochemically monitored by aid of an electrochemical detector under the potentiostatic condition (+800 mV vs. Ag/AgCl. The sensitivity of the detector was set at 64 nA full scale. For further explantions, see the text.

UA after the further co-elution of a mixture of UA and the deproteinized serum. As shown in Fig. 2, the method for the determination of UA in serum, which has been developed in this study, is not subject to interferences in other methods. The phosphotungstic acid method is subject to interference resulting from endogenous

nonurate chromogens, nutrients, and drugs (7). Urikase methods based on spectrophotometry are also not without problems, in that the specificity of the absorbance measurement is less than that of the enzyme, and several interferences have been noted (8). Content of UA in 0.5 ml of healthy human serum, as shown in Fig. 2, was found to be 21.6 μg (43.2 μg/ml). Since the sample preparation did not involve any transfer, the value for UA amount might be nearly absolute, indicative of an endogenous quantity.

As described above, we have developed a practical method for the determination of UA in human serum by RPHPLC with ECD. In addition, most recently, we have developed a method for the determination of xanthine and hypoxanthine by RPHPLC with ECD (Iwamoto, Yoshiura, and Iriyama, to be published in Jikeikai Med. J. with any other related experimental results). Xanthine, hypoxanthine, and UA are themselves produced either as a result of the breakdown of cellular material in toto, the turnover of nucleic acids in the cells, or as a result of the intermediary metabolism of various purine nucleotide derivatives. Modern biochemical investigators have found purine metabolism in general to be of great theoretical interest, and in addition to the problem there is hope the study of this metabolic system will provide answers to a number of ancilary enigmas of biochemistry. The biochemical and clinical importance of UA in gout and several other desease states was discussed by Balis (9) and Glynn et al. (10). As described by Glynn et al. (10), previous epidemiologic studies of UA have been limited by a cross-sectional design which precludes a determination of factors predictive changes in UA levels. Furthermore, some studies have not controlled for the

health status and drug intake of their populations. We believe that the well-known separation power of RPHPLC, combined with current state of the art in electrochemical monitoring will circumvent some of the problems presently encountered in the analysis of UA of biological and clinical importance. Because of its simplicity and applicability to small sample volumes, this method is useful in basic biomedical research. For example, we have determined concentrations of UA in rat tissues (e.g. brain, heart, stomach, and ren) according to the procedures developed in this study (Yoshiura, Iwamoto, and Iriyama, to be published elsewhere). This method can be applied for studying the change of UA levels in mammalian tissues.

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HIGH PRESSURE LIQUID CHROMATOGRAPHIC ASSAY OF CEFAMANDOLE IN SERUM FOLLOWING INTRAVENOUS AND INTRAPERITONEAL ADMINISTRATION

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ABSTRACT

Following cesarean section 102 women were treated with cefamandole by either perioperative intravenous administration or intraperitoneal irrigation. High-pressure liquid chromatographic (HPLC) methods for the quantitation of the low serum levels of cefamandole following intraperitoneal lavage were developed. The antibiotic was assayed in the serum using a standard microbiological assay and two types of reverse phase column technology for HPLC. The two HPLC systems were almost identical in performance. Both HPLC methods were at least 10-fold more sensitive than the microbiological assay. The correlation between the three methods was 0.9739. The half-life of cefamandole was 37 min, which was not significantly different from the half-life of the drug in serum of non-pregnant women. The peak serum levels were 47.6 ± 36.8 μg/ml and 1.98 ± 1.5 μg/ml for the intravenous and intraperitoneal methods of administration, respectively.

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INTRODUCTION

Intrauterine intraperitoneal (IU/IP) irrigation and intravenous (IV) administration of antibiotics have been used effectively as prophylaxis in high-risk cesarean section patients (1,2). Rudd and associates used IU/IP lavage with cefamandole nafate to reduce post-cesarean section endometritis (2). Thirty patients received antibiotic via IU/IP irrigation and none developed endometritis. The reported serum levels of cefamandole using IU/IP lavage were frequently less than 0.5 μg/ml. Although the microbiological assay may be capable of measuring these low levels, the coefficient of variation of the assay has been very large (3). The purpose of this investigation was to develop a phase extraction procedure for cefamandole and to develop sensitive high-pressure liquid chromatographic (HPLC) assays for the determination of the wide range of levels of cefamandole anticipated in serum subsequent to IU/IP lavage and IV administration of the drug. Two types of column technology were compared, the conventional C_{18} μ-Bondapak steel column, and the new Radial Compression System (RCM-Z) containing a C_{18} μ-Bondapak compressible column. Serum levels were then determined following both IU/IP irrigation and the IV administration of the drug. In addition, the half-life of cefamandole was calculated from these data to determine if physiologic changes associated with pregnancy affected the clearance of the drug from these women (4).

MATERIALS AND METHODS

Patients

One hundred and two women at high-risk for infection following cesarean section were entered into this study. Informed consent was obtained. The patients received either 2 g of cefamandole IV over a 20 min infusion or 2 g of cefamandole in 800 ml of saline as an IU/IP lavage of the uterus and pelvis commencing at repair of the uterine incision. Using a bulb syringe, the irrigant was applied and simultaneously suctioned as follows: 200

ml to the uterine incision before closure, 200 ml to the bladder flap and closed uterine incision, 200 ml to the colic gutters with the patient in reverse Trendelenburg position, and 200 ml to the subfascial and subcutaneous space. Blood samples were obtained at 30, 60, and 120 min following both the IU/IP irrigation and IV administration of the drug. The serum was separated and frozen at -20°C until assayed.

Microbiological Methods

The serum concentration of cefamandole was measured by the agar diffusion technique (5). The test organism was Bacillus subtilis ATCC 6633. All specimens were assayed in duplicate.

HPLC Assay

The serum concentration of cefamandole was measured by modification of an existing HPLC assay for other penicillin and cephalosporin antibiotics (6,7). The HPLC analysis was performed on an extract of 0.5 ml of serum. The sera were extracted using a two phase extraction procedure. Acetonitrile and dichloromethane were used in the phase extraction which eliminates dilution of the specimens. A 50 µl aliquot was injected into the HPLC. The HPLC system was an ALC/GPC Model 204 liquid chromatograph attached to a Model 710 B Waters Intelligent Sample Processor (WISP), (Waters Associates, Milford, Mass.). All analyses were performed utilizing a 30 cm µ-Bondapak C_{18} steel column (Waters Associates, Milford, Mass.). The eluate was monitored at 254 µm with a Waters 440 detector. The detector sensitivity was 0.10 absorbance units (AU) full scale for the analysis of serum extracts from patients receiving intravenous cefamandole, and 0.01 AU for analysis of serum extracts from patients receiving IU/IP irrigation with cefamandole. Peaks were recorded on a 10 mv chart recorder (Houston Instruments, Houston, Texas) at a chart speed of 0.5 cm/min. The mobile phase consisted of 0.1 M sodium phosphate (85%) and acetonitrile (15%) at a pH of 6.0. The flow rate was

3.0 ml/min. Prior to use the mobile phase was degassed by filtration with a 0.45 μm FHUP filter (Millipore Corp., Bedford, Mass.).

In addition, assays were performed using the radial compression Z module (RCM-Z) (Waters Assoc., Milford, Mass) equipped with a column 10 cm long with an inside diameter of 8 mm containing a 10 μ C_{18} packing which should produce a more rapid and efficient separation than the 30 cm C_{18} steel column. The mobile phase for this system consisted of 0.1 M sodium phosphate (83%) and acetonitrile (17%) at a pH of 6.0. The flow rate was 4.0 ml/min. Prior to use the mobile phase was degassed by filtration as described previously.

Standard curves for both HPLC assays of cefamandole in serum were generated by extracting and assaying normal human serum spiked with cefamandole ranging from 0 to 100 μg/ml. In addition, three serum specimens containing an unknown quantity of cefamandole were assayed with the patients' specimens each time the assay procedure was performed (the quantities were unknown to the person performing the assay). The controls and the unknowns were prepared by the addition of a stock solution of cefamandole to serum with a microliter syringe (Hamilton Co., Reno, Nevada). Between-batch and within-batch recoveries were determined throughout the study using the spiked controls prepared as mentioned previously.

Statistics

Friedman's Chi-square nonparametric analysis was used to compare the three assay methods. Correlation coefficients for the three methods were done using multiple least squares linear regression. Comparison of the between-batch and within-batch data were done by linear least squares regression.

RESULTS

A chromatogram of the separation of cefamandole is depicted in Figure 1. Chloramphenicol, vancomycin, aminoglycosides, penicillins, cephalosporins or theophylline were without effect on

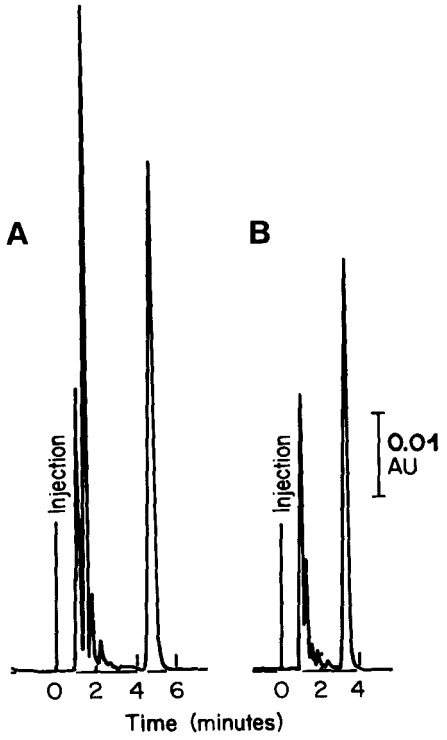

FIGURE 1. HPLC chromatograms of serum extracts of cefamandole (A) normal pooled human serum containing 47.5 µg/ml of cefamandole using a standard µ-Bondapak C_{18} steel column; (B) the same serum specimens using the Waters Radial Compression System (RCM-Z) equipped with a C_{18} column.

the separation when added to the specimens. Between-batch and within-batch reproducibility studies showed recoveries of at least 84% for the wide range of levels found using the two different methods of administering cefamandole. The within-batch and between-batch reproducibility data are depicted in Tables 1 and 2 and the correlation among the three methods of assay are shown in Table 3. When standard curves were prepared for quantitation of the low IU/IP irrigation levels, recoveries of greater than 90%

TABLE 1.

Results of Within-Batch Reproducibility With the C_{18} Column and the RCM-Z Module.

Cefamandole Concentration (µg/ml)	C_{18}				Average % Recovery	RCM-Z				Average % Recovery
	n	$\bar{x}$	SD	CV %		n	$\bar{x}$	SD	CV %	
1.0	9	0.84	0.05	5.95	84.0	8	0.90	0.04	4.44	90.0
5.0	7	4.93	0.18	3.65	98.6	6	4.76	0.17	3.45	95.2
24.4	9	23.80	0.74	3.11	97.9	10	24.5	1.00	4.08	100.0

$\bar{x}$, mean; SD, standard deviation; CV, coefficient of variation.

TABLE 2.

Results of Between-Batch Reproducibility With the C_{18} Column and the RCM-Z Module.

Cefamandole Concentration (µg/ml)	C_{18}				Average % Recovery	RCM-Z				Average % Recovery
	n	$\bar{x}$	SD	CV %		n	$\bar{x}$	SD	CV %	
1.0	5	0.87	0.13	14.83	87.0	4	0.83	0.10	12.05	83.0
5.0	5	4.57	0.49	10.72	91.4	4	4.77	0.38	7.97	95.4
24.4	3	24.28	0.88	3.62	99.5	4	23.74	1.45	6.11	97.3

$\bar{x}$, mean; SD, standard deviation; CV, coefficient of variation.

TABLE 3.

The Comparison of Concentration of Cefamandole by the Microbiological, the C_{18} Column and RCM-Z HPLC Methods.

Method of Administration	ASSAY		
	Microbiological	HPLC RCM-Z	HPLC C_{18}
IP	3.40	3.95	3.10
IP	2.60	2.23	1.70
IP	6.20	5.38	4.50
IP	4.50	2.72	2.30
IP	0.80	0.74	0.45
IP	2.95	2.80	2.10
IP	1.30	1.10	1.10
IP	0.60	0.76	0.45
IP	7.50	5.90	5.10
IP	3.60	5.10	4.20
IP	2.80	3.20	2.80
IP	1.30	1.00	0.86
IV	34.00	46.30	44.10
IV	15.20	21.00	18.60
IV	4.50	6.90	5.80
IV	26.50	21.90	17.10
IV	13.50	10.50	7.70
IV	4.30	3.50	2.80
IV	0.74	0.81	0.86
IP*	ND	0.31	0.67
IP*	ND	0.14	0.51
IV	46.00	34.90	33.80
IV	21.00	18.10	17.20
IV	4.20	5.10	3.90
IV	34.00	31.40	26.10
IV	14.00	13.60	11.00
IV	2.90	4.30	3.70
IV	53.00	55.50	45.40
IV	31.80	25.90	23.50
IV	102.00	100.50	106.90
IV	15.80	20.20	19.30
IV	47.50	37.00	36.00
IV	18.50	26.00	25.00

* Indicates that microbiological assay could not detect the presence of cefamandole in these specimens.

The correlation matrix for the above assays are as follows.

Microbiological to RCM-Z = 0.9792
Microbiological to C_{18} = 0.9739
RCM-Z to C_{18} = 0.9934

IP = Intraperitoneal.
IV = Intravenous.

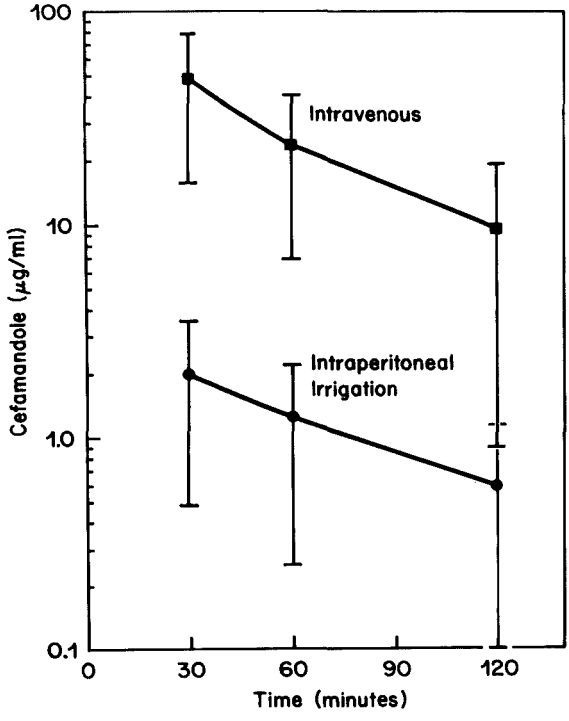

Figure 2. Semi-logarithmic plot of serum levels following the intravenous or intrauterine administration of 2 grams of cefamandole. Each time represents the mean and standard deviation. Forty-five or more values were used to achieve each point.

were obtained (data not shown). All methods were accurate and specific for cefamandole. The limit of detection for cefamandole by HPLC was < 0.05 µg/ml. The limit of the microbiological assay was approximately 0.50 µg/ml. Therefore the concentration of cefamandole in some specimens could not be measured by the microbiological assay method. The regression coefficient ($Y = \alpha + \beta X$; where X and Y represent the dependent and independent variables, β is the regression coefficient in the population and α is the value of Y when X is 0) for the comparisons of the RCM-Z method to the

TABLE 4.

Serum Concentration of Cefamandole Following Intravenous Infusion and Intrauterine Irrigation at Cesarean Section.

Administration	No. of Patients	30 Min.	60 Min.	120 Min.
IV Mean	53	47.6 ± 31.80**	23.67 ± 16.80	9.54 ± 9.67
Range		(15.0 - 165.6)	(3.5 - 80.9)	(0* - 34.7)
IU/IP irrigation Mean	49	1.98 ± 1.50	1.24 ± 0.99	0.58 ± .5.7
Range		0.1 - 5.1	(0.05 - 2.6)	(0 - 2.3)

** µg/ml ± standard deviation.
* Less than 0.01 µg/ml of cefamandole.

microbiological assay method, the C_{18} steel column method to the microbiological assay method, and the RCM-Z to the C_{18} steel column assay method was 0.51 = 0.953X, -0.87 + 0.957X, and 1.56 + 0.984X, respectively.

Friedman's Chi-square test indicated a significant difference in the comparison of the microbiological data with the HPLC C_{18} steel column data ($p < .001$). However, since these data had a very high correlation coefficient for all three methods, we do not feel that this significance was of practical importance.

Table 4 depicts the serum levels at the times collected. The serum levels decreased from 47.6 µg/ml at 30 min to 9.54 µg/ml at 120 min for the IV dose. The serum levels from the patients receiving cefamandole by IU/IP irrigation decreased from 1.98 µg/ml at 30 min to 0.58 µg/ml at 120 min. The disposition of cefamandole through the two hours is shown on Figure 2. The half-life for both methods of administration was 37 min.

DISCUSSION

The RCM-Z system provided a more rapid analysis of cefamandole than the standard C_{18} steel column. The C_{18} was slightly more sensitive than the RCM-Z as depicted in Figure 1. The decreased sensitivity of the RCM-Z is attributed to its shorter length and larger diameter. The C_{18} steel column has a lower flow rate, thus requiring less mobile phase for completion of the assay. Both HPLC assay methods worked well for the quantitation of cefamandole at the very low serum levels measured in this study.

In this report, IU/IP irrigation with cefamandole resulted in serum levels that were 4.5% of an equivalent IV dose. Although the cefamandole administered by lavage was present in the peritoneal cavity only momentarily, average serum concentrations of 1.98 µg/ml were present 30 minutes following irrigation. The serum concentrations measured in patients receiving cefamandole by IU/IP irrigation were 1.5 to 3 times higher than levels reported in two previous studies (1,8). This likely reflects idiosyncrasies in

the method of irrigation used as well as differences in the duration that the drug was in contact with the peritoneum before it was removed by suction. A second less likely explanation is that the microbiological assays used in the previous reports were less sensitive than the assays used in the present report. The sensitivity and precision of the microbiological assay probably decreases substantially at the low levels of cefamandole in the IU/IP lavage. Since the previous studies reported much lower cefamandole levels, this lack of sensitivity may also explain the discrepancy in results. Following either IU/IP irrigation or IV administration of cefamandole, large variations in serum concentration were measured in these women. We attributed this wide deviation to large differences in volume distribution in these patients who varied greatly in weight but who were all treated with a standard dose of the drug.

The serum half-life for cefamandole in non-pregnant individuals has been shown to be 34 minutes following an intravenous dose and 60 minutes following an intramuscular dose (9). In the present study a serum half-life of cefamandole of 37 minutes was measured in patients following either IU/IP irrigation or IV administration of the drug. It is well established that the glomerular filtration rate (GFR) increases during pregnancy, returning to normal only gradually over several days. It has been suggested that this change in renal function may affect the half-life of several drugs (4,5). From the data presented in this report, it would appear that pregnancy associated changes in GFR do not affect the renal excretion of cefamandole in patients treated with the drug in the peripartum period. The low but significant levels of cefamandole measured in the serum following rapid IU/IP irrigation likely reflects the rapid uptake of the drug across a large, well-vascularized space. Several reports have documented the efficacy of peritoneal lavage with antimicrobials at the time of cesarean section (1,2,8). The decreased incidence of postoperative infection in patients treated with IU/IP irrigation of cefamandole may be the result of either the

local bactericidal action of the drug or the result of a low but significant systemic level of cefamandole obtained in these patients. The results of this investigation justify the evaluation of the mean inhibitory concentrations of cefamandole for the bacteria recovered in these high-risk patients undergoing cesarean section.

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A RAPID HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS MEASUREMENT OF SIX TRICYCLIC ANTIDEPRESSANTS

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ABSTRACT

A reversed-phase high pressure liquid chromatographic procedure has been developed for the quantitation of the concentration of six different tricyclic antidepressants in the plasma of patients undergoing routine drug therapy. Plasma samples were extracted into a 97:3 hexane:isoamyl alcohol solution and then extracted back into dilute acid. A Supelcosil C-8 column with 5 micron packing was employed in combination with an acetonitrile/phosphate buffer/diethylamine mobile phase. At an optimized mobile phase pH of 7.22, baseline separation of all six tricyclic antidepressants plus the internal standard was achieved within 8 minutes. UV detection at 254 nm resulted in limits of detection of 2.5 μg/L for each drug. The potential interferences from 13 different benzodiazepines and neuroleptics was investigated. Five of the 13 parent drugs and three metabolites were found to interfere with this tricyclic antidepressant assay.

INTRODUCTION

The growing practice of monitoring the concentration of tricyclic antidepressants (TCAs) in plasma from patients undergoing antidepressant therapy has required many clinical laboratories to introduce procedures for quantitation of the common antidepressant drugs (1,2). Therapeutic plasma levels of antidepressants are usually in the μg/L range and a number of concurrently administered medications may possibly interfere with the analysis (3,4). These considerations have resulted in many laboratories using high-pressure liquid chromatography (HPLC) for the plasma monitoring of the six common drugs: doxepin, desmethyldoxepin, imipramine, desipramine, amitriptyline and nortriptyline.

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Several authors have published normal-phase chromatographic methods for the separation of one or more TCAs (5-14); however, only Sutheimer has accomplished baseline separation of all six drugs listed above (15). Sutheimer's method is based on the earlier work of Vandemark et al. (6) in which hydrophilic endogenous components were found to cause background interference. Sutheimer did not apply the method to patient samples and, further, presented no information on interferences, precision, or the extraction procedure.

Reversed-phase HPLC techniques have less interference from plasma extracts and have achieved more rapid chromatography of a few TCAs (16-29). Kabra et al. (19) described a procedure providing baseline resolution of all six drugs listed above; however, the retention time for the last eluting component was about 13 min. Bannister et al (21) described a rapid system for the automatic extraction and quantitation of TCAs in plasma samples but routine use required two chromatographic runs with two different standards because all TCAs could not be simultaneously separated in a single run.

Before chromatographic analysis, TCAs must be extracted from the plasma samples. Several liquid-solid and liquid-liquid extraction techniques have been published (3). The liquid-solid techniques have required a second liquid-liquid extraction (28) or evaporation step to concentrate the samples (13). The liquid-liquid extraction methods have required long mechanical shaking times (19), freezing steps for the isolation of the organic phase (11), evaporation of solvent to concentrate the samples (7), or an elaborate autoextraction system not available to many clinical laboratories (21). All of these techniques are time consuming. Additionally, the use of an evaporation step results in increased variability and requires two internal standards (10).

In this paper we report the development of a reversed-phase HPLC method for the simultaneous quantitation of six TCAs in the plasma from patients undergoing routine antidepressant therapy.

EXPERIMENTAL

Apparatus

A Waters HPLC system (Waters Associates, Milford, Ma.) equipped with a Model 441 UV absorbance detector (operated at 254 nm), a Model 6000A solvent-delivery system, a Model 710 autoinjector, a Model 720 integrator and a Model 730 System Controller was employed. A Supelcosil C-8 reversed-phase column (4.6 x 250 mm with 5 μm packing), fitted with a C-8 guard column (4.6 x 50 mm with 40 μm packing) was used (Supelco, Inc., Bellefonte, Pa.).

The mobile phase used for routine analysis was prepared by mixing 53.3 volumes of acetonitrile, 45.1 volumes deionized water, 1 volume diethylamine, and 0.4 volumes 85% phosphoric acid. The pH was adjusted to 7.2 by dropwise addition of phosphoric acid or sodium hydroxide. Other mobile phase combinations were prepared as needed. All mobile phases were degassed by ultrasonic vibration prior to use. Column mobile phase flow rate was 2.0 mL/min.

Reagents

HPLC grade hexane, acetonitrile, and isopropanol, and reagent grade potassium carbonate, diethylamine, and isoamyl alcohol were obtained from Fisher Scientific Co. (Atlanta, Georgia). A 25% solution of potassium carbonate containing 0.1% diethylamine was prepared and stored at room temperature. A 97:3 mixture of hexane:isoamyl alcohol was stored in a glass container at room temperature.

Standards

A standard stock solution of tricyclic antidepressant drugs contained 1 g/L of each of the following pure drugs was prepared in isopropanol: doxepin (Pfizer Laboratories), desmethyldoxepin (Pfizer Laboratories), imipramine (USV Laboratories), desipramine (USV Laboratories), amitriptyline (Merck, Sharp & Dohme Laboratories), and nortriptyline (Merck, Sharp & Dohme Laboratories). This stock standard was stored at -15°C.

A stock internal standard containing 1 g/L of Loxapine (American Cyanamid Co.) was prepared in isopropanol. This solution was stored at -15°C.

A tricyclic working standard (100 μg/L) was made by a 10^4-fold dilution of the TCA stock standard. To prevent adsorption of the TCAs onto the glass container, diethylamine was added to a final concentration of 0.1%. The tricyclic working solution was stored at 4°C.

The working internal standard was prepared by a 10^3-fold dilution of the stock internal standard to a final concentration of 1 mg/L with diethylamine added (0.1%); this solution was stored at 4°C.

Samples

Patient blood samples were collected in Dark Blue stoppered Vacutainers (Bectin-Dickinson, Rutherford, N.J.) and centrifuged within 2 hours of sampling to obtain the plasma. The plasma was then stored at -15°C in a clean Dark Blue Vacutainer tube until analyzed.

Sample Preparation

Two mL of plasma was transferred to a 15 x 150 mm borosilicate glass disposable test tube. Into this tube was added 100 µL of working internal standard, 250 µL of the 25% potassium carbonate solution, and 5 mL of the 97:3 hexane:isoamyl alcohol solution. Each tube was vortexed rapidly for 30 s and then centrifuged for 3 min at 500 x g to break the emulsion. The aqueous layer was removed by aspiration and the organic layer was transferred to a 15 mL glass conical centrifuge tube. A 100 µL aliquot of 0.25 mol/L HCl was added and the tube vortexed rapidly for 30 s. The organic layer was discarded and 50 µL of the aqueous layer was injected onto the column.

Quantitation

The quantity of each drug in a sample was calculated by determining the ratio of the peak absorbance of that tricyclic drug to that of the working internal standard. The concentration was then calculated by comparison with a TCA standard curve generated with each run.

RESULTS

Chromatography

The pH of the mobile phase was found to be a crucial factor in obtaining baseline separation of the six TCAs plus the internal standard. Over the pH range of 3.9 to 7.8 the retention times of the solutes showed considerable variation (Figure 1). At the low pH extreme several of the drugs coelute. Doxepin and nortriptyline reversed in elution order at pH 7. Amitriptyline and loxapine (the internal standard) also reversed in elution order at high mobile phase pH (pH 7.8). The optimal regions of chromatographic separation were determined from the retention data of Figure 1 using the "window diagram" technique popularized by Laub and Purnell (30) and applied to reverse-phase HPLC by Deming and Turoff (31). Third-order models were fitted to the retention data for each of the seven individual peaks. These fitted equations were then employed to calculate the relative retention ratios for all pairs of peaks (21 different pairs in this case) over the pH range investigated. These results are plotted in Figure 2. Regions of best separation for the worst separated pair of peaks are darkened in as "windows" in Figure 2. The highest window occurs at pH 7.22 where the worst separated pairs show a relative retention of about 1.10. At this pH all seven components are completely resolved as shown in the chromatogram of Figure 3.

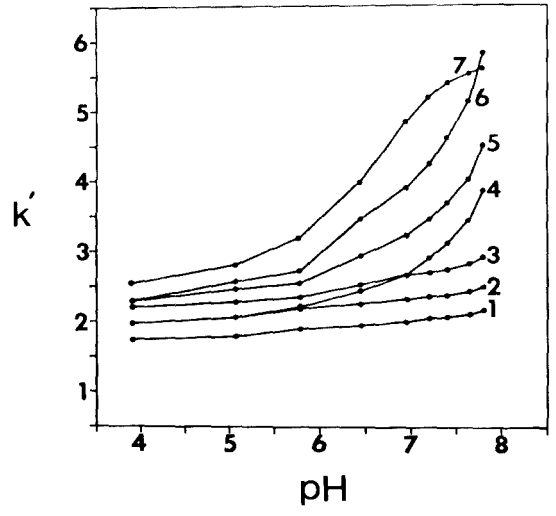

FIGURE 1. The relationship between k' and pH for the seven solutes (1 = desmethyldoxepin, 2 = desipramine, 3 = nortriptyline, 4 = doxepin, 5 = imipramine, 6 = amitriptyline, 7 = loxapine (internal standard)).

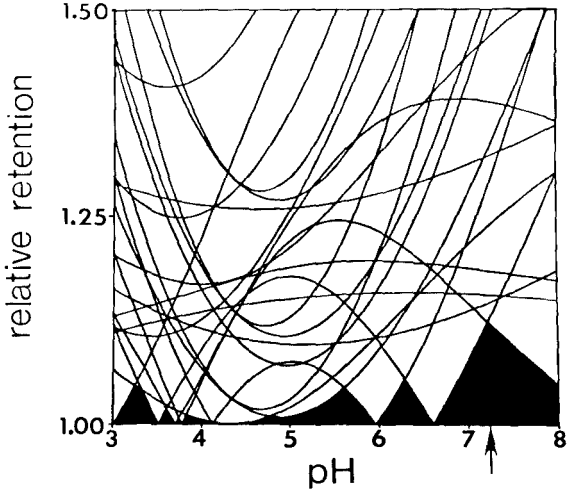

FIGURE 2. Window diagram of relative retention as a function of pH for all 21 pairs of tricyclic antidepressants. The location of the optimum pH is marked by an arrow.

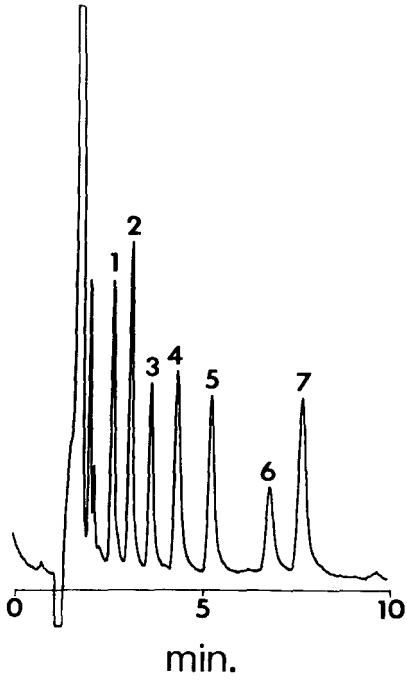

FIGURE 3. Chromatogram of a spiked plasma standard extract containing 100 µg/L of each of the tricyclic antidepressants. Peak identities: 1 = desmethyldoxepin, 2 = desipramine, 3 = nortriptyline, 4 = doxepin, 5 = imipramine, 6 = amitriptyline, 7 = loxapine (internal standard).

An amine modifier was employed to reduce peak tailing of the solutes. Diethylamine was chosen since it proved effective in improving peak symmetry at pH 7.2. The relatively high concentration of diethylamine (1%) not only improved peak shape, but also improved selectivity among solutes when compared with dibutylamine (Figure 4).

Precision, Accuracy and Recovery

The precision of analysis for a pooled serum sample spiked with 100 µg/L of each of the TCAs is shown in Table 1. The percent relative standard deviation ranged from 3.2 to 4.0%.

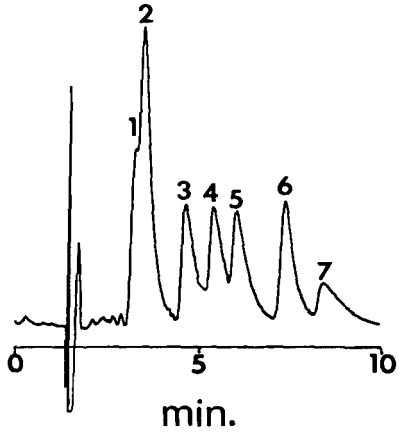

FIGURE 4. Chromatogram of tricyclic antidepressants with 1% dibutylamine as the amine modifier in the mobile phase. Peak identities as in Figure 3.

TABLE 1. Precision of the Method

	%RSD[a]	Mean[b]
Desmethyldoxepin	3.2	101.8
Desipramine	3.6	100.6
Nortriptyline	3.4	102.3
Doxepin	3.8	102.5
Imipramine	3.8	103.8
Amitriptyline	4.0	104.3

[a]n = 25 measurements; [b]μg/L

TABLE 2. Analytical Recovery of Tricycle Drugs from Pooled Serum

Drug	% Recovery[a]
Desmethyldoxepin	53.8
Desipramine	48.8
Nortriptyline	50.9
Doxepin	54.6
Imipramine	54.3
Amitriptyline	50.0

[a]concentration, 100μg/L

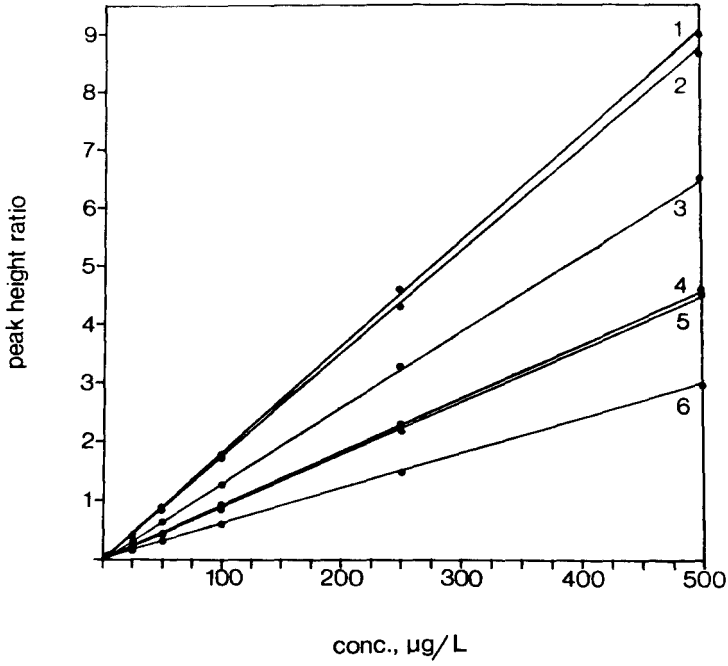

FIGURE 5. Calibration curves (1 = desmethyldoxepin, 2 = desipramine, 3 = nortriptyline, 4 = doxepin, 5 = imipramine, 6 = amitriptyline).

In Figure 5, a calibration curve is presented for each of the drugs over a concentration range of 2.5 to 500 µg/L. Using this technique, however, TCA concentrations up to 1000 µg/L can be measured without dilution.

The two stage liquid-liquid extraction can be completed within eight minutes. The percent recovery (Table 2) for these TCAs ranged from 48.8 to 54.6%.

Interference

Table 3 lists the k' of the TCAs and some additional psychotropic drugs which are often concurrently administered to patients receiving TCA therapy. To identify whether or not the antipsychotic drugs or metabolites interfered with the measurement of TCAs, plasma samples from patients receiving standard neuroleptic drug therapy were assayed. Each of the benzodiazepines was tested for interference using pooled human plasma

TABLE 3. k' for Tricyclic Drugs and Other Common Psychiatric Drugs and Metabolites

Tricyclic Antidepressants	k'
Desmethyldoxepin	2.50
Desipramine	2.95
Nortriptyline	3.45
Doxepin	4.18
Imipramine	5.00
Amitriptyline	6.41
Loxapine[a]	7.18

Benzoiazepines	k'
Desmethylchlordiazepoxide	2.06
Oxazepam	2.44
Chlordiazepoxide	2.53
Desmethyldiazepam	3.93
Diazepam	5.20

Neuroleptics	k'
Molindone	<2.00
Trifluoperazine	<2.00
Perphenazine	<2.00
Fluphenazine	2.67
Haloperidol	3.34
Thiothixene	3.39
Thioridazine metabolites	<2.00-4.14[b]
Chlorpromazine	8.23
Chlorpromazine metabolite (1)	3.02
Chlorpromazine metabolite (2)	3.65

[a]internal standard; [b]several unresolved peaks

spiked with drug standards since the metabolites of these compounds were available.

Among the benzodiazepines, three drugs were found to cause interference with this assay. Diazepam interferes with the measurement of imipramine, and the desmethyl metabolite of diazepam interferes with the measurement of doxepin. The third benzodiazepine, chlordiazepoxide, interferes with the analysis of desmethydoxepin.

Of the group of neuroleptic drugs tested, five were found to cause interference: Haloperidol, thiothixene, fluphenazine, chlorpromazine and thioridazine. Haloperidol and thiothixene elute with retention times which conflict with nortriptyline. Fluphenazine interferes with the measurement of desmethyldoxepin.

Although the parent compounds of chlorpromazine and thioridazine do not interfere with the measurement of TCAs, the metabolites do. Chlorpromazine metabolites interfere with the measurement of both desipramine and nortriptyline. The metabolites of thioridazine provide the greatest problems in this assay. In the plasma from a patient whose thioridazine treatment had been discontinued for seven days prior to the time the plasma sample was obtained, sufficient levels of thioridazine metabolites were still present to cause interference in the measurement of all six TCAs.

DISCUSSION

In this report, a rapid and sensitive HPLC technique is described for the simultaneous measurement of six tricyclic antidepressants (doxepin, desmethyldoxepin, imipramine, desipramine, amitriptyline, and nortriptyline) in human plasma. A simple extraction technique is used to prepare the samples for analysis by reversed-phase chromatography. The liquid-liquid extraction requires no evaporation step, small extraction volumes, and vortexing instead of mechanical shaking. The addition of the diethylamine to the potassium carbonate buffer prevents adsorption of the TCAs to the extraction glassware. These steps improve the reproducibility of the extraction procedure, with typical % RSDs for the complete assay ranging from 3.2 to 4.0% at a plasma concentration of 100 µg/L. Plasma samples can be prepared for chromatography within eight minutes.

Using this reversed-phase HPLC technique, the six tricyclic drugs and internal standard all elute within eight min. Thus, a total of only 16 minutes are required for both extraction and chromatography. Improvements in peak symmetry and selectivity were obtained by using the amine modifier diethylamine. The optimum mobile phase pH (7.22) was determined by conducting experiments over the pH range 3.9 to 7.8 and by plotting a window diagram of relative retention for each pair of peaks versus pH. Loxapine,

which provides acceptable extraction and chromatographic characteristics based on the structural similarities to the TCAs, was chosen as the internal standard.

It has been suggested that the use of a mobile phase pH above 7.0 in combination with an amine modifier may reduce the life of a silica based column by dissolving the silica (16). The method described in this report has been used for several years in both a routine clinical laboratory and in a research setting. Several thousand injections can be made on a single column before performance deteriorates. Degradation is primarily due to a buildup of lypophilic compounds on the column surface and is not due to the silica base of the column dissolving. The use of a guard column greatly extends the analytical column life by slowing lypophilic contamination.

Psychiatrists use TCAs as the drugs of choice for the treatment of affective disorders. TCA therapy is also sometimes combined with concurrent administration of other psychotropic agents. An evaluation, using the plasma from patients receiving some of these psychotropic drugs, showed limited interference from the parent compounds of three neuroleptics (thiothixene, haloperidol, and fluphenazine) and two benzodiazepines (diazepam and chlordiazepoxide). In addition, the metabolites of one of the benzodiazepines (diazepam) and two neuroleptics (chlorpromazine and thioridazine) caused interference in the assay.

This sensitive and rapid HPLC technique has been effectively applied for two years in a tricyclic drug monitoring program for both inpatients and outpatients at a major medical facility. Studies involving the pharmacokinetics and drug metabolism of TCAs are in progress.

ACKNOWLEDGMENT

This work was supported in part by NIH Biomedical Research Support Grant No. S07-RR-05815, University of South Carolina. Assistance from Dr. Sharon R. Batey, College of Pharmacy, University of South Carolina, and William S. Hall Psychiatric Institute, is gratefully acknowledged.

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DETERMINATION OF RITODRINE IN PLASMA USING HPLC

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ABSTRACT

An HPLC instrument coupled with an electrochemical detector was used to determine ritodrine (erythro-p-hydroxy-α-[1-[(p-hydroxyphenethyl)-amino]ethyl] benzyl alcohol hydrochloride) at nanogram levels in serum. Extraction of ritodrine was accomplished using a multistep ethyl acetate procedure, and the mobile phase consisted of acetonitrile, ammonium acetate, glacial acetic acid, and a counterion. The stationary phase was a Biophase ODS 5 μm column at ambient temperature. Nalbuphine hydrochloride (Nubain®) was used as an internal standard to quantitate the ritodrine levels of pregnant patients receiving ritodrine. The procedure's linearity for both ritodrine standards and spiked plasma samples was demonstrated. The precision of the assay was found to be 3.4% at 20 ng/ml ritodrine. The minimum detectable concentration, with a signal-to-noise ratio of 6, was determined to be 0.31 ng per 50 μl injected, corresponding to a concentration of 0.6 ng/ml plasma. The sensitivity, precision, and reproducibility of the assay were all found to be acceptable for determining ritodrine in patient serum.

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INTRODUCTION

Premature birth is the leading cause of neonatal mortality and morbidity(1). Ritodrine hydrochloride (Yutopar®) is the first product in the United States approved as tocolytic agent for the management of premature labor. However, little information is available describing its effective serum levels, pharmacokinetics, and side effects in pregnant women and neonates.

The only analytical technique available for determining ritodrine in serum is radioimmunoassay, a technique that requires the development of a specific antiserum(2). Therefore, the purpose of this study was to develop a high performance liquid chromatography (HPLC) procedure that would obviate the need for an antiserum and still provide a high level of sensitivity. Coupling HPLC with electrochemical detection (EC) provided a high level of sensitivity and precision, as well selectivity, for the analysis of ritodrine in serum. HPLC/EC methods similar in nature have been used to analyze tricyclic antidepressants(3).

MATERIALS AND METHODS

Reference Compounds

Reference ritodrine hydrochloride (Yutopar ®) was obtained from the Merrell Dow Research Center (Cincinnati, Ohio). The internal standard, nalbuphine hydrochloride (Nubain®), was donated by Endo Laboratories, Inc. (Garden City, New York).

Apparatus

An HPLC instrument (Perkin-Elmer Model 3B) coupled with an electrochemical detector (Bioanalytical Systems Model 4B) and a data terminal (Perkin-Elmer Model Sigma 10B) was used for determining ritodrine. The electrochemical detector was equipped with a glassy-carbon electrode; the chromatography column was a Biophase ODS 5 µm column. A Rheodyne injector (model 7125) was used with a 50 µl loop. The samples were centrifuged with an International centrifuge (model HN) and the samples were mixed in a Vortex shaker (model Vortex-Genie).

Chromatography Conditions

The mobile phase consisted of acetonitrile, ammonium acetate, and water (20/10/70) with 3mM ion-pair reagent (1-heptanesulfonicacid sodium salt) at pH 3.7. The ammonium acetate buffer (10x) contained 400mM ammonium acetate, 2.1M glacial acetic acid and water. Each liter of mobile phase contained 12ml of stock ion-pair solution (5.52gm ion-pair salt, 10ml glacial acetic acid, diluted to 100ml with water). The mobile phase was recirculated at a flow rate of 1ml/min.. The Biophase ODS 5 µm column was used at ambient temperature; the carbon electrode at 0.95 volts (vs. Ag/AgCl).

Treatment of Glassware

All test tubes and centrifuge tubes were surface treated with PROSIL-28 (PCR Research Chemicals, Inc.) an organosilane surface

treating agent. The procedure used was that recommended by the product's manufacturer.

Extraction Procedure

One ml of plasma was added to 1.0 ml of distilled water (Type 1, one megaohm) and 1.0 ml of 0.6 M K_2CO_3 in a 15 ml conical centrifuge tube with screw cap(3); this resulted in a pH of 9.4.. Then 8.0 ml of ethyl acetate was added. The tube was then mechanically shaken for 5 minutes, and then centrifuged at 2000 rpm for 15 minutes. The organic layer was transferred to a 15 ml conical centrifuge tube with a screw cap containing 1.2 ml of 0.1 M HCl. The tube was again shaken for 5 min. and centrifuged at 2000 rpm for 10 minutes. The top layer was aspirated to waste. Five-tenths ml of 0.6 M K_2CO_3 and 1.0 ml ethyl acetate was added (pH 9.4) and the tube was again shaken and centrifuged as mentioned above. The ethyl acetate layer was transferred to a 12 ml conical test tube and evaporated under a stream of purified nitrogen to dryness at room temperature. The residue was reconstituted in 300 µl mobile phase and 50 µl of the sample was injected into the liquid chromatograph.

RESULTS

LC Chromatograms

A chromatogram of ritodrine in the mobile phase (10 ng/ml) is shown in Figure 1. The retention time of the ritodrine was

approximately 9.33 min.; the contaminant came from the reagents but it was not further identified since it did not interfere with the procedure. Also shown in Figure 1 is a chromatogram from a plasma extracted sample; the concentration of ritodrine in this sample was also 10 ng/ml. The retention time of the internal standard, nalbuphine, was 13.58 min..

Standard Curves

The linearity for both rithodrine standards and plasma extracted samples over a range of 0-50ng/ml is demonstrated in Figure 2. The minimum detectable concentration was 0.6ng/ml in plasma, which yielded a signal-to-noise ratio of 6; this corresponds to 0.31 ng/ml per 50 µl injected.

Oxidation Potential

The oxidation potential for ritodrine (+0.95 volts) was optimized by plotting peak height versus applied potential. The appropriate range for the applied potential was first determined by Bioanalytical Systems of West Lafayette, Indiana.

Mobile Phase

Methanol was first used in the mobile phase; however, satisfactory results were not obtained. This led us to experiment with an acetonitrile solvent system. The mobile phase described in the methods section was found to be satisfactory after experimenting with the various solvent variables.

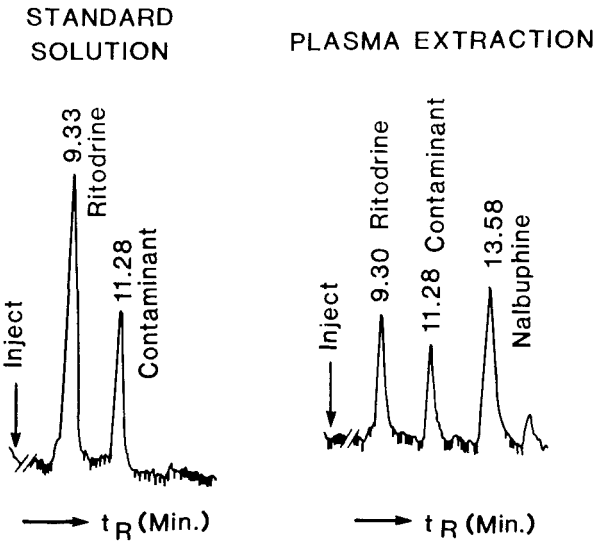

FIGURE 1 LC of Ritodrine Hudrochloride

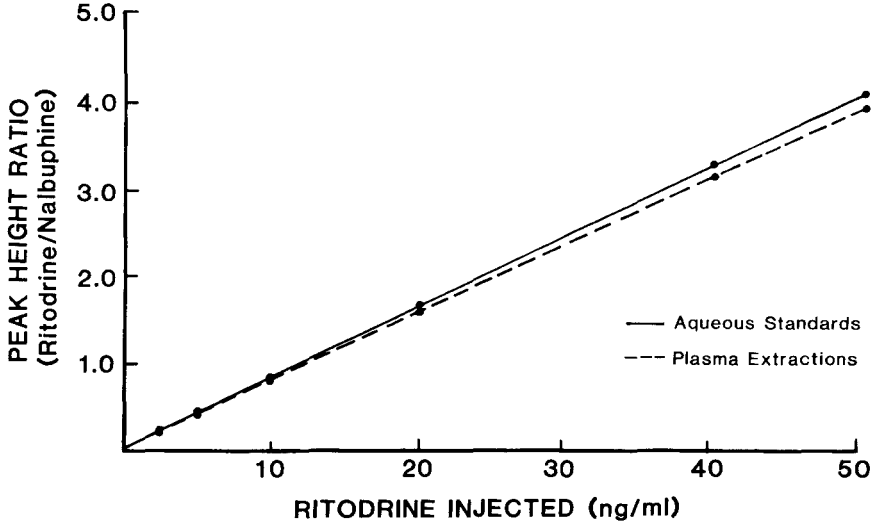

FIGURE 2 Linearity of Ritodrine Standards Using LCEC

TABLE I

Recovery of Ritodrine Added to Plasma

Tissue	Number	Recovery %	SD	CV%
Plasma (1 ml)	10	80.1	1.2	2.5%

TABLE II

Assay Precision for Plasma Ritodrine

Conditions	Concentration (ng/ml)	Mean + SD	CV%
Separate Extractions (n=12)	20	19.6 + 0.7	3.4

Recovery

The recovery of ritodrine was determined using spiked plasma samples (25ng/ml). The results are shown in Table 1. The mean recovery was 80.1% with a coefficient of variation of 2.5%. The extraction solvent used (ethyl acetate) was arrived at by experimenting with the solvents used in our laboratory for drug extraction (see Discussion).

Precision

The analytical precision at a plasma concentration of 20ng/ml was found to be excellent. The coefficient of variation was 3.4% (Table 2).

DISCUSSION

A radioimmunoassay technique for analyzing ritodrine was not used by us because of the difficulty in producing a suitable antiserum. Instead, an HPLC/EC technique was developed that gave a level of sensitivity (0.6ng/ml plasma) comparable to that reported by Gandar et al. (0.3ng/ml plasma)(2) using radioimmunoassay. We found the HPLC/EC technique to be sufficiently sensitive for analyzing ritodrine in pregnant women receiving the drug to prevent premature labor.

Since our lab does not have cyclic voltammetry, the oxidation potention for ritodrine was determined by Bioanalytical Systems of West Lafayette, Indiana. They found the optimum potential to be approximately +900 mv with a glassy carbon electrode. The potential used in our method was +950 mv; this value was arrived at experimentally and was within the appropriate range suggested by the voltammogram produced by Bioanalytical Systems.

A considerable amount of experimentation was done to determine which solvent system would provide the best results. The first system tried was that used by Merrell Laboratories, the previous manufacturer of ritodrine. Their mobile phase contained methanol, water, sodium-n-heptal sulphinate, and ammonium acetate. The solvent system we report here, using acetonitrile, etc., gave us good sensitivity and precision.

Several organic extraction solvents were tried before one was found that was satisfactory. Among those tried were: acetonitrile, methyl-t-butyl ether, diethyl ether, and ethyl acetate. Ethyl acetate gave an extraction efficiency of 80%; however, adjustment of

the pH as described in the procedure section is vital to obtain this efficiency (personal communication with Dr. Lan K. Wong of the University of Pittsburgh). The extraction procedure followed was that described by Suckow and Cooper with the exception of using ethyl acetate and adjustment of the pH.

Overall, the sensitivity, precision, and reproducibility of the assay were all found to be acceptable for determining ritodrine in the serum of pregnant mothers.

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Analysis of Indenolol In Biological Fluids By High Performance Liquid Chromatography.

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ABSTRACT

The analysis of indenolol in plasma and urine is described. The method involves extraction of the drug from plasma or urine using chloroform at basic pH. The separation was performed on CN column using methanol and 0.01M potassium dihydrogen phosphate solution 50:50. The efficiency of extraction was 97%. Minimum detectable amount by fluorescence was 20 ng/ml.

INTRODUCTION

Indenolol (1) a 1-(7-indenyloxy)-3-isopropylamino-2-propanol a relatively new beta-blocking agent is prescribed for the treatment of angina, cardiac arrhythmias and hypertension. Previous methods of analysis involved gas-liquid-chromatography, non-aqueous potentiometry, U.V. spectrophotometry and HPLC in dosage forms (1,2). The present study reports a simple method for the analysis of the drug in biological fluids using HPLC.

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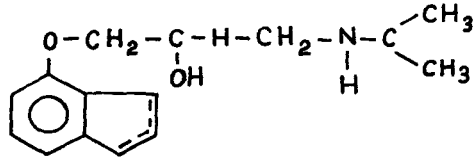

1

EXPERIMENTAL

Apparatus

The chromatographic equipment consisted of Model 6000A Pump with Flourescence detector model 420-C and 420-E from Water Associates (Bedford, Massachusetts, USA). The signal output was displayed on a Philipps PM 8251 single-pen recorder.

Chromatographic System

A 3.9ID X 30 cm commercially available stainless steel CN column made by chemically bonding, a cynogroup to PORASIL at 9% w/w was used (Waters Associates). Mobile phase consisted of methanol and 0.01M KH_2PO_4 (50:50). The mixture was degassed for 5 minutes by filteration: Flow rate was 1.5 ml/min. and detector gain was 64.

Reagents

Standard solutions were made by dissolving indenolol in the mobile phase. Methanol and chloroform (spectral grade) were obtained from Merck (61 Darmstadt Germany) and KH_2PO_4 (analytical grade) was obtained from BDH (Poole, England). Authentic sample (labelled purity 99.2% w/w) of indenolol hydrochloride was obtained from Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan.

Standard Curve

20 mg of indenolol hydrochloride was dissolved in 200 ml of distilled water. From this stock solution a series of dilutions were

made ranging from 2 µg/ml to 20 µg/ml. 25 µl of these solutions were injected onto the column in triplicate, the peak height was measured and plotted versus the concentration injected. The results are shown in Figure 1.

Extraction from the urine

Urine samples were collected from an apparently healthy adult male. In each run, various amount of the stock solution was added to 2 ml of urine giving final concentration ranging from 166 to 833 µg/L. Extraction was performed by adding 0.5 ml of 25% ammonia solution and the sample was then extracted with 5 ml of chloroform. The sample was then centrifugated for 10 minutes at 2500 r.p.m. The Chloroform layer was transferred and evaporated at 60° using water bath. The residual was dissolved in 2 ml of the mobile phase and 25 µl of this solution was injected in duplicate.

Extraction from plasma

Plasma was obtained from a whole citrated human blood which was then centrifugated for 10 minutes at 2000 r.p.m. and then pippted to another tube. In each run, various amounts of the stock solution was added to one ml of plasma giving final concentrations ranging also from 166 to 833 µg/L. The same procedure for urine was followed except that 0.1 ml of 25% ammonia solution was added instead of 0.5 ml.

RESULTS AND DISCUSSION

A simple method for the analysis of indenolol in biological fluids was developed using HPLC and fluorescence detector. A typical graph of the results when peak height was plotted versus concentration injected from both urine and plasma are shown in Figures 2 and 3 respectively. Typical chromatograms using this method are shown in Figures 4 and 5. The retention time for the drug was 4.2 min. The method is

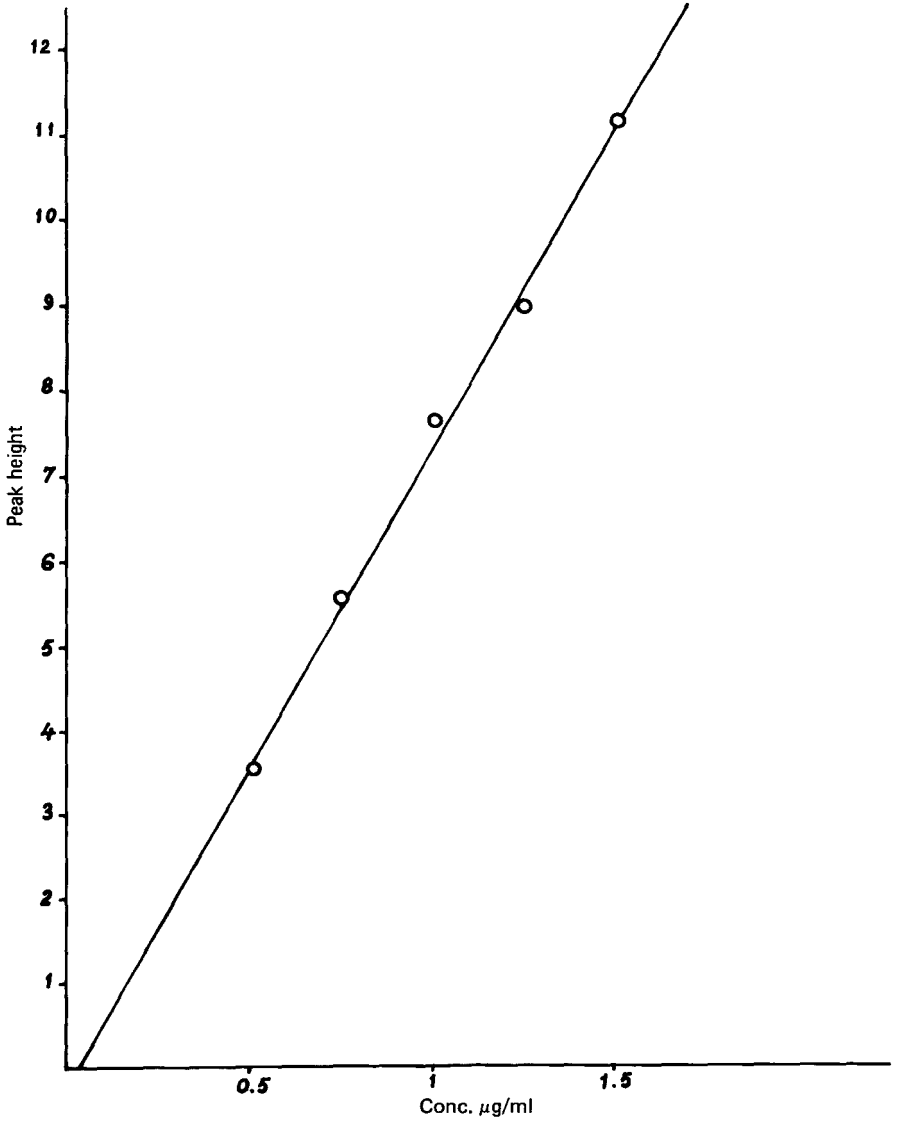

Fig. 1: Standard curve of indenolol when the peak height was plotted versus the Conc. injected.

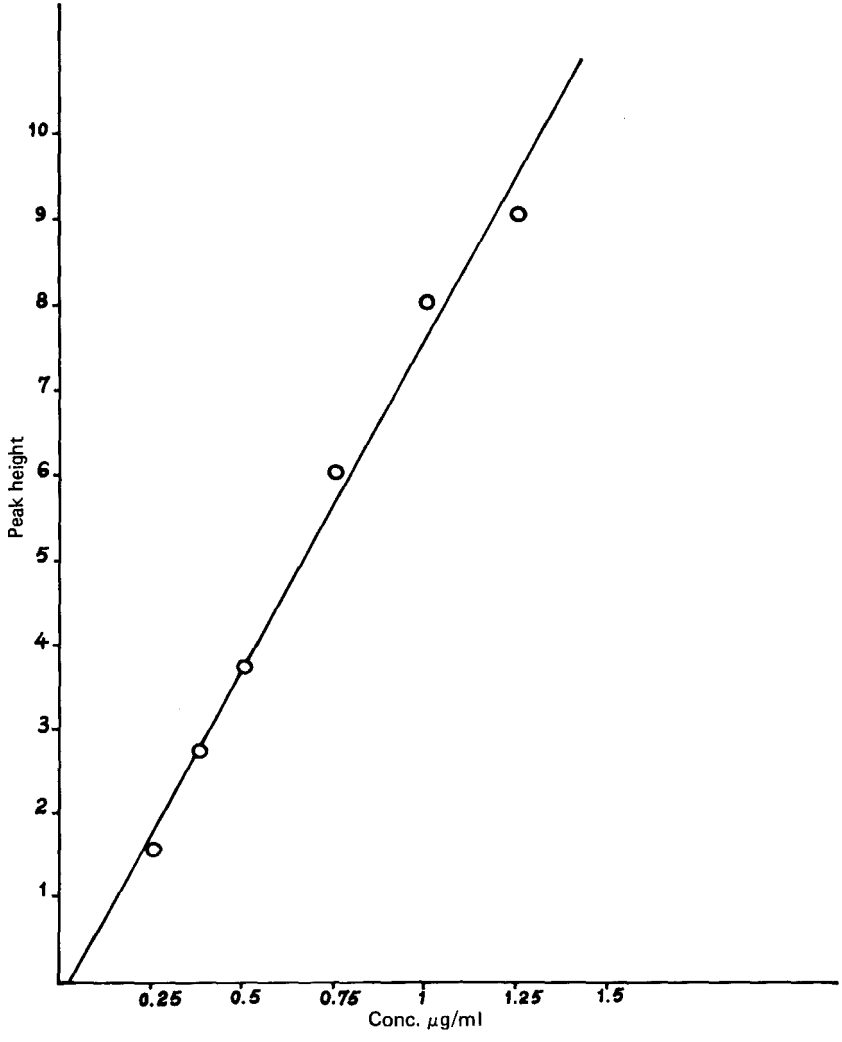

Fig. 2: Standard curve of indenolol extracted from plasma.

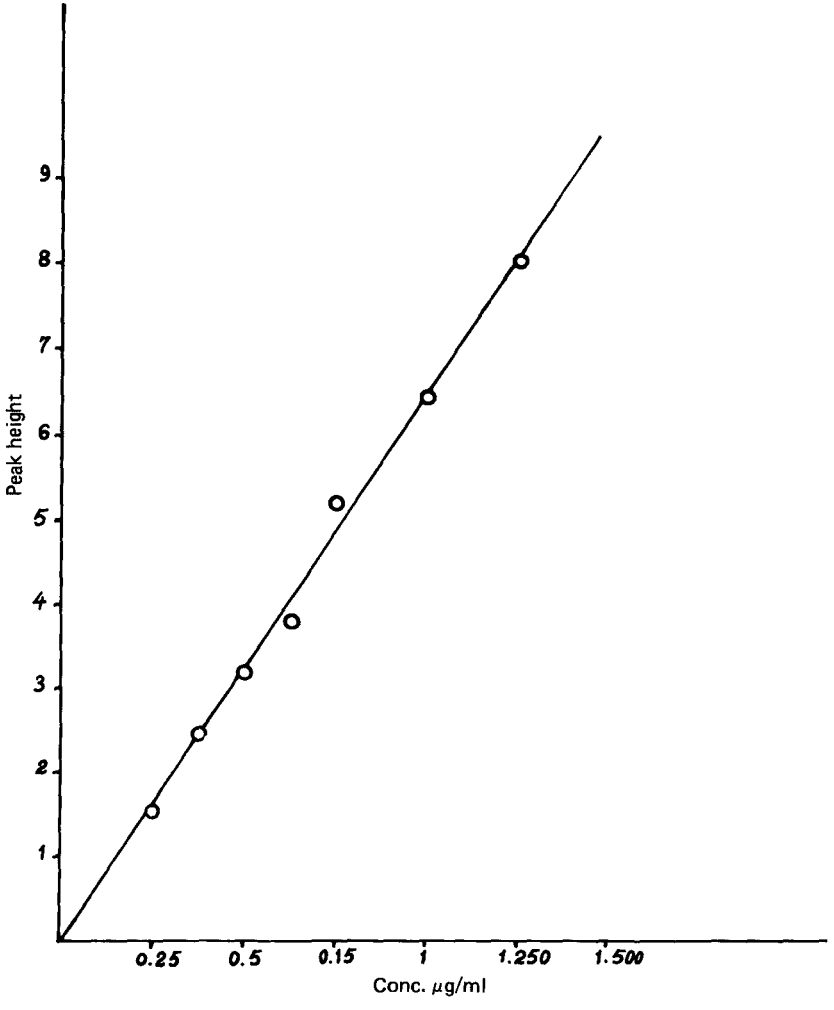

Fig. 3: Standard curve of indenolol extracted from urine.

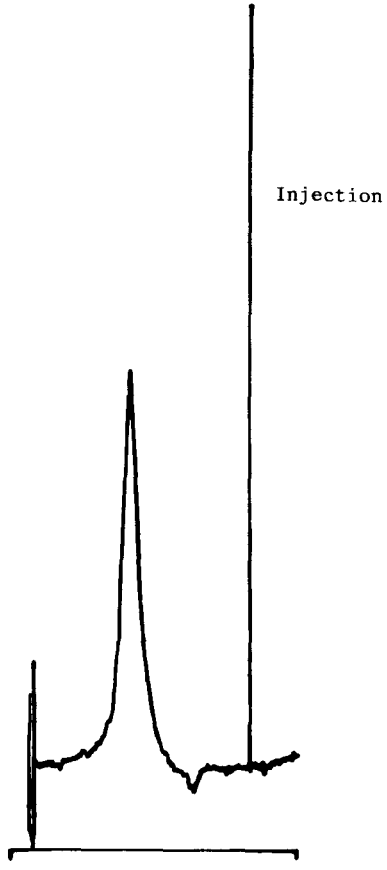

Fig. 4: A typical chromatogram of indenolol when 25 µl of the drug was injected.

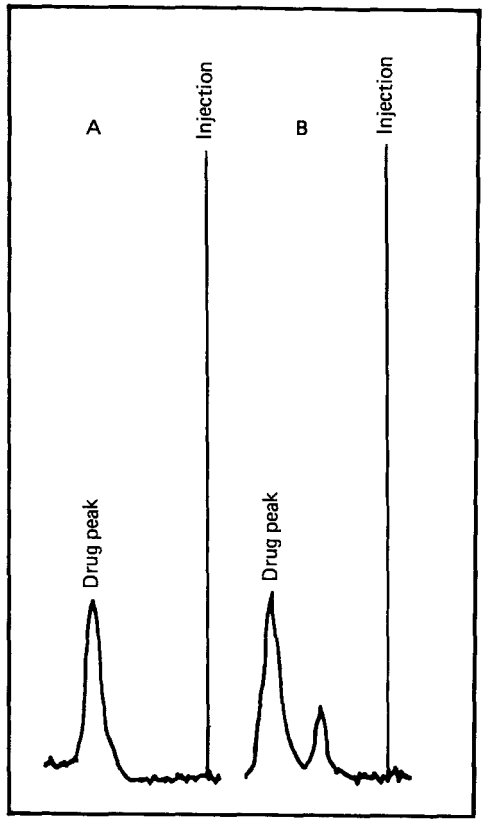

Fig. 5: Typical chromatograms of Indenolol extracted from Plasma (A) and Urine (B)

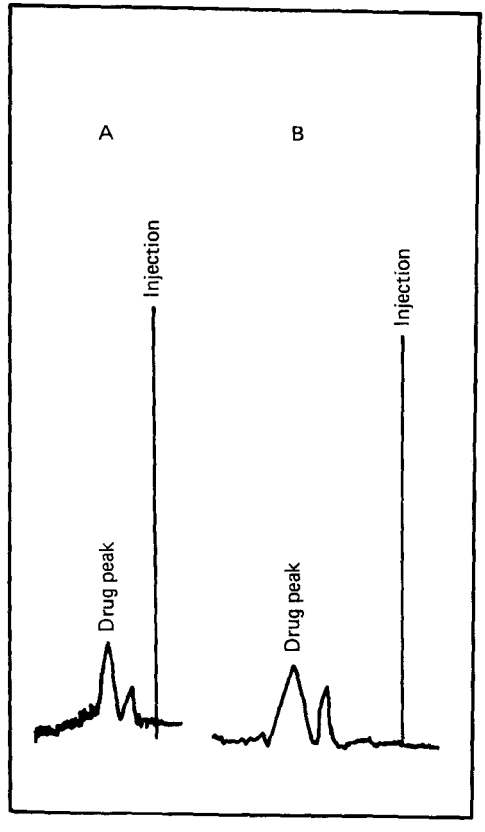

Fig.6: Typical chromatograms when 20 µg/L of Indenolol extracted from Plasma (A) and Urine (B)

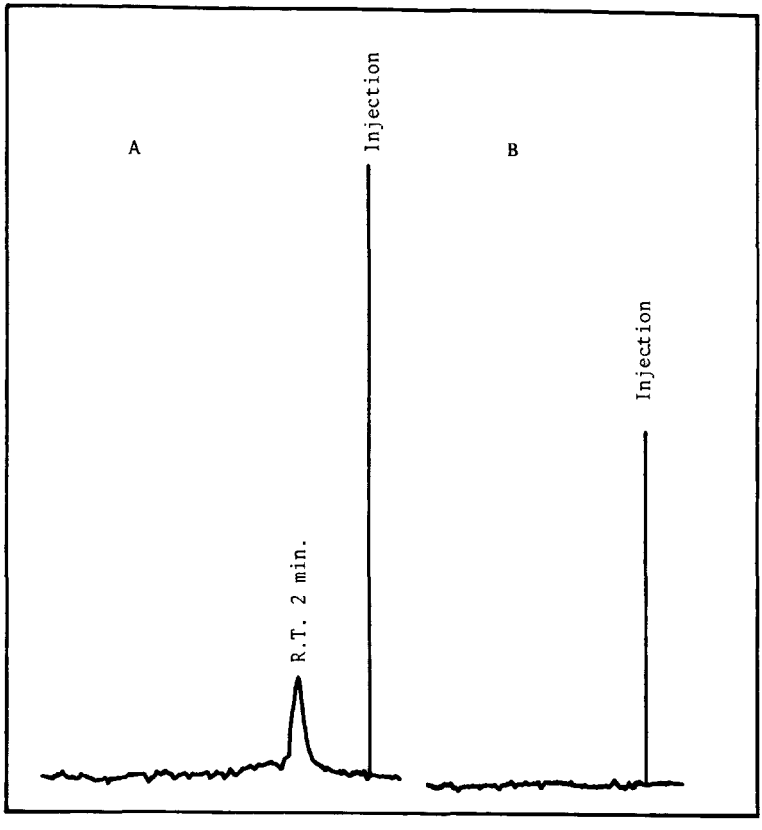

Fig. 7: Typical chromatograms when blank Urine (A) and blank Plasma (B) were extracted, and then injected onto the column.

very sensitive and concentrations as low as 20 µg/L (S/N > 2) can be detected (Figure 6). Peak height was linearily correlated to the drug concentration for the standard curves, urine and plasma with correlation coefficients of 0.995, 0.994 and 0.994. Intercepts of -0.35, -0.4, -0.1 and slopes of 0.007, 0.0066 and 0.0076 respectively. The interference from other plasma constituents was minimal and the recovery of the drug from the plasma or urine using this method of extraction was 97%. The sensitivity of the method can be considerably improved by dissolving the residual after evaporating the chloroform in 500 µl or even 100 µl of the mobile phase and injecting 50 or 75 µl of the reconstituted solution onto the column (Figure 7).

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HPLC ANALYSIS OF OLIGOMERIC ETHYLENE GLYCOL MIXTURES VIA BIS(2,4-DINITROPHENYLATION).

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ABSTRACT

Bis(2,4-dinitrophenylation) of oligomeric ethylene glycols of the formula $HO-(CH_2CH_2O)_n-H$ (n=4-16)to the corresponding DNP-$O(CH_2CH_2-O)_n$-DNP (where DNP stands for 2,4-dinitrophenyl) provides chromophoric derivatives, which are separated chromatographically on HPLC column.

The bis(2,4-dinitrophenyl) glycols are stable in presence of triethylamine, but undergo ethanolysis in presence of hydroxide ions. The quantitative removal of the DNP groups allows an integrated scheme to pure glycols from commercially available polyethylene glycol mixtures, by bis(2,4-dinitrophenylation), chromatographic separation, end-group removal, using HPLC of the bis-(2,4-dinitrophenyl) glycols for purity monitoring.

INTRODUCTION

Ethylene glycol oligomers are important starting materials in the synthesis of macrocyclic ethers (1-5). Their special solubility and chain folding properties are extensively studied (6-8). Considerable attention is also given to the use of oligoglycols as liquid polymeric supports in automatic liquid phase peptide synthesis (9).

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Our interest in facile synthetic methods to pure glycols has risen in the course of studies on the synthesis of polymeric crown (10) and pseudocrown ethers (11). This has also led us to develop a highly sensitive and accurate analytical method based on conversion of the glycols to bis(2,4-dinitrophenyl derivatives) (12), coupled with High Performance Liquid Chromatography Analysis (HPLC), which is reported in this paper.

Synthesis of Bis(2,4-Dinitrophenyl) Glycols: Chromophoric Ligands for HPLC Analysis.

The bis(2,4-dinitrophenylation) of glycols converts the water-soluble glycols, to hydrophotic derivatives incorporating high-extinction coefficient chromophores, which can be readily resolved by chromatographic methods using spectroscopic monitoring. The synthesis of bis(2,4-dinitrophenyl) glycols (abbreviated bis-DNP-glycols), the product composition are to be described elsewhere (13). The lower glycols (tri-to-penta glycol) yield exclusively the bis-DNP derivatives after 24 hours reaction. The reactivity of the glycol decreases as chain length increases, and longer reaction times are needed to convert the higher glycols to the bis-DNP derivatives.

High Performance Liquid Chromatography Analysis of Polyethylene Glycol Oligomer Mixtures.

The resolution of a prepared mixture of tetra, penta, hexa, hepta and octa glycols (as the bis-2,4-dinitrophenyl derivatives)

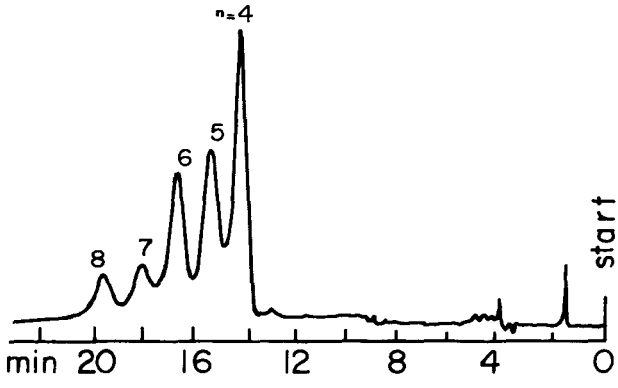


FIGURE 1
HPLC analysis of a prepared mixture of pure bis(2,4-dinitrophenyl) derivatives of tetra to octa glycol.

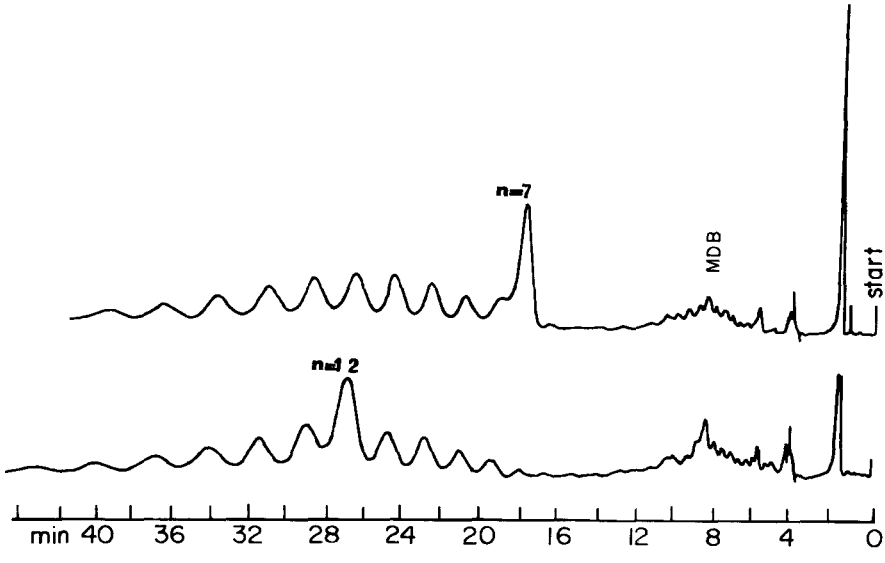


FIGURE 2

HPLC analysis of a mixture of bis(2,4-dinitrophenyl)polyethylene glycol 600, spiked with: (a) bis(2,4-dinitrophenyl)hepta glycol and (b) bis(2,4-dinitrophenyl)octa glycol.

on a nucleosil C_6H_5 (7μm) column is shown in Fig. 1. Next, the chromatogram of the bis(2,4-dinitrophenylated) mixture of polyethylene glycol (PEG)-600 is shown in Fig. 2. Bis(2,4-dinitrophenyl) heptaethylene glycol and bis(2,4-dinitrophenyl) dodecaethylene glycol were added as internal markers.

Under the above conditions, resolution between the bis(2,4-dinitrophenyl) derivatives of ethylene glycols from diglycol to pentadeca glycol is possible. The resolution of crude bis(2,4-dinitrophenylated) mixtures of polyethylene glycols (product of Fluka, Switzerland) are shown in Fig. 3. (Fig. 3A - PEG-200; Fig. 3B - PEG-300; Fig. 3C - PEG-400; Fig. 3D - PEG-600). Each chromatogram contains 2,4-dinitrophenol, 2,4-dinitrofluorobenzene (2,4 DNFB, R_t=6.0min), then a band of closely packed mono (2,4-dinitrophenyl) glycol homologes (abbreviated MDB, for mono-DNP-BAND). Then a band of the bis(2,4-dinitrophenyl) glycols, assigned by numerals identical with the integer n in $HO(CH_2CH_2O-)_nH$.

The identification of the components in Fig. 3 was through comparison with prepared mixtures of the pure glycols, such as a mixture of tetra to octa glycols shown in Fig. 1. Alternatively, samples were spiked with a known component, as shown in Fig. 2.

HPLC Purity Monitoring in Chromatographic Separation of Bis(2,4-Dinitrophenyl) Oligoethylene Glycol Mixtures.

Bis(2,4-dinitrophenyl) glycols are very sensitive to nucleophilic substitution by ethoxide in the system NaOH/EtOH/$CHCl_3$ [the reaction being completed within 5 minutes at 80°C (13)], and produce a mixture of liberated glycols and 2,4-dinitrophenol. This

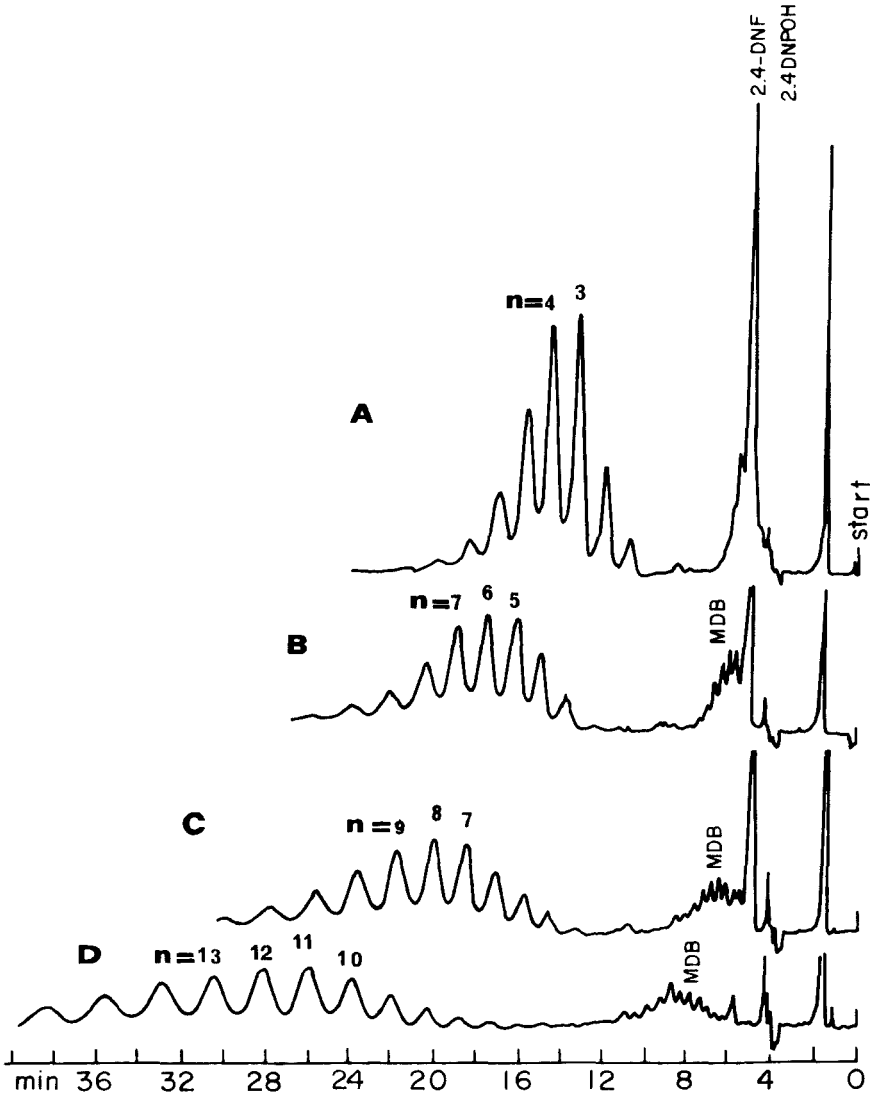


FIGURE 3

HPLC analysis of bis(2,4-dinitrophenyl) derivatives of polyethylene glycol mixtures: (a) PEG-200; (b) PEG-300; (c) PEG-400; (d) PEG-600. Numbers identify with n in $HO-(CH_2CH_2O)_n-H$.

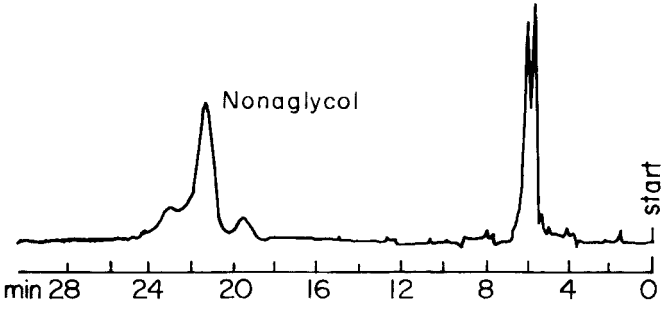


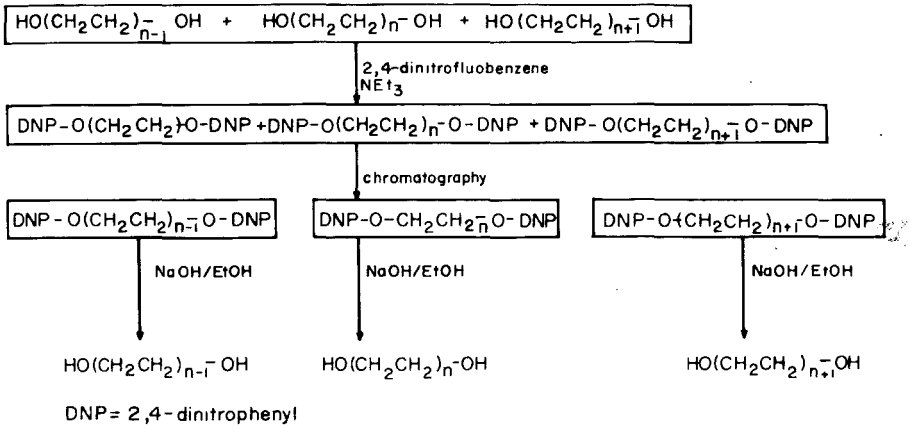
FIGURE 4

HPLC analysis of nonaglycol with octa glycol and decaglycols as impurities: PEG-300, starting material.

property allows the design of an integrated scheme leading from mixtures of polyethylene glycols, via bis-(2,4-dinitrophenylation) chromatographic separation and removal of the 2,4-dinitrophenyl protecting group to pure glycols (see scheme 1). The HPLC method described in this work allows this scheme by ensuring purity monitoring and control of the chromatographic separation procedure. From the composition of the commercial PEG-mixtures (see Fig. 3) it follows that PEG-200 is a source of tri, tetra and penta glycols, PEG-300 and PEG-400 are a source for penta to deca glycols and PEG-600 a source for octa to hexadeca glycols. Fig. 4 shows enriched nonaglycol obtained from PEG-300, and Fig. 5 shows enriched fractions of the Bis-(2,4-dinitrophenyl) derivatives of the higher glycols obtained from PEG-600 using scheme 1. Further chromatographic separation yields pure glycols.

In conclusion, in the present paper we have presented a highly sensitive HPLC method for the resolution and identification of

Scheme no. 1 : pure glycol via bis-(dinitrophenilation)of glycol mixtures, chromatographic separation and deblocking



oligoethylene glycols in synthetic samples or in their commercial admixtures, resulting from anionic polymerization of ethylene oxide. In addition, we have elaborated a method to pure oligo-ethyleneglycols (n=4-16) from commercially available polyethylene glycol mixtures by bis(2,4-dinitrophenylation) chromatographic separation and end-group removal. The HPLC analysis of the bis-DNP glycols enables purity monitoring of the glycols obtained by either route.

Making available high purity glycols will contribute to the synthesis of ultra pure crownethers, and will make evaluation of thermodynamic and biological measurements of various physical properties of crownethers more significant.

Although analysis of higher than hexadeca glycol was not attempted, the method should be applicable to higher glycols.

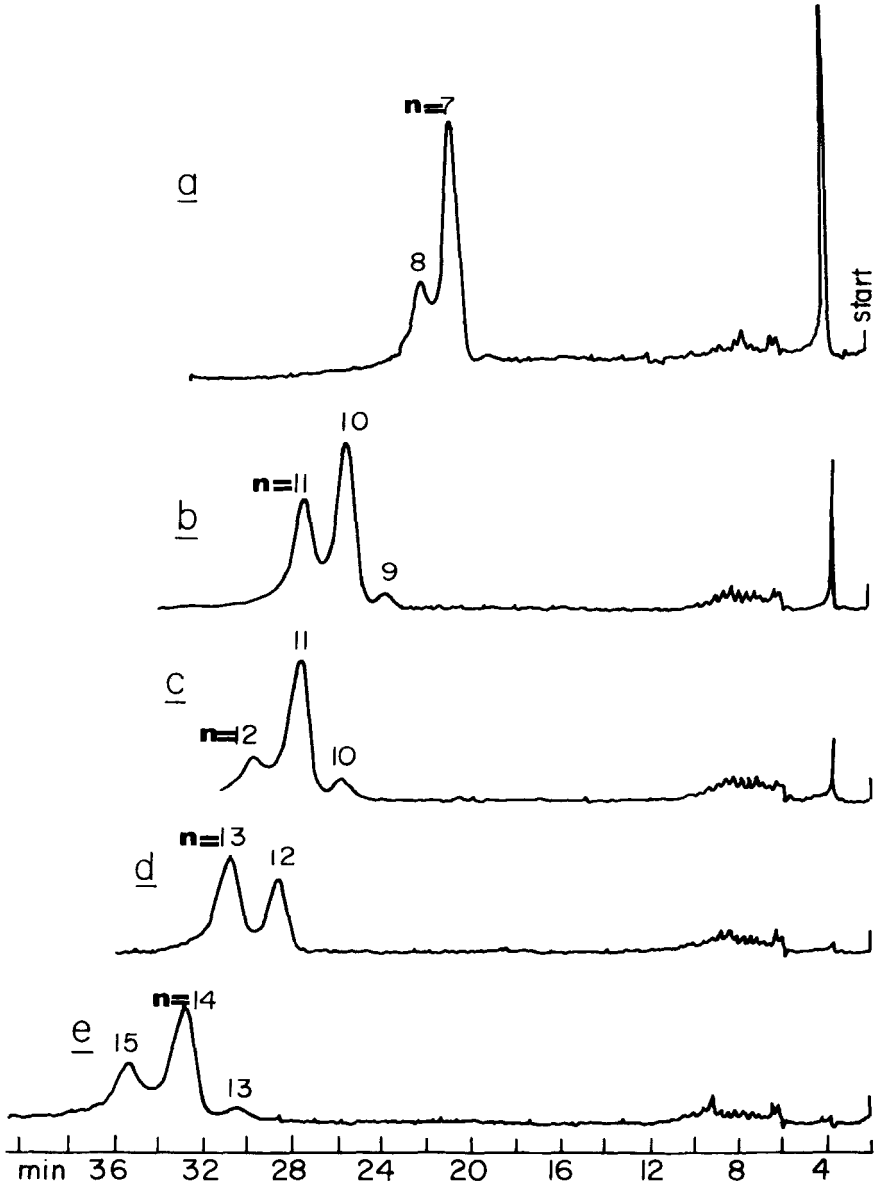


FIGURE 5

HPLC analysis of chromatographically separated bis(2,4-dinitrophenyl derivatives of: (a) hepta, octa; (b) nona, deca, undeca; (c) deca, undeca, dodeca; (d) dodeca, trideca and (e) trideca, tetradeca and pentadeca glycols. PEG-600, as starting material.

EXPERIMENTAL

Preparation of Bis(2,4-Dinitrophenyl) Derivatives.

The glycol (13.0 mmole) is dissolved in 30 ml distilled acetone, and 4.7 ml (66 mmoles) of triethylamine added, followed by 5.0 g (26.4 mmoles) of 2,4-dinitroflurobenzene (A.R). The solution is allowed to stand at room temperature for 24-48 hours in the case of the lower glycols (up to decaglycol), and up to 7 days with the higher glycols.

After evaporating the acetone, the product is taken in 100 ml $CHCl_3$, washed with 2x50 ml in HCl, and then with water to neutral pH. The solution is dried on $MgSO_4$, and the $CHCl_3$ removed by distillation. Chromatographic separation allows isolation of the bis(2,4-dinitrophenyl) derivatives in 45-76% yields (13).

High Performance Liquid Chromatography Analysis.

The HPLC analysis was performed on a Waters Associates Model 244 HPLC using a self-packed Nucleosil (~7 μm) C_6H_5 column of 250 by 4.6 mm, at room temperature. Several solvent elution systems were tried: (a) 20% dioxane in $CHCl_3$; (b) 20% dioxane + 1% isopropanol in $CHCl_3$; (c) 30% H_2O in CH_3OH. The last system was found to be superior and all the results reported here refer to the last system. Flow rate of 1 ml/min and chart speed 0.5 cm/min. Sample concentration in CH_2Cl_2: 10^{-4}M. Sample size: 5-20 μl, UV detector - 254 nm. All the HPLC chromatograms of the crude bis(2,4-dinitrophenyl) glycols contain 2,4-dinitrofluorobenzene (2,4-DNFB) and 2,4-dinitrophenol (2,4-DNPOH). In the case of the 2,4-dinitrophenylated polyethylene glycol mixtures a band corres-

ponding to the mono-2,4-dinitrophenyl derivatives is presented and is designated as MDB.

ACKNOWLEDGEMENTS

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DATA ACQUISITION AND PROCESSING FOR HIGH SPEED LIQUID CHROMATOGRAPHY

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SUMMARY

The effect of sampling rate and digital smoothing on data acquired from high speed liquid chromatography (HSLC) is explored. The amount of data required per peak is determined from the precision of area, height, and retention time measurements. The correct sampling rate is related mathematically to column characteristics and operating conditions. The effect of a modified moving average digital smoothing routine on peak width and height is investigated. Digital smoothing functions are shown to behave similarly to analog noise filters. The merits of raw data storage and post analysis processing are discussed in light of the short analysis times in HSLC and the decreased cost of computer memory.

INTRODUCTION

Short HPLC columns filled with 3 micron particles, when operated at high flow rates, are capable of performing many separations 5 - 50 times faster than with conventional HPLC columns, with little or no loss of resolution. Specialized or modified HPLC equipment has been developed to take full advantage of this technique. The high efficiency and low volume of the

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peaks generated in high speed liquid chromatography (HSLC) require reduced volume in injectors, connecting tubing, and detector flow cells.[1] Detector electronics must also be modified to cope with the sharp, relatively high frequency peaks. Noise filtering networks must have low time constants so as not to distort peak shape and thereby decrease resolution.[1-3] In a similar fashion, the data handling system employed for HSLC must be carefully designed to accurately represent the chromatographic data in a digital format without significantly distorting peak shape.

This paper describes a data handling system for use with HSLC based on the Apple II computer. The effects of sampling rate and digital smoothing routines on chromatographic data are investigated. The use of low cost RAM memory for raw data retention is described.

EXPERIMENTAL

CHROMATOGRAPHY

A Gilson Model 303 Pump (Gilson Medical Electronics, Middleton, WI, USA) in conjunction with an LDC Mark III Pulse Dampener (LDC, Riviera Beach, FL, USA) was used as the solvent delivery system. Samples were injected with a Rheodyne 7410 internal loop injector fitted with either a 2 or 5 ul sample loop (Rheodyne, Cotati, CA, USA). Separations were performed on 100mm x 4.6mm columns packed with 3 micron RoSiL C18

DA (Alltech Associates, Deerfield, IL, USA). Tubing used to connect the column to the injector and detector was 1/16" OD x .004" ID (Alltech Associates, Deerfield, IL, USA). A Kratos 773 UV-Visible Detector (Kratos Analytical Instruments, Westwood, NJ, USA), equipped with a 0.5 ul flow cell, was used to monitor column effluent at 254 nm. The square wave rise time for the detector's noise filter was set at 100 msec. Samples and mobile phases were as noted with each figure.

DATA SYSTEM

An Apple II Plus with 48K RAM memory was used as the host processor. Peripherals consisted of a single floppy disk drive with controller and an Apple DOT Matrix Printer (All obtained from Alltech Associates, Deerfield, IL, USA). Analog to digital (A/D) conversion and data integration was done with an Analytical Computers' Chromcard (Analytical Computers, Elmhurst, IL, USA) supplied with an optional 128K RAM memory expansion card.

The Chromcard consists of a printed circuit board that fits into one of the empty slots in the back of the Apple and receives the analog signal from the detector's recorder output. The accompanying software digitizes incoming data with 12 bit precision at up to 20 Hz. The chromatogram is displayed in real time on the Apple's CRT, using the high resolution graphics mode.

The incoming data is smoothed using 3, 5 or 9 points and is fitted to a quadratic curve. The first and second derivatives are calculated to define the onset of the peak, the position of valleys between peaks, and the proper peak end point. User defined valves for slope sensitivity, minimum peak height and minimum peak area are used to discriminate against noise and other artifacts. Raw data points are stored in RAM memory for subsequent re-analysis or for transfer to disk for permanent storage.

Once all peaks have been defined and raw areas calculated, retention times are compared with expected values supplied by the operator. If experimental values match expected values within a user defined window, peak names are assigned. Raw peak areas are then normalized, compared with an internal standard (if desired), and multiplied by response factors prior to generation of the final report.

The final report lists all chromatographic conditions, together with operator name, date, and sample identification. Each peak is listed with retention time, area, area %, normalized area and concentration. A typical report is shown in Figure 1. The chromatogram may then be reproduced on the line printer in a dot-matrix format, as shown in Figure 2.

DATE 06/22/83 OPERATOR DSB SAMPLE EXPT1 SIZE 20 ULITERS
METHOD N1.M METHOD DESCRIPTION NUCLEOTIDES

COLUMN DESCRIPTION:
COLUMN ID 8 MM OD PACKING C 18 REVERSE TYPE RADIAL PAK
MOBILE PHASE 50 MM AMM/PHOS PH6 FLOW RATE 1 ML/MIN PRESSURE 500 PSI

OPERATING CONDITIONS:
INITIAL TEMP ENDING TEMP TEMP RATE
DETECTOR TYPE UV 258NM DETECTOR SENS 0.2 DETECTOR TEMP

PK#	COMPOUND NAME	TIME	HT	WIDTH	AREA	AREA%	NORM	CONC
1	GTP	5.18	7.48	14.38	9472	14.88		0.
2	GDP	5.67	10.90	20.88	17709	27.82		0.
3	GMP	7.51	17.79	23.06	36467	57.29		0.

FIGURE 1

DOT MATRIX REPRODUCTION OF CHROMATOGRAM

FIGURE 2

RESULTS AND DISCUSSION

The extremely narrow peaks generated by HSLC present a unique problem to chromatography data systems. These narrow peaks are a result of the high efficiency of HSLC columns, coupled with the speed at which peaks elute. The speed arises from the use of short columns (30 - 150 mm) and operation at high mobile phase velocities. A comparison of HSLC with conventional HPLC is shown in Figure 3. The conventional column (150 mm, 5 micron) separates seven components in just under 9 minutes. The HSLC column (100 mm, 3 micron) separates the same mixture in just under 50 seconds, with little or no loss of resolution. The amount of information available from both chromatograms is the same, but the time base has been decreased almost twelvefold. The data system, to maintain the same integrity of information, must digitize the chromatogram twelve times faster, requiring higher sampling rates. Simultaneously, the peaks shift to a higher frequency, increasing the effect of digital smoothing routines on the peak shape.

A comparison may be made between these digital effects and their analog counterparts. Faster sampling rates correspond to faster strip chart recorder speeds. Improved digital smoothing routines parallel analog noise filters with shorter time constants.

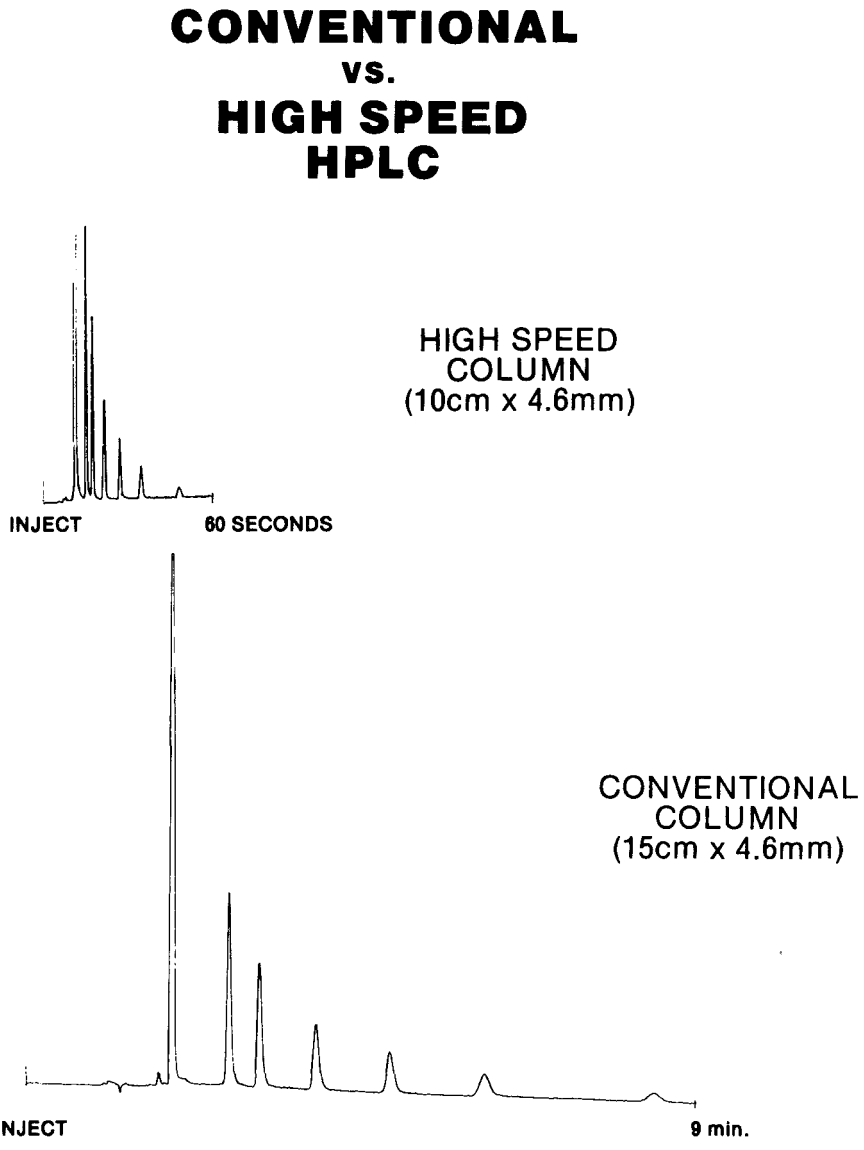

FIGURE 3 Conventional Column, 15cm x 4.6mm P/W RoSiL C18 HL 5 micron. High Speed Column 10cm x 4.6mm P/W RoSiL C18 HL 3 micron. Mobile Phase 75/25 Acetonitrile/Water. 1.0ml/min for Conventional Column, 3.0ml/min for High Speed Column. Sample - Anilides.

SAMPLING RATE

The sampling rate determines the interval between consecutive examinations of the analog signal. The speed of the analysis and the shape of the peaks determine the sampling rate required--the sharper the peaks, the greater the sampling rate. The sampling rate should be fast enough to provide an adequate digital representation of the peak profile. On the other hand, excessive sampling rates will generate large quantities of data. If raw data is being stored, this rapidly fills the computer's memory.

The sampling rate required for a given peak can be related to chromatographic parameters using Equation (1).

$$(1) \quad W = \frac{4\ Vo\ (1 + k^1)}{Q\ N^{\frac{1}{2}}}$$

W = Peak Width (at Base)

Vo = Column Void Volume

Q = Volume Flow Rate

N = Column Efficiency

k^1 = Capacity Factor

If the peak duration (W) is known, it is simple to calculate the sampling rate, using Equation (2).

$$(2) \quad F = \frac{C\ Q\ N^{\frac{1}{2}}}{4\ Vo\ (1 + k^1)}$$

F = Sampling Rate

C = The Number of Data Points Desired per Peak

Inspection of Equation (2) reveals that sampling rate increases with increased flow rate (Q), increased efficiency (N), and decreased column void volume (Vo). All three of these occur in HSLC. Smaller particles result in higher efficiencies; shorter columns have smaller void volumes, and shallow H/U curves for small particles allow operation at higher flow rates.

Choosing the correct value for C depends on the precision required from subsequent calculations. In most cases area, height, and retention time are extracted from raw peak data. Examining the precision of area, height, and retention time at various sampling rates may be used to determine the correct value for C.

The Chromcard system allows A/D conversion rates of 20 Hz, 10 Hz, 2 Hz, 1 Hz, and 0.5 Hz. For the high speed separation shown in Figure 4, A/D conversion rates of 20 Hz, 10 Hz, and 2 Hz were examined. Ten runs were collected at each rate. The earliest eluting peak (phenol) is the sharpest of the five, hence presenting the most demanding case to the data handling system. The mean values for area, height, and retention time, along with the relative standard deviation (RSD) for each quantity are shown in Table 1.

The mean values for retention time, peak height and peak area are nearly identical when calculated from data collected at 10 Hz and 20 Hz. As expected, the precision obtained from data collected at 20 Hz is slightly

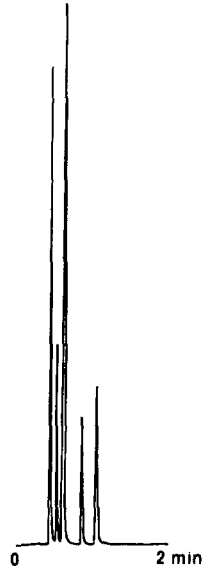

FIGURE 4 Column 10cm x 4.6mm P/W RoSiL C18 HL 3 micron. Mobile Phase 75/25 Acetonitrile/Water. Flow 3.0ml/min 3000 PSIG. Peak 1, Phenol; Peak 2, Benzaldehyde; Peak 3, N,N-Diethyl-m-Toulamide; Peak 4, Toluene; Peak 5, Ethyl Benzene.

TABLE 1

Integrator Precision at Various A/D Conversion Rates*

Sampling Rate (Points/Sec.)	Mean Value/% RSD		
	Retention Time (Seconds)	Peak Area	Peak Height
20	21.83 (0.771)	1522.55 (1.329)	2712.108 (1.271)
10	21.84 (0.418)	1536.92 (1.512)	2622.113 (1.497)
2	21.95 (1.293)	1623.90 (16.38)	1901.200 (12.34)

*10 runs at each rate. Data for Peak 1, phenol.

better, although the maximum RSD for 10 Hz is only 1.512%. This is acceptable precision for most applications. When the sampling rate is dropped to 2 Hz, however, the mean values for peak height and peak area differ greatly from those obtained at 10 and 20 Hz. The precision at the 2 Hz rate also becomes unacceptable (RSD>12%).

This information suggests that the number of points collected at the 10 Hz and 20 Hz rates provides an adequate data base for integration with good precision. Table 2 displays the number of data points obtained experimentally at each sampling rate, along with the theoretical value obtained by solving Equation (2) for C.

TABLE 2

Number of Data Points Collected at Various A/D Conversion Rates

Sampling Rate (Points/Second)	Number of Data Points	
	Experimental*	Calculated**
20	19.6	16.4
10	10.1	8.2
2	3.2	1.6

* Average values for 10 runs. Data for Peak 1, phenol.

** Calculated using $C = \frac{4 F Vo(1 + k1)}{Q N^{\frac{1}{2}}}$, from Eqn (2). N = 11,300 plates.

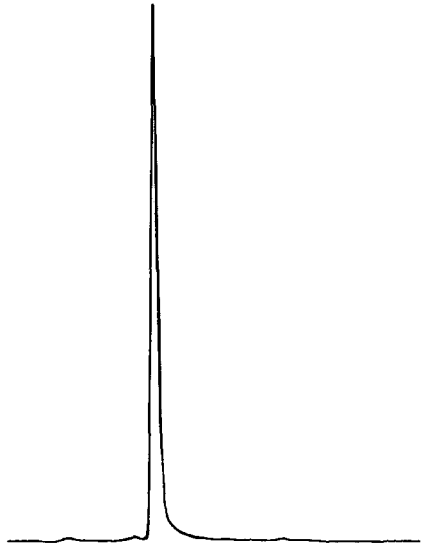

FIGURE 5 Phenol Peak Profile

The lack of correlation between the experimental and calculated values may be explained by the non-Gaussian shape of the phenol peak. The efficiency value used in calculating C by Equation (2) was obtained using bandwidth at 50% of peak height. This method assumes a Gaussian peak shape, and as Figure 5 reveals, the phenol peak has a significant tail. This leads to an inflated value for N and a reduced value for C. Nonetheless, Equation (2) provides a reasonable method for choosing sampling rate. This data indicates that a C value of 10 - 20 points/peak (minimum) will provide acceptable precision.

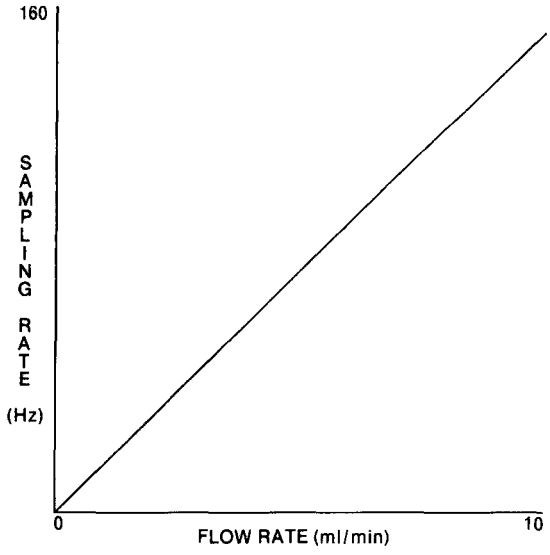

FIGURE 6 Sampling Rate vs. Flow Rate

For a typical 3 micron HSLC column 100 mm long with 12,000 plates and a Vo of 0.6 ml, then substituting in Equation (2) with C = 20, yields Equation (3).

$$(3) \quad F = \frac{15.22\ Q}{(1 + k^1)}$$

A peak eluting at the void volume ($k^1 = 0$) will require the fastest sampling rate. For HSLC with 4.6mm columns, flow rates of up to 6 ml/min may be encountered. Figure 6 displays the plot of sampling rate vs. flow rate, for a peak eluting at the void volume. For a flow rate of 6 ml/min, sampling rates of

close to 100 Hz may be required to generate 20 data points. Chromcard's current maximum sampling rate of 20 Hz should be adequate for most HSLC and for all conventional HPLC. However, to provide adequate precision and accuracy for the fastest analyses, a system with higher sampling rates would be desirable.

DIGITAL SMOOTHING

Most commercial LC detectors incorporate some type of analog noise filter as part of the signal electronics. The purpose of these filters is to eliminate noise at frequencies higher than that of the chromatographic peaks. Most of these filters can be characterized by their time constant. The time constant should be high enough to remove short term noise without distorting the peak shape. If a 5% increase in bandwidth due to noise filters is deemed acceptable, then the time constant should not exceed 32% of the peak's bandwidth.(3) Haddad, et al., quantifies the effects of RC filters on chromatographic efficiency and resolution.(2) As the peak's bandwidth decreases, the effect of the noise filters on the peak shape increases. Analog noise filters for HSLC have been designed with small time constants so as not to significantly degrade peak shape.

Chromatography data systems usually include software for smoothing digitized data. The purpose is similar to that of analog noise filters--to remove

spurious signals from chromatographic data. The simplest of these numeric noise suppression methods is the moving average. The values of several consecutive data points are summed and divided by the number of points collected. This average is used for the value of the middle point in the array. The oldest point is dropped; a new point is added, and the process is repeated for the new array. In this manner, a "window" moves through the data, assigning a value for the middle data point in the window based on the past and future points in the array. The number of points used in the average (the size of the window) determines the extent of the smoothing. The length of the smoothing array can be qualitatively compared with the magnitude of the time constant used in analog filters. The more points used in the smoothing function, the greater the distortion of peak shape. As the peak's bandwidth becomes smaller, the effect of the moving average on the peak shape becomes more significant.

The software used with Chromcard uses a modified moving average as described by Savitsky and Golay.(4) The software operates on a 3, 5, or 9 point window. Instead of calculating a simple average of the data points, the data in the array is fitted to a quadratic curve ($y = ax^2+bx+c$) using a least squares approximation. A quadratic curve fit is superior to a simple

moving average in that the quadratic function can be fitted more correctly to a rapidly changing signal such as a chromatographic peak. The result is better smoothing with less distortion.

The effect of the size of the window on peak shape is shown in Table 3. Using a 3, 5, or 9 point window, data from the phenol peak (Fig. 4) was acquired and smoothed with a quadratic curve fit. Peak height and peak bandwidth (at 50%) were measured for the smoothed data. Efficiency was calculated using the 1/2 height method.

Inspection of bandwidth and peak height values in Table 3 demonstrates that digital smoothing affects peak shape in a manner similar to analog noise filters. If the smoothing function begins to affect signals in the same frequency range as the chromatographic peaks, then the apparent bandwidth will increase, decreasing efficiency. Peak height will decrease, affecting

Table 3

Effect of Digital Smoothing on Peak Width and Height

Points Used in Smoothing Routine	Peak Height	Peak Width (50%)	Efficiency (Plates)
3	3529	6.750	12,100
5	3526	6.750	12,100
9	3385	7.125	11,000

sensitivity. In the case of the quadratic curve fit program applied in this work, no decrease in performance due to digital smoothing occurred for the 3 or 5 point windows. Using 9 points, however, reduced measured efficiency by 8.5% and peak height by 4.1%.

Unlike analog noise filters, whose characteristics are fixed by the value and nature of their electronic components, digital smoothing functions may take on almost any characteristic. The virtually unlimited ability to manipulate data through software makes this so. The Savitsky-Golay procedure, for example, may be modified to fit the raw data to virtually any mathematical function. In the future, it may be possible to dispense completely with analog signal processing in favor of more powerful, versatile digital methods.

When applying digital smoothing to chromatographic data, care must be taken to avoid distortion of peak shape. When the frequency of the peaks becomes closer to that of the noise to be eliminated, such as in HSLC, the design of the digital smoothing becomes increasingly important.

RAW DATA STORAGE

Chromatography data systems may be categorized according to the way they treat raw data. So called "on-the-fly" systems process data as it is received from the A/D converter, determining when peaks occur and

performing integration as the analysis takes place. Raw data values are discarded after they are processed. Small microprocessor based data systems usually have been limited to operation in this mode due to the high cost of solid state memory and the limited addressing capacity of 8 bit processors. In some cases, raw peak data can be stored in a small solid state memory buffer, then transferred to disc when the buffer is filled. The buffer is then filled with new data until the buffer once again requires "dumping" to disc. Unfortunately, transfer of data to a typical floppy disc can take up to one full second, during which time data acquisition must be interrupted. This places a severe limitation on sampling rate.

Larger computers with increased memory capacity have normally been employed for peak processing with retention of raw data. The major advantage of raw data retention is the ability to reanalyze the data under an alternate set of conditions. Peak processing parameters may be modified after the analysis to cope with unforeseen changes in the incoming data. For example, peak threshold and minimum area parameters can be adjusted to eliminate an unexpected peak from calculations.

In 1980 Reese predicted that data systems would be developed to operate with raw data retention as the cost of memory decreased.(5) Since that time, advances in

small personal computers have made fairly sophisticated computing power available at modest costs. Relatively inexpensive, high volume, solid state memory has also been developed making it possible to retain a fair amount of raw data with a microprocessor based system.

HSLC is an ideal candidate for systems of this nature since the amount of data generated is relatively small. Although the amount of data per unit time is high in HSLC due to the high sampling rates, the analyses themselves are typically quite short. In most cases, the entire separation takes place in less than ten minutes.

The data system employed in this work was equipped with a 128K RAM memory expansion card for the purpose of

Table 4

Data Storage Capacity at Various Sampling Rates*

Sampling Rate (Hz)	Approximate Storage Capacity (Minutes)
100	10
75	13.34
50	20
40	25
20	50
10	100

*Based on 128K system.

raw data storage. Table 4 shows the amount of raw chromatographic data (in minutes) that may be stored with 128K memory at various sampling rates. Even at sampling rates of 100 Hz, ten minutes of raw data may be stored in a 128K buffer. This is certainly adequate for even the most demanding HSLC.

CONCLUSION

With the advent of HSLC, the components of the LC system have been critically reviewed and modified to cope with the narrow, high speed peaks produced by HSLC columns. The data system must be viewed in the same light. Sampling rates should be adjusted to produce a minimum of 10 - 20 points per peak. Digital smoothing programs should be carefully evaluated to insure that no artificial decreases in performance are introduced. With the advent of low cost RAM memory to complement microcomputers, data processing with retention of raw data is possible.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 6(14), 2829-2837 (1983)

SEPARATION OF CHLORINATED PHENOLS BY ISOCRATIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON REVERSE PHASE COLUMN

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Carcinogenesis Division
Cancer Research Institute
Tata Memorial Centre
Parel, Bombay-400 012
India.

ABSTRACT

A method is described for isocratic high-performance liquid chromatographic separation of chlorinated phenols using methanol : phosphate buffer - pH 7.20 (50:50) solvent system. 13 out of 15 congeners of chlorophenols and phenol have been separated in 32 minutes at a flow rate of 0.8 ml per minute. The system is found to be useful for the separation of chlorophenols extracted from mouse liver fed with hexachlorocyclohexane.

INTRODUCTION

Hexachlorocyclohexane (HCH) is a widely used pesticide in developing countries and is reported to be carcinogenic in mice by many laboratories (1 - 3). We in our laboratory were interested in finding out the profile of the chlorophenols which are possible metabolites of HCH in mouse liver - the target tissue. Among the various separation techniques, high-performance liquid chromatography (HPLC) with reverse phase column is found to be very effective for

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the separation of chlorophenols in recent years. McLeod and Laver (4) have reported an isocratic system for the separation of 13 out of 19 congeners of chlorophenols using two reverse phase columns and acetonitrile : phosphate buffer - pH 9.2. Smit et al. (5) have separated 11 congeners of chlorophenols using tetrahydrofuran : perchloric acid - pH 3.0 solvent system. Ugland et al. (6) have successfully resolved 18 congeners of chlorophenols using a 30 minute linear gradient from methanol : phosphate buffer - pH 4.0 (56 : 44 to 80 : 20) as mobile phase. Lores et al. (7) separated several chlorophenols using methanol : acetonitrile : phosphate buffer - pH 4.0 (40:14:46) and acetonitrile : phosphate buffer (50:50).

The present communication reports an isocratic HPLC separation of chlorophenols using a solvent system consisting of methanol : phosphate buffer - pH 7.20 (50:50). Separation of 13 congeners of chlorophenols out of 15 and phenol is achieved by this system with a single C_{18} reverse phase column. The system has been successfully used to separate chlorophenols extracted from mouse liver fed with HCH. The clean up procedure of the chlorophenols from the liver and the chromatographic parameters like retention time, response factor are discussed.

MATERIALS AND METHODS

HPLC was carried out using a Waters Associates (Milford, Massachusettes, U.S.A.) HPLC system fitted with a model 6000 A solvent delivery system, μ Bondapak C_{18} reverse phase column (30 cm X 0.29 cm), U6K universal injector and a model 440 absorbance

detector fitted with 254 nm filter. Chromatograms were recorded on Omniscribe (Houston Instruments, Austin, Texas) strip-chart recorder.

The chlorophenols were obtained from Aldrich Chemical Co. and were purified before use, if necessary. Stock solutions were prepared by dissolving 4-10 mg of the compounds in 5 ml of methanol. The stock solutions were diluted in methanol singly or in mixture so as to get desired standard concentrations.

Distilled E.Merck (India) G.R. grade methanol and triple distilled water from all glass apparatus were used for preparing solvent system.

Solvent System : Two stock solutions of 50 mM dipotassium hydrogen phosphate and 50 mM of potassium dihydrogen phosphate were prepared from AnalaR grade reagent and stored at 4 ^{o}C. The stock solutions were mixed and diluted appropriately to make a buffer of 0.5 mM strength and pH 7.20 $\pm$ 0.02. Running solvent was prepared by mixing methanol and phosphate buffer (50:50) after filtering through appropriate Millipore filters.

Sample Preparation : Livers were collected from male Swiss mice (8-week-old) fed with a diet containing 500 ppm HCH continuously for 2 months. Livers were minced and the chlorophenols were extracted from it by alkaline and acid hydrolysis followed by distillation and extraction in toluene as described by Sackmauerova-veningerova et al. (8). Instead of 15 ml, 3 X 50 ml of toluene was used. The combined toluene extract was then concentrated to dryness using rotary vacuum evaporator at 60 ^{o}C. The residue was taken in 2 ml

benzene and cleaned up using Sephadex QAE Q-25-120 anion exchanger (Sigma) as described by Renberg (9). The final benzene solution was concentrated to dryness in a stream of nitrogen at 40 °C. The residue was then taken in 0.5 ml of running solvent and filtered before injection.

Procedure : C_{18} μ Bondapak column was first washed with methanol for 15 minutes and then with running solvent for 60 minutes at a rate of 2 ml per minute. The flow rate was then adjusted to 0.8 ml per minute. Suitable aliquots of standard mixture and the samples were injected (1-5 μl) by Hamilton syringe.

RESULTS AND DISCUSSION

Fig. 1 shows the separation of standard chlorophenols from a mixture using the present system. 13 out of 15 congeners of chlorophenols and phenol can be well separated. At a flow rate of 0.8 ml per minute 32 minutes were required to elute all chlorophenols under the present experimental condition. 2-chlorophenol eluted with 2,3,4,5-tetrachlorophenol while 2,4-dichlorophenol eluted with 2,3,4-trichlorophenol. The column required a minimum of 60 minutes washing for getting constant retention time of the chlorophenols.

The pH of the running solvent was very critical in the elution pattern of the chlorophenols. Change in pH caused change in the retention time and co-elution of several congeners. We have tried the separation with methanol : water (50:50) and methanol : phosphate buffer (50:50) ranging from pH 6 - 8. We found that pH 7.20 $\pm$ 0.02 was the optimum pH at which maximum separation of 13 congeners of chlorophenols and phenol was obtained. Methanol : water and methanol : phosphate buffer - pH < 6 caused

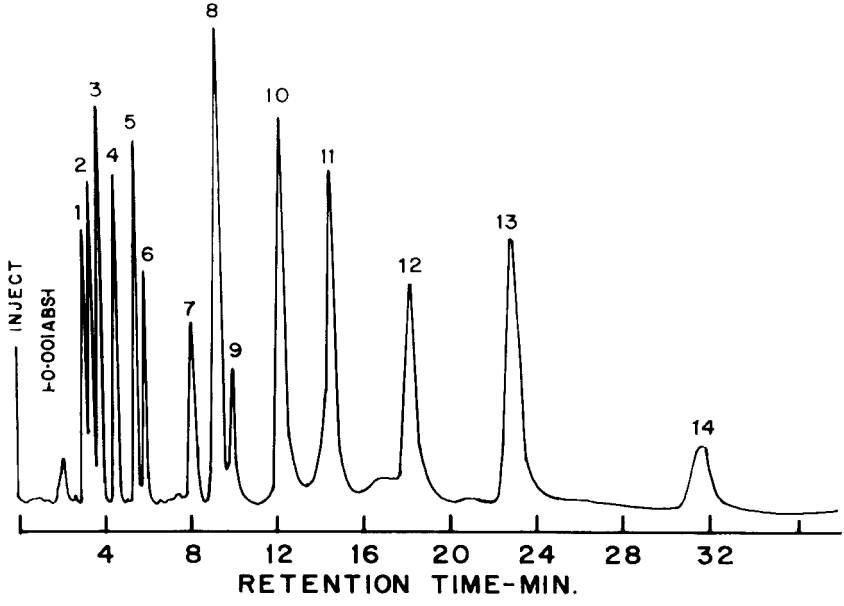

FIGURE 1. HPLC profile of the Chlorophenols and Phenol (Refer Table 1 for the Congeners of Chlorophenols).

poor resolution of the later eluting congeners. Although separation of chlorophenols have been reported using solvent with pH > 8 (4,10) we have not tried solvent system with higher pH as it might damage the reverse phase.

The retention time, response factor (peak area/ concentration) and the minimum detectable amounts of chlorophenols at 0.01 AUFS (254 nm) are given in Table 1. Pentachlorophenol has the maximum response factor followed by tetrachlorophenols, trichlorophenols and dichlorophenols. Phenol and monochlorophenols have the least response factor. It can be noted that ng of chlorophenols can be detected at 0.01 AUFS.

Table 1. Retention Time, Response Factor and Minimum Detectable Amounts of Chlorophenols at 0.01 AUFS (254 nm)

Peak No.	Phenols	Retention Time, Min.	Response Factor *	Minimum Detectable amount, ng.
1.	2,3,5,6-Tetrachloro-	3.00	72.85	5.0
2.	2,3,6-Trichloro-	3.26	67.40	5.0
3.	Pentachloro-	3.66	86.65	4.0
4.	2,4,6-Trichloro-	4.34	58.19	9.0
5.	2,6-Dichloro-	5.34	23.10	16.0
6.	Phenol	5.82	7.14	35.0
7.	2-Chloro-and	8.00	4.70	40.0
	2,3,4,5-Tetrachloro-	8.00	77.65	7.0
8.	2,3,5-Trichloro-	9.08	37.90	20.0
9.	4-Chloro-	9.90	4.72	45.0
10.	2,4,5-Trichloro-	12.08	46.76	9.0
11.	2,4-Dichloro-and	13.40	14.30	25.0
	2,3,4-Trichloro-	13.40	43.65	9.0
12.	3,4-Dichloro-	17.08	7.19	45.0
13.	3,5-Dichloro-	22.74	11.54	40.0
14.	3,4,5-Trichloro-	31.40	30.42	25.0

* Peak area $cm^2/mg \times 10^{-3}$, calculated from three concentrations.

Fig. 2 shows the HPLC profile of the chlorophenols extracted from mouse liver fed with HCH. It can be seen that 2,6-dichlorophenol was the major chlorophenol in the sample. Apart from this, 2,3,5,6-, 2,3,4,5-tetrachlorophenols, 2,3,6-, 2,4,6-, 2,3,5-trichlorophenols and pentachlorophenol were present to a lesser extent. Alkaline and acid hydrolysis

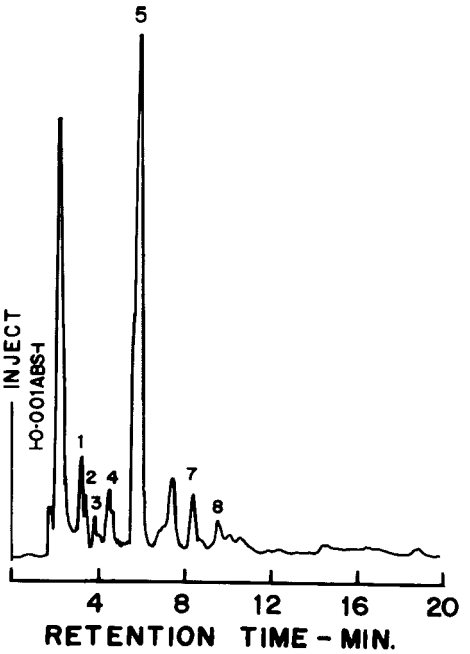

FIGURE 2. HPLC Profile of the Chlorophenols Extracted from HCH Treated Mouse Liver (Refer Table 1 for the Congeners of Chlorophenols).

followed by distillation and solvent extraction is reported to give good recovery of the chlorophenols from the biological samples (8). However, we found that some unknown compounds interfered with separation of chlorophenol if the samples were injected directly without QAE Sephadex ion exchange clean-up.

The present system is useful for separating most of the congeners of chlorophenols from a mixture. It can be used for estimating chlorophenols in biological samples where they are present as degraded products

of chlorinated pesticides. The method may further be useful for the quantitation of chlorophenols in environmental samples.

ACKNOWLEDGEMENT

The authors are thankful to the Government of India for the award of a Fellowship to one of them (K.M.M.).

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JOURNAL OF LIQUID CHROMATOGRAPHY, 6(14), 2839-2840 (1983)

ANNOUNCEMENT

The Biological Research Center of the Hungarian Academy of Sciences, the Chromatography Committee of the Hungarian Academy of Sciences, and the Hungarian Chemical Society announce a conference entitled

"ADVANCES IN LIQUID CHROMATOGRAPHY"

consisting of two parts:

- -Fourth Annual American-Eastern European Symposium on Liquid Chromatography,
- -International Symposium on Thin-Layer Chromatography with Special Emphasis on Overpressured Layer Chromatography (OPLC).

The conference will include invited papers, oral presentations, and posters in all fields of liquid chromatography and will be held in Szeged, Hungary on September 10-14, 1984.

All correspondence concerning the conference should be directed to:

- -Dr. Huba Kalasz, Department of Pharmacology, Semmelweis University of Medicine, P.O.Box 370, H-1445 Budapest, Hungary
- -Dr. E. Tyihak, Research Institute for Plant Protection, P.O. Box 102, H-1525 Budapest, Hungary.

ANNOUNCEMENT

14th NORTHEAST REGIONAL ACS MEETING

June 10-14, 1984

Fairfield University
Fairfield, Connecticut

Symposia

Applications of Molecular Orbital Theory	Chem'l Separations & Identifications
Chem'l Education Towards the 21st Century	The Future of the US Comodity & Specialty Chemicals Industry
Chemical Technicians	Hazardous Waste Management
Computers in Chemistry	Medicinal Chemistry
Organometallic Chemistry	Physical Organic Chemistry
Synthetic Organic Chemistry	Water Soluble Polymers

General Sessions

Analytical Chemistry, Biochemistry, Environmental, Inorganic Chemistry, Physical Chemistry.

The deadline for abstracts is January 15, 1984. Abstracts should be submitted to Miss Linda Somma, P.O.Box 2559, Waterbury, CT, 06725.

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(14), 2841-2843 (1983)

LC NEWS

FAST FLOW ION EXCHANGE GELS offer significant improvements in processing speed and througput for large scale use. The gels are derived from DEAE- and CM-Sepharose gels via modification of the cross-linking that results in improved mechanical strength. Pharmacia Fine Chemicals, Inc., JLC/83/14, 800 Centennial Avenue, Piscataway, NJ, 08854, USA.

ALKYLPHENONE HPLC INTERNAL STANDARD KIT consists of a family of neutral compounds whose retention volumes will not be aff fected by solvent modifiers or acidic/basic buffers. Due to their wide solubility range, they can be used with polar and non-polar solvents. Pierce Chemical Co., JLC/83/14, P.O.Box 117, Rockford, IL, 61105, USA

STRONG ANION EXCHANGE COLUMNS FOR HPLC are prepared by bonding an organic quaternary nitrogen via Si-C bonds to spherical silica. The columns are said to resist deterioration in aqueous, polar and low pH mobile phases. Phase Separations, Inc., JLC/83/14, River View Plaza, 16 River Street, Norwalk, CT, 06850, USA.

IMPROVED BIOPOLYMER YIELDS are claimed for an ion exchange 25 liter column bed capable of absorbing up to 2 kg of biopolymer. According to the manufacturer, packing and separation can be accomplished in a single shift. The column is made of transparent PVC to afford direct view of the column bed, allowing early detection of bed cracking. Whatman Chemical Separation, JLC/83/14, 9 Bridewell Place, Clifton, NJ, 07014, USA.

REACTIVATION SOLUTION RESTORES SILICA COLUMNS following water-deactivation during normal phase LC. The three-component solution provides a cost-saving treatment which avoids a lengthy series of multiple solvent washes and reduces down time. Burdick & Jackson Labs.,
LC/83/14, 1953 S. Harvey Street, Muekegon, MI, 49442, USA.

DIGITAL MICROPIPETTOR is a positive displacement device with a digital display, is easily set and secured at a precise volume. Sample volumes are unaffected by surface tension, viscosity, vapor pressure or density. No calibration for each reagent is necessary. Labindustries, JLC/83/14, 620 Hearst Avenue, Berkeley, CA, 94710, USA.

REVERSED-PHASE ODS COLUMNS are constructed of stainless steel with a highly polished interior surface. They are packed with Spherisorb ODS-II, a spherical silica of very narrow particle size distribution. It is coated with a C18 function and is fully end capped. Columns are guaranteed to deliver 50,000 to 80,000 plates per meter. HPLC Specialties, JLC/83/14, P. O. Box 484, Edmond, OK, 73083, USA.

PHENOLS IN WATER are determined by isocratic mode LC. A large volume of water is injected onto a reversed-phase column, and eluted with a 40:60 acetonitrile/water mobile phase. Detection is at 200 nm down to less than 0.001 ppm. Pye Unicam Ltd., JLC/83/14, York Street, Cambridge CB1 2PX, England.

ULTRA-RESOLUTION IN GPC ANALYSIS is offered by a new generation of GPC columns for polymer analysis and testing. They are available in 500, 1,000, 10,000, and 100,000 angstroms exclusion limits as well as a mixed-bed column that provides ultra-resolution from less than 100 to well over 20 million Daltons. They are available packed in any of several commonly used GPC solvents and in many polymer solvents not generally available with GPC columns. Jordi Associates, Inc., JLC/83/14, 397 Village Street, Millis, MA, 02054, USA.

PROTEIN ANALYSIS LC is described in a recent brochure. Four separation mechanisms are used--ion exchange, gel filtration, reverse phase, and hydroxyapatite fractionation. The methods allow identification and quantitation of components that might not be amenable to any single mechanism. Bio-Rad Labs, JLC/83/14, 2200 Wright Avenue, Richmond, CA, 94804, USA.

AUTOMATED TLC SAMPLE APPLICATOR consists of a control unit and the actual applicator. The control unit is a Z-80 microprocessor-based device that holds 8 sample application modes, five of which are user programmable. Sample volumes from 100 nL to 20 microL may be applied. Applied Analytical Industries, JLC/83/14, Route 6, Box 55, New Hanover Air Park, Wilmington, NC, 28405, USA.

THIN-LAYER CELL FOR ELECTROCHEMICAL DETECTION allows placement of the auxilliary electrode both downstream and across from the working electrode. A highly polished stainless steel top extends cell life, permits compatibility with new "high speed" columns, and allows for connection of low dead volume fittings for use with micro columns. Bioanalytical Systems, Inc., JLC/83/14, 1205 Kent Avenue, Purdue Research Park, West Lafayette, IN, 47906, USA.

LIQUID PROCESSING UNIT can be used to feed most analytical instruments. It performs all of the crucial sample pickup, mixing and dispensing operations. A pair of syringes whose plungers are driven by a stepper motor and precision ball lead screws, a mixing chamber, and a hand-held control unit are the principal working parts. A computer program directs all operations. Processing

parameters such as time, volume, ratios, and increments are entered thru the control unit and become a part of an individual routine program. Hamilton Company, JLC/83/14, P. O. Box 10030, Reno, NV, 89510, USA.

PREPARATIVE SPHERICAL SUPPORTS are said to provide resolution and loading characteristics approaching those of microparticulate columns. They are available in 30 micron particles for ion exchange, reversed phase, and hydrophobic interaction chromatography of proteins. SynChrom, Inc., JLC/83/14, P.O.Box 110, Linden, IN, 47955, USA.

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(14), 2845-2847 (1983)

LC CALENDAR

1983

DECEMBER 6-7 and 8-9: Electrofocusing and Electrophoresis Workshop, Los Angeles, CA, USA. Contact: Workshop Registrar, LKB Instruments, Inc., 9319 Gaither Road, Gaithersburg, MD, 20877, USA.

1984

JANUARY 19-20: Workshop: "Low Dispersion Liquid Chromatography," The Free University of Amsterdam, The Netherlands. Contact: LDLC Workshop Office, Dept. of Analytical Chem., The Free University, DeBoelelaan 1083, 1081 HV Amsterdam, The Netherlands.

FEBRUARY 12-16: 14th Australian Polymer Symposium, Old Ballarat Travel Inn, Ballarat, Australia, sponsored by the Polymer Div., Royal Australian Chemical Inst. Contact: Dr. G. B. Guise, RACI Polymer Div., P. O. Box 224, Belmont, Victoria 3216, Australia.

FEBRUARY 20-22: International Symposium on HPLC in the Biological Sciences, Regent Hotel, Melbourne, Australia. Contact: The Secretary, Int'l Symposium on HPLC in the Biological Sciences, St. Vincent's School of Medical Research, 41 Victoria Parade, Fitzroy 3065, Victoria, Australia.

APRIL 8-13: National ACS Meeting, St. Louis, MO. Contact: Meetings, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

MAY 20 - 26: 8th Intl. Symposium on Column Liquid Chromatography, New York Statler Hotel, New York City. Contact: Prof. Cs. Horvath, Yale University, Dept. of Chem. Eng., P. O. Box 2159, Yale Stn., New Haven, CT, 06520, USA.

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

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

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

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

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

1985

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

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

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

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

1986

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

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

1987

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

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

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

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(14), 2849-2855 (1983)

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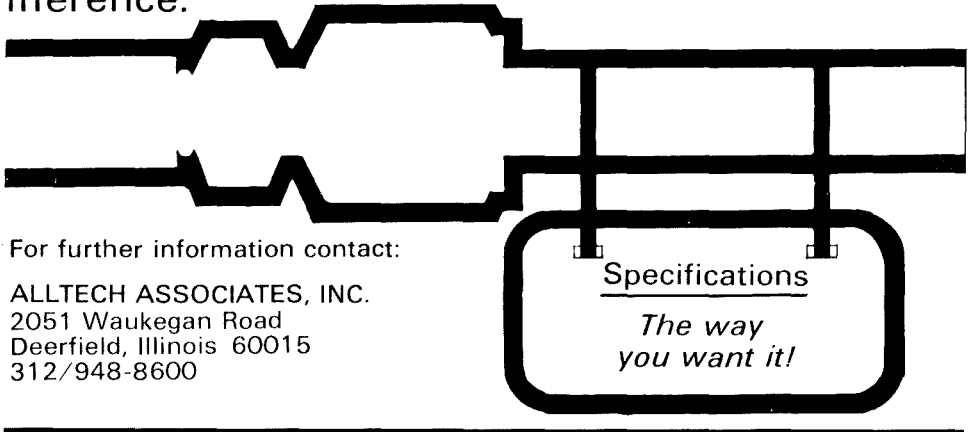

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