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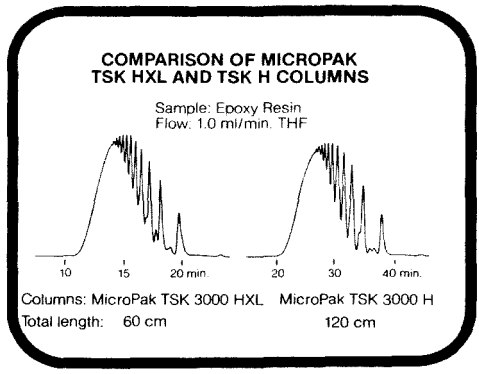

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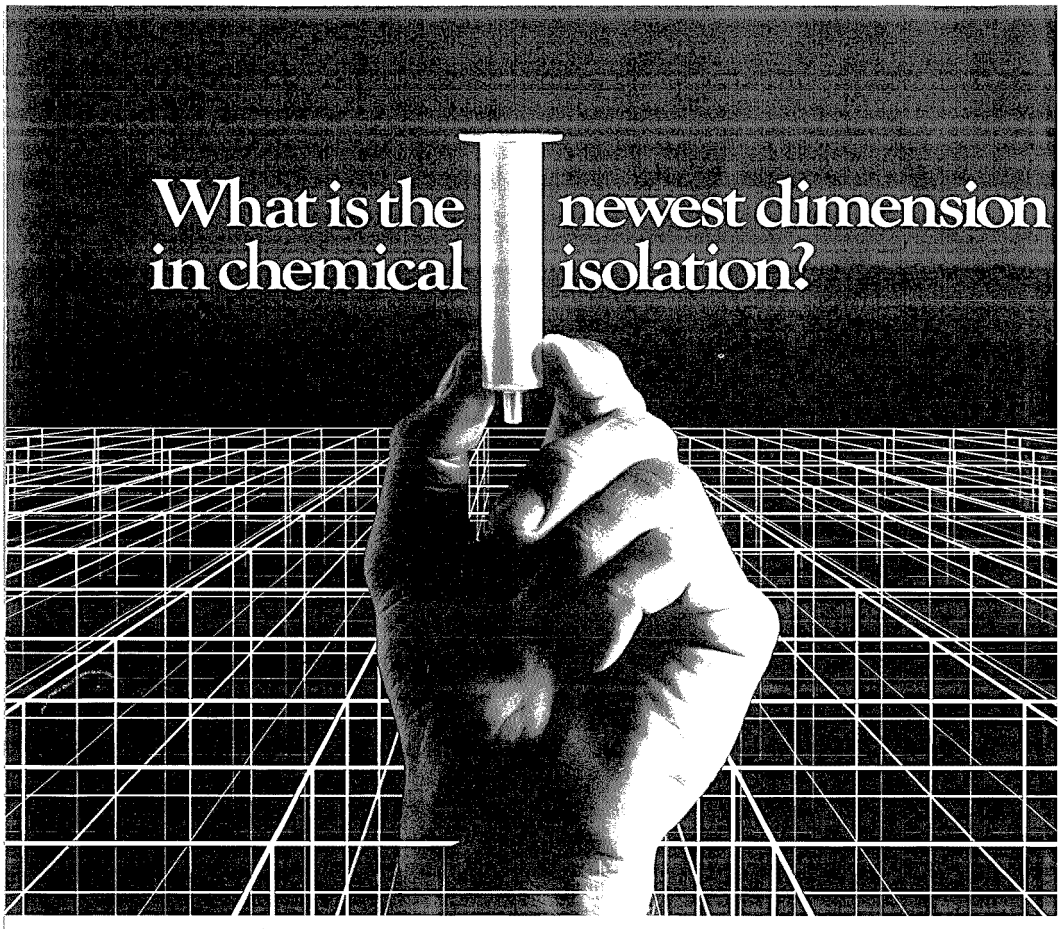

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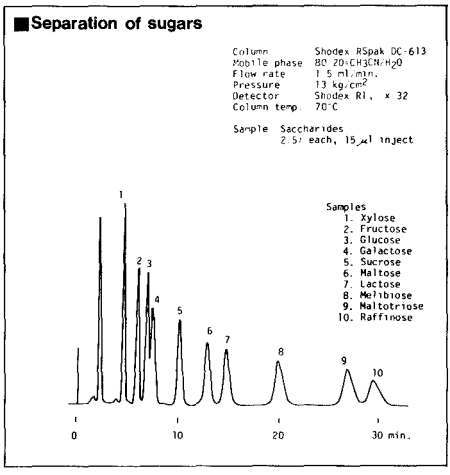
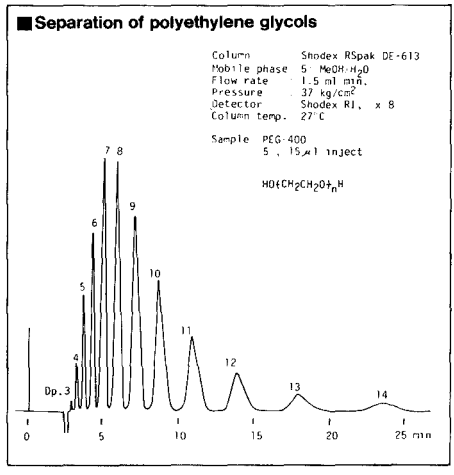


■Separation of polyethylene glycols



Nomenclature	Theoretical plates	Size	In-column eluent	Packed gel
DS-613	5,000	6mmφ×150mm	$CH_3N/THF/H_2O$ 40/30/30	Hydrophobic polystyrene gel
DE-613	5,000	〃	H_2O	Polymethacrylate gel
DM-614	4,000	〃	0.005M H_2PO_4	Hydrophilic polyester gel
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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(1), ix (1984)

ANNOUNCEMENT

A SPECIAL NOTE

This issue of the Journal of Liquid Chromatography is a very special one.....not only because it represents the beginning of a new year, but especially because it is the first time the Journal has had an Associate Editor.

It is with great pride and pleasure that I announce the appointment of Dr. Haleem J. Issaq as Associate Editor of the Journal. Dr. Issaq, of the NCI Frederick Cancer Research Facility, has made significant contributions to the success of the Journal as a member of the Editorial Board and as Guest Editor of the special issues on Thin Layer Chromatography.

Dr. Issaq brings many years of chromatographic expertise to the Journal. He is a pioneer in the development of significant chromatographic methodology and is at the forefront of the field involving prediction and optimization of chromatographic behavior.

I welcome Dr. Issaq as Associate Editor of the Journal of Liquid Chromatography. It will be an honor and a privelege to work with him.

Dr. Jack Cazes
Editor

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(1), 1-11 (1984)

POLYMER-SUBSTRATE INTERACTIONS IN SIZE EXCLUSION CHROMATOGRAPHY WITH SILICA GELS AND PURE SOLVENTS

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ABSTRACT

The retention volumes of polystyrene, poly(α-methylstyrene) and poly-(methylmethacrylate) molecules eluted with different pure solvents on silica size exclusion chromatography columns have been measured. Departures from the universal calibration plot due to polymer adsorption onto the silica substrate are found in some solvent systems, when the polymer gel interactions overcome the solvent gel ones. The competition between the interactions developed in the systems is adequately expressed through the solubility parameters of the polymers and of the solvents.

INTRODUCTION

In size exclusion chromatography secondary effects must be carefully avoided especially when the polymer molar mass distributions are calculated with the universal calibration curve, log (hydrodynamic size) vs. elution volume, based on the elution of polystyrene (PS) standards. If steric exclusion is not the only process controlling the polymer retention in the column, it is known that the elution volumes of the samples will not fit the universal calibration plot. The main processes which can superimpose to the

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size fractionation are the partition ones, where the solute molecules show a different solubility in the liquid stagnant phase, and the adsorption of solute molecules interacting with the gel structure.

When working with inert packings, such as the crosslinked polystyrene gels, it has been reported[1] that both partition and adsorption can be responsible for shifts towards higher elution volumes for the polymer samples and often it is difficult to distinguish between the two effects.

With active packings, like silica gels, displacements to both higher and lower elution volumes in respect of a reference system following the steric exclusion separation, have been reported[2]. The changes in the elution volumes of polystyrene, polydimethylsiloxane and polymethylmethacrylate samples eluted in several pure and mixed eluents on silica-based chromatographic columns have been thoroughly investigated by Figuerelo and coworkers[3,4], who took into account the interactions developed between the gel, the polymer and the eluent. The solvent-gel interactions were expressed through the eluent strength ε° [5], the polymer-solvent interactions through the exponent α of the viscometric equation for the given system, and the solute-gel interactions through the polymer solubility parameter, δ. The experimentally found lower elution volumes, in respect of the chosen reference system, occur in mixed solvent media with high ε° and low α values, and could be attributed to partitioning of the solute molecules preferring the mobile phase to the liquid stagnant phase. With solvents having low ε° values, higher retention volumes were found and partition plus adsorption effects were considered to be responsible for them. When the adsorption processes become predominant, the influence of hydrogen bonding was also stressed[6].

By looking at the interactions which can take place in the elution with pure solvents of neutral polymer molecules on active gels, it can be considered that any deviation occurring in respect of a purely steric exclusion mechanism results in higher retention volumes of the samples. As solvent composition changes cannot occur, partition will not play any role in the process; on the other hand the surface activity of the gel toward solute molecules can change according to the eluent and will then compete with the polymer-solvent interactions in the system. Retardation of the polymer

elution volumes will be then the result of polymer gel interactions prevailing over the polymer-solvent ones.

In this paper results on the elution behaviour of PS, poly(α-methylstyrene), (Pα MS), and poly(methylmethacrylate), (PMMA), samples in different pure solvents with microparticulate silica packings are reported, and the interactions developed in the different systems are discussed on the basis of the agreement, or disagreement, of the polymer retention volumes with the universal calibration plot of PS in tetrahydrofuran.

EXPERIMENTAL

Narrow distribution polymer standards were employed throughout this investigation. The PS standards were obtained from Arro Laboratories (Joliet, Illinois); Pα MS and PMMA samples were supplied from Polymer Laboratories (Shrewsbury, England).

Spherical silica gels having average particle diameter of about 10 μm (Lichrospher, E. Merck, Darmstadt, Germany) were slurry packed on stainless steel columns. Four individual columns (25 cm length, 0.46 cm I.D.) were used, with different pore size silicas. The mean pore size of the gels employed were 10 nm, 50 nm and 100 nm.

The measurement of the elution volumes of the polymers were run on two different column combinations: the first one consisted of 4 columns in series (2 x 100 nm, 1 x 50 nm, 1 x 10 nm); the second one was a two column system (100 nm, 50 nm).

All the solvents were analytical grade reagents (C. Erba, Milan, Italy) and were used as received, with the exception of tetrahydrofuran which was stored with KOH and distilled under CaH_2.

The chromatographic elutions were performed with a Waters M45 pump, a Rheodyne injection valve and a Siemens differential refractometer detector, at a flow rate of about 0.5 $cm^3\ min^{-1}$. Injection volumes of 50 μℓ on the 4 column system, and of 20 μℓ on the two column set were used, with polymer concentrations of 0.1% w/v.

The elution volumes of the samples were determined during each run by measuring the time for collecting a given amount of effluent. All the

measurements were done in triplicate, and the reproducibility of the elution volumes was better than 1%.

RESULTS AND DISCUSSION

The elution behaviour of the PS and P_α MS molecules in toluene was compared with that of PS in tetrahydrofuran. Both tetrahydrofuran and toluene are good solvents for PS, as indicated by the values of the exponent of the viscometric equations which are, at 25°C, α = 0.723 in tetrahydrofuran[7] and α = 0.73 in toluene[8]. Toluene is also a good solvent for P_α MS (α = 0.744[8]), therefore favourable polymer-solvents interactions are expected in all these systems. On the other hand, the eluent strength of tetrahydrofuran, ε° = 0.57[5], is higher than that of toluene, ε° = 0.29[5] meaning that the latter solvent has a lower affinity for the silica substrate. The results of the polymer elutions are reported in Fig. 1, in terms of log ($[\eta]$ M) vs. elution volumes, V_e. The solid line represents the curve for PS in tetrahydrofuran, and the points are for the elutions in toluene. It can be seen that the elution volumes of PS in toluene are generally displaced to higher values in respect of those of PS in tetrahydrofuran, the differences being small but, owing to the limited volumes involved in SEC with micropackings, significant. If the average molar masses of PS samples eluted in toluene ought to be calculated by means of the universal curve based on PS/tetrahydrofuran, significant errors would result.

The P^αMS samples eluted in toluene seem to agree better with the PS/tetrahydrofuran system in the lower part of the plot (molar masses $< 10^5$), whereas the high molar mass (7.6×10^5) sample shows a behaviour more similar to that of the system PS/toluene.

From the results of Fig. 1 it appears that, notwithstanding the favourable polymer-solvent interaction, PS molecules in toluene are interacting with the gel more than in tetrahydrofuran; according to the differences in the ε° values of the solvents, tetrahydrofuran will successfully compete with the PS molecules in the interactions with the silica.

The competition between the different binary interactions taking place in every solvent-polymer-gel system can be better expressed by considering the values of the solubility parameter, δ, of the solvents and of

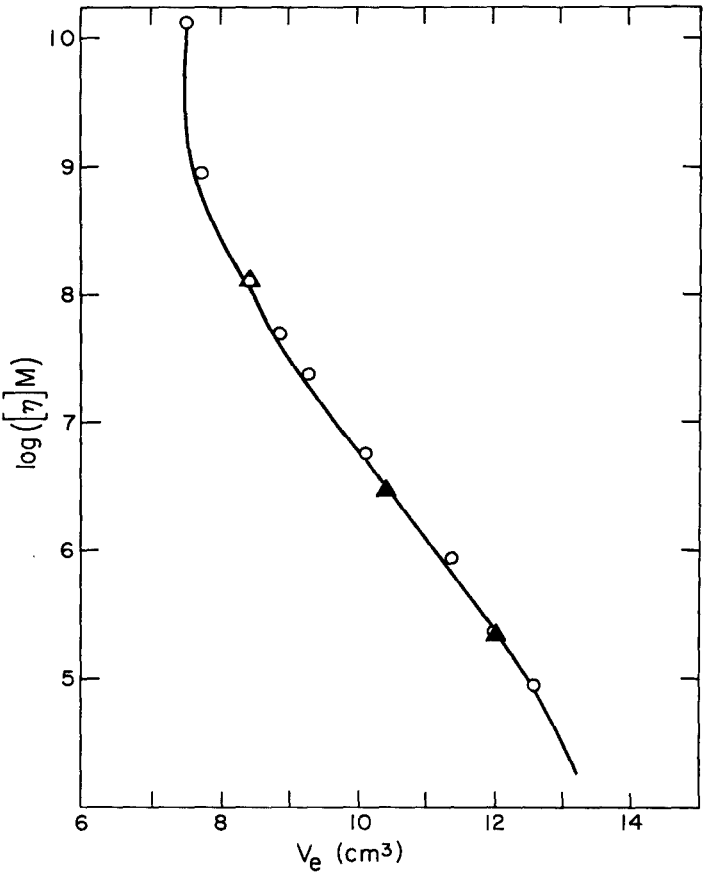

Figure 1 Elution behaviour of PS (o) and PαMS (▲) in toluene in conparison with PS in tetrahydrofuran (solid line). Column system: 2 x 100 nm, 50 nm, 10 nm.

the polymers[9]. In this way one can describe polymer-solvent, polymer-gel and solvent-gel interactions by using the same parameter, therefore allowing more direct comparisons.

For the systems of Fig. 1, the values of δ which are of interest are reported in Table 1. The δ for PαMS was not found in the literature, but its value should be close to that attributed to the monomer, α-methylstyrene.

TABLE 1

Values of the Solubility Parameters for the Polymers and Solvents Employed (from Ref. 8 and 10)

	δ	δ_d	δ_p	δ_h
		$(J \cdot cm^{-3})^{1/2}$		
Polystyrene	18.6	17.6	6.1	4.1
Poly(methylmethacrylate)	19.4	18.8	10.2	8.6
α-methylstyrene	17.4			
Toluene	18.2	17.3-18.1	1.4	2.0
Tetrahydrofuran	19.5	16.8-18.9	5.7	8.0
2-Ethoxyethanol	24.3	16.1	9.2	14.3
Acetonitrile	24.3	15.4-16.2	18.0	6.1

The affinity between PS, PαMS and the solvents is indicated by the similarity of the δ values, but as to the interactions with the gel, the strongest ones are those of tetrahydrodrofuran which has the highest δ value in the systems hereby considered. This is of course the result of the polarity of tetrahydrofuran, and the effect is particularly evident by looking at the values of the contributions to δ due to the polar forces and to hydrogen bonding, δ_p and δ_h respectively, which are also shown in Table 1 together with the contribution δ_d of the dispersion forces. The comparison of the solubility parameter values shows that in tetrahydrofuran the PS molecules will not preferentially interact with the silica stationary phase, therefore the retention volumes are only size-exclusion dependent. In toluene solutions, on the other hand, the interactions between PS and silica gel, as

expressed especially by the δ_p and δ_h values, are slightly prevailing over the solvent-gel ones, thus explaining the retardation of the polymer molecules in respect of the elutions in tetrahydrofuran. The values of the different contributions to the solubility parameter of α-methylstyrene are not known, and therefore the discussion for PαMS cannot go too far, but the comparison of the δ values in Table 1 seems to indicate that PαMS molecules in toluene shouldn't interact with the gel, and agreement with the elution curve of PS in tetrahydrofuran is expected.

In the case of polar solute molecules eluted on silica gel columns the retention volumes even in tetrahydrofuran can be affected by secondary non-exclusion effects. In Fig. 2 the behaviour of PMMA narrow distribution molecules in tetrahydrofuran is shown, compared with the system PS/tetrahydrofuran as a reference. The α value for PMMA/tetrahydrofuran at 25°C is 0.72[11], practically identical to that of PS/tetrahydrofuran, and the solubility parameter δ of PMMA is fully comparable with that of tetrahydrofuran (see Table 1), but the PMMA molecules are more retained than the PS ones. The explanation can be found by looking at the values of the contributions δ_p and δ_h for the polymers and for tetrahydrofuran: whereas the δ_h term for PS is negligible compared to that of the solvent, tetrahydrofuran, the d_h term for PMMA slightly overcomes that of tetrahydrofuran, and also δ_p is consistently higher for PMMA. The stronger polar interactions and hydrogen bonding capability of PMMA in respect of tetrahydrofuran can be therefore responsible of the polymer elution retardation in respect of the PS/tetrahydrofuran system. Retarded elutions of PMMA in several eluents, in respect of PS, were already discussed by Figuerelo et al.[4] but no data were reported on the elution of PMMA in tetrahydrofuran. The latter is the most common solvent employed in polymer SEC, and the results here reported show that with non deactivated silica packings molar mass distributions of PMMA in tetrahydrofuran cannot be correctly calculated by using the PS-based universal calibration plot.

The competition in developing specific polar interactions between the gel active sites and the eluent or the polymer molecules and the importance of these interactions in respect of the polymer-solvent ones can be further tested by considering the elution behaviour of a polar polymer in some polar

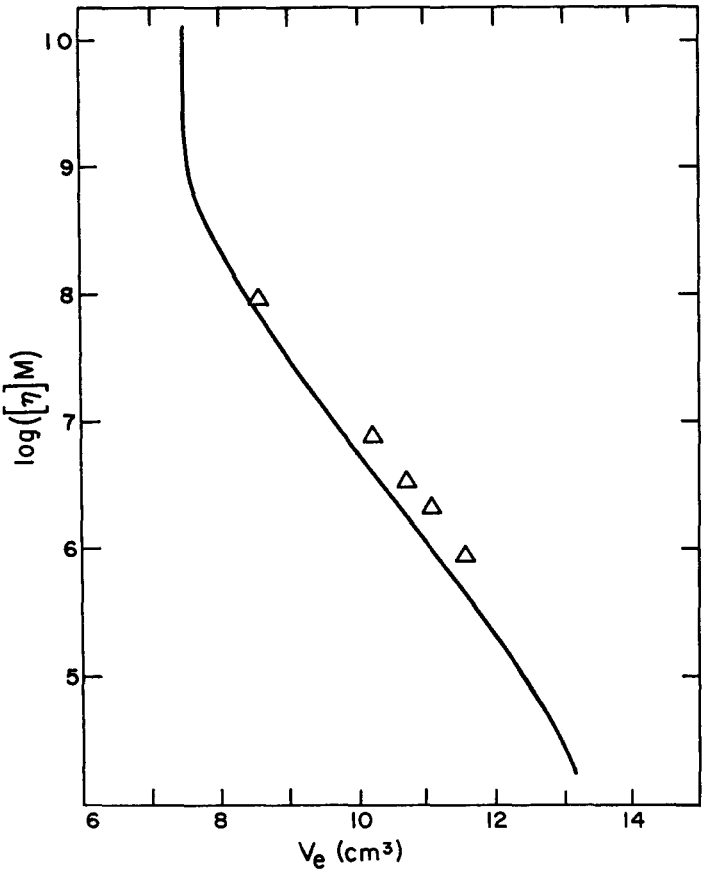

Figure 2 Elution behaviour of PMMA in tetrahydrofuran (△). Solid line represents PS in tetrahydrofuran. Column system: 2 x 100 nm, 50 nm, 10 nm.

eluent which at the same time is poor solvent for the polymer. For PMMA polymers two solvents have been found suitable for this comparison: acetonitrile and 2-ethoxyethanol. Both these eluents are practically theta solvents for PMMA at room temperature[12,13], (α = 0.5), and they have higher eluent strengths than tetrahydrofuran: ε° = 0.65 for acetonitrile, whereas for 2-ethoxyethanol ε° should be not very different from the value ε°

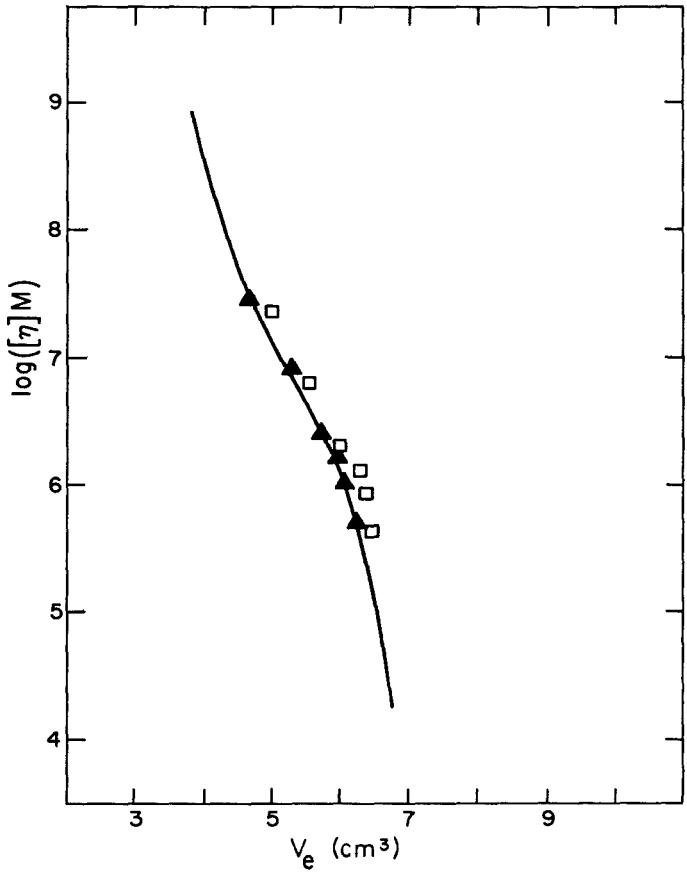

Figure 3 Elution of PMMA in 2-ethoxyethanol (▲) and in acetonitrile (□) compared with the PS/tetrahydrofuran line. Column system: 100 nm, 50 nm.

= 0.74[14] of the omologue compound 2-buthoxyethanol. Polymer-solvent interactions are then low, and solvent-gel interactions should be strong, as is also shown by the δ values in Table 1, which are higher than that of PMMA. But looking at the values of the hydrogen-bonding term δ_h for the two solvents in the same Table 1, it appears that 2-ethoxyethanol, being both proton acceptor and donor, has a definite higher affinity for the gel than

acetonitrile; the δ_h value of the latter solvent is also lower than that of PMMA. The retention volumes of PMMA in acetonitrile are then expected to be higher than those in 2-ethoxyethanol , and this is indeed experimentally found in the results reported in Fig. 3. Notwithstanding the high overall polarity of acetonitrile, adsorption of PMMA on the silica occurs in this solvent, whereas the opposite happens in 2-ethyoxyethanol solutions where the polymers elute regularly and the points fit the PS universal curve.

CONCLUSIONS

The retention volumes of polymer molecules eluted on silica gel columns with pure solvents can be adequately explained through the competition of interactions with the gel between the eluent and the solute, as expressed by the solubility parameters of solvents and polymers, and particularly by the contributions to δ due to polar and hydrogen-bonding interactions. Adsorption on the silica surface becomes particularly relevant with polar polymers, and therefore in these cases SEC should be performed with solvents having higher possibility than the polymers of forming hydrogen-bonds in order to completely suppress the polymer-gel interactions.

The adsorption of PMMA onto silica substrates from tetrahydrofuran, between other solvents, has been also recently measured with static experiments[15]; acid-base interactions between solvent, polymer and filler surface were proposed as a way of explaining all polar and hydrogen-bonding interactions. Yet the evaluation of the interactions through the solubility parameters as proposed here seems to be simpler and of more practical value for the chromatographic interpretation.

REFERENCES

1. Dawkins, J.V., Hemming, M., Gel Permeation Chromatography with Crosslinked Polystyrene Gels and Poor and Theta Solvents for Polystyrene, 1. Universal Calibration for Cyclohexane at 35°C. Makromol. Chem. 176, 1777, 1975.

2. Berek, D., Bakos, D., Bleha, T. Soltes, L., Gel Permeation Chromatography in Mixed Eluents. Effects of Sorption on Porasil Gels. Makromol. Chem. 176, 391, 1975.

3. Campos, A., Soria, V., Figuerelo, J.E., Polymer Retention Mechanism in GPC on Active Gels, 1. Polystyrene in Pure and Mixed Eluents. Makromol. Chem. 180, 1961, 1979.

4. Figuerelo, J.E., Soria, V., Campos, A., Polymer Retention Mechanisms in GPC on Active Gels, 3. Poly(dimethylsiloxane) and Poly(methylmethacrylate). Makromol. Chem. 182, 1525, 1981.

5. Snyder, L.R., Kirkland, J.J., Introduction to Modern Liquid Chromatography, J. Wiley and Sons, New York, 1979.

6. Figuerelo, J.E., Soria, V., Campos, A., Polymer Retention Mechanism in GPC on Active Gels, II. Low Molecular Weight Polystyrene. J. Liquid Chromatog. 3, 367, 1980.

7. Kolinsky, M., Janca, J., Some Applications of Gel Permeation Chromatography in Investigations of Structure and the Solution Properties of Radical-Initiated Poly(vinylchloride). J. Polym. Sci. A-1, 12, 1181, 1974.

8. Polymer Handbook, Bandrup J., Immergut, E.M., eds., J. Wiley and Sons, New York, 1975, p. IV-16.

9. Dawkins, J.V., Polymer-Gel Interactions in GPC with Organic Eluents, J. Liquid Chromatog. 1, 279, 1978.

10. van Krevelen, D.W., Properties of Polymers - Their Estimation and Correlation with Chemical Structures, Elsevier Scientific Publ. Company, Amsterdam 1976.

11. Evans, J.M., Gel Permeation Chromatography: A Guide to Data Interpretation. Polym. Eng. Sci. 13, 401, 1973.

12. Quadrat, O., Bohdanecky, M., Mrkvickova, L., The Configurational Characteristics of Conventional Poly(methyl methacrylate) in Acetonitrile, Makromol. Chem. 182, 445, 1981.

13. Quadrat, O., Mrkvickova, L., A Viscometric Study of Poly(methyl methacrylate) in 2-Alkoxyethanols, Europ. Polym. J. 17, 1155, 1981.

14. Snyder, L.R., Principles of Adsorption Chromatography, Dekker, New York, 1968.

15. Fowkes, F.M., Mostafa, M.A., Acid-Base Interactions in Polymer Adsorption, Ind. Eng. Chem. Prod. Res. Dev. 17, 3, 1978.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF 2,2-BIS(4-HYDROXYCYCLOHEXYL)PROPANE

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Phillips Petroleum Company

Phillips Research Center

Bartlesville, Oklahoma 74004

ABSTRACT

The three isomers of 2,2-bis(4-hydroxy-cyclohexyl)propane, and its major impurities were separated by high performance liquid chromatography. Isolated components were identified by mass spectrometry, nuclear magnetic resonance, and infrared spectroscopy. Preparative liquid chromatography effected the purification of 18g of pure 2,2-bis(4-hydroxycyclohexyl)propane in 30 minutes.

INTRODUCTION

We wanted to develop methods to characterize the impurities of commercial 2,2-bis(4-hydroxy-cyclohexyl)propane(Figure 1, structure b). We also

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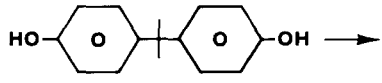
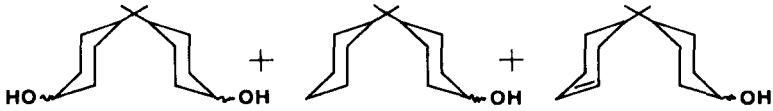
needed 2,2-bis(4-hydroxy-cyclohexyl)propane in its pure form(> 99.7%). Monofunctional impurities produced in its synthesis were undesirable. Commercial 2,2-bis(4-hydroxy cyclohexyl)propane formed by the hydrogenation of bisphenol A (Figure 1, structure a) possesses an average purity of about 90%. The hydrogenolysis and elimination side products (Figure 1, structures c and d) that are suspected to be formed, besides the expected 2,2-bis(4-hydroxycyclo-hexyl)propane (also called hydrogenated bisphenol A or (HBPA)) product, are shown in Figure 1.

In addition, to wanting pure HBPA, separation of the axial, axial (Figure 2, structure e), axial, equitorial (Figure 2, structure f) and equitorial, equitorial (Figure 2, structure g) HBPA isomers was desired. Standard methods of purifying HBPA such as recrystallization and distillation[1] were inadequate. Because we also wanted to isolate the pure

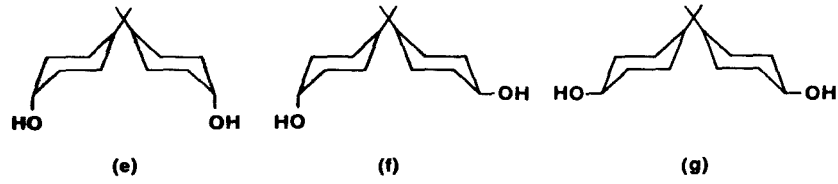


(a)



(b) (c) (d)

Figure 1
Products Formed From The Hydrogenation of Bisphenol A.



(e) (f) (g)

Figure 2
The Three Isomers of 2,2-bis(4-hydroxy-cyclohexyl)propane.

isomers of HBPA, a liquid chromatographic separation was in order.

The present work describes the separation of HBPA isomers and several impurities by liquid chromatographic techniques, followed by characterization of the material obtained.

MATERIALS AND METHODS

Instrumentation

Infrared (IR) spectra were obtained on a Perkin-Elmer 137 sodium chloride infrared spectrometer. Solid samples were run as potassium bromide KBr pellets using mixtures of 1% by weight. All absorptions were reported in wave numbers (cm^{-1}).

Mass spectra were obtained using a CEC 21-110 Mass Spectrometer equipped with a computerized data system. FIMS was used to get simpler spectra with fewer peaks, as too much fragmentation resulted using EIMS.

Nuclear magnetic resonance (NMR) proton spectra were obtained on a Varian EM 390 spectrometer,

operating at 90 MHz in the CW mode. Carbon 13 spectra were obtained on a Varian CFT 20 at 20 MHz in the FT mode. Chemical shifts are reported in parts per million (ppm down field from tetramethylsilane (Mallinkrodt). The spectra were obtained in $CDCl_3$ (Norell Chemical Co., Inc.) containing 1% by volume of the internal standard tetramethylsilane .

The analytical liquid chromatograph used in this study was a Waters Model 204 liquid chromatograph with a Model 6000-A pump, Model 401 refractive index detector, and Model 450 variable wavelength detector set at 254 nm and a dual channel 10 mv strip chart recorder. The column used was a 3.9 mm id x 30 cm μ-Porasil column packed with 10μm porous silica obtained from Waters Associates, Milford, Mass.

The preparative liquid chromatograph was a Waters Prep 500-A equipped with solvent delivery system, refractive index detector and strip chart recorder. The two cartridge μ-Porasil columns employed measured 5.7 x 30 cm and were packed with 325 g of 50-100 μ porous silica. These columns were obtained from Waters Associates, Milford, Massachusetts.

Thin Layer Chromatography

Thin layer chromatography (TLC) was effected using either 25 x 75 mm Baker Flex 1 B-F precoated silica gel sheets (J. T. Baker Company) or 25 x 75 mm precoated 60 F-254 silica gel plates (E. Merck Laboratories) in vapor saturated jars (6 x 12 cm). Visualization of the developed spots, using the Baker Flex plates, was accomplished by iodine staining.

Developed spots on the EM plates were visualized with 254 nm light followed by staining with a 10% solution of phosphomolybdic acid in 100% ethanol, followed by heating.

Reagents

All solvents used were reagent grade. Chloroform used in the high performance liquid chromatography work was filtered through a Millipore Type F-H 0.5 mm filter prior to use. HBPA, used for preparative separations, was obtained from commercially available sources..

RESULTS AND DISCUSSION

Thin Layer Chromatography

TLC was investigated for detecting impurities present in commercial HBPA and as a method of selecting an eluting solvent for preparative HPLC. Several solvent systems were evaluated using silica chromatographic plates for their ability to separate the impurities from HBPA. The solvent systems of 3% or 4% (v/v) methanol in chloroform were ideal for impurity detection by TLC. Monofunctional impurities, later identified as 2-cyclohexyl-2-(4[a]-hydroxy-cyclohexyl)propane (Figure 3, structure h) and 2-cyclohexyl-2-(4[e]-hydroxycyclohexyl)propane (Figure 3, structure i), were separated as well as the three isomers of HBPA.

When TLC was used to aid in the selection of a mobile phase for preparative HPLC, an important

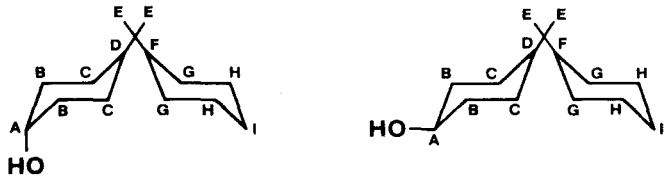


Figure 3

The two major impurities found in 2,2-bis(4-hydroxycylohexyl)propane (the letters correspond to ^{13}C nmr resonances listed in Table III).

criteria required the solvent system to elute the three isomers of HBPA to retardation factor (R_f) values between 0.15 and 0.35. This R_f range has been shown to be optimal in terms of sample loading, resolution, residence time and solvent usage(2). Also, the solvent system chosen had to dissolve acceptable amounts of HBPA so that 20g quantities of 90% pure material could be purified per injection. Two percent methanol in chloroform came closest to meeting the above-mentioned criteria. Experimentally, however, one percent methanol in chloroform turned out to be the best for preparative HPLC. Table I lists the HBPA R_f values for four solvent systems tried.

Identification of HBPA Isomers and Impurities

For the HPLC work with HBPA, the mobile phase was chloroform at 4mL/min. The amount of sample injected was 438-772 mg. Four commercial HBPA samples from different sources were evaluated. Two of the

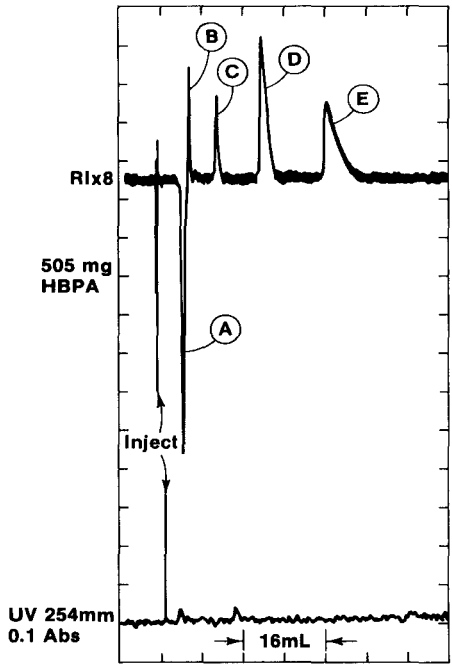
Table I

HBPA R_f VALUES

Solvent System MeOH in $CHCl_3$ (Volume %)	HBPA Isomer axial,axial	axial,equit.	equit,equit.
4%	0.43	0.29	0.20
3%	0.38	0.26	0.18
2%	0.27	0.17	0.12
1%	0.16	0.09	0.06

samples were completely chloroform soluble. There was a significant amount of chloroform insoluble material in the other two samples. This insoluble material was identified later as the equitorial,equitorial isomer of HBPA (Figure 2, structure g). Chromatograms of the chloroform soluble portions of the four samples are shown in Figures 4-7. These chromatograms show significant differences in impurities for the four samples.

For identification work, about 20 mg portions of each of the chloroform soluble material from the HBPA samples were injected onto the HPLC system and fractions corresponding to the peaks of interest were collected. These peaks are those labeled A-M in Figures 4-6. Material for unlabeled peaks was not collected. It was assumed that the peaks represented the major impurities and components present in the four samples, even though they were not collected for all four. The fractions were evaluated by mass spectrometry. The results of the identification work are summarized in Table II. In many cases, insufficient amounts of impurities were isolated for complete identification. The major impurities were character-



Figures 4-7
HPLC chromatogram of four differentcommercial HBPA samples, conditions as in text.

ized in more detail following preparative liquid chromatography. This is described in the following section.

Preparative LC Separation

The 1% methanol in chloroform solvent was pumped at a rate of 200 mL per minute. Sample preparation involved making a saturated solution of

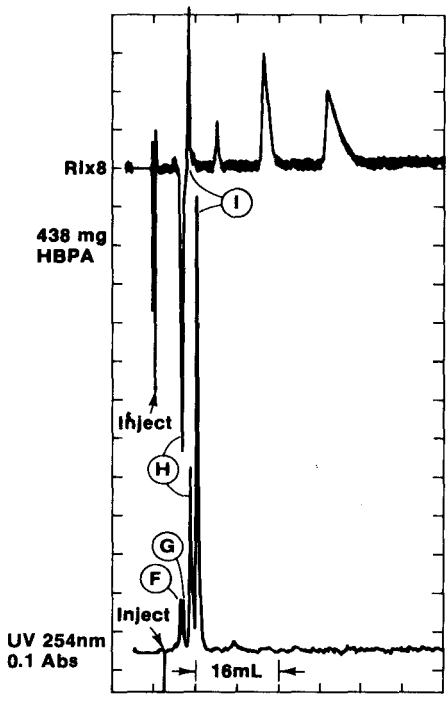


Figure 5

HBPA in the above-mentioned solvent which contained 20g of crude (90% pure) HBPA per 100 mL of solution. One hundred milliliter aliquots were injected by pumping the solution directly on the column. The first separation simply involved the removal of impurities from the three isomers of HBPA (Figure 8), affording 18g of pure (>99.7%) HBPA. Next, the isomers of HBPA were separated using the same solvent system, by collecting the appropriate fractions. Finally, the impurities were isolated in a separate operation using chloroform as the eluting solvent.

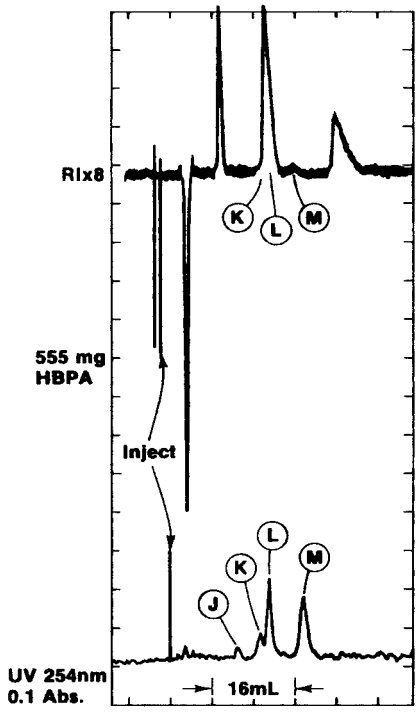


Figure 6

Pure material was obtained in each case by removing the solvent at reduced pressure. The first separation step allowed us to use HBPA as an isomer mixture. The second step allowed us to isolate and identify the three isomers of HBPA, while the last separation allowed us to isolate and identify the major impurities found in HBPA. The isomers of HBPA could now be used individually or as a given mixture.

Isolation of the isomers of HBPA, via a tedious multiple recrystallization sequence, was reported by Terada in 1966[1]. Our spectrographic

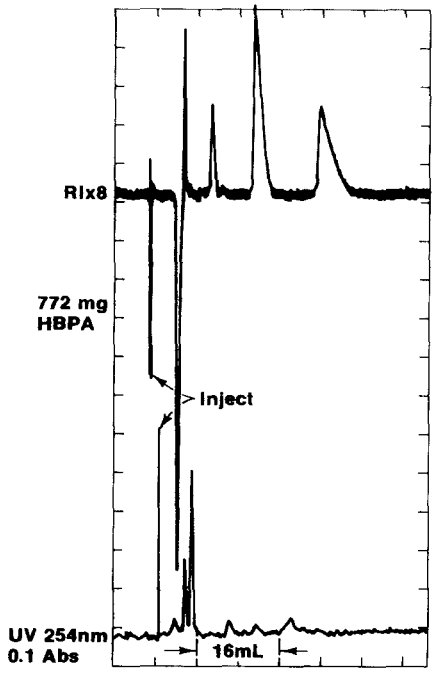
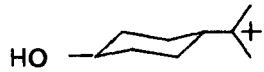
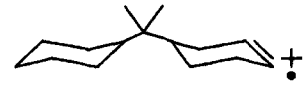


Figure 7

assignments of the three isomers were consistent with his and show up on the preparative HPLC chromatogram in Figure 8 as peak e (axial,axial); peak f, (axial,equitorial),and peak g (equitorial,equitorial). Figure 2 depicts these three isomers. The two impurities we found in commercial HBPA were different than those found in Terada's hydrogenated product. Therefore, we separated the two components of the impurity fraction and analyzed them by IR, ^{13}C NMR, ^{1}H NMR, and mass spectrometry. We identified the impurities as 2-cyclohexyl-2-(4[a]-hydroxycyclo-hexyl)propane (Figure 3,structure h) and 2-cyclohexyl-

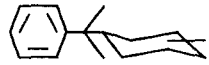
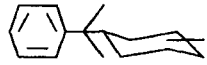
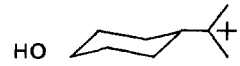
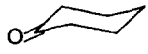
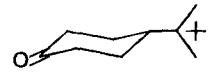
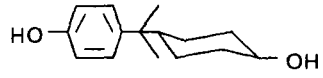
Table II
MASS SPECTROMETRIC RESULTS FOR PEAKS
A THROUGH M IN FIGURES 4 - 7

Peak[a]	Mass Spectrometric Results
A	Complete identification not possible. Two fragments identified were  m/e 125  m/e 123
B	Fragments identified were:  m/e 125  m/e 123  m/e 141  m/e 206  m/e 204 None of the above are believed to represent molecular ions.
C, D, E	HBPA isomers.
F, G	Insufficient material for identification

[a] Peaks as in Figures 4-6.

(continued)

Table II (continued)

Peak[a]	Mass Spectrometric Results
H	The UV absorbing impurity is  (isomer of I)
I	The UV-absorbing impurity is  (isomer of H)
J	Complete identification not possible. Fragments seen included.  m/e 123  m/e 141
K	Two major components - one is isomer of J; other showed the fragments.  m/e 97  m/e 139 Neither component could be identified.
L	Isomers of compounds in J and K, none completely identified.
M	The UV-absorbing impurity is 

[a] Peaks as in Figures 4-6

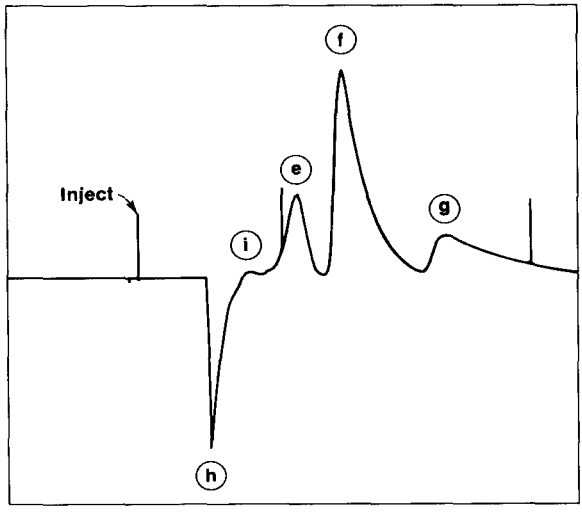


Figure 8
Prep LC chromatogram of HBPA, conditons as in text. Peak identifications h, i: two major impurities, (Figure 3); e,f,g: three HBPA isomers (Figure 2).

2-(4[e]-hydroxycyclohexyl)propane (Figure 3,structure i). Infrared spectroscopy showed that both impurities were alcohols by their large OH stretching frequencies at about 3350 cm-1. Proton NMR eliminated the possibility of any unsaturation in either impurity as there was only one resonance in both spectra beyond 4.2ppm. This broad peak at about 5.5 ppm was integrated as a single proton in both spectra and was identified as the alcohol proton. The definitive proof was found in ^{13}C nmr, which distinguished the axial and equitorial isomers and high resolution mass spectrometry, which showed that both impurities

possessed the same fragmentation pattern. The first impurity, h (Figure 3) , possessed the following spectra; (h) IR (film) 3350, 2940, 2890, 2865, 1449, 1378, 1270, 1100, 1019, 964, 800, and 698 cm^{-1}; ^{1}H nmr ($CDCl_3$) 5.32 (1.s [broad] alcohol)., 3.85 (1,m,hydroxymethine), 0.85 - 2.00 (20,m [broad] methylene and methine) and 0.60 ppm (6,s,gem dimethyls); ^{13}C nmr ($CDCl_3$) spectrum is listed in Table III;
field ionization mass spectra gave a base peak at m/e 125 [M-99] with characteristic peaks at 141 [M-83], 99[M-125] and m/e 83 [M-141].

CONCLUSION

Preparative LC allowed us to obtain quickly 18g quantities of pure HBPA from 20g of 90% pure HBPA.

TABLE III

CARBON 13 NMR OF AXIAL (h) AND EQUITORIAL (i) 2-CYCLOHEXYL-2-(4-HYDROXYCYCOLHEXYL) PROPANE

Carbon/Atom (Figure 3)	Chemical Shift Axial Isomer (ppm)	Chemical Shift Equitorial Isomer (ppm)
A	66.1	71.4
B	33.6	36.3
C	20.6	25.8
D	43.7	42.9
E	20.5	20.6
F	43.9	44.3
G	27.4	27.4
H	27.2	27.2
I	27.0	27.0

It also gave us the ability to separate each isomer of HBPA so that we could use each isomer by itself or as a mixture and allowed us to isolate and identify the two major impurities found in commercial HBPA.

ACKNOWLEDGEMENTS

We thank John E. Inda for the helpful ideas and effort he put into this research. We also wish to acknowledge Gil Greenwood for the mass spectrometric work, Mike Fuller for the infrared spectroscopy work, and Steve Wharry and Dan O'Donnell for the nmr work.

REFERENCES

1. Terada, A.; Bull. Chem. Soc. Japan, 1966, 39, 2194.
2. Rahn, P.C.; Woodman, M.; Beverung, W. Heckendorf, A., Waters Associates, "TLC to Prep", Waters Associates, Milford Massachusetts, 1979.

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A COMPARISON OF POLYMER SEPARATION EFFICIENCY AND RESOLUTION BY GRADIENT LC, GPC AND TLC.

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ABSTRACT

The specific resolution of gradient LC and reversed phase TLC methods for the separation of different molecular weight standards of poly(isoprene), poly(ethylene glycol), poly(ethylene oxide), poly(styrene) and poly(α-methylstyrene) were determined. It was found that gradient LC has an order of magnitude greater resolving power (for high polymers) than gel permeation chromatography (GPC) while TLC had from two to five times the resolving power of GPC in the molecular weight range investigated. This is a direct result of the greater selectivity of gradient LC and TLC techniques. The specific resolution is also dependent on the type of gradient used to achieve fractionation for the LC technique.

INTRODUCTION

Since the first successful fractionation of synthetic polymers using cross-linked poly(styrene) "gels" was demonstrated by Moore in 1964 (1), gel permeation chromatography (GPC) has experienced a phenomenal growth and has become one of the more widely used secondary techniques for determining the molecular weight and molecular weight distribution of polymers. In spite of

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its relative ease and popularity, GPC gives relatively poor resolution and has a limited peak capacity compared to conventional LC (2).

Recently an effective method for the fractionation of homopolymers by gradient LC and/or reversed phase TLC was demonstrated (3-6). These techniques are, in principle, modern versions of the earliest solvent-based fractionation methods (7,8). In addition, a theoretical and experimental evaluation of this technique and its "precipitation" mechanism was completed (4-6). The theory indicates that this technique should have excellent resolving power over a wide range of molecular weights (6). Although, resolving power is an important part of any polymer fractionation method, the ultimate use of a technique for the determination of molecular weights and molecular weight distribtuions must consider other factors as well (e.g., accuracy, precision, cost, speed, etc.). In this study we focus on the relative efficiency and resolution of homopolymer separation by gradient LC, GPC and TLC.

MATERIALS

KC18F reversed-phase TLC plates (5x20 cm) and Partisil 10, ODS-3 reversed-phase columns (25 cm long) were obtained from Whatman Chemical Separation Division, Inc. HPLC grade methanol and methylene chloride were obtained from Baker; Certified ACS grade 1,4 dioxane and ethylene glycol were obtained from Fisher Scientific Company. The following polymer standards were used: (1) poly(styrene) from Polysciences, Inc. mol. wt. = 10,000,000 (Mw/Mn = 1.2), mol. wt. = 390,000, (Mw/Mn = 1.04), mol. wt. = 100,000

(Mw/Mn = 1.06) mol. wt. = 35,000 (Mw/Mn = 1.04), mol. wt. = 9,000 (Mw/Mn = 1.08), mol. wt. = 3570 (Mw/Mn = 1.06), mol. wt. = 2,000 (Mw/Mn = 1.06), mol. wt. = 17,500 (Mw/Mn = 1.04), mol. wt. = 63,000 (Mw/Mn = 1.04), poly(isoprene); from Polymer Laboratories, mol. wt. = 1,360 (Mw/Mn = 1.11), mol. wt. = 11,100 (Mw/Mn = 1.08), Mol. wt. = 33,300 (Mw/Mn = 1.05), mol. wt. = 113,800 (Mw/Mn = 1.05), mol. wt. = 260,000 (Mw/Mn = 1.07), poly(ethylene glycol/oxide) from Polymer Laboratories, mol. wt. = 998 (Mw/Mn = 1.06), mol. wt. = 4820 (Mw/Mn = 1.04), mol. wt. = 11,250 (Mw/Mn = 1.07), mol. wt. = 73,000 (Mw/Mn = 1.02), poly(α-methyl styrene) from Polymer Laboratories, mol. wt. = 19,500 (Mw/Mn = 1.15), mol. wt. = 87,600 (Mw/Mn = 1.10), mol. wt. = 760,000 (Mw/Mn = 1.10), and styrene oligomers A1000 from Toyo Soda Manufacturing Company, LTD, Toso Building, 1-7-7 Akasaka, Minato-Ku, Tokyo, 107, Japan.

METHODS

All polymer standards were dissolved in the "good solvent" (5 mg/ml). Dioxane was the good solvent for poly(ethylene glycol/oxide) and methylene chloride was the good solvent for all other polymers. TLC fractionations were done in an 11 3/4 in. long, 4 in. wide, and 10 3/4 inch high chromaflex developing chamber without prior equilibration with the solvent vapor nor special treatment of the plates. Approximately 1 μℓ of solution was spotted on the TLC plates and the solvent front was allowed to move up the plate 10 cm from the spotting line. A Shimadzu dual wavelength TLC scanner Model CS910 was used in the reflectance mode to scan the plate. Other TLC chromatographic parameters and

Table I: Chromatographic Parameters of TLC Fractionations

Polymers	Solvents	Mobile phase Composition	Visualization Reagent	Scanning Wavelength (nm)
poly(styrene)	$MeCl_2$:MeOH	79:21 (V:V)	None	255
poly(isoprene)	$MeCl_2$:MeOH	75:25 (V:V)	None	200
poly(ethylene glycol/oxide)	Dioxan:Ethylene glycol	58:42 (V:V)	1%I_2/MeOH	405

the wavelengths at which the polymers were scanned are provided in Table I.

All gradient elution HPLC fractionations were done with a Varian 5020 liquid chromatograph coupled to a Water Associates variable wavelength detector. Since methylene chloride has a UV cut off wavelength of 235 nm, only higher wavelengths can be used. The use of composition gradient elution prevents the employment of a refractive index detector. Consequently, only UV absorbing polymers could be detected. This type of fractionation is not limited only to polymers that absorb UV radiation, however. With the recent commercial introduction of the mass detector marketed by Applied Chromatography Systems, the detection of any polymer separated via gradient elution can be easily achieved. The chromatographic parameters for the HPLC fractionation of polymers are provided in Table II.

The resolution of GPC, gradient LC and TLC methods of fractionating polymers are compared using the following equation

Table II: Chromatographic Parameters of HPLC Fractionations

Polymers	Solvents	Gradient	flow rate	UV Wavelength (nm)
poly(styrene)	$MeCl_2$:MeOH	55% $MeCl_2$ → 100%$MeCl_2$ in 27.5 minutes	1ml/min	254
poly(isoprene)	$MeCl_2$:MeOH	45% $MeCl_2$ → 100%$MeCl_2$ in 25 minutes	2ml/min	240
poly(α-methyl-styrene)	$MeCl_2$:MeOH	72.5% $MeCl_2$ → 100%$MeCl_2$ in 27.5 minutes	1ml/min	254

derived for calculating specific resolution (9):

$$Rsp = \frac{.576}{\sigma D_2} \qquad (1)$$

where σ is the standard deviation of the polymer standard peak and D_2 is the slope of the linear portion of the calibration curve. This equation has been used in GPC to compare the performance of GPC columns containing different types of packing materials (10). It should be noted that the specific resolutions used in this study (from equation 1) are independent of column length (9).

RESULTS AND DISCUSSION

A gradient LC separation of three poly(α-methylstyrene) standards is shown in Figure 1. A densitometric scan of five poly(styrene) standards separated by TLC is shown in Figure 2. Baseline separation is easily achieved for these and a variety of

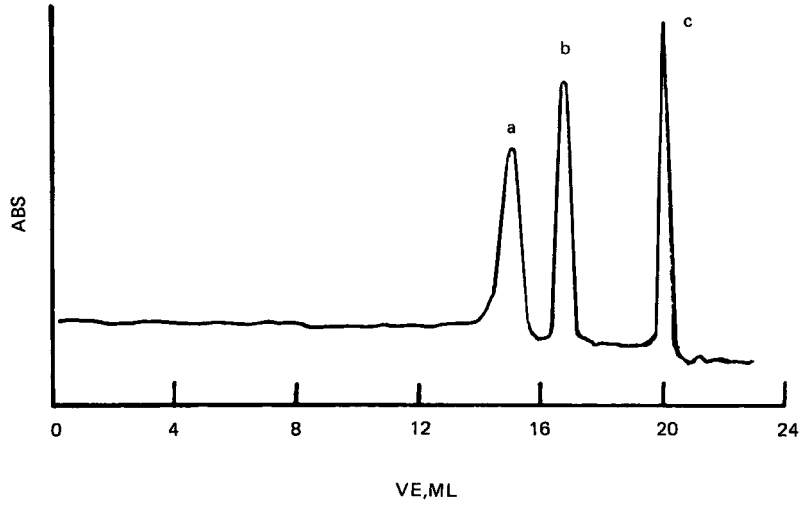

Figure 1: A gradient LC separation of three different molecular weight standards of poly(α-methylstyrene). The number average molecular weight of peak c = 760,000; b = 87,000 and a = 19,500.

other polymers (4). In all cases there is a linear relationship between the elution volume (Ve) in LC or R_f in TLC with the log of the molecular weight of the polymer (3-6). Only for very high molecular weight polymers does the relationship become nonlinear, as predicted by theory (4,6). As a result of the relationship between polymer retention and log molecular weight one can easily use either technique as a comparative method for the determination of molecular weights. One can also determine the distribution of molecular weight and different molecular weight averages using various techniques of peak slicing and integration, as will be

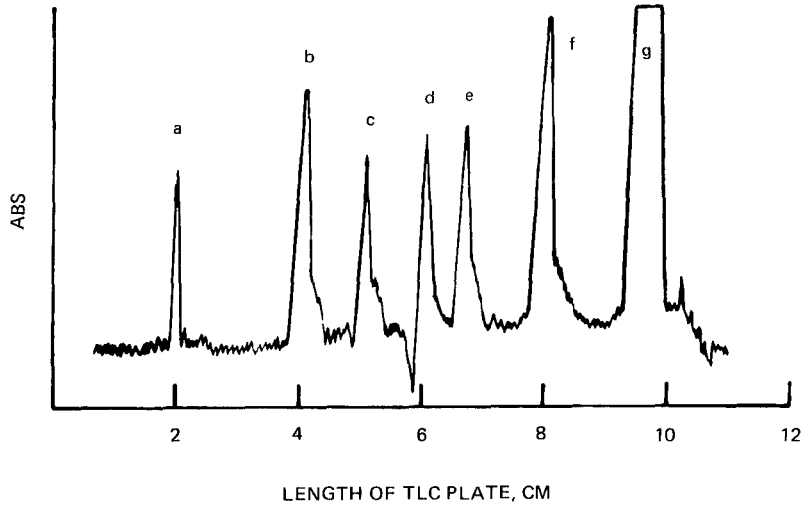

Figure 2: A densitometric scan (260 nm) of five styrene polymers separated by reversed phase TLC. The number average molecular weight of peak b = 10,000,000; c = 390,000; d = 100,000; e = 35,000 and f = 3,570. The "peak a" is the point on the TLC plate where the standards were spotted. The "peak g" is the discontinuity at the solvent front on the TLC plate. Impurities are frequently concentrated here.

shown in subsequent work. In addition one can determine the specific chromatographic resolution of each method by the same relationship originally derived for GPC (see Experimental Section). Consequently one can compare the resolving power of gradient LC, GPC and TLC techniques.

Specific resolutions (Rsp) of four different molecular weight poly(styrene) standards were determined for gradient LC and TLC

using equation 1 (see Experimental Section). These values are listed in Table III along with analogous values for eight different GPC packings. It is apparent that the resolution of both the gradient LC and TLC methods are better than that of GPC over the molecular weight range investigated. In fact the Rsp of the gradient LC technique increases to well over an order of magnitude of that for values obtained with comparable GPC techniques for high molecular weight polymers (Table III). The Rsp of TLC separations remains ~2 to 5 times that of GPC over the entire molecular weight range. It is also significant that only a single LC column or a single TLC plate was needed to achieve separation and generate data over the molecular weight range tested. Conversely, the Rsp values obtained for GPC required four separate columns (and experiments), each optimized for a specific molecular weight range (Table III).

Gradient LC produces characteristically high specific resolutions for other polymers such as poly(isoprene) and poly(α-methylstyrene) as well (Table IV). The relatively high Rsp for TLC also extends to a variety of different polymers (Table V). Indeed the TLC-Rsp for poly(ethylene glycol/oxide) approaches that of gradient LC.

The reason for the higher resolution of the gradient LC and TLC techniques is apparent upon examination of equation 1 (Experimental Section). The specific resolution is inversely proportional to the peak width (as expressed by the standard deviation, σ) and to the slope of the calibration curve (D_2). Consequently a smaller value of D_2 generally indicates greater selec-

Table III. COMPARISON OF THE SPECIFIC RESOLUTION OF GRADIENT LC, GPC, AND TLC METHODS OF POLY(STYRENE) FRACTIONATION

STATIONARY PHASE	TECHNIQUE	LINEAR MW FRACTIONATION RANGE	D_2	σ(ml)	MW	Rsp
Lichrospher 100[a]	GPC	$3 \times 10^3 - 5 \times 10^4$	1.28	0.229	5,000	1.95[a]
PSM-500[a]	GPC	$5 \times 10^3 - 4 \times 10^4$	2.17	0.147	5,000	1.75[a]
Whatman ODS-3(10μ)	HPLC	$10^3 - 10^7$	0.188	1.000	3,570	3.05
Whatman KC18F	TLC	$10^3 - 10^7$	7.509[b]	0.013[b]	3.570	5.94
Lichrospher 500[a]	GPC	$1.5 \times 10^4 - 1.5 \times 10^5$	1.32	0.275	51,000	1.60[a]
PSM-800[a]	GPC	$1.5 \times 10^4 - 1.5 \times 10^5$	2.25	0.118	51,000	2.15[a]
Whatman ODS-3(10μ)	HPLC	$10^3 - 10^7$	0.188	0.46	35,000	6.70
Whatman KC18F	TLC	$10^3 - 10^7$	7.509[b]	0.015[b]	35,000	5.14
Licrospher 1000[a]	GPC	$3 \times 10^4 - 2 \times 10^6$	1.98	0.142	97,000	2.05[a]
PSM-1500[a]	GPC	$4 \times 10^4 - 2 \times 10^6$	4.56	0.068	97,000	1.85[a]
Whatman ODS-3(10μ)	HPLC	$10^3 - 10^7$	0.188	0.31	100,000	9.95
Whatman KC18F	TLC	$10^3 - 10^7$	7.509[b]	0.018[b]	100,000	4.26
Licrospher 4000[a]	GPC	$10^5 \quad 7 \times 10^6$	3.84	0.144	390,000	1.05[a]
PSM-4000[a]	GPC	$7 \times 10^4 - 7 \times 10^5$	5.76	0.136	390,000	0.73[a]
Whatman ODS-3(10μ)	HPLC	$10^3 - 10^7$	0.414	0.19	390,000	7.32
Whatman KC18F	TLC	$10^3 - 10^7$	7.509[b]	0.019[b]	390,000	4.03

[a]GPC data taken from: J.J. Kirkland, J. Chromatogr., 125, 231 (1976).

[b]These numbers are unitless and cannot be directly compared to the analogous values obtained for HPLC and GPC. This is because the TLC parameters are normalized by dividing by the distance traveled by the solvent front (see Results and Discussion).

Table IV: Specific Resolution of Gradient LC Fractionations of Poly(isoprene) and Poly(α-methylstyrene).

Polymer	MW	σ (ml)	D_2	Rsp
poly(isoprene)	33300	1.20	0.0738	6.50
	113800	0.80	0.0738	9.76
	260000	0.70	0.0738	11.15
poly(α-methylstyrene)	19500	0.39	0.444	3.33
	87000	0.20	0.444	6.49
	76000	0.15	0.444	8.65

tivity. The standard peak deviation, σ, is thought to represent the efficiency of the technique. It is apparent from the values of D_2 and σ (Tables III, IV and V) that the greater resolution of the gradient LC method is largely the result of greater selectivity (smaller D_2) and not improvements in peak width (σ) which tend to be similar or somewhat greater for the low molecular weight polymers. Indeed it has been shown that band broadening for low molecular weight polymers is greater than for high molecular weight polymers in gradient "precipitation" LC (5). This is a result of two factors. First, mass transfer is significant for low MW polymers (which separate in the transition region) but is negligible for high MW polymers in this particular form of chromatography (5). Secondly, polymer standards are not monodisperse. The greater resolving power of gradient precipitation LC results in a greater bandwidth due to partial separation of the similar

Table V. Specific Resolution of TLC Fractionations of Poly(isoprene) and Poly(ethylene Glycol).

Polymer	MW	σ	D_2	Rsp
poly(isoprene)	1360	0.036	2.57	6.27
	11100	0.045	2.57	5.01
	33300	0.059	2.57	3.82
poly(ethylene glycol)	998	0.036	3.57	4.52
	4820	0.024	3.57	6.77
	11250	0.016	3.57	10.15
	73000	0.015	3.57	10.83

molecular weight polymers in a standard. This phenomenon has been demonstrated to be most pronounced for the lower molecular weight standards (5). This second factor is not as pronounced in GPC because of its lower resolving power. Although σ can be used as a measure of column efficiency, it must be understood that, technically, it only applies for a pure monodisperse polymer standard. The standard peak deviation, σ, has been utilized in GPC using polymer standards of narrow molecular weight distribution because the resolving power is such that there is generally little difference between these and an ideal monodisperse standard. One should note, however, that the use of polydisperse standards in a technique with higher fractionating power results in the seemingly ironic situation of having increased fractionation result in apparent decreased column efficiency (i.e., larger σ). In

reality, of course, this is an artifact of the standard used. This phenomena only affects the lower molecular weight standards for the gradient LC separations (Tables III and IV) (5). Consequently, the value of σ for the lowest molecular weight polymer is artificially high and the Rsp appears somewhat lower than it actually is.

One can compare the Rsp of the TLC technique directly with that of GPC and gradient LC; however, values of D_2 and σ are not directly comparable (Table III). This is because one plots R_f versus log molecular weight (in the TLC case) rather than retention volume. Consequently the quantity σ must be equal to the standard peak deviation divided by the distance the solvent traveled on the TLC plate.

In LC polymer separations the resolution is markedly affected by the type of gradient employed (Table VI). Although band broadening (σ) increases when a more gradual linear gradient is used, the selectivity (D_2) greatly increases. The net result is that Rsp increases with a more gradual gradient but analysis time also increases. One should also note that the linearity of log Mw vs. retention volume plots vary with the gradient used. More gradual gradients sometimes result in nonlinearity for the highest molecular weight polymers.

It is well known that conventional reversed phase LC can be used to separate styrene oligomers (9). Separation is achieved by a conventional partition-type mechanism and is superior to anything possible with GPC in this limited molecular weight range (9). This technique of nonaqueous gradient LC separates oligomers

Table VI: Effect of Different Mobile Phase Compositional Gradients on the Specific Resolution (Rsp) of Poly(styrene).

Gradient	3570			9000			35000			100,000			390,000		
	σ(ml)	D_2	Rsp	σ(ml)	D_2	Rsp	σ	D_2	Rsp	σ	D_2	Rsp	σ	D_2	Rsp
45 to 100% $MeCl_2$ in 55 minutes	1.58	0.090	4.00	1.13	0.090	5.65	0.55	0.090	11.65	0.75	0.090	8.55	0.21	0.144	19.04
45 to 100% $MeCl_2$ in 27.5 minutes	1.00	0.181	3.15	0.69	0.181	4.60	0.46	0.181	6.90	0.31	0.181	10.25	0.19	0.414	7.32
45 to 100% $MeCl_2$ in 18.3 minutes	0.67	0.338	2.55	0.40	0.338	4.25	0.19	0.338	8.95	0.18	0.338	9.45	0.14	0.833	5.10
45 to 100% $MeCl_2$ in 13.75 minutes	0.56	0.509	2.00	0.28	0.509	4.05	0.18	0.509	6.25	0.14	0.509	8.1	0.11	0.737	7.13

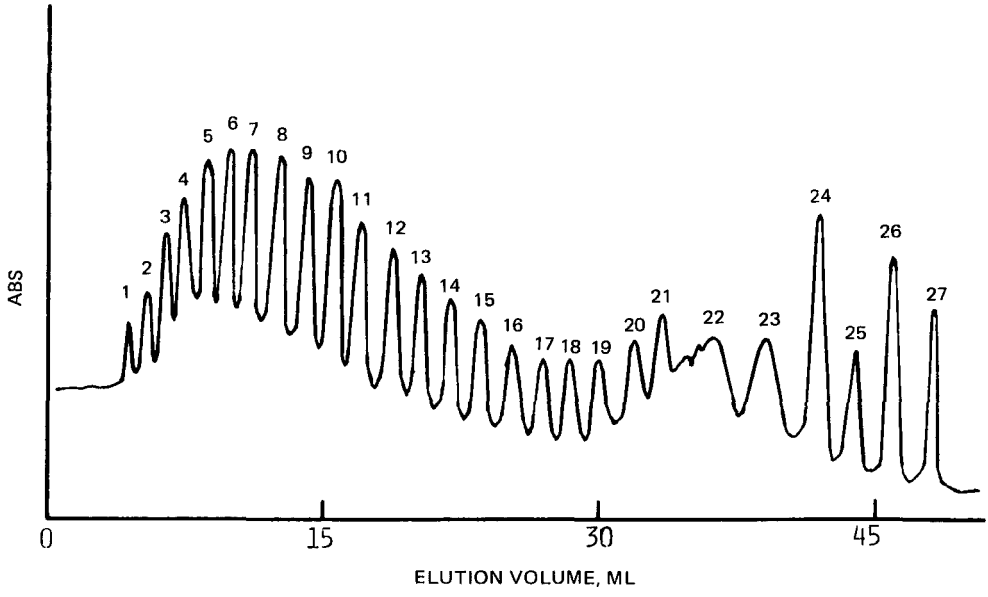

Figure 3: A combined phase (C_{18}) gradient LC separation of styrene oligomers and polymers. The mechanism of separation changes from traditional partitioning for the oligomers to a precipitation-dissolution process for the polymers. Peaks 1 through 20 are oligomers where the peak number is equivalent to the degree of polymerization (i.e., peak 2 = dimer, peak 8 = octamer, etc). Peaks 21 through 27 are polymer standards of low polydispersity (see Experimental Section). The number average molecular weight of peak 21 = 2,000; 22 = 3,570; 23 = 9,000; 24 = 17,500; 25 = 35,000; 26 = 100,000; 27 = 390,000.

in an identical manner and then continues to fractionate polymers with molecular weights well over a few thousand MW via a precipitation mechanism (3-5). Consequently, either gradient LC or TLC can be used to separate a variety of synthetic polymers over a wide range of molecular weights in a single efficient, high resolution run (Figure 3).

ACKNOWLEDGEMENT

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LITERATURE CITED

(1) Moore, J.C. J. Polymer Sci., A, 2, 835 (1964).

(2) Giddings, J.C. Anal. Chem., 39, 8, 1027 (1967).

(3) Armstrong, D.W. and Bui, K.H. Anal. Chem., 54, 4, 706 (1982).

(4) Armstrong, D.W., Bui, K.H.,and Boehm. R.E. J. Liq. Chrom., 6, 1, 1 (1983).

(5) Bui, K.H., Armstrong, D.W. and Boehm, R.E. J. Chromatogr. 1983, submitted.

(6) Boehm, R.E., Martire, D.C., Armstrong, D.W. and Bui, K.H. Macromolecules, 16, 466 (1983).

(7) Baker, C.A. and Williams, R.J.P., J. Chem. Soc. 2352 (1956).

(8) Cantow, M.J.R., Porter, R.S. and Johnson, J.F., J. Polymer Sci., 1, 187 (1963).

(9) Kirkland, J.J. J. Chromatogr., 125, 231 (1976).

(10) Yau, W.W., Kirkland, J.J. and Bly, D.D. "Modern Size Exclusion Liquid Chromatography" John Wiley & Sons, N.Y., 1979; pp. 97-122.

(11) Kato, Y., Kido, S., Watanbe, H., Yamamoto, M. and Hashimoto, T. J. Appl. Polymer Sci., 19, 629 (1975).

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DETERMINATION OF POLYMER MOLECULAR WEIGHT AND MOLECULAR WEIGHT DISTRIBUTION BY REVERSE PHASE THIN LAYER CHROMATOGRAPHY

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ABSTRACT

Reverse phase thin layer chromatography (RPTLC) and scanning densitometry was used to determine various molecular weight averages and the molecular weight distribution of broad molecular weight range samples of poly(styrene) and poly(methyl metharcylate). A basic program was developed which analyzes the analog signals from the scanner, calculates the desired parameters, prints the parameters, graphs the results and simultaneously displays the results on a CRT. The average molecular weight values obtained by this technique compare well to those obtained by other methods.

INTRODUCTION

The ability of RPTLC to efficiently fractionate a variety of synthetic polymers using a binary solvent mobile phase consisting of a thermodynamically "good" solvent and a thermodynamically "poor" solvent of the polymer was recently reported (1-3). The mechanism of fractionation was demonstrated to be a selective

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precipitation of the polymer resulting from the continuous change in mobile phase composition during development (1,2,4). In RPTLC the depletion of the less polar "good" solvent can occur naturally via selective absorption (during development) by the nonpolar stationary phase.

In addition to its high resolving power, RPTLC has several other advantages over conventional normal phase TLC methods for the separation of polymers. It is applicable to a greater variety of polymers as well as to a greater molecular weight range (2,5). In this work the applicability of RPTLC for the analysis of polymer molecular weights and polydispersity is evaluated.

MATERIALS

Whatman KC18F reversed phase TLC plates (5 x 20 cm and 20 x 20 cm) were used in all fractionations. HPLC grade methanol, methylene chloride, tetrahydrofuran (from Waters Associate), ethylene glycol (from Sigma Co.) and resublimed iodine (from Fisher Scientific Co.) were used as received. The polymer standards, their manufacturers and the various average molecular weight values supplied by the manufacturer are listed in Table I.

METHODS

All polymer standards were dissolved in methylene chloride (5 mg/ml) and 2 μℓ of the solution was deposited on the TLC plates via a Drummond 5 μℓ micropipette. All TLC fractionations were done in an 11 3/4 in. long, 4 in. wide and 10 3/4 in. high Chromaflex developing chamber. Both the narrow molecular weight range

TABLE I

Polymers Standards. The Average Molecular Weights are Certified by the Indicated Suppliers

Polymers	MW	Mw	Mn	Mw/Mn	Suppliers
Poly(styrene)	-	257800	-	2.1	NBS*
	-	37400	35800	1.04	NBS*
	900000	929000	850000	1.09	Poly Science
	233000	254000	217600	1.17	Poly Science
	100000	93050	926000	1.04	Poly Science
	100000	-	-	1.3	Poly Science
	3700000	-	-	1.2	Waters
	390000	-	-	1.04	Waters
	110000	-	-	1.1	Waters
	35000	-	-	1.04	Waters
	17500	-	-	1.04	Waters
Poly(methyl methacrylate)	-	81000	47000	1.7	Poly Science
	45000	-	-	1.09	Polymer Laboratories
	72000	-	-	1.08	Polymer Laboratories
	96000	-	-	1.10	Polymer Laboratories
	280000	-	-	1.15	Polymer Laboratories
	48000	-	-	1.16	Polymer Laboratories
	64000	-	-	1.16	Polymer Laboratories

*National Bureau of Standards

standards and the broad molecular weight range polymer (used as the unknown) were spotted on the same plate and the calibration curve was established using the elution data of the narrow molecular weight range standards. Typical calibration curves for poly(styrene) and poly(methyl methacrylate) are shown in Figure I.

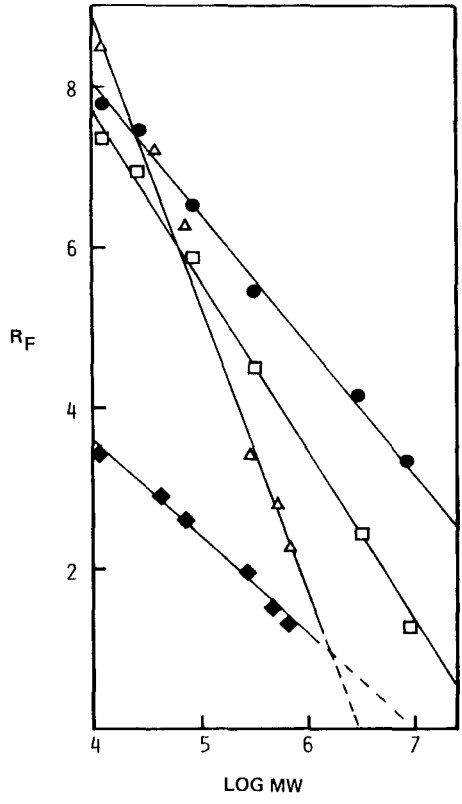

Figure 1. Typical TLC calibration curves for poly(styrene) developed with a 77.5:22.5 (v:v) $MeCl_2$:MeOH mobile phase (●) and with a 79:21 (v:v) $MeCl_2$:MeOH mobile phase (□). The poly(methyl methacrylate) calibration curves were obtained using a 76:24 (v:v) THF:ethylene glycol mobil phase (◆) and a 39:61 (v:v) $MeCl_2$:MeOH mobil phase (△).

It is apparent that for both polymer systems, there is a linear relationship between the R_f values (or elution distance) and the log of their molecular weight. Furthermore, this relationship holds for different mobile phase compositions and mobile phase systems. Consequently calibration can be done simply by linearly

correlating the molecular weight of the polymers to their elution values. Quantitative analysis of the polymer concentration along its elution path was performed via direct scanning densitometry using a Shimadzu model CS 910 dual wavelength TLC scanner. For polystyrene, maximum absorption occurs at wavelength of around 265 nm and almost no absorption occurs at 300 nm. Therefore, the sample wavelength was set at 265 nm and the reference wavelength was set at 300 nm. Detection was performed in the reflectance mode (Figure 3). Unusually high noise levels and baselines were observed (even under dual wavelength scan) when the KC 18 plates were scanned at 265 nm. This phenomenon is believed to be caused by the presence of the fluorescence indicator which has emission bands that extend into the UV. In the analysis of polydispersity of a polymer, this unusually high baseline is very deleterious since only the top portion of the peak (which protrudes above the base line) can be detected and the peak and hence the polydispersity of the polymer will appear to be artificially very narrow. Fortunately, the fluorescence indicator can be easily destroyed by spraying the plate with an 8% sulfuric acid/ethanol solution and heating the plate at 100°C for 10 minutes. Figure 2 shows that both the noise level and the baseline were considerably improved when the above treatment was performed on part of the plate. Consequently, the sensitivity of the detection is also enhanced with this treatment (Figure 3).

In order to visualize poly(methyl methacrylate), a 1% methanolic iodine solution was sprayed on the plate. After warming the plate at ∿50°C for a few minutes and letting the yellow background

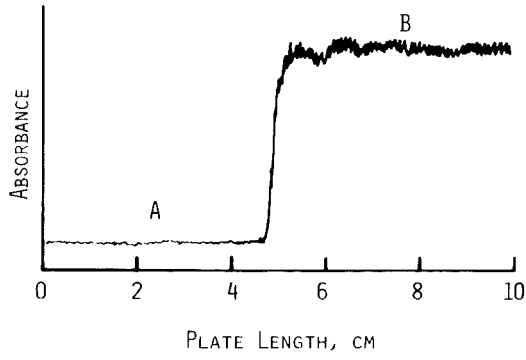

Figure 2: Scanning densitometric profile of a reversed phase TLC plate with fluorescent indicator. Part "A" of the plate was sprayed with H_2SO_4/EtOH solution and Part "B" was untreated. Note that the baseline is lower and more stable when the fluorescent indicator has been inactivated. The magnitude of this effect is dependent on the scanning wavelength used.

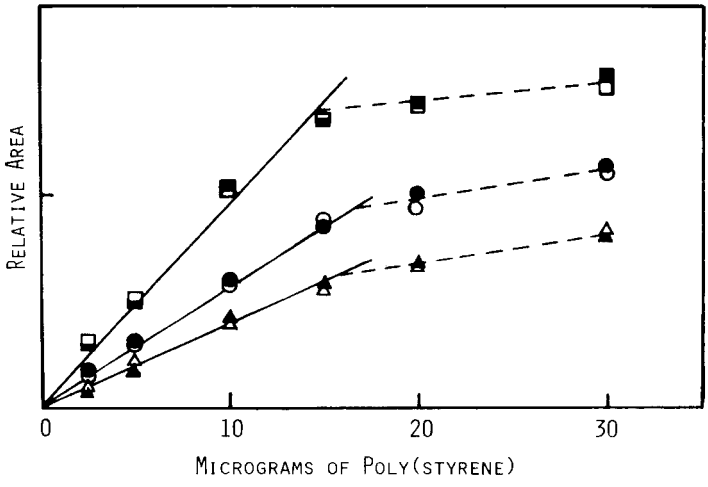

Figure 3: Plots of the weight of poly(styrene) spotted on a TLC plate versus densitometric peak area. The white symbols correspond to poly(styrene) of 35,000 Daltons and the dark symbols correspond to poly(styrene) of 10^6 Daltons. Three detection modes are shown:

□ , ■ = reflectance mode with H_2SO_4 treatment of plate

○ , ● = reflectance mode without treatment of plate

△ , ▲ = transmission mode without treatment of plate

of the plate fade, poly(methyl methacrylates) appeared as yellow-brown spots. The maximum absorption of these spots occured at 405 nm. Scanning densitometry of these spots, performed under single wavelength transmission mode provided the highest sensitivity (see Figure 4).

A Shimadzu Chromatopac model CR2A(X) data processor was used to analyze the analog data collected from the TLC scanner. The Chromatopac can function as a data processor as well as a per-

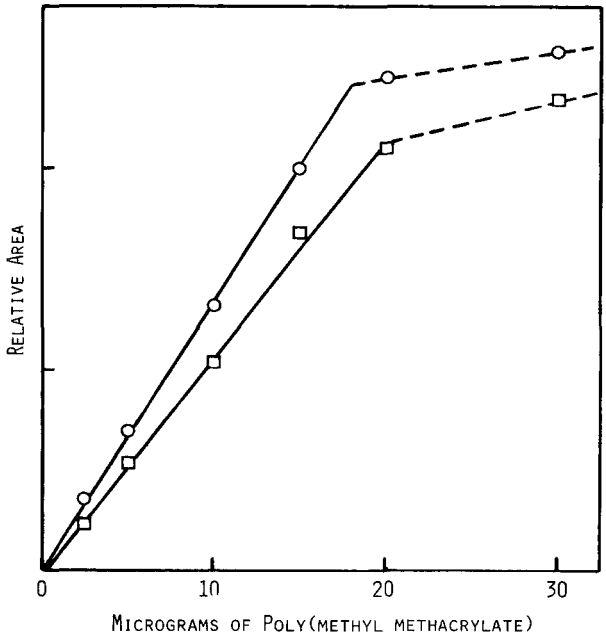

Figure 4: Plots of the weight of poly(methyl methacrylate) spotted on a TLC plate versus densitometric peak area. The circles (○) are for quantitation done in the transmission mode and the squares (□) are for quantitation of the same spots in the reflectance mode. The scanning wavelength was 405 nm (after spraying with iodine solution).

sonal computer. Data processed by the chromatopac such as peak retention time, peak area, area of a certain time band (slice area), retention time of the slice area, etc. can be manipulated freely as variables by a user defined Basic program. A Basic program was developed which permits calculations of the various average molecular weights and the polydispersity as well as the graphical display of the cumulative weight fraction molecular weight distribution and the weight fraction frequency distribution of the polymers. A listing of the program is provided in Appendix I.

RESULTS AND DISCUSSION

Figure 5 illustrates the scanning densitometric profile of a TLC chromatogram showing the fractionation of a mixture of 4 narrow disperse poly(styrene) standards and of a broad molecular weight range poly(styrene) standard (please note that these standards and standard mixtures were spotted on the same plate and developed under identical conditions). These scanning densitometric profiles are essentially a molecular weight size distribution of the polymer in weight concentration if and only if a detection method which is sensitive only to the weight concentration of the polymer and not the molecular weight of the polymer is used. Light absorption of polymers has been shown to be independent of the polymer molecular weight and to vary linearly with the concentration of the polymer in different solvent systems (when in sufficiently dilute solution) (6,7). Indeed Figure 3 shows that the UV absorption of poly(styrene) is independent of its molecular

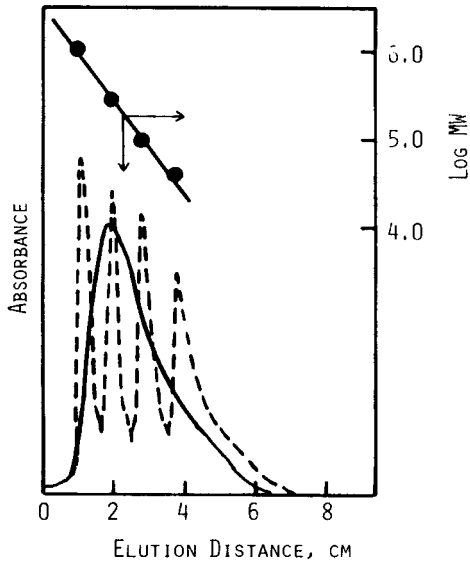

Figure 5: Two superimposed scanning densitometric profiles of a poly(styrene) unknown (i.e., the solid line, —) and poly(styrene) standards (i.e., the broken line, ---). Above the profiles is the calibration curve for the standards (i.e., log MW vs. elution distance).

weight and varies linearly with the concentration of the polymer (up to ∿15 μg). Using the scanning densitometric profile of the mixture of narrow disperse standards, a calibration curve can be readily established. From this calibration curve, the scanning densitometric profile of the broad molecular weight range standard is converted into a molecular weight distribution curve and the respective molecular weight averages can be calculated (i.e., including number average, weight average and z-average molecular weights. See Appendix I).

The various molecular weight averages of broad distribution poly(styrene) and poly(methyl methacrylate) standards determined

TABLE II.

A Comparison of Polymer Molecular Weight Averages Obtained by RPTLC and Other Traditional Methods

Polystyrene[1] (NBS)	Average Mw by RPTLC	Standard Deviation	Average MW given by Manufacturer
Mw	2.45×10^4	9000	2.58×10^4 (light scattering) 2.88×10^4 (sedimentation equilibrium)
Mn	1.20×10^4	9000	1.23 (fractionation)*
Mw/Mn	2.0	-	2.1
Polymethyl Methacrylate[2] (Poly Sciences)			
Mw	8.0×10^4	3000	8.1×10^4 (light scattering)
Mn	5.4×10^4	3000	4.7×10^4 (osmometry)
Mw/Mn	1.5	-	1.7

*Based on fractionation value of Mw/Mn multiplied by Mn value for light scattering

(1) Fractionated using 78:22 (v:v) $MeCl_2$/MeOH. Sample loading = 5 mg/ml. Analyzed in the reflectance mode at 265 nm after spraying with ethanolic sulfuric acid solution.

(2) Fractionated using 30:70 (v:v) ($MeCl_2$/MeOH). Sample loading = 5 mg/ml. Analyzed in the transmission mode at 405 nm after spraying with methanolic iodine solution.

TABLE III.

Polymers Which Have Been Fractionated by RPTLC

Polymers	Solvent Pairs	Reference
Poly(styrene)	$MeCl_2$/MeOH	1
Poly(α-styrene)	$MeCl_2$/MeOH	2
Poly(methyl methacrylate)	$MeCl_2$/MeOH*; THF:EG	2
Poly(ethylene glycol)	Dioxan/Ethylene glycol; MeOH/EG	2
Poly(ethylene oxide)	Dioxan/EG	2
Poly(vinyl chloride	THF:EG	8
Poly(vinyl acetate)	THF:EG	8
Poly(isoprene)	$MeCl_2$/MeOH	2
Poly(butadiene)	$MeCl_2$/MeOH	2
Poly(tetrahydrofuran)	THF:EG	2

*This work

by this technique are compared to those given by the manufacturers in Table II. It is apparent that the average molecular weight values determined by this technique compare well to those given by the manufacturers in spite of the fact that no correction for band broadening due to processes other than the fractionation process was made (in TLC fractionation of polymer, band broadening can be caused not only by the polydispersity of the polymer but also by other processes such as eddy diffusion and mass transfer).

Table III lists all the polymers which have been fractionated by RPTLC and the solvent pairs used to fractionate them. It is

apparent that except for those polymers which exist in the crystalline state (where elevated temperature is needed to break up the crystalline bond forces before dissolution of these polymers can occur) this technique is readily applicable to the analysis of a variety of macromolecules.

ACKNOWLEDGEMENT

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REFERENCES

1. Armstrong, D. W., Bui, K. H., Anal. Chem., 54, 706, (1982).

2. Armstrong, D. W., Bui, K. H., Boehm, R. E., J. Liq. Chrom., 6, 1, (1983).

3. Bui, K. H., Armstrong, D. W., submitted to J. Liq. Chromatogr.

4. Boehm, R. E., Martire, D. E., Armstrong, D. W., Bui, K. H., Macromolecules, 16, 466, (1983).

5. Octoka, E. P., Hellman, N. Y., Macromolecules, 3, 362, (1970).

6. Torkelson, J. M., Lipsky, S., Tirell, M., Tirrell, D., Macromolecules, 16, 326, (1983).

7. Lee, C. H., Waddell, W. H., Cassassa, E. F., Macromolecules, 14, 1021, (1981).

8. Author's unpublished results.

APPENDIX I

```
LIST
BASIC PROGRAM

5       PRINT "     *** MOLECULAR WEIGHT REPORT ***"
6       P.:P.
10      PRINT "CALIBRATION DATA"
20      PRINT "COEFFICIENT A";:INPUT A
```

```
30      PRINT "COEFFICIENT B";:INPUT B
40      PRINT "COEFFICIENT C";:INPUT C
50      PRINT "COEFFICIENT D";:INPUT D
60      P=MAXSL(1)
70      DIM AR(P),M(P),L(P),CU(P)
100     T=0:X=0:Y=0:ZA=0:ZB=0:ZC=0:AM=0
120     FOR J=1 TO P
130     T=T+SLAR(1,J)
140     NEXT J
145     PRINT
150     PRINT "Ret.     ";"Slice    ";"Mol.Wt    ";"Area ";"Cum"
155     PRINT "Time     ";" Area    ";" Slice    ";"  %   ";"AR."
156     PRINT "-----------------------------------"
157     PRINT
160     FOR I=1 TO P
170     M(I)=A+B*SLRT(1,I)+C*SLRT(1,I)^2+D*SLRT(1,I)^3
175     L(I)=10^M(I)
180     AR(I)=100*SLAR(1,I)/T
185     IF AM<AR(I) THEN AM=AR(I)
190     X=X+SLAR(1,I)
200     Y=Y+SLAR(1,I)/L(I)
210     ZA=ZA+SLAR(1,I)*L(I)
220     ZB=ZB+SLAR(1,I)*(L(I)^2)
230     ZC=ZC+SLAR(1,I)*L(I)^3
250     CU(I)=100*X/T
260     PRINT USING 261;SLRT(1,I),SLAR(1,I),L(I),AR(I),CU(I)
261     IMAGE;##.##   ####.# ######### ##.# ##.#
270     NEXT I
280     MW=ZA/X
290     MN=X/Y
300     MA=ZB/ZA
310     MB=ZC/ZB
315     MP=MW/MN
316     P.:P.:P.
317     PRINT "Distribution Averages:"
318     PRINT "Total Area =",T
320     PRINT "Mn =",MN
325     PRINT "Mw =",MW
330     PRINT "Mz =",MA
340     PRINT "MZ+1 =",MB
350     PRINT "MW/MN =",MP
355     P.:P.:P.
370     PRINT "Range of Log MW Plotted?"
380     PRINT "Minimum Log MW value";:INPUT MI
390     PRINT "Maximum Log MW value";:INPUT MX
392     P.:P.:P.
393     PRINT "4"
395     PRINT "                                        AREA %"
396     PRINT
400     Q=MX-MI
410     PVL=0
420     MOVE PRINTER,0,0
440     MOVE CRT 400,10
445     MOVE PRINTER,900,0
446     PRINT USING 447;AM
447     IMAGE;#.##
448     MOVE CRT 70,20
449     DRAW CRT 450,20
450     MOVE PRINTER,120,100
455     DRAW PRINTER,920,100
456     FOR I=10 TO 0 STEP -1
457     MOVE PRINTER,80*I+120,100
458     DRAW PRINTER,80*I+120,140
459     MOVE CRT 38*I+70,24
460     DRAW CRT 38*I+70,20
461     NEXT I
```

```
462     DRAW CRT 70,180
463     DRAW CRT 450,180
469     DRAW PRINTER 120,2300
470     DRAW PRINTER,920,2300
472     FOR I=10 TO 0 STEP -2
474     MOVE CRT 38*I+70,180
475     DRAW CRT 38*I+70,176
476     MOVE PRINTER,80*I+120,2300
477     DRAW PRINTER,80*I+120,2260
478     NEXT I
480     MOVE PRINTER,920,2300
481     DRAW PRINTER,920,100
482     MOVE CRT 450,180
483     DRAW CRT 450,20
500     FOR I=0 TO Q
510     MOVE PRINTER,50,(2200*I/Q)+100
512     MOVE CRT 30,(160*I/Q)+20
520     PRINT USING 530;MX-I
530     IMAGE;#
540     MOVE PRINTER,120,(2200*I/Q)+100
550     DRAW PRINTER,130,(2200*I/Q)+100
551     MOVE CRT 70,(160*I/Q)+20
552     DRAW CRT 75,(160*I/Q)+20
554     MOVE PRINTER,910,(2200*I/Q)+100
555     DRAW PRINTER,920,(2200*I/Q)+100
556     MOVE CRT 445,(160*I/Q)+20
557     DRAW CRT 450,(160*I/Q)+20
560     NEXT I
650     MOVE PRINTER,120,(2200*(MX-M(1))/Q)+100
651     MOVE CRT 70,160*(MX-M(1))/Q+20
660     FOR I=1 TO P
670     DRAW PRINTER,8*CU(I)+120,2200*(MX-M(I))/Q+100
671     DRAW CRT 3.8*CU(I)+70,160*(MX-M(I))/Q+20
690     NEXT I
695     MOVE PRINTER,120,2200*(MX-M(1))/Q+100
696     MOVE CRT 70,160*(MX-M(1))/Q+20
700     FOR I=1 TO P
710     DRAW PRINTER,800*AR(I)/AM+120,2200*(MX-M(I))/Q+100
711     DRAW CRT 380*AR(I)/AM+70,160*(MX-M(I))/Q+20
720     NEXT I
730     MOVE PRINTER,0,2400
731     MOVE CRT 0,190
740     P. USING 750;"LOG MW","0","20","40","60"
750     IMAGE;######  #         ##         ##         ##
760     MOVE PRINTER,720,2400
765     MOVE CRT 350,190
770     PRINT "  80         100"
775     PRINT
780     PRINT "                              CUM. AREA %"
900     END
```

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RELATIVE STRENGTH OF STRONGER SOLVENTS FOR SILICA, AMINO- AND CYANO-ALKYL BONDED SILICA COLUMNS IN NORMAL-PHASE LIQUID CHROMATOGRAPHY

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ABSTRACT

The strength and selectivity of solvents such as ethyl acetate, dioxan and ethyl alcohol in n-hexane binaries were determined using steroids as solutes in normal-phase liquid chromatography of silica gel, amino- and cyano-propyl silica columns. Based on the linear relationship between the logarithm of the capacity ratio and logarithm of solvent composition, the relative strength of solvents was determined from the experimental retention data described in our earlier articles. A micro-computer data base was compiled for filing the retention behavior of the steroids. Using this computer data base, the optimization process of binary solvents for a given sample was improved. An example of the phase system design is described.

INTRODUCTION

Optimization of a phase system has been usually carried out by a trial and error manner in liquid chromatography. This often involves tedious experimental procedures in designing a suitable system for a given sample mixture. A careful consideration of the retentivity and selectivity of packings and carrier solvents is recommended as a means for avoiding this problem. We examined the relative retentivity and

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TABLE I. Constant Ratios of the Linear Relationship between the Logarithms of Retention Index and Concentration of the Stronger Component in n-Hexane Binaries for a Pair of Solvents in Silica Gel and Chemically Bonded Silica Column Systems

	solvent	ethyl acetate/dioxan					
	column	silica		amino		cyano	
No	constant ratio / steroid	r_c	r_n	r_c	r_n	r_c	r_n
1	Estrone	1.25	1.28	1.69	1.75	1.03	1.16
2	Estradiol	1.06	1.04	1.22	1.17	1.00	1.05
3	Ethynylestradiol	1.05	1.11	1.27	1.24	0.94	1.02
4	Estriol	1.06	0.95	1.23	1.10	1.01	0.98
5	Testosterone	1.28	1.14	1.29	1.15	1.02	1.07
6	Methyltestosterone	1.26	1.12	1.34	1.18	1.06	1.14
7	Ethynyltestosterone	1.19	1.13	0.84	0.78	0.93	1.02
8	Testosterone propionate	1.60	1.45	1.62	1.46	0.96	1.13
9	Progesterone	1.36	1.21	1.23	1.12	1.07	1.23
10	Deoxycorticosterone acetate	1.41	1.28	1.57	1.45	0.93	1.01
11	Corticosterone	1.24	1.12	1.76	1.60	0.88	0.92
12	Cortisone	1.23	1.14	1.49	1.40	0.82	0.85
13	Cortisone acetate	1.20	1.12	1.12	1.04	0.84	0.88
14	Prednisone	1.26	1.15	1.18	1.10	0.87	0.90
15	Prednisolone	1.16	1.07	1.44	1.32	0.85	0.87
	mean value	1.24	1.15	1.35	1.26	0.95	1.02
	standard deviation	0.14	0.12	0.24	0.24	0.08	0.12

r_c and r_n : ratios of the intercepts and slopes in equation (1) for a pair of stronger solvents in the text.

(continued)

selectivity of various packing columns such as silica gel and chemically bonded silica columns [1-3]. In designing a phase system, characterization of the solvent role for a given column is also necessary and consequently solvent strength and selectivity have been determined. A comparative study of the strength of stronger solvents on various column packings in normal-phase operation was made in this paper.

Two proton acceptor solvents such as ethyl acetate and dioxan, and a proton donor-acceptor solvent, ethanol, were selected as typical stronger components in a binary system containing n-hexane as the diluent. The relative ratios of the solvent strength of ethyl acetate and ethyl alcohol to dioxan as the standard solvent on silica gel, amino-

TABLE I (continued)

solvent	ethyl alcohol/dioxan					
column	silica		amino		cyano	
constant ratio No steroid	r_c	r_n	r_c	r_n	r_c	r_n
1	0.58	0.69	0.58	0.69	1.04	1.12
2	0.58	0.67	0.65	0.72	0.84	0.87
3	0.56	0.64	0.63	0.67	0.90	0.91
4	0.49	0.54	0.49	0.54	0.57	0.60
5	0.55	0.62	0.45	0.55	0.69	0.75
6	0.55	0.61	0.48	0.60	0.73	0.80
7	0.50	0.58	0.52	0.59	0.77	0.84
8	0.45	0.64	0.28	0.53	0.64	0.85
9	0.48	0.55	0.42	0.64	0.55	0.57
10	0.47	0.52	0.35	0.47	0.53	0.53
11	0.52	0.56	0.48	0.52	0.54	0.56
12	0.49	0.54	0.40	0.45	0.48	0.48
13	0.46	0.51	0.38	0.44	0.50	0.50
14	0.47	0.52	0.39	0.43	0.48	0.50
15	0.44	0.48	0.47	0.53	0.50	0.54
mean value	0.51	0.58	0.46	0.56	0.65	0.69
standard deviation	0.05	0.06	0.10	0.09	0.17	0.20

and cyano-propylsilylated silica columns were determined using the systematic retention data of fifteen steroid hormones given in our earlier papers [1-3].

EXPERIMENTAL

The retention data from our earlier reports [1-3] have been used in this paper. A serial chromatographic study was carried out under strictly controlled conditions at constant temperature. The solutes are the fifteen steroids shown in Table I.

The experimental conditions for the capsaicin analogues are shown in the legend of Figure 4.

RESULTS AND DISCUSSION

The linear correlation between capacity ratio and molar concentration logarithms of the stronger solvent was confirmed in the normal-phase HPLC of binary solvents and chemically bonded silica such as amino- or cyano-propylsilylated silica and bare silica gel columns as follows: $\log k' = c - n \log Xs$ (1), where k' is the capacity ratio, Xs, the molar fraction of the stronger solvent and c and n are constants [1-3]. On the basis of this equation and experimental retention data obtained using various steroid hormones as solutes, the solvent strengths of ethyl acetate, dioxan as typical proton acceptor solvents and ethanol as a proton donor-acceptor solvent in n-hexane-binaries were compared.

To determine relative solvent strength, the mean values of the two constants in equation (1) for the fifteen steroids were initially calculated. Correlation between retentivity and solvent composition is illustrated in Figures 1 - 3 for silica, amino- and cyano-type columns, as calculated by the mean values of slopes and intercepts in equation (1). In these figures, the average retention of the steroids is presented by the ordinate. Thus, if the solvent composition is given by the abscissa, the relative retentivity of a particular stronger component in an n-hexane binary is suggested directly by the ordinate of the figure. This is illustrated by the arrow (a) in Figure 1.

The correlation lines were distributed in a fan shape and the increment in the relative strength of a pair of stronger solvents increased as the amount of solvent decreased. The relative strength of a solvent in the binary system varied with composition. For example, when the composition of the stronger solvent was ten percent, the relative solvent strengths of ethyl acetate and ethanol to dioxan as the standard were determined as follows: 0.7, 2.4 in silica (Figure 1), 0.7, 2.8 in the amino-type column (Figure 2) and 1.2, 1.8 in cyano-type column (Figure 3), respectively.

The relative strength of these three solvents varied markedly in the silica and amino columns, but hardly at all in the cyano column. The relative strength of two proton-acceptor solvents decreased in the cyano-column, although that of the proton acceptor-donor solvent remained at the same level for the three columns.

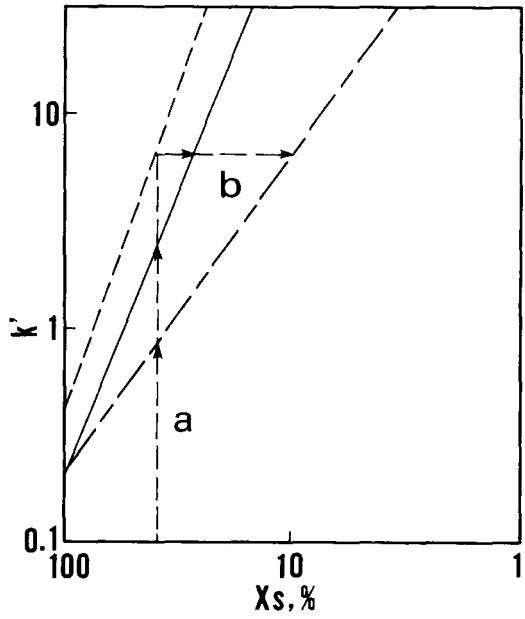

Figure 1 - 3. Logarithm of the Mean Values of the Capacity Ratios for Fifteen Steroids on Silica Gel and Chemically Bonded Silica Columns as a Function of the Logarithm of the Stronger Solvent-Concentration in n-Hexane-Binaries

Packing: silica gel (Figure 1); amino bonded silica (Figure 2); cyano bonded silica (Figure 3).

In Figures 1-3, the equi-eluotropic solvent composition is represented by the abscissa; for example, intercepts on horizontal dotted lines (b) in Figure 1 show the calculated values for the composition of three solvents which should have about the same retentivity.

These facts suggest that the above mathematical relationship between the average retention index and the solvent composition would greatly facilitate the systematic optimization of a mobile phase for a given solute mixture and column.

An attempt was made to evaluate the solvent selectivity in three columns using the retention indices of various steroids. To compare the characteristics of ethyl acetate and ethyl alcohol with dioxan as the standard solvent, the quotients of the two constants c and n in equation

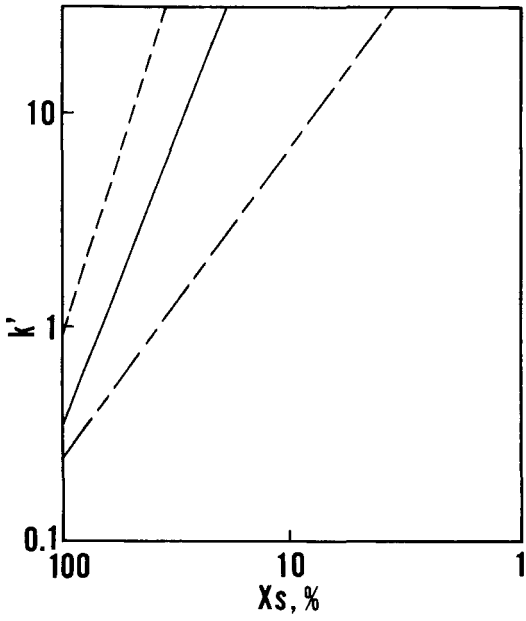

Figure 2

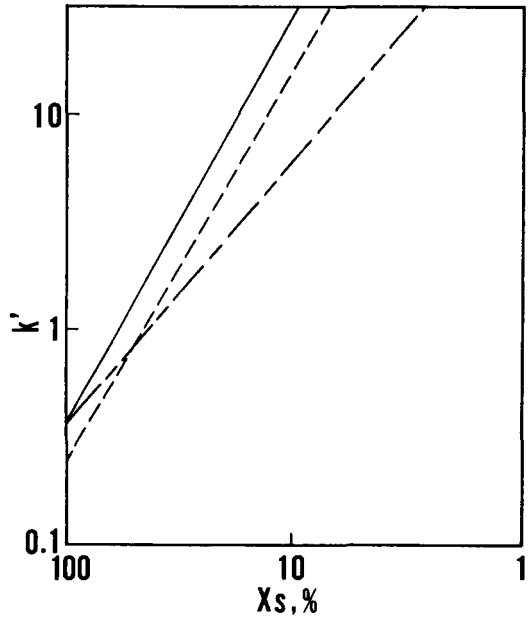

Figure 3

(1) for particular solutes were calculated. Table I shows the quotients of the constants (r_c and r_n) for silica and the two chemically bonded columns along with the mean values and the standard deviation.

In the case of the ratio for ethyl acetate-dioxan, the selectivity of solvents for steroids is suggested as follows: the strength of ethyl acetate decreases for acyl derivatives (8 - 10) as evident from larger constant values for c and n in the silica gel column and increases for corticosteroids (11 - 15) as indicated by smaller constants c and n in cyano column. However, in general, standard deviations of constant ratios in silica and cyano columns are smaller than that in the amino column and thus it is clear that selectivity resulting from changing the solvent from dioxan to ethyl acetate would be more effectively enhanced in the amino column than in the other two columns.

In the case of the ratios for ethyl alcohol-dioxan, solvent selectivity for steroids is suggested as follows: the strength of ethyl alcohol decreases for phenolic estrogens (1 - 3) by giving larger constant values of c and n in the amino and cyano bonded columns. The standard deviation of the constant ratios was least in silica gel column indicating that solvent change from dioxan to ethyl alcohol would possibly result in better selectivity of steroid retention in the other two chemically bonded columns.

From the results described above, selectivity through change can be expected to have the following order: amino $>$ cyano $\geqq$ non-bonded silica.

At our laboratory, the filing of retention data has been extensively examined by a micro-computer. The results obtained in this article as well as our earlier experimental data were compiled by a micro-computer data base. Using this data base, selection of a phase system for given samples can be carried out systematically. An example of micro-computer assisted optimization is shown below.

In order to identify the constituents of capsicum anuum tincture and related additives in cosmetic prepartions, the resolution of capsaicin analogues was examined [4]. At first, we obtained the two retention data using a phase system consisting of a silica gel column and n-hexane-dioxan binaries. According to equation (1), the solvent composition was calculated for a given capacity ratio. Using the micro-computer data base described above, the equi-eluotropic composition of a stronger solvent in n-hexane for silica, amino and cyano columns was ob-

tainable straightforward. For example, the calculated values of an equi-eluotropic solvent composition, $Xs_{(calc)}$ were obtained by incorporating the ratios of the two constants (mean values) in equation (1) for a pair of columns as well as the quotients of the constants for a pair of stronger solvents (mean values) as follows:

$$\log Xs_{(calc)} = {}^{1}/_{n(calc)} \times (c_{(calc)} - \log k') \qquad (2)$$

$$n \text{ or } c_{(calc\text{-}silica\text{-}ethyl\ alcohol)} = n \text{ or } c_{(exp\text{-}silica\text{-}dioxan)} \times r_n \text{ or } r_c(silica\text{-}ethyl\ alcohol/dioxan)$$

$$n \text{ or } c_{(calc\text{-}cyano\text{-}ethyl\ alcohol)} = n \text{ or } c_{(calc\text{-}silica\text{-}ethyl\ alcohol)} \times n\text{- or c-ratio}_{(cyano/silica\text{-}ethyl\ alcohol)} \qquad (3)$$

where calc and exp are the calculated and experimental values, respectively and the subscripts in parenthesis are phase systems. The constant ratios of r_n and r_c are from Table I and the n- or c-ratios for a pair of two columns are from our earlier paper [3]. The predicted values were found to be closely related to the experimental values and were thus helpful in quickly designing an optimum phase system. By both experiment and the use of the computer control of solvent composition and the design of a satisfactory phase system for the resolution of capsaicin homologues were possible. The calculated values and experimental results are shown in Table II. A mixture of solutes having closely re-

TABLE II. An Example of Micro-Computer Assisted-Phase System Optimixation for Capsaicin

		predicted value*			experimental value		
column	stronger component	c	n	Xs (k'=8)	c	n	Xs (k'=8)
silica	dioxan				4.51	2.54	26.3 %
cyano	dioxan	3.20	1.78	19.5 %	3.68	2.05	22.6 %
cyano	ethyl alcohol	2.08	1.23	9.1 %	2.03	1.24	8.1 %

* calculated from the experimental data obtained with the silica n-hexane-dioxan phase system and equations (2) and (3) in the text.

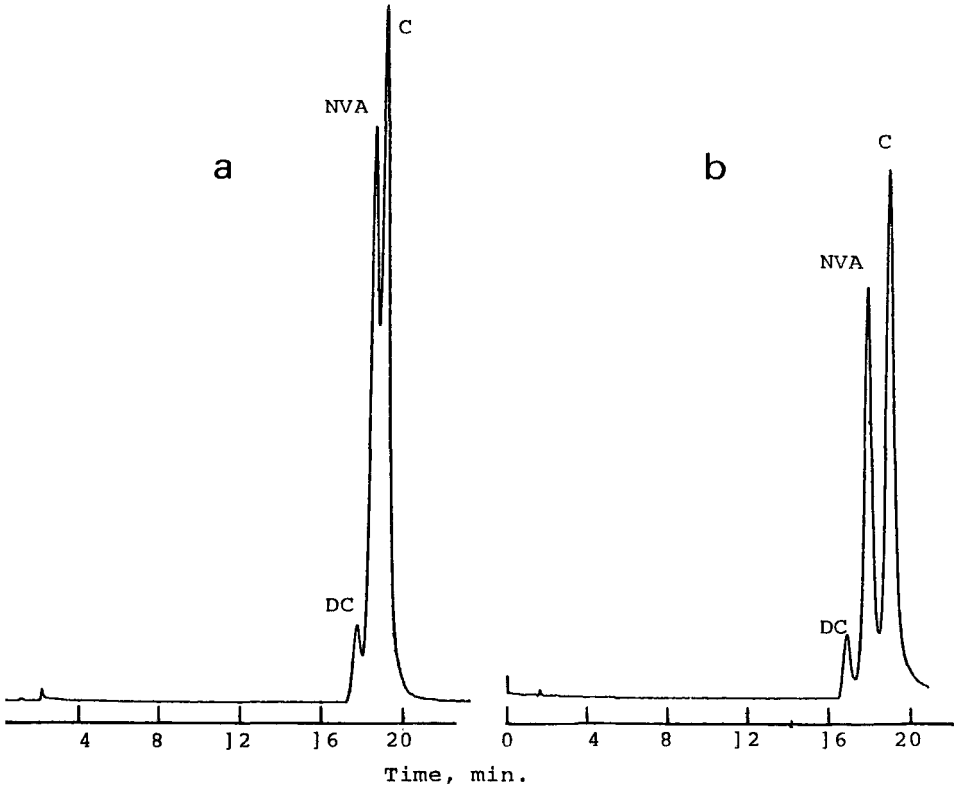

Figure 4. Chromatograms of Capsaicin Analogues
Samples: C, capsaicin; DC, dihydrocapsaicin; NVA, nonanoic acid vanillylamide. a: Column, Nucleosil 100-5; eluent, n-hexane-ethyl alcohol (23 : 2); flow rate, 1.0 ml/min; temp., 0°C. b: Column, Nucleosil 5CN (cyano-type); eluent, n-hexane-dioxan (4 : 1); flow rate, 1.2 ml/min; temp., 0°C.

lated structures was finally resolved by maintaining the capacity ratio at approximately eight and choosing a phase system with an n-hexane-dioxan binary solvent on a silica or cyano column. The chromatograms in Figure 4 show examples of incomplete (a) and an optimized (b) separations of analogous compounds.

Characterization of the solvent described in this text should be useful for finding an optimum solvent system and a suitable column in normal-phase liquid chromatography separation of given solute mixtures.

REFERENCES

1) S. Hara, S. Ohnishi, Yakugaku Zasshi, 103 (1983) 750.
2) S. Hara, S. Ohnishi, Yakugaku Zasshi, 103 (1983) 756.
3) S. Hara, S. Ohnishi, J. Liq. Chromatogr., 7 (1984) 69.
4) S. Ohnishi, T. Yoneya, S. Hara, Yakugaku Zasshi, 103 (1983) 236.

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CHARACTERIZATION OF AMINO-, CYANO-ALKYL BONDED SILICA COLUMNS IN NORMAL-PHASE LIQUID CHROMATOGRAPHY BY USING STEROIDS

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ABSTRACT

In order to characterize the chemically bonded phases in HPLC analysis, the retention behavior of fifteen steroids including estrogen, androgen, progestogen and corticoid were systematically examined using dioxan as the stronger component in an n-hexane-binary system. A linear relationship between the logarithm of the capacity ratio and logarithm of the molar concentration of the binary solvent was confirmed for amino- and cyano-type bonded as well as non-bonded silica gel columns. Based on the retention indices of these phases, the retentivity of the packing materials was determined as follows: the amino-type is similar to and the cyano-type is weaker (0.7 times) than bare silica gel when using dioxan as the stronger component. The specific retentivity of an amino column for polar steroids containing phenolic and alcoholic hydroxyl groups suggests a molecular interaction associated with hydrogen bonding between the polar packing surface and solute compounds. The selectivity of amino packing was found to be larger than cyano packing whose retention selectivity is similar to a bare silica gel.

INTRODUCTION

Applications of various chemically bonded silica gel columns have been developed recently and widely accepted by analytical laboratories probably because their favorable physical properties such as rigidity

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under high pressure, high speed performance and high efficiency. In various bonded phases, the octadecylsilylated silica gel has been used the most in recent HPLC analysis and involves a reversed-phase mode. However, aminopropyl- and cyanopropyl-silylated silica columns have also become important because of their wide range of selectivity in normal- and reversed-phase systems.

To clarify the retention characteristics of amino- and cyano-alkyl bonded phases, systematic studies using various solutes and binary solvents in the normal-phase mode have been carried out[1-5]. We examined the retention behavior of fifteen steroid hormones using binary solvents in liquid-solid chromatography. Medium polar compounds having hydroxyl, carbonyl and acyloxyl groups were selected as the solutes.

Dioxan was chosen in this paper as the stronger component in order to increase the polarity of the eluents to compete with active functional groups of sample molecules in the adsorption-desorption equilibrium process on the surface of the packing materials. Dioxan is an aprotic B-type solvent[6] and has no ultra-violet absorption in the detection region. It provides fairly large solvent strength for medium polar compounds[6,7] and thus has wide applicability as the stronger component in binary solvent systems.

Based on the linear relationship between the logarithm of the capacity ratio and the logarithm of solvent composition, as recently established by our cumulated experimental data[1-10], the retentivity of steroids on amino and cyano columns was determined and compared with the data obtained using a non-bonded silica gel column as the standard. The selectivity of three columns was evaluated on the basis of the systematic retention data for the steroid solutes.

EXPERIMENTAL

Apparatus The Liquid Chromatograph used was the 635 Hitachi model, Tokyo, equipped with a UV-detector, Uvilog 5 III, Oyo-Bunko, Tokyo.

Columns To examine the selectivity of the columns, we selected chemically bonded packing materials prepared from the same silica gel support. We used (A) silica gel, Nucleosil 100-5, (B) aminopropyl silica, Nucleosil 5NH2, (C) cyanopropyl silica, Nucleosil 5CN. The

particle size of these gels made by Macherey-Nagel was 5 μm. The columns were prepared using stainless steel tubes, 250 mm x 4 mm i.d. and a slurry of the packing materials in dioxan, methanol and carbon tetrachloride. The slurry packed columns were finally purged by chloroform.

Reagents Fifteen steroid hormones were used as the samples. The molecular structures are presented in Table I. The steroids were obtained as follows: estradiol, ethynylestradiol (Uclaf); estriol, corticosterone, cortisone and prednisolone (Sigma); others (Tokyo Kasei). Sample purity was checked by thin-layer chromatography which afforded a single spot on the silica gel plate.

Chromatography run Samples of 1 - 5 ng were dissolved in 10 μl of carrier solvent. Sample solutions of 0.2 - 2.0 μl were injected into the column. Flow rate was 1.2 ml/min. Column temperature was maintained at 40 °C. The capacity ratio, k' was calculated by the formula: $k' = (t_R - t_0)/t_0$ where t_R is retention time, t_0, the hold up time measured by injecting n-nonane as the non-retained sample.

RESULTS AND DISCUSSION

1. Correlation between the Capacity Ratio and Solvent Composition

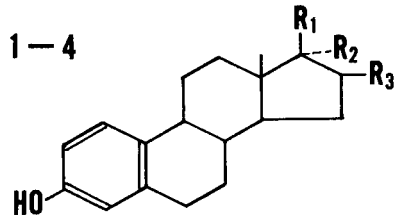
According to the mechanism involved in adsorption-desorption of the solute molecules and the silanol group as an active site on the silica gel surface, the mathematical relation between the capacity ratio logarithm for the silica gel column and molar ratio logarithm of the stronger solvent is proposed as follows:

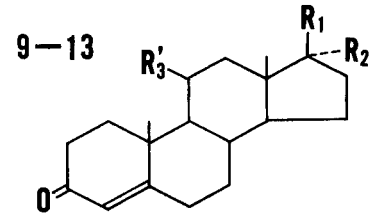
$$\log k' = c - n \log Xs \qquad (1)$$

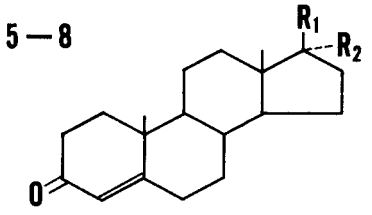
where k' is capacity ratio, Xs, the molar fraction of the stronger component in the binary solvent and c and n are constants. This relation was confirmed by the experimental results obtained from various solutes and solvent systems for silica gel columns[6-10]. Equation (1) was also established for amino- and cyano-type columns in normal-phase operation using n-hexane binaries containing ethyl acetate, ethanol as the stronger components [1-5].

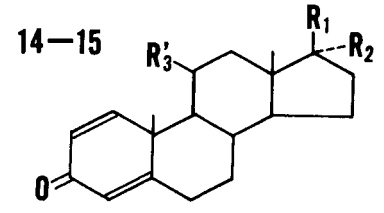
We examined systematically the retentivity of steroid samples using the dioxan-n-hexane binary solvent in this article. We classified the solutes into two groups, (a) four estrogens (1-4) and four androgens

TABLE I. Structural Formulas of the Steroid Hormones









Sample No.	R_1	R_2	R_3	Name
1	=O		H	Estrone
2	OH	H	H	Estradiol
3	OH	C≡CH	H	Ethynylestradiol
4	OH	H	OH	Estriol
5	OH	H		Testosterone
6	OH	CH3		Methyltestosterone
7	OH	C≡CH		Ethynyltestosterone
8	OCOC2H5	H		Testosterone propionate
9	COCH3	H	H	Progesterone
10	COCH2OCOCH3	H	H	Deoxycorticosterone acetate
11	COCH2OH	H	OH	Corticosterone
12	COCH2OH	OH	=O	Cortisone
13	COCH2OCOCH3	OH	=O	Cortisone acetate
14	COCH2OH	OH	=O	Prednisone
15	COCH2OH	OH	OH	Prednisolone

(5-8) and (b) seven progestogens and corticosteroids (9-15). The retention indices were measured at various solvent compositions. The results obtained using silica, amino and cyano columns are presented in Figure 1 - 3, respectively. The linear relation between the logarithms of capacity ratio and molar fraction of dioxan was determined for three phase systems and a correlation coefficient value of 0.98 - 1.00 was obtained. The mathematical relation confirmed here suggests that the retention mechanism of the chemically bonded packings is similar to that of silica gel in a normal-phase operation. The adsorption-desorption process may occur by a hydrogen bonding interaction between the active functional groups and solute molecules on the surface of the adsorbents.

Equation (1) should be quite useful for optimizing the phase system and thus the micro-computer program in equation (1) was compiled at our laboratory for data filing and a systematic approach to the design of an optimum phase system. The computer-assisted solvent selection is such that solvent composition output can be made by using at least two retention data values for input. The two constants of equation (1) and the correlation coefficient of the experimental data can also be calculated by computer. An example of filing data for estriol is shown in Figure 4. On the basis of six experimental data, solvent compositions corresponding to given capacity ratio values were determined.

2. Molecular Structures and the Retentivity

In Figures 1-3, the correlation lines representing the logarithm of capacity ratios for steroids versus the logarithm of solvent composition are distributed in a fan-shaped manner except for a few lines which cross over each other. The retention sequence of the steroids did not change with respect to solvent composition. This means that not only k', but also the increment of logarithm k' (logarithm of α, the separation factor) increases with concentration of the stronger component which decreased for nearly all solvent pairs. The two constants of equation (1) as the retention parameters for a class of compounds such as estrogens, androgens, progestogens and corticosteroids, vary in a similar manner for the three columns.

Retention sequence of the solutes in the silica gel column used as the standard is as follows: $1 < 3 < 2 \ll 4$ for estrogens, $8 \ll 6 < 7 < 5$ for androgens as shown in Figure 1a and $9 \ll 10 \ll 13 < 11 < 12 < 14 < 15$ for

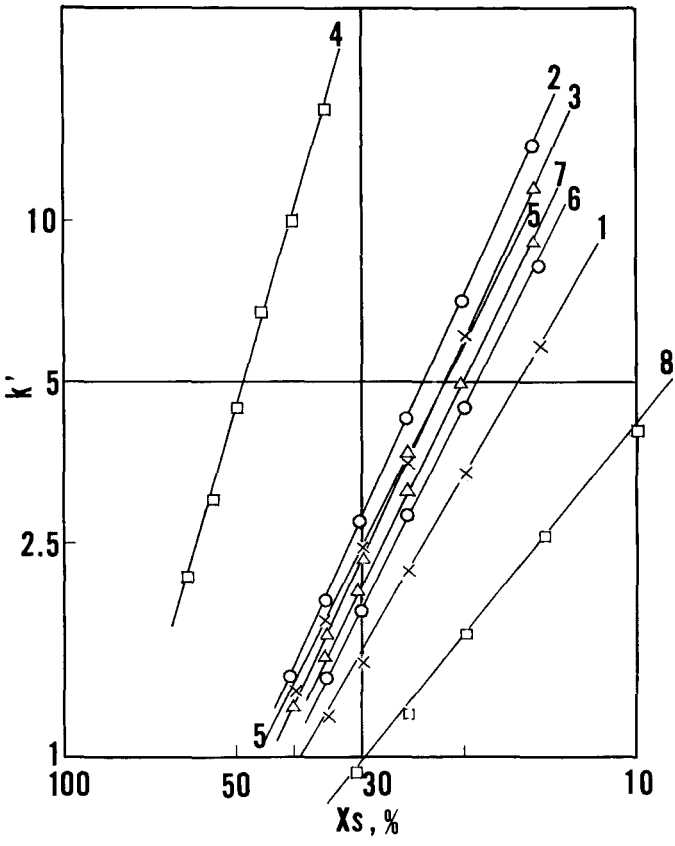


Fig. 1a

Fig. 1 - 3. Logarithm of Capacity Ratio of Silica Gel and Chemically Bonded Silica Columns as a Function of the Logarithm of Dioxan Concentration in n-Hexane

Packing: silica gel (Fig. 1); amino-bonded silica (Fig. 2); cyano-bonded silica (Fig. 3).

Samples in Table I: a, estrogen, androgen; b, progestogen, corticosteroid.

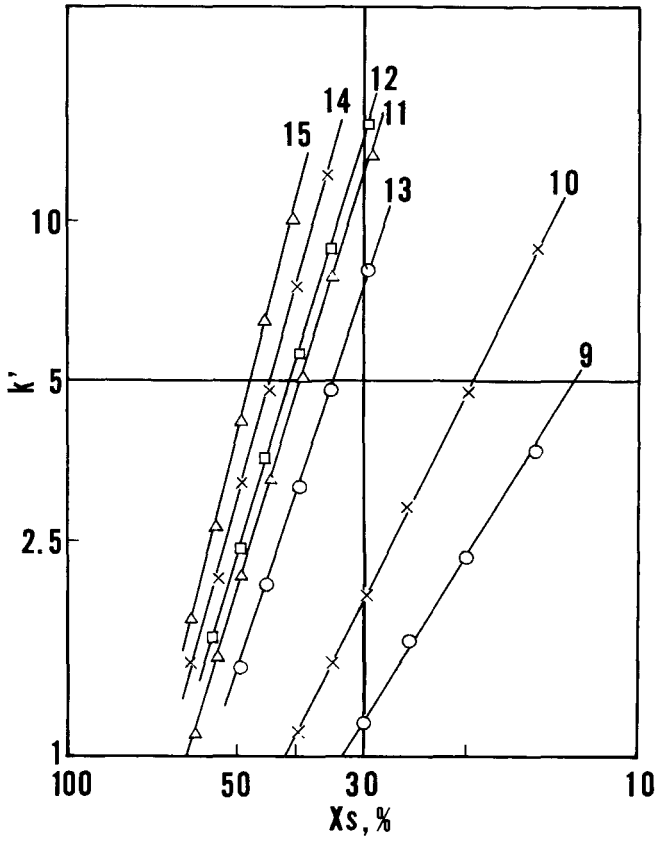


Fig. 1b

progestogens and corticosteroids as shown in Figure 1b. It was found that the capacity ratio increased when different functional groups were used in the following manner: the carbonyl group at 12 or 17 position was hydrogenated to a hydroxyl group, the O-acyl group was hydrolyzed to a hydroxyl group, the 4-en-3-one was dehydrogenated to 1,4-dien-3-one functional groups and additional groups accumulated about this molecule. These findings are in agreement with the retention mechanism involved in the hydrogen bonding association between polar groups in the solute and silanol group as the active site on the silica gel surface.

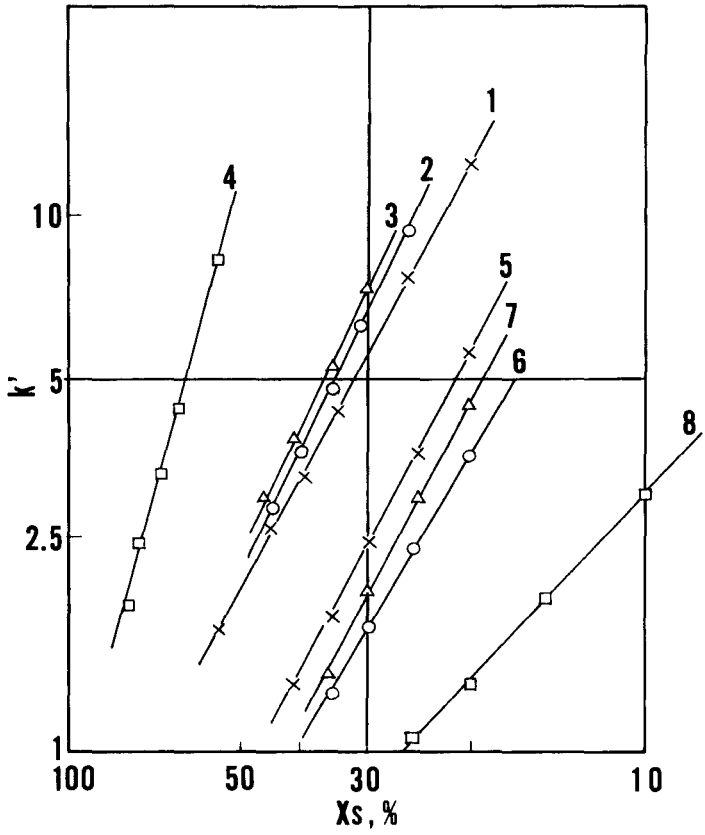


Fig. 2a

The retentivity of amino column increased in the following order: $1 < 2 < 3 \ll 4$ for estrogens, $8 \ll 6 < 7 < 5$ for androgens as shown in Figure 2a and $9 \ll 10 \ll 13 < 11 < 12 < 15 < 14$ for progestogens and corticosteroids as shown in Figure 2b. Sequence of steroid retentivity for the amino column was very close to that of the silica column used as the standard. Inversion of the retention order for the two columns was found only in the case of a few samples: 2, 3 and 14, 15.

Retention of the solutes in the cyano column also increased in a similar manner to the silica and amino columns, with the following se-

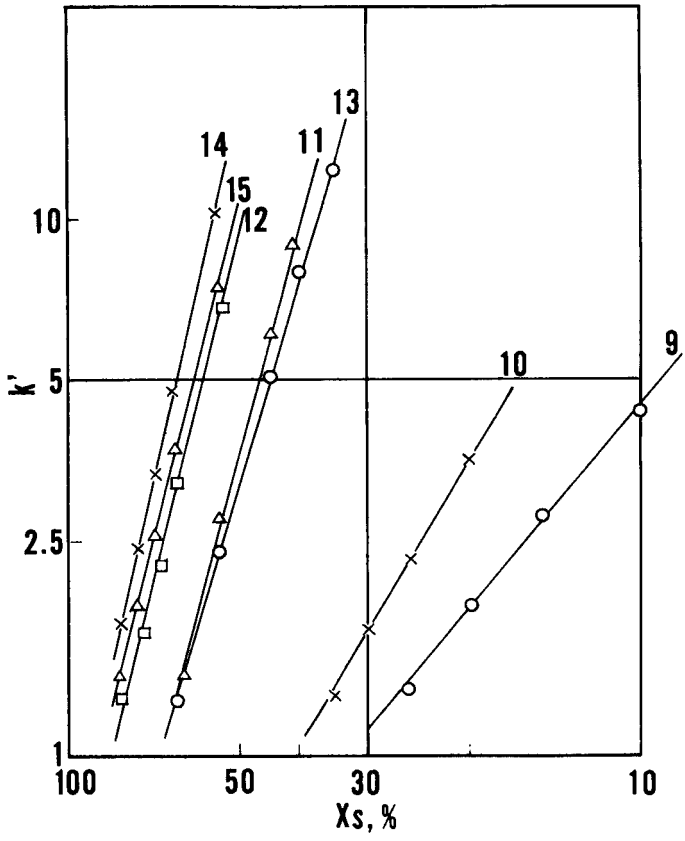


Fig. 2b

quence: $1 < 3 < 2 \ll 4$ for estrogens, $8 < 6 < 5 < 7$ for androgens as shown in Figure 3a and $9 \ll 10 \ll 11 < 13 < 12 < 14 < 15$ for progestogens and corticosteroids as shown in Figure 3b. The inversion of the retention order for the cyano and silica gel columns was observed for two pairs of samples: 5, 7 and 11, 13. Such chromatographic behavior of two packing materials was generally given and so retention mechanism for any of these solute samples in the three columns should be quite nearly the same.

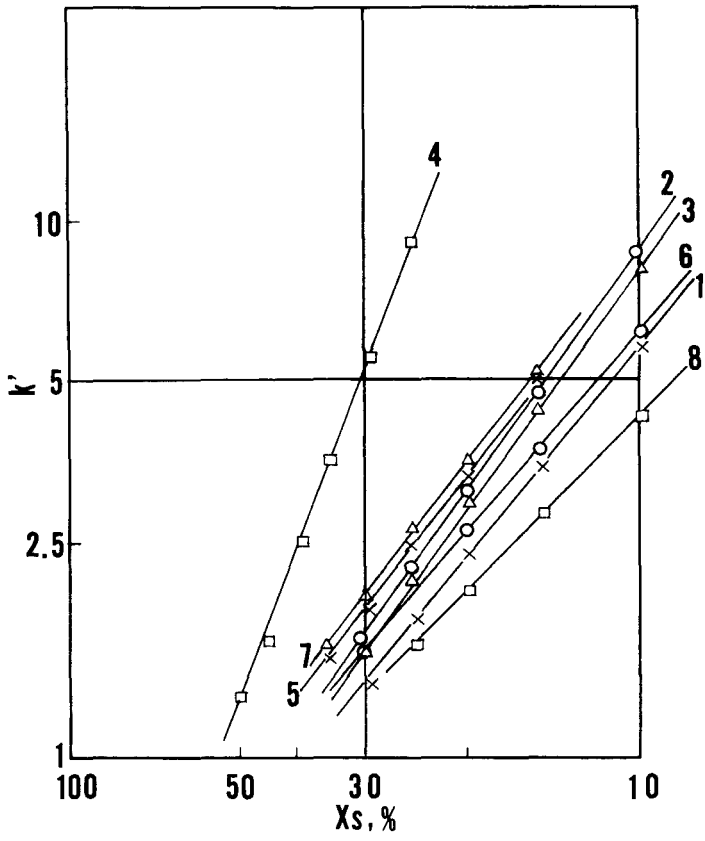


Fig. 3a

3. Selectivity of the Column Packing

Up to this point, observation has been made of different retentivities in three columns through use of binary mobile phases of the same composition and quantitative evaluation of column selectivity is examined in this section. The constants of equation (1) for a specific solute are considered to depend on the characteristics of the column and thus the quotients of the constants for a pair of columns by a using silica gel column as the standard were calculated as follows: the c-ratio and n-ratio for amino/silica and cyano/silica are the quotients of the two constants in equation (1) for the amino column/silica gel column

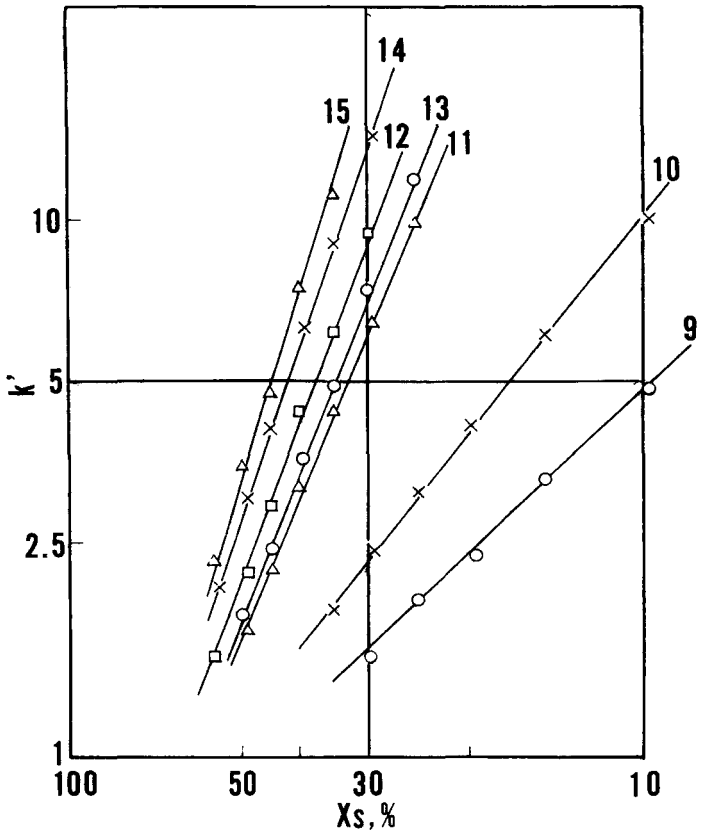


Fig. 3b

and cyano column/silica gel column, respectively. The constants c and n for the silica gel column and the quotients for the two chemically bonded columns to the silica column as the standard are tabulated in Table II. The mean values and standard deviation are also given in the table.

The mean values of the two constant ratios in Table II indicate that the relative retentivity of amino column is similar to that of the silica gel column and the retentivity of cyano column about thirty percent less than that of the silica column. As far as the column selectivities are concerned it was found that the retention indices of

****

*** Non-Bonded Silica (Estriol) ***

Dioxan(%)	k'	Log k'	Log Xs
35.00	16.05	1.2055	1.5441
40.00	10.16	1.0069	1.6021
45.00	6.74	0.8287	1.6532
50.00	4.42	0.6454	1.6990
55.00	3.00	0.4771	1.7404
60.00	2.16	0.3345	1.7782

Data N= 6
Log k'=c-nLog Xs

n= -3.7469
c= 7.0051
r= -0.9994

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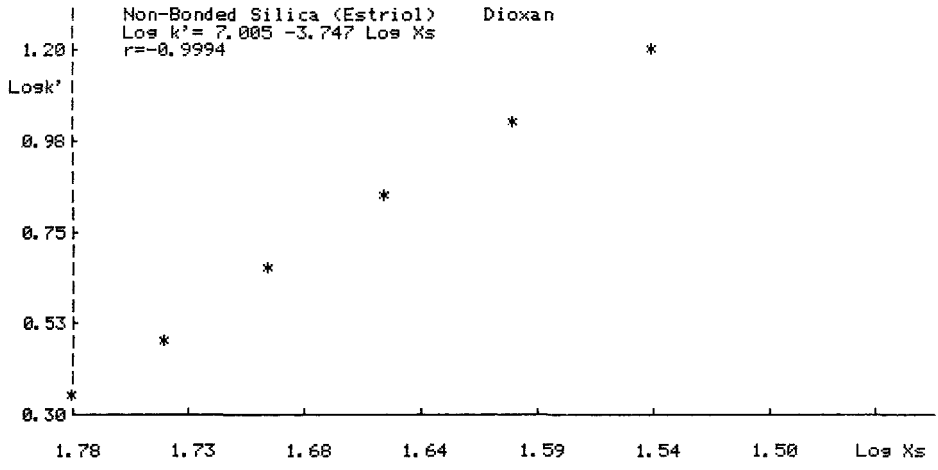

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k'	Xs Dioxan(%)
1.0	74.05
2.0	61.55
3.0	55.23
4.0	51.15
5.0	48.20
6.0	45.91
7.0	44.06
8.0	42.51
9.0	41.20
10.0	40.06

****

Fig. 4. Filing Example of Retention Data Obtained by a Micro-Computer

r is the correlation coefficient.

TABLE II. Constants and Constant Ratios of the Linear Relationship between the Retention Index and Solvent Composition of Dioxan in n-Hexane for a Silica Gel and Chemically Bonded Silica Columns

	constant		constant ratio			
Column	silica gel		amino/silica		cyano/silica	
No. steroid	c	n	c-ratio	n-ratio	c-ratio	n-ratio
1	2.71	1.69	1.29	1.10	0.72	0.72
2	3.56	2.11	1.13	1.02	0.70	0.74
3	3.38	2.03	1.25	1.13	0.72	0.74
4	7.01	3.75	1.13	1.07	0.71	0.76
5	3.66	2.21	0.94	0.93	0.62	0.61
6	3.39	2.10	0.83	0.84	0.55	0.53
7	3.44	2.11	0.90	0.90	0.66	0.63
8	2.05	1.41	0.87	0.91	0.74	0.65
9	2.56	1.68	0.71	0.72	0.61	0.55
10	3.43	2.11	0.85	0.86	0.70	0.64
11	6.48	3.61	1.06	1.03	0.69	0.69
12	6.63	3.68	1.32	1.24	0.83	0.82
13	5.81	3.32	1.17	1.11	0.83	0.81
14	7.18	3.94	1.26	1.17	0.81	0.80
15	8.03	4.36	1.08	1.03	0.80	0.80
mean value	4.62	2.67	1.05	1.00	0.71	0.70
standard deviation	1.99	0.98	1.19	0.14	0.08	0.09

c and n: intercept and slope of equation (1) in the text. c-ratio and n-ratio: quotients of constants c and n for a pair of columns.

estrogens (1 - 4) and corticosteroids (11 - 15) except for acetate (10) were larger and those of androgens (5 - 8) and progestogen (9) were smaller for the amino column than for the silica column. This suggests that a specific affinity between the surface of the amino packing and phenolic hydroxyl groups in the estrogens or alcoholic hydroxyl groups in the androgens and corticosteroids.

In Table II, the constants of the cyano column are always smaller than those of the silica column. The standard deviation of the constant ratios for the cyano and silica columns is fairly small. Thus the selectivity of the cyano column is approximately the same as that of the silica column. Consequently, the cyano column can be used as one similar to but having a retentivity weaker than the silica gel column.

CONCLUSIONS

The linear correlation between the logarithm of the capacity ratios for the chemically bonded amino- and cyano-propylsilylated silica and non-bonded silica gel columns versus the logarithm of the molar concentration of dioxan as the stronger component in an n-hexane-binary solvent was confirmed experimentally by the normal-phase liquid-solid chromatography of fifteen steroid hormones.

On the basis of the average retention indices of the steroids in these phase systems, column retentivity was evaluated quantitatively as follows: the retentivity of the amino column was similar to and that of the cyano column approximately 0.7 times more than that of the silica column as the standard. However, from the selectivity of the amino column, it was found that the retention of phenolic and alcoholic solutes was greater and that of acylated samples was smaller than retention of a silica gel column.

These results facilitate the design of an optimum phase system in binary solvent liquid-solid chromatography for samples of known molecular structure.

REFERENCES

1) S. Ohnishi, M. Ogawa, Y. Nishijima, Bunseki Kagaku, 28 (1979) 82.
2) S. Ohnishi, T. Sato, T. Yoneya, Bunseki Kagaku, 29 (1980) 272.
3) S. Ohnishi, T. Yoneya, S. Hara, Yakugaku Zasshi, 103 (1983) 236.
4) S. Hara, S. Ohnishi, Yakugaku Zasshi, 103 (1983) 750.
5) S. Hara, S. Ohnishi, Yakugaku Zasshi, 103 (1983) 756.
6) S. Hara, A. Ohsawa, J. Chromatogr., 200 (1980) 85.
7) S. Hara, A. Ohsawa, A. Dobashi, J. Liq. Chromatogr., 4 (1981) 409.
8) S. Hara, Y. Fujii, M. Hirasawa, S. Miyamoto, J. Chromatogr., 149 (1978) 143.
9) S. Hara, N. Yamauchi, C. Nakae, S. Sakai, 52 (1980) 33.
10) S. Hara, K. Kunihiro, H. Yamaguchi, E. Soczewinski, J. Chromatogr., 239 (1982) 687.

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DETERMINATION OF THE MAJOR FACTORS OF FERMENTATION OF THE NEBRAMYCIN COMPLEX BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

For the determination of the major factors (tobramycin, kanamycin B, apramycin) from the fermentation broth a new method has been developed with combination of some earlier published method. In this method the protein content of the mixture was removed by treatment with tris-(hydroxymethyl)-aminomethane followed by centrifuging then the antibiotic content was derivatized by 1-fluoro-2,4-dinitro-benzene. The mixture was analysed on a reversed phase column.

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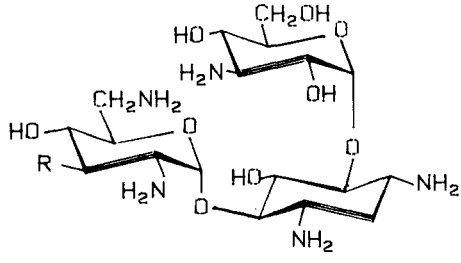
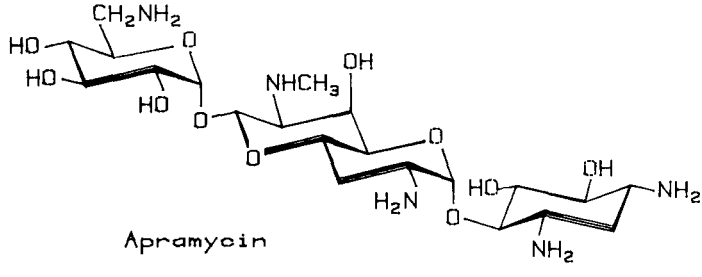
INTRODUCTION

The antibiotics tobramycin, kanamycin B and apramycin (Fig. 1), producing upon fermentation of the nebramycin complex, possess wide spectrum of activity and relatively low toxicity, providing favourable utilization in veterinary, and indeed, in human therapy. The control and qualification of the products obtained on fermentation are essential requirements of the application of such antibiotic substances.



Kanamycin B: R=OH
Tobramycin: R=H



Apramycin

Figure 1. Structures of the antibiotics

Several attempts have been made so far for the analysis of aminoglycoside-type antibiotics, involving a gas chromatographic method (1). One of the most severe difficulties of the high performance liquid chromatographic analysis of the aminoglycoside antibiotics is the detection. As these compounds do not have absorption in the ultraviolet range, sufficient chromatographic detection can be achieved only after derivatization. The prsence of amino groups in the molecule of these antibiotics allows the ion-exchange chromatographic separation and the detection can be performed either by refractive index detector or by fluorescence technique (2) preceiding derivatization of the separated components. For the separation and detection of the neomycin components Tsuji et al. (3) elaborated a method involving the reaction of the amino functions with 1-fluoro--2,4-dinitrobenzene (DNFB) and separation of the resulting N-dinitrophenyl derivatives on silicagel stationary phase using a chloroform-tetrahydrofurane-water system as the eluent. Although the separation was excellent, due to the large retention time of 2,4-dinitrophenol - the by-product of the derivatization reaction, formed from DNFB - the analysis required too long time. Barents at al. (4-6) and Elrod et al. (7) have reported on the analysis and determination of several aminoglycoside type antibiotics, also in serum, by the application of reversed phase column and derivatization with DNFB. For the detection and determination of several amino-

glycoside antibiotics a thin layer chromatographic method has been also reported recently (8).

Based on this background we attempted the separation and determination of the components formed upon nebramycin fermentation. First the isolated product mixture was investigated and then the method was extended to the examination of the fermentation broth, as well, assuring the fast and reliable control of the antibiotic level.

RESULTS AND DISCUSSION

Since there is no significant difference in the structure of the antibiotics to be separated (especially in the case of tobramycin and kanamycin B) the separation of the components of the mixture seemed to be difficult. No sufficient separation could be achieved using either ion exchange or ion-pair chromatographic technique (p-toluene sulfonic acid or pentane sulfonic acid ion pair forming agents, reversed phase column). In these cases the detection was very difficult and due to the low sensitivity of the refractive index detector the column was presumably overloaded.

The first successful experiments could be accomplished by using silicagel column and an eluent system similar to those reported in ref. 2, and these trials resulted in the sufficient separation of the three major components of the nebramycin complex (Fig. 2):

	k'	RT (min)
apramycin	3.64	6.40
kanamycin B	2.12	4.30
tobramycin	2.64	5.02

The retention time of the earlier mentioned by-product, 2,4-dinitrophenol, was 48 minutes, whereas the retention time of the last eluted component of the antibiotic mixture was below 10 minutes. Thus most part of the analysis involved the elution of 2,4-dinitrophenol making the

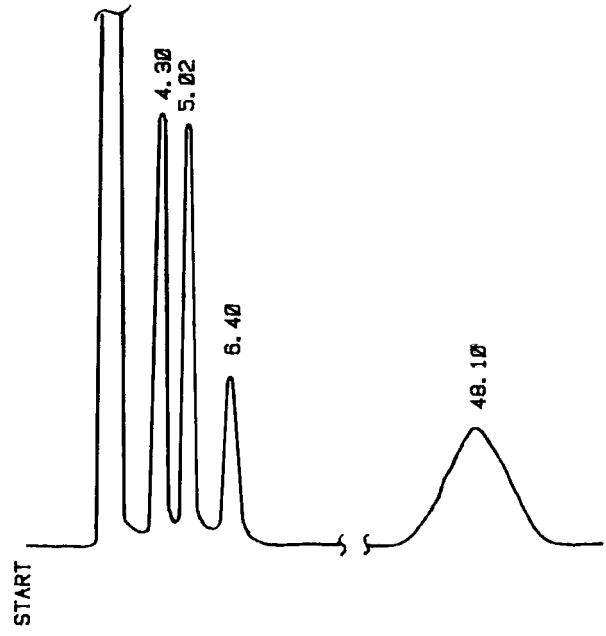


Figure 2. Separation of the major components of the nebramycin complex

method too long and tedious, therefore, another process was elaborated.

By the application of reversed phase technique and an eluent system (see Experimental) very similar to those reported for reversed phase column (4-7) satisfactory separation of the three antibiotics was obtained:

	k′	RT (min)
apramycin	6.97	9.56
kanamycin B	8.40	11.28
tobramycin	11.38	14.86

The excess of the derivatizing agent and the by-product of the derivatization reaction eluted at the outset of the chromatogram, so these materials did not disturb the determination. Using this procedure the three main components of the nebramycin complex could be simultaneously determined (Fig. 3).

The analysis of the antibiotics in the fermentation broth requires filtered and protein-free solution, as the presence of proteins and amino acides may disturb the determination process. In industry the removal of proteins from the fermentation broth is accomplished by heat-treatment under acidic conditions, followed by the filtering of precipitated proteins and suspended particles of the culture medium. The antibiotic components are then separated and isolated by means of ion-exchange chromatography on a large column.

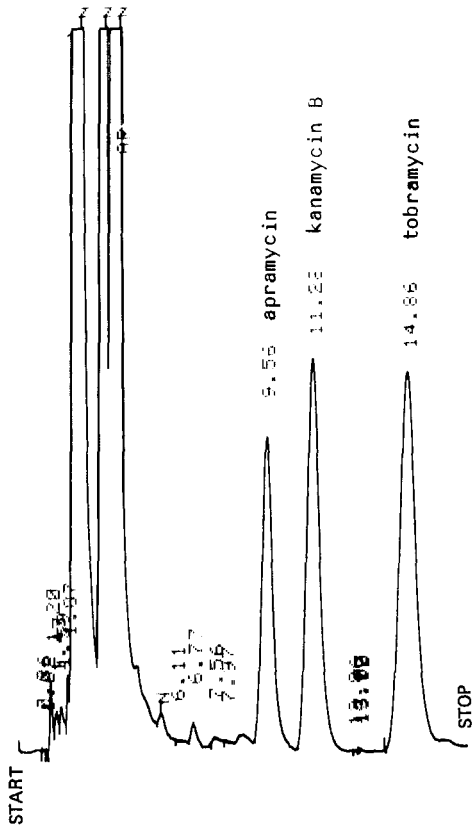


Fig. 3

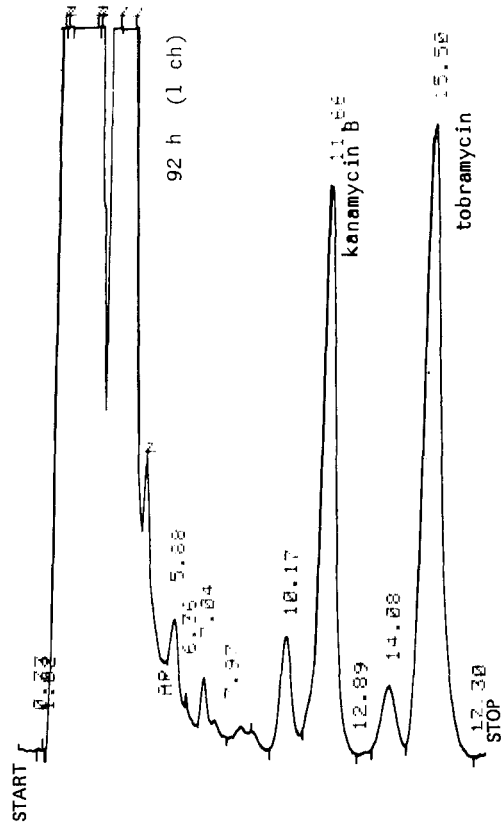


Fig. 4

Figures 3 & 4. Separation of apramycin, kanamycin B, and tobramycin on a reverse phase column from a mixture of the clear components (Fig. 3) and from a fermentation broth (Fig. 4).

For the liquid chromatographic analysis the removal of proteins could be achieved by treatment with tris-(hydroxymethyl)-aminomethane and subsequent centrifuging for a few minutes. Using this procedure several solid and colloid component of the system could be also removed.

The obtained clear fermentation broth was satisfactory for derivatization, i.e. for the determination of the antibiotic components (Fig. 4).

The analysis of these components in the fermentation broth allowed the monitoring of the antibiotic level upon fermentation. Taking samples in each 8th hour it was established that no detectable antibiotic substance is present after 24 hours of the inoculation of the producting strain (Streptomyces Tenebrarius). After that the antibiotic level quickly increases and reaches a maximum after 100-110 hours in the examined laboratory fermentations (Fig. 5). The fermentation has to be stopped and worked up at this point. Using this method the liquid chromatographic analysis, including the preparation of the samples, takes cca. 1.5 hours, so it is more quick and convenient than the microbiological evaluation.

EXPERIMENTAL

Preparation of the sample

To a 5 ml aliquot of the fermentation broth 5 ml aqueous solution of tris-(hydroxymethyl)-aminomethane (saturated) and 20 ml acetonitrile was added. The precipitated proteins were removed by centrifuging for 10 min. (at 3000 rpm) and the supernatant was decanted. For derivatization reaction 1 ml aliquot of this solution was used.

Preparation of the reference sample

To 100 ml of a "0 hr" fermentation broth the necessary amount of the antibiotics for the calibration was added

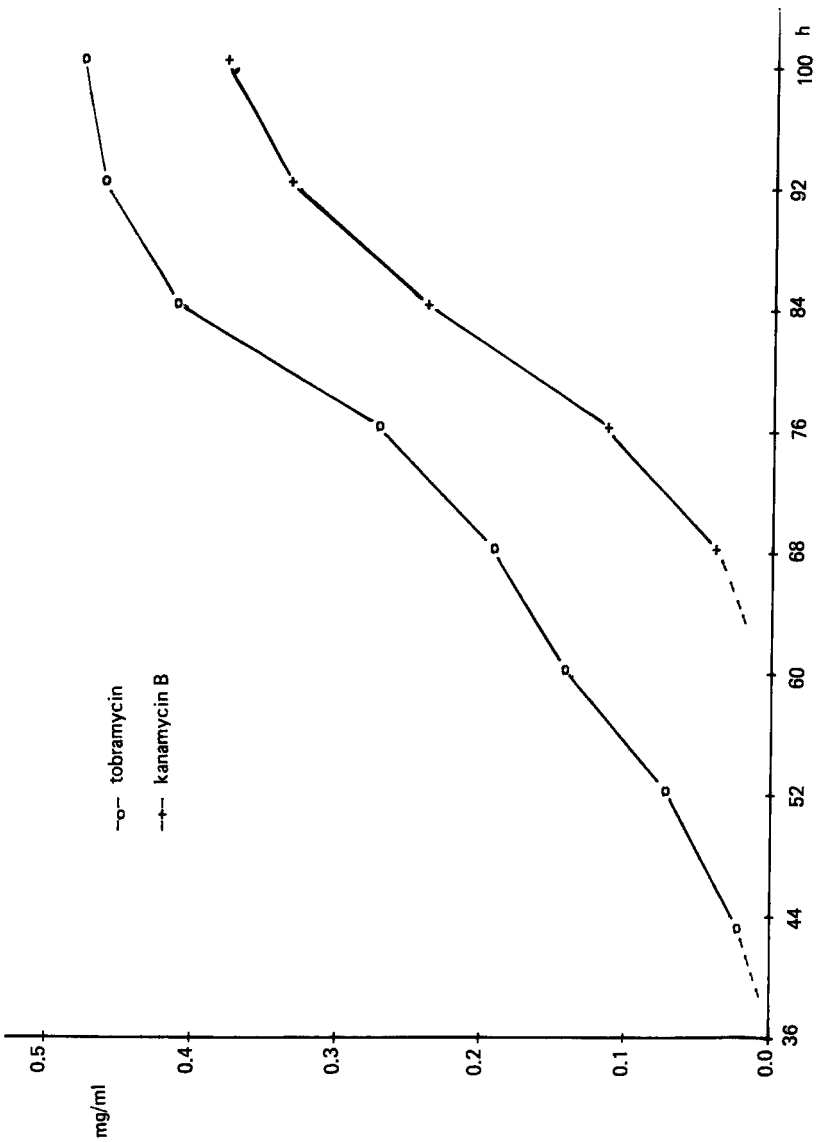


Figure 5. Concentration of the antibiotics during a fermentation. Apramycin was not detectable in this fermentation.

(apramycin: 0,2-1,0 mg, kanamycin B: 0,5-5,0 mg, tobramycin: 0,5-5,0 mg) and this solution was pre-treated similarly to that of the fermentation broth.

Derivatization

To 1 ml of a pre-threated fermentation broth 3 ml of 0,15 M methanolic DNFB solution was added. The mixture was heated at 100°C for 45 min. under reflux condenser, cooled, and the final volume of the solution was adjusted to 4 ml with eluent. This solution was applied for the determinations.

Liquid chromatographic determination

The analyses werw performed with a Hewlett-Packard 1081A isocratic instrument using a 20 ul loop Reodyne injector. The composition of the eluent was 55:45:0,15 acetonitrile-water-acetic acid, flow rate 1,2 ml/min. The separation was accomplished on a LiChrosorb RP-8 column (lenght: 20 cm, ID: 4,6 mm, particle size: 10 um, HP 79918B type). The detection was carried out at 350 nm, using an OE 308 type variable wave-lenght UV detector (Labor Műszeriapi Művek, Hungary). For the recording and integration a Hewlett-Packard 3385A type integrator was used. The quantitative determination was achieved according a calibration curve by measuring of the peak area.

REFERENCES

1. Tsuji, K. and Robertson, J. H., Methods in Enzymology, Vol. XLIII, edited by Hash, J. H., Academic Press, New York, San Francisco, London, 1975, p. 220.

2. Mays, D.L., VanApeldoorn, R. J., Laulack, L. G., J. Chromatogr., 120, 93, 1976.

3. Tsuji, K., Goetz, J. F., VanMeter, W., and Gusciora, K. A., J. Chromatogr., 175, 141, 1979.

4. Barends, D. M., Van der Sandt, J. S. F., and Hulshoff, A., J. Chromatogr., 182, 201, 1980.

5. Barends, D. M., Zwaan, C. L., and Hulshoff, A., J. Chromatogr., 222, 316, 1981.

6. Barends, D. M., Zwaan, C. L., and Hulshoff, A., J. Chromatogr., 225, 417, 1981.

7. Elrod, L., White, L. B., and Wong, C. F., J. Chromatogr., 208, 357, 1981.

8. Claes, P. J. and Vanderhaege, H., J. Chromatogr., 248, 483, 1982.

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MULTIRESIDUE ANALYSIS OF SOME INSECT GROWTH REGULATORS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY.

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ABSTRACT

A general procedure has been developed for the analysis of 8 different insect growth regulators (IGRs) by using reversed-phase high-performance liquid chromatography with gradient solvent systems. The method has been used to identify and separate 8 insect growth regulators from a mixture of the standards. The method has been evaluated with different column conditions and under different solvent systems. Best resolution was obtained by using a double column and methanol/water gradient system.

INTRODUCTION

Chitin synthesis inhibitors are gaining significance in insect control programs because of their favourable toxicological properties (1,7). Consequently, they are being evaluated extensively for controlling forestry pests. The benzoylphenyl ureas differ in their mechanism of action to conventional insecticides. They interfere with chitin deposition in the endocuticle, thus

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affecting the moulting process. These chemicals were first introduced in 1972 (3) and are referred to as moult inhibiting insect growth regulators (IGRs). Since then a number of benzoylated ureas have been synthesized by various pesticide manufacturers.

Work conducted at this Institute since 1974 has demonstrated the potential of this class of chemicals in forest pest control programs (4). To date, about 8 compounds (Table 1) have been screened for different types of forestry insects and some of them appear to be candidate materials for controlling spruce budworm, *Choristoneura fumiferana* (Clem.). The persistence, distribution and eventual fate of all chemicals released into the environment must be monitored. Consequently, the development of sensitive analytical methods to isolate, identify and quantify the materials at trace levels is a prerequisite for any such operation.

The gas chromatographic method has been the major way of analyzing trace levels of pesticide residues in biological and environmental samples since the mid-1950's. Most residue analysis procedures officially recognized by various governmental organizations employ gas chromatography. However, many of the newer types of pesticides, carbamates and insect growth regulators for example, are difficult to quantitate by gas chromatography. This is due to their nonvolatility and thermal instability. Although derivatization may overcome most of these problems (2,7), it is a rather time-consuming procedure. Also, this additional step usually introduces more experimental errors. During the past two decades the application of high-performance liquid chromatography (HPLC) to residue analysis has been expanded greatly and has become very popular.

Schaefer and Dupras (5,6) used the HPLC technique successfully to isolate BAY SIR 8514 (2-chloro-N-[[[4-(trifluoromethoxy) phenyl]amino]carbonyl]benzamide) and PH 60-40 or Dimilin® (2,6-difluoro-N-[[[4-chlorophenyl]amino]carbonyl]benzamide) from water and vegetation. This paper describes a multiresidue, reversed-phase HPLC method developed to identify and separate eight benzoyl urea derivatives from a mixture (Table 1). Due to the thermal

instability of some of these compounds, the use of HPLC appeared to be a convincing possibility for the identification and quantification of this class of compounds.

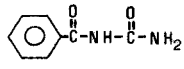
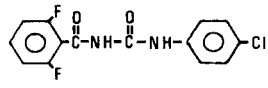
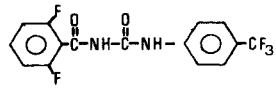
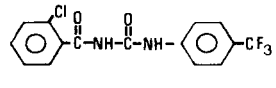
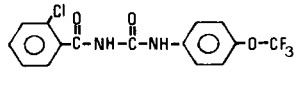
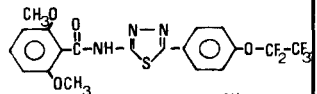
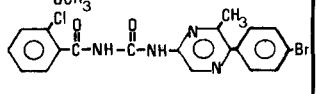
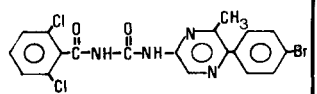
MATERIALS

The structural formulae, trade names and the manufacturers of the 8 IGRs are given in Table 1. The analytical grade materials

TABLE 1

Moult Inhibiting Insect Growth Regulators

Investigated Up To 1980

NUMBER	CHEMICAL STRUCTURE	COMPOUND	MANUFACTURER
1		BENZOYL UREA	----
2		PH 60-40	PHILIPS-DUPHAR
3		PH 60-44	"
4		PH 60-43	"
5		BAY SIR 8514	CHEMAGRO LTD.
6		L-1215	ELI LILLY & CO.
7		L-7063	"
8		EL-494	"

of these compounds used in the present study were supplied by the respective manufacturers.

All solvents (HPLC grade from J.T. Baker Chemical Co.) were filtered through appropriate Millipore filters and degassed prior to use. All compounds were stable in methanol and acetonitrile during the entire period (*ca.* 6 weeks) of this study. Standard stock solutions of the compounds were prepared in methanol and acetonitrile and subsequently diluted as required. All standards prepared were filtered through Millipore filters prior to injection into the HPLC system.

METHODS

A Hewlett-Packard model 1084B high-performance liquid chromatograph equipped with a variable wavelength detector (190-600 nm), microprocessor and electronic integrator was used for this study. The instrument also employed an automatic degassing system, dual solvent system and dual pumpheads with common drive which gave stable and reproducible flows. A Hewlett-Packard LC terminal (79850B) provided the chromatogram, area, area %, retention time (R.T.), etc., for each peak. The operating parameters were as follows:

Columns: (a) Hewlett-Packard RP-8, 10 µm, 20 cm x 4.6 mm ID.
(b) Hewlett-Packard RP-8, 7 µm, 10 cm x 4.6 mm ID.
(c) 2 of (a) connected together as a double column.

Column Pressure: 16-78 bars. (1 bar = 14.5 psig.)

Mobile Systems (V/V): (a) CH_3OH/H_2O
(b) CH_3CN/H_2O

Flow Rate: 1 ml/min and 1.5 ml/min.

Oven Temperature: ambient (24 ± 1°C)

Variable Wavelength: Sample (S):Reference (R) = 254:430 nm

Injection System: Rheodyne model 7120 syringe loading injector with 20 µl loop size.

Sample Size: 20 µl of 100 µg/ml standard stock solution.

Chart Speed: 0.1 cm/min.

Attenuation: 2^6.

Slope Sensitivity: 0.2.

An isocratic mobile system (Table 2) has been developed and used to obtain the basic chromatograms of 8 IGRs (Figs. 1 and 3) by the two solvent systems (CH_3OH/H_2O and CH_3CN/H_2O) chosen. Use of suitable gradient elution systems (Table 3) improved the resolution and separation of these compounds. The solutions of each IGR and their mixtures were injected several times to obtain reproducible results. The chromatograms obtained were well defined, having sharp peaks and a deviation in retention time (R.T.) for each injection of <1%. Under these experimental conditions, using the mixed standard, the minimum detection limit (MDL) for each IGR was found to be 10 ng. The stability of the instrument throughout the entire study was excellent.

TABLE 2

R.T. of IGRs Studied by Using a RP-8, 10 μm Column With Different Isocratic Solvent Systems. Flow Rate 1 ml/min.

No.	Compound	R.T. (min) $CH_3OH:H_2O$ = 65:35	R.T. (min) $CH_3OH:H_2O$ = 80:20	R.T. (min) $CH_3CN:H_2O$ = 65:35	R.T. (min) $CH_3CN:H_2O$ = 50:50
1	Benzoyl urea	3.37	2.91	2.85	3.21
2	PH 60-40	9.40	3.96	4.98	11.36
3	PH 60-44	11.43	4.03	5.49	14.56
4	PH 60-43	12.44	4.23	5.85	16.20
5	BAY SIR 8514	13.22	4.30	5.98	16.88
6	L-1215	16.61	4.32	6.21	19.90
7	L-7063	19.35	5.05	7.10	21.57
8	EL-494	25.39	5.47	8.27	29.19

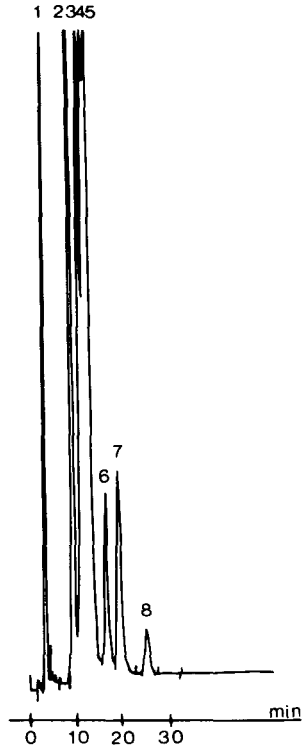


Figure 1. Separation of 8 IGR compounds using HPLC.
Column: RP-8, 10 μm.
Flow rate: 1 ml/min.
Solvent system: $CH_3OH:H_2O$ = 65:35.
Numbers on the chromatograms correspond to the IGRs given in Table 4.

RESULTS AND DISCUSSION

The average R.T.s of the IGRs studied are given in Tables 2, 4, 5, 6 and 7. The actual chromatograms obtained are given in Figs. 1-4. It is apparent from Table 2 and Fig. 2 that all the 8 IGRs studied gave well defined sharp peaks indicating that the HPLC is a viable tool to be exploited for the development of a suitable residue methodology for these compounds present in forestry substrates.

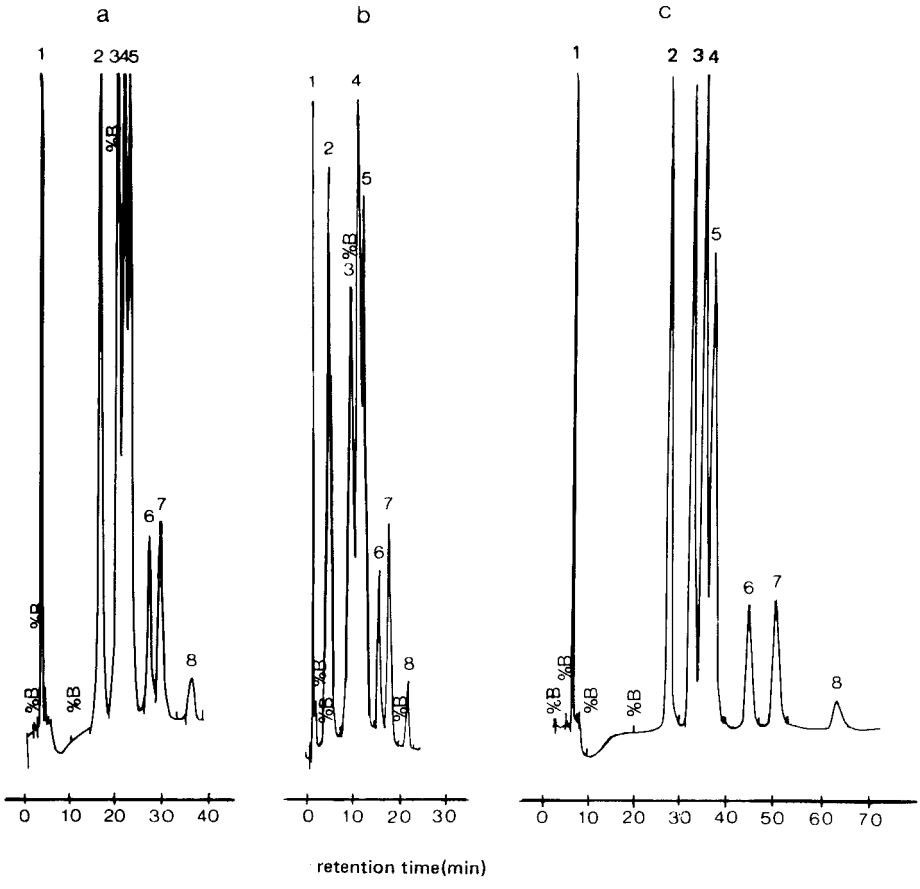


Figure 2. Separation of 8 IGRs by using 3 different HPLC columns.
(a) RP-8, 10 μm column.
(b) RP-8, 7 μm column.
(c) 2 of (a) connected together as a double column.
Flow rate: 1 ml/min.
Solvent systems: see Table 3A.
Numbers on the chromatograms correspond to the IGRs given in Table 5.

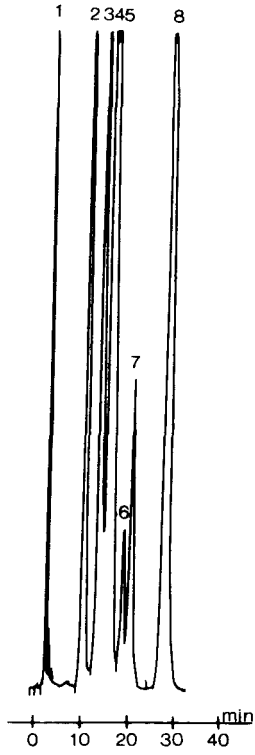


Figure 3. Separation of 8 IGR compounds using HPLC.
Column: RP-8, 10 μm.
Flow rate: 1 ml/min.
Solvent system: $CH_3CN:H_2O$ = 50:50.
Numbers on the chromatograms correspond to the IGRs given in Table 6.

The solubilities of these compounds in solvents such as acetonitrile, methanol and water varied considerably. Because of these differences, these solvents were found to be suitable in the present study to optimize the various HPLC conditions used.

Using 65:35 CH_3OH/H_2O as the solvent system, the eluting pattern obtained in the HPLC column (RP-8, 10 μm) showed that benzoyl urea, because of its low R.T. (3.37 min) is comparatively

TABLE 3

Solvent Systems for Separation of 8 IGRs.

Solvent System	Flow rate (ml/min)	Elution (min)	Time (min)	Methanol (%)	Water (%)
A	1	25-70	2	65	35
			3	50	50
			10	60	40
			20	65	35
Solvent System	**Flow rate (ml/min)**	**Elution (min)**	**Time (min)**	**Acetonitrile (%)**	**Water (%)**
B	1	60-80	3	45	55
			10	30	70
			20	25	75
			30	30	70
			40	45	55
			80	50	50
Solvent System	**Flow rate (ml/min)**	**Elution (min)**	**Time (min)**	**Acetonitrile (%)**	**Water (%)**
C	1.5	100	3	45	55
			10	30	70
			20	25	75
			30	30	70
			40	45	55
			80	45	55
			85	50	50
			90	55	45

the most soluble compound in this solvent system and EL-494 (R.T. 25.39 min) is the least soluble. This is also apparent from the other solvent systems (Table 2) used in the study. An examination of the structural patterns (polarity, molecular size, complexity, etc.) of the compounds (Table 1) qualitatively confirm this observation. Assigning a numerical value of 1 to benzoyl urea to

TABLE 4

Retention Times of 8 IGRs for Fig. 1.

No.	Compound	R.T. (min)
1	Benzoyl urea	3.37
2	PH 60-40	9.40
3	PH 60-44	11.43
4	PH 60-43	12.44
5	BAY SIR 8514	13.22
6	L-1215	16.61
7	L-7063	19.35
8	EL-494	25.39

TABLE 5

Retention Times of 8 IGRs for Fig. 2.

No.	Compound	Retention Time (min)		
		a	b	c
1	Benzoyl urea	3.37	1.73	6.67
2	PH 60-40	16.39	5.05	27.60
3	PH 60-44	20.23	9.95	32.48
4	PH 60-43	21.61	11.64	34.86
5	BAY SIR 8514	22.69	12.76	36.76
6	L-1215	27.02	16.08	44.69
7	L-7063	29.41	18.20	50.51
8	EL-494	36.00	22.24	63.44

TABLE 6

Retention Times of 8 IGRs for Fig. 3.

No.	Compound	R.T. (min)
1	Benzoyl urea	3.21
2	PH 60-40	11.36
3	PH 60-44	14.56
4	PH 60-43	16.20
5	BAY SIR 8514	16.88
6	L-1215	19.90
7	L-7063	21.57
8	EL-494	29.19

TABLE 7

Retention Times of 8 IGRs for Fig. 4.

No.	Compound	Retention Time (min) a	b	c
1	Benzoyl urea	3.43	1.77	6.12
2	PH 60-40	43.64	11.55	47.80
3	PH 60-44	50.71	40.29	57.66
4	PH 60-43	53.41	42.52	61.93
5	BAY SIR 8514	54.78	43.56	64.21
6	L-1215	61.65	48.58	76.50
7	L-7063	61.65	48.58	76.50
8	EL-494	71.81	56.42	92.11

represent its solubility (solubility factor SF = 1), the SF values of other compounds in 65:35 CH_3OH/H_2O solvent system are:

Compound	SF (R.T. of Benzoyl urea/R.T. of x)
Benzoyl urea	1.00 (R.T. 3.37 min)
PH 60-40	0.36 (R.T. 9.40 min)
PH 60-44	0.29 (R.T. 11.43 min)
PH 60-43	0.27 (R.T. 12.44 min)
BAY SIR 8514	0.25 (R.T. 13.22 min)
L-1215	0.20 (R.T. 16.61 min)
L-7063	0.17 (R.T. 19.35 min)
EL-494	0.13 (R.T. 25.39 min)

The SF values obtained from this study strongly demonstrated the close structural similarities of PH 60-40 versus PH 60-44 (Ar-Cl versus Ar-CF_3) and PH 60-43 versus BAY SIR 8514 (Ar-CF_3 versus Ar-O-CF_3). Also the low solubility of EL-494 compared to L-7063 is due to its increased molecular mass, because of the presence of an additional Cl on the aryl ring of the benzoyl

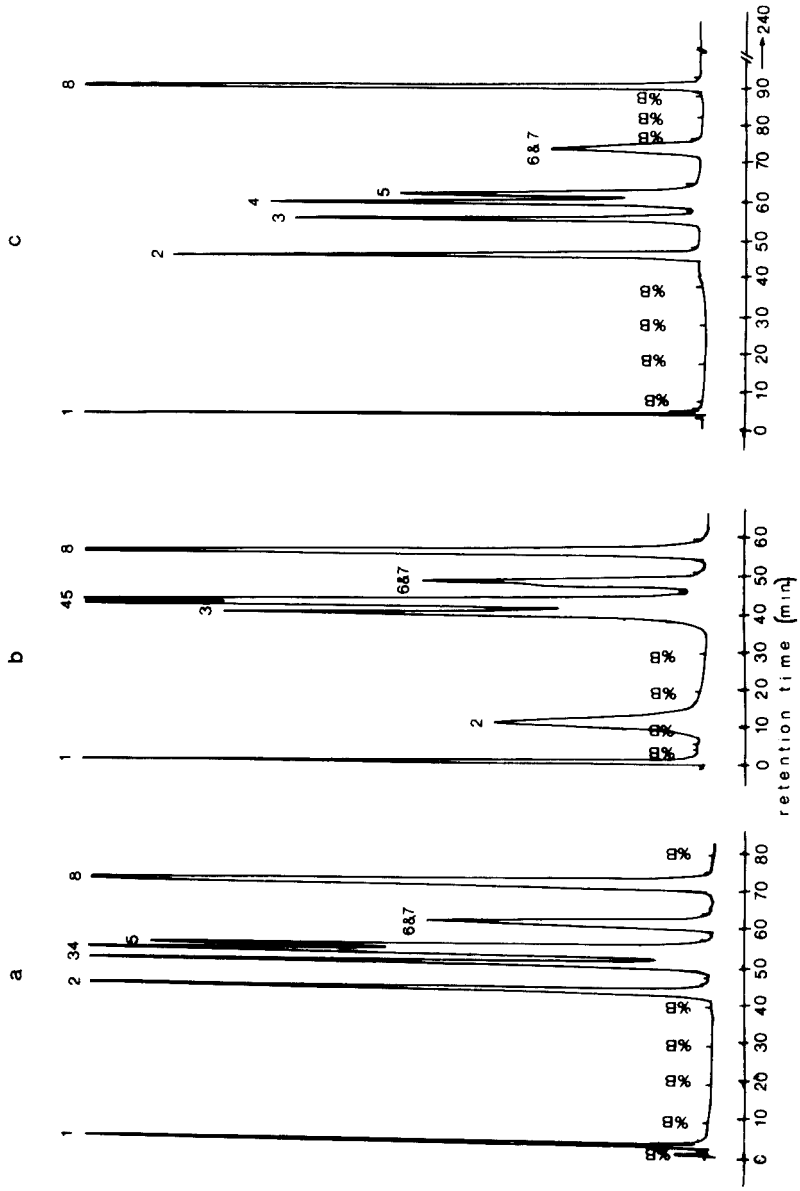


Figure 4. Separation of 8 IGRs by using 3 different HPLC columns. (a) RP-8, 10 µm column, solvent system B (Table 3). (b) RP-8, 7 µm column, solvent system B (Table 3). (c) 2 of (a) connected together as a double column, solvent system C (Table 3). Numbers on the chromatograms correspond to the IGRs given in Table 7.

moiety. Generally, the R.T. increases with diminishing solubility of the IGRs in both solvent systems used in this study.

The resolution of compounds in a chromatogram is determined by the type of column used and by its efficiency. Three different types of columns [(a) Hewlett-Packard RP-8, 10 µm, 20 cm x 4.6 mm I.D., (b) Hewlett-Packard RP-8, 7 µm, 10 cm x 4.6 mm I.D., (c) 2 of (a) connected together as a double column] along with two different solvent systems (CH_3OH/H_2O and CH_3CN/H_2O, both with or without gradient elution systems) (Tables 2 and 3), were tried to resolve all the eight IGRs satisfactorily (Figs. 1-4) and also to improve the separations among the 4 compounds namely, PH 60-40, PH 60-44, PH 60-43 and BAY SIR 8514.

It is evident (Fig. 1, Table 4) that by using the RP-8, 10 µm column and 65:35 CH_3OH/H_2O isocratic solvent system, the separation of these four IGRs (Peaks 2-5) was poor. Similar results were also obtained (Fig. 3, Table 6) by using CH_3CN/H_2O (50:50) gradient system. However, by choosing the gradient solvent system (Figs. 2a, 4a, Tables 5a, 7a), the same column showed a slightly better separation for all the IGRs.

Use of a 7 µm RP-8 column instead of the 10 µm RP-8, together with the gradient solvent system, gave a relatively good separation with low R.T.s (Figs. 2b, 4b, Tables 5b, 7b) for all the 8 IGRs studied. The minor drawback in this column is the poor resolution of the two structurally similar compounds *viz.* PH 60-43 and BAY SIR 8514.

The chromatogram in Fig. 2c was obtained by connecting in series two of the RP-8, 10 µm columns and using the CH_3OH/H_2O gradient solvent system (Table 3A) to elute the samples. Similar double column arrangement and the use of CH_3CN/H_2O gradient solvent system (Table 3C) yielded the chromatogram recorded in Fig. 4c. In this set up, the compounds L-1215 and L-7063 (Table 1) gave a single peak (Figs. 4a, 4b and 4c) and the elution was also longer compared to the CH_3OH/H_2O system. While comparing the two gradient solvent systems used in this study, it is evident that a double column (RP-8, 10 µm) with CH_3OH/H_2O gave the best resolution for all the 8 IGRs.

This study indicates that (1) the eluting pattern of the IGRs in the chosen solvent system is relatable to their solubilities in the system, i.e., benzoyl urea is more soluble than EL-494; (2) the molecular structure of the sample determines the elution order; the greater the complexity (steric effect, type and number of functional groups, etc.) the higher the R.T.; (3) a methanol/water gradient solvent system with a double column gives the best resolution; (4) the lowest amount of IGR we could detect in this study is 10 ng and (5) IGRs as a group respond extremely well to the HPLC technique reported herein and could be used as an analytical tool in the identification and quantification of these compounds from environmental samples.

ACKNOWLEDGEMENT

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REFERENCES

1. Hammann, I. and Sirrenberg, W. Laboratory Evaluation of BAY SIR 8514, a New Chitin Synthesis Inhibitor of the Benzoylated Urea Class. *Pflanzenschutz Nachrichten Bayer, 33(51),* 1(1980).

2. Lawrence, J.F. and Sundaram, K.M.S. Gas Chromatographic Analysis of TH 6040 Insecticide After Chemical Derivatization. *Jour. Assoc. Official Anal. Chem., 59(4),* 938(1975).

3. Plimmer, J.R. Pesticide Chemistry in the 20th Century. *ACS Symp. Series 37,* 237(1977).

4. Retnakaran, A. Toxicology and Efficacy of Insect Growth Regulators Aerially Applied Against the Spruce Budworm. *Env. Canada For. Serv., For. Pest Mngmt. Inst. Inf. Rept. FPM-X-45,* 1981. pp 63.

5. Schaefer, C.H. and Dupras, E.F., Jr. Factors Affecting the Solubility of SIR-8514 (2-chloro-N-[[[4-(trifluoromethoxy)

phenyl]amino]carbonyl]benzamide) under Laboratory and Field Conditions. *Jour. Agric. Food Chem., 27(5),* 1031(1979).

6. Schaefer, C.H. and Dupras, E.F., Jr. Residues of Diflubenzuron [1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea] in Pasture Soil, Vegetation, and Water Following Aerial Applications. *Jour. Agric. Food Chem. 25,* 1026(1977).

7. Sundaram, K.M.S. and Lawrence, J.F. GLC Analysis of TH 6040 Insecticide After Chemical Derivatization. *Can. Chem. Congr. Chem. Inst. Canada Abstr. Papers 59,* 9(1976).

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INVESTIGATION OF THE INFLUENCE OF HYDROPHOBIC IONS AS MOBILE PHASE ADDITIVES ON THE LIQUID CHROMATOGRAPHIC SEPARATION OF AMINO ACIDS AND PEPTIDES

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ABSTRACT

Tetraalkylammonium, R_4N^+, and alkylsulfonate, RSO_3^-, salts were evaluated as mobile phase additives for the separation of amino acids and peptides. The former were used in a basic mobile phase and the latter in an acidic one, conditions which convert the terminal carboxyl or amine groups in amino acids and peptides and the acidic or basic side chains if present into anionic or cationic forms, respectively. Because of the required strongly basic or acidic mobile phase pH, a polystyrene-divinylbenzene copolymer, PRP-1, was used as the reversed stationary phase. The retention is suggested to follow a dynamic interaction involving two major equilibria, namely retention of the hydrophobic ion and an ion exchange between the co-ion accompanying the hydrophobic ion and the amino acid or peptide ion of opposite charge. The effect of amino acid and peptide structure on retention is discussed. Key mobile phase variables are identified; a major one is the optimization of the hydrophobic ion concentration-mobile phase solvent composition to provide a sufficient number of charge sites on the stationary phase due to hydrophobic ion retention. Several separations are shown which focus on the advantages offered by using hydrophobic ions as mobile phase additives. In general,

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RSO_3^- salts appear to be more versatile than R_4N^+ salts in improving selectivity and resolution in amino acid and peptide separations.

INTRODUCTION

Reverse phase high performance liquid chromatography (RPLC) has emerged as a powerful technique for the sensitive, rapid, efficient separation of derivatized and underivatized amino acids (AA), peptides, and proteins (1-3). Even preparative applications are feasible since column loadings and sample recovery are often favorable (1,4,5).

The alkyl-modified silicas, in particular, have gained wide acceptance as the stationary phase for the LC separation of AA and peptides (1-3). However, polystyrene-divinylbenzene (PSDB) type stationary phases have also been shown to be useful for these kind of separations (6). Their major advantages lie in the fact that they are stable throughout the entire pH range, unlike the alkyl-modified silicas which have a useful pH range of about pH 2 to 8, and have favorable loading capacities for preparative applications (4,6). In addition to pH, other key mobile phase variables for both types of stationary phases that are manipulated to improve or change selectivity and resolution include mixed solvent ratio, type of solvents, ionic strength, type of buffer components and their concentration, and the use of hydrophilic or hydrophobic ions as mobile phase additives. The need for such a broad range of eluting conditions is important for several reasons. First, AA

and peptides vary widely in structural and functional features because of side chains and mobile phase conditions must have the potential to distinguish between major differences as well as minor differences. The latter are due to minor sequence changes in the peptide as the result of replacement or reduction of one or more subunits in the peptide. Second, the mobile phase conditions must be compatible with sensitive detection devices if analytical LC is the goal or with procedures that easily permit isolation of the peptide from the mobile phase if preparative LC is the goal.

The application of hydrophilic or hydrophobic ions as mobile phase additives has been particularly useful in AA and peptide separations. Typical additives used are alkylsulfonic (RSO_3^-) acids, alkyl sulfates, fluorinated alkyl carboxylic acids, amine salts, tetraalkylammonium (R_4N^+) salts, ClO_4^-, PO_4^{-3}, and others (1,7-16). Secondary equilibria between the additive and the AA or peptide will often bring about significant changes in retention characteristics. In general, retention is augmented due to the presence of the additive. Depending on the mobile phase and stationary phase modifications the nature of the secondary equilibria can range from ion pair formation to an ion exchange selectivity. In the former the interaction is viewed as first formation of an ion pair between the AA or peptide and the additive which is of opposite charge and then retention of the ion pair by the stationary phase. In the latter the additive (if

hydrophobic) is first retained and then the AA or peptide as a charged species exchanges with the co-ion that accompanies the charged additive. More recently it has been shown that under defined mobile phase conditions a dynamic ion exchange (also called ion interaction) type interaction occurs, the details of this and previous model studies are reviewed in detail elsewhere (7-9,17-20). Of particular interest is the recent studies which indicate that under defined mobile phase conditions the ion interaction type retention is present when using both the PSDB and alkyl-modified silica as the stationary phase and RSO_3^- salts or R_4N^+ salts as mobile phase additives (18,19).

Many studies in the past employed a mobile phase pH in the range of about 2.5 to 7 in part because of the pH limitation of the alkyl-modified silica. Thus, AA and peptides range from being partial cations to zwitterions and the full effect of using hydrophobic ions as mobile phase additives is not always realized. In this study we report the use of R_4N^+ salts as mobile phase additives and employ a mobile phase pH (pH > 10) that ensures that the carboxyl-terminus of an AA or peptide and acidic side chains, if present, are in their anionic form. The effect of RSO_3^- salts as mobile phase additives on the retention of AA and peptides from an acidic mobile phase, where the amine terminus and basic side chains, if present, are in their cation form, was also studied. PRP-1, which is a PSDB reverse stationary phase and is stable from pH 1 to 14, was used throughout this investigation.

EXPERIMENTAL

Chemicals and Instrumentation

Amino acids and peptides were obtained from Sigma Chemical Co., Chemalog, Vega Biochemicals, and Research Plus. Tetrapropyl-(TPABr), tetrabutyl-(TBABr), and tetrapentyl-(TPeABr) ammonium bromide and pentyl-(C_5SO_3H), heptyl-(C_7SO_3H), and octyl-(C_8SO_3H) sulfonic acids were purchased from Eastman Kodak and Aldrich Chemical Co. Different anion forms of the R_4N^+ salts were prepared by an anion exchange procedure (18) while the RSO_3H was converted to the salt form by titration with MOH or by cation exchange (19). Organic solvents and water were LC quality while all inorganic salts were analytical reagent grade.

PRP-1 is a 10 μm, spherical PSDV particle with a large surface area and porosity and was obtained as a prepacked column (4.1 mm x 150 mm) from Hamilton Co. A Waters Model 202 LC equipped with a Tracor Model 970 or Spectra Physics 770 variable wavelength detector was used.

Procedures

Peptide and AA sample solutions of about 1 mg/ml were prepared by dissolving mg quantities in H_2O, EtOH, or their mixture in septa sealed vials and refrigerated when not in use. Sample transfer was by syringe. Operating conditions, in general, involved 5 μl sample aliquots, 1 ml/min flow rate, inlet pressures of 700 to 1400 psi depending on mobile phase, detection

at 215 nm, and controlled temperature at 25°C. Mixed solvents are expressed as per cent by volume. Mobile phase pH was controlled by phosphate buffers, HCl, or NaOH. Column void volume, V_o, for a given mobile phase was determined by using analytes that were not retained. Capacity factor, k', was calculated by $k' = (v-v_o)/v_o$ where v is the retention volume of the analyte of interest. Breakthrough volumes were determined as previously described (18).

RESULTS AND DISCUSSION

Two major equilibria influence the retention of a charged analyte on PRP-1 when using hydrophobic ions as mobile phase additives (18-20). One describes the retention of the hydrophobic ion and its co-ion on the PRP-1 surface as a double layer where the hydrophobic ion makes up the primary layer and the co-ion the secondary layer. The second is an ion exchange selectivity between the co-ion and the analyte ion. For a R_4N^+ salt as the additive, A^- its co-anion, and K^- the analyte anion, the retention can be viewed as

$$\left.\begin{aligned} &(\text{PRP-1}) + R_4N^+A^- \rightleftharpoons (\text{PRP-1})\cdots R_4N^+A^- \quad (1a)\\ &(\text{PRP-1})\cdots R_4N^+A^- + X^- \rightleftharpoons (\text{PRP-1})\cdots R_4N^+X^- + A^- \quad (1b)\end{aligned}\right\} \quad (1)$$

If a RSO_3^- salt is the additive, C^+ its co-cation, and M^+ the analyte cation the process is

$$\left.\begin{array}{ll} (\text{PRP-1}) + RSO_3^-C^+ \rightleftharpoons (\text{PRP-1})\cdots RSO_3^-C^+ & (2a) \\ (\text{PRP-1})\cdots RSO_3^-C^+ + M^+ \rightleftharpoons (\text{PRP-1})\cdots RSO_3^-M^+ + C^+ & (2b) \end{array}\right\} \quad (2)$$

Since the hydrophobic salt is at a constant concentration in the mobile phase and the interaction is dynamic the overall process is given by the combination of equations 1a-1b and 2a-2b, respectively, as the analyte passes through the column.

The charge form of an AA and peptide is very dependent on pH because of their acidic and basic terminal groups. The pK_a values, which are also influenced by side chain groups, determine these pH conditions. In general, at pH < 2 the amine terminus is a cation. At a basic pH, pH > 10, the carboxyl terminus is an anion while at an intermediate pH the AA and peptides are zwitterions. If the side chains also contain acidic or basic groups they can provide additional charge sites depending on their pK_a values and the mobile phase pH.

It follows from equations 1 and 2 that in order to separate AA and peptides as anions, R_4N^+ salts and a basic mobile phase would be best while as cations, RSO_3^- salts and an acidic mobile phase would be best. Previous results (18) have already indicated that the zwitterion pH provides the lowest retention and often the poorest selectivity. For these reasons this mobile phase pH or one close to this, which is used most often (7-16) is the least useful of the three particularly when attempting to separate closely related AA and peptides.

Several mobile phase variables have been identified (18-20) which can be manipulated to alter analyte retention and subsequently improve resolution and selectivity while still maintaining favorable analysis times. The data reported in this paper for AA and peptide retention, as well as previous data for the retention of analyte cations and anions (18-20), on PRP-1 from mobile phases containing hydrophobic ions are consistent with these generalizations. Briefly, these are summarized in the following. 1) The structure and concentration of the R_4N^+ or RSO_3^- salt can be altered. As the alkyl chain length, R, increases, retention increases, due to the shift in the equilibrium shown in equations 1a and 2a, leading to an increase in the number of charge sites, which in turn increases analyte retention. Increasing the concentration of the additive in the mobile phase also increases its retention, number of charge sites, and analyte retention. 2) The ion exchange selectivity, indicated by the equilibrium in equation 1b and 2b is responsive to the concentration and type of co-ion in the mobile phase. Thus, the elution order, which is a measure of the selectivity of one ion over another and closely resembles the more traditional ion exchange selectivity (21), follows, in general, the order

$$NO_3^- > Br^- > NO_2^- > Cl^- > \text{formate} > F^- > OH^- \qquad (3)$$

$$M^{+2} > Na^+ > Li^+ > H^+ \qquad (4)$$

where a R_4N^+ salt or a RSO_3^- salt is used, respectively. 3) Mobile phase pH determines the charge on the peptide and AA; in general, as the charge increases retention increases in the presence of a hydrophobic ion of opposite charge. 4) Altering the organic solvent:water ratio sharply changes the retention of the additive on the stationary phase (equations 1a and 2a) which then influences the number of charge sites, and analyte retention; decreasing the organic modifier in the solvent mixture leads to increased additive retention, large number of charged sites, and increased analyte retention. 5) The type of organic modifier used influences the previous variable. The eluting power follows the order CH_3CN > EtOH > MeOH. 6) Ionic strength control can be used to influence analyte ion retention (equations 1b and 2b) since it can provide additional amounts of the same or an indifferent co-ion that accompanies the additive; increasing ionic strength will decrease analyte retention.

Table I demonstrates that AA and peptide retention increases as the hydrophobic ion alkyl chain length increases and that the additive significantly augments analyte retention. When using the R_4N^+ salts the mobile phase is basic (pH = 11) ensuring the analyte is an anion while with the RSO_3^- salts the mobile phase pH is acidic (pH = 2) so that the analytes are cations. If analyte retention is plotted versus C-number, where C-number is equal to the sum of all carbons in the alkyl chain of the R_4N^+ salts, retention increases rapidly and provides or approaches a

TABLE I

Effect of Hydrophobic Ion Alkyl Chain Length on the Retention of Amino Acids and Dipeptides on PRP-1

		Capacity Factor, k'					
		Tetraalkylammonium Salt[a]					
Analyte	No Salt[b]	TMABr	TEACl	TPABr	TBABr	TPeABr	THxABr
D,L-Phe	0.89	0.93	0.95	1.33	3.35	5.81	5.09
D,L-Trp	1.59	1.68	1.75	2.56	7.23	14.4	15.2
L-Leu-L-Tyr	0.63	0.66	0.66	1.19	4.17	12.1	14.3
D-Leu-L-Tyr	0.63	0.67	0.67	1.14	3.49	9.01	10.1

		Alkylsulfonic Acid[c]		
	No Salt[d]	C_5SO_3H	C_7SO_3H	C_8SO_3H
L-Ser	0	0.51	2.18	3.60
L-Thr	0.10	0.80	3.85	6.44
L-Gln	0.10	0.66	3.12	4.91
L-Ser-L-Ser	0.12	0.75	3.43	5.69
L-Ala-L-Ser	0.16	0.98	4.62	7.75
L-Ser-L-Ala	0.24	1.80	9.44	15.9

(a) A 1:9 CH_3CN, 1.00 x 10^{-3}M R_4N^+ salt, 1.00 x 10^{-2}M pH=11.0 (phosphate) buffer, and NaCl so that μ=0.10M mobile phase at 1.0 ml/min.

(b) Same as (a) except R_4N^+ salt is omitted.

(c) A 1.00 x 10^{-3}M RSO_3H, 0.01M HCl, and 100% H_2O mobile phase at 1.0 ml/min.

(d) Same as (c) except RSO_3H is omitted.

maximum. The maximum, which has been observed before (18), and its location relative to C-number is a function of the retention of the R_4N^+ salt. This retention increases as the CH_3CN concentration in the mobile phase decreases and therefore increases the number of charged sites (according to the equilibrium in equation 1a) on the PRP-1 (18) due to the retained R_4N^+ salt. As the number of sites increase, the maxima shifts to a lower C-number. Since only a few RSO_3^- salts were examined under comparable mobile phase conditions, it was not possible to ascertain whether a maximum was present when using RSO_3^- salts as mobile phase additives. These and other data not shown here, however, clearly indicate that increasing the alkyl chain in the RSO_3^- salt increases the number of charged sites (see equilibrium in equation 1b) due to RSO_3^- salt retention and enhances AA and peptide retention. Also, as the organic solvent concentration decreases in the mobile phase the number of RSO_3^- sites on the PRP-1 surface increases (19).

Tetraalkylammonium Salts

Table I and other preliminary experiments suggested that the optimum R_4N^+ salts were ones where R = propyl, butyl, or pentyl. Retention data for several AA at pH = 11 using these three R_4N^+ salts as additives are shown in Table II. The F^- salts were used because F^- is one of the weakest eluent co-anions (18,20) (see equation 1b; also compare data for Phe in Tables I and II). The presence of the R_4N^+ salt significantly enhances

TABLE II

Effect of R_4N^+ Salts on the Retention of Amino Acids on PRP-1

	Capacity Factor, k'			
	TPAF		TBAF	TPeAF
Amino Acid/% CH_3CN	0%	5%	10%	15%
Nonpolar				
L-Ala	2.15		2.60	5.05
L-Pro	10.8	1.10	2.59	6.11
L-Met		2.67	2.65	5.44
L-Phe		14.0	22.0	
Polar				
Gly	2.15		2.60	5.05
L-Ser	2.04		2.24	4.98
L-Thr	2.73		2.79	5.34
L-Cys		2.02	4.18	
L-Asn	2.18		2.58	4.88
L-Gln	2.63		2.34	4.80
Basic				
L-His	3.37		2.65	5.44
Acidic				
L-Asp		1.60	18.2	
L-Glu		1.64	17.7	
L-Tyr		2.11	16.6	

A 10 μm, 4.1 mm x 150 mm PRP-1 column using a $CH_3CN:H_2O$, 1.00×10^{-3}M R_4NF, 1.00×10^{-3}M NaOH, mobile phase at 1.0 ml/min with detection at 215 nm.

AA retention, however, the differences in retention for AA with polar and basic side chains tend to be small. For nonpolar AA differences are large only for those AA with very hydrophobic side chains such as with Phe and Leu.

If the AA side chain is acidic and the mobile phase pH is basic enough to convert this group into an anionic site then the AA is a divalent anion. As seen in Table II a marked increase in retention is found when a R_4N^+ salt is present as a mobile phase additive. In the absence of the R_4N^+ salt there is no retention on PRP-1 as the di-anion. If the alkyl chain length of the R_4N^+ salt is increased the retention of the acidic AA markedly increases in comparison to AA without acidic side chains. In Table II the number of sites (retained R_4N^+ salt) is approximately the same (10 to 13 μmoles of R_4N^+ salt retained/column as determined from breakthrough volumes) for TPAF, TBAF, and TPeAF when the CH_3CN:H_2O ratio is 5:95, 10:90, and 15:85, respectively. Thus, the observed retention trends are not due to differences in loading capacity.

The data in Table II indicate that acidic and very hydrophobic AA can be readily separated from other AA. Of particular interest are the separations involving the acidic AA. In the presence of a R_4N^+ salt these are highly retained and would elute after the polar, basic, or weakly hydrophobic AA; in the absence of the R_4N^+ salt and a mobile phase pH of 11 the acidic AA would elute from PRP-1 before or with these weakly retained AA.

Although not shown AA elution peaks are well defined and exhibit column efficiencies similar to peptide chromatographic peaks shown later.

Similar retention studies were carried out using dipeptides that contained polar subunits and tripeptides that contained weakly hydrophobic side chains as analytes. Often, these are difficult to separate in the absence of hydrophobic ions in the mobile phase. Table III lists retention data for several of the dipeptides. In general, even though retention is enhanced in the presence of the R_4N^+ salt relative to its absence, where in most cases retention on PRP-1 is zero or barely detectable, differences among the dipeptides are small except for those containing very hydrophobic side chains or acidic side chains (see Table IV).

If the mobile phase is at the zwitterion pH retention in the presence of the R_4N^+ salt is still enhanced relative to the absence of the R_4N^+ salt in the mobile phase. However, the level of enhancement is not comparable to that obtained when the mobile phase pH is basic and the carboxyl terminus and acidic side chains, if present, are ionized. The presence of the cationic site in the zwitterion is responsible for this difference.

Figure 1 shows the separation of several tripeptides that are poorly retained in the absence of the R_4N^+ salt and the improvement in resolution that can be obtained by optimizing the mobile phase variables. In Figure 1A poor resolution is obtained in the absence of a R_4N^+ salt. Marked improvement is

TABLE III

Effect of R_4N^+ Salts on the Retention of Polar Dipeptides on PRP-1

	Capacity Factor, k'			
	TPAF		TBAF	TPeAF
Dipeptide/% CH_3CN	0%	5%	10%	15%
Gly-Gly	2.85		2.47	5.87
Gly-L-Ser	2.57		2.39	5.61
Gly-L-Thr	4.33	0.84	2.64	6.06
Gly-L-Lys		1.10	2.72	5.43
Gly-L-Asn	2.65		2.29	5.28
Gly-L-Met		3.33	7.77	14.4
L-Ala-L-Ser	4.24		2.62	6.39
L-Ala-L-Thr			2.98	6.71
L-Ala-L-Lys		1.15	2.29	5.77
L-Ala-L-Asn	4.08		2.44	5.62
L-Ala-L-Met		6.07	11.3	17.9
L-Ala-L-Ala		1.05	3.14	7.03
L-Ser-L-Ser	2.39		2.29	5.25
L-Ser-L-Gly	2.51		2.31	5.34
L-Ser-L-Ala	3.23		2.46	5.57
L-Ser-L-Met		2.57	6.14	11.4
L-Ser-L-Leu		3.69	8.11	14.3
L-Met-L-Gly		4.34	9.00	15.0
L-Met-L-Ser		3.51	7.67	13.3

Column conditions are the same as Table II.

TABLE IV

Retention of Acidic Dipeptides on PRP-1 in the Presence of TPAF

	Capacity Factor, k'	
	pH	
Dipeptide	11.00	8.85
L-Tyr	3.43	1.31
Gly-L-Tyr	4.10	2.70
L-Tyr-Gly	5.01	4.58
L-Ala-L-Tyr	4.67	3.90
L-Tyr-L-Ala	6.79	6.00
L-Arg-L-Tyr	1.97	1.12
L-Tyr-L-Arg	2.98	1.87
L-Lys-L-Tyr	4.36	0.91
L-Tyr-L-Lys	5.04	1.45
L-Glu-L-Tyr	9.83	3.93
L-Tyr-L-Glu	11.8	6.17

Column conditions are the same as Table II except $CH_3CN:H_2O$ is 5:95 and pH=8.85 is by 1.0×10^{-2}M NH_3/NH_4^+ buffer with detection at 215 nm.

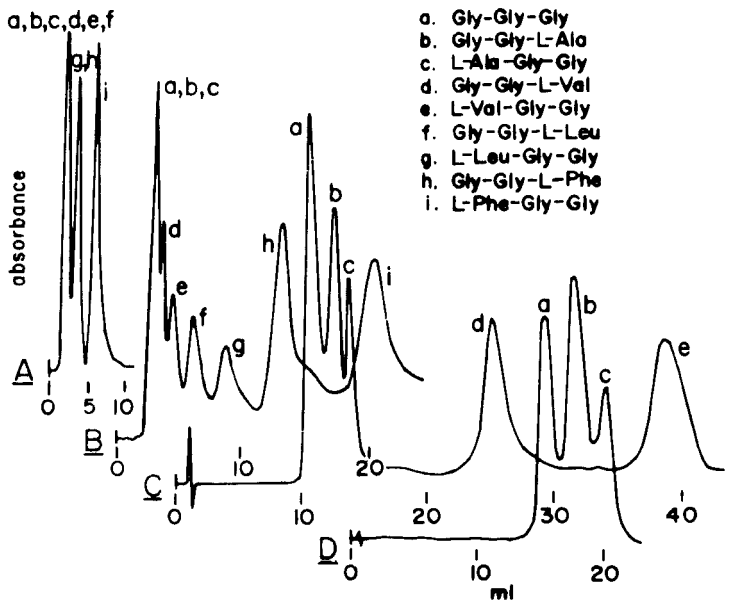

FIGURE 1

Effect of Mobile Phase Variables on Peptide Separations on PRP-1 Using a R_4N^+ Salt as a Mobile Phase Additive

A 4.1 mm x 150 mm, 10 μm, PRP-1 column and a 5:95 $CH_3CN:H_2O$ mobile phase solvent containing (A) pH=11.0 PO_4^{-3} buffer with no R_4N^+ salt, (B) 1.00×10^{-3}M TPeABr, pH=11.0 (PO_4^{-3}), (C) 1.00×10^{-3}M TPeABr, pH=11.0 (NaOH), and (D) 1.00×10^{-3}M TPeAF, pH=11.0 (NaOH) at a flow rate of 1.0 ml/min.

obtained, Figure 1B, when using TPeABr in the mobile phase. However, this mobile phase contains several strong eluent co-anions (18,20) (Br^-, PO_4^{-3}, and Cl^- provided by the R_4N^+ salt, the buffer which yields pH = 11, and NaCl which provides ionic strength control, respectively) which will reduce retention

and affect resolution. By switching to NaOH which provides the necessary pH, PO_4^{-3} is replaced by the very weak eluent co-anion, OH^- (18,20). Thus, resolution in Figure 1C, where only the least retained tripeptides are considered, is significantly improved as a result of increased retention and selectivity. An additional improvement is made by using TPeAF instead of the Br^- salt since F^- is also a very weak eluent co-anion (18,20); this is shown in Figure 1D where the three tripeptides that appear in the first peak in Figure 1B are now separated. Although not shown here a very modest change in CH_3CN:H_2O ratio will also strongly affect retention and resolution, particularly for those analytes that are highly retained.

The observation that the greatest effect on AA and peptide retention is due to ionization of additional acidic sites is consistent with the equilibria indicated in equation 1, particularly the exchange equilibrium shown in equation 1b. It is this exchange selectivity between the analyte anion and the co-anion provided by the R_4N^+ salt or any other salt in the mobile phase which appears to account for the major difference in retention. Although the presence of nonpolar, polar, and basic side chains can influence retention, this effect is small, except for very hydrophobic groups, and the major influence is the result of additional anionic charge sites.

The effect of one or two acidic side chains on dipeptide retention is illustrated in Table IV. When only one acidic

side chain is present the other AA subunit provides a very polar side chain and its effect would be one to also sharply decrease retention in the absence of the R_4N^+ salt. In all cases the mobile phase pH is basic enough so that both the acidic side chain group(s) and the carboxyl terminus are ionized. Thus, all analytes in Table IV are di-or trivalent anions and retention is significantly enhanced due to these additional anionic charges. At the $CH_3CN:H_2O$ ratio used and pH = 11 retention of all the dipeptides is negligible in the absence of a R_4N^+ salt; even at 100% H_2O, pH = 11 retention in most cases is small or barely detectable.

If the mobile phase pH in Table IV is reduced to a pH where the Tyr side chain is not ionized retention is reduced. Retention also correlates to the hydrophobicity provided by the second AA subunit (without the acidic side chain) in the dipeptide. The more hydrophobic the side chain the greater the retention is. Thus, retention for corresponding dipeptides is in the order Ala > Lys > Gly > Arg. For the Glu-Tyr dipeptides three anionic sites are present at pH = 11 and retention of these dipeptides is higher.

It should be noted that retention is always the largest when the acidic side chain is at subunit 1 in the dipeptide or when the side chain anionic site is far removed from the terminal $-CO_2^-$ site. Although not shown in Table IV, this trend was observed when comparing Asp-Gly to Gly-Asp and

Glu-Gly to Gly-Glu. These observations demonstrate that the behavior is not specific to Tyr.

Selectivity in Table IV is very favorable and many separations are possible via the use of a R_4N^+ salt as a mobile phase additive. The separation of a 8-component mixture of tyrosyl dipeptides is shown in Figure 2A where TPAF is used as the mobile phase additive. As in previous separations

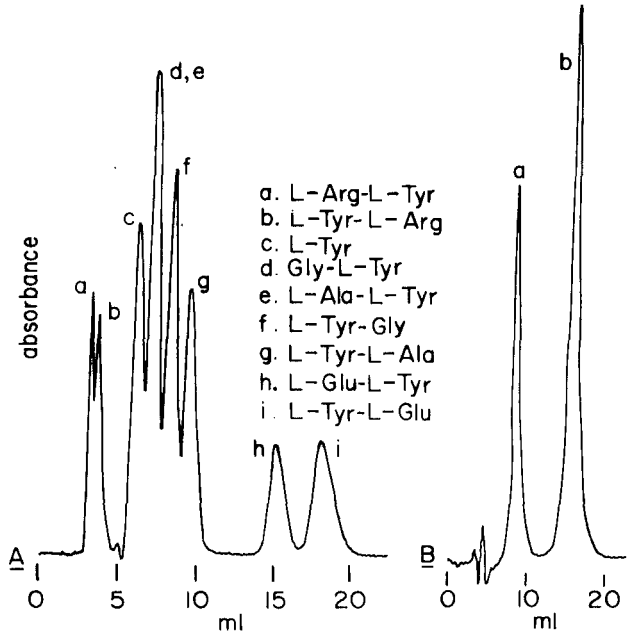

FIGURE 2

Separation of Tyr-Containing Acidic Dipeptides

A 4.1 mm x 150 mm, 10 μm, PRP-1 column and a (A) 5:95 $CH_3CN:H_2O$, 1.00×10^{-3}M TPAF, pH=11.0 (NaOH) mobile phase at a flow rate of 1.0 ml/min; (B) same as (A) except 100% H_2O.

excellent peak symmetry and favorable column efficiency is obtained. Resolution can be improved for given pairs of dipeptides by altering the $CH_3CN:H_2O$. For example, a baseline separation, Figure 2B, is obtained for the first two dipeptides in the separation of Figure 2A by using 100% H_2O. If the TPAF is omitted from the mobile phase in Figure 2 all of these very polar dipeptides would coelute with the dead volume peak on the PRP-1 when using a 5:95 $CH_3CN:H_2O$ mobile phase at pH = 11.0.

Table V lists retention data for several Tyr containing peptides in the presence and absence of TPAF. In basic solution retention of the peptides in the presence of TPAF is greatly

TABLE V

Retention of Tyrosine Peptides on PRP-1 in the Presence of TPAF

	k'			k'	
Peptide	No Salt[a]	TPAF[b]	Peptide	No Salt[a]	TPAF[b]
L-Tyr	0.29	4.46	Gly-Gly-Gly	0.29	1.15
Gly-Gly	0.28	1.07	Gly-Gly-L-Tyr	0.37	6.54
Gly-L-Tyr	0.35	5.86	Gly-L-Tyr-Gly	0.69	8.75
L-Tyr-Gly	0.35	8.00	L-Tyr-Gly-Gly	0.66	10.1

A 10 μm, 4.1 mm x 150 mm PRP-1 column using (a) 100% H_2O, 1.00 x 10^{-3}M NaOH, 1.0 x 10^{-1}M NaCl or (b) a 2.5:97.5 $CH_3CN:H_2O$, 1.00 x 10^{-3}M TPAF, 1.00 x 10^{-3}M NaOH mobile phase at 1 ml/min with detection at 215 nm.

enhanced due to the additional anionic charge of the Tyr side chain. Furthermore, the effect increases as this charged Tyr side chain is moved further from the charged $-CO_2^-$ terminus. Figure 3 demonstrates that TPAF in the mobile phase improves the resolution of peptide mixtures where the peptides have acidic side chains and that a modest change in solvent composition significantly changes retention and alters resolution (compare Figures 3A and 3B where the % CH_3CN is decreased by 2.5%, respectively). Very little retention is observed when attempting to separate the peptides in Figure 3 when omitting the TPAF from the mobile

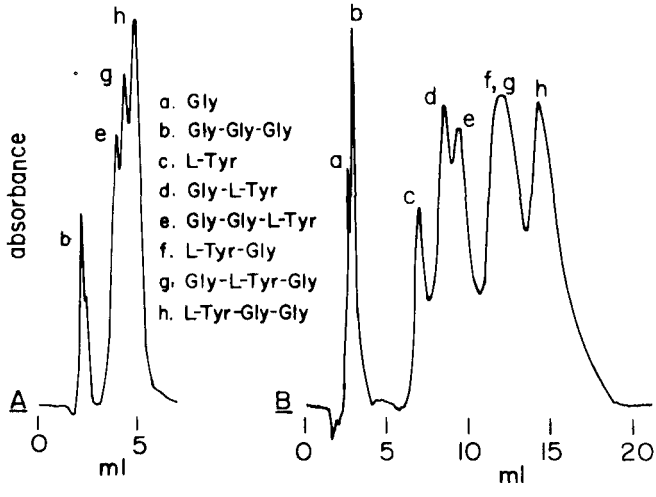

FIGURE 3

Effect of Mobile Phase Solvent Composition on Resolution of Tyr-Containing Acidic Peptides

A 4.1 mm x 150 mm, 10 μm, PRP-1 column and a 1.00 x 10^{-3}M TPAF, pH = 11.0 (NaOH), (A) 5:95 or (B) 2.5:97.5 CH_3CN:H_2O mobile phase at 1 ml/min.

phase. Although not shown in Table V, determination of peptide retention at the zwitterion pH and in the presence of TPAF indicated an enhanced retention over the absence of TPAF. However, the increase does not compare with that obtained at the basic pH.

Retention was determined for a series of Tyr peptides that also contain a Trp subunit as a function of solvent composition in the absence and presence of TPABr at pH = 11. At this pH all the Tyr-peptides are dianions. Since retention is high due to the very hydrophobic side chain from Trp, the Br^- salt was used to provide a stronger eluent co-anion. These data are summarized in Table VI. In the absence of the R_4N^+ salt retention is high and drops off as the CH_3CN concentration increases in the mobile phase. With the R_4N^+ salt present in the mobile phase retention is sharply enhanced and also drops rapidly with increasing CH_3CN in the mobile phase. These trends correlate with the decrease in retention of TPABr, these data are provided elsewhere (6), on the PRP-1 and are consistent with the equilibrium shown in equation 1a. That is, as the CH_3CN concentration increases the number of charged sites on the PRP-1 surface provided by the retained R_4N^+ salt decreases which then also decreases analyte retention. Thus, these data as well as others indicate that optimum retention and resolution require very careful control of the mobile phase solvent mixture and R_4N^+ concentration so that there are at least a minimum number of retained charge sites; our experiments

TABLE VI

The Effect of Solvent Composition on the Retention of Tyrosine Peptides on PRP-1

	Capacity Factor, k'					
	No TPABr[a]		1.00 x 10^{-3}M TPABr[b]			
Peptide 1% CH_3CN	2.5%	10%	7.5%	10%	13%	15%
L-Tyr		0	0.72	0.40	0.25	0.20
L-Trp	8.12	1.61	9.32	4.21	2.07	1.46
L-Trp-L-Tyr	11.5	1.31	27	8.02	2.85	1.85
L-Trp$(Gly)_1$L-Tyr	16.4	1.38	26	7.78	2.76	1.80
L-Trp$(Gly)_2$L-Tyr	18	1.41	26	7.46	2.62	1.79
L-Trp$(Gly)_3$L-Tyr	21	1.56	28	8.05	2.80	1.80
L-Trp$(Gly)_4$L-Tyr	25	1.77	30	8.38	2.87	1.75
	Encaphelins					
L-Tyr$(Gly)_2$L-Phe-L-Leu		3.95[a]			16.1[c]	
$(Gly)_2$L-Phe-L-Leu		6.46[a]			6.25[c]	
L-Tyr$(Gly)_2$L-Phe-L-Met		2.92[a]			12.2[c]	
$(Gly)_2$L-Phe-L-Met		4.77[a]			6.68[c]	

A 10 µm, 4.1 mm x 150 mm PRP-1 column using (a) a CH_3CN:H_2O, 1.00 x 10^{-3}M NaOH, 1.00 x 10^{-3}M NaF mobile phase, (b) a CH_3CN:H_2O, 1.00 x 10^{-3}M TPABr, 1.00 x 10^{-3}M NaOH mobile phase, or (c) same as (b) except TPAF and 12:88 CH_3CN:H_2O at a flow rate of 1 ml/min with detection at 215 nm.

indicate that for a 150 mm PRP-1 column the range is about 10 to 25 μmole/column. The retention minimum in Table VI that occurs in the presence of the TPABr when Gly units are inserted is probably the result of peptide coiling and the subsequent location of the terminal hydrophobic Trp subunit relative to the terminal divalent anionic charge due to the Tyr subunit.

Data for the 4 encaphelin peptides in Table VI illustrate how an R_4N^+ salt can be used to reverse elution order. In the absence of the TPAF the Tyr containing encaphelins are less retained on PRP-1 than the des-Tyr encaphelins at pH = 11. This is due to the anionic charge site from the Tyr subunit and its reducing effect (6) on the hydrophobic type retention that occurs in the absence of the TPAF. In the presence of the TPAF this same anionic charge site causes such a sharp increase in retention for the Tyr encaphelins that they are now more retained than the des-Tyr encaphelins whose retentions undergo little change. This is consistent with an increased exchange effect between the analyte di-anion and the retained TPAF as shown in equation 1b. Chromatograms illustrating this elution reversal, which is so favorable that baseline separations are easily obtained, are shown in Figure 4.

The effect of R_4N^+ salts on the retention of $(L\text{-}Ala)_n$, where n = 1 to 6, was studied. In the presence of TPAF retention was enhanced relative to its absence and increased as n, the number of Ala subunits in the peptide, increased. However, selectiv-

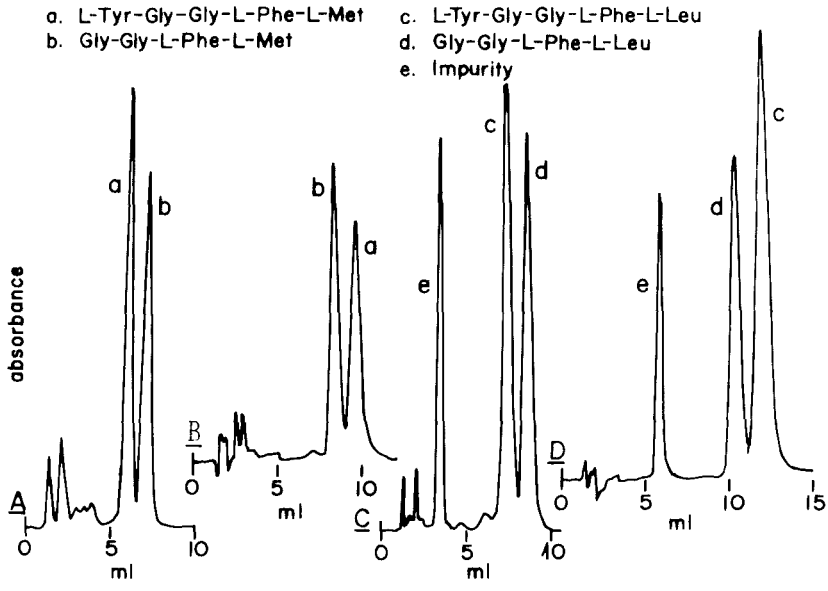

FIGURE 4

Separation of Encaphelin Peptides on PRP-1

A 4.1 mm x 150 mm, 10 μm, PRP-1 column and a (A,B) 13.5:86:5 CH_3CN:H_2O or (C,D) 1:9 CH_3CN:H_2O, pH = 11.0 $(NaOH)_2$ mobile phase in the presence (B,D) and absence (A,C) of 1.00×10^{-3} M TPAF at a flow rate of 1 ml/min.

ity, when compared to mobile phase conditions where the TPAF is omitted (6), does not improve.

When retention of dipeptide diastereomers were studied the presence of a R_4N^+ salt did not improve the separation of L,L and D,D enantiomers from L,D and D,L enantiomers even though retention was enhanced. In most cases selectivity was actually poorer than that obtained in the absence of a R_4N^+ salt (6).

Although the number of examples were limited it appeared that this was also true for dipeptide diastereomers that also contained an AA subunit with an acidic side chain.

Alkylsulfonate Salts

In the presence of a RSO_3^- salt and a mobile phase pH of pH < 2, AA retention is enhanced in comparison to the absence of the RSO_3^- salt with the largest increase occurring for AA with very hydrophobic or basic side chains. At this acidic pH both the amine terminus and basic side chains are in the cation form. The co-cation accompanying the RSO_3^- salt also influences retention. These two trends are illustrated in Figure 5 where retention of several polar AA can be compared in the presence of H^+, Li^+, and Na^+ salts of $C_8SO_3^-$ at pH = 2. Retention for the AA is significantly enhanced since in the absence of the $C_8SO_3^-$ salt retention is zero or barely detectable. Furthermore, unlike AA retention in the presence of R_4N^+ salts, retention in Figure 5 differs for the polar AA studied. This is a useful property since these AA are the most difficult to separate by RPLC in the absence of mobile phase additives. It should be noted that in Figure 5 the mobile phase solvent of 100% water is the weakest eluent solvent possible.

The effect of the added Na^+, Li^+, and H^+, which is provided by the $C_8SO_3^-$ salt, is compared at a 0.001M concentration in Figure 5. The full co-cation effect can not be realized since all three solutions also contain 0.01M HCl (pH = 2) so that the

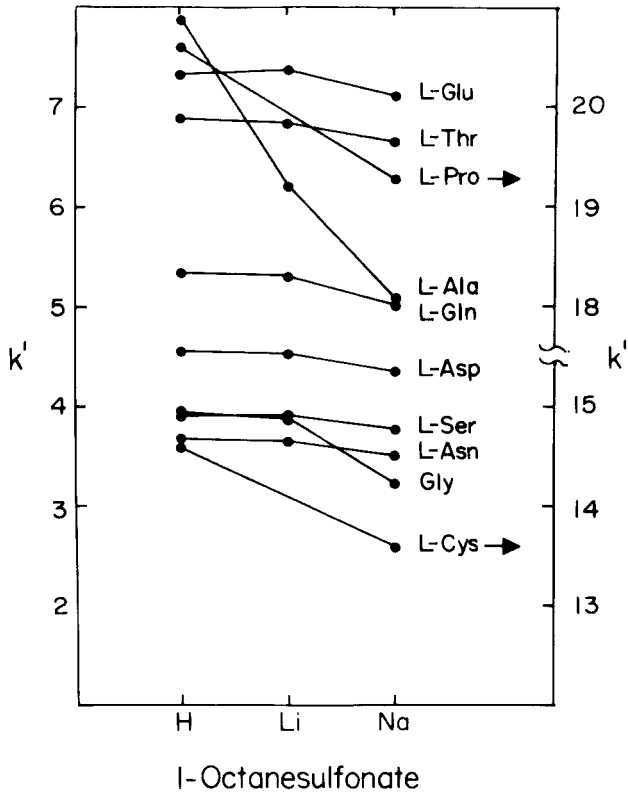

FIGURE 5

Effect of Co-Cation on Amino Acid Retention on PRP-1

A 4.1 mm x 150 mm, 10 µm, PRP-1 column and a 1.0×10^{-3}M $C_8SO_3^-M^+$ where $M^+ = H^+$, Li^+, or Na^+, 1.0×10^{-2} M HCl, 100% H_2O, mobile phase at a flow rate of 1 ml/min.

AA are in their cationic charged form. Even at this low level the co-cation effect, where the co-cation competes in the secondary layer with the AA cation for the $C_8SO_3^-$ anion site in the primary layer (see the exchange equilibria in equation 2b) is evident. Thus, the selectivity follows the order $Na^+ > Li^+ > H^+$, which is typical of the classical ion exchanger selectivity observed for these cations (21). As indicated by Table I, decreasing the alkyl chain length in the RSO_3^- salt decreases retention; the co-cation effect is also still observed.

Figure 6 illustrates the separation of several very polar AA as cations by using a C_8SO_3H, pH = 2, aqueous mobile phase. In the absence of the C_8SO_3H little or no retention is found for these AA, and in general, these are very difficult to separate by RPLC. As indicated in Figure 5, many other separations are also possible. Retention times and resolution are readily altered by using different RSO_3^- salts, modest addition of organic solvent to the mobile phase, and altering the RSO_3^- salt concentration.

Polar dipeptide retention is significantly enhanced on PRP-1 from an acidic, RSO_3^- salt containing mobile phase where the amine terminus is converted into the cationic form. Data illustrating this are shown in Table VII. In the absence of the C_8SO_3Na retention on PRP-1 is barely detected even when using 100% H_2O. The enhanced retention provides a sufficient selectivity so that many polar dipeptides are more easily separated. Two examples are shown in Figure 7. It should be noted that the

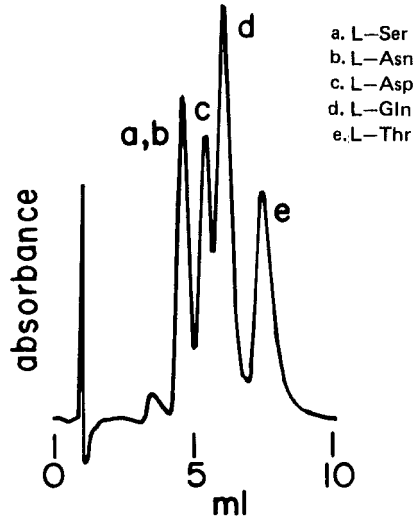

FIGURE 6

Separation of Polar Amino Acids on PRP-1

A 4.1 mm x 150 mm, 10 μm, PRP-1 column and a 1.0×10^{-2}M HCl, 1.00×10^{-3}M $C_8SO_3^-H^+$, 100% H_2O mobile phase at a flow rate of 1.0 ml/min.

polar diastereomers of DL-Ala-DL-Ser (Figure 7B) are readily separated. In both separations no retention is observed in the absence of the RSO_3^- salt. Although the number of examples are limited it appears that an acidic, RSO_3^- salt, mobile phase is superior to a basic, R_4N^+ salt, mobile phase for separating dipeptide diastereomers. The former mobile phase also appears to be superior to RPLC (6) for dipeptide diastereomer separation particularly when the dipeptides contain polar side chains. Also, there is an advantage over RPLC (6) for these kinds of

TABLE VII

Retention of Polar Dipeptides on PRP-1 in the Presence of C_8SO_3H

Peptide/C_8SO_3Na	Capacity Factor, k'	
	0[a]	0.001M[b]
L-Ser-L-Ser	0.12	2.38
Gly-L-Ser	0.13	2.39
L-Ser-Gly	0.12	2.68
L-Ala-L-Ser	0.16	2.77
L-Ser-L-Ala	0.24	4.27
Gly-L-Thr	0.23	3.40
L-Ala-L-Thr	0.24	3.80
Gly-L-Asn	0.12	2.28

A 10 μm, 4.1 mm x 150 mm PRP-1 column using (a) a $1.0x10^{-2}$M HCl, $1.0x10^{-2}$M NaCl, 100% H_2O mobile phase and (b) a $1.0x10^{-2}$M HCl, $1.0x10^{-3}$M C_8SO_3Na, 5:95 $CH_3CN:H_2O$ mobile phase at a flow rate of 1 ml/min with detection at 215 nm.

separations, particularly when separating dipeptide diastereomers that contain polar AA subunits since these are the most difficult to separate. The limited number of examples tested indicate that the L,L and D,D enantiomers always coelute first.

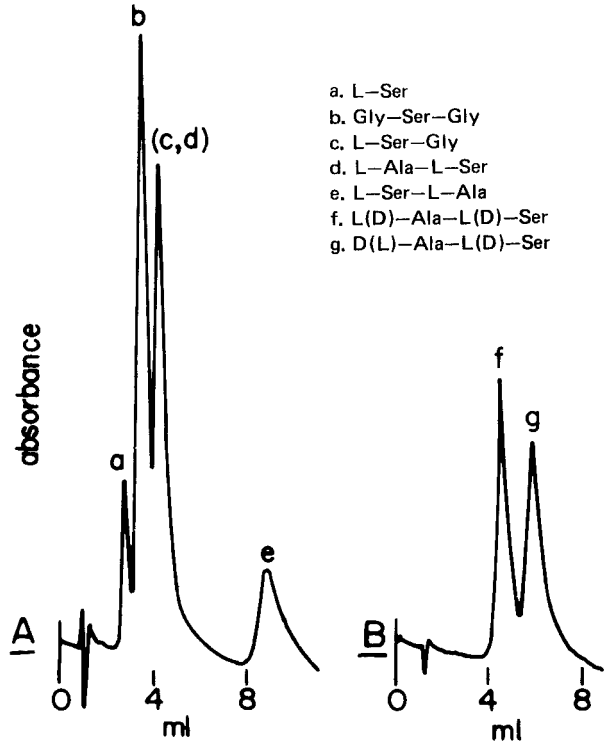

FIGURE 7

Separation of Polar and Diastereomeric Dipeptides on PRP-1

A 4.1 mm x 150 mm, 10 μm, PRP-1 column and a 2.5 x 10^{-2}M PO_4^{-3} pH = 2.2 buffer, 5.00 x 10^{-3}M $C_8SO_3^-Na^+$, 100% H_2O mobile phase at a 1.0 ml/min flow rate.

A major enhancement in retention occurs if the AA, or peptide contains a basic side chain and the mobile phase pH is acidic enough to convert this group, in addition to the terminal amine group, into its cationic form. This additional charge

site (see the exchange equilibrium in equation 2b) accounts for the enhancement. This is illustrated in Table VIII where retention data in the presence of C_5SO_3Na are shown; when the $C_5SO_3^-$ salt is omitted from the mobile phase ittle or no retention is found. The enhanced retention follows the order Arg > His > Lys and is followed (Table VIII) even when considering comparable Gly peptides of the three. Table VIII also reveals that the largest retention is favored when the charged basic side chain is in position 1 in the peptide or is in the same unit as that which provides the terminal $-NH_3^+$ charge site. This is opposite to the results found when using R_4N^+ salts, a basic mobile phase pH, and peptide analytes containing acidic side chain subunits. If the peptide contains several basic side chains, the increased number of charge sites sharply increases retention. This is particularly noticeable when comparing the retention of $(L\text{-}Lys)_n$, where n = 1 to 5, peptides. Data are shown in Table VIII only for n = 2. For n = 3 retention times appear to be at least 90 minutes; if the RSO_3^- salt is omitted no retention is observed.

Several chromatograms, which focus on the separation of peptides containing basic side chains, are shown in Figure 8. In addition to high retention and favorable selectivity (in the absence of the RSO_3^- salt no retention is observed), chromatographic peaks, column efficiency, and analysis times are generally favorable.

TABLE VIII

Retention of Basic Peptides on PRP-1 in the Presence of C_5SO_3Na

Peptide	k'[a]	Peptide	k'[a]
L-Lys	2.15	Gly-Gly-L-His	11.3
L-His	2.70	Gly-L-His-Gly	18
L-Arg	7.70	L-His-Gly-Gly	19
$(L\text{-}Lys)_2$	15.2[b]	L-Ala-L-His	12.9
Gly-L-Lys	6.62	L-His-L-Ala	22
L-Lys-Gly	6.92	L-His-L-His	35
Gly-Gly-L-Lys	8.30	Gly-L-Arg	15.5
Gly-L-Lys-Gly	9.10	L-Arg-Gly	19
L-Ala-L-Lys	8.60	L-Arg-L-Ala	42
L-Lys-L-Ala	16.1	L-Arg-Gly-Gly	32
Gly-L-His	9.50	Gly-L-Arg-Gly	24
L-His-Gly	10.4	Gly-Gly-L-Arg	19

A 10 μm, 4.1 mm x 150 mm PRP-1 column using (a) a 2.5×10^{-3}M C_5SO_3Na, 1.0×10^{-2}M HCl, 100% H_2O mobile phase at a flow rate of 2 ml/min and detection at 215 nm; for (b) the C_5SO_3Na was 1.0×10^{-3}M; no retention in the absence of C_5SO_3Na.

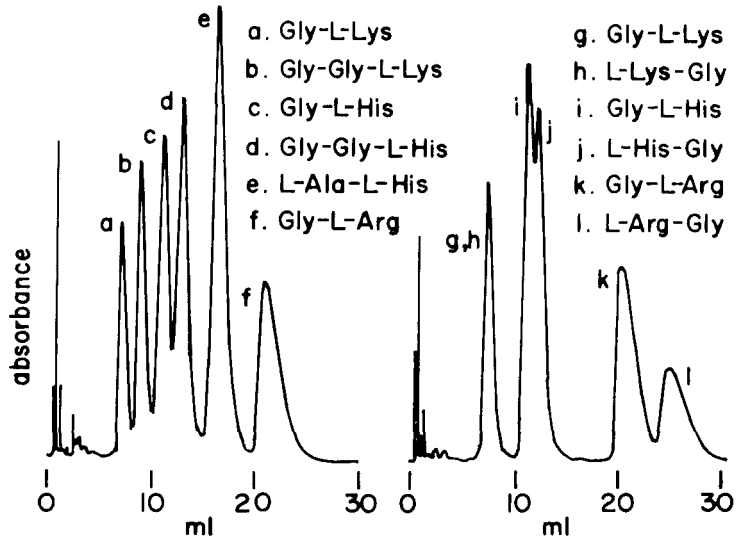

FIGURE 8

Separation of Basic Peptides on PRP-1

A 4.1 mm x 150 mm, 10μm, PRP-1 column and a 5.0×10^{-3}M $C_5SO_3^-Na^+$, 1.0×10^{-2}M HCl, 100% H_2O mobile phase at a flow rate of 2.0 ml/min.

Conclusions

The data reported here indicate that the major interactions influencing retention of AA and small chain peptides on PRP-1 from a mobile phase containing a hydrophobic ion are retention of the hydrophobic ion by the stationary phase and an exchange between its co-ion and the charged form of the AA or peptide. Evidence suggesting ion pair formation as a major contributing factor was not found. There is, however, the possibility that a hydrophobic interaction between the stationary phase and the AA

or peptide can also occur even though the analytes are charged as the result of mobile phase pH control. Since the amount of retained hydrophobic ion is small (usually less than 25 μmole/column), surface coverage on the PRP-1 (surface area is about 420 m^2/g and there is approximately 1 g PRP-1/column) is very small. Experiments with longer chain peptides particularly with those that also contain very hydrophobic side chains, suggest this possibility. Also, recent studies (22), where superficially charged ion exchangers were used as the stationary phase, strongly suggest this added interaction.

These data indicate several other generalizations, in addition to those cited earlier in this report, concerning AA and peptide separations. These are summarized in the following. It is best to start at a hydrophobic ion (R_4N^+ salt in a basic solution or a RSO_3^- salt in an acidic solution) concentration, organic solvent:water ratio, and an ionic strength that gives a loading of about 15 to 25 μmole of hydrophobic ion per column (15 cm). This is controlled by the hydrophobicity of the R groups, the concentration of the hydrophobic ion, the type of organic solvent, and the ratio of organic solvent to water in the mobile phase. From preliminary separations the concentrations of each of these variables can be adjusted to provide the best column performance in terms of resolution and analysis time. The largest effect of the hydrophobic ion in the mobile phase is realized when separating either acidic AA or basic AA or

peptides containing either of these side chains. In general, the RSO_3^- salts in an acidic mobile phase are more versatile than the basic, R_4N^+ salt mobile phases since the former has a more favorable effect on the selectivity of polar AA and peptides. Even diastereomers are more readily separated. Although F^-, OH^-, and H^+ are the weakest eluent co-ions when using R_4N^+ salts or RSO_3^- salts, respectively, and yield the most enhanced retention, stronger eluent co-ions can be used to reduce separation times, particularly when dealing with very highly retained AA or peptides. Finally, elution of AA and peptides in the presence of hydrophobic ions does not necessarily follow the same retention order as in the absence of the mobile phase additive; this is particularly true when the AA or peptides contain acidic or basic side chains and are ionized due to the mobile phase pH.

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REFERENCES

1. Majors, R. E., Barth, H.G., and Lochmüller, C. H., Anal. Chem., 54, 323R (1982).

2. Hearn, M. T. W., Regnier, F. E., and Wehr, C. T., Am. Lab., 14(10), 18 (1982).

3. Regnier, F. E., and Gooding, K. M., Anal. Biochem., 103, 1 (1980); 126, 1 (1982).

4. Pietrzyk, D. J., Cahill, Jr., W. R., and Stodola, J. D., J. Liq. Chromatogr., 5, 443 (1982).

5. Kullman, W., J. Liq. Chromatogr., 2, 1017 (1979).

6. Iskandarani, Z., and Pietrzyk, D. J., Anal. Chem., 53, 489 (1981).

7. Tomlinson, E., Jefferies, T. M., and Riley, C. M., J. Chromatogr., 159, 315 (1978).

8. Hearn, M. T. W., "Advances in Chromatography," J. C. Giddings, E. Grushka, J. Cazes, P. Brown, Eds., Dekker, New York, Vol. 18, 1980,pg 59; Vol. 20, 1982, pg 1.

9. Hearn, M. T. W. and Hancock, W. S., Biological/Biochemical Applications of Liquid Chromatography, Vol II, G. L. Hawk, Ed., M. Dekker, New York, 1979, pg. 243.

10. Kraak, J. C., Jonker, K. M., and Huber, J. F. K., J. Chromatogr., 142, 671 (1977).

11. Hancock, W. S., Bishop, C. A., Prestidge, R. L., Harding, D. R. K., and Hearn, M. T. W., J. Chromatogr., 153, 391 (1978).

12. Rivier, J. E., J. Liq. Chromatogr., 1, 343 (1978).

13. Hearn, M. T. W., Grego, B., and Hancock, W. S., J. Chromatogr., 185, 429 (1979).

14. Hancock, W. S., Bishop, C. A., Battersby, J. E., Harding, D. R. K., and Hearn, M. T. W., J. Chromatogr., 168, 377 (1979).

15. Bennett, H. P. J., Browne, C. A., Solomon, S., J. Liq. Chromatogr., 3, 1353 (1980).

16. Hearn, M. T. W., and Grego, B., J. Chromatogr., 203, 349 (1981).

17. Bidlingmeyer, B. A., J. Chromatogr.Sci., 18, 525 (1980).

18. Iskandarani, Z., and Pietrzyk, D. J., Anal. Chem., 54, 1065 (1982).

19. Smith, R. L., Iskandarani, Z., and Pietrzyk, D. J., Submitted Anal. Chem.

20. Iskandarani, Z., and Pietrzyk, D. J., Anal. Chem., 54, 2427 (1982).

21. Helfferich, F., Ion Exchange, McGraw-Hill, New York, 1962.

22. Iskandarani, Z., and Pietrzyk, D. J., Unpublished results.

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QUANTITATIVE ANALYSIS OF TUBERCIDIN IN STREPTOMYCES TUBERCIDICUS CULTURES BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

The tubercidin in Streptomyces tubercidicus cultures was extracted and detected by High Pressure Liquid Chromatography (HPLC). Using the methanol/water solvent (20/80), the column u-Bondapak C18 (Waters Associates) separated this antibiotic compound well. The detection was performed at 254nm where tubercidin was absorbed. This method provided a rapid and exact analysis for the amount of tubercidin present in cell free culture medium.

INTRODUCTION

The tubercidin, 7-deaza-adenosine ribonucleotide, has antimycobacterial and antitumor activity (1). Tubercidin structure is analogous to that of adenosine and differs by virtue of its unique 7-deaza adnosine base as shown in figure 1.

There are several methods available for determining tubercidin. Smulson (2) described that mixtures of tubercidin, guanosine, etc. were separated by paper chromatography. And Dekker(3) and Uematsu(4) described that nucleotide antibiotics were separated by column chromatography.

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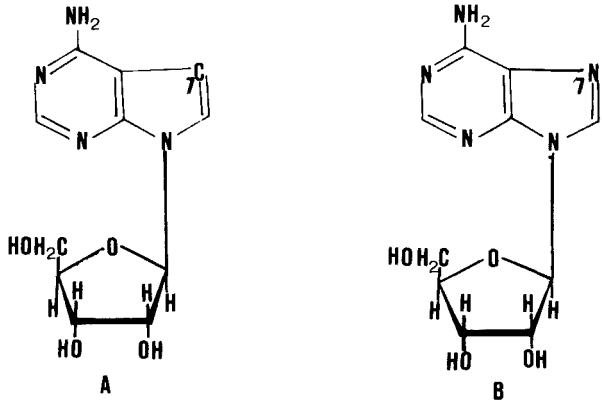

Figure 1
Structures of Tubercidin (A) and Adenosine (B)

The methods described above, however, were not suitable for the determination of tubercidin for several reasons; time-consuming, Complicated sample preparation and difficulty in exact quantitation in individual determination of biological mixtures. In this respect, a new method using HPLC for the quantitative analysis of tubercidin was developed in this present investigation.

MATERIALS AND METHODS

Bacterial Strains

S. tubercidicus ATCC 25502 which was obtained from Dr. Yim in our department, and three auxotrophic mutants, phe^-; Ile^-; val^-, $nico^-$; were used in this experiment. Three auxotrophic mutants were obtained from S. tubercidicus ATCC 25502 with UV and NTG by the procedure of Ochi et al. (5)

Bacterial Culture Medium

a) Seed culture medium ; William's (6) peptone-yeast extract-glucose broth was used.

100ml of seed culture medium contained ; peptone, 0.5g; yeast extract, 0.2g; glucose, 1g; casein hydrolyzate, 0.1g; NaCl, 0.5g. 10ml of seed culture medium was poured into 100ml Erlenmeyer flasks. After the medium was autoclaved at 121'C for 15min and cooled down to room temperature, the test strains were inoculated.

b) Fermentation culture ; modified Vavra's (7) medium was used. Two litres of fermentation medium contained ; peptone, 10g; glycerol, 40g; ammonium sulfate, 5g; Calcium carbonate, 1g; 250ml of culture medium was poured into 1 litre culture flasks. S. tubercidicus seed media were maintained aerobically at 27'C on a rotatory shaker. After 2 days, 0.1% inoculum was added to 250ml fermentation medium.

Sample Preparation

Cell free filtrate sample preparation was performed on the basis of Smulson's method (2).

Sixty hours after inoculation, the medium was filtered by centrifugation (Hitachi Automatic Refrigerated Centrifuge, Hitachi Koki Co., LTD) to obtain cell free sample. Activated charcoal (1g/100ml) was added to the filtrate, after adjustment to pH 8.0 with NH_4OH for 30min, and was removed by filtration. The charcoal was washed with 200ml of 80% acetone pH 2.0 (acidified with IN-HCl).

The aqueous-acetone solution was neutralized with ammonium hydroxide and taken dryness under vacuum. The residue was treated with hot absolute ethanol. The ethanol was evaporated to a small volume (5ml) and the insoluble material was removed. This sample solution was filtered through 0.45 um porosity nucleopore filter (Millipore corp.)

Standard Solution Preparation

Tubercidin (Sigma Co.) standard solution was prepared with 0.01mM and 0.1mM concentrations. Each guanosine, adenosine, cytosine (Sigma Co.) standard solutions were prepared with 0.1mM concentrations and standard mixture solution was prepared with several ratios of above standard solutions.

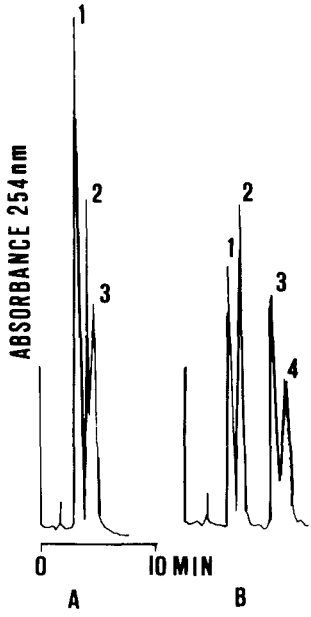

Figure 2

Tubercidin analysis pattern in standard mixture solution by HPLC using 30% Methanol solvent (A) and 20% Methanol solvent (B)
A 1, cytosine + guanosine ; 2, adenosine ; 3, tubercidine
B 1, cytosine ; 2, guanosine ; 3, adenosine ; 4, tubercidine

Analysis by HPLC

Operational conditions of HPLC (Waters Associates Inc. Milford, Mass 01757, USA) were as follow; column, u-Bondapak C18'; solvent, methanol/water (30/70, 20/80); flow rate, 1.0ml/min; detector U.V. model, 254nm; temperature, room temperature. Methanol solvents (for chromatographic grade, Merck) were degassed and filtered through Millipore filter prior to use.

RESULT AND DISCUSSION

As tubercidin is analogous to necleoside, adenosine, we attempted to separate tubercidin from adenosine, tubercidin, cytosine,

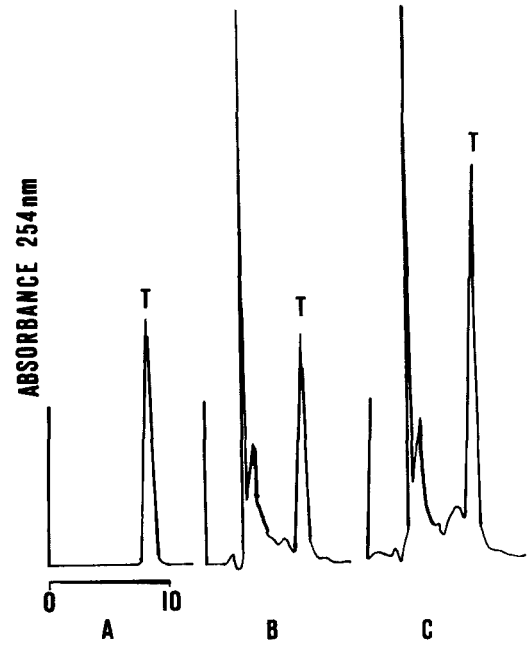

Figure 3

Tubercidin analysis pattern shown in cell filtrate sample by HPLC to show tubercidin peak (T) in tubercidin solution (A)[1], wild type cell filtrate solution (B)[2] and wild type cell filtrate solution plus tubercidin solution (C)[3].
1. 0.01mM tubercidin soln. 20 ul. 2. cell filtrate soln. 5 ul
3. cell filtrate soln. 5 ul + 0.01mM tubercidin soln. 20 ul

guanosine containing solution. Tubercidin in standard mixture solution could be well separated by HPLC using 20% methanol solvent could not separate four compounds completely as shown in figure 2-A. As shown in figure 3-B, tubercidin in cell filtrate samples was also detected by HPLC using 20% methanol solvent. To identify that the appeared peak is the true tubercidin one, standard tubercidin solution was added to the cell filtrate sample. The peak was increased as much as the amount of added tubercidin in turn as shown in figure 3-C. For exact quantitation of tubercidin, we

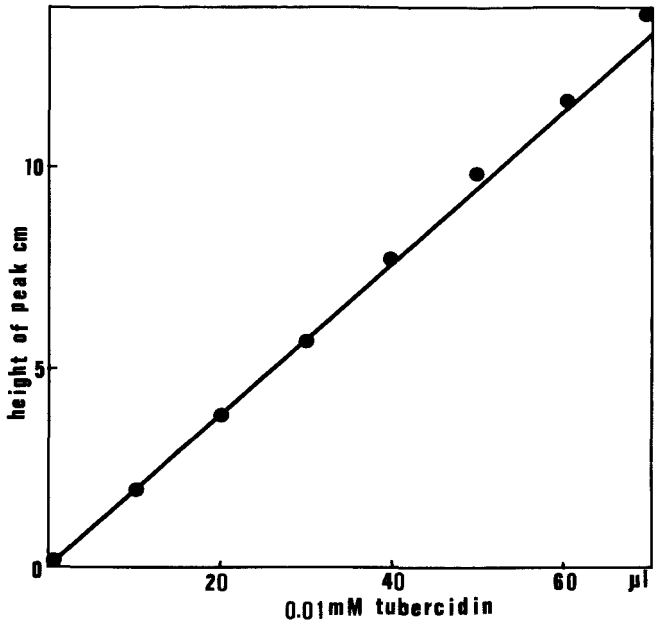

Figure 4

Tubercidin standard curve by HPLC chromatograms using 20% Methanol solvent. 10 ul aliguots of standard tubercidin solution (0.01mM) were injected.

prepared tubercidin standard curve by HPLC chromatograms using 20% methanol solvent, 0.01ml standard tubercidin solution. This result is shown in figure 4. On the basis of above result, we analyzed the tubercidin quantitatively in four strain culture filtrate samples. The result was as follow; Streptomyces tubercidicus ATCC 25502 (wild type), 0.51mg; phe^- auxotroph, 0.28mg; Ile^- auxotroph, 0.634mg; val^-, $Nico^-$ auxotroph 0.45mg per 100ml cell free filtrate samples. This result is shown in figure 5 to compare relative tubercidin amounts. One peculiar characteristic of above result was that Ile^- auxotroph produced more tubercidin than wild type strain.

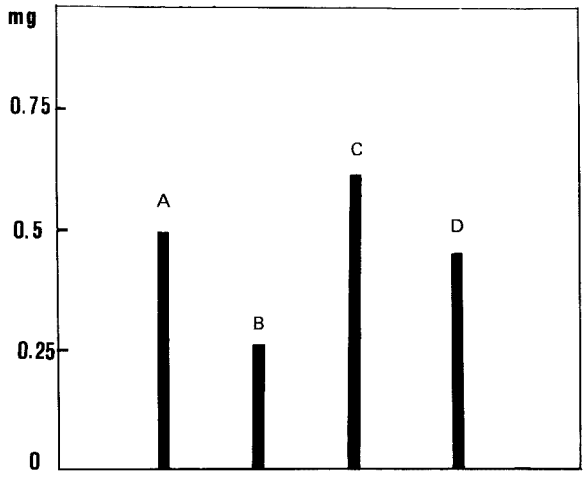

Figure 5

Relative tubercidin amounts produced by S. tubercidicus ATCC 25502 (wild type) and three auxotrophic mutants A, wild type strain; B, phe⁻ auxotrophic mutant ; C, Ile⁻ auxotrophic mutant ; D, val⁻ nico⁻ auxotrophic mutant.
(mg/ 100ml cell filtrate samples)

This fact suggests that strain can be improved by mutation as published by Wesseling et al (8). Other peaks appeared in cell filtrate sample (Figure 3) would be removed by intense purification procedure using paper chromatography or column chromatography etc., but it seems that these peaks do not influence on the quantitative analysis of tubercidin. In summury, the HPLC method described above can be useful for the detection as well as quantitative analysis of tubercidin and other nucleoside antibiotics present in bacterial cultures.

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REFERENCES

1. Buchanan, R. E., and N. E. Gibbon. Bergey's manual of determinative bacteriology. 8th ed. Williams & Wilkins comp. Baltimore 780 (1974)

2. Smulson, M. E., and R. J. Suhadolnik, J. Biol. Chem. 242(12), 2872 (1967)

3. Dekker, C. A., J. Am. Chem. Soc. 87, 4027 (1965)

4. Uematsu, F., and R. J. Suhadolnik, Biochemistry. 9(5), 1260 (1970)

5. Ochi, K., M. J. M. Hitchcock, and E. katz, J. Bacteriology, 139 (3), 984 (1979)

6. Williams, S. T., and T. Cross, Actinomycetes. In Method in Microbiology. C. Bootu, ed. 4 Academic Press, N. Y., 295 (1971)

7. Vavra, J. J., A. Dietz., and B. W. Churchill, Antibiotics and Chemotheraphy. 9, 426 (1959)

8. Wesseling, A. C., and D. L. Barbara, Developments in Industrial Microbiology. 20, 641 (1981)

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ANALYSIS OF THE ANTITUMOR AGENT BAY i 7433 (COPOVITHANE) IN PLASMA AND URINE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Copovithane (BAYi7433) is a synthetic polymer of molecular weight 7,800 daltons with antitumor activity. An analytical method for copovithane in biological fluids involving organic extraction, and hydrolysis and TNBS derivitization of generated methylamine was developed. This method utilizes HPLC for final quantitation of the TNBS-methylamine adduct. The lower limit of detection was 15 g/ml of either plasma or urine. This method was sensitive enough to monitor the pharmacokinetics of copovithane in patients receiving therapy.

INTRODUCTION

Bay i 7433 (copovithane, Fig. 1) is a synthetic copolymer of 1, 3 bis-(methylaminocarboxy)-2-methylene propane and N-vinylpyrrolidone with an average molecular weight of 5,800 daltons. Although uncharged, this high molecular weight material is highly soluble in aqueous solvents. Copovithane was found to have significant antitumor activity against sarcoma 180, P388 leukemia, carcinoma E0771 and fibrosarcoma F1026 in mice and activity against Walker 1098 tumor in rats. Copovithane was found to be a well-tolerated compound which exhibited antitumor activity in animal studies after single injection either prior to or after innoculation of tumor cells (1).

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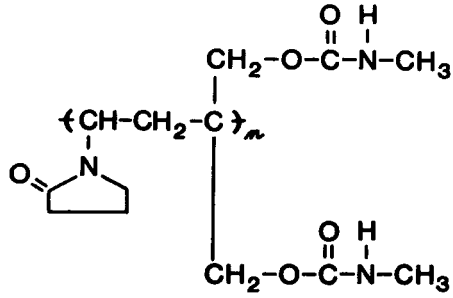

Bay I 7433
Copovithane

FIGURE 1: Subunit structure of copovithane polymer.

The mechanism of antitumor action of this agent has not yet been elucidated. However, copovithane is not cytotoxic in vitro, suggesting that this agent requires metabolic conversion or that this agent operates through changes in host control mechanisms (1). Copovithane is currently undergoing phase I clinical trials and in concert with these trials, we have developed a high performance liquid chromatographic method to assay copovithane in biological fluids in preparation for pharmacokinetic studies in man.

MATERIALS AND METHODS

All materials purchased from regular commercial suppliers were of reagent grade or higher. Distilled-in-glass acetonitrile was purchased from Burdick and Jackson, Muskegon, Michigan. All solvents were filtered, vacuum degassed and sparged with nitrogen immediately before use.

Chromatography. All analyses were performed with a Waters Associates (Milford, MA) liquid chromatograph consisting of a model 710B sample processor, a model M6000 pump, a model 720 system controller, a data module and a model 450 variable-wavelength UV detector. An analytical reverse phase (30 cm x 3.9 mm, 10 μ particle size) C-18 column from Waters Associates was used for all analyses. The mobile phase consisted of 30% acetonitrite and 70% water. The

flow rate was 2 ml/min. The column eluate was monitored for UV absorbance at 340 nm.

Plasma. Aliquots (2 ml) of plasma containing copovithane were placed in 15 ml Corex test tubes and cooled on ice for 5 minutes. Plasma proteins were precipitated by the addition of 200 µl of 10 N perchloric acid. The samples were vortexed vigorously and allowed to stand for 5 minutes on ice. The samples were spun at 17,000 x g for 15 minutes, the supernatents were transferred to glass (12 x 75 mm) test tubes and 200 µl of 10 N KOH was added to neutralize each sample. The samples were again cooled on ice for 5 minutes and then centrifuged for 3 minutes in a Serofuge II centrifuge. The supernatents were transferred to 15 ml Corex test tubes and 2 ml of hot (85°C) saturated sodium chloride solution was added to each sample. The drug was then extracted three times by addition of 3 ml of chloroform (Fisher Science Co., Fairlawn, New Jersey). The samples were vortexed vigorously and then spun at 17,000 x g for 15 minutes. The chloroform extracts were combined and then dried down under a nitrogen stream. The samples were then reconstituted with 1 ml of 5 N hydrochloric acid, quantitatively transferred to a 3.5ml screw-cap vial, sealed and placed in a 160°C oil bath. After 16 hours, the samples were removed, cooled on ice, and adjusted to neutrality with 1 ml of 5 N sodium hydroxide. The pH of each sample was further adjusted to 8.0 by the addition 3 ml of 1 M sodium bicarbonate buffer, pH 8.0. An aliquot (0.75 ml) of a 0.5% solution of trinitrobenzene sulphonic acid (TNBS, Sigma Chemical Co., MO) in acetone was added. The samples were then incubated in the dark for 150 min. and were extracted 3 times with 3 ml of ethyl acetate. The ethyl acetate extracts were combined and evaporated to dryness under a nitrogen stream. The samples were reconstituted in 250 µlof 0.2 M Na_2HPO_4 (pH 6.4) in acetonitrile and chromatographed as described.

RESULTS AND DISCUSSION

Copovithane (BAY i 7433) is a poly-n-methyl carbamate polymer. Since this molecule has little intrinsic UV character, chemical modification of its structure to incorporate a chromophore was necessary to increase sensitivity in the drug assay.

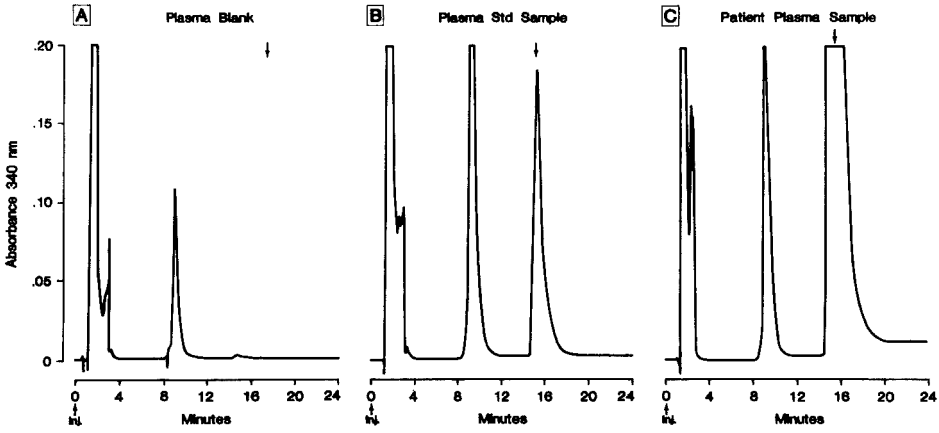

FIGURE 2: High performance liquid chromatography of copovithane.

2A: HPLC of plasma blank. This sample corresponds to injection of 2 ml of plasma.

2B: HPLC of plasma with added copovithane (400 µg/ml).

2C: HPLC of patient plasma sample after administration of copovithane. Quantitation of copovithane in this ample is approximately 150 µg/ml.

Figure 2A shows the high performance liquid chromatogram of a plasma blank. The arrow at 15 minutes indictes the position of the TNBS methylamine adduct derived from copovithane hydrolysis. At this figure shows, the plasma blank contains no peaks which interfere with chromatographic analysis. Figure 2B shows a plasma sample with copovithane added at a concentration of 400 µg/ml. Figure 2C shows a patient plasma sample obtained 1 hour after the administration of copovithane at a dose of 10g/m^2. Copovithane concentration in this sample was calculated to be 400 µg/ml. A standard curve for copovithane in plasma was constructed by the method of standard addition. The results are shown in Figure 3. The constructed curve closely fit (r^2 = 0.995) the calculated line, y = 1.22 x -10.2 over the concentration range 0 to 500 µg/ml. The calculated recovery rate using this method for plasma was 40%. Interassay and intra-assay variability of standards was 19% and 2.3% respectively. The functional lower limit of detection of this method in plasma was 15 g/ml.

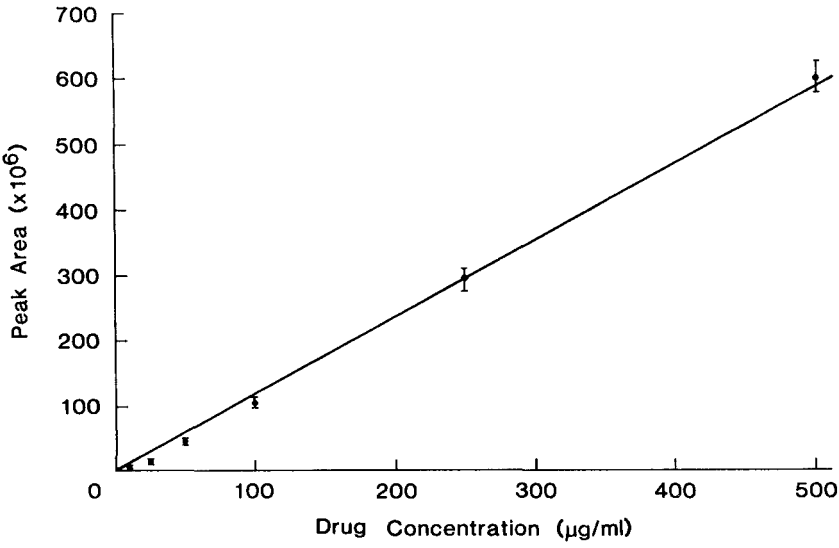

FIGURE 3: Standard curve for copovithane in plasma.

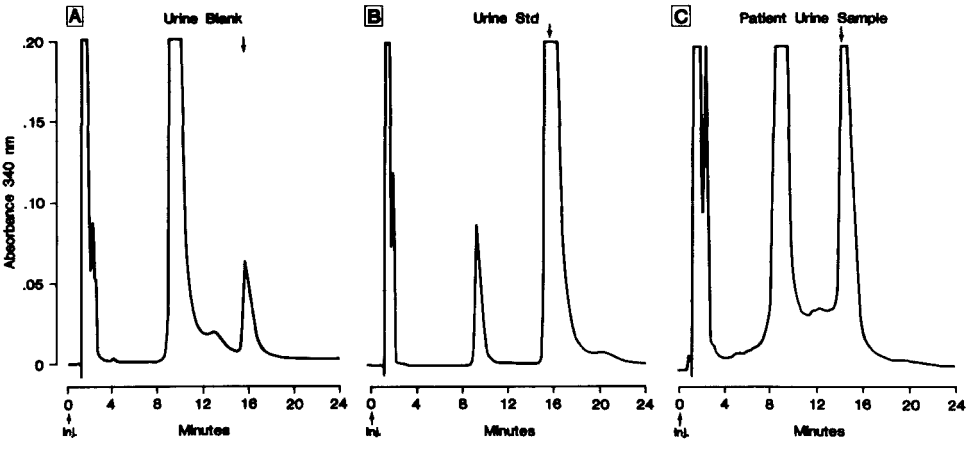

FIGURE 4: High performance liquid chromatography of copovithane in urine.

4A: HPLC of urine blank from a normal volunteer.

4B: HPLC of urine with added copovithane (100 µg/ml).

4C: HPLC of patient urine sample twenty-four hours after drug administration (75 µg/ml).

Incubation of copovithane with plasma at 37°C for 24 hours showed no decrease in drug concentration compared to a freshly prepared sample.

Figure 4A shows the HPLC profile of a urine blank. At approximately 16 min, figure 4A shows the presence of an endogenous peak which interferes with copovithane quantitation at low drug concentration. Attempts to further reduce this interference were unsuccessful.

Figure 4B shows a urine standard with added copovithane at a concentration of 100 µg/ml. The calculated recovery rate using this method was 80% for urine. Figure 4C shows the chromatogram of a urine sample collected from a patient in the first 24 hours after copovithane administration.

For quantitative purposes, a standard curve for urine was constructed also by this method of standard addition. As shown in figure 5, this assay was linear over the entire concentration range tested. Linear regression analysis showed

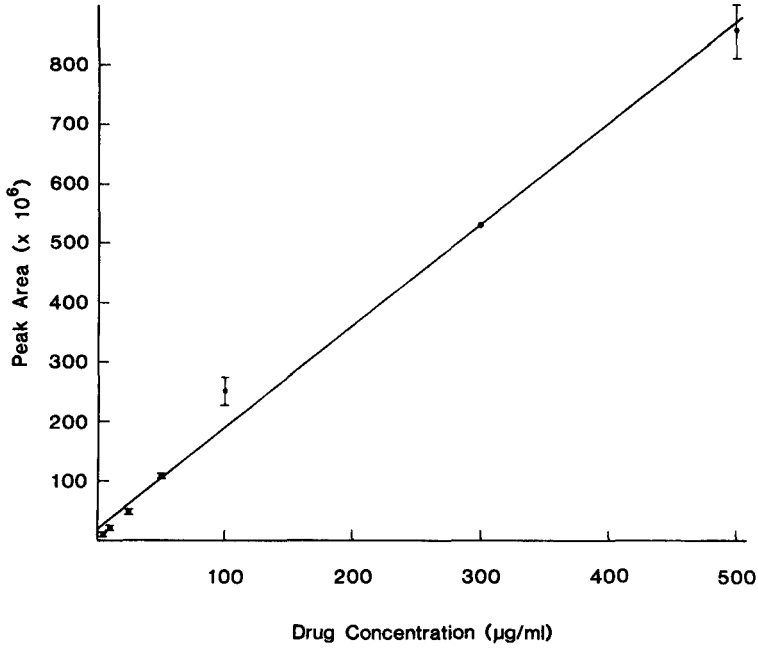

FIGURE 5: Standard curve for copovithane in urine.

that the points closely fit (r^2 = 0.95) a straight line. The lowest limit of detection using this method was approximately 15 μg/ml.

Preliminary pharmacokinetic analysis of plasma obtained from one patient who received copovithane as a 20 minute infusion at a dose of 10 g/m^2 is shown in figure 6. Assay sensitivity limits for plasma were reached after 12 hours. In this one patient, $t_{1/2-\alpha}$ was 18 minutes while the terminal phase $t_{1/2}$ was 180 minutes.

Complete pharmacokinetic analysis of copovithane administration will be presented upon completion of this clinical trial. However, this study shows that the method is sensitive enough to monitor plasma concentrations of copovithane at pharmacologically achievable doses.

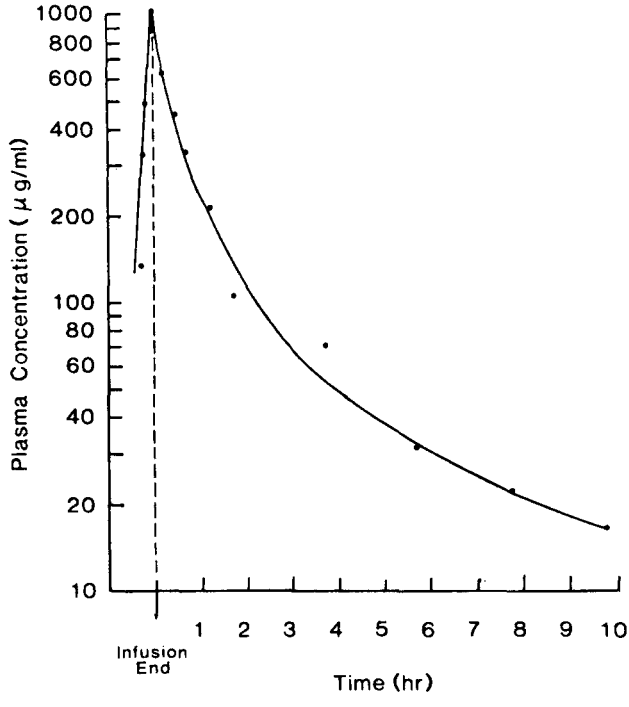

FIGURE 6: Plasma disappearance of copovithane in one patient who received 10 g/m^2 dose as a one-hour infusion.

REFERENCES

1. Schlumberger HD: Bayi7433, a synthetic polymer with antitumor activity. In: Augmenting Agents in Cancer Therapy - Current Status and Future Prospects. E. Hersh and M. Chirigos (eds). Raven Press, 1980.

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DETERMINATION OF FLECAINIDE IN HUMAN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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ABSTRACT

A simple, rapid, selective, and sensitive high-performance liquid chromatographic (HPLC) method for the monitoring of plasma flecainide levels in a therapeutic or research environment is described. The drug is first separated from plasma by a single-step extraction with hexane and then quantitated by HPLC with fluorescence detection. Two linear ranges have been established; 100-2000 ng/ml for drug monitoring in clinical management of patients and 3-300 ng/ml for pharmacokinetic studies. The intra-day variation is less than 6%.

INTRODUCTION

Flecainide acetate [R-818, N-(2-piperidylmethyl)-2,5-bis-(2,2,2-trifluoroethoxy)benzamide acetate] is a new antiarrhythmic agent. Its pharmacologic and therapeutic characteristics have been recently documented (1-3). Flecainide acetate is currently undergoing extensive clinical evaluation world-wide and is

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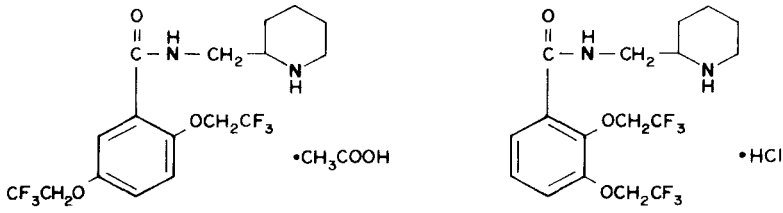

Figure 1. Structure of flecainide acetate.

marketed in West Germany and approved for marketing in the United Kingdom. Plasma level measurements are made to provide better therapeutic management of patients. Existing methods include gas-liquid chromatography (4), fluorometry (5, 6), and high-performance liquid chromatography (7, 8). The chromatographic methods are somewhat complex in procedure and are perhaps best suited for use in a research environment. The fluorometric methods are to be used under well controlled conditions due to their lack of selectivity.

A new method for the monitoring of flecainide levels in human plasma using a single liquid-liquid extraction step and HPLC-fluorescence detection is described. The method is simple, rapid, selective, sensitive, and especially suitable for therapeutic drug monitoring.

MATERIALS AND METHODS

Reagents

The methanol, acetonitrile, and hexane were HPLC grade. The phosphoric acid was analytical reagent grade. The water was deionized.

Chromatography System

The analysis was performed on a modular liquid chromatograph equipped with a ConstaMetric III pump (Laboratory Data Control), a Model 710B Intelligent Sample Processor (Waters Assoc., Inc.), a Model SP4100 Computing Integrator (Spectra-Physics), and a Model RF-530 Fluorescence Spectromonitor for high-speed liquid chromatography (Shimadzu Corp.). The excitation and emission wavelengths were 300 and 370 nm, respectively. A μBondapak phenyl column (30 cm x 3.9 mm, Waters Assoc., Inc.) was used. The mobile phase was prepared by mixing 400 ml acetonitrile and 600 ml aqueous 0.06% phosphoric acid. Prior to use the mobile phase was filtered through a Nylon 66 filter with a pore size of 0.45 μm. The flow rate was 2.2 ml/min.

Preparation of Standard Solutions

Standard solutions of flecainide were prepared by diluting a 10 mg flecainide acetate per liter aqueous stock solution. The concentrations used were 3, 5, 10, 25, 50, 100, 200, 300, 600, 1000, 1600, and 2000 ng in 0.5 ml; 3-300 ng standards were used for low range calibration and 100-2000 ng standards for high range calibration. A positional isomer of flecainide, [N-(2-piperidylmethyl)-2,3-bis(2,2,2-trifluoroethoxy)benzamide hydrochloride], was used as the internal standard. An internal standard solution of 1000 ng/0.5 ml or other concentrations were prepared by diluting a 10 mg per liter aqueous stock solution.

Extraction Procedure

Pipet 1 ml unknown human plasma into a 20 x 150 mm glass culture tube with a polyethylene-lined cap; add 0.5 ml deionized water (for calibration standards, add flecainide in

0.5 ml deionized water to one ml blank human plasma), internal standard in 0.5 ml deionized water, 1 ml 1N NaOH, and 10 ml hexane. Cap the tubes, shake on a reciprocal mechanical shaker (tubes in horizontal position) at a speed of ∿250 cycles per minute for 10 minutes and centrifuge for 5 minutes at ∿900 x g. Transfer 9.0 to 9.5 ml of the hexane phase to a 15 ml conical centrifuge tube. Evaporate to dryness at 60°C under N_2. Reconstitute the residue with 200 µl of the mobile phase and inject 50-150 µl into the liquid chromatograph.

Calibration

A least squares line of the peak height ratios (flecainide/internal standard) versus the flecainide concentrations in the calibration standards was obtained by linear regression. The slope and intercept of the least squares line were used to determine the flecainide concentrations in the unknown samples.

RESULTS AND DISCUSSION

The separation of flecainide and internal standard from the plasma was achieved by a single hexane extraction, and thus, considerably simplifies all existing procedures for sample preparation. Although the procedure of De Jong et al (7) using protein precipitation is also efficient, it does require a high speed centrifuge for the removal of precipitated proteins. Such a high speed centrifuge might not be available in clinical chemistry laboratories.

The internal standard, a positional isomer of flecainide, was well resolved from flecainide (Fig. 2) and there was no interference with either flecainide or the internal standard by endogenous materials from human plasma. The clean chromatogram

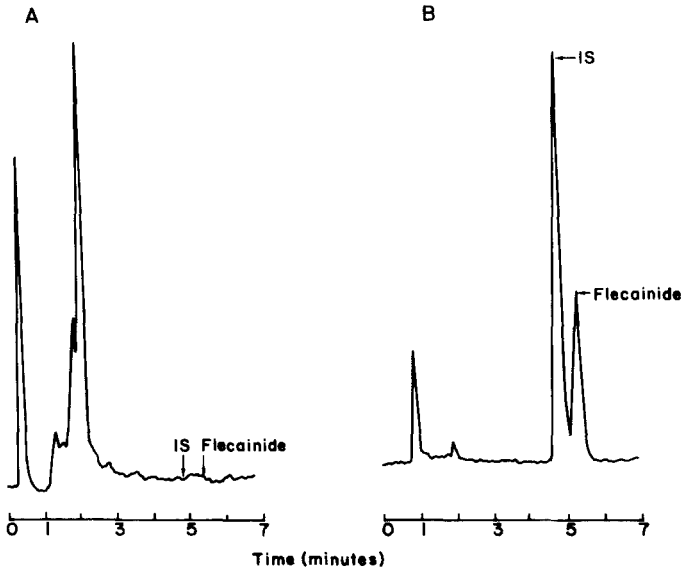

Figure 2. Chromatograms of human plasma samples; A. blank human plasma, 150 µl of total 200 µl of the reconstituted extract was injected; B. blank human plasma spiked with 100 ng flecainide and 5000 ng internal standard, 20 µl injected.

is largely due to the selectivity of fluorescence detection. Two linear ranges were established; 100-2000 ng/ml for therapeutic monitoring and 3-300 ng/ml for pharmacokinetic studies (Fig. 3). The minimal concentration quantifiable is 3 ng/ml with a one ml sample. At 3 ng/ml, the response was at least 10 times that of the background. The linearity was not tested beyond 2000 ng/ml. In the procedure described, 1000 ng internal standard was used for the high concentration range when a fixed aliquot was injected by the autosampler and peak heights measured by an integrator. However, when a strip chart recorder is used and all peaks are to be contained on scale by manipulating the

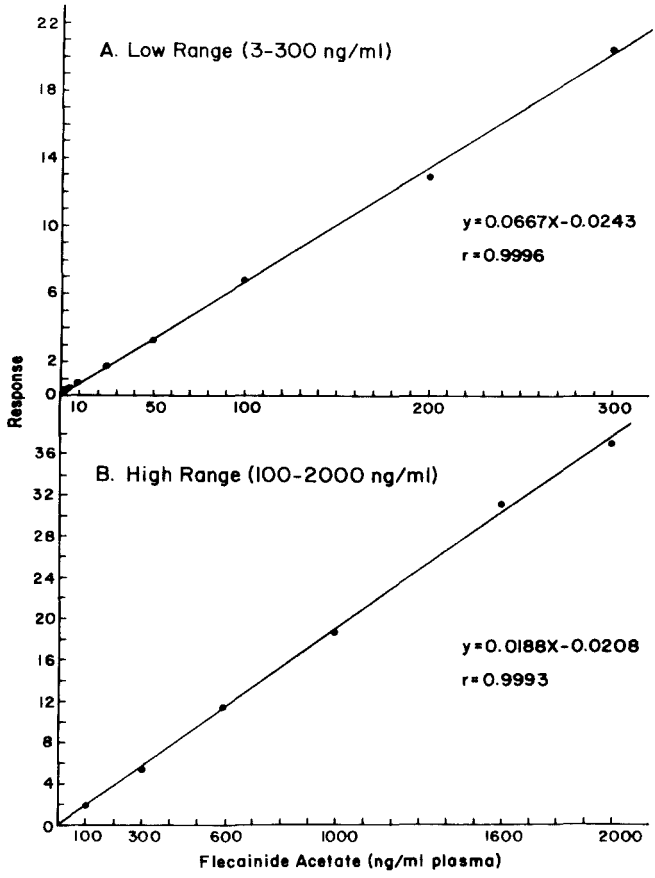

Figure 3. Calibration curves; A. low concentration range, 3-300 ng/ml; B. high concentration range, 100-2000 ng/ml.

size of the aliquot injected or range of the detector, then a larger amount of internal standard should be used -- 2500 to 5000 ng. For the low concentration range, 500 or 1000 ng internal standard is usually used.

Occasionally, some samples of blank human plasma showed a memory peak at about 12 min. after injection. The peak height was generally small and constituted no significant interference with flecainide in the high concentration range. However, for samples in the 3-25 ng/ml range, the injections should be spaced to avoid this memory peak.

Intra-day precision, expressed as the coefficient of variation (CV), was examined at 5, 10, 25, 50, 100, 200, 300, 600, 1000, and 1600 ng/ml. The CV was less than 4% at all levels except at 5 ng/ml, a CV of 6% was found. Accuracy, expressed as relative error, was +6.0 to -7.9% for concentration levels of 10-1600 ng/ml. At 5 ng/ml, the relative error was larger. The intra-day precision and accuracy data are shown in Table 1.

Procainamide, N-acetyprocainamide, propranolol, quinidine, lidocaine, salicylic acid, and disopyramide were tested for potential interference in the assay. These drugs were tested

TABLE 1

Precision and Accuracy

Sample Concentration (ng/ml)	Number of Samples	Coefficient of Variation (%)	Relative Error (%)
5	5	5.9	+30.4
10	5	2.9	+ 6.0
25	5	3.6	- 6.7
50	4	2.1	- 6.8
100	5	3.0	- 7.9
200	5	3.5	- 1.0
300	5	1.9	- 3.3
600	5	2.0	- 3.4
1000	3	1.9	- 3.1
1600	4	1.5	+ 0.4

by direct injection into the liquid chromatograph and their retention times were compared with those of flecainide and internal standard. None of these drugs were found to constitute any interference.

Among the existing methods for the quantitation of flecainide levels in human plasma, the GLC method (4), involving derivatization and detection by electron capture, has been most extensively used. More than 10,000 samples have been analyzed by this method in our laboratory. The new method described here was compared with the GLC method by analyzing 53 patient samples in a parallel manner. The results are depicted in Figure 4. The correlation coefficient (r) was 0.9962 and

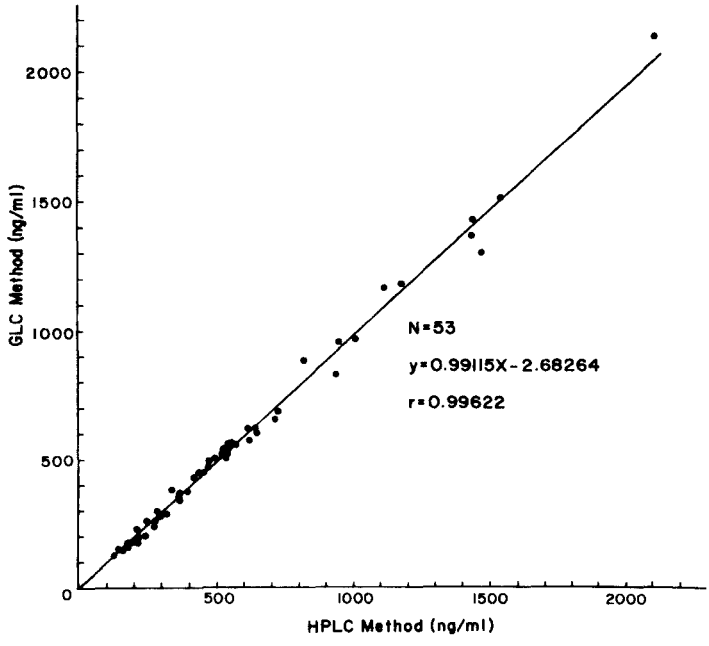

Figure 4. Comparison of the HPLC method using fluorescence detection with an established GLC method for the determination of flecainide in human plasma.

coefficient of determination (r^2) was 0.9924, indicating excellent agreement between the HPLC method and the well established GLC method.

In conclusion, this newly developed HPLC method with fluorescence detection is simple, rapid, sensitive, and selective, and is suitable for monitoring flecainide levels in plasma for the clinical management of patients.

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REFERENCES

1. Anderson, J.L., Stewart, J.R., Perry, B.A., Van Hamersveld, D.D., Johnson, T.A., Conard, G.J., Chang, S.F., Kvam, D.C., and Pitt, B., Oral flecainide acetate for the treatment of ventricular arrhythmias. N. Eng. J. Med., 305, 473 (1981).

2. Duff, H.J., Roden, D.M., Maffucci, R.J., Vesper, B.S., Conard, G.J., Higgins, S.B., Oates, J.A., Smith, R.F., and Woosley, R.L., Suppression of resistant ventricular arrhythmias by twice daily dosing of flecainide. Am. J. Cardiol., 48, 1133 (1981).

3. Hodges, M., Haugland, M.J., Granrud, G., Conard, G.J., Asinger, R.W., Mikell, F.L., and Krejci, J., Suppression of ventricular ectopic depolarizations by flecainide acetate, a new antiarrhythmic agent. Circulation, 65, 879 (1982).

4. Johnson, J.D., Carlson, G.L., Fox, J.M., Miller, A.M., Chang, S.F., and Conard, G.J., Quantitation of flecainide acetate (R-818), a new antiarrhythmic, in biological fluids by gas-liquid chromatography with electron capture detection. Submitted to J. Pharm. Sci., (1983).

5. Chang, S.F., Miller, A.M., Jernberg, M.J., Ober, R.E., and Conard G.J., Measurement of flecainide acetate in human

plasma by an extraction spectrophotofluorometric method. Arzneim. Forsh., 33 (1), 251 (1983).

6. Muhiddin, K.A., and Johnston, A., Spectrofluorometric assay and baccal absorption of flecainide (R-818). Br. J. Clin. Pharmacol., 12, 283P (1981).

7. DeJong, J.W, Hegge, J.A.J., Harmsen, E., and DeTombe, P.Ph., Fluorometric liquid chromatographic assay of the anti-arrhythmic agent flecainide in blood plasma. J. Chromatogr., 229, 498 (1982).

8. Chang, S.F, Welscher, T.M., Miller, A.M., and Ober, R.E., High performance liquid chromatographic method for the quantitation of flecainide, a new antiarrhythmic, in human plasma and urine. J. Chromatogr., 272, 341 (1983).

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PREPARATIVE HPLC FOR THE FACILE ISOLATION OF DRUG GLUCURONIDE CONJUGATES FROM CRUDE EXTRACTS OF URINE

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ABSTRACT

Reverse-phase preparative HPLC has been used to advantage for the isolation of crystalline quantities of drug glucuronide conjugates from crude urinary extracts. Following chronic administration of large doses of diazepam, levorphanol and hydroxyethylflurazepam to dogs, urine was collected and the water soluble drug conjugates adsorbed on a column of Amberlite XAD-2 resin. In each instance elution with methanol and solvent evaporation yielded a crude oil (approx. 3 g) which was chromatographed in one portion on either PrepPAK 500 C_{18} (Waters) or Magnum 40 ODS-3 (Whatman) columns using aqueous methanol solvent systems. A greater than 90% purification was achieved in this single initial chromatographic step. Employing a combination of subsequent semi-preparative HPLC steps on either C_{18} or silica gel columns, milligram quantities of the glucuronides of oxazepam, levorphanol and hydroxyethylflurazepam were isolated. The isolation procedures provide a general approach for obtaining milligram quantities of intact drug conjugates which may otherwise be difficult to obtain by chemical synthesis. Such conjugates can be used as authentic standards in the quantitation of certain drug metabolites in biological media during pharmacokinetic/biopharmaceutic studies.

INTRODUCTION

For many drugs and/or their metabolites conjugation with glucuronic acid to yield water soluble products represents a major

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pathway in their biotransformation and elimination from the body. Alcohols and phenols form ether-type glucuronides, aliphatic and aromatic acids form ester linkages while amines and thiols form N- and S-glucuronides, respectively. Although glucuronides are primarily excreted in the urine, the conjugates may also be excreted via the bile. Since glucuronides are more hydrophilic and usually more acidic than the parent drug they can less easily permeate cell membranes and generally have little, if any, pharmacologic activity compared to the parent drug.

However, although glucuronides may represent the end products in the metabolism of certain drugs in some instances their quantitation in either blood and/or urine provide the only feasible approach for evaluating the pharmacokinetics and/or bioavailability of the parent drug (1). For example a drug may undergo such extensive "first-pass" metabolism on oral administration that the plasma and urine concentrations of the intact drug are non-quantifiable with available methodologies while the glucuronide conjugate may be present in one or both media in high concentrations. Since the highly polar water soluble conjugates are usually difficult to extract into organic solvents without at the same time extracting large amounts of interfering biological substances, the most usual approach has been to cleave the conjugate by hydrolysis with β-glucuronidase and extract the drug moiety into a relatively non-polar solvent for subsequent quantitation.

A more simple approach is offered using reverse-phase HPLC whereby urine samples containing the unhydrolyzed conjugate can be

injected directly onto the column. However, such an approach requires the availability of the pure glucuronide as a chromatographic reference standard in the generation of calibration curves for quantitative purposes.

The objective of the present study was to investigate the utility of preparative HPLC in conjunction with both semi-preparative and analytical HPLC as a relatively simple means of obtaining milligram quantities of drug conjugates which could subsequently be used as analytical standards and thereby avoid certain difficult and time consuming chemical syntheses of such conjugates. The isolation of the glucuronides of oxazepam, levorphanol and hydroxyethylflurazepam, which were required for quantitative analytical purposes, were chosen to develop the chromatographic procedures.

MATERIALS and METHODS

HPLC instrumentation

Preparative chromatography was carried out using a Waters 500A preparative liquid chromatograph (Waters Associates, Milford, MA 01757) attached to an LDC DuoMonitor (LDC, Riviera Beach, Fla. 33404). Semi-preparative and analytical HPLC was performed with an LDC dual pump with a Model 1601 gradient master and a Hitachi variable wave length UV detector.

The types of columns used during the various chromatographies are noted in the text of the experimental sections.

Physico-chemical analysis methods

Proton NMR spectra were obtained with a Varian XL-200 spectrometer (Varian Associates, Palo Alto, Cal.) operating in the Fourier-transform mode.

Low resolution mass spectra (MS) were obtained with a Varian MAT CH5 instrument at 70 eV by direct insection probe. Liquid chromatography-mass spectrometry (LC-MS) was carried out using a Finnigan 1015 MS equipped with a high speed pumping system and a Hewlett-Packard direct liquid insertion (DLI) probe. Glucuronide conjugates were methylated with diazomethane prior to LC-MS.

Drug administration

Diazepam, levorphanol tartrate and hydroxyethylflurazepam were administered orally in solution to beagle dogs (11-15 kg body weight) in doses of 10, 10 and 50 mg/kg, respectively, for five consecutive days. The animals were housed in metabolic cages, allowed food and water ad lib and their urine collected for 7 days. Each separate urine pool was stored at 4° to await analysis.

Extraction of urine

Each urine pool (4-6 liters) was adjusted to pH9 with concentrated ammonium hydroxide and allowed to stand at room temperature overnight. The fine precipitate which formed was removed by filtration following the addition of ca. 20 g/liter of Celite 545 (Johns-Manville) to aid in the filtration process. The clear yellow filtrate was then allowed to flow under gravity through a

1.5 liter bed of Amberlite XAD-2 resin (Rohm & Haas Corp. Philadelphia, PA) in water contained in a 120x 6 cm (i.d.) column equipped with a coarse fritted-glass disc. The resin was then washed with 5 liters of water and any excess water remaining in the resin bed removed with suction through the column outlet. The adsorbed drug conjugates were then eluted from the resin with 3 x 1 liter portions of methanol. The combined methanolic eluates were evaporated in vacuo at 50° to yield a dark brown oil which was reconstituted in 100 ml of water, adjusted to pH9 with ammonium hydroxide and extracted twice with 2 volumes of ether to remove non-polar components. The aqueous phase was evaporated in vacuo to an oil by azeotropic distillation with an equal volume of n-butanol. The extract was stored at 4° to await preparative HPLC.

Monitoring preparative HPLC with analytical HPLC

Prior to any preparative isolation procedures small aliquots of each XAD-2 extract were used to develop a rapid analytical HPLC procedure for monitoring the elution profile of each conjugate from the preparative and semi-preparative columns. Reverse-phase chromatography with a Whatman Partisil-5 ODS-3 RAC column (10 cm x 9.4 mm i.d.) was used for monitoring purposes. Aliquots (10-50 µl) of fractions collected from the preparative columns were injected onto the analytical column using a WISP (Waters) automatic injector system. The solvent system was selected such that each fraction could be monitored in about 5 min.

Isolation of S-(+)-oxazepam glucuronide

The urine extract (3g) was dissolved in water (200 ml), filtered through a 1μ filter and applied to two PrepPAK 500 C_{18} cartridges, linked in series, through the pump of the Waters Prep 500 chromatographic unit. With UV detection at 254 nm the columns were eluted at 100 ml/min with a 1 liter step gradient starting with methanol:water:acetic acid (40:60:1) as shown in Figure 1. While the bulk of the chromogens eluted in fractions 1-10, the fractions (200 ml each) comprising the major peak (12-16) were pooled and evaporated in vacuo to 450 mg of an oil which was rechromatographed on four μBondapak C_{18} (Waters) columns (7.8 mm x 30 cm), linked in series, using a gradient of 20% methanol in 0.025M NaH_2PO_4 to 100% methanol. Elution was monitored with analytical HPLC, the main peak (fractions 95-122) evaporated in vacuo and the solid residue desalted on a column of sephadex LH-20 (Pharmacia) in methanol:water (7:3) to yield a pale yellow oil weighing 40 mg. The oil was rechromatographed on an M-9 Partisil (Whatman) column using chloroform:methanol:acetic acid (80:20:1) at a flow rate of 4 ml/min and fractions collected each min. A yellow chromagen eluted in fraction 4 and oxazepam glucuronide in fractions 6-9. Solvent evaporation yielded 26 mg of a white amorphous powder which could not be crystallized.

The NMR, MS, LC-MS (methyl ester) and UV spectra were consistent with the proposed structure and its HPLC characteristics in

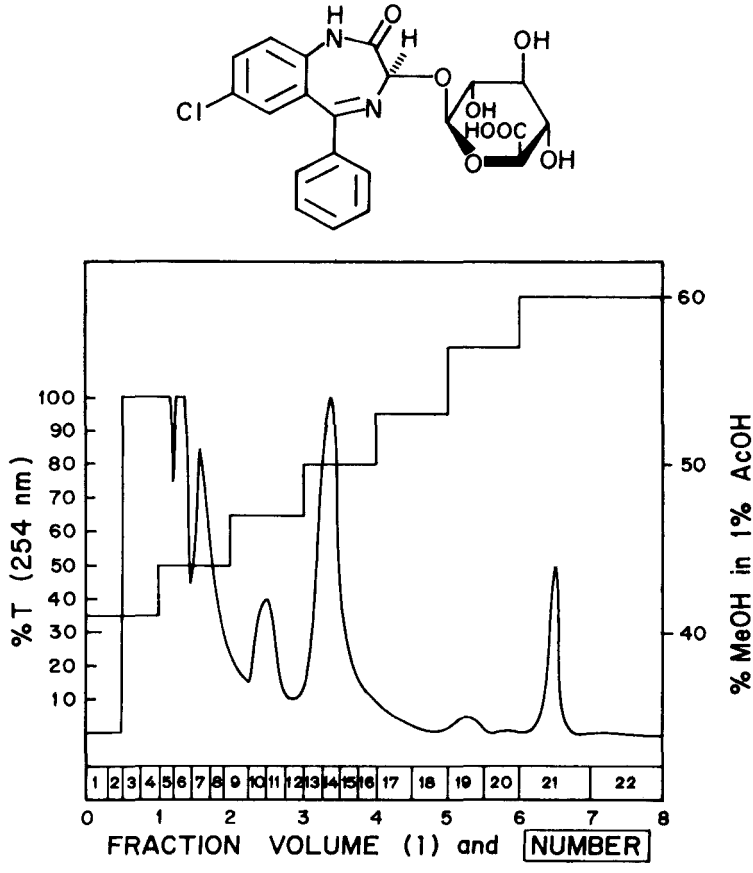

Figure 1. Structure of oxazepam glucuronide and the initial preparative chromatogram obtained using the Prep PAK 500 C_{18} column during its isolation from a crude extract of urine.

agreement with the findings of Ruelius et al. (2) for S-(+)-oxazepam glucuronide.

Isolation of levorphanol glucuronide

One-half of the urinary extract (1.6 g) was dissolved in water, filtered and applied to two PrepPak 500 C_{18} columns as

previously described. With detection at 280 nm, elution was carried out as shown in Figure 2 with a gradient of methanol in 0.01 M NaH_2PO_4 and 200 ml fractions collected at a flow rate of 100 ml/min. The main peak (fractions 26-30) was concentrated in vacuo and desalted on Diaion HP-20 (100-200 mesh: Mitsubishi Chemical Industries, NY, NY 10017) packed in a 24 x 1/2 in. stainless steel column. The sample, dissolved in 300 ml of water, was pumped onto the column, washed with water (100 ml) and eluted with methanol. The fractions containing the desired conjugate, as determined by analytical HPLC, were pooled and concentrated to give a light-brown solid (145 mg). The solid was dissolved in water and chromatographed on an M-9 ODS-2 (Whatman) 50 cm column again using a gradient of methanol in 0.01M NaH_2PO_4 at a flow rate of 2 ml/min. The major peak (280 nm) which eluted was concentrated and desalted on Diaion HP-20 as before and the product crystallized from methanol to yield 32 mg of levorphanol glucuronide mp 216-219°. The NMR, MS, UV and IR spectra were consistent with the proposed structure.

Isolation of hydroxyethylflurazepam glucuronide

The oily urine extract (3.5 g) was dissolved in 200 ml of water, filtered and applied to a 48 mm x 50 cm M-40 ODS-3 column (Whatman). Elution was carried out with an isocratic system of methanol:water:acetic acid (50:50:1) at a flow rate of 50 ml/min and 55 ml fractions collected. The desired metabolite was located by analytical HPLC (fractions 38-56), concentrated to 580 mg of an

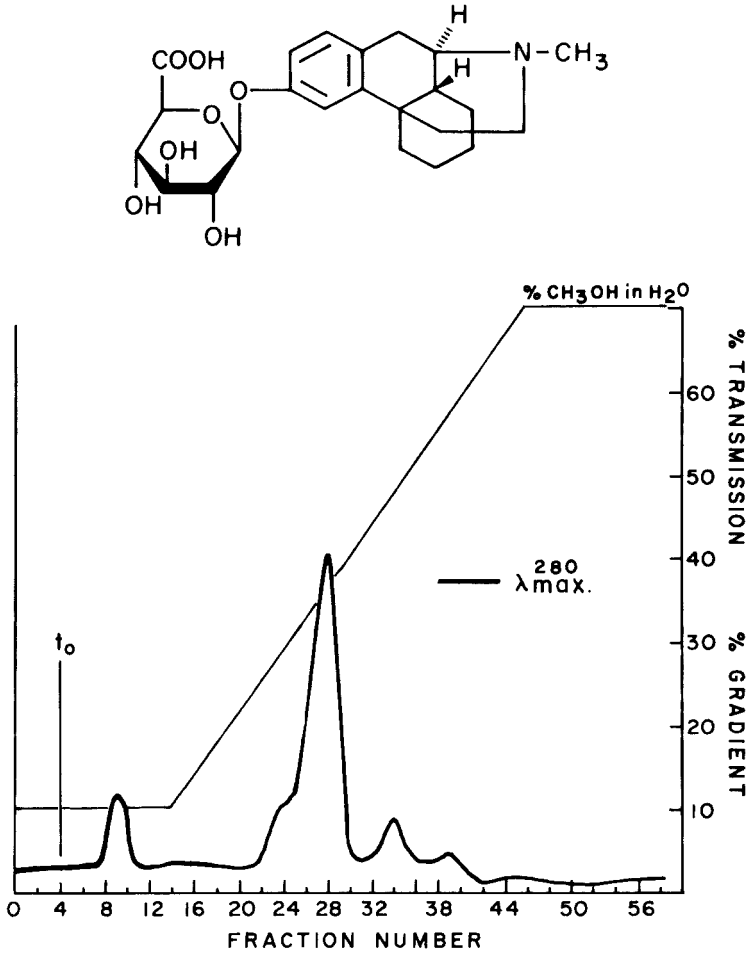

Figure 2. Structure of levorphanol glucuronide and the initial preparative chromatogram obtained using the Prep PAK 500 C_{18} column during its isolation from a crude extract of urine.

oily solid and rechromatographed on the same M-40 column using an isocratic system of methanol:acetonitrile:water:acetic acid (40:10:50:1) with detection at 254 nm. Concentration of the peak fractions yielded 150 mg of a yellow oil which was rechromatographed on an M-9 Partisil-10 column (Whatman) using an exponential gradient from 50% chloroform:hexane (6:4) to 100% chloroform:methanol:H_2O:acetic acid (90:10:1:1). The peak fractions were concentrated to an aqueous solution and lyophilized to give 46 mg of hydroxyethylflurazepam glucuronide as a white powder. Attempts at crystallization were unsuccessful. The NMR, LC-MS (methyl ester) and IR spectra were consistent with the proposed structure (Figure 3).

RESULTS AND DISCUSSION

The present studies demonstrate the utility of preparative HPLC in the isolation of water soluble glucuronide conjugates from crude urine extracts. In particular, preparative HPLC has the necessary capacity to handle such extracts in a single chromatographic run and to provide >90% purification in a single chromatographic step. Other approaches have involved the use of large ion exchange columns (2) or by partition column chromatography on celite (3). Both of the latter procedures are time consuming and have limited capacity in handling large crude extracts.

Two types of preparative C_{18} columns were evaluated; the Waters Prep PAK 500 C_{18} cartridge and the Whatman Magnum-40 ODS-3.

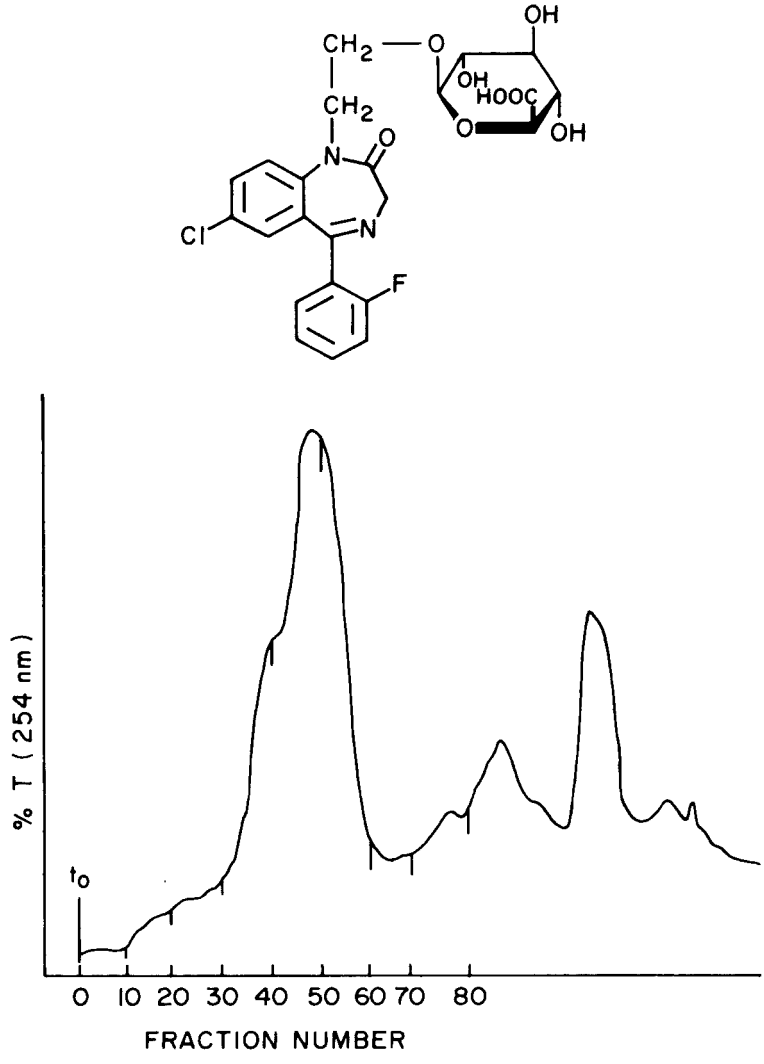

Figure 3. Structure of hydroxyethylflurazepam glucuronide and the chromatogram obtained on a preparative M-40 ODS-3 column during its isolation from a crude extract of urine.

Although satisfactory results were obtained with both types of columns, it has generally been noted, during both the present and other unpublished studies, that a single Magnum-40 column affords better resolution than two PrepPAK 500 columns linked in series, while their capacities are similar. The crude XAD-2 resin extracts were applied to the C_{18} columns as solutions in 200-300 ml of water. In this manner the extracts were evenly adsorbed to the top of the column prior to starting chromatography with the methanol gradient. In some instances it may be necessary to initially dissolve the crude extract in a small volume of methanol prior to dilution with water in order to achieve complete dissolution of the extract.

Following the initial chromatographic step, it now appears, in retrospect, that rechromatography on a Magnum-40 column using a different solvent system is the direction of choice since at this point the glucuronide is still grossly contaminated with urinary pigments and other interfering substances. The latter approach was used in the case of hydroxyethylflurazepam glucuronide. Subsequent chromatography can then be carried out on semi-preparative C_{18} or silica gel columns. One problem noted was that reverse phase chromatography was usually unsatisfactory as a final chromatographic purification step in completely removing urinary chromogens contaminating the glucuronides. For this reason, except in the case of levorphanol glucuronide which crystallized readily, the final chromatographic step was carried out on a silica

gel column which adequately resolves the interfering chromogens. Desalting of fractions containing buffering salts, as in the case of levorphanol glucuronide, was best achieved by HPLC on columns packed with Diaion HP-20 (100-200 mesh) which is similar in nature to XAD-2 as a high porosity copolymer of styrene and divinylbenzene. The procedure is rapid and also offers an advantage over open-column desalting on Sephadex LH-20 (oxazepam glucuronide) or XAD-2 in that a methanol gradient may be readily applied during the elution process which in certain instances affords additional chromatographic purification.

Ruelius et al. (2) have previously reported the isolation of the diastereoisomeric glucuronides of oxazepam from swine urine following chronic administration of large daily doses (50 mg/kg) of oxazepam to the animals. These investigators employed a combination of open-column ion exchange and adsorption chromatography for the isolation of the S(+)-and R(-)-isomers which was considerably more time consuming than the present HPLC procedure. More recently, while the present work was in progress, Seideman et al. (4) isolated S(+)-oxazepam glucuronide from dog urine using HPLC and a similar approach to that used in the present studies. However, it is not apparent as to whether single or multiple chromatographic runs were used at the initial purification step since the column employed has lower capacity than the large preparative columns used here.

In conclusion, preparative HPLC provides a convenient approach for the isolation of milligram quantities of water soluble conjugates from crude urinary extracts. With suitable choice of columns and solvent systems analytically pure material can be obtained in as few as three separate chromatographic runs.

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REFERENCES

1. Gibaldi, M. and Perrier, D. Pharmacokinetics (second edition), Marcel Dekker, New York 1982, p.180.

2. Ruelius, H. W., Tio, C. O., Knowles, J. A., McHugh, S. L., Schillings, R. T. and Sisenwine, S. F., Diasteriomeric glucuronides of oxazepam, Drug Metab. Disp., 7, 40, 1979.

3. Siiteri, P. K., Vande Wiele, R. L. and Lieberman, S., Occurrence of dehydroisoandrosterone glucuronide in normal human urine. J. Clin. Endocr. Metab. 23, 588, 1963.

4. Seideman, P., Ericsson, O., Groningsson, K. and von Bahr, C., Effect of phenobarbital on the formation of diasteriomeric oxazepam glucuronides in man: Analysis by HPLC, Acta Pharmacol. et Toxicol. 49, 200, 1981.

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RAPID DETERMINATION OF APOMORPHINE IN BRAIN AND PLASMA USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A new method for quantitative determination of apomorphine in mouse brain and rat plasma is described. The drug was extracted utilizing SEP-PAK C_{18} cartridge, and quantified by high performance liquid chromatography with electrochemical detector. The average recovery was 92 $\pm$ 2.8% with a day-to-day coefficient of variation of 10.2%. Apomorphine concentration in mouse brain and in rat plasma, as a function of dose and time, after injection with apomorphine-HCl were determined. The results indicate that the method is adequate for pharmacokinetic studies.

INTRODUCTION

Apomorphine (APO) is a drug that has been used for treatment of Parkinson's disease (1), Huntington's chorea (2), tardive dyskinesia (3), spasmodic torticollis (3), Gilles de la

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Tourette's syndrome (4), schizophrenia (5,6) and thalamic pain (7). It is also a direct dopamine receptor agonist in the central nervous system and an important tool in biochemical investigations of dopamine receptor function (8). Because relatively small doses of APO have been used, it has been difficult to measure this compound in biological fluids. Previously reported techniques for determining APO in biological matrixes include: spectrofluorimetry (9), gas chromatography (GC) (10-12), high-performance liquid chromatography (HPLC) (13,14), an enzymatic-radioisotopic method (15), and selected ion monitoring (16,17). All these methods have either a limitation in sensitivity, selectivity or simplicity.

This paper describes a new method for the determination of APO in rodent plasma and brain tissue using SEP-PAK C_{18} extraction procedure and HPLC with electrochemical detector. The method is simple, rapid, sensitive, and suitable for pharmacokinetic studies.

MATERIALS AND METHOD

Chemicals

Apomorphine hydrochloride was obtained from Merck & Co. Inc., Rahway, NJ. N-n-Propylnorapomorphine hydrochloride (PNAPO) was obtained from Sterling Winthrop Research Institute (Rensselaer, NY). SEP-PAK C_{18} cartridges were purchased from Waters Assoc. Inc., (Milford, MA). All

solvents, buffer components and chemicals were of analytical-reagent grade. Water was deionized and then double-distilled in glass.

Apparatus

The high performance liquid chromatography was constructed from four components: M45 solvent delivery system (Waters Assoc., Inc.); LC-3 electrochemical detector with a TL-5 glassy carbon electrode (Bioanalytical Systems, West Lafayette, IN); Model 7125 injection valve (Rheodyne Inc., Cotati, CA); Model LS-44 recorder (Linseis, Inc., Princeton Jct., NJ). A μBondapak C_{18} reverse phase column (Waters Assoc., Inc.) was used with an in-line guard column of 5 μm RP-18 (Brownlee Labs., Santa Clara, CA). The mobile phase was methanol -0.02 M Na_2HPO_4/0.03 M citric acid, pH 3.2 (34:66, v/v). The flow rate was fixed at 0.7 ml/min at ambient temperature and detector potential was set at 0.7 volts vs. the Ag/AgCl reference electrode.

Procedure

Brain. Mouse brain was rapidly removed following decapitation and immediately frozen at -70°C. The whole brain sample was weighed and put into a polypropylene tube containing 4 ml of 0.4 M perchloric acid, 2 μg of PNAPO, as (an internal standard) and 2 mg of sodium bisulfite The mixture was homogenized using Ultra-Turrax, (Janke and Kunkel, West Germany) for 30 seconds, vortexed, and then centrifuged at 27,000 RPM for 10 min (4°C). Extraction was performed using SEP-PAK C_{18} cartridge. The cartridge was

prepared by flushing with 5 ml of H_2O, 5 ml of MeOH through a glass syringe followed by 10 ml of H_2O. One ml of the supernatant of the homogenate was throughly mixed with 2 ml of 0.5 M Na_2HPO_4 buffer (pH 7.0) and then passed through the C_{18} cartridge at a flow rate not greater than 2 ml/min. The cartridge was washed with 5 ml of H_2O followed by 0.2 ml of a mixture prepared by mixing 1 part of MeOH and 1 part of 0.02 M Na_2HPO_4 + 0.03 M citric acid solution of (pH = 3.2). APO and PNAPO were eluted from the cartridge with 1.5 ml of the above mentioned MeoH-Na_2HPO_4-citric acid solution. The eluate (40 µl) was analyzed by HPLC.

Plasma. 0.5 µg of PNAPO was added to 1 ml of rat plasma, mixed thoroughly and passed through SEP-PAK C_{18} cartridge, which was then washed and eluted as described above.

With each set of brain or plasma samples, a standard curve was prepared by adding different amounts of APO and the same amount of PNAPO as samples into a drug-free brain or plasma. The standard samples were analyzed by HPLC as described above. The peak height ratio, APO/PNAPO, was calculated for the concentration of APO in samples. The cartridge was regenerated by flushing with 5 ml of MeOH and 10 ml of H_2O.

RESULTS AND DISCUSSION

Figures 1 and 2 show representative chromatograms of mouse brain and rat plasma. APO and PNAPO have retention

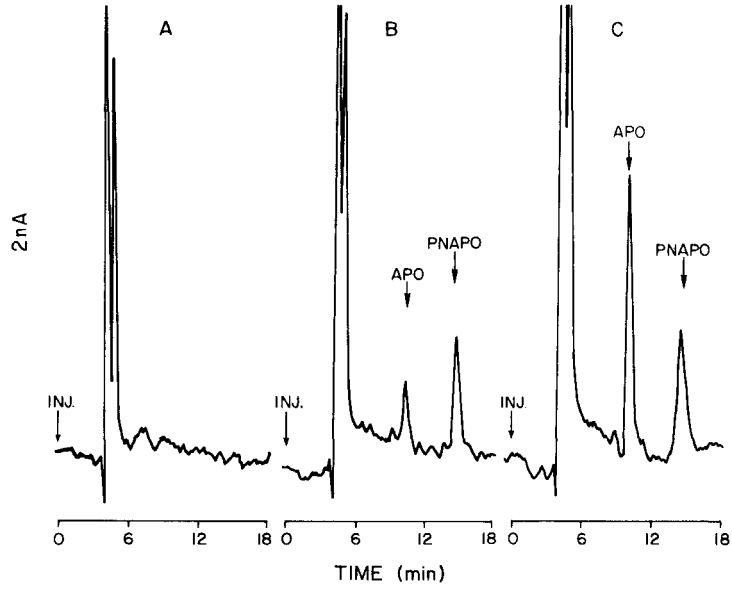

Fig. 1 Chromatograms of mouse brain samples:
(A) brain sample from a drug-free animal;
(B) brain sample after administration of 2 mg/kg of APO;
(C) brain sample after administration of 8 mg/kg of APO.
Conditions were as given in the Materials and Method section.

times of 9.9 and 14.4 min, respectively. No significant interference was found in samples from components not identifiable as APO and PNAPO.

To determine the optimum potential for the assay, a current-potential curve was generated (Fig. 3). The optimum operating potential for this assay was chosen to be 0.7 v. The use of the SEP-PAK C_{18} cartridge for extraction had several advantages over solvent extraction methods (9,14, 16-18). It provided chromatographically cleaner extracts in a single and

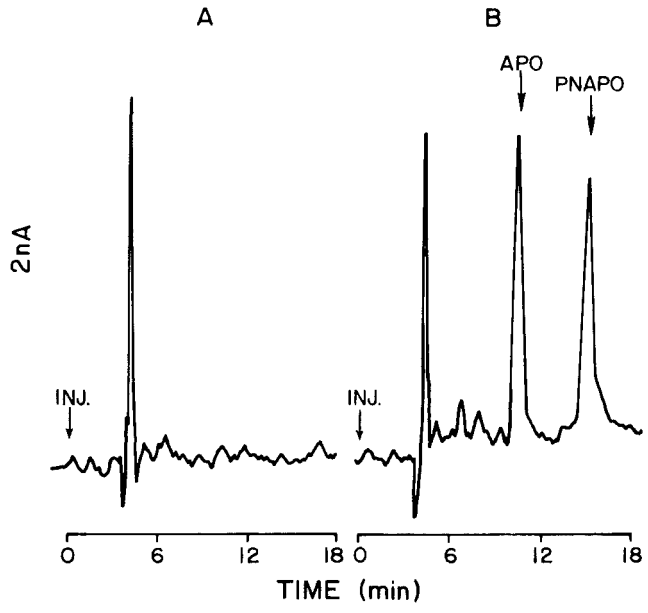

Fig. 2 Chromatograms of rat plasma samples:
(A) plasma sample from a drug-free animal;
(B) plasma sample, 10 minutes after administration of 10 mg/kg of APO.

quick step. Recovery utilizing this method was determined by comparing the current response of spiked drug-free brain homogenate or plasma extracts to that of a standard solution of APO. The average recovery was 92 $\pm$ 2.8% (M $\pm$ SD, N=20) over a concentration range of 0.3 - 3.0 μg/ml. The within-day and day-to-day precision, coefficient variation (CV) were 6.8% and 10.2% (n=10) respectively for samples spiked with 2 μg/ml of APO. The minimum detection, using a signal-to-noise ratio of 3:1, was determined to be 1 ng injected, corresponding to a concentration of 40 ng/ml of plasma and 500 ng/g of brain

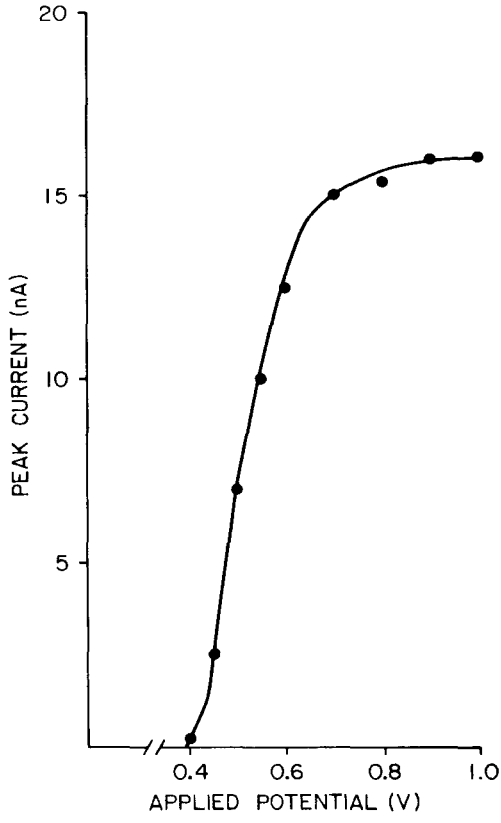

Fig. 3 Current-potential curve for APO.

tissue. Lower levels could be measured by eluting APO from SEP-PAK C_{18} cartridge with 1 ml of MeOH-Na_2HPO_4 buffer, and/or injecting of larger samples into the injection valve. The present method approaches the sensitivity found using a GC/MS method which selected ion monitoring technique (16,17).

As an application of the method to an APO diposition study, mouse brain APO levels 10 minutes after the

TABLE 1. Concentration of APO in the whole brain of two strains of mice after intraperitoneal injection.

	Mouse brain APO concentration*	
Dose of APO/kg	AKR/J	DBA/2J
Control	0	0
2 mg	1.65±0.37	1.33±0.23
8 mg	4.03±1.51	4.01±1.65

*Values are expressed in ug/g wet tissue and mean ± S.D. from 7 animals in each group.

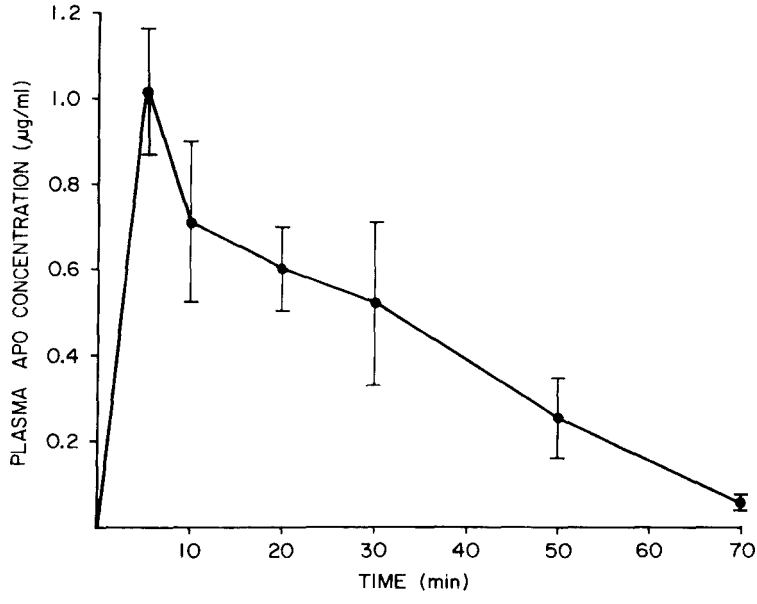

Fig. 4 Plasma APO concentration-time course in rats after intraperitoneal application of the drug 10 mg/kg. Each point represents the mean ± S.D. of four samples.

intraperitoneal administration of 2 mg/kg and 8 mg/kg of APO HCL were examined in different mouse strains. As shown in Table 1, there is no strain difference in APO concentrations, but clear dose effects. Figure 4 shows the time course of rat plasma APO levels following intraperitoneal injection of 10 mg/kg of this drug. These data are consistent with those reported by R.V. Smith et al (19), and H. Watanabe et al (16,20) who used HPLC with UV detector and a selected ion monitoring procedure, respectively. This essay is sensitive enough to detect brain and plasma levels of APO after administration of APO in doses commonly used in animal pharmacology ($\geq$2.0 mg/kg). For clinical doses ($\leq$0.1 mg/kg). Without modification, it is therefore unlikely that this assay will have the requisite sensitivity to detect APO levels in man after clinical doses.

REFERENCES

1. Cotzias, G.C, Papavasiliou, P.S., Fehling, C., Kaufman, B. and Mena, I., N. Engl. J. Med., 282, 31, 1970.

2. Tolosa, E.S. and Sparber, S.B., Life Sci., 15, 1371, 1974.

3. Tolosa, E.S., Arch. Neurol., 35, 459, 1978.

4. Feinberg, M. and Carroll, B.J., Arch. Gen. Psychiatry, 36, 979, 1979.

5. Tamminga, C.A., Schaffer, M.H., Smith, R.C. and Davis, J.M., Science, 200, 567, 1978.

6. Smith, R.C., Tamminga, C.A., and Davis, J.M., J. Neurol. Trans., 40, 171, 1977.

7. Miley, D.P., Abrams, A.A., Atkinson, J.H. and Janowsky, D.S., Am. J. Psychiatry, 135, 1230, 1978.

8. Seeman, P., Tedesco, Lee, J.L., Chan, T., Wong, M., Muller, P., Bowles, J., Whitaker, P.M., McManus, C., Tittler, M., Weinrich, P., Friend, W.C. and Brown, G.M., Federation Proc., 37, 130, 1978.

9. Van Tyle, W.K. and Burkman, A.M., J. Pharm. Sci., 60, 1736, 1971.

10. Smith, R.V. and Stocklinski, A.W., Anal. Chem., 47, 1321, 1975.

11. Miller, J.R., Blake, J.W. and Tobin, T., Res. Commun. Chem. in Pharmacol., 15, 447, 1976.

12. Baaske, D.M., Keiser, J.E. and Smith, R.V., J. Chromatogr., 140, 57, 1977.

13. Smith, R.V., Klein, A.E., Clark, A.M. and Humphrey, D.W., J. Chromatogr., 179, 195, 1979.

14. Smith, R.V., Humphrey, D.W., Szeinbach, S. and Glade, J.C., Anal. Lett., 12, 371, 1979.

15. Kebabian, J.W., J. Neurochem., 30, 1143, 1978.

16. Watanabe, H., Nakano, S. and Ogawa, N., Biomed. Mass Spectrom., 7, 160, 1980.

17. Watanabe, H., Nakano, S. and Ogawa, N., J. Chromatogr., 229, 95, 1982.

18. Eriksson, B-M. and Persson, B-A., J. Chromatogr. 185, 575, 1979.

19. Smith, R.V., Klein, A.E., Wilcox, R.E. and Riffee, W.H., J. Pharm. Sci., 70, 1144, 1981.

20. Watanabe, H., Nakano, S. and Ogawa, N., Pharmac. Biochem. Behav., 14, 493, 1981.

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A FAST CATION EXCHANGE SEPARATION OF IRON(II) AND IRON(III) ON ZIRCONIUM(IV) ARSENOPHOSPHATE COLUMNS USING POTASSIUM THIOCYANATE AS A COMPLEXING AGENT

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ABSTRACT

A new quantitative method has been developed for the separation of ferrous and ferric iron on zirconium(IV) arsenophosphate columns using the cation exchange properties of this material and the complex forming ability of potassium thiocyanate. The method is rapid and requires simply water as the eluant, the results obtained being precise and accurate within the experimental error range (±3%).

INTRODUCTION

Separation of the different valence states of iron has been the subject of several investigations during the past many years using various analytical techniques such as paper chromatography (1-3), electrophoresis (4), solvent extraction (5) and thin layer

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chromatography (6). Ion-exchange chromatography is a convenient and versatile analytical technique for such difficult separations and a survey of literature reveals that mostly anion exchangers have been used for the separation of ferrous and ferric iron (7-10). Only rarely a cation exchanger has been used for this separation (11). Moreover, as far as we are aware, inorganic ion-exchangers have not been used so far for the separation of different valence states of the same metal. The present article summarizes our efforts in this direction which have resulted a rapid and quantitative separation of Fe(II) and Fe(III). Zirconium(IV) arsenophosphate (ZAP) has been selected because it has earlier (12) shown excellent stability and ion-exchange behaviour.

EXPERIMENTAL

Reagents and Chemicals

Zirconyl chloride, tri-sodium orthophosphate, di-sodium arsenate, potassium thiocyanate, ferrous ammonium sulphate and ferric nitrate used in these studies were of AnalaR grade (98-99%) obtained either from the B.D.H.Poole (England) or E.Merck (Darmstadt).

Apparatus

A Pye unicam model SP 2900 atomic absorption spectrophotometer was used for all the determinations.

Synthesis and Ion-Exchange Behaviour of ZAP

ZAP was synthesized by the method reported earlier (12). It was subjected to heating at 200°C for 1 h in a muffle furnace to obtain α-ZAP as a

modified phase (13) showing the Na^+-exchange capacity as 1.03 meq/dry g. This phase has anunusually high selectivity for iron and shows an excellent reproducibility in its ion-exchange behaviour with a high regeneration power in addition to its improved chemical stability (14). It was boiled with 4M HNO_3 to remove any metallic or non-metallic impurities, washed with demineralized water (DMW) and dried as usual before use.

Separation Procedure

The sample solution (1 ml) containing Fe(II) and Fe(III) ions was treated with an excess of potassium thiocyanate (1M) solution and the red solution, thus obtained, was loaded on a column of the ion-exchange material (2 g) in a glass tube (internal diameter $\sim$ 0.6 cm). The elution was performed with 15 ml DMW to remove ferric ions while ferrous ions were eluted out with $\sim$ 25 ml of 2M HNO_3 at a flow rate of 3.5 ml per minute. The effluents were analyzed for their iron contents by atomic absorption spectrophotometry, the results being summarized in Table 1.

RESULTS AND DISCUSSION

The main feature of this study has been to devise a fast analytical procedure for the quantitative separation of Fe(II) and Fe(III). Depending upon its concentration thiocyanate gives a series of intensely red-coloured soluble complexes (15) with Fe(III), formulated as $[Fe(SCN)_n]^{3-n}$ where n = 1 .... 6. At a very high thiocyanate concentration, the complex formed is $[Fe(SCN)_6]^{3-}$. Thus, when to a mixture

TABLE 1

Quantitative Separation of Fe(II) and Fe(III) on α-ZAP Columns

Sl. No.	Amount of iron loaded (μg)		Amount of iron determined* after separation (μg)		Error, %		Standard deviation, %	
	Fe(II)	Fe(III)	Fe(II)	Fe(III)	Fe(II)	Fe(III)	Fe(II)	Fe(III)
1.	112	112	110.32	115.00	-1.50	+2.68	0.14	0.08
2.	168	168	165.20	171.36	-1.70	+2.00	0.03	0.07
3.	224	224	221.41	229.16	-1.16	+2.30	1.01	0.94
4.	280	280	273.10	286.70	-2.46	+2.39	0.67	0.38
5.	560	560	545.96	576.80	-2.51	+3.00	0.19	0.43
6.	56.0	280	53.410	283.10	-1.05	+1.11	0.29	1.81
7.	280	56.0	275.20	59.910	-1.71	+6.98	0.44	1.36

*Mean value of five replicates.

containing Fe(II) and Fe(III) ions, an excess of concentrated potassium thiocyanate solution is added, ferric iron is complexed to give negatively charged species. On passing this red colored solution through a column of α-ZAP in H^+-form, ferric iron is removed simply by washing with DMW. Ferrous is then eluted out with 2M HNO_3. The method is quite fast and specific for this separation. It requires only 10-15 minutes for the complete process.

As Table 1 indicates the method gives reproducible and accurate results. However, the assays tend to be slightly higher for iron(III) and lower for iron(II). Also, a higher Fe(II)/Fe(III) ratio in the sample solution results in a higher error range for Fe(III). It may be due to some auto-oxidation of Fe(II) by the residual air in the solvent, as observed earlier by other workers (4,5) for this separation.

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REFERENCES

1. Stevans,H.M., Anal.Chim.Acta, 15, 538, 1956.

2. Pollard,F.H., McOmie,J.F.W., Banister,A.J. and Nickless,G., Analyst, 82, 780, 1957.

3. Qureshi,M., Akhtar,I. and Varshney,K.G., Anal. Chem., 38, 1415, 1966.

4. Lingren,W.E., Reeck,G.R. and Marson,R.J., Anal. Chem., 40, 1585, 1968.

5. Lieser,K.H. and Schroeder,H., Z.Anal.Chem., 174, 1960.

6. Johri,K.N. and Mehra,H.C., Chromatographia, 4, 80, 1971.

7. Kraus,K.A. and Moore,G.E., J.Am.Chem.Soc., 75, 1460, 1953.

8. Pollard,F.H., McOmie,J.F.W., Nickless,G. and Hanson,P., J.Chromatogr., 4, 108, 1960.

9. Pollard,F.H. and Nickless,G., J.Chromatogr., 4, 196, 1960.

10. Sagortachew,B., Chem.Anal. (Warsaw), 17, 973, 1972.

11. Popa,G., Vladescu,L. and Preda,E., Rev.Chim., 28, 582, 1977.

12. Varshney,K.G. and Premadas,A., Sepn.Sci. & Technol., 16, 793, 1981.

13. Varshney,K.G., Varshney,K. and Agrawal,S., Sepn.Sci. & Technol., (in press).

14. Varshney,K.G., Agrawal,S. and Varshney,K., J.Liquid Chromatogr., (in press).

15. Vogel,A.I., A Textbook of Quantitative Inorganic Analysis, Longman Inc., New York, 1978, p. 741.

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PAPER CHROMATOGRAPHIC BEHAVIOR OF SOME TERTIARY AMINE POLLUTANTS AND AN ATTEMPT AT STRUCTURE-ACTIVITY-CORRELATION

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ABSTRACT

Ascending paper chromatography of some pesticides and toxicants containing a tertiary amino group has been performed to record their mobilities in acidic, basic and saline waters and some common organic solvents, Several binary separations of these pollutants have been achieved. The important separations are summarized below:

Amitrole from azobenzene, bavistin, calixin, 2,4-Lutidine, 2,6-Lutidine, N-ethylmorpholine, β-picoline, γ-picoline and quinoline; Azobenzene from bavistin, calixin, 2,4-Lutidine, 2,6-Lutidine, β-picoline, γ-picoline, pyridine and quinoline; Bavistin from 2,4-Lutidine, 2,6-Lutidine, β-picoline, γ-picoline and quinoline; and Quinoline from 2,4-Lutidine, 2,6-Lutidine, β-picoline, γ-picoline and N-ethylmorpholine from binary mixtures. An attempt at structure-activity-correlation reveals that the physical forces responsible for both the chromatographic

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behaviour as well as the toxicity are almost identical and the most important common physical interaction seems to be the hydrogen-bonding.

INTRODUCTION

Tertiary amine pollutants are found in varying quantities in samples of air, water and soil. One of their important characteristics is their carcinogenicity. Though the literature is replete with analytical, physico-chemical and chemical data on polycyclic aromatic hydrocarbons (PAH)(1) comparatively little progress has been made with tertiary amine pollutants and as far as we are aware there has been negligible attempt at structure-activity-correlations for this class of compounds. In primary attempts in this direction from our laboratories, a simple and inexpensive spot-test (2) was developed for the detection of these compounds in neutral, saline acidic and basic waters and a spectrophotometric method has been developed for the near-specific analysis of 2,6-Lutidine - an important member of this class (3).

The classical technique of chromatography i.e., column, paper and thin layer though on the decline due to the development of more powerful and sophisticated techniques i.e., HPLC and GC, certainly offer certain unique advantages as they can be used to separate complex mixtures into simpler fractions prior to further separation by high resolution techniques and identification of mixture constituents (4). Paper chromatography is a versatile technique due to its inherent simplicity and gives better resolution than column chromatography (5). Separations difficult in columns can be carried on papers often with success. A further advantage stems from the fact that paper techniques can often separate compounds with identical uv-absorption spectra (6). The disadvantages of paper chromatography are irreproducible papers,

often inadequate resolution and poor quantification (7). However, the disadvantages are not applicable to the development of structure-activity-correlations where only broad trends of various members of a family are considered. Furthermore the extreme simplicity and very modest cost makes it an ideal technique for routine analysis especially in third world countries.

The present communication summarises the separations and mobilities of tertiary amine pollutants by paper chromatography and an attempt has been made at a structure-activity-correlation.

EXPERIMENTAL

Reagents

Prepare aqueous or ethanolic solutions of the test substances (1M). When it is not possible to prepare 1M solution, prepare saturated solution. Prepare also aqueous saturated solution of phosphomolybdic acid, sodium carbonate and saturated solution of copper iodide in acetonitrile. All chemicals and reagents were of ANALAR grade obtained from BDH (England) and SIGMA (USA) except: bavistin [2-(methoxycabomoyl) benzimidazole 50% W/W; adjuvants add 100% W/W], and calixin (N-tridecyl-2,6-dimethylmorpholine 80% W/W; adjuvants 20% W/W), BASF, Aktiengesechaft, West Germany. Whatman No. 1 filter papers.

Apparatus

Ordinary 20 x 5 cm, glass jars were used to develop the paper 14 x 3 cm strips. UV-lamp was used for irradiating the spot to detect the fluorescent spot.

PROCEDURE

The ascending technique was applied in these studies. The papers were spotted for the pollutants, hung in the

glass jars and developed in the various types of waters and organic solvents. The pollutants were detected as yellow spots by dipping the strip in the solution of phosphomolybdic acid or by dipping in the solutions of copper iodide and sodium carbonate respectively followed by irradiation with uv-light.

RESULTS

The chromatographic behaviour of the following 12 pesticides (P) and toxicants (T) have been studied. 1. (P) Amitrol (3-Amino-1,2,4-triazole), 2. (P) Azobenzene (Diphenyldiazene), 3. (P) Bavistin (2-methoxy-carbamoyl benzimidazole), 4. (P) Calixin (N-tridecyl-2,6-dimethyl morpholine), 5. (T) 2,4-Lutidine (2,4-dimethyl pyridine), 6. (T) 2,6-Lutidine (2,6-dimethyl pyridine), 7. (T) N-Ethyl-morpholine, 8. (T) β-Picoline (3-methyl pyridine), 9. (T) γ-Picoline (4-methyl pyridine, 10. (T) Pyridine, 11. (T) quinoline and 12. (T) Trimethylamine. The solvent systems used were: 1. Distilled water, 2. 2%, 1% and 0.1% HCl, 3. 2%, 1% and 0.1% HNO_3, 4. 2%, 1% and 0.1% NaOH, 5. 2%, 1% and 0.1% NaCl, 6. 2% NaH_2PO_4, 7. 2% $KHSO_4$, 8. 2% Na_2CO_3, 9. 2% $NaNO_2$, 10. 2% $NaNO_3$, 11. 2% $Pb(NO_3)_2$. 12. Chloroform, 13. Carbontetrachloride, 14. Benzene, 15. Chlorobenzene and 16. Butyl alcohol.

The chromatographic behaviour of these compounds is illustrated in figure 1 by plotting R_f values against the solvents used. On the basis of the R_f values, several separations have been achieved.

DISCUSSION

Application of paper chromatography for the separation of organics containing a tertiary amino group using phosphomolybdic acid as a detector is documented in the Lederer's book of "Chromatography"(8) and the "Analytical Chemistry of Nitrogen and its Compounds"(9). Wagner and

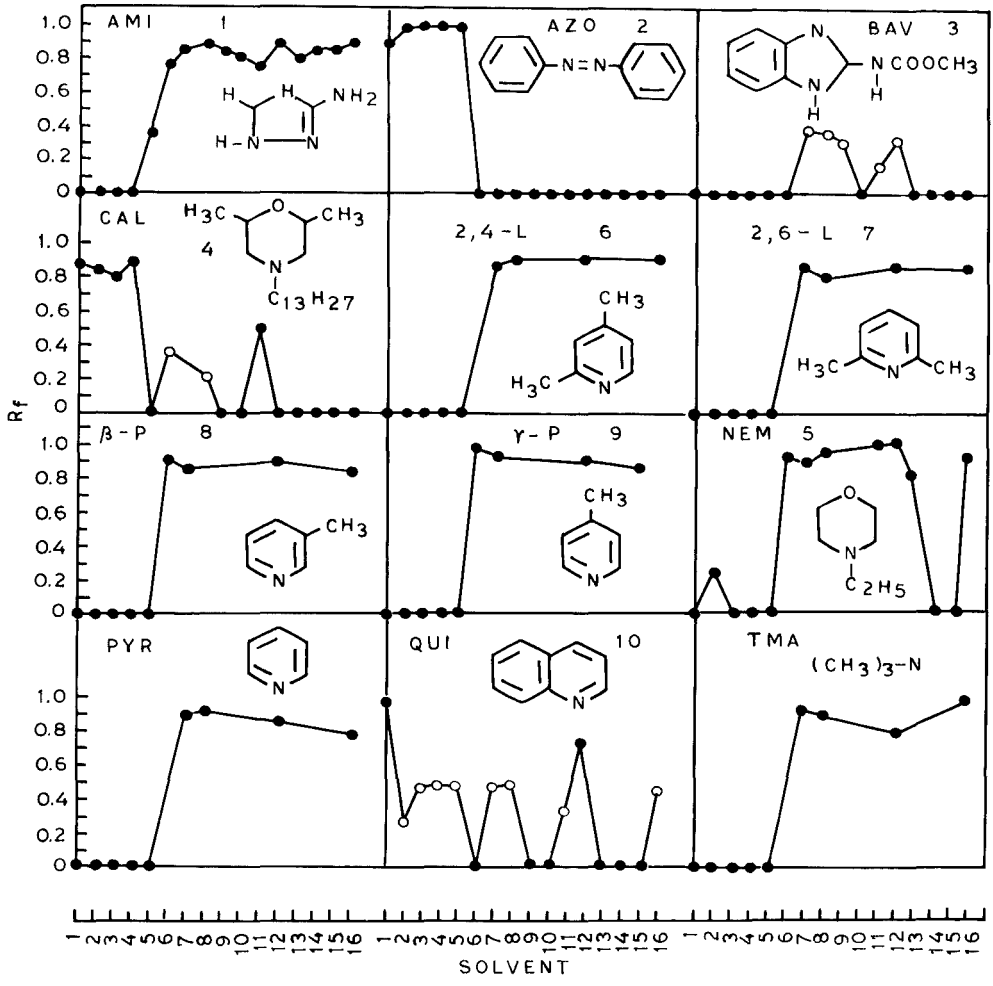

FIG. 1

ABBREVIATIONS:- 1 = $CHCl_3$, 2 = CCl_4, 3 = C_6H_6, 4 = C_6H_5Cl, 5 = $(CH_3)_3C$-OH
6 = DISTILLED WATER 7 = HCl, 8 = HNO_3 9 = NaOH, 10 = NaCl, 11 = NaH_2PO_4
12 = $KHSO_4$ 13 = Na_2CO_3, 14 = $NaNO_3$ 15 = $NaNO_2$ 16 = $Pb(NO_3)_2$
AMI = AMITROL, AZO = AZOBENZENE, BAV = BAVISTIN, CAL = CALIXIN,
2,4-L = 2,4-LUTIDINE, 2,6-L = 2,6-LUTIDINE, NEM = N-ETHYLMORPHOLINE
β-P = β-PICOLINE, γ-P = γ-PICOLINE, PYR = PYRIDINE, QUI = QUINOLINE
TMA = TRIMETHYLAMINE
● = R_f ○ = R_f OF TAILING COMPOUNDS

Lehmann have used saturated solution of copper iodide in acetonitrile, saturated solution of sodium carbonate in water followed by uv radiation for the detection of heterocyclic nitrogen compounds by fluorescence (10). These two detectors proved to be complementary for the pollutants under study as they give bright yellow colour and fluorescence respectively. The chromatographic data given in figure 1 shows that as the pH of the water decreases, detection and separation becomes easier. For example 27 separations can be achieved in strongly acidic solutions of HNO_3, HCl, $Pb(NO_3)_2$ while only two separations can be achieved in strongly alkaline solution of NaOH. This aspect is further discussed below.

Figure 1 also indicates that the mobility of organics under study except azobenzene, calixin and quinoline is very low in organic solvents. The mobility of these compounds is high in acidic waters, medium in neutral waters and low in alkaline waters. The results give the relevant information about several binary separations.

The next and perhaps the more important aspect of this work was to attempt a structure-activity-correlation. This attempt was due to the fact(figure 1) that trimethylamine, pyridine and its derivatives: 2,4-Lutidine, 2,6-Lutidine, β-picoline and γ-picoline give almost identical chromatographic behaviour and this made us suspect that the toxic/carcinogenic behaviour of these compounds is in some way localised on the lone-pair on the Nitrogen atom for this class of compounds. It also became clear that hydrogen bonding seems to play a crucial role in the chromatographic behaviour of this class. For example,(figure 1) the mobility of anitrole is essentially zero in non-hydrogen bond or weak hydrogen bond donor solvents i.e. $CHCl_3$, CCl_4, C_6H_6 and C_6H_5Cl while it has an intermediate value for $(CH_3)_3$-COH and a very high value for distilled water after which there is much less steep rise and levelling off as the pH of the media is decreased. These results are in consonance with the hydrogen-bond concept. It is

well known that hydrogen-bonding plays a crucial role in binding the carcinogen to nucleic acid analogue of DNA(11).

There was however no correlation between mobility in distilled water $\left[\rho_{H_2O} = R_f\right]$ and LD_{50} values and the scatter diagram is given in figure 2. LD_{50} values are a measure of toxicity (12) and defined as:

Toxicity is expressed as the LD_{50} based on oral feeding of male rats. The figure given is number of milligrams of the compounds required per kilogram of animal weight to produce mortality in 50% of the test animals. Thus lower the figure, the higher the toxicity.

We next attacked the problem in two ways. The number of nitrogen atoms per molecule of the tertiary amine pollutant was taken as a rough structural measure and denoted by n. First a plot was constructed of LD_{50} vs. n

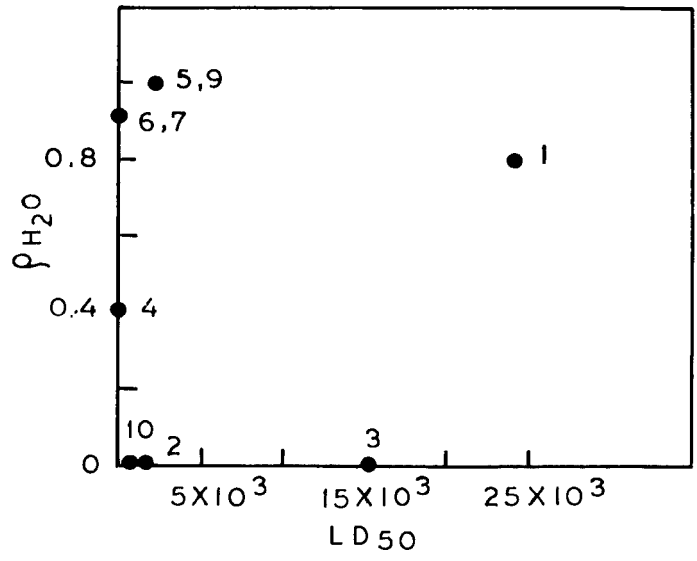

FIG. 2 SCATTER DIAGRAM OF AN ATTEMPTED CORRELATION BETWEEN ρ_{H_2O} AND LD50

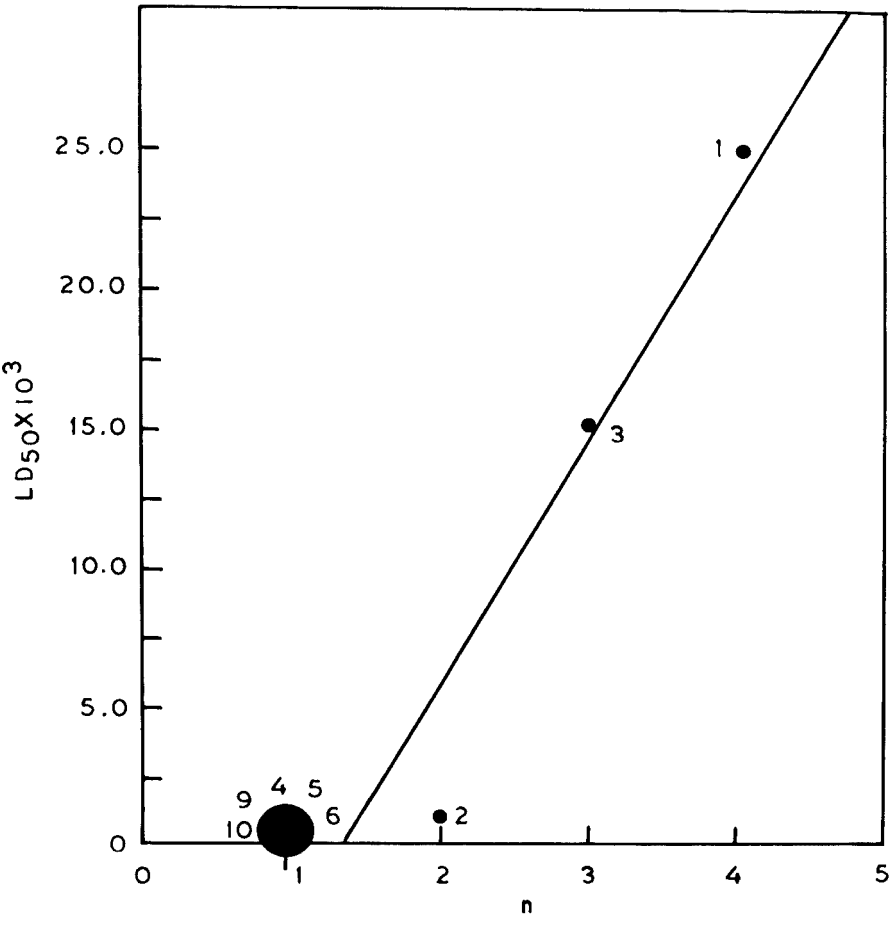

FIG. 3 CORRELATION FOR LD_{50} AND n

(figure 3) and a near linear correlation obtained. Next a plot was constructed of ρ_{H_2O} vs. n and a good parabolic relation analogous to equation 1 was obtained (figure 4)

$$\rho_{H_2O} = n - C_1 + \frac{C_2}{n - C_1} \qquad (1)$$

There is evidence to show that in many instances a parabolic relation is much more refined than a linear one.

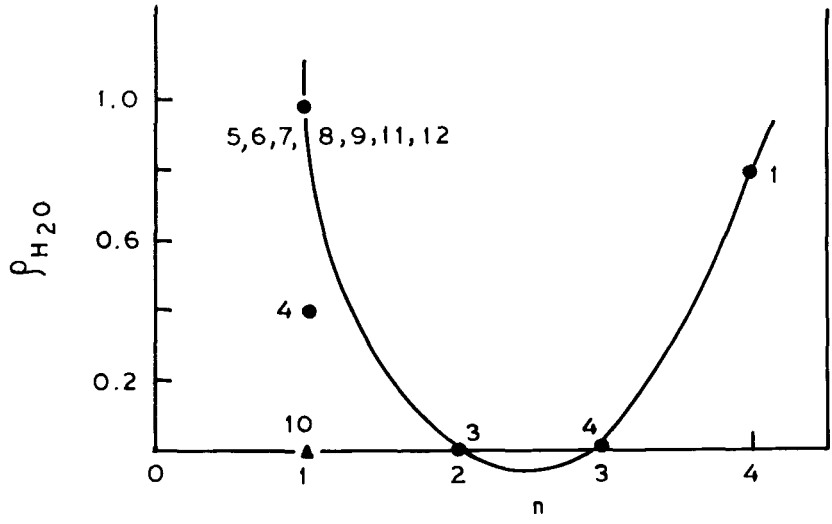

FIG. 4 CORRELATION FOR ρ_{H_2O} AND n

For example the linear (13) and parabolic (14) relations of the energy of the highest intermolecular charge transfer band ($h\nu_{CT}$) and ionisation potential of the donor (I^D) though ofcourse the theoretical considerations may be different. A characteristic common to both in the linear as well as the parabolic relationship is that compounds having one nitrogen atom per molecule have LD_{50} values closely spaced and ρ_{H_2O} almost identical, irrespective of the often severe structural modification of the molecule. The near- coincidence of ρ_{H_2O} values for n = 1 (figure 4) as compared to the small scatter of LD_{50} values again for n = 1 is surprising as the scale differs as 1 : 12,500 and hence a small deviation LD_{50} should not be apparent and hence the better coincidence of ρ_{H_2O} values as compared to LD_{50} values for n = 1 vindicates our belief that the parabolic relations are more refined than linear relationships. A final significant support for the parabolic relationship being better comes from the fact that quinoline (compound 10, figure 1) is the only compound of

the series that have a fused ring system and hence its toxicity will have a very significant contribution from its polycyclic nature and hence should deviate considerably from both relations. However, figure 4 as compared to figure 3 reveals that the deviation is much more pronounced in the parabolic relation (figure 4) than the linear one (figure 3).

Hence though apparently we have failed in the first instance to correlate structure with activity —— a very important conclusion emerges, n is related to LD_{50} values by a linear relationship and to ρ_{H_2O} values by perhaps a better parabolic relationship and for n = 1 the ρ_{H_2O} values are effectively constant. Hence, it seems a common mode of mechanism is responsible for the chromatographic as well as the toxic behaviour of these compounds and this common mode most certainly appears to be hydrogen bonding between nitrogen lone-pair and a proton of nucleic acid analogue of DNA. Therefore, there is certainly a good relation between structure and activity though not as simple a one as attempted herein

ACKNOWLEDGEMENT

The authors thank Professor W. Rahman, Chairman, Department of Chemistry for facilities and DOE under BRC project and CSIR (INDIA) for financial assistance.

REFERENCES

1. M. Lee, M.V. Norotny and K.D. Bartle, "Analytical Chemistry of Polycyclic Aromatic Compounds", Academic, New York, 1981.

2. M. Qureshi, H.S. Rathore and Afsar M. Sulaiman, Water Research, 1982, 16, 435-440.

3. P.M. Qureshi, M. Qureshi and Afsar M. Sulaiman, Talanta (Communicated) "A New Method for the Selective Spectrophotometric Determination of 2,6-Lutidine and its possible use for the Specific Analysis of a Physiologically important Pollutant".

4. Reference 1, p.123.

5. L. Duhois, A. Corkeny and J.L. Monkman, Int. J. Air Pollut., 1960, 2, 236.

6. F.A. Gunther and F. Buzzeti, Residue. Rev., 1964, 9, 90.

7. Reference 1, p.134.

8. E. Lederer and M. Lederer, "Chromatography", 1957, 2nd Edn., Elsevier Publ. Co., Lond., pp.203-206.

9. Streuli and Averell, "The analytical chemistry of nitrogen and its compounds", 1969, Parts I-II, 253-264, 568-581, Wiley Interscience, New York.

10. H. Wagner and H. Lehmann, Fresenius' Z. Anal.Chem., 1977, 283(2), 115-119.

11. D.E. Hathway and G.F. Kolar, Chem.Soc.Revs.,1980, 9 241.

12. M. Beroza, R.L. Caswell, R.R. Watts, 1981,"Analytical Reference Standars and Supplemental Data for Pesticides and other organic compounds", EPA, p.iv.

13. R. Foster, Nature, Lond., 1959, 183, 1253.

14. S.H. Hastings, J.L. Franklin, J.C. Schiller and F.A. Matsen, J. Am. Chem. Soc. 1953, 75, 2900.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(1), 219 (1984)

EASTERN ANALYTICAL SYMPOSIUM-1983

The 22nd Eastern Analytical Symposium, which was held in New York City on November 16th thru 18th was the overwhelming success we've come to expect. Included in the program were in-depth symposia and poster sessions in all areas of analytical chemistry where there have been significant advances, as well as an exposition of the latest instrumentation and laboratory equipment.

Of particular interest to liquid chromatographers were the outstanding sessions on Field-Flow Fractionation, HPLC, Ion and Countercurrent Chromatography, and Thin-Layer Chromatography. Additionally, many applications of liquid chromatography were presented at the sessions on Pharmaceutical Analysis, Forensics, Computers, Advances in Separation Techniques, and Food Analysis. In all, 60 papers on LC were included in the program, many of which will be published in The Journal of Liquid Chromatography.

The EAS Committee, under the leadership of General Chairperson Constance M. Paralusz and Program Chairman Haleem J. Issaq, are to be heartily commended for a job well done. With 4500 registrants this year, the majority of them in attendance for all three days, the Eastern Analytical Symposium has rapidly become a major gathering of analytical scientists. This year's attendance represents more than a 30% increase over last year and an 80% increase over two years ago. The EAS Committee has decided to expand the 23rd Eastern Analytical Symposium to 4 days to accomodate the program they're planning for next year.

Dr. Jack Cazes, Editor

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(1), 221-222 (1984)

LC NEWS

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/84/1, River View Plaza, 16 River Street, Norwalk, CT, 06850, 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/84/1, P. O. Box 484, Edmond, OK, 73083, USA.

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/84/1, 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/84/1, 2200 Wright Avenue, Richmond, CA, 94804, 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/84/1, 1205 Kent Avenue, Purdue Research Park, West Lafayette, IN, 47906, USA.

FIXED WAVELENGTH DETECTOR offers low cost and high performance. It can monitor the absorbance of one stream or the difference between two streams. A full 10mm path length is achieved with an 8 microliter cell volume. Wavelength conversion kits are available for operation at 254, 280, 350, 410, 440, and 550 nm. A 3mm path length cell is also available with a cell volume of either 1 or 47 microliters. LDC/Milton Roy Co., JLC/84/1, 3661 Interstate Industrial Park Rd., P. O. Box 10235, Riviera Beach, FL, 33461, USA

NEW HPLC COLUMN CONFIGURATIONS lead to faster analyses with minimal reduction in resolution. Phases available include C18, C8, C2, phenyl, cyclohexyl, CN, NH2, COOH, quaternary amine, sulfonic acid, diol, and silica. Analytichem International, JLC/84/1, 24201 Frampton Avenue, Harbor City, CA, 90710, USA

GPC ANALYSIS SYSTEM uses high efficiency GPC columns that generate up to 50,000 plates/meter and 80,000 plates/meter for 10 micron and 5 micron gels, respectively. Many separations can be performed in 4 to 6 minutes and molecular weights are calculated with reproducibility woithin 1 percent. Perkin-Elmer Corp., JLC/84/1, Main Avenue, Norwalk, CT, 06856, USA

HIGH PERFORMANCE SEPARATION OF BIOLOGICALS is accomplished with diphenyl TLC plates. The plates employ aromatics bonded to silica for reversed phase partition of biomaterials with molecular weights up to 100,000. Whatman, Inc., JLC/84/1, 9 Bridewell Place, Clifton, NJ, 07014, USA.

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LC CALENDAR

1984

JANUARY 17: "Process Scale HPLC," sponsored by the Chicago Chromatography Discussion Group. Contact: N. Armstrong, LC Company, P. O. Box 72125, Roselle, IL, 60172, USA.

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.

MARCH: "Basic GC School," a 3-day course (date to be announced), sponsored by the Chicago Chromatography Discussion Group. Contact: N. Armstrong, LC Company, P. O. Box 72125, Roselle, IL, 60172, USA.

MARCH 20: "New Developments in HPLC of Water Soluble Macromolecules," sponsored by the Chicago Chromatography Discussion Group. Contact: N. Armstrong, LC Company, P. O. Box 72125, Roselle, IL, 60172, USA.

APRIL: "HPLC of Water Soluble Polymers," a 2-day course (date to be announced) sponsored by the Chicago Chromatography Discussion Group. Contact: N. Armstrong, LC Company, P. O. Box 72125, Roselle, IL, 60172, USA.

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

APRIL 17: "New Developments in TLC," sponsored by the Chicago Chromatography Discussion Group. Contact: N. Armstrong, LC Company, P. O. Box 72125, Roselle, IL, 60172, 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 3-5: International Symposium on LCEC and Voltammetry, Indianapolis Hyatt Regency Hotel, Indianapolis, IN. Contact: The 1984 LCEC Symposium, P. O. Box 2206, West Lafayette, IN, 47906, USA.

JUNE 10-14: 14th Northeast Regional ACS Meeting, sponsored by the Western Connecticut and New Haven Sections, at Fairfield University, Fairfield, CT. Contact:D. L. Swanson, American Cyanamid Co., Stamford, CT, USA.

JUNE 18-20: Second International Conference on Chromatography & Mass Spectrometry in Biomedical Sciences, sponsored by the Italian Group for Mass Spectrometry in Biochemistry & Medicine, Milan, Italy. Contact: Dr. A. Frigerio, via Eustachi 36, I-20129 Milan, Italy, or Dr. H. Milon, P. O. Box 88, CH-1814 La Tour-de-Peilz, Switzerland.

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

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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

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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.

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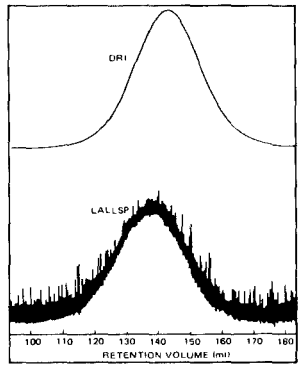

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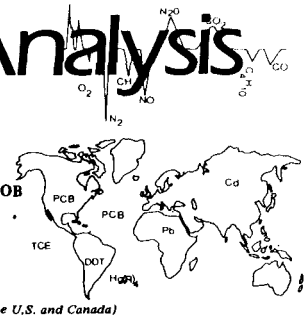

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